UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Control de *Listeria monocytogenes* en embutidos curado-madurados y quesos madurados tradicionales mediante cultivos protectores e hidrolizados proteicos

Irene Martín Tornero

Programa de Doctorado en Ciencia de los Alimentos

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UNIVERSITARIO DE INVESTIGACIÓN DE LA CARNE Y PRODUCTOS CÁRNICOS

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INFORMA:

Que el trabajo presentado por Dña. Irene Martín Tornero titulado "Control de *Listeria monocytogenes* en embutidos curado-madurados y quesos madurados tradicionales mediante cultivos protectores e hidrolizados proteicos", ha sido realizado bajo mi dirección en el departamento de Producción Animal y Ciencia de los Alimentos de esta Universidad. Todos los trabajos presentados en la sección de resultados y discusión han sido desarrollados integramente en el laboratorio y planta piloto por Dña. Irene Martín Tornero y sólo se presentarán en esta Tesis Doctoral. Hallándose concluido y reuniendo a mi entender las condiciones necesarias, autorizo su presentación ante el tribunal que ha de juzgarla.

Y para que así conste firmo el presente informe en Cáceres, a 08 de febrero de 2022.

La conformidad del director de la tesis consta en el original en papel de esta tesis doctoral

Fdo: Juan José Córdoba Ramos

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Fdo: Alicia Rodríguez Jiménez

Agradecimientos

En todos estos años he tenido la suerte de estar rodeada de personas maravillosas a las que me gustaría dedicar estos agradecimientos.

Agradezco a las entidades que han contribuido a la realización de esta Tesis Doctoral. Al Ministerio de Educación, Cultura y Deporte por la ayuda predoctoral concedida (FPU 16/05303). Del mismo modo agradecer al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) dependiente del Ministerio de Ciencia, Innovación y Universidades, a través del proyecto RTA-2017-00027-C03-03. A la Junta de Extremadura y al FEDER a través del proyecto IB16149 y las ayudas a Grupo GR15108 y GR18056 por la financiación parcial de actividades de esta Tesis Doctoral. Al Instituto de Investigación en Ciencias de la Alimentación (CIAL) y en especial a Dña. Rosina López-Fandiño y a D. Daniel Lozano Ojalvo por la cesión de hidrolizado proteico de lactuosuero ensayado en este Tesis Docotoral.

Gracias a mis directores de Tesis, los Dres. Juan José Córdoba Ramos y Alicia Rodríguez Jiménez, sin los cuales la realización de esta Tesis Doctoral no hubiera sido posible. Gracias por haber confiado en mí desde el principio, por todo el tiempo que me habéis dedicado, por vuestra entrega, dedicación y apoyo constante. Espero seguir aprendiendo de vosotros.

Y al resto de los integrantes del grupo de investigación de Higiene y Seguridad Alimentaria. A los Dres. Miguel Ángel Asensio, Mar Rodríguez, Elena Bermúdez y María J. Andrade por sus consejos y ayuda desinteresada. Al Dr. Félix Núñez, por acogerme en su despacho y estar siempre dispuesto a ayudarme. Al Dr. Josué Delgado por estar siempre disponible y darme consejos e ideas para futuras investigaciones. Y, por supuesto, a Librada, por tener siempre palabras de ánimo para mí.

Gracias también a todos los integrantes del grupo de Tecnología y Calidad de los Alimentos (TECAL), al Servicio de Innovación y Análisis de Productos de origen Animal (SIPA) y al Instituto Universitario de Investigación de Carne y Productos Cárnicos (IProCar).

Gracias a todos mis compañeros de laboratorio, a los que ya no están Patri, Alberto, Belén, Lourdes, Juanjo, Remi, Paco, María y Jose por haber sido un gran apoyo y por todo el cariño que me habéis dado. En especial a Patricia porque, aunque en la distancia, siempre estás y a Alberto, mi compi Listeria, por tu alegría y por haberme enseñado tanto, ha sido un placer aprender de ti.

Y, por supuesto, a los que siguen, Eva, Elia, Mariela, Juan Carlos, Alberto, Victor, Paula, Juan Carlos Pulido, Noelia, Silvia y Guada, por haber formado este grupo tan bonito, por alegrarme todos y cada uno de los días en el trabajo, por cada desayuno y por cada quedada.

En especial a Eva, porque más que una compañera eres una gran amiga, porque estos años trabajando a tu lado han sido un regalo y esta última etapa menos dura gracias a ti. Por nuestra complicidad, por hacer más fácil mi día a día, por todo lo que hemos compartido, por las risas y los momentos que nos quedan por vivir. Gracias a Elia porque no hay día que no me saques una sonrisa, a Mariela por tu cariño desde que empecé esta etapa, a Juan Carlos por haber estado ahí desde que te conocí y a Alberto, porque siempre tienes palabras bonitas para mí.

Obrigado à Dra. Paula Teixeira por me permitir realizar a minha estadia de investigação no seu grupo, pela orientação científica e por me permitir viver esta experiência. À Dra. Joana Barbosa pela ajuda constante desde que a conheci e pela sua estima. Agradeço também a todos os meus colegas de laboratório da Universidade Católica do Porto (Inês, Mónica, Marta, Claúdia, Joana, Lourenço, Liliana, Tiago, Teresa), por me terem feito sentir como um colega mais dos laboratórios desde o primeiro dia, pelos almoços e passeios à praia e por me terem ajudado em tudo o que precisei. Foi um prazer conhecer-vos.

A mi familia y amigos, por estar siempre a mi lado y cuidarme como lo habéis hecho. A César, por haberme alegrado todo este tiempo y haberme hecho este periodo más ameno.

A Sofía, mi ratonita, porque llegaste a nuestras vidas para llenarla de locura y felicidad, porque alegras a cualquiera que esté a tu lado, jamás pensé que algo tan pequeñito pudiera hacerme sentir tanto.

A mi hermana Eli, porque si hoy estoy escribiendo estas líneas en gran parte es por ti, gracias por haberme enseñado a amar la ciencia como lo hago, por haberme ayudado siempre y haber evitado tantas veces que me cayera, por hacerme el camino más fácil y la vida más bonita. Que suerte poder compartir todo contigo desde siempre porque no hay mejor regalo que una hermana como tú. A Juan Pablo por cuidarlas y quererlas tanto.

A mi abuela Dioni, por ser ejemplo de fuerza, bondad y superación. Por preocuparte siempre por mí y quererme como lo haces. Ojalá fueras eterna. A todas esas estrellas que me cuidan desde el cielo, sé que estaríais muy orgullosos de mí.

A mis PADRES, gracias por vuestro cariño, vuestro apoyo constante, por haberme dado lo mejor de vosotros, por valorarme tanto y haberme enseñado los valores que hacen que sea como soy. Sois el pilar en el que se sustenta todo. No puedo ser más afortunada de teneros y disfrutar de vosotros cada día

A ti Óscar, la suerte de mi vida, gracias por valorarme tanto, apoyarme en todo y alegrarte por cada logro que consigo como si fuera tuyo, por acompañarme al fin del mundo si supieras que eso me ayudaría a conseguir mi sueño. Eres mi felicidad y el mejor compañero de vida.

Gracias

A mis padres,
a mi hermana,
a Óscar.

“Aprendí que el coraje no es la
ausencia de miedo, sino el triunfo sobre él.
El hombre valiente no es aquel que no siente
miedo, si no el que lo conquista” (Nelson Mandela)

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I. INTRODUCCIÓN

I.1. ALIMENTOS MADURADOS TRADICIONALES

Dentro de las distintas formas de conservación de los alimentos, la maduración es una forma de conservación que se basa en la desecación en condiciones ambientales adecuadas para provocar, en el transcurso de una lenta y gradual reducción de la humedad, la evolución de los procesos naturales de fermentación o enzimáticos necesarios para aportar al producto cualidades organolépticas características y que garantice su estabilidad durante el proceso de comercialización. La maduración de los alimentos es una técnica que se lleva utilizando desde la época romana para la conservación de distintas carnes, siendo los alimentos madurados tradicionales muy valorados hoy en día.

Según el Reglamento (CE) nº 2073/2005, se define alimento listo para el consumo (RTE) como el destinado 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.

Dentro de estos se pueden destacar los derivados cárnicos como los embutidos curado-madurados, así como los quesos madurados.

I.1.1. Embutidos curado-madurados

Según la Norma de Calidad reguladora de los derivados cárnicos (Real Decreto 474/2014, de 13 de junio, por el que se aprueba la norma de calidad de derivados cárnicos), se define a los derivados cárnicos curado-madurados como aquellos sometidos a un proceso de salazón y de curado-maduración, suficiente para conferirles las características organolépticas propias y de estabilidad a temperatura ambiente. Los derivados cárnicos se dividen en derivados cárnicos tratados por calor y derivados

cárnicos no tratados por calor. Dentro de los derivados cárnicos no tratados por calor pueden ser:

- Derivados cárnicos curado-madurados.
- Derivados cárnicos oreados.
- Derivados cárnicos marinado-adobados.
- Derivados cárnicos salmuerizados.
- Derivados cárnicos no sometidos a tratamiento.

De acuerdo con el Real Decreto 474/2014 (B.O.E. A- 2014- 6435), los embutidos curado-madurados son los derivados cárnicos constituidos por trozos de carne o carne y grasa no identificables anatómicamente que, con carácter general y no limitativo, se han sometido a un proceso de picado más o menos intenso, mezclados con especias, ingredientes, condimentos y aditivos, embutidos o no en tripas naturales o envolturas artificiales, y sometidos a un proceso de salazón seguido de curado-maduración, acompañado o no de fermentación, suficiente para conferirles las características organolépticas propias y su estabilidad a temperatura ambiente. Las carnes podrán ser todas del mismo tipo o ser una mezcla de carnes de distinta procedencia, naturaleza, parte anatómica y especie animal.

En este grupo se engloban los chorizos y salchichones como embutidos curado-madurados. Ambos productos son embutidos elaborados con carne y grasa (generalmente de cerdo) con un grado de picado grueso o fino, sometidos a un proceso de salazón. Además, se añaden especias, condimentos, ingredientes y aditivos. La formulación del embutido variará según el tipo, en el chorizo es característica la incorporación del pimentón, mientras que, en el caso del salchichón, de la pimienta negra (Ordoñez y de la Hoz, 2001).

I.1.2. Quesos madurados

Según el Codex Alimentarius (CXS 283-1978), se entiende por queso el producto blando, semiduro, duro y extraduro, madurado o no madurado, y que puede estar recubierto, en el que la proporción entre las proteínas de suero y la caseína no sea superior a la de la leche, obtenido mediante:

(a) coagulación total o parcial de la proteína de la leche, leche desnatada/descremada, leche parcialmente desnatada/descremada, nata (crema), nata (crema) de suero o leche de mantequilla/manteca, o de cualquier combinación de estos materiales, por acción del cuajo u otros coagulantes idóneos, y por escurrimiento parcial del suero que se desprende como consecuencia de dicha coagulación, respetando el principio de que la elaboración del queso resulta en una concentración de proteína de la leche (especialmente la porción de caseína) y que por consiguiente, el contenido de proteína del queso deberá ser evidentemente más alto que el de la mezcla de los materiales lácteos ya mencionados en base a la cual se elaboró el queso; y/o

(b) técnicas de elaboración que comportan la coagulación de la proteína de la leche y/o de productos obtenidos de la leche que dan un producto final que posee las mismas características físicas, químicas y organolépticas que el producto definido en el apartado (a).

Haciendo referencia a la Norma de Calidad reguladora de los quesos (Real Decreto 1113/2006) se define queso madurado aquel que, tras el proceso de fabricación, requiere mantenerse durante cierto tiempo a una temperatura y en condiciones tales que se produzcan los cambios físicos y químicos característicos del mismo.

La palabra madurado en el queso madurado podrá sustituirse por los calificativos que se encuentran en la Tabla I.1 según el grado de maduración alcanzado por el producto a la salida de fábrica.

Tabla I.1. Denominación de los quesos en función del tiempo de maduración (RD 1113/2006).

Denominación	**Peso > 1,5 kg**	**Peso ≤ 1,5 kg**
	Maduración mínima en días	
Tierno	7	
Semicurado	35	20
Curado	105	45
Viejo	180	100
Añejo	270	-

De acuerdo con el contenido de grasa, los quesos se podrán denominar:

- Extragraso: el que contenga un mínimo de 60%
- Graso: el que contenga un mínimo de 45 y menos de 60%
- Semigraso: el que contenga un mínimo de 25 y menos de 45%
- Semidesnatado: el que contenga un mínimo de 10 y menos de 25%
- Desnatado: el que contenga menos de 10%

De acuerdo con el tipo de pasta se establecen 3 grupos de queso:

- Queso de pasta dura: después de 8 meses de maduración.
- Queso de pasta semidura: masa cerrada con un periodo de maduración de cuarenta y cinco a setenta y cinco días.
- Queso de pasta blanda: aproximadamente 15 días de maduración y pasta blanca.

Actualmente, en Extremadura, se producen tres tipos de quesos bajo Denominación de Origen Protegida (DOP) (MAPA, 2021). Uno de ellos, de pasta semidura, como es el "Queso Ibores" (Reglamento (CE) 205/2005), elaborados con leche cruda de cabra, y la coagulación de la leche se provocará con cuajo natural u otras enzimas coagulantes autorizadas y dos quesos de pasta blanda, "Torta del Casar" (Reglamento (CE) 1491/2003) y "Queso de la Serena" (Reglamento (CE) 1107/1996), reconocidos internacionalmente por su singularidad organoléptica. Su elaboración se sitúa en diferentes zonas geográficas de la comunidad autónoma extremeña y están elaborados con leche cruda de oveja, cuajo vegetal procedente de las flores secas del cardo *Cynara cardunculus* L. y sal. Además, ambos se caracterizan por tener una textura untuosa.

Las pequeñas diferencias entre el queso "Torta del Casar" y el "Queso de la Serena" se debe a la localización, la temperatura… Además, en la "*Torta del Casar*" se utiliza la leche de oveja de raza merina y churras mientras que en el "*Queso de la Serena*" solo se usa la leche de la oveja merina.

I.2. POBLACIÓN MICROBIANA EN EMBUTIDOS Y QUESOS MADURADOS

I.2.1. Población microbiana en embutidos curado-madurados

La población microbiana de los embutidos curado-madurados va variando durante su proceso de elaboración e incluye diferentes especies y cepas de bacterias, levaduras y mohos. Las bacterias ácido-lácticas (BAL) y los cocos Gram positivos, catalasa positivos, incluidos los micrococos y los estafilococos coagulasa negativos, son los grupos más predominantes en estos productos (Aquilanti y col., 2016).

Las BAL constituyen la población microbiana principal al final de la etapa de maduración de los embutidos curado-madurados. Incluso si sus niveles iniciales varían en la masa (3-4 log unidades formadoras de colonias (UFC)/g), crecen durante la etapa de fermentación y se vuelven dominantes, alcanzando poblaciones de 8 a 9 log UFC/ g (Talon y Leroy, 2014). Su capacidad para reducir el pH de la mezcla produciendo ácido láctico por la fermentación de los carbohidratos favorece la formación de características organolépticas deseables, que junto a la capacidad de producir otros compuestos como las bacteriocinas previenen el crecimiento de microorganismos patógenos y aseguran la estabilidad y seguridad del producto final (Prpich y col., 2021; Santa y col., 2012; Urso y col., 2006). Los principales géneros de BAL aislados de salchichón son el anteriormente denominado *Lactobacillus*, hoy en día dividido en 23 géneros (*Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacilus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus* and *Lentilactobacillus)* (Zheng y col., 2020), *Lactococcus*, *Pediococcus*, *Leuconoctoc*, *Weissella* y *Enterococcus* (Comi y col., 2005; Di Cagno y col., 2008; Urso y col., 2006), siendo generalmente el género dominante *Lactobacillus*. Tres especies de este género, *Lactilactobacillus sakei*, *Lactilactobacillus curvatus* y *Lactiplantibacillus plantarum*, son las especies que se suelen encontrar de forma dominante durante la maduración de los salchichones (Leroy y De Vuyst, 2015; Talon y Leroy, 2014).

Los cocos Gram positivos, catalasa positivos frecuentemente representan la segunda población microbiana dominante con niveles finales que varían de 5 a 7 log UFC/ g. Su

nivel inicial en masa varía de 3 a 4 log UFC/ g, y en ocasiones tienen dificultades para competir con BAL.

En los embutidos fermentados, los cocos Gram positivos, catalasa positivos son los principales responsables del desarrollo y estabilización del color, la proteólisis, la lipólisis y la descomposición de aminoácidos libres y peróxidos (Aquilanti y col., 2016; Iacumin y col., 2006; Urso y col., 2006).

Las especies cocos Gram positivos, catalasa positivos más frecuentemente aisladas en embutidos curado-madurados son *Staphylococcus xylosus*, *S. eqourum*, *S. saprophyticus* y *S. carnosus*. Las especies de *S. equorum, S. succinuss, S. warneri, S. vitulinus, S. pasteuri, S. epidermidis, S. lentus, S. haemoliticus, S. intermedius, S. saprophyticus, S. hominis* y *S.auricularis* también pueden aislarse de estos productos, pero con menor frecuencia (Babić y col., 2011; Milicevic y col., 2014; Sánchez Mainar y col., 2017).

La población de levaduras y mohos es muy variable y, aunque en menor medida, también juegan un papel relevante en la calidad de los embutidos curado-madurados, debido a que se encargan de la formación de una película superficial que ejerce una acción protectora tanto contra la deshidratación excesiva como contra la oxidación de la fracción lipídica debido a la acción del oxígeno y la luz (Cocolin y col., 2006; Gardini y col., 2001; Prpich y col., 2021). Además, estos microorganismos participan en el desarrollo del sabor, el aroma y la estabilización del color rojo de los salchichones (Visagie y col., 2014). Por otro lado, las especies de mohos y levaduras más habitualmente utilizadas como cultivos iniciadores y/o protectores pueden contribuir a aumentar la seguridad del producto mediante la actividad antagonista contra los mohos toxigénicos (Delgado y col., 2016).

Entre las levaduras, *Candida*, *Criptococus*, *Debaryomyces*, *Yarrowia*, *Rhodotorula*, *Pichia* y *Trichosporon* son los géneros más aislados (Murgia y col., 2019), siendo *Debaryomyces hansenii* la especie más frecuentemente aislada (Andrade y col., 2009, 2010; Delgado y col., 2019; Gardini y col., 2001).

En el caso de los mohos, estos tienen una mayor relevancia al final de la maduración de los embutidos, encontrándose principalmente los géneros *Penicillium* y *Aspergillus* (Prpich y col., 2021). Dentro del género *Penicillium* predominan las especies como *P.nalgiovense*, *P.solitum*, *P.comune*, *P.nordicum* y *P.chrisogenum* (Asefa y col., 2010; Castellari y col., 2010). En embutidos, también se han encontrado durante las etapas de maduración especies pertenecientes al género *Aspergillus* como *A. parasiticus*, *A. ochraceus*, *A. flavus* y *A. niger* (Castellari y col., 2010).

I.2.2. Población microbiana en quesos madurados

La población microbiana de los quesos madurados varía dependiendo del tipo de queso y del tiempo de maduración, aunque esta variación no es muy notoria. Varios microorganismos, incluidas bacterias, levaduras y mohos están presentes en el queso durante toda la maduración.

Las BAL son la población microbiana principal en el interior del queso durante todo el proceso de maduración (Coton y col., 2012; Crespo y col., 2022). Los niveles iniciales varían en torno a 8-9 log UFC/g manteniéndose constante hasta el final de la maduración (Ordiales, Benito, y col., 2013). La alta población de estas bacterias es extremadamente deseable en los quesos ya que estos microorganismos confieren características sensoriales típicas y contribuyen a la seguridad a través de la producción de compuestos antimicrobianos (Camargo y col., 2021).

Los principales géneros de BAL aislados de quesos son el anteriormente denominado *Lactobacillus*, *Lactococcus*, *Leuconostoc*, y *Enterococcus.* Dentro de estos géneros las especies mayormente aisladas de quesos son *Lc. casei*, *Lc. paracasei*, *Lp. plantarum*, *L. rhamnosus* y *L. curvatus* (Beresford y col., 2001; Sánchez-juanes y col., 2020). *Pediococcus acidilactici* y *Pe. pentosaceus* son los *Pediococos* más frecuentes aislados de quesos (Beresford y col., 2001).

Los cocos Gram positivos, catalasa positivos, levaduras, mohos y diversas bacterias Gram negativas (*Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp.) constituyen la población microbiana subdominante (Abriouel y col., 2008; Ordiales, Benito, ycol., 2013) o incluso codominante (Larpin-Laborde y col., 2011).

Además de estas bacterias hay que mencionar que, en quesos, especialmente los elbaorados con leche cruda, suele haber una población de enterobacterias que puede ser preocupante (Coton y col., 2012; Crespo y col., 2020; Gonçalves y col., 2018). Los altos niveles de este grupo microbiano durante todo el proceso de maduración contribuyen en el desarrollo de las características sensoriales de los quesos, incluyendo su textura y propiedades organolépticas (Crespo y col., 2020).

Con respecto a las levaduras, estas intervienen tanto directa como indirectamente en el proceso de maduración (Corsetti y col., 2001). Los quesos artesanales poseen una gran variedad de especies de levaduras, principalmente pertenecientes a los géneros *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Candida*, *Pichia* y *Yarrowia* (Banjara y col., 2015; Ceugniez y col., 2015; Gonçalves Dos Santos y col., 2017). La especie más abundante en la mayoría de los quesos es *D. hansenii* (Banjara y col., 2015), aunque la prevalencia de ciertas especies de levaduras puede verse influenciada por el tipo de queso (Dugat-Bony y col., 2016).

Los mohos más importantes que crecen en el queso pertenecen a los géneros *Penicillium*, *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor* y *Trichoderma* (Banjara y col., 2015), siendo el género predominante *Penicillium*. (Leggieri y col., 2020; Sengun y col., 2008).

I.3. PELIGROS MICROBIOLÓGICOS EN ALIMENTOS MADURADOS TRADICIONALES

I.3.1. Peligros microbiológicos en embutidos curado-madurados

Durante el proceso de elaboración de los embutidos curado-madurados se producen una serie de cambios bioquímicos y fisicoquímicos como resultado de las características del producto (carne, grasa y otros aditivos), la tecnología del procesamiento y duración del procesado, así como las interacciones entre los microorganismos y los componentes de dichos productos.

La estabilidad de los productos cárnicos fermentados está determinada principalmente por la acidificación, provocada por las bacterias ácido-lácticas, y la reducción de la actividad del agua (a_w) durante el curado y secado (Prpich y col., 2021).

Aunque la mayor parte de la población microbiana existente en los embutidos curado-madurados son las BAL, pueden estar presentes otros microorganismos que son indeseables como *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Clostridium perfringens* o incluso *Listeria monocytogenes*, lo que aumenta considerablemente el riesgo para la salud pública (Benito y col., 2007; Ferreira y col., 2007; Martín y col., 2007; Pereira y col., 2010).

Desde el punto de vista de la seguridad alimentaria, el riesgo de que el consumo de estos productos provoque alguna intoxicación o toxiinfección alimentaria se considera bajo, debido a que el número de bacterias disminuye de forma constante durante la maduración. De hecho, este proceso se considera esencial para inhibir microorganismos patógenos pertenecientes a los géneros *Salmonella* o *Shigella* y *E. coli*. Sin embargo, se ha demostrado la supervivencia de *Salmonella* y *E. coli* en este tipo de productos (Holck y col., 2017; Moore, 2004). Además, *S. aureus* es un patógeno bacteriano común que causa brotes de intoxicación alimentaria, y se aísla frecuentemente de la carne y embutidos curado-madurados (Benito y col., 2008; Casquete y col., 2012; Ferreira y col., 2007).

Además, el control de *L. monocytogenes* también es una preocupación importante para la seguridad alimentaria, ya que este patógeno puede estar presente en muchos productos alimenticios, incluida la carne y los derivados cárnicos curado-madurados (Doménech y col., 2015; Meloni, 2015). Además, se ha confirmado la implicación de estos productos en brotes de listeriosis (Benkerroum y col., 2005; Faber y Peterkin, 1991; Foodborne Ilness Outbreak Database, 2010; Schwartz y col., 1989).

Entre estos patógenos, *L. monocytogenes* es el más preocupante en la industria de los alimentos madurados tradicionales, principalmente por ser un microorganismo muy ubicuo, psicrótrofo y responsable de listeriosis.

I.3.2. Peligros microbiológicos en quesos madurados

Aunque la mayor parte de la población microbiana de los quesos madurados son las BAL, pueden estar presente otros microorganismos indeseables. Esto se ve potenciado en los quesos elaborados con leche cruda y, por lo tanto, sin la aplicación de un tratamiento

térmico que, unido a los diferentes procesos de ordeño y la variación de las condiciones higiénicas en las granjas, conduce a un mayor riesgo de contaminación con diferentes microorganismos patógenos (Ordiales, Benito, y col., 2013; Ordiales, Martín, y col., 2013). Entre las bacterias patógenas, los principales problemas de salud pública en los quesos son los siguientes: *Bacillus cereus*, *E. coli*, *Salmonella* y *L. monocytogenes* (Lobacz y Zulewska, 2021; Possas y col., 2021; Yoon y col., 2016).

La presencia de *E. coli* en los quesos podría deberse al uso de leche contaminada, de hecho, este microorganismo se usa como indicador de contaminación fecal directa o indirecta de los alimentos y, por lo tanto, de la posible presencia de patógenos entéricos (Gonçalves y col., 2018).

La contaminación por *Salmonella* en quesos es causada por el uso de leche contaminada y por contaminación directa de humanos (Lobacz y Zulewska, 2021).

En los quesos de leche cruda, la contaminación por *S. aureus* puede ocurrir a partir de leche cruda producida por animales que padecen mastitis y, en todos los quesos, por manipuladores de alimentos que son portadores de *S. aureus* o por malas prácticas de higiene (Kümmel y col., 2016). Uno de los patógenos de mayor preocupación para la industria láctea es *L. monocytogenes* siendo los quesos elaborados con leche cruda los de mayor riesgo (Camargo y col., 2021; Falardeau y col., 2021; Quigley y col., 2011) De hecho, la presencia de *L. monocytogenes* en los quesos de leche cruda europeos se ha informado con frecuencia (Amato y col., 2017; Knight y col., 2008).

Por lo que es fundamental comprender y cuantificar la prevalencia de *L. monocytogenes* en los quesos, ya que son un vehículo importante para la transmisión del

patógeno y la infección causa la tasa de mortalidad más alta entre las enfermedades zoonóticas (Martinez-Rios y Dalgaard, 2018).

I.4. LISTERIOSIS

L. monocytogenes es un peligro para la salud pública, ya que puede causar listeriosis en los consumidores (Vázquez-Boland y col., 2001). La listeriosis es una de las enfermedades transmitidas por los alimentos con mayor tasa de mortalidad (EFSA y ECDC, 2021), siendo según el último informe emitido por la Autoridad Europea de Seguridad Alimentaria (EFSA) de un 17,6% aumentando del 13,6% y 15,6% en 2017 y 2018, respectivamente. Además, la tasa de incidencia de la Unión Europea (UE) fue de 0,46 casos por 100.000 habitantes, que estaba al mismo nivel que en 2018 (0,47 casos por 100.000 habitantes), de los cuales, el 92,1 % requirieron hospitalización (EFSA y ECDC, 2021).

Los últimos informes publicados por la EFSA han demostrado una tendencia creciente de casos de listeriosis en la UE sin una causa claramente conocida, habiéndose incrementado desde el año 2010 hasta el 2019 (Buchanan y col., 2017; Figura I.1).

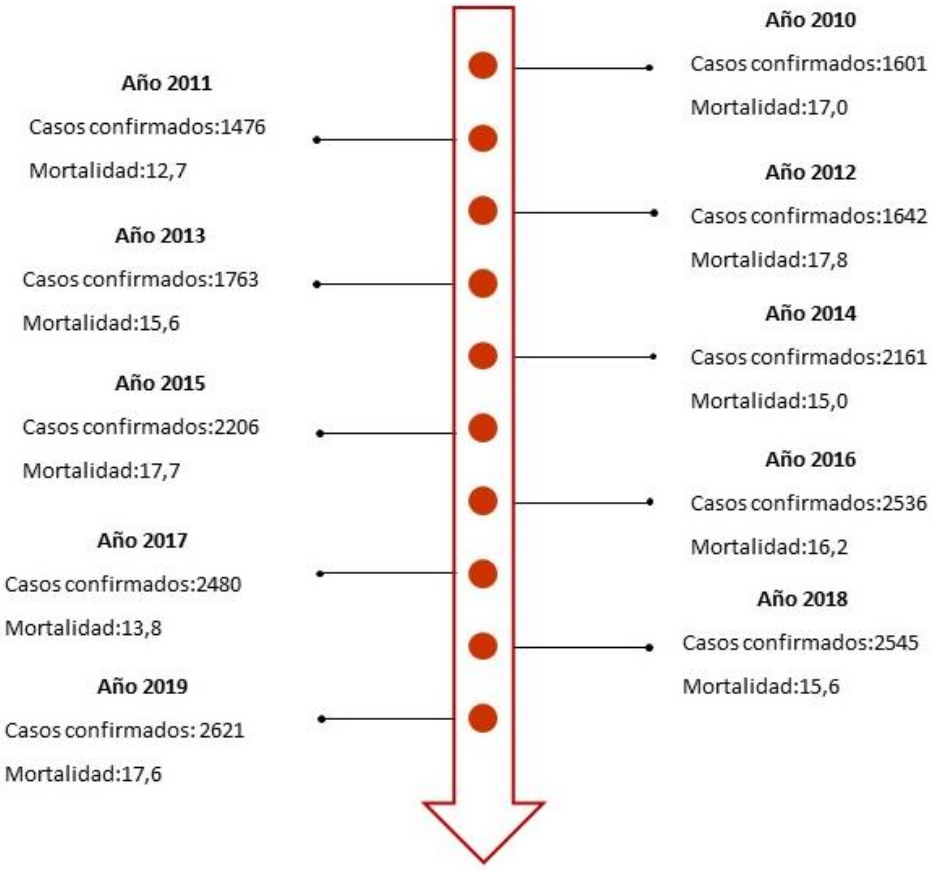

Figura I.1. Casos confirmados de listeriosis humana y mortalidad (%) en la Unión Europea desde el año 2010 al 2019. Elaborado en esta Tesis Doctoral y adaptada de EFSA y ECDC (2021,2019,2018, 2017, 2015a, 2015b, 2014, 2013, 2012).

La listeriosis es una enfermedad que afecta principalmente a ancianos, mujeres embarazadas, neonatos e inmunodeprimidos (Radoshevich y Cossart, 2018; Zhang y col., 2021). La incidencia de listeriosis es baja en la población general a pesar de la amplia distribución del microorganismo en el medio ambiente y la frecuencia relativamente alta de aislamiento en los alimentos (Buchanan y col., 2017; Scallan y col., 2011).

Aunque la enfermedad se puede expresar como una enfermedad leve y febril (listeriosis gastrointestinal no invasiva), *L. monocytogenes* también tiene la capacidad de sobrevivir en el cuerpo humano, atravesando la barrera intestinal, la barrera

hematoencefálica y la barrera fetoplacentaria, infectando órganos como el cerebro o el útero, provocando infecciones graves que amenazan la vida como meningitis, encefalitis o aborto espontáneo (listeriosis sistémica o invasiva) (Buchanan y col., 2017; Jordan y McAuliffe, 2018).

La forma invasiva es la forma predominante, que unido a la gravedad de la sintomatología y las elevadas tasas de hospitalización (superiores a 98%) y mortalidad (20-30%) (EFSA y ECDC, 2021; Osimani y Clementi, 2016), la convierten en el patógeno transmitido por los alimentos de mayor importancia (Bover y Garriga, 2014).

Sin embargo, esta forma se ve desarrollada en determinados grupos de riesgo como son ancianos, embarazadas, niños o individuos inmunodeprimidos (cáncer, diabetes mellitus, alcoholismo, enfermedades hepáticas, renales, cardiovasculares y autoinmunes y otras afecciones o tratamientos inmunosupresores) (EURL Lm, 2021; Mateus y col., 2013). La dosis infectiva mínima de esta forma de la enfermedad puede estar entre 100 a 1000 UFC (Warriner y Namvar, 2009) y el periodo de incubación es largo, transcurriendo normalmente entre 1-3 semanas, aunque puede prolongarse hasta un mes (EURL Lm, 2021).

La infección por *L. monocytogenes* notificada con mayor frecuencia en adultas es la que afecta al sistema nervioso central (SNC) (55 a 70% de los casos), desarrollándose como una meningoencefalitis acompañada de cambios severos en la conciencia, trastornos del movimiento y, en algunos casos, parálisis de los nervios del cráneo. La tasa de mortalidad por infección del SNC es de alrededor del 20%. pero puede ser tan alto como 40 a 60% si se asocia con concurrentes, enfermedad debilitante subyacente (Drevets y Bronze, 2008).

En las mujeres embarazadas, la listeriosis suele asociarse a una enfermedad leve o sin síntomas (Faber y Peterkin, 1991); sin embargo, en el caso de atravesar la placenta, puede darse un cuadro de listeriosis perinatal (McLauchlin y col., 2004; Wang y col., 2021) Su consecuencia es el aborto, nacidos de bebé o feto muerto o infección generalizada, o bien dar lugar a un cuadro de sepsis o meningitis en el neonato (listeriosis neonatal) (Allerberger y Wagner, 2010).

Por otro lado, la listeriosis no invasiva o gastrointestinal ocurre en personas sanas que consumen una alta dosis de *L. monocytogenes* (más de 10^8 UFC). El período de incubación es relativamente corto, oscilando entre 1 y 7 días (Warriner y Namvar, 2009). La mayoría de los pacientes cursan con síntomas de gastroenteritis febril como son diarrea, fiebre, dolor abdominal, escalofríos, dolor de cabeza y mialgias (Drevets y Bronze, 2008; Matle y col., 2020; Vázquez-Boland y col., 2001).

La preocupante repercusión de esta enfermedad en la salud pública hace que sea necesario conocer las características de dicha bacteria patógena y así poder establecer métodos de control en productos RTE como los embutidos curado-madurados y los quesos madurados.

I.5. *Listeria monocytogenes.* CARACTERÍSTICAS GENERALES

L. monocytogenes es una especie del género *Listeria* perteneciente a la familia *Listeriaceae* y actualmente está compuesta por 26 especies (LPSN, 2021), organizadas en dos grupos según su relación con *L. monocytogenes*. El primer grupo es el clado "*Listeria sensu stricto*" que consta de 9 especies que incluyen: *L. monocytogenes, L. innocua, L. welshimeri, L. ivanovii, L. seeligeri, L. marthii L. farberi, L. immobilis, L. cossartiae*

(Carlin y col., 2021; Figura I.2). Siendo este el grupo con mayor interés para la salud pública ya que estas especies se aíslan comúnmente del tracto intestinal de animales sin síntomas y productos alimenticios de origen animal (Carlin y col., 2021; Orsi y Wiedmann, 2016). El segundo grupo es el clado "*Listeria sensu lato*" y consta de 17 especies que incluyen: *L. weihenstephanensis, L. fleischmannii, L. rocourtiae, L. booriae, L. riparia, L. goaensis, L. thailandensis, L. valentina, L. grayi, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. grandensis y L. costaricensis, L. portnoyi* and *L. rustica* que se han aislado del medio ambiente o de las matrices alimentarias, pero no pueden colonizar huéspedes mamíferos (Carlin y col., 2021; Núñez-Montero y col., 2018; Olaimat y col., 2018; Figura I.2.).

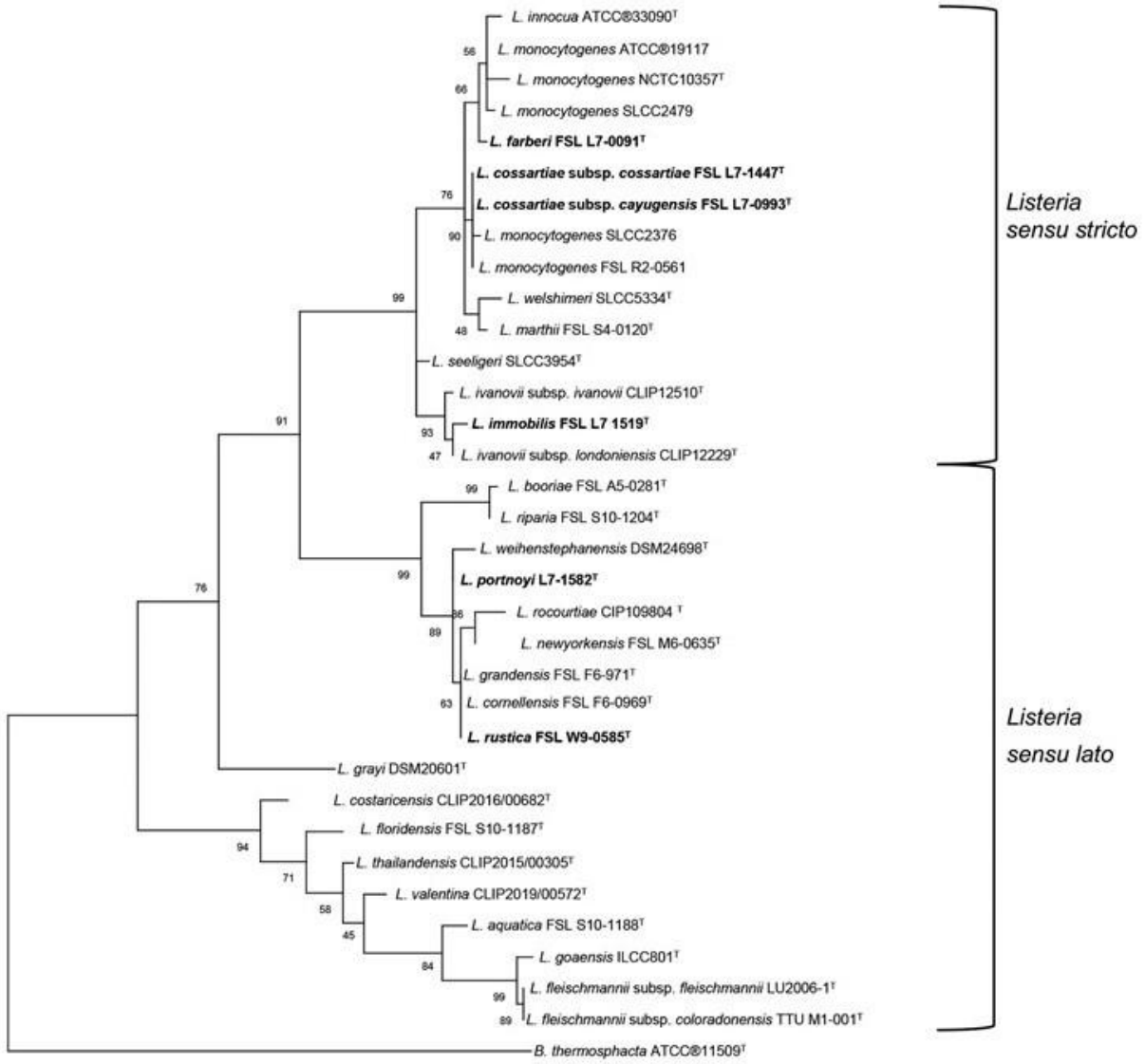

Figura I.2. Relaciones filogenéticas del género *Listeria* basadas en el análisis del gen 16S del ARN ribosómico (ARNr). Se incluyen en la figura una cepa de *Brochothrix* seleccionada como grupo externo al género *Listeria.* Adaptada de Carlin y col., 2021.

De todas las especies de *Listeria* estudiadas, sólo *L. monocytogenes* y *L. ivanovii* son patógenos tanto para seres humanos como para los animales (Terentjeva y col., 2021). Debido a la rareza de los casos de infección por *L. ivanovii* (Guillet y col., 2010), sólo *L. monocytogenes* representa un problema para la salud pública mundial (Abay y col., 2019; Núñez-Montero y col., 2018; Orsi y Wiedmann, 2016). En el caso de *L. innocua* se considera no patógena tanto para humanos como para animales. Sin embargo, esta especie

se ha aislado ocasionalmente de infecciones humanas (Favaro y col., 2014; Karli y col., 2014) y animales (Rocha y col., 2013).

Para diferenciar entre las distintas especies de *Listeria* y determinar la patogenicidad se utilizan las características bioquímicas (Carlin y col., 2021; Núñez-Montero y col., 2018; Orsi y Wiedmann, 2016; Tabla I.2).

Tabla I.2. Características bioquímicas de las distintas especies de *Listeria*. Adaptado de Carlin y col., 2021.

Características	***sensu stricto***									***sensu lato***																
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26
Reacción Voges-Proskauer	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	−	V	−	−	−	+	−	−
Reducción del nitrato	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	−	V*	−	+	+	+	+	+	+	+
Movilidad	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
Hemólisis	+	−	−	+	+	−	−	−	−	−	−	−	−	−	+(α)	−	−	−	−	−	−	−	−	−	−	−
D-Arilamidasa	−	+	V	V	+	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
α-Manosidasa	+	+	+	−	−	+	+	−	+	−	−	+	+	+	−	−	−	V	−	+	−	−	−	−	−	−
D-Arabitol	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	−	−	−	V	+	(+)	(+)
D-Xilosa	−	−	+	+	+	−	−	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
L-Ramnosa	+	V	V	−	+	−	+	−	−	+	+	+	+	+	+	+	+	−	+	+	V	−	−	+	+	+
D-ribosa	−	−	−	V*	−	−	−	+	V†	−	+	+	V	V	−	+	+	+	−	+	+	+	+	+	−	−
Glucosa-1-fosfato	−	−	−	V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Tagatosa	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	V	−	−	+	−	−	−	+	−	−
Glicerol	V	+	+	+	+	−	−	V	+	+	+	+	+	V	(+)	(+)	+	V	−	V	+	V	−	+	−	−
L-Arabinosa	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	+	+	+	V	−	−	−	+
D-Galactosa	V	−	−	V	−	−	−	−	−	−	+	+	+	+	−	−	−	+	+	−	+	−	−	+	+	+
D-Glucosa	V!	V!	+	V!	+	V!	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbosa	V!	V!	−	V!	−	V!	−	−	−	−	V!	−	−	−	−	−	−	V!	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	V	−	−	V	−	+	+	−	−	V	−	−	−	−	−	−
D-Manitol	−	−	−	−	−	−	−	−	−	+	V	+	+	V	−	−	−	+	−	−	+	−	−	−	+	+
Maltosa	+	+	+	+	+	+	+	V	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+	+	−	+
Lactosa	+	+	+	+	+	+	+	V	+	V!	+	+	+	+	+	−	−	+	+	−	+	(+)	−	+	(+)	+
Melobiosa	V!	V	−	−	−	V!	−	−	−	−	V	+	+	V	−	−	−	−	−	−	−	−	−	−	−	−
Sacarosa	+	+	+	+	+	−	−	V	−	−	V	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Inulina	V!	V!	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Melezitosa	V	V	V	V	V	−	−	V	−	−	V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Turanosa	−	V	−	−	−	+	−	−	−	−	V*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Lixosa	V	V	V	−	−	−	−	−	−	−	−	−	−	−	−	−	+	V	−	V	−	−	−	−	−	−

+: positivo; (+): débilmente positivo; -: negativo; V: variable entre réplicas y/o entre cepas; v!: variable entre estudios (posiblemente debido a diferencias en los tiempos de incubación y la temperatura) L1, *L. monocytogenes;* L2, *L. innocua;* L3, *L. welshimeri;* L4, *L. ivanovii;* L5, *L. seeligeri;* L6, *L. marthii*, L7, *L. farberi;* L8, *L. immobilis;* L9, *L. cossartiae;* L10, *L. weihenstephanensis;*L11, *L.fleischmannii;* L12, *L. rocourtiae;* L13, *L. booriae;* L14, *L. riparia;* L15, *L. goaensis*; L16, *L. thailandensis*; L17, *L. valentina*; L18, *L. grayi*; L19, *L. floridensis;* L20, *L. aquatica;* L21, *L. newyorkensis;* L22, *L. cornellensis*; L23, *L. grandensis;* L24, *L. costaricensis;* L25, *L. portnoyi*; L26, *L. rustica*

Dentro de las especies de *Listeria*, *L. monocytogenes* es un bacilo Gram positivo de pequeño tamaño (0,4-0,5 µm de diámetro y 1-1,5 µm de longitud), no esporulado, anaerobio facultativo y no formador de esporas. Son móviles debido a la producción de flagelina en un rango de temperatura de 20-25 ºC (EURL Lm, 2021), pero esta capacidad se reduce a temperaturas de 37 ºC. Las cepas de *L. monocytogenes* son catalasa positiva, oxidasa, urea e indol negativos e hidrolizan la esculina (Matle y col., 2020).

I.5.1. Serotipos de *L. monocytogenes*

L. monocytogenes ha sido dividido en 15 serotipos (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e 5, 6a, 6b y 7) basadas en el análisis de los grupos antígenos somático (O) y flagelar (H). De esta manera se conocen un total de 15 subtipos de antígeno O de *Listeria* (I-XV) y 4 subtipos (A-D) de antígeno H (Tabla I.3).

Tabla I.3. Clasificación serológica de *Listeria monocytogenes* basada en el análisis de los grupos antígenos somático(O) y flagelar (H). Adaptado de Chen y col., 2017.

Serotipo	Antígenos O	Antígenos H
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII, (IX)	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C
7	XII, XIII	A, B, C

Un gran número de estudios filogenéticos y de subtipificación han demostrado que los aislados de *L. monocytogenes* forman una población estructurada, compuesta de 4 linajes divergentes (I, II, III, and IV) (Orsi y col., 2011).

Las cepas del linaje I (serotipo 1/2b y 4b) y las cepas del linaje II (serotipos 1/2a y 1/2c) se aíslan de alimentos y pacientes y suponen la gran mayoría (>95%) de los casos notificados de listeriosis humana (Andritsos y col., 2021).

Por otro lado, no se tiene mucha información sobre las cepas pertenecientes al linaje III y IV debido al menor número de cepas aisladas y caracterizadas (Orsi y col., 2011; Senay y col., 2020).

Las cepas del linaje I están relacionadas con aislamientos de listeriosis en seres humanos, siendo el serotipo 4b el principal responsable de la mayor parte de los casos de

listeriosis humana (Borucki y Call, 2003; Chen y col., 2017); mientras que las cepas del linaje II (serotipos 1/2a y 1/2b) se aíslan comúnmente tanto de productos alimenticios como de pacientes y están relacionados principalmente con la infección esporádica de *L. monocytogenes* (Chen y col., 2017; Orsi y col., 2011). Siendo el serotipo 1/2a es el más frecuentemente aislado de los alimentos e industrias alimentarias (Borucki y Call, 2003).

Los casos de listeriosis humana relacionados con los aislados del linaje III son raros (solamente representan el 1%) y están más relacionados con aislados de origen animal poco adaptados a los procesados de alimentos (Orsi y col., 2011), mientras que de las cepas del linaje IV no se han estudiado con claridad y solo se han encontrado en un número muy reducido de muestras (den Bakker y col., 2012; Ward y col., 2008).

I.5.2. Condiciones ecológicas y persistencia de *L. monocytogenes* en alimentos madurados

L. monocytogenes se pueden encontrar en ambientes muy variados, como pueden ser el suelo (MacGowan y col., 1994), las aguas residuales (MacGowan y col., 1994), así como agua fresca estancada y corriente (Frances y col., 1991; Luppi y col., 1988), heces (animales y humanos) y materias primas usadas para la elaboración de alimentos (Faber y Peterkin, 1991; Milillo y col., 2012). Incluso se estima que entre el 2 y el 10% de la población general es portador de *L. monocytogenes* en el tracto intestinal sin consecuencias aparentes para la salud (Buchanan y col., 2017).

Aunque todos los ambientes citados anteriormente forman parte de la epidemiología de la listeriosis, son los alimentos el principal vehículo de transmisión de *L. monocytogenes* (Buchanan y col., 2017).

La supervivencia y el crecimiento de *L. monocytogenes* en los alimentos depende de factores intrínsecos al alimento, por ejemplo, el pH y la a_w, y factores extrínsecos como la humedad relativa, la temperatura de almacenamiento y la presencia de oxígeno (Jordan y McAuliffe, 2018; Tabla I.4).

Tabla I.4. Condiciones ecológicas en relación con el crecimiento y supervivencia de *Listeria monocytogenes*. Adaptada de EURL Lm, 2021.

Condiciones	**Crecimiento**			**Supervivencia (no crecimiento)**
	Mínimas	Óptimas	Máximas	
Temperatura (ºC)	-2	entre 30-37	45	-18
pH	entre 4,0-4,3	7,0	9,6	entre 3,3-4,2
Actividad de agua	0,92	0,99		< 0,90
Concentración de sal			12	> 20
Atmósfera	Anaerobio facultativo y microaerófilo (puede crecer en presencia o ausencia de oxígeno, como por ejemplo bajo condiciones a vacío o atmósfera modificada)			

L. monocytogenes es una bacteria psicotrofa capaz de sobrevivir durante largos periodos bajo condiciones de temperatura desfavorables, o incluso resistir la congelación en los cuales la concentración del patógeno se mantiene sin una reducción aparente o simplemente una ligera reducción durante la conservación en congelación (entre -18 y -20 ºC) (Ben Slama y col., 2013; Bover y Garriga, 2014). En matrices con un alto contenido en nutrientes y pH neutro, *L. monocytogenes* es capaz de crecer y sobrevivir a valores comprendidos entre -0,5 y 9,3 ºC (Bover-Cid y Garriga, 2014; Buchanan y col., 2017).

Además, *L. monocytogenes* es un microorganismo halotolerante, llegando a soportar concentraciones mayores del 20%, como salmueras para la elaboración de embutidos cárnicos o quesos (Bover y Garriga, 2014). Respecto al efecto de la a_w, se ha comprobado

que puede sobrevivir a valores de a_w 0,85 durante largos periodos en embutidos curado-madurados , especialmente a temperaturas de refrigeración(Bover y Garriga, 2014).

Por último, *L. monocytogenes* es también una bacteria microaerófila ya que puede crecer bien en condiciones aeróbicas y anaeróbicas. Además, hay que tener en cuenta la capacidad de *L. monocytogenes* para formar biopelículas. Estas biopelículas son agregados de células microbianas que se adhieren entre sí y/o a las superficies y están encerradas en una matriz polimérica extracelular (Costerton y Lewandowski, 1995; Ferreira y col., 2014).

Aunque la temperatura, el pH y el a_w en general son los factores más importantes, también hay que tener en cuenta otros factores como la composición de la atmósfera y la presencia de determinadas sustancias antimicrobianas y/o conservantes (Lungu y col., 2009; Roberts y col., 2020).

I.5.3. Genes implicados en la virulencia y resistencia al estrés

El proceso de infección de *L. monocytogenes* comprende varias etapas: adhesión e invasión de la célula hospedadora, escape de la vacuola, multiplicación intracelular y proliferación extracelular (Matle y col., 2020).

El primer paso (Figura I.3, etapa 1) en el ciclo de vida intracelular es la adhesión e invasión de la célula huésped por *L. monocytogenes*. La entrada de la bacteria a la célula hospedador está mediada por las interacciones de internalinas InlA e InlB (Matle y col., 2020). Una vez que *L. monocytogenes* invade la célula huésped, queda englobada en un fagosoma primario. Después la bacteria escapa de la vacuola por la acción de la hemolisina LLO (listeriolisina O) (Kyoui y col., 2014), fosfolipasa específica de fosfatidilinositol (plcA y plcB) (Gouin y col., 1994; Vázquez-Boland y col., 2001) y la

metaloproteasa Mpl. (O'Connor y col., 2010) (Figura I.3, etapa 2). Posteriormente, la bacteria se multiplica en el citosol gracias a varios factores como la internalina InlC, el sistema de absorción de azúcar Hpt y el O- acetiltransferasa OatA, que favorecen el crecimiento intracelular y el escape del sistema inmune (Figura I.3, etapa 3).

La bacteria patógena alcanza el citoplasma y lo logra mediante una proteína de superficie llamado ActA que induce la formación de filamentos llamados cola de cometa de actina (Matle y col., 2020). Luego, los filamentos facilitan el movimiento de *L. monocytogenes* tanto inter e intracelularmente (Klumpp y Loessner, 2013) (Figura I.3, etapa 4).

Además, la interacción de InlC con la proteína de unión a la actina Tub A y los filamentos de actina, permiten la propagación de la bacteria de célula a célula (proceso infeccioso) rodeándose de una vacuola de doble membrana (Yu y col., 2018). Escapar de la vacuola fagosómica es importante para una infección eficaz, y el hecho de no escapar de la membrana da como resultado una infección que se elimina rápidamente de los tejidos (Pushkareva y Ermolaeva, 2010). Durante el ciclo intracelular, LLO, PlcA y PlcB permiten la ruptura consecutiva de la vacuola de doble membranas que ha rodeado a la bacteria desde su fagocitosis en el paso de célula a célula (Figura I.3, etapa 6).

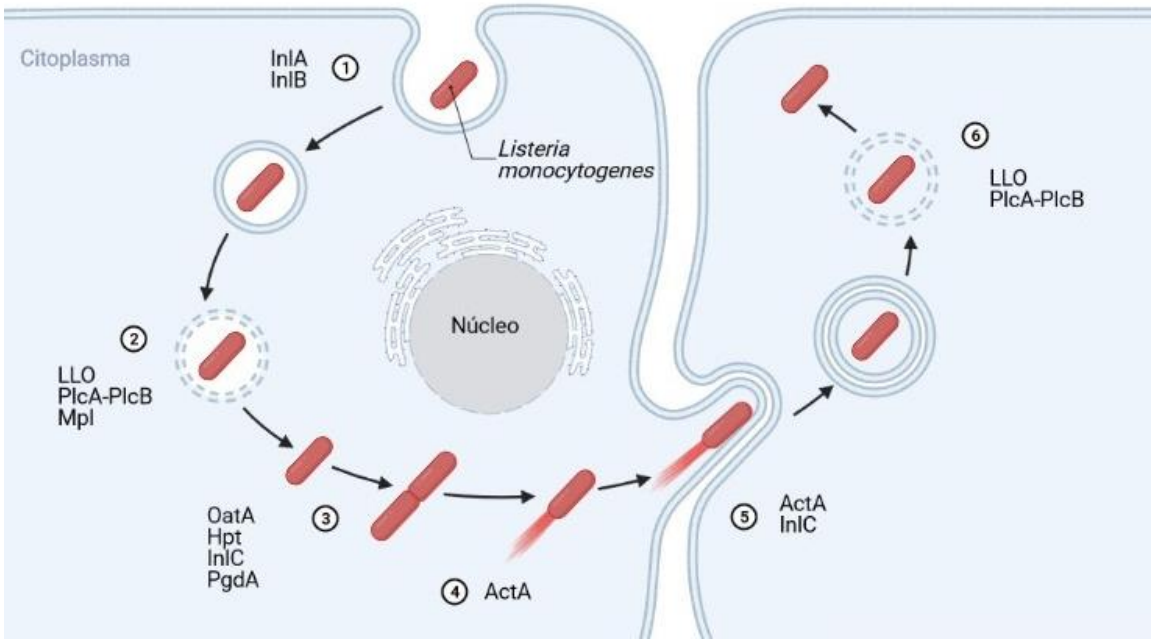

Figura I.3. Esquema del ciclo intracelular de *Listeria monocytogenes.* Esquema elaborado en esta Tesis Doctoral.

Cuando *L. monocytogenes* invade a un huésped debe realiza una coordinada regulación en la expresión de los genes de virulencia (*plcA, hly, mpl, actA, plcB, inlA, inlB, inlC* y *hpt).* Estos genes son regulados principalmente, por la proteína reguladora PrfA, la cual está involucrada directamente en la expresión de los genes (Dussurget, 2008).

Numerosos estudios han demostrado que la expresión génica puede estar influenciada por factores ambientales propios de los alimentos, como pueden ser el pH, la a_w, la temperatura o incluso la disponibilidad de nutrientes y espacio (Hadjilouka y col., 2016; Mataragas y col., 2015; Olesen y col., 2010) Además, se ha demostrado la activación de la expresión del gen *inlA* en condiciones experimentales ácidas que podrían darse en los embutidos curado-madurados y quesos madurados (Olesen y col., 2009; Sue y col., 2004).

También se ha demostrado cambios en la expresión génica tras la exposición a extractos antimicrobianos naturales derivados de plantas (Hadjilouka y col., 2017; Pieta y col., 2017) o tras la exposición de HPP (Bowman y col., 2008). La aplicación de cultivos protectores podría también afectar a la expresión génica y en consecuencia a la viabilidad y virulencia de *L. monocytogenes* (Wang y col., 2019; Yap y col., 2021).

I.5.4. Criterios de seguridad alimentaria

La UE establece los criterios de seguridad alimentaria respecto a *L. monocytogenes* de los productos RTE a través del Reglamento (CE) nº 2073/2005 modificado por el Reglamento (CE) 1441/2007. Para ello establece tres categorías de alimentos (Tabla I.5):

Tabla I.5. Criterios de seguridad Alimentaria para *L. monocytogenes*. Adaptado del Reglamento (CE) 1441/2007.

Categoría de Alimento	Límite
1. Alimentos RTE destinados a lactantes y con fines médicos especiales	Ausencia en 25 g durante toda su vida útil
2. Alimentos RTE que permiten el crecimiento de *L. monocytogenes*	Ausencia en 25 g antes de que el alimento deje de estar bajo el control inmediato del operador de la empresa productora
	Límite de 100 UFC/g durante toda su vida útil
3. Resto de alimentos RTE	Límite de 100 UFC/g durante toda su vida útil

I.6. CONTROL DE *L. monocytogenes* EN ALIMENTOS MADURADOS

Debido a su peligrosidad e incidencia es importante el control de *L. monocytogenes* en alimentos madurados. Entre las estrategias que pueden utilizarse para ello, se debe destacar la teoría de obstáculos de Leistner, entendiendo la inactivación microbiana como

una serie de factores y procesos de conservación (llamados obstáculos) que los microorganismos presentes en los alimentos no puedan superar (Ananou y col., 2007; Leistner, 1978). Los principales obstáculos empleados en la seguridad alimentaria son la temperatura, a_w, pH, el potencial redox, conservantes químicos, envasado al vacío o en atmósfera modificada, altas presiones hidrostáticas (HHP), ultravioleta (UV) y el biocontrol mediante microorganismos con actividad antagonista (Ananou y col., 2007; Reis y col., 2012).

En los alimentos madurados, como pueden ser los embutidos curado-madurados y los quesos madurados tradicionales los obstáculos van a ser las propias características intrínsecas (a_w, pH, concentración de NaCl y nitritos) y extrínsecas (temperatura y humedad durante la maduración, envasado) del producto, que contribuyen al control de los microorganismos patógenos (Díez y Patarata, 2013; Sadeghi-Mehr y col., 2016).

Los embutidos curado-madurados son productos cuyas condiciones de a_w y pH son suficientes para inhibir el crecimiento de *L. monocytogenes*. Sin embargo, cuando ha habido fallos en la maduración o contaminación posterior al procesado puede haber problemas sanitarios de listeriosis. De hecho, muchos autores han indicado la presencia de *L. monocytogenes* en embutidos curado-madurados (Doménech y col., 2015; EFSA, 2018; Ferreira y col., 2007; Meloni, 2015)

Además, en los derivados cárnicos curado-madurados como el salchichón se utilizan nitritos. A pesar de la demostrada eficacia antimicrobiana y tecnológica de este aditivo la tendencia actual tanto de consumidores como de las industrias es a su eliminación (Christieans y col., 2018).

Por otro lado, desde las Autoridades Competentes en materia de sanidad y alimentación, se han desarrollado iniciativas dirigidas a disminuir el consumo de sal en Europa. La reducción de sal unido a la reducción de nitritos en este tipo de productos podría incrementar la frecuencia de aparición de los peligros microbiológicos lo cual conlleva la necesidad de ajustar otros factores estabilizadores como la sustitución parcial o total por otras sales, utilización de agentes antimicrobianos o modificación de las condiciones de procesado y almacenamiento (Fraqueza y col., 2021).

Los quesos son productos cuyas condiciones de acidez, a_w y concentración de sal influyen en la supervivencia y crecimiento de patógenos (Gould y col., 2014). Sin embargo, el uso de leche cruda y, por lo tanto, la ausencia de un tratamiento térmico, unido a los diferentes procesos de ordeño y la variación de las condiciones higiénicas en las granjas, conduce a un mayor riesgo de contaminación con diferentes microorganismos patógenos (Pereira y col., 2010).

Partiendo entonces de las singularidades que tiene los embutidos curado-madurados y los quesos madurados y de la premisa de aplicar obstáculos que impidan el crecimiento de los microorganismos patógenos, el desarrollo de las denominadas tecnologías emergentes adquiere mayor protagonismo en el ámbito de estos productos para eliminar *L. monocytogenes* (Bover-Cid y col., 2011; Hereu y col., 2012).

Por lo que es necesario, el control del crecimiento de *L. monocytogenes* en este tipo de productos RTE. Aunque las técnicas de conservación convencionales (tratamientos térmicos intensos, salazón, acidificación, secado y conservación química) se han utilizado ampliamente para controlar y minimizar los riesgos asociados con la presencia de *L. monocytogenes* en los alimentos tradicionales (Amit y col., 2017; Jan y col., 2017), la tendencia actual de los consumidores al consumo de productos alimenticios menos

adulterados, sin conservantes químicos y menos procesados, sin perder su calidad microbiológica y su seguridad (Meloni, 2019; Pérez-Baltar y col., 2019) hace que el desarrollo de nuevos métodos sean necesarios para el control de *L. monocytogenes* en este tipo de productos. Todo esto ha producido que estas técnicas empiecen a ser reemplazados por nuevos tratamientos no térmicos que incluyen HHP, irradiaciones ionizantes con electrones acelerados (E-beam), campos eléctricos pulsados, nuevos sistemas de envasado y biocontrol (Barba y col., 2018; Jan y col., 2017).

El procesamiento de HHP es una tecnología de post-envasado que permite extender la vida útil, con un efecto mínimo sobre la temperatura y por tanto, sobre las características sensoriales y nutricionales, mejorando la seguridad alimentaria en aquellos productos donde otras tecnologías, como el tratamiento térmico, no son convenientes (Cava y col., 2021; Grossi y col., 2014).

Además, es una tecnología no térmica recomendada y aceptada por organizaciones y autoridades internacionales para el control de *L. monocytogenes* en productos cárnicos y lácticos RTE después de su envasado (USDA-FSIS, 2012).

La alta presión daña las membranas celulares y también puede afectar a las proteínas y los ácidos nucleicos. En combinación, estas lesiones celulares conducen a la inactivación celular y la muerte (Bowman y col., 2008).

Sin embargo, la letalidad del tratamiento está relacionada con la composición y las propiedades físico-químicas de la matriz alimentaria, como el pH, la a_w, presencia de sal o altas concentraciones de grasas (Bover-Cid y col., 2015). Además, el tratamiento con presión por sí solo no suele ser suficiente para erradicar por completo *L. monocytogenes*

de los alimentos (Misiou y col., 2018), por lo que sería conveniente combinarlo con otros tratamientos.

La irradiación con electrones acelerados es una alternativa eficaz en la reducción de microorganismos patógenos, reduciendo los riesgos de la contaminación de productos RTE (Huang y col., 2019). Este tipo de radiación daña las moléculas de ADN de los microorganismos de forma eficaz por lo que impide la reproducción de células vivas.

Sin embargo, dependiendo de la intensidad de la radiación, esta técnica puede producir efectos negativos en las propiedades sensoriales, tales como el aroma, color, sabor y textura en algunos productos alimentarios como la carne (Ahn y col., 2000; Lee y Ahn, 2005). Además, no es una de las formas de irradiación más efectivas, por eso sería interesante la combinación de irradiación a bajas dosis y la utilización de agentes antimicrobianos o métodos de biocontrol (Ibrahim, 2013).

Por lo que respecta a los tratamientos de alimentos mediante la aplicación de pulsos de luz sobre la superficie del producto y material de envasado en contacto con los alimentos, han demostrado ser eficaces para la descontaminación de *L. monocytogenes* (Rajkovic y col., 2010). Este tratamiento se suele usar en alimentos líquidos como lácteos y zumos y en superficies de contacto con los alimentos (Rajkovic y col., 2010).

Sin embargo, todas estas son para aplicar en producto acabado una vez que está contaminado. Si se quiere controlar y/o evitar el crecimiento de microorganismos patógenos en los alimentos, como es el caso de *L. monocytogenes*, la aplicación de estrategias de biocontrol mediante cultivos protectores o la utilización de otras estrategias como los hidrolizados proteicos representan otra gran alternativa dentro de la teoría de obstáculos.

I.6.1. Cultivos protectores para el control de *L. monocytogenes*

I.6.1.1. Levaduras

Entre las especies de levaduras, *D. hansenii* es la predominante en salchichón (Andrade y col., 2010; Gardini y col., 2001; Murgia y col., 2019), y su utilidad como agente de biocontrol frente a mohos toxigénicos ha sido demostrada en derivados cárnicos curado-madurados (Andrade y col., 2014; Iacumin y col., 2017; Núñez y col., 2015; Peromingo y col., 2019; Simoncini y col., 2014).

Además, *D. hansenii* es considerada como un "microorganismo seguro" por el grupo de expertos sobre Seguridad Biológica de la EFSA (EFSA BIOHAZ Panel, 2021), se ha demostrado su efecto probiótico en estudios *in vitro* (Ochangco y col., 2016) y sus efectos beneficiosos sobre las características sensoriales de los derivados cárnicos curado-madurados han sido igualmente demostrados (Andrade y col., 2010; Cano-García y col., 2013; Corral y col., 2014, 2015; Martín y col., 2006).

El principal mecanismo de acción de las levaduras frente a diferentes microorganismos patógenos parece ser la competición por los nutrientes y el espacio (Andrade y col., 2014; Simoncini y col., 2014; Virgili y col., 2012; Zhao y col., 2008). Algunos estudios han demostrado el efecto inhibidor de *D. hansenii* frente a ciertos microorganismos patógenos y en concreto frente a *L. monocytogenes* (Alía y col., 2020).

I.6.1.2. Bacterias ácido-lácticas

Taxonómicamente, las BAL se dividen en 6 familias, 38 géneros y todas pertenecen al orden *Lactobacillales*, clase *Bacilli* y filo *Firmicutes* (Agriopoulou y col., 2020). Morfológicamente, son cocos o bacilos Gram positivas, no formadoras de esporas, catalasa negativa y la mayoría de las especies no son móviles y tienen la capacidad de

catabolizar azúcares principalmente en ácido láctico y de tolerar pH bajos (Papadimitriou y col., 2016; Reis y col., 2012) Además, las BAL también poseen un bajo contenido de guanina y citosina (G + C) en su molécula de ADN (<55%) (Ananou y col., 2007; Tumbarski y col., 2018).

La utilización de BAL ha sido ampliamente desarrollada por tratarse de un grupo de microorganismos presente de forma natural en muchos alimentos de origen animal y vegetal y estar tradicionalmente implicados en los procesos de fermentación de alimentos tales como productos lácteos, cárnicos, de panadería o en vegetales (Coelho y col., 2014; Quijada y col., 2018) En general, se reconocen como bacterias seguras (GRAS) (del inglés generally recognized as safe) según la Food and Drug Administration (FDA) de Estados Unidos y el estado de Presunción de seguridad calificada por la EFSA (Yap y col., 2021) y se aplican ampliamente como cultivos iniciadores para la producción de una serie de productos lácteos y embutidos curado-madurados (Tumbarski y col., 2018) ya que juegan un papel importante en el desarrollo de las propiedades organolépticas del producto, a través de sus actividades metabólicas (lipólisis y proteólisis), producción de importantes compuestos de aroma y sabor, contribución a la textura y su destacada actividad bactericida contra el deterioro y las bacterias patógenas (Mokoena, 2017). Todo esto las hacen aptas para aplicación como cultivos protectores en los alimentos (Papadimitriou y col., 2016).

El mecanismo de acción de las BAL se basa principalmente en la competición por nutrientes con el microorganismo antagónico y en la producción de uno o más metabolitos activos, como los ácidos orgánicos (ácido láctico, acético, fórmico, propiónico y butírico), que intensifican su acción reduciendo el pH del medio, y otras sustancias, tales como etanol, ácidos grasos, acetona, peróxido de hidrógeno, diacetilo, compuestos

antifúngicos, bacteriocinas y sustancias inhibidoras de tipo bacteriocina (Favaro y col., 2015; Reis y col., 2012).

Las bacteriocinas son un grupo de péptidos antimicrobianos o inhibidores proteicos sintetizados ribosómicamente y producidos por algunos microorganismos, incluidas las BAL, principalmente activos contra organismos estrechamente relacionados, en su mayoría bacterias Gram positivas (Mokoena, 2017; Yang y col., 2014). La producción de estos compuestos se lleva a cabo para obtener una ventaja competitiva tanto por el espacio como por los nutrientes en el medio ambiente.

Las bacteriocinas se clasifican en función de sus estructuras primarias, pesos moleculares, modificaciones postraduccionales y características genéticas en 4 clases.

Las bacteriocinas de la clase I, lantibióticos, son péptidos pequeños (<5 kDa) termoestables, que se caracterizan en función de su modificación postraduccional, siendo la nisina y lactocina los principales representantes de este grupo. De ellas la nisina A producida por *Lactococcus lactis* subsp. *lactis*, se utiliza con frecuencia como conservante en quesos procesados (Ray y col., 2014; Yang y col., 2014). Esta clase de bacteriocinas actúan mediante la formación de poros, a través de la membrana. despolarización, de la membrana citoplasmática de las especies (Yang y col., 2014; Zacharof y Lovitt, 2012).

Las bacteriocinas de la clase II (bacteriocinas que no contienen lantionina), por lo general, son también péptidos termoestables pequeños no modificados de <10 kDa, que se subdividen en dos grupos (Cotter y col., 2005; Ray y col., 2014). El primer grupo (Clase IIa) está compuesto por bacteriocinas similares a la pediocina con actividad anti-listerial. Las pediocinas son producidas por *Pediococcus* spp. y aunque no son muy

eficaces contra las esporas, son más eficaces que la nisina en algunos sistemas alimentarios como la carne (Leroy y col., 2013). Numerosos estudios informan de la inhibición de *L. monocytogenes* por pediocinas o cultivos productores de pediocina en salchichón (García Fontán y col., 2007). El segundo grupo (Clase IIb) requieren la actividad sinérgica de dos péptidos complementarios para ejercer actividad antimicrobiana. La lactacina F y la lactococina G son miembros de este grupo (Yang y col., 2014).

Las bacteriocinas de la clase III son el grupo menos caracterizado y consisten en proteínas termolábiles que son generalmente grandes con un tamaño superior a 30 kDa. El grupo incluye helveticina J producida por *Lactobacillus helveticus* y enterolisina producida por *Enterococcus faecium* (Cotter y col., 2005; Mokoena, 2017; Parada y col., 2007).

Por último, las bacteriocinas de la clase IV consisten en grandes complejos de proteínas con otras macromoléculas, y se han desestablecido, y los péptidos miembros se reclasificaron como bacteriolisinas, es decir, polipéptidos hidrolíticos; dejando solo tres clases de bacteriocinas, basado en las características genéticas y bioquímicas de sus miembros (Mokoena, 2017).

Una de las grandes ventajas del biocontrol con BAL es que este grupo de microorganismos se encuentra durante todo el proceso de elaboración de los embutidos curado-madurados y quesos madurados (Casquete y col., 2011; Coton y col., 2012; Crespo y col., 2022; Frédéric Leroy y De Vuyst, 2004; Talon y Leroy, 2014).

I.6.2. Selección de bacterias ácido-lácticas con actividad anti-*L. monocytogenes*

Las BAL autóctonas procedentes de embutidos curado-madurados y quesos madurados están especialmente adaptadas a la ecología de estos productos, controlando los procesos de maduración e inhibiendo el crecimiento de microorganismos espontáneos (Hugas y Monfort, 1997). El aislamiento y selección de la población microbiana autóctona de embutidos curado-madurados tradicionales y quesos madurados para su uso como cultivos iniciadores en la fermentación de estos productos son de gran interés para la estandarización de la calidad, aportando a la vez características sensoriales deseables como consecuencia de la composición y actividad metabólica de esta población microbiana autóctona y la restricción de las tasas de multiplicación de patógenos (Benito y col., 2008; Prpich y col., 2015).

I.6.3. Hidrolizados proteicos en el control de *L. monocytogenes*

Los péptidos o hidrolizados proteicos derivados de proteínas alimentarias hidrolizadas han atraído recientemente un gran interés ya que han demostrado tener actividad antiinflamatoria, antioxidante, antialérgica, antihipertensiva y antimicrobiana (Hernández-Ledesma y col., 2014). Estos hidrolizados proteicos pueden considerarse como conservantes seguros y naturales porque generalmente se obtienen utilizando enzimas gastrointestinales, y como aditivos naturales para preservar la seguridad alimentaria (Demers-Mathieu y col., 2013; Lozano-Ojalvo y col., 2017).

Además, han demostrado una fuerte capacidad para controlar o incluso inhibir el crecimiento de ciertas bacterias patógenas y no patógenas como *L. monocytogenes*, *B. subtilis*, *E. coli* y *Pseudomonas aeruginosa* (Clare y col., 2005; Demers-Mathieu y col., 2013; Hernández-Ledesma y col., 2014; López Expósito y Recio, 2006).

Las actividades antimicrobianas de los péptidos derivados de proteínas de la leche son muy diversas, desde aquellos con efecto prebiótico, péptidos con la capacidad de prevenir la adhesión o invasión de microorganismos patógenos, hasta péptidos que inhiben el crecimiento de microorganismos (Hernández-Ledesma y col., 2014).

La relevancia industrial del uso de hidrolizados proteicos se basa en su tiempo de producción a corto plazo, la accesibilidad de la fuente de proteína (proteína de suero) en la industria láctea, su bajo precio, y que está en concordancia con las directrices europeas sobre lactantes y fórmulas de seguimiento relacionadas con la reducción de riesgos de alergia a las proteínas de la leche, además de aportar un valor añadido a los productos de la industria láctea (Lozano-Ojalvo y col., 2017).

II. OBJETIVOS

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II.1. OBJETIVOS

El trabajo realizado durante la Tesis Doctoral titulada "Control de *Listeria monocytogenes* en embutidos curado-madurados y quesos madurados tradicionales mediante cultivos protectores e hidrolizados proteicos" está integrado dentro de un estudio más amplio que se está realizando en el Grupo de Investigación de Higiene y Seguridad Alimentaria perteneciente al Instituto Universitario de Investigación de la Carne y Productos Cárnicos (IProCar) de la Universidad de Extremadura y que está financiado a través del proyecto INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria) RTA2017-00027-C03-03 e IB16149 (Junta de Extremadura-Consejería de Economía e Infraestructuras, Fondo Europeo de Desarrollo Regional- "Una manera de hacer Europa").

Los objetivos de esta Tesis Doctoral son los siguientes:

1. Aislar bacterias ácido-lácticas de embutidos curado-madurados y quesos madurados.
2. Seleccionar las bacterias ácido-lácticas con actividad anti-*Listeria monocytogenes* en medio de cultivo.
3. Seleccionar las bacterias ácido-lácticas con actividad anti-*L. monocytogenes* en sistemas modelo de medios elaborados con embutidos curado-madurados (salchichón) y quesos madurados.
4. Evaluar los cambios en la expresión del gen *inlA* relacionado con el estrés acídico mediante un método de PCR en tiempo real de transcripción inversa diseñado y optimizado en este trabajo.
5. Evaluar la actividad frente a *L. monocytogenes* de un hidrolizado proteico de suero lácteo.

6. Determinar el efecto de las bacterias ácido-lácticas seleccionadas para ser utilizadas como estrategia de biocontrol frente al crecimiento de *L. monocytogenes* en embutidos curado-madurados y quesos madurados.

7. Evaluar el efecto de los tratamientos de biocontrol con bacterias ácido-lácticas sobre las propiedades sensoriales de los embutidos curado-madurados y quesos madurados tradicionales.

8. Caracterizar las bacterias ácido-lácticas seleccionadas la actividad antimicrobiana y su inocuidad para poderlas proponerlas como cultivos protectores.

II.2. OBJETIVES

The work carried out during the Doctoral Thesis entitled "Control of *Listeria monocytogenes* in dry-cured fermented sausages and ripened cheeses by means of protective cultures and protein hydrolysates" is integrated into a broader study that is being carried out in the Hygiene and Safety Research Group Alimentaria belonging to the University Institute for Meat and Meat Products Research (IProCar) of the University of Extremadura and which is financed through the INIA project (National Institute for Agricultural and Food Research and Technology) RTA2017-00027-C03-03 and IB16149 (Junta de Extremadura-Ministry of Economy and Infrastructure, European Regional Development Fund- “A way to make Europe”).

The objectives of this Doctoral Thesis are described below:

1. To isolate lactic-acid bacteria from dry-cured fermented sausages and ripened cheeses.
2. To select lactic-acid bacteria with anti-*Listeria monocytogenes* activity in culture medium.
3. To select lactic-acid bacteria with anti-*L. monocytogenes* in model systems made with dry-cured fermented sausages (*salchichón*) and ripened cheeses.
4. To evaluate changes in the expression of the *inlA* gene related to acidic stress by means of a reverse transcription real-time PCR method designed and optimized in this work.
5. To evaluate the activity against *L. monocytogenes* of a whey protein hydrolysate.

6. To determine the effect of lactic-acid bacteria to be used as a biocontrol strategy against the growth and gene expression of *L. monocytogenes* in dry-cured fermented sausages and ripened cheeses.

7. To evaluate the effect of biocontrol treatments with lactic-acid bacteria on the sensory properties of dry-cured fermented sausages and ripened cheeses.

8. To characterize the antimicrobial activity and harmlessness of the selected lactic-acid bacteria to propose them as protective cultures.

III. MATERIAL Y MÉTODOS

III.1. MATERIAL

III.1.1. Reactivos Químicos

Para la realización de esta Tesis Doctoral se utilizaron reactivos químicos suministrados por las casas comerciales SCHARLAB, S.L., PANREAC QUÍMICA S.L.U., THERMO FISHER SCIENTIFIC™, LABKEM, CHROMagar™, LONZA, Laboratorios CONDALAB, ALFA AESAR.

El etanol absoluto 99,9% (v/v) y el isopropanol 99,9% (v/v) utilizados fueron suministrados por la casa comercial SCHARLAB, S.L.

Para modificar la a_w de los medios de cultivo se utilizó glicerol 99,5% (p/v) de la casa comercial THERMO FISHER SCIENTIFIC™ y para modificar el pH de los medios de cultivos se utilizó ácido láctico 88-92% (v/v) y ácido acético glacial de la casa comercial SCHARLAB, S.L.

Para el desarrollo de las técnicas moleculares, los cebadores y sondas empleados fueron adquiridos en la casa comercial METABION INTERNATIONAL AG. Para la extracción de ADN y ARN se utilizó el kit comercial MasterPure™ Complete DNA and RNA Purification Kit de la casa comercial EPICENTRE®.

Los reactivos empleados para el desarrollo de las reacciones de PCR en tiempo real o cuantitativa (qPCR) (SYBR® Premix Ex Taq™ II y Premix Ex Taq™ Probe qPCR) y de transcripción inversa (kit Prime Script™ RT Reagent, Perfect Real Time) fueron de la casa comercial TAKARA BIO INC.

Los reactivos empleados para la PCR convencional (dNTP´s, PCR buffer, Taq polimerasa) fueron de la casa comercial MBI fermentas.

Para la tinción de los geles de agarosa se utilizaron los reactivos SYBR™ Safe DNA Gel Stain y SYBR™ Gold Nucleic Acid Gel Stain (10.000X) de la marca comercial INVITROGEN.

El tinte de carga Blue/Orange Loading Dye 6X empleado para la preparación de las muestras y marcadores de ADN para cargar en geles de agarosa, fue suministrado por la casa comercial PROMEGA y el marcador de peso molecular GeneRuler 100 bp DNA Ladder™ fue de la casa comercial THERMO FISHER SCIENTIFIC™.

Las enzimas de restricción utilizadas en los protocolos de PFGE fueron *SgsI*, *NotI* y *XbaI* pertenecieron a la casa comercial THERMO FISHER SCIENTIFIC™. Cada enzima de restricción necesitó un tampón específico para su funcionamiento siguiendo las indicaciones del fabricante. Para ello, para las enzimas *SgsI* y *XbaI* se utilizó el tampón *Tango* 10X y para la enzima *NotI* se utilizó el tampón *O* 10X, ambos de THERMO FISHER SCIENTIFIC™.

En los protocolos de PFGE también se utilizó Albumina Sérica Bovina o BSA (del inglés, *Bovine Serum Albumin*) de la casa comercial SIGMA-ALDRICH.

Durante la estancia en el Departamento de "Centro de Biotecnologia e Química Fina" Universidad Católica de Porto (Universidade Católica Portuguesa, Oporto, Portugal), se utilizaron los siguientes reactivos:

El NaOH requerido para neutralizar el sobrenadante de las BAL fue obtenido en Pronalab.

Las enzimas utilizadas fueron obtenidas de la marca comercial SIGMA-ALDRICH y Boehringer Mannhein.

Todos los precursores de amina fueron de la marca SIGMA-ALDRICH mientras que el piridoxal-5-fosfato usado como suplemento fue de la marca comercial FLUKA.

Los antibióticos utilizados fueron adquiridos de las marcas comerciales SIGMA-ALDRICH, FISHER SCIENTIFIC y LABESFAL.

III.1.2. Tampones

Para la realización de esta Tesis Doctoral fueron utilizados diferentes tampones cuya composición se muestra a continuación:

Tampón TE pH 8 (tampón necesario para el método de PFGE):

10 mM TRIS

1 mM EDTA

Tampón TBE 5X pH 8,4 (tampón necesario para el método de PFGE):

0,45 M TRIS

0,45 M ácido bórico

10 mM EDTA

Tampón de Lisis Celular o *Cell Lysis buffer* (CLB) pH 8,0 (tampón necesario para el método de PFGE):

50 mM TRIS

50 mM EDTA

1% sarcosina

0,1 mg/mL de proteinasa K

TAE 50X pH: 8,0 (tampón utilizado en geles de agarosa):

242 g/L TRIS Base

57,1 mL ácido acético glacial

100 mL EDTA 0,5 M

III.1.3. Medios de cultivo

Los medios de cultivo utilizados en este trabajo se prepararon siguiendo las recomendaciones de los fabricantes pertenecientes a las diferentes casas comerciales: Laboratorios CONDALAB, OXOID, bioTRADING. A continuación, se detallan los distintos medios de cultivo utilizados en este trabajo y la composición de los mismos.

III.1.3.1. Medios de cultivo de uso general

Para la parte experimental de este trabajo se emplearon diversos medios de cultivo que se elaboraron siguiendo las fórmulas comerciales. Estos medios aparecen detallados en la Tabla III.1:

Tabla III.1. Medios de cultivos utilizados.

Medio de cultivo	Siglas	Casa comercial	Microorganismos/actividad
Infusión Cerebro-Corazón	BHI	Conda	Medio de uso general
Agar Rogosa y Sharpe	MRS	Oxoid	BAL
Agar Manitol Salado	MSA	Oxoid	Estafilococos
Agar Bilis Glucosa con cristal violeta y rojo neutro	VRBG	Conda	*Enterobacteriaceae*
Agar para Métodos Estándar	PCA	Conda	Microorganismos aerobios totales
CHROMAgar™ Listeria		bioTRADING	*L. monocytogenes*
Agua de Peptona		Conda	Medio de uso general
Luria-Bertani Modificado	MLB	Sigma	Actividad gelatinasa
DNAsa agar		Pronadisa	Actividad ADNasa
Agar Columbia		Oxoid	Actividad hemolítica
Agar de Soja Tríptica	TSA	Pronadisa	Medio de uso general
Agar Extracto de Malta	AEM	Scharlab	Levaduras y mohos

Todos los medios de cultivo sólidos de uso general se esterilizaron a 121 ºC (103 KPa) durante 16 min antes de su uso. Una vez esterilizados, se enfriaron hasta alcanzar una temperatura de 45-50 ºC para ser finalmente repartidos en placas Petri de 9 cm de diámetro. Las placas de Petri con los medios de cultivo se conservaron en refrigeración hasta su utilización. Los medios de cultivo líquidos fueron repartidos en tubos y esterilizados a 121 ºC durante 16 min. Posteriormente se enfriaron y se conservaron en refrigeración hasta su uso.

III.1.3.2. Medios de cultivo elaborados con derivados cárnicos y quesos liofilizados

III.1.3.2.1. Proceso de liofilización

El proceso de liofilización se realizó con lonchas de salchichón y queso tipo Torta con un grosor aproximado de 1 cm y 0,5 mm, respectivamente. Estas lonchas se colocaron sobre una malla plástica de polietileno de alta densidad y se congelaron a -82 °C durante al menos 2 h antes de introducirlas en el liofilizador (LABCONCO). Tras su liofilización, las muestras se trituraron en una picadora y envasaron a vacío conservándose a -20 °C hasta su uso.

III.1.3.2.2. Elaboración y composición

Para evaluar la actividad anti-*L. monocytogenes* de las BAL y el hidrolizado proteico, se utilizaron dos medios de cultivo:

- **Agar queso:** se elaboró según el procedimiento previamente descrito por Gori y col., (2007) y cuya composición se indica en la Tabla III.2. Además, el pH del medio de cultivo fue ajustado a 6 usando ácido láctico para simular los valores de pH típicos de los quesos tipo Torta y se comprobó que el valor de la a_w era de 0,975.

Tabla III.2. Composición del medio elaborado con Torta del Casar

Componentes	Cantidad
Queso (Torta del Casar) liofilizado	200 g
Tri-sodio citrato dihidrato	12,5 g
Agar bacteriológico	20 g
Agua destilada	900 mL

- **Agar salchichón:** se elaboró siguiendo el procedimiento descrito por Rodríguez y col. (2014) y cuya composición se muestra en la Tabla III.3. Además, el pH del medio se ajustó a 5,5 con ácido láctico y se midió la a_w del medio de cultivo que era de 0,98.

Tabla III.3. Composición del medio elaborado con salchichón.

Componentes	Cantidad
Salchichón liofilizado	250 g
Agar bacteriológico	20 g
Agua destilada	1000 mL

III.1.4. Equipos

Las pesadas de los componentes de los medios de cultivo y otras pesadas rutinarias se realizaron en una balanza electrónica COBOS mod. CB COMPLET con precisión de 10 mg. Para las pesadas de precisión se utilizó una balanza analítica de SARTORIOUS mod. LA310S con una precisión de 0,1 mg.

El agua destilada y el agua ultrapura utilizadas para elaborar los medios de cultivo y los reactivos, así como los cebadores y sondas utilizados en este trabajo fueron obtenidos

utilizando el sistema de purificación de agua Elix Technology Inside Milli-Q® Integral 5 Water System de EMD MILLIPORE.

Para la preparación de los distintos medios de cultivo se utilizaron agitadores magnéticos con placa calefactora JP SELECTA® mod. Agimatic-N y un baño termostático JP SELECTA® mod PRECISTERM S-140.

La liofilización del salchichón y del queso, previamente loncheados, utilizados en la preparación de los medios de cultivo agar salchichón y agar queso se llevó a cabo en un liofilizador LABCONCO® mod. Freezone-6 y el picado posterior de los productos liofilizados se realizó en una picadora JATA PC-123 de 700 W.

El almacenamiento del salchichón y queso liofilizados, así como de muestras y reactivos se hizo a -20 °C en congeladores de las marcas LYNX y LIEBHERR MEDline.

La esterilización de los medios de cultivo, reactivos, tampones y material de laboratorio se llevó a cabo en autoclave P-SELECTA® mod. Presoclave III 80.

Para atemperar los medios de cultivos y/o llevar a cabo el desarrollo de aquellos protocolos laboratoriales que requerían condiciones de temperatura controlada se utilizó un baño termoregulado de la marca P-SELECTA® mod. PRESCIDIG y un baño termoregulado con agitación de vaivén P-SELECTA® mod. UNITRONIC OR.

Las placas de Petri utilizadas para los medios de cultivo fueron de 9 cm y de 5,5 cm de diámetro de la casa comercial THERMO FISHER SCIENTIFIC™.

Una vez elaborados y depositados en placa Petri, los medios de cultivo se conservaron a 5 ºC en una cámara refrigerada por un equipo de frío RivaCold mod. RC325-45ED.

Las mediciones de la a_w se realizaron con un medidor LabMASTER-a_w de NOVASINA AG mod. a_w SPRINT-TH 300.

Las medidas de pH se realizaron en un pH-metro CRISON mod. BASIC 20.

Las medidas de textura se realizaron con un texturómetro TA XT (StableMicro Systems Ltd.) equipado con una sonda cilíndrica de 5 cm de diámetro.

Las medidas del color se realizaron con un colorímetro Minolta mod. CR-300 (Konica Minolta).

Todos los ensayos que requerían condiciones de esterilidad se realizaron en una campana de flujo laminar TELSTAR mod. BIO-II-A con lámpara UV SILVANA 46320 (1X15 W) con tubo PHILIPS ultra-violet de 15 W.

El material de laboratorio que se utilizó en los ensayos tales como espátulas de aluminio, cucharas tipo espátula o puntas de bisturí fueron de la marca SCHARLAB, S.L.

Las pipetas automáticas de 0,5-10 µL, 2-20 µL, 10-100 µL, 100-1000 µL y 1000-5000 µL utilizadas para llevar a cabo los ensayos con microorganismos, así como el desarrollo de las técnicas moleculares pertenecían a la casa comercial THERMO FISHER SCIENTIFIC™ mod. FINNPIPETTE. Para el manejo de los microorganismos se utilizaron puntas de pipeta sin filtro de DASLAB®, mientras que para las técnicas moleculares se utilizaron puntas con filtro FISHERBRAND® SURE ONE de THERMO FISHER SCIENTIFIC™.

Para la conservación y manipulación de los microorganismos, así como el material genético se utilizaron microtubos tipo Eppendorf de tapa con cierre seguro de distintos

volúmenes (0,2, 0,5, 1,5 y 2 mL) de las marcas comerciales DASLAB®, AXYGEN SCIENTIFIC y DELTALAB.

Los microorganismos que fueron incubados a 37 °C lo hicieron en una estufa P-SELECTA mod. 207 con temperatura regulable. Para la incubación de microorganismos a las temperaturas de 30 y 25 °C se utilizaron estufas refrigeradas VELP SCIENTIFICA mod. FOC 225 de SCHARLAB, S.L.

Para la homogeneización de los microtubos tipo Eppendorf y tubos de laboratorio se utilizaron agitatubos HEIDOLPH mod. REAX TOP.

Para el aislamiento de bacterias de superficies de equipos y utensilios de las industrias de embutidos y quesos, se utilizaron toallitas estériles humedecidas de la casa comercial AES CHEMUNEX. Una vez utilizadas, cada toallita se guardó en bolsas estériles de la marca BAGPAGE sin filtro.

Para la homogeneización y posterior detección o recuentos microbianos de las toallitas, medios elaborados con salchichón o queso y de los propios productos se usaron bolsas Stomacher con filtro de la marca BAGPAGE y un homogeneizador STOMACHER mod. STOMACHER R400 CIRCULATOR.

Para los procedimientos de centrifugación se empleó una microcentrífuga refrigerada mod. CENTRIFUGE 5430R de EPPENDORF.

La incubación de las muestras a temperaturas de 65 °C se llevó a cabo en un termobloque mod. DRY BATH FB15101 de FISHER SCIENTIFIC.

Los salchichones y los quesos fueron madurados en una cámara con humedad relativa y temperatura controlada de la casa comercial Pecomark.

Durante todo el proceso de extracción del material genético se utilizó hielo para mantener las condiciones óptimas de las muestras y reactivos químicos utilizados, que se elaboró con una máquina de hielo triturado mod. IMS-85 Automatic Flake Ice Maker de LABREEZ.

La cantidad y calidad del ADN y ARN extraídos fueron determinadas mediante un espectrofotómetro THERMO SCIENTIFIC NANODROP mod. 2000C (THERMO FISHER SCIENTIFIC™), conectado a un ordenador portátil DELL mod. LATITUDE D505.

Para llevar a cabo las mezclas de reacción para su análisis mediante las técnicas moleculares de PCR se utilizó una campana TELSTAR mod. Mini-V/PCR.

Las reacciones de PCR convencional y de transcripción inversa se llevaron a cabo en un termociclador mod. MASTERCYCLER® EP GRADIENT de EPPENDORF.

La fusión de los geles de agarosa se realizó en un microondas mod. SMW-3 de SCHNEIDER.

Las electroforesis de los ácidos nucleicos se llevaron a cabo en una cubeta horizontal mod. Sub-Cell® GT, alimentada por una fuente mod. PowerPac™ Basic ambos de BIO-RAD LABORATORIES, INC.

Las reacciones de qPCR se llevaron a cabo en un equipo qPCR Viia™ 7 Real-Time System de APPLIED BIOSYSTEMS, conectado a un ordenador DELL mod. OPTIPLEX XE. Además, se utilizaron placas de reacción de 96 pocillos (0,1 mL) MicroAmp™ Fast Optical con películas adhesivas MicroAmp™ de APPLIED BIOSYSTEMS™.

El almacenamiento de las muestras de ARN extraído se hizo en un congelador ECONOFREEZER de THERMO SCIENTIFIC mod. 900 series (-82 °C). Por otra parte, las muestras de ADN extraídas, así como la mayor parte de los reactivos químicos empleados en las técnicas moleculares fueron almacenadas a temperatura de -20 °C en congeladores No Frost de SVAN mod. SCV1863FFDX.

Para ajustar la concentración de los cultivos bacterianos mediante la medida de la turbidez se utilizó un biofotómetro mod. 6131 de EPPENDORF.

Para la digestión enzimática de muestras en los análisis de PFGE se utilizó un agitador térmico mod. TS-100 de BIOSAN.

La técnica de PFGE se llevó a cabo en el equipo mod. CHEF Mapper® XA Pulsed Field Electrophoresis System de BIO-RAD LABORATORIES, INC. Para la elaboración y procesamiento de los moldes se utilizó un molde mod. 50-Well Plug y un filtro mod. Plug Cap ambos de la casa comercial BIO-RAD LABORATORIES, INC. Tras la electroforesis, los geles se tiñeron con bromuro de etidio (10 mg/mL) o SYBR™ Gold Nucleic Acid Gel Stain (10.000X).

Las imágenes de los geles fueron obtenidas con un equipo Gel Doc 2000 Image Documentation System de BIO-RAD LABORATORIES, INC. y el equipo de la casa comercial LABNET mod. ENDURO™ GDS GEL DOCUMENTATION SYSTEM.

Los compuestos volátiles se analizaron por cromatografía de gases-espectrometría de masas (GC-MS) en un cromatógrafo de gases de la marca Agilent Technologies, equipado con una columna HP-5 (5% fenil-95% dimetilpolisiloxano) y acoplado a un detector de espectrómetro de masas (MS), 5975C (Agilent Technologies).

III.1.5. Programas informáticos

Para el desarrollo de experimental de esta Tesis Doctoral se usaron la siguiente relación de programas informáticos:

Para el diseño de cebadores se utilizó el programa Primer3web versión 4.1.0 de la casa comercial ELIXIR.

El manejo, desarrollo y obtención de los resultados obtenidos en los ensayos de qPCR se realizó mediante la utilización del programa ViiA™ 7 RUO V 1.2.4 de APPLIED BIOSYSTEMS.

La medida de la cantidad y calidad de ácidos nucleicos se realizó con el programa NanoDrop™ 2000/2000C versión 1.2.4 de THERMO FISHER SCIENTIFIC™.

Para el análisis de datos, elaboración de tablas y obtención de las diferentes gráficas se utilizó el programa de MICROSOFT Excel 2016.

Para la elaboración y adaptación de figuras se usó el programa de MICROSOFT PowerPoint 2016.

El tratamiento estadístico se llevó a cabo mediante el programa IBM SPSS v.22.

III.1.6. Material biológico

La cepa de referencia de *L. monocytogenes* utilizada en este trabajo fue S7-2 (serotipo 4b), aislada de industria cárnica y cedida por el Instituto Nacional de Investigaciones y Tecnología Agraria y Alimentaria (INIA).

También se utilizaron 6 BAL, tres pertenecientes a *Lactiplantibacillus plantarum* (B2, B5 y B6), dos cepas de *Leuconostoc mesenteroides* (B1 y B3) y una cepa atribuida

a *Lactiplantibacillus* spp. (B4) aisladas e identificadas en un trabajo previo por el grupo de investigación.

Por otra parte, se realizó el aislamiento de cepas de BAL en 3 industrias que elaboran embutidos curado-madurados y 12 industrias que elaboran quesos situadas en la Comunidad Autónoma de Extremadura (ver apartado III.2.2). Se obtuvieron un total de 371 cepas.

La cepa utilizada como control en los procedimientos de PFGE fue *S. enterica* subsp. *enterica* serovar Braenderup (ATCC® BAA-664™).

En este trabajo se empleó un hidrolizado proteico hipoalergénico de proteína de leche con posible actividad antimicrobiana, suministrado por el Grupo de Investigación "Bioactividad y Alergenicidad de Proteínas y Péptidos Alimentarios" (BIOPEP) del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), a través de un programa de colaboración.

Durante la estancia en la Universidad de Porto (Universidade Católica Portuguesa, Porto, Portugal), se utilizaron los microorganismos que se detallan en la Tabla III.4.

Tabla III.4. Microorganismos utilizados durante la estancia predoctoral, así como la fuente de obtención.

Microorganismos	**Fuente**
Bacillus cereus ESB014	Colección de cultivo ESB
Salmonella Enteritidis ESB008	
Salmonella Typhimurium ESB009	
Acinetobacter spp ESB260	
Acinetobacter baumannii ESB028	
Pediococcus acidilactici HA-6111-2	
Listeria monocytogenes SCOTT A	
Enterococcus faecalis ATCC 29212	ATCC
Staphylococcus aureus ATCC 25213	
Staphylococcus aureus ATCC 6538	
Listeria monocytogenes EDG-e	
Escherichia coli ATCC 25922	
Escherichia coli ATCC 8739	
Enterococcus faecium DSMZ 13590	DSMZ
Listeria monocytogenes NCTC 11994	NCTC

ESB – colección de cultivos de la Escola Superior de Biotecnologia; ATCC – Colección Americana de Cultivos Tipo; DSMZ – Colección Alemana de Microorganismos y Cultivos Celulares; NCTC - Colección Nacional de Cultivos Tipos

III.2. MÉTODOS

III.2.1. Diseño experimental

Para la realización de esta Tesis Doctoral se planteó el diseño experimental que se muestra en la Figura III.1. En primer lugar, se llevó a cabo el aislamiento de BAL procedente de industrias de embutidos curado-madurados y quesos madurados tradicionales. Posteriormente, se caracterizaron los aislados de BAL mediante métodos moleculares. Una vez caracterizadas las BAL, se evaluó su efecto frente a *L. monocytogenes* de forma aislada y en conjunto con un hidrolizado proteico de suero lácteo. Finalmente, se llevaron a cabo los tratamientos con BAL como métodos de biocontrol de *L. monocytogenes* en salchichón y quesos tipo Torta, y se evaluó dicho efecto sobre la viabilidad del microorganismo mediante la evaluación de la expresión génica de genes de virulencia y de respuesta al estrés del microorganismo patógeno, así como su efecto en las características sensoriales y fisicoquímicas del producto. Adicionalmente, se ha puesto a punto un método de PCR en tiempo real para evaluar cambios en la expresión de un gen que varía ante situaciones de estrés acídico. Finalmente, durante la estancia en la Universidade Católica Portuguesa se seleccionaron las cepas con capacidad para producir compuestos proteicos y se caracterizaron las bacteriocinas producidas por las BAL seleccionadas.

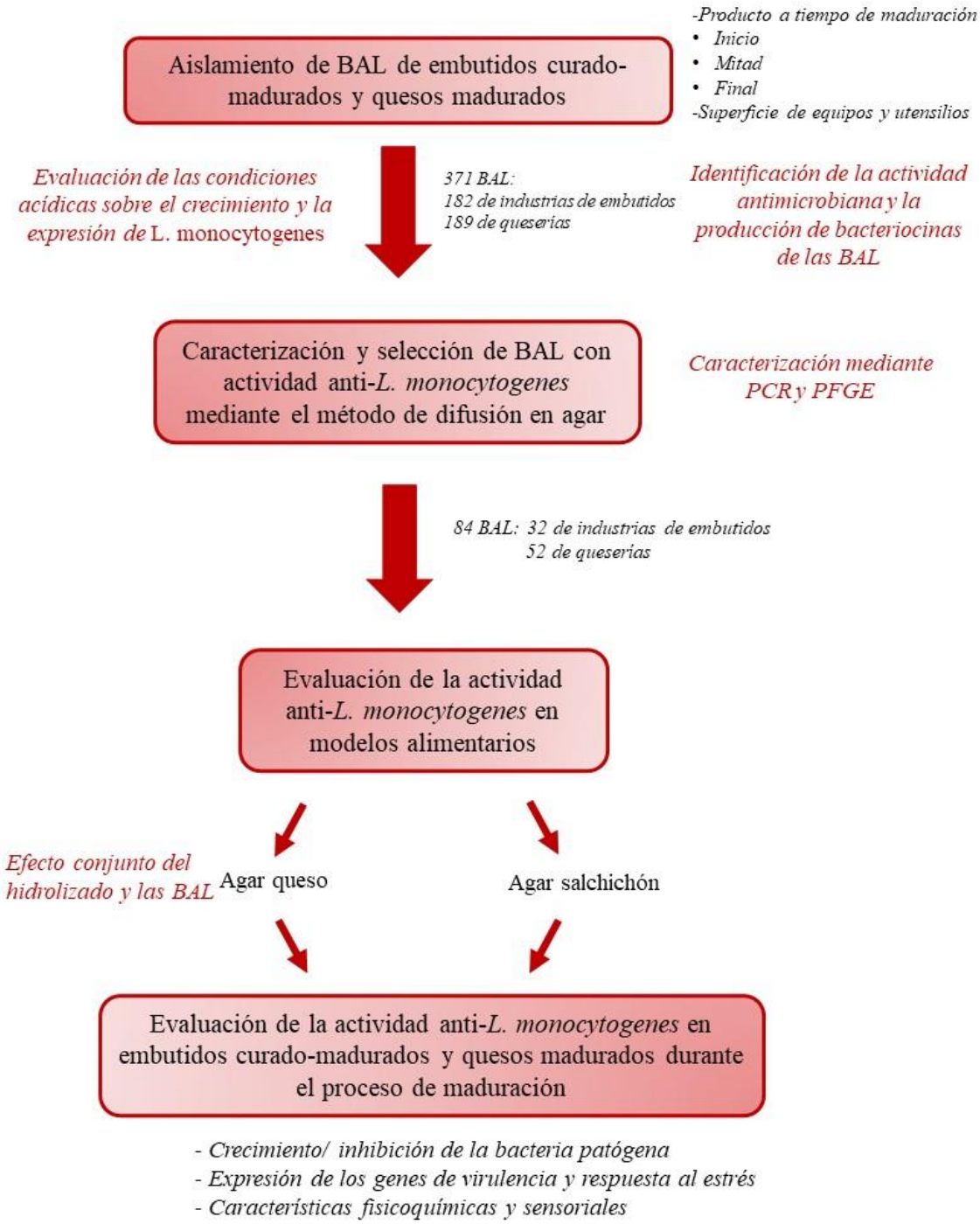

Figura III.1. Diseño experimental del trabajo desarrollado en esta Tesis Doctoral.

III.2.2. Toma de muestra para el aislamiento de bacterias ácido-lácticas

El muestreo para el aislamiento de bacterias ácido-lácticas se llevó a cabo en 12 queserías situadas en la comunidad autónoma de Extremadura y pertenecientes a 3 DOP: 3 industrias encuadradas en la DOP "Torta del Casar" (A, B, C), 4 industrias encuadradas en la DOP "Queso de la Serena" (D, E, F, G) y 5 industrias encuadradas en la DOP "Queso Ibores" (H, I, J, K, L), y en 3 industrias cárnicas elaboradoras de embutidos curado-madurados (M, N, O) de Extremadura y Castilla León. En cada industria se realizaron aislamientos de la leche y cuajada (en las de quesos) o de producto antes de embutir (en las cárnicas) y de producto a mitad y final de maduración. Además, se muestreó algunas de las superficies de las industrias.

Para el muestreo de las superficies de las diferentes industrias se utilizó una toallita humedecida estéril que se arrastró por la superficie a muestrear utilizando guantes estériles con la finalidad de evitar la contaminación de la muestra. El uso de este material permite acceder a zonas o recovecos difícilmente accesibles con otros utensilios de muestreo, además de poder muestrear un área o superficie de gran tamaño. Una vez que se aplicó la toallita en la zona de interés, ésta se introdujo en una bolsa estéril, se identificó y se cerró. A continuación, las muestras fueron almacenadas a temperatura ambiente utilizando neveras portátiles de poliestireno expandido hasta ser procesadas.

Para el aislamiento de BAL, se homogenizaron 10 g de producto o la toallita humedecida con 90 mL de agua de peptona 1% (p/v) y se realizaron diluciones seriadas que posteriormente se inocularon en placas que contenían agar MRS que se incubaron a 30 ºC durante 24-48 h. Las colonias se aislaron hasta obtener un cultivo puro bacteriano a través de pases sucesivos en el mismo agar. Posteriormente, se tomó una colonia y se

cultivó en caldo MRS a 37 ºC durante 24-48 h para finalmente tomar 1 mL del cultivo líquido y conservarlo en una solución de glicerol al 10 % (v/v) a -80 ºC.

III.2.3. Caracterización de bacterias ácido-lácticas

La caracterización de los aislados de BAL obtenidas en los muestreos de las industrias de embutidos y quesos se llevó a cabo, en primer lugar, mediante secuenciación de la región 16S del ARN ribosómico (ARNr) y posteriormente, mediante el método PFGE. Los aislados se procesaron siguiendo el protocolo descrito por el Centro de Control y Prevención de Enfermedades o CDC (*Center for Disease Control and Prevention*): *"Standard Operating Procedure for Pulsenet PFGE of Listeria monocytogenes"* con algunas modificaciones.

III.2.3.1. Secuenciación de la región 16s del ARN ribosómico

En primer lugar, se llevó a cabo la extracción de ADN a partir de 1 mL de un cultivo puro de BAL. Posteriormente, se centrifugó a 10.000 rpm durante 10 min en condiciones de refrigeración (4 ºC) y se eliminó el sobrenadante. El sedimento obtenido se empleó para la extracción del ADN usando el kit comercial de extracción MasterPure™ Complete DNA and RNA Purification Kit. Finalmente, el ADN obtenido fue diluido en 35 µL de tampón TE y se mantuvo a -20 ºC hasta su utilización.

La cantidad y calidad del ADN obtenido fueron determinadas espectrofotométricamente. La calidad del ADN aislado se determinó mediante la relación de absorbancia A_{260}/A_{280}, considerándose que un ADN es de buena calidad cuando dicha relación tiene un valor en torno a 2,0 (Sambrook y col., 1989).

Posteriormente, se realizó la identificación de las cepas de BAL mediante análisis de secuenciación de la región 16S ARNr según la metodología propuesta por (Walter y col.

2000). Los productos de la reacción de secuenciación del ciclo se purificaron con una columna Sephadex. El análisis de secuencias de los productos de PCR fue realizado por el Servicio de Técnicas de Biociencias Aplicadas de la Universidad de Extremadura (STAB, Badajoz, España). Los resultados de las secuencias obtenidos se alinearon con la base de datos de secuencias de nucleótidos en GenBank usando la herramienta BLAST de NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Por último, se analizaron las secuencias y se utilizó un 97% de similitud como criterio para la identificación de especies.

III.2.3.2. Caracterización de las bacterias ácido-lácticas mediante PFGE

Como control del peso molecular se utilizó el control estándar *S. enterica* subsp. *enterica* serovar *Braenderup* (ATCC® BAA-664™).

III.2.3.2.1. Preparación de los bloques de agarosa

Para la extracción del ADN genómico y obtención de los bloques de agarosa se empleó un cultivo de cada una de las cepas de bacterias ácido-lácticas con actividad antimicrobiana frente a *L. monocytogenes* en agar MRS incubado durante 48 h a 30 ºC. Se arrastró parte del cultivo con una torunda estéril, se resuspendió en tampón TE y se ajustó la concentración a 1 densidad óptica mediante espectrofotometría a 600 nm de longitud de onda. Posteriormente, se transfirieron 200 µL de dicha solución a un tubo estéril y fueron incubados con 10 µL de lisozima (20 mg/mL) durante 20 min a 55 °C. Una vez terminada dicha incubación, se añadieron 20 µL de proteinasa K (20 mg/mL).

A continuación, se prepararon los bloques de agarosa con 200 µL de agarosa SeaKem® Gold al 1% (p/v) y 0,5% de SDS en tampón TE (previamente calentado en el microondas y estabilizado en una estufa a 50 °C). La agarosa se mezcló con la solución

de 200 µL tratada con lisozima y proteinasa K, transfiriéndose posteriormente este volumen al interior de un molde para formar 4 bloques de agarosa de cada una de las cepas.

III.2.3.2.2 Lisis y lavado celular de los bloques de agarosa

Pasado el tiempo necesario para que solidificaran los bloques de agarosa, se retiró el adhesivo de la parte interior del molde y aquellos moldes de una misma cepa se introdujeron en 5 mL de tampón CLB. Los tubos con los bloques de agarosa inmersos en CLB se incubaron en un baño de agua a 55 °C durante 2 h con agitación constante a 175 rpm.

Tras el proceso de lisis celular de los bloques de agarosa, se eliminó el tampón CLB filtrando los bloques a través de un filtro específico y se llevaron a cabo dos procesos de lavado con agua destilada y 4 con tampón TE. Finalmente, los moldes de agarosa se depositaron en 1 mL de tampón TE y se conservaron a 4 °C hasta su utilización.

III.2.3.2.3. Digestión de los bloques de agarosa con enzimas de restricción

Para llevar a cabo la digestión con las enzimas de restricción se utilizó una porción de aproximadamente 1/3 del molde de agarosa. Para la digestión se utilizó una enzima de restricción primaria *SgsI* y una enzima de restricción secundaria *NotI*, las cuales llevan a cabo cortes en la secuencia del ADN de las BAL de forma específica (Tabla III.5). El uso de enzimas secundarias está recomendado en las situaciones en las que los patrones de PFGE obtenidas con la enzima primaria son indistinguibles. Para la digestión de la cepa o control estándar (*S. enterica* subsp. *enterica* serovar *Braenderup*) se utilizó la enzima de restricción *XbaI* (Tabla III.5).

Tabla III.5. Secuencia de reconocimiento y corte de las enzimas de restricción utilizadas.

Enzima	Secuencias
NotI	5′…GC ✂CGCCGC...3′ 3′…CGGCGG ✂CG...5′
SgsI	5′…GG ✂CGCGCC...3′ 3′…CCGCGC ✂GG...5′
Xbal	5′…T ✂ CTAGA…3′ 3’…AGATC ✂ T…5’

Para el proceso de restricción enzimática se llevaron a cabo dos pasos:

a) Etapa de pre-digestión o equilibrado

Se recomienda este paso para equilibrar los moldes en la solución tampón en la cual se va a llevar a cabo el proceso de digestión (Tabla III.6). Los moldes se incubaron durante 15-20 min a temperatura ambiente en esta solución de equilibrado.

Tabla III.6. Volumen utilizado de cada reactivo para la solución de equilibrado y la solución de digestión de las distintas enzimas utilizadas.

Reactivo	Solución de equilibrado	Solución de digestión		
		NotI	*SgsI*	*XbaI*
		µL/molde		
Agua destilada estéril	180	176.5	176.5	176.5
Tampón de restricción 10X	20	20	20	20
BSA (20mg/mL)		1	1	1
NotI (10U/µL)		2.5		
SgsI (10U/µL)			2.5	
XbaI (10U/µL)				2.5
Total	200	200	200	200

b) Etapa de digestión

Tras retirar la solución de equilibrado, los moldes se sumergieron en una solución con la enzima de restricción que lleva a cabo el proceso de digestión (Tabla III.6). La

temperatura de incubación fue de 37 ºC para todas las enzimas debido a que ésta es la temperatura óptima de funcionamiento de las enzimas de restricción, incubándose los moldes durante 3 h en agitación continua.

III.2.3.2.4. Preparación del gel y condiciones de la electroforesis

Para llevar a cabo la electroforesis se preparó un gel de 100 mL de agarosa SeaKem® Gold al 1% en una solución de tampón TBE 0,5X, preparada a partir del tampón TBE 5X. Para ello, la agarosa fue derretida en un microondas y posteriormente atemperada en un baño a 55 °C durante 20 min aproximadamente.

Previamente a la electroforesis, los moldes se retiraron de la solución con la enzima de restricción y se introdujeron en 200 µL de tampón TBE 0,5X, incubándose a temperatura ambiente durante 10 min para posteriormente depositarse en cada uno de los 15 pocillos del gel. Se colocó un control en cada extremo del gel y uno más en el pocillo central (pocillo 8). Se añadieron los 100 mL de agarosa en el molde y se dejó solidificar a temperatura ambiente cubriendo el gel con una superficie limpia para evitar la aparición de polvo o impurezas sobre la superficie del mismo.

Una vez solidificado se introdujo en la cubeta de electroforesis inmerso en aproximadamente 2,3 L de tampón TBE 0,5X que se mantuvieron circulando a 70 recirculaciones/min y a una temperatura de 14 °C. Para llevar a cabo la electroforesis se utilizó el equipo CHEF Mapper® XA Pulsed Field Electrophoresis System. El tiempo total de la electroforesis fue de 17 h con pulsos cada 1-15 s. El voltaje empleado fue de 6 V/cm, los ángulos de 120° y el factor rampa lineal.

III.2.3.2.5. Tinción del gel y análisis de los perfiles

Posteriormente, el gel se depositó en un recipiente con 360 mL de agua destilada y 40 μL de SYBR™ gold Nucleic acid Gel Strain (10.000X) durante 20 min en agitación constante. Finalmente, se visualizó y fue fotografiado en un transiluminador.

III.2.4. Métodos moleculares para estudiar cambios en la expresión de genes de virulencia y de respuesta al estrés de *L. monocytogenes*

III.2.4.1. Optimización de un método de PCR en tiempo real para determinar cambios en la expresión de un gen relacionado con el estrés

III.2.4.1.1. Diseño de cebadores

En este trabajo, se diseñó una pareja de cebadores para el desarrollo de un método de qPCR para detertar cambios en la expresión del gen *inlA*. Este diseño se hizo de acuerdo a los requisitos publicados por Rodriguez y col. (2015) (Tabla III.7). Este paso es fundamental para la correcta optimización de un nuevo método de qPCR.

Tabla III.7. Requisitos mínimos para el diseño de los cebadores utilizados en los ensayos de PCR en tiempo real (qPCR). Adaptado de Rodríguez y col. (2015).

Parámetros	Cebadores
Contenido de G-C (%)	30-80%
Tm (teórica)	50-60ºC (siempre >55ºC)
Longitud de la secuencia	15-30 pb
Longitud del producto de qPCR	Entre 50-150 pb
Regla en el extremo 3´	Máximo 2 G o C en las últimas 5 bases
Serie de nucleótidos idénticos	Máximo 3 (nunca 3 G seguidas)

Los cebadores fueron diseñados utilizando el software Primer3web (Untergasser y col., 2012) a partir de secuencias conocidas del gel *InlA* de *L. monocytogenes* para estudiar el impacto de las condiciones ácidas en la expresión génica relacionada con este

patógeno. En la Tabla III.8 se muestra las secuencias de nucleótidos de los cebadores diseñados, así como otra información de interés.

Tabla III.8. Secuencia de nucleótidos, tamaño del producto de qPCR, posición dentro del gen y referencia GenBank de los cebadores diseñados a partir del gen *inlA* para su amplificación mediante PCR en tiempo real (qPCR).

Cebadores	**Secuencia (5´-3´)**	**Tamaño del producto de PCR**	**Posición dentro del gen**	**Referencia GenBank**
inlA-F	ACGGCAAAGAAACAACCAAA	65	1514	FJ495194
inlA-R	TACGGGCTTAGCTGGTTCAG		1578	

Finalmente, para comprobar la especificidad de los cebadores diseñados, se compararon sus secuencias con las secuencias de nucleótidos de la base de datos GenBank del Centro Nacional de Información Biotecnológica (NCBI) utilizando la herramienta BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Se comprobó la especificidad de los mismos ya que la similitud fue superior al 99 %.

III.2.4.1.2. Optimización de las condiciones de la reacción

Para la optimización de las condiciones de reacción del método de qPCR se utilizaron las cuatro cepas de referencia S2, S4-2, S12-1 y S7-2. Cada una de las reacciones de amplificación se realizó por triplicado para todas las cepas analizadas, en un volumen final de 12,5 µL y en placas MicroAmp de 96 pocillos cubiertas con una lámina óptica adhesiva. Además, en todas las reacciones se incluyeron tres muestras control con agua destilada estéril en lugar de ADN. Para optimizar las condiciones de la reacción de qPCR, se utilizó un rango de concentraciones de cebadores que osciló entre 50 y 300 nM y unas condiciones de temperatura de hibridación y tiempos comprendidos entre 55 y 60 ºC y desde 30 s a 1 min, respectivamente.

La mezcla de reacción optimizada referida a un volumen final de 12,5 µL contenía 6,25 µL de SYBR® Green, 0,125 µL de 50x ROX™, 2,5 µL de ADN (10 ng/µL). La concentración optimizada de los cebadores fueron 300 nM y las condiciones de amplificación fueron 1 ciclo de 95 ºC durante 10 min seguido de 30 ciclos de 95 ºC durante 15 s y 60 ºC durante 1 min.

Finalmente, se llevó a cabo el análisis de la curva de disociación del producto de PCR mediante el calentamiento del producto en temperaturas comprendidas en un rango de 72 a 95 °C, durante el cual se obtuvieron medidas de fluorescencia de manera continua, calculándose de forma automática la temperatura de fusión o Tm (*Melting temperature*). Posteriormente, se llevó a cabo la comparación del valor de Tm obtenido con el valor de Tm esperado para la secuencia amplificada y así determinar la especificidad de los cebadores diseñados.

III.2.4.1.3. Sensibilidad del método de qPCR diseñado. Curvas estándar.

A partir de la extracción de ARN y posterior síntesis del ADN complementario (ADNc) (apartado III.2.4.2.1 y III.2.4.2.2) de las muestras de referencia se elaboraron curvas estándar con el objetivo de evaluar la sensibilidad y eficiencia de los métodos de qPCR desarrollados.

Para ello, se utilizaron 10 diluciones seriadas de ADNc de cada una de las cepas de referencia usando concentraciones que variaron entre 500 y 0,005 ng/µL, aplicando las condiciones de reacción para la amplificación de este cebador (apartado III.2.4.1.2). Una vez obtenidos los valores de Cq, estos fueron enfrentados a los valores del $\log_{10}$ de las distintas concentraciones de ADNc utilizadas, construyéndose así las curvas estándar correspondientes (Rodríguez y col., 2015).

Para el ensayo de sensibilidad y eficiencia de los resultados obtenidos mediante qPCR se utilizaron tres réplicas de cada dilución del ADNc. El límite de detección fue definido como la menor concentración de ADNc capaz de ser detectada en la reacción. Los criterios considerados para determinar la eficiencia de la reacción fueron el coeficiente de correlación (R^2) de las curvas estándar y la eficiencia de la reacción, calculada mediante la fórmula $E = 10^{-1}/S^{-1}$, siendo "S" la pendiente de la curva estándar. Se consideró que el método de qPCR estaba optimizado cuando $R^2 \geq 0{,}98$ y las eficiencias de las curvas estándar estaban cercanas al 100% (Rodriguez y col., 2015).

III.2.4.2. Métodos moleculares para el análisis de la expresión de genes de virulencia y de respuesta al estrés de L. monocytogenes en salchichón y quesos

Para estudiar la expresión de los genes de virulencia *hly, iap* y *plcA* y de respuesta al estrés *sigB* de *L. monocytogenes* se utilizaron las metodologías TaqMan® y SYBR Green™.

III.2.4.2.1. Extracción de ARN de medios de cultivo y productos

La extracción de ARN se realizó a partir de 1 mL de una muestra homogeneizada almacenada a -82 ºC procedente de cada tratamiento (apartado III.2.1, Figura III.1). Posteriormente, se centrifugó a 10.000 rpm durante 10 min en condiciones de refrigeración (4 ºC) y se eliminó el sobrenadante. El sedimento obtenido se empleó para la extracción de todo el material genético usando el kit comercial de extracción MasterPure™ Complete DNA and RNA Purification Kit. Una vez centrifugado, se realizó un tratamiento con ADNasa, para eliminar el contenido de ADN genómico de la muestra. Para ello, se utilizó una solución que contenía la enzima ADNasa (1 U/µL) proporcionada por el kit comercial.

En la extracción de ARN procedente del análisis sobre salchichones y quesos, fue necesario incluir una etapa inicial adicional al protocolo de extracción para eliminar la materia grasa del producto. Para ello, se añadieron 200 µL de cloroformo sobre el homogeneizado del salchichón o queso, previo al tratamiento enzimático. A continuación, la muestra se centrifugó, para separar la grasa del resto del contenido celular. Esta grasa fue eliminada junto con el cloroformo y se continuó la extracción siguiendo las instrucciones del kit de extracción.

Finalmente, el ARN obtenido fue diluido en 35 µL de tampón TE y se mantuvo a -82 ºC hasta su utilización. La cantidad y calidad del ARN obtenido fueron determinadas espectrofotométricamente. La calidad del ARN aislado se determinó mediante la relación de absorbancia A_{260}/A_{280}, que al igual que en el caso del ADN, se considera que un ARN es de buena calidad cuando dicha relación tiene un valor en torno a 2,0 (Sambrook y col., 1989).

III.2.4.2.2. Síntesis del ADNc

Antes de realizar el proceso de transcripción inversa (RT) del ARN total extraído para la obtención del ADNc, fue necesario normalizar la concentración de las muestras de ARN a una concentración de 100 ng/µL. La síntesis del ADNc se llevó a cabo utilizando el kit comercial PrimeScrip^{RT} Reagent. La mezcla de la reacción estaba compuesta por 5 µL de ARN, 2 µL de los cebadores aleatorios Random 6 mers (100 µM), 0,5 µL de OligoDT (50 µM), 0,5 µL de la enzima transcriptasa inversa PrimerScript^{RT} Enzyme Mix I y agua ultrapura estéril para llevar el volumen de la reacción final a 10 µL.

Las condiciones de amplificación de la reacción de RT consistieron en un ciclo de 15 min a 37 ºC para la transcripción inversa, un ciclo de 5 s a 85 ºC para la inactivación de la enzima y una etapa final de enfriamiento a 4 ºC. El ADNc obtenido se conservó a -

20 ºC hasta su uso. En todas las reacciones de RT se incluyó un control negativo que contenía todos los componentes de la mezcla de reacción, excepto el material genético.

Finalmente, previo a la utilización del ADNc para los ensayos de expresión génica, éste se diluyó 1:10 para ser utilizado en las reacciones de qPCR.

III.2.4.2.3. Genes de virulencia y de respuesta al estrés de L. monocytogenes

Para llevar a cabo la cuantificación de la expresión génica absoluta y relativa de genes de virulencia y de respuesta al estrés de *L. monocytogenes*, se utilizaron las metodologías TaqMan® y SYBR Green™ respectivamente, utilizando los cebadores y sondas que se detallan en la Tabla III.9.

Tabla III.9. Secuencia de nucleótidos de los cebadores y sondas utilizados para los métodos de PCR en tiempo real de transcripción inversa (RT-qPCR) para el análisis de la expresión de los genes de virulencia y de respuesta al estrés de *Listeria monocytogenes.*

Genes	Cebadores y sondas	Secuencias (5´- 3´)	Tamaño del producto de qPCR	Referencia
hly	hly-f	CATGGCACCACCAGCATCT	64	Rodríguez-Lázaro y col. (2004)
	hly-r	ATCCGCGTGTTTCTTTTCGA		
	hly probe	[HEX]-CGC CTG CAA GTC CTA AGA CGC CA-[TAMRA]		
iap	iap-f	AATCTGTTAGCGCAACTTGGTTAA	78	Rodríguez-Lázaro y col. (2004)
	iap-r	CACCTTTGATGGACGTAATAATACTGTT		
	iap probe	[HEX]-CAACACCAGCGCCACTACGGACG-[TAMRA]		
sigB	sigB-f	CCAAGAAAATGGCGATCAAGAC	166	Rantsiou y col. (2012)
	sigB-r	CGTTGCATCATATCTTCTAATAGCT		
	sigB probe	[HEX]-TGTTCATTACAAAAACCTAGTAGAGTCCAT-[TAMRA]		
plcA	plcA-f	CTAGAAGCAGGAATACGGTACA	115	Rantsiou y col. (2012)
	plcA-r	ATTGAGTAATCGTTTCTAAT		
	plcA probe	HEX]-AATTTATTTAAATGCATCACTTTCAGGT-[TAMRA]		
16S	16S-f	ACGAACGGAGGAAGAGCTTG	82	Alía y col. (2020)
	16S-r	CCCCAACTTACAGGCAGGTT		

El gen 16S del ARNr se utilizó como control endógeno.

III.2.4.2.4. Estudios de expresión génica absoluta

Los componentes de la mezcla de reacción empleada para la amplificación de cada uno de los genes de virulencia y de respuesta al estrés de *L. monocytogenes* aparecen detallados en la Tabla III.10.

Tabla III.10. Concentración de los cebadores y sondas y el volumen necesario de cada uno de los componentes de la reacción para el análisis de la expresión génica absoluta de *Listeria monocytogenes* utilizando la metodología TaqMan®.

Gen	Concentración (nM)		Volumen (µL/muestra)						
	Cebador	Sonda	Agua	TaqMan®	ROX	$MgCl_2$	Cebador directo	Cebador reverso	Sonda
hly	450	100	1,75	7	0,125	0,75	0,5625	0,5625	0,125
iap	300	300	2,875	7	0,125	-	0,375	0,375	0,375
sigB	300	300	2,875	7	0,125	-	0,375	0,375	0,375
plcA	300	200	2,875	7	0,125	-	0,375	0,375	0,250

Además, para todos los genes se utilizó una cantidad de 2,5 µL de ADNc.

Las condiciones utilizadas para la amplificación de los genes de virulencia y de respuesta al estrés se detallan en la Tabla III.11.

Tabla III.11. Condiciones de reacción para la amplificación de los genes de virulencia y respuesta al estrés de *Listeria monocytogenes* utilizando la metodología TaqMan®.

Genes	Etapas	
	Primera (1 ciclo)	Segunda (40 ciclos)
hly	95 ºC,10 min	95 ºC, 15s / 55 ºC, 1min
iap		60 ºC, 1 min
sigB		60 ºC, 1 min
plcA		60 ºC, 1 min

III.2.4.2.4.1. Elaboración de las curvas estándar para el cálculo de la expresión génica absoluta

Para el estudio de la expresión génica absoluta, el número de copias de los genes objeto de estudio se determinó mediante la comparación de los valores de Cq con una curva estándar.

En primer lugar, se obtuvieron las curvas estándar de las copias de cada uno de los genes en estudio a partir del ADN genómico y utilizando las condiciones previamente optimizadas se llevó a cabo una reacción de qPCR, donde los productos de la reacción fueron medidos espectrofotométricamente (Mayer y col., 2003). Estos fragmentos se consideraron como la disolución patrón.

Posteriormente, el número de copias fue calculado siguiendo la fórmula descrita por Hyeon y col. (2010):

$$Número\ de\ copias = \frac{\text{WqPCR}}{(660\frac{\text{g}}{\text{mol}})}\ x\ (\text{LqPCR})\ x\ (6{,}023\ \text{x}10^{23})$$

donde WqPCR es el peso del fragmento de qPCR (g/µL), 660 g/mol es el peso molecular de un par de bases (ADN de doble cadena), LqPCR es el número de pares de bases (pb) del fragmento de PCR y 6,023x10^{23} es el número de Avogadro.

A partir de la disolución patrón, se prepararon diluciones decimales seriadas. De esta manera, las curvas estándar se construyeron relacionando el número de copias de los diferentes genes con el valor de Cq obtenido para cada uno de esos patrones analizados. Para llevar a cabo la amplificación mediante qPCR, cada dilución de las copias de gen fue ensayada por triplicado. Los criterios considerados para determinar la fiabilidad de los resultados fueron los mismos que los establecidos para el método de qPCR desarrollado anteriormente (apartado III.2.3.1.4).

Una vez elaboradas las curvas estándar, el número total de copias del gen de interés se calculó extrapolando los valores de Cq de las muestras analizadas con los valores obtenidos en las curvas estándar.

III.2.4.2.5. Estudios de expresión génica relativa

Para la cuantificación relativa de la expresión de los genes de virulencia y de respuesta al estrés *hly, iap, sigB y plcA* así como del gen 16S del ARNr utilizado como control endógeno basados en la metodología SYBR™ Green se utilizaron las condiciones descritas previamente en la metodología TaqMan® (apartado III.2.4.2.4), pero añadiendo una curva de disociación después de las reacciones de qPCR. Las reacciones de amplificación se llevaron a cabo por triplicado en un volumen final de 12,5 µL (Tabla III.12).

Tabla III.12. Concentración de los cebadores y volumen de los componentes de la reacción para el análisis de la expresión génica relativa de *Listeria monocytogenes* utilizando la metodología SYBR™ Green.

Genes	**Concentración (nM)**	**Volumen (µL/muestra)**						
		Agua	**ROX**	**SYBR**	**$MgCl_2$**	**Cebador directo**	**Cebador reverso**	**ADNc**
hly	450	1	0,125	7	0,75	0,5625	0,5625	2,5
iap	300	2,125	0,125	7	-	0,375	0,375	2,5
sigB	300	2,125	0,125	7	-	0,375	0,375	2,5
plcA	300	2,125	0,125	7	-	0,375	0,375	2,5
16S	300	2,875	0,125	6,25	-	0,375	0,375	2,5

Para llevar a cabo la expresión génica relativa se evaluó la expresión de los genes objeto de estudio en relación con un calibrador o muestra control y un gen endógeno o estructural (gen 16S del ARNr) que mantiene una expresión génica constante. Los genes diana fueron los genes de virulencia (*plcA, hly* e *iap)* y de respuesta al estrés *(sigB)* de *L. monocytogenes.*

El análisis de los resultados obtenidos mediante RT-qPCR se llevó a cabo mediante el método del $2^{-\Delta\Delta Ct}$ o método de cuantificación relativa de los niveles de expresión de los genes diana (*hly, iap, sigB y plcA*) respecto al gen constitutivo o control endógeno 16S

(Livak y Schmittgen, 2001). Este gen se utiliza para normalizar las diferencias existentes entre la concentración de ARNm de las distintas muestras analizadas mediante RT-qPCR. Las condiciones control (o calibrador) variaron en función del experimento, en el caso del estudio del efecto conjunto de los hidrolizados proteicos con las bacterias ácido-lácticas sobre la expresión de *L. monocytogenes* en un medio de queso fue el medio en el que sólo se añadió *L. monocytogenes* o en el caso del ensayo de los salchichones fue la expresión de *L. monocytogenes* en los salchichones en los que solo se añadió *L. monocytogenes.*

III.2.5. Selección de bacterias ácido-lácticas con efecto anti-*L. monocytogenes*

III.2.5.1. Preparación inicial de los inóculos y recuento de los microorganismos

La cepa de *L. monocytogenes* utilizada para los tratamientos de biocontrol fue la cepa de referencia S7-2 (serotipo 4b). Para preparar esta cepa de trabajo, ésta fue revitalizada mediante 2 pases sucesivos de 24 h en caldo BHI a 37 ºC. Para llevar a cabo los experimentos, se ajustó la concentración del cultivo inicial mediante diluciones decimales en agua de peptona al 1% a la concentración definida en cada uno de los experimentos realizados y que se detallan en el apartado correspondiente.

Para llevar a cabo los estudios de biocontrol se utilizaron las 84 cepas de BAL seleccionadas. Cada una de las cepas utilizadas fueron incubadas en caldo MRS en agitación constante a 30 °C durante 48 h. Finalizado el tiempo de incubación, se ajustó la concentración del cultivo inicial mediante diluciones decimales en agua de peptona al 1%, a la concentración definida en cada uno de los experimentos realizados. Luego, el

cultivo con la concentración requerida se centrifugó a 10.000 g durante 5 min y se descartó el sobrenadante. El sedimento se lavó y se resuspendió en PBS.

Finalmente, para llevar a cabo el recuento de los microorganismos, se utilizó agar cromogénico CHROMagar^{TM} Listeria para el recuento de *L. monocytogenes,* agar MRS para el recuento de bacterias ácido-lácticas, agar VRBG para el recuento de enterobacterias, agar MSA para el recuento de *Staphylococcos,* agar MEA para el recuento de levaduras y mohos y agar PCA para el recuento de aerobios totales.

Para ello, al final del periodo de incubación de cada experimento, el medio elaborado con salchichón o queso fueron homogeneizados con 30 mL de agua de peptona al 1% y el salchichón o el queso en 90 ml de agua de peptona al 1%. Posteriormente, se hicieron diluciones seriadas en el mismo diluyente para determinar el crecimiento de los distintos microorganismos, inoculando para ello, 100 µL de las mismas en el medio específico para el recuento de cada uno. El medio cromogénico CHROMagar^{TM} Listeria y VRBG fueron incubados a 37 ºC durante 24 h, el medio MRS, MSA y PCA fueron incubados a 30 ºC durante 48 h y el medio MEA fue incubado a 25 ºC durante 5 días.

La ratio de crecimiento de *L. monocytogenes* en presencia de las bacterias ácido-lácticas, expresada como log UFC, se calculó como la diferencia del crecimiento del lote problema (sometido al tratamiento) respecto al lote control (sin ser sometido al tratamiento).

III.2.5.2. Selección inicial en medio de cultivo

Como paso inicial para la selección de BAL con actividad frente a *L. monocytogenes* se utilizó el método de difusión en agar. Para ello, tras la activación de las BAL como de *L. monocytogenes* como se indica en el apartado III.2.5.1, se colocó un volumen de 10

µL de cada BAL en la superficie del medio BHI agar y posteriormente estas placas se cubrieron con 10 mL de agar BHI que contenían 10^6 UFC/mL de *L. monocytogenes*. La actividad anti-*L. monocytogenes* se determinó mediante la observación de halos de inhibición alrededor del crecimiento de las distintas BAL.

III.2.6. Crecimiento y expresión génica de L. monocytogenes en presencia de bacterias ácido-lácticas en embutidos curado-madurados y quesos madurados

III.2.6.1. Evaluación de la actividad anti-L. monocytogenes de las bacterias ácido-lácticas en salchichón

En los estudios de biocontrol se evaluó la capacidad de las BAL utilizadas para reducir o inhibir el crecimiento de *L. monocytogenes* en salchichón simulando las condiciones del producto, utilizando inicialmente un medio elaborado con salchichón y una cepa del serotipo 4b de *L. monocytogenes*. Posteriormente se seleccionaron aquellos aislados de BAL con mayor capacidad de reducir el crecimiento de *L. monocytogenes* para evaluar su capacidad antagonista sobre el propio salchichón.

III.2.6.1.1. Ensayos in vitro

En primer lugar, se llevó a cabo un primer análisis *in vitro* utilizando un medio de cultivo elaborado con salchichón y evaluando los aislados de BAL que habían presentado un efecto frente a *L. monocytogenes* en agar BHI (apartado III.2.5.2). Cada una de las BAL aisladas de embutidos curado-madurados y que presentaron actividad anti-*L. monocytogenes* (32) se co-inoculó con el serotipo 4b de *L. monocytogenes* en el medio elaborado con salchichón. Para ello, sobre la superficie del medio se inocularon 50 µL en total de una solución que contenía 3 log UFC/cm^2 de BAL y 50 µL de una solución de *L. monocytogenes* que contenía 3 log UFC/cm^2, preparados tal y como se describe en el

apartado III.2.5.1. Los inóculos se extendieron por toda la placa mediante un asa Drigalski.

En este ensayo, además, se utilizó un lote control inoculado únicamente con *L. monocytogenes* para evaluar el crecimiento del microorganismo sobre el medio elaborado con salchichón, sin la presencia de BAL. Cada experimento se realizó por triplicado. Las placas inoculadas se incubaron a temperatura de refrigeración (7 ºC) durante 7 días. Finalizado el periodo de incubación, se llevaron a cabo los recuentos tal y como se describe en el apartado III.2.5.1.

A partir de los resultados obtenidos, se seleccionó un aislado de BAL que mostró el mejor resultado sobre la inhibición del crecimiento de *L. monocytogenes*.

III.2.6.1.2. Ensayo en salchichones

Para llevar a cabo este estudio, se adquirió la mezcla para la elaboración del salchichón en una empresa cárnica de la región de Extremadura y estaba formada por carne picada de cerdo (90%) y lomo ibérico de cerdo (7%), con una adicción de NaCl (1,8%), azúcar (0,4%), nitrato de potasio (120 ppm), nitrito sódico (100 ppm), pimienta negra y especias.

A partir de los resultados obtenidos del ensayo in vitro se seleccionó una cepa de *Lactilactobacillus sakei* 205 que mostró los mejores resultados sobre la inhibición del crecimiento de *L. monocytogenes.*

Figura III.2. Fotografía de los salchichones antes del proceso de maduración

Para ello, se prepararon soluciones de la cepa de *L. monocytogenes* S7-2 a dos concentraciones (10^4 UFC/g y 10^7 UFC/g) y de la cepa de *Ll. sakei* 205 a una concentración de 10^6 UFC/g. Estas soluciones de microorganismos fueron preparadas para ser resuspendidas en un volumen total de 150 mL de PBS y ser añadidas a la masa cárnica antes de ser embutida.

Para llevar a cabo este ensayo se utilizaron, además, dos lotes control inoculado únicamente con *L. monocytogenes* a las dos concentraciones estudiadas y un lote control inoculado únicamente con *Ll. sakei* 205. Además, se utilizó un lote no inoculado.

Los embutidos obtenidos fueron madurados en cámaras de secado controlado en la Facultad de Veterinaria de la Universidad de Extremadura siguiendo las condiciones industriales tradicionales de estos productos tal y como se especifica en la Tabla III.13.

Tabla III.13. Condiciones de temperatura y humedad relativa empleada en la maduración de los salchichones.

Días de maduración	Temperatura (ºC)	Humedad relativa (%)
1 al 3	5	85
3 al 20	7	80
20 al 30	9	75
30 al 90	12	70

El experimento se realizó por quintuplicado, para ello se tomaron 5 embutidos de cada lote a los 0, 15, 30, 60 y 90 días para realizar los recuentos de todos los microorganismos estudiados tal y como se describe en el apartado III.2.5.1.

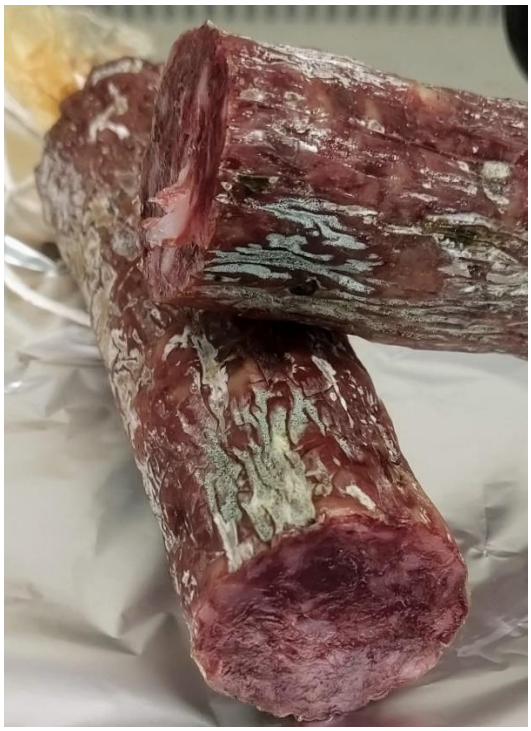

Figura III.3. Fotografía de los salchichones al final del proceso de maduración

Además, en todos los tiempos de maduración estudiados, se evaluaron los parámetros fisicoquímicos y se tomó una muestra de 1 mL del homogeneizado de los

salchichones de cada uno de los lotes en agua de peptona al 1% que se almacenó a -82 ºC para la extracción de ARN y posterior evaluación de la expresión génica.

III.2.6.1.3. Estudios de expresión génica absoluta de L. monocytogenes en salchichón sometida a tratamientos de biocontrol con Ll. sakei

A partir de las muestras conservadas a -82 ºC procedentes de los tratamientos de biocontrol en salchichón, se llevaron a cabo los estudios de expresión absoluta de los genes de virulencia *plcA*, *hly* e *iap* de *L. monocytogenes*, siguiendo el protocolo desarrollado en el apartado III.2.4.2.4.

*III.2.6.2. Evaluación de la actividad anti-*L. monocytogenes *de las bacterias ácido-lácticas e hidrolizados proteicos en queso*

Se evaluó la capacidad de las BAL de forma aislada y en combinación con un hidrolizado proteico de suero lácteo para reducir o inhibir el crecimiento de *L. monocytogenes* en queso, simulando las condiciones del producto. Para ello, en primer lugar, se utilizó un medio elaborado con queso y una cepa del serotipo 4b de *L. monocytogenes.* Después, se seleccionaron los tratamientos con mayor actividad anti-*L. monocytogenes* para evaluar su capacidad antagonista en queso tipo Torta.

III.2.6.2.1. Ensayos in vitro

En primer lugar, se utilizó un medio de cultivo elaborado con queso tipo Torta y se evaluaron las BAL aisladas de industrias lácteas con inhibición frente a *L. monocytogenes* en agar BHI (52) (apartado III.2.5.2). Además, se evaluó la capacidad de inhibir dicha bacteria patógena de un hidrolizado proteico junto con 6 BAL previamente aisladas y caracterizadas en nuestro grupo de investigación.

En primer lugar, se inocularon 100 µL del hidrolizado proteico a una concentración de 0,5 µg/ µL sobre la superficie del agar elaborado de queso y se secaron durante 5-10 min para evaluar la actividad anti-*L. monocytogenes*. Una vez seco, cada una de las BAL se co-inoculó con el serotipo 4b de *L. monocytogenes*. Para ello, sobre la superficie del medio se inocularon 50 µL en total de una solución que contenía 3 log UFC/cm^2 de bacterias ácido-lácticas y 50 µL de una solución de *L. monocytogenes* que contenía 3 log UFC/cm^2, preparados tal y como se describe en el apartado III.2.5.1. Los inóculos se extendieron por toda la placa mediante un asa Drigalski.

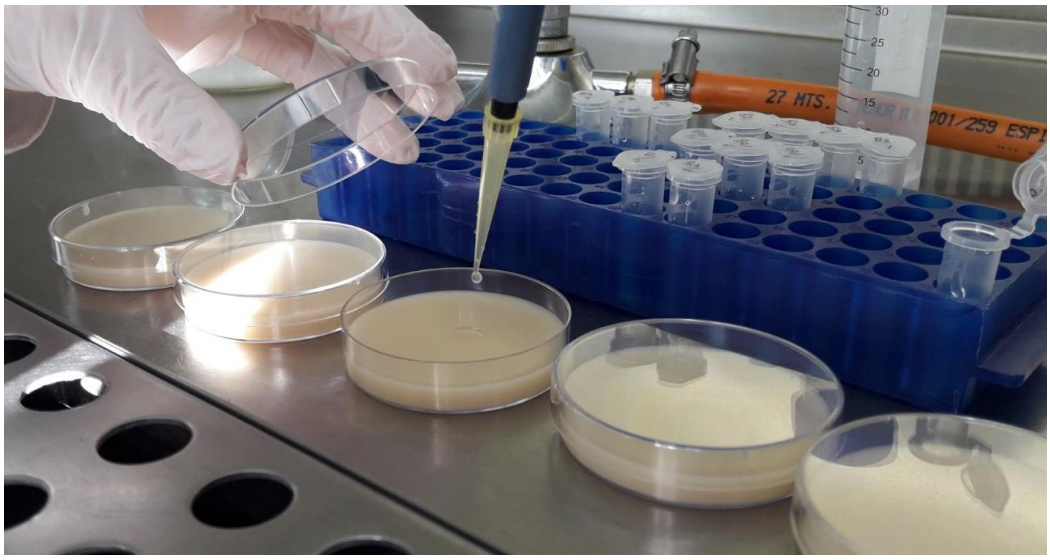

Figura III.4. Fotografía de la inoculación del medio de agar queso con bacterias ácido-lácticas y/o hidrolizado proteico.

En este ensayo, además, se utilizó un lote control inoculado únicamente con *L. monocytogenes* para evaluar el crecimiento del microorganismo sobre el medio elaborado con queso, sin la presencia de BAL ni de hidrolizado proteico. Cada experimento se realizó por triplicado. Las placas inoculadas se incubaron a temperatura de refrigeración (7 ºC) durante 7 días. Finalizado el periodo de incubación, se llevaron a cabo los recuentos tal y como se describe en el apartado III.2.5.1.

Por una parte, a partir de los resultados obtenidos, se seleccionaron 2 de los 52 aislados de BAL que mostraron los mejores resultados sobre la inhibición del crecimiento de *L. monocytogenes*. Por otra parte, en el ensayo en el que se evaluaron el efecto conjunto del hidrolizado proteico con las 6 BAL, se determinó la influencia de los mismos en la expresión génica de *L. monocytogenes.*

III.2.6.2.2. Ensayo en quesos

Para la realización de este estudio, se adquirieron quesos recién cuajados en una empresa elaboradora de quesos Torta del Casar dentro de la DOP.

A partir de los resultados obtenidos del ensayo in vitro se seleccionaron dos cepas: una cepa de *Lacticaseibacillus casei* 116 y otra cepa de *Lactococcus garviae* 151 que mostraron las mayores reducciones en el crecimiento de *L. monocytogenes.*

Figura III.5. Fotografía de los quesos inoculados al inicio del proceso de maduración

Para ello, se prepararon soluciones de la cepa de *L. monocytogenes* S7-2 a dos concentraciones (10^4 UFC/g y 10^7 UFC/g) y de las cepas de BAL, *Lc. casei* y *Lco. garviae*

a la concentración de 10^7 UFC/g. Estas soluciones de microorganismos fueron preparadas para ser resuspendidas en un volumen total de 1 mL de PBS. La inoculación se realizó en el centro de la cuajada (en un cubo de 16 cm^2 de superficie, 6 cm de profundidad y 100 g de peso).

Para llevar a cabo este ensayo se utilizaron, además, dos lotes control inoculados únicamente con *L. monocytogenes* a las dos concentraciones estudiadas y dos lotes control inoculados únicamente con *Lc. casei y Lco. garviae.* Además, se utilizó un lote no inoculado.

Los quesos fueron madurados en cámaras de secado controlado en la Facultad de Veterinaria de la Universidad de Extremadura siguiendo las condiciones industriales tradicionales de estos productos y detalladas en la Tabla III.14.

Tabla III.14. Condiciones de temperatura y humedad relativa empleada en la maduración de los quesos.

Días de maduración	**Temperatura (ºC)**	**Humedad relativa (%)**
1 al 35	6	90
35 al 45	8	80
45 al 55	9	80
55 al 90	10	80

El experimento se realizó por quintuplicado, para ello se tomaron 5 quesos de cada lote a los 0, 30, 45, 60 y 90 días para realizar los recuentos de todos los microorganismos estudiados tal y como se describe en el apartado III.2.5.1.

Figura III.6. Fotografía de los quesos inoculados al final del proceso de maduración

III.2.7. Evaluación de la influencia de las bacterias ácido-lácticas sobre los parámetros fisicoquímicos y sensoriales de los embutidos curado-madurados y quesos madurados

III.2.7.1. Evaluación de los parámetros fisicoquímicos

Para evaluar el efecto de la inoculación de las BAL en las características fisicoquímicas de los embutidos curado-madurados y quesos madurados, se midió la a_w, humedad y pH a lo largo de la maduración de dichos productos.

La a_w se determinó a 25 ºC utilizando un medidor de a_w y la calibración se realizó utilizando varias soluciones saturadas de a_w conocida. El contenido de humedad se determinó siguiendo los métodos oficiales de la Asociación de Químicos Analíticos Oficiales (AOAC, 2000). Este parámetro se determinó gravimétricamente. El pH se midió con un pH-metro que se calibró con 3 soluciones estándar de pH diferentes (4, 7 y 9,25). El pH se determinó después de homogeneizar 3 g de cada muestra. con 27 mL de agua destilada durante 30 s utilizando un homogeneizador.

El cloruro de sodio se determinó por duplicado para cada uno de los embutidos y quesos al final del período de maduración mediante valoración química utilizando el método Volhard (AOAC, 2016). Los resultados se expresan en g de NaCl/ 100g de muestra.

Los nitritos se determinaron únicamente en los embutidos al final del periodo de maduración según el método descrito por la AOAC (2005). La medición del nitrito residual se realizó espectrofotométricamente a partir de un tinte rosado producido mediante el acoplamiento de sulfanilamida con diclorhidrato de NED. Se obtuvo una curva de calibración diluyendo la solución estándar (100 mg/L $NaNO_2$) con agua destilada para cubrir un rango de concentración de 0,1 a 0,8 mg/L $NaNO_2$. El contenido de nitrito residual se calculó usando una curva estándar de solución de nitrito como mg de nitrito por kg de muestra.

III. 2.7.2. Evaluación de las características sensoriales

Para evaluar el efecto de la inoculación de BAL en las características sensoriales del producto se midió la textura y el color, así como los compuestos volátiles.

El análisis de textura se realizó al final del proceso de maduración de los quesos con un texturómetro. Este análisis se realizó a temperatura ambiente utilizando un análisis de perfil de textura (TPA). Para llevar a cabo esta medida fue necesario cortar trozos de queso de aproximadamente 1 cm de espesor y se obtuvieron resultados de las siguientes medidas: dureza (N), elasticidad (cm), cohesión, gomosidad (N), masticabilidad (N cm) y adhesividad (N s). Todos los análisis de textura se realizaron por triplicado de cada quintuplicado.

El color se determinó al final de la maduración en la superficie de corte de cada muestra utilizando un colorímetro con un iluminante D65, un observador estándar de 0° y un puerto/área de visualización de 2,5 cm, que fue calibrado antes de su uso con un azulejo blanco que tiene los siguientes valores: L* = 93.5, a* = 1.0 y b* =0.8. El color se expresó de acuerdo con el sistema de la Comisión Internacional de l'Eclairage (CIE) y se expresó como CIE L* (luminosidad), CIE a* (rojez), CIE b* (amarillez), en el que el croma y el ángulo de matiz se calcularon como: $(a^{*2}+b^{*2})^{0,5}$ y $\tan^{-1}$ (b*/a*), respectivamente.

Los compuestos volátiles de los embutidos curado-madurados y quesos madurados se extrajeron mediante microextracción en fase sólida (SPME) después de calentar a 37 ºC durante 30 min, utilizando una fibra de Divinilbenceno-Carboxeno-Polidimetilsiloxano (DVB/CAR/PDMS) de 50/30 µm. Luego se analizaron por cromatografía de gases-espectrometría de masas (GC-MS) en un cromatógrafo de gases 6890 GC equipado con una columna HP-5 (5% fenil-95% dimetilpolisiloxano) y acoplado a un detector de espectrómetro de masas (MS), 5975C. La temperatura del horno se inició a 40 ºC por 5 min y se fue aumentando hasta 280 ºC, con una tasa de 7 ºC/min. El tiempo de desorción fue de 30 min a 250 ºC. La temperatura de la línea de transferencia se estableció en 280 ºC. El gas portador fue helio con un caudal de 1,2 mL/min. La detección de MS se realizó en exploración completa (50–350 amu). Para el tratamiento de los datos se utilizó la búsqueda automática de picos y la deconvolución espectral, y la identificación de los compuestos volátiles se logró comparando sus espectros de masas con la biblioteca NIST/EPA/NIH.

III.2.8. Evaluación de la actividad antimicrobiana y de la producción de bacteriocinas de las bacterias ácido-lácticas aisladas

Durante la estancia en la Universidad de Oporto (Portugal) se ha llevado a cabo un estudio profundo sobre la caracterización de BAL en base a su seguridad y capacidad de producir compuestos proteicos con capacidad antimicrobiana.

III.2.8.1. Detección de actividad antimicrobiana

Para determinar la naturaleza de la inhibición de las cepas positivas, los cultivos (C) de BAL se centrifugaron a 7000 rpm durante 10 min a 4 ºC y se obtuvo los sobrenadantes libres de células (CFS), posteriormente se ajustó el pH a 6,0 con NaOH estéril (1 M) y se incubó a 80 ºC durante 10 min (CFSn). Para determinar si la inhibición se debía a la producción de peróxido de hidrógeno o a compuestos proteicos, el sobrenadante se trató durante 1 h a 37 ºC con 0,1 mg/mL de catalasa (500 UI/mL, estéril; CFSnC) y 0,1 mg/mL de proteinasa K (CFSnCK). Por último, C, CFS, CFSn, CFSnC, CFSnCK se inocularon sobre una placa de TSAYE crecida durante 24 h a 37ºC de cada uno de los distintos patógenos *L. monocytogenes, E. faecalis* ATCC 29212, *S. aureus* ATCC 25213 y *E. coli* ATCC 25922 y se incubaron durante la noche a 30 °C. Los resultados fueron positivos si se observó una zona de halo translúcido alrededor del lugar. *Pe. acidilactici* HA-6111-2 se utilizó como una cepa de control positiva anti-*L. monocytogenes* (Albano y col., 2009).

III.2.8.2. Presencia de factores de virulencia

III.2.8.2.1. Producción de aminas biógenas

Para determinar la producción de aminas biógenas (tiramina, histamina, putrescina y cadaverina) se utilizó el método desarrollado por Bover-Cid y Holzapfel, (1999). Para ello, los aislados de BAL se cultivaron 7 veces en caldo MRS suplementado con 0,1%

(p/v) de cada aminoácido precursor. Estos precursores fueron: base libre de tirosina para la tiramina, monoclorhidrato de histidina para la histamina, monoclorhidrato de ornitina para la putrescina y monoclorhidrato de lisina para la cadaverina, y todos se suplementaron con 0,005% de piridoxal-5-fosfato para promover la inducción enzimática. Después, los aislados se colocaron por duplicado en el medio con cada aminoácido y se incubaron durante 4 días a 30 ºC. Para este ensayo se utilizaron placas sin aminoácidos como control negativo. Los resultados fueron positivos cuando apareció un color púrpura, o desapareció el precipitado de tirosina, alrededor de las colonias.

III.2.8.2.2. Producción de enzimas hidrolíticas

La actividad gelatinasa se evaluó siguiendo el método propuesto por Tiago y col. (2004) utilizando el caldo Luria-Bertani Modificado (MLB) suplementado con 50,0 g/L de gelatina. Los tubos se incubaron a 30 ºC durante 7 días y después se refrigeraron durante aproximadamente 30 min a 4 ºC. Los resultados se consideraron positivos si el medio permanecía sin solidificar después del enfriamiento.

La actividad ADNasa se determinó siguiendo el método propuesto por Ben Omar y col. (2004) y utilizando medio agar ADNasa con 0,05 g/L de verde de metilo. El resultado se consideró positivo cuando tras incubar las placas a 30 ºC durante 48 h se observó un halo claro alrededor de las colonias. Ambas determinaciones se realizaron por duplicado y se utilizó *S. aureus* ATCC 25213 como control positivo.

III.2.8.2.3. Actividad hemolítica

La producción de hemolisina se evaluó sembrando aislados en placas de agar Columbia con sangre de oveja desfibrilada al 5 %. Las placas se incubaron a 30 ºC durante 24 h y tras este tiempo se consideró actividad hemolítica positiva cuando se observó la

presencia de halos claros alrededor de las colonias (hemólisis β), y actividad hemolítica negativa cuando se observaron zonas verdosas (hemólisis α) o ausencia de zonas claras (hemólisis γ) alrededor de las colonias. *S. aureus* ATCC 25213 se utilizó como control positivo.

III.2.8.3. Presencia de genes de virulencia

Para llevar a cabo la detección de los genes de virulencia de las BAL, se utilizaron los cebadores que se detallan en la Tabla III.15.

Tabla III.15. Secuencia de nucleótidos de los cebadores utilizados para el análisis de los genes de virulencia de las bacterias ácido-lácticas.

Genes	Secuencias (5´-3´)	Tamaño (pb)	Controles positivos	Referencia
ace	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	1008	*E.faecalis* DS16;*E.faecalis* F2;*E.faecalis* P1	Martín-Platero y col. (2009)
agg	AAG AAA AAG AAG TAG ACC AAC AAA CGG CAA GAC AAG TAA ATA	1553	*E.faecalis* P1	Eaton y Gasson (2001)
gelE	ACC CCG TAT CAT TGG TTT ACGCATTGCTTTTCCATC	419	*E.faecalis* P1	
efaAfs	GACAGACCCTCACGAATA AGTTCATCATGCTGT AGT A	705	*E.faecalis* F2	
efaAfm	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735	*E.faecalis* F10	
cylA	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTCGTCA	517	*E.faecalis* F2	Semedo y col. (2003)
cylB	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	843	*E.faecalis* F2	
cylM	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	742	*E.faecalis* F2	
cylLL	GATGGAGGGTAAGAATTATGG GCTTCACCTCACTAAGTTTTATAG	253	*E.faecalis* DS16	
cylLS	GAAGCACAGTGCTAAATAAGG GTATAAGAGGGCTAGTTTCAC	240	*E.faecalis* DS16	
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	*E.faecalis VanB*	Vankerckhoven y col. (2004)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1	
hdc1	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	367	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1	De Las Rivas y col. (2005)
tcd	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	924		
odc	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	1446	-	

Los componentes de la mezcla de reacción, así como las condiciones empleadas para la amplificación de cada uno de los genes de virulencia aparecen detallados en la Tabla III.16.

Tabla III.16. Volumen necesario de cada uno de los componentes de la reacción, así como las condiciones para llevar a cabo las reacciones de la PCR convencional.

Genes	PCR	Condiciones PCR		
		Etapa 1		Etapa 2
ace	0,5µl dNTPs (10mM); 2,5 µl tampón NH_4; 2,5 µl MgCl (25mM); 0,25µl cebador F/R (10pM);0,4 µl Taq polimerasa (5U)	95ºC, 1min	30 ciclos	94ºC, 1min, 55ºC 1min, 72ºC,1 min
agg gelE efaAfs efaAfm	0,25µl dNTPs (10mM); 2,5 µl tampón NH_4; 2,5 µl MgCl (25mM); 1,25µl cebador F/R (10pM);0,25 µl Taq polimerasa (5U)	94ºC, 1min	35 ciclos	94ºC, 1min, 55ºC, 1min, 72ºC, 2 min
cylA cylB cylM cylLL cylLS	0,25µl dNTPs (10mM); 2,5 µl tampón NH_4; 2,5 µl MgCl (25mM); 1,25µl cebador F/R (10pM);0,25 µl Taq polimerasa (5U)	95ºC, 1min	35 ciclos	94ºC, 1min, 55ºC, 1min, 72ºC, 2 min
hyl asa1	0,5µl dNTPs (10mM); 2,5 µl tampón NH_4; 2,5 µl MgCl (25mM); 0,35µl cebador F/R(10pM);0,4 µl Taq polimerasa(5U)	94ºC, 1min	30 ciclos	94ºC, 1min, 56ºC 1min, 72ºC,1 min
hdc1 tcd odc	2,5 µl Tris HCl; 0,5µl dNTPs (10mM); 5 µl tampón KCl; 2,5 µl MgCl (25mM); 0,75µl cebador hdc F/R (10pM);2µl cebador tdc F/R (10pM), 1µl cebador odc F/R (10pM);0,4 µl Taq polimerasa (5U)	95ºC, 1min	30 ciclos	95ºC, 30s, 52ºC, 30s, 72ºC 2 min

Los productos de la PCR se analizaron mediante electroforesis en geles de agarosa al 0,8 % con tampón Tris-Acetato-EDTA 1X (tampón TAE; Bio-Rad). Para cada reacción de PCR se incluyó un control negativo (muestra sin ADN) y un control positivo (muestra con ADN de cada cepa según el gen estudiado).

III.2.8.4. Evaluación de la susceptibilidad a antibióticos

Los antibióticos utilizados en este ensayo fueron ampicilina, cloranfenicol, eritromicina, tetraciclina, gentamicina, estreptomicina y kanamicina. Las concentraciones ensayadas se basaron en los valores de corte microbiológicos establecidos por la

Comisión Técnica de Aditivos y Productos o Sustancias utilizados en los Piensos para Animales (FEEDAP) y también por el Clinical & Laboratory Standards Institute CLSI, (2017), para enterococos y algunos antibióticos. Los valores de corte microbiológicos (μg/mL) se determinaron por el método de dilución en agar según el CLSI, (2012) para enterococos, y por el método de microdilución en caldo según Klare y col. (2005) para las otras especies de BAL. Todos los aislamientos se cultivaron en agar Müeller-Hinton sin antibióticos añadidos como control negativo. Además, se utilizó la cepa *E. faecalis* ATCC 29212 como control para monitorear la precisión de las CIM (CLSI, 2012).

III.2.8.5. Caracterización de las bacteriocinas producidas por bacterias ácido-lácticas seleccionadas

III.2.8.5.1. Producción de bacteriocina durante el crecimiento

Para determinar la producción de bacteriocina durante el crecimiento se inoculó caldo MRS (100 ml) con 1% (v/v) de un cultivo crecido de la cepa productora de bacteriocina y se incubó a 30 ºC durante 24 h. Cada hora se registró los cambios en el pH y la densidad óptica (600 nm). Los recuentos (UFC/mL) y la actividad de la bacteriocina frente a *L. monocytogenes* ScottA, *L. monocytogenes* EDG-e, *L. monocytogenes* NCTC 11994 and *E. faecalis* ATCC 29212 se registró como describen Van Reenen y col. (1998) cada 2 h durante 24 h. La cepa *Pe. acidilactici* HA-6111-2 se usó como control. La actividad antimicrobiana se expresó como unidades arbitrarias por mL (UA/mL), siendo UA el recíproco de la dilución más alta que muestra una clara zona de inhibición del crecimiento.

III.2.8.5.2. Métodos moleculares para detectar genes que codifican la producción de bacteriocinas

Para llevar a cabo la identificación de los genes que codifican la producción de bacteriocinas se utilizaron los cebadores que se detallan en la Tabla III.17.

Tabla III.17. Secuencia de nucleótidos de los cebadores utilizados para los métodos de PCR de los genes de bacteriocinas.

Genes	Cebadores	Secuencias (5´-3´)	Tamaño del producto de PCR	Referencia
nisin	nisZ-prom-F nisP3	CTCGACGATACCATCACTCTTC TCTTTCCCATTAACTTGTACTGTG	1010	Ho y col. (2018)
lacticin 481	lact-481-F lact481-R	TCTGCACTCACTTCATTAGTTA AAGGTAATTACACCTCTTTTAT	366	Rodriguez y col. (2000)
lactococcin 972	lcn972-F lcn972-R	TTGTAGCTCCTGCAGAAGGAACATGG GCCTTAGCTTTGAATTCTTACCAAAAG	312	Alegría y col. (2010)
lactococcin G and Q	lactGQ-F lactGQ-R	GAAAGAATTATCAGAAAAAG CCACTTATCTTTATTTCCCTCT	620	Alegría y col. (2010)
lactococcin A and B	lcnAB-F lcnA-R lcnB-R	GAAGAGGCAATCAGTAGAG GTGTTCTATTTATAGCTAATG CCAGGATTTTCTTTGATTTACTTC	771	Alegría y col. (2010)
Brevicin	brevicin 174A-F brevicin 174A-R	GTCTTAAATGCTAGGCTTGTCA CTGGCAAGACAAACGGTTAG	766	Noda y col. (2015)
plantaracin A	pnlA-F pnlA-R	TAGAAATAATTCCTCCGTACTTC ATTAGCGATGTAGTGTCATCCA	573	Xie y col. (2011)
plantaracin EF	plnEF-F pnlEF-R	TATGAATTGAAAGGGTCCGT GTTCCAAATAACATCATACAAGG	516	Xie y col. (2011)
pediocin PA	pediocin PA1-F pediocin PA1-R	AAAGATACTGCGTTGATAGG GAGAAGCCATGCTGAAAG	1120	Xie y col. (2011)
plantaracin NC8	pnlNC8-F pnlNC8-R	GGTCTGCGTATAAGCATCGC AAATTGAACATATGGGTGCTTTAAATTCC	159	Maldonado y col. (2004)
plantaracin S	pnlS-F pnlS-R	GCCTTACCAGCGTAATGCCC CTGGTGATGCAATCGTTAGTTT	320	Stephens y col. (1998)
plantaricin W	pnlW-F pnlW-R	TCACACGAAATATTCCA GGCAAGCGTAAGAAATAAATGAG	44	Holo y col. (2001)

La mezcla de reacción optimizada referida a un volumen de 50 µl contenía 2,5 mM de $MgCl_2$, 1 µl de tampón de reacción, 100 µM de cada desoxinucleósido trifosfato (dNTP), 100 pmol de cada cebador, 5 U de ADN polimerasa Taq y se utilizaron 250 ng de ADN genómico de cepas de BAL seleccionadas.

Las condiciones de amplificación optimizadas de cada cebador se detallan en la Tabla III.18.

Tabla III.18. Condiciones necesarias para la amplificación de los genes de bacteriocinas.

<table>
<tr><th rowspan="2">Genes</th><th colspan="2">Condiciones PCR</th></tr>
<tr><th>Etapa 1 (1 ciclo)</th><th>Etapa 2 (30 ciclos)</th></tr>
<tr><td>Nisin
Lacticin 481
Lactococcin 972
Lactococcin G and Q
Lactococcin A and B</td><td rowspan="11">95ºC, 6 min</td><td>95ºC, 30 s</td><td>50ºC, 30s</td></tr>
<tr><td>Brevicin</td><td>96ºC, 1 min</td><td>56ºC, 30 s</td></tr>
<tr><td>Plantaracin A</td><td rowspan="3">94ºC, 30s</td><td>55ºC, 1 min</td></tr>
<tr><td>Plantaracin EF</td><td>54ºC, 1 min</td></tr>
<tr><td>Pediocin PA</td><td>50ºC, 1 min</td></tr>
<tr><td>Plantaracin NC8</td><td rowspan="3">94ºC, 1 min</td><td>60ºC, 1 min</td></tr>
<tr><td>Plantaracin S</td><td>60ºC, 1 min</td></tr>
<tr><td>Plantaricin W</td><td>55.5ºC,1 min</td></tr>
</table>

Al terminar la amplificación de los productos de la PCR se separaron por electroforesis en geles de agarosa al 1,5 % (p/v) utilizando Tris acetato EDTA 1x a 90 V durante 1 h. Los geles se tiñeron con 3 µl de solución de tinción de ácidos nucleicos. Las bandas de ADN fueron visualizadas en un transiluminador. Para conocer el tamaño de los productos se utilizó un marcador de peso molecular de ADN de 0,05-1,5 Kpb.

III.2.8.5.3. Efecto de enzimas, temperatura, pH, tensioactivos y proteasa inhibidores de la actividad bacteriocina

Para ver el efecto de diversos factores en la inhibición de la actividad de la bacteriocina, las cepas se cultivaron en caldo MRS durante 18 h a 30 ºC y tras este tiempo, se centrifugaron a 8000 g durante 10 min a 4 ºC. Posteriormente, el sobrenadante libre de células se ajustó a pH 6,0 con NaOH 1 M. Se incubó 1 mL de cada sobrenadante libre de células durante 2 h en presencia de 1 mg/ mL y 0,1 mg/ mL de cada una de proteinasa K, papaína, pepsina, tripsina, α-amilasa y catalasa.

En un experimento separado, se añadieron 1% (p/ v) de SDS, EDTA, Tween 20, Tween 80, urea, Triton X-114, Triton X-100, Oxbile y NaCl a los sobrenadantes libres de células que contienen bacteriocinas. Se añadió EDTA a los sobrenadantes libres de células para producir concentraciones finales de 0,1, 2,0 y 5,0 mM. Los sobrenadantes libres de células sin tratar y los detergentes en agua a las mismas concentraciones se utilizaron como controles. Todas las muestras se incubaron a 30 °C durante 5 h.

Para determinar el efecto del pH sobre la bacteriocina se ajustó los sobrenadantes libres de células con NaOH 1 M o HCl 1 M estéril desde un pH 2 a 12 (aumentando en 2 unidades de pH). Después de 1 h de incubación a temperatura ambiente (25 °C), las muestras se reajustaron a pH 6,5 con NaOH 1 M o HCl 1 M estéril y se trataron a 80 ºC durante 10 min. El efecto de la temperatura sobre la actividad de la bacteriocina se probó a 4, 25, 30, 37, 45, 60, 80 y 100 ° C durante 60 y 120 min. La actividad de la bacteriocina también se probó después de 20 minutos a 121 °C. En todas las pruebas, la actividad antimicrobiana se controló mediante el método de prueba de la mancha de agar Van Reenen y col. (1998) y las cepas *L. monocytogenes* ScottA, *L. monocytogenes* EDG-e, *L. monocytogenes* NCTC 11994 and *E. faecalis* ATCC 29212 se utilizaron como controles.

III.2.8.5.4. Lisis celular

Para ello, 20 mL del sobrenadante libre de células (3200 UA/ml, pH 6,0 analizado en *L. monocytogenes* ScottA, *L. monocytogenes* EDG-e y *L. monocytogenes* NCTC 11994 y 6400 AU/ml en *E. faecalis* ATCC 29212) se esterilizaron por filtración y se añadieron a 100 mL de fase exponencial temprana de *L. monocytogenes* ScottA (5 h de edad; OD = 0,6), *L. monocytogenes* EDG-e (4 h; OD = 0,5), *L. monocytogenes* NCTC 11994 (6 h; OD=0,4) y *E. faecalis* ATCC 29212 (4 h; OD=0,6). Posteriormente, se tomaron lecturas de densidad óptica a 600 nm cada hora durante 12 h. Cada cultivo diana de *L. monocytogenes* y *E. faecalis* sin bacteriocinas añadidas se utilizó como control y se realizaron dos réplicas independientes.

III.2.8.5.5. Purificación parcial de bacteriocinas.

Las cepas productoras de bacteriocinas se cultivaron en 400 ml de caldo MRS a 30 °C hasta la fase estacionaria temprana (cultivos de 18 h). Las células se recolectaron por centrifugación (10.000 rpm, 20 min, 4 °C) y se añadió gradualmente sulfato de amonio al sobrenadante hasta una saturación del 60 % y 80%. Después de 4 h de agitación lenta a 4 °C, se recolectaron las proteínas (10.000 rpm, 1 h, 4 °C). Las proteínas precipitadas en el sedimento y flotando en la superficie se recogieron y solubilizaron en un décimo de volumen de tampón de acetato de amonio 25 mM (pH 6,5) (Sambrook y col., 1989). Todas las muestras se almacenaron a -20 °C.

III.2.8.5.6. Tamaño molecular de las bacteriocinas.

Para obtener el tamaño de las bacteriocinas, las muestras se separaron mediante tricina-SDS-PAGE, como describen (Schägger y von Jagow, 1987). Se utilizó un marcador de bajo peso molecular con tamaños de 6,5 a 270 kDa, los geles se fijaron y se tiñeron la mitad con Coomassie Brilliant Blue R250. Las posiciones de las bacteriocinas

activas se determinaron superponiendo la otra mitad del gel (no teñido y prelavado extensamente con agua destilada estéril) con células de *L. monocytogenes* ScottA (10^6 UFC / ml) en agar BHI y se incubaron a 37ºC durante 24h.

III.2.9. Tratamiento estadístico

En primer lugar, se determinó si los datos obtenidos en este trabajo seguían una distribución normal mediante la prueba de Shapiro-Wilk. Para aquellos datos que siguieron una distribución normal, se aplicó el Test ANOVA para determinar diferencias significativas entre las medias y el test Tukey como prueba Post hoc. En el caso de la distribución no normal de los datos analizados, se utilizó la prueba no paramétrica de Kruskal-Wallis para determinar diferencias significativas entre las medias. Posteriormente, se aplicó el test U de Man-Whitney como pruebas Post hoc para determinar que medias diferían. Para establecer las correlaciones entre dos variables independientes en el análisis sensorial de las muestras se determinaron los coeficientes de correlación de Spearman (datos no normales). El nivel de significancia se estableció en $p \leq 0,05$

IV. RESULTADOS

IV.1. SELECCIÓN Y EVALUACIÓN DE BACTERIAS ÁCIDO-LÁCTICAS E HIDROLIZADOS PROTEICOS CON ACTIVIDAD ANTI-*L. monocytogenes*

Journal of the Science of Food and Agricultural (2022). Enviado para su publicación

International Journal of Food Microbiology (2022)

Food Research International (2022). Enviado para su publicación

Selection and characterization of lactic acid bacteria with activity against *Listeria monocytogenes* from traditional RTE ripened foods

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Abstract

Lactic-acid bacteria isolated throughout the ripening process from traditional RTE soft cheeses and dry-cured fermented sausages were characterized and selected for their anti-*L. monocytogenes* activity. For this, the LAB isolates were first screened for their activity against this pathogen in agar spot assay and further evaluated in cheese and dry-cured fermented sausage models simulating conditions of ripening of these products. From a total of 371 LAB isolates, 84 showed anti-listerial activity in agar spot assay and only 10 of them were selected for reducing *L. monocytogenes* counts in food models. The selected LAB strains belonged to the species *Enterococcus faecium*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lactilactobacillus sakei* and *Lactococcus garviae.* All of them have at least one gene encoding known bacteriocins. The most active strains *Lc. casei* 116 and *Ll. sakei* 205 provoked reductions higher than 2 log cycles of *L. monocytogenes* levels food models, respectively. The selected LAB strains may be utilized to control *L. monocytogenes* throughout the ripening process in ripened soft cheeses and dry-cured fermented sausages. The combination of 16S rRNA sequencing with PFGE analysis with restriction *SgsI* and *NotI* enzymes could be useful to control the implantation of these selected strains throughout the ripening process.

Keywords: lactic-acid bacteria, anti-*Listeria monocytogenes* activity, PFGE analysis

1. INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive, facultatively anaerobic, rod-shaped intracellular bacterium (Filipello et al., 2017; Rodriguez et al., 2021). Due to its resistance to high salinity and low pH environments, ability to form biofilms, and growth at low temperatures, *L. monocytogenes* is found in raw, unpasteurized milk or cheeses, although in the last decade, other foods have also been involved in several outbreaks, including meatloaf, smoked fish, fermented raw sausages, or vegetables, especially in ready-to-eat (RTE) processed foods (Shamloo et al., 2019; Smith et al., 2018). The presence of *L. monocytogenes* in ripened products such as traditional RTE dry-cured fermented sausages and cheeses is of great public health concern because it causes listeriosis. The invasive form of this disease has a high mortality rate and even if patients survive, severe neurological sequelae may possess (García et al., 2020). Undoubtedly, the control of *L. monocytogenes* in traditional RTE ripened foods constitutes a challenge for fermented food-related industries.

An alternative to control this pathogenic bacterium in these RTE products is the use of selected lactic-acid bacteria (LAB) with anti-*L. monocytogenes* activity as a protective culture (Siedler et al., 2019; Singh, 2018). These antimicrobial properties of LAB are attributed to different modes of action including competition for nutrients and/or space and the production of one or more antimicrobial active metabolites such as organic acids (mainly lactic and acetic acids), hydrogen peroxide and also other compounds, such as bacteriocins (Vieco-Saiz et al., 2019; Yap et al., 2021). They have been widely used in traditional fermentation processes since they are Generally Recognized As Safe (GRAS) and have important technological properties, such as inhibition of the growth of *L. monocytogenes* (Reis et al., 2012). Before using LAB in traditional RTE ripened foods, it is necessary a selection of strains adapted to these products with activity against *L.*

monocytogenes. LAB can be found naturally in different food products (Carr et al., 2002; Gajbhiye & Kapadnis, 2016) including dry-cured fermented sausages and ripened cheeses (Perin et al., 2017). However, not all LAB strains present as natural contamination capable of surviving in these traditional ripened foods have anti-*L. monocytogenes* activity. Thus, it is necessary to conduct an appropriate methodology for the selection and characterization of active LAB against *L. monocytogenes* adapted to traditional ripened products. In this work, the methodology followed to isolate and characterize LAB with anti-*L. monocytogenes* activity in traditional dry-cured fermented sausage and in traditional soft-ripened cheeses industries will be described (Figure 1).

The objective of this work was to isolate and characterize LAB from traditional RTE ripened soft cheeses and dry-cured fermented sausages throughout their ripening process with anti-*L. monocytogenes* activity.

2. MATERIALS AND METHODS

2.1. *Listeria monocytogenes* culture and conditions

To evaluate the anti-*L. monocytogenes* activity of the LAB isolates in agar spot assay and LAB strains in food models, the strain of *L. monocytogenes* S7-2 (serotype 4b) was used. This strain belongs to the National Institute of Agricultural and Food Research and Technology (INIA) collection (Madrid, Spain) and was isolated from ripened foods. To prepare the *L. monocytogenes* inoculum, 100 µL of a stock culture (stored in Brain Heart Infusion (BHI) broth (Pronadisa, Madrid, Spain) containing 20 % (w/v) glycerol at −80 °C) was sub-cultured twice onto BHI broth at 37 °C for 24 h. To check the level of inoculation, serial dilutions were plated onto Chromagar™ Listeria agar plates (Scharlab, Madrid, Spain) and incubated at 37 °C for 48 h.

2.2. Obtention of LAB isolates in ripened cheeses and dry-cured fermented sausages industries

This study was carried out in 12 factories of traditionally Protected Designation of Origin (PDO) soft-bodied cheeses from "Torta del Casar" (A, B and C), "Queso de la Serena" (D, E, F and G) and "Queso Ibores" (H, I, J, K and L) and 3 dry-cured fermented meat sausages factories (M, N and O). In cheese industries, samples for LAB isolation were aseptically taken from milk, curd and half-ripened and ripened cheeses. In the dry-cured fermented sausages factories, samples for LAB isolates were aseptically taken from the mix of meat before stuffing and the product at the middle and end of ripening. Additionally, work and equipment surfaces were sampled with sterile swabs (Aes Chemunex, France).

All taken samples, including swabs and food products, were put into sterile stomacher bags under suitable conditions and transported under refrigeration conditions to the lab to be processed. Then, 10 g of products or swabs were mixed with 90 mL of 1 % (w/v) peptone water (Conda, Spain) and homogenized in a Stomacher device (Seward, model 400 Circulator, UK) at 300 rpm for 2 min. Several decimal dilutions were prepared in 1 % (w/v) sterile peptone water for LAB enumeration on Man Rogosa Sharpe agar (MRS; Conda) and incubated at 30 ºC for 48 h, under microaerophilic conditions (ISO 15214:1998). The characteristic colonies in the MRS agar were picked from the agar plates, re-streaked to obtain pure culture and preliminarily characterized as LAB isolates according to the Gram stain reaction, morphology in MRS plates and microscopic observation. All LAB isolates were cultured in MRS broth (Conda) and stored at −80 °C in the same medium with 10% (v/v) glycerol (Fisher Scientific, USA).

2.3. Screening of anti-*L. monocytogenes* activity of LAB isolates

The anti-*L. monocytogenes* activity of all LAB isolates obtained was firstly tested by using the agar spot assay (Figure 1). For this, LAB isolates were anaerobically grown in 10 mL of MRS broth and incubated for 48 h at 30 °C. After incubation, the cultures were centrifuged for 3–5 min at 10,000 rpm. Subsequently, the pellet obtained was resuspended in 200 µL MRS broth and 10 µL was spotted onto the surface of BHI agar (Conda). Then, these plates were overlaid with 10 mL of BHI agar containing 10^6 CFU/mL of *L. monocytogenes* S7-2. The anti-listerial activity was determined by observation of clearing zones of inhibition around LAB spot. Only LAB isolates showing strong zones of inhibition were selected.

2.4. Identification of selected LAB isolates by 16S rRNA sequencing

The selected LAB isolates were inoculated in a MRS broth medium and then incubated at 30 °C for 24 h. For each isolate studied, the DNA was extracted according to the instructions of the commercial DNA extraction MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA). The obtained DNA was eluted in 35 µL of TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA; Epicentre) and kept at −20 °C until required. The DNA concentration (ng/µL) and purity (A_{260}/A_{280} ratio) were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Then, the identification of the LAB strains was performed by sequencing analysis of the 16S rRNA region according to the methodology proposed by Walter et al. (2000). Sequence analysis of PCR products was carried out by the Applied Bioscience Techniques Service of the University of Extremadura (STAB, Badajoz, Spain). The sequence results obtained were aligned with the nucleotide sequence database in GenBank using the BLAST tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were analyzed and 97% similarity was used as the criterion for species identification.

2.5. Evaluation of anti-*L. monocytogenes* activity of selected LAB strains in food models

2.5.1. Preparation of food models and inocula of tested microorganisms

The LAB strains preliminarily selected by their anti-*L. monocytogenes* activity in agar spot assay were further evaluated for their efficiency to inhibit this pathogen in food models (Figure 1). For this, model systems relied on "Torta del Casar" soft cheese and dry-cured fermented sausage were used. The conditions used were those that these products usually have at the first stages of the ripening process when microorganisms could be inoculated into the products.

To prepare "Torta del Casar" cheese model system, ripened cheeses were firstly lyophilized in a freeze dryer (Labconco®, USA). Then, the "Torta del Casar" cheese-based agar was carried out by autoclaving 20 g of bacteriological agar (Pronadisa, Spain) in 500 mL of deionized water for 20 min at 121 °C (103kPa) and mixed with 200 g of freeze-dried "Torta del Casar" cheese and 12.5 g of trisodium citrate dehydrate (Scharlab, Spain) heated in 400 mL of deionized water at 100 ºC for 1 h (Gori et al., 2007; Martín et al., 2022). Finally, the pH was adjusted to 6.0 using lactic acid by using a pH meter (Crison, Spain) and the water activity (a_w) value was checked to confirm a value of 0.975 by an a_w meter (Novasina AG, Switzerland). This was done before cooling down the medium up to 45-50 ºC in a water bath with shaken vigorously prior to pouring 15 mL into 25 cm^2 Petri plates.

The dry-cured fermented sausage model system was prepared with dry-cured fermented "salchichón", as the main constituent, lyophilized in a freeze dryer from Labconco®. Next, the dry-cured fermented "salchichón"-based agar was prepared by autoclaving 250 g/L of lyophilized dry-cured fermented sausage "salchichón" and 20 g/L of

bacteriological agar. The pH was adjusted to 5.5 with lactic acid and the a_w was found to be 0.98 by using a pH meter (Crison) and an a_w meter (Novasina AG), respectively. After autoclaving for 20 min at 121 ºC, the culture medium was cold down up to 45–50 ºC and vigorously shaken prior to pouring into 25 cm^2 Petri plates.

For preparing the inoculum of *L. monocytogenes* S7-2, the working cultures of this microorganism were consecutively grown twice in BHI broth at 37 °C for 24 h each. The concentration of the fresh cultures was adjusted to reach a concentration of 4 log CFU/mL in the inoculum.

The inoculum of each one of the selected LAB strains was prepared after anaerobically growing twice in 10 mL of MRS broth at 30 °C each. After the second incubation, the cultures were centrifuged for 3–5 min at 10,000 rpm. Subsequently, the pellet obtained was resuspended in 200 µL MRS broth and diluted with BHI to reach a concentration of 4 log CFU/mL in the inoculum.

2.5.2. Experimental settings

L. monocytogenes S7-2 and the selected LAB strains were co-inoculated in "Torta del Casar" cheese-based agar or dry-cured fermented-based agar depending on the LAB industry (cheese or dry-cured fermented sausage) origin. For this, the inoculum of *L. monocytogenes* 4b (50 µL) and LAB strains (in a total volume of 50 µL) were inoculated to obtain final concentrations of approximately 3 log CFU/cm^2. Samples were incubated for 7 days at 7 ºC. This temperature was used to simulate cooling temperature according to the maximum temperature allowed for the "Torta del Casar" cheese and dry-cured fermented sausages at the first stages of ripening (Regulation (EC) nº 853/2004). All assays were conducted in quintuplicate.

2.5.3. Enumeration of inoculated microorganisms

187 Levels of inoculated microorganisms (*L. monocytogenes* and LABs) were determined at
188 days 0 and 7 of incubation time. For this, inoculated "Torta del Casar" cheese-based agar
189 and dry-cured fermented sausage-based agar were mixed with 30 mL of 1 % (w/v)
190 peptone water and homogenized in a Stomacher device at 300 rpm for 1 min. Decimal
191 serial dilutions of the homogenate were subsequently carried out in 1% (w/v) peptone
192 water and then 100 μL of the cell suspensions were spread onto Chromagar™ *Listeria*
193 agar plates to determine *L. monocytogenes* counts and on MRS for LAB counts. Plates of
194 Chromagar™ *Listeria* agar were incubated at 37 ºC for 24-48 hours and characteristic *L.*
195 *monocytogenes* colonies (green colonies with a surrounded opaque halo) were counted,
196 and results expressed as log CFU/cm^2. MRS plates were incubated at 30 ºC for 48 h, under
197 microaerophilic conditions (ISO 15214:1998) and results were expressed as log CFU/
198 cm^2. Those LAB strains showing the highest activity against *L. monocytogenes* were
199 further selected for the next assays.

200 **2.6. Detection of genes encoding known bacteriocins**

201 The LAB strains showing the highest anti-*L.monocytogenes* activity in food model
202 systems were tested by PCR based on genes related to bacteriocins production (nisin,
203 lacticin 481, lactococcins A, B, G, Q and 972, brevicin, plantaracins A, E, F, S, NC8 and
204 pediocin PA) production. For this, total genomic DNA from overnight cultures of these
205 LAB strains was extracted according to the instructions of the MarterPure™ Complete
206 DNA and RNA Purification kit (Epicentre). The DNA obtained was eluted in TE Buffer
207 (10 mM Tris-HCl [pH 7.5], 1 mM EDTA; Epicentre) and kept at −20 °C until required.
208 The DNA concentration (ng/μL) and
209 purity (A_{260}/A_{280} ratio) were determined using a 1.5 μL aliquot on a NanoDrop
210 spectrophotometer (Thermo Fisher Scientific).

All primers used in the PCR reactions (Table 1) were previously reported for bacteriocin gene detection in LAB (Alegría et al., 2010; Ho et al., 2018; Holo et al., 2001; Maldonado et al., 2004; Rodríguez et al., 2000; Stephens et al., 1998; Xie et al., 2011). They were purchased from Sigma. For amplification of DNA amplicons from 44 to 1110 bp (Table 1), 50 µL reaction mixtures containing 2.5 mM $MgCl_2$, 1 µL reaction buffer, 100 µM each deoxynucleoside triphosphate (dNTP), 100 pmol of each primer, 5 U of Taq DNA polymerase (Thermo Fisher Scientific), and 250 ng of genomic DNA of selected LAB strains as the template were used, with a thermal cycler MASTER CYCLER® (Eppendorf). Several protocols were optimized for each gene encoding known bacteriocins (Table 2). After amplification, PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels using 1x Tris acetate EDTA (Scharlab S.L., Spain) at 90 V for 1 h. Gels were stained with 3 µL Red Safe Nucleic Acid Staining Solution (iNtRon Biotechnology, Korea). DNA bands were visualized with a UV transilluminator Gel Doc 2000 Image Documentation System (Bio-Rad Laboratories, USA) and then photographed by means of the camera GeneSnap and analyzed by the software equipment GeneTools (Syngene, UK). A DNA molecular size marker of 0.05–1.5 kbp (NZYTech Lda., Portugal) was used to determine the size of the PCR products.

2.7. Final characterization of the selected LAB strains with anti-*L. monocytogenes* by Pulsed-field Gel Electrophoresis typing

The most active LAB strains against *L. monocytogenes* were further characterized by Pulsed-field Gel Electrophoresis (PFGE) digesting the DNA with the restriction *SgsI* and *NotI* enzymes (Thermo Fisher Scientific) and the subsequent electrophoresis on the Chef Mapper® XA Pulsed Field Electrophoresis System (Bio-Rad Laboratories) (Figure 1). The protocol described by PulseNet (Graves & Swaminathan, 2001) was used with some modifications. Bacterial cells embedded in agarose Seakem® Gold (Lonza, Switzerland)

(plugs) with lysozyme (Sigma-Aldrich, USA) (20 mg/mL) were lyzed in lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0, 1% sarcosine, 0.1 mg/mL proteinase K [Thermo Fisher Scientific]) by incubating for 2 h at 55 °C in a water bath with agitation. The plugs were then washed twice in sterile ultrapure water and 4 times in TE buffer. The digestion of each DNA sample in the plugs was carried out with the *SgsI* (10 U/μL) and *NotI* (10 U/μL) enzymes for 3 h at 37 °C. The digestion of the control *Salmonella enterica* subsp. *enterica* serovar *Braenderup* (ATCC® BAA-664™) was carried out with the enzyme Xbal (10 U/μL)The PFGE was performed in 1% (w/v) agarose Seakem® Gold in TBE buffer (0.45 M TRIS, 0.45 M boric acid, 10 mM EDTA) in the Chef Mapper® XA Pulsed Field Electrophoresis System at 6 V/cm and 14 °C for 17 h with switch times of 1 s–15 s. Images were obtained with a Gel Doc 2000 Image Documentation System (Bio-Rad Laboratories), after staining with SYBR™ Gold Nucleic Acid Gel Stain (10.000X; Invitrogen, USA) for 15 min. The combined *SgsI/ NotI* PFGE pulsotypes were analyzed using the BioNumerics software (Applied Maths, Belgium).

2.8. Statistical analyses

The statistical treatment was carried out using the software IBM SPSS Statistic version 20 (IBM, USA). For the statistical analysis of data, LAB evaluated conditions were used as independent variables. The counts of *L. monocytogenes* (log CFU/ cm^2) were analyzed as dependent variables. Once dependent and independent variables of the analysis were determined, a normal distribution of obtained data was studied by using the Shapiro Wilk test. Subsequently, analyses were conducted using the Mann–Whitney test (Mann & Whitney, 1947) and statistical significance was established at $p \leq 0.05$.

3. RESULTS

3.1. Preliminary identification of LAB isolates and screening of anti-*L. monocytogenes* activity

A total of 371 isolates (189 obtained from cheese factories and 182 from dry-cured meat industries) were preliminarily considered as LAB. Most of these isolates were obtained from products at different stages of ripening (initial, half, and final maturation stages). From these LAB isolates, 84 showed inhibition zones (from 2 to 12 mm) against *L. monocytogenes* S7-2 in the initial screening using the agar spot assay. Of these, 52 were derived from cheese factories and 32 were isolated in the dry-cured fermented sausages industries (Table 3). In all of the sampled industries, active isolates were obtained, except in factory E, where no LAB isolates with anti-*L. monocytogenes* activity were found.

3.2. Molecular identification of selected LAB isolates by 16S rRNA sequencing

The 84 selected LAB isolates with anti- *L. monocytogenes* activity were identified by 16S rRNA sequencing. All of them belonged to the species *Enterococcus durans, Enterococcus faecium, Enterococcus hirae, Leuconostoc pseudomesenteroides, Leuconostoc mesenteroides, Lacticaseibacillus casei, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Lactilactobacillus sakei, Lactococcus garviae* and *Lactococcus lactis* (Table 3). The most frequent species isolated in the 12 raw ewe's milk cheeses factories tested was *Le. mesenteroides* while *Ll. sakei* was the predominant species between the isolates of the 3 dry-cured fermented sausages industries. The A cheese factory and the M dry-cured fermented sausage industry were the industries where more diversity of active species was found (Table 3).

3.3. Selection of LAB strains by their anti-*L. monocytogenes* activity on food models

Initial levels of both inoculated microorganisms (*L. monocytogenes* and LABs) were found around 3 log CFU/cm^2 at day 0 of inoculation and all of the tested LAB strains reaching values of about 5 log CFU/cm^2 after incubation of the inoculated food models for 7 days at 7 ºC (data not shown).

The growth/reduction of *L. monocytogenes* during the incubation time on the corresponding food model system depended on the inoculated LAB. Thus, it was observed that only 5 out of the 52 strains isolates from cheese industries had a significant ($p \leq 0.05$) reduction of *L. monocytogenes* compared to the control without LAB inoculation (Figure 2). These strains were *Lc. casei* 31, *Lc. casei* 116, *Lco. garviae* 151, *Lco. garviae* 156 and *Lc. paracasei* 185, observing the highest inhibition of *L. monocytogenes* in "Torta del Casar" cheese-based agar when co-inoculated with *Lc. casei* 116 (approximately of 2.35 log CFU/cm^2). The same occurs with LAB strains from dry-cured fermented sausage industries since only 5 out of the 32 strains tested showed a significant reduction of *L. monocytogenes* counts compared to the control (Figure 2). These strains were *Lc. paracasei* 13, *E. faecium* 188, *Ll. sakei* 197, *Ll. sakei* 204 and *Ll. sakei* 205. The highest inhibition of *L. monocytogenes* growth (2.5 log CFU/cm^2) was found with *Ll. sakei* 205. These 10 LAB strains were selected and further investigated for bacteriocin encoding gene detection.

3.4. Detection of bacteriocin structural genes

All the 10 selected LAB strains showed amplicons of the expected size for at least one of the genes encoding known bacteriocins (Table 4). Most of these strains amplified the gene pediocin PA. However, no PCR products were obtained with the specific primers based on lacticin 481, brevicin and plantaracin W (Table 4). *Lc casei* 116 and *Lco garviae* 151,

isolated in cheese industries, showed the highest number (5) of amplicons (Table 4). Thus, in *Lc casei* 116 the genes encoding nisin, lactacins A, B, G and Q and pediocin PA were detected, while in *Lco garviae* amplicons of the appropriate sizes for lactacins 972, B, G and Q, plantaracin EF and pediocin PA were found. It is also remarkable that the strains *Ll sakei* 31, from cheese origin, and *Lc paracasei* 13, from fermented sausage origin, showed amplicons for 3 of the bacteriocin encoding genes tested in this work (Table 4).

3.5. Final characterization by PFGE of the selected strains with anti-*L. monocytogenes* activity

The selected LAB strains showing the highest activity in food model systems were further characterized by the PFGE restriction analysis (Figure 3). PFGE profiles with the restriction *NotI* and *SgsI* enzymes of the most active LAB strains (*Ll. sakei* 31, *Lc. casei* 116 and *Lco. garviae* 151 from cheese origin and *Ll sakei* 197, 204 and 205 isolated from dry-cured fermented sausages industries), are shown in Figure 3. The PFGE profiles obtained with *Not I* showed 8-16 restriction fragments (depending on the strains), between 33 and 668 Kpb, that allow a clear differentiation between all of the tested strains. The enzyme *SgsI* generated PFGE profiles of restriction fragments that ranged from 10 to 16 depending on the strains (Figure 3), which also allow the differentiation of the selected LAB strains.

4. DISCUSSION

In this study, isolation, selection, and characterization of autochthonous LAB strains from traditional soft cheeses and dry-cured fermented sausages were carried out to be considered as bioprotective cultures for their ability to inhibit the *L. monocytogenes* growth. Traditional ripened foods constitute rich ecological niches for screening LAB

with anti-*L. monocytogenes* activity (Cocolin et al., 2007; Xiraphi et al., 2008). A total of 371 isolates obtained from different steps of processing of these products were preliminarily characterized as LAB. Although a higher number of cheese factories than meat industries was tested, the number of LAB isolates obtained was very similar. These LAB isolates were firstly tested for the anti-*L. monocytogenes* activity using a screening method based on agar spot assay. This method has been reported as appropriate for the screening of LAB strains with antimicrobial activities (Albano et al., 2007; Balouiri et al., 2016; Campagnollo et al., 2018). A 22.64% of the LAB isolates tested showed inhibition zones of *L. monocytogenes* higher than 2 mm, which could be because of competition for nutrients and space and/or production of antimicrobial compounds such as lactic acid and other organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide and bacteriocins (Kasra-Kermanshahi & Mobarak-Qamsari, 2015). The percentage of LAB isolates with anti-listerial activity was in the range of active strains reported with the agar spot assay in different foods (Fontana et al., 2015; Macaluso et al., 2016). When the origin of active isolates was analyzed, a higher percentage was obtained in cheese industries (61.9%) than in dry-cured fermented sausages factories (38.1%), in spite of the number of LAB isolates tested from the two types of industries was similar (189 from cheese factories and 182 from meat industries). It is possible that the ecological niche of ripened cheeses made with raw milk, usually with relative high levels of microbial contamination, favors that native LAB strains develop antimicrobial activity to be competitive in this food environment (Arqués et al., 2014).

When the selected active LAB isolates were characterized by 16S rRNA sequencing a total of eleven different LAB species was found. This method has been reported for the appropriate characterization of LAB strains isolated from ripened foods (Domingos-Lopes et al., 2017). All the species found have been extensively reported with

antimicrobial activity (Mohammed & Çon, 2021; Pellegrino et al., 2019; Rodríguez et al., 2000). Most of the eleven active species were found in both types of industries, except *Lc lactis* and *E. hirae* which were only detected in cheese factories, while *Ll. sakei* was only found in dry-cured fermented sausages factories, probably because this species is more adapted to this specific ecological niche (Nomura et al., 2006; Zagorec & Champomier-Vergès, 2017).

All the 84 different LAB strains selected by their anti-listerial activity belonging to the described eleven species were further tested by anti-*L. monocytogenes* activity in food models. Only 10 of the 84 LAB strains tested showed activity in the assays of co-inoculation in the food models tested. In the cheese model the most active LAB strain was *Lc. casei* 116 showing a reduction of 2.35 log CFU/cm^2 of *L. monocytogenes* while in dry-cured fermented "salchichón" model *Ll. sakei* 205 achieved a reduction of 2.5 log CFU/cm^2. Reductions in counts of this pathogen of 2 log CFU/ mL or cm^2 have also been reported with selected LAB strains in milk and cheese agar medium (García et al., 2020; Martín et al., 2022). Panebianco et al. (2021) reported reductions of *L. monocytogenes* levels from 0.5 to 1 log CFU/g both *in vitro* and in soft cheese by using selected *Ll. sakei* and *Lp. plantarum*. Although many LAB strains were selected for their ability to inhibit *L. monocytogenes*, in vitro assays, not all of them have been effective in real RTE ripened food systems (Martín et al., 2021; Panebianco et al., 2021). The food models used in the present work simulate conditions of temperature, water activity and pH conditions of cheese and dry-cured fermented sausages. This step in the selection of active LAB is of utmost importance to discard those strains lacking activity or with low effectiveness in the processing or storage conditions of ripened foods. After this evaluation 10 LAB strains able to reduce *L. monocytogenes* in conditions of ripening of cheeses and dry-cured fermented sausages, were obtained. These 10 LAB strains were investigated for

382 genes encoding known bacteriocins. The results justify the reduction of *L. monocytogenes*
383 counts in the food models since all these strains have at least one gene encoding known
384 bacteriocins. Selected LAB strains with anti-microbial activity isolated from ripened
385 products have been reported to produce different bacteriocins such as nisin lactococcins
386 and pediocins (Alegría et al., 2010; Salas et al., 2017; Xie et al., 2011). In the present
387 work, *Lc. casei* 116 showing the highest activity in the cheese model amplified the genes
388 encoding nisin, lactacins A, B, G and Q and pediocin PA. Regarding active LAB strains
389 from dry-cured fermented sausages origin, the most active strain in the "salchichón"
390 model was *Ll. sakei* 205 that only amplified the gene encoding Pediocin PA, probably
391 because the activity of this strain is not only due to the production of bacteriocins, but
392 also, to the production of organic acids, diacetyl, carbon dioxide, ethanol or hydrogen
393 peroxide (Kasra-Kermanshahi & Mobarak-Qamsari, 2015). In addition, other active
394 strains such as *Lc. paracasei* 13 showed amplicons for 3 of genes encoding bacteriocins
395 tested in this work.
396 Considering the activity in the food models and the presence of genes encoding known
397 bacteriocins, 6 LAB strains including *Ll. sakei* 31, *Lc. casei* 116 and *Lco. garviae* 151
398 from cheese origin and *Ll. sakei* 197, 204 and 205 isolated from dry-cured fermented
399 sausages industries, were selected as the most active strains against *L. monocytogenes*.
400 Thus, the procedure followed in the present work including first screening in agar spot
401 assay and further selection in food models allowed the selection of 6 LAB actives strains
402 active against *L. monocytogenes*, all of them possessing genes encoding known
403 bacteriocins. These strains could be proposed to be used in ripened cheeses or dry-cured
404 fermented sausages to control *L. monocytogenes*. In fact, the most active strain *Ll. sakei*
405 205 produced a reduction higher than 2 log CFU/g of *L. monocytogenes* counts during
406 ripening of dry-cured fermented "salchichón" elaborated according to the traditional

processing (Martín et al., 2021) and the most active strain *Lc. casei* 116 has been reported to reduce 2 log CFU/g of *L. monocytogenes* levels during ripening "Torta del Casar" soft cheese following an industrial process (unpublished data). This reduction could be sufficient to guarantee the elimination of *L. monocytogenes* throughout the processing of RTE ripened foods at the level of hypothetic contamination n (usually below 2 log CFU/g). This allows to minimize the risk of listeriosis and meet the microbiological criteria for this pathogen of ripened foods during their shelf-life as established by The European Union (Regulation (EC) nº 1441/2007). In addition, the selected LAB strains in this work have belonged to species with the recognition of "Qualified Presumption of Safety" (QPS) from the European Food Safety Authority (EFSA).

To evaluate the implantation of the selected actives strains throughout the food ripening process it is necessary a characterization method that allows distinguishing to the strain level. In this work, further characterization of the 6 selected strains was carried out by PFGE analysis using the restriction *SgsI* and *NotI* enzymes. With these two enzymes, clear differentiation of the 6 selected strains was obtained. This technique but using the restriction enzyme *Sfi*I has been reported to allow differentiation of some LAB species at strain level (Adesulu-Dahunsi et al., 2021). The combination of 16S rRNA sequencing with PFGE with restriction *SgsI* and *NotI* enzymes allows successful intraspecific differentiation to be used to control the implantation of these selected strains throughout the ripening process.

5. CONCLUSION

The procedure followed including first screening in agar spot and further selection in food models allowed selection of 6 LAB active strains actives against *L. monocytogenes*, all of them with genes encoding known bacteriocins. The most active strains *Lc. casei* 116

and *Ll. sakei* 205 provoked reductions higher than 2 log cycles of *L. monocytogenes* in cheeses and dry-cured fermented sausages models, respectively. Considering the safety and antimicrobial potential of the selected LAB, they may be utilized to control *L. monocytogenes* throughout the ripening process in ripened soft cheese and dry-cured fermented sausages. The combination of 16S rRNA sequencing with PFGE analysis using restriction *SgsI* and *NotI* enzymes could be useful to control the implantation of these selected strains throughout the ripening process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions: Conceptualization, J.J.C. and A.R.; methodology, I.M, A.A., R.M.; formal analysis, I.M.; investigation, I.M.; resources, I.M., A.R. and J.J.C.; data curation, I.M..; writing—original draft preparation, I.M. and J.J.C.; writing—review and editing, I.M., A.R., A.A., R.M. and J.J.C.; supervision, A.R. and J.J.C.; project administration, J.J.C and A.R..; funding acquisition, J.J.C. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This work has been funded by the INIA throughout the projects RTA-2017-00027-C03-03 and the Junta de Extremadura and FEDER by IB16149. GR15108 and GR18056. I. Martín is supporting by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

References

Adesulu-Dahunsi, A. T., Sanni, A. I., & Jeyaram, K. (2021). Diversity and technological characterization of *Pediococcus pentosaceus* strains isolated from Nigerian traditional fermented foods. *Lwt*, *140*, 110697. https://doi.org/10.1016/j.lwt.2020.110697

Albano, H., Oliveira, M., Aroso, R., Cubero, N., Hogg, T., & Teixeira, P. (2007). Antilisterial activity of lactic acid bacteria isolated from "Alheiras" (traditional Portuguese fermented sausages): In situ assays. *Meat Science*, *76*(4), 796–800. https://doi.org/10.1016/j.meatsci.2007.01.019

Alegría, Á., Delgado, S., Roces, C., López, B., & Mayo, B. (2010). Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk. *International Journal of Food Microbiology*, *143*(1–2), 61–66. https://doi.org/10.1016/j.ijfoodmicro.2010.07.029

Arqués, J. L., Rodríguez, E., Langa, S., Landete, J. M., & Medina, M. (2014). Antimicrobial activity of lactic acid bacteria in dairy products and gut: Effect on pathogens. *BioMed Research International*. https://doi.org/10.1155/2015/584183

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Campagnollo, F., Margalho, L., Kamimura, B., Feliciano, M., Freire, L., Lopes, L., Alvarenga, V., Cadavez, V., Gonzales-Barron, U., Schaffner, D., & Sant'Ana, A. (2018). Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. *Food Microbiology*, *73*, 288–297.

https://doi.org/10.1016/j.fm.2018.02.006

Carr, F. J., Chill, D., & Maida, N. (2002). The lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology*, *28*(4), 281–370. https://doi.org/10.1080/1040-840291046759

Cocolin, L., Foschino, R., Comi, G., & Grazia Fortina, M. (2007). Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. *Food Microbiology*, *24*(7–8), 752–758. https://doi.org/10.1016/j.fm.2007.03.001

Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. *Official Journal of the European Communitites*, *1441*.

Domingos-Lopes, M. F. P., Stanton, C., Ross, P. R., Dapkevicius, M. L. E., & Silva, C. C. G. (2017). Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. *Food Microbiology*, *63*, 178–190. https://doi.org/10.1016/j.fm.2016.11.014

Filipello, V., Gallina, S., Amato, E., Losio, M. N., Pontello, M., Decastelli, L., & Lomonaco, S. (2017). Diversity and persistence of *Listeria monocytogenes* within the Gorgonzola PDO production chain and comparison with clinical isolates from the same area. *International Journal of Food Microbiology*, *245*, 73–78. https://doi.org/10.1016/j.ijfoodmicro.2017.01.012

Fontana, C., Cocconcelli, P. S., Vignolo, G., & Saavedra, L. (2015). Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria. *Food Control*, *47*, 53–59. https://doi.org/10.1016/j.foodcont.2014.06.021

Gajbhiye, M. H., & Kapadnis, B. P. (2016). Antifungal-activity-producing lactic acid bacteria as biocontrol agents in plants. *Biocontrol Science and Technology*, *26*(11), 1451–1470. https://doi.org/10.1080/09583157.2016.1213793

García, M. J., Ruíz, F., Asurmendi, P., Pascual, L., & Barberis, L. (2020). Searching potential candidates for development of protective cultures: Evaluation of two *Lactobacillus* strains to reduce *Listeria monocytogenes* in artificially contaminated milk. *Journal of Food Safety*, *40*(1). https://doi.org/10.1111/jfs.12723

Gori, K., Mortensen, H. D., Arneborg, N., & Jespersen, L. (2007). Ammonia production and its possible role as a mediator of communication for *Debaryomyces hansenii* and other cheese-relevant yeast species. *Journal of Dairy Science*, *90*(11), 5032–5041. https://doi.org/10.3168/jds.2006-750

Graves, L. M., & Swaminathan, B. (2001). PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. *International Journal of Food Microbiology*, *65*(1–2), 55–62. https://doi.org/10.1016/S0168-1605(00)00501-8

Ho, V. T. T., Lo, R., Bansal, N., & Turner, M. S. (2018). Characterisation of *Lactococcus lactis* isolates from herbs, fruits and vegetables for use as biopreservatives against *Listeria monocytogenes* in cheese. *Food Control*, *85*, 472–483. https://doi.org/10.1016/j.foodcont.2017.09.036

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S., & Nes, I. F. (2001). Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology*, *147*(3), 643–651. https://doi.org/10.1099/00221287-147-3-643

ISO 15214:1998. Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of mesophilic lactic acid bacteria-Colony-count technique at 30

527 degrees C.

528 Kasra-Kermanshahi, R., & Mobarak-Qamsari, E. (2015). Inhibition effect of lactic acid
529 bacteria against food born pathogen, *Listeria monocytogenes*. *Applied Food*
530 *Biotechnology*, *2*(4), 11–19. https://doi.org/10.22037/afb.v2i4.8894

531 Macaluso, G., Fiorenza, G., Gaglio, R., Mancuso, I., & Scatassa, M. L. (2016). In vitro
532 evaluation of bacteriocin like inhibitory substances produced by lactic acid bacteria
533 isolated during traditional sicilian cheese making. *Italian Journal of Food Safety*,
534 *5*(1), 20–22. https://doi.org/10.4081/ijfs.2016.5503

535 Maldonado, A., Jiménez-Díaz, R., & Ruiz-Barba, J. L. (2004). Induction of plantaricin
536 production in *Lactobacillus plantarum* NC8 after coculture with specific Gram-
537 positive bacteria is mediated by an autoinduction mechanism. *Journal of*
538 *Bacteriology*, *186*(5), 1556–1564. https://doi.org/10.1128/JB.186.5.1556-
539 1564.2004

540 Mann, H. B., & Whitney, D. . (1947). On a test of whether one of two random variables
541 is stochastically larger than the other. *The Annals of Mathematical Statistics*, *2*(5),
542 347–370. http://projecteuclid.org/euclid.aop/1176996548

543 Martín, I., Rodríguez, A., Alía, A., Martínez-Blanco, M., Lozano-Ojalvo, D., & Córdoba,
544 J. J. (2022). Control of *Listeria monocytogenes* growth and virulence in a traditional
545 soft cheese model system based on lactic acid bacteria and a whey protein
546 hydrolysate with antimicrobial activity. *International Journal of Food*
547 *Microbiology*, *361*(March 2021), 109444.
548 https://doi.org/10.1016/j.ijfoodmicro.2021.109444

549 Martín, I., Rodríguez, A., Sánchez-Montero, L., Padilla, P., & Córdoba, J. J. (2021).
550 Effect of the dry-cured fermented sausage “salchichón” processing with a selected

Lactobacillus sakei in *Listeria monocytogenes* and microbial population. *Foods*, *10*(4). https://doi.org/10.3390/foods10040856

Mohammed, S., & Çon, A. H. (2021). Isolation and characterization of potential probiotic lactic acid bacteria from traditional cheese. *Lwt*, *152*(April), 112319. https://doi.org/10.1016/j.lwt.2021.112319

Noda, M., Miyauchi, R., Danshiitsoodol, N., Higashikawa, F., Kumagai, T., Matoba, Y., & Sugiyama, M. (2015). Characterization and mutational analysis of a two-polypeptide bacteriocin produced by citrus Iyo-derived *Lactobacillus brevis* 174A. *Biological and Pharmaceutical Bulletin*, *38*(12), 1902–1909. https://doi.org/10.1248/bpb.b15-00505

Nomura, M., Kobayashi, M., Narita, T., Kimoto-Nira, H., & Okamoto, T. (2006). Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. *Journal of Applied Microbiology*, *101*(2), 396–405. https://doi.org/10.1111/j.1365-2672.2006.02949.x

Panebianco, F., Giarratana, F., Caridi, A., Sidari, R., De Bruno, A., & Giuffrida, A. (2021). Lactic acid bacteria isolated from traditional Italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese. *Lwt*, *137*(July 2020), 110446. https://doi.org/10.1016/j.lwt.2020.110446

Pellegrino, M. S., Frola, I. D., Natanael, B., Gobelli, D., Nader-Macias, M. E. F., & Bogni, C. I. (2019). In vitro characterization of lactic acid bacteria isolated from bovine milk as potential probiotic strains to prevent bovine mastitis. *Probiotics and Antimicrobial Proteins*, *11*(1), 74–84. https://doi.org/10.1007/s12602-017-9383-6

Perin, L. M., Savo Sardaro, M. L., Nero, L. A., Neviani, E., & Gatti, M. (2017). Bacterial ecology of artisanal Minas cheeses assessed by culture-dependent and -independent

methods. *Food Microbiology*, *65*, 160–169. https://doi.org/10.1016/j.fm.2017.02.005

Regulation (EC) No 853/2004 of the European Parliament and of the Council. In *Official Journal of the European Union* (Issue L139).

Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews*, *4*(2), 124–140. https://doi.org/10.1007/s12393-012-9051-2

Rodriguez, C., Taminiau, B., García-Fuentes, E., Daube, G., & Korsak, N. (2021). *Listeria monocytogenes* dissemination in farming and primary production: Sources, shedding and control measures. *Food Control*, *120*, 107540. https://doi.org/10.1016/j.foodcont.2020.107540

Rodríguez, E., González, B., Gaya, P., Nuñez, M., & Medina, M. (2000). Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal*, *10*(1–2), 7–15. https://doi.org/10.1016/S0958-6946(00)00017-0

Salas, L. M., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal microbial agents for food biopreservation—a review. *Microorganisms*, *5*(3), 1–35. https://doi.org/10.3390/microorganisms5030037

Shamloo, E., Hosseini, H., Moghadam, A. Z., Larsen, H. M., Haslberger, A., & Alebouyeh, M. (2019). Importance of *Listeria monocytogenes* in food safety: A review of its prevalence, detection, and antibiotic resistance. *Iranian Journal of Veterinary Research*, *20*(4), 241–254.

Siedler, S., Balti, R., & Neves, A. R. (2019). Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. *Current Opinion in Biotechnology*, *56*, 138–

146. https://doi.org/10.1016/j.copbio.2018.11.015

Singh, P. (2018). Recent approaches in food bio-preservation-a review. *Open Veterinary Journal*, *8*(1), 2226–4485. https://doi.org/10.4314/ovj.v8i1.16

Smith, A., Moorhouse, E., Monaghan, J., Taylor, C., & Singleton, I. (2018). Sources and survival of *Listeria monocytogenes* on fresh, leafy produce. *Journal of Applied Microbiology*, *125*(4), 930–942. https://doi.org/10.1111/jam.14025

Stephens, S. K., Floriano, B., Cathcart, D. P., Bayley, S. A., Witt, V. F., Jiménez-Díaz, R., Warner, P. J., & Ruiz-Barba, J. L. (1998). Molecular analysis of the locus responsible for production of plantaricin S, a two-peptide bacteriocin produced by *Lactobacillus plantarum* LPCO10. *Applied and Environmental Microbiology*, *64*(5), 1871–1877. https://doi.org/10.1128/aem.64.5.1871-1877.1998

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Frontiers in Microbiology*, *10*(57). https://doi.org/10.3389/fmicb.2019.00057

Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., & Alatossava, T. (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Applied and Environmental Microbiology*, *66*(1), 297–303. https://doi.org/10.1128/AEM.66.1.297-303.2000

Xie, Y., An, H., Hao, Y., Qin, Q., Huang, Y., Luo, Y., & Zhang, L. (2011). Characterization of an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. *Food Control*, *22*(7), 1027–1031.

https://doi.org/10.1016/j.foodcont.2010.12.007

Xiraphi, N., Georgalaki, M., Rantsiou, K., Cocolin, L., Tsakalidou, E., & Drosinos, E. H. (2008). Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroides* E131. *Meat Science*, *80*(2), 194–203. https://doi.org/10.1016/j.meatsci.2007.11.020

Yap, P.-C., MatRahim, N.-A., AbuBakar, S., & Lee, H. Y. (2021). Antilisterial potential of lactic acid bacteria in eliminating *Listeria monocytogenes* in host and ready-to-eat food application. *Microbiology Research*, *12*(1), 234–257. https://doi.org/10.3390/microbiolres12010017

Zagorec, M., & Champomier-Vergès, M.-C. (2017). *Lactobacillus sakei:* A starter for sausage fermentation, a protective culture for meat products. *Microorganisms*, *5*(3), 56. https://doi.org/10.3390/microorganisms5030056.

Table 1. Primers used for PCR amplification of the bacteriocin-related genes

Target gene	Primers' name	Primer sequence (5´-3´)	Size amplicon (bp)	References
Nisin	nisZ-prom-F nisP3	CTCGACGATACCATCACTCTTC TCTTTCCCATTAACTTGTACTGTG	1010	(Ho et al., 2018)
Lacticin 481	lact-481-F lact481-R	TCTGCACTCACTTCATTAGTTA AAGGTAATTACACCTCTTTTAT	366	(Rodríguez et al., 2000)
Lactococcin 972	lcn972-F lcn972-R	TTGTAGCTCCTGCAGAAGGAACATGG GCCTTAGCTTTGAATTCTTACCAAAAG	312	(Alegría et al., 2010)
Lactococcin G and Q	lactGQ-F lactGQ-R	GAAAGAATTATCAGAAAAAG CCACTTATCTTTATTTCCCTCT	620	(Alegría et al., 2010)
Lactococcin A and B	lcnAB-F lcnA-R lcnB-R	GAAGAGGCAATCAGTAGAG GTGTTCTATTTATAGCTAATG CCAGGATTTTCTTTGATTTACTTC	771	(Alegría et al., 2010)
Brevicin	brevicin 174A-F brevicin 174A-R	GTCTTAAATGCTAGGCTTGTCA CTGGCAAGACAAACGGTTAG	766	(Noda et al., 2015)
Plantaracin A	pnlA-F pnlA-R	TAGAAATAATTCCTCCGTACTTC ATTAGCGATGTAGTGTCATCCA	573	(Xie et al., 2011)
Plantaracin EF	plnEF-F pnlEF-R	TATGAATTGAAAGGGTCCGT GTTCCAAATAACATCATACAAGG	516	(Xie et al., 2011)
Pediocin PA	pediocin PA-1-F pediocin PA-1-R	AAAGATACTGCGTTGATAGG GAGAAGCCATGCTGAAAG	1120	(Xie et al., 2011)
Plantaracin NC8	pnlNC8-F pnlNC8-R	GGTCTGCGTATAAGCATCGC AAATTGAACATATGGGTGCTTTAAATTCC	159	(Maldonado et al., 2004)
Plantaracin S	pnlS-F pnlS-R	GCCTTACCAGCGTAATGCCC CTGGTGATGCAATCGTTAGTTT	320	(Stephens et al., 1998)
Plantaricin W	pnlW-F pnlW-R	TCACACGAAATATTCCA GGCAAGCGTAAGAAATAAATGAG	44	(Holo et al., 2001)

Table 2. Optimized conditions used for PCR amplification of the bacteriocin-related genes.

Target gene	Stages		
	Stage 1 (1 cycle)	Stage 2 (30 cycles)	
Nisin	95 ºC, 6 min	95 ºC, 30 s	50 ºC, 30s
Lacticin 481			
Lactococcin 972			
Lactococcin G and Q			
Lactococcin A and B			
Brevicin		96 ºC, 1 min	56 ºC, 30 s
Plantaracin A		94 ºC, 30s	55 ºC, 1 min
Plantaracin EF			54 ºC, 1 min
Pediocin PA			50 ºC, 1 min
Plantaracin NC8			60 ºC, 1 min
Plantaracin S		94 ºC, 1 min	60 ºC, 1 min
Plantaricin W			55.5 ºC,1 min

All PCR reactions have a final extension step consisting in 72ºC for 5 min.

Table 3. Distribution of the lactic-acid bacteria isolates from cheese and meat industries showing anti-*Listeria monocytogenes* activity in the agar plot assay.

Identification	Cheese												Dry-cured fermented sausages			Total
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
Enterococcus durans	1	2	-	-	-	-	-	-	-	-	-	-	-	3	-	6
Enterococcus faecium	1	1	-	-	-	-	-	-	-	-	-	-	2	3	2	9
Enterococcus hirae	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Leuconostoc pseudomesenteroides	1	-	-	-	-	2	-	-	-	-	-	-	1	-	-	4
Leuconostoc mesenteroides	-	1	3	-	-	1	12	-	-	2	-	-	-	-	1	20
Lacticaseibacillus casei	6	-	1	-	-	-	-	-	1	-	-	-	-	1	-	9
Lactiplantibacillus plantarum	-	-	-	1	-	-	1	-	-	-	-	-	1	-	-	3
Lacticaseibacillus paracasei	1	1	5	-	-	-	-	-	-	-	1	-	1	-	-	9
Lactilactobacillus sakei	-	-	-	-	-	-	-	-	-	-	-	-	-	5	6	11
Lactococcus garvieae	1	1	-	-	-	-	-	-	-	-	-	-	2	3	-	7
Lactococcus lactis	-	-	-	-	-	-	-	3	-	1	-	1	-	-	-	5
Total isolates	11	6	9	1	0	3	13	3	1	3	1	1	8	15	9	84
	52												32			

Table 4. Bacteriocin encoding genes of the ten selected LAB with the highest anti-*L. monocytogenes* activity

	LAB strains	Target gene												
		nisin	lact481	lcn972	lcnG and Q	lcnA	lcnB	brevi	plnA	plnEF	PedioPA	pnlNC8	pnlS	pnlW
Cheeses	*Ll. sakei* 31	-	-	-	-	-	-	-	-	X	X	-	X	-
	Lc. casei 116	X	-	-	X	X	X	-	-	-	X	-	-	-
	Lco. garviae 151	-	-	X	X	-	X	-	-	X	X	-	-	-
	Lco. garviae 156	-	-	-	-	-	-	-	-	-	-	X	X	-
	Lc. paracasei 185	-	-	-	-	-	-	-	-	-	-	-	X	-
Dry-cured fermented sausages	*Lc. paracasei* 13	-	-	-	-	-	-	-	X	-	X	X	-	-
	E. faecium 188	X	-	-	-	-	-	-	-	-	-	-	-	-
	Ll. sakei 197	-	-	-	-	-	-	-	-	-	-	-	X	-
	Ll. sakei 204	-	-	-	-	-	-	-	-	X	X	-	-	-
	Ll. sakei 205	-	-	-	-	-	-	-	-	-	X	-	-	-

Nisin (nisin), lact481 (lacticin 481), lcn972 (lactococcin 972) lcnG and Q (lactococcin G, Q), lcnA(lactococcin A), lcnB (lactococcins B), brevi (brevicin), plnA (plantaracin A), plnEF (plantaracin EF), pedioPA(pediocin PA),pnlNC8(plantaracin NC8), pnlS (plantaracin S) and pnlW (plantaracin W).

Figure 1. Diagrammatic flowchart for the isolation and selection of lactic-acid bacteria strains with anti- *L. monocytogenes* activity from dry-cured fermented sausages and ripened cheeses.

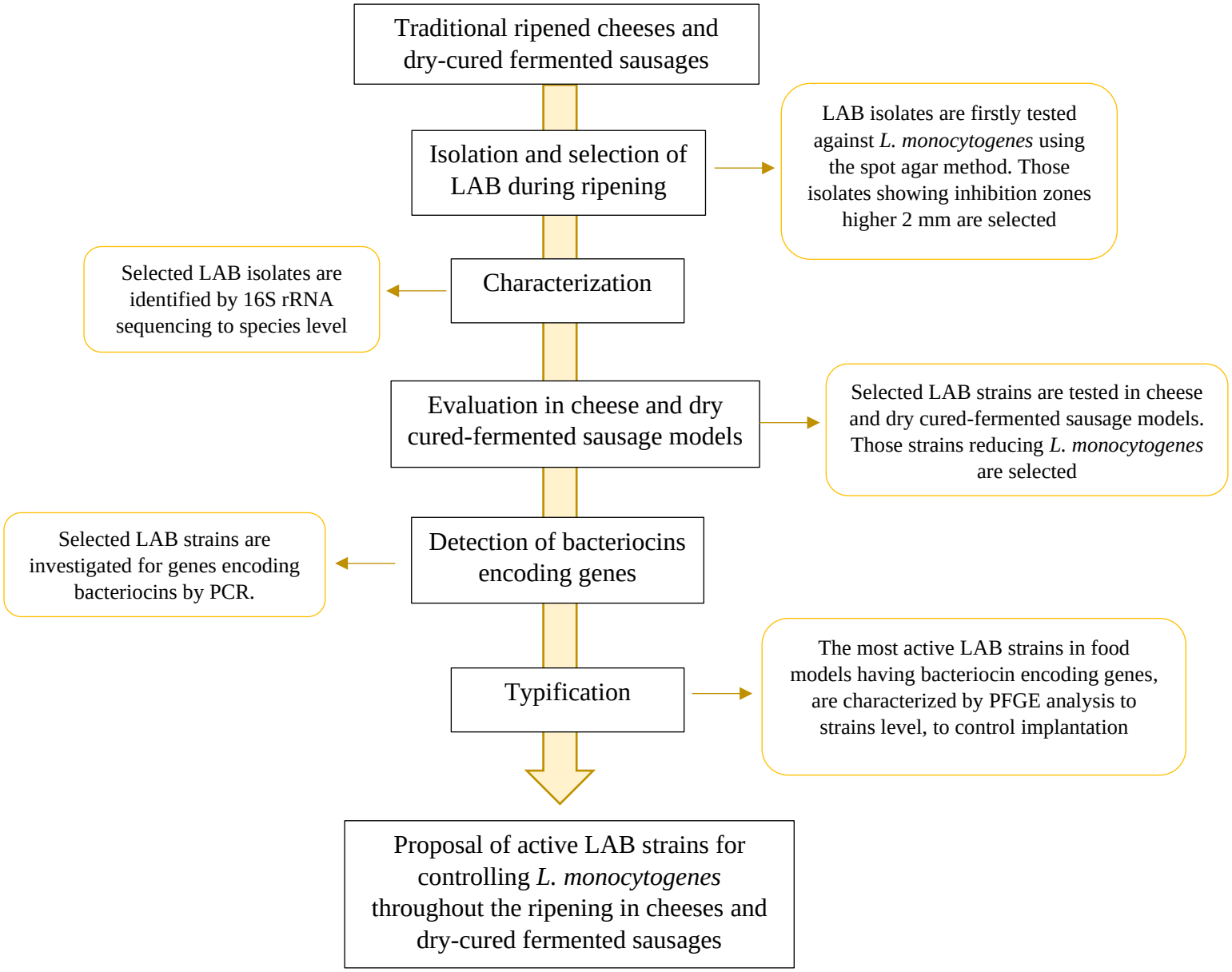


Figure 2. Reduction of the levels of *L. monocytogenes* counts in food models (cheese-based agar and dry-cured fermented sausages-based agar) after incubation for 7 days at 7ºC. *Lacticaseibacillus casei* 31, *Lacticaseibacillus casei* 116, *Lactococus garviae* 151, *Lactococus garviae* 156 and *Lacticaseibacillus paracasei* 185, *Lacticaseibacillus paracasei* 13, *Enterococcus faecium* 188, *Lactilactobacillus sakei* 197, *Lactilactobacillus sakei* 204 and *Lactilactobacillus sakei* 205.

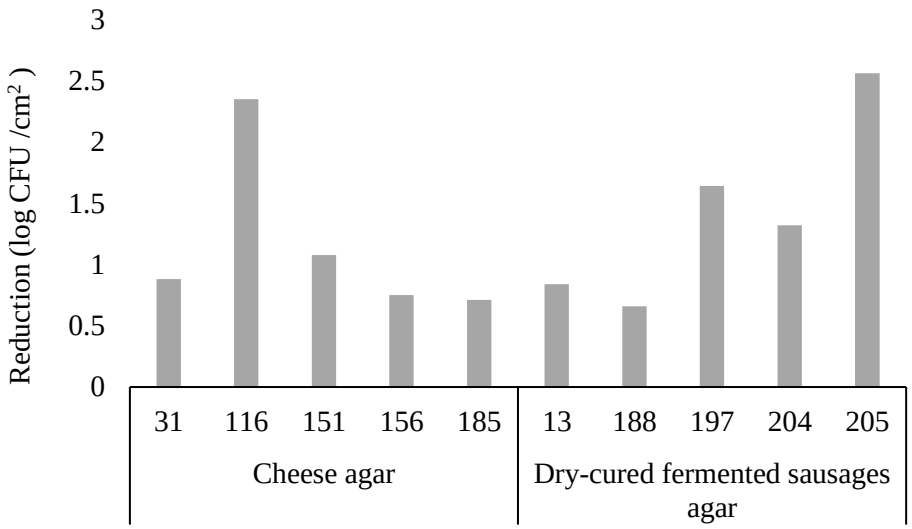
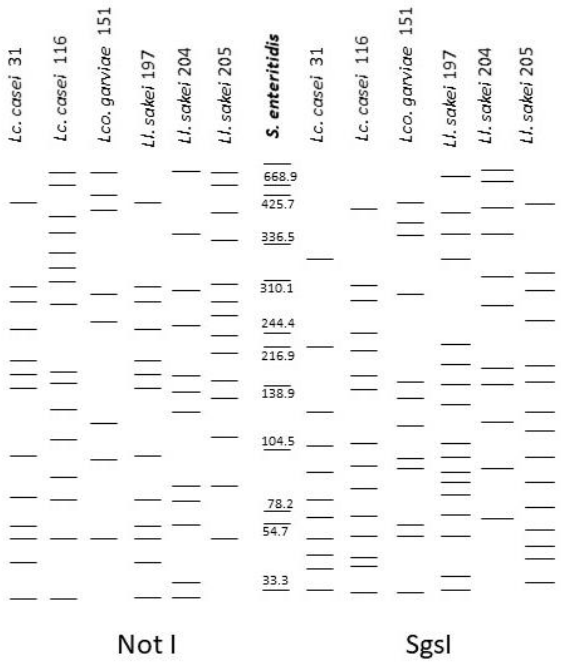


Figure 3. Pulsed Field Gel Electrophoresis (PFGE) profiles of selected lactic-acid bacteria by using the restriction enzymes *NotI* and *SgsI*. *Lacticaseibacillus casei* 31, *Lacticaseibacillus casei* 116, *Lactococus garviae* 151, *Lactilactobacillus sakei* 197, *Lactilactobacillus sakei* 204 and *Lactilactobacillus sakei* 205. *Salmonella enterica* subsp. *enterica* serovar *Braenderup* was used as control.



International Journal of Food Microbiology 361 (2022) 109444

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Control of *Listeria monocytogenes* growth and virulence in a traditional soft cheese model system based on lactic acid bacteria and a whey protein hydrolysate with antimicrobial activity

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ARTICLE INFO

Keywords:
L. monocytogenes
Biocontrol
Traditional cheese
Growth reduction
Virulence

ABSTRACT

"Torta del Casar" is a Spanish soft-ripened cheese made with sheep's raw milk and subjected to a short ripening process, which favors the growth of pathogenic microorganisms including *Listeria monocytogenes*. The development of strategies to control pathogens and minimize health risks associated with the presence of *L. monocytogenes* in these products is of great interest. In this regard, the anti-*Listeria* activity of a whey protein hydrolysate (ProH) alone or combined with six lactic acid bacteria strains isolated from cheese was evaluated in this study as a biocontrol strategy using a "Torta del Casar" cheese-based medium. The most active combinations of lactic acid bacteria assayed induced a reduction higher than two logarithmic units in the growth of *L. monocytogenes* (serotype 4b) compared to their respective control when they were co-inoculated in "Torta del Casar" cheese-based medium at 7 °C for 7 days. In addition, the observed downregulation of some key virulence genes of *L. monocytogenes* suggests that the strain *Lactiplantibacillus plantarum* B2 alone and combined with the strain *Lactiplantibacillus* spp. B4 are good candidates to be used as biocontrol agents against *L. monocytogenes* growth in traditional soft cheeses based on raw milk during their storage at refrigeration temperatures.

1. Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, psychotropic, facultative anaerobic, catalase positive, and non-spore-forming bacterium (Rodriguez et al., 2021). This bacterium is an opportunistic foodborne pathogen causative of listeriosis, which is a human disease that induces life-threatening infections primarily in the immune-suppressed populations including pregnant women, newborns, the elderly, AIDS patients, and organ transplant recipients (Bhunia, 2008; Vitullo et al., 2013). In the United States, this bacterium causes approximately 1600 hospitalizations each year that result in 260 deaths (Shamloo et al., 2019), and 2621 confirmed cases of human listeriosis have been reported in the European Union in 2019 (EFSA and ECDC, 2021).

The ability of *L. monocytogenes* to survive and grow when is exposed to adverse conditions such as moderate pH and reduced temperature as well as high concentrations of sodium chloride, makes difficult to control this microorganism in food (Faber and Peterkin, 1991; Lebreton and Cossart, 2017). This foodborne pathogen can be present in a wide range of raw and processed foods being several of them ready-to-eat products including seafood, dairy products, meat products, fruits, soft cheeses, ice creams, raw milk, candied apples, frozen vegetables, and poultry (CDC, 2020). Due to its ability to resist adverse conditions, *L. monocytogenes* is the pathogen most commonly found in cheeses, not only in raw milk cheeses, but also pasteurized milk cheeses, and in most cases, it derives from cross-contamination (Campagnollo et al., 2018; Di Ciccio et al., 2012; Santorum et al., 2012).

Elimination of *L. monocytogenes* in cheese is highly important since it has been demonstrated that this bacterium persists throughout the cheese food chain from farm to fork (Lahou and Uyttendaele, 2017). For this reason, different strategies able to control and reduce the presence of this pathogen in foods are being currently investigated (Tumbarski et al., 2018). The fight against the presence of this hazard is more serious and challenging in traditional high-quality soft-ripened cheeses elaborated with raw milk in Spain because they are produced under Protected

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https://doi.org/10.1016/j.ijfoodmicro.2021.109444
Received 14 March 2021; Received in revised form 8 October 2021; Accepted 17 October 2021
Available online 21 October 2021

Designations of Origin (P.D.O.) such as "*Torta del Casar*" which is a traditional cheese elaborated in Extremadura, a southwest region located in Spain, in accordance with the Regulation (CE) 1491/2003 of the European Commission. P.D.O. regulations normally forbid practices or approaches such as the addition of substances or microorganisms which do not belong to the product itself.

Although conventional preservation techniques such as intense heat treatments, salting, acidification, drying, and chemical preservation have been widely used to control and minimize risks associated with the presence of *L. monocytogenes* in traditional foods (Amit et al., 2017; Jan et al., 2017), they have started to be replaced by new conservation techniques including high hydrostatic pressure, pulsed electric fields, new packaging systems, and biopreservation (Barba et al., 2018; Devlieghere et al., 2004; Jan et al., 2017). In this regard, biopreservation, understood as the use of safe natural or controlled or their metabolites as a way to improve food safety and extending its shelf life (Singh, 2018), has been described as a good alternative compared to other strategies or methods since its effects on organoleptic and sensorial characteristics of cheeses is negligible (Demers-Mathieu et al., 2013; Ho et al., 2018). Nowadays, consumers reject food with chemical additives and they look for safer products made with natural and minimally processed ingredients (Yang et al., 2014). Thus, biopreservation may assure fulfillment of consumers' expectations.

The use of native microorganisms to reduce foodborne pathogens could be a very attractive approach for their control because they are ecologically adapted to the food products. In this regard, the use of LAB strains has been proposed as a strategy to inhibit *L. monocytogenes* (Cintas et al., 1998; Mataragas et al., 2003; Scatassa et al., 2017) in different foods including soft and semi-hard cheeses. Some LAB strains are safe for their use in the food industry as they are included in the European Food Safety Authority (EFSA) list of qualified presumptions of safety (EFSA, 2021). The high adaptability of some of these bacteria to a wide range of environmental conditions occurring during cheese manufacture, together with the fact that they are generally recognized as safe (GRAS), facilitate their use to control *L. monocytogenes* growth in this dairy product (Cleveland et al., 2001; Gálvez et al., 2007; Quinto et al., 2016).

On another side, some studies have recently reported that antimicrobial peptides derived from the hydrolysis of milk proteins using gastrointestinal enzymes could be considered as natural additives to preserve food safety (Demers-Mathieu et al., 2013; Lozano-Ojalvo et al., 2017). These peptides have shown a strong ability to control or even inhibit the growth of pathogenic and non-pathogenic bacteria (Clare et al., 2005; López Expósito and Recio, 2006).

In this work, we aimed to evaluate the potential effect of different LAB strains alone, combined, or together with an already described hydrolysate of whey proteins containing antimicrobial peptides (ProH, Lozano-Ojalvo et al., 2017) on *L. monocytogenes* growth throughout the processing of a traditional soft-ripened cheese. However, this evaluation cannot be directly conducted in the industry due to the sanitary risk caused by the experimental use of *L. monocytogenes* as well as the difficulties derived from the production of sterile conditions that allow the direct evaluation of *L. monocytogenes* growth without interferences induced by other microbial populations present during the cheese ripening in the industry.

For these reasons, to properly evaluate the individual and/or combined effect of LAB strains and ProH on *L. monocytogenes* in a traditional soft-ripened cheese obtained from raw milk, a system based on a homemade "Torta del Casar" cheese-based agar that can be easily sterilised has been designed. The objective of this study was to investigate the effect of different combinations of six LAB strains and a whey protein hydrolysate (ProH) on the growth and the expression of genes involved in virulence and stress response of *L. monocytogenes* in a "Torta del Casar" cheese-based agar following conventional processing and storage conditions used in the industry.

2. Materials and methods

2.1. Microorganisms

Three strains belonging to *Lactiplantibacillus plantarum* (B2, B5 and B6), two strains from *Leuconostoc mesenteroides* (B1 and B3) and one strain ascribed to *Lactiplantibacillus spp.* (B4) were selected due to their anti-*listerial* activity from more than 80 isolates belong to traditional soft-ripened cheeses ("*Torta del Casar*") processed in three industries located in Cáceres (Spain) after conducting initial experiments. Sequential dilutions of the samples were homogenized in 1% peptone water (Pronadisa, Madrid, Spain), plated onto de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, UK), and anaerobically incubated at 30 °C for 72 h. Then the isolates were maintained at −80 °C in MRS Broth containing 20% (*w/v*) glycerol (Fisher Bioreagent, Belgium) for long storage. The identification of the LAB strains was performed by sequencing analysis of the 16S rRNA region according to the methodology proposed by Walter et al. (2000).

The strain *L. monocytogenes* S7–2 (serotype 4b) whose origin is ripened products and belonging to National Institute of Agricultural and Food Research and Technology (INIA) collection (Madrid, Spain) was used to evaluate the antilisterial activity determined in the present work. This strain was then maintained at −80 °C in Brain Heart Infusion (BHI) Broth (Pronadisa, Madrid, Spain) containing 20% (*w/v*) glycerol for long storage.

2.2. Preparation of inocula

To prepare the individual LAB inoculum, each isolate was inoculated in MRS Broth and incubated for 24 h at 30 °C. After incubation, cultures were centrifuged for 3–5 min at 10,000 rpm. The pellet obtained was then resuspended in 200 μL MRS Broth and prepared at a final concentration of 10^9 CFU /mL.

To prepare the *L. monocytogenes* inoculum, 100 μL of the stock of *L. monocytogenes* S7–2 (serotype 4b) were transferred to 10 mL BHI Broth and incubated for 24 h at 37 °C. Finally, 100 μL of this culture were then transferred to a second tube of 10 mL BHI, incubated overnight for 24 h at 37 °C, and prepared at a final concentration of ≈ 10^8 CFU /mL.

2.3. Protein hydrolysate

The ProH was produced from a whey protein isolate (Arla Foods Ingredients, Sønderhøj, Denmark) dissolved in 50 mM citrate buffer, pH 1.5, at a concentration of 5 mg/mL. The protein solution was pre-incubated at 37 °C for 10 min prior to the addition of 172 U/mg protein of porcine pepsin (EC 3.4.23.1, 3440 U/mg; Sigma-Aldrich, St. Louis, MO, USA). The mixture was vacuum-sealed in polyethylene bags and pressurized at 400 MPa and 37 ± 2 °C using an Iso-lab 900 High-Pressure Food Processor (Mod FPG7 100:9/2C, Stansted Fluid Power Ltd. Essex, UK) with water as pressure-transmitting fluid. The pressure was raised at a rate of 600 MPa/min, maintained for 30 min, and released in <4 s. After removal from the high-pressure unit, the pepsin reaction was stopped by raising the pH to 7.0 with 2 N NaOH. The protein content of the hydrolysate (ProH) was determined by the Kjeldahl method and it was lyophilized and stored at −20 °C until further use.

Whey protein solution and ProH were characterized by RP-HPLC using an Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany). Absorbance was recorded at 214 nm with an Agilent 1100 Series variable wavelength detector. Chromatographic separations were performed with a Hi-Pore® Reversed-Phase RP-318 Column (Bio-Rad Laboratories, CA, USA). Operating conditions were column at ambient temperature; flow rate, 0.8 mL/min; injection volume, 50 μL (1 mg/mL); solvent A, 0.37 mL/L trifluoroacetic acid (TFA) in Milli-Q water; solvent B, 0.27 mL/L of TFA in HPLC-grade acetonitrile

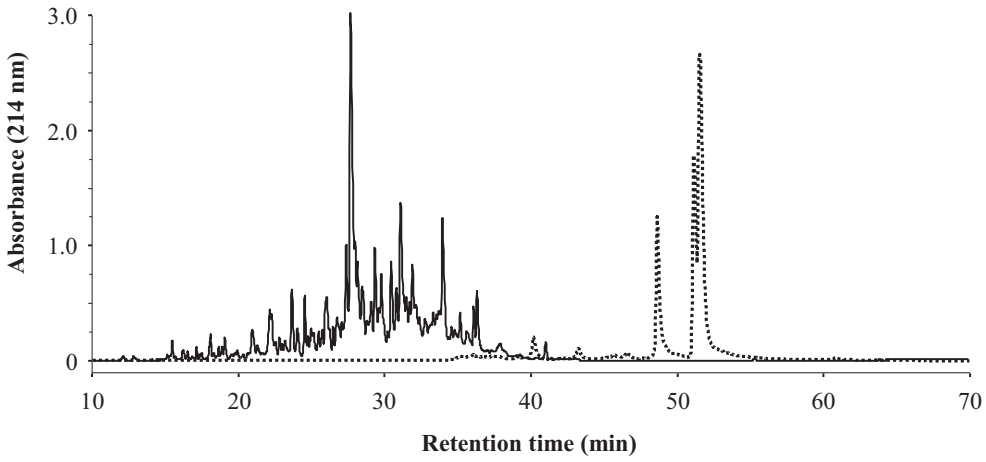

Fig. 1. RP-HPLC pattern of whey proteins hydrolyzed with pepsin under high pressure (ProH). The profile of the intact whey proteins is drawn with a discontinuous grey line.

(Scharlau Chemie, Barcelona, Spain). The elution was performed with a linear gradient of solvent B in A going from 0 to 70% in 70 min.

The chromatographic pattern of ProH was determined by RP-HPLC, showing complete disappearance of the intact whey proteins after their hydrolysis under the high-pressure conditions assayed (Fig. 1).

2.4. Preparation of a "Torta del Casar" cheese-based agar

"Torta del Casar" cheese-based agar has been used as a model system to evaluate the effectiveness of LAB strains and/or ProH against *L. monocytogenes*. This "Torta del Casar" cheese-based agar was prepared by autoclaving 20 g of bacteriological agar (Pronadisa, Madrid, Spain) in 500 mL of deionized water for 20 min at 121 °C (103 kPa). Next, it was mixed with 200 g of freeze-dried "Torta del Casar" cheese and 12.5 g of trisodium citrate dehydrate (Scharlab, Barcelona, Spain) heated in 400 mL of deionized water at 100 °C for 1 h (Gori et al., 2007). Finally, the pH was adjusted to 6.0 using lactic acid and the water activity (a_w) value was 0.975 a_w before cooling down the medium to 45–50 °C in a water bath with shaken vigorously prior to pouring 15 mL into a 25 cm^2 Petri dish.

2.5. Experimental settings

The effect of LAB strains and ProH on *L. monocytogenes* growth was evaluated in the "Torta del Casar" cheese-based agar following 20 different combinations (Table 1). As shown in Table 1, firstly the effect of 4 LAB (B1-B4) alone and in combination with ProH in individual and grouped ways was checked (1st step). Secondly, two new LAB strains (B5 and B6) were tested alone, together with ProH and combined with B2 and B4 strains which were strains with the best antagonistic effect results against *L. monocytogenes* in the 1st step (2nd step). Finally, the influence of the LAB strains B2 and B4 in the absence and the presence of ProH was evaluated (3rd step).

Briefly, 100 μL of the ProH at a concentration of 0.5 μg/μL were firstly spread plated on the soft cheese-based agar surface and dry out for 5–10 min to evaluate anti-*L. monocytogenes* activity. The evaluation of antimicrobial agents against different microorganisms on the surface of culture medium has been reported as appropriate methods (Balouiri et al., 2016). For this, the inoculum of *L. monocytogenes* 4b (50 μL) and/or LAB strains (in a total volume of 50 μL) were inoculated reaching final concentrations of 10^3 CFU/cm^2. For this, the original inocula of both types of microorganisms were diluted in 1% (*w/v*) peptone water. Samples were incubated for 7 days at 7 °C. This temperature was used to simulate cooling temperature according to the maximum temperature allowed for the "Torta del Casar" cheese storage (Official Journal of the European Union, 2004). After incubation time, "Torta del Casar" cheese-based agar was mixed with 30 mL of 1% (*w/v*) peptone water and homogenized in a Stomacher device (Seward, model 400 Circulator, West Sussex, UK) at 300 rpm for 1 min. Finally, 1 mL of this homogenate was used for RNA extraction and 1 mL for preparing decimal serial dilution to enumerate *L. monocytogenes*. All experiments were conducted in quintuplicate.

Table 1
Batches names tested in this study including microorganisms and the experimental step number.

Batch name	Microorganisms	Step number
Control	*L. monocytogenes* alone	1
B1	*L. monocytogenes* combined with B1	1
B2	*L. monocytogenes* combined with B2	1
B3	*L. monocytogenes* combined with B3	1
B4	*L. monocytogenes* combined with B4	1
B5	*L. monocytogenes* combined with B5	2
B6	*L. monocytogenes* combined with B6	2
ProH	*L. monocytogenes* combined with ProH	1
ProH+B1	*L. monocytogenes* combined with ProH and B1	1
ProH+B2	*L. monocytogenes* combined with ProH and B2	1
ProH+B3	*L. monocytogenes* combined with ProH and B3	1
ProH+B4	*L. monocytogenes* combined with ProH and B4	1
ProH+B5	*L. monocytogenes* combined with ProH and B5	2
ProH+B6	*L. monocytogenes* combined with ProH and B6	2
B1+B2+B3+B4	*L. monocytogenes* combined with B1, B2, B3, B4	1
ProH+B1+B2+B3+B4	*L. monocytogenes* combined with ProH and B1, B2, B3, B4	1
B2+B4+B5+B6	*L. monocytogenes* combined with B2, B4, B5, B6	2
ProH+B2+B4+B5+B6	*L. monocytogenes* combined with ProH and B2, B4, B5, B6	2
B2+B4	*L. monocytogenes* combined with B2 and B4	3
ProH+B2+B4	*L. monocytogenes* combined with ProH and B2 and B4	3

2.6. Enumeration of L. monocytogenes

Decimal serial dilutions of the homogenate were subsequently carried out in 1% (*w/v*) of peptone water and then 100 μL of the cell suspensions were spread onto Chromagar™ *Listeria* agar plates. Plates were

incubated at 37 °C for 24 h, characteristic *L. monocytogenes* colonies (green colonies with a surrounded opaque halo) counted, and results expressed as Log CFU/cm^2.

2.7. Gene expression analysis

2.7.1. RNA extraction

Extraction of RNA was performed using MasterPure™ DNA & RNA Purification Kit (Epicentre, Madison, Wisconsin, USA), according to the manufacturer's instructions. After extraction, RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and stored at −80 °C.

2.7.2. cDNA synthesis

Synthesis of cDNA was carried out using 5 μL of total RNA (100 ng/μL) according to the Prime Script™ RT Reagent kit protocol (Takara Bio Inc., Japan) as described by the manufacturer and samples were stored at −20 °C until their further use for real-time PCR (qPCR).

2.7.3. Quantitative real-time PCR conditions

The ViiA™ 7 Real-Time System (Applied Biosystems, USA) was used to carry out the qPCR amplification and detection. Reactions were prepared in triplicate in MicroAmp Optical 96-well Reaction Plates (Applied Biosystems, USA) and sealed with optical adhesive covers (Applied Biosystems, USA). Three replicates of RNA control samples and a template-free negative controls were also included in each plate. The sequences of primers based on the genes *plcA*, *hly, iap, sigB* and *16S rRNA*, the PCR mixtures and amplification conditions are shown in Tables 2, 3 and 4, respectively. After the final PCR cycle, melting curve analyses of the PCR products were performed by heating to 72–95 °C including continuous measurement of the fluorescence for verification. Quantitative results of qPCR assays were expressed as an exponential cycle number and threshold cycle (Ct) determined.

2.7.4. Gene expression analysis

Relative quantification of the expression of the *hly, sigB, iap,* and *plcA* genes was calculated using the housekeeping gene *16S* rRNA as an endogenous control (reference gene) to normalise the quantification of the target mRNA. The expression ratio was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The necessary requirements to use the mentioned method were firstly fulfilled. Therefore, it was shown that the experimental treatment did not affect the expression of the housekeeping gene. Control conditions corresponded to *L. monocytogenes* grown in the absence of neither LAB nor ProH.

Table 2
Sequences of primers used in this study.

Genes	Primers	Sequence (5′-3′)
16S	16S-f	ACGAACGGAGGAAGAGCTTG
	16S-r	CCCCAACTTACAGGCAGGTT
plcA	plcA-f	CTAGAAGCAGGAATACGGTACA
	plcA-r	ATTGAGTAATCGTTTCTAAT
hly	hly-f	CATGGCACCACCAGCATC T
	hly-r	ATCCGCGTGTTTCTTTTCGA
iap	iap-f	AAT CTG TTA GCG CAA CTT GGT TAA
	iap-r	CAC CTT TGA TGG ACG TAA TAA TAC TGT T
sigB	sigB-f	CCAAGAAAATGGCGATCAAGAC
	sigB-r	CGTTGCATCATATCTTCTAATAGCT

Table 3
Real-time quantitative PCR (qPCR) mixtures used for gene expression analyses.

Gene	Primer concentration (nM)	Volume (μL/sample)						
		Water	ROX	SYBR® Green	$MgCl_2$	Forward primer	Reverse primer	cDNA
16S	300	2.875	0.125	6.25	–	0.375	0.375	2.5
plcA	300	2.125	0.125	7	–	0.375	0.375	2.5
hly	450	1	0.125	7	0.75	0.5625	0.5625	2.5
iap	300	2.125	0.125	7	–	0.375	0.375	2.5
sigB	300	2.125	0.125	7	–	0.375	0.375	2.5

Table 4
Real-time quantitative PCR (qPCR) conditions used for gene expression analyses.

Gene	Stages		
	Stage 1 (1 cycle)	Stage 2 (40 cycles)	
16S	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min
plcA			55 °C, 1 min
hly			60 °C, 1 min
iap			60 °C, 1 min
sigB			55 °C, 1 min

2.8. Statistical analyses

The statistical treatment was carried out using the software IBM SPSS Statistic version 20 (IBM, USA). For the statistical analysis of data, LAB and ProH evaluated conditions were used as independent variables. The counts of *L. monocytogenes* (Log CFU /cm^2) and Log_2 values of relative gene expression were analysed as dependent variables. Once dependent and independent variables of the analysis were determined, a normal distribution of obtained data was studied by using the Shapiro Wilk test. Subsequently, analyses were conducted using the Mann–Whitney test (Mann and Whitney, 1947) and statistical significance was established at $p \leq 0.05$.

3. Results

3.1. Effect on the Listeria monocytogenes growth

Growth levels of *L. monocytogenes* in presence or absence of LAB strains and/or ProH are illustrated in Fig. 2. Counts of both kind of microorganisms (*L. monocytogenes* and LAB) reached counts around 5 log CFU/cm^2 after incubation for 7 days at 7 °C (Data not shown). Regarding the experimental conditions where *L. monocytogenes* was inoculated together with each of the six LAB strains used in this study, it was observed that only in two out of the six strains had a significant growth inhibition of *L. monocytogenes* compared to the control. These strains were B2 and B4, being this inhibition approximately of 1.35 and 0.7 Log CFU/cm^2, respectively. The remaining LAB strains (B1, B3, B5, B6) did not induce neither significant growth reduction of the foodborne pathogen nor stimulation of its growth.

The combination of two or four LAB strains (B1+B2+B3+B4, B2+B4+B5+B6, and B2+B4) showed a significant inhibitory potential against *L. monocytogenes* ($p \leq 0.05$). Specifically, after 7 days of incubation at 7 °C, inhibition of *L. monocytogenes* was 0.6, 0.92, and 2 Log CFU/cm^2 for the batches B1+B2+B3+B4, B2+B4, and B2+B4+B5+B6, respectively. This last batch showed the greatest growth reduction of *L. monocytogenes* (Supplementary Table).

The remaining 10 batches applied together with ProH in presence or absence of individual or combined LAB strains displayed variable results on its effectiveness to control *L. monocytogenes* growth. When ProH was inoculated alone (batch ProH), it did not cause significant differences compared to the control. However, when the hydrolysate was combined

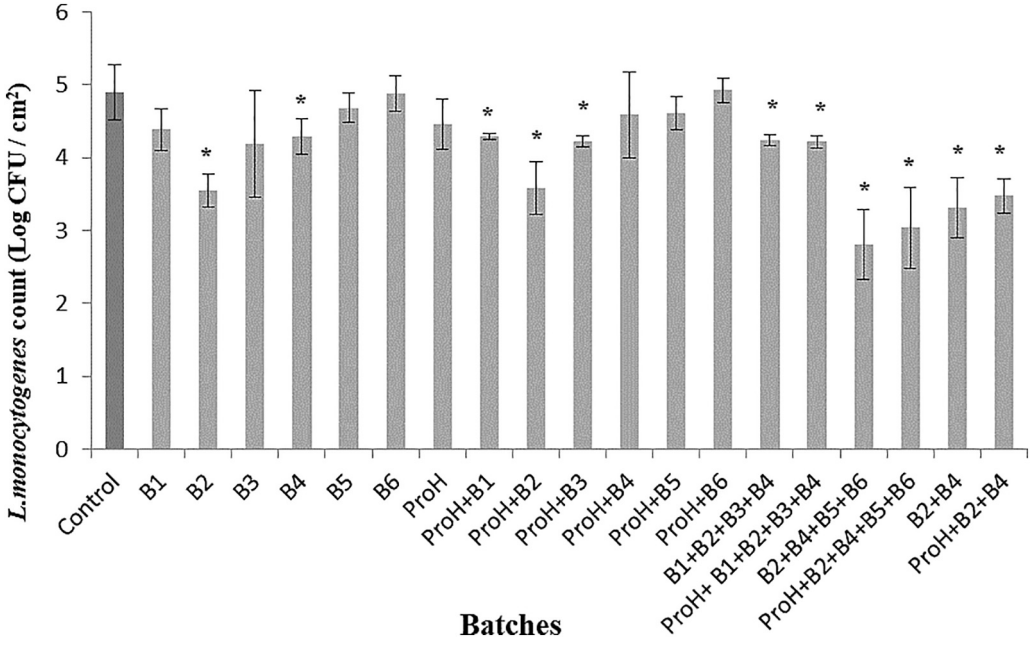

Fig. 2. Changes in the population of *Listeria monocytogenes* grown in "Torta del Casar" cheese-based agar at 7 °C for 7 days in the presence of different combinations of LAB strains and ProH. The assays were conducted in quintuplicate. Bars represent standard deviation of the mean values. Change in the population with asterisk indicates significant differences ($p \leq 0.05$) compared to the control (*L. monocytogenes* alone).

with the bacterial strains B1, B2 and B3 (batches ProH+B1, ProH+B2 and ProH+B3), a significant inhibitory effect on the growth of *L. monocytogenes* was observed (Supplementary Table). Specifically, reduction of *L. monocytogenes* in those batches was 0.61, 1.32, and 0.68 Log CFU/cm^2, respectively. The remaining batches (ProH+B4, ProH+B5, and ProH+B6) did not induce either inhibition or stimulation of the pathogen growth. However, when the ProH was inoculated with the three combinations of four LAB strains (ProH+B1+B2+B3+B4,

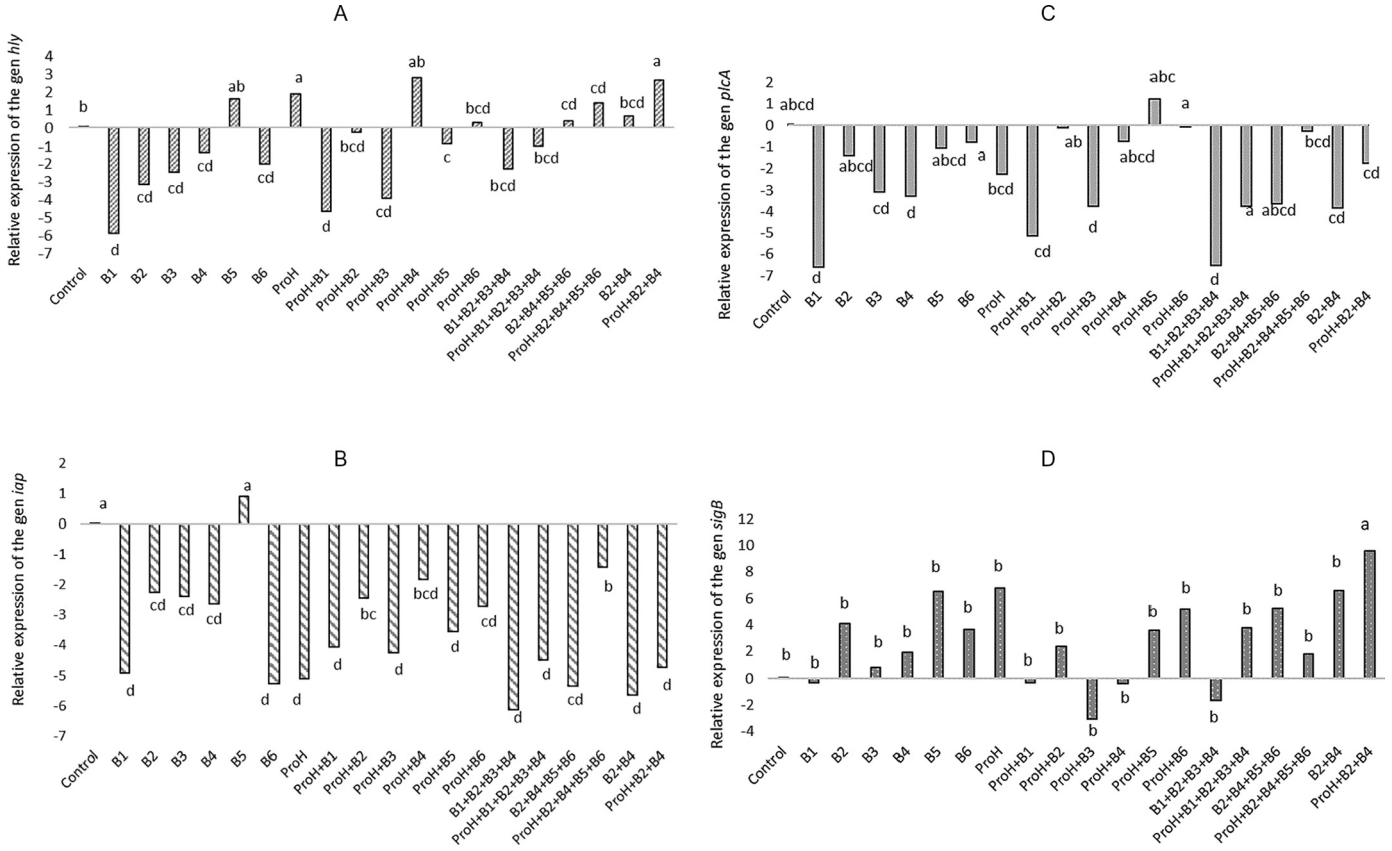

Fig. 3. Effect of different LAB strains and ProH combinations on the relative expression of the *hly* (A), *iap* (B), *plcA* (C), and *sigB* (D) genes of *Listeria monocytogenes* grown in "Torta del Casar" cheese-based agar at 7 °C for 7 days compared to the control (control batch without LAB and ProH, value = 0). Mean values of the *hly, iap*, *plcA* and *SigB* gene transcription with different letters indicate significant differences ($p \leq 0.05$).

ProH+B2+B4+B5+B6, and ProH+B2+B4) a significant antagonistic effect was observed ($p \leq 0.05$). Indeed, counts of *L. monocytogenes* were reduced at 0.68, 1.86, and 1.42 Log CFU/cm^2, respectively.

3.2. Effect on the relative expression of hly, iap, plcA and sigB genes by L. monocytogenes

Fig. 3 shows the effects of different combinations of LAB strains and/or ProH on the relative expression of the *hly*, *iap*, *plcA,* and *sigB* gene by *L. monocytogenes*. The relative expression of the target genes under the different conditions evaluated (*L. monocytogenes* combined with LAB strains and/or ProH) was compared to the control (*L. monocytogenes* alone).

3.2.1. hly gene

Several batches showed a tendency to increase the expression of this gene (B5, ProH, ProH+B4, ProH+B6, B2+B4+B5+B6, ProH+B2+B4+B5+B6, B2+B4, and ProH+B2+B4), but only batches ProH and ProH+B2+B4 significantly increased ($p \leq 0.05$) the expression of *hly* gene compared to the control. In addition, some batches (B1, B2, B3, B4, B6, ProH+B1) showed relative expression values of *hly* gene lower than the control ($p \leq 0.05$).

In general, the presence of ProH did not increase the expression of this gene compared to the batch inoculated with the specific LAB strain in the absence of ProH, except in the case of ProH+B4. In addition, batch ProH showed an upregulation of this gene compared to the control batch.

In those batches where two or more LAB strains were inoculated together with ProH, the relative expression of the *hly* gene related to the virulence of L. *monocytogenes* was always higher than when ProH was not added (ProH+B1+B2+B3+B4, ProH+B2+B4+B5+B6, and ProH+B2+B4). Indeed, the batch ProH+B2+B4 presented significant differences ($p \leq 0.05$) compared to the batch B2+B4, in which ProH was not added.

3.2.2. iap gene

Although batch B5 showed a slight activation of the expression of this gene, it was not significantly different ($p > 0.05$) compared to the control. Most of the remaining batches showed a significant inhibition of the relative expression of this gene referred to the control. Batches inoculated with ProH increased their expression of the *iap* gene, but it was only significant ($p \leq 0.05$) for ProH+B1+B2+B3+B4 compared to the batch combining the same LAB strains in absence of ProH (batch B1+B2+B3+B4).

3.2.3. plcA gene

None of the batches analysed showed significant differences compared to the control ($p > 0.05$). Only in the case of the batch ProH+B5 there was a tendency to increase the expression of this gene, while in the remaining batches data pointed out an inhibition of its expression.

3.2.4. sigB gene

Fourteen out of the 20 batches showed an activation of this gene expression, but only the batch ProH+B2+B4 showed significant differences ($p \leq 0.05$) compared to the control. The presence of ProH did not produce significant changes in the expression of *sigB* gene compared to the batches without the hydrolysate, except for the batch ProH+B2+B4, where the expression of *sigB* gene was statistically higher than batch B2+B4.

4. Discussion

In this study the effectiveness of two antagonist strategies (LAB isolated from "Torta del Casar" and ProH) against *L. monocytogenes* was checked in a "Torta del Casar" cheese-based agar following conventional processing and storage conditions used in the industry.

Results have shown that only two of the LAB strains, *L. plantarum* B2 and *Lactiplantibacillus spp* B4, had an antimicrobial effect against *L. monocytogenes* when LAB were inoculated alone. Other studies showed similar results when assayed single inoculations of LAB ascribed to the same species (García et al., 2020; Quinto et al., 2016). Other authors have shown the ability of LAB to compete with *L. monocytogenes* avoiding its growth (Arena et al., 2016; Campagnollo et al., 2018; García et al., 2020; Scatassa et al., 2017). Independently of the combination of LAB strains used, a reduction in the growth of this pathogenic microorganism was observed compared to the control without LAB. Specifically, the highest inhibition was observed when B2+B4+B5+B6 was applied. However, the use of combined LAB strains to inhibit the growth of *L. monocytogenes* has not been enough investigated (Melia et al., 2017; Morandi et al., 2019; Scatassa et al., 2017).

On the other hand, peptides derived from hydrolysed food proteins have recently attracted a great interest since they have exhibited various physiological activities such as anti-inflammatory, anti-allergic, antihypertensive, and anti-microbial activities (Hernández-Ledesma et al., 2014). Indeed, several hydrolysates derived from milk proteins have shown an inhibitory activity against certain microorganisms including *L. monocytogenes*, *Bacillus subtilis*, *Escherichia coli,* and *Pseudomonas aeruginosa* (Demers-Mathieu et al., 2013; Hernández-Ledesma et al., 2014) and they may provide an added value to by-products of the dairy industry. Besides the antibacterial activity observed in milk-derived hydrolysates, Lozano-Ojalvo et al. (2017) have recently demonstrated that ProH (whey proteins hydrolysed with pepsin under high hydrostatic pressure) is hypoallergenic not only by showing a reduced ability to induce sensitization to whey proteins in a mouse model of food allergy, but also by its capacity to retained sufficiently immunogenicity to stimulate immune Th2 responses without generating milk-specific IgE antibodies, pointing out that this hypoallergenic hydrolysate may maintain peptides with different bioactivities (Lozano-Ojalvo et al., 2017). In addition, the industrial relevance of ProH is based on its short-term production time, accessibility of the protein source (whey protein) in the dairy industry, its low price, and that it is in concordance with the European guidelines on infants and follow-on formulas related to the reduction of risks to allergy to milk proteins.

In this study, the utilisation of ProH has been shown to be effective against *L. monocytogenes* growth in a cheese-based medium when combined with the LAB strains B1, B2, and B3 but not either alone or in combination with other LAB strains. In addition, the effect of ProH in presence of B1 suggests that the hydrolysate may enhance the effect of this strain increasing its ability to compete for nutrients with *L. monocytogenes*, producing undissociated lactic acid, or decreasing a_w due to salt diffusion (Gonzales-Barron et al., 2020; Kapetanakou et al., 2017).

The influence that strategies combining LAB strains and the antimicrobial peptide ProH has on gene transcription profiles of *L. monocytogenes* growing on cheeses has been scarcely studied. This type of study may help in understanding the mode of action of LAB strains combined with ProH as well as their effect on the control of *L. monocytogenes* growth and virulence. Therefore, one of the purposes of this work was to evaluate the impact of both control strategies (LAB and ProH) on the relative expression of four genes involved in virulence and stress response (*plcA*, *hly*, *iap*, and *sigB*) of *L. monocytogenes*. The results suggest that the expression of *hly, plcA* and *iap* genes were repressed or similar to the control in most of the batches analysed. This behavior of *L. monocytogenes* was previously observed after the exposure of the pathogen to essential oils (Hadjilouka et al., 2017; Pieta et al., 2017), as well as *Debaryomyces hansenii* in sliced dry-cured ham (Alía et al., 2020).

In addition, *L. monocytogenes* may respond rapidly to adverse conditions or environment changes based on the alternative sigma factor, SigB (σ^B), encoded by *sigB*. This gene contributes to the ability of *L. monocytogenes* to adapt and survive underlying stress conditions encountered in foods such as acidic or osmotic conditions (Ferreira

et al., 2001; Hadjilouka et al., 2016). In this work, the upregulation of *sigB* gene expression was observed in most of the batches studied, but only the batch ProH+B2+B4 showed significant differences compared to the control. This result agrees with those previously reported in dry-cured fermented sausages, with decreasing values of pH and a_w during the manufacturing process, and also when the pathogen was exposed to 3% NaCl (Mataragas et al., 2015; Olesen et al., 2010).

Taking together these results, they suggest that although the combination of ProH and two LAB strains (B2 and B4) (batch ProH+B2+B4) repressed the growth of *L. monocytogenes* in a cheese-based agar, it activated substantially the expression of both *hly* and *sigB* genes, increasing the virulence of this pathogen. It has been previously observed that *sigB* gene plays a key role in the expression of other virulence genes such as *hly* (Hadjilouka et al., 2016). In the case of other batches that significantly inhibited the growth of *L. monocytogenes* cells such as batches B2+B4 and more concisely B2, the expression of the *hly* and *sigB* genes was not overexpressed compared to the control (*L. monocytogenes* alone). In these batches, surviving pathogen cells could be highly injured, which may lead to an increased expression of genes involved in cell damage repair mechanisms and to repress the expression of virulence genes.

From the results, the strain *Lactiplantibacillus plantarum* B2 alone and combined with the strain *Lactiplantibacillus* B4 could be considered as a biocontrol strategy to control *L. monocytogenes*. The anti-*L. monocytogenes* activity of both strains should be further tested in soft cheeses made with raw milk throughout the ripening process in a pilot plant following industrial conditions. In this evaluation, the effect of microbial competition should be checked, to understand in soft cheeses made with raw milk the mechanisms of microbial interaction to perform a quantitative risk assessment process, as has been reported by Panebianco et al. (2021) in soft cheese made with pasteurized milk. So far, some strains of LAB isolated from soft-ripened cheeses and selected by their anti-*L. monocytogenes* activity in cheese-based agar following the method used in the present work, have shown to be effective reducing up to 2–3 log Log CFU/cm^2 the counts of this pathogen throughout the ripening (≈ 60–90 days) of the "Torta del Casar" cheese in the presence of competitive microbial population (Martin et al., 2021).

In conclusion, our findings suggest that the use of the LAB *Lactiplantibacillus plantarum* B2 alone and combined with the strain *Lactiplantibacillus* B4 could be proposed as a biocontrol strategy to counteract the presence of *L. monocytogenes* in traditional cheeses produced with raw milk during their storage at refrigeration temperatures. However, the application of the hydrolysate ProH together with LAB strains seems to be not advisable for controlling *L. monocytogenes* in this sort of cheeses since it appears to enhance the virulence of this pathogenic bacterium.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109444.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been funded by the INIA throughout the projects RTA-2017-00027-C03-03 and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. We thank the Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM) for supplying the hydrolysate obtained from whey proteins. I. Martín is supporting by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

References

Alía, A., Córdoba, J.J., Rodríguez, A., García, C., Andrade, M.J., 2020. Evaluation of the efficacy of *Debaryomyces hansenii* as protective culture for controlling *Listeria monocytogenes* in sliced dry-cured ham. Lwt 119, 108886. https://doi.org/10.1016/j.lwt.2019.108886.

Amit, S.K., Uddin, M.M., Rahman, R., Islam, S.M.R., Khan, M.S., 2017. A review on mechanisms and commercial aspects of food preservation and processing. Agric. Food Secur. 6, 1–22. https://doi.org/10.1186/s40066-017-0130-8.

Arena, M.P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., Fiocco, D., 2016. Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. Front. Microbiol. 7, 1–10. https://doi.org/10.3389/fmicb.2016.00464.

Balouiri, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for in vitro evaluating antimicrobial activity: a review. J. Pharm. Anal. 6, 71–79. https://doi.org/10.1016/j.jpha.2015.11.005.

Barba, F.J., SantAna, A.S., Orlien, V., Koubaa, M., 2018. Innovative technologies for food preservation: Inactivation of spoilage and pathogenic microorganisms. Elsevier. https://doi.org/10.1016/C2016-0-00177-8.

Bhunia, A.K., 2008. Foodborne microbial pathogens: Mechanisms and pathogenesis. Springer, New York. https://doi.org/10.1007/978-0-387-74537.

Campagnollo, F., Margalho, L., Kamimura, B., Feliciano, M., Freire, L., Lopes, L., Alvarenga, V., Cadavez, V., Gonzales-Barron, U., Schaffner, D., Sant'Ana, A., 2018. Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. Food Microbiol. 73, 288–297. https://doi.org/10.1016/j.fm.2018.02.006.

CDC (Centers for Disease Control and Prevention), 2020. *Listeria* (listeriosis): Prevention. Available online. https://www.cdc.gov/listeria/prevention.html. (Accessed 26 February 2021).

Cintas, L.M., Casaus, P., Fernández, M.F., Hernández, P.E., 1998. Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin a and lactocin S against spoilage and foodborne pathogenic bacteria. Food Microbiol. 15, 289–298. https://doi.org/10.1006/fmic.1997.0160.

Clare, D., Catignani, G., Swaisgood, H., 2005. Biodefense properties of milk: the role of antimicrobial proteins and peptides. Curr. Pharm. Des. 9, 1239–1255. https://doi.org/10.2174/1381612033454874.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Review article. Int. J. Food Microbiol. 71, 1–20. https://doi.org/10.1016/S0168-1605(01)00560-8.

Demers-Mathieu, V., Gauthier, S.F., Britten, M., Fliss, I., Robitaille, G., Jean, J., 2013. Inhibition of *Listeria monocytogenes* growth in Cheddar cheese byananionic peptides-enriched extract from whey proteins. Int. Dairy J. 32, 6–12. https://doi.org/10.1016/j.idairyj.2013.03.008.

Devlieghere, F., Vermeiren, L., Debevere, J., 2004. New preservation technologies: possibilities and limitations. Int. Dairy J. 14, 273–285. https://doi.org/10.1016/j.idairyj.2003.07.002.

Di Ciccio, P., Conter, M., Zanardi, E., Ghidini, S., Vergara, A., Paludi, D., Festino, A.R., Ianieri, A., 2012. *Listeria monocytogenes*: biofilms in food processing. Ital. J, Food Sci.

EFSA, ECDC, 2021. The European Union one health 2019 zoonoses report. EFSA J. 19 (2), 1–286. https://doi.org/10.2903/j.efsa.2021.6406.

Faber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511 (doi:0146-0749/91/030476-36$02.00/0).

Ferreira, A., O'Byrne, C.P., Boor, K.J., 2001. Role of σ B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. 67, 4454–4457. https://doi.org/10.1128/AEM.67.10.4454-4457.2001.

Gálvez, A., Abriouel, H., López, R.L., Omar, N.Ben, 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120, 51–70. https://doi.org/10.1016/j.ijfoodmicro.2007.06.001.

García, M.J., Ruíz, F., Asurmendi, P., Pascual, L., Barberis, L., 2020. Searching potential candidates for development of protective cultures: evaluation of two lactobacillus strains to reduce *Listeria monocytogenes* in artificially contaminated milk. J. Food Saf. 40 https://doi.org/10.1111/jfs.12723.

Gonzales-Barron, U., Campagnollo, F.B., Schaffner, D.W., Sant'Ana, A.S., Cadavez, V.A.P., 2020. Behavior of *Listeria monocytogenes* in the presence or not of intentionally-added lactic acid bacteria during ripening of artisanal Minas semi-hard cheese. Food Microbiol. 91.

Gori, K., Mortensen, H.D., Arneborg, N., Jespersen, L., 2007. Ammonia production and its possible role as a mediator of communication for *Debaryomyces hansenii* and other cheese-relevant yeast species. J. Dairy Sci. 90, 5032–5041. https://doi.org/10.3168/jds.2006-750.

Hadjilouka, A., Molfeta, C., Panagiotopoulou, O., Paramithiotis, S., Mataragas, M., Drosinos, E.H., 2016. Expression of *Listeria monocytogenes* key virulence genes during growth in liquid medium, on rocket and melon at 4, 10 and 30 °C. Food Microbiol. 55, 7–15. https://doi.org/10.1016/j.fm.2015.11.008.

Hadjilouka, A., Mavrogiannis, G., Mallouchos, A., Paramithiotis, S., Mataragas, M., Drosinos, E.H., 2017. Effect of lemongrass essential oil on *Listeria monocytogenes* gene expression. LWT - Food Sci. Technol. 77, 510–516. https://doi.org/10.1016/j.lwt.2016.11.080.

Hernández-Ledesma, B., García-Nebot, M.J., Fernández-Tomé, S., Amigo, L., Recio, I., 2014. In: Dairy protein hydrolysates : Peptides for health benefits, 38, pp. 82–100. https://doi.org/10.1016/j.idairyj.2013.11.004.

Ho, V.T.T., Lo, R., Bansal, N., Turner, M.S., 2018. Characterisation of lactococcus lactis isolates from herbs, fruits and vegetables for use as biopreservatives against *Listeria*

monocytogenes in cheese. Food Control 85, 472–483. https://doi.org/10.1016/j.foodcont.2017.09.036.
Jan, A., Sood, M., Sofi, S.A., Norzom, T., 2017. Non-thermal processing in food applications: a review. Int. J. Food Sci. Nutr. 2, 171–180.
Kapetanakou, A.E., Gkerekou, M.A., Vitzilaiou, E.S., Skandamis, P.N., 2017. Assessing the capacity of growth, survival, and acid adaptive response of *Listeria monocytogenes* during storage of various cheeses and subsequent simulated gastric digestion. Int. J. Food Microbiol. 246, 50–63. https://doi.org/10.1016/j.ijfoodmicro.2017.01.015.
Lahou, E., Uyttendaele, M., 2017. Growth potential of *Listeria monocytogenes* in soft, semi-soft and semi-hard artisanal cheeses after post-processing contamination in deli retail establishments. Food Control 76, 13–23. https://doi.org/10.1016/j.foodcont.2016.12.033.
Lebreton, A., Cossart, P., 2017. RNA- and protein-mediated control of *Listeria monocytogenes* virulence gene expression. RNA Biol. 14, 460–470. https://doi.org/10.1080/15476286.2016.1189069.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
López Expósito, I., Recio, I., 2006. Antibacterial activity of peptides and folding variants from milk proteins. Int. Dairy J. 16, 1294–1305. https://doi.org/10.1016/j.idairyj.2006.06.002.
Lozano-Ojalvo, D., Pérez-Rodríguez, L., Pablos-Tanarro, A., López-Fandiño, R., Molina, E., 2017. Pepsin treatment of whey proteins under high pressure produces hypoallergenic hydrolysates. Innov. Food Sci. Emerg. Technol. 43, 154–162. https://doi.org/10.1016/j.ifset.2017.07.032.
Mann, H.B., Whitney, D., 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Stat. 2, 347–370.
Martin, I., Rodríguez, A., Alía, A., Gómez, F., Pulido, J.C., Bermúdez, E., Córdoba, J.J., 2021. Control of *Listeria monocytogenes* in ripened ready-to-eat foods. In: XXVIII National Congress of the Spanish Society of Microbiología (SEM), 28-2 July 2021, p. 65. https://congresosem21.es/wp-content/uploads/2021/07/XVIII-Congreso-Nacional-deMicrobiologi%CC%81a-FINAL-7.pdf. (Accessed 10 January 2021).
Mataragas, M., Drosinos, E.H., Metaxopoulos, J., 2003. Antagonistic activity of lactic acid bacteria against *Listeria monocytogenes* in sliced cooked cured pork shoulder stored under vacuum or modified atmosphere at 4 ± 2 °C. Food Microbiol. 20, 259–265. https://doi.org/10.1016/S0740-0020(02)00099-0.
Mataragas, M., Bellio, A., Rovetto, F., Astegiano, S., Greci, C., Hertel, C., Decastelli, L., Cocolin, L., 2015. Quantification of persistence of the food-borne pathogens *Listeria monocytogenes* and salmonella enterica during manufacture of italian fermented sausages. Food Control 47, 552–559. https://doi.org/10.1016/j.foodcont.2014.07.058.
Melia, S., Purwati, E., Yuherman, Jaswandi, Aritonang, S.N., Silaen, M., 2017. Characterization of the antimicrobial activity of lactic acid bacteria isolated from buffalo milk in west sumatera (Indonesia) against *Listeria monocytogenes*. Pakistan J. Nutr. 16, 645–650. https://doi.org/10.3923/pjn.2017.645.650.
Morandi, S., Silvetti, T., Battelli, G., Brasca, M., 2019. Can lactic acid bacteria be an efficient tool for controlling *Listeria monocytogenes* contamination on cheese surface? The case of Gorgonzola cheese. Food Control 96, 499–507. https://doi.org/10.1016/j.foodcont.2018.10.012.
Official Journal of the European Union, 2004. Regulation (EC) No 853/2004 of the European Parliament and of the Council. Off. J. Eur. Union. L139/55.
Olesen, I., Thorsen, L., Jespersen, L., 2010. Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. Int. J. Food Microbiol. 141, S60–S68. https://doi.org/10.1016/j.ijfoodmicro.2010.01.042.
Panebianco, F., Giarratana, F., Caridi, A., Sidari, R., De Bruno, A., Giuffrida, A., 2021. Lactic acid bacteria isolated from traditional italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese. LWT Food Sci. Technol. 137, 110446 https://doi.org/10.1016/j.lwt.2020.110446.
Pieta, L., Escudero, F.L.G., Jacobus, A.P., Cheiran, K.P., Gross, J., Moya, M.L.E., Soares, G.L.G., Margis, R., Frazzon, A.P.G., Frazzon, J., 2017. Comparative transcriptomic analysis of *Listeria monocytogenes* reveals upregulation of stress genes and downregulation of virulence genes in response to essential oil extracted from baccharis psiadioides. Ann. Microbiol. 67, 479–490. https://doi.org/10.1007/s13213-017-1277-z.
Quinto, E.J., Marín, J.M., Schaffner, D.W., 2016. Effect of the competitive growth of *Lactobacillus sakei* MN on the growth kinetics of *Listeria monocytogenes* Scott a in model meat gravy. Food Control 63, 34–45. https://doi.org/10.1016/j.foodcont.2015.11.025.
Regulation (CE) 1491, 2003. of European Commission of 25 of August: Registration of the Protected Designation of Origin "Torta del Casar" in the Register of Protected Designations of Origin and the Protected Geographic Indications.
Rodriguez, C., Taminiau, B., García-Fuentes, E., Daube, G., Korsak, N., 2021. *Listeria monocytogenes* dissemination in farming and primary production: sources, shedding and control measures. Food Control 120, 107540. https://doi.org/10.1016/j.foodcont.2020.107540.
Santorum, P., García, R., López, V., Martínez-Suárez, J.V., 2012. Review. Dairy farm management and production practices associated with the presence of *Listeria monocytogenes* in raw milk and beef. SpanishJ. Agric. Res. 10, 360. https://doi.org/10.5424/sjar/2012102-314-11.
Scatassa, M.L., Gaglio, R., Cardamone, C., Macaluso, G., Arcuri, L., Todaro, M., Mancuso, I., 2017. Anti-listeria activity of lactic acid bacteria in two traditional Sicilian cheeses. Ital. J. Food Saf. 6, 13–17. https://doi.org/10.4081/ijfs.2017.6191.
Shamloo, E., Hosseini, H., Moghadam, A.Z., Larsen, H.M., Haslberger, A., Alebouyeh, M., 2019. Importance of *Listeria monocytogenes* in food safety: a review of its prevalence, detection, and antibiotic resistance. Iran. J. Vet. Res. 20, 241–254.
Singh, V.P., 2018. Recent approaches in food bio-preservation-a review. Open Vet. J. 8 (1), 104–111. https://doi.org/10.4314/ovj.v8i1.16.
Tumbarski, Y., Lante, A., Krastanov, A., 2018. Immobilization of bacteriocins from lactic acid bacteria and possibilities for application in food biopreservation. Open Biotechnol. J. 12, 25–32. https://doi.org/10.2174/1874070701812010025.
Vitullo, M., Grant, K.A., Sammarco, M.L., Tamburro, M., Ripabelli, G., Amar, C.F.L., 2013. Real-time PCRs assay for serogrouping *Listeria monocytogenes* and differentiation from other Listeria spp. Mol. Cell. Probes 27, 68–70. https://doi.org/10.1016/j.mcp.2012.10.001.
Walter, J., Tannock, G.W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D.M., Munro, K., Alatossava, T., 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66, 297–303. https://doi.org/10.1128/AEM.66.1.297-303.2000.
Yang, S.C., Lin, C.H., Sung, C.T., Fang, J.Y., 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Front. Microbiol. 5, 1–10. https://doi.org/10.3389/fmicb.2014.00241.

Evaluation of the influence of acidic conditions on the growth and expression of two virulence genes of *Listeria monocytogenes*

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Abstract

Fermented foods are preserved by the production of acids by beneficial bacteria producing a reduction in pH in the range between 4.5 and 6. In this work, the effect of these acidic conditions on the growth and expression of the *hly* and *inlA* genes of *Listeria monocytogenes* was evaluated. For the evaluation of the *inlA* gene, a novel real-time PCR (qPCR) method using SYBR® Green methodology was developed. In general, *L. monocytogenes* counts increased as the pH did. The highest growth of this pathogen was observed at 12 hours and a pH value = 6.8 (control conditions). At pH 4.5, close to the pH limit of the bacterium growth, levels of *L. monocytogenes* were kept constant throughout the incubation time. However, a significant increase of the relative expression of the virulence genes, *hly* and *inlA*, in most of the acidic conditions in all the incubation times, was detected. At pH 4.5 the most pronounced upregulation of the relative expression of the virulence genes was found and this could increase the pathogenicity of surviving *L. monocytogenes* The efficient *inlA*-based qPCR method could be of interest to check changes in the expression of such virulence gene of this pathogenic bacterium in acidic environments.

Keywords: *L. monocytogenes*, *inlA* gene, acidic conditions, qPCR

1. INTRODUCTION

Listeria monocytogenes is one of the most important foodborne pathogens (Chen, Regan, Laksanalamai, Healey, & Hu. 2017) which is responsible for causing listeriosis, a disease that affects particularly pregnant women, newborns, the elderly, and individuals with compromised immune systems (Rodriguez, Taminiau, García-Fuentes, Daube, & Korsak, 2021). This illness causes septicemia and meningitis with high mortality rates (from 12.8 to 17% of cases) (Lomonaco et al., 2009; Melo, Andrew, & Faleiro, 2015; Ramaswamy et al., 2007). This bacterium is well-known for its ability to survive and grow on different stressful environmental conditions including a wide range of pH (4.0–9.5), and temperatures (1–45 °C) values and water activity (a_w) values as low as 0.92 a_w (Carpentier & Cerf, 2011; Chen et al., 2018; Lianou & Koutsoumanis, 2013). Moreover, *L. monocytogenes* can grow in high salt concentration environments (up to 10% [w/v] NaCl) and, as a facultative anaerobic microbe, withstands several and varied food packaging conditions (Melo et al., 2015).

In general, bacteria can change lifestyles and develop resistance to extreme conditions when there is a modification of environmental conditions (Lebreton & Cossart, 2017). *L. monocytogenes* colonizing foods may be exposed to numerous stress factors such as heat, freezing, oxidation, dehydration, osmolites, and acid in food environments throughout the processing and storage in the industry (Alía, Rodríguez, Andrade, & Córdoba, 2021). This microorganism may normally confront more than one type of stress at the same time to survive in foods. Thus, low temperatures storages or freezing processes (-4ºC) and processes employing high temperatures (>45ºC) lead to microorganisms needing to acclimate to the thermal stress environment and adapt their growth to these conditions (Bucur, Grigore-Gurgu, Crauwels, Riedel, & Nicolau, 2018). Acidification with lactic acid is frequently used in the food industry to control the growth of pathogenic microorganisms including *L. monocytogenes* in foods (Jones, Vail, & McMullen, 2013).

The use of organic acids as food preservatives and the compounds produced by lactic-acid bacteria used as protective cultures may also provoke an acidic environment in foods that *L. monocytogenes* needs to face to survive (Alía et al., 2021). It should be noted that the capacity of *L. monocytogenes* to counteract and deal with the environmental challenges encountered in food greatly affects the survival and the virulence of the pathogen in the human body, where it also encounters adverse stress conditions (e.g. the stomach) (Werbrouck et al., 2009).

It has been described that the capacity of *L. monocytogenes* to counteract stress may be related to the induction of virulence or stress-associated genes. Thus, some virulence genes such as *hly* are also associated with osmotic stress, *iap* also plays an important role in salt adaptation (Olesen, Vogensen, & Jespersen, 2009), and *prfA* induces an acidic stress response (Bowman, Lee Chang, Pinfold, & Ross, 2010). In the case of the stress-related gene, *sigB*, it contributes to the ability of *L. monocytogenes* to multiply and survive under non-host-associated environmental stress conditions such as acid, osmotic, oxidative, cold, nutrient limitation, and energy stresses. This gene plays a main role in the expression of the virulence gene *hly* (Chaturongakul & Boor, 2006). In addition, the *inlA* gene is involved in the virulence response of *L. monocytogenes* whose principal roles imply adhesion and invasion (Gaillard, Berche, Frehel, Gouln, & Cossart, 1991; Lecuit et al., 2001). Sue, Fink, Wiedmann, & Boor, (2004) and Olesen et al. (2009) demonstrated the increase in the transcription of this important virulence gene after exposing this pathogen to acidic stress. Thus, analysis of the expression of *hly* and *inlA* genes could be of great interest to evaluate the response of *L. monocytogenes* under acid stress conditions. To know the unique effect of the acidic conditions on growth, and expression analysis of *L. monocytogenes* in these environments, a model system simulating food conditions

should be used. It is not advisable to conduct this kind of experiment directly in foods to avoid thus interference of microbial population from contamination origin.

The aim of the present study was to evaluate the growth and the expression of the virulence *hly* and *inlA* genes of *L. monocytogenes* under acidic conditions that may occur in food commodities. For this, a novel real-time PCR (qPCR) method designed on the basis of *inlA* gene was also optimized.

2. MATERIALS AND METHODS

2.1. *L. monocytogenes* strain

The strain *L. monocytogenes* S7-2 (serotype 4b) belonging to the National Institute of Agricultural and Food Research and Technology (INIA) collection (Madrid, Spain) was used in this study. The strain was then maintained at -80 ºC in Brain Heart Infusion (BHI) broth (Pronadisa, Madrid, Spain) containing 20 % glycerol.

2.2. Preparation of inoculum

To prepare the *L. monocytogenes* working solution, 100 µL of the stored culture at -80 ºC was inoculated in 10 mL BHI broth and incubated for 24 h at 37 °C. One hundred µL of such culture were transferred to a second tube of 10 mL BHI and incubated overnight for 24 h at 37 °C. At the end of the incubation, fresh cultures contained ≈ 8 log CFU /mL of cells. The concentration of the fresh cultures was adjusted to reach 3 log CFU/mL in the inoculum.

2.3. Experimental design

The effect of acidic conditions on *L. monocytogenes* growth and gene expression was evaluated in this study. For this, 100 µL of *L. monocytogenes* (3 log CFU/mL) were

inoculated in BHI broth adjusted to pH values of 6, 5.5, 5, and 4.5 with lactic acid (Scharlab S.L., Spain). BHI broth without the addition of lactic acid was used as a control (pH = 6.8). Samples were incubated with shaking at 37ºC for 4, 8, and 12 hours. The assay was performed in triplicate and repeated twice.

2.4. Enumeration of *L. monocytogenes* counts

At the end of the incubation time, 1 mL aliquot of each BHI broth adjusted to the different pH evaluated was transferred to a tube of 9 mL peptone water 1% (w/v) (Pronadisa, Spain). Next, serial decimal dilutions were made in the same diluent and then 0.1 mL of the cell suspensions were spread onto Chromagar™ *Listeria* agar plates (Chromagar, France). The Petri dishes containing the *L. monocytogenes* selective medium were incubated for 24 hours at 37ºC. After the incubation period, the characteristic *L. monocytogenes* colonies, those green colonies with a surrounded opaque halo were counted. Counts were expressed as log CFU/mL.

2.5. Gene expression analysis

2.5.1. RNA extraction

After centrifuging 1 mL aliquot of the previously obtained culture (10,000 rpm, 10 min, 4 °C), the resultant supernatant was removed, and the pellet was processed to extract RNA according to the instructions of the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, USA). The obtained RNA was eluted in 35 µL of TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA; Epicentre) and kept at −20 °C until required. The RNA concentration (ng/µL) and purity (A_{260}/A_{280} ratio) were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.5.2. Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized using 5 µL of total RNA (100 ng/µl) according to the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan) as described by the manufacturer. Next, it was stored at -20ºC until being used for qPCR reactions.

2.5.3. Primers

2.5.3.1. 16S rRNA and hly *primers*

The primer pair, F-16S/R-16S, previously designed from the 16S rRNA region (Alía, Andrade, Córdoba, Martín, & Rodríguez, 2020) was used as the reference gene (Table 1) The primer pair, hlyQFa/hlyQRa, previously designed from the virulence *hly* gene (Rodríguez-Lázaro et al., 2004) of *L. monocytogenes* was also used (Table 1).

2.5.3.2. inlA *primers*

A specific primer pair inlA-F/R (Table 1) was designed from the *inlA* gene sequence of *L. monocytogenes* strain 4140 (accession number FJ495194) retrieved from GenBank from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The design of primers was conducted using the Primer3 Software version 0.4.0 (Koressaar & Remm, 2007; Untergasser et al., 2012) according to guidelines provided by Rodríguez, Rodríguez, Córdoba, & Andrade,. (2015). Primers' specificity was tested by comparing their oligonucleotide sequences with the nucleotide sequence database in GenBank using the BLAST tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5.4. Real-time PCR conditions

The ViiA™ 7 Real-Time System (Applied Biosystems, USA) was used for qPCR reactions performance. The reactions were prepared in MicroAmp™ Fast optical 96-well Reaction Plates (Applied Biosystems) and sealed with optical adhesive films. Three replicates of RNA of the control batch (*L. monocytogenes* in BHI broth) and template-

free negative control (ultra-pure water instead of cDNA) were also included in the runs. The qPCR reaction mixtures and conditions used for amplification of 16S rRNA and *hly* genes were those described by Alía et al. (2020) and Rodríguez-Lázaro et al. (2004), respectively (Table 2). Regarding amplification of the *inlA* gene, mixture (Table 2) and conditions were optimized in this work. The qPCR amplification conditions used for *inlA* gene were: a single step of 10 min at 95 °C, 30 cycles of 95 °C for 15 s and 60 °C for 1 min. After the final PCR cycle, the specific binding of SYBR® Green to the amplicon was tested by analysis the melting curve analysis of the PCR products performed by heating from 60 to 95 °C and continuous measurement of the fluorescence to verify the PCR products. Quantitative results of the qPCR assay were expressed as a fractional cycle number, and then the quantitative cycle (Cq) value was determined, which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal. No Template Control (NTC) Cq value was established in 30 cycles.

The criteria considered for reliability of the designed method based on *inlA* gene were the correlation coefficient (R^2) and the amplification efficiency calculated from the formula $E = 10^{-1/S}-1$ (S being the slope of the linear fit). Optimization was made when $R^2 \geq 0.98$ and efficiencies of standard curves were close to 100 % (Rodríguez et al., 2015).

2.5.5. Gene expression analysis

The relative quantification of the expression of the *inlA* and *hly* genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The requirements necessary to use the mentioned method regarding the effect of experimental conditions on housekeeping gene (16S rRNA) expression and < 10% difference between efficiencies of the standard curves of the target and housekeeping genes were first met. To calculate the relative expression of the target genes (*hly* and *inlA*), the calibrator (control sample) was *L.*

monocytogenes inoculated in BHI broth without pH modification (pH value = 6.8) at the different sampling times (4, 8, and 12 hours).

2.6. Statistical analyses

The statistical treatment was conducted by using the software IBM SPSS Statistic version 20 (IBM, USA). For the statistical analysis of the data, the pH of the BHI broth and the incubation time were used as independent variables. The counts of *L. monocytogenes* (log CFU /mL) and gene expression data were analyzed as dependent variables The normality study of the different data populations was carried out using the Shapiro Wilk test. Subsequently, the analysis of the data was conducted using the Mann–Whitney test (Mann & Whitney, 1947). The statistical significance was established at $p \leq 0.05$.

3. Results and discussion

To the best of our knowledge, this is the first study that in-depth investigates the influence of acidic conditions on *L. monocytogenes* growth and the expression of two of their virulence genes (*hly* and *inlA*). This type of study is of great importance in order to control the growth and virulence of this hazardous pathogen in foods, especially in ready-to-eat (RTE) foods covering dairy products.

3.1. Effect of acidic conditions on the growth of *L. monocytogenes*

In general, at the same incubation time, levels of *L. monocytogenes* increased as the pH did. After 4 hours at 37 °C, *L. monocytogenes'* population ranged between 2.9 and 4.5 log CFU/mL being significantly highest ($p \leq 0.05$) at the two most basic conditions evaluated (pH 6 and pH 6.8 in the control; Fig 1). After 8 and 12 hours of incubation, *L. monocytogenes* counts were in the range of 3-5.7 log CFU/ mL, and 2.9-8 log CFU/mL, respectively. Results showed that independently of the incubation time when the pH

decreased, a reduction in the growth of this pathogenic microorganism was observed. This was expected as has been reported by Vermeulen et al. (2007).

In addition, it was also observed that at the same pH value, *L. monocytogenes* levels got higher as the incubation time did, except in the case of pH 4.5 ($p > 0.05$), where the counts were kept constant (≈ 3 log CFU/ml) throughout the incubation time which could be due to the fact that the pH of the medium was close to the limit of the bacterium growth which is 4.2 (EURL Lm, 2021). Therefore, in this acidic condition, *L. monocytogenes* does not have the ability to grow but it has the ability to survive. In fact, Bucur et al. (2018) and Skandamis et al. (2012) have shown the ability of *L. monocytogenes* to resist moderate pH values.

3.2. Effect of acidic conditions on the relative gene expression of *L. monocytogenes*

3.2.1. *inlA* gene

The *inlA* gene is one of the virulence genes of *L. monocytogenes* and it has been demonstrated the activation of its expression under acidic experimental conditions (Olesen et al., 2009; Sue et al., 2004). For this reason, this gene was one of the target genes used for studying the impact of acidic conditions on gene expression related to this pathogen. Firstly, a fast and reliable qPCR method based on the *inlA* gene using SYBR® Green methodology for detecting acid stress changes was designed and developed. This is the first method relied on this fluorescent dye developed for detecting changes in the transcription of this gene. Other previous works used TaqMan™ methodology to determine the expression of the *inlA* gene under different nutritional and environmental conditions (Hanna & Wang, 2006; Olesen et al., 2009). The SYBR® Green methodology has numerous advantages regarding the use of other technologies usually employed in qPCR such as lower cost, easier to perform and optimise and higher sensitivity (Rodriguez, Rodríguez, Luque, Justesen, & Córdoba, 2011; Tajadini, Panjehpour, &

Javanmard, 2014). Secondly, this method was utilized to analyze the trend of the expression of the *inlA* gene of this pathogenic bacterium under acidic stress conditions.

3.2.1.1. Design of primers and optimization of a real-time PCR method based on the *inlA* gene

The design of specific primers is one of the critical steps in the development of a successful qPCR assay. The specific primer pair (inlA-F/R) was designed based on the *inlA* gene sequence of *L. monocytogenes*. The predicted characteristics of the primer designed in this study are described as follows: the length of both primers was 20 bp, % GC of the forward primer was 40% while that for the reverse primer was 55%. The melting temperature (Tm) values of the inlA-F and inlA-R were 60.51 and 60.40 ºC, respectively, being the difference in the Tm values between primers 0.11 ºC. Regarding secondary structure analysis of the primers, neither hairpins nor self-dimers were found. With respect to the specificity of the primers by comparing their oligonucleotide sequences with the nucleotide sequence database in GenBank using the BLAST tool from NCBI showed that they were specific being more than 99 %. Both primers amplified a PCR product whose size is 65 bp. The analysis of the melting curve of the PCR product showed just one amplified product obtaining a Tm value of 78.5 ºC (Fig. 2). Therefore, this primer pair was appropriate for qPCR since only one amplicon was observed and primer dimers were not detected. This confirmed its specificity in spite of using SYBR® Green, reducing significantly the cost of analyses of high number of samples. This methodology has been widely used in previous studies with similar results (Azinheiro Carvalho, Prado, & Garrido-Maestu, 2020; Cordero, Córdoba, Bernáldez, Rodríguez, & Rodríguez, 2015; Rodríguez et al., 2012).

The standard curve generated showed linearity across all the range of concentrations used with a high (R^2 correlation coefficient > 0.99) indicating very low inter-assay variability.

The slope of the line of the standard curve was -3.45 (Fig. 3) which corresponded to efficiency of 95%. The parameters related to sensitivity of the method obtained from the standard curve indicated that the method was robust and reproducible. The linearity (R^2) and the slope were within the recommended range reported by Rodriguez, Rodriguez, Córdoba, & Andrade, (2015). These results indicated that the designed qPCR method could be appropriate to quantify changes in the expression of the *inlA* gene linked to the acid stress responses by *L. monocytogenes.*

3.2.1.2. Relative *inlA* gene expression

The effect of acidic conditions modifying pH on the relative expression of the *inlA* gene by *L. monocytogenes* in relation to the incubation time (4, 8 and 12 hours) was evaluated (Fig. 4). The relative expression of the target gene was determined and compared with the control batch (*L. monocytogenes* inoculated in BHI broth). In general, the relative expression of this gene was higher at pH values lower than that for the control (Fig. 4). At 4 hours of incubation, a significant increase ($p \leq 0.05$) of the relative expression of *inlA* gene at the two most acidic conditions (pH values 4.5 and 5) with respect to the control, was observed. In addition, at 8 and 12 hours of incubation, a significant increase of the relative expression of such gene with respect to the control was also observed at pH 5.5 and 6, respectively ($p \leq 0.05$). At all incubation times evaluated, the highest relative expression of *inlA* gene was found at pH 4.5 ($p \leq 0.05$; Fig. 4B). In general, the activation of the expression of the *inlA* gene of *L. monocytogenes* rose as the acidity of the medium increased. Our results described here are consistent with the observations of Olesen et al. (2009).

Previous studies have investigated the *inlA* gene transcription in *L. monocytogenes* because of acidic-type stress (Conte et al., 2002, Olesen et al., 2009). However, in this study, the effect of a much wider range of pH conditions that can be found in foods

≈ 7 to 4.5) on the expression of such gene was evaluated instead of an only pH value. Besides, Olesen et al. (2009) tested the influence of the acidic-stress conditions on short-term (3h) and long-term stress (18h) while in this study different incubation times including short, mid and long-term stress were checked.

3.2.2. Relative *hly* gene expression

In this work the relative expression of the *hly* gene was also measured under the experimental conditions related to acidic stress because such gene has been demonstrated to be intimately involved in the virulence of this pathogen and its transcription seems to be affected by various type of stress including acidic changes (Li, Carpenter, & Broadbent, 2021).

Regarding the expression of the *hly* gene, an upregulation was observed at stronger acidic conditions in most of cases. After 4 and 8 hours of incubation, only when *L. monocytogenes* grew in BHI broth with the two lowest pH values, a significant increase in the expression of the *hly* gene compared to the control was found (Fig. 4A and 4B; $p \leq 0.05$). At 8 hours, the highest level of the gene expression was found for pH 4.5. At the largest incubation time (12 hours), the relative expression values of the *hly* gene of *L. monocytogenes* in all the experimental conditions evaluated were higher ($p \leq 0.05$) than in the control (Fig. 4C) but there were no differences between the different pH values tested.

The overexpression of the *hly* gene was also described by Alía, Rodríguez, Andrade, Gomez, & Cordoba (2019) when exposing *L. monocytogenes* to osmotic stress. In addition, our results described here are consistent with the observations of Li et al. (2021) who observed that the *hly* gene expression increased when the pH was decreased after 4 hours at 37ºC in a TSB medium with 4.75 mM of L-lactic or acetic acid (pH values from 7.4 to 6).

In this study, the *inlA* and *hly* genes of *L. monocytogenes* showed similar expression patterns in response to acid exposure showing that the rising of acidic conditions stimulated the expression of both genes. This is quite interesting since both genes affect virulence of this pathogenic and they seem to be activated under acidic conditions. Findings of this study are like those encountered by Li et al. (2021) who demonstrated the activation of both genes in organic acid habituated cells compared to the baseline control or pH control. Focusing on the two genes tested, the *inlA* gene of *L. monocytogenes* was more affected by the changes occurred in the acidity of the medium. Thus, the developed method using the SYBR® Green methodology could be of interest to check changes in the virulence gene expression of this pathogenic bacterium in acidic environments.

The results have shown that there is an inverse relationship between the relative expression of both *hly* and *inlA* genes and growth of *L. monocytogenes* under acidic simulated conditions by using BHI broth modifying with lactic acid. Under more acidic conditions, the growth of the pathogen was more limited, and the expression of both virulence genes was more pronounced. This means that this pathogen needs to activate its virulence by activation of both genes in order to counteract stress provoked by the reduction of the pH of the culture medium as has been described by Li et al. (2021).

In this work, it has been demonstrated that the acidic stress provoked by the addition of lactic acid inhibits the growth of *L. monocytogenes* and favors the transcription of the *inlA* and *hly* genes, virulence genes of this foodborne pathogen. This is of utmost interest since lactic acid is a metabolite produced by a wide range of bacteria, and more specifically by lactic-acid bacteria, during fermentation and ripening processes of different RTE foods that can be contaminated easily by *L. monocytogenes* during its processing and commercialization such as RTE cheeses and dry-cured fermented sausages (Prpich, Camprubí, Cayré, & Castro, 2021; Ray & Joshi, 2014). In these

products, *L. monocytogenes* could be present throughout the entire commercialization process, since according to the food safety criteria in some countries such as those of the European Union a maximum level of 100 CFU/g for this pathogen in RTE food products throughout their shelf-life is allowed (Commission, 2007). Therefore, the information obtained in this research is useful to understand the growth and virulence of this hazardous pathogen in order to control it during the processing or commercialization of foods, especially fermented or ripened RTE products.

In conclusion, although the growth of *L. monocytogenes* under acidic conditions usually found in fermented foods including dry-cured fermented sausages or cheeses (pH values ranging between 4.5 and 6) is limited, an upregulation of the relative expression of the virulence genes, *hly* and *inlA*, is produced. Thus, there is an inverse relationship between the growth and relative expression of virulence genes of this pathogenic bacterium in acidic conditions. To evaluate changes in the expression of the *inlA* gene in acidic conditions an efficient method using SYBR® Green methodology has been developed. This method could be of great interest to check modifications of the expression of the mentioned virulence gene of this pathogenic bacterium in acidic food environments.

Acknowledgements

This work was supported by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. I. Martín is supporting by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

References

Alía, A., Andrade, M.J., Córdoba, J.J., Martín, I., & Rodríguez, A. (2020). Development of a multiplex real-time PCR to differentiate the four major *Listeria monocytogenes* serotypes in isolates from meat processing plants. *Food Microbiology*, 87. https://doi.org/10.1016/j.fm.2019.103367

Alía, A., Rodríguez, A., Andrade, M.J., & Córdoba, J.J. (2021). Molecular mechanisms of *Listeria monocytogenes*. Stress responses, in: Dongyou Liu (Ed.), *Molecular Food Microbiology*. pp. 169–186.

Alia, A., Rodriguez, A., Andrade, M. J., Gomez, F. M., & Cordoba, J. J. (2019). Effect of water activity, NaCl content and temperature on the growth and gene expression of *Listeria monocytogenes* in a dry-cured ham model system. *Meat Science, 155*, 16–19. https://doi.org/10.1016/j.meatsci.2019.04.017

Azinheiro, S., Carvalho, J., Prado, M., & Garrido- Maestu, A. (2020). Multiplex detection of *Salmonella* spp., *E. coli* O157 and *L. monocytogenes* by qPCR melt curve analysis in spiked infant formula. *Microorganisms,* 4;8(9):1359. https://doi.org/10.3390/microorganisms8091359.

Bowman, J.P., Lee Chang, K.J., Pinfold, T., & Ross, T. (2010). Transcriptomic and phenotypic responses of *Listeria monocytogenes* strains possessing different growth efficiencies under acidic conditions. *Applied Environmental Microbiology Journal*, 76, 4836–4850. https://doi.org/10.1128/AEM.00315-10

Bucur, F.I., Grigore-Gurgu, L., Crauwels, P., Riedel, C.U., & Nicolau, A.I. (2018). Resistance of *Listeria monocytogenes* to stress conditions ancountered in food and food processing environments. *Frontiers in Microbiology*, 9, 1–18. https://doi.org/10.3389/fmicb.2018.02700

Carpentier, B., & Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journalof Food Microbiology* https://doi.org/10.1016/j.ijfoodmicro.2011.01.005

Chaturongakul, S., & Boor, K.J. (2006). σ^B activation under environmental and energy stress conditions in *Listeria monocytogenes. Applied Environmental Microbiology Journal*, 72(8), 5197-5203. http://doi.org/10.1128/AEM.03058-05

Chen, J.Q., Regan, P., Laksanalamai, P., Healey, S., & Hu, Z. (2017). Prevalence and methodologies for detection, characterization and subtyping of *Listeria monocytogenes* and *L. ivanovii* in foods and environmental sources. *Food Science and Humman Wellness*, 6, 97–120. https://doi.org/10.1016/j.fshw.2017.06.002

Chen, M., Cheng, J., Wu, Q., Zhang, J., Chen, Y., Xue, L., Lei, T., Zeng, H., Wu, S., Ye, Q., Bai, J., & Wang, J. (2018). Occurrence, antibiotic resistance, and population diversity of *Listeria monocytogenes* isolated from fresh aquatic products in China. *Frontiers in Microbiology*, 9, 1–11. https://doi.org/10.3389/fmicb.2018.02215.

Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. Official Journal of the European Communitites, 1441.

Conte, M.P., Petrone, G., Di Biase, A.M., Longhi, C., Penta, M., Tinari, A., Superti, F., Fabozzi, G., Visca, P., & Seganti, L. (2002). Effect of acid adaptation on the fate of *Listeria monocytogenes* in THP-1 human macrophages activated by gamma interferon. *Infection and Immunity.* 70(8): 4369–4378. https://doi.org/10.1128/IAI.70.8.4369-4378.2002

Cordero, M., Córdoba, J.J., Bernáldez, V., Rodríguez, M., & Rodríguez, A. (2015).

Quantification of *Penicillium nalgiovense* on dry-cured sausage 'Salchichón' using a SYBR Green-Based Real-Time PCR. *Food Analitical Methods*, 8, 1582–1590. https://doi.org/10.1007/s12161-014-0078-2

EURL Lm, (2021). Technical guidance document on challenge test and durability studies for assessing shelf-life of ready-to- eat foods related to *Listeria monocytogenes* in ready-to-eat foods [WWW Document]. https://ec.europa.eu/food/system/files/2021-07/biosafety_fh_mc_tech-guide-doc_listeria-in-rte-foods_en_0.pdf

Gaillard, J.L., Berche, P., Frehel, C., Gouln, E., & Cossart, P. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell*, 65, 1127–1141. https://doi.org/10.1016/0092-8674(91)90009-N

Hanna, S., & Wang, H.H. (2006). Assessment of environmental factors on *Listeria monocytogenes* Scott A gene expression by relative quantitative Taqman Real-Time reverse transcriptase PCR. *Journal of Food Protection*, 69, 2754–2757. https://doi.org/10.4315/0362-028x-69.11.2754

Jones, T.H., Vail, K.M., & McMullen, L.M. (2013). Filament formation by foodborne bacteria under sublethal stress. *International Journal of Food Microbiology*, 165, 97–110. https://doi.org/10.1016/j.ijfoodmicro.2013.05.001

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer. *Bioinformatics*, 23, 1289–1291. https://doi.org/10.1093/bioinformatics/btm091

Lebreton, A., & Cossart, P. (2017). RNA- and protein-mediated control of *Listeria monocytogenes* virulence gene expression. *RNA Biology*, 14, 460–470.

https://doi.org/10.1080/15476286.2016.1189069

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., & Cossart, P. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science*. 292, 1722-1725. https://doi.org/10.1126/science.1059852

Li, M., Carpenter, C. E., & Broadbent, J.R. (2021). Organic acid exposure enhances virulence in some *Listeria monocytogenes* strains using the *Galleria mellonella* infection model. *Frontiers in Microbiology*, 12: 675241. https://doi.org/10.3389/fmicb.2021.675241

Lianou, A., & Koutsoumanis, K.P. (2013). Strain variability of the behavior of foodborne bacterial pathogens: A review. *International Journal of Food Microbiology*, 167, 310–321. https://doi.org/10.1016/j.ijfoodmicro.2013.09.016

Livak, K.J., & Schmittgen, T.D. (2001). Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lomonaco, S., Decastelli, L., Nucera, D., Gallina, S., Manila Bianchi, D., & Civera, T., (2009). *Listeria monocytogenes* in Gorgonzola: Subtypes, diversity and persistence over time. *International Journal of Food Microbiology*, 128, 516–520. https://doi.org/10.1016/j.ijfoodmicro.2008.10.009

Mann, H.B., & Whitney, D. (1947). On a test of whether one of two random variables is stochastically larger than the other. *Annals of Mathematical Statistics*, 2, 347–370.

Melo, J., Andrew, P.W., & Faleiro, M.L. (2015). *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. *Food Research Internatinal*, 67, 75–90.

https://doi.org/10.1016/j.foodres.2014.10.031

Olesen, I., Vogensen, F.K., & Jespersen, L., (2009). Gene transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathogen*. Dis. 6, 669–680. https://doi.org/10.1089/fpd.2008.0243

Prpich, N. Z. P., Camprubí, G. E., Cayré, M. E., & Castro, M. P. (2021). Indigenous microbiota to leverage traditional dry sausage production. *International Journal of Food Science*, https://doi.org/10.1155/2021/6696856

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., & Vijila, H.M. (2007). *Listeria* - Review of epidemiology and pathogenesis. *Journal Microbiology Immunology and Infection*, 40, 4–13.

Ray, R.C., & Joshi, V., (2014). Fermented foods: Past, present and future. http://doi.org/10.13140/2.1.1849.8241

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J.A., & Pla, M., (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by Real-Time PCR: assessment of *hly, iap,* and *lin02483* targets and AmpliFluor technology. *Applied and Environmental Microbiology Journal*, 70, 1366–1377. https://doi.org/10.1128/AEM.70.3.1366-1377.2004

Rodriguez, A., Rodriguez, M., Córdoba, J.J., & Andrade, M.J., (2015). Design of primers and probes for quantitative Real-Time PCR methods, in: *Methods in molecular biology* (Clifton, N.J.). pp. 31–56. https://doi.org/10.1007/978-1-4939-2365-6

Rodriguez, A., Rodríguez, M., Luque, M.I., Justesen, A.F., & Córdoba, J.J. (2011). Quantification of ochratoxin A-producing molds in food products by SYBR Green

and TaqMan real-time PCR methods. *International Journal of Food Microbiology*, 149, 226–235. https://doi.org/10.1016/j.ijfoodmicro.2011.06.019

Rodríguez, A., Rodríguez, M., Luque, M.I., Martín, A., & Córdoba, J.J. (2012). Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. *Food Microbiology*, 31, 89–99. https://doi.org/10.1016/j.fm.2012.02.009

Rodriguez, C., Taminiau, B., García-Fuentes, E., Daube, G., & Korsak, N. (2021). *Listeria monocytogenes* dissemination in farming and primary production: Sources, shedding and control measures. *Food Control* 120, 107540. https://doi.org/10.1016/j.foodcont.2020.107540

Skandamis, P.N., Gounadaki, A.S., Geornaras, I., & Sofos, J.N. (2012). Adaptive acid tolerance response of *Listeria monocytogenes* strains under planktonic and immobilized growth conditions. *International Journal of Food Microbiology*, 159, 160–166. https://doi.org/10.1016/j.ijfoodmicro.2012.07.027

Sue, D., Fink, D., Wiedmann, M., & Boor, K.J. (2004). σ^B-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. *Microbiology* 150, 3843–3855. https://doi.org/10.1099/mic.0.27257-0

Tajadini, M., Panjehpour, M., & Javanmard, S. (2014). Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Advanced Biomedical Research*, 3, 85. https://doi.org/10.4103/2277-9175.127998

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., & Rozen, S.G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Research*. 40, 1–12. https://doi.org/10.1093/nar/gks596

Vermeulen, A., Gysemans, K.P.M., Bernaerts, K., Geeraerd, A.H., Van Impe, J.F., Debevere, J., & Devlieghere, F. (2007). Influence of pH, water activity and acetic acid concentration on *Listeria monocytogenes* at 7ºC: data collection for the development of a growth/no growth model. *International Journal of Food Microbiology*, 114, 332–41. https://doi.org/10.1016/j.ijfoodmicro.2006.09.023

Werbrouck, H., Vermeulen, A., Van Coillie, E., Messens, W., Herman, L., Devlieghere, F., & Uyttendaele, M. (2009). Influence of acid stress on survival, expression of virulence genes and invasion capacity into Caco-2 cells of *Listeria monocytogenes* strains of different origins. *International Journal of Food Microbiology*, 134, 140–146. https://doi.org/10.1016/j.ijfoodmicro.2009.03.022

Table 1

Nucleotide sequences of primers used for reverse-transcription real-time PCR performance

Genes	Primer pairs	Nucleotide sequences (5´-3´)	Reference
Target genes			
inlA	inlA-F	ACGGCAAAGAAACAACCAAA	This study
	inlA-R	TACGGGCTTAGCTGGTTCAG	
hly	hlyQFa	CATGGCACCACCAGCATC T	Rodríguez-Lázaro et al. (2004)
	hlyQRa	ATCCGCGTGTTTCTTTTCGA	
Reference gene			
16 S	F-16S	ACGAACGGAGGAAGAGCTTG	Alía et al. (2020)
	R-16S	CCCCAACTTACAGGCAGGTT	

Table 2

Real-time PCR (qPCR) mix used for gene expression analysis of *inlA* and *hly*.

Genes	Primers	Volumes (µL)					Primer concentrations (nM)
		H_2O	SYBR Green	ROX	$MgCl_2$	cDNA	
Target genes							
inlA	inlA-F inlA-R	2.875	6.25	0.125	-	2.5	300
hly	hly- F hly-R	1.75	6.25	0.125	0.75	2.5	450
Reference gene							
16 S	F-16S R-16S	2.875	6.25	0.125	-	2.5	300

Figure legends

Figure 1. Growth of *Listeria monocytogenes* (expressed as counts, log CFU/ml) at different combinations of pH (6.8, 6, 5.5, 5, and 4.5) and incubation time (4, 8, and 12 hours). The experiments were carried out in triplicate. The bars represent the standard deviation of the mean values. Mean values of counts with different letters (a-e) indicate significant differences ($p \leq 0.05$) between different pH values at the same incubation time. Mean values of counts with different numbers (1-3) indicate significant differences ($p \leq 0.05$) between different incubation times at the same pH value.

Figure 2. Representative fluorescence melting curve of the amplification of the *inlA* gene region of *Listeria monocytogenes* with a specific primer pairs inlA-F/R designed in this work.

Figure 3. Representative standard curve showing the correlation between the cDNA concentration of *Listeria monocytogenes* grew in BHI broth without modifying pH and the quantification cycle (Cq) values of the amplification of the *inlA* gene of *Listeria monocytogenes* obtained using the SYBR Green-based qPCR method optimized in this study.

Figure 4. Effect of different pH on the relative expression of the *hly* and *inlA* genes on the *Listeria monocytogenes* at 4 hours (A), 8 h (B) and 12 h (C) with respect to the control (control batch without changes in the pH value=0). Mean values of the relative expression of the *hly* and *inlA* gene with asterisk indicate significant differences ($p \leq 0.05$) with respect to the calibrator. Mean values of the relative expression of the *hly* and *inlA* gene with different letters (a-d) indicate significant differences ($p \leq 0.05$) between the different pH values at the same time of incubation.

Figure 1. Martín et al. 2021

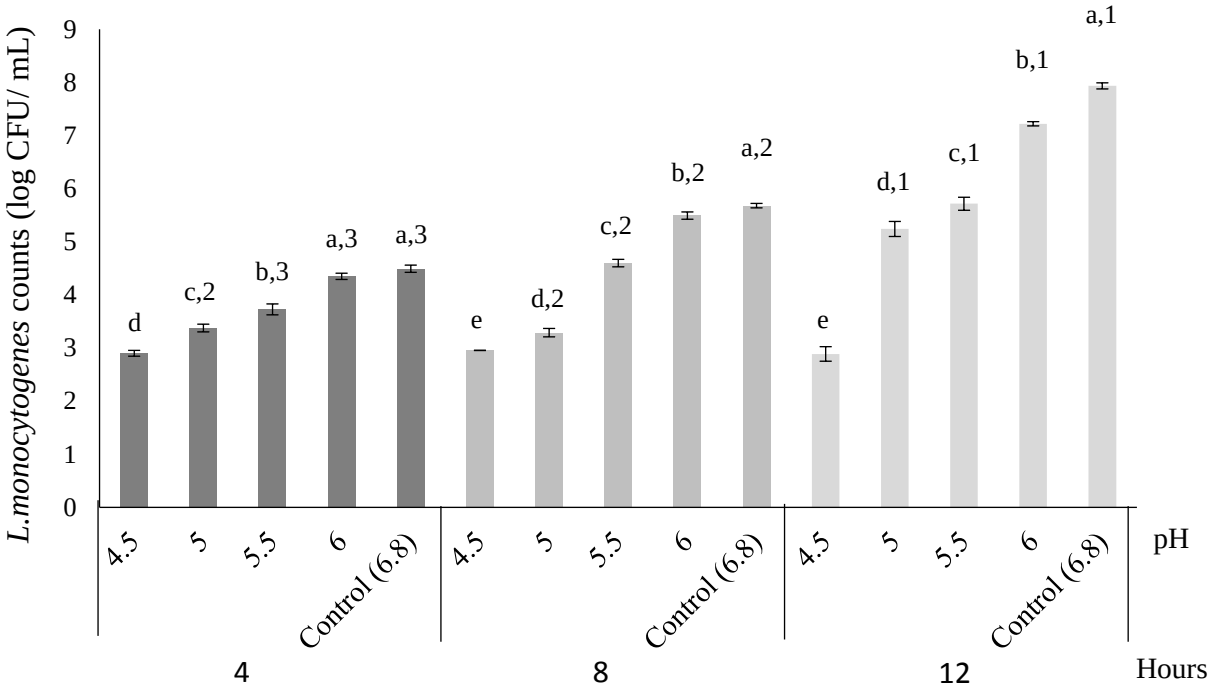


Figure 2. Martín et al. 2021

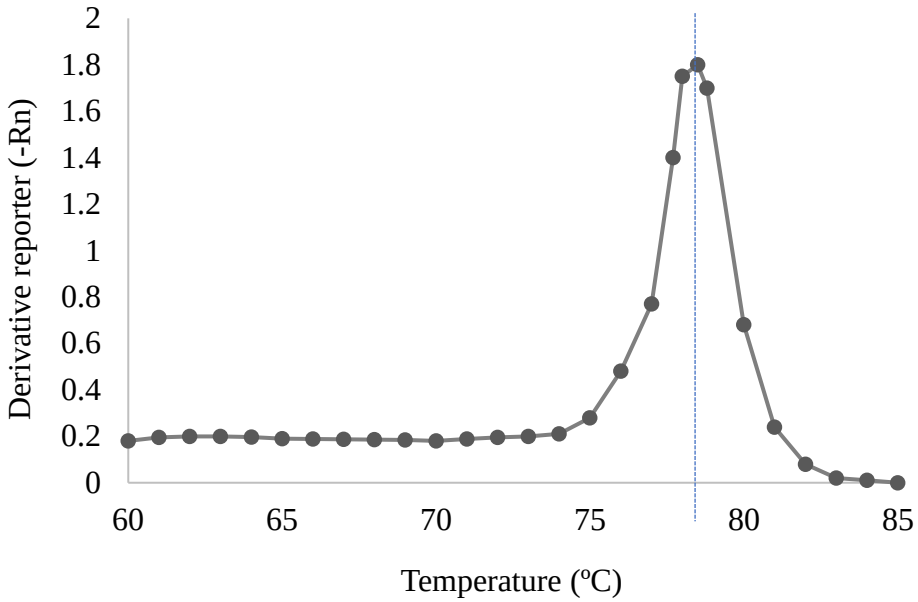


Figure 3. Martín et al. 2021

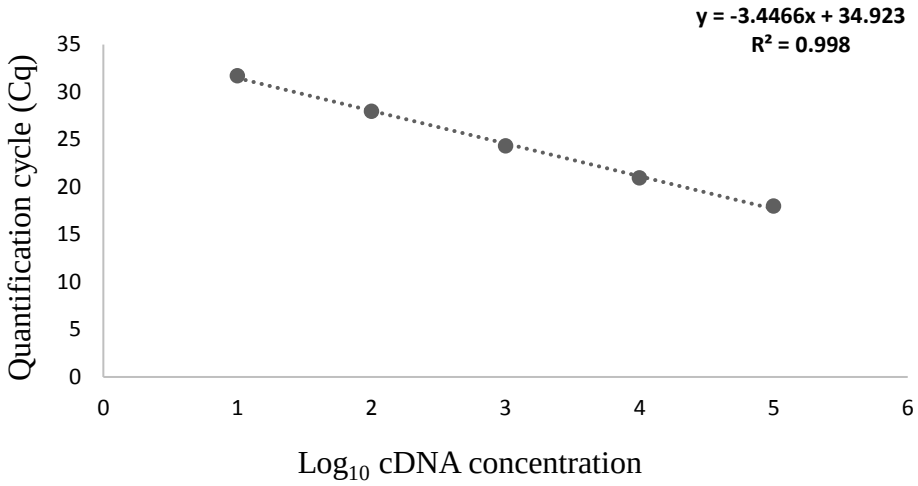
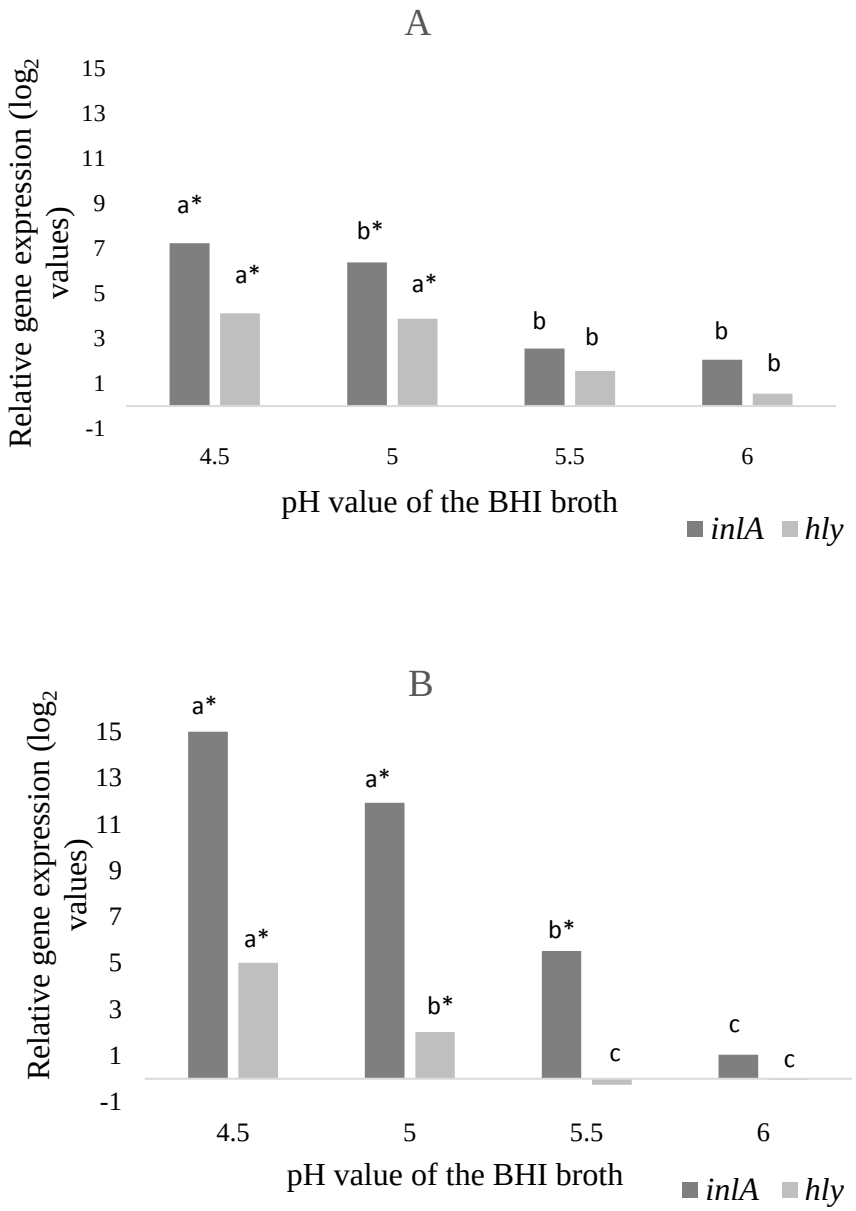
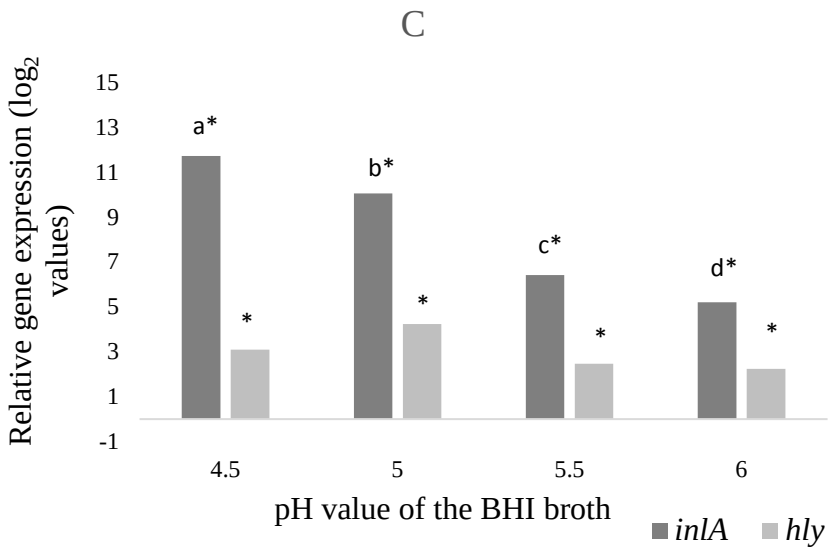


Figure 4. Martín et al., 2021




C
Relative gene expression (log2 values)
15
13
11
9
7
5
3
1
-1
a*
b*
c*
d*
*
*
*
*
4.5
5
5.5
6
pH value of the BHI broth
inlA
hly

IV.2. EVALUACIÓN DE BACTERIAS ÁCIDO-LÁCTICAS SELECCIONADAS EN EMBUTIDOS CURADO-MADURADOS

Foods (2021)

Biology (2021)

Food Chemistry (2022). Enviado para su publicación

foods

MDPI

Article

Effect of the Dry-Cured Fermented Sausage "Salchichón" Processing with a Selected *Lactobacillus sakei* in *Listeria monocytogenes* and Microbial Population

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Abstract: In the present work, the effect of processing of dry-cured fermented sausage "salchichón" spiked with the selected *Lactobacillus sakei* 205 was challenge-tested with low and high levels of *L. monocytogenes*. The evolution of the natural microbial population throughout the "salchichón" ripening was also evaluated. For this, a total of 150 "salchichón" were elaborated and divided into six equal cases which were inoculated with different levels of *L. monocytogenes*, and *L. sakei* 205. Afterwards, sausages were ripened for 90 days according to a typical industrial process. Moisture content (%) and water activity (a_w) decreased throughout the ripening up to values around 26% and 0.78, respectively. No differences for moisture content, a_w, pH, NaCl and nitrite concentration were observed between the analyzed cases. Lactic acid bacteria counts in the *L. sakei* 205 inoculated cases were always higher than 6 log CFU g^{-1} during ripening. *Enterobacteriaceae* counts were reduced during ripening until non-detectable levels at the end of processing. Reductions in *L. monocytogenes* counts ranged from 1.6 to 2.2 log CFU g^{-1}; therefore, the processing of "salchichón" itself did not allow the growth of this pathogen. Reduction in *L. monocytogenes* was significantly higher in the cases inoculated with *L. sakei* 205.

Keywords: *L. monocytogenes* reduction; dry-cured fermented sausages; *L. sakei*; challenge test

Citation: Martín, I.; Rodríguez, A.; Sánchez-Montero, L.; Padilla, P.; Córdoba, J.J. Effect of the Dry-Cured Fermented Sausage "Salchichón" Processing with a Selected *Lactobacillus sakei* in *Listeria monocytogenes* and Microbial Population. *Foods* **2021**, *10*, 856. https://doi.org/10.3390/foods10040856

Academic Editor: Andreas Kimon Karatzas

Received: 28 March 2021
Accepted: 13 April 2021
Published: 15 April 2021

1. Introduction

Dry-cured fermented sausages are Mediterranean products that are widely consumed in Spain and well known in international markets [1,2]. Among the Spanish dry-cured fermented sausages, "salchichón" is a typical dry-cured fermented sausage classed as a ready-to-eat (RTE) food that is manufactured with traditional technologies without adding starter cultures [3]. This product is usually made from comminuted meat and fat, mixed with salt and other spices, and filled into casings, before their ripening in drying chambers, at temperature and relative humidity (RH) conditions that may vary between 7 and 14 °C, and 80 and 85%, respectively [4,5]. At these conditions, a decrease in water activity (a_w) values below 0.90 and a slight reduction in pH to levels ranging from 5.4 to 6.0 have usually been reported for this kind of meat product [6,7].

The food-handling involved in the manufacture of "salchichón" increases the risk of microbial contamination, with *Listeria monocytogenes* being the most hazardous pathogenic microorganism in this RTE product. *L. monocytogenes* is the causative agent of listeriosis, one of the most serious foodborne diseases caused mainly by food consumption [8]. Dry-cured fermented sausages are considered products with low risk for foodborne listeriosis [9,10]; however, the presence of this pathogenic bacterium has been reported in ripened sausages [11–13], and in some cases, it has even been involved in listeriosis outbreaks [14,15].

In the European Union (EU), the food safety criteria for *L. monocytogenes* in RTE food products have been published in the Regulation (CE) 2073/2005 [16] amended by Regulation (CE) 1441/2007 [17]. This regulation set a maximum level of 100 CFU g^{-1} for *L. monocytogenes* in RTE foods throughout their shelf life. However, it has been found that dry-cured fermented sausages contaminated by *L. monocytogenes* at levels higher than 100 CFU g^{-1} are consumed without any further heat treatment in the EU. Thus, the development of *L. monocytogenes* in this RTE meat product should be considered a great public health concern [13].

In order to minimize the risk that the presence of *L. monocytogenes* implies in dry-cured fermented sausages, the implementation of a process addressing the survival of this pathogenic bacteria throughout the manufacturing procedure is of great importance [18]. Thus, reducing or eliminating *L. monocytogenes* from meat products is a real challenge for the meat industry and food safety authorities.

There are few references in the literature of a challenge test to evaluate the effect of processing of "salchichón" in the growth/inactivation of *L. monocytogenes*. Thus, it is not clear if *L. monocytogenes* can be diminished throughout the processing of this product. Different studies have indicated that this pathogen could survive during the dry-cured fermented sausage's manufacturing and may not be completely eliminated [19], and in some cases, the growth of this microorganism was reported during ripening [20].

In the evaluation of the growth/inactivation of *L. monocytogenes* throughout the processing of "salchichón", the effect of lactic acid bacteria (LAB) should be considered because selected LAB strains have demonstrated antimicrobial effect against this pathogenic bacterium [21,22]. Among LAB, *Lactobacillus sakei* has a technological use in the preservation of several dry-cured fermented sausages due to its capacity to produce organic acids, hydrogen peroxide, and bacteriocins [23]. In fact, *L. sakei* has shown protective antimicrobial effect against both pathogenic and spoiler microorganisms in meat products [24,25].

In addition to the evaluation growth/inactivation of *L. monocytogenes*, the effect of processing of "salchichón" on the evolution of a natural microbial population should be determined, because unusual development of some microbial groups may lead to irregular microbial quality of the sausages and even spoilage during ripening. For example, growth of some *Enterobacteriaceae* strains throughout ripening in dry-cured meat products has been reported [26], which may reduce the microbiological quality of the sausage pieces.

With the aim of evaluating potential food safety implications, "salchichón" spiked with selected strains of *L. sakei* was challenge-tested with low and high levels of *L. monocytogenes*. In addition, the effect of dry-cured fermented sausage "salchichón" processing on the evolution of the natural microbial population of this product was evaluated.

2. Materials and Methods

2.1. Microbial Cultures

The strain *L. sakei* 205 from the Food Hygiene and Safety Collection at the University of Extremadura was used for the inoculation of "salchichón", with LAB as a protective culture. This strain was isolated from traditional dry-cured fermented sausages and selected by its antagonist activity against *L. monocytogenes* in agar "salchichón" (unpublished data). To prepare the inoculum of *L. sakei* 205, 100 µL of a stock culture (stored in brain heart infusion (BHI) broth (Conda, Spain) containing 20% (*w*/*v*) glycerol at −80 °C) were inoculated onto 10 mL of de Man–Rogosa–Sharpe (MRS) broth (Fisher Bioreagents, Belgium) and incubated for 48 h at 30 °C. At the end of the incubation, ≈8.0 log CFU mL^{-1} cells were obtained and an aliquot of this was diluted in 1% (*w*/*v*) peptone water (Conda, Spain) to reach a final concentration of approximately 6.0 log CFU mL^{-1}. Then, the culture was centrifuged at 10,000× *g* for 5 min, and the supernatant was discarded. The sediment was then washed and resuspended in phosphate-buffered saline (PBS) and used for the inoculation of the "salchichón" mix before stuffing. To determine the final concentration (CFU mL^{-1}) of *L. sakei* 205 in PBS in order to adjust the level of inoculation, serial dilutions in 1% (*w*/*v*) peptone water were inoculated onto MRS agar (Oxoid, UK) and incubated anaerobically at

30 °C for 72 h. In addition, initial levels of LAB on the sausages at day 0 of processing were determined as described previously.

For the inoculation of the "salchichón" with *L. monocytogenes*, strain S7-2 (serotype 4b) belonging to National Institute of Agricultural and Food Research and Technology (INIA) collection (Madrid, Spain) was used. To prepare the *L. monocytogenes* inoculum, 100 µL of a stock culture (stored in BHI broth containing 20% (*w*/*v*) glycerol at −80 °C) were transferred to 10 mL BHI broth and incubated for 24 h at 37 °C. A total of 100 µL of such culture were then transferred to a second tube of 10 mL BHI and incubated overnight at 37 °C. At the end of the incubation period, ≈8.0 log CFU mL^{-1} cells were obtained and aliquots of this were diluted to reach final concentrations of approximately 7.0 log CFU mL^{-1} and 4.0 log CFU mL^{-1}. Then, the cultures were centrifuged at 10,000× *g* for 5 min, the supernatants discarded, and the sediments were washed and resuspended in PBS and used for the inoculation of the "salchichón" mix before stuffing. To verify the levels of inoculation, serial dilutions were inoculated onto Chromagar™ *Listeria* agar plates and incubated at 37 °C for 48 h. In addition, the real initial levels (CFU g^{-1}) of *L. monocytogenes* on the sausages were determined at day 0 of processing.

2.2. Preparation of Dry Fermented Sausages "Salchichón"

The mixture used for the manufacture of dry fermented sausages "salchichón" was purchased from a meat company in the Extremadura region (Cáceres) and its composition consisted of minced Iberian pork meat (90%) and Iberian pig fatback (7%), with an addition of NaCl (1.8%), cane sugar (0,4%), potassium nitrate (120 ppm), sodium nitrite (100 ppm), black paper and spices. This mixture was transported in refrigerated conditions (<2 °C) from the company to the meat pilot plant located at the Faculty of Veterinary of the University of Extremadura in order to prepare the sausages. Then, the mixture was divided into six equal cases of 10 kg each for the inoculation: (1) B (inoculated only with *L. sakei* at ≈6 log CFU g^{-1}); (2) LI (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1}); (3) LI+LAB (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1} combined with *L. sakei* at ≈6 log CFU g^{-1}); (4) HI (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1}); (5) HI+LAB (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1} combined with *L. sakei* at ≈6 log CFU g^{-1}); and (6) C (uninoculated control).

In all cases, except in C, respective microorganism inocula were adjusted and prepared to be resuspended in a total volume of 150 mL of PBS (as described in Section 2.1) that were added to the ingredients and mixed with an automatic kneader, that was cleaned and sanitized between cases. In the case of C, 150 mL of sterilized PBS were added instead of the bacterium inocula.

The meat dough of each case was stuffed into regenerated collagen casings (40 mm in diameter) supplied by Viscofan (Navarra, Spain). The final weight of each sausage was approximately 500 g. The sausages obtained were ripened in controlled drying chambers at the Faculty of Veterinary Science of the University of Extremadura according to the industrial traditional conditions of "salchichón": 5 °C at 85% relative humidity (RH) for 3 days, then 7 °C and 80% RH for the 17 days, 9 °C and 75% RH for 10 days, and finally, the sausages were kept at 12 °C and 70% RH to reach 90 days of ripening.

Five sausages of each case were taken at 0, 15, 30, 60, and 90 days of the ripening time for microbiological and physicochemical analysis. Before analysis, casings were aseptically removed in a laminar flow cabinet (Telstar, Spain). The experiment, consisting of 6 different cases, 5 sampling times, and 5 different analyzed sausages for each case and sampling time, was evaluated once, according to the European Union Reference Laboratory Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (such as "salchichón") where no growth or the growth probability of this pathogen is ≤10% [27].

2.3. Microbiological Analysis

2.3.1. Confirmation of Absence on *L. monocytogenes* Contamination in Control

Control C was tested to confirm the absence of natural contamination of *L. monocytogenes*. For this, 25 g of each of the 5 sausages were taken at every sampling time and evaluated for the presence or absence of this pathogen according to ISO 11290-1 (International Organization for Standardization [28]).

2.3.2. Estimation of Microbiological Levels

For the remaining microbiological analysis, 10 g of each of the 5 sausages were sampled at every sampling time, mixed with 90 mL of 1% (*w*/*v*) peptone water, and homogenized in a Stomacher machine (Seward, model 400 Circulator, West Sussex. UK) at 300 rpm for 1 min. Decimal serial dilutions were subsequently carried out in 1% (*w*/*v*) of peptone water, and then 100 µL of the cell suspensions were spread onto the surface of different agar plates according to the microbial group analyzed.

In all the inoculated cases with *L. monocytogenes* (LI, LI+LAB, HI, and HI+LAB), viable counts of this pathogen were enumerated on CHROMagar™ *Listeria* Chromogenic media (CH-L, Scharlab, Barcelona, Spain) in duplicate. Each plate was seeded with 0.1 mL, incubated at 37 °C for 24 and 48 h. After the incubation period, the characteristic *L. monocytogenes* colonies, green colonies with a surrounded opaque halo, were counted.

In all cases, 5 groups of microorganisms were determined by using different culture media: the total viable microorganism counts on plate count agar (PCA; Conda Spain), LAB in MRS agar, *Enterobacteriaceae* on Violet Red Bile Glucose agar (VRBG, Conda, Spain), *Staphylococci* in Mannitol salt agar (MSA; Oxoid, UK) and mold and yeast counts on malt extract agar (MEA; 20 g/L of malt extract (Scharlab, Spain), 1 g/L of peptone water, 20 g/L of D (+) glucose monohydrate (Scharlab, Spain), bacto agar 20 g/L (Scharlab, Spain). All the above inoculated media were incubated at 30 °C for 48 h, except MEA and VRBG, which were cultured at 25 °C for 5 days and 37 °C for 24 h, respectively.

2.3.3. Evaluation of Implantation of *L. sakei* 205

In addition, to evaluate the implantation of *L. sakei* 205, MRS plates at the last sampling time (90 days) of *L. sakei* 205 inoculated cases were taken, and 50% of the characteristic LAB colonies were randomly isolated and inoculated in MRS broth and streaked on fresh MRS agar plates and incubated at 37 °C. The cultures were sub-cultured to obtain pure cultures. Pure cultures were maintained in a sterilized MRS broth and kept at −20 °C until characterization by molecular analysis. This procedure was also followed to evaluate LAB colonies on MRS plates in case C at day 90 of ripening to confirm the absence of *L. sakei* 205. The identification of the LAB strains was performed by sequencing analysis of the 16S rRNA region according to the methodology proposed by Walter et al. [29], and PFGE analysis of the DNA with the restriction *NotI* and *SgsI* enzymes (Thermo Fisher Scientific, Waltham, MA., USA) following procedures previously described by Alía et al. [30].

2.4. Physicochemical Analysis

All the analyses for determination of the physicochemical characteristics of the sausages were made in quintuplicates in those cases in which *L. monocytogenes* was not inoculated (B and C).

2.4.1. Water Activity Determination

The a_w of dry fermented sausages "salchichón" was determined at 25 °C by using a Novasina Lab Master Water activity meter model AW SPRINT-TH 300 (Novasina AG, Switzerland). Calibration was performed by using several saturated solutions of known a_w.

2.4.2. Moisture Content Determination

Moisture content was determined following the official methods of the Association of Official Analytical Chemists [31]. This parameter was determined gravimetrically.

2.4.3. pH Determination

The pH was measured with a pH-meter (Model 340, Mettler-Toledo GmbH, Greifensee, Switzerland) that was calibrated with 3 different standard pH solutions (4.0, 7.0 and 9.25). The pH was determined after homogenizing 3 g of each sample with 27 mL of distilled water for 30 s using a homogenizer.

2.4.4. Sodium Chloride Determination

NaCl was determined in duplicate for each of the 5 dry-cured sausages of each case at the end of the ripening period using the Volhard method [32].

2.4.5. Nitrite Determination

Nitrite content was determined in each of the 5 dry-cured sausages of each case at the end of the ripening period according to the method described by AOAC (2005) [33]. Measurement of residual nitrite was spectrophotometrically conducted from pinkish dye produced by coupling sulfanilamide with NED dihydrochloride. A calibration curve was obtained by diluting the standard solution (100 mg/l $NaNO_2$) with distilled water to cover a concentration range from 0.1 to 0.8 mg/l $NaNO_2$. The residual nitrite content was calculated using a standard curve of nitrite solution as mg nitrite per kg sample.

2.5. Statistical Analyses

The statistical treatment was carried out using the software IBM SPSS Statistic version 20 (IBM, New York, NY, USA). For the statistical analysis of the data, different cases and days of ripening were used as independent variables. The counts (Log CFU g^{-1}), a_w, pH, moisture content, sodium chloride, and nitrite values were analyzed as dependent variables. Once the dependent and independent variables of the analysis were determined, a study of the normality of the different data populations was carried out using the Shapiro–Wilk test. Subsequently, the analysis of the data was conducted using the Mann–Whitney test [34]. Statistical significance was established at $p \leq 0.05$.

3. Results

3.1. Enumeration of Microorganisms

Microbiological analysis of the dry-cured sausages to monitor the dynamic changes in the populations responsible for the ripening was carried out. The results obtained from the enumeration of microorganisms of the six cases are shown in Table 1. Total aerobic microorganism counts showed average levels higher in the cases inoculated with *L. sakei* 205 (B, LI+LAB, HI+LAB) than in the uninoculated control (C) or in the cases only inoculated with *L. monocytogenes* (LI, HI). There was not an increase in the levels of the total aerobic microorganisms throughout the ripening of "salchichón" in any of the analyzed cases.

Initial *Enterobacteriaceae* counts ranged between 4.09 and 3.82 log CFU g^{-1} in all analyzed cases (Table 1). However, the number of *Enterobacteriaceae* always significantly decreased ($p \leq 0.05$) throughout the ripening and were below the detection limit at the end of the maturation process.

Counts on MSA agar (*Staphylococci*) showed initial levels of ≈ 5 log CFU g^{-1} in C and B (Table 1). In both cases, an increase ($p \leq 0.05$) in this microbial group of about 1 log CFU g^{-1} was observed until day 30 of ripening. In C, such counts were kept constant until the end of the processing; however, in B, the levels of *Staphylococci* decreased until initial levels at the two last sampling days. Although in the cases where *L. monocytogenes* + *L. sakei* 205 were inoculated there were no data at days 0 and 15 of analysis, the evolution of *Staphylococci* at days 30, 60 and 90 was similar to that the observed in case B, showing levels at the end of the ripening period of at least 1 log CFU/g lower ($p \leq 0.05$) than in the uninoculated control C.

Table 1. Evolution of the different microbial groups in the different cases of dry-cured fermented sausages "salchichón" throughout the ripening process. Microorganisms determined in each media were: Total aerobic counts on plate count agar (PCA), *Enterobacteriaceae* on Violet Red Bile Glucose agar (VRBG), *Staphylococci* on Mannitol salt agar (MSA), and molds and yeasts on Malt extract agar (MEA).

		Days of Ripening				
Cases		**0**	**15**	**30**	**60**	**90**
C	PCA	$5.45 \pm 0.209^{c,2}$	$6.56 \pm 0.159^{a,1}$	$6.86 \pm 0.101^{a,1}$	$6.62 \pm 0.266^{b,1}$	$6.68 \pm 0.306^{a,b,1}$
	VRBG	$4.01 \pm 0.153^{a,1}$	$3.48 \pm 0.360^{d,2}$	2.11 ± 0.312^{3}	$1.99 \pm 0.007^{a,3}$	nd
	MSA	5.04 ± 0.226^{2}	$5.94 \pm 0.217^{b,1}$	$6.08 \pm 0.077^{b,1}$	$6.02 \pm 0.161^{a,1}$	$5.90 \pm 0.185^{a,1}$
	MEA	$5.32 \pm 0.281^{b,3}$	$6.38 \pm 0.188^{b,1,2}$	$6.68 \pm 0.074^{a,b,1}$	$6.66 \pm 0.158^{a,1}$	$6.24 \pm 0.100^{b,2}$
B	PCA	$6.98 \pm 0.135^{b,1}$	$6.43 \pm 0.328^{a,2}$	$6.43 \pm 0.328^{b,2}$	$6.89 \pm 0.170^{a,b,1}$	$7.10 \pm 0.118^{a,1}$
	VRBG	$3.85 \pm 0.109^{a,b,1}$	$3.54 \pm 0.130^{c,d,2}$	2.11 ± 0.271^{3}	$1.97 \pm 0.012^{a,b,3}$	nd
	MSA	4.59 ± 0.428^{2}	$6.33 \pm 0.074^{a,1}$	$6.33 \pm 0.074^{a,1}$	$5.08 \pm 0.397^{b,2}$	$5.05 \pm 0.208^{b,2}$
	MEA	$7.16 \pm 0.083^{a,1}$	$7.04 \pm 0.151^{a,1,2}$	$6.92 \pm 0.097^{a,1,2,3}$	$6.70 \pm 0.209^{a,3}$	$6.81 \pm 0.092^{a,2,3}$
LI+LAB	PCA	$7.10 \pm 0.049^{a,b,1}$	$6.20 \pm 0.068^{a,3}$	$6.20 \pm 0.068^{b,3}$	$6.75 \pm 0.281^{b,2}$	$6.54 \pm 0.199^{b,2}$
	VRBG	$3.82 \pm 0.057^{b,1}$	$3.91 \pm 0.047^{b,1}$	2.07 ± 0.211^{2}	$1.97 \pm 0.007^{a,b,2}$	nd
	MSA	-	-	$5.26 \pm 0.320^{e,1}$	$4.99 \pm 0.243^{b,1}$	$4.50 \pm 0.225^{c,2}$
	MEA	-	-	$6.46 \pm 0.313^{b,c,1}$	$6.27 \pm 0.205^{b,1,2}$	$5.92 \pm 0.102^{c,3}$
HI+LAB	PCA	$7.36 \pm 0.212^{a,1}$	$6.46 \pm 0.084^{a,2}$	$6.46 \pm 0.084^{b,2}$	$7.36 \pm 0.372^{a,1}$	$6.87 \pm 0.237^{a,b,2}$
	VRBG	$4.09 \pm 0.215^{a,1}$	$3.83 \pm 0.069^{b,c,2}$	2.05 ± 0.132^{3}	$1.98 \pm 0.014^{a,b,3}$	nd
	MSA	-	-	$5.60 \pm 0.024^{d,1}$	$4.20 \pm 0.117^{c,3}$	$4.84 \pm 0.172^{b,c,2}$
	MEA	-	-	$6.73 \pm 0.093^{a,b,1}$	$6.66 \pm 0.091^{a,1}$	$6.06 \pm 0.155^{b,c,2}$
LI	PCA	$5.19 \pm 0.251^{c,3}$	$5.29 \pm 0.327^{c,2,3}$	$5.82 \pm 0.245^{c,1}$	$5.72 \pm 0.256^{c,1,2}$	$5.81 \pm 0.271^{c,1}$
	VRBG	$3.84 \pm 0.246^{a,b,2}$	$4.28 \pm 0.139^{a,1}$	$<1.97 \pm 0.018^{3}$	$<1.95 \pm 0.033^{b,3}$	nd
	MSA	-	-	$5.42 \pm 1.120^{e,1}$	$3.66 \pm 0.335^{c,3}$	$4.83 \pm 0.094^{b,c,2}$
	MEA	-	-	$5.38 \pm 0.267^{d,2}$	$6.05 \pm 0.163^{b,1}$	$5.98 \pm 0.278^{b,c,1}$
HI	PCA	$6.47 \pm 0.010^{b,1}$	$5.75 \pm 0.118^{b,3}$	$5.44 \pm 0.130^{d,4}$	$6.10 \pm 0.089^{c,2}$	$5.95 \pm 0.219^{c,2,3}$
	VRBG	$4.05 \pm 0.131^{a,1}$	$3.98 \pm 0.167^{b,2}$	$<2.18 \pm 0.212^{3}$	$<1.98 \pm 0.005^{a,b,3}$	nd
	MSA	-	-	$5.84 \pm 0.157^{c,1}$	$3.76 \pm 0.362^{c,3}$	$5.06 \pm 0.115^{b,2}$
	MEA	-	-	6.09 ± 0.193^{c}	6.02 ± 0.135^{b}	$6.04 \pm 0.077^{b,c}$

C (uninoculated control), B (inoculated with *L. sakei*), LI+LAB (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1} combined with *L. sakei*), HI+LAB (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1} combined with *L. sakei*), LI (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1}), HI (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1}). Values are expressed as mean ± standard deviation. The means with different letters (a–e) in the same column indicate significant differences ($p \leq 0.05$) between cases on the same day. Mean values with different numbers (1–4) in the same row indicate significant differences ($p \leq 0.05$) between days in the same case and the same culture medium. nd: not detected (below the detection limit). (-): not determined.

Counts on MEA agar (molds and yeasts) ranged between 5.3 and 7.1 log CFU/g during the ripening process in all the analyzed cases (Table 1). No significant effect of the ripening on levels of molds and yeasts was observed.

The LAB levels are shown in Figure 1. The microbiological analysis revealed significant differences at the beginning of the ripening between cases inoculated with LAB (B, LI+LAB, HI+LAB) and those which were not inoculated with LAB (C, LI, HI). Counts on MRS agar were around 7 log CFU g^{-1} for all cases inoculated with *L. sakei* (B, LI+LAB, HI+LAB) and 4–5 log CFU g^{-1} for those not inoculated with *L. sakei* (C, LI, HI). LAB counts of the cases inoculated with *L. sakei* remained constant (≈7 log CFU g^{-1}) until 30 days of ripening, then they decreased slightly but their levels were always higher than 6 log CFU g^{-1}. The levels of LAB in those cases that were not inoculated with *L. sakei* showed an increase in these counts at days 15 and 30 of ripening, reaching counts around 7 log CFU g^{-1} (Figure 1). Thereafter, counts decreased throughout the ripening process, except in C, where the counts on MRS agar were more similar to those found in the cases inoculated with *L. sakei* 205. However, the cases inoculated only with *L. monocytogenes* (LI and HI) showed lower LAB counts throughout the maturation process, and were significantly different from the remaining cases at the end of the ripening.

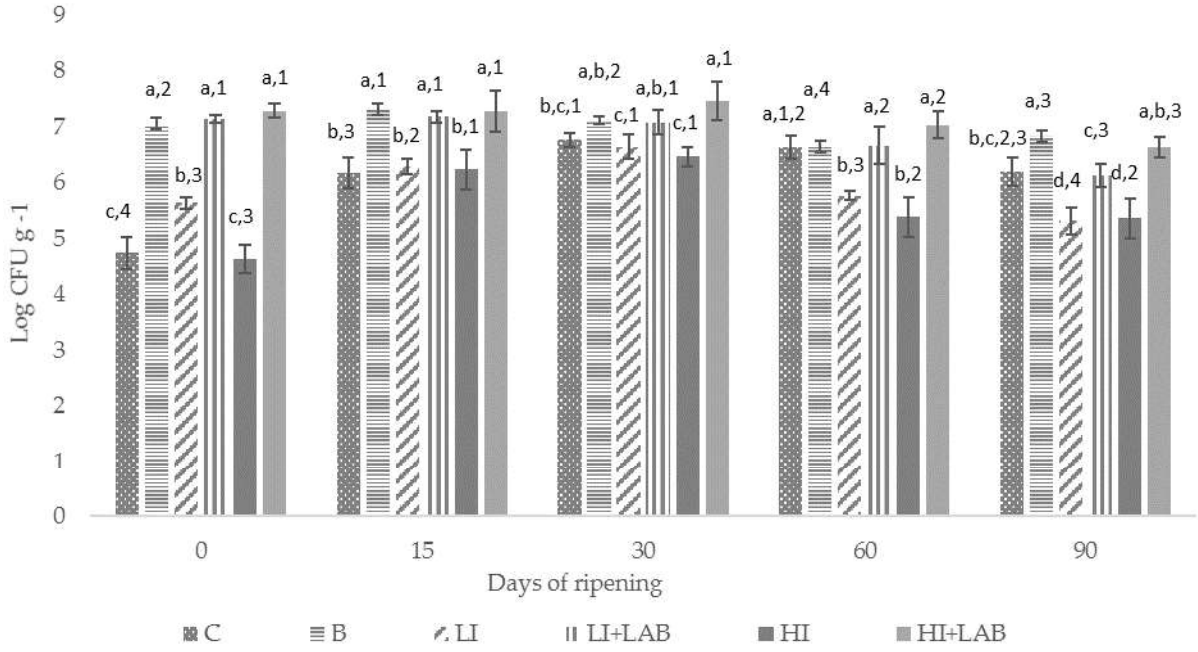

Figure 1. Evolution of lactic acid bacteria counts throughout the ripening process of "salchichón". C (uninoculated control), B (inoculated with *L. sakei*), LI+LAB (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1} combined with *L. sakei*), HI+LAB (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1} combined with *L. sakei*), LI (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1}), HI (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1}). Bars with different letters (a–d) indicate significant differences ($p \leq 0.05$) between cases on the same day. Bars with different numbers (1–4) indicate significant differences ($p \leq 0.05$) between days in the same case.

When isolates of the cases inoculated with *L. sakei* were analyzed to evaluate the implantation of this strain, most of the investigated isolates (85.7%) were identified as *L. sakei* (100% identity) by sequencing analysis of the 16S rRNA region. In addition, these isolates showed the same pattern of *L. sakei* 205 in the PFGE analysis. The remaining strains were *Lactobacillus plantarum* group (7.15%) and *Lactobacillus curvatus* (7.15%). However, the isolates of C, LI and HI were identified as *Lactobacillus curvatus* (50%), *Enterococcus faecalis* (36%), and *Lactobacillus plantarum* groups (14%).

The inoculation of *L. monocytogenes* resulted in an initial concentration of 6.63–6.60 log CFU g^{-1} in HI and HI+LAB and 4.09–4.18 CFU g^{-1} in LI and LI+LAB (Table 2). *L. monocytogenes* did not grow in any of the inoculated cases at any of the ripening time. Levels of this pathogenic bacterium showed significant ($p \leq 0.05$) decreases in comparison with the initial ones, at day 15 (LI+BAL) and at days 30, 60 and 90 (all cases inoculated with *L. monocytogenes*; Table 2). After 90 days of ripening, the reduction in *L. monocytogenes* counts in LI was 1.61 log CFU g^{-1}, while in LI+LAB it was significantly higher (2.03 log CFU g^{-1}, Figure 2). In HI, the reduction was 1.64 log CFU g^{-1}, while in HI+LAB it was significantly higher (1.77 log CFU g^{-1}, Figure 2).

Table 2. Evolution of *L. monocytogenes* counts on inoculated cases of dry-cured fermented sausages "salchichón" throughout the ripening process.

Cases	Days of Ripening				
	0	**15**	**30**	**60**	**90**
LI	4.09 ± 0.091^{1}	$3.68 \pm 0.451^{1,2}$	3.45 ± 0.233^{2}	2.89 ± 0.136^{3}	$2.49 \pm 0.231^{b,3}$
LI+LAB	4.18 ± 0.089^{1}	3.84 ± 0.103^{2}	3.42 ± 0.215^{3}	2.74 ± 0.117^{4}	$2.14 \pm 0.127^{a,5}$
HI	6.63 ± 0.056^{1}	$6.43 \pm 0.179^{1,2}$	$6.31 \pm 0.075^{b,2}$	5.12 ± 0.214^{3}	$5.00 \pm 0.104^{b,3}$
HI+LAB	6.60 ± 0.107^{1}	6.43 ± 0.136^{1}	$6.04 \pm 0.129^{a,2}$	4.99 ± 0.111^{3}	$4.83 \pm 0.091^{a,3}$

LI+LAB (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1} combined with *L. sakei*), HI+LAB (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1} combined with *L. sakei*), LI (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1}), HI (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1}). Values are expressed as mean ± standard deviation. Mean values with different letters (a,b) in the same column indicate significant differences ($p \leq 0.05$) between cases on the same incubation day. Mean values with different numbers (1–4) in the same row indicates significant differences ($p \leq 0.05$) between incubation days in the same case.

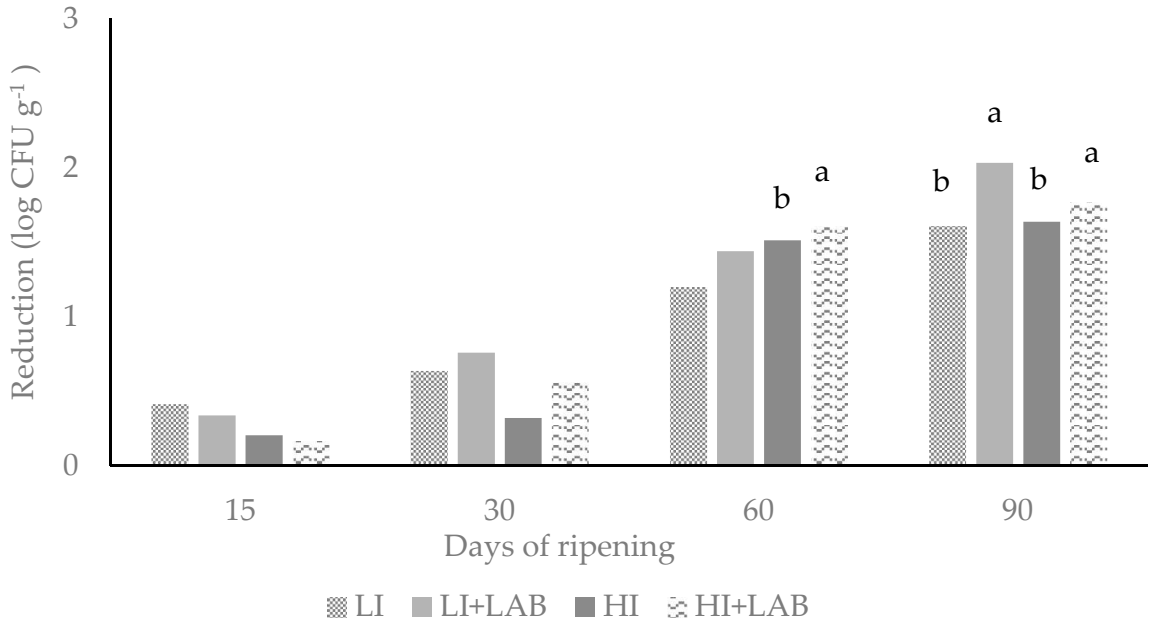

Figure 2. Reduction in the *L. monocytogenes* levels throughout the processing of dry-cured fermented "salchichón". LI (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1}), LI+LAB (inoculated with *L. monocytogenes* at ≈4 log CFU g^{-1} combined with *L. sakei*), HI (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1}), HI+LAB (inoculated with *L. monocytogenes* at ≈7 log CFU g^{-1} combined with *L. sakei*). Bars with different letters (a,b) indicate significant differences ($p \leq 0.05$) between cases on the same day.

3.2. Physicochemical Parameters

The evolution of moisture content (%), a_w, and pH throughout the ripening process of cases C and B of "salchichón" is shown in Table 3. Initial moisture content in both cases was above 85%, decreasing throughout the ripening process until values of 25–26% ($p \leq 0.05$; Table 3). However, there were no significant differences ($p > 0.05$) between both cases at any of the ripening times. The a_w decreased ($p \leq 0.05$) from initial values (0.946–0.947) found in the raw product to values below 0.790 at day 90 (Table 3). No significant differences ($p > 0.05$) among control and inoculated cases were observed in this parameter.

Table 3. Moisture content, water activity (a_w) and pH in uninoculated control (C) and case inoculated only with *L. sakei* 205 (case B) of dry-cured fermented sausages "salchichón" throughout the ripening process.

	Cases	Days of Ripening				
		0	**15**	**30**	**60**	**90**
Moisture content (%)	C	89.67 ± 0.656 [1]	56.35 ± 2.525 [2]	43.16 ± 1.396 [3]	32.34 ± 2.155 [3,4]	26.04 ± 1.484 [4]
	B	85.84 ± 0.235 [1]	61.19 ± 3.052 [2]	42.70 ± 1.387 [3]	31.65 ± 1.188 [4]	25.17 ± 1.484 [5]
a_w	C	0.947 ± 0.001 [1]	0.905 ± 0.009 [2]	0.874 ± 0.003 [3]	0.821 ± 0.003 [4]	0.785 ± 0.008 [5]
	B	0.946 ± 0001 [1]	0.914 ± 0.007 [2]	0.877 ± 0.005 [3]	0.817 ± 0.003 [4]	0.779 ± 0.010 [5]
pH	C	5.83 ± 0.226 [1,2]	5.76 ± 0.058 [a,2]	5.89 ± 0.030 [a,1,2]	5.99 ± 0.071 [1]	5.87 ± 0.105 [1,2]
	B	5.82 ± 0.021 [1,2]	5.48 ± 0.058 [3]	5.73 ± 0.085 [2]	5.98 ± 0.050 [1]	5.91 ± 0.041 [1]

Values are expressed as mean ± standard deviation. Mean values with different letters (a) in the same column indicate significant differences ($p \leq 0.05$) between cases on the same day. Mean values with different numbers (1–4) in the same row indicate significant differences ($p \leq 0.05$) between incubation days in the same case.

The evolution of pH differed between cases C and B, showing lower values in the case inoculated with *L. sakei* than in the uninoculated control at days 15 and 30 of ripening (Table 3). However, at day 30, an increase in the pH value was observed in C up to values close to 5.9, and they were kept constant until the end of ripening. There were no significant differences between both cases at days 60 and 90 of processing.

NaCl and nitrite contents were only determined in ripened "salchichón" (at day 90), showing both cases (C and B) with similar values, higher than 3.2% NaCl and around 6.9–7.4 ppm of nitrite (Table 4). No differences ($p > 0.05$) between both cases in these parameters were found.

Table 4. Sodium chloride (NaCl) and nitrite contents in uninoculated control (C) and case inoculated only with *L. sakei* 205 (B) in dry-cured fermented sausages "salchichón".

Cases	NaCl (%)	Nitrites (ppm)
C	3.29 [1] ± 0.100	7.49 ± 1.019
B	3.36 ± 0.099	6.96 ± 0.770

[1] Values are expressed as mean ± standard deviation. Mean values with different letters in the same column indicate significant differences ($p \leq 0.05$) between cases.

4. Discussion

In this work, the survival and control of *L. monocytogenes* throughout the processing of dry-cured fermented sausage "salchichón" was evaluated by using the challenge test [27]. For this study, an industrial traditional procedure for the production of "salchichón" was carried out [35]. Differences in the reduction/inactivation of *L. monocytogenes* in dry-cured fermented sausages in relation to the initial load have been reported [19,36]; therefore, two levels of inoculation of *L. monocytogenes* were used for this study: high level (about 7 log CFU g^{-1}) and low level (around 4 log CFU g^{-1}). In addition, exploring greater effectiveness in reducing *L. monocytogenes*, a selected strain of *L. sakei* (205) isolated from dry-cured fermented sausages and previously selected because of its antagonist activity against *L. monocytogenes* (unpublished data), was evaluated in the challenge test.

Physicochemical evaluation of case B inoculated with *L. sakei* 205 and uninoculated control (C) showed that the ripening process was correctly conducted. Thus, moisture content and a_w decreased throughout the ripening process, from initial values around 85% to 25–26% and from 0.94 to 0.78 a_w, respectively, reaching values similar to those usually reported for dry-cured fermented sausages [37–39]. The decrease in a_w in dry-cured fermented sausages such as "salchichón" is important for extending the shelf life and safety of the product [40]. No differences for moisture content or a_w parameters were observed between both cases. This evidences that *L. sakei* 205 does not provoke any modification in any of the two above-mentioned parameters, as has been reported for other LAB assayed [41,42]. However, the strain *L. sakei* 205 provoked a slight reduction

in pH in the first 30 days of ripening, in comparison with uninoculated control, probably due to the increase in the lactic acid content, as a result of carbohydrate breakdown by microbial metabolism [43]. There was no reduction in pH in the uninoculated control (C), in spite of LAB counts from natural contamination reaching similar levels as those found in B (inoculated with *L. sakei* 205). In the last stages of processing, an increase in pH was detected, mainly in B (inoculated with *L. sakei* 205), which could be explained by the accumulation of non-protein nitrogen and amino acid catabolism products [43,44]. Consequently, no differences between B and C were detected for this parameter at day 90 of ripening. There were also no differences between both cases (B and C) in the remaining physical–chemical parameters analyzed, NaCl and nitrite content. Levels of NaCl found at day 90 were similar to those reported for dry-cured fermented sausages [45] and in accordance with the level of salt used in the manufacture of sausages. Regarding nitrite content, low levels (lower than 10 ppm) were detected at the end ripening. Nitrite values obtained were in levels usually found in ripened sausages, because it is a very reactive compound and only residual nitrite that has not reacted with myoglobin is detected in finished products [46].

Besides the physicochemical parameter, the evolution of the different microbial groups in the six tested cases throughout the processing of "salchichón" was evaluated.

Levels of total aerobic microorganisms were higher in sausages inoculated with *L. sakei* 205 than in the remaining cases at the beginning of ripening, due to the effect of the addition of the protective cultures [22]. However, during processing there was not an increase in this microbiological parameter in any of the analyzed cases, mainly due to the decrease in the a_w until values around 0.78 at the end of maturation but also by the slight decrease in pH, and the presence of NaCl and nitrites. These data are consistent with those observed in other dry-cured fermented sausages [46–48].

LAB counts of the cases inoculated with *L. sakei* 205 remained constant, with values of about 7 log CFU g^{-1} until 30 days of ripening and then a slight decrease was observed, probably due to the reduction in a_w, but counts were always higher than 6 log CFU g^{-1}. In addition, the characterization of the LAB isolates in these sausages showed that *L. sakei* 205 was the strain mostly detected, which means that this strain was properly implanted throughout the ripening. The levels of LAB in cases that were not inoculated with *L. sakei* 205 showed an increase in this microbial group at the beginning of processing to reach similar counts to those detected in the inoculated cases with the selected strain of LAB. However, in the analysis of the isolates no *L. sakei* was detected, which suggests that this strain only was present in cases inoculated with this selected LAB.

During the evolution of the remaining microbial groups, the decrease in *Enterobacteriaceae* counts until non-detectable levels at day 90 of ripening in all analyzed cases and the decrease in *Staphylococci* only in cases inoculated with *L. sakei* 205 at days 60 and 90 of ripening is relevant. The *Enterobacteriaceae* results are consistent with those reported by Cocolin et al. [49], who demonstrated the persistence of this microbial group until day 60 of ripening of dry-fermented sausages. It should be emphasized that no growth of *Enterobacteriaceae* during processing was found in this work; however, punctual growth of this microbial group during the processing of dry-cured fermented sausages has previously been reported [46,50]. The decrease in a_w, the various metabolites excreted by LAB, and the slight drop in pH may partially explain the reduction and disappearance of *Enterobacteriaceae* in this kind of meat products [46]. The reduction in levels of *Staphylococci* only in cases inoculated with *L. sakei* 205 at the end of ripening may be related to the effect of the synergist action of this strain together with the reduction in a_w throughout the processing.

The evaluation of the growth/inactivation rate of *L. monocytogenes* in both high and low inoculation levels showed no growth of this pathogen at any of the ripening times evaluated. This aspect is relevant because at the first 15 days of ripening there could be conditions of temperature (7 °C), a_w (0.947), and pH (5.8–5.4) which can favor the growth of *L. monocytogenes*, but the synergistic effect of the above parameters and the presence of

NaCl, nitrite, and LAB inoculated or from natural contamination inhibit the growth of this pathogen.

Throughout the ripening process, reductions in *L. monocytogenes* ranging from 1.6 to 2.2 log CFU g^{-1} were observed at both high and low inoculation levels of "salchichón". These values are lower than reductions of up to 5 log CFU g^{-1} reported in inoculated Portuguese "linguiça" smoked dry-cured sausages [51], although in that work the reduction was mainly due to the smoking and the high temperatures employed in the process. However, reduction levels found in the present work are similar to those found for this pathogen in other dry-cured meat products [52]. Several authors have reported that a long ripening period, which is related to a high decrease in a_w, leads to a higher reduction in *L. monocytogenes* counts in dry-cured meat products [53–55]. However, short ripening periods in dry-cured fermented sausages have been associated with a greater survival of *L. monocytogenes* [19,45]. Nitrite used in the formulation of "salchichón" at a concentration lower than 150 ppm, as occurred in the present work, also contributes to the reduction in *L. monocytogenes*, as has been reported in different types of dry-fermented sausages [56,57].

There were no differences in reductions in relation to the level of inoculation of *L. monocytogenes*. However, the reduction in the pathogen counts was significantly higher in HI+LAB and LI+LAB than in HI and LI. This additional reduction provoked by the presence of *L. sakei* 205 was 0.42 log CFU g^{-1} in LI. This proves that *L. sakei* 205 has anti-*L. monocytogenes* activity during the ripening of "salchichón", even considering that in the cases inoculated only with *L. monocytogenes*, indigenous LAB were found, probably with some antimicrobial effect. *L. sakei* is highly adapted to the fermented meat matrix [39], and many studies have determined that *L. sakei* has been widely used as a biocontrol for *L. monocytogenes* in dry fermented sausages [24,58].

Although the additional reduction in *L. monocytogenes* provoked by the selected *L. sakei* was not very high, it could be sufficient to guarantee the elimination of this pathogenic bacterium throughout the processing of "salchichón" when this pathogen contaminates this product at the usual levels (below 2 log CFU g^{-1}). This is very important, because minimizing the risk of listeriosis caused by the consumption of "salchichón" improves food safety and meets the microbiological criteria of RTE foods throughout their shelf life in the EU [1,2].

5. Conclusions

The processing of "salchichón" does not allow the growth of *L. monocytogenes.* On the contrary, it provokes a reduction in this pathogen that could be even higher by using the strain *L. sakei* 205 as a protective culture. The manufacturing of "salchichón" also allows minimization of *Enterobacteriaceae* until non-detectable levels. These findings may be of great interest both for the safety and extending the shelf-life of "salchichón".

Author Contributions: All the authors contributed significantly to the research. Conceptualization, J.J.C. and A.R.; data curation, I.M.; formal analysis, I.M., L.S.-M. and P.P.; funding acquisition, J.J.C.; methodology, I.M.; project administration, J.J.C.; resources, A.R. and J.J.C.; supervision, A.R. and J.J.C.; writing—original draft preparation, I.M. and J.J.C.; and writing—review and editing, I.M., A.R., L.S.-M., P.P. and J.J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been funded by the Spanish Ministry of Economy, Industry, and Competitiveness, the Spanish Ministry of Education, Culture and Sports and the Junta De Extremadura and Feder.

Data Availability Statement: Not applicable.

Acknowledgments: This work was supported by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. I. Martín is supporting by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

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

References

1. Peromingo, B.; Andrade, M.J.; Delgado, J.; Sánchez-Montero, L.; Núñez, F. Biocontrol of aflatoxigenic *Aspergillus parasiticus* by native *Debaryomyces hansenii* in dry-cured meat products. *Food Microbiol.* **2019**, *82*, 269–276. [CrossRef] [PubMed]
2. Bonilauri, P.; Merialdi, G.; Ramini, M.; Bardasi, L.; Taddei, R.; Grisenti, M.S.; Daminelli, P.; Cosciani-Cunico, E.; Dalzini, E.; Frustoli, M.A.; et al. Modeling the behavior of *Listeria innocua* in Italian salami during the production and high-pressure validation of processes for exportation to the U.S. *Meat Sci.* **2021**, *172*, 108315. [CrossRef] [PubMed]
3. Casquete, R.; Benito, M.J.; Martín, A.; Ruiz-Moyano, S.; Aranda, E.; Córdoba, M.G. Microbiological quality of salchichón and chorizo, traditional Iberian dry-fermented sausages from two different industries, inoculated with autochthonous starter cultures. *Food Control* **2012**, *24*, 191–198. [CrossRef]
4. Bernáldez, V.; Córdoba, J.J.; Rodríguez, M.; Cordero, M.; Polo, L.; Rodríguez, A. Effect of *Penicillium nalgiovense* as protective culture in processing of dry-fermented sausage "salchichón". *Food Control* **2013**, *32*, 69–76. [CrossRef]
5. Delgado, J.; Rondán, J.J.; Núñez, F.; Rodríguez, A. Influence of an industrial dry-fermented sausage processing on ochratoxin A production by *Penicillium nordicum*. *Int. J. Food Microbiol.* **2021**, *339*. [CrossRef] [PubMed]
6. Utrilla, M.C.; García Ruiz, A.; Soriano, A. Effect of partial replacement of pork meat with an olive oil organogel on the physicochemical and sensory quality of dry-ripened venison sausages. *Meat Sci.* **2014**, *97*, 575–582. [CrossRef]
7. Soriano, A.; Cruz, B.; Gómez, L.; Mariscal, C.; García Ruiz, A. Proteolysis, physicochemical characteristics and free fatty acid composition of dry sausages made with deer (*Cervus elaphus*) or wild boar (*Sus scrofa*) meat: A preliminary study. *Food Chem.* **2006**, *96*, 173–184. [CrossRef]
8. Schoder, D. *Listeria*: Listeriosis. *Encycl. Food Health* **2015**, 561–566. [CrossRef]
9. Barmpalia-Davis, I.M.; Geornaras, I.; Kendall, P.A.; Sofos, J.N. Survival of *Listeria monocytogenes* in a Simulated Dynamic Gastrointestinal Model during Storage of Inoculated Bologna and Salami Slices in Vacuum Packages. *J. Food Prot.* **2008**, *71*, 2014–2023. [CrossRef]
10. Simpson, C.A.; Geornaras, I.; Yoon, Y.; Scanga, J.A.; Kendall, P.A.; Sofos, J.N. Effect of inoculum preparation procedure and storage time and temperature on the fate of *Listeria monocytogenes* on inoculated salami. *J. Food Prot.* **2008**, *71*, 494–501. [CrossRef]
11. Ferreira, V.; Barbosa, J.; Silva, J.; Felício, M.T.; Mena, C.; Hogg, T.; Gibbs, P.; Teixeira, P. Characterisation of alheiras, traditional sausages produced in the North of Portugal, with respect to their microbiological safety. *Food Control* **2007**, *18*, 436–440. [CrossRef]
12. Doménech, E.; Jimenez -Belenguer, A.; Amoros, J.A.; Ferrus, M.A.; Escriche, I. Prevalence and antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain. *Food Control* **2015**, *47*, 120–125. [CrossRef]
13. Meloni, D. Presence of *Listeria monocytogenes* in Mediterranean-Style Dry Fermented Sausages. *Foods* **2015**, *4*, 34–50. [CrossRef]
14. Schwartz, R.H.; Eaton, J.; Bower, B.D.; Aynsley-Green, A. Ketogenic Diets in the T Epilepsy: Short-Term Clinical E. *Dev. Med. Child Neurol.* **1989**, *31*, 149–151. [CrossRef]
15. Foodborne Ilness Outbreak Database Siena Foods Salame. Available online: http://www.outbreakdatabase.com/details/assi-market-pickles-2010/?vehicle=cucumber (accessed on 2 February 2021).
16. Commission, E. Commission Regulation (EC) N0. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Communitites* **2005**, *338*, 1–26.
17. Commission, E. Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. *Off. J. Eur. Communitites* **2007**, *322*, 12–29.
18. USDA-FSIS Salmonella Compliance Guidelines for Small and Very Small Meat and Poultry Establishments that Produce Ready-to-Eat (RTE) Products and Revised Appendix A. 2017; Volume 7. Available online: https://www.fsis.usda.gov/guidelines/2017-0008 (accessed on 3 February 2021).
19. Nightingale, K.K.; Thippareddi, H.; Phebus, R.K.; Marsden, J.L.; Nutsch, A.L. Validation of a traditional Italian-style salami manufacturing process for control of *Salmonella* and *Listeria monocytogenes*. *J. Food Prot.* **2006**, *69*, 794–800. [CrossRef]
20. Nissen, H.; Holck, A. Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella kentucky* in Norwegian fermented, dry sausage. *Food Microbiol.* **1998**, *15*, 273–279. [CrossRef]
21. Cenci-Goga, B.T.; Rossitto, P.V.; Sechi, P.; Parmegiani, S.; Cambiotti, V.; Cullor, J.S. Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Sci.* **2012**, *90*, 599–606. [CrossRef] [PubMed]
22. Pedonese, F.; Torracca, B.; Mancini, S.; Pisano, S.; Turchi, B.; Cerri, D.; Nuvoloni, R. Effect of a *Lactobacillus sakei* and *Staphylococcus xylosus* protective culture on *Listeria monocytogenes* growth and quality traits of Italian fresh sausage (salsiccia) stored at abusive temperature. *Ital. J. Anim. Sci.* **2020**, *19*, 1363–1374. [CrossRef]
23. Chaillou, S.; Champomier-Vergès, M.C.; Cornet, M.; Crutz-Le Coq, A.M.; Dudez, A.M.; Martin, V.; Beaufils, S.; Darbon-Rongère, E.; Bossy, R.; Loux, V.; et al. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nat. Biotechnol.* **2005**, *23*, 1527–1533. [CrossRef]
24. Zagorec, M.; Champomier-Vergès, M.-C. *Lactobacillus sakei*: A Starter for Sausage Fermentation, a Protective Culture for Meat Products. *Microorganisms* **2017**, *5*, 56. [CrossRef]
25. Gelinski, J.M.L.N.; Baratto, C.M.; Casagrande, M.; de Oliveira, T.P.; Megiolaro, F.; de Martini Soares, F.A.S.; de Souza, E.M.B.; Vicente, V.A.; Fonseca, G.G. Control of pathogens in fresh pork sausage by inclusion of *Lactobacillus sakei* BAS0117. *Can. J. Microbiol.* **2019**, *65*, 831–841. [CrossRef]

26. García, C.; Martín, A.; Timón, M.L.; Córdoba, J.J. Microbial populations and volatile compounds in the "bone taint" spoilage of dry cured ham. *Lett. Appl. Microbiol.* **2000**, *30*, 61–66. [CrossRef]
27. Beaufort, A.; Cornu, M.; Bergis, H.; Lardeux, A.L.; Lombard, B. EURL Lm Technical Guiadance Document for Conducing Shelf-Life Studies on *Listeria monocytogenes* in Ready to Eat Foods. Available online: http://www.fsai.ie/uploadedFiles/EURL%0ALm_TechnicalGuidanceDocument%0ALmshelf-lifestudies_V3_2014-%0A06-06(2).pdf (accessed on 24 January 2021).
28. *ISO 11290-2: Método Horizontal para el Recuento de* L. monocytogenes *y Listeria spp.*; International Organization for Standardization: Geneva, Switzerland, 2017.
29. Walter, J.; Tannock, G.W.; Tilsala-Timisjarvi, A.; Rodtong, S.; Loach, D.M.; Munro, K.; Alatossava, T. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* **2000**, *66*, 297–303. [CrossRef]
30. Alía, A.; Andrade, M.J.; Rodríguez, A.; Martín, I.; Pérez-Baltar, A.; Medina, M.; Córdoba, J.J. Prevalence and characterization of *Listeria monocytogenes* in deboning and slicing areas of Spanish dry-cured ham processing. *LWT Food Sci. Technol.* **2020**, *128*, 109498. [CrossRef]
31. AOAC. *Moisture in Malt Gravimetric Method (935.29)*, 17th ed.; AOAC: Gaithersburg, MD, USA, 2000.
32. AOAC. *Official Methods of Analysis of AOAC International*, 20th ed.; AOAC: Gaithersburg, MD, USA, 2016; Volume 52, ISBN 0935584870.
33. AOAC. *Official Methods of Analysis of AOAC International*, 18th ed.; AOAC: Gaithersburg, MD, USA, 2005; Volume 3172.
34. Mann, H.B.; Whitney, D. On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Stat.* **1974**, *2*, 347–370. [CrossRef]
35. Delgado, J.; Peromingo, B.; Rodríguez, A.; Rodríguez, M. Biocontrol of *Penicillium griseofulvum* to reduce cyclopiazonic acid contamination in dry-fermented sausages. *Int. J. Food Microbiol.* **2019**, *293*, 1–6. [CrossRef] [PubMed]
36. Thévenot, D.; Delignette-Muller, M.L.; Christieans, S.; Vernozy-Rozand, C. Fate of *Listeria monocytogenes* in experimentally contaminated French sausages. *Int. J. Food Microbiol.* **2005**, *101*, 189–200. [CrossRef] [PubMed]
37. Casquete, R.; Benito, M.J.; Martín, A.; Ruiz-Moyano, S.; Hernández, A.; Córdoba, M.G. Effect of autochthonous starter cultures in the production of "salchichón", a traditional Iberian dry-fermented sausage, with different ripening processes. *LWT Food Sci. Technol.* **2011**, *44*, 1562–1571. [CrossRef]
38. Álvarez, M.; Andrade, M.J.; García, C.; Rondán, J.J.; Núñez, F. Effects of preservative agents on quality attributes of dry-cured fermented sausages. *Foods* **2020**, *9*, 1505. [CrossRef]
39. El Adab, S.; Essid, I.; Hassouna, M. Microbiological, biochemical and textural characteristics of a tunisian dry fermented poultry meat sausage inoculated with selected starter cultures. *J. Food Saf.* **2015**, *35*, 75–85. [CrossRef]
40. Chevallier, I.; Ammor, S.; Laguet, A.; Labayle, S.; Castanet, V.; Dufour, E.; Talon, R. Microbial ecology of a small-scale facility producing traditional dry sausage. *Food Control* **2006**, *17*, 446–453. [CrossRef]
41. Najjari, A.; Boumaiza, M.; Jaballah, S.; Boudabous, A.; Ouzari, H.I. Application of isolated *Lactobacillus sakei* and *Staphylococcus xylosus* strains as a probiotic starter culture during the industrial manufacture of Tunisian dry-fermented sausages. *Food Sci. Nutr.* **2020**, *8*, 4172–4184. [CrossRef] [PubMed]
42. Pavli, F.G.; Argyri, A.A.; Chorianopoulos, N.G.; Nychas, G.J.E.; Tassou, C.C. Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. *LWT* **2020**, *118*. [CrossRef]
43. Aleson-Carbonell, L.; Fernández-López, J.; Pérez-Alvarez, J.A.; Kuri, V. Functional and sensory effects of fibre-rich ingredients on breakfast fresh sausages manufacture. *Food Sci. Technol. Int.* **2005**, *11*, 89–97. [CrossRef]
44. Pérez-Alvarez, J.A.; Sayas-Barberá, M.E.; Fernández-López, J.; Aranda-Catalá, V. Physicochemical characteristics of Spanish-type dry-cured sausage. *Food Res. Int.* **1999**, *32*, 599–607. [CrossRef]
45. Gonzales-Barron, U.; Cadavez, V.; Pereira, A.P.; Gomes, A.; Araújo, J.P.; Saavedra, M.J.; Estevinho, L.; Butler, F.; Pires, P.; Dias, T. Relating physicochemical and microbiological safety indicators during processing of linguiça, a Portuguese traditional dry-fermented sausage. *Food Res. Int.* **2015**, *78*, 50–61. [CrossRef]
46. Fernández-López, J.; Sendra, E.; Sayas-Barberá, E.; Navarro, C.; Pérez-Alvarez, J.A. Physico-chemical and microbiological profiles of "salchichón" (Spanish dry-fermented sausage) enriched with orange fiber. *Meat Sci.* **2008**, *80*, 410–417. [CrossRef]
47. Capita, R.; Llorente-Marigómez, S.; Prieto, M.; Alonso-Calleja, C. Microbiological profiles, pH, and titratable acidity of chorizo and salchichón (Two Spanish dry fermented sausages) manufactured with ostrich, deer, or pork meat. *J. Food Prot.* **2006**, *69*, 1183–1189. [CrossRef]
48. Metaxopoulos, J.; Samelis, J.; Papadelli, M. Technological and microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry fermented sausage in Greece. *Ital. J. Food Sci.* **2001**, *13*, 3–18.
49. Cocolin, L.; Dolci, P.; Rantsiou, K.; Urso, R.; Cantoni, C.; Comi, G. Lactic acid bacteria ecology of three traditional fermented sausages produced in the North of Italy as determined by molecular methods. *Meat Sci.* **2009**, *82*, 125–132. [CrossRef] [PubMed]
50. Lizaso, G.; Chasco, J.; Beriain, M.J. Microbiological and biochemical changes during ripening of salchichon, a Spanish dry cured sausage. *Food Microbiol.* **1999**, *16*, 219–228. [CrossRef]
51. Hajmeer, M.N.; Tajkarimi, M.; Gomez, E.L.; Lim, N.; O'Hara, M.; Riemann, H.P.; Cliver, D.O. Thermal death of bacterial pathogens in linguiça smoking. *Food Control* **2011**, *22*, 668–672. [CrossRef]

52. Taddei, R.; Giacometti, F.; Bardasi, L.; Bonilauri, P.; Ramini, M.; Fontana, M.C.; Bassi, P.; Castagnini, S.; Ceredi, F.; Pelliconi, M.F.; et al. Effect of production process and high-pressure processing on viability of *Listeria innocua* in traditional Italian dry-cured coppa. *Ital. J. Food Saf.* **2020**, *9*, 104–109. [CrossRef]
53. Reynolds, A.E.; Harrison, M.A.; Rose-Morrow, R.; Lyon, C.E. Validation of dry cured ham process for control of pathogens. *J. Food Sci.* **2001**, *66*, 1373–1379. [CrossRef]
54. Montiel, R.; Peirotén, Á.; Ortiz, S.; Bravo, D.; Gaya, P.; Martínez-Suárez, J.V.; Tapiador, J.; Nuñez, M.; Medina, M. Inactivation of *Listeria monocytogenes* during dry-cured ham processing. *Int. J. Food Microbiol.* **2020**, *318*, 108469. [CrossRef]
55. Barbuti, S.; Grisenti, M.S.; Frustoli, M.A.; Parolari, G. Validation of the Manufacturing Process of Italian Dry-Cured Ham(Prosciiutto) for the Inactivation of *Listeria monocytogenes* and *Salmonella* spp. *Int. Congr. Meat Sci. Technol.* **2009**, *PE6.10*, 361.
56. Sebranek, J.G. Natural and organic cured meat products: Regulatory, manufacturing, marketing, quality and safety issues. *Am. Meat Sci. Assoc.* **2007**, *1*, 1–15.
57. Lücke, F.K. Utilization of microbes to process and preserve meat. *Meat Sci.* **2000**, *56*, 105–115. [CrossRef]
58. Bošković, M.; Tadić, V.; Dordević, J.; Glišić, M.; Lakićević, B.; Dimitrijević, M.; Ž Baltić, M. Effect of starter cultures on survival of *Listeria monocytogenes* in Čajna sausage. *IOP Conf. Ser. Earth Environ. Sci.* **2017**, *85*, 012074. [CrossRef]

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Growth and Expression of Virulence Genes of *Listeria monocytogenes* during the Processing of Dry-Cured Fermented "Salchichón" Manufactured with a Selected *Lactilactobacillus sakei*

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Simple Summary: During the ripening process of the dry-cured fermented sausage "salchichón", *Listeria monocytogenes* could fail to be eliminated. In addition, the food safety criterion for *L. monocytogenes* in the European Union sets up a maximum level of 100 units of this microorganism per gram in ready-to-eat products throughout their shelf-life. Thus, since *L. monocytogenes* could be present in this product, it is necessary to evaluate the impact of the dry-cured fermented processing in the potential virulence of this pathogen, even considering the possible effect of the usual microbiota (lactic-acid bacteria) of "salchichón". In this work, the effect of the processing of "salchichón", inoculated with a selected strain of *Lactilactobacillus sakei*, on the growth of *L. monocytogenes* and on the expression of its virulence genes, was evaluated. The processing of "salchichón" provoked a relevant reduction in *L. monocytogenes*, but this pathogen was not completely eliminated. However, a downregulation in the expression of the tested virulence genes was found, which could suppose a reduction in the pathogenic effect of this microorganism. These findings could be of great interest to consider the dry-cured ripening of "salchichón" as a safe process to control the pathogen *L. monocytogenes*.

Abstract: The effect of the dry-cured fermented processing of "salchichón" inoculated with a selected strain of *Lactilactobacillus sakei* (205) on the growth and transcriptional response of three virulence genes (*plcA*, *hly*, and *iap*) of *Listeria monocytogenes* was evaluated. For this, three different batches of "salchichón" were analyzed: batch B (inoculated only with *L. sakei*), batch L (inoculated only with *L. monocytogenes*), and batch L + B (inoculated with both microorganisms). Sausages were ripened for 90 days according to a traditional industrial process. The processing of "salchichón" provoked a reduction in *L. monocytogenes* counts of around 2 log CFU/g. The downregulation of the expression of the three genes was found at the end of ripening when the water activity (a_w) of "salchichón" was <0.85 a_w. The combined effect on the reduction in *L. monocytogenes* counts together with the downregulation in the expression of the virulence genes throughout the "salchichón" processing could be of great interest to control the hazard caused by the presence of this pathogenic bacterium.

Keywords: *Listeria monocytogenes*; dry-cured fermented sausage; *Lactilactobacillus sakei*; virulence gene expression

Citation: Martín, I.; Alía, A.; Rodríguez, A.; Gómez, F.; Córdoba, J.J. Growth and Expression of Virulence Genes of *Listeria monocytogenes* during the Processing of Dry-Cured Fermented "Salchichón" Manufactured with a Selected *Lactilactobacillus sakei*. *Biology* **2021**, *10*, 1258. https://doi.org/10.3390/biology10121258

Academic Editors: Huizhong Chen and Cormac Gahan

Received: 6 October 2021
Accepted: 30 November 2021
Published: 2 December 2021

1. Introduction

L. monocytogenes is a Gram-positive, facultative intracellular bacterium responsible for human listeriosis, one of the most significant foodborne diseases in industrialized countries [1] that has well-known adverse health effects [2]. *L. monocytogenes* may contaminate food products at different steps of the manufacturing process, since this organism is able to survive on equipment and in production facilities [3,4]. It has been also reported that

L. monocytogenes can pull through stressful environments, such as low temperature, high acidity, and salt contents [2,5,6]. This ability is a serious concern for the dry-cured fermented sausage industry. This is because dry-cured fermented meat products elaborated with meat and fat, mixed with salt, nitrate and/or nitrite, sugar, and spices like black pepper, which is stuffed into a casing and subjected to fermentation and drying processes, form a nutrient-rich but still restricted ecological niche that can favour the growth of *L. monocytogenes* [7].

The dry-cured fermented sausage "salchichón" is one of the most typical Spanish dry-cured meat ready-to-eat (RTE) products, characterized by a bacterial fermentation process followed by a ripening period [8]. Although the consumption of dry-cured fermented sausages is considered at low risk for foodborne listeriosis [9], the presence of this pathogenic bacterium has been reported in ripened sausages [10–12], and in some cases, it has also been involved in listeriosis outbreaks [13].

During the ripening of dry-cured fermented sausages such as "salchichón", a reduction of *L. monocytogenes* has been reported, but its presence has not been completely eliminated [14]. In addition, the food safety criterion for *L. monocytogenes* sets up a maximum level of 100 CFU/g for this pathogen in RTE-food products throughout their shelf-life [15].

Since *L. monocytogenes* can be present in the dry-cured fermented sausage "salchichón", it is necessary to evaluate the impact of the dry-cured fermented processing on the potential virulence of this pathogen. The intracellular parasitism of *L. monocytogenes* requires the coordinated expression of several genes that encode virulence factors, such as the *plcA*, *hly*, and *iap* genes [16]. The transcriptional response of *L. monocytogenes* under a variety of pH, water activity (a_w), temperature conditions [17,18], or upon exposure to protective cultures [19] could result in changes in the *L. monocytogenes* viability and virulence [20–25].

To evaluate changes in the *L. monocytogenes* virulence during the processing of "salchichón", the usual microbiota present in this product should be considered. This is mainly composed of lactic-acid bacteria (LAB) such as *Lactilactobacillus* spp. together with yeast and moulds [26]. LAB in conjunction with fermentation and ripening processes contributes to the organoleptic characteristics of the products as well as their stability [27–29]. *Lactilactobacillus sakei* is the most frequently isolated LAB in meat products, in particular on dry-cured fermented sausages [8,30–35] whose main functions in sausage fermentation are the acidification and inhibition of undesired autochthonous microbiota [36]. This bacterium can grow and survive under the conditions encountered during meat storage and processing [37]. In fact, *L. sakei* possesses the ability to use nutrients encountered in meat [30]. Furthermore, it has been proposed as a protective culture against *L. monocytogenes* in dry-cured fermented sausages [38–40].

Current knowledge regarding the effect of an antagonistic *L. sakei* strain against *L. monocytogenes* should be integrated with information concerning the molecular response of the pathogen to such treatments. Therefore, the objective of this work was to investigate the effect of the processing of the dry-cured fermented sausage "salchichón", inoculated with a selected strain of *L. sakei*, on the growth of *L. monocytogenes* and the transcriptional response of some of its virulence genes (*plcA*, *hly*, and *iap*).

2. Materials and Methods

2.1. Bacterial and Culture Conditions

To evaluate the effect of the processing of "salchichón" on the growth and expression of the key virulence genes of *L. monocytogenes*, a strain of serotype 4b isolated from dry-cured meat products was used, since this serotype is the most frequently involved in clinical cases of listeriosis. In addition, this serotype has been reported as the highest transcriptomic response under hostile environments [41]. Thus, the strain *L. monocytogenes* S7-2 (serotype 4b), belonging to the National Institute of Agricultural and Food Research and Technology (INIA) collection (Madrid, Spain), has been used for the inoculation of "salchichón".

To evaluate the effect on LAB against *L. monocytogenes*, a selected strain of *L. sakei* 205 from the Food Hygiene and Safety Culture Collection at the University of Extremadura was used. This strain was isolated from traditional dry-cured fermented sausages and selected by its antagonistic activity against *L. monocytogenes* in a sterilized "salchichón"-based agar and in the dry-cured fermented sausages "salchichón" [14]. Thus, in the present work, the effect of the selected *L. sakei* strain on the expression of the virulence genes of *L. monocytogenes* in a real food system (dry-cured fermented "salchichón") in the presence of the natural microbiological population of this product was evaluated.

To prepare the *L. monocytogenes* and LAB inocula, 100 µL of a stock culture (stored in Brain Heart Infusion (BHI) broth (Pronadisa, Madrid Spain) containing 20 % (*w*/*v*) glycerol at −80 °C) was sub-cultured twice onto BHI broth at 37 °C for 24 h and Man Rogosa Sharpe (MRS) broth (Fisher Bioreagents, Belgium) at 30 °C for 48 h, respectively (Figure 1). At the end of the incubation period, a suspension containing ≈ 8.0 log CFU/mL cells was obtained and an aliquot of this was diluted in 1% (*w*/*v*) peptone water to reach final concentrations of ≈7.0 and 6.0 log CFU/mL for *L. monocytogenes* and *L. sakei*, respectively. Next, after centrifuging cultures at 10,000× *g* for 5 min, the supernatants were discarded, and the sediments were washed and resuspended in PBS to be used for the inoculation of the "salchichón" mix before stuffing. To check the level of inoculation, serial dilutions were plated onto Chromagar™ *Listeria* agar plates (Scharlab, Madrid, Spain) and incubated at 37 °C for 48 h for *L. monocytogenes*, while for LAB, dilutions were spread on MRS (Oxoid, Basingstoke, UK) agar plates and anaerobically incubated at 30 °C for 72 h. In addition, the real initial levels (log CFU/g) of *L. monocytogenes* and *L. sakei* 205 on the sausages were determined at day 0 of processing.

2.2. Elaboration of Dry-Cured Fermented "Salchichón"

The "salchichón" sausages were elaborated in a pilot plant located at the Faculty of Veterinary of the University of Extremadura (Spain) according to the industrial processing of this product [14], the mixture composition proceeding as follows: minced Iberian pork meat (90%) and Iberian pig fatback (7%), with an addition of NaCl (1.8%), cane sugar (0.4%), potassium nitrate (120 ppm), sodium nitrite (100 ppm), black pepper, and spices (Figure 1). This mixture was divided into 3 different batches, to which 150 mL of PBS with the corresponding microorganism's inoculum, prepared as has been described in Section 2.1, were added. The three batches were: batch B (control inoculated only with *L. sakei* at a concentration of ≈6 log CFU/g); batch L (inoculated only with *L. monocytogenes* at a concentration of ≈7 log CFU/g); batch L + B (inoculated with *L. monocytogenes* at a concentration of ≈7 log CFU/g combined with *L. sakei* at a concentration of ≈6 log CFU/g). All ingredients and corresponding inoculum were then mixed using an automatic kneader that was cleaned and sanitized between batches. The mixes of the different batches were then stuffed into regenerated collagen casings of 40 mm in diameter (Viscofan, Navarra, Spain), reaching each sausage an approximate weight of 500 g.

The sausages were ripened for 90 days in controlled drying chambers of the pilot plant of the Faculty of Veterinary Science following the conditions used in typical traditional processing of "salchichón" [14]: 5 °C at 85% relative humidity (RH) for 3 days, then 7 °C and 80% RH for the 17 days, 9 °C and 75% RH for 10 days, and 12 °C and 70% RH to reach 90-day ripening (Figure 1).

For physicochemical, microbial, and gene expression analyses, five sausages of each batch were taken at the beginning of processing (day 0) and at 15, 30, 45, 60, and 90 days of ripening. All of them were aseptically removed from the casings in a laminar flow cabinet (Telstar, Spain) before analytical determinations. Thus, the experiment consisting of 3 different batches × 5 sampling times × 5 different analyzed sausages/each batch and sampling time was evaluated once according to the European Union Reference Laboratory Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (such as "salchichón"), where no growth or the growth probability of this pathogen is ≤10% [42].

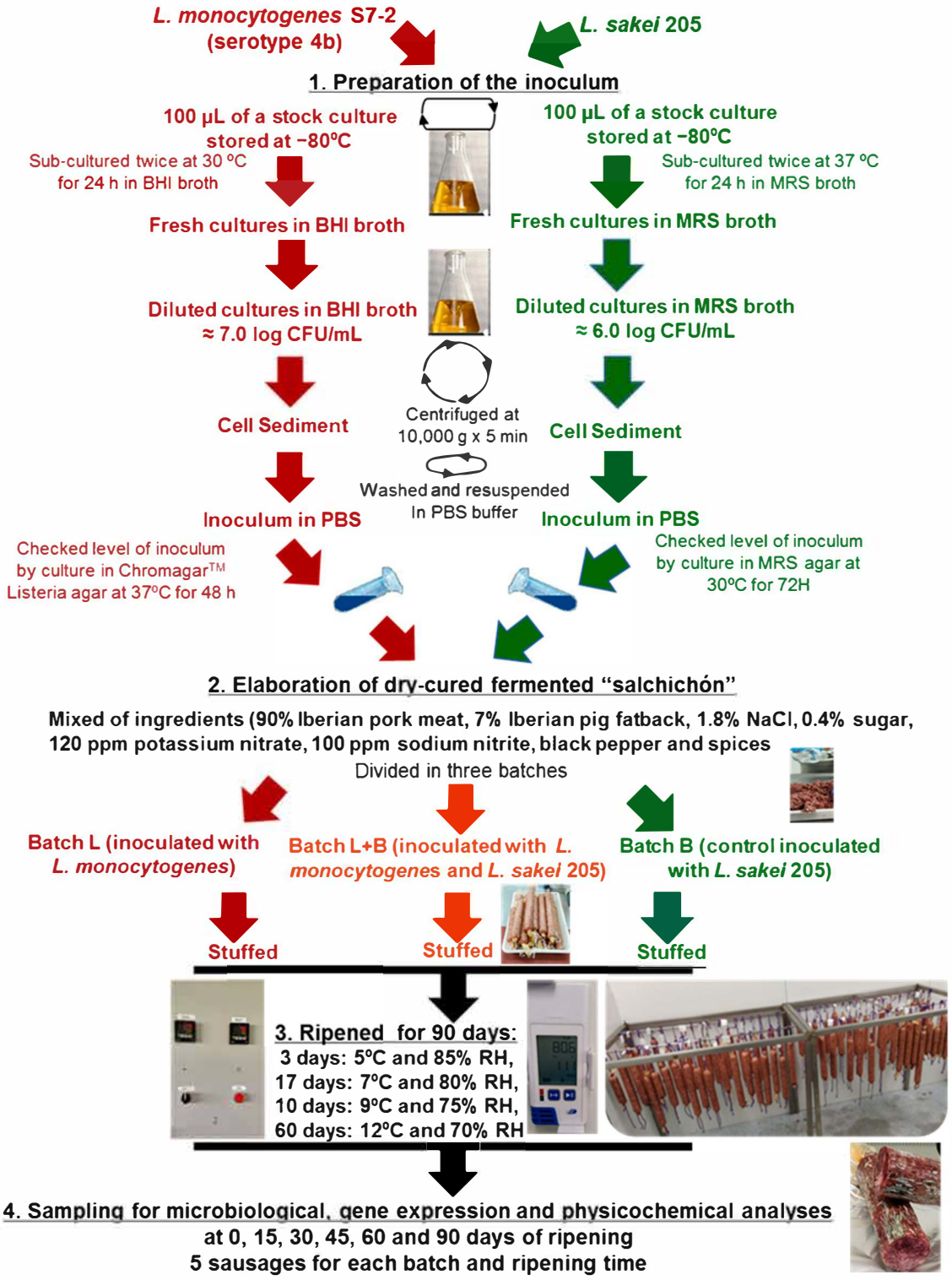

Figure 1. Scheme of the methodology followed in this work for preparing the inoculum, elaboration, and ripening conditions of the inoculated "salchichón" and sampling.

2.3. Microbiological Analysis

At each sampling time, 10 g of each of the 5 dry-cured sausages "salchichón" were aseptically taken from the center of the product and mixed with 90 mL of 1% (*w*/*v*) peptone water and homogenized in a Stomacher machine (Seward, model 400 Circulator, West Sussex, UK) at 300 rpm for 1 min. One mL of this homogenate was frozen at −80 °C and

stored for further RNA extraction and gene expression analysis. Another one mL aliquot was used to make serial dilutions for the estimation of *L. monocytogenes* and LAB counts. For this, decimal serial dilutions were subsequently carried out in 1% (*w*/*v*) of peptone water, and 100 µL of the cell suspensions were then spread onto the surface of the MRS agar and CHROMagar™ *Listeria* Chromogenic medium to determine LAB counts and *L. monocytogenes*, respectively. Their incubation was performed for 24–48 h at 30 °C for LAB counts and for 24–48 h at 37 °C for *L. monocytogenes* counts. To evaluate the implantation of *L. sakei* 205 in all batches and at the last sampling time (90 days), 50% of the characteristic LAB colonies were randomly isolated from MRS plates and characterized by a sequencing analysis of the 16S rRNA region and PFGE analysis of the DNA with the restriction *NotI* and *SgsI* enzymes (Thermo Fisher Scientific, Waltham, MA, USA) [14].

2.4. RNA Extraction and Gene Expression Assay

One mL of each of the samples stored at −80 °C for gene expression analysis purposes was thawed in refrigeration (4 °C). Later, RNA was extracted according to the instructions of the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), after centrifuging 1 mL aliquot (10,000 rpm, 10 min at 4 °C) and removing the resultant supernatant. To remove genomic DNA contamination, samples were treated with the RNAse-Free Dnase I (Epicentre). RNA concentration and purity ($A_{260/280}$) were then measured using the Nanodrop™ (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using about 500 ng of the total extracted RNA according to the manufacturer's instructions for the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Kusatsu, Shiga, Japan).

Quantitative PCR (qPCR) based on TaqMan® methodology was then used to amplify the virulence-related genes of *L. monocytogenes*, *plcA*, *hly*, and *iap* following the method described by Alía et al. [17]. In addition, a RT-qPCR based on SYBR® Green methodology was also performed to amplify the constitutive 16S rRNA gene used to ensure that both RNA extraction and cDNA synthesis processes were properly carried out according to Alía et al. [43]. The ViiA™ 7 system (Applied Biosystems, Waltham, MA, USA) was used for qPCR performance. The reactions were prepared in MicroAmp® Fast Optical 96-Well Reaction plates (Applied Biosystems). Five replicates of RNA from control samples (only *L. monocytogenes*) and template-free negative controls (ultra-pure water instead of cDNA) were also included in the runs. Data analysis on the absolute expression of the target genes were determined using ViiA™ 7 V.1.2.2 Software (Thermo Fisher Scientific). The quantification cycle (Cq), the intersection between each fluorescence curve and a threshold line, was automatically calculated by the instrument using default parameters. The absolute expression levels of the three target genes from the different batches were extrapolated from the standard curves built for each gene as described by Alía et al. [17] by using the Cq values obtained for the samples. The absolute gene expression of each sample was evaluated in quintuplicate.

2.5. Physicochemical Analysis

The determination of pH and a_w was carried out only in the batch inoculated with *L. sakei* 205 (batch B). The pH value was recorded after homogenizing 3 g of each sample with 27 mL of distilled water with a digital pH-meter Crison Basic 20 (Crison, Barcelona, Spain), while a_w was determined on sausage slices using a Novasina Lab Master Water activity meter model AW SPRINT-TH 300 (Novasina AG, Lachen, Switzerland).

2.6. Statistical Analysis

Statistical analyses were performed using the software IBM SPSS Statistic for Windows v.22.0 (IBM, New York, NY, USA). The different batches and days of ripening were used as independent variables. The counts (Log CFU/g), a_w, pH values, and absolute expression were analyzed as dependent variables. Once the dependent and independent variables of the analysis were determined, a study of the normality of the different data populations

was carried out using the Shapiro Wilk test. The analysis of the data was conducted using the Mann–Whitney test [44], and the statistical significance was set at $p \leq 0.05$.

3. Results and Discussion

3.1. Evolution of Water Activity and pH during Ripening of "Salchichón"

The a_w decreased ($p \leq 0.05$) from an initial value of 0.947 a_w in the raw product to values below 0.790 a_w at day 90 (Figure 2). The evolution of a_w was similar to that reported for dry-cured fermented sausages by previous studies [45,46].

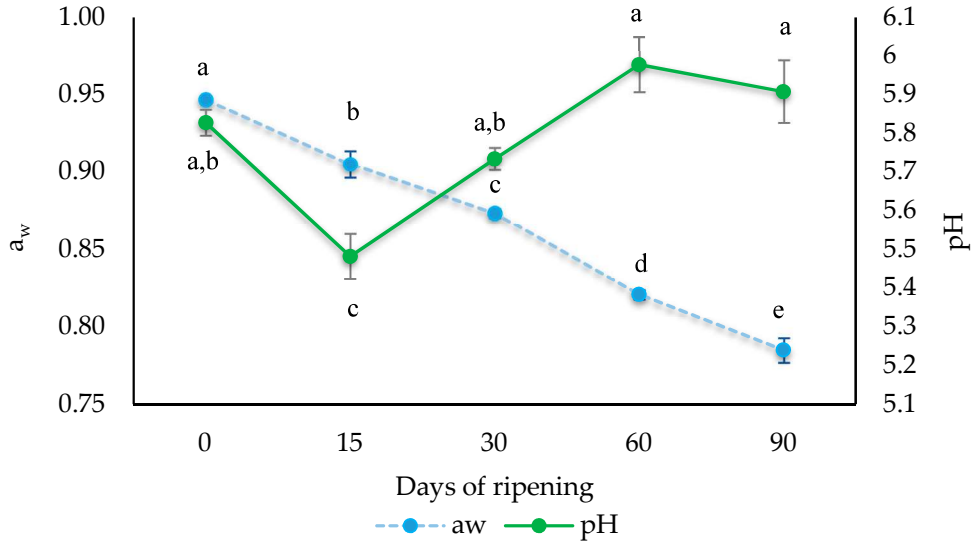

Figure 2. Evolution of water activity (a_w) and pH of dry-cured fermented sausages "salchichón" throughout the ripening process. Different letters indicate significant differences in the same parameter at the different ripening days ($p \leq 0.05$).

The evolution of pH throughout the ripening process of "salchichón" is shown in Figure 2. A decrease in pH values is observed after 15 days of ripening from 5.82 to 5.48 ($p \leq 0.05$), probably due to the growth of the inoculated *L. sakei* 205. However, at day 30 of ripening, an increase in the pH value was observed (close to 5.8), and it then remained constant until the end of maturation. The increase in pH at the end of the ripening time may be due to the accumulation of non-protein nitrogen and amino acid catabolism products [47,48].

3.2. Evolution of Lactic-Acid Bacteria and L. monocytogenes Counts throughout the Ripening Process of "Salchichón"

The evolution of LAB counts in *L. sakei* 205-inoculated batches (batches B and L + B) was very similar, since levels varied between 6.5 and 7.5 log CFU/g in both batches during all the ripening times (Figure 3). There were no significant differences ($p \leq 0.05$) in LAB counts between these two batches. However, in the batch inoculated only with *L. monocytogenes* (batch L), LAB counts were always lower than 6.5 log CFU/g, and for most of the ripening times were significantly lower ($p \leq 0.05$) than those found in *L. sakei* 205-inoculated batches. These results were expected because of the inoculation with the selected *L. sakei* strain of dry-cured fermented sausages "salchichón" composing batches B and L + B. In batches inoculated with *L. sakei* (B and L + B), most of the tested isolates (86%) were identified as *L. sakei* (100% identity) by a sequencing analysis of the 16S rRNA region. In the PFGE analysis, these isolates showed the same pattern of *L. sakei* 205. The remaining strains were *Lactilactobacillus plantarum* group (7%) and *Lactilactobacillus curvatus* (7%). In batch L, none of the isolates were identified as *L. sakei*. Thus, the inoculated *L. sakei* 205 was the predominant strain in *L. sakei*-inoculated bathes (B and B + L) and it was not detected in the batch inoculated only with *L. monocytogenes* (L).

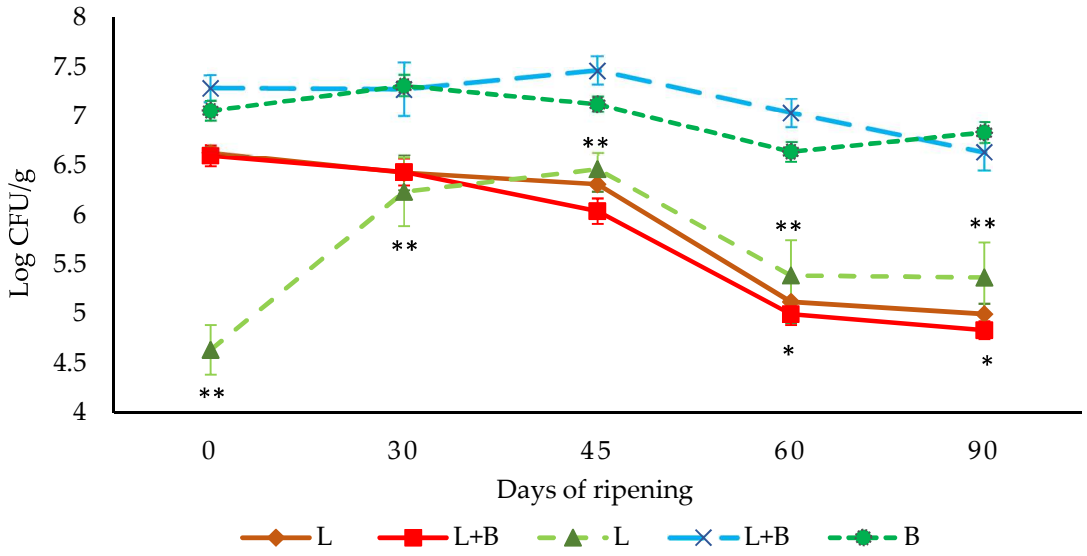

Figure 3. Growth curves of the LAB populations (dashed line) during the ripening process of dry-fermented sausages for batch B (●), L (▲) and batch L + B (X) and *L. monocytogenes* populations (solid line) for batch L (♦) and batch L + B (■). (*) indicates significant differences ($p \leq 0.05$) between batches of *L. monocytogenes* and (**) between batches of LAB.

Levels of *L. monocytogenes* decreased ($p \leq 0.05$) in both inoculated batches (L and L + B) throughout the ripening process of the dry-cured fermented sausage "salchichón" (Figure 3). No increase in *L. monocytogenes* growth during the processing was observed in batches inoculated with this pathogen alone (L) or together with *L. sakei* 205 (L + B) (Figure 3), even considering that during the first 15 days of ripening, there could be certain conditions such as temperature (7 °C), a_w (0.947–0.914), and pH (5.8–5.4) that may allow the growth of *L. monocytogenes*. Likely, the synergistic effect of a_w, temperature, and pH reduction together with the effect of NaCl and nitrite added, and the presence of LAB inoculated or from contamination, prevent the growth of *L. monocytogenes* in the first days of ripening [38,49,50]. After 15 days of processing, the a_w values of "salchichón", ranging from 0.914 to 0.779 a_w, did not allow the growth of *L. monocytogenes* [51,52]. From day 45 until the end of ripening, levels of *L. monocytogenes* were significantly lower in the batch inoculated with *L. sakei* 205 (L + B) than in the batch only inoculated with *L. monocytogenes* (L). This additional reduction in *L. monocytogenes* can be associated with the presence of the strain *L. sakei* 205. Different strains of this species have shown anti-microbial effects when they have been used as protective cultures [30,38,40]. In this work, a reduction of about 2 log CFU/g was achieved during the processing of dry-cured fermented sausage "salchichón". This means that this pathogenic bacterium could be detected after 90 days of ripening if the levels of contamination in raw materials or during processing are higher than 2 log CFU/g. Thus, it is of great importance to evaluate the effect of processing in the expression of virulence genes of *L. monocytogenes*.

3.3. Effect of Processing and Presence of L. sakei on the Absolute Transcription Levels of L. monocytogenes Virulence Genes in Dry-Cured Fermented Sausages

Findings on this work revealed a high and significant relationship between the transcriptomic response and counts of *L. monocytogenes* (*r*-values: 0.792, 0.821, and 0.820 for the *plcA*, *hly*, and *iap* genes, respectively). The two virulence genes *plcA* and *hly* showed higher expression values than those found for the *iap* gene in all the tested conditions ($p \leq 0.05$; Table 1). In addition, at 0, 15, and 30 days of processing, no differences ($p > 0.05$) in the gene expression values were found for all the analysed batches (Table 1). However, at days 60 and 90 of ripening, a significant decrease in the transcription levels regarding days 0, 15, and 30 was detected for all the analysed genes.

Table 1. Absolute expression values (mean log copies of gene ± standard deviation) of the virulence (*plcA*, *hly*, and *iap*) genes of *Listeria monocytogenes* in dry-cured fermented sausages "salchichón" throughout the ripening process. Batches: L: *L. monocytogenes*, L + B: *L. monocytogenes* + *Lactilactobacillus sakei* 205. The assays were conducted in quintuplicate.

Genes	Batches	Days of Ripening				
		0	15	30	60	90
hly	L	3.01 ± 0.372^{1}	3.07 ± 0.099^{1}	3.26 ± 0.216^{1}	$1.89 \pm 0.226^{2,b}$	1.82 ± 0.370^{2}
	L + B	$3.28 \pm 0.132^{1,2}$	2.96 ± 0.180^{2}	3.42 ± 0.208^{1}	$2.48 \pm 0.272^{3,a}$	1.49 ± 0.316^{4}
plcA	L	4.17 ± 0.286^{1}	$4.32 \pm 0.282^{1,a}$	4.41 ± 0.410^{1}	2.47 ± 0.236^{2}	3.06 ± 0.505^{2}
	L + B	4.01 ± 0.265^{1}	$3.74 \pm 0.440^{1,b}$	4.35 ± 0.271^{1}	2.91 ± 0.494^{2}	2.68 ± 0.483^{2}
iap	L	2.72 ± 0.181^{1}	2.69 ± 0.067^{1}	2.82 ± 0.283^{1}	1.65 ± 0.368^{2}	1.96 ± 0.249^{2}
	L + B	2.92 ± 0.165^{1}	2.70 ± 0.076^{1}	3.16 ± 0.378^{1}	1.66 ± 0.433^{2}	1.53 ± 0.491^{2}

Within column, different superscript letters denote significant differences for the same gene in each batch at the different ripening days ($p \leq 0.05$). Within row, different superscript numbers denote significant differences for the same gene and batch at each ripening day ($p \leq 0.05$).

These results showed that the virulence gene expression values decreased at the end of the ripening period, likely due to the stress conditions created due to the processing and composition of "salchichón" mainly characterized by the decrease in a_w throughout the ripening until values below 0.85 a_w. A decrease in the *plcA*, *hly*, and *iap* gene expression values has been reported in a dry-cured ham model system as a consequence of a_w decrease [19]. Furthermore, a downregulation in the expression of *plcA* and *hly* genes has been reported in different stress conditions on dry-cured meat products, likely due to the *prfA* gene repression, the major regulator of *L. monocytogenes* virulence [6,53]. Thus, at the end of "salchichón" ripening with a_w below 0.85 a_w, the expression of stress-related genes is likely enhanced, and the transcription of other non-stress-related genes such as virulence genes is repressed to facilitate the *L. monocytogenes* survival in highly stressful environments, as has been previously reported [6,54]. This agrees with the results obtained in this study, since it has been observed that the expression of the three virulence genes tested decreased. These findings are very interesting, since they correspond to real food samples. Most published works that showed an up-regulation in virulence genes of *L. monocytogenes* were in vitro-conducted with laboratory-based media [6]. However, the food matrix, especially a meat-based one, may influence the virulence potential of *L. monocytogenes*, possibly through the downregulation of virulence genes [6,21,55]. In addition, Bowman et al. [53] reported the suppression of *prfA* and *sigB* genes because of the non-thermal treatment on *L. monocytogenes*; this may also explain the decrease in the expression levels of *plcA* and *hly* genes.

When the effect of the inoculated *L. sakei* 205 on the expression of virulence genes tested was analyzed, the expression values of the three genes remained constant compared to those obtained in the control batch (L), except at day 60 of maturation, when significantly higher expression values were detected for the *hly* gene in batch L + B (Table 1). In addition, no differences between these two batches were found at the end of the ripening time (Table 1). These results contrast with the demonstrated effect of some bacteriocinogenic LAB such as *Enterococcus faecium* on the inhibition of the expression of the virulence genes of *L. monocytogenes* [56]. In the present work, *L. sakei* 205, which provokes an additional decrease in *L. monocytogenes* counts to those produced by the action of the reduction in pH and a_w throughout the ripening of "salchichón", does not show any appreciable effect on the virulence gene expression of *L. monocytogenes* throughout this processing. To be used as a protective culture, it is important that *L. sakei* 205 does not cause an increase in the virulence gene expression of this pathogenic bacterium, since in some cases, various protective cultures such as selected strains of *Debaryomyces hansenii* have been reported to enhance the expression of virulence genes on *L. monocytogenes* [19].

In the present work, it has been demonstrated that a reduction in *L. monocytogenes* counts throughout the ripening process and a downregulation in the expression of virulence genes of the pathogenic bacterium cells could survive dry-cured fermented processing, but this effect was not increased by the addition of the strain *L. sakei* 205. The last effect is of great importance, since reports the relevance of the virulence gene transcription on the invasive and survival ability of *L. monocytogenes* in human models or cell lines [20–24]. Although further studies should be carried out to evaluate the effect of *L. monocytogenes* surviving "salchichón" ripening in culture cells, the processing of this product to reach a_w values below 0.85 a_w seems to be effective to control the microbial hazard caused by the presence of *L. monocytogenes*.

4. Conclusions

This study describes for the first time the combined effect of the processing of "salchichón" manufactured with a selected protective culture of *L. sakei* on minimizing the growth and expression of three virulence genes (*plcA*, *hly*, and *iap*) of *L. monocytogenes*. The processing of "salchichón" provokes a reduction of 2 log CFU/g of *L. monocytogenes*. *L. sakei* 205 provokes an additional decrease in *L. monocytogenes* counts to those produced by the action of the reduction in pH and a_w throughout the ripening of "salchichón". In addition, when the a_w of the product is lower than 0.85 a_w, a downregulation in the expression of the above virulence genes was found. However, *L. sakei* 205 does not show any appreciable effect on the virulence gene expression of *L. monocytogenes* throughout "salchichón" processing. The combined effects of the reduction in *L. monocytogenes* throughout the procession of "salchichón" and the downregulation of the virulence gene expression of the surviving strains of this pathogen are relevant to control *L. monocytogenes* in this product.

Author Contributions: Conceptualization, J.J.C. and A.R.; methodology, I.M.; software, A.A.; validation, I.M., A.A. and F.G.; formal analysis, I.M., A.A. and F.G.; investigation, I.M.; resources, A.R. and J.J.C.; data curation, I.M.; writing—original draft preparation, I.M. and J.J.C.; writing—review and editing, I.M., A.R., A.A., F.G. and J.J.C.; visualization, I.M.; supervision, A.R. and J.J.C.; project administration, J.J.C. and A.R.; funding acquisition, J.J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Economy, Industry, and Competitiveness (RTA-2017-00027-C03-03); the Spanish Ministry of Education, Culture, and Sports (FPU 16/05303); and the Junta De Extremadura and Feder (IB16149, GR15108 and GR18056).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This work was supported by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108, and GR18056. I. Martín is supported by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

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

References

1. Filipello, V.; Gallina, S.; Amato, E.; Losio, M.N.; Pontello, M.; Decastelli, L.; Lomonaco, S. Diversity and persistence of *Listeria monocytogenes* within the Gorgonzola PDO production chain and comparison with clinical isolates from the same area. *Int. J. Food Microbiol.* **2017**, *245*, 73–78. [CrossRef] [PubMed]
2. Kathariou, S. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* **2002**, *65*, 1811–1829. [CrossRef] [PubMed]
3. Alía, A.; Andrade, M.J.; Rodríguez, A.; Martín, I.; Pérez-Baltar, A.; Medina, M.; Córdoba, J.J. Prevalence and characterization of *Listeria monocytogenes* in deboning and slicing areas of Spanish dry-cured ham processing. *LWT-Food Sci. Technol.* **2020**, *128*, 109498. [CrossRef]

4. Samelis, J.; Bedie, G.K.; Sofos, J.N.; Belk, K.E.; Scanga, J.A.; Smith, G.C. Control of *Listeria monocytogenes* with combined antimicrobials after postprocess contamination and extended storage of frankfurters at 4 °C in vacuum packages. *J. Food Prot.* **2002**, *65*, 299–307. [CrossRef]
5. EURL Lm Technical Guidance Document on Challenge Test and Durability Studies for Assessing Shelf-Life of Ready-to Eat Foods Related to Listeria monocytogenes. 2021. Available online: https://ec.europa.eu/food/system/files/2021-07/biosafety_fh_mc_tech-guide-doc_listeria-in-rte-foods_en_0.pdf (accessed on 28 July 2021).
6. Mataragas, M.; Rovetto, F.; Bellio, A.; Alessandria, V.; Rantsiou, K.; Decastelli, L.; Cocolin, L. Differential gene expression profiling of *Listeria monocytogenes* in cacciatore and felino salami to reveal potential stress resistance biomarkers. *Food Microbiol.* **2015**, *46*, 408–417. [CrossRef]
7. Janßen, D.; Eisenbach, L.; Ehrmann, M.A.; Vogel, R.F. Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in a fermented sausage model. *Int. J. Food Microbiol.* **2018**, *285*, 188–197. [CrossRef]
8. Martín, B.; Jofré, A.; Garriga, M.; Pla, M.; Aymerich, T. Rapid quantitative detection of *Lactobacillus sakei* in meat and fermented sausages by real-time PCR. *Appl. Environ. Microbiol.* **2006**, *72*, 6040–6048. [CrossRef]
9. Barmpalia-Davis, I.M.; Geornaras, I.; Kendall, P.A.; Sofos, J.N. Survival of *Listeria monocytogenes* in a simulated dynamic gastrointestinal model during storage of inoculated Bologna and Salami slices in vacuum packages. *J. Food Prot.* **2008**, *71*, 2014–2023. [CrossRef]
10. Doménech, E.; Jimenez-Belenguer, A.; Amoros, J.A.; Ferrus, M.A.; Escriche, I. Prevalence and antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain. *Food Control.* **2015**, *47*, 120–125. [CrossRef]
11. Ferreira, V.; Barbosa, J.; Silva, J.; Felício, M.T.; Mena, C.; Hogg, T.; Gibbs, P.; Teixeira, P. Characterisation of alheiras, traditional sausages produced in the North of Portugal, with respect to their microbiological safety. *Food Control.* **2007**, *18*, 436–440. [CrossRef]
12. Meloni, D. Presence of *Listeria monocytogenes* in mediterranean-style dry fermented sausages. *Foods* **2015**, *4*, 34–50. [CrossRef]
13. Foodborne Ilness Outbreak Database Siena Foods Salame. Available online: http://www.outbreakdatabase.com/details/assi-market-pickles-2010/?vehicle=cucumber (accessed on 26 June 2021).
14. Martín, I.; Rodríguez, A.; Sánchez-Montero, L.; Padilla, P.; Córdoba, J.J. Effect of the dry-cured fermented sausage "salchichón" processing with a selected *Lactobacillus sakei* in *Listeria monocytogenes* and microbial population. *Foods* **2021**, *10*, 856. [CrossRef]
15. Commission, E. Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. *Off. J. Eur. Communities* **2007**, *322*, 12–29.
16. Rolhion, N.; Cossart, P. How the study of *Listeria monocytogenes* has led to new concepts in biology. *Futur. Microbiol. Futur. Med.* **2017**, *12*, 621–638. [CrossRef] [PubMed]
17. Alía, A.; Rodríguez, A.; Andrade, M.J.; Gómez, F.; Córdoba, J.J. Combined effect of temperature, water activity and salt content on the growth and gene expression of *Listeria monocytogenes* in a dry-cured ham model system. *Meat Sci.* **2019**, *155*, 16–19. [CrossRef] [PubMed]
18. Hadjilouka, A.; Molfeta, C.; Panagiotopoulou, O.; Paramithiotis, S.; Mataragas, M.; Drosinos, E.H. Expression of *Listeria monocytogenes* key virulence genes during growth in liquid medium, on rocket and melon at 4, 10 and 30 °C. *Food Microbiol.* **2016**, *55*, 7–15. [CrossRef]
19. Alía, A.; Córdoba, J.J.; Rodríguez, A.; García, C.; Andrade, M.J. Evaluation of the efficacy of Debaryomyces hansenii as protective culture for controlling *Listeria monocytogenes* in sliced dry-cured ham. *LWT* **2020**, *119*, 108886. [CrossRef]
20. Olesen, I.; Vogensen, F.; Jespersn, L. Gene Transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathog. Dis.* **2009**, *6*, 669–680. [CrossRef]
21. Olesen, I.; Thorsen, L.; Jespersen, L. Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. *Int. J. Food Microbiol.* **2010**, *141*, S60–S68. [CrossRef] [PubMed]
22. Garner, M.R.; James, K.E.; Callahan, M.C.; Wiedmann, M.; Boor, K.J. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Appl. Environ. Microbiol.* **2006**, *72*, 5384–5395. [CrossRef]
23. Kang, J.; Burall, L.; Mammel, M.K.; Datta, A.R. Global transcriptomic response of *Listeria monocytogenes* during growth on cantaloupe slices. *Food Microbiol.* **2019**, *77*, 192–201. [CrossRef] [PubMed]
24. Lin, C.S.; Wang, C.; Tsai, H.J.; Chou, C.H. Growth of *Listeria monocytogenes* on a RTE-meat matrix enhances cell invasiveness to mouse J774A.1 macropahges. *Int. J. Food Microbiol.* **2010**, *144*, 199–201. [CrossRef]
25. Upadhyay, A.; Johny, A.K.; Amalaradjou, M.A.R.; Baskaran, S.A.; Kim, K.S.; Venkitanarayanan, K. Plant-derived antimicrobials reduce *Listeria monocytogenes* virulence factors in vitro, and down-regulate expression of virulence genes. *Int. J. Food Microbiol.* **2012**, *157*, 88–94. [CrossRef]
26. Delgado, J.; Peromingo, B.; Rodríguez, A.; Rodríguez, M. Biocontrol of *Penicillium griseofulvum* to reduce cyclopiazonic acid contamination in dry-fermented sausages. *Int. J. Food Microbiol.* **2019**, *293*, 1–6. [CrossRef]
27. Mataragas, M.; Alessandria, V.; Rantsiou, K.; Cocolin, L. Evaluation of the *Listeria monocytogenes* inactivation during post-process storage of fermented sausages: A basis for the development of a decision support tool. *Food Control.* **2015**, *50*, 568–573. [CrossRef]
28. Cano-García, L.; Belloch, C.; Flores, M. Impact of *Debaryomyces hansenii* strains inoculation on the quality of slow dry-cured fermented sausages. *Meat Sci.* **2014**, *96*, 1469–1477. [CrossRef] [PubMed]
29. Martín, A.; Córdoba, J.J.; Aranda, E.; Córdoba, M.G.; Asensio, M.A. Contribution of a selected fungal population to the volatile compounds on dry-cured ham. *Int. J. Food Microbiol.* **2006**, *110*, 8–18. [CrossRef]

30. Zagorec, M.; Champomier-Vergès, M.-C. *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms* **2017**, *5*, 56. [CrossRef]
31. Kurbakov, K.A.; Konorov, E.A.; Minaev, M.Y.; Kuznetsova, O.A. Multiplex real-time PCR with HRM for detection of *Lactobacillus sakei* and *Lactobacillus curvatus* in Food Samples. *Food Technol. Biotechnol.* **2019**, *57*, 97–104. [CrossRef]
32. Prado, N.; Sampayo, M.; González, P.; Lombó, F.; Díaz, J. Physicochemical, sensory and microbiological characterization of Asturian Chorizo, a traditional fermented sausage manufactured in Northern Spain. *Meat Sci.* **2019**, *156*, 118–124. [CrossRef]
33. Quijada, N.M.; De Filippis, F.; Sanz, J.J.; García-Fernández, M.D.C.; Rodríguez-Lázaro, D.; Ercolini, D.; Hernández, M. Different Lactobacillus populations dominate in "Chorizo de León" manufacturing performed in different production plants. *Food Microbiol.* **2018**, *70*, 94–102. [CrossRef]
34. Sidira, M.; Mitropoulou, G.; Galanis, A.; Kanellaki, M.; Kourkoutas, Y. Effect of Sugar Content on Quality Characteristics and Shelf-Life of Probiotic Dry-Fermented Sausages Produced by Free or Immobilized *Lactobacillus casei* ATCC 393. *Foods* **2019**, *8*, 219. [CrossRef] [PubMed]
35. Sobrino, O.J.; Rodríguez, J.M.; Moreira, W.L.; Fernández, M.F.; Sanz, B.; Hernández, P.E. Antibacterial activity of *Lactobacillus* strains isolated from dry fermented sausages. *Int. J. Food Microbiol.* **1993**, *75*, 344–349. [CrossRef]
36. Lucke, F.K. Utilization of microbes to process and preserve meat. *Meat Sci.* **2000**, *56*, 105–115. [CrossRef]
37. Chaillou, S.; Champomier-Vergès, M.C.; Cornet, M.; Crutz-Le Coq, A.M.; Dudez, A.M.; Martin, V.; Beaufils, S.; Darbon-Rongère, E.; Bossy, R.; Loux, V. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nat. Biotechnol.* **2005**, *23*, 1527–1533. [CrossRef] [PubMed]
38. Bošković, M.; Tadić, V.; Dordević, J.; Glišić, M.; Lakićević, B.; Dimitrijević, M.; Ž Baltić, M. Effect of starter cultures on survival of *Listeria monocytogenes* in Čajna sausage. *IOP Conf. Ser. Earth Environ. Sci.* **2017**, *85*, 012074. [CrossRef]
39. Prpich, N.Z.P.; Garro, O.A.; Romero, M.; Judis, M.A.; Cayré, M.E.; Castro, M.P. Evaluation of an autochthonous starter culture on the production of a traditional dry fermented sausage from Chaco (Argentina) at a small-scale facility. *Meat Sci.* **2016**, *115*, 41–44. [CrossRef]
40. Prpich, N.Z.P.; Castro, M.P.; Cayré, M.E.; Garro, O.A.; Vignolo, G.M. Indigenous starter cultures to improve quality of artisanal dry fermented sausages from chaco (Argentina). *Int. J. Food Sci.* **2015**, *2015*, 1–9. [CrossRef] [PubMed]
41. Mataragas, M.; Greppi, A.; Rantsiou, K.; Cocolin, L. Gene Transcription patterns of pH and salt stressed *Listeria monocytogenes* cells in simulated gastric and pancreatic conditions. *J. Food Prot.* **2014**, *77*, 254–261. [CrossRef]
42. Beaufort, A.; Cornu, M.; Bergis, H.; Lardeux, A.L.; Lombard, B. EURL Lm Technical Guidance Document for Conducing Shelf-life Studies on Listeria monocytogenes in Ready to Eat Foods. Available online: https://www.fsai.ie/uploadedFiles/EURL%20Lm_Technical%20Guidance%20Document%20Lm%20shelf-life%20studies_V3_2014-06-06%20(2).pdf (accessed on 6 November 2021).
43. Alía, A.; Andrade, M.J.; Córdoba, J.J.; Martín, I.; Rodríguez, A. Development of a multiplex real-time PCR to differentiate the four major *Listeria monocytogenes* serotypes in isolates from meat processing plants. *Food Microbiol.* **2020**, *87*, 856. [CrossRef] [PubMed]
44. Mann, H.B.; Whitney, D. On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Stat.* **1947**, *2*, 347–370. [CrossRef]
45. Casquete, R.; Benito, M.J.; Martín, A.; Ruiz-Moyano, S.; Hernández, A.; Córdoba, M.G. Effect of autochthonous starter cultures in the production of "salchichón", a traditional Iberian dry-fermented sausage, with different ripening processes. *LWT-Food Sci. Technol.* **2011**, *44*, 1562–1571. [CrossRef]
46. El Adab, S.; Essid, I.; Hassouna, M. Microbiological, biochemical and textural characteristics of a tunisian dry fermented poultry meat sausage inoculated with selected starter cultures. *J. Food Saf.* **2015**, *35*, 75–85. [CrossRef]
47. Aleson-Carbonell, L.; Fernández-López, J.; Pérez-Alvarez, J.A.; Kuri, V. Functional and sensory effects of fibre-rich ingredients on breakfast fresh sausages manufacture. *Food Sci. Technol. Int.* **2005**, *11*, 89–97. [CrossRef]
48. Pérez-Álvarez, J.A.; Sayas-Barberá, M.E.; Fernández-López, J.; Aranda-Catalá, V. Physicochemical characteristics of Spanish-type dry-cured sausage. *Food Res. Int.* **1999**, *32*, 599–607. [CrossRef]
49. Benkerroum, N.; Daoudi, A.; Hamraoui, T.; Ghalfi, H.; Thiry, C.; Duroy, M.; Evrart, P.; Roblain, D.; Thonart, P. Lyophilized preparations of bacteriocinogenic *Lactobacillus curvatus* and *Lactococcus lactis* subsp. lactis as potential protective adjuncts to control *Listeria monocytogenes* in dry-fermented sausages. *J. Appl. Microbiol.* **2005**, *98*, 56–63. [CrossRef] [PubMed]
50. Pavli, F.G.; Argyri, A.A.; Chorianopoulos, N.G.; Nychas, G.J.E.; Tassou, C.C. Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. *LWT* **2020**, *118*, 108810. [CrossRef]
51. Nolan, D.A.; Chamblin, D.C.; Troller, J.A. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. *Int. J. Food Microbiol.* **1992**, *16*, 323–335. [CrossRef]
52. Vermeulen, A.; Gysemans, K.P.M.; Bernaerts, K.; Geeraerd, A.H.; Van Impe, J.F.; Debevere, J.; Devlieghere, F. Influence of pH, water activity and acetic acid concentration on *Listeria monocytogenes* at 7 °C: Data collection for the development of a growth/no growth model. *Int. J. Food Microbiol.* **2007**, *114*, 332–341. [CrossRef]
53. Bowman, J.P.; Bittencourt, C.R.; Ross, T. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology* **2008**, *154*, 462–475. [CrossRef]
54. Jiang, L.; Olesen, I.; Andersen, T.; Fang, W.; Jespersen, L. Survival of *Listeria monocytogenes* in simulated gastrointestinal system and transcriptional profiling of stress- and adhesion-related genes. *Foodborne Pathog. Dis.* **2010**, *7*, 267–274. [CrossRef] [PubMed]

55. Mahoney, M.; Henriksson, A. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. *Int. J. Food Microbiol.* **2003**, *84*, 255–261. [CrossRef]
56. Ye, K.; Zhang, X.; Huang, Y.; Liu, J.; Liu, M.; Zhou, G. Bacteriocinogenic *Enterococcus faecium* inhibits the virulence property of *Listeria monocytogenes*. *Food Sci. Technol.* **2018**, *89*, 87–92. [CrossRef]

Effect of a *Lactilactobacillus sakei* strain on the evolution of volatile compounds and on the final sensorial characteristics of traditional Iberian dry-cured fermented "salchichón".

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Abstract

In this work the effect of *Lactilactobacillus sakei* 205 on the evolution of volatile compounds throughout the ripening process and in the final sensorial characteristics of traditional Iberian dry-cured fermented "salchichón", was evaluated. The inoculation of *L. sakei* 205 does not suppose modification of the physicochemical parameters covering humidity, water activity, and pH and those related to texture and color of ripened "salchichón". However, an increase of volatile compounds derived from amino acid catabolism and microbial esterification and a decrease of compounds derived from lipid oxidation, mainly hexanal, was observed throughout the ripening time in *L. sakei* inoculated "salchichón" in comparison with uninoculated control. These differences could have a possible positive effect on the final flavor of the inoculated product, although were not enough to be differentiated from non-inoculated ones after sensorial analysis. Thus, the use *L. sakei* 205 as a protective culture could be recommendable to improve the safety and quality of traditional "salchichón".

Keywords: *L. sakei*, Iberian dry-cured fermented sausages, volatile compounds, texture, sensorial analysis.

1. INTRODUCTION

Traditional Iberian dry-cured fermented sausage "salchichón" is a high-value product obtained from autochthonous Iberian pigs in Spain. This meat product is usually elaborated following traditional recipes without using starter cultures and ripened for at least 2-3 months to allow the formation of characteristic flavor. Since this type of product has been spontaneously fermented depending on the natural contaminant microbial population, the flavor of the final product is difficult to guarantee, as it has been reported in meat products of similar characteristics (Xiao et al., 2020). This problem could be solved by the addition of safe starter cultures. Lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) are the most popular starter cultures for the production of dry-cured fermented sausages (Pavli et al., 2020). Several *Lactilactobacillus* spp. possess lipolytic and proteolytic activities, which may contribute to the characteristic and pleasant flavor development of the dry-cured fermented sausages (Leroy & De Vuyst, 2004). In addition, LAB play an important role in meat preservation as they can accelerate the acidification of the raw material through the production of organic acids, mainly lactic acid (Kargozari et al., 2014). They can also produce bacteriocins, which must prevent the growth of some pathogens and spoilage microorganisms and provide a diversity of sensory properties by modifying the raw material (Leroy & De Vuyst, 2004).

Indigenous LAB belonging to dry-cured fermented sausages have been reported as especially well adapted to the ecological conditions of meat fermentation, controlling the ripening processes and inhibiting the growth of spontaneous microorganisms (Hugas & Monfort, 1997). Thus, the use of selected LAB as protective cultures isolated from indigenous microbial population of Iberian dry-cured fermented sausages could be of great interest to control *Listeria monocytogenes,* the most worrying microorganism in this kind of products (Laranjo et al., 2019). The strain *Lactilactobacillus sakei* 205 isolated

from traditional Iberian dry-cured fermented sausages has been selected by its effect against *L. monocytogenes* both *in vitro* and *in vivo* assays, being the last throughout the ripening process of Iberian dry-cured fermented "salchichón" (Martín et al., 2021). Before to propose the use of *L. sakei* 205 as a protective culture in the elaboration of traditional Iberian dry-cured fermented "salchichón", it is necessary to evaluate its effect on the evolution of volatile compounds involved in flavor development throughout the ripening and on the sensorial characteristics of this product. This evaluation should be done in real conditions of processing, taking in account that in addition of the selected *L. sakei* strain, microorganisms from contamination origin, mainly indigenous LAB, could be present in the product throughout the ripening.

This study was aimed to determine the effect of the selected strain *L. sakei* 205 and indigenous LAB on the evolution of volatile compounds throughout the ripening process and on the final sensorial characteristics of traditional Iberian dry-cured fermented "salchichón".

2. MATERIALS AND METHODS

2.1. Lactic-acid bacterium culture

The strain *L. sakei* 205 was isolated from Iberian dry-cured fermented sausages and selected by its anti-*L. monocytogenes* activity in vitro and throughout the ripening process of dry-cured fermented sausages (Martín et al., 2021). The inoculum of LAB was obtained by inoculating 100 µL of a culture preserved at -80 ºC on Man Rogosa Sharpe (MRS) broth (Fisher Bioreagents, Belgium) and incubating at 30 ºC for 48 h. Later, 100 µL of were plated on MRS agar plate and incubated again at 30 ºC for 48 h and, the counts of the microorganism were conducted. Once the concentration of cells was known, the appropriate dilutions were made to achieve the concentration of 7 log CFU/mL which was

used as inoculum. Then, the culture was centrifuged at 10,000 g for 5 min, and the supernatant was discarded. The sediment was then washed and resuspended in phosphate-buffered saline (PBS) and used for the inoculation of the Iberian "salchichón" mix before stuffing. To determine the final concentration (CFU/mL) of *L. sakei* 205 in PBS in order to adjust the level of inoculation, serial dilutions in 1% (w/v) peptone water (Conda, Spain) were inoculated onto MRS agar (Oxoid, UK) and incubated anaerobically at 30 °C for 72 h. In addition, initial levels of LAB on the sausages at day 0 of processing were determined as described before.

2.2. Preparation of Iberian dry-cured fermented sausages "salchichón"

Meat dough used for the manufacture of "salchichón" was purchased from a meat company in the Extremadura region (Cáceres) and made with Iberian pork meat and Iberian pig fatback, salt, sugar, potassium nitrate, sodium nitrite, black pepper, and spices. The effect of *L. sakei* 205 on the evolution of volatile compounds throughout the ripening and the final sensory characteristics of "salchichón" was evaluated. For this, two equal batches of 10 kg each of the mixture were manufactured: a non-inoculated batch (Control) and another batch inoculated with *L. sakei* at 7 log CFU/g (Ls).

The inoculum of *L. sakei* was adjusted and prepared to be resuspended in a total volume of 150 mL of PBS. In the case of Control batch, 150 mL of sterilized PBS were added instead of the bacterium inoculum.

Then the mixtures of each batch after addition of PBS (Control batch) and inoculation (Ls batch) were stuffed into regenerated collagen casings (40 mm in diameter) and the sausages were ripening in the Research Pilot Plant facility at Faculty of Veterinary Science, University of Extremadura (Cáceres, Spain) for 90 days adjusting the relative humidity (RH) and temperature to a traditional processing in the meat industry for this type of product. Thus, the ripening started with 3 days at 5 °C and 85% RH, followed by

17 days at 7 °C and 80 % RH, 10 days at 9 °C and 75% RH, and, finally, the sausages were kept at 12 °C and 70 % RH to reach 90 days of ripening.

The microbiological analysis, water activity, pH, moisture content and volatile compounds analysis were determined at 0, 15, 30, 60 and 90 days of ripening; however, the color, texture, sodium chloride, and nitrite contents were determined only at the end of the processing (90 days). For all the analysis five sausages were taken in each sampling time. Thus, all the tests were carried out in quintuplicate.

2.3. Microbial analysis

The total viable microorganism counts were determined in Plate Count Agar (PCA; Pronadisa, Spain), while LAB ones in MRS (Oxoid). Both agar plates were incubated at 30 ºC for 48 h. *Enterobacteriaceae* were determined on Violet Red Bile Dextrose (VRBG, Oxoid) agar, and the incubation was carried out at 37 ºC for 48 h in anaerobic conditions. Counts were expressed as log CFU/g.

2.4. Physico-chemical analysis

The pH, water activity (a_w) and moisture content were measured in quintuplicate throughout the ripening of the sausages to assure their correct processing. The pH values of the Iberian dry-cured fermented sausages were evaluated using a pH-meter, model 340 (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). The a_w was determined using a Novasina LabMaster a_w meter (Novasina AG, Switzerland). The moisture content was analyzed following the official methods of the Association of Official Analytical Chemists (AOAC, 2000) being gravimetrically determined.

2.5. Instrumental texture

The texture analysis was performed at room temperature using a Texture Profile Analysis (TPA). The instrument used was a TA XT Plus Texture Analyzer (StableMicro Systems Ltd., Godalming, UK) equipped with a cylindrical probe of 5 cm in diameter. This

148 procedure involved cutting slices approximately 1 cm thick. Hardness (N), springiness
149 (cm), cohesiveness, gumminess (N), chewiness (N cm), and adhesiveness (N s) were
150 evaluated at the end of the ripening process. All the texture analysis were carried out in
151 triplicate of each quintuplicate.

152 **2.6. Instrumental color**

153 Color was determined on the cut surface of each sample using a Minolta CR-300
154 colorimeter (Konica Minolta, Inc; Nieuwegein, The Netherlands) with an illuminant D65,
155 a 0° standard observer and one port/ display area of 2.5 cm. that was calibrated before use
156 with a white tile having the following values: L* = 93.5, a* = 1.0 and b* =0.8. Color was
157 expressed according to the Commission International de l'Eclairage (CIE) system and
158 reported as CIE L* (lightness), CIE a* (redness), CIE b* (yellowness), in which the
159 chroma and hue angle were calculated as $(a^{*2}+b^{*2})^{0.5}$ and $\tan^{-1}$ (b*/a*), respectively.

160 **2.7. Volatile compound analysis**

161 The volatile compounds in dry-cured sausages were extracted by solid-phase
162 microextraction (SPME) after heating to 37 ºC for 30 min, using a divinylbenzene-
163 carboxen-polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm fiber (Merck; Darmstadt,
164 Germany). They were then analyzed by gas chromatography-mass spectrometry (GC-
165 MS) in a Gas Chromatograph 6890 GC (Agilent Technologies; Santa Clara, CA, USA)
166 equipped with an HP-5 column (5% phenyl−95% dimethylpolysiloxane) and coupled to
167 a mass spectrometer (MS) detector, 5975C (Agilent Technologies). Oven temperature
168 started at 40 ºC for 5 min and was increased to 280 ºC, with a rate of 7 ºC/min. The
169 desorption time was 30 min at 250 ºC. The transfer line temperature was established at
170 280 ºC. The carrier gas was helium with a flow rate of 1.2 mL/min. MS detection was
171 performed in full scan (50–350 amu). Automated peaks search and spectral deconvolution

were used for data treatment, and the dentification of the volatile compounds was achieved by comparing their mass spectra with the NIST/EPA/NIH library.

2.8. Sodium chloride and nitrite determination

Sodium chloride (NaCl) and nitrite were determined in duplicate for each of the five dry-cured sausages of each batch at the end of the ripening period using the Volhard (AOAC, 2016) and an AOAC (2005) methods, respectively.

2.9. Sensory evaluation

A triangular sensory analysis was carried out in this study with semi-trained panel (24), including students and lectures at the Faculty of Veterinary Sciences (University of Extremadura, Caceres, Spain),

Three samples were presented to each panelist member, marked with random three digits codes, and served at room temperature on white plates, and they rated which sample is different. Cookies (with no added salt) and about 200 mL of water were also provided to the panelists to rinse between samples. The panel sessions were held around 2 h before lunch in the sensory panel booth room of the Faculty of Veterinary Sciences of the University of Extremadura in Caceres (Spain). Data about sex and age were also asked.

2.10. Statistical analyses

The statistical treatment was carried out using SPSS IBM v.22 software (IBM, USA). Once the dependent and independent variables of the analysis were determined (microbiological level, a_w, pH, moisture content, texture, color, sodium chloride, nitrite values, volatile compounds and different batches and days of ripening, respectively), a study of the normality of the different data populations was carried out using the Shapiro Wilk test. Subsequently, the analysis of the data was conducted using the non-parametric

Kruskal-Wallis and Mann-Whitney test. Statistical significance was established at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Enumeration of microorganisms

The results obtained from the enumeration of microorganisms are shown in Figure 1. Total aerobic counts showed similar levels to those detected in MRS agar (lactic acid bacteria) in the two analyzed batches and all days of ripening (4.7-7 log CFU/g). Thus, the evolution of the microbial population was as expected for dry-cured fermented sausages (Najjari et al., 2020), being LAB the predominant microbial group. The microorganisms growing on VRBG (*Enterobacteriaceae*) showed higher levels (around 4 log CFU/g) in both Control and Ls batches at day 0 than in the remaining days whose levels decreased to values below to 2 log CFU/g after day 30 of ripening (Figure 1), probably due to the combined effect of the reduction of a_w and the antimicrobial activity of LAB (Martín et al., 2021). The LAB levels were around 7 log CFU/g for the inoculated batch and 4 log CFU/g for Control, at day 0 of ripening. The presence of LAB in Control batch at a level of 4-5 log CFU/g from microbial contamination has been reported as usual in dry-cured fermented sausages processing (Najjari et al., 2020). The evolution of LAB throughout the ripening showed that the level of this microbial group remained at 6-7 log CFU/g during all the ripening time in Ls batch while in the Control batch LAB counts were increased from 4 to 6 log CFU/g at the end of the processing (Figure 1). In the final product, the *L. sakei* inoculated batch (Ls batch) showed a significant ($p \leq 0.05$) higher level of LAB than the Control batch, which could be justified by the addition of *L. sakei* 205 adapted to dry-cured fermented sausage environment from where it was isolated.

3.2. Effect of *L. sakei* 205 on the "salchichón" characteristics

3.2.1 Moisture content, water activity, and pH changes

The moisture content (%) decreased ($p \leq 0.05$) during ripening from initial values around 85% to 25-26% at the end of ripening for the two batches. Finally, a_w ranged from initial values of 0.946-0.947 to 0.790 at day 90 (Figure 2a). The pH values decreased from about 5.8 to 5.4 during the first 15 days of ripening, and then rose up to 6 at the end of processing in both batches (Figure 2b). Moisture content, a_w, and pH values did not differ significantly between batches Control and Ls throughout the ripening period except for pH in the first 30 days of processing where pH was significantly lower in batch Ls (Figure 2). The lowest pH at days 15 and 30 of ripening in batch Ls could be due to the release of lactic acid by the inoculated *L. sakei* (Benito et al., 2007). In both batches, the moisture content, a_w, and pH values were similar to those reported in dry-cured fermented sausages (Casquete et al., 2011). Thus, the addition of *L. sakei* 205 did not suppose a decrease in the pH of "salchichón", contrarily to the decrease of pH values reported in dry-cured fermented sausages in which selected starter cultures of *L. sakei* were added (Najjari et al., 2020). Neither did it suppose a modification of the evolution of parameters such as moisture content and a_w that could influence sensorial characteristics of the final products.

3.2.2. Sodium chloride, nitrites and color determination

Sodium chloride and nitrite content were only determined in ripened "salchichón" (at day 90) showing both batches (Control and Ls) similar values of approximately 3.3% (w/w) and around 6.9-7.4 mg/Kg in NaCl and nitrite, respectively (Table 1). No differences ($p > 0.05$) between both batches in these parameters were found. These values are similar to those usually found in dry-cured fermented sausages (Casquete et al., 2011; Gonzales-Barron et al., 2015).

Regarding color determination, this is the first parameter that affects consumer acceptance of meat products. In the CIELAB parameters, no differences between the inoculated and Control batches were observed except for the parameter L*(lightness) which was significantly ($p \leq 0.05$) higher in Ls than in Control batch. A positive correlation between fat content and L* value, as well as a negative correlation between drying/ripening time, was previously reported (Olivares et al., 2010). However, the a* (redness) and b* (yellowness) values of Ls batch were very similar to those found in the Control. Thus, there are no differences in color of sausages between batches that could be attributed to *L. sakei* 205 inoculation.

3.2.3. Texture analysis

Results concerning the texture profile analysis of "salchichón" at the end of ripening showed similar values between control and inoculated sausages. The only difference ($p \leq 0.05$) between batches was the lower value of cohesiveness found in batch Ls than in the Control batch (Table 2). Both batches showed higher levels of adhesiveness than others previously reported, from -0.6 and -1.3 N s (Herrero et al., 2007; Álvarez et al., 2020). The profile of texture analysis of both Control ad Ls batches ranges in usual values of dry-cured fermented sausages (El Adab et al., 2015; Herrero et al., 2007).

3.2.4. Analysis of volatile compounds

A total of fifty-two volatile compounds were identified throughout the ripening time in the two different batches of "salchichón" (Table 3). Most of these compounds have previously reported in different types of dry-cured fermented sausages (Andrade et al., 2010; Bianchi et al., 2007; Flores et al., 2004). These volatile compounds were classified according to their most probable origin as from lipid oxidation (25), microbial esterification (3), carbohydrate fermentation (1), amino acid catabolism (6) and spices (13) and exposed as summatory of accumulated area of these groups in Figure 3.

However, some of the compounds could have more than one source or they are derived of secondary reactions between substances generated in different catabolic routes (Berdagué et al., 1993). When the accumulated area of these groups of compounds was analyzed, it can be observed that levels of those derived from lipid oxidation showed a decrease throughout the ripening time in both Control and inoculated batches in comparison with day 0 (Figure 3). However, the accumulated area of compounds derived from amino acid catabolism and spices increased throughout the ripening time in both analyzed batches, showing the highest mean values at day 60 (derived from amino acid catabolism) and at day 90 of ripening (derived from species). Compounds derived from microbial esterification showed highest levels at day 90 of ripening in both analyzed batches (Figure 3).

When specific volatile compounds in each group were analyzed, it can be observed that most of the significant ($p \leq 0.05$) differences were detected between days of ripening in each Control and Ls batch (Table 3). Thus, in group of **compounds derived from lipid oxidation** is remarkable the significant ($p \leq 0.05$) decrease of hexanal, heptanal and 1-pentanol throughout the ripening time. However, there was a significant ($p \leq 0.05$) increase of 2-octanone and octanoic acid during ripening in both inoculated and Control batches. There were not consistent differences between Control and *L. sakei* inoculated batches in volatile compounds derived from lipid oxidation. Linear aldehydes such as hexanal have been reported as the most important aldehyde in dry-cured fermented sausages (de Lima Alves et al., 2020; Vargas-Ramella et al., 2020), and it has also been chosen as an index of the level of oxidation (Sidira et al., 2015). Given that the aldehydes, hexanal and heptanal, and the alcohol 1-pentanol are formed by oxidation of unsaturated fatty acids, their reduction during ripening could be related to the microbial catalase activity of the inoculated *L. sakei* in Ls batch and lactic acid bacteria from contamination

297 origin in Control batch, that suppose a retard in autoxidation due to decomposition of
298 hydrogen peroxide (Martín et al., 2003). Since hexanal gives to the dry-cured fermented
299 meat products a typical flavor of rancid, pungent and toasty (Iacumin et al., 2020; Pinna
300 et al., 2009), the reduction observed for this compound in both batches throughout the
301 ripening process may be related to activity of LAB in both batches. This reduction it is
302 very interesting for the flavor of final product since may contribute to reduce the note of
303 rancidity of ripened "salchichón". The increases observed during ripening in 2-octanone
304 and octanoic acid may derived from oxidation of aldehydes from linoleic acid during the
305 ripening time (Shahidi et al., 2006; Martín et al., 2003).
306 **Compounds derived from amino acid catabolism** showed higher levels at days 60 and
307 90 than at days 0 and 30 of ripening, probably due to the increase in the proteolytic activity
308 during ripening, since these compounds derived mostly from amino acids by Strecker
309 degradation and/or microbial metabolism (Barbieri et al., 1992; Durá et al., 2004; Martín
310 et al., 2006). It is remarkable that in both analyzed batches most of these compounds were
311 detected only at days 60 or 90 of ripening, although in batch Ls some compounds like 2-
312 methyl-1-propanol and 3-methyl-butanal were also detected at the beginning of ripening.
313 From all of these compounds the branched aldehyde 3-methyl-butanal has been reported
314 to contribute considerably to the overall flavor of the dry-cured fermented sausages
315 (Andrade et al., 2010; Domínguez et al., 2019). The 2-methyl-propanoic acid and 3-
316 methyl-butanoic acid that were detected at the end of ripening in Ls and Control batches,
317 have been reported as derived from microbial metabolism of Val and Leu, respectively
318 (Ansorena et al., 2001). These compounds have been attributed to a characteristic "sweet"
319 odour (Mateo & Zumalacárregui, 1996) and may also have a positive impact on aroma of
320 dry-cured fermented sausages due to their conversion into fruity esters (Stahnke, 1994).
321 Thus, the inoculated *L. sakei* 205 could contribute to the generation of the compounds

derived from amino acid catabolism, although there were not consistent differences with respect to Control batch because of probable action of LAB from microbial contamination found in high level after day 15 of ripening in this batch (Figure 1).

Compounds derived from microbial esterification showed also higher levels at days 60 and 90 of ripening than at the beginning of maturation (Table 3). In addition, in Ls batch all these compounds, except hexanoic acid ethyl ester, were detected in all days of ripening while in Control batch most of them were only detected only at days 60 and 90 of ripening. Ethyl esters, usually present in dry-cured fermented sausages, may arise from the action of lactic-acid bacteria inoculated in the Ls batch but also from LAB contamination in Control batch. This could justify the compounds from microbial esterification were detected throughout the ripening in Ls batch, where levels of LAB were always higher than 6 log CFU/g, while in Control batch were detected mainly at the end of ripening when this microbial group reach levels higher than 6 log CFU/g (Figure 1). Ethyl esters contribute to a proper dry-cured fermented sausages flavor due to their characteristic fruity notes, their low odour threshold values and their contribution to mask rancid odours (Careri et al., 1993; Flores et al., 2004; Stahnke, 1994). Their presence, together with 3-methylbutanal, has been associated with a ripened flavor (Andrade et al., 2010).

Regarding to **compounds derived from carbohydrate fermentation** only acetic acid was found (Table 3). This compound was detected in all days of ripening in Ls batch, and only in some of the days of ripening in Control batch. Acetic acid derives from the microbial fermentation of sugars mainly LAB or the Maillard reaction (Andrade et al., 2010). The fact that not significant differences in this compound between Control and Ls batches were detected seems to indicate that inoculation of *L. sakei* 205 do not suppose an increase of acidity of "salchichón".

Most of volatile **compounds from the added spices** were detected in both analyzed batches of "salchichón" in all days of ripening (Table 3). Terpenes were most of the compounds derived from spices, D-limonene, caryophyllene and 3-carene being predominant in both batches. Particularly, α and β- pinene, D-limonene and carene (Meynier et al., 1999) were identified in the essential oil of black pepper, ingredient added to raw meat and fat during the mixing process. However, the presence of terpenes has no relation to the ripening and microbial action, so they were related to raw material and ingredients which supports that no significant differences were detected between batches in most of the compounds. The quantity of terpenes studied had a tendency to increase during the ripening process, probably due to the dehydration: the loss of water causes the increase of fat level, in which terpenes are easily dissolved (Iacumin et al., 2020).

3.2.5. Sensory evaluation

According to the levels of significance for the triangular test (Harry & Heymann, 2010), there were no significant differences ($p > 0.05$) between both Control and Ls batches. Thus, the selected strain of *L. sakei* 205 at the levels 7 log CFU/g inoculated has no significant effect on the sensory quality of the finished "salchichón". No effect has also been found by the use of selected LAB in sensorial analysis by triangular test in other meat products (Amézquita and Brashears, 2002). These results are consistent with the no differences observed in physicochemical parameters (pH, a_w, humidity content), color and texture analysis due to *L. sakei* 205 inoculation as compared with the uninoculated control. In addition, the increase of volatile compounds derived from amino acid catabolism and the decrease of compounds derived from lipid oxidation, observed throughout the ripening time, due to *L. sakei* 205 inoculation, was not enough to be differentiated from non-inoculated ones after sensorial analysis. In any case, it is relevant

371 the obtained results about the non-modification of the sensorial characteristics of the
372 "salchichón" in order to propose *L. sakei* 205 as a protective culture.

373 **CONCLUSION**

374 The inoculation of *L. sakei* 205 allow that "salchichón" has a maintained level of LAB
375 between 6-7 log CFU/g throughout the ripening process, being LAB, the dominant
376 microbial population avoiding growth of undesirable microorganisms such as
377 *Enterobacteriacea*e. The inoculation of this LAB strain does not suppose a modification
378 of the physicochemical parameters covering moisture content, water activity and pH and
379 those related to texture and color of ripened sausages. Increase of volatile compounds
380 derived from amino acid catabolism and microbial esterification and decrease of
381 compounds derived from lipid oxidation was observed throughout the ripening time and
382 the inoculation of *L. sakei* 205 could contribute to this positive effect on the flavor of
383 "salchichón". The "salchichón" inoculated with *L. sakei* 205 is not differentiated from
384 non-inoculated "salchichón" after sensorial analysis. Thus, the use of *L. sakei* 205 as a
385 protective culture could be advisable to improve the safety of the product and to increase
386 the generation of volatile compounds associated to characteristic flavor of ripened dry-
387 cured fermented sausages.

388 **CRediT authorship contribution statement**

389 **Irene Martín:** Data curation, Investigation, Writing – original draft, Writing – review &
390 editing. **Carmen García:** Writing – review & editing. **Alicia Rodríguez:** Investigation,
391 Writing – review & editing. **Juan J. Córdob**a**:** Writing – original draft, Writing – review
392 & editing, Supervision.

393

394 **Declaration of Competing Interest**

395 The authors declare that they have no known competing financial interests or personal
396 relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been funded by by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. I. Martín was supported by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sport (FPU 16/05303).

References

Álvarez, M., Andrade, M. J., García, C., Rondán, J. J., & Núñez, F. (2020). Effects of preservative agents on quality attributes of dry-cured fermented sausages. *Foods*, *9*, 1505. https://doi.org/10.3390/foods9101505

Amézquita, A & Brashears, M.M. 2002. Competitive inhibition of *Listeria monocytogenes* in Ready-to-Eat meat products by lactic acid bacteria. *Journal of Food Protection* 65, 2, 316–325. https://doi.org/10.4315/0362-028x-65.2.316

Andrade, M. J., Córdoba, J. J., Casado, E. M., Córdoba, M. G., & Rodríguez, M. (2010). Effect of selected strains of *Debaryomyces hansenii* on the volatile compound production of dry fermented sausage "salchichón". *Meat Science*, *85*(2), 256–264. https://doi.org/10.1016/j.meatsci.2010.01.009

Ansorena, D., Gimeno, O., Astiasarán, I., & Bello, J. (2001). Analysis of volatile compounds by GC-MS of a dry fermented sausage: Chorizo de Pamplona. *Food Research International*, *34*(1), 67–75. https://doi.org/10.1016/S0963-9969(00)00133-2

AOAC. (2000). Moisture in malt gravimetric method (935.29). In *Official methods of analysis of AOAC international* (17th ed.).

AOAC. (2005). Official Methods of Analysis of AOAC International, 18th Edition. In *Association of Official Analytical Chemists* (Volume 3172).

AOAC. (2016). Official Methods of Analysis of AOAC International - 20th Edition. In *Association of Official Analytical Chemists.* (Vol. 52, Issue 2). https://doi.org/10.1021/ac50052a726

Barbieri, G., Bolzoni, L., Parolari, G., Virgili, R., Buttini, R., Careri, M., & Mangia, A. (1992). Flavor compounds of dry-cured ham. *Jorunal of Agricultural and Food Chemistry*, *40*(July), 2389–2394.

Benito, M. J., Martín, A., Aranda, E., Pérez-Nevado, F., Ruiz-Moyano, S., & Córdoba, M. G. (2007). Characterization and selection of autochthonous lactic acid bacteria isolated from traditional Iberian dry-fermented salchichón and chorizo sausages. *Journal of Food Science*, *72*(6), 193–201. https://doi.org/10.1111/j.1750-3841.2007.00419.x

Betdague, L.J., Denoyer, C., Le Quere, J.L., Semon, E. (1991). Volatile components of dry-cured ham. *Agricultural and Food Chemistry, 39*(7), 1257-1261. https://doi.org/10.1021/jf00007a012

Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M., & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, *72*(4), 1552–1563. https://doi.org/10.1016/j.talanta.2007.02.019

Careri, M., Mangia, A., Barbieri, G., Bouoni, L., Virgili, R., Parolari, G. (1993). Sensory property relationships to chemical data of italian-type dry-cured ham. *Journal of Food Science,58*(5), 968-972. https://doi.org/10.1111/j.1365.2621.1993.tb06090.x

444 Casquete, R., Benito, M. J., Martín, A., Ruiz-Moyano, S., Córdoba, J. J., & Córdoba, M.
445 G. (2011). Role of an autochthonous starter culture and the protease EPg222 on the
446 sensory and safety properties of a traditional Iberian dry-fermented sausage
447 "salchichón". *Food Microbiology*, *28*(8), 1432–1440.
448 https://doi.org/10.1016/j.fm.2011.07.004

449 de Lima Alves, L., Donadel, J. Z., Athayde, D. R., da Silva, M. S., Klein, B., Fagundes,
450 M. B., de Menezes, C. R., Barin, J. S., Campagnol, P. C. B., Wagner, R., &
451 Cichoski, A. J. (2020). Effect of ultrasound on proteolysis and the formation of
452 volatile compounds in dry fermented sausages. *Ultrasonics Sonochemistry*,
453 *67*(April), 105161. https://doi.org/10.1016/j.ultsonch.2020.105161

454 Domínguez, R., Purriños, L., Pérez-Santaescolástica, C., Pateiro, M., Barba, F. J.,
455 Tomasevic, I., Campagnol, P. C. B., & Lorenzo, J. M. (2019). Characterization of
456 volatile compounds of dry-cured meat products using HS-SPME-GC/MS
457 technique. *Food Analytical Methods*, *12*(6), 1263–1284.
458 https://doi.org/10.1007/s12161-019-01491-x

459 Durá, M. A., Flores, M., & Toldrá, F. (2004). Effect of growth phase and dry-cured
460 sausage processing conditions on *Debaryomyces* spp. generation of volatile
461 compounds from branched-chain amino acids. *Food Chemistry*, *86*(3), 391–399.
462 https://doi.org/10.1016/j.foodchem.2003.09.014

463 El Adab, S., Essid, I., & Hassouna, M. (2015). Microbiological, biochemical and
464 textural characteristics of a tunisian dry fermented poultry meat sausage inoculated
465 with selected starter cultures. *Journal of Food Safety*, *35*(1), 75–85.
466 https://doi.org/10.1111/jfs.12164

467 Flores, M., Durá, M. A., Marco, A., & Toldrá, F. (2004). Effect of *Debaryomyces* spp.

on aroma formation and sensory quality of dry-fermented sausages. *Meat Science*, *68*(3), 439–446. https://doi.org/10.1016/j.meatsci.2003.04.001

Gonzales-Barron, U., Cadavez, V., Pereira, A. P., Gomes, A., Araújo, J. P., Saavedra, M. J., Estevinho, L., Butler, F., Pires, P., & Dias, T. (2015). Relating physicochemical and microbiological safety indicators during processing of linguiça, a Portuguese traditional dry-fermented sausage. *Food Research International*, *78*, 50–61. https://doi.org/10.1016/j.foodres.2015.11.007

Harry, L. T., & Heymann, H. (2010). *Sensory Evaluation of Food* (Dennis R. Heldman (ed.); Second Edi, Vol. 6). Springer. https://doi.org/10.1017/CBO9781107415324.004

Herrero, A. M., Ordóñez, J. A., de Avila, R., Herranz, B., de la Hoz, L., & Cambero, M. I. (2007). Breaking strength of dry fermented sausages and their correlation with texture profile analysis (TPA) and physico-chemical characteristics. *Meat Science*, *77*(3), 331–338. https://doi.org/10.1016/j.meatsci.2007.03.022

Hugas, M., & Monfort, J. M. (1997). Bacterial starter cultures for meat fermentation. *Food Chemistry*, *59*(4), 547–554. https://doi.org/10.1016/S0308-8146(97)00005-8

Iacumin, L., Osualdini, M., Bovolenta, S., Boscolo, D., Chiesa, L., Panseri, S., & Comi, G. (2020). Microbial, chemico-physical and volatile aromatic compounds characterization of Pitina PGI, a peculiar sausage-like product of North East Italy. *Meat Science*, *163*(September 2019), 108081. https://doi.org/10.1016/j.meatsci.2020.108081

Kargozari, M., Moini, S., Basti, A. A., Emam-Djomeh, Z., Gandomi, H., Martin, I. R., Ghasemlou, M., & Carbonell-Barrachina, Á. A. (2014). Effect of autochthonous starter cultures isolated from Siahmazgi cheese on physicochemical,

492 microbiological and volatile compound profiles and sensorial attributes of sucuk, a
493 Turkish dry-fermented sausage. *Meat Science*, *97*(1), 104–114.
494 https://doi.org/10.1016/j.meatsci.2014.01.013

495 Laranjo, M., Potes, M.E., Elias, M. (2019). Role of starter cultures on the safety of
496 fermented meat products. *Frontiers in Microbiology,10, 853.*
497 https://doi.org/10.3389/fmicb.2019.00853

498 Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for
499 the food fermentation industry. *Trends in Food Science and Technology*, *15*(2),
500 67–78. https://doi.org/10.1016/j.tifs.2003.09.004

501 Martín, A., Córdoba, J. J., Aranda, E., Córdoba, M. G., & Asensio, M. A. (2006).
502 Contribution of a selected fungal population to the volatile compounds on dry-
503 cured ham. *International Journal of Food Microbiology*, *110*(1), 8–18.
504 https://doi.org/10.1016/j.ijfoodmicro.2006.01.031

505 Martín, A., Córdoba, J. J., Benito, M. J., Aranda, E., & Asensio, M. A. (2003). Effect of
506 *Penicillium chrysogenum* and *Debaryomyces hansenii* on the volatile compounds
507 during controlled ripening of pork loins. *International Journal of Food*
508 *Microbiology*, *84*(3), 327–338. https://doi.org/10.1016/S0168-1605(02)00474-9

509 Martín, I., Rodríguez, A., Sánchez-Montero, L., Padilla, P., & Córdoba, J. J. (2021).
510 Effect of the dry-cured fermented sausage "salchichón" processing with a selected
511 *Lactobacillus sakei* in *Listeria monocytogenes* and microbial population. *Foods*,
512 *10*(4). https://doi.org/10.3390/foods10040856

513 Mateo, J., & Zumalacárregui, J. M. (1996). Taste compounds in chorizo and their
514 changes during ripening. *Meat Science*, *44*(4), 245–254.
515 https://doi.org/10.1016/S0309-1740(96)00098-8

516 Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E., & Gandemer, G. (1999). Volatile
517 compounds of commercial Milano salami. *Meat Science*, *51*(2), 175–183.
518 https://doi.org/10.1016/S0309-1740(98)00122-3

519 Najjari, A., Boumaiza, M., Jaballah, S., Boudabous, A., & Ouzari, H. I. (2020).
520 Application of isolated *Lactobacillus sakei* and *Staphylococcus xylosus* strains as a
521 probiotic starter culture during the industrial manufacture of Tunisian dry-
522 fermented sausages. *Food Science and Nutrition*, *8*(8), 4172–4184.
523 https://doi.org/10.1002/fsn3.1711

524 Olivares, A., Navarro, J. L., Salvador, A., & Flores, M. (2010). Sensory acceptability of
525 slow fermented sausages based on fat content and ripening time. *Meat Science*,
526 *86*(2), 251–257. https://doi.org/10.1016/j.meatsci.2010.04.005

527 Pavli, F. G., Argyri, A. A., Chorianopoulos, N. G., Nychas, G. J. E., & Tassou, C. C.
528 (2020). Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on
529 physicochemical, microbiological and sensorial characteristics of dry-fermented
530 sausages. *Lwt*, *118*(October 2019). https://doi.org/10.1016/j.lwt.2019.108810

531 Pinna, A., Quintavalla, S., Simoncini, N., Toscani, T., & Virgili, R. (2009). Composti
532 organici volatili di un sistema modello simil- prosciutto inoculato con lieviti
533 autoctoni isolati da prosciutti tipici. *Microbiologia/Prodotti Carnei*, 93–105.

534 Shahidi, F., Rubin, L.J., D´Souza, L.A., Teranishi, R., Buttery, R.G. (2009). Meat flavor
535 volatiles: A review of the composition, techniques of analysis, and sensory
536 evaluation. *Critical Reviews in Food Science and Nutrition*, 24, 1986.
537 https://doi.org/10.1080/10408398609527435

538 Sidira, M., Kandylis, P., Kanellaki, M., & Kourkoutas, Y. (2015). Effect of immobilized
539 *Lactobacillus casei* on the evolution of flavor compounds in probiotic dry-

fermented sausages during ripening. *Meat Science*, *100*, 41–51. https://doi.org/10.1016/j.meatsci.2014.09.011

Stahnke, L. H. (1994). Aroma components from dried sausages fermented with *Staphylococcus xylosus*. *Meat Science*, *38*(1), 39–53. https://doi.org/10.1016/0309-1740(94)90094-9

Vargas-Ramella, M., Munekata, P. E. S., Gagaoua, M., Franco, D., Campagnol, P. C. B., Pateiro, M., Barretto, A. C. da S., Domínguez, R., & Lorenzo, J. M. (2020). Inclusion of healthy oils for improving the nutritional characteristics of dry-fermented deer sausage. *Foods*, *9*(10). https://doi.org/10.3390/foods9101487

Xiao, Y., Liu, Y., Chen, C., Xie, T., & Li, P. (2020). Effect of *Lactobacillus plantarum* and *Staphylococcus xylosus* on flavour development and bacterial communities in Chinese dry fermented sausages. *Food Research International*, *135*(December 2019), 109247. https://doi.org/10.1016/j.foodres.2020.109247

Table 1.

Sodium chloride, nitrite content and CIELAB parameters of dry-cured fermented sausages (salchichón) at the end of the ripening process.

Parameters	Batches	
	Control	Ls
NaCl (%)	3.29 ± 0.100	3.36 ± 0.099
Nitrites (ppm)	7.49 ± 1.019	6.96 ± 0.77
L*	36.62 ± 0.617	37.83 ± 0.789*
a*	12.38 ± 1.137	11.63 ± 0.276
b*	4.23 ± 0.491	4.55 ± 0.557

Control (uninoculated batch), Ls (batch inoculated with *L. sakei*). Values are expressed as mean ± standard deviation. Asterisks indicate significant differences with respect to the control ($p \leq 0.05$)

560 **Table 2.**

561 Values of instrumental texture parameters (hardness, adhesiveness, springiness, cohesiveness and
562 chewiness) of dry-cured fermented sausages (salchichón) at the end of the ripening process.

Parameters	Batches	
	Control	Ls
Hardness (N)	214.78 ± 45.427	220.32 ± 29.131
Adhesiveness (N/s)	-7.77 ± 1.406	-8.43 ± 2.054
Springiness	0.67 ± 0.085	0.63 ± 0.074
Cohesiveness	0.61 ± 0.019	0.60 ± 0.014*
Chewiness(N)	89.44 ± 26.752	82.82 ± 16.565

563 Control (uninoculated batch), Ls (batch inoculated with *L. sakei*). Values are expressed as mean ± standard deviation.
564 Asterisks indicate significant differences with respect to the control ($p \leq 0.05$)

565

566 **Table 3.**

567 Profiles of volatile compounds of Control and Ls batches during the ripening process of dry-cured fermented sausages (Arbitrary Area Units x 10^6)

Origin/ Compound	Control				Ls			
	0	30	60	90	0	30	60	90
Lipid oxidation								
Tridecane	0.49 ± 0.095	0.35 ± 0.127	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dodecane	0.48 ± 0.066	0.34 ± 0.123	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.157
1-Pentanol	0.79 ± 0.202^{a}	0.59 ± 0.235^{a}	0.13 ± 0.001^{b}	0.16 ± 0.150^{b}	0.79 ± 0.041^{a}	0.71 ± 0.259^{ab}	0.13 ± 0.011^{c}	0.34 ± 0.241^{bc}
1-Hexanol	0.14 ± 0.129^{b2}	0.08 ± 0.170^{b2}	0.52 ± 0.188^{ab}	1.06 ± 0.647^{a}	2.94 ± 0.468^{a1}	2.32 ± 1.159^{a1}	0.41 ± 0.059^{b}	1.66 ± 1.299^{ab}
1-Heptanol	0.05 ± 0.113 ^{2}	0.09 ± 0.117 ^{2}	n.d.	0.07 ± 0.133	0.25 ± 0.041^{a1}	0.07 ± 0.146^{ab}	0.04 ± 0.094^{ab}	0.20 ± 0.208^{b}
1-Octanol	0.25 ± 0.052	0.11 ± 0.153	0.21 ± 0.070	0.08 ± 0.174	0.18 ± 0.100	0.16 ± 0.146	0.17 ± 0.058	0.39 ± 0.344
1-Octen-3-ol	1.60 ± 0.206^{a}	1.20 ± 0.400^{a}	0.57 ± 0.018^{b1}	0.42 ± 0.336^{b}	1.47 ± 0.112^{a}	1.37 ± 0.261^{a}	0.49 ± 0.071^{b2}	0.76 ± 0.559^{b}
2-Octen-1-ol, (E)-	0.33 ± 0.057 ^{1}	0.27 ± 0.029	n.d.	n.d.	0.24 ± 0.017 ^{2}	0.25 ± 0.030	n.d.	n.d.
Terpinen-4-ol	1.50 ± 0.022	1.33 ± 0.143	n.d.	n.d.	1.17 ± 0.042	n.d.	n.d.	n.d.
Pentanal	0.53 ± 0.123^{a1}	0.38 ± 0.233^{ab}	0.13 ± 0.003^{b}	0.25 ± 0.001^{b}	0.35 ± 0.068 ^{2}	0.35 ± 0.068	0.18 ± 0.056	0.29 ± 0.283
Hexanal	12.36 ± 2.447^{a1}	9.88 ± 3.050^{a}	2.40 ± 0.096^{b}	1.91 ± 2.244^{b}	8.06 ± 1.404^{a2}	8.00 ± 1.335^{a}	1.94 ± 0.664^{b}	5.14 ± 3.760^{ab}
Heptanal	0.76 ± 0.101^{a2}	0.66 ± 0.187^{ab}	0.30 ± 0.095^{bc}	0.40 ± 0.327^{c}	0.93 ± 0.071^{a1}	0.83 ± 0.195^{a}	0.23 ± 0.057^{b}	0.74 ± 0.580^{ab}
2-Heptenal, (Z)-	0.39 ± 0.046^{a}	0.36 ± 0.133^{a}	0.21 ± 0.027^{b}	n.d.	0.37 ± 0.037^{b}	0.55 ± 0.093^{a}	0.19 ± 0.045^{c}	n.d.
2-Undecenal	0.31 ± 0.059^{b}	0.26 ± 0.042^{b}	0.46 ± 0.100^{a2}	0.23 ± 0.023^{b}	0.29 ± 0.022^{b}	0.27 ± 0.042^{b}	0.63 ± 0.073^{a1}	0.93 ± 0.999^{ab}
2-Nonenal, (E)-	0.80 ± 0.153	0.60 ± 0.240	n.d.	n.d.	0.86 ± 0.061	0.79 ± 0.210	n.d.	n.d.
Piperonal	0.31 ± 0.081^{a}	0.25 ± 0.025^{ab}	0.16 ± 0.028^{b}	n.d.	0.31 ± 0.081	0.24 ± 0.028	0.23 ± 0.087	2.00 ± 1.777
2-Decenal,(Z)-	0.43 ± 0.042	0.36 ± 0.086	0.36 ± 0.114	n.d.	0.41 ± 0.024	0.31 ± 0.176	0.45 ± 0.076	0.30 ± 0.125
2-Octenal,(E)-	1.01 ± 0.284^{a}	0.71 ± 0.354^{ab}	0.22 ± 0.041^{b}	0.39 ± 0.015^{b}	0.68 ± 0.062^{a}	0.62 ± 0.163^{a}	0.24 ± 0.063^{b}	0.52 ± 0.603^{ab}

2,4-Decadienal,(E,E)-	0.24 ± 0.019	0.21 ± 0.016	0.32 ± 0.223	n.d.	0.23 ± 0.019	0.23 ± 0.016	0.35 ± 0.064	0.22 ± 0.020
2-Heptanone	n.d.	n.d.	0.56 ± 0.026	0.55 ± 0.056	0.87 ± 0.136^{a}	0.53 ± 0.359^{b}	0.48 ± 0.064^{b}	0.52 ± 0.012^{b}
2-Octanone	n.d.	n.d.	0.17 ± 0.031^{b}	0.32 ± 0.100^{a}	0.30 ± 0.072^{a}	0.25 ± 0.022^{a}	0.14 ± 0.004^{b}	0.28 ± 0.009^{a}
2-Nonanone	n.d.	n.d.	0.30 ± 0.065	0.28 ± 0.007	0.31 ± 0.083^{a}	0.19 ± 0.111^{b}	0.20 ± 0.116^{b}	0.28 ± 0.007^{b}
Hexanoic acid	1.89 ± 0.559^{a}	1.73 ± 0.708^{a}	0.59 ± 0.103^{b}	0.68 ± 0.171^{b}	1.96 ± 0.214^{a}	1.96 ± 0.548^{a}	0.60 ± 0.105^{b}	1.52 ± 0.847^{ab}
Octanoic acid	n.d.	n.d.	0.50 ± 0.488^{a}	0.31 ± 0.173^{b}	n.d.	n.d.	0.50 ± 0.459^{a}	0.19 ± 0.262^{b}
Butanoic acid	0.19 ± 0.109^{b2}	0.28 ± 0.021^{a2}	0.13 ± 0.009^{b}	0.13 ± 0.115^{b}	0.57 ± 0.035^{a1}	0.64 ± 0.083^{a1}	0.13 ± 0.007^{b}	0.23 ± 0.146^{b}
Microbial esterification								
Hexanoic acid, ethyl ester	n.d.	n.d.	n.d.	0.34 ± 0.044 ^{1}	0.44 ± 0.110^{a}	0.41 ± 0.064^{a}	n.d.	0.26 ± 0.052^{b2}
n-Caproic acid vinyl ester	0.45 ± 0.089^{a1}	0.41 ± 0.094^{ab}	0.17 ± 0.022^{c}	0.24 ± 0.002^{bc}	0.31 ± 0.015^{a2}	0.34 ± 0.062^{a}	0.19 ± 0.066^{b}	0.27 ± 0.030^{ab}
Octanoic acid, ethyl ester	n.d.	n.d.	0.69 ± 0.143	0.91 ± 0.272 ^{1}	0.21 ± 0.009^{b}	0.21 ± 0.002^{b}	0.66 ± 0.276^{a}	0.56 ± 0.124^{ab2}
Carbohydrate fermentation								
Acetic acid	0.04 ± 0.091^{b2}	n.d.	0.27 ± 0.027^{a}	n.d.	0.42 ± 0.041^{a1}	0.43 ± 0.097^{a}	0.22 ± 0.038^{b}	0.12 ± 0.167^{b}
Amino acid catabol								
2-methyl-1-propanol	n.d.	n.d.	1.99 ± 0.149^{a}	0.98 ± 0.110^{b1}	0.27 ± 0.043^{b}	0.09 ± 0.126^{b}	1.99 ± 0.439^{a}	0.55 ± 0.165^{b2}
3-methyl-butanal	n.d.	n.d.	1.40 ± 0.724^{a}	0.10 ± 0.214^{b}	0.83 ± 0.157^{b}	n.d.	0.88 ± 0.849^{a}	n.d.
3-methyl-1-butano	n.d.	n.d.	1.03 ± 0.104	1.35 ± 0.570	n.d.	0.84 ± 0.154^{b}	1.25 ± 0.196^{a}	1.18 ± 0.274^{a}
2-methyl-propanoic acid	n.d.	n.d.	0.13 ± 0.003	0.10 ± 0.145	n.d.	n.d.	0.52 ± 0.298	0.51 ± 0.378
3-methyl-butanoic acid	n.d.	n.d.	0.18 ± 0.054	0.28 ± 0.392	n.d.	n.d.	0.71 ± 0.629	0.74 ± 0.446
2-methyl-1-butanol	n.d.	n.d.	0.31 ± 0.051^{a2}	0.24 ± 0.022^{b}	n.d.	n.d.	0.40 ± 0.025^{a1}	0.09 ± 0.130^{b}
Spices								

α-pinene	0.22 ± 0.206	0.17 ± 0.198	0.53 ± 0.121	0.56 ± 0.090	0.18 ± 0.214	0.27 ± 0.196	0.24 ± 0.334	0.25 ± 0.098
β-pinene	1.04 ± 0.086^{b}	1.01 ± 0.249^{b}	1.87 ± 0.260^{a}	2.12 ± 0.651^{a}	1.07 ± 0.102^{b}	1.10 ± 0.280^{b}	2.18 ± 0.461^{a}	1.75 ± 0.197^{a}
α-terpineol	0.34 ± 0.017^{a1}	0.28 ± 0.020^{b}	0.25 ± 0.017^{b}	0.28 ± 0.044^{b}	0.25 ± 0.025 ^{2}	0.20 ± 0.112	0.26 ± 0.030	0.31 ± 0.065
Safrole	0.58 ± 0.074 ^{1}	0.55 ± 0.084	0.58 ± 0.084	0.57 ± 0.032	0.48 ± 0.036^{b2}	0.47 ± 0.048^{b}	0.56 ± 0.015^{a}	0.51 ± 0.027^{ab}
D-limonene	2.28 ± 0.132^{b}	2.19 ± 0.433^{b}	4.45 ± 0.974^{b}	5.94 ± 2.365^{a}	2.20 ± 0.299^{b}	2.30 ± 0.715^{b}	4.44 ± 1.124^{b}	12.67 ± 8.630^{a}
o-Cymene	0.55 ± 0.026 ^{2}	0.45 ± 0.049 ^{2}	0.76 ± 0.212	0.76 ± 0.677	1.39 ± 0.340^{ab1}	1.33 ± 0.386^{ab1}	0.60 ± 0.165^{b}	3.37 ± 2.749^{a}
3-carene	1.85 ± 0.101^{b}	1.72 ± 0.333^{b}	2.65 ± 0.341^{a}	2.73 ± 0.101^{a}	1.66 ± 0.323^{b}	1.65 ± 0.619^{b}	2.68 ± 0.239^{a}	1.86 ± 1.127^{ab}
(+)-4-Carene	n.d.	0.24 ± 0.002^{b}	0.19 ± 0.029^{c}	0.32 ± 0.003^{a}	n.d.	0.24 ± 0.002^{b}	0.20 ± 0.014^{c}	0.32 ± 0.010^{a}
α-phellandrene	n.d.	0.04 ± 0.098^{b}	0.40 ± 0.092^{a}	0.39 ± 0.109^{a}	n.d.	0.05 ± 0.112^{b}	0.48 ± 0.081^{a}	0.43 ± 0.265^{a}
β-phellandrene	0.33 ± 0.212^{ab}	0.21 ± 0.141^{b}	0.53 ± 0.158^{a}	0.63 ± 0.394^{a}	0.26 ± 0.034^{bc}	0.25 ± 0.062^{c}	0.55 ± 0.021^{ab}	0.55 ± 0.191^{a}
α-copaene	0.31 ± 0.030 ^{1}	0.28 ± 0.036	0.27 ± 0.043	0.27 ± 0.015	0.24 ± 0.026 ^{2}	0.24 ± 0.025	0.26 ± 0.012	0.24 ± 0.017
Caryophyllene	3.58 ± 0.480 ^{1}	3.56 ± 0.651	4.12 ± 0.570	4.15 ± 0.311 ^{1}	2.92 ± 0.337^{b2}	2.96 ± 0.352^{b}	4.07 ± 0.134^{a}	3.15 ± 0.528^{b2}
Humulene	0.24 ± 0.018	0.25 ± 0.053	0.26 ± 0.040	0.23 ± 0.022 ^{1}	0.20 ± 0.004	0.20 ± 0.002	n.d	0.21 ± 0.011 ^{2}
Others								
Caryophyllene oxide	0.69 ± 0.031^{a1}	0.40 ± 0.043^{b}	n.d	n.d.	0.41 ± 0.073 ^{2}	0.39 ± 0.104	n.d.	0.41 ± 0.133
Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	0.29 ± 0.026^{b}	0.25 ± 0.030^{b}	0.54 ± 0.041^{a}	0.29 ± 0.017^{b}	0.22 ± 0.022^{b}	0.25 ± 0.010^{b}	0.49 ± 0.052^{a}	0.24 ± 0.007^{b}
1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)-	0.97 ± 0.117^{b}	0.93 ± 0.107^{b}	1.08 ± 0.453^{a}	0.96 ± 0.076^{b}	0.84 ± 0.035^{b}	0.80 ± 0.076^{b}	0.93 ± 0.051^{a}	n.d.

Control (uninoculated batch), Ls (batch inoculated with *L. sakei*). Values are expressed as mean ± standard deviation. The means with different letters (a–c) in the same row indicate significant differences ($p \leq 0.05$) between days in the same batch and the same compound. Mean values with different numbers (1–2) in the same column indicate significant differences ($p \leq 0.05$) between batches on the same day.

572

573 **Figure legends**

574 **Figure 1.** Evolution of the total aerobic microorganism (a), lactic acid bacteria (b) and
575 *Enterobacteriaceae* (c) in both batches studied of "salchichón" throughout the ripening
576 process. Different letters indicate significant differences ($p \leq 0.05$) between days in the
577 same batch and different numbers (1–2) indicate significant differences ($p \leq 0.05$)
578 between batches at the sampling time.

579

580 **Figure 2.** Evolution of water activity (a_w) and moisture content (A), and pH (B) during
581 ripening of dry-fermented "salchichón". Values with statistically significant differences
582 ($p \leq 0.05$) between batches at each sampling day are indicated by an asterisk.

583

584 **Figure 3.** Accumulated area of volatile compounds according to their origin, throughout
585 the ripening of dry-cured fermented "salchichón" in Control and inoculated with *L.sakei*
586 (Ls) batches.

587

588

Figure 1. Martín et al., 2022

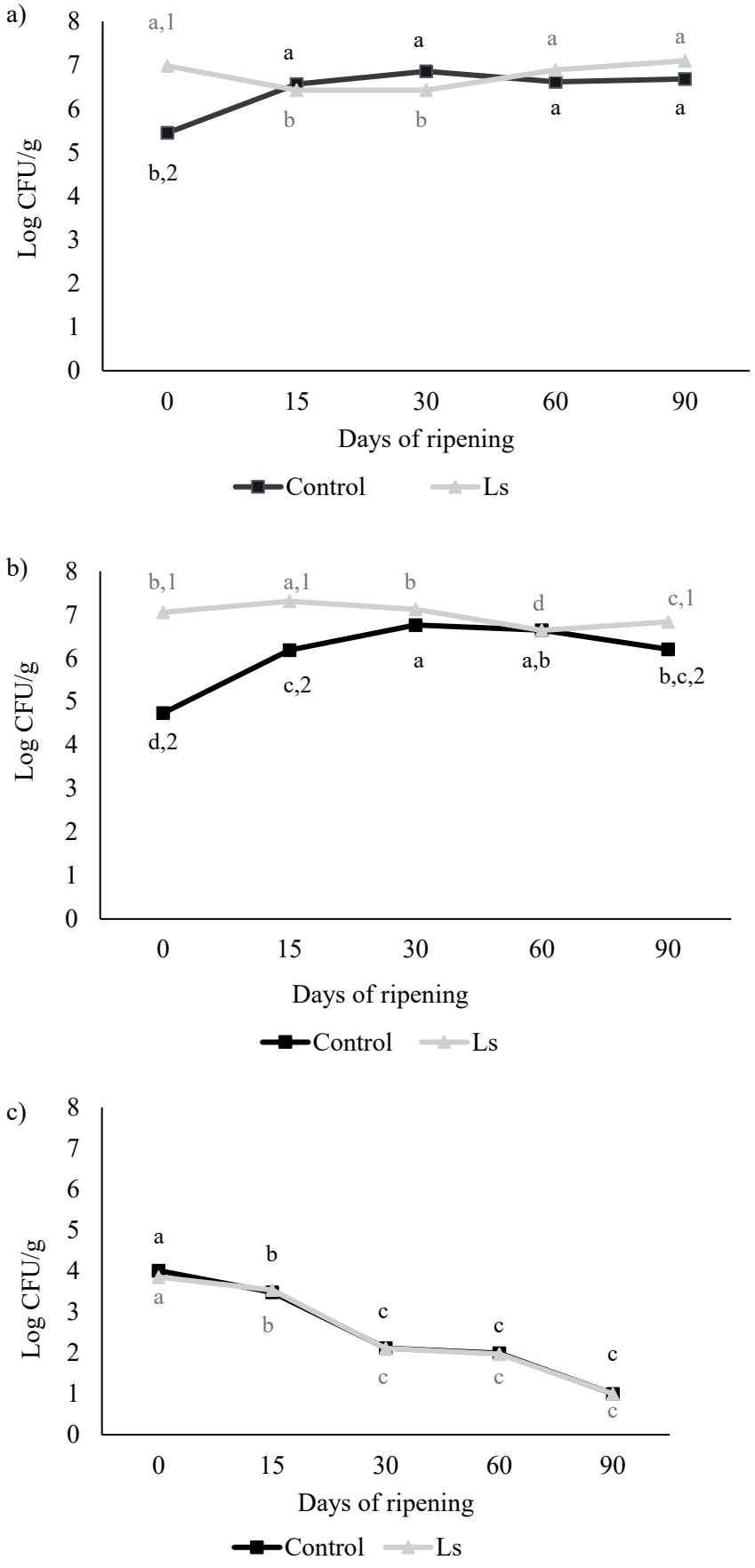
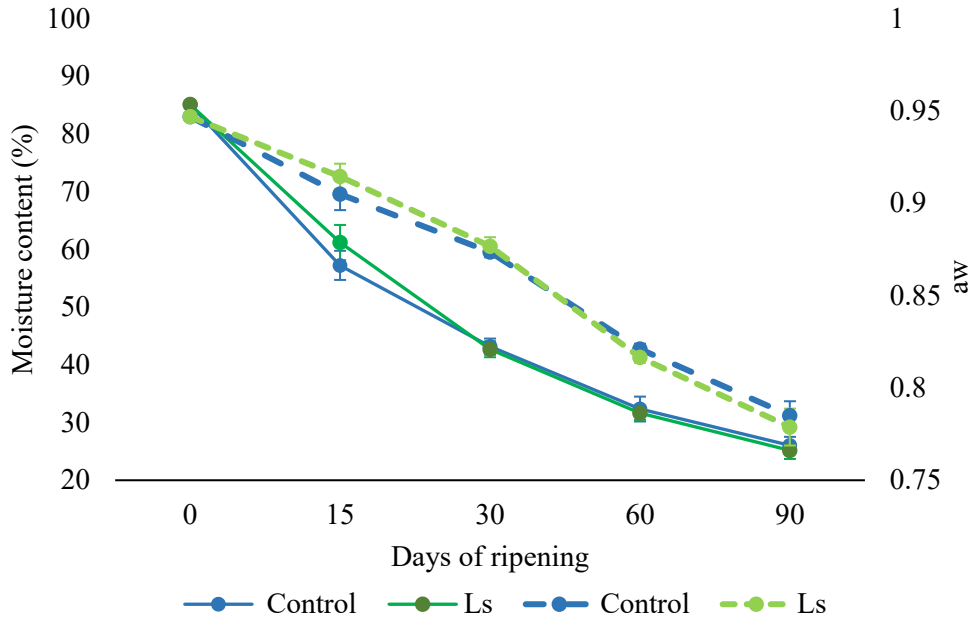


Figure 2. Martín et al., 2022

2.a)



2.b)

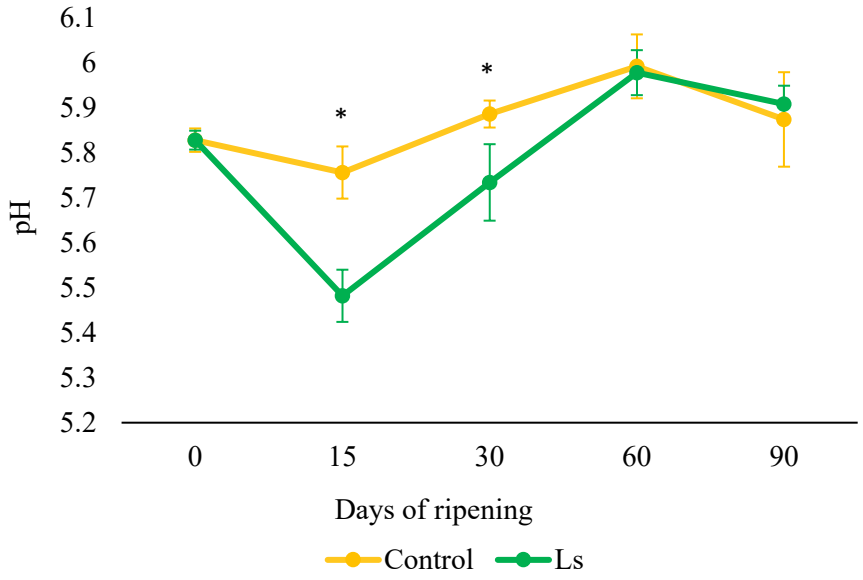
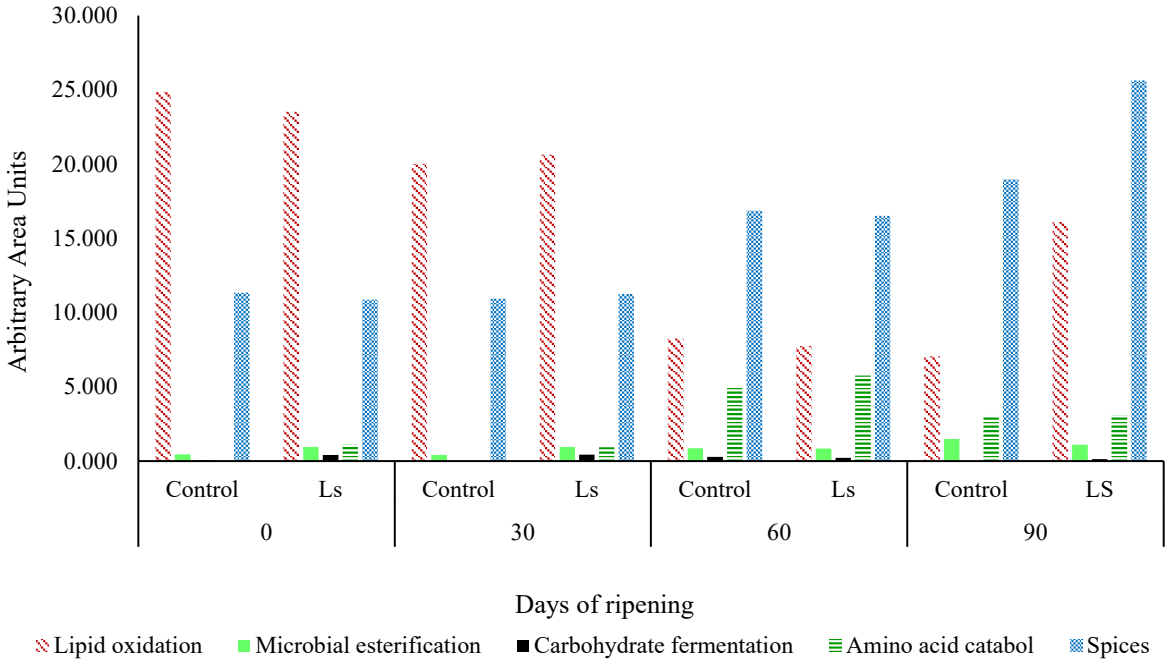


Figure 3. Martín et al., 2022



IV.3. EVALUACIÓN DE BACTERIAS ÁCIDO-LÁCTICAS SELECCIONADS EN QUESOS MADURADOS

International Journal of Food Microbiology (2022). Enviado para su publicación

Food Microbiology (2022). Enviado para su publicación

Registro de Patente

Application of selected lactic-acid bacteria to control *Listeria monocytogenes* in soft-ripened "Torta del Casar" cheese

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Abstract

In this work, the effect of two selected protective cultures of *Lacticaseibacillus casei* 116 and *Lactococcus garviae* 151 on the reduction of *L. monocytogenes* reduction throughout the processing of the traditional "Torta del Casar" soft cheese was evaluated. A total of 185 "Torta del Casar" cheeses were made and divided into nine batches which were inoculated with different levels of *L. monocytogenes* and lactic-acid bacteria (LAB). Moisture content (%) and water activity (a_w) decreased throughout the cheese ripening up to values around 35-38% and 0.93-0.94, respectively. The addition of the selected LAB did not affect physicochemical parameters covering moisture content, a_w, pH and NaCl content. LAB counts were always higher than 8 log CFU/g during ripening, even in the non-inoculated LAB batches. Both inoculated LAB strains did not have influence on the growth of *Enterobacteriaceae* and yeasts; however, provoked a relevant reduction in *L. monocytogenes* counts during the ripening of the traditional and non-standardized "Torta del Casar" cheese (up to 5 log CFU/g). Thus, the utilization of any of both strains, *L. casei* 116 and *L. garviae* 151, as a protective culture could be recommendable to ensure and improve the hygienic-sanitary quality of non-heat treated soft-ripened cheeses such as "Torta del Casar" cheese.

Keywords: Torta del Casar, *L. monocytogenes, L. casei, L. garviae*

1. INTRODUCTION

Listeria monocytogenes is a Gram-positive microorganism that can cause foodborne listeriosis, an infection of great concern, especially for young, old, pregnant, and immune-compromised people. Some properties of *L. monocytogenes*, such as tolerance to acidic conditions (it can survive but not grow at pH values from 3.3 to 4.2) and high salt concentrations (from 0.5 to 12%), and the ability to grow at refrigeration temperatures, allow the survival of this pathogen in ready-to-eat (RTE) foods covering dairy products (Lomonaco et al., 2009).

Cheeses are considered to be one of the dairy products most frequently contaminated with *L. monocytogenes* (Morandi et al., 2020). The use of raw milk, the absence of any standardizing thermal process, coupled with different milking and handling processes, and the hygienic conditions prevailing in the farmhouses, lead to an increased risk of contamination with *L. monocytogenes* (Pereira et al., 2010). An example of such products is "Torta del Casar" that is a traditional high-quality Spanish cheese marketed under the Registry of the Protected Designation of Origin (PDO) "Torta del Casar" (Casar de Cáceres, Spain), in accordance with Regulation (EC) 1491/2003 (European Commission, 2003). This type of soft-ripened cheese is made from raw ewe´s milk using only vegetable coagulant (flowers of the plant *Cynara cardunculus L.*) as rennet and without any added starter culture. The absence of a standardizing thermal process means that the final microbiological characteristics depend on the indigenous microbiota present (Ordiales et al., 2013a; Ordiales et al., 2013b) and if *L. monocytogenes* contaminates milk or pre-processed cheeses during processing, it could not be eliminated.

For this reason, it is imperative the development of strategies to control the presence and survival of *L. monocytogenes* in traditional high-quality cheeses including "Torta del Casar", which may suppose an important and essential economic income to the producer

region. These strategies may fulfill the requirements established by the PDO and their application must ensure the maintenance of the quality standards of the cheese which in turn they must protect consumers' health.

One of the most interesting strategies is the use of selected protective cultures of microorganisms that are commonly found in this product because they have a greater ability to adapt in this niche than others. Within the characteristic microbiota of these cheeses, the dominant microbial population corresponds to lactic acid bacteria (LAB) (Pereira et al., 2010; Perin et al., 2017), and to a lesser extent by cocci Gram-positive catalase-positive bacteria, yeast, moulds, and diverse Gram-negative bacteria (Gonçalves et al., 2018). In addition, undesired microorganisms as coliforms, *Staphylococcus* spp., *Pseudomona*s or even *Listeria* spp. can be present in this type of product and pose a risk for consumers (Pereira et al., 2010). Among LAB, *Lacticaseibacillus casei* is commonly isolated from cheeses. *Lactococci* are LAB that are important contributors to the production of fermented dairy products (Ordiales et al., 2013a), and *L. garviae* is found in raw milk, cheese, and other dairy products (Abdelfatah and Mahboub, 2018). In particular, the contribution of LAB to the cheese final organoleptic characteristics is a consequence of their ability to ferment lactose and their proteolytic activity (Menéndez et al., 2000; Ordiales et al., 2013a). In addition, it has been described that they have the ability to compete against *L. monocytogenes* avoiding its growth (Arena et al., 2016; Campagnollo et al., 2018; García et al., 2020; Scatassa et al., 2017). However, it has been reported the presence of this pathogen in ripened cheese in spite of the existence of indigenous LAB (Pereira et al., 2010). Thus, it is necessary to use selected strains of LAB with anti-*L. monocytogenes* activity as protective cultures. Several LAB strains have been selected by their ability to inhibit *L. monocytogenes* growth in a culture medium made with "Torta del Casar*"* (Martín et al., 2022), but it is necessary to evaluate this activity

throughout the ripening process of this cheese protected under a PDO in presence of natural indigenous microbial population by using the challenge test methodology.

Therefore, this research aimed to evaluate the reduction in *L. monocytogenes* counts during the production of traditional "Torta del Casar" cheese, in the presence of two selected protective cultures of *Lacticaseibacillus casei* and *Lactococcus garviae*.

2. MATERIALS AND METHODS

2.1. Origin of the strains and growth conditions

The strains, *L. casei* 116 (B1; patent number P202131120) and *L. garviae* 151 (B2), from the Food Hygiene and Safety Culture Collection at the University of Extremadura, were used. These strains were isolated from ripened cheeses of Extremadura (Spain) and selected by their antagonist activity against *L. monocytogenes* in cheese-based agar following the methodology described by Martín et al. (2022). The experimental contamination of "Torta del Casar" with *L. monocytogenes* was conducted by inoculating the strain S7-2 (serotype 4b) belonging to National Institute of Agricultural, and Food Research and Technology (INIA) Culture Collection (Madrid, Spain).

To prepare LAB and *L. monocytogenes* inocula, 100 μL of the stock culture (stored in Brain Heart Infusion (BHI) broth (Conda, Spain) containing 20% (w/v) glycerol at -80°C) were sub-cultured twice onto 10 mL of Man Rogosa Sharpe (MRS) broth (Fisher Bioreagents, Belgium) at 30ºC for 48 hours and BHI broth at 37ºC for 24 hours, respectively.

At the end of the incubation period, ≈8.0 log CFU/mL cells were obtained and aliquots of these were diluted in 1% (w/v) peptone water (Conda) to reach final concentrations of about 7.0 log CFU/mL in the case of LAB and, in the case of the pathogen, 7.0 log CFU/mL (high inoculation level) and 4.0 log CFU/mL (low inoculation level). Afterward, cultures were centrifugated at 10,000 g for 5 min, supernatants were discarded before

114 washing sediments which were resuspended in phosphate-buffered saline (PBS) to be
115 used for the inoculation of the "Torta del Casar" cheese curd. To verify the level of
116 inoculation, serial dilutions were inoculated onto MRS agar (Oxoid, UK) at 30 °C for 72
117 hours in the case of LAB, and onto Chromagar™ *Listeria* agar plates (Scharlab, Spain)
118 at 37 °C for 48 hours, in the case of *L. monocytogenes*. In addition, the initial counts
119 (CFU/g) of *L. casei* 116, *L. garviae* 151 and *L. monocytogenes* S7-2 on the curd after
120 inoculation were determined at day 0 of processing.
121
122 **2.2. Preparation, inoculation and ripening of traditional soft cheeses "*Torta del***
123 ***Casar*"**
124 To follow an industrial process, "*Torta del Casar*" cheeses used in this study were
125 elaborated from raw milk of sheep in a cheese dairy located in Cáceres (Extremadura
126 region, Spain). In these facilities, the curds with an approximate weight of 0,5 kg were
127 elaborated by pressing for 2 hours and salting in brine for 3 hours. Since the handling of
128 pathogenic microorganisms is forbidden in the food industry for safety reasons, the
129 inoculation and ripening of the salted curds were done in the pilot plant at the Faculty of
130 Veterinary of the University of Extremadura (Spain). Thus, after salting, curds were
131 transported under refrigerated conditions (< 2 ºC) to the Faculty of Veterinary for the
132 inoculation and posterior ripening. In all cases, the different microorganisms were
133 inoculated in a laminar flow cabinet (Telstar, Spain) at a final volume of 1 mL of PBS
134 with sterile micropipettes. The inoculation was done in the centre of the curd (in a cube
135 of 16 cm^2 of surface and 6 cm deep [the entire volume of curd], and ≈ 100 g of weight).
136 Previously, to estimate the level of inoculation, a cube of the abovementioned measures
137 in several curds was extracted and weighed which were not used for the experiment.

In this work, 185 pressed and salted curds "Torta del Casar" were used and divided into the following 9 batches: (1) batch C (uninoculated control, only with 1 mL of sterile PBS); (2) batch 7B1 (inoculated only with *L. casei* at ≈ 7 log CFU/g); (3) batch 7B2 (inoculated only with *L. garviae* at ≈ 7 log CFU/g); (4) batch 4Lm +7B1 (inoculated with *L. monocytogenes* at ≈ 4 log CFU/g combined with *L. casei* at ≈7 log CFU/g); (5) batch 4Lm +7B2 (inoculated with *L. monocytogenes* at ≈ 4 log CFU/g combined with *L. garviae* at ≈ 7 log CFU/g); (6) batch 7Lm + 7B1 (inoculated with *L. monocytogenes* at ≈ 7 log CFU/g combined with *L. casei* at ≈ 7 log CFU/g); (7) batch 7Lm+ 7B2 (inoculated with *L. monocytogenes* at ≈7 log CFU/g combined with *L. garviae* at ≈ 7 log CFU/g); (8) batch 4Lm (inoculated with *L. monocytogenes* at ≈ 4 log CFU/g); (9) batch 7Lm (inoculated with *L. monocytogenes* at ≈ 7 log CFU/g).

After inoculation, the cheeses' curds were ripened in a chamber of the pilot plant at the Faculty of Veterinary following the industrial conditions used for this product: 35 days at 6°C and 90% relative humidity (RH), 10 days at 8ºC and 80 % RH, 10 days at 9ºC and 80 % RH and 35 days at 10°C and 80% RH.

For microbiological and physicochemical analysis, five cheeses of each batch were taken at 0, 45, 60 and 90 days of ripening. An additional sampling time was done at 30 days only for physicochemical analysis. In all cheese samples, the entire inoculated cube of cheese of 16 cm^2 of surface, 6 cm deep and 100 g weight, was always processed and aseptically mixed for analysis in a laminar flow cabinet (Telstar). Thus, the experiment consisted of 9 batches x 4 sampling times x 5 different cheeses/each batch and sampling time which were evaluated once, according to the European Union Reference Laboratory Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (such as soft-ripened cheeses) where no growth or the growth probability of this pathogen is ≤ 10% (Beaufort et al., 2014).

163

164 **2.3. Confirmation of the absence of natural contamination of *L. monocytogenes***

165 The absence of contamination of *L. monocytogenes* in the uninoculated control (batch C)
166 was tested by taking 25 g of each of the 5 cheeses from this batch at every sampling time
167 and evaluated according to ISO 11290-1 standard (International Organization for
168 Standardization, ISO, 2017).

169

170 **2.4. Estimation of microbiological levels**

171 For the microbiological analysis, samples of 10 g each of the 5 "Torta del Casar" cheeses
172 were analyzed at every sampling time, mixed with 90 mL of 1 % (w/v) peptone water and
173 homogenized at 300 rpm for 120 s using a laboratory Stomacher machine (Seward, model
174 400 Circulator, UK). Decimal serial dilutions were subsequently prepared in 1% (w/v) of
175 peptone water and then 100 μL of the cell suspensions were spread onto the surface of
176 different agar plates according to the microbial group analyzed. The total viable
177 microorganism counts were determined on Plate Count Agar (PCA, Conda) and incubated
178 at 30 ºC for 48 hours. *Enterobacteriaceae* were grown on Violet Red Bile Glucose Agar
179 (VRBG, Conda) and incubated at 37ºC for 48 hours. Determination of *Staphylococci* was
180 conducted on Mannitol Salt Agar (MSA; Conda) and incubated for 48 hours at 30 ºC. The
181 yeast colonies were counted on Malt Extract Agar (MEA; 20 g/L of malt extract
182 (Scharlab), 1 g/L of peptone water, 20 g/L of D (+) glucose monohydrate (Scharlab),
183 bacto agar 20 g/L (Scharlab)) and incubated at 25 °C for 5 days while LAB were grown
184 on MRS agar and incubated at 30 °C for 48 hours. Finally, *L. monocytogenes* counts were
185 determined in Chromagar™ *Listeria* Chromogenic agar plates and incubated at 37 ºC for
186 24 and 48 hours. After the incubation period, the characteristic *L. monocytogenes*
187 colonies, those green colonies with a surrounded opaque halo were counted. For

188 enumeration of the different microorganisms in culture media, only those agar plates with
189 colonies ranging between 300 and 30 colonies were considered and counts were
190 expressed as log CFU/g.
191
192 **2.5. Physicochemical analysis**
193 All the analyses for the determination of the physicochemical characteristics of the
194 cheeses were made in quintuplicate in those batches in which *L. monocytogenes* was not
195 inoculated (batches C, 7B1 and 7B2). The water activity (a_w) of "Torta del Casar" cheeses
196 were determined at 25°C by using a Novasina Lab Master Water activity meter model
197 A_W SPRINT-TH 300 (Novasina AG, Switzerland). The pH was measured using a pH-
198 meter (Model 340, Mettler-Toledo GmbH, Switzerland) after homogenizing 3 g of each
199 sample with 27 mL of distilled water for 30 s using a homogenizer. Moisture content (%)
200 was analyzed by dehydration at 100 ºC to a constant weight following the official methods
201 of the Association of Official Analytical Chemists (AOAC, 2000). Sodium chloride
202 content was determined only at the end of the ripening period using the Volhard method
203 (AOAC, 2016).
204
205 **2.6. Statistical analyses**
206 For the statistical treatment, the software IBM SPSS Statistic version 20 (IBM, USA) was
207 used. The different batches and days of ripening were analyzed as independent variables.
208 The counts (log CFU/g), a_w, pH, moisture content and sodium chloride values were
209 analyzed as dependent variables. Once the dependent and independent variables of the
210 analysis were established, a study of the normality of the different data populations was
211 carried out using the Shapiro Wilk test. Subsequently, the analysis of the data was

conducted using the Mann–Whitney test (Mann and Whitney, 1947). Statistical significance was established at $p \leq 0.05$.

3.RESULTS

3.1. Physicochemical changes of cheeses during ripening

The evolution of moisture content (%), a_w and pH throughout the ripening process of "Torta del Casar" cheeses was measured (Table 1). Initial moisture content in all batches decreased progressively ($p \leq 0.05$) during the ripening process, from 88-87% to 45-43% at 60 days, and to 35-38% at the end of the process (90 days). At 30 and 45 days of ripening, the moisture content of the batch in which *L. garviae* was inoculated (batch 7B2) was significantly lower than that found in the control batch (batch C). However, there were no significant differences ($p > 0.05$) between batches at the end of the ripening time. In the case of a_w, these values decreased from 0.975-0.971 at day 0 to 0.943-0.938 at day 90 (Table 1). No significant differences ($p > 0.05$) between control and LAB inoculated batches were observed for this parameter.

Sodium chloride content was only determined in ripened cheeses (at day 60) showing all batches similar values, higher than 2.6 % in NaCl, without differences between batches ($p > 0.05$) (data not shown).

Regarding pH, there were not significant differences in the mean values (5.20-5.45) of the three analyzed batches at days 0, 30 and 45 ($p > 0.05$; Table 1). However, at day 60, an increase of the pH value was observed in the batches in which *L. casei* (batch 7B1) and *L. garviae* (batch 7B2) were added, reaching values around 5.75. At the end of the ripening period, only the batch in which *L. casei* was inoculated (batch 7B1) showed a pH significantly ($p \leq 0.05$) higher than that found in the control batch (batch C).

3.2. Evaluation of the microbial population during cheese ripening

238 The results obtained from the enumeration of microorganisms of the 9 batches during the
239 cheese ripening process are shown in Table 2. Total aerobic microbial counts were higher
240 in the inoculated batches than in the control batch being in all of them counts higher than
241 8 log CFU/g. Furthermore, there was an increase in the levels of the total aerobic
242 microbial counts at the end of cheese maturation in the uninoculated batch (batch C).
243 Initial *Enterobacteriaceae* counts were around 6.8 CFU/g in all analyzed batches at the
244 beginning of ripening (Table 2). However, the number of *Enterobacteriaceae*
245 significantly decreased ($p \leq 0.05$) throughout the ripening in all the analyzed batches
246 (from 6.71 to 5.49 log CFU/g).
247 With regard to *Staphylococci*, initial levels of about 5 log CFU/g in batches C, 7B1 and
248 7B2 (Table 2) were encountered. In these batches, the counts significantly decreased
249 throughout cheese ripening from day 0 to day 60; however, at the end of the maturation
250 period (90 days), there was a significant rise ($p \leq 0.05$) in most of the batches studied,
251 reaching *Staphylococci* counts around 5.6 log CFU/g, except in batch 7B2 whose values
252 were lower at the end of ripening.
253 Regarding counts on MEA agar (yeasts), they ranged between 6.4 and 8.8 log CFU/g
254 during the ripening process in all the analyzed batches (Table 2), being significantly ($p \leq$
255 0.05) higher at the end of ripening (day 90) in all the batches studied. No significant effect
256 was observed on the levels of yeasts in the different batches at any of the sampling days
257 studied ($p > 0.05$).
258

259 **3.3. Lactic-acid bacteria counts during cheese ripening**

260 The LAB levels are shown in Table 3. The mean counts of LAB were greater than 8 log
261 CFU/g in all batches; however, the microbiological analysis did not reveal significant
262 differences ($p > 0.05$) at the beginning of the ripening between batches inoculated with

L. casei and *L. garviae* (batches 7B1, 7B2, 4Lm+ 7B1, 4Lm+ 7B2, 7Lm+ 7B1 and 7Lm+ 7B2) and those which were not inoculated with LAB (batches C, 4Lm, 7Lm). No differences in LAB counts were also found during ripening between inoculated and uninoculated LAB batches.

3.4. Effect of *L. casei* and *L garviae* on *L. monocytogenes* growth during cheese ripening

Microbiological analysis of the uninoculated batches revealed that *L. monocytogenes* was not presented in any of the batches at any of the ripening time, excluding thus the natural presence of this microorganism in the samples used. The inoculation of *L. monocytogenes* resulted in an initial level of 7.5 log CFU/g in batches 7Lm, 7Lm+ 7B1 and 7Lm+ 7B2 and 4.6-4.7 log CFU/g in batches 4Lm, 4Lm+ 7B1 and 4Lm+ 7B2. Levels of this pathogenic bacterium showed significant ($p \leq 0.05$) decreases in comparison with the initial ones, at day 45 (all batches except 4Lm) and at days 60 and 90 (all batches inoculated with *L. monocytogenes*; Figure 1). After 90 days of ripening, the reduction in *L. monocytogenes* counts in batch 4Lm was 1.14 log CFU/g while in batch 4Lm+ 7B1 and 4Lm+ 7B2 was significantly higher (3.47 and 3.57 log CFU/g, respectively) (Figure 1.a). In batch 7Lm the reduction in *L. monocytogenes* counts was about 3 log CFU/g, while in batch 7Lm+ 7B1 and 7Lm+ 7B2 was significantly higher (5.01 and 4.07 log CFU/g) (Figure 1.b).

4.DISCUSSION

L. monocytogenes contaminating soft-ripened cheeses represents a real risk for food safety (Lahou and Uyttendaele, 2017). Therefore, it is necessary to use strategies to control this pathogenic bacterium in cheeses. In this regard, the use of LAB strains with

anti-*L. monocytogenes* activity has been proposed as a strategy to inhibit this pathogen in soft-ripened cheeses (Campagnollo et al., 2018; Scatassa et al., 2017). In this study, the control of *L. monocytogenes* throughout the ripening of "*Torta del Casar*" with two selected strains LAB isolates, *L. casei* 116 and *L. garviae* 151, from soft-ripened cheeses and previously selected by its anti-*L. monocytogenes* activity, was evaluated.

Physicochemical evaluation of batches inoculated with both LAB strains (batches 7B1 and 7B2) and the uninoculated control batch (batch C) showed that the ripening process was correctly carried out. Thus, the moisture content and a_w values showed similar results to those reported by other authors for this type of cheese (Ordiales et al., 2014; Ordiales et al., 2013a). No differences for the a_w parameter were observed between the batches inoculated with LAB (batches 7B1 and 7B2) and the uninoculated batch (batch C) during all the ripening process. However, the strain *L. garviae* (batch 7B2) provoked a slight reduction in the moisture content at 30 and 45 days of ripening in comparison with the uninoculated control (batch C). At the end of maturation, there were no significant differences ($p > 0.05$) between all the batches studied. This evidences that the use of starter cultures was not associated ($p > 0.05$) with any differences observed at the end of the ripening process in any of the two abovementioned parameters. The addition of *L. casei* and *L. garviae* did not provoke a significant reduction of pH, but on the contrary, it seems to provoke a slight increase in the pH values at 60 days of maturation; however, at 90 days of ripening only the batch in which *L. casei* was inoculated (batch 7B1), the pH was significantly higher than in the control batch. The amount of sodium chloride was not modified by the addition of LAB, and, in addition, the percentages are within the values established by the DOP Torta del Casar (< 3%) (Estrada et al., 2019). Thus, these results seem to indicate that the addition of the selected LAB used in the present study did not cause changes in physicochemical parameters including moisture content, a_w, pH

and salt concentration. The a_w, pH and salt concentration are parameters that have a great influence on cheese stability and they condition the development of the microorganisms, the enzymatic activity and the rate of the biochemical reactions during the ripening time (Sanjuán et al., 2002).

Regarding counts of microorganisms, during processing there was not an increase in total aerobic microbial counts in any of the analyzed batches, except in batch C. The *Enterobacteriaceae* counts decreased significantly during the ripening process in all batches studied, although the final values are still very high (6.5-5.5 log CFU/g). The presence of high levels of *Enterobacteriaceae* is usual in soft cheeses made from raw milk as has been previously reported (Coton et al., 2012; Crespo et al., 2020; Gonçalves et al., 2018). These levels during all the ripening process suggest a relevant contribution of their associated activity in the development of the sensory characteristics of "Torta del Casar" cheese, including its texture and organoleptic properties (Crespo et al., 2020). The reduction in levels of *Staphylococci* only when *L. garviae* was inoculated (batch 7B2) at the end of ripening may be related to the effect of the synergistic action of this strain together with the reduction in moisture content (Otunba et al., 2021). Yeasts are in high levels in this kind of cheese made with raw milk and they increased during the ripening process of "Torta del Casar" because of the slight reduction of humidity content and aw throughout the processing. Thus, similar levels of yeast have been reported in soft cheese made with raw milk (Banjara et al., 2015).

LAB counts were greater than 8 log CFU/g throughout ripening, with no variability among batches despite in some of the batches one of the two selected LAB strains (*L. casei* 116 or *L. garviae* 151), was intentionally added. This is because the indigenous LAB population counts are around 8 log CFU/g while the two selected LAB strains were added at a concentration of about 7 log CFU/g. The LAB counts found in this study were

similar to those found in "Torta del Casar" cheese (Ordiales et al., 2014). From the results, it can be observed that LAB is the microbial group that shows the highest microbial levels throughout the ripening in all the analyzed batches.

With respect to *L. monocytogenes* counts, at the end of maturation (90 days), a reduction in the pathogen counts of about 1.1 and 3 log CFU/g was found in the batches 4Lm and 7Lm, respectively. Although this reduction in *L. monocytogenes* counts was quite important, the decrease in the levels of this pathogen in batches in which the two LAB strains were inoculated was more impressive. Thus, counts around 1.2 and 1.1 log CFU/g for the batches 4Lm+ 7B1 and 4Lm+ 7B2, respectively (Figure 1a), indicating percentages reduction over the *L. monocytogenes* population with respect to the control batch (batch 4Lm) of 65.08 and 67.74%, respectively. Levels of *L. monocytogenes* in 7Lm+ 7B1 and 7Lm+ 7B2 were 2.61 and 3.55 log CFU/g, respectively (Figure 1b). The reduction in *L. monocytogenes* counts achieved in batches with high inoculation level (7.5 log CFU/g) was similar to that obtained in batches with low inoculation level (4.5 log CFU/g). The highest antagonistic effect against *L. monocytogenes* was observed in batch 7Lm+ 7B1 in which *L. casei* was added, reducing the counts of the pathogen up to 5 log CFU/g from the beginning of maturation. In the present study, it is clear that LAB did not cause a reduction in pH during cheese ripening, so the observed inhibition cannot be associated with more acidic conditions in the food substrate. A likely explanation for the observed inhibition of the pathogen could be competition by nutrient and/or space between pathogen and the starter culture or production of compounds like bacteriocins by the selected LAB, considering that it has been described that dairy-related strains of *L. casei* and *L. garviae* produce active bacteriocins against *L. monocytogenes* (Deegan et al., 2006; Parada et al., 2007; Tosukhowong et al., 2012). It has been demonstrated that LAB of dairy origin can exert an anti-*L. monocytogenes* activity during production and

storage of different cheeses (Campagnollo et al., 2018; Ortolani et al., 2010; Panebianco et al., 2021).

It should be noted that the levels of the pathogen inoculum used in this research (4.5 log CFU/g and 7.5 log CFU/g) were similar to the ones found contaminated dairy products and cheeses (1-5 log CFU/g) according to the studies conducted by (Rudolf and Scherer, 2001) in different soft and semi-soft cheeses. This was done because we wanted to in-depth investigate the capacity of the selected LAB strains to control or even inhibit the growth of *L. monocytogenes* during ripening of a soft-ripened cheese. In this study it has been also demonstrated that from day 45 of ripening, and more strongly, from day 60, both LAB strains reduce the levels of this pathogenic bacterium, maintaining this effect until the end of ripening. Thus, it could be sufficient to guarantee the elimination of this pathogenic bacterium throughout the processing of "Torta del Casar" cheese when this pathogen contaminates this product at the usual levels (< 2-3 log CFU/g). This is very important since minimizing the risk of listeriosis caused by the consumption of soft and semi-soft cheeses, food safety improves and the microbiological criteria of RTE foods throughout their shelf life in the EU are met (European Commission, 2007). Consequently, we may hypothesize that by applying such a concentration of selected *L. casei* and *L. garviae* and simulating the low levels of contamination that commonly occur with this pathogenic microorganism in dairy products, the control and more likely the inhibition of *L. monocytogenes* could be achieved.

5.CONCLUSION

The inoculation of *L. casei* 116 and *L. garviae* 151 seems to not affect in general to physicochemical parameters of the traditional "Torta del Casar" cheese covering moisture content, a_w, pH and sodium chloride content. Both LAB strains do not have an influence

on the growth of other indigenous microorganisms including other autochthonous LAB, *Enterobacteriaceae* and yeasts; however, they provoked a relevant reduction in *L. monocytogenes* levels during the ripening of the traditional and non-standardized "Torta del Casar" cheese (up to 5 log CFU/g) despite that the cheese ripening itself can control the development of *L. monocytogenes* (1.14-3 log CFU/g). Thus, the use of any of both strains, *L. casei* 116 and *L. garviae* 151, as a protective culture could be recommendable to ensure and improve the hygienic-sanitary quality of non-heat treated soft-ripened cheeses such as "Torta del Casar".

Acknowledgements

This work was supported by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. I. Martín is supporting by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

References

Abdelfatah, E. N., Mahboub, H. H. H., 2018. Studies on the effect of *Lactococcus garvieae* of dairy origin on both cheese and Nile tilapia (O. niloticus). Int. J. Vet. 6(2), 201–207. https://doi.org/10.1016/j.ijvsm.2018.11.002

AOAC., 2000. Moisture in malt gravimetric method (935.29). In *Official methods of analysis of AOAC international* (17th ed.).

AOAC., 2016. Official Methods of Analysis of AOAC International - 20th Edition. In *Association of Official Analytical Chemists.* (Vol. 52, Issue 2). https://doi.org/10.1021/ac50052a726

412 Arena, M. P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., Fiocco, D.,
413 2016. Use of *Lactobacillus plantarum* strains as a bio-control strategy against
414 food-borne pathogenic microorganisms. Front. Microbiol. *7*(APR), 1–10.
415 https://doi.org/10.3389/fmicb.2016.00464

416 Banjara, N., Suhr, M.J., Hallen-Adams, E., 2015. Diversity of yeast and mold species
417 from a variety of cheese types. Curr. Microbiol. https://doi.org/10.1007/s00284-
418 015-0790-1

419 Beaufort, A., Cornu, M., Bergis, H., Lardeux, A. L., Lombard, B., 2014. *EURL* Lm
420 Technical guiadance document for conducing shelf-life studies on *Listeria*
421 *monocytogenes* in ready to eat foods. Community Reference Laboratory for
422 *Listeria monocytogenes*. http://www.fsai.ie/uploadedFiles/EURL
423 %0ALm_Technical Guidance Document%0A Lm shelf-life studies_V3_2014-
424 %0A06-06 (2).pdf

425 Campagnollo, F., Margalho, L., Kamimura, B., Feliciano, M., Freire, L., Lopes, L.,
426 Alvarenga, V., Cadavez, V., Gonzales-Barron, U., Schaffner, D., Sant'Ana, A.,
427 2018. Selection of indigenous lactic acid bacteria presenting anti-listerial activity,
428 and their role in reducing the maturation period and assuring the safety of
429 traditional Brazilian cheeses. Food Microbiol. 73, 288–297.
430 https://doi.org/10.1016/j.fm.2018.02.006

431 Commission, E. Commission Regulation (EC) No 1441/2007 of 5 December 2007
432 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff.
433 Off. J. Eur. Communitites 2007

434 Coton, M., Delbés-Paus, C., Irlinger, F., Desmasures, N., Le Fleche, A., Stahl, V.,
435 Montel, M. C., Coton, E., 2012. Diversity and assessment of potential risk factors

of Gram-negative isolates associated with French cheeses. Food Microbiol. *29*(1), 88–98. https://doi.org/10.1016/j.fm.2011.08.020

Crespo, A., Martín, A., Ruiz-Moyano, S., Benito, M. J., Rufo, M., Paniagua, J. M., Jiménez, A., 2020. Application of ultrasound for quality control of Torta del Casar cheese ripening. Int. J. Dairy Sci. 103(10), 8808–8821. https://doi.org/10.3168/jds.2020-18160

Deegan, L. H., Cotter, P. D., Hill, C., Ross, P., 2006. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. Int. Dairy J. 16(9), 1058–1071. https://doi.org/10.1016/j.idairyj.2005.10.026

Estrada, O., Ariño, A., Juan, T., 2019. Salt distribution in raw sheep milk cheese during ripening and the effect on proteolysis and lipolysis. Foods, 8(3), 1–14. https://doi.org/10.3390/foods8030100

García, M. J., Ruíz, F., Asurmendi, P., Pascual, L., Barberis, L., 2020. Searching potential candidates for development of protective cultures: Evaluation of two *Lactobacillus* strains to reduce *Listeria monocytogenes* in artificially contaminated milk. J. Food Saf. 40(1). https://doi.org/10.1111/jfs.12723

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. de G., Egas, C., Merchán, A. V., Galván, A. I., Ruiz-Moyano, S., 2018. Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. J. Food Sci. 83(5), 1333–1341. https://doi.org/10.1111/1750-3841.14141

ISO., 2017. *ISO 11290-1: Método horizontal para la detección de* L. monocytogenes *y* Listeria *spp*.

Lahou, E., Uyttendaele, M., 2017. Growth potential of *Listeria monocytogenes* in soft, semi-soft and semi-hard artisanal cheeses after post-processing contamination in deli retail establishments. Food Control, *76*, 13–23. https://doi.org/10.1016/j.foodcont.2016.12.033

Lomonaco, S., Decastelli, L., Nucera, D., Gallina, S., Manila Bianchi, D., Civera, T., 2009. *Listeria monocytogenes* in Gorgonzola: Subtypes, diversity and persistence over time. Int. J. Food Microbiol. 128(3), 516-520 https://doi.org/10.1016/j.ijfoodmicro.2008.10.009

Mann, H. B., Whitney, D., 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Statist. 2(5), 347–370. http://projecteuclid.org/euclid.aop/1176996548

Martín, I., Rodríguez, A., Alía, A., Martínez-Blanco, M., Lozano-Ojalvo, D., Córdoba, J.J., 2022. Control de *Listeria monocytogenes* growth and virulence in a traditional soft cheese model system based on lactic acid bacteria and a whey protein hydrolysate with antimicrobial activity. Int. J. Food Microbiol. 361, 109444. https://doi.org/10.1016/j.ijfoodmicro.2021.109444

Menéndez, S., Centeno, J. A., Godínez, R., Rodríguez-Otero, J. L., 2000. Effects of *Lactobacillus* strains on the ripening and organoleptic characteristics of Arzua-Ulloa cheese. Int. J. Food Microbiol. 59(1–2), 37–46. https://doi.org/10.1016/S0168-1605(00)00286-5

Morandi, S., Silvetti, T., Vezzini, V., Morozzo, E., Brasca, M., 2020. How we can improve the antimicrobial performances of lactic acid bacteria? A new strategy to control *Listeria monocytogenes* in Gorgonzola cheese. Food Microbiol. 90, 103488. https://doi.org/10.1016/j.fm.2020.103488

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., Córdoba, M de G., 2014. Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of "Torta del Casar" cheese. Int. J. Dairy Technol. 67(3), 402–409. https://doi.org/10.1111/1471-0307.12129

Ordiales, Elena, Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., Córdoba, M de G., 2013a. Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. Food Control 33(2), 448–454. https://doi.org/10.1016/j.foodcont.2013.03.027

Ordiales, Elena, Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., Córdoba, M. de G., 2013b. Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. J. Dairy Sci. 96(9), 5477–5486. https://doi.org/10.3168/jds.2013-6587

Ortolani, M. B. T., Yamazi, A. K., Moraes, P. M., Viçosa, G. N., Nero, L. A., 2010. Microbiological quality and safety of raw milk and soft cheese and detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*. Foodborne Pathog. Dis. 7(2), 175–180. https://doi.org/10.1089/fpd.2009.0390

Otunba, A. A., Osuntoki, A. A., Olukoya, D. K., Babalola, B. A., 2021. Genomic, biochemical and microbial evaluation of probiotic potentials of bacterial isolates from fermented sorghum products. Heliyon, 7(12), e08536. https://doi.org/10.1016/j.heliyon.2021.e08536

Panebianco, F., Giarratana, F., Caridi, A., Sidari, R., De Bruno, A., Giuffrida, A., 2021. Lactic acid bacteria isolated from traditional Italian dairy products: activity against

Listeria monocytogenes and modelling of microbial competition in soft cheese. Lwt 137, 110446. https://doi.org/10.1016/j.lwt.2020.110446

Parada, J. L., Caron, C. R., Medeiros, A. B. P., Soccol, C. R., 2007. Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. Braz. Arch. Biol. Technol. 50(3), 521–542. https://doi.org/10.1590/s1516-89132007000300018

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M. P., Xavier Malcata, F., 2010. Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. Food Microbiol. 27(3), 339–346. https://doi.org/10.1016/j.fm.2009.10.012

Perin, L. M., Savo Sardaro, M. L., Nero, L. A., Neviani, E., Gatti, M., 2017. Bacterial ecology of artisanal Minas cheeses assessed by culture-dependent and -independent methods. Food Microbiol. 65, 160–169. https://doi.org/10.1016/j.fm.2017.02.005

Regulation (CE) 1491/2003 of European Commissión of 25 of August 2003: Registration of the protected designation of origin "Torta del Casar" in the register of protected designations of origin and the protected geographic indications.

Rudolf, M., Scherer, S., 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. Int. J. Food Microbiol. 63(1–2), 91–98. https://doi.org/10.1016/S0168-1605(00)00413-X

Sanjuán, E., Millán, R., Saavedra, P., Carmona, M. A., Gómez, R., Fernández-Salguero, J., 2002. Influence of animal and vegetable rennet on the physicochemical characteristics of Los Pedroches cheese during ripening. Food Chem. 78(3), 281–

289. https://doi.org/10.1016/S0308-8146(02)00098-5

Scatassa, M. L., Gaglio, R., Cardamone, C., Macaluso, G., Arcuri, L., Todaro, M., Mancuso, I., 2017. Anti-*Listeria* activity of lactic acid bacteria in two traditional Sicilian cheeses. Ital. J. of Food Saf. 6(1), 13–17. https://doi.org/10.4081/ijfs.2017.6191

Tosukhowong, A., Zendo, T., Visessanguan, W., Roytrakul, S., Pumpuang, L., Jaresitthikunchai, J., Sonomoto, K., 2012. Garvieacin Q, a novel class II bacteriocin from *Lactococcus garvieae* BCC 43578. Appl. Environ. Microbiol. 78(5), 1619–1623. https://doi.org/10.1128/AEM.06891-11

Table 1. pH, moisture content (%) and water activity (a_w) in uninoculated batch (batch C), batch inoculated only with *L. casei* (batch 7B1), and batch inoculated only with *L. garviae* (batch 7B2) of "Torta del Casar" cheeses throughout ripening process.

	Batches	Days of ripening				
		0	30	45	60	90
pH	C	5.33 ± 0.009 [3]	5.20 ± 0.118 [2,3]	5.44 ± 0.091 [2]	5.65 ± 0.063[b,1]	5.73 ± 0.057[b,1]
	7B1	5.36 ± 0.008[3,4]	5.24 ± 0.054 [4]	5.45 ± 0.122 [3]	5.77 ± 0.043[a,2]	6.04 ± 0.098[a,1]
	7B2	5.34 ± 0.008 [3]	5.25 ± 0.086 [3,4]	5.33 ± 0.097 [4]	5.73 ± 0.023[a,b,2]	5.84 ± 0.039[b,1]
Moisture content (%)	C	88.68 ± 2.125 [1]	49.70 ± 1.263[a,2]	47.29 ± 0.468[a,2,3]	44.79 ± 1.611 [3]	37.64 ± 2.981 [4]
	7B1	88.21 ± 3.128 [1]	48.05 ± 0.903[a,2]	46.32 ± 1.493[a,b,2,3]	45.54 ± 2.341 [3]	38.17 ± 1.766 [4]
	7B2	87.77 ± 5.106 [1]	45.99 ± 1.733[b,2]	44.72 ± 1.411[b,2]	43.81 ± 0.534 [2]	35.52 ± 1.264 [3]
a_w	C	0.975 ± 0.003 [1]	0.968 ± 0.007 [1,2]	0.962 ± 0.003 [2]	0.966 ± 0.003 [2]	0.943 ± 0.005 [3]
	7B1	0.974 ± 0.001 [1]	0.968 ± 0.003 [1,2]	0.964 ± 0.002 [3]	0.967 ± 0.001 [2]	0.943 ± 0.004 [4]
	7B2	0.975 ± 0.002 [1]	0.970 ± 0.002 [1,2]	0.960 ± 0.003 [3]	0.965 ± 0.003 [2,3]	0.938 ± 0.006 [4]

C (uninoculated control), 7B1 (inoculated with *L. casei*), 7B2 (inoculated with *L. garviae*). Values are expressed as mean ± standard deviation. Mean values with different letters (a–b) in the same column indicate significant differences ($p \leq 0.05$) between batches on the same sampling day. Mean values with different numbers (1–4) in the same row indicate significant differences ($p \leq 0.05$) between days in the same batch and the same physicochemical parameter.

547 **Table 2.** Evolution of the different microbial groups in the different batches of "Torta del Casar" cheeses throughout their ripening process.
548

Batches	Microorganism	Days of ripening			
		0	45	60	90
C	TAM	$8.08 \pm 0.034^{d,2}$	$8.29 \pm 0.237^{c,1,2}$	$8.36 \pm 0.199^{c,1}$	$8.37 \pm 0.065^{b,c,1}$
	E	6.81 ± 0.294^{1}	$6.17 \pm 0.293^{b,c,d,1,2}$	$6.13 \pm 0.245^{a,b,c,1,2}$	$5.54 \pm 0.629^{c,2}$
	S	$5.46 \pm 0.186^{a,1}$	$4.32 \pm 0.416^{b,2}$	$4.27 \pm 0.144^{d,2}$	$5.87 \pm 0.190^{a,1}$
	Y	6.45 ± 0.028^{3}	6.28 ± 0.133^{4}	$7.75 \pm 0.047^{a,b,2}$	$8.25 \pm 0.084^{c,1}$
7B1	TAM	$8.28 \pm 0.046^{c,2}$	$8.62 \pm 0.215^{a,b,c,2}$	$9.06 \pm 0.283^{a,1}$	$8.33 \pm 0.141^{b,c,2}$
	E	6.74 ± 0.307^{1}	$6.02 \pm 0.182^{d,2}$	$5.81 \pm 0.261^{c,2}$	$5.71 \pm 0.323^{a,b,c,2}$
	S	$4.92 \pm 0.292^{b,2}$	$3.95 \pm 0.280^{b,3}$	$4.53 \pm 0.400^{d,2}$	$5.87 \pm 0.078^{a,1}$
	Y	6.46 ± 0.011^{3}	6.20 ± 0.125^{4}	$7.74 \pm 0.086^{a,b,2}$	$8.31 \pm 0.094^{c,1}$
7B2	TAM	$8.41 \pm 0.050^{a,b,1,2}$	$8.50 \pm 0.192^{b,c,1}$	$8.53 \pm 0.355^{b,c,1}$	$7.99 \pm 0.316^{d,2}$
	E	6.96 ± 0.144^{1}	$6.13 \pm 0.370^{c,d,2}$	$5.97 \pm 0.097^{b,c,2,3}$	$5.54 \pm 0.255^{c,3}$
	S	$5.62 \pm 0.185^{a,1,2}$	$5.82 \pm 0.144^{a,1}$	$5.21 \pm 0.089^{c,3}$	$5.30 \pm 0.280^{c,2,3}$
	Y	6.44 ± 0.018^{2}	6.25 ± 0.119^{3}	$7.70 \pm 0.023^{b,1}$	$8.21 \pm 0.061^{c,1}$
4Lm+ 7B1	TAM	$8.48 \pm 0.030^{a,2}$	$8.73 \pm 0.136^{a,b,1}$	$8.71 \pm 0.175^{a,b,c,1,2}$	$8.48 \pm 0.149^{a,b,c,2}$
	E	–	$6.73 \pm 0.22^{a,b,1}$	$6.32 \pm 0.246^{a,b,2}$	$6.71 \pm 1.005^{a,1,2}$
	S	–	5.55 ± 0.183^{a}	5.31 ± 0.287^{c}	$5.62 \pm 0.247^{a,b,c}$
	Y	–	6.16 ± 0.093^{3}	$7.79 \pm 0.066^{a,b,2}$	$8.79 \pm 0.033^{a,1}$
4Lm+ 7B2	TAM	$8.38 \pm 0.037^{b,2}$	$8.67 \pm 0.183^{a,b,1}$	$8.59 \pm 0.118^{a,b,c,1}$	$8.38 \pm 0.075^{b,c,2}$
	E	–	$6.92 \pm 0.112^{a,1}$	$6.38 \pm 0.193^{a,b,2}$	$6.59 \pm 0.195^{a,b,2}$
	S	–	$4.05 \pm 0.357^{b,2}$	$3.60 \pm 0.281^{e,2}$	$5.61 \pm 0.285^{a,b,c,1}$

	Y	–	6.27 ± 0.107 [3]	7.83 ± 0.048[a,2]	8.76 ± 0.068[a,b,1]
7Lm+ 7B1	TAM	8.38 ± 0.055[b,2]	8.85 ± 0.104[a,1]	8.55 ± 0.113[b,c,2]	8.73 ± 0.096[a,1]
	E	–	6.77 ± 0.146[a,1]	6.26 ± 0.152[a,b,c,2]	5.69 ± 0.392[b,c,3]
	S	–	5.81 ± 0.142[a,2]	6.51 ± 0.135[a,b,1]	5.79 ± 0.262[a,b,2]
	Y	–	6.28 ± 0.130 [3]	7.83 ± 0.047[a,2]	8.61 ± 0.111[a,b,1]
7Lm+ 7B2	TAM	8.41 ± 0.060[a,b,2]	8.91 ± 0.140[a,1]	8.85 ± 0.143[a,b,1]	8.44 ± 0.130[a,b,c,2]
	E	–	6.62 ± 0.162[a,b,c]	6.59 ± 0.409[a]	5.49 ± 0.397[b,c]
	S	–	5.39 ± 0.199[a,3]	6.54 ± 0.068[a,1]	5.82 ± 0.231[a,2]
	Y	–	6.20 ± 0.157 [3]	7.69 ± 0.043[b,2]	8.33 ± 0.108[c,1]
4Lm	TAM	8.44 ± 0.038[a,b,2]	8.77 ± 0.123[a,b,1]	8.73 ± 0.364[a,b,c,1]	8.62 ± 0.110[a,b,1]
	E	–	6.61 ± 0.130[a,b,c,d,1]	6.12 ± 0.051[a,b,c,2]	6.26 ± 0.142[a,b,c,2]
	S	–	5.85 ± 0.234[a,1]	5.99 ± 0.207[b,1]	5.32 ± 0.145[b,c,2]
	Y	–	6.34 ± 0.149 [3]	7.74 ± 0.039[a,b,2]	8.58 ± 0.118[b,1]
7Lm	TAM	8.41 ± 0.024[a,b,3]	8.81 ± 0.108[a,b,1]	8.59 ± 0.107[a,b,c,2]	8.28 ± 0.092[c,d,3]
	E	–	6.15 ± 0.643[b,c,d,1,2]	5.94 ± 0.123[b,c,2]	6.59 ± 0.142[a,b,1]
	S	–	5.27 ± 0.520[a,2]	4.30 ± 0.375[d,3]	6.05 ± 0.156[a,1]
	Y	–	6.18 ± 0.168 [3]	7.73 ± 0.029[a,b,2]	8.39 ± 0.095[c,1]

Batches: C (uninoculated control), 7B1 (inoculated with *L. casei*), 7B2 (inoculated with *L. garviae*), 4Lm+7B1 (inoculated with *L. monocytogenes* at ≈4 log CFU/ g combined with *L. casei*), 4Lm+7B2 (inoculated with *L. monocytogenes* at ≈4 log CFU/ g combined with *L. garviae*), 7Lm+7B1 (inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L. casei*), 7Lm+7B2 (inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L. garviae*), 4Lm (inoculated with *L. monocytogenes* at ≈4 log CFU/ g), 7Lm (inoculated with *L. monocytogenes* at ≈7 log CFU/ g). Microorganisms: TAM (Total aerobic microbial), *E*nterobacteriaceae (E), *Staphylococci* (S), and yeasts (Y). Values are expressed as mean values ± standard deviation. Mean values with different letters (a–d) in the same column indicate significant differences ($p \leq 0.05$) between batches on the same day. Mean values with different numbers (1–3) in the same row indicate significant differences ($p \leq 0.05$) between days in the same batch and the same microbial group.

556 **Table 3.** Evolution of lactic acid bacteria counts throughout the ripening process of
557 "*Torta del Casar*" cheeses.

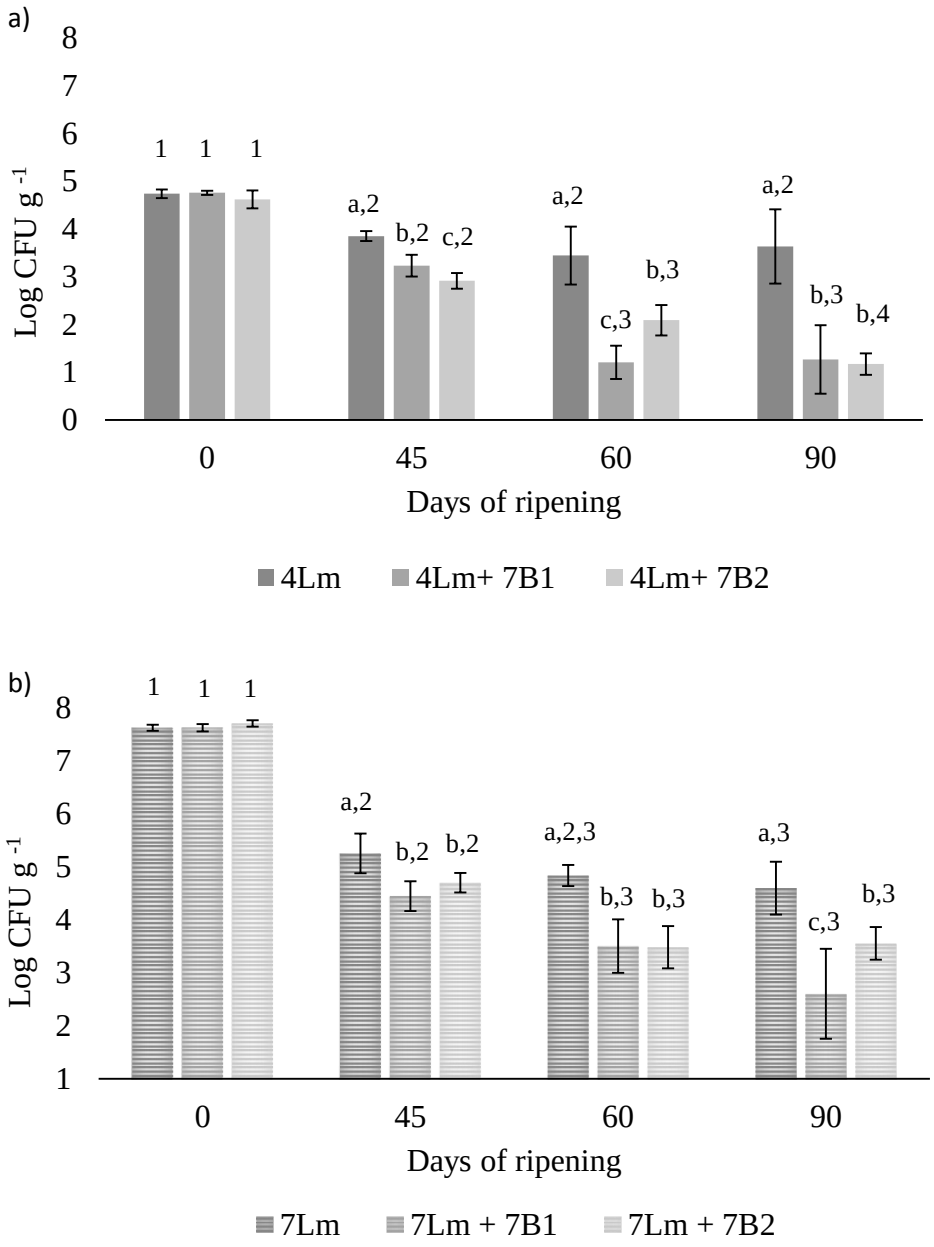
Batches	Days of ripening			
	0	45	60	90
C	8.14 ± 0.079^{c}	8.11 ± 0.216 ^{c}	8.24 ± 0.159^{a,b}	8.32 ± 0.132^{a,b}
7B1	8.30 ± 0.193^{a,b,c,1,2}	8.55 ± 0.158^{a,b,1,2}	8.68 ± 0.399^{a,1}	8.14 ± 0.150^{b,c,2}
7B2	8.23 ± 0.057^{b,c,1,2}	8.31 ± 0.293^{b,c,1,2}	8.50 ± 0.229^{a,b,1}	7.94 ± 0.314^{c,2}
4Lm+ 7B1	8.26 ± 0.081^{a,b,c,3}	8.82 ± 0.124^{a,1}	8.54 ± 0.160^{a,b,2}	8.63 ± 0.122^{a,1,2}
4Lm+ 7B2	8.35 ± 0.033^{a,b,2}	8.71 ± 0.136^{a,1}	8.63 ± 0.140^{a,b,2}	8.30 ± 0.223^{a,b,c,1}
7Lm+ 7B1	8.36 ± 0.072^{a,b,2}	8.73 ± 0.092^{a,1}	8.39 ± 0.216^{a,b,2}	8.49 ± 0.102^{a,b,1,2}
7Lm+ 7B2	8.28 ± 0.045^{a,b,c,2}	8.49 ± 0.087^{a,b,1}	8.22 ± 0.150^{b,2}	8.18 ± 0.078^{b,c,2}
4Lm	8.44 ± 0.054^{a,1,2}	8.57 ± 0.117^{a,b,1,2}	8.65 ± 0.207^{a,b,1}	8.30 ± 0.219^{a,b,c,2}
7Lm	8.30 ± 0.060^{a,b,c,2,3}	8.66 ± 0.166^{a,1}	8.40 ± 0.077^{a,b,2}	8.20 ± 0.104^{b,c,3}

558 C (uninoculated control), 7B1 (inoculated with *L. casei*), 7B2 (inoculated with *L. garviae*), 4Lm+7B1 (inoculated with
559 *L. monocytogenes* at ≈4 log CFU/g combined with *L. casei*), 4Lm+7B2 (inoculated with *L. monocytogenes* at ≈4 log
560 CFU/ g combined with *L. garviae*), 7Lm+7B1 (inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L.*
561 *casei*), 7Lm+7B1 (inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L. garviae*), 4Lm (inoculated
562 with *L. monocytogenes* at ≈4 log CFU/ g), 7Lm (inoculated with *L. monocytogenes* at ≈7 log CFU/ g). Values are
563 expressed as mean ± standard deviation. The means with different letters (a–c) in the same column indicate significant
564 differences ($p \leq 0.05$) between batches on the same day. Mean values with different numbers (1–3) in the same row
565 indicate significant differences ($p \leq 0.05$) between days in the same batch..

Figure legends

Figure 1. Evolution of *L. monocytogenes* levels throughout the processing of "*Torta del Casar*" cheeses. (a). 4Lm (batch inoculated with *L. monocytogenes* at ≈4 log CFU/ g), 4Lm + 7B1 (batch inoculated with *L. monocytogenes* at ≈4 log CFU/ g combined with *L. casei*), 4Lm + 7B2 (batch inoculated with *L. monocytogenes* at ≈4 log CFU/ g combined with *L. garviae*); (b). 7Lm (batch inoculated with *L. monocytogenes* at ≈7 log CFU/ g), 7Lm + 7B1 (batch inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L. casei*), 7Lm + 7B2 (batch inoculated with *L. monocytogenes* at ≈7 log CFU/ g combined with *L. garviae*). Different letters (a-c) indicate significant differences ($p \leq 0.05$) between batches on the same day. Bars indicate standards deviations.

Figure 1.



Evolution of volatile compounds during ripening and final sensory changes of raw ewe's milk cheese "Torta del Casar" inoculated with selected protective lactic acid bacteria

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Abstract

The effect of selected *Lactocaseibacillus casei* 116 and *Lactococcus garviae* 151 on the evolution of volatile compounds throughout maturation and on the final sensory characteristics of "Torta del Casar" cheeses, was evaluated. For this, both strains were separately inoculated in cheeses (batches Lc and Lg), and together with an uninoculated control batch, were ripened for 90 days. Physicochemical and microbiological parameters, volatile compounds, and the sensory characteristics in the final product, were analysed. Results showed that the selected LAB strains did not affect physicochemical parameters, including texture and colour of the ripened cheeses. *Lc. casei* and *Lco. garviae* could have a positive effect on the aroma, for their contribution to the generation of methyl branched acids and for the reduction of compounds derived from β-oxidation of fatty acids. Thus, *Lc. casei* and *Lco. garviae* could be proposed as protective cultures in "Torta del Casar" cheese.

Keywords: "Torta del Casar" cheeses, LAB, volatile compound, texture, sensorial analysis

I. INTRODUCTION

"Torta del Casar" is a high-quality Spanish cheese marketed under the Registry of the Protected Designation of Origin "Torta del Casar" (Casar de Cáceres, Cáceres, Spain) in accordance with Regulation (CE) 1491/2003 of the European Commission. This type of cheese is made from whole raw Merino ewe´s milk using only the dried flowers of the plant *Cynara cardunculus* as rennet. The absence of any standardizing thermal process leads to the presence of undesirable microorganisms throughout ripening (Pereira et al., 2010). In recent decades soft cheeses such as "Torta del Casar" have been linked to many outbreaks of illnesses in Europe and worldwide (Martinez-Rios and Dalgaard, 2018). Due to its ubiquity and ability to survive and even grow at refrigeration temperatures, most of the outbreaks related to the consumption of soft cheeses have been caused by *Listeria. monocytogenes* (Amato et al., 2017; Possas et al., 2021).

The use of bioprotective cultures represents an additional hurdle to avoid *L. monocytogenes* proliferation and persistence in these products (Martinez-Rios and Dalgaard, 2018; Rolim et al., 2020). Lactic-acid bacteria (LAB) are the most frequent microbial population of raw milk (Gezginc et al., 2022) and can be used as protective cultures in this product.

Lacticaseibacillus casei 116 and *Lactococcus garviae* 151 strains have been previously selected by their antagonistic effect against *L. monocytogenes* in "Torta del Casar" cheese (Martín et al., 2022). However, before to be proposed some of these strains as possible protective cultures in this kind of cheese it is necessary to evaluate that there are no negative effects on volatile compounds generation throughout the ripening and in the final sensorial characteristics of this product.

The aim of the present work was to evaluate the effect of the addition of selected LAB strains isolated from raw ewe´s milk cheeses on the evolution of the volatile compounds throughout the ripening process and the final sensory traits of "Torta del Casar" cheese.

2. MATERIALS AND METHODS

2.1. Origin of the strains and growth conditions

The *Lc. casei* 116 (No. P202131120) and *Lco. garviae* 151 strains maintained at the Food Hygiene and Safety Culture Collection at the University of Extremadura (Cáceres, Spain), have been used for the inoculation of traditional raw ewe´s milk cheese. These strains were isolated from traditional ripened soft cheeses of Extremadura (Spain) and selected by its antagonist activity against *L. monocytogenes* in cheese-based agar following the methodology described by Martín et al. (2022).

To prepare LAB inoculum, 100 μL of the stock culture (stored in Man Rogosa Sharpe (MRS) broth (Fisher Bioreagents, Belgium) containing 20% (w/v) glycerol at -80 °C) were inoculated onto 10 mL of MRS broth and incubated for 48 h at 30 °C. At the end of the incubation, ≈8.0 log CFU/mL cells were obtained and an aliquot of this was diluted in 1% (w/v) peptone water (Conda) to reach a final concentration of approximately 7.0 log CFU/mL. Then, cultures were centrifugated at 10,000 g for 5 min, and the supernatants were discarded. The sediments were then washed and resuspended in phosphate-buffered saline (PBS) and used for the inoculation of the "Torta del Casar" cheese curds. To verify the level of inoculation, serial dilutions were poured onto MRS agar (Oxoid, UK) and incubated anaerobically at 30 °C for 72 h. In addition, the initial counts (CFU/g) of *Lc. casei* 116 and *Lco. garviae* 151 on the curd were determined at day 0 of processing.

2.2. Preparation of "Torta del Casar" cheese

"Torta del Casar" cheeses used in this study were elaborated from raw milk of sheep in a cheese industry located in Cáceres (Extremadura region, Spain). In this factory, the curds with an approximate weight of 0.5 kg were elaborated by pressing for 1.5 h and salting in brine for 1 h. After salting, curds were transported from the factory to a pilot plant placed at the Faculty of Veterinary of the University of Extremadura under refrigerated conditions (< 2 ºC) for their inoculation and posterior ripening due to safety reasons. The inoculation was done in the centre of the curd (in a cube of 16 cm^2 of surface and 6 cm deep [the entire volume of curd], and ≈ 100 g of weight). According to the inoculated microorganisms, three batches of cheeses were manufactured: batch C (uninoculated control), batch Lc (inoculated only with *Lc. casei* at ≈7 log CFU/g) and batch Lg (inoculated only with *Lco. garviae* ≈7 log CFU/g). The two bacterium inocula were prepared each in a final volume of 1 mL of PBS with sterile micropipettes in a laminar flow cabinet (Telstar, Spain). In batch C, 1 mL of sterilized PBS was added instead of the bacterium inoculum.

After inoculation, the cheeses' curds were ripened in a chamber of the pilot plant following the industrial conditions used for this product: 35 days at 6°C and 90% relative humidity (RH), 10 days at 8ºC and 80 % RH, 10 days at 9ºC and 80 % RH. Finally, cheeses were kept at 10 °C and 80% RH for 35 days.

Five cheeses of each batch were taken at 0, 30, 45, 60 and 90 days of ripening for microbiological, physicochemical, and volatile compounds analysis. In ripened cheeses, colour, texture, and sensory analysis were also determined. All analysis were carried out in quintuplicate.

2.3. Microbiological analysis

The total viable aerobic microbial and LAB counts were determined on Plate Count Agar (PCA; Pronadisa, Spain) and MRS (Oxoid) agar, respectively. Both agar media were incubated at 30 °C for 48 h. Finally, the *Enterobacteriaceae* were counted on Violet Red Bile Glucose (VRBG, Oxoid) agar, and the incubation was carried out at 37ºC for 48 h. After incubation, colonies with the expected characteristics were counted and results were expressed as log CFU/g.

2.4. Physicochemical analysis

The water activity (a_w) of "Torta del Casar" cheeses was determined at 25 °C by using a Novasina Lab Master meter (Novasina AG, Switzerland). Calibration was done by using several saturated solutions of known a_w. The pH was measured using a pH-meter model 340 (Mettler-Toledo GmbH, Switzerland) calibrated with 3 different standard pH solutions (4.0, 7.0 and 9.25). Moisture content (%) was gravimetrically analysed following the official method of the Association of Official Analytical Chemists (AOAC, 2000).

2.5. Instrumental texture

The texture analysis was performed at room temperature using a Texture Profile Analysis (TPA) and was carried out in triplicate of each of the five cheese samples composing each batch. The instrument used was a TA XT Plus Texture Analyzer (StableMicro Systems Ltd., Godalming, UK) equipped with a cylindrical probe of 5 cm in diameter. This procedure involved cutting slices approximately 1 cm thick. Hardness (N), springiness (cm), cohesiveness, gumminess (N), chewiness (N cm), and adhesiveness (N s) were evaluated at the end of the ripening process.

2.6. Instrumental colour

Colour was determined on the cut surface of each sample using a Minolta CR-300 colorimeter (Konica Minolta, Inc; Nieuwegein, The Netherlands) with an illuminant D65, a 0° standard observer and one port/ display area of 2.5 cm. that was calibrated before use with a white tile having the following values: L* = 93.5, a* = 1.0 and b* =0.8. Colour was expressed according to the Commission International de l'Eclairage (CIE) system and reported as CIE L* (lightness), CIE a* (redness), CIE b* (yellowness). In which the chroma and hue angle were calculated as $(a^{*2}+b^{*2})^{0.5}$ and $\tan^{-1}(b^*/a^*)$, respectively.

2.7. Volatile compound analysis

The volatile compounds in soft cheeses were extracted by solid-phase microextraction (SPME) after heating to 37 ºC for 30 min, using a divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm fiber (Merck; Darmstadt, Germany). They were then analysed by gas chromatography-mass spectrometry (GC-MS) in a Gas Chromatograph 6890 GC (Agilent Technologies; Santa Clara, CA, USA) equipped with a HP-5 column (5% phenyl−95% dimethylpolysiloxane) and coupled to a mass spectrometer (MS) detector, 5975C (Agilent Technologies). Oven temperature started at 40 ºC for 5 min and was increased to 280 ºC, with a rate of 7 ºC/min. The desorption time was 30 min at 250 ºC. The transfer line temperature was established at 280 ºC. The carrier gas was helium with a flow rate of 1.2 mL/min. MS detection was performed in full scan (50–350 amu). Automated peaks search and spectral deconvolution were used for data treatment, and the identification of the volatile compounds was achieved by comparing their mass spectra with the NIST/EPA/NIH library.

2.8. Sensory evaluation

A triangular of olfactory analysis was carried out in this study with semi- trained panel (24), including students and lectures at the Faculty of Veterinary Sciences (University of Extremadura, Caceres, Spain). Three samples were presented to each volunteer, marked with random three digits' codes, and served at room temperature on white plastic plates**.** The panel sessions were held around 2 h before lunch in the sensory panel booth room at the Faculty of Veterinary Science of the University of Extremadura in Caceres (Spain). Information about sex and age of each volunteer was required.

2.9. Statistical analyses

For the statistical analysis of the data, the software IBM SPSS Statistic version 20 (IBM, USA) was used. Once the dependent (microbiological level, a_w, pH, moisture content, texture, colour, volatile compounds) and independent (different batches and days of ripening) variables of the analysis were determined, a study of the normality of the different data populations was carried out using the Shapiro Wilk test. Subsequently, the analysis of the data was conducted using the Mann–Whitney test (Mann and Whitney, 1947). Statistical significance was established at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Physicochemical parameters

The evolution of the physicochemical parameters during ripening is presented in Figure 1. The moisture content (%) decreased significantly ($p \leq 0.05$) during ripening from initial levels around 88% to 35-38% at the end of ripening in all batches studied (Figure 1a). These values are similar to those found in soft-ripened Torta del Casar cheese by Ordiales et al. (2013b). There were no differences between batches C and Lc throughout the

ripening period; however, the moisture content values of the batch inoculated with *Lco. garviae* (Lg) were significantly lower at days 30 and 45 (Figure 1a). However, there were no significant differences ($p > 0.05$) between batches at the end of the ripening time.

The a_w ranged from initial values of 0.975 to 0.938-0.943 at the end of ripening (Figure 1b). Similar evolution in the a_w was reported by Ordiales et al., (2013b) in "Torta del Casar" cheese. No significant differences ($p \leq 0.05$) in a_w values were found between batches during the ripening.

The pH values decreased until day 30 of ripening in the three analysed batches to reach levels of around 5.20 (Figure 1c). However, at days 45, 60 and 90 the pH increased to reach levels around 5.8 at the end of ripening. The increase in pH values of ripened cheese might be due to the decomposition of lactic acid and the formation of basic compounds derived from the hydrolysis of proteins (Özer and Kesenkaş, 2019; Jia et al., 2021). Small differences in the pH values between batches were found, only at day 90, the batch in which *Lc. casei* was inoculated (batch Lc) showed a pH significantly ($p \leq 0.05$) higher than that found in the control batch (batch C).

Thus, only little and punctual differences over the physicochemical parameters of "Torta del Casar" cheese (humidity and pH) because of the inoculation of the selected LAB strains *Lc. casei* and *Lco. Garviae* were observed, but in general, these microorganisms did not affect these parameters on soft-ripened cheeses. These results contrast with those found by Jia et al. (2021) in semihard goat cheese, who reported that the addition of selected starter cultures could decrease the pH of matured cheese.

3.2. Enumeration of microorganisms

Table 1 shows the results obtained from the enumeration of microorganisms. The total viable aerobic microbial and LAB counts showed similar levels and, higher than 8 log

CFU/g, in all batches studied and sampling days. The total viable microbial and LAB counts were similar to those found in other soft cheeses clotted with vegetable rennet (Ordiales et al., 2014; Crespo et al., 2022). Thus, LAB is the predominant microbial group during the ripening of soft ewe's milk cheeses made with raw milk and clotted with vegetable rennet, such as "Serra da Estrela", "Serpa", "Queso de la Serena" and "Torta del Casar" cheese (Ordiales et al., 2013a; Gonçalves et al., 2018).

On the other hand, the levels of *Enterobacteriaceae* were high (≈ 6.8 log CFU/g) in all batches at the beginning of the ripening (day 0), probably this is because this kind of cheese is made with raw milk. A significant ($p \leq 0.05$) decrease of this microbial group was observed throughout the ripening (Table 1), probably due to the antimicrobial effect of the dominant LAB population. Similar levels of *Enterobacteriaceae* have been previously reported in "Torta del Casar" cheese (Chaves-López et al., 2006; Ordiales et al., 2013b; Tabla et al., 2016).

3.3. Analysis of volatile compounds

A total of 34 volatile compounds was identified in all the three analysed batches of "Torta del Casar" cheese throughout the 90-day ripening including carboxylic acids, alcohols, aldehydes, ketones and esters (Tables 2, 3 and 4). Most of the identified compounds have also been reported in other studies of "Torta del Casar" cheeses and other types of similar soft-bodied cheeses (Ferreira et al., 2009; Jia et al., 2021; Ordiales et al., 2013b).

3.3.1. Acids

Carboxylic acids were the most abundant volatile compounds found (Table 2).. This wide range of carboxylic acids found might be due to the lipolytic LAB activity (Jia et al., 2021). Within these compounds, acetic, hexanoic and 3-methyl-butanoic acids were found in higher levels in all batches (Table 2). In the LAB-inoculated batches Lc and Lg,

2-methyl-propanoic acid was also found as the most abundant one at the end of the ripening period (Table 2). Acetic and hexanoic acids have been reported as the acids found in the highest amounts in ripened "Torta del Casar" cheese (Ordiales et al., 2013b). In general, most acids showed significant ($p \leq 0.05$) increases throughout the ripening process in both the uninoculated control and inoculated batches, especially the methyl-branched acids that were detected at low levels or non-detected at day 0 of ripening (Table 2). The formation of acids in "Torta del Casar" cheese is caused by enzymes, mainly from vegetal rennet, and microbial activity (Curioni and Bosset, 2002; Delgado et al., 2010b). Regarding linear n-acids evolution, only scarce differences were found between batches throughout the ripening process. Thus, only acetic acid at day 0, butanoic acid at 45 and 60 and octanoic and propanoic acids at day 30 showed significantly higher amounts in one of the inoculated batches than in the control one (Table 2). In addition, hexanoic acid showed a lower level in batch Lg than in the control batch at 45 days of ripening. However, none of these differences between batches in n-acids abundance was found at the end of ripening, except for propanoic acid that showed a significantly higher amount in batch Lg than in the control batch (Table 2). Acetic and propanoic acids could have a microbial origin as a result of lactose fermentation, mainly some LAB(Delgado-Martínez et al., 2019). Short-chain fatty acids have low perception thresholds and provide typical aroma notes to cheeses such as Cheddar, Roncal, Emmental, Camembert and Grana Padano (Curioni and Bosset, 2002).

When the evolution of methyl-branched acids is studied, it is remarkable that at day 90 of ripening most of these compounds (2-methyl-propanoic acid, 3-methyl-butanoic acid and 2-methyl-butanoic acid) showed significantly ($p \leq 0.05$) higher abundance in Lc and Lg than in control batches. Branched-chain carboxylic acids such as 2-methyl-propanoic, 3-methylbutanoic and 2-methylbutanoic acids, derived from the catabolism of valine,

leucine and isoleucine, respectively (Yvon and Rijnen, 2001), mainly from microbial origin (Calzada et al., 2015). In the present work the microbial activity of the selected *Lc. casei* and *Lco. garviae* strains to generate branched-chain carboxylic acids seems to be demonstrated since they were detected in higher amounts in inoculated than in control batches. Thus, inoculation of the selected *Lc. casei* and *Lco. garviae* could contribute to the aroma of "Torta del Casar" cheese since the 2-methylbutanoic acid and 3-methylbutanoic acids make contributions to the overall aroma and flavour (Qian and Reineccius, 2002).

3.3.2. Alcohols, ketones and aldehydes

Table 3 shows the results for alcohols, ketones and aldehydes. The alcohols 2-butanol, 2-methyl-1-butanol, 2,3-butanediol, 2-butoxy-ethanol and 2,6-dimethyl-4-heptanol increased their levels throughout the ripening process, while 2-methyl-1-propanol, 3-methyl-1-butanol and phenyl-ethyl-alcohol showed higher abundance at day 0 of ripening and decreased during maturation. The most abundant alcohols at the end of the ripening were 2-butanol and 2,3-butanediol. The 2-butanol derived from 2,3-butanediol by the action of LAB(Ghiaci et al., 2014), while the microbial reduction of acetoin could be the origin of 2,3-butanediol (Morales et al., 2004).

Branched-chain alcohols 2-methyl-1-propanol and 3-methyl-1-butanol come from the reduction of branched-chain aldehydes and can be found in raw milk cheeses with intense proteolysis due to vegetable rennet (Carbonell et al., 2002) as is the case of "Torta del Casar". In fact, the former authors found 3-methyl-1-butanol as the major alcohol in "La Serena" cheese also made with vegetable rennet.

The evolution of the production of alcohol compounds throughout the ripening process was very similar in the three analysed batches (Table 3). In ripened cheeses the only

significant ($p \leq 0.05$) differences between batches were the lower amount of 2-butanol and 2-methyl-1-propanol in batch Lc than in the control and Lg batches and the lower amount of 2,3-butanediol and 2-butoxy-ethanol in batch Lg than in the remaining batches. Probably the activity of the selected *Lc. casei* and *Lco. garviae* in the production of the former compounds is lower than that of the natural contaminant LAB.

A low abundance of **ketones** was detected at the beginning of ripening, but significant increases for all detected compounds of this group were found after 90 days of ripening (Table 3). Ketones are abundant constituents of most dairy products and have typical odours and low perception thresholds (Delgado et al., 2010a; Jia et al., 2021). Thus, they could play an important role in the final aroma of "Torta del Casar" cheese. The 2-butane-one and 2-heptane-one were the most abundant ketones detected during ripening. Similar results have been found in "Torta del Casar" (Delgado et al., 2010a) and in other cheeses made with raw sheep's milk such as "La Serena" cheeses (Carbonell et al., 2002). The inoculated and uninoculated batches showed the same evolution of ketones formation and only a few differences in levels of these compounds were detected at the end of ripening time. Thus, only 2-nonanone showed significantly higher abundance in batches Lc and Lg than in control batches, while 2-heptanone and 2-butanone were encountered in higher amounts in the control than in the LAB-inoculated batches (Table 3). These compounds are derived from β-oxidation of fatty acids, that are first oxidized to α-ketoacids, which are further decarboxylated to their corresponding methyl-ketones with one carbon atom less, such as 2-heptanone, 2-nonanone and 2-butanone and finally the methyl-ketones can be reduced to secondary alcohols (van Mastrigt et al., 2018). These compounds are necessary to cheese aroma, but since they are derived from β-oxidation of fatty acids, their production should be not stimulated, as could happen with the inoculation of selected LAB assayed, to avoid rancidity notes.

As aldehydes, only 3-methylbutanal was identified throughout the ripening process. The aldehydes have been reported as minor compounds in this kind of soft cheeses, probably because of their instability since they are reduced to alcohols or oxidized to acids (Delgado et al., 2010a). High concentrations of aldehydes are associated with the development of off-flavours in cheese (Chen et al., 2019) so an increase throughout the processing would be negative for the global aroma of this product. In the present work, a significant ($p \leq 0.05$) increase of 3-methylbutanal was observed throughout the ripening process in all the analysed batches. The 3-methylbutanal, derived from the degradation of amino acid leucine (Castada et al., 2019; Meng et al., 2021), is probably a consequence of proteolysis from vegetal rennet and microbial origin. Nevertheless, since no significant differences ($p > 0.05$) between batches were detected in 3-methylbutanal in the present work, it seems that the selected *Lc. casei* and *Lco. garviae* have a low impact on the production of this compound, at least no difference of the probable effect of LAB present in control samples from contamination origin.

3.3.3. Esters and other compounds

A decrease in most of the **esters** throughout the ripening process was observed in all batches (Table 4). Most of the identified esters were ethyl esters, which could be important contributors to the typical aroma of "Torta del Casar" cheeses (Delgado et al., 2010a, Ferreira et al., 2009). Some LAB, as well as chemical reactions, are involved in ester formation in soft-ripened cheeses (Delgado et al., 2015). In the present work, few differences between batches were detected throughout the ripening process, and only it is noticeable that in the final product the abundance of hexanoic acid, ethyl ester, octanoic acid, ethyl ester, decanoic acid, ethyl ester and 1-butanol, 3-methyl-, acetate was lower in batch Lc than in control and Lg batches (Table 4). Although some of these esters have a

sweet, fruity and ice-cream flavour and could contribute positively to the aroma of ripened cheese, it should be considered that also may have a negative influence, depending on the concentration and type of cheese (Jia et al., 2021). In the present work, the inoculated LAB strains and especially *Lc. casei*, did not contribute to ester formation, at least no difference of the probable effect of LAB present in control samples from contamination origin.

Other compounds were also detected throughout the processing in most of the analysed batches including two pyrazines (trimethyl-pyrazine and 2,5-dimethyl-pyrazine), dimethyl disulfide, and 1,5,9-decatriene, 2,3,5,8-tetramethyl- (Table 4). Only dimethyl disulfide and the alkyl pyrazines increased during the maturation (Table 4). Dimethyl disulfide detected at similar levels in all analysed batches has been reported in ripened cheeses as degradation of methional that derived from amino acid methionine, but its contribution to the aroma of cheeses is only marginal (Frank et al., 2003). Alkyl pyrazines are produced in cheese via the condensation of aminoketones, which are formed mainly through Maillard and Strecker degradation reactions (Frank et al., 2003). From the results, only trimethyl-pyrazine showed differences between batches in ripened cheeses. Therefore, batch Lc showed a higher abundance of trimethyl-pyrazine than the remaining batches (Table 4). Trimethyl-pyrazine has been reported in ripened cheeses and it has been associated with strong savory to musty potato-like (Frank et al., 2003).

From the analysis of the above volatile compounds, it can be deduced that the addition to "Torta del Casar" cheese of the selected *Lc. casei* and *Lco. garviae* could have a positive effect on the aroma, for their contribution to the generation of methyl branched compounds (mainly methyl branched acids) and for not increasing and even reducing oxidation compounds from β-oxidation of fatty acids, as it can be observed in Figure 2

where the addition of abundance area of methyl branched, and β-oxidation compounds are represented.

3.4. Texture and colour analysis

The texture is an important characteristic of cheese in deciding consumer acceptability (Delgado et al., 2010a). The results obtained from the texture and colour parameters of the cheese batches at the end of ripening are shown in Table 5. No differences in texture analysis were observed between control (batch C) and LAB-inoculated cheeses (Lc and Lg batches). Medved'ová et al., (2020) also did not find significant differences ($p \leq 0.05$) in the texture of the cheeses when *Lc. ramhnosus* was added. However, these results contrast with the pronounced effect found by Jia et al., (2021) in semihard goat cheeses inoculated with selected LAB starter cultures. Regarding colour determination, no differences ($p > 0.05$) were observed between the inoculated with *Lc. casei* (Lc) and control (C) batches in any of the parameters studied. Only, differences ($p \leq 0.05$) were observed between the inoculated batch with *Lco. garviae* (Lg) and the control batch in the parameter L * (lightness). The a*(redness) and b*(yellowness) values of all batches were very similar ($p > 0.05$).

3.5. Sensory evaluation

In the triangular olfactory analysis, no differences between batches were found**,** and no negative effect in the aroma was encountered for any of the panelists in any of the batches analysed. Thus, the addition of the selected *Lc. casei* and *Lco. garviae* to "Torta del Casar" cheese did not provoke detectable changes in the aroma of the product.

4. CONCLUSION

LAB is the predominant microbial group during the ripening of inoculated and non-inoculated "Torta del Casar" cheeses.. The inoculation of selected *Lc. casei* and *Lco. garviae* did not affect physicochemical parameters covering humidity, a_w, pH and texture and colour of the ripened cheeses. Carboxylic acids were the most abundant volatile compounds found during ripening and the methyl-branched acids were detected in a higher abundance in inoculated ripened cheeses. Although no effect in the olfactory evaluation was detected by panelists, *Lc. casei* and *Lco. garviae* could have a positive effect on the aroma, for their contribution to the generation of methyl branched compounds (mainly methyl branched acids) and for not increasing and even reducing oxidation compounds from β-oxidation of fatty acids. Thus, these microorganisms could be proposed as protective culture in soft-ripened "Torta del Casar" cheese.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been funded by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108 and GR18056. I. Martín was supported by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sport (FPU 16/05303).

Martín, I., Rodríguez, A and Córdoba, J.J, inventor 2021. Nueva cepa de *Lacticaseibacillus casei* 116 con actividad antagonista frente a *Listeria monocytogenes* para su uso como cultivo protector en quesos madurados. No. P202131120.

References

Amato, E., V. Filipello, M. Gori, S. Lomonaco, M.N. Losio, A. Parisi, P. Huedo, S.J. Knabel, and M. Pontello., 2017. Identification of a major *Listeria monocytogenes* outbreak clone linked to soft cheese in Northern Italy - 2009-2011. BMC Infect. Dis. 17:1–7. http://doi.org/10.1186/s12879-017-2441-6.

AOAC. 2000. Moisture in Malt Gravimetric Method (935.29). 17th ed.Calzada, J., A. del Olmo, A. Picon, and M. Nuñez., 2015. Effect of high pressure processing on the lipolysis, volatile compounds, odour and colour of cheese made from unpasteurized milk. Food Bioprocess Technol. 8:1076–1088. http://doi.org/10.1007/s11947-015-1473-4.

Carbonell, M., M. Nuñez, and E. Fernández-García., 2002. Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavour characteristics. Lait 82:683–698. http://doi.org/10.1051/lait:2002042.

Castada, Z. H.; Hanas, K.; Barringer, A. S., 2019. Swiss Cheese Flavor Variability Based on Correlations of Volatile Flavor Compounds, Descriptive Sensory Attributes, and Consumer Preference. Foods. 8, 78, http://doi.org/10.3390/foods8020078

Chaves-López, C., M. De Angelis, M. Martuscelli, A. Serio, A. Paparella, and G. Suzzi., 2006. Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). J. Appl. Microbiol. 101:353–360. http://doi.org/10.1111/j.1365-2672.2006.02941.x.

Chen, C., Y. Lu, H. Yu, Z. Chen, and H. Tian., 2019. Influence of 4 lactic acid bacteria

on the flavor profile of fermented apple juice. Food Biosci. 27:30–36. http://doi.org/10.1016/j.fbio.2018.11.006.

Crespo, A., A. Jiménez, S. Ruiz-Moyano, A. V. Merchán, A.I. Galván, M.J. Benito, and A. Martín., 2022. Low-frequency ultrasound as a tool for quality control of soft-bodied raw ewe's milk cheeses. Food Control 131. http://doi.org/10.1016/j.foodcont.2021.108405.

Curioni P.M.G., and J.O. Bosset., 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12:959–984. http://doi.org/10.1016/S0958-6946(02)00124-3.

Delgado-Martínez, F.J., A.I. Carrapiso, R. Contador, and M.R. Ramírez., 2019. Volatile compounds and sensory changes after high pressure processing of mature "Torta del Casar" (raw ewe's milk cheese) during refrigerated storage. Innov. Food Sci. Emerg. Technol. 52:34–41. http://doi.org/10.1016/j.ifset.2018.11.004.

Delgado, F.J., J. González-Crespo, R. Cava, J. García-Parra, and R. Ramírez., 2010a. Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. Food Chem. 118:182–189. http://doi.org/10.1016/j.foodchem.2009.04.081.

Delgado, F.J., J. Rodríguez-Pinilla, J. González-Crespo, R. Ramírez, and I. Roa., 2010b. Proteolysis and texture changes of a Spanish soft cheese ('Torta del Casar') manufactured with raw ewe milk and vegetable rennet during ripening. Int. J. Food Sci. Technol. 45:512–519. http://doi.org/10.1111/j.1365-2621.2009.02157.x.

Delgado, F.J., J. Rodríguez-Pinilla, G. Márquez, I. Roa, and R. Ramírez., 2015. Physicochemical, proteolysis and texture changes during the storage of a mature soft cheese treated by high-pressure hydrostatic. Eur. Food Res. Technol. 240:1167–1176. http://doi.org/10.1007/s00217-015-2420-3.

European Commission. 2003. Regulation (EC) 1491/2003 of European Commission of 25 of August: Registration of the Protected Designation of Origin "Torta del Casar" in the Register of Protected Designations of Origin and the Protected Geographic Indications.

Ferreira, I.., O. Pinho, and P. Sampaio., 2009. Volatile fraction of DOP "Castelo Branco" cheese: Influence of breed. Food Chem. 112:1053–1059. http://doi.org/10.1016/j.foodchem.2008.06.048.

Frank, O., M. Jezussek, and T. Hofmann., 2003. Sensory activity, chemical structure, and synthesis of Mail-lard generated bitter-tasting 1-oxo-2,3-dihydro-1H-indolizinium-6-olates. J. Agric. Food Chem. 51, 2693–2699. http://doi.org/10.1021/jf026098d

Gezginc, Y., T. Karabekmez-Erdem, H.D. Tatar, E.C. Dağgeçen, S. Ayman, and İ. Akyol., 2022. Metagenomics and volatile profile of Turkish artisanal Tulum cheese microbiota. Food Biosci. 45:101497. http://doi.org/10.1016/j.fbio.2021.101497.

Ghiaci, P., F. Lameiras, J. Norbeck, and C. Larsson., 2014. Production of 2-butanol through meso-2,3-butanediol consumption in lactic acid bacteria. FEMS Microbiol. Lett. 360:70–75. http://doi.org/10.1111/1574-6968.12590.

Gonçalves, M.T.P., M.J. Benito, M. de G. Córdoba, C. Egas, A. V. Merchán, A.I. Galván, and S. Ruiz-Moyano., 2018. Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. J. Food Sci. 83:1333–1341. http://doi.org/10.1111/1750-3841.14141.

Jia, R., Zhang, F., Song, Y., Lou, Y., Zhao, A., Liu, Y., Peng, H., Hui, Y., Ren, Y., and Wang, B., 2021. Physicochemical and textural characteristics and volatile compounds of semihard goat cheese as affected by starter cultures. J. Dairy Sci. 104:270–280. http://doi.org/10.3168/jds.2020-18884.

Mann, H.B., and D.. Whitney., 1947. On a test of whether one of two random variables

is stochastically larger than the other. Ann. Math. Stat. 2:347–370.

Martín, I., A. Rodríguez, A. Alía, M. Martínez-Blanco, D. Lozano-Ojalvo, and J.J. Córdoba., 2022. Control of *Listeria monocytogenes* growth and virulence in a traditional soft cheese model system based on lactic acid bacteria and a whey protein hydrolysate with antimicrobial activity. Int. J. Food Microbiol. 361:109444. http://doi.org/10.1016/j.ijfoodmicro.2021.109444.

Martinez-Rios, V., and P. Dalgaard., 2018. Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis. Food Control 84:205–214. http://doi.org/10.1016/j.foodcont.2017.07.020.

Medved'ová, A., M. Koňuchová, K. Kvočiková, I. Hatalová, and L. Valík., 2020. Effect of lactic acid bacteria addition on the microbiological safety of Pasta-filata types of cheeses. Front. Microbiol. 11:1–16. http://doi.org/10.3389/fmicb.2020.612528.

Meng, H.Y., M. Piccand, P. Fuchsmann, S. Dubois, A. Baumeyer, M. Tena Stern, and U. Von Ah., 2021. Formation of 3-methylbutanal and 3-methylbutan-1-ol recognized as malty during fermentation in swiss raclette-type cheese, reconstituted milk, and de Man, Rogosa, and Sharpe broth. J. Agric. Food Chem. 69:717–729. http://doi.org/10.1021/acs.jafc.0c06570.

Morales, P., I. Feliu, E. Fernández-García, and M. Nuñez., 2004. Volatile compounds produced in cheese by *Enterobacteriaceae* strains of dairy origin. J Food Prot 67, 567–573. http://doi.org/10.4315/0362-028x-67.3.567.

Ordiales, E., M.J. Benito, A. Martín, R. Casquete, M.J. Serradilla, and M.G. Córdoba., 2013a. Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. Food Control 33:448–454. http://doi.org/10.1016/j.foodcont.2013.03.027.

Ordiales, E., A. Martín, M.J. Benito, M. Fernández, R. Casquete, and M. de Guía

Córdoba., 2014. Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of "Torta del Casar" cheese. Int. J. Dairy Technol. 67:402–409. http://doi.org/10.1111/1471-0307.12129.

Ordiales, E., A. Martín, M.J. Benito, A. Hernández, S. Ruiz-Moyano, and M. de G. Córdoba., 2013b. Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. J. Dairy Sci. 96:5477–5486. http://doi.org/10.3168/jds.2013-6587.

Özer, E., and H. Kesenkaş., 2019. The effect of using different starter culture combinations on ripening parameters, microbiological and sensory properties of Mihaliç cheese. J. Food Sci. Technol. 56:1202–1211. http://doi.org/10.1007/s13197-019-03583-2.

Pereira, C.I., J.A. Graça, N.S. Ogando, A.M.P. Gomes, and F. Xavier Malcata., 2010. Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. Food Microbiol. 27:339–346. http://doi.org/10.1016/j.fm.2009.10.012.

Possas, A., O.M. Bonilla-Luque, and A. Valero., 2021. From cheese-making to consumption: Exploring the microbial safety of cheeses through predictive microbiology models. Foods 10. http://doi.org/10.3390/foods10020355.

Qian, M., and G. Reineccius., 2002. Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J. Dairy Sci. 85:1362–1369. http://doi.org/10.3168/jds.S0022-0302(02)74202-1.

Rolim, F.R.L., O.C. Freitas Neto, M.E.G. Oliveira, C.J.B. Oliveira, and R.C.R.E. Queiroga., 2020. Cheeses as food matrixes for probiotics: In vitro and in vivo tests. Trends Food Sci. Technol. 100:138–154. http://doi.org/10.1016/j.tifs.2020.04.008.

Tabla, R., A. Gómez, A. Simancas, J.E. Rebollo, F. Molina, and I. Roa. 2016., *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. Small Rumin. Res. 145:123–129. http://doi.org/10.1016/j.smallrumres.2016.11.008.

van Mastrigt, O., Gallegos Tejeda, D., Kristensen, M. N., Abee, T., and Smid, E. J., 2018. Aroma formation during cheese ripening is best resembled by *Lactococcus lactis* retentostat cultures. *Microbial Cell Factories*, *17*(1), 1–8. https://doi.org/10.1186/s12934-018-0950-7

Yvon, M., and L. Rijnen., 2001. Cheese flavour formation by amino acid catabolism. Int. Dairy J. 11:185–211. http://doi.org/10.1016/S0958-6946(01)00049-8.

Table legends

Table 1. Counts of total aerobic microorganism (TAM), lactic-acid bacteria (LAB) and *Enterobacteriacea*e (E) throughout "Torta del Casar" cheese ripening.

Table 2. Acids (AU×10^6) of "Torta del Casar" cheese during the ripening process

Table 3. Alcohols, ketones and aldehydes (AU×10^6) of "Torta del Casar" cheese during the ripening process

Table 4. Esters and others volatile compounds (AU×10^6) of "Torta del Casar" cheese during the ripening process

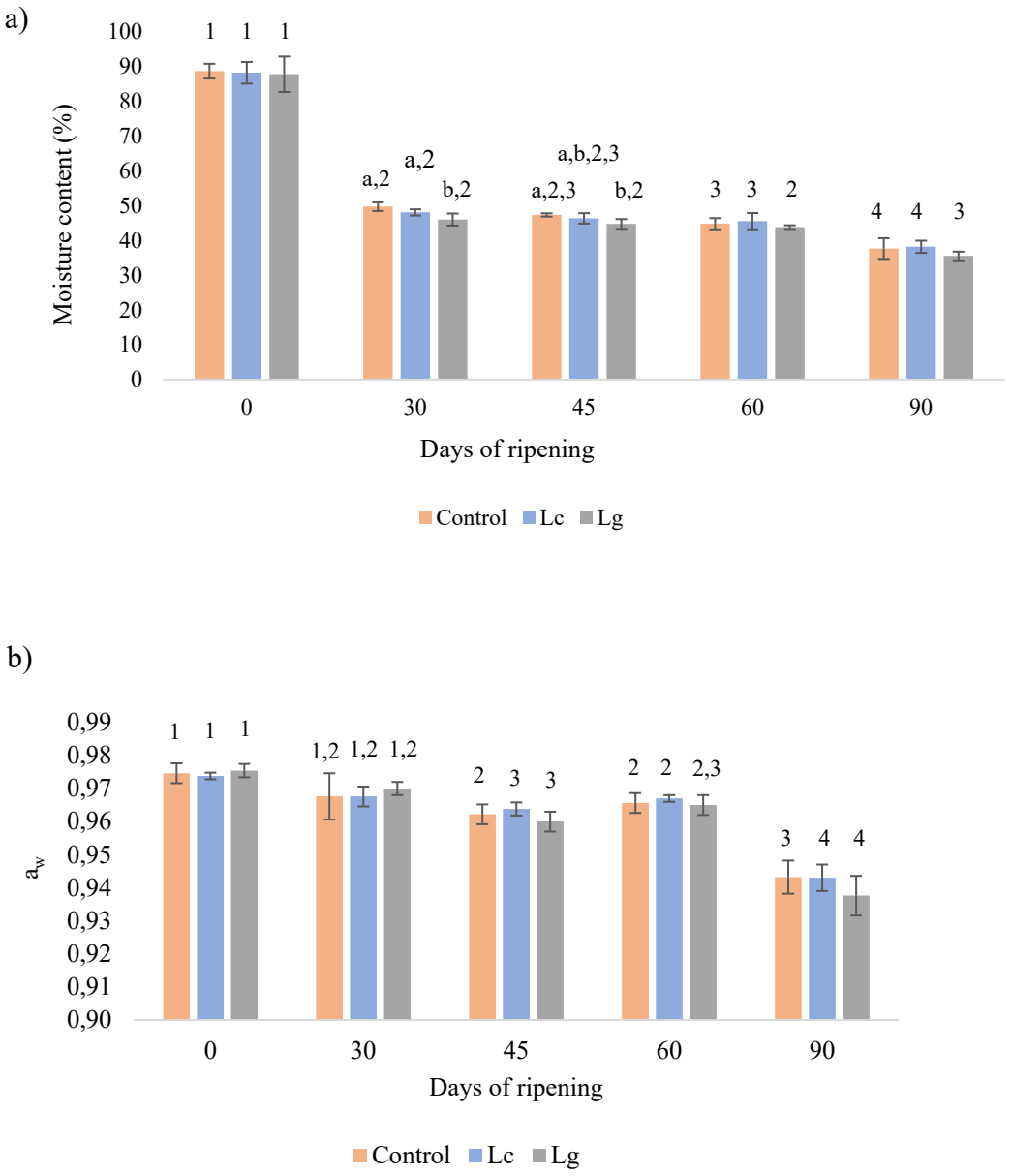
Table 5. Values of instrumental texture (hardness, adhesiveness, springiness, cohesiveness and chewiness) and colour parameters of "Torta del Casar" cheese at the end of the ripening process.

Figure legends

Figure 1. Evolution of moisture content (a), water activity (b) and pH (c) during ripening of "Torta del Casar" cheese. Mean values with different letters indicate significant differences between batches at the same incubation day. Mean values with different numbers indicate significant differences between days within the same batch.

Figure 2. Accumulated area (AU x10^6) of n-compounds and methyl branched compounds throughout the ripening of "Torta del Casar" cheese in Control, inoculated with *Lacticaseibacillus casei* (Lc) and inoculated with *Lactococcus garviae* (Lg) batches.

Figure 1. Martín et al., 2022



c)

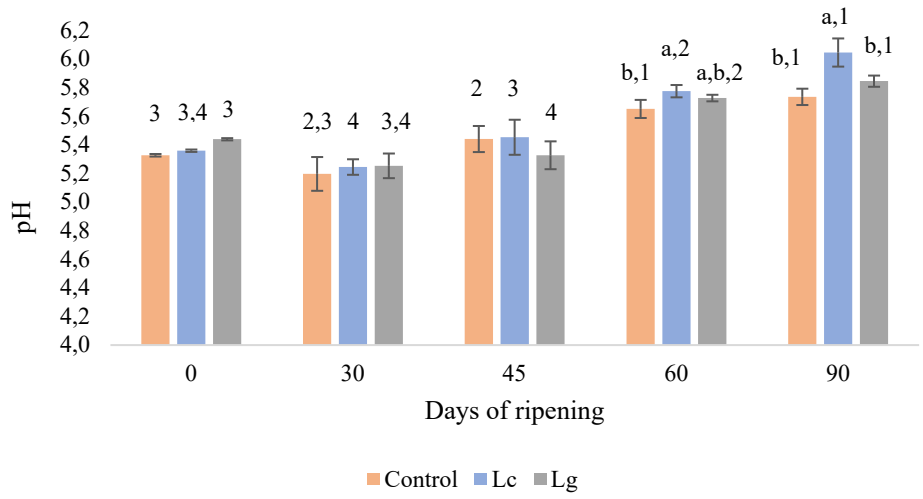

6,2
6,0
5,8
5,6
5,4
5,2
5,0
4,8
4,6
4,4
4,2
4,0
pH
3
3,4
3
2,3
4
3,4
2
3
4
b,1
a,2
a,b,2
b,1
a,1
b,1
0
30
45
60
90
Days of ripening
Control
Lc
Lg

Figure 2. Martín et al., 2022

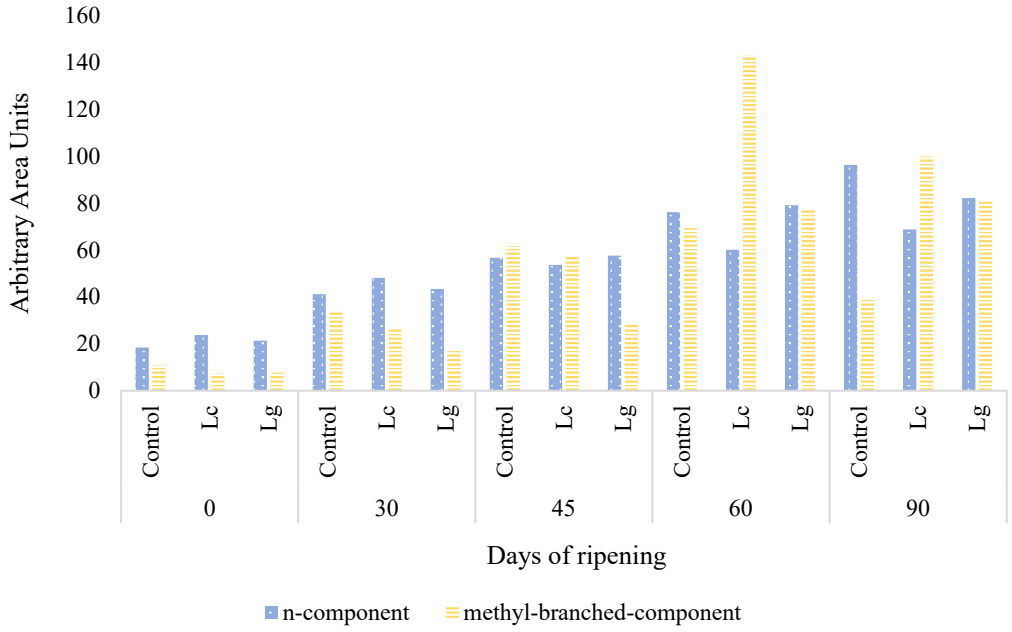


Table 1.

Batches	Microorganism	Days of ripening			
		0	45	60	90
C	TAM	8.08 ± 0.034^{c2}	8.29 ± 0.237^{1,2}	8.36 ± 0.199^{b,1}	8.37 ± 0.065^{a,1}
	LAB	8.14 ±0.079	8.11 ± 0.216	8.24 ± 0.159	8.32 ± 0.132
	E	6.81 ± 0.294^{1}	6.17 ± 0.293^{1,2}	6.13 ± 0.245^{1,2}	5.54 ± 0.629^{2}
Lc	TAM	8.28 ± 0.046^{b2}	8.62 ± 0.215^{2}	9.06 ± 0.283^{a,1}	8.33 ± 0.141^{a2}
	LAB	8.30 ± 0.193	8.55 ± 0.158	8.68 ± 0.399	8.14 ± 0.150
	E	6.74 ± 0.307^{1}	6.02 ± 0.182^{2}	5.81 ± 0.261^{2}	5.71 ± 0.323^{2}
Lg	TAM	8.41 ± 0.050^{a,1,2}	8.5 ± 0.192^{1}	8.53 ± 0.355^{b,1}	7.99 ± 0.316^{b,2}
	LAB	8.23 ± 0.057	8.31 ± 0.293	8.50 ± 0.229	7.94 ± 0.314
	E	6.96 ± 0.144^{1}	6.13 ± 0.370^{2}	5.97 ± 0.097^{2,3}	5.54 ± 0.255^{3}

C (uninoculated batch), Lc (inoculated with *Lacticaseibacillus casei*) and Lg (inoculated with *Lactococcus garviae*). Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($P \leq 0.05$) between batches at the same day and different numbers indicate significant differences ($P \leq 0.05$) between days of ripening at the same batch.

Table 2.

Origin/ Compound	Batches	Days of ripening				
		0	30	45	60	90
Acids						
Acetic acid	C	5.88 ± 1.114^{b,3}	14.44 ± 5.803^{1}	15.44 ± 3.855^{1}	16.88 ± 4.768^{1}	8.54 ± 0.059^{2}
	Lc	10.50 ± 2.972^{a,2,3}	16.94 ± 0.681^{1}	13.66 ± 1.638^{1,2}	14.33 ± 3.647^{1,2}	7.42 ± 0.678^{3}
	Lg	7.72 ± 1.183^{b,1,2}	14.58 ± 2.117^{1,2}	16.12 ± 6.524^{1}	18.90 ± 6.212^{1}	9.25 ± 0.356^{3}
Butanoic acid	C	1.86 ± 0.429^{2}	3.00 ± 0.258^{2}	2.97 ± 0.406^{b,2}	2.99 ± 0.138^{b,2}	5.46 ± 1.380^{1}
	Lc	1.88 ± 0.349^{2}	3.14 ± 0.143^{1,2}	2.93 ± 0.450^{b,1,2}	3.46 ± 0.649^{b,1,2}	4.90 ± 1.446^{1}
	Lg	1.95 ± 0.615^{3}	3.01 ± 0.012^{2}	4.35 ± 0.439^{a,1,2}	4.97 ± 0.497^{a,1}	5.45 ± 0.757^{1}
Hexanoic acid	C	4.52 ± 0.937^{3}	4.44 ± 0.948^{3}	8.19 ± 0.618^{a,b,2}	9.67 ± 0.778^{1,2}	11.32 ± 2.618^{1}
	Lc	5.06 ± 1.212^{2}	7.86 ± 0.986^{1,2}	8.83 ± 1.899^{a,1,2}	8.83 ± 0.963^{1,2}	11.68 ± 4.520^{1}
	Lg	5.81 ± 1.496^{2}	7.08 ± 0.789^{2}	6.84 ± 0.646^{b,2}	9.75 ± 1.755^{1}	9.99 ± 1.534^{1}
Octanoic acid	C	0.60 ± 0.148^{b,2}	0.38 ± 0.049^{b,2}	1.71 ± 0.313^{1}	2.13 ± 0.252^{1}	2.34 ± 0.692^{1}
	Lc	0.99 ± 0.330^{a,2}	1.62 ± 0.466^{a,1,2}	2.60 ± 1.631^{1,2}	2.57 ± 0.714^{1,2}	4.64 ± 3.324^{1}
	Lg	0.95 ± 0.255^{a,1,2}	1.43 ± 0.362^{a,1,2}	0.47 ± 0.246^{2}	1.62 ± 1.301^{1,2}	1.94 ± 0.493^{1}
Propanoic acid	C	n.d.	0.28 ± 0.118^{b,2}	0.97 ± 0.446^{1,2}	1.53 ± 0.440^{1}	0.72 ± 0.529^{b,1,2}
	Lc	n.d.	0.88 ± 0.560^{a}	1.47 ± 0.955	1.42 ± 0.467	1.45 ± 0.410^{a,b}
	Lg	n.d.	0.25 ± 0.074^{b,2}	0.41 ± 0.155^{2}	1.26 ± 0.412^{1}	1.71 ± 0.401^{a,1}
2- methyl-propanoic acid	C	0.12 ± 0.002^{3}	2.64 ± 1.918^{a,2}	6.01 ± 2.742^{1}	6.40 ± 2.368^{1}	3.45 ± 0.702^{b,2}
	Lc	0.12 ± 0.002^{3}	3.87 ± 1.506^{a,2}	8.21 ± 1.757^{1,2}	7.18 ± 2.259^{1,2}	11.94 ± 3.156^{a,1}
	Lg	0.13 ± 0.004^{3}	1.02 ± 0.375^{b,3}	6.83 ± 1.688^{2}	6.65 ± 2.955^{2}	11.02 ± 4.210^{a,1}

3-methyl-butanoic acid	C	2.00 ± 0.265^{a,3}	23.93 ± 1.513^{a,2}	47.41 ± 5.618^{a,1}	54.71 ± 2.382^{b,1}	28.59 ± 4.543^{b,2}
	Lc	0.69 ± 0.113^{b,4}	16.41 ± 2.777^{a,3}	41.59 ± 9.609^{a,2,3}	126.91 ± 12.188^{a,1}	79.43 ± 3.102^{a,2}
	Lg	1.33 ± 0.973^{a,b,3}	10.13 ± 2.629^{b,2}	15.85 ± 2.838^{b,2}	63.26 ± 3.974^{b,1}	61.67 ± 2.608^{a,1}
2-methyl-butanoic acid	C	n.d.	0.93 ± 0.541^{2}	3.17 ± 1.271^{a,1}	3.78 ± 0.614^{1}	2.75 ± 0.631^{b,1}
	Lc	n.d.	1.37 ± 0.874^{3}	2.36 ± 0.771^{a,b,2,3}	5.08 ± 2.271^{1}	4.35 ± 0.768^{a,1,2}
	Lg	n.d.	0.60 ± 0.145^{2}	1.04 ± 0.258^{b,2}	3.11 ± 1.435	3.83 ± 0.749^{a,b,1}
3-methyl-2-butenioc acid	C	n.d.	n.d.	0.19 ± 0.040	0.29 ± 0.068	0.17 ± 0.041
	Lc	n.d.	n.d.	0.22 ± 0.058	0.35 ± 0.172	0.20 ± 0.055
	Lg	n.d.	n.d.	0.14 ± 0.002	0.20 ± 0.078	0.16 ± 0.020

Control (uninoculated batch), Lc (batch inoculated with *Lacticaseibacillus casei*) and Lg (batch inoculated with *Lactococcus garviae*). Values are expressed as mean ± standard deviation. Mean values with different letters indicate significant differences ($P \leq 0.05$) between batches at the same day and compound studied. The mean values with different numbers indicate significant differences ($P \leq 0.05$) between days at the same batch and compound studied. n.d. not detected.

Table 3.

Origin/ Compound	Batches	Days of ripening				
		0	30	45	60	90
Alcohols						
2-butanol, (R)-	C	n.d.	n.d.	4.27 ± 1.14^{b,2}	12.42 ± 0.860^{a,1}	12.94 ± 1.005^{a,1}
	Lc	n.d.	n.d.	7.58 ± 1.94^{a}	5.27 ± 1.198^{b}	5.26 ± 0.827^{b}
	Lg	n.d.	n.d.	2.21 ± 0.311^{c,2}	10.21 ± 3.759^{a,1}	12.70 ± 1.434^{a,1}
2-methyl-1-propanol	C	0.19 ± 0.014^{1,2}	0.20 ± 0.011^{1,2}	0.17 ± 0.031^{2}	0.14 ± 0.032^{2}	0.25 ± 0.081^{a,1}
	Lc	0.18 ± 0.026	0.17 ± 0.027	0.16 ± 0.038	0.14 ± 0.035	0.14 ± 0.002^{b}
	Lg	0.17 ± 0.010	0.18 ± 0.052	0.16 ± 0.044	0.15 ± 0.058	0.17 ± 0.034^{a,b}
3-methyl-1-butanol	C	8.29 ± 0.538^{a,1}	5.70 ± 0.712^{2}	3.92 ± 0.426^{3}	3.20 ± 0.267^{3}	2.97 ± 0.607^{3}
	Lc	6.14 ± 0.192^{b,1}	4.63 ± 0.755^{2}	4.09 ± 1.013^{2}	2.61 ± 0.804^{3}	2.57 ± 0.211^{3}
	Lg	5.93 ± 0.219^{b,1}	5.14 ± 0.410^{1}	3.94 ± 0.818^{2}	3.40 ± 0.890^{2,3}	2.75 ± 0.286^{3}
2-methyl-1-butanol	C	n.d.	0.11 ± 0.016	n.d.	n.d.	0.16 ± 0.042
	Lc	n.d.	n.d.	n.d.	0.12 ± 0.022	0.21 ± 0.015
	Lg	n.d.	n.d.	0.12 ± 0.009	0.11 ± 0.000	0.15 ± 0.027
Phenylethyl alcohol	C	1.36 ± 0.137^{1}	0.86 ± 0.094^{b,3}	1.04 ± 0.151^{2,3}	1.02 ± 0.173^{1,2}	0.93 ± 0.089^{3}
	Lc	1.25 ± 0.039	1.08 ± 0,075^{a}	1.07 ± 0.308	0.99 ± 0.145	0.89 ± 0.207
	Lg	1.30 ± 0.404^{1}	1.21 ± 0.180^{a,1,2}	0.92 ± 0.164^{1,2}	0.97 ± 0.149^{1,2}	0.80 ± 0.149^{2}
2,3-butanediol, [R-(R*,R*)]-	C	2.80 ± 0.853^{2}	15.42 ± 1.458^{1}	10.01 ± 1.038^{b,1}	9.59 ± 1.204^{a,b,1,2}	10.93 ± 0.449^{a,1}
	Lc	2.54 ± 0.822^{2}	14.50 ± 1.181^{1}	4.20 ± 0.973^{c,1,2}	6.19 ± 0.959^{b,1,2}	3.09 ± 0.144^{b,2}
	Lg	2.58 ± 0.738^{3}	14.32 ± 1.674^{1,2}	14.81 ± 0.634^{a,1}	12.59 ± 1.500^{a,1}	5.77 ± 0.528^{b,2,3}
2-butoxy-ethanol	C	n.d.	0.27 ± 0.054^{1}	0.20 ± 0.089^{b,1,2}	0.15 ± 0.021^{b,2}	0.23 ± 0.083^{b,1,2}

	Lc	n.d.	0.30 ± 0.056 [1]	0.24 ± 0.082[b,1,2]	0.20 ± 0.033[b,2]	0.22 ± 0.039[b,1,2]
	Lg	n.d.	0.32 ± 0.052 [2]	0.39 ± 0.036[a,1]	0.37 ± 0.041[a,1]	0.36 ± 0.042[a,1]
2,6-dimethyl-4-heptanol	C	n.d.	0.26 ± 0.004	0.37 ± 0.462	0.19 ± 0.022	0.18 ± 0.037
	Lc	n.d.	0.19 ± 0.017[2]	0.66 ± 0.146[1]	0.28 ± 0.159[2]	0.37 ± 0.029[2]
	Lg	n.d.	0.24 ± 0.023	0.35 ± 0.211	0.18 ± 0.048	0.19 ± 0.015
Ketones						
2-nonanone	C	0.38 ± 0.086[a,b,2]	0.14 ± 0.038[2]	0.41 ± 0.339[2]	0.19 ± 0.002[2]	0.61 ± 0.021[b,1]
	Lc	0.54 ± 0.182[a,2]	0.13 ± 0.006[2]	0.21 ± 0.089[2]	0.18 ± 0.056[2]	0.95 ± 0.050[a,1]
	Lg	0.23 ± 0.028[b,2]	0.16 ± 0.036[2]	0.18 ± 0.051[2]	0.11 ± 0.006[2]	0.79 ± 0.152[a,b,1]
2-heptanone	C	0.32 ± 0.009[1,2]	0.12 ± 0.032[2]	0.26 ± 0.078[1,2]	0.16 ± 0.052[2]	5.23 ± 2.880[a,1]
	Lc	0.38 ± 0.138[1,2]	0.12 ± 0.004[2]	0.19 ± 0.091[1,2]	0.18 ± 0.060[1,2]	1.10 ± 0.096[b,1]
	Lg	0.27 ± 0.057[2]	0.13 ± 0.004[2]	0.28 ± 0.155[2]	0.13 ± 0.009[2]	0.98 ± 0.147[b,1]
2,3-butanedione	C	0.25 ± 0.040[3]	0.57 ± 0.104[a,1,2,3]	0.88 ± 0.322[1]	0.72 ± 0.221[1,2]	0.40 ± 0.115[a,b,2,3]
	Lc	0.27 ± 0.066[3]	0.43 ± 0.075[a,b,3]	1.00 ± 0.128[1]	0.69 ± 0.140[2]	0.34 ± 0.080[b,3]
	Lg	0.26 ± 0.079[3]	0.39 ± 0.104[b,2,3]	1.16 ± 0.202[1]	0.56 ± 0.066[2]	0.41 ± 0.037[a,2,3]
2-pentanone	C	0.16 ± 0.038	0.12 ± 0.012	0.15 ± 0.004	n.d.	1.27 ± 0.103
	Lc	n.d.	n.d.	n.d.	n.d.	0.87 ± 0.098
	Lg	n.d.	n.d.	0.16 ± 0.095	n.d.	0.40 ± 0.021
2-butanone	C	0.30 ± 0.099 [4]	1.12 ± 0.626 [4]	10.17 ± 1.942 [3]	18.70 ± 1.575[a,2]	35.37 ± 4.673[a,1]
	Lc	0.31 ± 0.096 [4]	1.13 ± 0.403 [4]	9.62 ± 1.403 [3]	15.76 ± 1.897[b,2]	25.99 ± 3.316[b,1]
	Lg	0.28 ± 0.117 [4]	0.53 ± 0.220 [4]	9.30 ± 3.246 [3]	17.64 ± 1.161[a,b,1]	31.63 ± 1.967[a,b,1]
Aldehydes						
3-methyl-butanal	C	0.17 ± 0.020 [2]	0.38 ± 0.112 [1,2]	0.34 ± 0.142[a,b,1,2]	0.61 ± 0.202 [a,1]	0.60 ± 0.049[1,2]
	Lc	0.17 ± 0.020 [2]	0.33 ± 0.151 [1,2]	0.55 ± 0.100[a,1]	0.41 ± 0.185 [b,1,2]	0.69 ± 0.027 [1]

Lg	0.16 ± 0.013 [2]	0.22 ± 0.039 [2]	0.21 ± 0.088 [b,2]	0.31 ± 0.155 [b,2]	0.68 ± 0.130 [1]

Control (uninoculated batch), Lc (batch inoculated with *Lacticaseibacillus casei*) and Lg (batch inoculated with *Lactococcus garviae*). Values are expressed as mean ± standard deviation. Mean values with different letters indicate significant differences ($P \leq 0.05$) between batches at the same day and compound studied. The means with different numbers indicate significant differences ($P \leq 0.05$) between days at the same batch and compound studied. n.d. not detected.

Table 4.

Origin/Compound	Batches	Days of ripening				
		0	30	45	60	90
Esters						
Butanoic acid, ethyl ester	C	1.67 ± 0.156 [1]	0.40 ± 0.151 [a,2]	0.17 ± 0.027 [2,3]	0.12 ± 0.003 [3]	0.14 ± 0.002 [3]
	Lc	1.69 ± 0.058 [1]	0.24 ± 0.028 [b,2]	0.15 ± 0.024 [3]	0.18 ± 0.061 [2,3]	0.13 ± 0.017 [3]
	Lg	2.00 ± 0.449 [1]	0.26 ± 0.089 [a,b,2]	0.21 ± 0.052 [2]	0.19 ± 0.046 [2]	0.15 ± 0.010 [2]
Hexanoic acid, ethyl ester	C	2.57 ± 0.439 [1]	0.79 ± 0.233 [2]	0.54 ± 0.069 [a,b,2]	0.43 ± 0.042 [2]	0.53 ± 0.090 [a,2]
	Lc	2.08 ± 0.057 [1]	0.57 ± 0.058 [2]	0.40 ± 0.067 [b,3]	0.46 ± 0.148 [2,3]	0.32 ± 0.032 [b,3]
	Lg	2.61 ± 0.856 [1]	0.69 ± 0.130 [2]	0.66 ± 0.158 [a,2]	0.58 ± 0.113 [2]	0.56 ± 0.050 [a,2]
Octanoic acid, ethyl ester	C	1.85 ± 0.305 [1]	0.40 ± 0.087 [2]	0.41 ± 0.030 [2]	0.41 ± 0.018 [a,2]	0.49 ± 0.053 [a,2]
	Lc	1.60 ± 0.134 [1]	0.42 ± 0.044 [2]	0.40 ± 0.041 [2]	0.35 ± 0.040 [b,2]	0.35 ± 0.028 [b,2]
	Lg	1.97 ± 0.757 [1]	0.42 ± 0.038 [2]	0.36 ± 0.055 [2]	0.36 ± 0.032 [a,b,2]	0.45 ± 0.040 [a,2]
Decanoic acid, ethyl ester	C	2.18 ± 0.258 [b,1]	0.56 ± 0.135 [2]	0.62 ± 0.053 [a,2]	0.65 ± 0.030 [a,2]	0.64 ± 0.073 [a,2]
	Lc	2.79 ± 0.577 [a,b,1]	0.65 ± 0.075 [2]	0.68 ± 0.102 [a,2]	0.53 ± 0.044 [b,2]	0.45 ± 0.094 [b,2]
	Lg	2.97 ± 0.856 [a,1]	0.62 ± 0.065 [2]	0.48 ± 0.058 [b,2]	0.52 ± 0.051 [b,2]	0.56 ± 0.111 [a,b,2]
Dodecanoic acid, ethyl ester	C	0.18 ± 0.025	n.d.	n.d.	n.d.	n.d.
	Lc	0.21 ± 0.044	n.d.	n.d.	n.d.	n.d.
	Lg	0.24 ± 0.069	n.d.	n.d.	n.d.	n.d.
1-butanol, 3-methyl-, acetate	C	0.37 ± 0.015 [1]	0.37 ± 0.035 [a,1]	0.28 ± 0.043 [a,b,1,2]	0.23 ± 0.042 [2]	0.33 ± 0.093 [a,1,2]
	Lc	0.35 ± 0.039 [1]	0.27 ± 0.065 [b,1,2]	0.18 ± 0.025 [b,2]	0.19 ± 0.095 [2]	0.19 ± 0.025 [b,2]
	Lg	0.37 ± 0.027	0.27 ± 0,046 [b]	0.34 ± 0,087 [a]	0.30 ± 0.099	0.35 ± 0.025 [a]

Others compounds						
1,5,9-decatriene, 2,3,5,8-tetramethyl-	C	0.60 ± 0.089^{b,3}	0.61 ± 0.108^{b,3}	0.77 ± 0.048^{a,b,1,2}	0.89 ± 0.025^{a,1}	0.67 ± 0.023^{2,3}
	Lc	0.71 ± 0.084^{a,b,1,2}	0.85 ± 0.068^{a,1}	0.85 ± 0.073^{a,1}	0.70 ± 0.051^{b,1,2}	0.64 ± 0.133^{2}
	Lg	0.76 ± 0.017^{a,1,2}	0.80 ± 0.053^{a,1}	0.68 ± 0.032^{b,2}	0.74 ± 0.048^{b,1,2}	0.66 ± 0.089^{2}
Dimethyl ether	C	29.18 ± 3.598^{1}	5.55 ± 3.239^{2}	1.39 ± 0.562^{2,3}	1.18 ± 0.068^{a,b,2,3}	0.66 ± 0.278^{b,2,3}
	Lc	31.91 ± 1.945^{1}	3.78 ± 0.655^{2}	1.30 ± 0.699^{3}	0.41 ± 0.075^{b,3}	0.25 ± 0.093^{c,3}
	Lg	31.42 ± 0.338^{1}	3.50 ± 1.693^{2}	1.63 ± 0.826^{3}	1.75 ± 0.689^{a,2,3}	1.50 ± 0.080^{a,3}
trimethyl-pyrazine	C	n.d.	n.d.	0.13 ± 0.009^{2}	0.14 ± 0.012^{2}	0.42 ± 0.129^{a,b,1}
	Lc	n.d.	n.d.	n.d.	n.d.	0.68 ± 0.099^{a}
	Lg	n.d.	n.d.	n.d.	n.d.	0.28 ± 0.081^{b}
2,5-dimethyl-pyrazine	C	n.d.	n.d.	n.d.	n.d.	0.18 ± 0.007
	Lc	n.d.	n.d.	n.d.	n.d.	0.37 ± 0.054
	Lg	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.008
Dimethyl disulfide	C	0.15 ± 0.020	0.52 ± 0.085^{a,1}	0.36 ± 0.094^{a,1,2}	0.21 ± 0.038^{2}	0.17 ± 0.006^{2}
	Lc	n.d.	0.50 ± 0.089^{a,1}	0.35 ± 0.011^{a,1,2}	0.29 ± 0.022^{2}	0.16 ± 0.023^{2}
	Lg	n.d.	0.27 ± 0.044^{b,1}	0.16 ± 0.039^{b,2}	0.28 ± 0.082^{1}	0.14 ± 0.002^{2}

Control (uninoculated batch), Lc (batch inoculated with *Lacticaseibacillus casei*) and Lg (batch inoculated with *Lactococcus garviae*). Values are expressed as mean ± standard deviation. Mean values with different letters indicate significant differences ($P \leq 0.05$) between batches at the same day and compound studied. The means with different numbers indicate significant differences ($P \leq 0.05$) between days at the same batch and compound studied. n.d. not detected.

Table 5.

Parameters	Batches		
	C	Lc	Lg
Hardness (N)	5.05 ± 2.663	5.18 ± 1.579	4.44 ± 1.702
Adhesiveness (N/s)	-0.36 ± 0.192	-0.55 ± 0.312	-0.47 ± 0.262
Springiness	0.74 ± 0.052	0.76 ± 0.068	0.75 ± 0.079
Cohesiveness	0.64 ± 0.049	0.64 ± 0.033	0.65 ± 0.039
Chewiness (N)	2.66 ± 0.831	2.57 ± 0.885	2.54 ± 0.828
CIE L*	101.09 ± 6.250[a]	100.51 ± 2.730[a]	97.11 ± 3.740[b]
CIE a*	-1.43 ± 0.859	-1.26 ± 0.920	-1.72 ± 0.640
CIE b*	5.49 ± 2.510	6.26 ± 1.190	5.17 ± 2.211

C (uninoculated batch), Lc (inoculated with *Lacticaseibacillus casei*) and Lg (inoculated with *Lactococcus garviae*). Values are expressed as mean ± standard deviation. Different letters indicate significant differences between batches.

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Título:	NUEVA CEPA DE LACTICASEIBACILLUS CASEI 116 CON ACTIVIDAD ANTAGONISTA FRENTE A LISTERIA MONOCY-TOGENES PARA SU USO COMO CULTIVO PROTECTOR EN QUESOS MADURADOS	
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(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.ºSOLICITUD: FECHA SOLICITUD:	
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(7) TÍTULO DE LA INVENCIÓN:	NUEVA CEPA DE LACTICASEIBACILLUS CASEI 116 CON ACTIVIDAD ANTAGONISTA FRENTE A LISTERIA MONOCYTOGENES PARA SU USO COMO CULTIVO PROTECTOR EN QUESOS MADURADOS
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(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
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NO	[✓]
(10) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA:	
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NO	[ ]
(11-1) DEPÓSITO 1:	
REFERENCIA DE IDENTIFICACIÓN:	116
INSTITUCIÓN DE DEPÓSITO:	Colección Española de Cultivos Tipo (CECT) Edificio 3 CUE, Parc Cientific Universitat de Valencia, Catedrático Agustín Escardino, 9, 46980 Paterna (Valencia), Spain
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(15) DECLARACIONES DE PRIORIDAD:	
PAÍS DE ORIGEN:	
CÓDIGO PAÍS:	
NÚMERO:	
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(16) REMISIÓN A UNA SOLICITUD ANTERIOR:	
PAÍS DE ORIGEN:	
CÓDIGO PAÍS:	
NÚMERO:	
FECHA:	
(17) REPRESENTANTE:	
APELLIDOS:	
NÚMERO DE PODER:	
(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE	
DOMICILIO:	Vicerrectorado de Investigación y Transferencia. Avda. de Elvas, s/n
LOCALIDAD:	Badajoz
PROVINCIA:	06 Badajoz
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MEDIO PREFERENTE DE COMUNICACIÓN	Correo electrónico
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN:	
DESCRIPCIÓN:	[✓] N.º de páginas: 10
REIVINDICACIONES:	[✓] N.º reivindicaciones: 4
DIBUJOS:	[✓] N.º de dibujos: 1
RESUMEN:	[✓] N.º de páginas: 1
FIGURA(S) A PUBLICAR CON EL RESUMEN:	[✓] N.º de figura(s): 1
ARCHIVO DE PRECONVERSION:	[✓]
DOCUMENTO DE REPRESENTACIÓN:	[ ] N.º de páginas:
JUSTIFICANTE DE PAGO (1):	[✓] N.º de páginas: 3
JUSTIFICANTE DEL DEPÓSITO BIOLOGICO (1):	[✓] N.º de páginas: 3
LISTA DE SECUENCIAS PDF:	[ ] N.º de páginas:
ARCHIVO PARA LA BUSQUEDA DE LS:	[ ]
OTROS (Aparecerán detallados):	
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]

(21) NOTAS:	
(22) FIRMA:	
FIRMA DEL SOLICITANTE O REPRESENTANTE:	09167700S PEDRO MARIA FERNÃ#NDEZ (R: Q0618001B)
LUGAR DE FIRMA:	Badajoz
FECHA DE FIRMA:	02 Diciembre 2021

DESCRIPCION

Nueva cepa de *lacticaseibacillus casei* 116 con actividad antagonista frente a *listeria monocytogenes* para su uso como cultivo protector en quesos madurados

SECTOR DE LA TÉCNICA

La presente invención pertenece al sector agroalimentario, en particular a la industria quesera. Más concretamente, la presente invención se refiere a una bacteria ácido-láctica del género *Lacticaseibacillus* y a su uso como inhibidor de la bacteria patógena *Listeria monocytogenes* que puede contaminar el queso durante su proceso de elaboración, cumpliendo así con los objetivos de seguridad alimentaria.

ESTADO DE LA TÉCNICA

El estilo de vida actual y la demanda de los consumidores han conducido a la producción masiva de alimentos listos para su consumo (RTE, del inglés *ready to eat*). Entre estos productos se encuentran alimentos madurados tradicionales de amplio arraigo como el queso madurado, elaborado con leche cruda con Denominación de Origen Protegida (D.O.P.) (Torta del Casar [Reglamento CE 1491/2003], Queso de la Serena [Reglamento CE 1107/1996] y Queso de los Ibores [Reglamento CE 205/2005]). Se trata de alimentos con procesos de maduración cortos (en general no superior a 2 meses), que no garantizan la eliminación de microorganismos patógenos que pueden llegar a las materias primas o al producto durante el proceso de elaboración.

Listeria monocytogenes es posiblemente el microorganismo patógeno que más preocupa, tanto a la industria alimentaria como a las autoridades sanitarias, siendo el objetivo principal de la higienización de alimentos RTE. Es una bacteria ubicua muy común en el ambiente de las industrias alimentarias e incluso en la maquinaria y utensilios insuficientemente desinfectados. Su alta persistencia en los ambientes de procesado se debe a su capacidad de formar biofilms o biopelículas, así como a su resistencia a la aplicación de desinfectantes. Puede multiplicarse en distintos tipos de alimentos, incluso cuando se almacenan a temperaturas de refrigeración (<5˚C) y tolera condiciones de estrés, como valores de pH bajos (4,4), presencia de nitritos, concentraciones de NaCl de hasta un 12% y valores relativamente bajos de actividad de agua (a_w) (0,92-0,95) (*Lado, B.H., Yousef, A.E. 2007. Listeria, listeriosis and food safety (3rd ed.) pp. 157-214, Boca Raton, FL: CRC Press*). A una temperatura de

4˚C crece en el producto RTE a una velocidad de aproximadamente 0,15-0,30 unidades logarítmicas/día, pudiendo multiplicarse por un factor de 10 si se produce un abuso (aumento incontrolado) moderado (2-4ºC) en la temperatura de almacenamiento, alcanzando niveles peligrosos en muy pocos días. La exposición del microorganismo patógeno a un tipo de estrés suave puede causar protección cruzada frente a otros factores de estrés, modificar su virulencia y dar lugar a cepas más persistentes.

L. monocytogenes causa listeriosis, una enfermedad de baja morbilidad comparada con la de otras enfermedades de origen alimentario, como las causadas por *Salmonella spp.* y *Campylobacter jejuni* (*CDC. 2013. Vital Signs: Listeria Illnesses, Deaths, and Outbreaks - United States, 2009–2011. MMWR 62, 448-452.; Autoridad Europea de Seguridad Alimentaria [EFSA] 2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. EFSA J. 13, 3991 [165 pp.]*). A pesar de su baja incidencia, la enfermedad se considera de gran importancia debido a su alta tasa de mortalidad en la Unión Europea. Durante 2019 (*EFSA, 2021. The European Union One Health 2019 Zoonoses Report. European Food Safety Authority. European Centre for Disease Prevention and Control. EFSA Journal 2021;19[2]:6406*), la mortalidad por listeriosis fue del 17,6%, 80 veces superior a Salmonellosis (0,22%) y más de 580 veces superior a Campilobacteriosis (0,03%). Además, hay que tener en cuenta que, en los grupos de riesgo, esta tasa de mortalidad puede llegar al 22-30% (*Gillespie, I.A., McLauchlin, J., Grant, K.A., Little, C.L., Mithani, V., Penman, C., Lane, C., Regan, M. 2006. Changing pattern of human listeriosis, England and Wales, 2001-2004. Emerg. Infect. Dis. 12, 1361-1366)* e incluso al 75% en individuos ancianos e inmunocomprometidos (*Gellin, B.G., Broome, C.V. 1989. Listeriosis. J. Am. Med. Assoc. 261, 1313-1320*). En EEUU, la listeriosis representa un 19% de las muertes causadas por patógenos alimentarios (*Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L. 2011. Foodborne illness acquired in the United States—major pathogens. Emerg. Infect Dis. 17, 7-15).* El 99% de los casos de listeriosis se asocian al consumo de alimentos contaminados con el patógeno, siendo los alimentos RTE los implicados en el mayor número de brotes (*FAO/WHO. 2004. Risk assessment of Listeria monocytogenes in ready-to-eat foods. Microbiological Risk Assessment Series 4 [78 pp].*). De ellos, los productos cárnicos y quesos RTE son una de las principales fuentes de listeriosis en Europa.

La listeriosis en la forma invasiva se manifiesta por fiebre súbita, mareos, abortos y encefalitis, meningitis y, finalmente, septicemia. *L. monocytogenes* también causa una enfermedad leve cuyos síntomas son parecidos a los de la gripe (fiebre, dolores musculares, náuseas). Puede afectar a individuos sanos, pero no es muy probable, ya que, dada su ubicuidad, es ingerida

regularmente con los alimentos sin producir síntomas de enfermedad. Si bien no se conoce la dosis infectiva, se estima que es elevada, aunque está relacionada con la virulencia de la cepa. De los 13 serotipos conocidos de *L. monocytogenes*, únicamente tres, los serotipos 1/2a, 1/2b y 4b, causan más del 90% de los casos de listeriosis en el hombre (*McLauchlin, J. 1990. Distribution of serovars of Listeria monocytogenes isolated from different categories of patients with listeriosis. Eur. J. Clin. Microbiol. Infect. Dis. 9, 210-213*; *Farber, J.M., Peterkin, P.I. 1991. Listeria monocytogenes, a food-borne pathogen. Microbiol. Rev. 55, 476-511*).

Los datos epidemiológicos indican que es muy improbable que menos de 100 UFC/g de *L. monocytogenes* puedan causar la enfermedad en la población general, aunque en individuos inmunocomprometidos, embarazadas, recién nacidos, ancianos, enfermos de SIDA o personas con tratamientos con inmunosupresores, la dosis infectiva se sitúa entre 100 a 1000 UFC/g, por lo que no sería necesaria la multiplicación del microorganismo en los alimentos.

El Reglamento CE 2073/2005 establece un máximo de 100 UFC/g durante la vida útil de los productos no destinados a la población de riesgo (lactantes y usos médicos especiales). Considera que pertenecen a esta categoría los productos RTE que tengan, o bien valores de pH ≤ 4,4, valores de a_w ≤0,92 o valores de pH ≤ 5,0 y a_w ≤ 0,94, así como aquellos productos que tengan una vida útil inferior a 5 días. En cambio, en EEUU, Japón o Corea, el criterio de seguridad alimentaria para *L. monocytogenes,* en alimentos RTE, es de ausencia de este patógeno en 25 gramos. Esta política supone una barrera comercial a la exportación de alimentos madurados tradicionales extremeños, dado que en estos países sería suficiente detectar la presencia de este patógeno para rechazar el producto.

La contaminación de alimentos madurados en las plantas de procesado se atribuye principalmente a cepas de *L. monocytogenes* presentes en el ambiente *(Chasseignaux, E., Gérault, P., Toquin, M.T., Salvat, G., Colin, P., Ermel, G. 2002. Ecology of Listeria monocytogenes in the environment of raw poultry meat and raw pork meat processing plants. FEMS Microbiol. Lett. 210, 271–275*.; *López, V., Villatoro, D., Ortiz, S., López, P., Navas, J., Martínez-Suárez, J.V. 2008. Molecular tracking of Listeria monocytogenes in an Iberian pig abattoir and processing plant. Meat Sci. 78 130–134*.). Algunos subtipos de *L. monocytogenes* pueden persistir durante meses o años en plantas de procesado de alimentos (*Wulff, G., Gram, L., Ahrens, P., Vogel, B.F. 2006. One group of genetically similar Listeria monocytogenes strains frequently dominates and persists in several fish slaughter- and smokehouses. Appl. Environ. Microbiol. 72, 4313-4322*.), y parecen ser característicos de cada planta (*Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., Corby, J., Wiedmann, M. 2007. Pulsed-field gel electrophoresis (PFGE) analysis of temporally matched Listeria*

monocytogenes isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source-associated as well as widely distributed PFGE types. J. Clin. Microbiol. 45, 865-873.). Estas cepas no se inactivan con el descenso de la a_w que se produce durante la maduración, lo que obliga a establecer medidas de control que actúen sinérgicamente.

Entre las posibles estrategias están la utilización de tecnologías no térmicas, como altas presiones hidrostáticas y electrones acelerados (*Bajovic, B., Bolumar, T., Heinz, V. 2012. Quality considerations with high pressure processing of fresh and value added meat products. Meat Sci. 92, 280-289.*; *Cabeza, M.C., Cambero, I., de la Hoz, L., Ordóñez, J.A. 2007. Optimization of the E-beam irradiation treatment to eliminate Listeria monocytogenes from ready-to-eat (RTE) cooked ham. Inn. Food Sci. Emerg. Technol. 8, 299-305; Cabeza, M.C., de la Hoz, L., Ordóñez, J.A., Cambero, M.I. 2009. Safety and quality of ready-to-eat dry fermented sausages subjected to E-beam radiation. Meat Sci. 83, 320-327.*; *Hoz, L., Cambero, I., Cabeza, M.C., Herrero, A.M., Ordóñez, J.A. 2008. Elimination of Listeria monocytogenes from vacuum-packed dry-cured ham by E-beam irradiation. J. Food Prot. 71, 2001-2006.*; *Velasco, R., Ordóñez, J.A., Cambero, M.I., Cabeza, M.C. 2015. Use of E-beam radiation to eliminate Listeria monocytogenes from surface mould cheese. Int. Microbiol.18, 33-40.*, *Bover-Cid, S., Belletti, N., Aymerich, T., Garriga, M. 2015. Modeling the protective effect of aw and fat content on the high pressure resistance of Listeria monocytogenes in dry-cured ham. Food Res. Int. 75, 194–199*). Si bien estas tecnologías han mostrado ser eficaces en la inactivación de *L. monocytogenes* en alimentos, su aplicación en industrias pequeñas, que elaboran productos madurados tradicionales, plantea problemas económicos y de infraestructura, dado que necesitan grandes equipos con un coste económico elevado. Además, su aplicación puede provocar cambios sensoriales en los productos e inactivar al resto de la población microbiana no patógena de estos alimentos madurados tradicionales, limitando así su posible efecto beneficioso, tanto en las características sensoriales, como en las propiedades funcionales del producto.

Una alternativa a las anteriores tecnologías es el uso de estrategias de biocontrol, basada en la utilización de cultivos protectores con actividad anti-*L. monocytogenes*. Dentro de los microorganismos que pueden ser utilizados como cultivos bioprotectores, lo más adecuado es utilizar aquellos que se encuentran de forma habitual en estos alimentos madurados debido a que tendrán una buena capacidad de adaptación y, además, su aplicación no debe suponer una modificación de las características sensoriales. Entre estos microorganismos se encuentran las bacterias ácido-lácticas y sus compuestos extracelulares, cuya eficacia se ha estudiado en diversos tipos de alimentos como derivados de carne *(Quinto, E.J., Marín, J.M.,*

Schaffner, D.W. 2016. Effect of the competitive growth of Lactobacillus sakei MN on the growth kinetics of Listeria monocytogenes Scott A in model meat gravy. Food Control 63, 34-45.), embutidos crudos-curados (*Raimondi, S., Popovic, M., Amaretti, A., Gioia, D.D., Rossi, M. 2014. Anti-Listeria Starters: In Vitro Selection and Production Plant Evaluation. J. Food Prot., 77, 837–842*; *Rubio R., Martín, B., Aymerich, T., Garriga, M. 2014. The potential probiotic Lactobacillus rhamnosus CTC1679 survives the passage through the gastrointestinal tract and its use as starter culture results in safe nutritionally enhanced fermented sausages. Int. J. Food Microbiol. 186, 55–60*; *Barbosa, M.S., Todorov, S.D., Ivanova, I., Chovert, J.M., Haertlé, T., Franco, B.D.G. 2015. Improving safety of salami by application of bacteriocins produced by an autochthonous Lactobacillus curvatus isolate. Food Microbiol. 46, 254-262*; *Kargozari, M., Emam-Djomeh, Z., Gandomi, H., Partovi, R., Ghasemlou, R., Revilla-Martín, I. 2015. Identification of selected Lactobacillus strains isolated from Siahmazgi cheese and study on their behavior after inoculation in fermented-sausage model medium. LWT - Food Sci. Technol. 62, 1177-1183*) y quesos (*Arqués, J.L., Rodríguez, E., Gaya, P., Medina, M., Nuñez, M. 2005. Effect of combinations of high-pressure treatment and bacteriocin-producing lactic acid bacteria on the survival of Listeria monocytogenes in raw milk cheese. Int. Dairy J. 15, 893-900.*; *Valero, A., Hernandez, M., De Cesare, A., Manfreda, G., González-García, P., Rodríguez-Lázaro, D. 2014. Survival kinetics of Listeria monocytogenes on raw sheep milk cured cheese under different storage temperatures. Int. J. Food Microbiol. 184, 39-44.*; Morandi, S., Silvetti, T*., Miranda Lopez, J.M., Brasca, M. 2015. Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk valtellina casera cheese. J. Food Safety, 35, 193-205).* Además de los anteriores autores, otros investigadores han aislado bacterias ácido-lácticas de embutidos tradicionales de Extremadura y del queso Torta del Casar, con el objetivo de seleccionar cultivos probióticos (*Fernández, M., Ruiz-Moyano, S., Benito, M.J., Martín, A., Hernández, A., Córdoba, M.G. 2016. Potential antimicrobial and antiproliferative activities of autochthonous starter cultures and protease EPg222 in dry-fermented sausages. Food Funct. 7, 2320-2330.*). También se ha evaluado su actividad frente a microorganismos patógenos en medios de cultivo (*Casquete, R., Benito, M.J., Martín, A., Ruiz-Moyano, S., Aranda, E., Córdoba, M.G. 2012. Use of autochthonous Pediococcus acidilactici and Staphylococcus vitulus starter cultures in the production of "chorizo" in 2 different traditional industries. J. Food Sci. 71, M-70-M79*), habiéndose propuesto la utilización de algunas de las cepas seleccionadas, pero fundamentalmente como cultivos probióticos.

Si bien en quesos madurados se utilizan cepas de bacterias acido-lácticas comerciales como cultivos iniciadores, no existe en el mercado ninguna cepa con actividad frente a *L. monocytogenes* probada en quesos madurados.

BREVE DESCRIPCIÓN DE LAS FIGURAS

Figura 1. Reducción de *L. monocytogenes* durante la maduración de los quesos con respecto a los dos niveles, alta (7 log [10^7] UFC/g) y baja (4 log [10^4] UFC/g), de inoculación de esta bacteria patógena al día 0 de maduración. Control B1 (control con sólo *L. monocytogenes* con baja inoculación), B1+116 (inoculado con *L. monocytogenes* con bajo nivel de inoculación y *L. casei 116*), Control A1 (control de *L. monocytogenes* con alto nivel de inoculación), A1+116 (inoculado con *L. monocytogenes* con alto nivel de inoculación y *L. casei 116*). Barras con diferentes letras indican diferencias significativas ($p \leq 0,05$) entre lotes.

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

En respuesta a las necesidades planteadas en el estado de la técnica, los autores de la presente invención han logrado aislar una nueva cepa de la especie *Lacticaseibacillus casei* con efecto protector en la elaboración de quesos madurados para así cumplir con los objetivos de seguridad alimentaria respecto a *Listeria monocytogenes*.

Así, en un aspecto principal de la invención se contempla una nueva cepa de bacteria del género *Lacticaseibacillus*, denominada *Lacticaseibacillus casei 116* (en adelante *L. casei* 116), depositada con el número CECT 30488 en la institución Colección Española de Cultivos Tipo, depósito realizado de acuerdo con los términos del Tratado de Budapest. La cepa *L. casei* 116 pertenece a una especie bacteriana categorizada por la EFSA como QPS (presunción cualificada de seguridad), (*EFSA, 2021. Updated list of QPS status recommended biological agents in support of EFSA risk assessments. EFSA Journal 2021;19[7]:6689.*)

En un segundo aspecto principal de la invención se contempla una composición que comprende un cultivo microbiano biológicamente puro de la cepa *L. casei 116*, depositada con el número CECT 30488, o cualquier producto o microorganismo derivado de dicho cultivo, incluso mezclado con otros microorganismos, y un vehículo para su dispersión y/o multiplicación.

El término *"vehículo"* se refiere a cualquier sustancia o diluyente inocuo, autorizado para la elaboración de complementos alimenticios, que no provoca irritación significativa a las plantas o a los productos agrícolas, y no anula la actividad biológica y las propiedades del ingrediente activo administrado. Como tal, el término incluye específicamente, pero no se limita a, soluciones y dispersiones acuosas, tales como medios de cultivo, polvos inertes, y disolventes inertes (por ejemplo, agua).

La utilización de *L. casei 116* como cultivo protector permite cumplir con el criterio microbiológico establecido para quesos madurados en el Reglamento (CE) n° 1441/2007 de la Comisión, de 5 de diciembre de 2007, que modifica el Reglamento (CE) n° 2073/2005 relativo a los criterios microbiológicos aplicables a los productos alimenticios. De esta forma la aplicación como cultivo protector de esta nueva cepa de bacteria ácido-láctica permite conseguir los objetivos de seguridad alimentaria en quesos madurados.

Así, en un otro aspecto principal de la invención, se contempla el empleo de la cepa L. casei 116, o de la composición que comprende la cepa *L. casei 116,* como cultivo protector frente a *L. monocytogenes* en la elaboración de quesos madurados, para poder cumplir así los objetivos de seguridad alimentaria respecto a este patógeno.

La aplicación de la cepa de la invención sobre los quesos madurados no afecta a sus parámetros físico-químicos ni sensoriales.

Los ejemplos que siguen a continuación ilustran la presente invención e indican como se ha obtenido y seleccionado la cepa de *L. casei 116*, pero no deben ser considerados como limitaciones a los aspectos esenciales del objeto de la misma, tal como han sido expuestos en los apartados anteriores de esta descripción.

Ejemplo 1

Procedimiento para el aislamiento y selección de la nueva cepa de L. casei 116

1. Cultivo y aislamiento de bacterias lácticas a partir de quesos elaborados con leche cruda de oveja de las D.O.P Torta del Casar, quesos de la serena y quesos de Ibores. Los aislamientos de cepas de bacterias lácticas se realizaron en distintas fases de maduración y diferentes industrias. Tras realizar diluciones decimales seriadas de las muestras de quesos, éstas se inocularon en agar Man Rogosa Sharpe (MRS) (Oxoid, Reino Unido) y se incubaron en condiciones de microaerofilia a 37ºC durante 48 h. A continuación, los aislados se mantuvieron a -80ºC en caldo MRS que contenía 40% de glicerol.

2. Evaluación de la actividad anti-L. monocytogenes mediante el método de difusión en agar en el medio BHI (*Budde, B.B., Hornbaek, T., Jacobsen, T., Barkholt, V., Koch, A.G. 2003. Leuconoctoc carnosus 4010 has the potential for use as a pretective culture for vacuum-packet meats: culture isolation, bacteriocin identification, and meat application experiments. Int. J. Food microbiol. 83, 171-184*). Partiendo del cultivo de BAL incubado durante 24h a 30ºC, se inocularon 10 µl sobre la superficie de agar BHI (Conda, España) y posteriormente, estas

placas se cubrieron con 10 ml de agar BHI que contenían 10^6 UFC/ ml de L. monocytogenes. La actividad anti-Listeria se determinó mediante la observación de zonas claras de inhibición alrededor de la mancha de la bacteria ácido-láctica. Las cepas que mostraron actividad se seleccionaron para una segunda evaluación de actividad anti-*L. monocytogenes* en agar queso, elaborado siguiendo el procedimiento descrito por Gori y col. (*Gori, K., Mortensen, H.D., Arneborg, N., Jespersen, L., 2007. Ammonia production and its possible role as a mediator of communication for Debaryomyces hansenii and other cheese-relevant yeast species. J. Dairy Sci. 90, 5032–5041*). Para ello, se inocularon conjuntamente *L. monocytogenes* y cada una de las bacterias lácticas seleccionadas a una concentración de 10^3 UFC/cm^2. Se seleccionó un aislado de bacterias lácticas con alta actividad frente a *L. monocytogenes.*

3. Este aislado fue caracterizado mediante métodos moleculares. En primer lugar, se llevó a cabo el análisis de secuenciación de la región 16S del ARNr para la identificación a nivel de especie. Este método ha sido utilizado con éxito en la identificación de bacterias ácido-lácticas de interés alimentario. Concretamente se ha empleado para la identificación de bacterias aisladas de diferentes tipos de alimentos (*Benito, M.J., Serradilla, M.J., Ruiz-Moyano, S., Martín, A., Pérez-Nevado, F., Córdoba, M.G. 2008. Rapid differentiation of lactic acid bacteria from autochthonous fermentation of Iberian dry- fermented sausages. 80(3): 656-61*). La amplificación de esta región permite la identificación de las bacterias a nivel de especie, e incluso permite ver variaciones dentro de una misma especie. También se llevó a cabo la tipificación mediante electroforesis en campo pulsante y se determinaron los genes de producción de bacteriocinas. El aislado con elevada actividad anti-*L. monocytogenes* fue caracterizado por los métodos anteriores como *L. casei 116*. Finalmente se evaluó el efecto anti-*L. monocytogenes* de la cepa caracterizada *L. casei 116* en quesos de pasta blanda durante la maduración.

4. *L. casei 116* provocó una reducción de *L. monocytogenes* durante la maduración de 2 log (10^2) UFC/g y de hasta 5 log (10^5) UFC/g actuando sinérgicamente *L. casei 116* con la reducción de la a_w y de pH de los quesos.

Ejemplo 2

Efecto de L. casei 116 en queso de pasta blanda "Torta del Casar" durante el proceso de maduración.

Se inoculó esta cepa en cuajada a niveles de aproximadamente 10^6 UFC/g al inicio de la maduración junto con dos niveles de inoculación de *L. monocytogenes*: alta (lote AI, nivel aproximado 10^7 UFC/g) y baja (lote BI, nivel aproximado 10^4 UFC/g). Se dejó madurar durante 90 días variando las condiciones de humedad y temperatura como sigue:

- 6ºC y 90% de humedad; 35 días
- 8ºC y 80% de humedad; 10 días
- 9ºC y 80% de humedad; 10 días
- 10ºC y 80% de humedad; 35 días

Tabla 1. Parámetros físico-químicos del lote control y el lote inoculado con L. casei 116 a lo largo del proceso de maduración de los quesos

	Lotes	Días de Maduración				
		0	30	45	60	90
pH	Control	5,34 ± 0,021^{3}	5,20 ± 0,118 ^{2,3}	5,44 ± 0,091 ^{2}	5,65 ± 0,063^{b,1}	5,73 ± 0,057^{b,1}
	L. casei 116	5,36 ± 0,008^{3,4}	5,24 ± 0,054 ^{4}	5,45 ± 0,122 ^{3}	5,77 ± 0,043^{a,2}	6,04 ± 0,098^{a,1}
a_w	Control	0,975 ± 0,003^{1}	0,968 ± 0,007 ^{1,2}	0,962 ± 0,003 ^{2}	0,966 ± 0,003 ^{2}	0,943 ± 0,005 ^{3}
	L. casei 116	0,974 ± 0,001^{1}	0,968 ± 0,003 ^{1,2}	0,964 ± 0,002 ^{2}	0,967 ± 0,001 ^{2}	0,943 ± 0,004 ^{3}
Humedad (%)	Control	88,68 ± 2,125 ^{1}	49,70 ± 1,263 ^{2}	47,29 ± 0,468 ^{2,3}	44,79 ± 1,611 ^{3}	37,64 ± 2,981 ^{4}
	L. casei 116	88,21 ± 3,128 ^{1}	48,05 ± 0,903^{2}	46,32 ± 1,493 ^{2,3}	45,54 ± 2,341 ^{3}	38,17 ± 1,766 ^{4}

Se evaluaron los parámetros físico-químicos (humedad, a_w, pH y concentración de NaCl) durante la maduración. También se evaluaron los parámetros microbiológicos, así como los niveles de *L. monocytogenes* y de bacterias ácido-lácticas durante la maduración del queso. Para ello, se maduraron lotes control sin inoculación de cualquier microorganismo y con la inoculación *L. casei 116*. Los parámetros físico-químicos en los lotes inoculados sólo con *L. casei 116* evolucionaron igual que en los lotes control sin esta bacteria ácida-láctica, observándose que los quesos madurados con esta bacteria láctica tienes igual o menor acidez que los quesos control sin *L. casei* 116 (Tabla 1).

La inoculación de la bacteria láctica seleccionada no supuso ninguna modificación con respecto al control sin inocular en el contenido acuso ni en la actividad de agua. No se observaron tampoco diferencias en cuanto a los parámetros sensoriales en los lotes inoculados con *L. casei 116* y los controles sin inocular (Tabla 2).

Tabla 2. Parámetros de textura y color del lote control y el lote inoculado con *L. casei* 116 al final del procesado de los quesos

Parámetros	Lotes	
	Control	*L. casei* 116
Dureza (N)	5,05 ± 2,663	5,18 ± 1,579
Adhesividad (N/s)	-0,36 ± 0,192 [a]	-0,55 ± 0,312[b]
Elasticidad	0,74 ± 0,052	0,76 ± 0,068
Cohesión	0,64 ± 0,049	0,64 ± 0,033
Masticabilidad (N)	2,66 ± 0,831	2,57 ± 0,885
CIE L*	101,09 ± 6,250	100,51 ± 2,730
CIE a*	-1,43 ± 0,859	-1,26 ± 0,920
CIE b*	5,49 ± 2,510	6,26 ± 1,190

Diferentes letras indican diferencias significativas (p≤0,05) entre lotes.

Sin embargo, en relación con el control de *L. monocytogenes* se observó que en los lotes inoculados con *L. casei 116* hubo una reducción en los recuentos de esta bacteria patógena, que variaba de 2 log (10^2) UFC/g hasta 5 log (10^5) UFC/g, cuando *L. casei 116* actúa sinérgicamente junto con la reducción de la a_w y de pH que tiene lugar durante la maduración (Figura 1). Esto supone que, en los casos en los que se produzca, durante la elaboración del queso, una contaminación de *L. monocytogenes* a niveles inferiores a 100 UFC/g (niveles permitidos en la Unión Europea en alimentos RTE de acuerdo al reglamento CE 2073/2005), la adición de *L. casei* 116 podría eliminar a *L. monocytogenes* permitiendo cumplir el criterio de seguridad alimentaria de ausencia de esta bacteria patógena.

REIVINDICACIONES

1. Cepa 116 de *Lacticaseibacillus casei* con número de depósito CECT30488.

2. Composición que comprende un cultivo biológicamente puro de la cepa de la reivindicación 1, o cualquier producto o mezcla de microorganismos derivado del mismo.

3. Composición, según la reivindicación 2, que además comprende un vehículo para su dispersión y/o multiplicación.

4. Uso de la cepa, según la reivindicación 1, o de una composición, según la reivindicación 2 o 3, como cultivo protector en la elaboración de quesos madurados frente a *Listeria monocytogenes*.

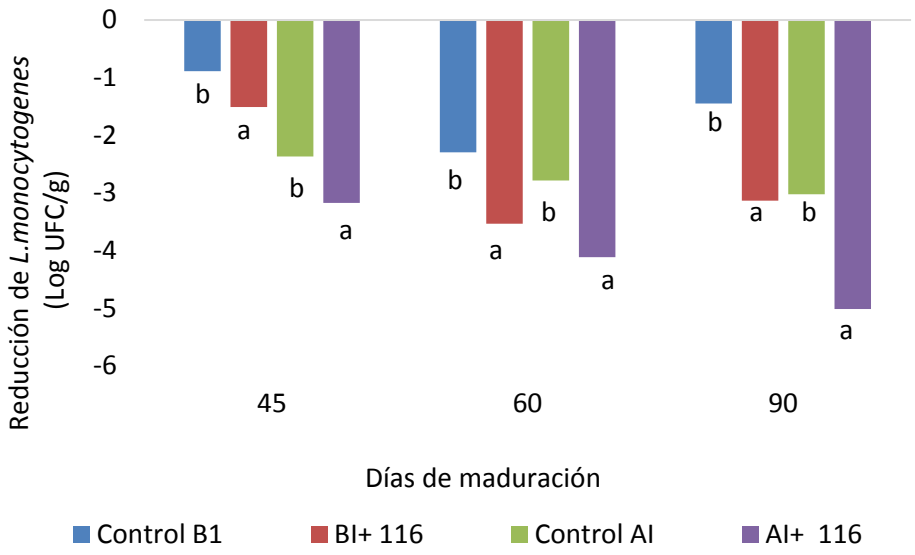
0
-1
-2
-3
-4
-5
-6
Reducción de L.monocytogenes (Log UFC/g)
b
a
b
a
b
a
b
a
b
a
b
a
45
60
90
Días de maduración
Control B1
BI+ 116
Control AI
AI+ 116

FIGURA 1

RESUMEN

Nueva cepa de *lacticaseibacillus casei* 116 con actividad antagonista frente a *listeria monocytogenes* para su uso como cultivo protector en quesos madurados

La presente invención se refiere a una nueva cepa de *Lacticaseibacillus casei* con número de depósito CECT 30488. Asimismo, se refiere a una composición que comprende dicha cepa y a su empleo como cultivo protector en la elaboración de quesos madurados frente a *L. monocytogenes*.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO Universidad de Extremadura Avda. de Elvas, s/n 06006 Badajoz Spain NAME AND ADDRESS OF DEPOSITOR	RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the 116	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CECT 30488

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION
The microorganism identified under I was accompanied by: ☒ a scientific description ☒ a proposed taxonomic designation (Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 9th November 2021 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: COLECCIÓN ESPAÑOLA DE CULTIVOS TIPO (CECT). Address: Edificio 3 CUE. Parc Científic Universitat de Valencia Catedrático Agustín Escardino, 9 46980 Paterna (Valencia) ESPAÑA	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 16 Nov 2021 Mari Carmen Macián Rovira, PhD

[1]Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO Universidad de Extremadura Avda. de Elvas, s/n 06006 Badajoz Spain NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS MADE	VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page
I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Universidad de Extremadura Avda. de Elvas, s/n 06006 Badajoz Spain	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CECT 30488 Date of the deposit or of the transfer[1]: 2021-11-09
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2021-11-12[2]. On that date the said microorganism was ☒ [3] viable ☐ [3] no longer viable	

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

Form BP/9 (first page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: COLECCIÓN ESPAÑOLA DE CULTIVOS TIPO (CECT). Address: Edificio 3 CUE. Parc Científic Universitat de Valencia Catedrático Agustín Escardino, 9 46980 Paterna (Valencia) ESPAÑA	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2021-11-16 MariCarmen Macián Rovira, PhD.

[4] Fill in if the information has been requested and if the results of the test were negative.

IV.4. CARACTERIZACIÓN DE CEPAS DE BACTERIAS ÁCIDO-LÁCTICAS SELECCIONADAS

Food Control (2022). Enviado para su publicación

Biopreservative potential of lactic acid bacteria isolated from fermented foods

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Declarations of interest: none

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Abstract

In this study, the antimicrobial activity of lactic acid bacteria (LAB) isolated from dry-cured fermented sausages and ripened cheeses against various foodborne pathogens was evaluated and the bacteriocins produced by the selected LAB of interest were characterized. For this, 33 bacteria were initially selected for their ability to produce proteinaceous compounds with antimicrobial activity. Subsequently, only three isolates (*Lacticaseibacillus casei* 116, *Lactococcus garviae* 151 and *Latilactobacillus sakei* 205) were selected since they did not have virulence factors or were resistant to antibiotics. Due to the greater bacteriocin activity spectrum, *Lb. sakei* 205 was selected and its bacteriocin characterized. The highest level of activity (6400 AU/mL) was observed against *Enterococcus faecalis* ATCC 29212 during growth of *Lb. sakei* 205 for 18-20 h. The maximum activity of the bacteriocin against *Listeria monocytogenes* NCTC 11994 and *L. monocytogenes* EDG-e (3200 AU/ml) was reached after 18 h and for *L. monocytogenes* ScottA (3200 AU/mL) after 20 h. The activity of *Lb. sakei* 205 bacteriocin remained stable at temperatures ranging from 25 ºC to 60 ºC, at pH values between 4.0 and 6.0. The bacteriocin was sensitive to all the detergents and totally inactivated with the proteolytic enzyme proteinase K, confirming its proteinaceous nature. The bacteriocin of *Lb. sakei* 205, with an approximate molecular size of 24 kDa, also affected the viability of all the studied pathogens and repressed their growth for at least 12 h.

The identified properties of *Lb. sakei* 205 strain and its produced bacteriocin indicate its potential application as a biopreservative in the food industry.

Keywords: Bacteriocin, Biopreservation, Dry-cured fermented sausage, Lactic acid bacteria, *Latilactobacillus sakei*, pediocin PA-1

1. INTRODUCTION

Lactic acid bacteria (LAB) constitute a microbial group naturally present in the autochthonous microbiota of dry-cured sausages and ripening cheeses (Prpich et al., 2021). Food industries are particularly interested in this bacterial group due to their technological properties, being often used as starter cultures to produce fermented products (Perin et al., 2014; Talon & Leroy, 2014). Furthermore, given their long history of consumption in fermented foods, LAB have been considered to have the status of ‘‘generally regarded as safe” (GRAS) by the American Food and Drug Administration (Silva et al., 2018).

Fermented products such as dry-cured fermented sausages and traditional soft cheeses present a complex microbiota (Talon & Leroy, 2014). Lactic acid bacteria are responsible for the taste, aroma, texture and microbial stability of these products, leading to several studies to characterise their technological properties to select possible starter cultures (Prpich et al., 2021). Furthermore, biopreservation using LAB and/or their antimicrobial metabolites represents an alternative for controlling foodborne pathogens and improving food safety (Ananou et al., 2007; Castellano et al., 2008). These antimicrobial properties of LAB are derived from competition for nutrients and/or space and the production of antimicrobial active metabolites such as organic acids (mainly lactic and acetic acid), diacetyl, hydrogen peroxide, and other compounds, such as bacteriocins and antifungal peptides (Kavitha et al., 2020; Pinto et al., 2020; Reis et al., 2012). Indigenous LAB from fermented products are considered a potential source of bacteriocins, being these products often studied to isolate and identify strains capable of producing different bacteriocins with different antimicrobial potential (Albano et al., 2009; Joana Barbosa et al., 2014; Bassi et al., 2015). Many authors have studied the use of LAB or their metabolites in dry-cured fermented sausages and traditional soft cheeses against several

Listeria monocytogenes strains (Golmoradi Zadeh et al., 2022; Gonzales-Barron et al., 2020; Martín et al., 2021) since this is one of the most worrying microorganisms in the meat and dairy industries (Mazaheri et al., 2021).

Despite their GRAS status, some LAB can carry virulence genes and express them in food products, which poses a risk for the consumers (Nieto-Arribas et al., 2011). Moreover, LAB can also present resistance to distinct antibiotics and carry genes related to this characteristic, enhancing their virulence potential (Ammor et al., 2007). For these reasons, the safety aspects of novel LAB strains to be proposed as protective cultures need to be carefully examined, and the possibility of delivering virulence factors should be excluded (Todorov et al., 2017a).

The objective of this work was to determine the antimicrobial activity of LAB isolated from dry-cured fermented sausages (182) and ripened cheeses (189) against several foodborne pathogens, mainly *L. monocytogenes*, and characterize the bacteriocins produced by the selected LAB.

2. MATERIALS AND METHODS

2.1. Microorganism and growth conditions

In this study, 371 LAB isolates previously isolated from dry-cured fermented sausages and ripened cheeses, belonging to the culture collection of the Food Hygiene and Safety group (Universidad de Extremadura) were used. Each LAB isolate was grown on de Man, Rogosa and Sharpe (MRS) agar (Lab M, UK) at 30 ºC for 48 h.

To test the inhibitory effects of LAB isolates, seven *L. monocytogenes* (FSL N1-227, FSL N3-013, FSL R2-499, FSL J1-031, FSL J1-177, MF 4077, 2542) belonging to the culture collection of *Escola Superior de Biotecnologia* (ESB), and three strains belonging to other important foodborne pathogens and taken from the American Type Culture

Collection (ATTC; *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25213 and *Escherichia coli* ATCC 25922), were used as targets. All the targets were grown on TSAYE- Trypticase Soy Agar (TSA, Pronadisa, Spain) with 6 g/L of yeast extract (YE, Lab M) at 37 ºC for 24 h.

2.2. Screening of antimicrobial activity

Each target microorganism was grown in TSBYE (Trypticase Soy Broth with 6 g/L of yeast extract) and spread on TSAYE. To determine the nature of the inhibition, culture broths (C) of each LAB were centrifuged at 7000 rpm for 10 min at 4 °C (Hettich Zentrifugen, Rotina 35R, Germany), and cell- free- supernatants (CFS) were adjusted to pH 6.0 with sterile NaOH (1 M; Pronalab, Portugal) and treated at 80 ºC for 10 min (CFSn). To determine whether the inhibition was due to hydrogen peroxide production or to proteinaceous compounds, neutralised supernatant was treated for 1 h at 37 ºC with 0.1 mg/mL of catalase (500 IU/mL, sterile; CFSnC) and 0.1 mg/mL of proteinase K (CFSnCK), both from Sigma (Germany). Grown culture (C), CFS, CFSn, CFSnC, and CFSnCK were spotted on the lawns of targets and incubated overnight at 30 °C. Results were recorded as positive if a translucent halo zone was observed around the spot (Joana Barbosa et al., 2014). *Pediococcus acidilactici* HA- 6111- 2 was used as a positive control (Albano et al., 2009).

2.3. Presence of virulence factors

2.3.1. Screening-test of biogenic amines production

In this study, the production of tyramine, histamine, putrescine and cadaverine was evaluated according to the method developed by Bover-Cid and Holzapfel (1999). Each LAB isolate was subcultured seven times in MRS broth containing 0.1% (w/v) of each

precursor amino acid (all from Sigma Aldrich). These precursors were: tyrosine- free base for tyramine, histidine monohydrochloride for histamine, ornithine monohydrochloride for putrescine, and lysine monohydrochloride for cadaverine, and all were supplemented with 0.005% of pyridoxal- 5- phosphate (Fluka, Germany) to promote the enzyme induction. Then, isolates were spotted in duplicate on the medium with each amino acid and incubated for 4 days at 30 ºC. Plates without amino acid were used as a negative control. The results were positive when a purple colour appeared, or tyrosine precipitate disappeared, around the colonies.

2.3.2. Production of hydrolytic enzymes

Gelatinase activity was evaluated using the Modified Luria–Bertani (MLB, Sigma) broth supplemented with 50.0 g/L of gelatin. Tubes were incubated at 30 ºC for 7 days and then refrigerated for ≈ 30 min at 4 ºC. The results were considered gelatinase positive if the medium did not solidify after cooling (Tiago et al., 2004).

DNase activity was determined using a DNase agar medium (Pronadisa, Spain) with 0.05 g/L of methyl green (Sigma). The result was considered positive when after incubating the plates at 30 ºC for 48 h, a clear halo was observed around the colonies (Ben Omar et al., 2004).

All experiments were performed in duplicate, and *S. aureus* ATCC 25213 was used as a positive control.

2.3.3. Hemolytic activity

Hemolysin production was assessed by streaking isolates on Columbia Agar plates with 5% defibrinated sheep blood (Oxoid, UK). Plates were incubated at 30 ºC for 24 h after examining plates for hemolysis activity. Positive hemolytic activity was considered when

147 the presence of clear halos around the colonies occurred (β- hemolysis), and negative
148 hemolytic activity was considered when greenish zones (α- hemolysis) or the absence of
149 clear zones (γ- hemolysis) around the colonies were observed. *Staphylococcus aureus*
150 ATCC 25213 was used as a positive control.
151
152 *2.3.4. Presence of virulence genes*
153 The presence of virulence genes was investigated according to Barbosa et al. (2010) and
154 Perin et al. (2014): surface adhesin genes (*esp, ace, efaAfs* and *efaAfm*), aggregation
155 substance (*agg*), extracellular metalloendopeptidase gene (*gelE),* cytolytic activity (*cylA,*
156 *cylB, cylM, cylL$_L$* and *cylL$_S$),* vancomycin resistance genes *(vanA and vanB),*
157 hyaluronidase gene (*hly*), aggregation substance precursor (*asa1*) and genes related to
158 biogenic amines (*hdc1, tdc* and *odc*). The DNA of LAB isolates was extracted according
159 to the protocol for total DNA purification from Gram-positive bacteria of the GRS
160 genomic DNA kit-Bacteria (Grisp, Portugal). PCR amplifications were performed in a
161 ThermoCycler (Bio- Rad, USA) in 0.2 mL reaction tubes each with 25 μL of mixtures
162 using 0.5 mM of each primer, 0.1 mM of deoxynucleoside triphosphates (dNTP's,
163 ABGene, Surrey, UK), 1X of PCR Buffer, 2.5 mM of $MgCl_2$, 2U of Taq polymerase (all
164 from MBI Fermentas, France) and 100 ng/μL of DNA. The PCR program consisted of a
165 primary DNA denaturation step at 94 °C for 1 min, followed by 35 cycles of 1 min at 94
166 °C, 1 min at 55 °C, and 2 min at 72 °C, with an extension of the amplified product at 72
167 ºC for 7 min. After the last cycle, the products were cooled to 4 °C. The PCR products
168 were analysed by electrophoresis in 0.8% agarose gels with 1X Tris-Acetate-EDTA
169 buffer (TAE buffer; Bio- Rad). A negative control (sample without template) and a
170 positive control (sample with DNA from each strain according to the studied gene) were
171 included for each PCR reaction.

2.4. Antibiotic susceptibility testing

The antibiotics used were ampicillin and chloramphenicol (Fluka), erythromycin, tetracycline and gentamicin (all from Labesfal, Portugal), streptomycin and kanamycin (Sigma). The concentrations tested were based on the microbiological cut-off values established by the Panel on Additives and Products or Substances in Animal Feed (FEEDAP) and also by Clinical and Laboratory Standards Institute (CLSI, 2017), for enterococci. Microbiological cut- off values (μg/mL) were determined by the agar dilution method according to the CLSI (2012) for enterococci, and by broth microdilution method according to Klare et al. (2005) for the other LAB species. All isolates were grown in Müeller- Hinton Agar (MHA, BioMérieux, France) with no added antibiotic as a negative control. In addition, the quality control strain *E. faecalis* ATCC 29212 was used to monitor the accuracy of MICs (CLSI, 2012).

2.5. Bacteriocin activity spectrum

Antimicrobial activity by bacteriocin LAB-producing strains was screened against a large number of microorganisms (Table 1) according to the method described by Van Reenen et al. (1998). The bacteriocin activity was tested following the method described in section 2.2. Treated cell-free supernatant (CFSn) was screened against bacteria listed in Table 1. The confirmation of antimicrobial activity was assumed if a translucent halo zone was observed around the spots.

2.6. Characterization of the bacteriocin produced by *Lactilactobacillus sakei* 205

Due to the previous tests, the *Lb. sakei* 205 strain was selected.

2.6.1. Identification of genes encoding bacteriocin production

197 DNA from *Lb. sakei* 205 was tested for presence of genes encoding plantaricin NC8,
198 plantaricin S and plantaricin W according to the methodology proposed by Winkelströter
199 et al. (2015), nisin, lacticin A, lactococcin 972, lactococcin G and Q, lactococcin A and
200 B according to Ho et al. (2018), brevicin, plantaracin A, plantaracin EF and pediocin
201 according to Azizi et al. (2017).
202

203 *2.6.2. Maximum bacteriocin production (AU/mL) during growth of* Lb. sakei *205*

204 To determine the maximum bacteriocin production during its growth, 1% (v/v) of an
205 overnight *Lb. sakei* 205 culture was inoculated in MRS broth (100 mL) and incubated at
206 30 ºC. Samples were taken at regular intervals during 24 h of incubation. Changes in pH
207 and optical density (O.D., 600 nm) were recorded every hour and viable counts (Colony
208 Forming Units (CFU)/mL) and bacteriocin activity (AU/mL) against *L. monocytogenes*
209 ScottA, *L. monocytogenes* EDG-e, *L. monocytogenes* NCTC 11994 and *E. faecalis* ATCC
210 29212 were studied every 2 h, as described by Van Reenen et al. (1998). Two independent
211 replicates were performed.
212 To determine the bacteriocin activity (AU/mL), each treated CFSn was successively
213 diluted in sterile Ringer's solution (Lab M), and 10 µL aliquots of each dilution were
214 spotted onto a soft agar plate (BHI with 0.7% w/v agar) seeded with approximately 10^6
215 CFU/mL of each target *L. monocytogenes* strain and *E. faecalis*. Plates were incubated at
216 30 ºC from 24 h to 48 h. *Pediococcus acidilactici* HA-6111-2 was used as a control.
217 Antimicrobial activity was expressed as arbitrary units per mL (AU/mL), being AU the
218 reciprocal of the highest dilution showing a clear growth inhibition zone.
219

220 *2.6.3. Effect of temperature, enzymes, pH, and surfactants on bacteriocin activity*

Latilactobacillus sakei 205 was grown in MRS broth overnight at 30 ºC. Cells were harvested (8000 x g, 10 min, 4 ºC), and the CFS was adjusted to pH 6.5 with 1 M NaOH (CFSn). One millilitre of sterile CFSn was incubated for 2 h in the presence of proteinase K, trypsin, papain, pepsin, α-amylase and catalase (all from Boehringer Mannheim GmbH, Germany) at both 1 mg/mL and 0.1 mg/mL (final concentrations). The remaining antimicrobial activity was monitored by the agar spot test method. In a separate experiment, 1% (w/v) sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic (EDTA), Tween 20, Tween 80, urea, Triton X-100, Triton X-114, ox-bile, and NaCl were added to bacteriocin-containing CFSn. EDTA was added to CFSn in final concentrations of 1.0, 2.0 and 5.0 mM. Untreated CFSn and detergents, at the respective concentrations in water, were used as controls. All samples were incubated at 30 ºC for 5 h and then tested for antimicrobial activity. The effect of pH on bacteriocin activity was tested by adjusting the pH of sterile CFS from 2.0 to 12.0 (at increments of 2 pH units) with sterile 1M NaOH or 1M HCl. After 1 h of incubation at room temperature (25 ºC), samples were readjusted to pH 6.5 with sterile 1 M NaOH or 1 M HCl, heated to 80 ºC for 10 min, and tested for antimicrobial activity. The effect of temperature on bacteriocin activity was tested by incubating CFSn at 4, 25, 30, 37, 45, 60, 80, and 100 ºC for 120 min. Bacteriocin activity was also tested after 15 min at 121 ºC. Antimicrobial activity (expressed as AU/mL) was monitored for all conditions using the agar-spot test method (Van Reenen et al., 1998), and *L. monocytogenes* ScottA, *L. monocytogenes* EDG-e, *L. monocytogenes* NCTC 11994 and *E. faecalis* ATCC 29212, were used as target strains.

2.6.4. Cell Lysis

Twenty millilitres of the bacteriocin-containing cell-free supernatant (3200 AU/mL, pH 6.0 assayed on *L. monocytogenes* ScottA, *L. monocytogenes* EDG-e, *L. monocytogenes*

NCTC 11994 and 6400 AU/mL on *E. faecalis* ATCC 29212) was filter-sterilized and added to 100 mL early exponential phase of *L. monocytogenes* ScottA (5 h old; OD= 0.6), *L. monocytogenes* EDG-e (4 h old; OD=0.5), *L. monocytogenes* NCTC 11994 (6 h old; OD=0.4) and *E. faecalis* ATCC 29212 (4 h old; OD=0.6). Optical density readings at 600 nm were taken every hour for 12 h. Each target *L. monocytogenes* and *E. faecalis* cultures without added bacteriocins were used as controls, and two independent replicates were performed.

2.6.5. Adsorption studies, partial purification, and determination of the molecular size of Lb. sakei *205 bacteriocin*

Adsorption of *Lb. sakei* 205 bacteriocin was conducted according to the method described by Yang et al. (1992). Briefly, after pH adjusted to 6.0, bacteriocin producing cells (cultured for 15 h to 18 h at 30 ºC) were harvested by centrifugation (7000 rpm, 15 min, 4 ºC) and washed with sterile 0.1M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 mL of 100 mM NaCl (pH 2.0) and agitated for 1 h at 4 ºC to allow delaminating bacteriocin from the cells. Then, cells were harvested, and the cell-free supernatant was neutralised and tested for bacteriocin activity as described by Van Reenen et al. (1998). The supernatant from the first centrifugation was kept at 4 ºC for partial purification. Then, ammonium sulphate was added gradually to the stored supernatant to reach 60% and 80% of saturation, and each solution was kept at slow stirring for 4 h at 4 ºC. After centrifugation (12000 rpm, 20 min, 4 ºC), precipitated proteins in the pellet and floating on the surface were collected and dissolved in 25mM ammonium acetate buffer (pH 6.5) following the method described by Sambrook et al. (1989). All samples were stored at -20 ºC.

To determine the molecular size of *Lb. sakei* 205 bacteriocin, samples were separated by tricine-SDS-PAGE as described by Schägger and von Jagow (1987). A low molecular weight marker was used with sizes ranging from 6.5 kDa to 270 kDa (GRS Protein Marker PLUs; Grisp). Samples were added to the acrylamide gel in duplicate and, after running, the gel was split in two. One-half of the gels were fixed with 20% isopropanol and 10% acetic acid, and the other half was stained with Coomassie Brilliant Blue R250 (Bio-Rad, USA) to visualise the position of the peptide band and the other half was not stained and extensively pre-washed with the sterile distilled water to determine the position of the active bacteriocin. The non-stained gels were overlaid with 10^6 CFU/mL of *L. monocytogenes* ScottA, first embedded in BHI soft agar (0.7% agar w/v; Biokar) and incubated at 37 ºC for 24 h.

3. RESULTS AND DISCUSSION

3.1. Screening of antimicrobial activity

Many strains belonging to the LAB group are widely isolated from various fermented products such as dry-cured fermented sausages and semi and ripened cheeses (Campagnollo et al., 2018; A. A. T. De Carvalho et al., 2006; Garcia-Gonzalez et al., 2021; Panebianco et al., 2021). LAB improve the nutritional and technological characteristics of the products but also have the ability to produce antimicrobial substances that inhibit foodborne pathogens such as *L. monocytogenes*, *Bacillus cereus* and *S. aureus* (Kanwal et al., 2021).

From 371 LAB isolates from traditional dry-cured fermented sausages and ripened cheeses, 43 inhibited *L. monocytogenes*, *E. faecalis*, *S. aureus* and *E. coli* strains by cell-to-cell competition, eight inhibited by low pH and 33 inhibited by proteinaceous

295 compounds against *L. monocytogenes* and *E. faecalis*. These 33 isolates were selected for
296 further experiments (Table 2).
297
298 **3.2. Presence of virulence factors**
299 Safety is essential for bio-preservative cultures (Hossain et al., 2021). The capacity to
300 produce hydrolytic enzymes DNase and gelatinase was not found in any of the isolates
301 investigated. Similar results were reported for other LAB isolates of the same species
302 (Barbosa et al., 2010; Gómez et al., 2016; Hossain et al., 2021; Perin et al., 2014; Valledor
303 et al., 2022). Moreover, lack of hemolytic activity is a key demand (Li et al., 2020; Oh &
304 Jung, 2015). Of the 33 LAB isolates studied, none was β-hemolytic, 18 (54.5%) were α-
305 hemolytic, and 15 (45.5%) displayed no hemolysis (ɣ-hemolytic). Bermudez-Humaran
306 and Langella (2012) demonstrated that hemolytic activity is regarded as a disadvantage;
307 therefore, only the LAB isolates that did not show hemolysis (ɣ-hemolytic) were selected
308 for further studies.
309 Major amines found in high concentrations in fermented foods are histamine, tyramine,
310 putrescine, and cadaverine (Durak-Dados et al., 2020). However, none of the selected
311 LAB strains had the ability to produce histamine, putrescine, and cadaverine and only
312 two isolates (*Enterococcus faecium* 40 and *Lb. sakei* 175), could produce tyramine.
313 Bover-Cid and Holzapfel (1999) also observed that some strains of *E. faecium* and *Lb.*
314 *sakei* produced tyramine but not histamine, putrescine, and cadaverine. More recently,
315 Valledor et al. (2022) evaluated the safety of two *E. faecium* strains (*E. faecium* ST20Kc
316 and ST41Kc) and found that both produced tyramine; Mrkonjic Fuka et al. (2020) also
317 reported that 14.81% of the studied *Lb. sakei* strains (n=57) harboured the gene encoding
318 for a tyrosine decarboxylase (*tcd*). Histamine and tyramine are the biogenic amines most
319 studied in fermented foods and starter cultures due to their toxic effects caused by their

vasoactive and psychoactive properties (Bover-Cid & Holzapfel, 1999). For this reason, these two bacteria were not selected for further assays.

3.3. Presence of virulence genes

Screening for genes related to different virulence factors can also be used to discard strains from preliminary screening to avoid the application of potentially dangerous strains.

Despite their beneficial potential, the 13 tested strains presented a variable pattern of virulence-related genes: none of the strains presented evidence for harbouring *hyl*, *agg*, *esp*, *cylA*, *cylB*, *cylM*, *cylLS*, *ace*, *asa1*, *hdc* and *odc*, but other virulence-related genes evaluated in this study *gelE*, *EfaAfs*, *EfaAfm*, *cylLL* and *tcd* were detected at different frequencies (Table 3).

The presence of specific genes does not automatically translate into their expression and potential virulence of the strain that harbors this genetic material. For instance, the presence of the *gelE* gene may not be sufficient for gelatinase activity since the complete *fsr* operon seems to be essential for its expression. Lopes et al. (2006) found that all enterococci isolates with an incomplete *fsr* operon were unable to produce gelatinase, but 12 isolates with the complete *fsr* operon were gelatinase negative. According to de Castilho et al. (2019), the *fsr* operon seems to be easily damaged, deleted and lost mainly during the freezing of cells under laboratory conditions. As with the isolates in this study, Barbosa et al. (2010) also reported the presence of "silent" genes, i.e., isolates that carried the *gelE* gene but did not express it phenotypically (gelatinase production).

EfaA is a cell wall adhesin associated with endocarditis found in clinical and food enterococci isolates (Eaton & Gasson, 2001). Although antigen A proteins efaAfs and efaAfm are commonly found in *E. faecalis* and *E. faecium* isolates, respectively, other

345 authors have already reported the absence of the *efaAfs* gene in *E. faecalis* strains and
346 also found *E. faecalis* and other enterococcal species with the *efaAfm* gene (J. Barbosa et
347 al., 2010; Semedo et al., 2003). Similar results were found in the present study, with
348 *Enterococcus hirae* 15 and *Enterococcus durans* 159 harbouring both *efaAfs* and *efaAfm*
349 genes, as well as isolates belonging to other genera (*Lactiplantibacillus*,
350 *Lacticaseibacillus*, *Latilactobacillus*, and *Leuconostoc*).
351 Although γ-hemolytic, four isolates (*Lco. garviae* 151, *Lb. sakei* 197, *Lacticaseibacillus*
352 *casei* 31 and *Lacticaseibacillus paracasei* 185) harboured the *cylLL* gene. Despite the
353 association between β-hemolysis and the presence of the complete cyl operon, Semedo et
354 al. (2003) hypothesized that high levels of sequence divergence might exist in genes
355 encoding the structural subunits of cytolysin, which may prevent amplification. However,
356 the same authors also suggest that molecular screening of *cyl* genes should be performed
357 for non-hemolytic strains in order to assess their pathogenic potential.
358 In the present study, none of the isolates harboured the *hdc1* and *odc* genes (Table 3),
359 and, as previously mentioned, none had the ability to produce histamine and putrescine.
360 Furthermore, although most isolates showed the *tcd* gene associated with tyramine
361 production, only two isolates (*E. faecium* 40 and *Lb. sakei* 175) produced tyramine. Other
362 authors have reported a high number of enterococci and *L. sakei* strains that harboured
363 the *tcd* gene (Mrkonjic Fuka et al., 2020; Muñoz-Atienza et al., 2011; Todorov et al.,
364 2017b) and, in addition, also strains harbouring the *tcd* gene but not producing tyramine
365 (Bover-Cid et al., 2001; Muñoz-Atienza et al., 2011). These results emphasize the
366 importance of simultaneously studying both phenotypic and genotypic characteristics of
367 an isolate.
368
369 **3.4. Antibiotic susceptibility**

The minimal inhibitory concentrations (MICs, μg/mL) and antibiotic susceptibility of LAB isolates were evaluated according to the stated guidelines (CLSI, 2017; EFSA, 2012). The results are shown in Table 4.

In general, the isolates were sensitive to the antibiotics tested. Only *Enterococcus*, *Lcb. paracasei*, *Lpb. plantarum* and *Lc. mesenteroides* strains showed resistance to at least one antibiotic. Thus, only three isolates (*Lcb. casei* 116, *Lco. garviae* 151 and *Lb. sakei* 205) were sensitive to all the antibiotics tested (Table 4). Other authors also reported a low occurrence of antibiotic resistance by the same species (Mrkonjic Fuka et al., 2020; Valledor et al., 2022).

This susceptibility is an advantage, and according to FEEDAP Panel (EFSA, 2012), these three sensitive isolates are acceptable to be used as a feed additive. Consequently, only *Lcb. casei* 116, *Lco. garviae* 151 and *Lb. sakei* 205 were selected for further characterization.

3.5. Bacteriocin activity spectrum

The inhibitory activity of bacteriocins may vary given that some only inhibit taxonomically related Gram-positive bacteria, and others are active against a broader range of Gram-positive and Gram-negative microorganisms (Harzallah & Belhadj, 2013). Therefore, the activity of the three selected bacteriocin-producing LAB was screened against nine Gram-positive and six Gram-negative bacteria listed in Table 1.

All LAB isolates inhibited at least one of the *L. monocytogenes* strains studied. The neutralised supernatant of *Lb. sakei* 205 showed high anti-listerial activity, but it was also effective against other important microorganisms, such as *S.* Typhimurium ESB009. Also De Carvalho et al. (2010) found a *L. sakei* subsp. *sakei* strain able to inhibit *Listeria seeligeri*, *Listeria innocua* and *L. monocytogenes* ScottA, in addition to other

395 microorganisms such as *Staphylococcus epidermidis* and different enterococci. Most of
396 the bacteriocins described for *Lb. sakei* are active against a broad range of genera and
397 species (Todorov et al., 2011; Urso et al., 2006).
398 This significant advantage contributes to making this microorganism even more
399 appealing to the food industry. Due to its broad-spectrum antimicrobial activity, only *Lb.*
400 *sakei* 205 was selected and bacteriocin(s) produced by this strain were characterized. In
401 addition to the characteristics already mentioned, *Lb. sakei* 205 has been tested as a
402 biocontrol of *L. monocytogenes* in dry-cured fermented sausages production, and the
403 counts of this foodborne pathogen were significantly reduced (Martín et al., 2021). With
404 this, the use of *Lb. sakei* 205 appears to be of great interest for the safety of dry-cured
405 fermented sausages.
406

407 **3.5. Characterization of *Lb. sakei* 205-produced bacteriocin(s)**
408

409 *3.5.1. Identification of several genes encoding bacteriocin production*

410 In the test for identifying genes encoding bacteriocin(s) from *Lb. sakei* 205, none of the
411 target genes analyzed for brevicin, plantaricin A, plantaricin EF, plantaricin W,
412 plantaricin NC8, lactococcin 972, lactococcin G and Q, lactococcin A and B, or nisin
413 were found. However, amplicons of the corresponding size of pediocin PA-1 and
414 plantaricin S were generated. Other bacteriocin genes have been detected in other *Lb.*
415 *sakei* strains, such as curvacin, enterocin, sakacin (Martinez et al., 2015; Todorov et al.,
416 2011; Todorov et al., 2013), and carnocin (Li et al., 2022).
417 This preliminary screening does not exclude the need for PCR products sequencing or the
418 analysis of each strain by whole-genome sequencing for a deeper study.
419

3.5.2. Growth and bacteriocin production

Figure 1 shows the antimicrobial activity (AU/mL) of treated cell-free supernatant of *Lb. sakei* 205 against *L. monocytogenes* ScottA, EDG-e and NCTC 11994 and *E. faecalis* ATCC 29212 over time, as well the viable cell counts of *Lb. sakei* 205 and the pH changes of the solution.

The higher bacteriocin activity was observed against *E. faecalis* ATCC 29212 with 6400 AU/ml during growth of *Lb. sakei* 205 for 18-20 h. The maximum activity of the bacteriocin for *L. monocytogenes* NCTC 11994 and *L. monocytogenes* EDG-e (3200 AU/ml) was reached after 18 h and for *L. monocytogenes* ScottA (3200 AU/mL) after 20 h. Also, the changes in pH values decreased from 6.25 (at the beginning) to 4.8 (at the end of the screening), reaching its minimum (pH 4.68) after 22 h of growth. The number of viable cells of *Lb. sakei* 205 increased approximately 3 log CFU/mL (maximum cell growth at 16 h of growth). Regarding the characterization of other bacteriocins produced by other *Lb. sakei*-producing strains, Todorov et al. (2011) found a maximal bacteriocin production of 800 AU/mL against *L. innocua* F after 11 h of *Lb. sakei* R1333 growth and Todorov et al. (2013) reported the highest production of bacteriocin by *Lb. sakei* ST22Ch and ST153Ch strains of 1600 AU/mL after 19 h of growth and 800 AU/mL after 12 h of *Lb. sakei* ST154Ch growth against *E. faecium* ATCC 19433.

3.5.3. Effect of enzymes, temperature, pH, and surfactants on bacteriocin(s) activity

The impact of certain detergents, enzymes, pH, and temperature values on bacteriocin(s) activity against *L. monocytogenes* NCTC 11994, *L. monocytogenes* EDG-e, *L. monocytogenes* Scott A and *E. faecalis* ATCC 29212 is shown in Tables 5 and 6.

The activity of *Lb. sakei* 205 bacteriocin remained stable at temperatures ranging from 25 ºC to 60 ºC (Table 5). However, residual activity was observed at lower temperatures

(below 30 ºC) and higher temperatures (higher than 80 ºC). Similar results were observed for other bacteriocins produced by *Pediococcus* strains (Albano et al., 2007; Ramos et al., 2016), but not for those produced by *Lactobacillus* strains (Barbosa et al., 2021; Todorov et al., 2011; Todorov et al., 2013; Wen et al., 2016; Zhao et al., 2022).

It is possible to observe that the antimicrobial activity of *Lb. sakei* 205 bacteriocin was affected (50%-100% of reduction) at pH values below 2.0 and above 8.0, suggesting that the peptide is sensitive to acidic and alkaline conditions (Table 6). The bacteriocin remained stable after incubation for 1 h at pH 4.0 and 6.0 (0% reduction). Heredia-Castro et al. (2015) indicated that the antimicrobial activity of different LAB strains had a wide pH range (4.0-8.0). The acidification can result in proteins denaturation and also cause solubilization of metals in some LAB, being toxic at some concentrations (Papadimitriou et al., 2016), which confirms the data found. Although to less extent, also a reduction in antimicrobial activity of other bacteriocins, such as plantaricins and pediocins, have been reported by others (Albano et al., 2007; Barbosa et al., 2021; Ramos et al., 2016).

Lactilactobacillus sakei 205 bacteriocin was sensitive to all the detergents tested but remained active against *L. monocytogenes* NCTC 11994 and *L. monocytogenes* EDG-e after treatment with surfactants (Tween-20 and Tween-80). Similar results were observed against *L. monocytogenes* ScottA and *E. faecalis* ATCC 29212 since bacteriocin remained active after treatment with NaCl. However, almost a total loss of activity against the other tested strains was observed after treatment with all the detergents (Table 6). This could indicate a high sensitivity of the bacteriocin to protease inhibitors, corroborating the reported data for other LAB species (Benmouna et al., 2018). In fact, high inactivation of antimicrobial activity was observed after treatment with the proteolytic enzyme, proteinase K, confirming its proteinaceous nature (Table 6). Overall, less activity reduction was observed after treatment with other proteases tested (papain and pepsin)

and after the antioxidant enzyme activity (catalase). Other authors have been reporting the inhibition of several studied bacteriocins when treated with proteinase K (Albano et al., 2007; Barbosa et al., 2021; Oliveira et al., 2020; Ramos et al., 2016; Todorov et al., 2013) but also with pepsin, papain and catalase (Jiang et al., 2012; Ramos et al., 2016; Todorov et al., 2013).

3.5.4. Cell lysis

The effect of *Lb. sakei* 205 bacteriocin on the growth of *L. monocytogenes* ScottA, EDG-e and NCTC 11994, and *E. faecalis* ATCC 29212 is presented in Figure 2.

When the bacteriocinogenic *Lb. sakei* 205 supernatant was added to a mid-log culture (5h-old) of *L. monocytogenes* ScottA, a decrease of approximately 2 log CFU/mL was observed (Figure 2.a). Adding the proteinaceous substance against a mid-log culture of *L. monocytogenes* EDG-e (4h-old; Figure 2.b) and *L. monocytogenes* NCTC 11994 (6h-old; Figure 2.c), the cell growth of both pathogens was suppressed. However, the reduction was not so noticeable (approx. 1 log CFU/mL). On the contrary, when the supernatant was added to a mid-log culture (4h-old) of *E. faecalis* ATCC 29212, a great decrease (2.2 log CFU/mL) in the viability of this bacterium was observed (Figure 2.d). No change in cell number of all *L. monocytogenes* was recorded for untreated (control) samples. Although the more significant inhibition against *L. monocytogenes* ScottA and *E. faecalis* ATCC 29212, the treated cell-free supernatant of *Lb. sakei* 205 affected the viability of all the studied pathogens and repressed their growth for at least 12 h. These results were similar to those reported by Todorov et al. (2011) in which the numbers of *L. monocytogenes* and *E. faecium* were decreased after the addition of the bacteriocin (800 AU/mL) produced by *Lb. sakei* R1333. Also, Barbosa et al. (2021) reported a

decrease of approximately 2 log cycles of *L. monocytogenes* 7947 after adding the bacteriocin-containing cell-free supernatant (12800 AU/mL) of *Lpb. plantarum* R23.

3.5.5. Adsorption studies and molecular size determination

The bacteriocin did not adhere to the surface of producer cells since it was not detected after the treatment of *Lb. sakei* 205 with 100 mM NaCl at pH 2.0. According to tricine/SDS-PAGE, the approximate molecular size of this bacteriocin was 24 kDa (Figure 3), from the association between the position of the peptide band and the clear zone of growth inhibition of *L. monocytogenes* Scott A. This molecular size is higher than most bacteriocins previously described for *Lb sakei*. However, a molecular weight of around 14 kDa for *Lactobacillus pentosus* bacteriocin ST712BZ (Todorov & Dicks, 2007) and 17 kDa for pediocins (Papagianni & Anastasiadou, 2009; Ramos et al., 2016) have also been reported. As previously mentioned, although more tests are needed, these results may be in agreement with the amplicons of the corresponding size of pediocin PA-1 found.

4. CONCLUSION

Among 371 LAB isolates screened, 33 had the ability to produce proteinaceous compounds with antimicrobial activity against important foodborne pathogens, but only three (*Lc. casei* 116, *Lco. garviae* 151 and *Lb. sakei* 205) did not show virulence factors and were sensitive to all the antibiotics studied. *Latilactobacillus sakei* 205, isolated from dry-cured fermented sausage, was finally chosen due to its high bacteriocin activity spectrum. The bacteriocin produced by this strain has an approximately molecular size of 24 kDa and its activity remained stable at environmental factors. In addition, it inhibited the growth of important foodborne pathogens, such as *L. monocytogenes*, commonly

found in this type of products. It is essential to highlight the large activity spectrum of *Lb. sakei* 205 strain, especially against the Gram-negative foodborne pathogen *Salmonella*, which deserves to be addressed in future trials. All the mentioned factors indicate the potential effectiveness of *Lb. sakei* 205 strain and its produced bacteriocin as a biopreservative in the food industry.

Acknowledgements

The authors would like to thank the scientific collaboration under the *Fundação para a Ciência e a Tecnologia* (FCT) project UIDP/00329/2020. Also thank the projects INIA throughout the projects RTA-2017-00027-C03-03 and the Junta de Extremadura and FEDER by IB16149 and GR18056.

Funding

This work was supported by the project "Safe meat products" GO77 PDR2020-1.0.1-FEADER-031359. I. Martín is supported by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303). Financial support for author J. Barbosa was provided by a post-doctoral fellowship SFRH/BPD/113303/2015 (FCT).

Reference

Albano, H., Pinho, C., Leite, D., Barbosa, J., Silva, J., Carneiro, L., Magalhães, R., Hogg, T., & Teixeira, P. (2009). Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage. *Food Control*, *20*(8), 764–770. https://doi.org/10.1016/j.foodcont.2008.09.021

Albano, H., Todorov, S. D., van Reenen, C. A., Hogg, T., Dicks, L. M. T., & Teixeira, P. (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology*, *116*(2), 239–247. https://doi.org/10.1016/j.ijfoodmicro.2007.01.011

Ammor, M. S., Belén Flórez, A., & Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*, *24*(6), 559–570. https://doi.org/10.1016/j.fm.2006.11.001

Ananou, S., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (2007). Biopreservation, an ecological approach to improve the safety and shelf-life of foods. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 475–486.

Azizi, F., Habibi Najafi, M. B., & Edalatian Dovom, M. R. (2017). The biodiversity of *Lactobacillus* spp. from Iranian raw milk Motal cheese and antibacterial evaluation based on bacteriocin-encoding genes. *AMB Express*, *7*(1). https://doi.org/10.1186/s13568-017-0474-2

Barbosa, J., Gibbs, P. A., & Teixeira, P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control*, *21*(5), 651–656. https://doi.org/10.1016/j.foodcont.2009.10.002

Barbosa, J., Albano, H., Silva, B., Almeida, M. H., Nogueira, T., & Teixeira, P. (2021). Characterization of a *Lactiplantibacillus plantarum* R23 isolated from arugula by whole-genome sequencing and its bacteriocin production ability. *International Journal of Environmental Research and Public Health*, *18*(11).

https://doi.org/10.3390/ijerph18115515

Barbosa, J., Borges, S., & Teixeira, P. (2014). Selection of potential probiotic *Enterococcus faecium* isolated from Portuguese fermented food. *International Journal of Food Microbiology*, *191*, 144–148. https://doi.org/10.1016/j.ijfoodmicro.2014.09.009

Bassi, D., Puglisi, E., & Cocconcelli, P. S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Current Opinion in Food Science*, *2*, 118–122. https://doi.org/10.1016/j.cofs.2015.03.002

Ben Omar, N., Castro, A., Lucas, R., Abriouel, H., Yousif, N. M. K., Franz, C. M. A. P., Holzapfel, W. H., Pérez-Pulido, R., Martínez-Cañamero, M., & Gálvez, A. (2004). Functional and safety aspects of Enterococci isolated from different Spanish foods. *Systematic and Applied Microbiology*, *27*(1), 118–130. https://doi.org/10.1078/0723-2020-00248

Benmouna, Z., Dalache, F., Karam, N., & Zadi-Karam, H. (2018). Optimization and some characteristics of bacteriocin produced by *Enterococcus* sp. CM9 collected from Mauritanian Camel milk. *Emirates Journal of Food and Agriculture*, *30*(4), 275–282. https://doi.org/10.9755/ejfa.2018.v30.i4.1662

Bermudez-Humaran, L. G., & Langella, P. (2012). Importance of commensal and probiotic bacteria in human health. *Current Immunology Reviews*, *8*(3), 248–253. https://doi.org/10.2174/157339512800671994

Bover-Cid, S., & Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology*, *53*(1), 33–41. https://doi.org/10.1016/S0168-1605(99)00152-X

590 Campagnollo, F., Margalho, L., Kamimura, B., Feliciano, M., Freire, L., Lopes, L.,
591 Alvarenga, V., Cadavez, V., Gonzales-Barron, U., Schaffner, D., & Sant'Ana, A.
592 (2018). Selection of indigenous lactic acid bacteria presenting anti-listerial activity,
593 and their role in reducing the maturation period and assuring the safety of
594 traditional Brazilian cheeses. *Food Microbiology*, *73*, 288–297.
595 https://doi.org/10.1016/j.fm.2018.02.006

596 Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of
597 bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat
598 produced in Argentina. *Meat Science*, *79*(3), 483–499.
599 https://doi.org/10.1016/j.meatsci.2007.10.009

600 Clinical and Laboratory Standards Institute. (2012). Performance Standards for
601 Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement.
602 In *M100-S22* (Vol. 32, Issue 3).

603 CLSI - Clinical & Laboratory Standards Institute. (2017). *Performance Standards for*
604 *Antimicrobial Susceptibility Testing* (27th Editi).

605 De Carvalho, A. A. T., De Paula, R. A., Mantovani, H. C., & De Moraes, C. A. (2006).
606 Inhibition of *Listeria monocytogenes* by a lactic acid bacterium isolated from
607 Italian salami. *Food Microbiology*, *23*(3), 213–219.
608 https://doi.org/10.1016/j.fm.2005.05.009

609 De Carvalho, K. G., Bambirra, F. H. S., Kruger, M. F., Barbosa, M. S., Oliveira, J. S.,
610 Santos, A. M. C., Nicoli, J. R., Bemquerer, M. P., De Miranda, A., Salvucci, E. J.,
611 Sesma, F. J. M., & Franco, B. D. G. M. (2010). Antimicrobial compounds
612 produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated
613 from a Brazilian meat product. *Journal of Industrial Microbiology and*

Biotechnology, *37*(4), 381–390. https://doi.org/10.1007/s10295-009-0684-y

de Castilho, N. P. A., Nero, L. A., & Todorov, S. D. (2019). Molecular screening of beneficial and safety determinants from bacteriocinogenic lactic acid bacteria isolated from Brazilian artisanal calabresa. *Letters in Applied Microbiology*, *69*(3), 204–211. https://doi.org/10.1111/lam.13194

Durak-Dados, A., Michalski, M., & Osek, J. (2020). Histamine and other biogenic amines in food. *Journal of Veterinary Research (Poland)*, *64*(2), 281–288. https://doi.org/10.2478/jvetres-2020-0029

Eaton, T. J., & Gasson, M. J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology*, *67*(4), 1628–1635. https://doi.org/10.1128/AEM.67.4.1628-1635.2001

EFSA. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance by EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). *EFSA Journal*, *10*, 2740–2749. https://doi.org/10.2903/j.efsa.2012.2740

Garcia-Gonzalez, N., Battista, N., Prete, R., & Corsetti, A. (2021). Health-promoting role of *Lactiplantibacillus plantarum* isolated from fermented foods. *Microorganisms*, *9*(2), 1–30. https://doi.org/10.3390/microorganisms9020349

Golmoradi Zadeh, R., Asgharzadeh, S., Darbandi, A., Aliramezani, A., & Masjedian Jazi, F. (2022). Characterization of bacteriocins produced by *Lactobacillus* species against adhesion and invasion of *Listeria monocytogenes* isolated from different samples. *Microbial Pathogenesis*, *162*(November 2021), 105307. https://doi.org/10.1016/j.micpath.2021.105307

638 Gómez, N. C., Ramiro, J. M. P., Quecan, B. X. V., & de Melo Franco, B. D. G. (2016).
639 Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of
640 *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157: H7
641 biofilms formation. *Frontiers in Microbiology*, *7*(JUN), 1–15.
642 https://doi.org/10.3389/fmicb.2016.00863

643 Gonzales-Barron, U., Campagnollo, F. B., Schaffner, D. W., Sant'Ana, A. S., &
644 Cadavez, V. A. P. (2020). Behavior of *Listeria monocytogenes* in the presence or
645 not of intentionally- added lactic acid bacteria during ripening of artisanal Minas
646 semi-hard cheese. *Food Microbiology*, *91*(September 2019).
647 https://doi.org/10.1016/j.fm.2020.103545

648 Harzallah, D., & Belhadj, H. (2013). *Lactic acid bacteria as probiotics : Characteristics*
649 *, selection criteria and role in immunomodulation of human GI muccosal barrier*.

650 Heredia-Castro, P. Y., Méndez-Romero, J. I., Hernández-Mendoza, A., Acedo-Félix, E.,
651 González-Córdova, A. F., & Vallejo-Cordoba, B. (2015). Antimicrobial activity
652 and partial characterization of bacteriocin-like inhibitory substances produced by
653 *Lactobacillus* spp. isolated from artisanal Mexican cheese. *Journal of Dairy*
654 *Science*, *98*(12), 8285–8293. https://doi.org/10.3168/jds.2015-10104

655 Ho, V. T. T., Lo, R., Bansal, N., & Turner, M. S. (2018). Characterisation of
656 *Lactococcus lactis* isolates from herbs, fruits and vegetables for use as
657 biopreservatives against *Listeria monocytogenes* in cheese. *Food Control*, *85*, 472–
658 483. https://doi.org/10.1016/j.foodcont.2017.09.036

659 Hossain, M. I., Kim, K., Rahaman Mizan, M. F., Toushik, S. H., Ashrafudoulla, M.,
660 Roy, P. K., Nahar, S., Jahid, I. K., Choi, C., Park, S. H., & Ha, S. Do. (2021).
661 Comprehensive molecular, probiotic, and quorum-sensing characterization of anti-

listerial lactic acid bacteria, and application as bioprotective in a food (milk) model. *Journal of Dairy Science*, *104*(6), 6516–6534. https://doi.org/10.3168/jds.2020-19034

Jiang, J., Shi, B., Zhu, D., Cai, Q., Chen, Y., Li, J., Qi, K., & Zhang, M. (2012). Characterization of a novel bacteriocin produced by *Lactobacillus sakei* LSJ618 isolated from traditional Chinese fermented radish. *Food Control*, *23*(2), 338–344. https://doi.org/10.1016/j.foodcont.2011.07.027

Kanwal, H., Di Cerbo, A., Zulfiqar, F., Sabia, C., Nawaz, A., Siddiqui, F. M., Aqeel, M., & Ghazanfar, S. (2021). Probiotic characterization and population diversity analysis of gut-associated *Pediococcus acidilactici* for its potential use in the dairy industry. *Applied Sciences (Switzerland)*, *11*(20). https://doi.org/10.3390/app11209586

Kavitha, S., Harikrishnan, A., & Jeevaratnam, K. (2020). Characterization and evaluation of antibacterial efficacy of a novel antibiotic-type compound from a probiotic strain Lactobacillus plantarum KJB23 against food-borne pathogens. *Lwt*, *118*(August 2019), 4–11. https://doi.org/10.1016/j.lwt.2019.108759

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., & Witte, W. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of *Lactobacilli*, <i>Pediococci<i>, *Lactococci*, and *Bifidobacteria*. *Applied and Environmental Microbiology*, *71*(12), 8982–8986. https://doi.org/10.1128/AEM.71.12.8982-8986.2005

Li, M., Wang, Y., Cui, H., Li, Y., Sun, Y., & Qiu, H. J. (2020). Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Frontiers in Veterinary Science*, *7*(February), 1–10.

https://doi.org/10.3389/fvets.2020.00049

Li, Z., Li, Y., Xiao, C., Yan, Z., Pan, R., Gao, Y., Li, B., Wei, J., Qiu, Y., Liu, K., Shao, D., & Ma, Z. (2022). Genomic and metabolic features of the *Lactobacillus sakei* JD10 revealed potential probiotic traits. *Microbiological Research*, *256*(July 2021), 126954. https://doi.org/10.1016/j.micres.2021.126954

Lopes, M. de F. S., Simões, A. P., Tenreiro, R., Marques, J. J. F., & Crespo, M. T. B. (2006). Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *International Journal of Food Microbiology*, *112*(3), 208–214. https://doi.org/10.1016/j.ijfoodmicro.2006.09.004

Martín, I., Rodríguez, A., Sánchez-Montero, L., Padilla, P., & Córdoba, J. J. (2021). Effect of the dry-cured fermented sausage "salchichón" processing with a selected *Lactobacillus sakei* in *Listeria monocytogenes* and microbial population. *Foods*, *10*(4). https://doi.org/10.3390/foods10040856

Martinez, R. C. R., Staliano, C. D., Vieira, A. D. S., Villarreal, M. L. M., Todorov, S. D., Saad, S. M. I., & Franco, B. D. G. de M. (2015). Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei* subsp. sakei 2a in a potentially synbiotic cheese spread. *Food Microbiology*, *48*, 143–152. https://doi.org/10.1016/j.fm.2014.12.010

Mazaheri, T., Cervantes-Huamán, B. R. H., Bermúdez-Capdevila, M., Ripolles-Avila, C., & Rodríguez-Jerez, J. J. (2021). *Listeria monocytogenes* biofilms in the food industry: Is the current hygiene program sufficient to combat the persistence of the pathogen? *Microorganisms*, *9*(1), 1–19. https://doi.org/10.3390/microorganisms9010181

Mrkonjic Fuka, M., Tanuwidjaja, I., Zgomba Maksimovic, A., Zunabovic-Pichler, M.,

Kublik, S., Hulak, N., Domig, K. J., & Schloter, M. (2020). Bacterial diversity of naturally fermented game meat sausages: Sources of new starter cultures. *Lwt*, *118*(October 2019), 108782. https://doi.org/10.1016/j.lwt.2019.108782

Muñoz-Atienza, E., Landeta, G., de las Rivas, B., Gómez-Sala, B., Muñoz, R., Hernández, P. E., Cintas, L. M., & Herranz, C. (2011). Phenotypic and genetic evaluations of biogenic amine production by lactic acid bacteria isolated from fish and fish products. *International Journal of Food Microbiology*, *146*(2), 212–216. https://doi.org/10.1016/j.ijfoodmicro.2011.02.024

Nieto-Arribas, P., Seseña, S., Poveda, J. M., Chicón, R., Cabezas, L., & Palop, L. (2011). Enterococcus populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. *Food Microbiology*, *28*(5), 891–899. https://doi.org/10.1016/j.fm.2010.12.005

Oh, Y. J., & Jung, D. S. (2015). Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented milletalcoholic beverage in Korea. *Lwt*, *63*(1), 437–444. https://doi.org/10.1016/j.lwt.2015.03.005

Oliveira, M., Barbosa, J., Albano, H., & Teixeira, P. (2020). Bacteriocinogenic activity of *Leuconostoc lactic* RK18 isolated from fermented food. In O. Kovalyov (Ed.), *Fermented Foods:Nutrition and Role in Health and Disease* (pp. 159–181). Nova Science Publishers.

Panebianco, F., Giarratana, F., Caridi, A., Sidari, R., De Bruno, A., & Giuffrida, A. (2021). Lactic acid bacteria isolated from traditional Italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese. *Lwt*, *137*(July 2020), 110446. https://doi.org/10.1016/j.lwt.2020.110446

734 Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem,
735 M., Lemos, J. A., Linares, D. M., Ross, P., Stanton, C., Turroni, F., van Sinderen,
736 D., Varmanen, P., Ventura, M., Zúñiga, M., Tsakalidou, E., & Kok, J. (2016).
737 Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology*
738 *Reviews*, *80*(3), 837–890. https://doi.org/10.1128/mmbr.00076-15

739 Papagianni, M., & Anastasiadou, S. (2009). Pediocins: The bacteriocins of Pediococci.
740 Sources, production, properties and applications. *Microbial Cell Factories*, *8*(1),
741 1–16. https://doi.org/10.1186/1475-2859-8-3

742 Perin, L. M., Miranda, R. O., Todorov, S. D., Franco, B. D. G. de M., & Nero, L. A.
743 (2014). Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic
744 lactococci and enterococci isolated from goat milk. *International Journal of Food*
745 *Microbiology*, *185*, 121–126. https://doi.org/10.1016/j.ijfoodmicro.2014.06.001

746 Pinto, A., Barbosa, J., Albano, H., Isidro, J., & Teixeira, P. (2020). Screening of
747 bacteriocinogenic lactic acid bacteria and their characterization as potential
748 probiotics. *Microorganisms*, *8*(3). https://doi.org/10.3390/microorganisms8030393

749 Prpich, N. Z. P., Camprubí, G. E., Cayré, M. E., & Castro, M. P. (2021). Indigenous
750 microbiota to leverage traditional dry sausage production. *International Journal of*
751 *Food Science*, *2021*, 1–15. https://doi.org/10.1155/2021/6696856

752 Ramos, B., Ferreira, V., Brandao, T. R. S., Teixeira, P., & Silva, C. L. M. (2016).
753 Antilisterial active compound from lactic acid bacteria present on fresh iceberg
754 lettuce. *Acta Alimentaria*, *45*(3), 416–426.
755 https://doi.org/10.1556/066.2016.45.3.13

756 Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria
757 antimicrobial compounds: characteristics and applications. *Food Engineering*

Reviews, *4*(2), 124–140. https://doi.org/10.1007/s12393-012-9051-2

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2º Edition).

Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, *166*(2), 368–379. https://doi.org/10.1016/0003-2697(87)90587-2

Semedo, T., Almeida Santos, M., Silva Lopes, M. F., Figueiredo Marques, J. J., Barreto Crespo, M. T., & Tenreiro, R. (2003). Virulence factors in food, clinical and reference Enterococci: A common trait in the genus? *Systematic and Applied Microbiology*, *26*(1), 13–22. https://doi.org/10.1078/072320203322337263

Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. *Frontiers in Microbiology*, *9*(APR). https://doi.org/10.3389/fmicb.2018.00594

Talon, R., & Leroy, S. (2014). Fermented foods: Fermented meat products and the role of starter cultures. In *Encyclopedia of Food Microbiology: Second Edition* (Second Edi, Vol. 1). Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00116-6

Tiago, I., Teixeira, I., Silva, S., Chung, P., Veríssimo, A., & Manaia, C. M. (2004). Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-ε-caprolactones. *Current Microbiology*, *49*(6), 407–414. https://doi.org/10.1007/s00284-004-4353-0

Todorov, S. D., & Dicks, L. M. T. (2007). Bacteriocin production by *Lactobacillus pentosus* ST712BZ isolated from boza. *Brazilian Journal of Microbiology*, *38*(1),

166–172. https://doi.org/10.1590/S1517-83822007000100034

Todorov, S. D., Rachman, C., Fourrier, A., Dicks, L. M. T., van Reenen, C. A., Prévost, H., & Dousset, X. (2011). Characterization of a bacteriocin produced by Lactobacillus sakei R1333 isolated from smoked salmon. *Anaerobe*, *17*(1), 23–31. https://doi.org/10.1016/j.anaerobe.2010.01.004

Todorov, S. D., Holzapfel, W., & Nero, L. A. (2017a). In Vitro Evaluation of Beneficial Properties of Bacteriocinogenic *Lactobacillus plantarum* ST8Sh. *Probiotics and Antimicrobial Proteins*, *9*(2), 194–203. https://doi.org/10.1007/s12602-016-9245-7

Todorov, S. D., Stojanovski, S., Iliev, I., Moncheva, P., Nero, L. A., & Ivanova, I. V. (2017b). Technology and safety assessment for lactic acid bacteria isolated from traditional Bulgarian fermented meat product "lukanka." *Brazilian Journal of Microbiology*, *48*(3), 576–586. https://doi.org/10.1016/j.bjm.2017.02.005

Todorov, S. D., Vaz-Velho, M., de Melo Franco, B. D. G., & Holzapfel, W. H. (2013). Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from salpicao, a fermented meat product from North-West of Portugal. *Food Control*, *30*(1), 111–121. https://doi.org/10.1016/j.foodcont.2012.07.022

Urso, R., Rantsiou, K., Cantoni, C., Comi, G., & Cocolin, L. (2006). Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. *International Journal of Food Microbiology*, *110*(3), 232–239. https://doi.org/10.1016/j.ijfoodmicro.2006.04.015

Valledor, S. J. D., Dioso, C. M., Bucheli, J. E. V., Park, Y. J., Suh, D. H., Jung, E. S., Kim, B., Holzapfel, W. H., & Todorov, S. D. (2022). Characterization and safety evaluation of two beneficial, enterocin-producing *Enterococcus faecium* strains

isolated from kimchi, a Korean fermented cabbage. *Food Microbiology*, *102*(September 2021), 103886. https://doi.org/10.1016/j.fm.2021.103886

Van Reenen, C. A., Dicks, L. M. T., & Chikindas, M. L. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*, *84*(6), 1131–1137. https://doi.org/10.1046/j.1365-2672.1998.00451.x

Wen, L. S., Philip, K., & Ajam, N. (2016). Purification, characterization and mode of action of plantaricin K25 produced by *Lactobacillus plantarum*. *Food Control*, *60*, 430–439. https://doi.org/10.1016/j.foodcont.2015.08.010

Winkelströter, L. K., Tulini, F. L., & De Martinis, E. C. P. (2015). Identification of the bacteriocin produced by cheese isolate *Lactobacillus paraplantarum* FT259 and its potential influence on *Listeria monocytogenes* biofilm formation. *LWT - Food Science and Technology*, *64*(2), 586–592. https://doi.org/10.1016/j.lwt.2015.06.014

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359. https://doi.org/10.1128/aem.58.10.3355-3359.1992

Zhao, D., Wang, Q., Lu, F., Bie, X., Zhao, H., Lu, Z., & Lu, Y. (2022). A novel plantaricin 827 effectively inhibits *Staphylococcus aureus* and extends shelf life of skim milk. *Lwt*, *154*, 112849. https://doi.org/10.1016/j.lwt.2021.112849

Figure Captions

Figure 1. Production of bacteriocin by *L. sakei* 205 in MRS broth. The antimicrobial activity of cell-free supernatants is presented as AU/mL (bars) for *L. monocytogenes* NCTC 11994 (L1), *L. monocytogenes* EDG-e (L2), *L. monocytogenes* ScottA (L3) and *E. faecalis* ATCC 29212 (E1) strains. Viable cell counts of *L. sakei* 205 are presented as log (CFU/mL) and pH changes are also presented.

Figure 2. Effect of *Lb. sakei* 205 bacteriocin(s) on the growth of *L. monocytogenes* ScottA (a), *L. monocytogenes* EDG-e (b), *L. monocytogenes* NCTC 11994 (c) and *E. faecalis* ATCC 29212 (d) presented as log (CFU/mL). The darkest line represents target cultures without added bacteriocins. The arrow indicates the point at which the bacteriocin was added.

Figure 3. Tricine/SDS-PAGE of *Lb. sakei* 205 bacteriocin. Lanes 1 and 4: Molecular mass marker (M); Lane 2: peptide bands in the stained gel with Coomassie Blue R250; Lane 3: growth inhibition zone, corresponding to the position of the peptide band in lane 2. The gel was covered with viable cells of *L. monocytogenes* Scott A (10^6 CFU/mL), embedded in BHI soft agar and incubated at 37 °C for 24 h.

Table 1. Target microorganisms and their source, used for bacteriocin activity spectrum test

Species	Source
Bacillus cereus ESB014	ESB culture collection
Salmonella Enteritidis ESB008	
Salmonella Typhimurium ESB009	
Acinetobacter spp. ESB260	
Acinetobacter baumannii ESB028	
Pediococcus acidilactici HA- 6111- 2	
Listeria monocytogenes SCOTT A	
Enterococcus faecalis ATCC 29212	ATCC
Staphylococcus aureus ATCC 25213	
Staphylococcus aureus ATCC 6538	
Listeria monocytogenes EDG-e	
Escherichia coli ATCC 25922	
Escherichia coli ATCC 8739	
Enterococcus faecium DSMZ 13590	DSMZ
Listeria monocytogenes NCTC 11994	NCTC

ESB –*Escola Superior de Biotecnologia;* ATCC – American Type Culture Collection; DSMZ – German Collection of Microorganisms and Cell Culture; NCTC – National Collection of Types cultures

Table 2. Identification of 33 lactic-acid bacteria isolates selected for further experiments

Code	Product /Surface	Identification
15	Surface meat industry	*Enterococcus hirae*
31	Ripened cheese	*Lacticaseibacillus casei*
37	Dry-cured fermented sausage	*Enterococcus faecium*
38	Dry-cured fermented sausage	*Enterococcus faecium*
40	Dry-cured fermented sausage	*Enterococcus faecium*
89	Dry-cured fermented sausage	*Lactiplantibacillus plantarum*
116	Ripened cheese	*Lacticaseibacillus casei*
151	Ripened cheese	*Lactococcus garvieae*
156	Ripened cheese	*Lactococcus garvieae*
159	Dry-cured fermented sausage	*Enterococcus durans*
174	Dry-cured fermented sausage	*Lactilactobacillus sakei*
175	Dry-cured fermented sausage	*Lactilactobacillus sakei*
185	Ripened cheese	*Lacticaseibacillus paracasei*
187	Ripened cheese	*Enterococcus faecium*
189	Surface meat industry	*Enterococcus faecium*
193	Dry-cured fermented sausage	*Leuconostoc mesenteroides*
194	Dry-cured fermented sausage	*Lactilactobacillus sakei*
197	Dry-cured fermented sausage	*Lactilactobacillus sakei*
205	Dry-cured fermented sausage	*Lactilactobacillus sakei*
258	Ripened cheese	*Leuconostoc mesenteroides*
262	Ripened cheese	*Lacticaseibacillus paracasei*
270	Curd	*Leuconostoc pseudomesenteoroides*
272	Ripened cheese	*Leuconostoc mesenteroides*
273	Ripened cheese	*Leuconostoc mesenteroides*
274	Ripened cheese	*Leuconostoc mesenteroides*
284	Ripened cheese	*Lactiplantibacillus plantarum*
288	Ripened cheese	*Leuconostoc mesenteroides*
297	Ripened cheese	*Leuconostoc mesenteroides*
304	Ripened cheese	*Leuconostoc mesenteroides*
306	Curd	*Leuconostoc mesenteroides*
307	Ripened cheese	*Leuconostoc mesenteroides*
313	Curd	*Leuconostoc pseudomesenteroides*
314	Ripened cheese	*Leuconostoc mesenteroides*

Table 3. Virulence genes of selected lactic-acid bacteria isolates.

Isolates	Virulence genes															
	agg	*esp*	*gelE*	*efaAfs*	*efaAfm*	*cylA*	*cylB*	*cylM*	*cylLL*	*cylLS*	*ase*	*hyl*	*asa1*	*hdc*	*tcd*	*odc*
E. hirae 15	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
Lcb.casei 31	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–
Lcb. casei 116	–	–	+	+	+	–	–	–	–	–	–	–	–	–	+	–
Lco. garviae 151	–	–	+	+	+	–	–	–	+	–	–	–	–	–	+	–
E. durans 159	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	–
Lb. sakei 174	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Lcb. paracasei 185	–	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–
Lb. sakei 197	–	–	+	+	+	–	–	–	+	–	–	–	–	–	+	–
Lb. sakei 205	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Lcb. paracasei 262	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Lpb. plantarum 284	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	–
Lc. mesenteroides 307	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	–
Lc. mesenteroides 313	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	–

+: positive for virulence gene; −: negative for virulence gene

873 **Table 4.** Minimal inhibitory concentrations (MIC; μg/mL) and respective susceptibility
874 to eight antibiotics by thirteen LAB isolates.

Isolates	MIC (Susceptibility)							
	Amp	**Chl**	**Gen**	**Kan**	**Str**	**Tet**	**Van**	**Ery**
E. hirae 15	8(S)	1(S)	≤4(S)	256(S)	≤4(S)	2(S)	4(S)	2(**I**)
Lcb. casei 31	8(S)	1(S)	≤4(S)	16(S)	≤4(S)	2(S)	n.r.	0.5(S)
Lcb. casei 116	≤2(S)	0.5(S)	≤4(S)	8(S)	≤4(S)	2(S)	n.r.	≤0.25(S)
Lco. garviae 151	≤2(S)	0.5(S)	≤4(S)	16(S)	4(S)	4(S)	n.r.	0.25(S)
E. durans 159	4(S)	0.5(S)	≤4(S)	256(S)	4(S)	16(**R**)	4(S)	2(I)
Lb. sakei 174	≤2(S)	0.5(S)	≤4(S)	16(S)	≤4(S)	4(S)	n.r.	1(S)
Lcb. paracasei 185	8(**R**)	0.5(S)	≤4(S)	16(S)	≤4(S)	≤0.5(S)	n.r.	1(S)
Lb. sakei 197	0.5(S)	0.5(S)	≤4(S)	8(S)	≤4(S)	4(S)	n.r.	1(S)
Lb. sakei 205	0.5(S)	0.5(S)	≤4(S)	4(S)	≤4(S)	≤0.5(S)	n.r.	≤0.25(S)
Lcb. paracasei 262	4(S)	0.5(S)	≤4(S)	16(S)	≤4(S)	4(S)	n.r.	2(**R**)
Lpb. plantarum 284	8(**R**)	0.5(S)	≤4(S)	16(S)	n.r.	4(S)	n.r.	1(S)
Lc. mesenteroides 307	≤2(S)	0.5(S)	≤4(S)	32(**R**)	≤4(S)	≤0.5(S)	n.r.	2(S)
Lc. mesenteroides 313	8(**R**)	0.5(S)	≤4(S)	32(**R**)	≤4(S)	1(S)	n.r.	2(S)

875 Amp- ampicillin; Chl- chloramphenicol; Gen- gentamicin; Kan- kanamycin: Str-streptomycin;
876 Tet- tetracycline; Van- vancomycin; Ery- erytromycin; R- resistant; I – intermediate; S –
877 sensitive; n.r. – not required.

878

879 **Table 5.** Reduction of antimicrobial activity of *Lb. sakei* 205 bacteriocin (expressed in
880 percentage values) against four pathogens, under the effect of temperature

881

		L1		**L2**		**L3**		**E1**	
		1h	2h	1h	2h	1h	2h	1h	2h
T (ºC)	4	0.00%	50.00%	50.00%	75.00%	75.00%	87.50%	75.00%	87.50%
	25	0.00%	0.00%	25.00%	25.00%	25.00%	50.00%	50.00%	50.00%
	30	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	37	0.00%	0.00%	0.00%	0.00%	0.00%	50.00%	0.00%	0.00%
	60	50.00%	50.00%	0.00%	0.00%	0.00%	50.00%	25.00%	25.00%
	80	50.00%	50.00%	75.00%	75.00%	50.00%	50.00%	50.00%	75.00%
	100	75.00%	87.50%	75.00%	87.50%	50.00%	75.00%	87.50%	87.50%
	121	100.00%		100.00%		100.00%		100.00%	

882 (L1) *L. monocytogenes* NCTC 11994; (L2) *L. monocytogenes* EDG-e; (L3) *L. monocytogenes*
883 ScottA and (E1) *E. faecalis* ATCC 29212

884 **Table 6**. Reduction of antimicrobial activity of *Lb. sakei* 205 bacteriocin (expressed in
885 percentage values) against *L. monocytogenes* ScottA, EDG-e and NCTC 11994 and an *E.*
886 *faecalis*, under the effect of pH, detergents, surfactants and protease inhibitors

		L1	L2	L3	E1
pH	2	75.00%	75.00%	50.00%	50.00%
	4	0.00%	0.00%	0.00%	0.00%
	6	0.00%	0.00%	0.00%	0.00%
	8	50.00%	25.00%	75.00%	50.00%
	10	50.00%	87.50%	75.00%	75.00%
	12	93.75%	96.88%	87.50%	93.75%
Enzymes (mg/mL)	Proteinase K 1.0	87.50%	100.00%	100.00%	96.88%
	Proteinase K 0.1	75.00%	100.00%	100.00%	87.50%
	Papain 1.0	75.00%	75.00%	75.00%	50.00%
	Papain 0.1	0.00%	25.00%	25.00%	0.00%
	Pepsin 1.0	50.00%	50.00%	50.00%	50.00%
	Pepsin 0.1	0.00%	25.00%	25.00%	0.00%
	Catalase 1.0	0.00%	75.00%	87.50%	75.00%
	Catalase 0.1	0.00%	50.00%	75.00%	50.00%
Detergents	Tween 20	0.00%	0.00%	87.50%	25.00%
	Tween 80	0.00%	0.00%	87.50%	50.00%
	Triton X-100	87.50%	93.75%	87.50%	50.00%
	SDS	50.00%	93.75%	87.50%	87.50%
	EDTA	87.50%	75.00%	87.50%	75.00%
	Ox-Bile	93.75%	93.75%	100.00%	96.88%
	Urea	75.00%	87.50%	87.50%	25.00%
	NaCl	50.00%	75.00%	0.00%	0.00%
	Sodium carbonate	75.00%	75.00%	93.75%	75.00%
	Sodium deoxycholate	87.50%	87.50%	87.50%	87.50%

887 (L1) *L. monocytogenes* NCTC 11994; (L2) *L. monocytogenes* EDG-e; (L3) *L. monocytogenes*
888 ScottA and (E1) *E. faecalis* ATCC 29212

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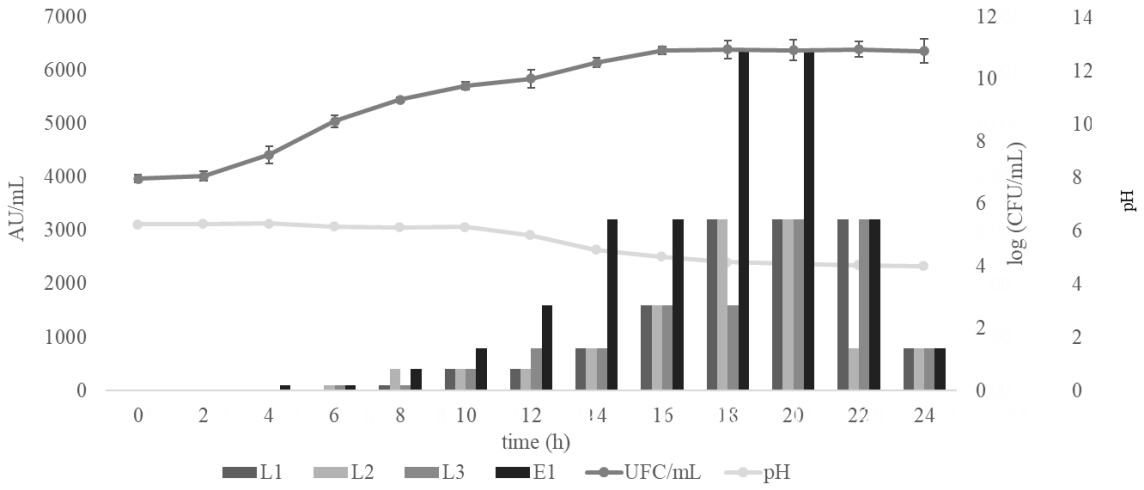


Figure 1. Production of bacteriocin by *L. sakei* 205 in MRS broth. The antimicrobial activity of cell-free supernatants is presented as AU/mL (bars) for *L. monocytogenes* NCTC 11994 (L1), *L. monocytogenes* EDG-e (L2), *L. monocytogenes* ScottA (L3) and *E. faecalis* ATCC 29212 (E1) strains. Viable cell counts of *L. sakei* 205 are presented as log (CFU/mL) and pH changes are also presented.

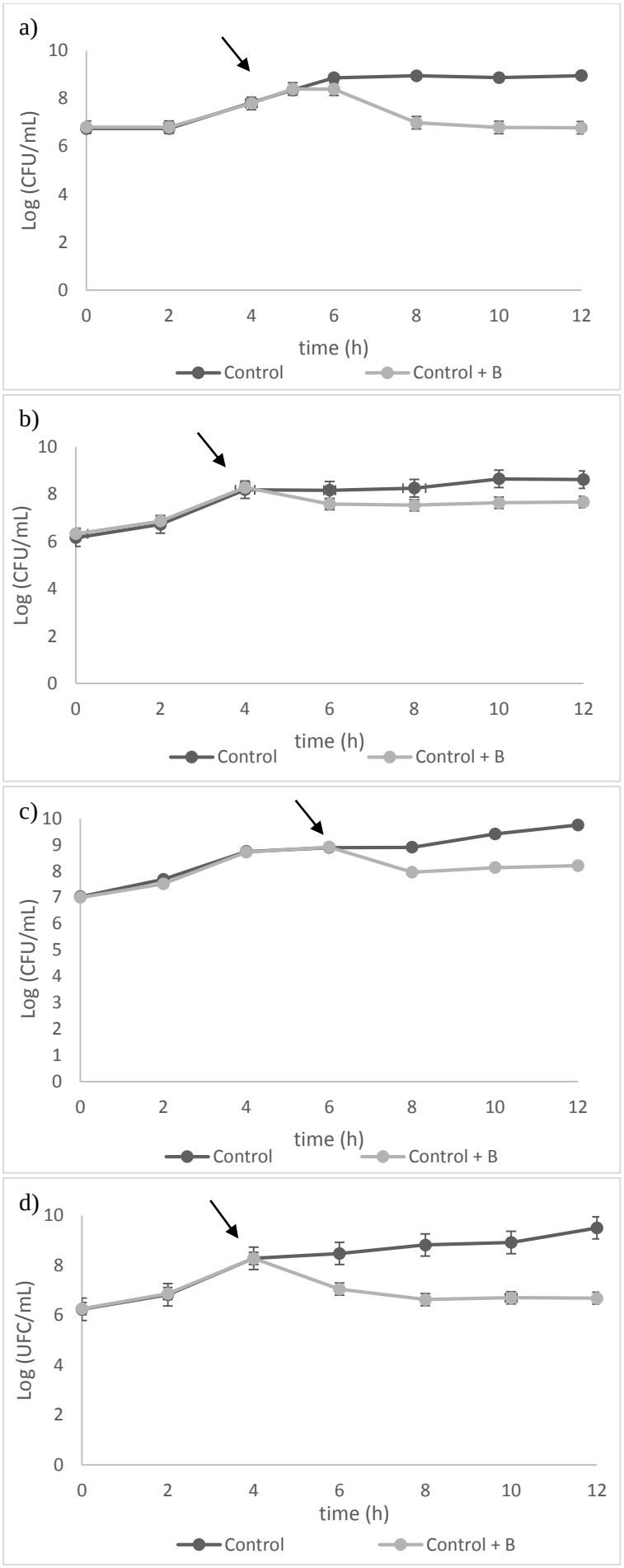
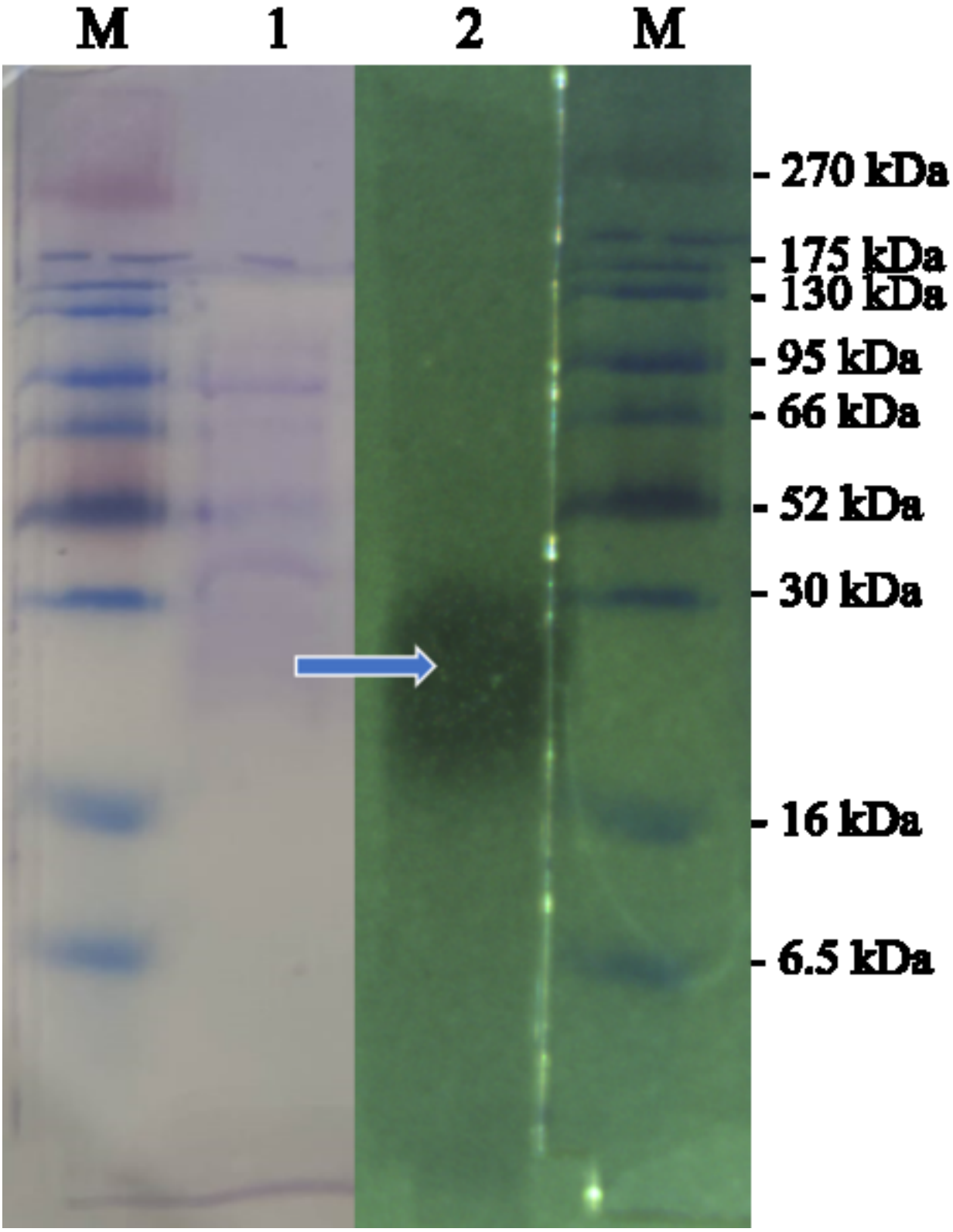


Figure 2. Effect of *Lb. sakei* 205 bacteriocin(s) on the growth of *L. monocytogenes* ScottA (a), *L. monocytogenes* EDG-e (b), *L. monocytogenes* NCTC 11994 (c) and *E. faecalis* ATCC 29212 (d) presented as log (CFU/mL). The darkest line represents target cultures without added bacteriocins. The arrow indicates the point at which the bacteriocin was added.

Figure 3

Click here to access/download;Figure;renamed_e07c7.png



V. DISCUSIÓN

V.1. SELECCIÓN Y EVALUACIÓN DE BACTERIAS ÁCIDO-LÁCTICAS E HIDROLIZADOS PROTEICOS COMO ESTRATEGIAS DE CONTROL DE *L. monocytogenes* EN EMBUTIDOS CURADO-MADURADOS Y QUESOS MADURADOS.

El cambio en los hábitos de consumo y el consiguiente aumento en la demanda de alimentos RTE, sin conservantes ni aditivos, hace que sea más complicado garantizar la calidad microbiológica y la seguridad del producto.

En productos RTE como los embutidos curado-madurados y quesos madurados puede haber contaminación y desarrollo de microorganismos patógenos durante su proceso de elaboración y comercialización (Fretz y col., 2010; Magalhães y col., 2015). Entre ellos, *L. monocytogenes* es el microorganismo patógeno que más preocupa en este tipo de industrias (EFSA, 2018; Kurpas y col., 2018; Martinez-Rios y Dalgaard, 2018). Por todo esto, es necesario la búsqueda de alternativas que garanticen la seguridad de estos productos y además no modifiquen sus características sensoriales.

La utilización de cultivos protectores para inhibir el crecimiento de microorganismos patógenos ha sido objeto de numerosos estudios (García y col., 2020; Mokoena y col., 2021; Vieco-Saiz y col., 2019), siendo una de las estrategias principales frente a *L. monocytogenes*. En este sentido, la utilización de BAL es uno de los métodos más utilizados para el control de este microorganismo patógeno en derivados cárnicos curado-madurados y quesos madurados de distintos grados de maduración (Barcenilla y col., 2022; Wemmenhove y col., 2018). Además, también pueden utilizarse hidrolizados proteícos que tienen péptidos con efecto microbiano. De hecho, los péptidos derivados de proteínas alimentarias hidrolizadas han despertado recientemente un gran interés ya que

han mostrado diversas actividades fisiológicas como actividades antiinflamatorias, antialérgicas, antihipertensivas y antimicrobianas contra ciertos microorganismos, incluidos *L. monocytogenes, B. subtilis, E. coli* y *Ps. aeruginosa* (Demers-Mathieu y col., 2013; Hernández-Ledesma y col., 2014) y pueden aportar un valor añadido a los subproductos de la industria láctea. Esta estrategia puede utilizarse sóla o en combinación con los cultivos protectores.

Los embutidos curado-madurados y quesos madurados tradicionales constituyen un importante nicho ecológico de BAL autóctonas entre las que podrían encontrarse cepas con importante potencial frente a *L. monocytogenes* (Cocolin y col., 2007; Sip y col., 2012; Xiraphi y col., 2008). Por ello, el aislamiento y selección de BAL con actividad anti- *L. monocytogenes* y su aplicación en este tipo de productos madurados es de gran interés y se ha tratado con profundidad en esta Tesis Doctoral.

V.1.1. Aislamiento de bacterias ácido-lácticas de quesos madurados y embutidos curado-madurados tradicionales.

Un amplio número de cepas de BAL han sido aisladas de productos madurados, como embutidos curado-madurados y quesos madurados (Campagnollo y col., 2018; De Carvalho y col., 2006; Garcia-Gonzalez y col., 2021; Panebianco y col., 2021), aunque no todas han demostrado actividad antimicrobiana en los diferentes procesados en los que se han ensayado.

La selección de BAL con actividad anti-*L. monocytogenes* de embutidos curado-madurados y quesos madurados para ser utilizados como cultivos protectores debe realizarse a partir de cepas adaptadas al nicho ecológico de estos productos, ya que deben sobrevivir y ser competitivos en las condiciones de procesado y posterior almacenamiento frente a los microorganismos patógenos que se desean eliminar, en este caso, *L.*

monocytogenes. Por lo tanto, las cepas de BAL deben aislarse durante las distintas etapas del procesado incluyendo la maduración, así como en el almacenamiento de los embutidos curado-madurados y quesos madurados.

En esta Tesis Doctoral, se aislaron 371 cepas de BAL, de las cuales 182 pertenecieron a embutidos de 3 industrias tradicionales de cerdo ibérico y 189 pertenecieron principalmente a quesos de 12 queserías tradicionales incluidas en las diferentes denominaciones de origen protegidas "Torta del Casar", "Quesos de la Serena" y "Quesos de Ibores". La mayoría de estos aislamientos se obtuvieron de productos en diferentes etapas de procesado (inicio, mitad y final de maduración), siendo algunos de distintas superficies de equipos y utensilios (6). Posteriormente, los aislados se caracterizaron preliminarmente como BAL mediante distintas pruebas como tinción de Gram, reacción de catalasa, observación de la forma por microscopía y características bioquímicas, tal y como se han llevado a cabo en otros estudios cuyo objetivo era el aislamiento de cepas de BAL de quesos de pasta blanda (Campagnollo y col., 2018; Panebianco y col., 2021) o embutidos curado-madurados (Papamanoli y col., 2003).

V.1.2. Evaluación de la actividad anti-*L. monocytogenes* de los aislados de bacterias ácido-lácticas

Se conoce ampliamente que las BAL mejoran las características sensoriales, nutricionales y tecnológicas del producto, pero también es conocida la capacidad que tiene este grupo bacteriano de producir metabolitos secundarios con actividad antimicrobiana que permiten la prevención de microorganismos patógenos transmitidos por alimentos como *L. monocytogenes*, *B. cereus* y *S. aureus* (Kanwal y col., 2021).

En el desarrollo de este trabajo se ha procedido inicialmente a realizar una selección de aislados con actividad anti-*L. monocytogenes* mediante el método de difusión en agar

utilizando medios de cultivo de uso común en el laboratorio. Otros autores también utilizaron este método para evaluar la capacidad antimicrobiana de las BAL para inhibir microorganismos patógenos (Sip y col., 2012; Todorov y Dicks, 2005). Como resultados de esta primera selección se obtuvo que 84 (22,64%) de los 371 aislados de BAL mostraron halos de inhibición de *L. monocytogenes* de al menos 2 mm. De estos, 52 procedían de queserías y 32 fueron aislados de las industrias de embutidos curado-madurados (Tabla V.1). Esta inhibición puede deberse a la competición por el medio de estos aislados con *L. monocytogenes* o a que produzcan compuestos que inhiben a esta bacteria patógena como ácido láctico y otros ácidos orgánicos, etanol, diacetilo, dióxido de carbono, peróxido de hidrógeno o bacterias lácticas (Kasra-Kermanshahi y Mobarak-Qamsari, 2015).

El porcentaje encontrado de aislados activos tras esta primera selección en medios de cultivo está en el mismo rango que el observado por otros autores cuando utilizaron esta metodología para la selección de BAL activas en diferentes alimentos (Fontana y col., 2015; Macaluso y col., 2016). Atendiendo al origen de los aislados, el porcentaje de activos fue mayor en los procedentes de las queserías, 61,9%, frente al 38,1% encontrado entre los obtenidos en industrias cárnicas, a pesar de que el número de aislados ensayados de cada industria fue muy similar (189 de quesos frente a 182 de embutidos). Es probable que en el nicho ecológico de quesos elaborados con leche cruda, donde hay una población importante de BAL desde el principio del procesado, se produzca una selección natural hacia el predominio de estas bacterias con actividad antimicrobiana que las haga más competitivas (Arqués y col., 2014).

Los 84 aislados de BAL seleccionados con actividad anti-*L. monocytogenes* fueron identificados mediante la secuenciación de la subunidad ribosomal ARNr 16S y posterior

análisis de dichas secuencias. Se determinó que los distintos aislados pertenecían a las especies *E. durans*, *E. faecium*, *E. hirae*, *Le. pseudomesenteroides*, *Le. mesenteroides*, *Lc. casei*, *Lp. plantarum*, *Lc. paracasei*, *Ll. sakei*, *Lco. garviae* y *Lco. lactis*. Otros autores también han encontrado estas especies de BAL con actividad antimicrobiana en embutidos curado-madurados (Ammor y col., 2005) y quesos madurados (Duan y col., 2008). La mayoría de estas especies correspondieron tanto a aislados de industrias de embutidos curado-madurados como de queserías, excepto *Ll. sakei* que sólo se aisló en industrias cárnicas y *Lc Lactis* y *E. hirae*, solo detectaron en queserías, probablemente porque estas últimas especies están más adaptadas a nichos ecológicos específicos (Nomura y col., 2006; Zagorec y Champomier-Vergès, 2017).

Tabla V.1. Identificación de las BAL aisladas de diferentes tipos de productos, tiempo de maduración e industrias que presentaron actividad anti-*Listeria monocytogenes*.

Código	Tipo de producto/superficie de aislamiento	Tiempo de maduración	Industria	Identificación
1	Chorizo	Final	M	*Lactococcus garvieae*
13	Chorizo	Final	M	*Lacticaseibacillus paracasei*
14	Superficie	-	M	*Leuconostoc pseudomesenteroides*
15	Superficie	-	M	*Enterococcus hirae*
17	Salchichón	Inicio	N	*Lacticaseibacillus casei*
18	Queso "Torta del Casar"	Final	A	*Leuconostoc pseudomesenteroides*
19	Salchichón	Final	N	*Lactilactobacillus sakei*
21	Salchichón	Mitad	N	*Enterococcus faecium*
22	Salchichón	Mitad	N	*Enterococcus faecium*
31	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
37	Salchichón	Mitad	N	*Enterococcus faecium*
38	Salchichón	Mitad	M	*Enterococcus faecium*
40	Chorizo	Final	M	*Enterococcus faecium*
74	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus paracasei*
88	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
89	Salchichón	Mitad	M	*Lactiplantibacillus plantarum*
114	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
115	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
116	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
117	Queso "Torta del Casar"	Final	A	*Lacticaseibacillus casei*
118	Salchichón	Mitad	M	*Lactococcus garviae*
126	Queso "Torta del Casar"	Mitad	A	*Enterococcus faecium*
128	Salchichón	Inicio	N	*Lactilactobacillus sakei*
134	Salchichón	Final	N	*Enterococcus durans*
151	Queso "Torta del Casar"	Mitad	B	Lactococcus garvieae
156	Queso "Torta del Casar"	Final	A	*Lactococcus garvieae*
158	Salchichón	Final	N	*Lactococcus garvieae*
159	Salchichón	Final	N	*Enterococcus durans*
161	Queso "Torta del Casar"	Final	A	*Enterococcus durans*
162	Salchichón	Final	N	*Lactococcus garvieae*
163	Salchichón	Final	N	*Lactococcus garvieae*
165	Chorizo	Final	N	*Enterococcus durans*
173	Chorizo	Final	O	*Lactilactobacillus sakei*
174	Chorizo	Final	O	*Lactilactobacillus sakei*
175	Chorizo	Final	N	*Lactilactobacillus sakei*
185	Queso "Torta del Casar"	Final	B	*Lacticaseibacillus paracasei*
187	Queso "Torta del Casar"	Mitad	B	*Enterococcus faecium*
188	Superficie	-	O	*Enterococcus faecium*
189	Superficie	-	O	*Enterococcus faecium*
193	Chorizo	Final	O	*Leuconostoc mesenteroides*
194	Chorizo	Final	O	*Lactilactobacillus sakei*

195	Chorizo	Final	O	*Lactilactobacillus sakei*
197	Chorizo	Final	O	*Lactilactobacillus sakei*
199	Queso "Torta del Casar"	Mitad	B	*Enterococcus durans*
200	Queso "Torta del Casar"	Mitad	B	*Leuconostoc mesenteroides*
203	Salchichón	Final	N	*Lactilactobacillus sakei*
204	Chorizo	Final	N	*Lactilactobacillus sakei*
205	Chorizo	Final	O	*Lactilactobacillus sakei*
206	Queso "Torta del Casar"	Mitad	B	*Enterocuccus durans*
223	Queso "Torta del Casar"	Final	C	*Leuconostoc mesenteroides*
239	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus casei*
242	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus paracasei*
244	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus paracasei*
246	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus casei*
248	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus paracasei*
251	Queso "Torta del Casar"	Final	C	*Leuconostoc mesenteroides*
258	Queso "Torta del Casar"	Final	C	*Leuconostoc mesenteroides*
262	Queso "Torta del Casar"	Final	C	*Lacticaseibacillus paracasei*
270	Cuajada "Queso de la Serena"	Cuajada	F	*Leuconostoc pseudomesenteoroides*
272	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
273	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
274	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
275	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
284	"Queso de la Serena"	Mitad	G	*Lactiplantibacillus plantarum*
288	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
295	"Queso de la Serena"	Final	D	*Lactiplantibacillus plantarum*
297	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
298	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
299	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
300	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
304	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
306	Cuajada "Queso de la Serena"	Cuajada	F	*Leuconostoc mesenteroides*
307	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
313	Cuajada "Queso de la Serena"	Cuajada	F	*Leuconostoc pseudomesenteroides*
314	"Queso de la Serena"	Mitad	G	*Leuconostoc mesenteroides*
335	"Queso Ibores"	Final	I	*Lacticaseibacillus paracasei*
340	"Queso Ibores"	Leche	J	*Lactococcus lactis*
344	"Queso Ibores"	Mitad	J	*Lactococcus lactis*
352	"Queso Ibores"	Mitad	K	*Lactococcus lactis*
353	Leche "Queso de Ibores"	Leche	H	*Lactococcus lactis*
364	"Queso Ibores"	Final	L	*Lacticaseibacillus casei*
365	"Queso Ibores"	Mitad	J	*Lactococcus lactis*
369	Superficie	-	H	*Lactococcus lactis*
370	Superficie	-	H	*Leuconostoc mesenteroides*

Una vez identificadas las especies, se procedió a evaluar la actividad antagonista de las distintas cepas frente a *L. monocytogenes* mediante ensayo de co-inoculación con este

patógeno en modelos alimentarios que simulan la composición, a_w y pH de los embutidos curado-madurados y de los quesos madurados incubándolos a temperaturas características de las etapas críticas del procesado y almacenamiento de estos alimentos. Este paso es de suma importancia para descartar cepas de BAL carentes de actividad o con baja actividad en las condiciones de procesado o almacenamiento de los alimentos madurados RTE.

Para llevar a cabo dicho ensayo, se utilizó un medio elaborado con salchichón dónde se evaluaron las BAL seleccionadas de industrias cárnicas mientras que las cepas aisladas de queserías se evaluaron en un medio elaborado con queso "Torta del Casar". Los resultados mostraron que el aumento o la reducción de los recuentos de *L. monocytogenes* en los modelos alimentarios correspondientes dependía de la BAL inoculada. Así, se observó que sólo 5 de las 52 cepas de BAL aisladas de industrias queseras provocaron una reducción importante de los recuentos de *L. monocytogenes* en el medio elaborado con queso en comparación con el medio de queso control donde solamente se inoculó el microorganismo patógeno. Estas cepas fueron *Lc. casei* 31, *Lc. casei* 116, *Lco. garviae* 151, *Lco. garviae* 156 y *Lc. paracasei* 185, observándose una inhibición del crecimiento del microorganismo patógeno de hasta aproximadamente 2,35 log UFC/cm^2 en el medio de cultivo elaborado con queso "Torta del Casar" (con la cepa *Lc. casei* 116). Lo mismo ocurrió en el ensayo en que se evaluó la capacidad antagonista de las cepas de BAL aisladas de industrias de embutidos curado-madurados, ya que sólo en 5 de las 32 cepas de BAL ensayadas mostraron una reducción importante de los recuentos de *L. monocytogenes* respecto al medio control. Estas cepas fueron *Lc. paracasei* 13, *E. faecium* 188, *Ll. sakei* 197, *Ll. sakei* 204 y *Ll. sakei* 205. La inhibición más alta (2,5 log UFC/cm^2) se obtuvo cuando se co-inoculó *L. monocytogenes* con *Ll. sakei* 205. De esta manera, se seleccionaron un total de 10 cepas de BAL activas frente a *L. monocytogenes*

en sistemas modelos que simulaban los alimentos RTE de interés en esta Tesis Doctoral. Otras cepas de BAL seleccionas han mostrado reducciones similares e incluso inferiores de *L. monocytogenes* en leche y en medio de agar queso (García y col., 2020; Martín y col., 2022). Panebianco y col. (2021) encontraron reducciones de entre 0,5 y 1 log UFC/g tanto in vitro como en queso de pasta blanda.

A continuación, se evaluó la presencia de genes que codifican la producción de estos metabolitos secundarios (nisin, lacticin 481, lactococcin 972, G y Q, Ay B, brevicin, plantaracin A, EF, NC, S y W, pediocin A) en las 10 cepas de BAL que mostraron una mayor actividad antagonista en los modelos alimentario, dado que la actividad antagonista de algunas de estas BAL puede deberse a la producción de bacteriocinas. La producción de bacteriocinas supone un valor añadido a la BAL, ya que está demostrada su eficacia en cuanto a la inhibición de bacterias patógenas en diversas matrices alimentarias incluidos los embutidos curado-madurados y quesos madurados (Soltani y col., 2021). Estas cepas mostraron amplicones del tamaño esperado para al menos uno de los genes que codifican la producción de bacteriocinas. Otros autores observaron que cepas de BAL seleccionadas con actividad antimicrobiana aisladas de productos madurados producen diferentes bacteriocinas, como lactococinas, nisina y pediocinas (Alegría y col., 2010; Salas y col., 2017; Xie y col., 2011). En el presente trabajo, *Lc. casei* 116 que mostró la actividad más alta en el modelo de queso, presentó los genes que codifican nisina, lactacinas A, B, G y Q y pediocina PA. En cuanto a las cepas BAL activas de embutidos curado-madurados, la cepa más activa en el modelo de "salchichón" *Ll. sakei* 205 solo presentó el gen de pediocina PA, probablemente debido a que la actividad de esta cepa no solo se debe a la producción de bacteriocinas, sino también a la producción de ácidos orgánicos, diacetilo, dióxido de carbono, etanol o peróxido de hidrógeno (Kasra-Kermanshahi y Mobarak-Qamsari, 2015).

Considerando la actividad anti-*L. monocytogenes* en los modelos de salchichón y de queso y la evaluación de genes codifican para la síntesis de bacteriocinas se seleccionaron las 6 cepas más activas, *Ll. sakei* 31, *Lc. casei* 116 y *Lco. garviae* 151 procedentes de quesos y *Ll. sakei* 197, 204 y 205 aisladas de industrias cárnicas. Estas cepas están disponibles para su utilización en productos madurados. Para controlar la implantación durante la maduración de las mismas se procedió al análisis de restricción PFGE utilizando las enzimas de restricción *SgsI* y *NotI*. Picozzi y col. (2010) indicó que los análisis de PFGE son un método apropiado para la diferenciación de cepas de BAL. Con estas dos enzimas se obtuvo una clara diferenciación de las 6 cepas seleccionadas. Adesulu-Dahunsi y col., (2021) también consiguieron diferenciar a nivel de cepa diferentes especies de BAL utilizando la técnica de PFGE con otra enzima de restricción (*Sfi*I) diferente a las anteriores. Por lo tanto, esta técnica y el procedimiento seguido parecen útiles para la diferenciación a nivel de cepa de las BAL seleccionadas. Para controlar la implantación de las cepas seleccionadas se propone la utilización de la secuenciación 16S ARNr para la caracterización a nivel de especie y el análisis de PFGE con enzimas de restricción *SgsI* y *NotI* para la diferenciación a nivel de cepa.

V.1.3. Evaluación del comportamiento de *L. monocytogenes* en condiciones experimentales ácidas

Otro de los mecanismos de acción de las BAL para controlar e incluso inhibir el crecimiento y la proliferación de *L. monocytogenes* es, tal como se ha señalado, la producción de ácidos orgánicos que actúan acidificando el medio en el que se encuentra esta bacteria patógena. Se conoce que, aunque valores de pH más bajos pueden retrasar el crecimiento de *L. monocytogenes* (Vermeulen y col., 2007), también dichas condiciones extremas pueden causar estrés a este microorganismo activando factores de virulencia. Por este motivo, en esta Tesis Doctoral, se ha evaluado el efecto que tienen

distintas condiciones de pH ácidos (entre 4,5 y 6) similares a las provocadas por la presencia de BAL en embutidos curado-madurados y quesos madurados en el crecimiento y en la expresión de dos genes de virulencia (*hly* e *inlA*) de *L. monocytogenes*. Adicionalmente, se ha optimizado un método de PCR en tiempo real mediante la metodología SYBR® Green para detectar cambios en la expresión del gen *inlA*.

El gen *inlA* es uno de los genes de virulencia de *L. monocytogenes* y se ha demostrado la activación de su expresión en condiciones experimentales ácidas (Olesen y col., 2009; Sue y col., 2004). Por este motivo se diseñó y desarrolló un método qPCR rápido y fiable basado en el gen *inlA* utilizando la metodología SYBR® Green para detectar cambios en el estrés ácido.

En primer lugar, se procedió al diseño de los cebadores basados en la secuencia del gen *inlA* de *L. monocytogenes*. Los cebadores diseñados en esta Tesis Doctoral cumplieron los requisitos recomendados para poder ser utilizados de forma eficiente en reacciones de qPCR (Rodriguez y col., 2015). A continuación, la optimización del método de qPCR se llevó a cabo satisfactoriamente teniendo en cuenta los resultados obtenidos en relación con la linealidad y la eficiencia de la curva estándar. Ésta mostró una linealidad superior al 0,99 y un valor de eficiencia del 95%, lo cual es indicativo de que el método es robusto y reproducible. La linealidad (R^2) y la pendiente estuvieron dentro del rango recomendado (Rodríguez y col., 2015). Estos resultados indicaron que el método qPCR diseñado podría ser apropiado para cuantificar los cambios en la expresión del gen *inlA* vinculado a las respuestas al estrés ácido de *L. monocytogenes*.

Una vez optimizado el método de qPCR, se evaluó el efecto de condiciones experimentales ácidas (en medio de cultivo BHI con ácido láctico con valores de pH =

6;5,5;5 y 4,5), sobre el crecimiento y la expresión relativa de los genes *inlA* y *hly* de *L. monocytogenes* con respecto al tiempo de incubación (4, 8 y 12 horas).

En este estudio, se observó un aumento en la expresión de los genes *inlA* y *hly* de *L. monocytogenes* a medida que aumenta la acidez del medio. Esto es muy interesante ya que ambos genes afectan a la virulencia de este patógeno y parecen activarse en condiciones ácidas. Los resultados de este estudio son similares a los encontrados por Li y col. (2021) quienes demostraron la activación de ambos genes en cepas de *L. monocytogenes* habituadas a ácidos orgánicos. Comparando los dos genes evaluados, el gen *inlA* de *L. monocytogenes* se vio más afectado por los cambios ocurridos en la acidez del medio que el gen *hly*. Por tanto, el método desarrollado con la metodología SYBR® Green podría ser de interés para comprobar cambios en la expresión de la virulencia de esta bacteria patógena en ambientes ácidos como podemos encontrar en embutidos curado-madurados y quesos madurados. Además, los resultados han demostrado que existe una relación inversa entre la expresión relativa de los genes *hly* e *inlA* y el crecimiento de *L. monocytogenes* en condiciones ácidas simuladas. Esto significa que este patógeno necesita activar su virulencia mediante la activación de ambos genes para contrarrestar el estrés provocado por la reducción del pH del medio de cultivo como ha sido descrito por Li y col. (2021). Siendo esto de gran importancia ya que el ácido láctico es un metabolito producido por una amplia gama de bacterias, y más específicamente por las BAL, durante los procesos de maduración de diferentes alimentos RTE que pueden contaminarse fácilmente por *L. monocytogenes* durante su procesado y comercialización como los embutidos curado-madurados y quesos madurados (Prpich y col., 2021; Ray y col., 2014). En conclusión, aunque el crecimiento de *L. monocytogenes* en condiciones ácidas que normalmente se encuentran en los alimentos fermentados, incluidos los embutidos curado-madurados o quesos curados (valores de pH que oscilan entre 4,5 y 6)

es limitado, se produce un aumento de la expresión relativa de los genes de virulencia, *hly* e *inlA*, que podría incrementar la virulencia de este patógeno. Por lo tanto, la información obtenida puede ser de gran utilidad para evaluar posibles incrementos de virulencia en cepas de *L. monocytogenes* que puedan sobrevir en los ambientes ácidos de alimentos madurados RTE en el que se utilicen las BAL seleccionadas.

V.1.4. Evaluación de hidrolizado proteico de lactosuero

Tal como se había indicado anteriormente los hidrolizados proteicos sólos o en combinación con BAL seleccionadas son otra estrategia para el control de *L. monocytogenes*. En esta Tesis Doctoral se evaluó el efecto de un hidrolizado proteico elaborado y suministrado por CIAL a partir del suero obtenido en queserías sobre el crecimiento y la expresión de genes de virulencia y respuesta al estrés de *L. monocytogenes* cuando se inoculó en un medio elaborado con queso "Torta del Casar" solo o en combinación con 6 BAL aisladas e identificadas en un trabajo previo por el grupo de investigación (*Lp. plantarum* [B2, B5 y B6], dos cepas de *Leuconostoc mesenteroides* [B1 y B3] y una cepa adscrita a *Lactiplantibacillus* spp. [B4]). Los resultados obtenidos mostraron que independientemente de la combinación de cepas de BAL utilizada, se observó una reducción en el crecimiento de este microorganismo patógeno en comparación con el medio de cultivo control solamente inoculado con *L. monocytogenes* y que la utilización del hidrolizado proteico es efectiva frente al crecimiento de esta bacteria patógena en un medio elaborado con queso cuando se combina con el efecto de algunas de las BAL seleccionadas (B1, B2, y B3), pero no presenta ningún efecto solo o en combinación con otras cepas de BAL.

Además, el efecto del hidrolizado proteico en presencia de *Le. mesenteroides* sugiere que éste puede potenciar el efecto de esta cepa aumentando su capacidad para competir

por nutrientes con *L. monocytogenes*, produciendo ácido láctico no disociado o disminuyendo la a_w debido a la difusión de la sal (Gonzales-Barron y col., 2020; Kapetanakou y col., 2017).

Además, *L. monocytogenes* tiene la capacidad de responder rápidamente a condiciones adversas o cambios ambientales regulados mediante la expresión del gen *sigB* (Ferreira y col., 2001; Hadjilouka y col., 2016). Se observó un aumento de la expresión del gen *sigB* en la mayoría de los lotes estudiados, aunque solo fue significativa cuando se inoculó el hidrolizado junto a *Lp. plantarum* B2 y la cepa *Lactiplantibacillus* spp. B4. Este resultado concuerda con los resultados observados por otros autores en distintos embutidos curado-madurados con procesos de elaboración diferentes en los que se produce un descenso del pH y a_w durante el proceso de elaboración y tenian un contenido de sal de alrededor del 3% (Mataragas y col., 2015; Olesen y col., 2010).

Los resultados obtenidos en esta Tesis Doctoral confirmaron que la utilización de BAL podría proponerse como una estrategia de biocontrol de *L. monocytogenes* en quesos tradicionales elaborados con leche cruda. Sin embargo, la utilización de los hidrolizados proteicos no parecen ser adecuados, dado que su utilización de forma individual no prococa ninguna reducción de *L. monocytogenes* en los ensayos realizados en modelos alimentarios y cuando se utiliza de forma combinada con cepas de BAL seleccionadas podría potenciar la virulencia de esta bacteria patógena. Éste fue el motivo por el que se decidió no continuar evaluando la estrategia basada en la utilización de los hidrolizados proteicos para controlar y/o minimizar el desarrollo de *L. monocytogenes* en alimentos madurados. Se procedió sólo a la evalución en productos madurados de las cepas de BAL seleccionadas utilizando la metodología de “challenge test”.

V. 2. EVALUACIÓN DE CEPAS SELECCIONADAS EN MATRICES ALIMENTARIAS MEDIANTE "CHALLENGE TEST".

Antes de la propuesta final de las cepas de BAL seleccionadas como cultivos protectores, es fundamental evaluar su actividad anti-*L. monocytogenes* de embutidos y quesos madurados en las condiciones del procesado y en presencia de la contaminación habitual de estos productos. Esto permitirá conocer no sólo su efecto en la reducción de esta bacteria patógena, sino además las posibles consecuencias sobre las características sensoriales del producto que pudieran derivarse de la utilización de las BAL seleccionadas como cultivos protectores.

Una vez realizada la selección de las 6 cepas con actividad anti-*L. monocytogenes* en función de su actividad en los modelos alimentarios y de la presencia de genes relacionados con la síntesis de bacteriocinas, se procedió a investigar la capacidad de las cepas de BAL más efectivas para controlar el crecimiento de *L. monocytogenes* en el producto alimenticio mediante la metodología de "challenge test" o test de desafío. Estos tests han sido utilizados en la selección de BAL para reducir o eliminar *L. monocytogenes* en los alimentos (Bungenstock y col., 2020; Campagnollo y col., 2018).

Con la metodología del "challenge test" se han evaluado las cepas de BAL seleccionadas más activas en salchichón o queso "Torta del Casar" según la procedencia de la cepa. Durante la maduración de estos productos se han procesado diferentes lotes en función de tipo, combinación y nivel de microorganismo inoculado, pero todo el proceso de maduración se hizo sólo una vez en cada uno de los productos, siguiendo las recomendaciones para el desarrollo de tests de desafío en alimentos donde no hay

probabilidad de crecimiento de *L. monocytogenes* o ésta es inferior al 10% (Beaufort y col., 2014).

V.2.1. Efecto de *Ll. sakei* sobre el crecimiento y expresión génica de *L. monocytogenes* en embutidos curado-madurados. Influencia de *Ll. sakei* sobre las características sensoriales del producto.

Durante la maduración, se evaluó *Ll. sakei* 205 por ser la cepa más activa en los modelos de salchichón. Además, *Ll. sakei* forma parte de la población microbiana predominante en los embutidos curado-madurados y está adaptada a este nicho ecológico, de tal manera que es un microorganismo que podría colonizar el producto de manera efectiva y competir potencialmente frente a *L. monocytogenes* (El Adab y col., 2015). Además, esta bacteria ha sido considerada como un "microorganismo seguro" por los miembros del comité de expertos sobre Seguridad Biológica de la EFSA (EFSA BIOHAZ Panel, 2021).

En cuanto a su capacidad como agente de biocontrol, *Ll. sakei* ha sido ampliamente utilizada frente a microorganismos patógenos como *Salmonella*, *S. aureus* y *L. monocytogenes* en productos cárnicos (Bošković y col., 2017; Gelinski y col., 2019; Zagorec y Champomier-Vergès, 2017).

Cuando se evaluó el efecto de *Ll. sakei* 205 sobre la población microbiana contaminante de los embutidos curado-madurados durante el proceso de maduración, se observó una disminución de los recuentos de enterobacterias hasta niveles no detectables al final de la maduración. Estos resultados concuerdan con los obtenidos por Cocolin y col. (2009) donde se demostró la persistencia de este grupo microbiano hasta el día 60 de maduración de los embutidos curado-madurados. Aunque en el ensayo realizado durante esta Tesis Doctoral no se observó crecimiento de enterobacterias durante el procesado,

otros autores han indicado el crecimiento puntual de este grupo microbiano durante el procesado de embutidos curado-madurados (Fernández-López y col., 2008; Lizaso y col., 1999). La disminución o incluso la eliminación de las enterobacterias en este tipo de productos puede explicarse por el descenso de la a_w, los diversos metabolitos excretados por las BAL y un ligero descenso en los valores de pH (Fernández-López y col., 2008). Por otro lado, la reducción de los niveles de estafilococos únicamente en los casos inoculados con *Ll. sakei* 205 al final de la maduración puede estar relacionada con el efecto de la acción sinérgica de esta cepa junto con la reducción de la a_w a lo largo del procesado.

Durante la maduración del salchichón, no se observó en ningún caso crecimiento de *L. monocytogenes*. Esto es de gran importancia porque en los primeros 15 días de maduración pueden existir condiciones de temperatura, a_w y pH que pueden favorecer el crecimiento de este microorganismo patógeno; sin embargo, el efecto sinérgico de los parámetros anteriores y la presencia de NaCl, nitritos y la adición de las BAL inoculadas o bien las que se encuentran de forma natural en el producto parecen inhibir el crecimiento de este patógeno.

Cuando se estudió el efecto de *Ll. sakei* 205 frente a *L. monocytogenes* a lo largo del proceso de maduración de los embutidos curado-madurados, se observaron reducciones significativas en los recuentos de este patógeno (hasta 2,2 log UFC/g). Estos resultados concuerdan con los obtenidos en estudios previos para este patógeno en otros productos cárnicos curados (Taddei y col., 2020). Varios estudios previos han demostrado que durante la maduración de los embutidos curado-madurados se produce una importante disminución de a_w, que favorece la reducción de los recuentos de *L. monocytogenes* en estos productos (Barbuti y col., 2009; Montiel y col., 2020; Reynolds y col., 2001).

Además, el nitrito utilizado en la formulación del salchichón de este trabajo también contribuye al control de *L. monocytogenes*, tal y como han indicado otros autores para diferentes tipos de embutidos curado-madurados (Lucke, 2000; Sebranek, 2007). Sin embargo, la reducción en los recuentos de este patógeno fue significativamente mayor cuando se inoculó *Ll. sakei* 205. Esto confirma que *Ll. sakei* 205 tiene una importante actividad anti-*L. monocytogenes* durante la maduración del salchichón, aun considerando que en los embutidos curado-madurados en los que se inoculó únicamente *L. monocytogenes* también se encontraban BAL autóctonas, probablemente con algún efecto antimicrobiano.

Aunque la reducción adicional de *L. monocytogenes* provocada por *Ll. sakei* 205 no fue muy elevada, podría ser suficiente para garantizar la eliminación de esta bacteria patógena durante el proceso de elaboración del salchichón cuando este patógeno pudiera llegar al producto en los niveles habituales de contaminación (inferior al 2 log UFC/g). Este aspecto es muy importante, no solo por reducir o eliminar el riesgo de listeriosis vinculado al consumo de salchichón, sino además porque permite cumplir los criterios microbiológicos de los alimentos RTE a lo largo de su vida útil en la UE (Bonilauri y col., 2021; Peromingo y col., 2019).

Además de evaluar el efecto sobre el crecimiento de *L. monocytogenes*, se determinó el efecto de *Ll. sakei* 205 sobre la expresión de tres genes de virulencia de la bacteria patógena, lo cual puede ser de gran utilidad para comprender la respuesta de este microorganismo en presencia de este potencial cultivo protector y, por lo tanto, diseñar estrategias de control eficaces frente al mismo (Hadjilouka y col., 2017). En este sentido, se evaluó el efecto de *Ll. sakei* 205 sobre la expresión absoluta de los genes de virulencia *plcA*, *hly* e *iap* de *L. monocytogenes* en el salchichón. Para ello, se estudió la relación

entre el crecimiento y la expresión génica de *L. monocytogenes* mediante un análisis de correlación, obteniéndose una buena correlación entre ambos parámetros (valores r: 0,792, 0,821 y 0,820 para los genes *plcA, hly* e *iap,* respectivamente).

Los resultados obtenidos revelaron que los dos genes de virulencia *plcA* y *hly* mostraron valores de expresión superiores a los encontrados para el gen *iap* en todas las condiciones evaluadas. Además, se obtuvo que los valores de expresión de los genes de virulencia disminuyeron al final del período de maduración, probablemente debido a las condiciones de estrés creadas por el proceso de elaboración y la composición del salchichón, caracterizados principalmente por la disminución de la a_w a lo largo de la maduración. Estos resultados concuerdan con los obtenidos es un estudio llevado a cabo por Alía y col. (2020), en el que observaron que una disminución en los valores de expresión de los genes *plcA, hly* e *iap* en un sistema modelo de jamón curado como consecuencia de la disminución de a_w.

De forma generalizada, la no modificación en la expresión de los genes de virulencia de *L. monocytogenes* cuando se inoculó junto con *Ll. sakei* 205 en los embutidos curado-madurados contrastan con el efecto demostrado de algunas BAL bacteriocinogénicas como *E. faecium* sobre la inhibición de la expresión de los genes de virulencia de *L. monocytogenes* (Ye y col., 2018).

Como se ha comentado con anterioridad, además de la disminución adicional de los recuentos de *L. monocytogenes* que provocó la presencia de *Ll. sakei* 205, también se demostró en esta Tesis Doctoral, que esta BAL no mostró ningún efecto apreciable sobre la expresión génica del microorganismo patógeno a lo largo del proceso de elaboración del salchichón. Esto es muy importante ya que un cultivo protector no debe provocar un aumento de la expresión de un gen de virulencia del microorganismo patógeno pues puede

suponer un problema desde el punto de vista de la seguridad alimentaria. En algunos estudios, se ha demostrado que la utilización de cultivos protectores, como distintas cepas de *D. hansenii*, pueden aumentar la expresión de genes de virulencia en *L. monocytogenes* en un modelo alimentario elaborado con jamón curado (Alía y col., 2020), no siendo aconsejable su utilización como cultivo protector en alimentos RTE.

Finalmente, una vez comprobada la eficacia de un cultivo protector frente al microorganismo patógeno de interés, es necesario realizar una evaluación de los parámetros fisicoquímicos (pH, contenido de humedad, a_w), bioquímicos (generación de compuestos volátiles) y sensoriales del producto inoculado, aspectos que también se evaluaron en esta Tesis Doctoral. Esta evaluación permite descartar algunas cepas de BAL que podrían afectar negativamente a las propiedades fisicoquímicas y organolépticas de los productos RTE y proponer únicamente aquellas cepas sin efecto o con repercusión positiva sobre sus características físicoquímicas y sensoriales.

La evaluación de los parámetros fisicoquímicos (humedad, a_w y pH) de los salchichones durante la maduración demostró que la inoculación de *Ll. sakei* 205 no afecto a estos parámetros. De hecho, el contenido de humedad y la a_w de los salchichones disminuyeron a lo largo del proceso de maduración, alcanzando valores similares a los habitualmente encontrados para este tipo de productos (Álvarez y col., 2020; Casquete y col., 2011; El Adab y col., 2015). Esta disminución en los valores del contenido de humedad y a_w es muy importante para prolongar la vida útil y controlar la inocuidad del producto (Chevallier y col., 2006). Los resultados obtenidos muestran que *Ll. sakei* no provocó ninguna modificación en el contenido de humedad y de a_w, concordando estos resultados con los obtenidos en otras BAL (Najjari y col., 2020; Pavli y col., 2020). Sin embargo, la cepa *Ll. sakei* 205 provocó una ligera reducción en el pH en los primeros 30

días de maduración, en comparación con los embutidos sin inocular, probablemente debido al aumento en el contenido de ácido láctico, como resultado de la degradación de carbohidratos por el metabolismo microbiano (Aleson-Carbonell y col., 2005). Sin embargo, al final de la maduración no se encontraron diferencias en el pH por la adicción de la BAL seleccionada. Los contenidos de NaCl y nitritos fueron similares en todos los lotes estudiados siendo estos valores similares a los obtenidos en otros estudios (Fernández-López y col., 2008; Gonzales-Barron y col., 2015).

En cuanto a los parámetros del color y la textura de los embutidos curado-madurados, la adición de la BAL seleccionada no provocó modificaciones en relación al lote control (salchichones sin inocular), obteniéndose valores similares a los habituales en estos productos (El Adab y col., 2015; Herrero y col., 2007).

Finalmente, se realizó un análisis de los compuestos volátiles de los salchichones inoculados con *Ll. sakei* 205 y no inoculados a lo largo de la maduración de los embutidos curado-madurados. De esta forma, se identificaron un total de 52 compuestos volátiles, habiéndose encontrado la mayoría de estos compuestos previamente en diferentes tipos de embutidos curado-madurados (Andrade y col., 2010; Bianchi y col., 2007; Flores y col., 2004). Estos compuestos volátiles fueron clasificados de acuerdo con su origen más probable: oxidación de lípidos, esterificación microbiana, fermentación de carbohidratos, catabolismo de aminoácidos y especias.

No hubo diferencias consistentes entre los salchichones inoculados con *Ll. sakei* 205 y los lotes sin inocular en la mayoría de los compuestos volátiles. En vista a los resultados, se observó un aumento de compuestos volátiles derivados del catabolismo de aminoácidos y esterificación microbiana y una disminución de los compuestos derivados

de la oxidación de lípidos a lo largo del tiempo de maduración. Además, la inoculación de *Ll. sakei* 205 podría contribuir a este efecto positivo sobre el sabor del salchichón.

En la evaluación sensorial de los salchichones inoculados con *Ll. sakei* 205, se evaluó si los catadores podían diferenciar el salchichón control y el inoculado con la BAL seleccionada. Analizando los resultados obtenidos se obtuvo que la aplicación de *Ll. sakei* en la masa de los embutidos no modificó las características sensoriales del producto ya que los catadores no fueron capaces de diferenciar entre ambos productos.

En vista a todos estos resultados, la aplicación de *Ll. sakei* 205 como cultivo protector sería de gran interés para la reducción de *L. monocytogenes* en embutidos curado-madurados y, en general, para mejorar inocuidad del producto, además de aumentar la generación de compuestos volátiles asociados al aroma a curado de este tipo de productos.

V.2.2. Efecto de *Lc. casei* y *Lco. garviae* sobre el crecimiento de *L. monocytogenes* y las características sensoriales en queso "Torta del Casar"

Tras la selección de las 5 BAL con mayor actividad anti-*L. monocytogenes* en el medio de cultivo elaborado con queso, se evaluó en queso durante la maduración las dos cepas que presentaron mayor efecto en el crecimiento del microorganismo patógeno: *Lc. casei* 116 *y Lco.garviae* 151. Para ello se elaboraron quesos tipo "Torta del Casar" en una quesería de la D.O.P. "Torta del Casar" y tras el cuajado y prensado se trasnsportaron a la planta piloto de la Facultad de Veterinaria de la UEx para su división en lotes en los que se inocularon los distintos microoorganismos a evaluar y se maduraron en cámaras siguiendo el procesado industrial. Se evaluó el efecto de las BAL inoculadas sobre el crecimiento de *L. monocytogenes* y las características sensoriales en los quesos

madurados. Es necesario considerar que *Lc. casei* se suele aislar de los quesos y los *Lactococos* contribuyen de manera importante a la producción de productos lácteos fermentados (Ordiales y col., 2013a). Además, se ha descrito que *Lco. garviae* se encuentra en la leche cruda, el queso y otros productos lácteos (Abdelfatah y Mahboub, 2018). Por otro lado, se ha descrito que estas dos especies de BAL tienen la capacidad de competir contra *L. monocytogenes* limitanto su desarrollo (Arena y col., 2016; Campagnollo y col., 2018; García y col., 2020; Scatassa y col., 2017). *Lc casei* está entre las especies consideradas como "microorganismos seguros" por miembros del comité de expertos sobre Seguridad Biológica de la EFSA (EFSA BIOHAZ Panel, 2021).

Cuando se evaluó el efecto de *Lc. casei* 116 y *Lco. garviae* 151 sobre distintos grupos microbianos en los quesos "Torta del Casar", los recuentos de enterobacterias disminuyeron durante el proceso de maduración en todos los lotes evaluados, y especialmente en los lotes inoculados con las BAL seleccionadas. No obstante, los valores finales de este grupo microbiano continuaron siendo altos al final de la maduración. Hay que tener en cuenta que se trata de un queso elaborado con leche cruda. En este y otros tipos de quesos de pasta blanda elaborados con leche cruda se detectan habitualmente niveles altos de enterobacterias, tal como ha sido previamente descrito (Coton y col., 2012; Crespo y col., 2020; Gonçalves y col., 2018). La presencia de las BAL seleccionadas sin duda están limitando el desarrollo de este grupo microbiano.

Al evaluar los recuentos de BAL, estos fueron superiores a 8 log UFC/g durante todo el proceso de maduración, sin variabilidad entre los quesos inoculados con alguna de la BAL seleccionada y los quesos sin inocular. Esto se debe a que los recuentos de la población autóctona de BAL son de alrededor de 8 log UFC/g, mientras que la inoculación de las BAL seleccionadas se realizaron a una concentración de

aproximadamente 7 log UFC/g. Los valores encontrados en este estudio fueron similares a los encontrados en otro estudio sobre el queso "Torta del Casar" (Ordiales y col., 2014).

La capacidad de *Lc. casei* 116 y *Lco. garviae* 151 para controlar el desarrollo de *L. monocytogenes* en quesos madurados fue evaluado en esta Tesis Doctoral. Para llevar a cabo dicho ensayo, *L. monocytogenes* fue inoculada a dos concentraciones diferentes, una baja y otra elevada (4,5 y 7,5 log UFC/g), respectivamente. Estos niveles están dentro de los encontrados en otros productos lácteos y quesos contaminados con este microorganismo patógeno (1-5 log UFC/g), (Rudolf y Scherer, 2001) en diferentes quesos blandos y semiblandos. La inoculación del microorganismo a dos niveles se realizó porque se quería investigar en profundidad la capacidad de las cepas de BAL seleccionadas para reducir el crecimiento de *L. monocytogenes* durante la maduración de quesos de pasta blanda.

Los recuentos de *L. monocytogenes*, al final de la maduración (90 días), se redujeron alrededor de 1,1 y 3 log UFC/g en los lotes inoculados únicamente con este patógeno a baja y a alta concentración, respectivamente. A pesar de que esta reducción fue bastante importante, fue significativamente superior en los lotes en los que se inocularon alguna de las dos cepas de BAL. Así, observamos una reducción adicional de 1,2 y 1,1 log UFC/g cuando se co-inoculó *Lc. casei* 116 o *Lco. garviae* 151 junto con *L. monocytogenes* a la concentración más baja y, de la misma manera, se observó una reducción adicional de 2,61 y 3,55 log UFC/g cuando se inocularon con el patógeno a la concentración más alta.

De las dos cepas de BAL utilizadas la que mayor efecto antagónico frente a *L. monocytogenes* tuvo fue la cepa *Lc. casei* 116, llegando a reducciones de los recuentos del patógeno de hasta 5 log UFC/g durante el proceso de maduración. Estos resultados concuerdan con resultados de investigación previos que han demostrado que las BAL de

origen lácteo pueden ejercer una acción anti-*L. monocytogenes* durante la producción y almacenamiento de diferentes quesos (Campagnollo y col., 2018; Ortolani y col., 2010; Panebianco y col., 2021). Estos resultados son muy prometedores ya que la reducción de los recuentos de este patógeno podría ser suficiente para garantizar la eliminación de esta bacteria patógena a lo largo de la elaboración del queso "Torta del Casar" cuando este patógeno contamina este producto en los niveles habituales (≤ 2-3 log UFC/g).

Como se ha indicado anteriormente, antes de proponer la utilización de estos cultivos para el control de *L. monocytogenes* en quesos madurados es necesario realizar una evaluación de los parámetros fisicoquímicos (pH, contenido de humedad, a_w), bioquímicos (generación de compuestos volátiles) y sensoriales del producto inoculado, aspectos que también se evaluaron en esta Tesis Doctoral.

La evaluación de los parámetros fisico-químicos durante el proceso de maduración reflejó que la inoculación de las BAL no afectó de forma general al proceso de maduración de los quesos, que en todos los lotes evolucionó correctamente. De hecho, los valores del contenido de humedad y a_w fueron similares a los encontrados por otros autores para este tipo de queso (Ordiales y col., 2014; Ordiales y col., 2013). Además, la adicción de las BAL seleccionadas no modificaron los valores de a_w durante todo el proceso de maduración.

En los lotes inoculados con *Lco. garviae* 151 se observó mayor reducción del contenido de humedad a los 30 y 45 días de maduración en comparación con los quesos sin inocular. No obtante, al final de la maduración, no se observó diferencias en el contenido acuso entre los lotes inoculados *Lco. garviae* 151 y el control sin inocular.

La adición de las BAL seleccionadas no provocó una reducción significativa del pH, sino por el contrario, parece provocar un ligero aumento en los valores de pH a los 60 días de maduración; sin embargo, a los 90 días de maduración solo en el lote en el que se inoculó *Lc. casei* 116, el pH fue significativamente mayor que en los quesos control. El contenido de NaCl fue similar en todos lotes estudiados estando dentro de los establecidos por la D.O.P. Torta del Casar (< 3%) (Estrada y col., 2019).

Una vez expuesto lo anterior, se puede afirmar que la inhibición observada no puede asociarse al hecho de que haya unas condiciones más ácidas en los quesos madurados inoculados con alguna de las dos BAL seleccionadas. Una posible explicación para la reducción en los niveles del microorganismo patógeno podría ser la competencia por nutrientes y/o espacio entre el patógeno y el cultivo protector o la producción de compuestos como bacteriocinas por las BAL seleccionadas, ya que se ha descrito que las cepas de *Lc. casei* y *Lco. garviae* producen bacteriocinas activas frente a *L. monocytogenes* (Deegan y col., 2006; Parada y col., 2007; Tosukhowong y col., 2012).

La textura es una característica muy importante en este tipo de quesos e influye en la aceptabilidad del consumidor (Delgado y col., 2010). Los resultados obtenidos del parámetro de textura muestran que la adicción de BAL seleccionadas no provocaron una modificación de este parámetro respecto a los quesos no inoculados. Medved'ová y col. (2020) tampoco encontraron diferencias en la textura de los quesos cuando se añadió una cepa de *Lc. ramhnosus.*

Con respecto a la determinación del color, tampoco se observaron diferencias relevantes entre los quesos inoculados con BAL y los quesos no inoculados. Únicamente se detectaron algunas diferencias en el lote inoculado con *Lco. garviae* 151 en el parámetro L* (luminosidad).

Por lo tanto, estos resultados parecen indicar que la adición de las BAL utilizada en este trabajo no provocó cambios en los parámetros fisicoquímicos, teniendo estos una gran influencia en la estabilidad del queso y condicionando el desarrollo de los microorganismos, la actividad enzimática y la velocidad de las reacciones bioquímicas durante el tiempo de maduración (Sanjuán y col., 2002).

En cuanto al análisis de los compuestos volátiles, se identificaron un total de 34 compuestos en los quesos analizados a lo largo de los 90 días de maduración, incluidos ácidos carboxílicos, alcoholes, aldehídos, cetonas y ésteres. La mayoría de los compuestos identificados también han sido encontrados en estudios de quesos "Torta del Casar" y otros quesos de pasta blanda (Ferreira y col., 2009; Jia y col., 2021; Ordiales y col., 2013). En el presente trabajo, se demostró la capacidad de las BAL seleccionadas para generar ácidos carboxílicos de cadena ramificada, ya que se detectaron en cantidades más altas en los lotes inoculados que en el lote control. Así, la inoculación de *Lc. casei* y *Lco. garviae* podría contribuir al aroma del queso "Torta del Casar" ya que los ácidos 2-metilbutanoico y 3-metilbutanoico contribuyen al aroma y sabor general (Qian y Reineccius, 2002).

La evolución de la producción de compuestos alcohólicos a lo largo del proceso de maduración fue muy similar en los quesos inoculados con BAL y los quesos sin inocular. Con respecto a las cetonas se detectó una baja abundancia de estos compuestos al comienzo de la maduración, aumentando de forma significativa todos los compuestos detectados de este grupo después de 90 días de maduración. Las cetonas son constituyentes abundantes de la mayoría de los productos lácteos y tienen olores típicos y umbrales de percepción bajos (Delgado y col., 2010; Jia y col., 2021) y, por tanto, podrían jugar un papel importante en el aroma final del queso "Torta del Casar". Los lotes

inoculados y sin inocular mostraron la misma evolución en la formación de cetonas y solo se detectaron algunas diferencias en los niveles de estos compuestos al final del tiempo de maduración. Así, solo 2-nonanona mostró una abundancia significativamente mayor en los quesos inoculados con BAL que en los lotes de control, mientras que 2-heptanona y 2-butanona se encontraron en cantidades más altas en el control que en los lotes inoculados con BAL. Estos compuestos son necesarios para el aroma del queso, pero dado que derivan de la β-oxidación de ácidos grasos, no se debe estimular su producción (Güler y Gürsoy-Balci, 2011). La inoculación de las BAL seleccionadas no contribuyen al incremento de estos compuestos.

Como aldehídos, solo se identificó 3-metilbutanal durante todo el proceso de maduración. Las altas concentraciones de aldehídos están asociadas con el desarrollo de sabores desagradables en el queso (Chen y col., 2019; Moio y Addeo, 1998), por lo que un aumento a lo largo del procesado sería negativo para el aroma global de este producto. En este trabajo se observó un aumento significativo de 3-metilbutanal a lo largo del proceso de maduración. Sin embargo, dado que no se detectaron diferencias entre los quesos inoculados con BAL y los quesos sin inocular, parece que la adicción de *Lc. casei* y *Lco. garviae* tienen un impacto bajo en la producción de este compuesto.

Por otro lado, se observó una disminución en la mayoría de los ésteres a lo largo del proceso de maduración en todos quesos estudiados. La mayoría de los ésteres identificados eran ésteres etílicos, que podrían contribuir de manera importante al aroma típico de los quesos “Torta del Casar” (Delgado y col., 2010; Ferreira y col., 2009). En el presente trabajo las cepas de BAL inoculadas y especialmente *Lc. casei*, no contribuyó a la formación de ésteres, o al menos no hubo diferencias con los quesos no inoculados (aunque estos tienen BAL de forma natural). Aunque algunos de estos ésteres tienen un

sabor dulce, afrutado y podrían contribuir positivamente al aroma del queso madurado, es importante considerar que también pueden tener una influencia negativa, dependiendo de la concentración y tipo de queso (Jia y col., 2021).

De forma general se puede deducir del análisis de los compuestos volátiles que la adición al queso "Torta del Casar" de *Lc. casei* y *Lco. garviae* podría tener un efecto positivo sobre el aroma, por su contribución a la generación de compuestos ramificados (principalmente ácidos ramificados de metilo) y por no aumentar e incluso reducir los compuestos de oxidación resultantes de la β-oxidación de los ácidos grasos.

En la evaluación sensorial de los quesos inoculados con *Lc. casei* y *Lco. garviae* se consideró una característica principal a la hora de consumir estos productos como es el olor del producto. Analizando los resultados obtenidos se puede deducir que la aplicación de cualquiera de las dos cepas BAL seleccionadas en los quesos "Torta del Casar" no generó modificaciones en el olor ya que los catadores no pudieron diferenciarlos de los lotes sin inocular.

En vista de los resultados, se puede plantear la aplicación tanto de *Lc. casei* 116 como de *Lco. garviae* 151, especialmente la primera de las cepas, como cultivo protector para el control de *L. monocytogenes* en quesos madurados, minimizando el riesgo de listeriosis provocada por el consumo de quesos blandos y semiblandos y así garantizar la seguridad alimentaria y el cumplimiento de los criterios microbiológicos de los alimentos RTE durante toda su vida útil en la UE (Reglamento (CE) nº 2073/2005), sin modificar las características sensoriales del producto.

Debido a su capacidad anti-*L. monocytogenes* y a sus características tecnológicas de *Lc. casei* 116 se ha llevado a cabo el registro de una patente con el título de "Nueva cepa

de *Lacticaseibacillus casei* 116 con actividad antagonista frente a *Listeria monocyotogenes* para su uso como cultivo protector en quesos madurados"

V.3. ESTUDIO DEL MECANISMO DE LA ACTIVIDAD DE LAS CEPAS DE BAL SELECCIONADAS

Adicionalmente al trabajo anteriormente descrito, durante la estancia de investigación en la Universidad de Oporto (Portugal) se ha llevado a cabo un estudio profundo sobre la caracterización de BAL en base a su seguridad y capacidad de producir compuestos proteicos con capacidad antimicrobiana recogido en la Figura V.1, con el objetivo fundamental de la caracterización de la actividad antimicrobiana de las cepas previamente seleccionadas en la UEx, especialmente la que ha sido tramitada como las cepas finalmente seleccionadas *Ll sakei* 205 de embutidos y *Lc. casei* 116 de quesos.

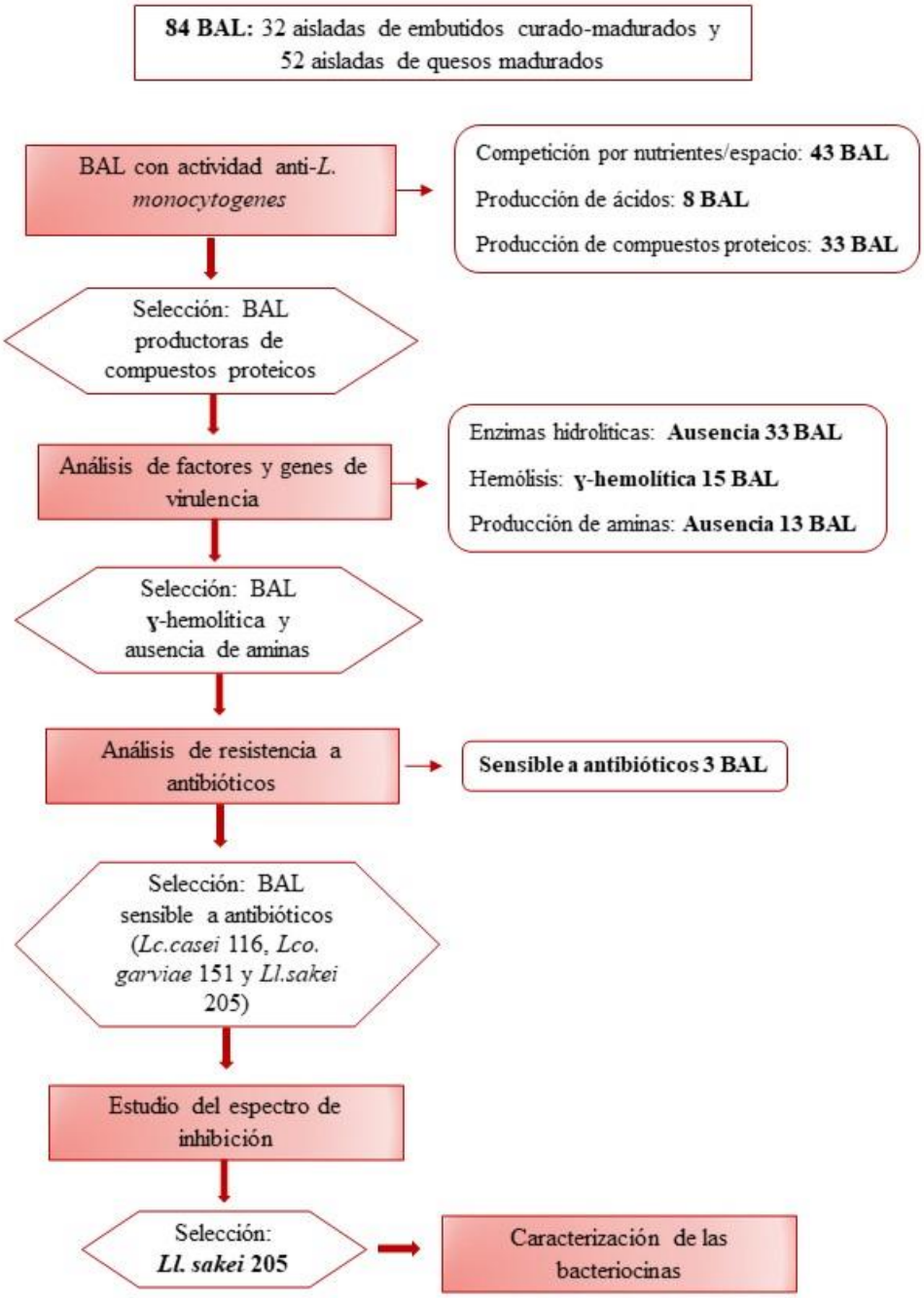


Figura V.I. Diagrama de flujo de la selección de BAL en base a su seguridad y capacidad de producir compuestos proteicos con capacidad antimicrobiana

Para llevar a cabo este estudio, se utilizaron las 84 BAL aisladas de embutidos curado-madurados y quesos madurados con actividad anti-*L. monocytogenes* (Tabla V.1). Se obtuvo que 43 de ellas inhibieron a dicho patógeno por competencia por nutrientes y/o

espacio, 8 BAL controlaron el desarrollo del microorganismo patógeno por la producción de ácidos que disminuyen el pH del medio y 33 evitaron el crecimiento de los microorganismos patógenos debido a la producción de compuestos proteicos. Por lo tanto, las 33 cepas que utilizaron este último mecanismo, entre las que estaban las 10 más activas en los modelos de alimentos, fueron seleccionados para el resto de los estudios.

En estas cepas seleccionadas se evaluaron la presencia de factores y genes de virulencia para descartar aquellas cepas que no son seguras. La ausencia de factores patógenos son criterios esenciales para la selección de posibles microorganismos que se van a proponer como cultivos protectores o probióticos (Hossain y col., 2021). Los resultados obtenidos mostraron que ninguna de las cepas evaluadas mostró capacidad para producir enzimas hidrolíticas ADNasa y gelatinasa. Lo mismo observaron otros autores al estudiar otros aislados de la mismas especies evaluadas (Barbosa y col., 2010; Gómez y col., 2016; Perin y col., 2014; Valledor y col., 2022). Además, la ausencia de actividad hemolítica es una demanda clave en la propuesta de cepas como cultivos protectores (Oh y Jung, 2015). De las 33 cepas de BAL evaluadas, ninguna tuvo actividad β-hemolítica, 18 (54,5 %) fueron α-hemolíticas y 15 (45,5 %) no presentaron hemólisis (ɣ-hemolítico). Ya que esta actividad se considera una desventaja en la selección de cultivos protectores y probióticos (Bermudez-Humaran y Langella, 2012), solo se seleccionaron las 15 cepas que no presentaron actividad hemolítica (ɣ-hemolítica).

En las 15 cepas seleccionadas se evaluó la capacidad de formación de aminas como aspecto negativo para descartar aquellas que tienen actividad decarbolixasa y conducen a la formación de estos metabolitos. Las principales aminas que se encuentran en altas concentraciones en los alimentos fermentados son histamina, tiramina, putrescina y cadaverina (Durak-Dados y col., 2020). Ninguna de las cepas evaluadas tiene la capacidad

de producir histamina, putrescina y cadaverina y solo dos de ellas (*E. faecium* 40 y *Ll. sakei* 175) podrían producir tiramina. Debido a los efectos tóxicos que presenta la producción de aminas, estas dos cepas no fueron seleccionados para los siguientes estudios, seleccionándose sólo 13 para los siguientes ensayos.

La detección de genes relacionados con diferentes factores de virulencia también se puede utilizar para evitar la aplicación de cepas potencialmente peligrosas. A pesar de su potencial beneficioso, las cepas probadas presentaron un patrón variable de genes relacionados con la virulencia. Sin embargo, debemos destacar que la presencia de genes específicos no se traduce automáticamente en su expresión y potencial virulencia de la cepa que alberga este material genético. De hecho, aunque algunas cepas albergaban los genes *hdc1* y *odc*, ninguno de las BAL tuvo la capacidad de producir histamina y putrescina. Lo mismo ocurrió con el gen *gelE* y la producción de gelatinasa. A pesar de que la mayoría de las cepas mostraron el gen *tcd*, solo las dos indicadas anteriormente (*E. faecium* 40 y *Ll. sakei* 175) produjeron tiramina.

La resistencia a los antibióticos también es otro factor que debe evaluarse antes de la posible propuesta como cultivo protector. En este trabajo se estudió las principales clases de antibióticos y en general, las cepas de *Enterococcus* mostraron mayor resistencia a los antibióticos que el resto de cepas. Además, hay que destacar que sólo 3 (*Lc. casei* 116, *Lco. garviae* 151 y *Ll. sakei* 205) de las 13 cepas finalmentes seleccionadas fueron sensibles a todos los antibióticos probados, lo que indica que no tienen factores de resistencia a antibióticos. Estas 3 cepas fueron seleccionados para su posterior caracterización.

Un aspecto que destacar es la capacidad de las BAL de inhibir patógenos. Esta actividad inhibitoria puede variar dado que algunas solo inhiben bacterias Gram-positivas

relacionadas taxonómicamente, y otras son activas contra una gama más amplia de microorganismos Gram-positivos y Gram-negativos (Harzallah y Belhadj, 2013).

De las 3 BAL seleccionadas, todas inhibieron al menos una de las cepas de *L. monocytogenes* estudiadas y, además, la mayoría de ellas inhibieron todas las cepas de este patógeno. Además, la bacteriocina producida por *Ll. sakei* 205 mostró una importante actividad anti-*L. monocytogenes*, pero también fue eficaz contra otros microorganismos importantes, como *Acinetobacter baumannii* ESB028, *S. aureus* ATCC 6538, *S. aureus* ATCC, *S. typhimurium* ESB009 y *S. enteritidis* ESB008417536(405) y *E. faecalis* ATCC 29212. Otros autores estudiaron la capacidad de *Ll. sakei* de inhibir una amplia gama de microorganismos patógenos (De Carvalho y col., 2010; Todorov y col., 2011; Urso y col., 2006).

Esta es una importante ventaja que contribuye a hacer aún más atractivo el uso de este microorganismo en la industria alimentaria y por eso esta bacteria fue seleccionada para la caracterización de las bacteriocinas.

Es importante estudiar el crecimiento y la producción de bacteriocina, así como la resistencia a la temperatura, el pH, enzimas y ciertos detergentes además de su actividad frente a patógenos. En este trabajo se estudió la actividad de las bacteriocinas producida por *Ll. sakei* 205 frente a *L. monocytogenes* NCTC 11994, *L. monocytogenes* EDG-e, *L. monocytogenes* Scott A y *E. faecalis* ATCC 29212.

La mayor actividad antimicrobiana de las bacteriocinas producidas por *Ll. sakei* 205 se observó contra *E. faecalis* con 6400 UA/ml durante 18-20 h de crecimiento. El número de células viables de *Ll. sakei* 205 aumentó aproximadamente, 3 log UFC/mL (crecimiento celular máximo a las 16 h de crecimiento). Todorov y col. (2011) también

observaron un gran crecimiento de la bacteriocina producida por *Ll. sakei;* sin embargo, obtuvo la máxima producción entre las 6 a 11 h, siendo esta determinada 1600 UA/mL contra *L. innocua*.

La actividad de la bacteriocina producida por *Ll. sakei* 205 permaneció estable a temperaturas que oscilaron entre 25 ºC y 60 ºC; sin embargo, se observó sólo actividad residual a temperaturas superior a 80 ºC. Resultados similares fueron observados por Albano y col. (2007) en la bacteriocina producida por *Pe. acidilactici* aislado de "Alheira", un embutido fermentado producido tradicionalmente en Portugal. Estos autores encontraron una reducción de la actividad de esta bacteriocina cuando se expuso a altas temperaturas. Sin embargo, esto no supone un problema ya que los embutidos curado-madurados no son tratados por el calor.

La bacteriocina producida *Ll. sakei* 205 se mantuvo estable después de la incubación durante 1 h a pH 4,0 y 6,0 (0% de reducción) por lo que mantendría su actividad en embutidos curado-madurados. Heredia-Castro y col. (2015) indicaron que la actividad antimicrobiana de diferentes cepas de BAL presentaba un amplio rango de pH (4.0-8.0). La bacteriocina fue sensible a todos los detergentes probados, pero se mantuvo estable frente a *L. monocytogenes* NCTC 11994 y *L. monocytogenes* EDG-e después del tratamiento con detergentes tensoactivos (Tween-20 y Tween-80). Se observaron resultados similares contra *L. monocytogenes* ScottA y *E. faecalis* ya que la bacteriocina se mantuvo estable después del tratamiento con NaCl. Se observó una alta inactivación de la actividad antimicrobiana después del tratamiento con la enzima proteolítica proteinasa K, lo que confirma su naturaleza proteica. En general, se observó una menor reducción de la actividad después del tratamiento con otras proteasas probadas (papaína

y pepsina) y después de la actividad de la enzima antioxidante (catalasa). Estos resultados fueron similares a los obtenidos por Albano y col. (2007).

El efecto del sobrenadante libre de células tratado de *Ll. sakei* 205 sobre el crecimiento de *L. monocytogenes* ScottA, EDG-e y NCTC 11994, y *E. faecalis* también fue evaluado en esta Tesis Doctoral. En vista a los resultados obtenidos, se puede afirmar que el sobrenadante libre de células tratado de *Ll. sakei* 205 afectó a la viabilidad de todos los patógenos estudiados. Sin embargo, este efecto fue más significativo frente a *L. monocytogenes* ScottA y *E. faecalis*, con reducciones superiores a 2 log UFC/mL. Estos resultados fueron similares a los estudiados por Todorov y col., (2011) en los que *L. monocytogenes* y *E. faecium* disminuyeron en presencia de la bacteriocina (800 AU/mL) producida por *Ll. sakei* R1333.

Cuando se determinó el peso molecular de la bacteriocina de *Ll. sakei* 205 mediante electroforesis en gel de poliacrilamida se observó que tiene un peso molecular de 24 kDa. Este tamaño molecular es mayor que la mayoría de las bacteriocinas descritas previamente para *Ll. sakei*. Sin embargo, también se ha informado un peso molecular de alrededor de 14 kDa para la bacteriocina de *Lactobacillus pentosus* ST712BZ (Todorov y Dicks, 2007) y de 17 kDa para las pediocinas (Papagianni y Anastasiadou, 2009; Ramos y col., 2016). Por lo que, estos resultados pueden estar de acuerdo con los amplicones del tamaño correspondiente de pediocina PA-1 encontrados.

En definitiva, en esta Tesis Doctoral se han seleccionado 2 cepas de bacterias lácticas (*Ll. sakei* 205 de embutidos y *Lc. casei* 116 de quesos capaces de provocar reducciones de 2 log UFC/g de *L. monocytogenes* durante la maduración de estos productos. Se ha demostrado que estas cepas son seguras dado que no tienen genes ni factores de virulencia, no provocan hemolisis, no tienen capacidad de producir aminas y no presentan

resistencia a antibióticos. Las dos cepas tienen genes relacionados con la síntesis de bacteriocinas y las bacteriocinas producida por *Ll. sakei* 205 han sido caracterizadas. Estas dos cepas no producen cambios sensoriales negativos en los respectivos productos madurados en los que han sido ensayados, al contrario, podrían favorecer el desarrollo de compuestos volátiles de interés en estos productos, repercutiendo así positivamente en el aroma de embutidos y quesos madurados. Por tanto, se proponen estas dos cepas para su utilización como cultivos protectores en alimentos madurados tradicionales.

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Review

Strategies for Biocontrol of *Listeria monocytogenes* Using Lactic Acid Bacteria and Their Metabolites in Ready-to-Eat Meat and Dairy-Ripened Products

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Abstract: *Listeria monocytogenes* is one of the most important foodborne pathogens. This microorganism is a serious concern in the ready-to-eat (RTE) meat and dairy-ripened products industries. The use of lactic acid bacteria (LAB)-producing anti-*L. monocytogenes* peptides (bacteriocins) and/or lactic acid and/other antimicrobial system could be a promising tool to control this pathogen in RTE meat and dairy products. This review provides an up to date about the strategies of use of LAB and their metabolites in RTE meat products and dairy foods by selecting the most appropriate strains, by analysing the mechanism by which they inhibit *L. monocytogenes* and methods of effective application of LAB, and their metabolites in these kinds of products to control this pathogen throughout the processing and storage. The selection of LAB with anti-*L. monocytogenes* activity allows to dispose of effective strains in meat and dairy-ripened products, achieving reductions form 2–5 logarithmic cycles of this pathogen throughout the ripening process. The combination of selected LAB strains with antimicrobial compounds, such as acid/sodium lactate and other strategies, as the active packaging could be the next future innovation for eliminating risk of *L. monocytogenes* in meat and dairy-ripened products.

Keywords: *L. monocytogenes;* LAB; bacteriocins; protective cultures

Citation: Martín, I.; Rodríguez, A.; Delgado, J.; Córdoba, J.J. Strategies for Biocontrol of *Listeria monocytogenes* Using Lactic Acid Bacteria and Their Metabolites in Ready-to-Eat Meat and Dairy Ripened Products. *Foods* **2022**, *11*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: 20 January 2022
Accepted: 7 February 2022
Published: date

1. Introduction

Listeria monocytogenes is one of the most important pathogenic microorganisms and is responsible for causing listeriosis, an illness that affects mainly pregnant women, newborns, the elderly, and individuals with compromised immune systems [1]. Although it is a relatively rare illness, with a notification rate of 0.46 cases per 100,000 people in 2019 in the European Union (EU), most of the infections required hospitalisation (92.1%) [2]. This microorganism is a serious concern in the ready-to-eat (RTE) meat and dairy products industries, including dry-cured fermented sausages or ripened cheeses [3–5], since it could colonize and grow in raw material and pre-processed products throughout the processing and/or storage of these products, posing a risk for the consumers and/or also provoking non-compliance of microbiological criteria for this pathogen bacterium. Although in most of these RTE ripened foods, the reduction of water activity (a_w) and pH throughout the ripening are hurdles that aid to control *L. monocytogenes*, this pathogen has been involved in many outbreaks linked to the consumption of the above products [3,6–8].

The use of lactic acid bacteria (LAB) as protective cultures could be an additional tool to control *L. monocytogenes* in RTE meat and dairy-ripened products. LAB have been frequently used as starter or protective cultures due to their natural ability to dominate the microbial population of many foods where they naturally occur due to their ability to catabolize carbohydrates to lactic acid and produce other biologically active compounds,

such as organic acids, diacetyl, hydrogen peroxide, and antibacterial peptides and flavour precursors [9]. In addition, screening natural LAB strains to find the ones able to produce antimicrobial molecules is a promising strategy. An important number of either bacteriostatic or bactericidal compounds produced by LAB has been described [10].

The LAB genera are *Carnobacterium*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus,* and the former *Lactobacillus* genus, which has been recently reclassified into 25 new genera [11,12]. Most of them have the status Generally Recognised as Safe (GRAS) according to the U.S. Food and Drug Administration (FDA). In addition, many LAB species have the recognition of Qualified Presumption of Safety (QPS) from the European Food Safety Authority (EFSA) (Table 1); thus, they have this presumptive qualification of being safe to be used as protective cultures in foods.

Table 1. LAB included in the 2020 updated list of QPS status recommended biological agents for safety risk assessments carried out by EFSA Scientific Panels and Units [11].

Bifidobacterium adolescentis	*Lactobacillus delbruechkii*	*Ligilactobacillus animalis*
Bifidobacterium animalis	*Lactobacillus gallinarum*	*Ligilactobacillus aviaries*
Bifidobacterium bifidum	*Lactobacillus gasseri*	*Ligilactobacillus salivarius*
Bifidobacterium breve	*Lactobacillus helveticus*	*Liminosilactobacillus fermentum*
Bifidobacterium longum	*Lactobacillus johnsonii*	*Liminosilactobacillus mucosae*
Carnobacterium divergens	*Lactobacillus kefiranofaciens*	*Liminosilactobacillus panis*
Companilactobacillus alimentarius	*Lactococcus lactis*	*Liminosilactobacillus pontis*
Companilactobacillus farciminis	*Lapidilactobacillus dextrinicus*	*Liminosilactobacillus reuteri*
Corynebacterium ammoniagenes	*Latilactobacillus curvatus*	*Loigolactobacillus coryniformis*
Corynebacterium glutamicum	*Latilactobacillu sakei*	*Microbacterium imperial*
Fructilactobacillus sanfranciscensis	*Lentilactobacillus buchneri*	*Oenococcus oeni*
Lacticaseibacillus casei	*Lentilactobacillus diolivorans*	*Pasteuria nishizawae*
Lacticaseibacillus paracasei	*Lentilactobacillus hilgardii*	*Pediococcus acidilactici*
Lacticaseibacillus rhamnosus	*Lentilactobacillus kefiri*	*Pediococcus parvulus*
Lactiplantibacillus pentosus	*Lentilactobacillus parafarraginis*	*Pediococcus pentosaceus*
Lactiplantibacillus plantarum	*Lentilactobacillus paraplantarum*	*Propionibacterium acidipropionici*
Lactobacillus acidophilus	*Leuconostoc citreum*	*Propionibacterium freudenreichii*
Lactobacillus amylolyticus	*Leuconostoc lactis*	*Secundilactobacillus collinoides*
Lactobacillus amylovorus	*Leuconostoc mesenteroides*	*Streptococcus thermophilus*
Lactobacillus cellobiosus	*Leuconostoc pseudomesenteroides*	
Lactobacillus crispatus	*Levilactobacillus brevis*	

Although many LAB strains have been isolated and selected for their ability to in vitro inhibit *L. monocytogenes*, not all of them have been effectives in real RTE ripened food systems. In the present work, the strategies of selection of effective LAB species against *L. monocytogenes* to be used in RTE meat products and dairy-ripened products will be reviewed. In addition, we will also review the mechanism by which they inhibit *L. monocytogenes* as well as effective methods of LAB application and their metabolites in these kinds of products to control this pathogen throughout their processing and storage.

2. Selection and Evaluation of LAB from RTE Meat and Dairy-Ripened Products with Anti-*L. monocytogenes* Activity

Many traditional RTE fermented foods constitute rich ecological niches for screening LAB with anti-*L. monocytogenes* activity [13–15]. The selection of LAB with anti-*L. monocytogenes* activity from these products to be used as protective cultures should be performed from strains adapted to the ecological niche of these products since they must survive and are competitive in conditions of processing and/or storage. Thus, LAB strains should be isolated during ripening and/or storage conditions of meat and dairy-ripened products following different steps (Figure 1). First, LAB isolate should be obtained from meat and dairy-ripened products, testing different days of ripening (for example initial, half, and final time) or in different days of storage, with the purpose to obtain strains adapted to characteristics and processing conditions of the chosen foods. Then, isolated strains should be preliminary characterized by Gram staining, catalase reaction, shape by microscopic observation of overnight cultures, and biochemical features, as it has been reported in LAB strains isolation from soft cheese [16,17] or dry-cured fermented sausages [18]. After the first preliminary characterization, the isolates are screened for anti-L. monocytogenes in culture media usually by the agar spot-on-a-lawn method [14,19]. Thus, preliminary active isolates against L. monocytogenes are obtained, which are characterized by 16S rRNA sequencing [17] and further evaluated by co-inoculation with *L. monocytogenes* for anti-listerial activity in food models simulating temperature, water activity, and pH conditions of RTE products, as it has been reported by Martín et al., 2022 (Unpublished data) in soft cheese model for the selection of active anti-listerial LAB strains (Figure 1). This step is of the utmost importance to discard LAB strains lacking activity or with low activity in the processing or storage conditions of RTE foods. Thus, active LAB strains against L. monocytogenes able to be finally evaluated in RTE products are selected. In parallel, the selected LAB strains should be finally characterized by some additional method to the 16 rRNA sequencing, which allow the differentiation at strain level. This should be of great value to evaluate implantation of selected strains in RTE foods in the next step. Pulsed-field gel electrophoresis analysis (PFGE) has been reported as an appropriate method for the differentiation of LAB strains [20]. In addition, the RFLP analysis of the tuf gene has also been described as a suitable tool for the differentiation of LAB strains [21].

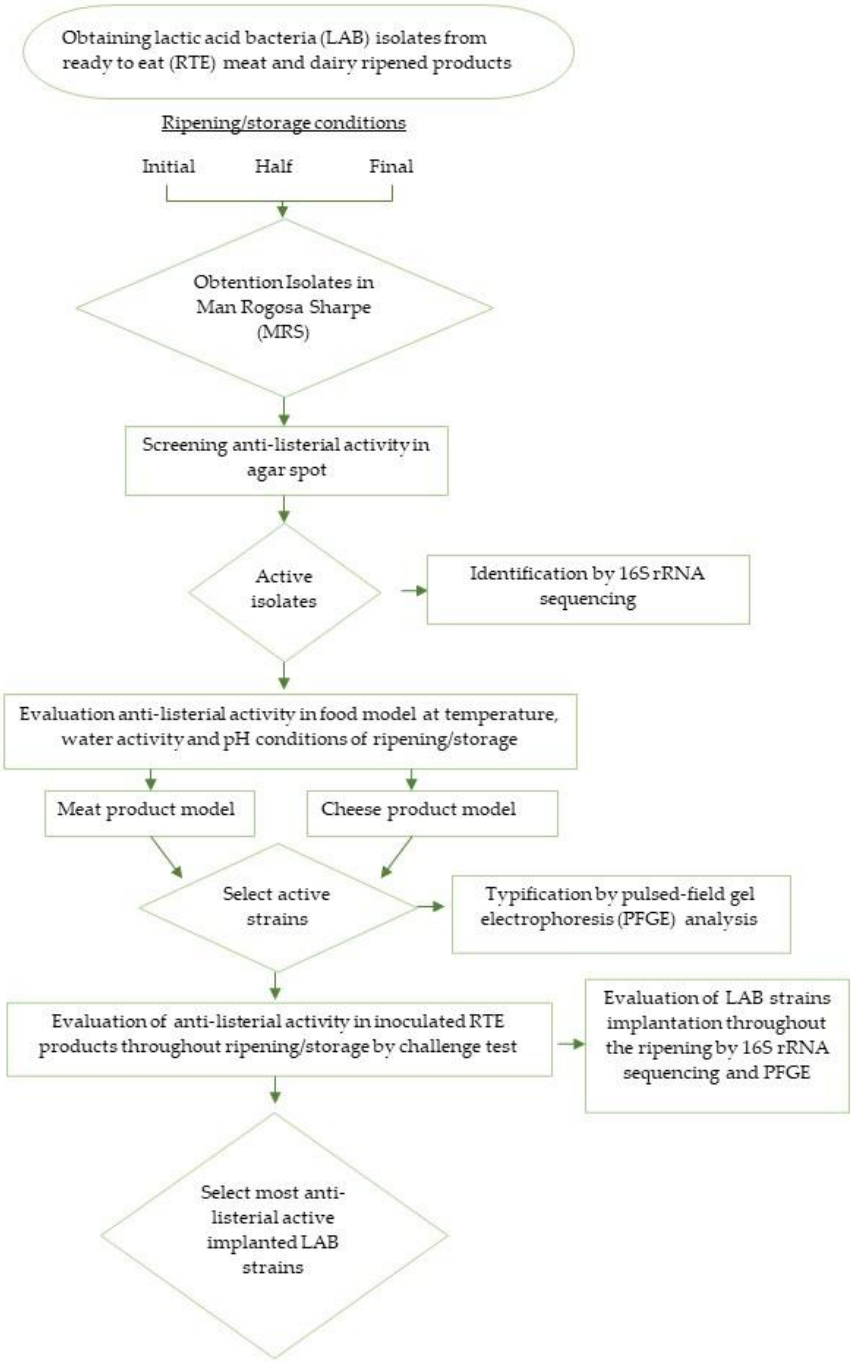

Figure 1. Diagrammatic flowchart for the isolation and selection of LAB strains with anti-listerial activity from RTE meat and dairy foods.

Finally, it should be investigated the ability of the most effective LAB strains to control the growth of *L. monocytogenes* in the food product by the challenge test methodology (Figure 1), as it has been reported in dry-cured fermented sausages [22] and ripened cheeses [16]. These authors have found the ripening reduction of *L. monocytogenes* from 2 to 5 log CFU/g because of the action of selected LAB strains. In addition, the assayed strains were successfully implanted in the food matrices.

Many studies have focused on the selection of LAB to reduce or eliminate *L. monocytogenes* in foods [23]. Campagnollo et al. [16] reported the isolation and characterization of LAB strains with anti-listerial activity and their effects on *L. monocytogenes* during

refrigerated shelf-life of soft and ripening of semi-hard cheese. De Carvalho et al. [24] tested several LAB isolated from naturally fermented Italian salami for antagonistic activity against *L. monocytogenes*. Pedonese et al. [25] stated that *Lactilactobacillus* (*Ll*) *sakei* is capable of suppressing the growth of pathogenic and spoilage microorganisms and improving the sensory quality of fresh meat preparations and products.

Most of the selected LAB strains with anti-*L. monocytogenes* activity have also been evaluated for possible modification of physicochemical (pH, humidity content, a_w), biochemical (proteolysis, lipolysis, volatile compounds generation), and sensorial parameters before being proposed as protective or starter cultures [16,26]. This evaluation allows to rule out some LAB strains that could negatively affect the physicochemical properties of RTE products and propose only those strains without effect or with positive repercussion on sensorial characteristic. All the former works have selected and characterized LAB strains that are available to be used as protective cultures in RTE meat and dairy-ripened products due to their effect against *L. monocytogenes*.

3. Effect of Selected LAB Strains on *L. monocytogenes* Inhibition

LAB mechanisms for *L. monocytogenes* inhibition in RTE foods include (a) production of inhibitory compounds, (b) competition for nutrients, (c) prevention of pathogen adhesion, and (d) competition for space or niche competition.

3.1. Production of Inhibitory Compounds

LAB have the ability to produce antimicrobial compounds, such as lactic acid and other organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide, bacteriocins, or bactericidal proteins [27]. Table 2 shows a summary of these compounds and their mechanisms of action.

Table 2. Inhibitory compounds produced by selected LABs and their mechanisms of action against pathogens microorganisms, such as *L. monocytogenes*.

Inhibitory Compound	Mechanism of Action	References
Lactic acid and other volatile acids	Disruption of cellular metabolism	[27]
Ethanol	Membrane fluidity and integrity	[28]
Hydrogen peroxide	Inactivation of essential biomolecules by superoxide anion chain reaction	[29]
Carbon dioxide	Anaerobic environment and/or inhibition of enzyme decarboxylation and/or disruption of the cell membrane	[30]
Diacetyl	Interference with arginine utilization	[29]
Bacteriocins	Disruption of cytoplasmic membrane	[27,31]

Homofermentative LAB ferment carbohydrates to produce lactic acid as the major metabolic product, leading to pH reduction of food and also directly to growth inhibition of many microorganisms [32]. It has been described that the principal antimicrobial compound responsible for their activity against pathogens is synthesis of organic acids, mainly lactic and acetic acids [28,33]. Organic acids act by acidifying the intracellular pH, generating an unfavourable local microenvironment for pathogenic bacteria [28,34]. They also act by inhibiting the active transport of excess internal protons that leads to the depletion of cellular energy [35]. The bacterial cell wall and the cytoplasmic membrane are the main targets of organic acids provoking alteration and death and metabolic functions of pathogenic microorganisms [36]. It has been proven that concentrations of 0.5% (*v*/*v*) of lactic acid could completely disrupt the growth of pathogenic microorganisms, such as *Salmonella* spp., *Escherichia coli*, or *L. monocytogenes* [37]. Wemmenhove et al. [38] tested the effect of lactic acid against *L. monocytogenes* in Gouda cheese. In addition, it has been

studied that the short-chain fatty acids produced by LAB in food fermentation improve the integrity of the barrier and prevent the adhesion of pathogenic bacteria or indirectly inhibit the expression of the virulence genes at the transcriptional level of *L. monocytogenes*.

However, heterofermentative LAB produce lactic acid and additional compounds, such ethanol and carbon dioxide [39,40]. Ethanol produced by heterofermentative LAB affects membrane fluidity and integrity, leading to plasma membrane leakage and causing bacterial death [28]. Barker and Park [41] found that a 5% ethanol concentration inhibited the replication of *L. monocytogenes*.

Some LAB strains produce hydrogen peroxide (Table 2) that provokes inactivation of essential biomolecules of the pathogens, such as *L. monocytogenes,* by superoxide anion chain reaction negatively affects its viability [29]. Thus, in vitro inhibition of *L. monocytogenes* mainly due to production of hydrogen peroxide was reported by Ghalfi et al. [42] with an *Ll. curvatus* selected strain from meat origin.

Heterofermentative LAB strains also produce carbon dioxide as a by-product of sugar fermentation that inhibits growth of *L. monocytogenes* [27,30].

Some LAB strains produce diacetyl, which interferes with arginine utilization of pathogen microorganisms, such as *L. monocytogenes* [29]. In fact, the combination of some bacteriocins, such as reuterin and diacetyl, have been reported to be anti-microbial additives with effects against *L. monocytogenes* [43].

Bacteriocins are produced by some LAB strains and contribute to the biological control of pathogenic and spoilage microorganisms. Bacteriocins and their effect against *L. monocytogenes* are detailed in the following section (Section 4).

3.2. Competition for Nutrients

One of the main mechanisms of action of non-pathogenic bacteria against pathogenic bacteria is competition for nutrients in a specific niche, leading to depletion [44]. The metabolic activity of *L. monocytogenes* may not be affected by antimicrobial compounds produced by LAB (bacteriocins, organic acids including lactic, and acetic acids) due to its acid tolerance and synthesis of proteolytic enzymes. Therefore, the growth rate of LAB is of great importance in their role in competing for nutrients with *L. monocytogenes* [45].

However, under stress conditions provoked by the lack of nutrients or acid stress caused by the organic acids synthesized by LAB, *L. monocytogenes* can express some of its virulence factors, such as InlA and InlB protein, to confront this stress [46]. In the same way, LAB may induce the synthesis of bacteriocins as a method to minimize the stress caused by insufficient nutrients [44].

3.3. Competition for Space

Another mechanism of action of LAB includes the competitive exclusion of pathogenic microorganisms from space [47]. Adhesion of *L. monocytogenes* on host cells is of great importance for their invasion and virulence [44,48]. LAB can prevent the binding of *L. monocytogenes* on host cells by colonizing the host cells and/or saturating the pathogen binding receptor [44]. Corr et al. [49] showed that pre-treatment of intestinal epithelial cells with LAB before infection with *L. monocytogenes* resulted in a significant decrease in its invasion (60–90%). When there is a direct cell-to-cell competition between *L. monocytogenes* and LAB to the binding sites, LAB inhibit the attachment of the pathogen, being reduced by 4.38 and 3.22 log CFU/g after 24 h and 72 h, respectively [50]. According to Pilchová et al. [51], a significant inhibition of the adhesion, invasion, and transepithelial translocation of *L. monocytogenes* was obtained using *Lacticaseibacillus (Lc) paracasei* but only if this strain was recombined to obtain the expression of the adhesion protein of *L. monocytogenes.*

Competitive inhibition of selected LAB strains has been reported in biofilm formation of *L. monocytogenes,* which poses a risk factor in the food industry [52]. These authors found that selected *Ll. curvatus*, *Lactococcus (La) lactis*, *Lactobacillus helveticus*, and *Weissella*

viridescens isolated from Brazilian's foods developed protective biofilms against *L. monocytogenes* hampering the biofilm formation by this pathogen, mostly due the exopolysaccharide production by these LAB strains. Thus, selected LAB strains could be promissory candidates for controlling the presence of *L. monocytogenes* biofilms in food-processing facilities [50].

3.4. Reduction of L. monocytogenes Virulence by LAB

Many authors have studied the use of LAB to reduce the expression of virulence of pathogens by modulating the expression of genes or proteins through bacterial signalling mechanisms. Thus, Dutra et al. [53] reported that *Lc. casei* and *Lc. rhamnosus* significantly reduced the binding (10–13%) and invasion (40–50%) of *L. monocytogene*s into cells, indicating that LAB are effective in reducing this pathogen colonization both when administered prophylactically and during infection. Another study by Upadhyay et al. [54] demonstrated that *Liminosilactobacillus (Li) reuteri*, *Li. fermentum*, *Lactiplantibacillus (Lp) plantarum,* and *La. lactic* reduced the adhesion and invasion of Caco-2 cell of *L. monocytogenes,* down-regulating the expression of the majority of virulence genes of this pathogen (*plcA*, *plcB*, *iap*, *hly*, *inlA*, *inlB*, *actA,* and *prfA*).

A significant reduction in *L. monocytogenes* virulence on epithelial cells was observed when the cell monolayers were mixed with *Carnobacterium divergens* V41 cultures during 1 or 4 h. The ability to control foodborne pathogenic microorganism virulence has previously been evaluated for probiotic LAB and found to be strain specific. For example, Garriga et al. [55] reported a that bacteriocinogenic *Ll. sakei* strain significantly decreased the adhesion of *L. monocytogenes*.

Winkelströter and De Martinis [56] showed that the bacteriocins produced by *Enterococcus* (E) *faecium*, *Leuconostoc (Le) mesenteroides,* and *Ll. sakei* significantly decreased the expression of *inlA* gene from different *L. monocytogenes* strains.

4. Bacteriocins with Activity against *L. monocytogenes*

Antimicrobial peptides or proteins produced by LAB are small, ribosomally synthesized, and possess activity against closely related gram-positive bacteria, whereas producer bacteria are immune to their own proteinaceous metabolites [57]. In general, bacteriocin-producing strains mostly belong to the formerly named *Lactobacillus* and *Lactococcus* genera and are well-proven to have Generally Recognised as Safe (GRAS) status [58].

The antibacterial spectrum of bacteriocins frequently includes spoilage microorganisms and foodborne pathogens, such as *L. monocytogenes* and *Staphylococcus aureus*. In addition to their antimicrobial action towards these unwanted species, bacteriocins are believed to contribute to increasing the competitiveness of the producer strain [59].

So far, bacteriocins have been classified into four general classes attending to their composition and structural properties. The first class, termed as lantibiotics, contains unusual amino acids (i.e., lanthionines and β-methyllanthionines). The production of this class of bacteriocins involves post-translational modifications, which are well described for nisin [60]. The second one is comprehended by bacteriocins that do not contain lanthionine residues, being characterised by heat stability and their site of action as the cell membrane. The third one is composed by the large and heat-sensitive bacteriocins, and finally, the fourth class is bacteriocins containing other chemical moieties (carbohydrates and lipids) [61]. Subsequent subclassifications into these groups has been performed, as shown in Table 3.

Table 3. Bacteriocins classification, main features, examples of different bacteriocins, and their producer microorganisms.

Class	Characteristics	Example	Producer	Reference
Ia	Lantibiotics (<5 KDa)	Nisin	*Lactococcus lactis*	[62]
Ib	Carbacyclic lantibiotics	Labyrinthopeptien A1	*Actinomadura nambiensis*	[63]
Ic	Sactibiotics	Subtilosin A	*Bacillus subtilis*	[64]
IIa	Heat-stable peptides with N terminal-YGNGV	Pediocin PA-1, sakacins A and P, leucocin A, garviecin LG34	*Pediococcus pentosaceus, Pediococcus acidilactici, Lactilactobacillus sakei, Lactococcus garvieae*	[65–69]
IIb	Two-peptide bacteriocins	Lactococcin G, plantaricin EF and JK	*Lactiplantibacillus plantarum, Lactococcus* spp.	[70–72]
IIc	Circular bacteriocins	Enterocin AS-48, gassericin A	*Lactococcus gasseri, Enterococcus faecalis*	[73,74]
IId	Single, linear, nonpediocin-like bacteriocins	Thuricin S, bactofencin A	*Bacillus thuringensis, Ligilactobacillus salivarius*	[75,76]
IIIa	Heat labile, >30 KDa with hydrolase activity	Lysostaphin	*Staphylococcus. simulans* biovar *staphylolyticus*	[77]
IIIb	Heat labile, >30 KDa without hydrolase activity	Helveticin	*Lactobacillus helveticus*	[78]
IV	Large complexes with carbohydrate or lipid moieties	Enterocin F4-9	*Enterococcus faecalis*	[79]

Among all bacteriocins, the post-translationally modified class Ia nisin is probably the best-known bacteriocin with listericidal effect. Nisin's mechanism of action involves membrane permeabilization through binding to lipid II, the phenyl chain-linked donor of the peptidoglycan building blocks [80]. This lipid II is believed crucial to peptidoglycan synthesis, and nisin is considerably more active towards peptidoglycan-rich, gram-positive microorganisms than gram-negative ones, the latter being only affected by nisin in conjunction with chemically induced damage of the outer membrane [81]. This bacteriocin is approved as a preservative by the European Commission, named as E 234, intended for use in various dairy products, among others [82].

Apart from the well-known nisin, the II class bacteriocins and most concretely IIa class are the most commonly active against *L. monocytogenes*. Some of these bacteriocins include garviecin LG34, bifidocin A, leucocin C-607, pediocin GS4, plantaricin LPL-1, or pediocin PA-1 or sakacins [65,83–87]. These pediocin-like class IIa bacteriocins deploy great bacterial inhibition at nanomolar concentrations in relation to the high affinity to specific receptors or docking molecules [83]. One of these target receptors is the called mannose-phosphotransferase system (man-PTS), which phosphorylates and transports carbohydrates and other related substances, and the membrane components, ManY/IIC and ManZ/IID, belonging to man-PTS, form a membrane-located complex [88–90]. Additionally, these class IIa bacteriocins act on the cytoplasmic membrane of gram-positive microorganisms, dissipating the transmembrane electrical potential and resulting in intracellular ATP depletion. Furthermore, they induce the leak of ions, amino acids, proteins, and nucleic acids by forming hydrophilic pores in target membranes [87,91].

Although the nature of these compounds is able to inhibit *L. monocytogenes*, several strains from this pathogen have been able to develop a certain degree of resistance against bacteriocins. The two strategies deployed by resistant bacteria to counteract the bacteriocins effect are membrane surface charge and membrane fluidity [92,93].

For class I bacteriocins, such as nisin, changes in membrane lipid composition are involved in *L. monocytogenes* resistance [94], as well as phospholipids charges in interactions between artificial membranes and nisin [95,96]. For class IIa bacteriocins, their target, the man-PTS receptor, plays a key role in the resistance against this type of bacteriocins [97]. On one hand, the low expression of genes related to this receptor is directly linked to IIa-class bacteriocin resistance [98]. On the other hand, as occurs for leucocin A, changes in membrane fatty acid composition, increase in D-alanine content of wall teichoic acid, and increase in L-lysine content of membrane phospholipids are other common strategies elicited by class-IIa resistant *L. monocytogenes*.

To overcome the limitation of bacteriocin resistance by *L. monocytogenes* and maximize its inhibitory activity, the use of different bacteriocins combined or even a given bacteriocin in combination with technological or chemical treatments could provide an alternate approach to tackle this problem [99], enhancing the antimicrobial effect as discussed in Section 6.

Selection of Bacteriocin-Producing Lactic Acid Bacteria and Bacteriocin Characterization

A common requirement for any protective culture is the safety of these organisms irrespectively of the production of antimicrobial metabolites. The ability to produce biogenic amines, such as 2-phenylethylamine, putrescine, cadaverine, agmatine, spermine, spermidine, histamine, and tyramine, should also be ruled out by gene analyses [100]. Additionally, other unwanted genes involved in virulence, such as *asa1*, *agg*, *efaA*, *hyl*, *esp*, *cylL*$_L$, *cylL*$_S$, *ace*, and *gelE*, should also be tested with the aim to ensure the lack of virulence in the selected strain [101]. Finally, the antibiotic resistance must be evaluated both for the possible involvement of some of these bacteria, mainly *E. faecalis* and *E. faecium*, in human infections [102] and the induction of potential antimicrobial resistance through horizontal gene transference [103].

Once the safety characterisation has been considered, the first step to evaluate the production of bacteriocins would entail a screening based on the assessment of the antimicrobial activity of the cell-free medium (CFM) in which the candidate LAB has been grown. The most recommended conditions to maximize the bacteriocin in vitro production for LAB are Man Rogosa Sharpe broth, pH 5.5–6.5, at 30–37 °C for 24–48 h [104]. After obtaining the CFM, the in vitro antimicrobial activity against *L. monocytogenes* should be tested by co-culturing, in which CFM is simultaneously added with *L. monocytogenes*, or by delayed culturing, in which broth medium is inoculated with the pathogen and incubated, followed by CFM addition after 6 h of incubation [105].

A relatively quick and cheap technique for bacteriocin characterization for those CFM showing any degree of inhibition on *L. monocytogenes* is the tricine SDS-PAGE analysis [106] after protein precipitation with 40, 60, and 80% ammonium sulphate [107]. This tool is useful for a primary characterization given that it informs about the presence or absence of any proteinaceous compound as well as displays information about the potential bacteriocin molecular weight if any band is found. Thus, it serves to categorize the potential bacteriocin within some of the compatible classes attending to this feature. Although it could be thought that the main limitation of this technique is the degree of purity of the proteinaceous precipitation, since generally a complex of proteinaceous compounds is excreted to the CFM, a simple method based on the evaluation of the anti-listerial activity of every band from a given sample by setting the tricine-SDS-PAGE onto a solid medium inoculated with *L. monocytogenes* is commonly used [108].

Whether the tricine-SDS-PAGE reveals a single band linked to a sample with anti-listerial activity, apart from the information about the molecular weight, this band could be excised, digested, and analysed by high-resolution mass spectrometry to identify its

aminoacidic sequence and similarities with other previously published bacteriocins by means of software analyses such as MASCOT [109].

With the aim to genetically characterize the ability of bacteriocin production by LAB, the detection of genes that encode for these metabolites deserves to be exploited. There are numerous target genes to evaluate its presence in food products and even the bacteriocin production through transcriptional analysis. Some of these genes have been used for characterizing LAB ability to produce bacteriocins [110,111]. However, to completely characterize the ability of bacteriocin production, the most recommended approach consists of whole-genome sequencing to evaluate the presence of any reported bacteriocin [107]. This tool additionally offers the possibility of sweeping the currently sequenced genome in the future with the aim to detect genes encoding for ulteriorly discovered bacteriocins.

The bacteriocins as bioprotective tool against *L. monocytogenes* could be split into two different applications: (a) the addition of the purified bacteriocin to the food and (b) the inoculation of the bacteriocin-producing LAB in the food. The former has been assayed with the well-known nisin to successfully inhibit *L. monocytogenes* in milk although the anti-listerial effect depends on its chemical composition and the technological process at which the food has undergone [112]. Additionally, intrinsic mechanisms from milk to inhibit pathogens, such as lactoperoxidase, seem to work synergistically with this bacteriocin, resulting in maximizing the anti-listerial effect [113].

5. Application of Selected LAB or Bacteriocins in RTE Dry-Cured Meat Products

The meat industry has carried out extraordinary research efforts to minimize the appearance of outbreaks caused by foodborne *L. monocytogenes*. The application of selected LAB and/or their purified antimicrobial metabolites for the biopreservation of RTE dry-cured meat products has been increasing in the last years with promising results. Selected LAB strains or their metabolites have been directly incorporated into the meat products throughout the processing to reduce the hazard posed by the presence and growth of *L. monocytogenes* in these products.

With this aim, *Ll. sakei* has been widely employed in several studies with different results. García-Diez and Patarata [114] concluded that the addition of *Ll. sakei* at a concentration of 6 log CFU/g did not provoke significant reduction in *L. monocytogenes* counts in a Portuguese dry-fermented sausage. However, Ortiz et al. [115] showed that *Ll. sakei*, when added to meat batter in Iberian chorizo, showed an anti-listerial activity at either 7 or 20 °C, reducing by 2 $\log_{10}$ units the pathogen counts. In addition, Vaz-Velho et al. [116] demonstrated that *Ll. sakei* was enough to minimise *L. monocytogenes* counts (up to 2 log CFU/g) in a Portuguese salami-like product, Alheira. Selected *Lp. plantarum* has also been used to inhibit and control *L. monocytogenes* in RTE meat products. Thus, Kamiloglu et al. [117] evaluated the effect of five *Lp. plantarum* (initially inoculated at 7 log CFU/g) against *L. monocytogenes* in sucuk, a traditional dry-fermented sausage from Turkey. They observed a decrease in *L. monocytogenes* counts from 1 to 2.7 log CFU/g for the different *Lp. plantarum* strains tested during ripening. In such work, they determined that acidification and production of bacteriocins and/or bacteriocin like peptides were the cause for the control of this pathogenic microorganism. Zanette et al. [118] tested the anti-listerial activity of two *Lp. plantarum* strains (one bacteriocin-producing strain and one bacteriocin non-producing strain) and found they were equally effective to limit *L. monocytogenes* growth (≈ 1.7 log CFU/g reduction) from the initial levels of the pathogen (4 log CFU/g).

The combination of selected active LAB, such as *Ll. sakei* (CRL1862), with bacteriocin combination and 2.5% lactic acid and acetic acid diminished the *L. monocytogenes* counts at levels lower than 2 log CFU/g (from initial counts at 3–4 log CFU/g) in frankfurters from day 6 to day 36 at 10 °C [119]. However, no significant additional reductions were observed when selected *Ll. sakei* was evaluated in combination of packing under vacuum or modified atmosphere packaging. Nikodinoska et al. [120] tested the antagonistic activity of *Lp. plantarum* alone and combined with nitrite (at two concentrations) against the

pathogenic bacterium in a chorizo sausage model. Counts of *L. monocytogenes* were reduced with the addition of the LAB strain (ranging from 2.6 to 3.8 log CFU/g depending on the nitrite concentration used). In samples where nitrite was not added, *Lp. plantarum* reduced *L. monocytogenes* growth but not until the end of ripening. On the contrary, Macieira et al. [121], who used bacteriocinogenic *Lp. plantarum* cultures (at a concentration of 6 log CFU /g) in a traditional Portuguese fermented dry-cured sausage, did not have any antagonistic activity against *L. monocytogenes* (initially inoculated at 5 log CFU/g).

In the study carried out by Sadaghiani et al. [122], they checked the effect of one strain of *Lp. plantarum* (initially inoculated at 7 log CFU/g) in ground raw beef alone and in combination with a garlic extract (1%). The LAB strain alone decreased the counts of the pathogen at 0.7 log CFU/g, but when combined with the garlic extract, this reduction was 1.5 log CFU/g.

Pediococcus (P) acidilactici has also been quite utilised as a biopreservative to control the development of *L. monocytogenes* in RTE meat products. Cosansu et al. [123] demonstrated that the bacteriocin-producing *P. acidilactici* possessed a significant anti-listerial activity on sucuk but not on sliced turkey bread. *P. acidilactici* produced a reduction of 3.3 log CFU/g *L. monocytogenes* counts after 8 days of sucuk fermentation at mild temperatures (22–24 °C). On the other hand, Ortiz et al. [115] showed that a starter culture containing *P. acidilactici* in Iberian chorizo provoked an anti-listerial effect at 7 °C.

Other researchers have focused on looking for other LAB species as biopreservatives to counteract and minimize the growth of *L. monocytogenes* in RTE meat products. Regarding *P. pentosaceus*, it was added individually and in combination with *P. acidilacti* in sliced fresh beef samples [124]. This study concluded that the use of *P. pentosaceus* alone or combined with *P. acidilacti* is promising since they limited the *L. monocytogenes* counts <2 log CFU/g on day 2. *Li. reuteri* is another LAB species used as biopreservative in the meat industry. Sadaghiani et al. [122] checked the anti-*L. monocytogenes* activity of a *Li. reuteri* strain in conjunction with garlic extract (1%) in beef, concluding that the combination of garlic extract with *Li. reuteri* caused a 1.4 log count reduction, while *Li. reuteri* alone only provoked a 0.5 log reduction. Orihuel et al. [125] reported that a bacteriocinogenic *E. mundtii* strain had limited anti-*L. monocytogenes* activity in beef sausage when applied alone, but in combination with curing additives, reductions of 2 log CFU/g counts were achieved. Finally, Castellano et al. [119] showed that the bacteriocin synthesized by *Ll. curvatus* possessed some bacteriostatic effect in frankfurters but lower than that shown by the bacteriocin produced by *Ll. sakei.*

Some metabolites synthesized by LAB have also been utilised as a biopreservative to control *L. monocytogenes* in RTE meat products. Trinetta et al. [126] studied the antagonistic effect of sakacin A, a bacteriocin produced by *Aureobasidium pullulans*, when it was directly added to RTE turkey breasts and when incorporated in a pullulan film to package this product. Results showed that sakacin A directly applied to turkey decreased the *L. monocytogenes* counts by more than 2 log CFU/g, while sakacin A-containing pullulan films diminished its counts 3 log CFU/g. Another bacteriocin that displayed anti-*L. monocytogenes* activity was nisin when was added in RTE turkey ham [127]. This bacteriocin was used in different concentrations (from 0.2 to 0.5%), and its antagonistic effect increased as the concentration did, keeping the *L. monocytogenes* counts lower than the control in all treatments. Leucocin A is another bacteriocin used for *L. monocytogenes* control purposes in RTE meat products. This bacteriocin produced by *Le. gelidum* has been employed in wieners (sausages) to counteract *L. monocytogenes* [128]. The antimicrobial activity of this bacteriocin was lower than the previous ones, obtaining only a reduction of 1 log CFU/g after 16 days of incubation at refrigeration temperatures.

6. Application of Selected LAB or Bacteriocins in RTE Dairy-Ripened Products

Most of the application of LAB species in dairy-ripened products have been reported in cheese throughout the ripening or storage. Thus, selected strains of *Ll. sakei* and *Lp. plantarum* used as protective cultures in soft cheese reduced the loads of *L. monocytogenes*

from 0.5 to almost 1 log CFU/g during 1375 h of storage at 15 °C [17]. Higher reduction was found in semi-hard cheeses ripened with *L. brevis*, *Lp. plantarum*, and *E. faecalis*, where *L. monocytogenes* counts were reduced by 4 log CFU/g after 15 days of ripening in cheeses made with raw milk and after 21 days in cheese made with pasteurized milk [16].

Selected *Lactococcus* spp. has been widely used as protective cultures in cheese. Thus, Kondrotiene et al. [129] found a significant reduction in *L. monocytogenes* counts when three nisin A-producing *La. lactis* strains were applied to fresh cheese. In addition, selected strains of *La. lactis* subsp. *lactis* and *E. durans* as individual or mixed cultures have also been reported to provoke a reduction of 2–3 log CFU/g of *L. monocytogenes* during 35 days of storage at 4 °C of ultrafiltered cheese [130]. These authors underlined the potential application of the above LAB strains in bio-control of this pathogen bacterium during storage of ultrafiltered cheese.

Ll. sakei, *La. lactis*, and *Carnobacterium* strains selected from Gorgonzola cheese have been reported to provoke a notable inhibition at low level of contamination of *L. monocytogenes* (2 log CFU/g) in this kind of cheese [10]. This inhibition was found during the first stage of ripening (6 days), and *L. monocytogenes* cells were maintained below the EC limit (<2 log CFU/g) for 60 days. However, these authors reported that when *L. monocytogenes* was inoculated on the cheese surface at the end of ripening process (after 50 days; pH: 6.7), only one of the selected *La. lactis* strains exerted a significant inhibition on the growth of this pathogen if the cheese was strictly maintained at 4 °C.

Morandi et al. [10] underlined that the susceptibility of *L. monocytogenes* biotypes to LAB antimicrobial activity is strain dependent. Thus, a blend of different LAB strains could represent a more effective tool to develop protective culture for ripened cheeses. In this sense, combinations of different LAB strains have been proposed to be used as protective cultures in cheese. The combination of *Lp. plantarum* strain (initially inoculated at 8 log CFU/mL) with a nisin producer reduced *L. monocytogenes* to undetectable levels in cheese by day 28 of ripening [131]. Furthermore, these authors found that *Lp. plantarum* was much more effective in inhibiting *L. monocytogenes* when the nisin producer was attached than when it was alone.

Some studies have reported the use of bacteriocin produced by LAB for biopreservation of cheeses [132,133]. Nisin is the most frequently used although it has been reported as efficient in control *L. monocytogenes* only in fresh cheese [134,135]. An increase in anti-*L. monocytogenes* activity has been suggested when combining nisin with a second bacteriocin [134]. Therefore, the use of nisin in combination with the IIa class bovicin HC5 in fresh cheese against *L. monocytogenes* has been reported to provoke a 4 log reduction of this pathogen after 9 days at refrigeration storage [136]. In ripened cheese, it has been proposed as most effective to use nisin-producing strain of *Lc. lactis* subsp. lactis for the milk before cheese production, provoking an initial reduction higher than 2 log CFU/g [137] since the use of nisin could have the problem of the regrowth during ripening of the surviving *L. monocytogenes* [134]. Other bacteriocins, such as pediocins, enterocins, and lactacins, have also been used on the surface of cheese and mainly in fresh cheese [138–140], but their utility in ripened cheese is limited. Thus, although it has been highlighted that the utilization of bacteriocins could contribute to the creation of low-salt and healthier formulations of cheeses and to the optimization of processing conditions without compromising the microbiological safety of these RTE foods [141], the problem of the regrowth during ripening of surviving *L. monocytogenes* should be considered, which it makes more effective the use of selected LAB than the direct addition of bacteriocins.

Furthermore, combinations of different preservation methods may act synergistically or provide higher protection than a single method alone [142]. Thus, the combination of selected LAB with antimicrobial compounds has been proposed. In this sense, it has been proposed that selected *La. lactis* be used in combination with acid/sodium lactate (LASL-L-lactic acid 61% (*w*/*w*) and L-sodium lactate 21% (*w*/*w*)) [143]. The former authors found a total inhibition of *L. monocytogenes* strains in the first 50 days of ripening of Gorgonzola cheese when this combination was used, while LASL with selected *C. divergens* was more

effective in the second part of ripening when the pH was raised. These authors encouraged the use of LASL along with antimicrobial LAB rotation schemes during cheese ripening for the prevention and/or control of the *L. monocytogenes* on the cheese surface of Gorgonzola cheese.

Finally, the use of active packaging with bacteriocins produced by selected LAB species is a promising strategy to control *L. monocytogenes* in packaged cheeses. In fact, Contessa et al. [144] described a film based on agar-agar incorporated with bacteriocin produced by a selected *Lc. casei* to be used as active packaging in curd cheese. This active packaging provokes a reduction of 3 $\log_{10}$ units of pathogen bacteria, such as *L. monocytogenes*.

7. Conclusions and Future Remarks

L. monocytogenes is a serious concern in the RTE meat and dairy-ripened products industries. The use of LAB as protective cultures and/or their metabolites could be a promising tool to control *L. monocytogenes* in these kinds of products. Although LAB strains are present in most of the ripened foods as the natural microbial population, to find strains with anti-*L. monocytogenes* activity able to survive in conditions of ripened products, an appropriated selection methodology is necessary. This includes recovery of LAB isolates from different ripening/storage conditions and evaluation of the anti-listerial activity in food models simulating temperature, a_w, and pH conditions of the processing. Then, final selection should be performed after evaluation of the most active strains in food matrices, following the challenge test methodology. As a result of the proposed isolation and selection methods for LAB strains with the ability to produce antimicrobial compounds, such as lactic acid and other organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide, bacteriocins, are available. In addition, the selected LAB strains can compete for nutrients and space with *L. monocytogene,s* and some of them are able to eliminate this pathogen bacterium from biofilm and reduce its virulence and the ability of *L. monocytogenes* to survive. These strains have showed effectivity in meat and dairy-ripened products, achieving reductions form 2–5 $\log_{10}$ units of *L. monocytogenes* throughout the ripening process. This could be sufficient to guarantee the elimination of this pathogenic bacterium throughout the ripening/storage of RTE meat and dairy-ripened products when this pathogen contaminates these products at the usual levels (below 2 log CFU/g). This is of utmost importance since minimizing the risk of listeriosis caused by the consumption of these products improves food safety and meets the microbiological criteria of RTE foods throughout their shelf life. Bacteriocins could be also used to control *L. monocytogenes*, but their activity in these products could be limited by the regrowth during ripening or storage of the surviving strains of this pathogen. Thus, the combination of different active LAB strains and those bacteriocigenic ones could be the most appropriate strategies to control *L. monocytogenes* in ripened foods. Furthermore, the combination of selected LAB strains with antimicrobial compounds, such as acid/sodium lactate, and other strategies for active packaging could be the next step to eliminate the risk posed by *L. monocytogenes* in meat and dairy-ripened products.

Author Contributions: Conceptualization, J.J.C. and A.R.; methodology, I.M.; writing—original draft preparation, I.M., J.D., A.R., and J.J.C.; writing—review and editing, I.M., J.D., A.R., and J.J.C.; funding acquisition, J.J.C.; supervision, J.J.C. and A.R.; project administration, J.J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been funded by the Spanish Ministry of Economy, Industry, and Competitiveness; the Spanish Ministry of Education, Culture, and Sports; and the Junta De Extremadura and Feder.

Institutional Review Board Statement:

Informed Consent Statement:

Data Availability Statement: Not applicable

Acknowledgments: This work was supported by the INIA throughout the projects RTA-2017-00027-C03-03 (Spanish Ministry of Economy, Industry, and Competitiveness) and the Junta de Extremadura and FEDER by IB16149, GR15108, and GR18056. I. Martín is supported by a pre-doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU 16/05303).

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

References

1. Baka, M.; Noriega, E.; Mertens, L.; Van Derlinden, E.; Van Impe, J.F.M. Protective role of indigenous *Leuconostoc carnosum* against *Listeria monocytogenes* on vacuum packed Frankfurter sausages at suboptimal temperatures. *Food Res. Int.* **2014**, *66*, 197–206, doi:10.1016/j.foodres.2014.08.011.
2. EFSA and ECDC The European Union One Health 2019 Zoonoses Report. *EFSA J.* **2021**, *19*, doi:10.2903/j.efsa.2021.6406.
3. EFSA and ECDC Scientific opinion on the *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA J.* **2018**, *16*, 1–173, doi:10.2903/j.efsa.2018.5134.
4. Kurpas, M.; Wieczorek, K.; Osek, J. Ready-to-eat meat products as a source of *Listeria monocytogenes*. *J. Vet. Res.* **2018**, *62*, 49–55, doi:10.2478/jvetres-2018-0007.
5. Martinez-Rios, V.; Dalgaard, P. Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis. *Food Control* **2018**, *84*, 205–214, doi:10.1016/j.foodcont.2017.07.020.
6. Fretz, R.; Sagel, U.; Ruppitsch, W.; Pietzka, A.T.; Stöger, A.; Huhulescu, S.; Heuberger, S.; Pichler, J.; Much, P.; Pfaff, G.; et al. Listeriosis outbreak caused by acid curd cheese "Quargel", Austria and Germany 2009. *Eurosurveillance* **2010**, *15*, 1–2, doi:10.2807/ese.15.05.19477-en.
7. Cartwright, E.J.; Jackson, K.A.; Johnson, S.D.; Graves, L.M.; Silk, B.J.; Mahon, B.E. Listeriosis outbreaks and associated food vehicles, United States, 1998-2008. *Emerg. Infect. Dis.* **2013**, *19*, 1–9, doi:10.3201/eid1901.120393.
8. Magalhães, R.; Almeida, G.; Ferreira, V.; Santos, I.; Silva, J.; Mendes, M.M.; Pita, J.; Mariano, G.; Mâncio, I.; Sousa, M.M. Cheese-related listeriosis outbreak, Portugal, march 2009 to february 2012. *Eurosurveillance* **2015**, *20*, 21104, doi:10.2807/1560-7917.ES2015.20.17.21104.
9. Egan, K.; Field, D.; Rea, M.C.; Ross, R.P.; Hill, C.; Cotter, P.D. Bacteriocins: Novel solutions to age old spore-related problems? *Front. Microbiol.* **2016**, *7*, 461, doi:10.3389/fmicb.2016.00461.
10. Morandi, S.; Silvetti, T.; Battelli, G.; Brasca, M. Can lactic acid bacteria be an efficient tool for controlling *Listeria monocytogenes* contamination on cheese surface? The case of Gorgonzola cheese. *Food Control* **2019**, *96*, 499–507, doi:10.1016/j.foodcont.2018.10.012.
11. EFSA BIOHAZ Panel Updated list of QPS status recommended biological agents in support of EFSA risk assessments. *EFSA J.* **2021**, *19*, 1–5.
12. Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C.M.A.P.; Harris, H.M.B.; Mattarelli, P.; O'toole, P.W.; Pot, B.; Vandamme, P.; Walter, J.; et al. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus beijerinck* 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 2782–2858, doi:10.1099/ijsem.0.004107.
13. Cocolin, L.; Foschino, R.; Comi, G.; Grazia Fortina, M. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. *Food Microbiol.* **2007**, *24*, 752–758, doi:10.1016/j.fm.2007.03.001.
14. Sip, A.; Wieckowicz, M.; Olejnik-Schmidt, A.; Grajek, W. Anti-*Listeria* activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. *Food Control* **2012**, *26*, 117–124, doi:10.1016/j.foodcont.2012.01.014.
15. Xiraphi, N.; Georgalaki, M.; Rantsiou, K.; Cocolin, L.; Tsakalidou, E.; Drosinos, E.H. Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroide*s E131. *Meat Sci.* **2008**, *80*, 194–203, doi:10.1016/j.meatsci.2007.11.020.
16. Campagnollo, F.B.; Margalho, L.P.; Kamimura, B.A.; Feliciano, M.D.; Freire, L.; Lopes, L.S.; Alvarenga, V.O.; Cadavez, V.A.P.; Gonzales-Barron, U.; Schaffner, D.W.; et al. Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. *Food Microbiol.* **2018**, *73*, 288–297, doi:10.1016/j.fm.2018.02.006.
17. Panebianco, F.; Giarratana, F.; Caridi, A.; Sidari, R.; De Bruno, A.; Giuffrida, A. Lactic acid bacteria isolated from traditional Italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese. *Lwt* **2021**, *137*, 110446, doi:10.1016/j.lwt.2020.110446.
18. Papamanoli, E.; Tzanetakis, N.; Litopoulou-Tzanetaki, E.; Kotzekidou, P. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Sci.* **2003**, *65*, 859–867, doi:10.1016/S0309-1740(02)00292-9.
19. Todorov, S.D.; Dicks, L.M.T. Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *J. Basic Microbiol.* **2005**, *45*, 312–322, doi:10.1002/jobm.200410532.
20. Picozzi, C.; Bonacina, G.; Vigentini, I.; Foschino, R. Genetic diversity in Italian *Lactobacillus sanfranciscensis* strains assessed by multilocus sequence typing and pulsed-field gel electrophoresis analyses. *Microbiology* **2010**, *156*, 2035–2045,

doi:10.1099/mic.0.037341-0.
21. Park, S.H.; Jung, J.H.; Seo, D.H.; Lee, H.L.; Kim, G.W.; Park, S.Y.; Shin, W.C.; Hong, S.; Park, C.S. Differentiation of lactic acid bacteria based on RFLP analysis of the *tuf* gene. *Food Sci. Biotechnol.* **2012**, *21*, 911–915, doi:10.1007/s10068-012-0119-9.
22. Martín, I.; Rodríguez, A.; Sánchez-Montero, L.; Padilla, P.; Córdoba, J.J. Effect of the dry-cured fermented sausage "salchichón" processing with a selected *Lactobacillus sakei* in *Listeria monocytogenes* and microbial population. *Foods* **2021**, *10*, doi:10.3390/foods10040856.
23. Bungenstock, L.; Abdulmawjood, A.; Reich, F. Evaluation of antibacterial properties of lactic acid bacteria from traditionally and industrially produced fermented sausages from Germany. *PLoS One* **2020**, *15*, 1–15, doi:10.1371/journal.pone.0230345.
24. De Carvalho, A.A.T.; De Paula, R.A.; Mantovani, H.C.; De Moraes, C.A. Inhibition of *Listeria monocytogenes* by a lactic acid bacterium isolated from Italian salami. *Food Microbiol.* **2006**, *23*, 213–219, doi:10.1016/j.fm.2005.05.009.
25. Pedonese, F.; Torracca, B.; Mancini, S.; Pisano, S.; Turchi, B.; Cerri, D.; Nuvoloni, R. Effect of a *Lactobacillus sakei* and *Staphylococcus xylosus* protective culture on *Listeria monocytogenes* growth and quality traits of Italian fresh sausage (salsiccia) stored at abusive temperature. *Ital. J. Anim. Sci.* **2020**, *19*, 1363–1374, doi:10.1080/1828051X.2020.1844084.
26. Cruxen, C.E. dos S.; Funck, G.D.; Haubert, L.; Dannenberg, G. da S.; Marques, J. de L.; Chaves, F.C.; da Silva, W.P.; Fiorentini, Â.M. Selection of native bacterial starter culture in the production of fermented meat sausages: Application potential, safety aspects, and emerging technologies. *Food Res. Int.* **2019**, *122*, 371–382, doi:10.1016/j.foodres.2019.04.018.
27. Kasra-Kermanshahi, R.; Mobarak-Qamsari, E. Inhibition effect of lactic acid bacteria against food born pathogen, *Listeria monocytogenes*. *Appl. Food Biotechnol.* **2015**, *2*, 11–19, doi:10.22037/afb.v2i4.8894.
28. Vieco-Saiz, N.; Belguesmia, Y.; Raspoet, R.; Auclair, E.; Gancel, F.; Kempf, I.; Drider, D. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Front. Microbiol.* **2019**, *10*, doi:10.3389/fmicb.2019.00057.
29. Leroy, F.; De Vuyst, L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* **2004**, *15*, 67–78, doi:10.1016/j.tifs.2003.09.004.
30. Singh, V.P. Recent approaches in food bio-preservation-A review. *Open Vet. J.* **2018**, *8*, 104–111, doi:10.4314/ovj.v8i1.16.
31. Simons, A.; Alhanout, K.; Duval, R.E. Bacteriocins, antimicrobial peptides from bacterial origin: Overview of their biology and their impact against multidrug-resistant bacteria. *Microorganisms* **2020**, *8*, 639, doi:10.3390/microorganisms8050639.
32. Mokoena, M.P.; Omatola, C.A.; Olaniran, A.O. Applications of lactic acid bacteria and their bacteriocins against food spoilage microorganisms and foodborne pathogens. *Molecules* **2021**, *26*, 7055, doi:10.3390/molecules26227055.
33. Russo, P.; Arena, M.P.; Fiocco, D.; Capozzi, V.; Drider, D.; Spano, G. *Lactobacillus plantarum* with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *Int. J. Food Microbiol.* **2017**, *247*, 48–54, doi:10.1016/j.ijfoodmicro.2016.04.027.
34. Dittoe, D.K.; Ricke, S.C.; Kiess, A.S. Organic acids and potential for modifying the avian gastrointestinal tract and reducing pathogens and disease. *Front. Vet. Sci.* **2018**, *5*, 1–12, doi:10.3389/fvets.2018.00216.
35. Ricke, S.C. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poult. Sci.* **2003**, *82*, 632–639, doi:10.1093/ps/82.4.632.
36. Nair, S.M.; Amalaradjou, M.A.; Venkitanarayanan, K. Antivirulence properties of probiotics in combating microbial pathogenesis; *Adv. Appl. Microbiol.* , **2017**; 98, 1-29, doi: 10.1016/bs.aambs.2016.12.001.
37. Wang, C.; Chang, T.; Yang, H.; Cui, M. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella Enteritidis*, *Escherichia coli* and *Listeria monocytogenes*. *Food Control* **2015**, *47*, 231–236, doi:10.1016/j.foodcont.2014.06.034.
38. Wemmenhove, E.; van Valenberg, H.J.F.; van Hooijdonk, A.C.M.; Wells-Bennik, M.H.J.; Zwietering, M.H. Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid. *Food Control* **2018**, *84*, 413–418, doi:10.1016/j.foodcont.2017.08.028.
39. Mokoena, M.P.; Mutanda, T.; Olaniran, O. Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages Mduduzi. *Food Nutr. Res.* **2016**, *60*, 29630, doi:10.3402/fnr.v60.29630.
40. Zúñiga, M.; Pardo, I.; Ferrer, S. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *Int. J. Food Microbiol.* **1993**, *18*, 37–42, doi:10.1016/0168-1605(93)90005-2.
41. Barker, C.; Park, S.F. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. *Appl. Environ. Microbiol.* **2001**, *67*, 1594–1600, doi:10.1128/AEM.67.4.1594-1600.2001.
42. Ghalfi, H.; Thonart, P.; Benkerroum, N. Inhibitory activity of *Lactobacillus curvatus* CWBI-B28 against *Listeria monocytogenes* and ST2-verotoxin producing *Escherichia coli* O157. *African J. Biotechnol.* **2006**, *5*, 2303–2306, doi:10.4314/ajb.v5i22.55976.
43. Langa, S.; Martín-Cabrejas, I.; Montiel, R.; Landete, J.M.; Medina, M.; Arqués, J.L. Short communication: Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens. *J. Dairy Sci.* **2014**, *97*, 6116–6121, doi:10.3168/jds.2014-8306.
44. Yap, P.-C.; MatRahim, N.-A.; AbuBakar, S.; Lee, H.Y. Antilisterial potential of lactic acid bacteria in eliminating *Listeria monocytogenes* in host and ready-to-eat food application. *Microbiol. Res. (Pavia).* **2021**, *12*, 234–257, doi:10.3390/microbiolres12010017.

45. Saraoui, T.; Fall, P.A.; Leroi, F.; Antignac, J.P.; Chéreau, S.; Pilet, M.F. Inhibition mechanism of *Listeria monocytogenes* by a bioprotective bacteria *Lactococcus piscium* CNCM I-4031. *Food Microbiol.* **2016**, *53*, 70–78, doi:10.1016/j.fm.2015.01.002.
46. Zilelidou, E..; Rychli, K.; Manthou, E.; Ciolacu, L.; Wagner, M.; Skandamis, P.N. Highly invasive *Listeria monocytogenes* strains have growth and invasion advantages in strain competition. **2015**, 10, doi:10.1371/journal.pone.0141617.
47. Bermudez-Brito, M.; Plaza-Díaz, J.; Muñoz-Quezada, S.; Gómez-Llorente, C.; Gil, A. Probiotic mechanisms of action. *Ann. Nutr. Metab.* **2012**, *61*, 160–174, doi:10.1159/000342079.
48. Herich, R.; Levkut, M. Lactic acid bacteria, probiotics and immune system. *Vet. Med. (Praha).* **2002**, *47*, 169–180, doi:10.17221/5821-VETMED.
49. Corr, S.C.; Gahan, C.G.M.; Hill, C. Impact of selected *Lactobacillus* and *Bifidobacterium* species on *Listeria monocytogenes* infection and the mucosal immune response. *FEMS Immunol. Med. Microbiol.* **2007**, *50*, 380–388, doi:10.1111/j.1574-695X.2007.00264.x.
50. Ndahetuye, J.B.; Koo, O.K.; O'Bryan, C.A.; Ricke, S.C.; Crandall, P.G. Role of lactic acid bacteria as a biosanitizer to prevent attachment of *Listeria monocytogenes* F6900 on deli slicer contact surfaces. *J. Food Prot.* **2012**, *75*, 1429–1436, doi:10.4315/0362-028X.JFP-12-072.
51. Pilchová, T.; Pilet, M.F.; Cappelier, J.M.; Pazlarová, J.; Tresse, O. Protective effect of *Carnobacterium* spp. against *Listeria monocytogenes* during host cell invasion using in vitro HT29 model. *Front. Cell. Infect. Microbiol.* **2016**, *6*, 88, doi:10.3389/fcimb.2016.00088.
52. Gómez, N.C.; Ramiro, J.M.P.; Quecan, B.X.V.; de Melo Franco, B.D.G. Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Escherichia coli* O157: H7 biofilms formation. *Front. Microbiol.* **2016**, *7*, 1–15, doi:10.3389/fmicb.2016.00863.
53. Dutra, V.; Silva, A.C.; Cabrita, P.; Peres, C.; Malcata, X.; Brito, L. *Lactobacillus plantarum* LB95 impairs the virulence potential of Gram-positive and Gram-negative food-borne pathogens in HT-29 and vero cell cultures. *J. Med. Microbiol.* **2016**, *65*, 28–35, doi:10.1099/jmm.0.000196.
54. Upadhyay, A.; Upadhyaya, I.; Mooyottu, S.; Venkitanarayanan, K. Eugenol in combination with lactic acid bacteria attenuates *Listeria monocytogenes* virulence in vitro and in invertebrate model *Galleria mellonella*. *J. Med. Microbiol.* **2016**, *65*, 443–455, doi:10.1099/jmm.0.000251.
55. Garriga, M.; Rubio, R.; Aymerich, T.; Ruas-Madiedo, P. Potentially probiotic and bioprotective lactic acid bacteria starter cultures antagonise the *Listeria monocytogenes* adhesion to HT29 colonocyte-like cells. *Benef. Microbes* **2015**, *6*, 337–343, doi:10.3920/BM2014.0056.
56. Winkelströter, L.K.; De Martinis, E.C.P. Effect of bacteriocins and conditions that mimic food and digestive tract on biofilm formation, in vitro invasion of eukaryotic cells and Internalin gene expression by *Listeria monocytogenes*. *Probiotics Antimicrob. Proteins* **2013**, *5*, 153–164, doi:10.1007/s12602-013-9135-1.
57. De Vuyst, L.; Leroy, F. Bacteriocins from lactic acid bacteria: Production, purification, and food applications. *J. Mol. Microbiol. Biotechnol.* **2007**, *13*, 194–199, doi:10.1159/000104752.
58. Pessione, E. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 86, doi:10.3389/fcimb.2012.00086.
59. Vogel, R.F.; Pohle, B.S.; Tichaczek, P.S.; Hammes, W.P. The competitive advantage of *Lactobacillus curvatus* LTH 1174 in sausage fermentations is caused by formation of curvacin A. *Syst. Appl. Microbiol.* **1993**, *16*, 457–462, doi:10.1016/S0723-2020(11)80280-8.
60. Cheigh, C.I.; Pyun, Y.R. Nisin biosynthesis and its properties. *Biotechnol. Lett.* **2005**, *27*, 1641–1648, doi:10.1007/s10529-005-2721-x.
61. Klaenhammer, T.R. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **1993**, *12*, 39–85, doi:10.1016/0168-6445(93)90057-G.
62. Deegan, L.H.; Cotter, P.D.; Hill, C.; Ross, P. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int. Dairy J.* **2006**, *16*, 1058–1071, doi:10.1016/j.idairyj.2005.10.026.
63. Férir, G.; Petrova, M.I.; Andrei, G.; Huskens, D.; Hoorelbeke, B.; Snoeck, R.; Vanderleyden, J.; Balzarini, J.; Bartoschek, S.; Brönstrup, M.; et al. The lantibiotic peptide labyrinthopeptin A1 demonstrates broad anti-HIV and anti-HSV activity with potential for microbicidal applications. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0064010.
64. Kawulka, K.E.; Sprules, T.; Diaper, C.M.; Whittal, R.M.; McKay, R.T.; Mercier, P.; Zuber, P.; Vederas, J.C. Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α-carbon cross-links: Formation and reduction of α-Thio-α-Amino acid derivatives. *Biochemistry* **2004**, *43*, 3385–3395, doi:10.1021/bi0359527.
65. Gao, Y.; Li, D.; Liu, S.; Zhang, L. Garviecin LG34, a novel bacteriocin produced by *Lactococcus garvieae* isolated from traditional Chinese fermented cucumber. *Food Control* **2015**, *50*, 896–900, doi:10.1016/j.foodcont.2014.10.040.
66. Fimland, G.; Jack, R.; Jung, G.; Nes, I.F.; Nissen-Meyer, J. The bactericidal activity of pediocin PA-1 is specifically inhibited by a 15-mer fragment that spans the bacteriocin from the Center toward the C terminus. *Appl. Environ. Microbiol.* **1998**, *64*, 5057–5060, doi:10.1128/aem.64.12.5057-5060.1998.
67. Chen, H.; Tian, F.; Li, S.; Xie, Y.; Zhang, H.; Chen, W. Cloning and heterologous expression of a bacteriocin sakacin P from

Lactobacillus sakei in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1061–1068, doi:10.1007/s00253-012-3872-z.
68. Trinetta, V.; Morleo, A.; Sessa, F.; Iametti, S.; Bonomi, F.; Ferranti, P. Purified sakacin A shows a dual mechanism of action against *Listeria* spp: Proton motive force dissipation and cell wall breakdown. *FEMS Microbiol. Lett.* **2012**, *334*, 143–149, doi:10.1111/j.1574-6968.2012.02630.x.
69. Ceuppens, S.; De Coninck, D.; Bottledoorn, N.; Van Nieuwerburgh, F.; Uyttendaele, M. Microbial community profiling of fresh basil and pitfalls in taxonomic assignment of enterobacterial pathogenic species based upon 16S rRNA amplicon sequencing. *Int. J. Food Microbiol.* **2017**, *257*, 148–156, doi:10.1016/j.ijfoodmicro.2017.06.016.
70. Ekblad, B.; Nissen-Meyer, J.; Kristensen, T. Whole-genome sequencing of mutants with increased resistance against the two-peptide bacteriocin plantaricin JK reveals a putative receptor and potential docking site. *PLoS One* **2017**, *12*, 1–11, doi:10.1371/journal.pone.0185279.
71. Ekblad, B.; Kyriakou, P.K.; Oppegård, C.; Nissen-Meyer, J.; Kaznessis, Y.N.; Kristiansen, P.E. Structure-function analysis of the two-peptide bacteriocin plantaricin EF. *Biochemistry* **2016**, *55*, 5106–5116, doi:10.1021/acs.biochem.6b00588.
72. Oppegård, C.; Rogne, P.; Kristiansen, P.E.; Nissen-Meyer, J. Structure analysis of the two-peptide bacteriocin lactococcin G by introducing D-amino acid residues. *Microbiology* **2010**, *156*, 1883–1889, doi:10.1099/mic.0.038430-0.
73. Gálvez, A.; Maqueda, M.; Martínez-Bueno, M.; Valdivia, E. Bactericidal and bacteriolytic action of peptide antibiotic AS-48 against gram-positive and gram-negative bacteria and other organisms. *Res. Microbiol.* **1989**, *140*, 57–68, doi:10.1016/0923-2508(89)90060-0.
74. Pandey, N.; Malik, R.K.; Kaushik, J.K.; Singroha, G. Gassericin A: A circular bacteriocin produced by Lactic acid bacteria *Lactobacillus gasseri*. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1977–1987, doi:10.1007/s11274-013-1368-3.
75. Chehimi, S.; Pons, A.M.; Sablé, S.; Hajlaoui, M.R.; Limam, F. Mode of action of thuricin S, a new class IId bacteriocin from *Bacillus thuringiensis*. *Can. J. Microbiol.* **2010**, *56*, 162–167, doi:10.1139/W09-125.
76. O'Shea, E.F.; O'Connor, P.M.; O'Sullivan, O.; Cotter, P.D.; Ross, R.P.; Hill, C. Bactofencin A, a new type of cationic bacteriocin with Unusual Immunity. *MBio* **2013**, *4*, doi:10.1128/mBio.00498-13.
77. Schindler, C.A.; Schuhardt, V.T. Lysostaphin; a new bacteriolytic agent for the Staphylococcus. *Proc. Natl. Acad. Sci.* **1964**, *51*, 414–421, doi:10.1073/pnas.51.3.414.
78. Vaughan, E.E.; Daly, C.; Fitzgerald, G.F. Identification and characterization of helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. *J. Appl. Bacteriol.* **1992**, *73*, 299–308, doi:10.1111/j.1365-2672.1992.tb04981.x.
79. Maky, M.A.; Ishibashi, N.; Zendo, T.; Perez, R.H.; Doud, J.R.; Karmi, M.; Sonomoto, K. Enterocin F4-9, a novel O-linked glycosylated bacteriocin. *Appl. Environ. Microbiol.* **2015**, *81*, 4819–4826, doi:10.1128/AEM.00940-15.
80. Breukink, E.; Van Heusden, H.E.; Vollmerhaus, P.J.; Swiezewska, E.; Brunner, L.; Walker, S.; Heck, A.J.R.; De Kruijff, B. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. *J. Biol. Chem.* **2003**, *278*, 19898–19903, doi:10.1074/jbc.M301463200.
81. Breukink, E.; De Kruijff, B. The lantibiotic nisin, a special case or not? *Biochim. Biophys. Acta - Biomembr.* **1999**, *1462*, 223–234, doi:10.1016/S0005-2736(99)00208-4.
82. European Commission. Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Off. J. Eur. Union* **2011**, *L295*, 1–177, doi:10.3000/19770677.L_2011.295.eng.
83. Zhu, L.; Zeng, J.; Wang, C.; Wang, J. Structural basis of pore formation in the mannose phosphotransferase system (man-PTS) by pediocin PA-1. *Appl. Environ. Microbiol.* **2021**, doi:10.1128/AEM.01992-21.
84. Schillinger, U.; Kaya, M.; Lücke, F.-K. Behaviour of Listeria monocytogenes in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J. Appl. Bacteriol.* **1991**, *70*, 473–478, doi:10.1111/j.1365-2672.1991.tb02743.x.
85. Chen, Y.; Wu, H.; Kuo, C.; Chen, Y.; Ho, S.; Yanagida, F. Leucocin C-607, a Novel Bacteriocin from the multiple-bacteriocin-producing *Leuconostoc pseudomesenteroides* 607 isolated from persimmon. *Probiotics Antimicrob. Proteins* **2018**, *10*, 148–156, doi:10.1007/s12602-017-9359-6.
86. Liu, G.; Ren, L.; Song, Z.; Wang, C.; Sun, B. Purification and characteristics of bifidocin A, a novel bacteriocin produced by Bifidobacterium animals BB04 from centenarians' intestine. *Food Control* **2015**, *50*, 889–895, doi:10.1016/j.foodcont.2014.10.049.
87. Wang, Y.; Qin, Y.; Zhang, Y.; Wu, R.; Li, P. Antibacterial mechanism of plantaricin LPL-1, a novel class IIa bacteriocin against *Listeria monocytogenes*. *Food Control* **2019**, *97*, 87–93, doi:10.1016/j.foodcont.2018.10.025.
88. Diep, D.B.; Skaugen, M.; Salehian, Z.; Holo, H.; Nes, I.F. Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 2384–2389, doi:10.1073/pnas.0608775104.
89. Jeckelmann, J.M.; Erni, B. The mannose phosphotransferase system (Man-PTS) - Mannose transporter and receptor for bacteriocins and bacteriophages. *Biochim. Biophys. Acta - Biomembr.* **2020**, *1862*, 183412, doi:10.1016/j.bbamem.2020.183412.
90. Liu, X.; Zeng, J.; Huang, K.; Wang, J. Structure of the mannose transporter of the bacterial phosphotransferase system. *Cell Res.* **2019**, *29*, 680–682, doi:10.1038/s41422-019-0194-z.
91. Chikindas, M.L.; Garcia-Garcera, M.J.; Driessen, A.J.M.; Ledeboer, A.M.; Nissen- Meyer, J.; Nes, I.F.; Abee, T.; Konings, W.N.; Venema, G. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl. Environ. Microbiol.* **1993**, *59*, 3577–3584, doi:10.1128/aem.59.11.3577-3584.1993.

92. Moll, G.N.; Konings, W.N.; Driessen, A.J.M. Bacteriocins: Mechanism of membrane insertion and pore formation. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **1999**, *76*, 185–198, doi:10.1023/A:1002002718501.
93. Rashid, R.; Veleba, M.; Kline, K.A. Focal targeting of the bacterial envelope by antimicrobial peptides. *Front. Cell Dev. Biol.* **2016**, *4*, 1–13, doi:10.3389/fcell.2016.00055.
94. Mazzotta, A.S.; Montville, T.J. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. *J. Appl. Microbiol.* **1997**, *82*, 32–38, doi:10.1111/j.1365-2672.1997.tb03294.x.
95. Demel, R.A.; Peelen, T.; Siezen, R.J.; De Kruijff, B.; Kuipers, O.P. Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity: A monolayer study. *Eur. J. Biochem.* **1996**, *235*, 267–274, doi:10.1111/j.1432-1033.1996.00267.x.
96. Van Kraaij, C.; Breukink, E.; Noordermeer, M.A.; Demel, R.A.; Siezen, R.J.; Kuipers, O.P.; De Kruijff, B. Pore formation by nisin involves translocation of its C-terminal part across the membrane. *Biochemistry* **1998**, *37*, 16033–16040, doi:10.1021/bi980931b.
97. Gravesen, A.; Ramnath, M.; Rechinger, K.B.; Andersen, N.; Jänsch, L.; Héchard, Y.; Hastings, J.W.; Knøchel, S. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. *Microbiology* **2002**, *148*, 2361–2369, doi:10.1099/00221287-148-8-2361.
98. Opsata, M.; Nes, I.F.; Holo, H. Class IIa bacteriocin resistance in *Enterococcus faecalis* V583: The mannose PTS operon mediates global transcriptional responses. *BMC Microbiol.* **2010**, *10*,224, doi:10.1186/1471-2180-10-224.
99. Kumariya, R.; Garsa, A.K.; Rajput, Y.S.; Sood, S.K.; Akhtar, N.; Patel, S. Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microb. Pathog.* **2019**, *128*, 171–177, doi:10.1016/j.micpath.2019.01.002.
100. Barbieri, F.; Montanari, C.; Gardini, F.; Tabanelli, G. Biogenic amine production by lactic acid bacteria: A review. *Foods* **2019**, *8*, 1–27, doi:10.3390/foods8010017.
101. Biscola, V.; Choiset, Y.; Rabesona, H.; Chobert, J.M.; Haertlé, T.; Franco, B.D.G.M. Brazilian artisanal ripened cheeses as sources of proteolytic lactic acid bacteria capable of reducing cow milk allergy. *J. Appl. Microbiol.* **2018**, *125*, 564–574, doi:10.1111/jam.13779.
102. Bintsis, T. Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS Microbiol.* **2018**, *4*, 665–684, doi:10.3934/microbiol.2018.4.665.
103. Marshall, B.M.; Levy, S.B. Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.* **2011**, *24*, 718–733, doi:10.1128/CMR.00002-11.
104. Oliveira, M.; J. Barbosa; Albano, H.; Teixeira, P. Bacteriocinogenic activity of *Leuconostoc lactis* RK18 isolates from fermented food. In *Fermented Foods*; Oliver, Kovalyov, Eds.; Nova Science Publishers, Inc, NY, USA, **2020** Chapter 3, p.159.
105. Mariam, S.H.; Zegeye, N.; Tariku, T.; Andargie, E.; Endalafer, N.; Aseffa, A. Potential of cell-free supernatants from cultures of selected lactic acid bacteria and yeast obtained from local fermented foods as inhibitors of *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus*. *BMC Res. Notes* **2014**, *7*, 1–9, doi:10.1186/1756-0500-7-606.
106. Schägger, H.; von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368–379, doi:10.1016/0003-2697(87)90587-2.
107. Barbosa, J.; Albano, H.; Silva, B.; Almeida, M.H.; Nogueira, T.; Teixeira, P. Characterization of a *Lactiplantibacillus plantarum* r23 isolated from arugula by whole-genome sequencing and its bacteriocin production ability. *Int. J. Environ. Res. Public Health* **2021**, *18*, doi:10.3390/ijerph18115515.
108. Abrams, D.; Barbosa, J.; Albano, H.; Silva, J.; Gibbs, P.A.; Teixeira, P. Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from "Alheira." *Food Control* **2011**, *22*, 940–946, doi:10.1016/j.foodcont.2010.11.029.
109. Nebbia, S.; Lamberti, C.; Lo Bianco, G.; Cirrincione, S.; Laroute, V.; Cocaign-Bousquet, M.; Cavallarin, L.; Giuffrida, M.G.; Pessione, E. Antimicrobial potential of food lactic acid bacteria: Bioactive peptide decrypting from caseins and bacteriocin production. *Microorganisms* **2021**, *9*, 1–19, doi:10.3390/microorganisms9010065.
110. Azizi, F.; Habibi Najafi, M.B.; Edalatian Dovom, M.R. The biodiversity of *Lactobacillus* spp. from Iranian raw milk Motal cheese and antibacterial evaluation based on bacteriocin-encoding genes. *AMB Express* **2017**, *7*, doi:10.1186/s13568-017-0474-2.
111. Ho, V.T.T.; Lo, R.; Bansal, N.; Turner, M.S. Characterisation of *Lactococcus lactis* isolates from herbs, fruits and vegetables for use as biopreservatives against *Listeria monocytogenes* in cheese. *Food Control* **2018**, *85*, 472–483, doi:10.1016/j.foodcont.2017.09.036.
112. Bhatti, M.; Veeramachaneni, A.; Shelef, L.A. Factors affecting the antilisterial effects of nisin in milk. *Int. J. Food Microbiol.* **2004**, *97*, 215–219, doi:10.1016/j.ijfoodmicro.2004.06.010.
113. Zapico, P.; Medina, M.; Gaya, P.; Nuñez, M. Synergistic effect of nisin and the lactoperoxidase system on *Listeria monocytogenes* in skim milk. *Int. J. Food Microbiol.* **1998**, *40*, 35–42, doi:10.1016/S0168-1605(98)00008-7.
114. García-Díez, J.; Patarata, L. Influence of salt level, starter culture, fermentable carbohydrates, and temperature on the behaviour of *L. monocytogenes* in sliced chouriço during storage. *Acta Aliment.* **2017**, *46*, 206–213, doi:10.1556/066.2017.46.2.10.

115. Ortiz, S.; López, V.; Garriga, M.; Martínez-Suárez, J. V. Antilisterial effect of two bioprotective cultures in a model system of Iberian chorizo fermentation. *Int. J. Food Sci. Technol.* **2014**, *49*, 753–758, doi:10.1111/ijfs.12362.
116. Vaz-Velho, M.; Jácome, S.; Noronha, L.; Todorov, S.; Fonseca, S.; Pinheiro, R.; Morais, A.; Silva, J.; Teixeira, P. Comparison of antilisterial effects of two strains of lactic acid bacteria during processing and storage of a portuguese salami-like product alheira. *Chem. Eng. Trans.* **2013**, *32*, 1807–1812, doi:10.3303/CET1332302.
117. Kamiloglu, A.; Kaban, G.; Kaya, M. Effects of autochthonous *Lactobacillus plantarum* strains on *Listeria monocytogenes* in sucuk during ripening. *J. Food Saf.* **2019**, *39*, 1–8, doi: 10.1111/jfs.12618.
118. Zanette, C.M.; Dalla Santa, O.R.; Bersot, L.S. Effect of *Lactobacillus plantarum* starter cultures on the behavior of *Listeria monocytogenes* during sausage maturation. *Int. Food Res. J.* **2015**, *22*, 844–848.
119. Castellano, P.; Peña, N.; Ibarreche, M.P.; Carduza, F.; Soteras, T.; Vignolo, G. Antilisterial efficacy of *Lactobacillus* bacteriocins and organic acids on frankfurters. Impact on sensory characteristics. *J. Food Sci. Technol.* **2018**, *55*, 689–697, doi:10.1007/s13197-017-2979-8.
120. Nikodinoska, I.; Baffoni, L.; Di Gioia, D.; Manso, B.; García-Sánchez, L.; Melero, B.; Rovira, J. Protective cultures against foodborne pathogens in a nitrite reduced fermented meat product. *Lwt* **2019**, *101*, 293–299, doi:10.1016/j.lwt.2018.11.022.
121. Macieira, A.; Barros, D.; Vaz-Velho, M.; Pinheiro, R.; Fonseca, S.; Albano, H.; Teixeira, P. Effects of *Lactobacillus plantarum* bacteriocinogenic culture on physicochemical, microbiological, and sensorial characteristics of "Chouriço Vinha d'Alhos", A traditional Portuguese Sausage. *J. Food Qual. Hazards Control* **2018**, *5*, 118–127, doi:10.29252/jfqhc.5.4.2.
122. Khalili Sadaghiani, S.; Aliakbarlu, J.; Tajik, H.; Mahmoudian, A. Anti-*Listeria* activity and shelf life extension effects of *Lactobacillus* along with garlic extract in ground beef. *J. Food Saf.* **2019**, *39*, 1–8, doi:10.1111/jfs.12709.
123. Cosansu, S.; Georgaras, I.; Ayhan, K.; Sofos, J.N. Control of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus acidilactici* 13 and its antimicrobial substance in a dry fermented sausage sucuk and in turkey breast. *J. Food Nutr. Res.* **2010**, *49*, 206–214.
124. Olaoye, O.A.; Onilude, A.A. Investigation on the potential application of biological agents in the extension of shelf life of fresh beef in Nigeria. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1445–1454, doi:10.1007/s11274-010-0319-5.
125. Orihuel, A.; Bonacina, J.; Vildoza, M.J.; Bru, E.; Vignolo, G.; Saavedra, L.; Fadda, S. Biocontrol of *Listeria monocytogenes* in a meat model using a combination of a bacteriocinogenic strain with curing additives. *Food Res. Int.* **2018**, *107*, 289–296, doi:10.1016/j.foodres.2018.02.043.
126. Trinetta, V.; Floros, J.D.; Cutter, C.N. Sakacin a-containing pullulan film: An active packaging system to control epidemic clones of *Listeria monocytogenes* in ready-to-eat foods. *J. Food Saf.* **2010**, *30*, 366–381, doi:10.1111/j.1745-4565.2010.00213.x.
127. Ruiz, A.; Williams, S.K.; Djeri, N.; Hinton, A.; Rodrick, G.E. Nisin affects the growth of *Listeria monocytogenes* on ready-to-eat turkey ham stored at four degrees Celsius for sixty-three days. *Poult. Sci.* **2010**, *89*, 353–358, doi:10.3382/ps.2008-00503.
128. Balay, D.R.; Dangeti, R. V.; Kaur, K.; McMullen, L.M. Purification of leucocin A for use on wieners to inhibit *Listeria monocytogenes* in the presence of spoilage organisms. *Int. J. Food Microbiol.* **2017**, *255*, 25–31, doi:10.1016/j.ijfoodmicro.2017.05.016.
129. Kondrotiene, K.; Kasnauskyte, N.; Serniene, L.; Gölz, G.; Alter, T.; Kaskoniene, V.; Maruska, A.S.; Malakauskas, M. Characterization and application of newly isolated nisin producing *Lactococcus lactis* strains for control of *Listeria monocytogenes* growth in fresh cheese. *LWT - Food Sci. Technol.* **2018**, *87*, 507–514, doi:10.1016/j.lwt.2017.09.021.
130. Ivanovic, M.; Mirkovic, N.; Mirkovic, M.; Miocinovic, J.; Radulovic, A.; Knudsen, T.S.; Radulovic, Z. Autochthonous *Enterococcus durans* PFMI565 and *Lactococcus lactis* subsp. *lactis* BGBU1–4 in bio-control of *Listeria monocytogenes* in ultrafiltered cheese. *Foods* **2021**, *10*, 1448, doi: 10.3390/foods10071448.
131. Mills, S.; Serrano, L.M.; Griffin, C.; O'Connor, P.M.; Schaad, G.; Bruining, C.; Hill, C.; Ross, R.P.; Meijer, W.C. Inhibitory activity of *Lactobacillus plantarum* LMG P-26358 against *Listeria innocua* when used as an adjunct starter in the manufacture of cheese. *Microb. Cell Fact.* **2011**, *10*, 1–11, doi:10.1186/1475-2859-10-S1-S7.
132. Hernández, D.; Cardell, E.; Zárate, V. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin like substance produced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol.* **2005**, *99*, 77–84, doi: 10.1111/j.1365-2672.2005.02576.x.
133. O'Sullivan, L.; O'Connor, E.; Ross, R..; Hill, C. Evaluation of live-culture producing lacticin 3147 as a treatment for the control of *Listeria monocytogenes* on the surface of smear-ripened cheese. *J. Appl. Microbiol.* **2006**, *100*, 135–143, doi: 10.1111/j.1365-2672.2005.02747.x.
134. Falardeau, J.; Trmčić, A.; Wang, S. The occurrence, growth, and biocontrol of *Listeria monocytogenes* in fresh and surface-ripened soft and semisoft cheeses. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 4019–4048, doi:10.1111/1541-4337.12768.
135. Lourenço, A.; Kamnetz, M.B.; Gadotti, C.; Diez-Gonzalez, F. Antimicrobial treatments to control *Listeria monocytogenes* in queso fresco. *Food Microbiol.* **2017**, *64*, 47–55, doi:10.1016/j.fm.2016.12.014.
136. Pimentel-Filho, N. de J.; Mantovani, H.C.; de Carvalho, A.F.; Dias, R.S.; Vanetti, M.C.D. Efficacy of bovicin HC5 and nisin combination against *Listeria monocytogenes* and *Staphylococcus aureus* in fresh cheese. *Int. J. Food Sci. Technol.* **2014**, *49*, 416–422, doi:10.1111/ijfs.12316.
137. Dal Bello, B.; Cocolin, L.; Zeppa, G.; Field, D.; Cotter, P.D.; Hill, C. Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. *Int. J. Food Microbiol.* **2012**, *153*, 58–65,

doi:10.1016/j.ijfoodmicro.2011.10.016.
138. Loessner, M.; Guenther, S.; Steffan, S.; Scherer, S. A pediocin-producing *Lactobacillus plantarum* strain inhibits *Listeria monocytogenes* in a multispecies cheese surface microbial ripening consortium. *Appl. Environ. Microbiol.* **2003**, *69*, 1854–1857, doi:10.1128/AEM.69.3.1854-1857.2003.
139. Ribeiro, S.C.; O'Connor, P.M.; Ross, R.P.; Stanton, C.; Silva, C.C.G. An anti-listerial *Lactococcus lactis* strain isolated from Azorean Pico cheese produces lacticin 481. *Int. Dairy J.* **2016**, *63*, 18–28, doi:10.1016/j.idairyj.2016.07.017.
140. Ribeiro, S.C.; Ross, R.P.; Stanton, C.; Silva, C.C.G. Characterization and application of antilisterial enterocins on model fresh cheese. *J. Food Prot.* **2017**, *80*, 1303–1316, doi:10.4315/0362-028X.JFP-17-031.
141. Possas, A.; Bonilla-Luque, O.M.; Valero, A. From cheese-making to consumption: Exploring the microbial safety of cheeses through predictive microbiology models. *Foods* **2021**, *10*, 335, doi:10.3390/foods10020355.
142. Arqués, J.L.; Rodríguez, E.; Langa, S.; Landete, J.M.; Medina, M. Antimicrobial activity of lactic acid bacteria in dairy products and gut: Effect on pathogens. *Biomed Res. Int.* **2015**, doi:10.1155/2015/584183.
143. Morandi, S.; Silvetti, T.; Vezzini, V.; Morozzo, E.; Brasca, M. How we can improve the antimicrobial performances of lactic acid bacteria? A new strategy to control *Listeria monocytogenes* in Gorgonzola cheese. *Food Microbiol.* **2020**, *90*, 103488, doi:10.1016/j.fm.2020.103488.
144. Contessa, C.R.; Souza, N.B. De; Batt, G.; Moura, C.M. De; Silveira, G.; Moraes, C.C. Development of active packaging based on agar-agar incorporated with bacteriocin of *Lactobacillus sakei.* **2021**, *11*, 1–9, doi:10.3390/biom11121869.

VI. CONCLUSIONES

VI.1. CONCLUSIONES

El trabajo de investigación llevado a cabo en esta Tesis Doctoral ha permitido obtener las siguientes conclusiones:

1. El procedimiento utilizado de evaluación inicial de la actividad anti-*L. monocytogenes in vitro* mediante ensayos de difusión en agar, seguido con evaluación de la actividad antimicrobiana en sistemas modelo que simulan la composición y las condiciones de maduración de los productos y evaluación final en alimentos madurados, permite seleccionar de forma efectiva cepas de bacterias ácido-lácticas con actividad relevante frente a esta bacteria patógena.

2. El método SYBR Green qPCR diseñado permite evaluar cambios en la expresión del gen de virulencia *inlA* de *L. monocytogenes*, lo cual es de gran utilidad para detectar un aumento en la expresión de este gen, que puede ocurrir en cepas resistentes de esta bacteria patógena en condiciones de acidez de embutidos curado-madurados y quesos curados, lo que podría conducir a un aumento de su patogenicidad.

3. Los hidrolizados proteicos de lactosuero con potencial aplicación en quesos madurados presentan actividad antimicrobiana limitada en sistemas modelo de quesos madurados y además incrementan la expresión de genes de virulencia *L. monocytogenes* por lo que no son adecuados como estrategia de control de esta bacteria patógena.

4. Las bacterias ácido-lácticas seleccionadas *Ll. sakei* 205 aislada de embutidos y *Lc. casei* 116 procedente de quesos, son cepas inocuas, sin resistencia a antibióticos y con capacidad demostrada para reducir *L. monocytogenes* tanto en sistemas modelo de alimentos madurados como durante la maduración de estos productos, por lo que son propuestas como cultivos protectores.

5. *Ll. sakei* 205 y *Lc. casei* 116 no provocan modificación negativa alguna en las características sensoriales de los alimentos madurados y pueden favorecer el desarrollo del sabor y aroma característicos de embutidos curado-madurados y quesos madurados.

6. La combinación de la caracterización de las bacterias ácido-lácticas seleccionadas mediante el método de secuenciación de la región 16S ARNr con el análisis de huella genética usando el protocolo optimizado de PFGE con los enzimas de restricción *SgsI* y *NotI* permite la diferenciación a nivel de cepa, lo cual de gran utilidad para controlar la implantación de las cepas seleccionadas durante la maduración de embutidos y quesos madurados.

VI.2. CONCLUSIONS

The research work carried out in this Doctoral Thesis has allowed the following conclusions to be obtained:

1. The procedure used for initial *in vitro* evaluation of anti-*L. monocytogenes* by using the agar spot assay, followed by evaluation of antimicrobial activity in model systems that simulate product ripening composition and conditions and final evaluation in ripened foods, allows effective selection of lactic-acid bacteria strains with relevant activity against this pathogenic bacterium.

2. The SYBR Green qPCR method designed allows evaluating changes in the expression of the virulence gene *inlA* of *L. monocytogenes,* which is very useful for detecting an increase in the expression of this gene, which can occur in resistant strains of this pathogenic bacterium in acidity conditions of dry-cured fermented sausages and ripened cheeses, which could lead to an increase in their pathogenicity.

3. Whey protein hydrolysates with potential application in ripened cheeses have limited antimicrobial activity in model systems of ripened cheeses and also increase the expression of *L. monocytogenes* virulence genes, so they are not suitable as a control strategy for this pathogenic bacterium.

4. Selected lactic-acid bacteria *Ll. sakei* 205 isolated from dry-cured fermented sausages and *Lc. casei* 116 from cheese, are innocuous strains, without resistance to antibiotics and with a demonstrated capacity to reduce *L. monocytogenes* both

in model systems of matured foods and during the ripening of these products, for which they are proposed as protective cultures.

5. *Ll. sakei* 205 and *Lc. casei* 116 do not cause any negative modification in the sensory characteristics of ripened foods and can favor the development of the characteristic flavor and aroma of dry-cured fermented sausages and ripened cheeses.

6. The combination of the characterization of the selected lactic-acid bacteria by the 16S rRNA sequencing method with the genetic fingerprint analysis using the optimized PFGE protocol with the restriction enzymes *SgsI* and *NotI* allows differentiation at the strain level, which is very useful to control the implantation of the selected strains during the ripening of dry-cured meats and ripened cheeses.

VII. BIBLIOGRAFÍA

Abay, S., Çakır Bayram, L., Aydin, F., Müştak, H. K., Diker, K. S., & Erol, İ. (2019). Pathogenicity, genotyping and antibacterial susceptibility of the *Listeria* spp. recovered from stray dogs. *Microbial Pathogenesis*, *126*, 123–133.

Abdelfatah, E. N., & Mahboub, H. H. H. (2018). Studies on the effect of *Lactococcus garvieae* of dairy origin on both cheese and Nile tilapia (O. niloticus). *International Journal of Veterinary Science and Medicine*, *6*(2), 201–207.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *127*(3), 200–208.

Adesulu-Dahunsi, A. T., Sanni, A. I., & Jeyaram, K. (2021). Diversity and technological characterization of *Pediococcus pentosaceus* strains isolated from Nigerian traditional fermented foods. *Lwt*, *140*, 110697.

Agriopoulou, S., Stamatelopoulou, E., Sachadyn-Król, M., & Varzakas, T. (2020). Lactic acid bacteria as antibacterial agents to extend the shelf life of fresh and minimally processed fruits and vegetables: Quality and safety aspects. *Microorganisms*, *8*(6), 1–23.

Ahn, D. U., Jo, C., Du, M., Olson, D. G., & Nam, K. C. (2000). Quality characteristics of pork patties irradiated and stored in different packaging and storage conditions. *Meat Science*, *56*(2), 203–209.

Albano, H., Pinho, C., Leite, D., Barbosa, J., Silva, J., Carneiro, L., Magalhães, R., Hogg, T., & Teixeira, P. (2009). Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage. *Food Control*, *20*(8), 764–770.

Albano, H., Todorov, S. D., van Reenen, C. A., Hogg, T., Dicks, L. M. T., & Teixeira, P. (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology*, *116*(2), 239–247.

Alegría, Á., Delgado, S., Roces, C., López, B., & Mayo, B. (2010). Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk. *International Journal of Food Microbiology*, *143*(1–2), 61–66.

Aleson-Carbonell, L., Fernández-López, J., Pérez-Alvarez, J. A., & Kuri, V. (2005). Functional and sensory effects of fibre-rich ingredients on breakfast fresh sausages manufacture. *Food Science and Technology International*, *11*(2), 89–97.

Alía, A, Andrade, M. J., Córdoba, J. J., Martín, I., & Rodríguez, A. (2020). Development of a multiplex real-time PCR to differentiate the four major *Listeria monocytogenes* serotypes in isolates from meat processing plants. *Food Microbiology*, *87*.

Alía, Alberto, Córdoba, J. J., Rodríguez, A., García, C., & Andrade, M. J. (2020). Evaluation of the efficacy of *Debaryomyces hansenii* as protective culture for controlling *Listeria monocytogenes* in sliced dry-cured ham. *Lwt*, *119*, 108886.

Allerberger, F., & Wagner, M. (2010). Listeriosis: A resurgent foodborne infection. *Clinical Microbiology and Infection*, *16*(1), 16–23.

Álvarez, M., Andrade, M. J., García, C., Rondán, J. J., & Núñez, F. (2020). Effects of preservative agents on quality attributes of dry-cured fermented sausages. *Foods*, *9*, 1505.

Amato, E., Filipello, V., Gori, M., Lomonaco, S., Losio, M. N., Parisi, A., Huedo, P., Knabel, S. J., & Pontello, M. (2017). Identification of a major *Listeria monocytogenes* outbreak clone linked to soft cheese in Northern Italy - 2009-2011. *BMC Infectious Diseases*, *17*(1), 1–7.

Amit, S. K., Uddin, M. M., Rahman, R., Islam, S. M. R., & Khan, M. S. (2017). A review on mechanisms and commercial aspects of food preservation and processing. *Agriculture and Food Security*, *6*(1), 1–22.

Ammor, S., Rachman, C., Chaillou, S., Prévost, H., Dousset, X., Zagorec, M., Dufour, E., & Chevallier, I. (2005). Phenotypic and genotypic identification of lactic acid bacteria isolated from a small-scale facility producing traditional dry sausages. *Food Microbiology*, *22*(5), 373–382.

Ananou, S., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (2007). Biopreservation, an ecological approach to improve the safety and shelf-life of foods. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 475–486.

Andrade, M. J., Córdoba, J. J., Casado, E. M., Córdoba, M. G., & Rodríguez, M. (2010).

Effect of selected strains of *Debaryomyces hansenii* on the volatile compound production of dry fermented sausage "salchichón". *Meat Science*, *85*(2), 256–264.

Andrade, M. J., Rodríguez, M., Casado, E. M., Bermúdez, E., & Córdoba, J. J. (2009). Differentiation of yeasts growing on dry-cured Iberian ham by mitochondrial DNA restriction analysis, RAPD-PCR and their volatile compounds production. *Food Microbiology*, *26*(6), 578–586.

Andrade, M. J., Thorsen, L., Rodríguez, A., Córdoba, J. J., & Jespersen, L. (2014). Inhibition of ochratoxigenic moulds by *Debaryomyces hansenii* strains for biopreservation of dry-cured meat products. *International Journal of Food Microbiology*, *170*, 70–77.

Andritsos, N. D., Paramithiotis, S., Mataragas, M., & Drosinos, E. H. (2021). *Listeria monocytogenes* serogroup 1/2 strains have a competitive growth advantage over serotype 4b during refrigerated storage of an artificially contaminated ready-to-eat pork meat product. *Applied Sciences (Switzerland)*, *11*(13).

AOAC. (2000). Moisture in malt gravimetric method (935.29). In *Official methods of analysis of AOAC international* (17th ed.).

AOAC. (2005). Official Methods of Analysis of AOAC International 18th Edition. *Association of Official Analytical Chemists.*, 3172. https://www.techstreet.com/standards/official-methods-of-analysis-of-aoac-international-20th-edition-2016?product_id=1937367

AOAC. (2016). Official Methods of Analysis of AOAC International - 20th Edition. In *Association of Official Analytical Chemists.* (Vol. 52, Issue 2). https://doi.org/10.1021/ac50052a726

Aquilanti, L., Garofalo, C., Osimani, A., & Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: An overview. *International Food Research Journal*, *23*(2), 429–445.

Arena, M. P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., & Fiocco, D. (2016). Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers in Microbiology*, *7*, 1–10.

Arqués, J. L., Rodríguez, E., Langa, S., Landete, J. M., & Medina, M. (2014). Antimicrobial activity of lactic acid bacteria in dairy products and gut: Effect on pathogens. *BioMed Research International*.

Asefa, D. T., Kure, C. F., Gjerde, R. O., Omer, M. K., Langsrud, S., Nesbakken, T., & Skaar, I. (2010). Fungal growth pattern, sources and factors of mould contamination in a dry-cured meat production facility. *International Journal of Food Microbiology*, *140*(2–3), 131–135.

Babić, I., Markov, K., Kovačević, D., Trontel, A., Slavica, A., Dugum, J., Čvek, D., Svetec, I. K., Posavec, S., & Frece, J. (2011). Identification and characterization of potential autochthonous starter cultures from a Croatian "brand" product "Slavonski kulen." *Meat Science*, *88*(3), 517–524.

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of yeast and mold species from a variety of cheese types. *Current Microbiology*, *70*(6), 792–800.

Barba, F. J., SantAna, A. S., Orlien, V., & Koubaa, M. (2018). Innovative technologies for food preservation: *Inactivation of spoilage and pathogenic microorganisms*. Elsevier.

Barbosa, J., Gibbs, P. A., & Teixeira, P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control*, *21*(5), 651–656.

Barbuti, S., Grisenti, M. S., Frustoli, M. A., & Parolari, G. (2009). Validation of the manufacturing process of italian dry-cured ham(Prosciiutto) for the inactivation of *Listeria monocytogenes* and *Salmonella* spp. *Int Congr Meat Sci Technol*, PE6.10.

Barcenilla, C., Ducic, M., López, M., Prieto, M., & Álvarez-Ordóñez, A. (2022). Application of lactic acid bacteria for the biopreservation of meat products: A systematic review. *Meat Science*, *183*.

Beaufort, A., Cornu, M., Bergis, H., Lardeux, A. L., & Lombard, B. (2014). *EURL* Lm Technical guiadance document for conducing shelf-life studies on *Listeria monocytogenes* in ready to eat foods. Community Reference Laboratory for Listeria Monocytogenes. http://www.fsai.ie/uploadedFiles/EURL %0ALm_Technical Guidance Document%0A Lm shelf-life studies_V3_2014-%0A06-06 (2).pdf

Ben Omar, N., Castro, A., Lucas, R., Abriouel, H., Yousif, N. M. K., Franz, C. M. A. P., Holzapfel, W. H., Pérez-Pulido, R., Martínez-Cañamero, M., & Gálvez, A. (2004). Functional and safety aspects of *Enterococci* isolated from different Spanish foods. *Systematic and Applied Microbiology*, *27*(1), 118–130.

Ben Slama, R., Miladi, H., Chaieb, K., & Bakhrouf, A. (2013). Survival of *Listeria monocytogenes* cells and the effect of extended frozen storage (-20 °C) on the expression of its virulence gene. *Applied Biochemistry and Biotechnology*, *170*(5), 1174–1183.

Benito, M. J., Martín, A., Aranda, E., Pérez-Nevado, F., Ruiz-Moyano, S., & Córdoba, M. G. (2007). Characterization and selection of autochthonous lactic acid bacteria isolated from traditional Iberian dry-fermented salchichón and chorizo sausages. *Journal of Food Science*, *72*(6), 193–201.

Benito, María J., Serradilla, M. J., Ruiz-Moyano, S., Martín, A., Pérez-Nevado, F., & Córdoba, M. G. (2008). Rapid differentiation of lactic acid bacteria from autochthonous fermentation of Iberian dry-fermented sausages. *Meat Science*, *80*(3), 656–661.

Benkerroum, N., Daoudi, A., Hamraoui, T., Ghalfi, H., Thiry, C., Duroy, M., Evrart, P., Roblain, D., & Thonart, P. (2005). Lyophilized preparations of bacteriocinogenic *Lactobacillus curvatus* and *Lactococcus lactis* subsp. lactis as potential protective adjuncts to control *Listeria monocytogenes* in dry-fermented sausages. *Journal of Applied Microbiology*, *98*(1), 56–63.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal*, *11*(4–7), 259–274.

Bermudez-Humaran, L. G., & Langella, P. (2012). Importance of commensal and probiotic bacteria in human health. *Current Immunology Reviews*, *8*(3), 248–253.

Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M., & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, *72*(4), 1552–1563.

Bonilauri, P., Merialdi, G., Ramini, M., Bardasi, L., Taddei, R., Grisenti, M. S., Daminelli, P., Cosciani-Cunico, E., Dalzini, E., Frustoli, M. A., Giacometti, F., Tomasello, F., Piva, S., & Serraino, A. (2021). Modeling the behavior of *Listeria*

innocua in Italian salami during the production and high-pressure validation of processes for exportation to the U.S. *Meat Science*, *172*, 108315.

Borucki, M. K., & Call, D. R. (2003). *Listeria monocytogenes* serotype identification by PCR. *Journal of Clinical Microbiology*, *41*(12), 5537–5540.

Bošković, M., Tadić, V., Dordević, J., Glišić, M., Lakićević, B., Dimitrijević, M., & Ž Baltić, M. (2017). Effect of starter cultures on survival of *Listeria monocytogenes* in Čajna sausage. *IOP Conference Series: Earth and Environmental Science*, *85*(1).

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

Bover-Cid, S., Belletti, N., Garriga, M., & Aymerich, T. (2011). Model for *Listeria monocytogenes* inactivation on dry-cured ham by high hydrostatic pressure processing. *Food Microbiology*, *28*(4), 804–809.

Bover-Cid, Sara, & Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology*, *53*(1), 33–41.

Bover, S., & Garriga, M. (2014). Condiciones que determinan el crecimiento y la supervivencia de *Listeria monocytogenes* en alimentos listos para el consumo.

Bowman, J. P., Bittencourt, C. R., & Ross, T. (2008). Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology*, *154*(2), 462–475.

Buchanan, R. L., Gorris, L. G. M., Hayman, M. M., Jackson, T. C., & Whiting, R. C. (2017). A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control*, *75*, 1–13.

Bungenstock, L., Abdulmawjood, A., & Reich, F. (2020). Evaluation of antibacterial properties of lactic acid bacteria from traditionally and industrially produced fermented sausages from Germany. *PLoS ONE*, *15*(3), 1–15.

Camargo, A. C., de Araújo, J. P. A., Fusieger, A., de Carvalho, A. F., & Nero, L. A. (2021). Microbiological quality and safety of Brazilian artisanal cheeses. *Brazilian Journal of Microbiology*, *52*(1), 393–409.

Campagnollo, F., Margalho, L., Kamimura, B., Feliciano, M., Freire, L., Lopes, L., Alvarenga, V., Cadavez, V., Gonzales-Barron, U., Schaffner, D., & Sant'Ana, A. (2018). Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. *Food Microbiology*, *73*, 288–297.

Cano-García, L., Flores, M., & Belloch, C. (2013). Molecular characterization and aromatic potential of *Debaryomyces hansenii* strains isolated from naturally fermented sausages. *Food Research International*, *52*(1), 42–49.

Carlin, C. R., Liao, J., Weller, D., Guo, X., Orsi, R., & Wiedmann, M. (2021). *Listeria cossartiae* sp. Nov., *Listeria immobilis* sp. nov., *Listeria portnoyi* sp. nov. and *Listeria rustica* sp. nov., isolated from agricultural water and natural environments. *International Journal of Systematic and Evolutionary Microbiology*, *71*(5).

Casquete, R., Benito, M. J., Martín, A., Ruiz-Moyano, S., Aranda, E., & Córdoba, M. G. (2012). Microbiological quality of salchichón and chorizo, traditional Iberian dry-fermented sausages from two different industries, inoculated with autochthonous starter cultures. *Food Control*, *24*(1–2), 191–198.

Casquete, R., Benito, M. J., Martín, A., Ruiz-Moyano, S., Hernández, A., & Córdoba, M. G. (2011). Effect of autochthonous starter cultures in the production of "salchichón", a traditional Iberian dry-fermented sausage, with different ripening processes. *LWT - Food Science and Technology*, *44*(7), 1562–1571.

Castellari, C., Quadrelli, A. M., & Laich, F. (2010). Surface mycobiota on Argentinean dry fermented sausages. *International Journal of Food Microbiology*, *142*(1–2), 149–155.

Cava, R., Higuero, N., & Ladero, L. (2021). High-pressure processing and storage temperature on *Listeria monocytogenes* , microbial counts and oxidative changes of two traditional dry-cured meat products. *Meat Science*, *171*(August 2020), 108273.

Ceugniez, A., Drider, D., Jacques, P., & Coucheney, F. (2015). Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. *Food Microbiology*, *52*, 177–184.

Chen, C., Lu, Y., Yu, H., Chen, Z., & Tian, H. (2019). Influence of 4 lactic acid bacteria on the flavor profile of fermented apple juice. *Food Bioscience*, *27*(August 2018),

30–36.

Chen, J. Q., Regan, P., Laksanalamai, P., Healey, S., & Hu, Z. (2017). Prevalence and methodologies for detection, characterization and subtyping of *Listeria monocytogenes* and *L. ivanovii* in foods and environmental sources. *Food Science and Human Wellness*, *6*(3), 97–120.

Chevallier, I., Ammor, S., Laguet, A., Labayle, S., Castanet, V., Dufour, E., & Talon, R. (2006). Microbial ecology of a small-scale facility producing traditional dry sausage. *Food Control*, *17*(6), 446–453.

Cholakov, R., Tumbarski, Y., Yanakieva, V., Dobrev, I., Salim, Y., & Denkova, Z. (2017). Antimicrobial activity of *Leuconostoc lactis* strain BT17, isolated from a spontaneously fermented cereal beverage (Boza). *Journal of Microbiology, Biotechnology and Food Sciences*, *7*(1), 47–49.

Christieans, S., Picgirard, L., Parafita, E., Lebert, A., & Gregori, T. (2018). Impact of reducing nitrate/nitrite levels on the behavior of *Salmonella Typhimurium* and *Listeria monocytogenes* in French dry fermented sausages. *Meat Science*, *137*, 160–167.

Clare, D., Catignani, G., & Swaisgood, H. (2005). Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Current Pharmaceutical Design*, *9*(16), 1239-1255.

Clinical and Laboratory Standards Institute. (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. In *M100-S22* (Vol. 32, Issue 3).

CLSI - Clinical & Laboratory Standards Institute. (2017). *Performance Standards for Antimicrobial Susceptibility Testing* (27th Editi).

Cocolin, L., Dolci, P., Rantsiou, K., Urso, R., Cantoni, C., & Comi, G. (2009). Lactic acid bacteria ecology of three traditional fermented sausages produced in the North of Italy as determined by molecular methods. *Meat Science*, *82*(1), 125–132.

Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C., & Comi, G. (2006). Dynamics and characterization of yeasts during natural fermentation of Italian sausages. *FEMS Yeast Research*, *6*(5), 692–701.

Codex Alimentarius Commission. (1978). Codex General Standard for Cheese, CODEXSTAN283–1978. Codex Alimentarius Commission.

Coelho, M. C., Silva, C. C. G., Ribeiro, S. C., Dapkevicius, M. L. N. E., & Rosa, H. J. D. (2014). Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria. *International Journal of Food Microbiology*, *191*, 53–59.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., & Cocolin, L. (2005). Characterisation of naturally fermented sausages produced in the North East of Italy. *Meat Science*, *69*(3), 381–392.

Comisión Europea. (2007). Reg

Corral, S., Salvador, A., Belloch, C., & Flores, M. (2014). Effect of fat and salt reduction on the sensory quality of slow fermented sausages inoculated with *Debaryomyces hansenii* yeast. *Food Control*, *45*, 1–7.

Corral, S., Salvador, A., Belloch, C., & Flores, M. (2015). Improvement the aroma of reduced fat and salt fermented sausages by *Debaromyces hansenii* inoculation. *Food Control*, *47*, 526–535.

Corsetti, A., Rossi, J., & Gobbetti, M. (2001). Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *International Journal of Food Microbiology*, *69*(1–2), 1–10.

Costerton, J. W., & Lewandowski, Z. (1995). Microbial biofilms. *Annual Review of Microbiology*, 341–357.

Coton, M., Delbés-Paus, C., Irlinger, F., Desmasures, N., Le Fleche, A., Stahl, V., Montel, M. C., & Coton, E. (2012). Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiology*, *29*(1), 88–98.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology*, *3*(10), 777–788.

Crespo, A., Jiménez, A., Ruiz-Moyano, S., Merchán, A. V., Galván, A. I., Benito, M. J., & Martín, A. (2022). Low-frequency ultrasound as a tool for quality control of soft-bodied raw ewe's milk cheeses. *Food Control*, *131*.

Crespo, A., Martín, A., Ruiz-Moyano, S., Benito, M. J., Rufo, M., Paniagua, J. M., & Jiménez, A. (2020). Application of ultrasound for quality control of Torta del Casar cheese ripening. *Journal of Dairy Science*, *103*(10), 8808–8821.

De Carvalho, A. A. T., De Paula, R. A., Mantovani, H. C., & De Moraes, C. A. (2006). Inhibition of *Listeria monocytogenes* by a lactic acid bacterium isolated from Italian salami. *Food Microbiology*, *23*(3), 213–219.

De Carvalho, K. G., Bambirra, F. H. S., Kruger, M. F., Barbosa, M. S., Oliveira, J. S., Santos, A. M. C., Nicoli, J. R., Bemquerer, M. P., De Miranda, A., Salvucci, E. J., Sesma, F. J. M., & Franco, B. D. G. M. (2010). Antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian meat product. *Journal of Industrial Microbiology and Biotechnology*, *37*(4), 381–390.

De Las Rivas, B., Marcobal, Á., & Muñoz, R. (2005). Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. *FEMS Microbiology Letters*, *244*(2), 367–372.

Deegan, L. H., Cotter, P. D., Hill, C., & Ross, P. (2006). Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, *16*(9), 1058–1071.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J., & Ramírez, R. (2010). Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. *Food Chemistry*, *118*(1), 182–189.

Delgado, J., Peromingo, B., Núñez, F., & Asensio, M. A. (2016). Use of molds and their antifungal proteins for biocontrol of toxigenic molds on dry-ripened cheese and meats. *Current Opinion in Food Science*, *11*, 40–45.

Delgado, J., Peromingo, B., Rodríguez, A., & Rodríguez, M. (2019). Biocontrol of *Penicillium griseofulvum* to reduce cyclopiazonic acid contamination in dry-fermented sausages. *International Journal of Food Microbiology*, *293*(August 2018), 1–6.

Demers-Mathieu, V., Gauthier, S. F., Britten, M., Fliss, I., Robitaille, G., & Jean, J. (2013). Inhibition of *Listeria monocytogenes* growth in Cheddar cheese byananionic peptides-enriched extract from whey proteins. *International Dairy Journal*, *32*(1),

6–12.

den Bakker, H. C., Bowen, B. M., Rodriguez-Rivera, L. D., & Wiedmann, M. (2012). FSL J1-208, a virulent uncommon phylogenetic lineage IV *Listeria monocytogenes* strain with a small chromosome size and a putative virulence plasmid carrying internalin-like genes. *Applied and Environmental Microbiology*, *78*(6), 1876–1889.

Di Cagno, R., Chaves Lòpez, C., Tofalo, R., Gallo, G., De Angelis, M., Paparella, A., Hammes, W. P., & Gobbetti, M. (2008). Comparison of the compositional, microbiological, biochemical and volatile profile characteristics of three Italian PDO fermented sausages. *Meat Science*, *79*(2), 224–235.

Díez, J. G., & Patarata, L. (2013). Behavior of *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in chouriço de vinho, a dry fermented sausage made from wine-marinated meat. *Journal of Food Protection*, *76*(4), 588–594.

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

Drevets, D. A., & Bronze, M. S. (2008). *Listeria monocytogenes*: Epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunology and Medical Microbiology*, *53*(2), 151–165.

Duan, Y., Tan, Z., Wang, Y., Li, Z., Li, Z., Quin, G., Huo, Y., & Cai, Y. (2008). Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese. *The Journal of General and Applied Microbiology*, *54*(4), 51–60.

Dugat-Bony, E., Garnier, L., Denonfoux, J., Ferreira, S., Sarthou, A. S., Bonnarme, P., & Irlinger, F. (2016). Highlighting the microbial diversity of 12 French cheese varieties. *International Journal of Food Microbiology*, *238*, 265–273.

Durak-Dados, A., Michalski, M., & Osek, J. (2020). Histamine and other biogenic amines in food. *Journal of Veterinary Research (Poland)*, *64*(2), 281–288.

Dussurget, O. (2008). New insights into determinations of *Listeria monocytogenes* virulence. *International Review of Cell and Molecular Biology*, *270*, 1–38.

Eaton, T. J., & Gasson, M. J. (2001). Molecular screening of *Enterococcus* virulence

determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology*, *67*(4), 1628–1635.

EFSA, ECDC,2021. he European Union One Health 2019 Zoonoses Report. EFSA J. 19, 1-286

EFSA, ECDC, 2019. The European Union One Health 2018 Zoonoses Report. EFSA J. 17, 1-276

EFSA, ECDC, 2018a. The European Union Summary report on trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16, 1–262.

EFSA, ECDC, 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. 15, 1–228.

EFSA, ECDC, 2015a. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J. 16, 1–165.

EFSA, ECDC, 2015b. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA J. 13, 1–190.

EFSA, ECDC, 2014. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J. 16, 1–312.

EFSA, ECDC, 2013. The European Union Summary Report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. EFSA J. 11, 1–250.

EFSA, ECDC, 2012. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. EFSA J. 10, 1–442.

EFSA. (2018). *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA Journal*, *16*(1).

EFSA and ECDC. (2021). The European Union One Health 2019 Zoonoses Report. *EFSA Journal*, *19*(2).

EFSA BIOHAZ Panel. (2021). Updated list of QPS status recommended biological agents in support of EFSA risk assessments. *EFSA Journal*, *19*(7), 1–5.

El Adab, S., Essid, I., & Hassouna, M. (2015). Microbiological, biochemical and textural characteristics of a tunisian dry fermented poultry meat sausage inoculated with selected starter cultures. *Journal of Food Safety*, *35*(1), 75–85.

Estrada, O., Ariño, A., & Juan, T. (2019). Salt distribution in raw sheep milk cheese during ripening and the effect on proteolysis and lipolysis. *Foods*, *8*(3), 1–14.

EURL Lm. (2021). EURL Lm TECHNICAL GUIDANCE DOCUMENT on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *Listeria monocytogenes*. 60.

Faber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes* , a Food-Borne Pathogen. *MICROBIOLOGICAL REVIEWS*, *55*(3), 476–511.

Falardeau, J., Trmčić, A., & Wang, S. (2021). The occurrence, growth, and biocontrol of *Listeria monocytogenes* in fresh and surface-ripened soft and semisoft cheeses. *Comprehensive Reviews in Food Science and Food Safety*, *20*(4), 4019–4048.

Favaro, L., Barretto Penna, A. L., & Todorov, S. D. (2015). Bacteriocinogenic LAB from cheeses - Application in biopreservation? *Trends in Food Science and Technology*, *41*(1), 37–48.

Favaro, M., Sarmati, L., Sancesario, G., & Fontana, C. (2014). First case of *Listeria innocua* meningitis in a patient on steroids and eternecept. *JMM Case Reports*, *1*(2).

Fernández-López, J., Sendra, E., Sayas-Barberá, E., Navarro, C., & Pérez-Alvarez, J. A. (2008). Physico-chemical and microbiological profiles of "salchichón" (Spanish dry-fermented sausage) enriched with orange fiber. *Meat Science*, *80*(2), 410–417.

Ferreira, A., O'byrne, C. P., & Boor, K. J. (2001). Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Applied and Enviromental Microbiology*, *67*(10),4454-4457.

Ferreira, I., Pinho, O., & Sampaio, P. (2009). Volatile fraction of DOP "Castelo Branco" cheese: Influence of breed. *Food Chemistry*, *112*(4), 1053–1059.

Ferreira, V., Wiedmann, M., Teixeira, P., & Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: Epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection*, *77*(1), 150–170.

Ferreira, Vânia, Barbosa, J., Silva, J., Felício, M. T., Mena, C., Hogg, T., Gibbs, P., & Teixeira, P. (2007). Characterisation of alheiras, traditional sausages produced in the North of Portugal, with respect to their microbiological safety. *Food Control*, *18*(5), 436–440.

Flores, M., Durá, M. A., Marco, A., & Toldrá, F. (2004). Effect of *Debaryomyces* spp. on

aroma formation and sensory quality of dry-fermented sausages. *Meat Science*, *68*(3), 439–446.

Fontana, C., Cocconcelli, P. S., Vignolo, G., & Saavedra, L. (2015). Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria. *Food Control*, *47*, 53–59.

Foodborne Ilness Outbreak Database. (2010). *Siena Foods Salame*. http://www.outbreakdatabase.com/details/assi-market-pickles-2010/?vehicle=cucumber

Frances, N., Hornby, H., & Hunter, P. R. (1991). The isolation of *Listeria* species from fresh-water sites in Cheshire and North Wales. *Epidemiology and Infection*, *107*(1), 235–238.

Fraqueza, M. J., Laranjo, M., Elias, M., & Patarata, L. (2021). Microbiological hazards associated with salt and nitrite reduction in cured meat products: control strategies based on antimicrobial effect of natural ingredients and protective microbiota. *Current Opinion in Food Science*, *38*, 32–39.

Fretz, R., Sagel, U., Ruppitsch, W., Pietzka, A. T., Stöger, A., Huhulescu, S., Heuberger, S., Pichler, J., Much, P., Pfaff, G., Stark, K., Prager, R., Flieger, A., Feenstra, O., & Allerberger, F. (2010). Listeriosis outbreak caused by acid curd cheese "Quargel", Austria and Germany 2009. *Eurosurveillance*, *15*(5), 1–2.

Garcia-Gonzalez, N., Battista, N., Prete, R., & Corsetti, A. (2021). Health-promoting role of *Lactiplantibacillus plantarum* isolated from fermented foods. *Microorganisms*, *9*(2), 1–30.

García Fontán, M. C., Lorenzo, J. M., Parada, A., Franco, I., & Carballo, J. (2007). Microbiological characteristics of "androlla", a Spanish traditional pork sausage. *Food Microbiology*, *24*(1), 52–58.

García, M. J., Ruíz, F., Asurmendi, P., Pascual, L., & Barberis, L. (2020). Searching potential candidates for development of protective cultures: Evaluation of two *Lactobacillus* strains to reduce *Listeria monocytogenes* in artificially contaminated milk. *Journal of Food Safety*, *40*(1).

Gardini, F., Suzzi, G., Lombardi, A., Galgano, F., Crudele, M. A., Andrighetto, C.,

Schirone, M., & Tofalo, R. (2001). A survey of yeasts in traditional sausages of southern Italy. *FEMS Yeast Research*, *1*(2), 161–167.

Gelinski, J. M. L. N., Baratto, C. M., Casagrande, M., de Oliveira, T. P., Megiolaro, F., de Martini Soares, F. A. S., de Souza, E. M. B., Vicente, V. A., & Fonseca, G. G. (2019). Control of pathogens in fresh pork sausage by inclusion of *Lactobacillus sakei* BAS0117. *Canadian Journal of Microbiology*, *65*(11), 831–841.

Gómez, N. C., Ramiro, J. M. P., Quecan, B. X. V., & de Melo Franco, B. D. G. (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157: H7 biofilms formation. *Frontiers in Microbiology*, *7*, 1–15.

Gonçalves Dos Santos, M. T. P., Benito, M. J., Córdoba, M. de G., Alvarenga, N., & Ruiz-Moyano Seco de Herrera, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. *International Journal of Food Microbiology*, *262*, 63–70.

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. de G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. *Journal of Food Science*, *83*(5), 1333–1341.

Gonzales-Barron, U., Cadavez, V., Pereira, A. P., Gomes, A., Araújo, J. P., Saavedra, M. J., Estevinho, L., Butler, F., Pires, P., & Dias, T. (2015). Relating physicochemical and microbiological safety indicators during processing of linguiça, a Portuguese traditional dry-fermented sausage. *Food Research International*, *78*, 50–61.

Gonzales-Barron, Ursula, Campagnollo, F. B., Schaffner, D. W., Sant'Ana, A. S., & Cadavez, V. A. P. (2020). Behavior of *Listeria monocytogenes* in the presence or not of intentionally- added lactic acid bacteria during ripening of artisanal Minas semi-hard cheese. *Food Microbiology*, *91*.

Gori, K., Mortensen, H. D., Arneborg, N., & Jespersen, L. (2007). Ammonia production and its possible role as a mediator of communication for *Debaryomyces hansenii* and other cheese-relevant yeast species. *Journal of Dairy Science*, *90*(11), 5032–5041.

Gouin, E., Mengaud, J., & Cossart, P. (1994). The virulence gene cluster of *Listeria*

monocytogenes is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species. *Infection and Immunity*, *62*(8), 3550–3553.

Gould, L. H., Mungai, E., & Behravesh, C. B. (2014). Outbreaks attributed to cheese: Differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. *Foodborne Pathogens and Disease*, *11*(7), 545–551.

Grossi, A., Bolumar, T., Søltoft-Jensen, J., & Orlien, V. (2014). High pressure treatment of brine enhanced pork semitendinosus: Effect on microbial stability, drip loss, lipid and protein oxidation, and sensory properties. *Innovative Food Science and Emerging Technologies*, *22*, 11–21.

Guillet, C., Join-Lambert, O., Le Monnier, A., Leclercq, A., Mechaï, F., Mamzer-Bruneel, M. F., Bielecka, M. K., Scortti, M., Disson, O., Berche, P., Vazquez-Boland, J., Lortholary, O., & Lecuit, M. (2010). Human listeriosis caused by *Listeria ivanovii*. *Emerging Infectious Diseases*, *16*(1), 136–138.

Güler, Z., & Gürsoy-Balci, A. C. (2011). Evaluation of volatile compounds and free fatty acids in set types yogurts made of ewes', goats' milk and their mixture using two different commercial starter cultures during refrigerated storage. *Food Chemistry*, *127*(3), 1065–1071.

Hadjilouka, A., Mavrogiannis, G., Mallouchos, A., Paramithiotis, S., Mataragas, M., & Drosinos, E. H. (2017). Effect of lemongrass essential oil on *Listeria monocytogenes* gene expression. *LWT - Food Science and Technology*, *77*, 510–516.

Hadjilouka, A., Molfeta, C., Panagiotopoulou, O., Paramithiotis, S., Mataragas, M., & Drosinos, E. H. (2016). Expression of *Listeria monocytogenes* key virulence genes during growth in liquid medium, on rocket and melon at 4, 10 and 30 °C. *Food Microbiology*, *55*, 7–15.

Harzallah, D., & Belhadj, H. (2013). Lactic acid bacteria as probiotics : Characteristics , selection criteria and role in immunomodulation of human GI muccosal barrier.

Heredia-Castro, P. Y., Méndez-Romero, J. I., Hernández-Mendoza, A., Acedo-Félix, E., González-Córdova, A. F., & Vallejo-Cordoba, B. (2015). Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese. *Journal of Dairy Science*,

98(12), 8285–8293.

Hereu, A., Bover-Cid, S., Garriga, M., & Aymerich, T. (2012). High hydrostatic pressure and biopreservation of dry-cured ham to meet the Food Safety Objectives for *Listeria monocytogenes*. *International Journal of Food Microbiology*, *154*(3), 107–112.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). *Dairy protein hydrolysates : Peptides for health benefits*. *38*, 82–100.

Herrero, A. M., Ordóñez, J. A., de Avila, R., Herranz, B., de la Hoz, L., & Cambero, M. I. (2007). Breaking strength of dry fermented sausages and their correlation with texture profile analysis (TPA) and physico-chemical characteristics. *Meat Science*, *77*(3), 331–338.

Ho, V. T. T., Lo, R., Bansal, N., & Turner, M. S. (2018). Characterisation of *Lactococcus lactis* isolates from herbs, fruits and vegetables for use as biopreservatives against *Listeria monocytogenes* in cheese. *Food Control*, *85*, 472–483.

Holck, A., Axelsson, L., Mcleod, A., Rode, T. M., & Heir, E. (n.d.). *Health and Safety Considerations of Fermented Sausages*.

Hossain, M. I., Kim, K., Rahaman Mizan, M. F., Toushik, S. H., Ashrafudoulla, M., Roy, P. K., Nahar, S., Jahid, I. K., Choi, C., Park, S. H., & Ha, S. Do. (2021). Comprehensive molecular, probiotic, and quorum-sensing characterization of anti-listerial lactic acid bacteria, and application as bioprotective in a food (milk) model. *Journal of Dairy Science*, *104*(6), 6516–6534.

Huang, M., Zhang, M., & Bhandari, B. (2019). Recent development in the application of alternative sterilization technologies to prepared dishes: A review. *Critical Reviews in Food Science and Nutrition*, *59*(7), 1188–1196.

Hugas, M., & Monfort, J. M. (1997). Bacterial starter cultures for meat fermentation. *Food Chemistry*, *59*(4), 547–554.

Hyeon, J. Y., Park, C., Choi, I. S., Holt, P. S., & Seo, K. H. (2010). Development of multiplex real-time PCR with internal amplification control for simultaneous detection of Salmonella and Cronobacter in powdered infant formula. *International Journal of Food Microbiology*, *144*(1), 177–181.

Iacumin, L., Comi, G., Cantoni, C., & Cocolin, L. (2006). Ecology and dynamics of coagulase-negative cocci isolated from naturally fermented Italian sausages. *Systematic and Applied Microbiology*, *29*(6), 480–486.

Iacumin, L., Manzano, M., Andyanto, D., & Comi, G. (2017). Biocontrol of ochratoxigenic moulds (*Aspergillus ochraceus* and *Penicillium nordicum*) by *Debaryomyces hansenii* and *Saccharomycopsis fibuligera* during speck production. *Food Microbiology*, *62*, 188–195.

Ibrahim, H. M. (2013). *Prediction of meat and meat products by gamma rays , electron beams and X-ray irradiations-A Review*. *3*(5), 521–534.

Jan, A., Sood, M., Sofi, S. A., & Norzom, T. (2017). Non-thermal processing in food applications : A review. *International Journal of Food Science and Nutrition*, *2*(6), 171–180.

Jia, R., Zhang, F., Song, Y., Lou, Y., Zhao, A., Liu, Y., Peng, H., Hui, Y., Ren, R., & Wang, B. (2021). Physicochemical and textural characteristics and volatile compounds of semihard goat cheese as affected by starter cultures. *Journal of Dairy Science*, *104*(1), 270–280.

Jordan, K., & McAuliffe, O. (2018). *Listeria monocytogenes* in Foods. In *Advances in Food and Nutrition Research* (1st ed., Vol. 86). Elsevier Inc.

Kanwal, H., Di Cerbo, A., Zulfiqar, F., Sabia, C., Nawaz, A., Siddiqui, F. M., Aqeel, M., & Ghazanfar, S. (2021). Probiotic characterization and population diversity analysis of gut-associated *Pediococcus acidilactici* for its potential use in the dairy industry. *Applied Sciences (Switzerland)*, *11*(20).

Kapetanakou, A. E., Gkerekou, M. A., Vitzilaiou, E. S., & Skandamis, P. N. (2017). Assessing the capacity of growth, survival, and acid adaptive response of *Listeria monocytogenes* during storage of various cheeses and subsequent simulated gastric digestion. *International Journal of Food Microbiology*, *246*, 50–63.

Karli, A., Sensoy, G., Unal, N., Yanik, K., Cigdem, H., Belet, N., & Sofuoglu, A. (2014). Ventriculoperitoneal shunt infection with *Listeria innocua*. *Pediatrics International*, *56*(4), 621–623.

Kasra-Kermanshahi, R., & Mobarak-Qamsari, E. (2015). Inhibition effect of lactic acid

bacteria against food born pathogen, *Listeria monocytogenes*. *Applied Food Biotechnology*, *2*(4), 11–19.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., & Witte, W. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of *Lactobacilli*, *Pediococci*, *Lactococci*, and *Bifidobacteria*. *Applied and Environmental Microbiology*, *71*(12), 8982–8986.

Klumpp, J., & Loessner, M. J. (2013). *Listeria* phages. *Bacteriophage*, *3*(3), e26861.

Knight, A. J., Worosz, M. R., Todd, E. C. D., Bourquin, L. D., & Harris, C. K. (2008). *Listeria* in raw milk soft cheese: A case study of risk governance in the united states using the IRGC framework .

Kümmel, J., Stessl, B., Gonano, M., Walcher, G., Bereuter, O., Fricker, M., Grunert, T., Wagner, M., & Ehling-Schulz, M. (2016). *Staphylococcus aureus* entrance into the dairy chain: Tracking S. aureus from dairy cow to cheese. *Frontiers in Microbiology*, *7*(OCT), 1–11.

Kurpas, M., Wieczorek, K., & Osek, J. (2018). Ready-to-eat meat products as a source of *Listeria monocytogenes*. *Journal of Veterinary Research (Poland)*, *62*(1), 49–55.

Kyoui, D., Takahashi, H., Miya, S., Kuda, T., & Kimura, B. (2014). Comparison of the major virulence-related genes of *Listeria monocytogenes* in Internalin A truncated strain 36-25-1 and a clinical wild-type strain. *BMC Microbiology*, *14*(1), 1–7.

Larpin-Laborde, S., Imran, M., Bonaïti, C., Bora, N., Gelsomino, R., Goerges, S., Irlinger, F., Goodfellow, M., Ward, A. C., Vancanneyt, M., Swings, J., Scherer, S., Guéguen, M., & Desmasures, N. (2011). Surface microbial consortia from Livarot, a French smear-ripened cheese. *Canadian Journal of Microbiology*, *57*(8), 651–660.

Lee, E. J., & Ahn, D. U. (2005). Quality characteristics of irradiated turkey breast rolls formulated with plum extract. *Meat Science*, *71*(2), 300–305.

Leggieri, M. C., Pietri, A., & Battilani, P. (2020). Modelling fungal growth, mycotoxin production and release in grana cheese. *Microorganisms*, *8*, 69.

Leistner, L. (1978). Hurdle effect and energy saving. In *Food Quality and Nutrition* (W.K. Donke, pp. 553–557). Applied Science Publisher.

Leroy, F., & De Vuyst, L. (2015). Fermented Foods: Fermented Meat Products. In F. T. Benjamin Caballero, Paul M. Finglas (Ed.), *Encyclopedia of Food and Health* (pp. 656–660). Academic Press.

Leroy, Frédéric, & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology*, *15*(2), 67–78.

Leroy, Frédéric, Geyzen, A., Janssens, M., De Vuyst, L., & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, *31*(2), 130–137.

Li, M., Carpenter, C. E., & Broadbent, J. R. (2021). Organic acid exposure enhances virulence in some *Listeria monocytogenes* strains using the *Galleria mellonella* infection model. *Frontiers in Microbiology*, *12*(July), 1–10.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using Real-Time Quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*, 402–408.

Lizaso, G., Chasco, J., & Beriain, M. J. (1999). Microbiological and biochemical changes during ripening of salchichon, a Spanish dry cured sausage. *Food Microbiology*, *16*(3), 219–228.

Lobacz, A., & Zulewska, J. (2021). Fate of *Salmonella* spp. in the fresh soft raw milk cheese during storage at different temperatures. *Microorganisms*, *9*(5).

López Expósito, I., & Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal*, *16*(11), 1294–1305.

Lozano-Ojalvo, D., Pérez-Rodríguez, L., Pablos-Tanarro, A., López-Fandiño, R., & Molina, E. (2017). Pepsin treatment of whey proteins under high pressure produces hypoallergenic hydrolysates. *Innovative Food Science and Emerging Technologies*, *43*(July), 154–162.

LPSN, 2021. List of prokaryotic names with stading in nomenclature. https://lpsn.dsmz.de/genus/listeria LPSN, 2021.

Lucke, F. K. (2000). Utilization of microbes to process and preserve meat. *Meat Science*, *56*(2), 105–115.

Lungu, B., Ricke, S. C., & Johnson, M. G. (2009). Growth, survival, proliferation and pathogenesis of *Listeria monocytogenes* under low oxygen or anaerobic conditions: A review. *Anaerobe*, *15*(1–2), 7–17.

Luppi, A., Bucci, G., Maini, P., & Rocourt, J. (1988). Ecological survey of *Listeria* in the Ferrara Area (Northern Italy). *Zentralblatt Fur Bakteriologie, Mikrobiologie, Und Hygiene*, *269*(2), 266–275.

Macaluso, G., Fiorenza, G., Gaglio, R., Mancuso, I., & Scatassa, M. L. (2016). In vitro evaluation of bacteriocin like inhibitory substances produced by lactic acid bacteria isolated during traditional sicilian cheese making. *Italian Journal of Food Safety*, *5*(1), 20–22.

MacGowan, A. P., Bowker, K., McLauchlin, J., Bennett, P. M., & Reeves, D. S. (1994). The occurrence and seasonal changes in the isolation of *Listeria* spp. in shop bought food stuffs, human faeces, sewage and soil from urban sources. *International Journal of Food Microbiology*, *21*(4), 325–334.

Magalhães, R., Almeida, G., Ferreira, V., Santos, I., Silva, J., Mendes, M. M., Pita, J., Mariano, G., Mâncio, I., Sousa, M. M., Farber, J., Pagotto, F., & Teixeira, P. (2015). Cheese-related listeriosis outbreak, Portugal, march 2009 to february 2012. *Eurosurveillance*, *20*(17), 21104.

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2009). Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *International Journal of Food Microbiology*, *132*(1), 24–32.

Martín, A., Córdoba, J. J., Aranda, E., Córdoba, M. G., & Asensio, M. A. (2006). Contribution of a selected fungal population to the volatile compounds on dry-cured ham. *International Journal of Food Microbiology*, *110*(1), 8–18.

Martín, Alberto, Colín, B., Aranda, E., Benito, M. J., & Córdoba, M. G. (2007). Characterization of *Micrococcaceae* isolated from Iberian dry-cured sausages. *Meat Science*, *75*(4), 696–708.

Martín, I., Rodríguez, A., Alía, A., Martínez-Blanco, M., Lozano-Ojalvo, D., & Córdoba, J. J. (2022). Control of *Listeria monocytogenes* growth and virulence in a traditional soft cheese model system based on lactic acid bacteria and a whey protein hydrolysate with antimicrobial activity. *International Journal of Food*

Microbiology, *361*, 109444.

Martinez-Rios, V., & Dalgaard, P. (2018). Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis. *Food Control*, *84*, 205–214.

Mataragas, M., Bellio, A., Rovetto, F., Astegiano, S., Greci, C., Hertel, C., Decastelli, L., & Cocolin, L. (2015). Quantification of persistence of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* during manufacture of Italian fermented sausages. *Food Control*, *47*, 552–559.

Mataragas, Marios, Rovetto, F., Bellio, A., Alessandria, V., Rantsiou, K., Decastelli, L., & Cocolin, L. (2015). Differential gene expression profiling of *Listeria monocytogenes* in cacciatore and felino salami to reveal potential stress resistance biomarkers. *Food Microbiology*, *46*, 408–417.

Mateus, T., Silva, J., Maia, R. L., & Teixeira, P. (2013). Listeriosis during Pregnancy: A Public Health Concern. *ISRN Obstetrics and Gynecology*, *2013*, 1–6.

Matle, I., Mbatha, K. R., & Madoroba, E. (2020). A review of *Listeria monocytogenes* from meat and meat products: Epidemiology, virulence factors, antimicrobial resistance and diagnosis.

Mayer, Z., Bagnara, A., Färber, P., & Geisen, R. (2003). Quantification of the copy number of *nor*-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. *International Journal of Food Microbiology*, *82*(2), 143–151.

McLauchlin, J., Mitchell, R. T., Smerdon, W. J., & Jewell, K. (2004). *Listeria monocytogene*s and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *International Journal of Food Microbiology*, *92*(1), 15–33.

Medved'ová, A., Koňuchová, M., Kvočiková, K., Hatalová, I., & Valík, L. (2020). Effect of lactic acid bacteria addition on the microbiological safety of Pasta-filata types of cheeses. *Frontiers in Microbiology*, *11*, 1–16.

Meloni, D. (2015). Presence of *Listeria monocytogenes* in mediterranean-style dry fermented sausages. *Foods*, *4*(4), 34–50.

Meloni, D. (2019). High-hydrostatic-pressure (HHP) processing technology as a novel control method for *Listeria monocytogenes* occurrence in mediterranean-style dry-fermented sausages. *Foods*, *8*(12).

Milicevic, B., Danilovic, B., Zdolec, N., Kozachinski, L., Dobranic, V., & Savic, D. (2014). Microbiota of the fermented sausages: Influence to product quality and safety. *Bulgarian Journal of Agricultural Science*, *20*(5), 1061–1078.

Milillo, S. R., Friedly, E. C., Saldivar, J. C., Muthaiyan, A., O'Bryan, C., Crandall, P. G., Johnson, M. G., & Ricke, S. C. (2012). A review of the ecology, genomics, and stress response of *Listeria innocua* and *Listeria monocytogenes*. *Critical Reviews in Food Science and Nutrition*, *52*(8), 712–725.

Misiou, O., van Nassau, T. J., Lenz, C. A., & Vogel, R. F. (2018). The preservation of *Listeria*-critical foods by a combination of endolysin and high hydrostatic pressure. *International Journal of Food Microbiology*, *266*, 355–362.

Moio, L., & Addeo, F. (1998). Grana Padano cheese aroma. *Journal of Dairy Research*, *65*(2), 317–333.

Mokoena, M. P. (2017). Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review. *Molecules*, *22*(8).

Mokoena, Mduduzi P., Omatola, C. A., & Olaniran, A. O. (2021). Applications of lactic acid bacteria and their bacteriocins against food spoilage microorganisms and foodborne pathogens. *Molecules*, *26*(22).

Mokoena, Mduduzi Paul. (2017). Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review. *Molecules*, *22*(8).

Montiel, R., Peirotén, Á., Ortiz, S., Bravo, D., Gaya, P., Martínez-Suárez, J. V., Tapiador, J., Nuñez, M., & Medina, M. (2020). Inactivation of *Listeria monocytogenes* during dry-cured ham processing. *International Journal of Food Microbiology*, *318*, 108469.

Murgia, M. A., Marongiu, A., Aponte, M., Blaiotta, G., Deiana, P., & Mangia, N. P. (2019). Impact of a selected *Debaryomyces hansenii* strain's inoculation on the

quality of Sardinian fermented sausages. *Food Research International*, *121*(March), 144–150.

Najjari, A., Boumaiza, M., Jaballah, S., Boudabous, A., & Ouzari, H. I. (2020). Application of isolated *Lactobacillus sakei* and *Staphylococcus xylosus* strains as a probiotic starter culture during the industrial manufacture of Tunisian dry-fermented sausages. *Food Science and Nutrition*, *8*(8), 4172–4184.

Nomura, M., Kobayashi, M., Narita, T., Kimoto-Nira, H., & Okamoto, T. (2006). Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. *Journal of Applied Microbiology*, *101*(2), 396–405.

Núñez-Montero, K., Leclercq, A., Moura, A., Vales, G., Peraza, J., Pizarro-Cerdá, J., & Lecuit, M. (2018). *Listeria costaricensis* sp. Nov. *International Journal of Systematic and Evolutionary Microbiology*, *68*(3), 844–850.

Núñez, F., Lara, M. S., Peromingo, B., Delgado, J., Sánchez-Montero, L., & Andrade, M. J. (2015). Selection and evaluation of *Debaryomyces hansenii* isolates as potential bioprotective agents against toxigenic penicillia in dry-fermented sausages. *Food Microbiology*, *46*, 114–120.

O'Connor, L., O'Leary, M., Leonard, N., Godinho, M., O'Reilly, C., Egan, J., & O'Mahony, R. (2010). The characterization of *Listeria* spp. isolated from food products and the food-processing environment. *Letters in Applied Microbiology*, *51*(5), 490–498.

Ochangco, H. S., Gamero, A., Smith, I. M., Christensen, J. E., Jespersen, L., & Arneborg, N. (2016). In vitro investigation of *Debaryomyces hansenii* strains for potential probiotic properties. *World Journal of Microbiology and Biotechnology*, *32*(9), 141.

Oh, Y. J., & Jung, D. S. (2015). Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented milletalcoholic beverage in Korea. *Lwt*, *63*(1), 437–444.

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018). Emergence of antibiotic resistance in *Listeria monocytogenes* isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Olesen, I., Thorsen, L., & Jespersen, L. (2010). Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. *International Journal of Food Microbiology*, *141*, S60–S68.

Olesen, I., Vogensen, F., & Jespersn, L. (2009). Gene Transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathogens and Disease*, *669–680*.

Ordoñez J.A. y De la Hoz L. (2001). Embutidos crudos curados. Tipos, fenómenos madurativos y alteraciones. Enciclopedia de la carne y los productos cárnicos. Ediciones Marín y Macías. Madrid, España.

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., & de Guía Córdoba, M. (2014). Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of "Torta del Casar" cheese. *International Journal of Dairy Technology*, *67*(3), 402–409.

Ordiales, Elena, Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, *33*(2), 448–454.

Ordiales, Elena, Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., & Córdoba, M. de G. (2013). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of Dairy Science*, *96*(9), 5477–5486.

Orsi, R. H., Bakker, H. C. de., & Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*, *301*(2), 79–96.

Orsi, R. H., & Wiedmann, M. (2016). Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. *Applied Microbiology and Biotechnology*, *100*(12), 5273–5287.

Ortolani, M. B. T., Yamazi, A. K., Moraes, P. M., Viçosa, G. N., & Nero, L. A. (2010). Microbiological quality and safety of raw milk and soft cheese and detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*. *Foodborne Pathogens*

and Disease, *7*(2), 175–180.

Osimani, A., & Clementi, F. (2016). The occurrence of *Listeria monocytogenes* in mass catering: An overview in the European Union. *International Journal of Hospitality Management*, *57*, 9–17.

Palavecino Prpich, N. Z., Castro, M. P., Cayré, M. E., Garro, O. A., & Vignolo, G. M. (2015). Autochthonous starter culture selection to keep traditions in the manufacture of dry sausages alive. *Annals of Microbiology*, *65*(3), 1709–1719.

Panebianco, F., Giarratana, F., Caridi, A., Sidari, R., De Bruno, A., & Giuffrida, A. (2021). Lactic acid bacteria isolated from traditional Italian dairy products: activity against *Listeria monocytogenes* and modelling of microbial competition in soft cheese. *Lwt*, *137*, 110446.

Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., Lemos, J. A., Linares, D. M., Ross, P., Stanton, C., Turroni, F., van Sinderen, D., Varmanen, P., Ventura, M., Zúñiga, M., Tsakalidou, E., & Kok, J. (2016). Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, *80*(3), 837–890.

Papagianni, M., & Anastasiadou, S. (2009). Pediocins: The bacteriocins of *Pediococci*. Sources, production, properties and applications. *Microbial Cell Factories*, *8*(1), 1–16.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E., & Kotzekidou, P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science*, *65*(2), 859–867.

Parada, J. L., Caron, C. R., Medeiros, A. B. P., & Soccol, C. R. (2007). Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. *Brazilian Archives of Biology and Technology*, *50*(3), 521–542.

Pavli, F. G., Argyri, A. A., Chorianopoulos, N. G., Nychas, G. J. E., & Tassou, C. C. (2020). Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. *Lwt*, *118*.

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M. P., & Xavier Malcata, F. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, *27*(3), 339–346.

Pérez-Baltar, A., Serrano, A., Bravo, D., Montiel, R., & Medina, M. (2019). Combined effect of high pressure processing with enterocins or thymol on the inactivation of *Listeria monocytogenes* and the characteristics of sliced dry-cured ham. *Food and Bioprocess Technology*, *12*(2), 288–297.

Perin, L. M., Miranda, R. O., Todorov, S. D., Franco, B. D. G. de M., & Nero, L. A. (2014). Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. *International Journal of Food Microbiology*, *185*, 121–126.

Peromingo, B., Andrade, M. J., Delgado, J., Sánchez-Montero, L., & Núñez, F. (2019). Biocontrol of aflatoxigenic *Aspergillus parasiticus* by native *Debaryomyces hansenii* in dry-cured meat products. *Food Microbiology*, 293, 1–6

Picozzi, C., Bonacina, G., Vigentini, I., & Foschino, R. (2010). Genetic diversity in Italian *Lactobacillus sanfranciscensis* strains assessed by multilocus sequence typing and pulsed-field gel electrophoresis analyses. *Microbiology*, *156*(7), 2035–2045.

Pieta, L., Escudero, F. L. G., Jacobus, A. P., Cheiran, K. P., Gross, J., Moya, M. L. E., Soares, G. L. G., Margis, R., Frazzon, A. P. G., & Frazzon, J. (2017). Comparative transcriptomic analysis of *Listeria monocytogenes* reveals upregulation of stress genes and downregulation of virulence genes in response to essential oil extracted from Baccharis psiadioides. *Annals of Microbiology*, *67*(7), 479–490.

Possas, A., Bonilla-Luque, O. M., & Valero, A. (2021). From cheese-making to consumption: Exploring the microbial safety of cheeses through predictive microbiology models. *Foods*, *10*(2).

Prpich, N. Z. P., Camprubí, G. E., Cayré, M. E., & Castro, M. P. (2021). Indigenous microbiota to leverage traditional dry sausage production. *International Journal of Food Science*, *2021*, 1–15.

Pushkareva, V. I., & Ermolaeva, S. A. (2010). *Listeria monocytogenes* virulence factor Listeriolysin O favors bacterial growth in co-culture with the ciliate Tetrahymena pyriformis, causes protozoan encystment and promotes bacterial survival inside

cysts. *BMC Microbiology*, *10*.

Qian, M., & Reineccius, G. (2002). Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. *Journal of Dairy Science*, *85*(6), 1362–1369.

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2011). Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology*, *150*(2–3), 81–94.

Quijada, N. M., De Filippis, F., Sanz, J. J., García-Fernández, M. del C., Rodríguez-Lázaro, D., Ercolini, D., & Hernández, M. (2018). Different *Lactobacillus* populations dominate in "Chorizo de León" manufacturing performed in different production plants. *Food Microbiology*, *70*, 94–102.

Radoshevich, L., & Cossart, P. (2018). *Listeria monocytogenes*: Towards a complete picture of its physiology and pathogenesis. *Nature Reviews Microbiology*, *16*(1), 32–46.

Rajkovic, A., Tomasevic, I., Smigic, N., Uyttendaele, M., Radovanovic, R., & Devlieghere, F. (2010). Pulsed UV light as an intervention strategy against *Listeria monocytogenes* and *Escherichia coli* O157:H7 on the surface of a meat slicing knife. *Journal of Food Engineering*, *100*(3), 446–451.

Ramos, B., Ferreira, V., Brandao, T. R. S., Teixeira, P., & Silva, C. L. M. (2016). Antilisterial active compound from lactic acid bacteria present on fresh iceberg lettuce. *Acta Alimentaria*, *45*(3), 416–426.

Rantsiou, K., Mataragas, M., Alessandria, V., & Cocolin, L. (2012). Expression of virulence genes of *Listeria monocytogenes* in food. *Journal of Food Safety*, *32*(2), 161–168.

Ray, R. C., Studies, E., & Joshi, V. (2014). Fermented foods: Past, present and future. In *Microorganisms and Fermentation of Traditional Foods* (1st Editio, p. 36).

Real Decreto 474/2014, de 13 de junio, por el que se aprueba la norma de calidad de derivados cárnicos.

REGLAMENTO (CE) nº 1491/2003 DE LA COMISIÓN de 25 de agosto de 2003 por el que se completa el Reglamento (CE) nº 2400/96 (Ficodindia del l'Etna, Monte Etna,

Colline di Romagna, Pretuziano delle Colline Teramane, Torta del Casar, Manzana de Girona o Poma de Girona)

Reglamento (CE) n° 1107/96 de la Comisión de 12 de junio de 1996 relativo al registro de las indicaciones geográficas y de las denominaciones de origen con arreglo al procedimiento establecido en el artículo 17 del Reglamento (CEE) N° 2081/92 con la rectificación publicada en el Diario Oficial de las CE el 13 de noviembre de 1996

Reglamento (CE) nº 2073/2005 de la Comisión de 15 de noviembre de 2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios. D. Of. la Unión Eur. 1–26.

Reglamento (CE) nº 205/2005 de la Comisión, de 4 de febrero de 2005, que completa el anexo del Reglamento (CE) nº 2400/96 en lo que atañe al registro de determinadas denominaciones en el Registro de Denominaciones de Origen Protegidas y de Indicaciones Geográficas Protegidas (Valdemone [DOP], Queso Ibores [DOP], Pera de Jumilla [DOP], Aceite de Terra Alta u Oli de Terra Alta [DOP], Sierra de Cádiz [DOP], Requeijão Serra da Estrela [DOP], Zafferano dell'Aquila [DOP], Zafferano di San Gimignano [DOP], Mantecadas de Astorga [IGP] y Pan de Cea [IGP]).

Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews*, *4*(2), 124–140.

Reynolds, A. E., Harrison, M. A., Rose-Morrow, R., & Lyon, C. E. (2001). Validation of dry cured ham process for control of pathogens. *Journal of Food Science*, *66*(9), 1373–1379.

Roberts, B. N., Chakravarty, D., Gardner, J. C., Ricke, S. C., & Donaldson, J. R. (2020). *Listeria monocytogenes* response to anaerobic environments. *Pathogens*, *9*(3), 1–9.

Rocha, P. R. D. A., Dalmasso, A., Grattarola, C., Casalone, C., Del Piero, F., Bottero, M. T., & Capucchio, M. T. (2013). Atypical cerebral listeriosis associated with *Listeria innocua* in a beef bull. *Research in Veterinary Science*, *94*(1), 111–114.

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J. A., & Pla, M. (2004). Quantitative Detection of *Listeria monocytogenes* and *Listeria innocua* by Real-Time PCR: assessment of *hly*, *iap*, and *lin02483* targets and AmpliFluor technology. *Applied and Environmental Microbiology*, *70*(3), 1366–

1377.

Rodríguez, A., Medina, Á., Córdoba, J. J., & Magan, N. (2014). The influence of salt (NaCl) on ochratoxin A biosynthetic genes, growth and ochratoxin A production by three strains of *Penicillium nordicum* on a dry-cured ham-based medium. *International Journal of Food Microbiology*, *178*, 113–119.

Rodriguez, A., Rodriguez, M., Córdoba, J. J., & Andrade, M. J. (2015). Design of Primers and Probes for Quantitative Real-Time PCR Methods. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 1275, pp. 31–56).

Rudolf, M., & Scherer, S. (2001). High incidence of *Listeria monocytogenes* in European red smear cheese. *International Journal of Food Microbiology*, *63*(1–2), 91–98.

Sadeghi-Mehr, A., Lautenschlaeger, R., & Drusch, S. (2016). Behavior of *Salmonella* spp. and *Listeria monocytogenes* throughout the manufacture and shelf-life of dry-cured formed ham. *Food Control*, *64*, 22–28.

Salas, L. M., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal microbial agents for food biopreservation—a review. *Microorganisms*, *5*(3), 1–35.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2º Edition).

Sánchez-juanes, F., Teixeira-martín, V., González-buitrago, J. M., Velázquez, E., & Flores-félix, J. D. (2020). Identification of species and subspecies of lactic acid bacteria present in spanish cheeses type “torta” by maldi-tof ms and phes gene analyses. *Microorganisms*, *8*(2).

Sánchez Mainar, M., Stavropoulou, D. A., & Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. *International Journal of Food Microbiology*, *247*, 24–37.

Sanjuán, E., Millán, R., Saavedra, P., Carmona, M. A., Gómez, R., & Fernández-Salguero, J. (2002). Influence of animal and vegetable rennet on the physicochemical characteristics of Los Pedroches cheese during ripening. *Food Chemistry*, *78*(3), 281–289.

Santa, O. R. D., Alvarez, D. C., Santa, H. S. D., Zanette, C. M., Freitas, R. J. S. de, Macedo, R. E. F. de, & Terra, N. N. (2012). Microbiota of sausages obtained by spontaneous fermentation produced in the South of Brazil. *Food Science and Technology*, *32*(4), 653–660.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-Major pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15.

Scatassa, M. L., Gaglio, R., Cardamone, C., Macaluso, G., Arcuri, L., Todaro, M., & Mancuso, I. (2017). Anti-listeria activity of lactic acid bacteria in two traditional sicilian cheeses. *Italian Journal of Food Safety*, *6*(1), 13–17.

Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, *166*(2), 368–379.

Schwartz, R. H., Eaton, J., Bower, B. D., & Aynsley-Green, A. (1989). Ketogenic Diets in the T Epilepsy : Short-Term Clinical E. *Developmental Medicine and Child Neurology*, *31*, 149–151.

Sebranek, J. G. (2007). Natural and organic cured meat products: Regulatory, manufacturing, marketing, quality and safety issues. *American Meat Science Association*, 1–15.

Semedo, T., Santos, M. A., Martins, P., Silva Lopes, M. F., Figueiredo Marques, J. J., Tenreiro, R., & Barreto Crespo, M. T. (2003). Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the *cyl* operon in enterococci. *Journal of Clinical Microbiology*, *41*(6), 2569–2576.

Senay, T. E., Ferrell, J. L., Garrett, F. G., Albrecht, T. M., Cho, J., Alexander, K. L., Myers-morales, T., Grothaus, O. F., & Orazio, E. F. D. (2020). *Neurotropic Lineage III Strains of Listeria monocytogenes Disseminate to the Brain without Reaching High Titer in the Blood*. *5*(5), 1–20.

Sengun, I. Y., Yaman, D. B., & Gonul, S. a. (2008). Mycotoxins and mould contamination in cheese: a review. *World Mycotoxin Journal*, *1*(3), 291–298.

Simoncini, N., Virgili, R., Spadola, G., & Battilani, P. (2014). Autochthonous yeasts as

potential biocontrol agents in dry-cured meat products. *Food Control*, *46*, 160–167.

Sip, A., Wieckowicz, M., Olejnik-Schmidt, A., & Grajek, W. (2012). Anti-*Listeria* activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. *Food Control*, *26*(1), 117–124.

Soltani, S., Hammami, R., Cotter, P. D., Rebuffat, S., Said, L. Ben, Gaudreau, H., Bédard, F., Biron, E., Drider, D., & Fliss, I. (2021). Bacteriocins as a new generation of antimicrobials: Toxicity aspects and regulations. *FEMS Microbiology Reviews*, *45*(1), 1–24.

Sue, D., Fink, D., Wiedmann, M., & Boor, K. J. (2004). σB-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. *Microbiology*, *150*(11), 3843–3855.

Taddei, R., Giacometti, F., Bardasi, L., Bonilauri, P., Ramini, M., Fontana, M. C., Bassi, P., Castagnini, S., Ceredi, F., Pelliconi, M. F., Serraino, A., Tomasell, F., Piva, S., Mondo, E., & Merialdi, G. (2020). Effect of production process and high-pressure processing on viability of *Listeria innocua* in traditional Italian dry-cured coppa. *Italian Journal of Food Safety*, *9*(2), 104–109.

Talon, R., & Leroy, S. (2014). Fermented foods: Fermented meat products and the role of starter cultures. In *Encyclopedia of Food Microbiology: Second Edition* (Second Edi, Vol. 1). Elsevier.

Terentjeva, M., Šteingolde, Ž., Meistere, I., Elferts, D., Avsejenko, J., Streikiša, M., Gradovska, S., Alksne, L., Ķibilds, J., & Bērziņš, A. (2021). Prevalence, genetic diversity and factors associated with distribution of *Listeria monocytogenes* and other *Listeria* spp. In cattle farms in Latvia. *Pathogens*, *10*(7).

Tiago, I., Teixeira, I., Silva, S., Chung, P., Veríssimo, A., & Manaia, C. M. (2004). Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-ε-caprolactones. *Current Microbiology*, *49*(6), 407–414.

Todorov, S. D., & Dicks, L. M. T. (2005). Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *Journal of Basic Microbiology*, *45*(4), 312–322.

Todorov, S. D., Rachman, C., Fourrier, A., Dicks, L. M. T., van Reenen, C. A., Prévost, H., & Dousset, X. (2011). Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe*, *17*(1), 23–31.

Tosukhowong, A., Zendo, T., Visessanguan, W., Roytrakul, S., Pumpuang, L., Jaresitthikunchai, J., & Sonomoto, K. (2012). Garvieacin Q, a novel class II bacteriocin from *Lactococcus garvieae* BCC 43578. *Applied and Environmental Microbiology*, *78*(5), 1619–1623.

Tumbarski, Y., Lante, A., & Krastanov, A. (2018). Immobilization of bacteriocins from lactic acid bacteria and possibilities for application in food biopreservation. *The Open Biotechnology Journal*, *12*(1), 25–32.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), 1–12.

Urso, R., Comi, G., & Cocolin, L. (2006). Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. *Systematic and Applied Microbiology*, *29*(8), 671–680.

Urso, R., Rantsiou, K., Cantoni, C., Comi, G., & Cocolin, L. (2006). Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. *International Journal of Food Microbiology*, *110*(3), 232–239.

USDA-FSIS. (2012). High pressure processing (HPP) and inspection program personnel (IPP) verification responsibilities. Directive 6120.2. Available in: https://www.fsis.usda.gov/wps/wcm/connect/a64961fa-ed6f-44d1-b637-62232a18f9 98/6120.2.pdf?MOD=AJPERES

Valledor, S. J. D., Dioso, C. M., Bucheli, J. E. V., Park, Y. J., Suh, D. H., Jung, E. S., Kim, B., Holzapfel, W. H., & Todorov, S. D. (2022). Characterization and safety evaluation of two beneficial, enterocin-producing *Enterococcus faecium* strains isolated from kimchi, a Korean fermented cabbage. *Food Microbiology*, *102*, 103886.

Van Reenen, C. A., Dicks, L. M. T., & Chikindas, M. L. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by

Lactobacillus plantarum. *Journal of Applied Microbiology*, *84*(6), 1131–1137.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., & Goossens, H. (2004). Development of a multiplex PCR for the detection of *asaI*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among european hospital isolates of *Enterococcus faecium*. *Journal of Clinical Microbiology*, *42*(10), 4473–4479.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, *14*(3), 584–640.

Vermeulen, A., Gysemans, K. P. M., Bernaerts, K., Geeraerd, A. H., Van Impe, J. F., Debevere, J., & Devlieghere, F. (2007). Influence of pH, water activity and acetic acid concentration on *Listeria monocytogenes* at 7 °C: data collection for the development of a growth/no growth model. *International Journal of Food Microbiology*, *114*(3), 332–341.

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Frontiers in Microbiology*, *10*(57).

Virgili, R., Simoncini, N., Toscani, T., Camardo Leggieri, M., Formenti, S., & Battilani, P. (2012). Biocontrol of *Penicillium nordicum* growth and ochratoxin A production by native yeasts of dry cured ham. *Toxins*, *4*(12), 68–82.

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H. W., Perrone, G., Seifert, K. A., Varga, J., Yaguchi, T., & Samson, R. A. (2014). Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology*, *78*(1), 343–371.

Wang, Y., Qin, Y., Zhang, Y., Wu, R., & Li, P. (2019). Antibacterial mechanism of plantaricin LPL-1, a novel class IIa bacteriocin against *Listeria monocytogenes*. *Food Control*, *97*(17), 87–93.

Wang, Z., Tao, X., Liu, S., Zhao, Y., & Yang, X. (2021). An update review on *Listeria* infection in pregnancy. *Infection and Drug Resistance*, *14*, 1967–1978.

Ward, T. J., Ducey, T. F., Usgaard, T., Dunn, K. A., & Bielawski, J. P. (2008). Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. *Applied and Environmental Microbiology*, *74*(24), 7629–7642.

Warriner, K., & Namvar, A. (2009). What is the hysteria with *Listeria*? *Trends in Food Science and Technology*, *20*(6–7), 245–254.

Wemmenhove, E., van Valenberg, H. J. F., van Hooijdonk, A. C. M., Wells-Bennik, M. H. J., & Zwietering, M. H. (2018). Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid. *Food Control*, *84*(1662), 413–418.

Xie, Y., An, H., Hao, Y., Qin, Q., Huang, Y., Luo, Y., & Zhang, L. (2011). Characterization of an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. *Food Control*, *22*(7), 1027–1031.

Yang, S. C., Lin, C. H., Sung, C. T., & Fang, J. Y. (2014). Antibacterial activities of bacteriocins: Application in foods and pharmaceuticals. *Frontiers in Microbiology*, *5*, 1–10.

Yap, P.-C., MatRahim, N.-A., AbuBakar, S., & Lee, H. Y. (2021). Antilisterial potential of lactic acid bacteria in eliminating *Listeria monocytogenes* in host and ready-to-eat food application. *Microbiology Research*, *12*(1), 234–257.

Ye, K., Zhang, X., Huang, Y., Liu, J., Liu, M., & Zhou, G. (2018). Bacteriocinogenic *Enterococcus faecium* inhibits the virulence property of *Listeria monocytogenes*. *LWT - Food Science and Technology*, *89*(October 2017), 87–92.

Yoon, Y., Lee, S., & Choi, K. H. (2016). Microbial benefits and risks of raw milk cheese. *Food Control*, *63*, 201–215.

Yu, T., Jiang, X., Zhang, Y., Ji, S., Gao, W., & Shi, L. (2018). Effect of Benzalkonium Chloride adaptation on sensitivity to antimicrobial agents and tolerance to environmental stresses in *Listeria monocytogenes*. *Frontiers in Microbiology*, *9*.

Zacharof, M. P., & Lovitt, R. W. (2012). Bacteriocins produced by lactic acid bacteria a review article. *APCBEE Procedia*, *2*, 50–56.

Zagorec, M., & Champomier-Vergès, M.-C. (2017). *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms*, *5*(3), 56.

Zhang, X., Wang, S., Chen, X., & Qu, C. (2021). Review controlling *Listeria monocytogenes* in ready-to-eat meat and poultry products: An overview of outbreaks, current legislations, challenges, and future prospects. *Trends in Food Science and Technology*, *116*, 24–35.

Zhao, Y., Tu, K., Shao, X., Jing, W., & Su, Z. (2008). Effects of the yeast *Pichia guilliermondii* against *Rhizopus nigricans* on tomato fruit. *Postharvest Biology and Technology*, *49*(1), 113–120.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus beijerinck* 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858.

VIII. RESUMEN

VIII.1. RESUMEN

Los cambios en el estilo de vida de la población han incrementado el consumo de productos listos para el consumo (RTE), entre los que se encuentran los alimentos madurados tradicionales como embutidos curado-madurados y quesos. *Listeria monocytogenes* es el microorganismo patógeno más preocupante en este tipo de productos por su ubicuidad y patogenicidad. Esta bacteria patógena provoca listeriosis que, aunque tiene una incidencia baja, presenta elevadas tasas de mortalidad y hospitalización. Por todo ello, el control de *L. monocytogenes* en alimentos madurados RTE es un objetivo prioritario para las industrias y las autoridades sanitarias. Es necesario pues, el desarrollo de estrategias de control de esta bacteria patógena en embutidos curado-madurados y quesos madurados. Entre estas estrategias están los protocolos de biocontrol basados en la utilización de cultivos protectores de bacterias ácido-lácticas e hidrolizados proteicos. Los alimentos madurados tradicionales son una fuente importante de BAL autóctonas, aunque no todas tienen capacidad para inhibir a *L. monocytogenes*, por lo que es necesario su evaluación y selección antes de proponer la utilización de determinadas cepas activas como cultivos protectores. Así mismo, se ha encontrado recientemente que determinados hidrolizados proteícos procedentes de subproductos alimentarios como por ejemplo el lactosuero pueden tener efecto antimicrobiano y podrían ser también de utilidad en control de esta bacteria patógena, tras su consiguiente evaluación. La utilización de estas estrategias de biocontrol requieren además de estudios de posibles efectos en las características sensoriales de los productos.

Los objetivos de esta Tesis Doctoral consistieron en: a) aislar bacterias ácido-lácticas de embutidos curado-madurados y quesos madurados, b) seleccionar bacterias ácido-lácticas con actividad anti-*L. monocytogenes* en medio de cultivo BHI, c) seleccionar las

BAL con actividad anti-*L. monocytogenes* en medios elaborados con queso y salchichón, d) determinar el efecto de las bacterias ácido-lácticas utilizadas como estrategia de biocontrol frente al crecimiento y expresión génica de *L. monocytogenes* en embutidos curado-madurados y quesos madurados e) evaluar el efecto de un hidrolizado de suero lácteo frente a *L. monocytogenes*, f) evaluar el efecto de los tratamientos de biocontrol con bacterias ácido-lácticas sobre las propiedades sensoriales de los embutidos curado-madurados.

En esta Tesis Doctoral, se aislaron 371 cepas de BAL, de las cuales 182 pertenecieron a embutidos y 189 pertenecieron principalmente a quesos. Se realizó una primera selección de aislados con actividad anti-*L. monocytogenes* mediante el método de difusión en agar. Como resultados de esta primera selección se obtuvo que 84 (22,64%) de los 371 aislados de BAL mostraron halos de inhibición de *L. monocytogenes* de al menos 2 mm. Los 84 aislados de BAL seleccionados con actividad anti-*L. monocytogenes* fueron identificados mediante la secuenciación de la subunidad ribosomal ARNr 16S y posterior análisis de dichas secuencias. Se determinó que los distintos aislados pertenecían a las especies *E. durans*, *E. faecium*, *E. hirae*, *Le. pseudomesenteroides*, *Le. mesenteroides*, *Lc. casei*, *Lp. plantarum*, *Lc. paracasei*, *Ll. sakei*, *Lco. garviae* y *Lco. lactis*. Se realizó una segunda selección de cepas con actividad antagonista frente a *L. monocytogenes* mediante ensayo de co-inoculación con este patógeno en modelos alimentarios que simulan la composición, a_w y pH de los embutidos curado-madurados y de los quesos madurados. Sólo 5 cepas *Lc. casei* 31, *Lc. casei* 116, *Lco. garviae* 151, *Lco. garviae* 156 y *Lc. paracasei* 185, provocaron una reducción de *L. monocytogenes* de hasta aproximadamente 2,35 log UFC/cm^2 en el medio de cultivo elaborado con queso "Torta del Casar". Lo mismo ocurrió en el ensayo en que se evaluó la capacidad antagonista de las cepas de BAL aisladas de industrias de embutidos curado-madurados, ya que sólo en

5 de las 32 cepas de BAL ensayadas (*Lc. paracasei* 13, *E. faecium* 188, *Ll. sakei* 197, *Ll. sakei* 204 y *Ll. sakei* 205) mostraron una reducción importante de los recuentos de *L. monocytogenes* de hasta 2,5 log UFC/cm^2. En estas cepas se evaluó la presencia de genes que codifican la producción de bacteriocinas conocidas (nisina, lacticina 481, lactococcina 972, G y Q, Ay B, brevicina, plantaracina A, EF, NC, S y W, pediocina A). Todas ellas mostraron amplicones del tamaño esperado para al menos uno de los genes que codifican la producción de bacteriocinas. Considerando la actividad anti-*L. monocytogenes* en los modelos de salchichón y de queso y la evaluación de genes que codifican para la síntesis de bacteriocinas se seleccionaron las 6 cepas más activas, *Ll. sakei* 31, *Lc. casei* 116 y *Lco. garviae* 151 procedentes de quesos y *Ll. sakei* 197, 204 y 205 aisladas de industrias cárnicas. Estas cepas están disponibles para su utilización en productos madurados. Para controlar la implantación de las cepas seleccionadas se propone la utilización de la secuenciación 16S ARNr para la caracterización a nivel de especie y el análisis de PFGE con enzimas de restricción *SgsI* y *NotI* para la diferenciación a nivel de cepa. También se evaluaron en el modelo de queso la actividad anti-*L. monocytogenes* de un hidrolizado de lactosuero. Este hidrolizado proteico presentó actividad antimicrobiana limitada y además incrementó la expresión de genes de virulencia de *L. monocytogenes* por lo que no es adecuado como estrategia de control de esta bacteria patógena. En esta tesis doctoral se desarrolló un método de qPCR para evaluar cambios de expresión del gen de virulencia *inlA* de *L. monocytogenes*, de gran utilidad para detectar un aumento en la expresión de este gen, que pueden producirse en cepas resistentes de esta bacteria patógena en condiciones de acidez de embutidos curado-madurados y quesos madurados, lo que podría suponer un incremento de su patogenicidad.

Las bacterias ácido-lácticas seleccionadas *Ll. sakei* 205 aislada de embutidos y *Lc. casei* 116 procedente de quesos, fueron finalmente caracterizadas resultando ser cepas inocuas, sin resistencia a antibióticos y con capacidad demostrada para reducir *L. monocytogenes* tanto en sistemas modelo de alimentos madurados como durante la maduración de estos productos, por lo que son propuestas como cultivos protectores. Estas cepas no provocan modificación negativa alguna en las características sensoriales de los alimentos madurados y pueden favorecer el desarrollo del sabor y aroma característicos de embutidos curado-madurados y quesos madurados.

VIII.2. ABSTRACT

Changes in the lifestyle of the population have increased the consumption of ready-to-eat (RTE) products, among which are traditional ripened foods such as dry-cured fermented sausages and cheeses. *Listeria monocytogenes* is the most worrying pathogenic microorganism in this type of product due to its ubiquity and pathogenicity. This pathogenic bacterium causes listeriosis which, although it has a low incidence, has high mortality and hospitalization rates. For all these reasons, the control of *L. monocytogenes* in RTE-ripened foods is a priority objective for industries and health authorities. Therefore, it is necessary to develop strategies to control this pathogenic bacterium in dry-cured fermented sausages and ripened cheeses. Among these strategies are biocontrol protocols based on the use of protective cultures of LAB and protein hydrolysates. Traditional ripened foods are an important source of autochthonous LAB, although not all of them have the capacity to inhibit *L. monocytogenes*, so their evaluation and selection are necessary before proposing the use of certain active strains as protective cultures. Likewise, it has recently been found that certain protein hydrolysates from food by-products, such as whey, for example, can have an antimicrobial effect and could also be useful in controlling this pathogenic bacterium, after subsequent evaluation. The use of these strategies of biocontrol also requires studies of possible effects on the sensory characteristics of the products. The objectives of this Doctoral Thesis were: a) to isolate LAB from dry-cured fermented sausages and ripened cheeses, b) to select lactic acid bacteria with anti-*L.monocytogenes* activity in BHI culture medium, c) to select LAB with anti-*L. monocytogenes* activity in culture media made with cheeses and dry-cured fermented sausages, d) to determine the effect of lactic-acid bacteria used as a biocontrol strategy against the growth and gene expression of *L. monocytogenes* in dry-cured fermented sausages and ripened cheeses and e) to evaluate the effect of a whey

hydrolyzate against *L.monocytogenes*, f) to evaluate the effect of biocontrol treatments with LAB on the sensory properties of dry-cured sausages.

In this Doctoral Thesis, 371 strains were LAB isolated, of which 182 belonged to sausages and 189 belonged mainly to cheese. The first selection of isolates with anti-*L. monocytogenes* was carried out by the agar diffusion method. As a result of this selection, it was obtained that 84 (22.64%) of the 371 LAB isolates showed inhibition halos of *L. monocytogenes* of at least 2 mm. The 84 selected LAB isolates with anti-*L. monocytogenes* were identified by sequencing the 16S rRNA ribosomal subunit and subsequent analysis of these sequences. It was determined that the different isolates belonged to the species *E. durans*, *E. faecium*, *E. hirae*, *Le. pseudomesenteroides*, *Le. mesenteroides*, *Lc. casei*, *Lp. plantarum*, *Lc. paracasei*, *Ll. sakei*, *Lco. garviae* and *Lco. lactis*. The second selection of strains with antagonistic activity against *L. monocytogenes* was carried out by co-inoculation test with this pathogen in food models that simulate the composition, a_w and pH of dry-cured fermented sausages and ripened cheeses. Only the 5 strains *Lc. casei* 31, *Lc. casei* 116, *Lco. garviae* 151, *Lco. garviae* 156 and *Lc. paracasei* 185, caused a reduction of *L. monocytogenes* of up to approximately 2.35 log CFU/cm^2 in the culture medium made with "Torta del Casar" cheese. Similar results were obtained in the assay in which the antagonistic capacity of the LAB strains isolated from dry-cured fermented sausage industries was evaluated, since only 5 of the 32 BAL strains tested (*Lc. paracasei* 13, *E. faecium* 188, *Ll. sakei* 197, *Ll. sakei* 204 and *Ll. sakei* 205), showed a significant reduction in *L. monocytogenes* counts down to 2.5 log CFU/cm^2. In these strains, the presence of genes encoding the production of known bacteriocins (nisin, lacticin 481, lactococcin 972, G and Q, Ay B, brevicin, plantarazin A, EF, NC, S and W, pediocin A) was evaluated. All of them showed amplicons of the expected size for at least one of the genes encoding the production of bacteriocins. Considering the anti-*L.*

monocytogenes in the sausage and cheese models and the evaluation of genes that code for the synthesis of bacteriocins, the 6 most active strains were selected, *Ll. sakei* 31, *Lc. casei* 116 and *Lco. garviae* 151 from cheeses and *Ll. sakei* 197, 204 and 205 isolated from meat industries. These strains are available for its use in ripened product. To control the implantation of the selected strains, the use of 16S rRNA sequencing is proposed for characterization at the species level and PFGE analysis with restriction enzymes *SgsI* and *NotI f*or differentiation at the strain level. Anti-*L. monocytogenes* activity was also evaluated in the cheese model from a whey hydrolyzate. This protein hydrolyzate presented limited antimicrobial activity and also increased the expression of virulence genes of L. monocytogenes, so it is not suitable as a control strategy for this pathogenic bacterium. In this Doctoral Thesis, a qPCR method was developed to evaluate changes in the expression of the virulence gene *inlA* of *L. monocytogenes*, which is very useful for detecting an increase in the expression of this gene, which can occur in resistant strains of this pathogenic bacterium in acidity conditions of dry-cured sausages and ripened cheeses, which could lead to an increase in their pathogenicity.

The selected lactic acid bacteria *Ll. sakei* 205 isolated from sausages and *Lc. casei* 116 from cheeses, were finally characterized, since they were innocuous strains, without resistance to antibiotics and with demonstrated capacity to reduce *L. monocytogenes* counts both in model systems of fermented foods and during the ripening of these products, for which they are proposed as protective cultures. These strains do not cause any negative modification in the sensory characteristics of ripened foods and can favor the development of the characteristic flavor of dry-cured sausages and ripened cheeses.

IX. ANEXOS

ANEXO 1. OTRAS PUBLICACIONES DERIVADAS DE LA TESIS DOCTORAL

ARTÍCULOS NACIONALES

1.AUTORES: **Martín, I.,** Rodríguez, A., Gómez, F.M., Pulido, J.C., Córdoba, J.J.

TÍTULO: Control de *Listeria monocytogenes* en embutidos curado-madurados con bacterias ácido-lácticas seleccionadas

REF. REVISTA/LIBRO: Eurocarne

CAPÍTULOS DE LIBRO NACIONALES

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TÍTULO: Efecto del procesado de embutidos curado-madurados y bacterias lácticas seleccionadas en el control de *Listeria monocytogenes.*

REF. REVISTA/LIBRO: Productos cárnicos seguros, nutritivos y saludables. Editado por: Núñez, M., Jiménez-Colmenero, F., Córdoba, J.J.