Evaluación del crecimiento de Salmonella enterica y Listeria monocytogenes en pera mínimamente procesada e influencia de las condiciones de conservación en el potencial patogénico de Listeria monocytogenes

Pilar Colás Medà

Universitat de Lleida

TESIS DOCTORAL

Evaluación del crecimiento de *Salmonella enterica* y *Listeria monocytogenes* en pera mínimamente procesada e influencia de las condiciones de conservación en el potencial patogénico de *Listeria monocytogenes*

Pilar Colás Medà

Memoria presentada para optar al grado de Doctor por la Universitat de Lleida Programa de **Doctorado en Ciencia y Tecnología Agraria y Alimentaria**

Directoras
Dra. Maribel Abadias Seró
Dra. Inmaculada Viñas Almenar

Lleida, Noviembre de 2016

Los estudios presentados en esta tesis doctoral se realizaron en el laboratorio de Microbiología de productos vegetales del Fruitcentre-IRTA de Lleida.

Para la realización de este trabajo se recibió apoyo económico del Ministerio de Economía y Competitividad mediante el proyecto nacional AGL-2012-38671, del Fondo Europeo de Desarrollo Regional y de la Universitat de Lleida, Grupo Alimentario Citrus y Banco Santander mediante la concesión de una beca pre-doctoral enmarcada dentro del plan UdL Impuls (2011). También se recibió apoyo económico de la Universitat de Lleida para la realización de una estancia pre-doctoral en el laboratorio del "Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM)" en Bilthoven (Holanda).

Índice

RESUMEN/ RESUM/ SUMMARY

RESUMEN

En los últimos años se ha visto incrementada la demanda de alimentos listos para el consumo que sean nutricionalmente equilibrados, con bajo aporte calórico y alto aporte de compuestos con capacidad antioxidante. Esto se refleja en el incremento de ventas de los vegetales mínimamente procesados. En la actualidad, existe en el mercado un mayor número de productos basados en hortalizas mínimamente procesadas que en frutas mínimamente procesadas.

En primer lugar, en esta tesis se desarrolló un producto mínimamente procesado a base de pera, debido a que Lleida es la mayor zona productora de pera del Estado. El procesado mínimo de fruta consta de unas etapas básicas. Sin embargo, existen numerosos factores que pueden afectar a la calidad del producto final. Por ello, es necesaria una etapa previa de desarrollo del producto, para establecer las condiciones óptimas de materia prima, proceso y conservación. Se evaluó la aptitud al procesado de cuatro variedades de pera producidas en la zona, "Conference", "Ercolini", "Flor de Invierno" y "Passa-Crassana" (*Capítulo 1*). Se concluyó que bajo las condiciones ensayadas, la pera de la variedad "Conference" presentó los mejores atributos para el procesado mínimo. El pardeamiento enzimático superficial de la pera mínimamente procesada es una alteración no deseada y por este motivo también se ensayaron distintas alternativas para reducir su incidencia. Los resultados obtenidos mostraron que un tratamiento antioxidante-estabilizante de la textura compuesto por ascorbato de calcio al 2% y cloruro de calcio al 1% mantuvo el color y textura de la pera "Conference" mínimamente procesada conservada en atmosfera modificada pasiva a 5 °C durante 14 días. La calidad a lo largo de la vida útil de la pera mínimamente procesada obtenida en el proceso de desarrollo de esta tesis se evaluó bajo condiciones comerciales simuladas (8 días a 4 °C) y en condiciones simuladas de rotura de la cadena de frío (3 días a 4 °C más 5 días a 8 °C).

En segundo lugar, se evaluaron aspectos de calidad microbiológica de la pera mínimamente procesada. Se evaluó la capacidad de supervivencia/crecimiento de *Salmonella enterica* subsp. Enteritidis y *Listeria monocytogenes* en pera "Conference" mínimamente procesada tratada con ascorbato de calcio al 2% y cloruro de calcio al 1% envasada en atmosfera modificada pasiva y conservada bajo condiciones comerciales simuladas (4 °C) y en condiciones simuladas de rotura de la cadena de frío (*Capítulo 2*). El tiempo y las temperaturas de conservación estudiadas inhibieron el crecimiento de las cepas evaluadas de *S. enterica* en pera mínimamente procesada, mientras que la población de las cepas

de *L. monocytogenes* estudiadas se incrementó al aumentar el tiempo de conservación. En base a estos resultados, se seleccionó *L. monocytogenes* como patógeno de mayor riesgo y se empleó en los posteriores estudios. Se evaluó el efecto del estado de madurez de la pera en el momento del procesado en el comportamiento de esta bacteria durante su conservación (*Capítulo 3)*. Se observó que el estado de madurez tuvo un efecto en la capacidad de crecimiento de *L. monocytogenes* cuando la pera se conservó en aire a 10 y 20 °C. A estas temperaturas el patógeno presentó un mayor potencial de crecimiento en pera procesada en estado maduro (31-42 N) o sobremaduro (< 31 N) que en el parcialmente maduro (43-53 N). En general, el potencial de crecimiento de *L. monocytogenes* en pera se incrementó al aumentar la temperatura de conservación.

Se evaluó si la supervivencia de *L. monocytogenes* después de someterse a una simulación gastrointestinal se podía ver afectada en función de la matriz (pera parcialmente madura y melón mínimamente procesados) y temperatura de conservación (*Capítulo 4*). En pera y melón inoculados y conservados a 1 °C se observaron las menores reducciones de población después de la simulación gastrointestinal. Cuando pera y melón inoculados con *L. monocytogenes* se conservaron a 10 °C, la capacidad de supervivencia a la simulación gastrointestinal de la bacteria se redujo al incrementar el tiempo de conservación. La capacidad de supervivencia a la simulación gastrointestinal de *L. monocytogenes* en melón después de la conservación no refrigerada (20 °C) se redujo con el tiempo de conservación, mientras que en pera la bacteria presentó la misma capacidad de supervivencia a lo largo del tiempo. El crecimiento de *L. monocytogenes* en pera mínimamente procesada pudo representar un estrés subletal para la bacteria que ocasionó que se adaptara al entorno y presentara una mayor resistencia frente a un estrés letal posterior como el de la fase gástrica de la simulación gastrointestinal.

Si la matriz ha podido ocasionar en *L. monocytogenes* cambios fisiológicos, estos cambios también podrían afectar el potencial patogénico de la bacteria, incrementando el riesgo de ocasionar una listeriosis. Por ello, se determinó si el tiempo de conservación y la matriz (pera y melón mínimamente procesados) podían tener un efecto en la supervivencia de *L. monocytogenes* después de la simulación gastrointestinal y en la posterior habilidad para adherirse e invadir a las células Caco-2 (*Capítulo 5*). En el momento de la inoculación de pera y melón, y después de la simulación gastrointestinal, *L. monocytogenes* en pera presentó una mayor capacidad de adhesión sobre células Caco-2 que la bacteria en melón. Sin embargo, la capacidad de invasión de la bacteria fue la misma en ambas matrices.

L. monocytogenes crecida en pera y melón y expuesta a la simulación gastrointestinal, presentó una mayor capacidad de invasión a los 2 días que a tiempo inicial o después de 7 días de conservación.

Finalmente, se evaluó el potencial de virulencia que presentaban 53 cepas de *L. monocytogenes* de distintos orígenes y serotipos (*Capítulo 6*). Se evaluaron características fenotípicas como la supervivencia al estrés gástrico, la capacidad de crecimiento y la capacidad de invasión, a la vez que se secuenciaron tres genes relacionados con la capacidad de virulencia (*inlA* y *prfA*) y el estrés (*sigB*) de *L. monocytogenes*, todos ellos relacionados con el riesgo de causar listeriosis. Se concluyó que las cepas de *L. monocytogenes* estudiadas del serotipo 4b de ambos orígenes (humano y alimentario) presentaron una mayor capacidad de invasión sobre células Caco-2 pero una menor capacidad de crecimiento. Ninguna de las cepas 4b presentó mutaciones en la secuencia de nucleótidos del gen *inlA* que codificaran por un codón de terminación prematuro ("premature stop codón", PMSC). De forma contraria, las cepas de *L. monocytogenes* estudiadas del serotipo 1/2c presentaron una elevada capacidad de crecimiento pero una reducida capacidad de invasión sobre células Caco-2. En las cepas de los serotipos 1/2a y 1/2c sí se observaron mutaciones PMSC en la secuencia de *inlA*. Se pudo relacionar una menor capacidad de invasión sobre las células Caco-2 con la presencia de mutaciones PMSC en el gen *inlA*.

En el producto de pera mínimamente procesada estudiado en esta tesis, que no recibe ningún tratamiento posterior antes de su consumo, en el que *L. monocytogenes* puede crecer en atmosfera modificada, en presencia de antioxidantes, a temperaturas de refrigeración (incluso 1 °C), que presenta una elevada supervivencia al paso gastrointestinal y con una capacidad de invasión destacable, se hace indispensable prevenir la contaminación, desde el campo hasta el momento de consumo.

RESUM

En els últims anys, la demanda d'aliments llestos per ser consumits que siguin nutricionalment equilibrats, amb un baix contingut calòric i amb un alt contingut en compostos amb capacitat antioxidant s'ha vist incrementada. Aquesta tendència s'ha vist reflectida en l'increment de les vendes del vegetals mínimament processats. En la actualitat, existeix en el mercat un major nombre de productes compostos per hortalisses mínimament processades que per fruites mínimament processades.

En primer lloc, en aquesta tesi es va desenvolupar un producte mínimament processat a base de pera, degut a que Lleida és la major zona productora de pera de l'Estat. El processat mínim de fruita consta d'unes etapes bàsiques. Però existeixen nombrosos factors que poden afectar a la qualitat del producte final. Per tot això, es necessària una etapa prèvia de desenvolupament del producte, per establir les condicions òptimes de la matèria prima, procés i conservació. Es va avaluar la aptitud al processat de quatre varietats de pera produïdes a la zona, "Conference", "Ercolini", "Flor de Invierno" i "Passa-Crassana" (*Capítol 1*). Es va concloure que sota les condicions assajades, la pera de la varietat "Conference" va presentar els millors atributs per al processat mínim. L'enfosquiment enzimàtic superficial de la pera mínimament processada és una alteració no desitjada i per aquest motiu es van avaluar diferents alternatives per reduir-ne la incidència. Els resultats obtinguts van mostrar que un tractament antioxidant-estabilitzant de la textura format per ascorbat de calci al 2% i clorur de calci al 1%, va mantenir el color i textura de la pera "Conference" mínimament processada conservada en atmosfera modificada passiva a 5 °C durant 14 dies. La qualitat al llarg de la vida útil de la pera mínimament processada obtinguda en aquesta tesi es va avaluar sota condicions comercials simulades (8 dies a 4 °C) i condicions de simulació del trencament de la cadena de fred (3 dies a 4 °C més 5 dies a 8 °C).

En segon lloc, es van avaluar alguns aspectes de qualitat microbiològica de pera mínimament processada. Es va estudiar la capacitat de supervivència/creixement de *Salmonella enterica* subsp. Enteritidis i *Listeria monocytogenes* en pera "Conference" mínimament processada tractada amb ascorbat de calci al 2% i clorur de calci al 1% envasada en atmosfera modificada passiva i conservada sota condiciones comercials simulades i condicions simulades del trencament de la cadena de fred (*Capítol 2*). El temps i les temperatures de conservació estudiades van inhibir el creixement de les soques de *S. enterica* avaluades en pera mínimament processada, mentre que la població de les soques de *L. monocytogenes* estudiades va incrementar al augmentar

el temps de conservació. Després dels resultats observats, es va seleccionar *L. monocytogenes* com a patogen de major risc i es va utilitzar en els posteriors estudis. Es va avaluar l'efecte de l'estat de maduresa de la pera en el moment del processat en el comportament d'aquesta bactèria durant la seva conservació (*Capítol 3*). Es va observar que l'estat de maduresa en el moment del processat va tenir un efecte en la capacitat de creixement de *L. monocytogenes* quan la pera es va conservar a 10 i 20 °C. Sota aquestes temperatures el patogen va presentar un major potencial de creixement en pera processada en estat madur (31-42 N) o sobremadur (< 31 N) que en el parcialment madur (43-53 N). En general, el potencial de creixement de *L. monocytogenes* en pera es va incrementar al augmentar la temperatura de conservació.

Es va avaluar si la supervivència de *L. monocytogenes* després de sotmetre's a una simulació gastrointestinal podia veure's afectada en funció de la matriu (pera parcialment madura i meló mínimament processats) i temperatura de conservació (*Capítol 4*). En pera i meló mínimament processats i conservats a 1 °C es van observar les menors reduccions de població després de la simulació gastrointestinal. Quan la pera i meló inoculats amb *L. monocytogenes* es van conservar a 10 °C, la capacitat de supervivència a la simulació gastrointestinal de la bactèria es va reduir a l'incrementar el temps de conservació. La capacitat de supervivència a la simulació gastrointestinal de *L. monocytogenes* en meló després de la conservació no refrigerada (20 °C) es va reduir amb el temps de conservació, mentre que en pera la bactèria va presentar la mateixa capacitat de supervivència al llarg del temps. El creixement en pera mínimament processada va poder representar un estrès subletal per a la bactèria que va ocasionar que *L. monocytogenes* s'adaptés al entorn i presentés una major resistència enfront a un estrès letal com el de la fase gàstrica de la simulació gastrointestinal.

Partint del plantejament que la matriu ha pogut ocasionar en *L. monocytogenes* canvis fisiològics, aquest canvis també podrien afectar el potencial patogènic de la bactèria, incrementant el risc d'ocasionar uns listeriosis. Es va determinar si el temps de conservació i la matriu (pera i meló mínimament processats) podrien tenir un efecte en la supervivència de *L. monocytogenes* després de la simulació gastrointestinal i en la posterior habilitat per adherir-se i envair a les cèl·lules Caco-2 (*Capítol 5*). En el moment de la inoculació de pera i meló, i després de la simulació gastrointestinal, *L. monocytogenes* en pera va presentar una major capacitat d'adhesió sobre cèl·lules Caco-2 que la bactèria en meló, tot i que la capacitat d'invasió de la bactèria va ser la mateixa en ambdues matrius. *L. monocytogenes* crescuda en pera i meló i exposada a

la simulació gastrointestinal, va presentar una major capacitat d'invasió de les cèl·lules Caco-2 als 2 dies que a temps inicial o després de 7 dies de conservació.

Finalment, es va avaluar el potencial de virulència que presentaven 53 soques de *L. monocytogenes* de diferents orígens i serotips (*Capítol 6*). Es van avaluar característiques com la supervivència al estrès gàstric, la capacitat de creixement i la capacitat d'invasió, a la vegada que es van seqüenciar tres gens, relacionats amb la capacitat de virulència (*inlA* i *prfA*) i l'estrès (*sigB*) de *L. monocytogenes*, tots ells relacionats amb el risc de causar listeriosis. Es va concloure que les soques de *L. monocytogenes* del serotip 4b estudiades d'ambdós orígens (humà i alimentari) van presentar una major capacitat d'invasió de les cèl·lules Caco-2 però una menor capacitat de creixement. Cap de les soques 4b estudiades van presentar mutacions en la seqüència de nucleòtids del gen *inlA* que codifiquessin per un codó de terminació prematur ("premature stop codón", PMSC). De forma contraria, les soques de *L. monocytogenes* del serotip 1/2c van presentar una elevada capacitat de creixement però una reduïda capacitat d'invasió de les cèl·lules Caco-2. En les soques dels serotips 1/2a i 1/2c sí es van observar mutacions PMSC en la seqüència d' *inlA*. Es va poder relacionar una menor capacitat d'invasió de les cèl·lules Caco-2 amb la presència de mutacions PMSC en el gen *inlA*.

En el producte de pera mínimament processat estudiat en aquesta tesi, que no rebrà cap tractament posterior abans de ser consumit, en el que *L. monocytogenes* pot créixer en atmosfera modificada, en presència d'antioxidants, a temperatures de refrigeració (inclús a 1 °C), que presenta una elevada supervivència al pas gastrointestinal i amb una capacitat d'invasió destacable, es fa indispensable prevenir la contaminació, des del camp fins al moment del consum.

SUMMARY

In recent years, consumers demand ready-to-eat foods that are nutritionally balanced, low in calories and high in compounds with antioxidant capacity. Therefore, the consumption and sales of fresh-cut fruit and vegetables have risen. Currently, the number of fresh-cut products containing vegetables, that are available, in the market is higher than the fresh-cut products containing fruits.

For these investigation firstly, a new minimally processed product was developed based on pear since the Lleida-area (Catalonia) is the main producer of pear in the country. The minimal processing of fruits involves basic unit operations, such as peeling slicing, and packaging. However, before launching a product to the market, a product-development step should be carried out because factors like raw material, process and storage conditions could affect the shelf life of fresh-cut fruit. The suitability of four different cultivars of pear ('Conference', 'Ercolini', 'Flor de Invierno' and 'Passa-Crassana') was studied (*Chapter 1*). After evaluation, the 'Conference' pear appeared the most suitable for minimal processing. In order to minimize the visual deterioration of fresh-cut pear caused by the enzymatic browning, different antioxidant treatments were evaluated. The use of calcium ascorbate (2%) plus calcium chloride (1%) as antioxidant treatment delivered colour and texture stability for fresh-cut 'Conference' pear for 14 days at 5 °C. The physicochemical quality, nutritional changes, microbial quality and sensorial quality of fresh-cut 'Conference' pear dipped in calcium ascorbate (2%) plus calcium chloride (1%) and stored under modified atmosphere were evaluated under conditions simulating either commercial application (constant storage at 4 °C) or a simulated cold chain break (temperature abuse conditions, 3 days at 4 °C plus 5 days at 8 °C)

Secondly, microbial quality of the fresh-cut 'Conference' pear was studied. The impact of storage temperature on growth and survival of *Salmonella enterica* subsp. Enteritidis and *Listeria monocytogenes* on fresh-cut 'Conference' pear (dipped in the antioxidant solution and packaged under modified atmosphere) under conditions simulating commercial application stored at constant temperature and under conditions mimicking temperature abuse was evaluated (*Chapter 2*). After 8 days of storage, both constant temperature (4 °C) and temperature abuse conditions caused a significant decrease of *S. enterica* populations on pear wedges. In contrast, *L. monocytogenes* grew at both storage conditions. *L. monocytogenes* was able to grow on fresh-cut pear processed under the conditions evaluated. Thus, this pathogen presents higher health risk for ready-to-eat food consumers and was

selected and was selected for further studies. The next studies focused on *L. monocytogenes* behaviour. The effect of pear ripeness during processing on the survival and growth of *L. monocytogenes* on fresh-cut 'Conference' pear slices at different temperatures was studied (*Chapter 3*). At the end of the storage period, significant differences in the *L. monocytogenes* populations were observed between different ripeness stages at 10 and 20 °C. At these temperatures, the growth of *L. monocytogenes* was significantly higher on ripe (31-42 N) or overripe (< 31 N) pears than on mature-green pears (43-53 N). The growth potential of *L. monocytogenes* on fresh-cut 'Conference' pear increased with increasing storage temperature.

The effect of storage temperature and fruit matrix (fresh-cut mature-green pear and melon) on *L. monocytogenes* survival along gastrointestinal simulation was evaluated (*Chapter 4*). The lowest pathogen reductions after through a gastrointestinal simulation model were observed in both matrices at 1 °C. At 10 °C, *L. monocytogenes* showed noteworthy reduction throughout the gastrointestinal simulation regardless of the support matrix when the storage time increased. When the pathogen behaviour was evaluated at 20 °C (without cold stress), *L. monocytogenes* on fresh-cut pear was more resistant in the gastrointestinal simulation after 1 and 2 days of contact than the pathogen on melon. The mild-low pH of pear flesh could have switched on the acid tolerance response of *L. monocytogenes* in the stressful environment and subsequently enhanced pathogen survival to a lethal stress, caused during the gastric step of the gastrointestinal simulation.

After these results, the hypothesis that *L. monocytogenes* might increase its pathogenic potential during their shelf life on fresh-cut fruits was evaluated. The pathogenic potential of *L. monocytogenes*, inoculated on fresh-cut pear and melon during the shelf life, was investigated (*Chapter 5*). The pathogenic potential was expressed as the capability for the organism to adhere to and invade differentiated Caco-2 cells after passage through the gastrointestinal simulation model. After inoculation, pathogens on fresh-cut pear showed more capacity to adhere to Caco-2 cells than pathogen on fresh-cut melon. In spite of the higher adhesive capacity of pathogen grown on pear, these cells exhibit similar invasive capacity as cells grown on melon. After 2 days of storage, *L. monocytogenes* grown on fresh-cut melon showed similar adhesion capacity as cells grown on pear, but cells grown on melon had a higher invasive capacity. *L. monocytogenes*' ability to adhere to Caco-2 cells showed a weak reduction with increasing storage time in both matrices.

Finally, diversity of virulence potentials of 53 isolates of *L. monocytogenes* from different origins and serotypes were evaluated (*Chapter 6*). Phenotypic characterization determined as maximum growth rate, acid tolerance and invasion capacity of 53 strains of *L. monocytogenes* were studied in relation to different serotypes and allelic variants of genes encoding InternalinA (*inlA*), the transcriptional regulation PrfA (*prfA*) and the stress response factor SigmaB (*sigB*). Overall, serotype 4b strains were characterised by a relative high invasion capacity but low growth potential, and no premature stop codon (PMSC) mutations in *inlA* sequence were observed in these isolates. In contrast, strains of serotype 1/2c were characterised by a relative low invasion capacity but high growth potential. Strains of serotype 1/2a and 1/2c harboured different PMSC mutations in *inlA* sequence. The strains with lower invasion capacity to Caco-2 were characterised by high prevalence of mutations PMSC in *inlA*.

The fresh-cut pear evaluated during this thesis allowed growth of *L. monocytogenes* despite the application of modified atmosphere packaging, antioxidant solution and storage under refrigerate storage (even at 1 °C). In addition, the pathogen showed high survival in the gastrointestinal simulation model and an outstanding invasion capacity. Therefore, is very important to prevent the contamination of the fresh-cut products throughout the entire food chain production.

INTRODUCCIÓN GENERAL

INTRODUCCIÓN GENERAL

En las últimas décadas, la sociedad ha sufrido dos cambios notables en cuanto a las tendencias de consumo. El primero, tuvo lugar en el momento en que la mujer ocupó su lugar en el mundo laboral fuera del hogar y el tiempo dedicado a la preparación de la comida se vio reducido. Esto ocasionó que se incrementara la demanda de productos listos para el consumo, sabrosos y fáciles de preparar. El segundo cambio, éste mucho más cercano a la actualidad, fue ocasionado porque la sociedad se concienció de la importancia que tiene cuidar el cuerpo y la mente, pasando a un primer plano, en la elección del consumidor, el valor nutricional del alimento y la reducción de los aditivos alimentarios (Sillani y Nassivera, 2015). Por estos motivos, ha aumentado la demanda de productos listos para el consumo que sean nutricionalmente equilibrados, con bajo aporte calórico y alto aporte de compuestos de alto interés, como los compuestos con capacidad antioxidante.

Las frutas y hortalizas son una excelente fuente de nutrientes desde un punto de vista nutricional por su elevado contenido en vitaminas y minerales, y el bajo contenido en lípidos y proteínas. Sustancias con propiedades antioxidantes como los compuestos fenólicos y antocianos, están presentes en frutas y hortalizas. El consumo de estos alimentos puede ayudar a la prevención de algunas enfermedades como las cardiovasculares, el cáncer, la diabetes u la obesidad (Crozier *et al.*, 2009; Xue *et al.*, 2016). Algunas autoridades, como la Organización Mundial de la Salud (OMS) o la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), han realizado campañas promocionales en más de 40 países recomendando el consumo de 400 g diarios de frutas y hortalizas (5aldia, 2016).

Una alternativa al consumo de frutas y hortalizas frescas enteras son los productos mínimamente procesados, que principalmente mantienen las propiedades nutricionales de las frutas y hortalizas enteras pero con la característica de estar listas para ser consumidas. En una investigación reciente se observó que cuando a niños y adolescentes se les ofrecía fruta mínimamente procesada, el consumo de fruta incrementaba en un 70% (Wansink *et al.*, 2013).

1. Generalidades de los productos vegetales mínimamente procesados

Las frutas y hortalizas mínimamente procesadas son aquellos productos vegetales, limpios, pelados enteros y/o cortados en diferentes formatos, cuyo mínimo procesado permite mantener sus propiedades naturales, con facilidad de ser utilizadas por el consumidor para el consumo directo en crudo o para preparaciones culinarias. Puede emplearse el envasado al vacío o en atmosfera modificada con o sin utilización de gases.

Existen múltiples nombres para referirse a este tipo de productos: mínimamente procesados, productos de cuarta gama (IV gama, originariamente del francés "4ème Game"), productos listos para el consumo o productos frescos cortados (originariamente del inglés "ready-to-eat" o "ready-to-use", o "fresh-cut"), etc.

En el año 2014 los 5 países europeos que ocuparon la mayor parte del mercado de frutas y hortalizas mínimamente procesadas, con un total de 3 millones de euros, fueron Reino Unido en primera posición (33%) seguido de Italia (26%) y Francia (18%), España en cuarta posición (12%) y Alemania en quinta (11%) (VVA Brussels, 2015). En cuanto a consumo per cápita anual de este tipo de productos, España todavía se encuentra por debajo de la media europea con un consumo de 2,8 kg/persona/año, mientras que en Reino Unido se consumen 12 kg y en Francia e Italia, 6 kg y 4 kg, respectivamente (Luna, 2014). En España, se estima que en el año 2015 se comercializaron 91.000 toneladas de productos vegetales mínimamente procesados, observándose un crecimiento del 11% respecto al año anterior. Del total de este volumen, el 96,9% lo ocuparon las hortalizas mínimamente procesadas, mientras que el 3,1% restante fue para frutas mínimamente procesadas (Monje, 2016).

En los últimos años, se ha hecho patente en los lineales de los supermercados españoles que cada vez las hortalizas mínimamente procesadas (verduras de hoja cortadas, lechugas cortadas, brotes, mezcla de lechugas y/o brotes, preparado para sopas, ensaladas con "toppings", etc.) ocupan un mayor espacio en las zonas refrigeradas. Por otro lado, las frutas mínimamente procesadas también han mostrado un incremento en la demanda pero todavía ocupan un espacio pequeño del lineal. Según los datos de Alimarket, el 80% de la producción de frutas y hortalizas mínimamente procesadas se comercializó en supermercados y/o grandes superficies, mientras que el 20% de la producción se distribuyó en el canal HORECA (Hoteles, restaurantes y catering) (Monje, 2016). Debe destacarse

que las estadísticas se elaboran a partir de los datos que facilitan los productores mayoritarios del estado español, pero estos no representan a todos los productores. De este modo alguno de los proveedores del canal HORECA no están contabilizados y esto hace que el porcentaje real sea probablemente superior al 20%.

Lleida es la provincia con mayor producción de pera a nivel español, produciendo 176.649 toneladas de pera el año 2014. Según variedades, las más producidas fueron "Conference" (87.167 toneladas), "Blanquilla" (17.901 toneladas), "Ercolini" (6.960 toneladas) y "Limonera" (2.839 toneladas) (DAAM, 2015). Principalmente, esta fruta se vende para el consumo en fresco o para la elaboración de zumos. El procesado de la pera como producto mínimamente procesado o de IV gama puede aportar un nuevo valor añadido a la fruta fresca para los productores de la zona.

La pera presenta un contenido rico en azúcares como fructosa, sorbitol y sacarosa, mientras que un bajo contenido en glucosa, proteínas y lípidos. Su composición también contiene ácidos orgánicos como el málico, cítrico y quínico; minerales, entre los que destacan el yodo y el potasio, y una gran cantidad de fibra (12-15% en materia seca), fundamentalmente insoluble (Barroca *et al.*, 2006; Chen *et al.*, 2007). También presenta un contenido de compuestos fenólicos destacable, principalmente ácidos fenólicos y flavonoides (flavonoles y favanoles) (Amiot *et al.*, 1995), los cuales están relacionados con un efecto beneficioso para la salud debido a sus propiedades antioxidantes (Crozier *et al.*, 2009).

2. Frutas mínimamente procesadas

Un producto mínimamente procesado debe presentar una óptima calidad organoléptica y ha de ser seguro para el consumidor. La calidad inicial del fruto es de gran importancia para obtener un producto mínimamente procesado de calidad, ya que solo vamos a mantener esta propiedad y no a mejorarla con el procesado. Existen una serie de factores como son la variedad y tamaño del fruto, su estado de madurez antes del procesado, la calidad higiénico-sanitaria y la temperatura de almacenamiento de éste que tienen un impacto en la calidad y vida útil de la fruta mínimamente procesada (Gorny *et al.*, 2000).

La fruta mínimamente procesada es un producto más difícil de obtener que las hortalizas mínimamente procesadas debido a que algunos frutos requieren unos

valores de maduración óptimos antes de ser procesados. En consecuencia, el estado de madurez en el que se procesa la fruta tiene un efecto en la calidad sensorial y en la vida útil del producto (Gorny *et al.*, 1998; Oms-Oliu *et al.*, 2009; Soliva-Fortuny *et al.*, 2002). Gorny *et al.* (1998) observaron que las nectarinas procesadas en un estado de madurez parcialmente verde (valores de penetromía, > 40-53 N) presentaron una mayor vida útil que las nectarinas procesadas en un estado de madurez más avanzado (> 27-40 N). Sin embargo, este último presentó una mayor valoración por parte del consumidor. Es decir, se debe llegar a un equilibrio entre el sabor y el tiempo de vida útil para poder tener un producto aceptado por el consumidor.

Por otro lado, existen variedades de fruta más sensibles al procesado y, por lo tanto, menos adecuadas para transformarse en un producto mínimamente procesado de calidad (Gorny *et al.*, 2000). Algunas variedades presentan un mayor potencial de pardeamiento, mayor tendencia a sufrir fisiopatologías o un mayor ratio respiratorio (Altisent *et al.*, 2014). Por este motivo la selección de la variedad más apta para el procesado es de gran importancia cuando se pretende desarrollar un producto.

2.1. Etapas del procesado mínimo

El diagrama de flujo que se presenta a continuación describe las distintas etapas por las que va a pasar el fruto durante el procesado mínimo antes de llegar al consumidor (Figura 1). Cuando la fruta llega del campo a la industria procesadora se hace una selección previa del producto. A continuación, debe pasar por una etapa previa de **pre-enfriamiento** para reducir la temperatura interna del fruto y evitar la pérdida de agua de éste. Existen distintos métodos para llevar al fruto de su "temperatura de campo" (calor latente) a la temperatura de pre-enfriamiento. Esta temperatura se encuentra unos grados por encima de la temperatura óptima de almacenamiento. Existen distintos sistemas de pre-enfriamiento como el enfriamiento por aire, enfriamiento por agua, enfriamiento por vacío y enfriamiento por hielo (Edeogu *et al.*, 1997; Sánchez, 2016). La técnica más adecuada a emplear dependerá del tipo de producto, su valor y su calidad inicial. A partir de este momento todo el procesado de la fruta se va a llevar a cabo a baja temperatura ambiental (< 15 °C). En el caso que la fruta ya recolectada se suministre refrigerada, esta etapa inicial no será necesaria realizarla.

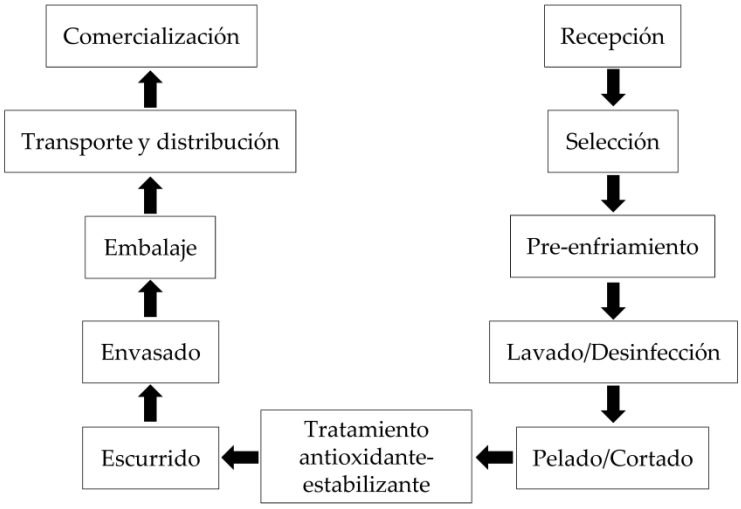


***Figura 1**. Etapas del proceso de elaboración de la fruta mínimamente procesada, adaptación de Oria (2016).*

La **desinfección superficial** del fruto entero en la elaboración de frutas mínimamente procesada es imprescindible, debido a que no existe ninguna otra etapa durante el proceso que permita una reducción de la carga microbiana. Existen distintas opciones en cuanto a desinfección del fruto entero, la más extendida en la industria agroalimentaria es el uso del hipoclorito sódico. La población microbiana de hortalizas y frutas frescas enteras que se desinfectan con este producto suelen presentar reducciones de 2 unidades logarítmicas (Gil *et al.*, 2009). Sin embargo, el hipoclorito sódico presenta una serie de inconvenientes debido a que es reactivo con la materia orgánica y pueden generarse trihalometanos (THM) que son substancias potencialmente cancerígenas (López-Gálvez *et al.*, 2010). En algunos países se viene prohibiendo o poniendo limitaciones a este tipo de desinfección, por todo ello, se han estudiado ampliamente otras alternativas. Existen otros tratamientos alternativos de origen químico como el ácido peroxiacético, dióxido de cloro, peróxido de hidrógeno, ácidos orgánicos, agua electrolizada, ozono u otros métodos físicos como ultrasonidos, altas presiones, radiofrecuencias, radiación ultravioleta (UV-C, radiación no ionizante), etc. (Artés *et al.*, 2009; Gil *et al.*, 2009; Goodburn y Wallace, 2013).

El **pelado y corte** es una de las etapas del proceso que se encuentra menos tecnificada debido a que por las características de algunos frutos es necesaria la presencia de operarios que realicen los cortes o que revisen que el equipo auxiliar

haya eliminado toda la piel del fruto. En esta etapa se rompe la integridad de la fruta y se liberan exudados del producto, que en una etapa posterior se eliminarán mediante inmersión o ducha.

La aplicación de un **tratamiento antioxidante-estabilizante** de la textura tiene el objetivo de aplicar mediante una solución acuosa a 5 °C de temperatura una serie de compuestos químicos como ácido ascórbico, ácido cítrico, ascorbato de calcio, cloruro de calcio, etc., que evitaran el pardeamiento y la pérdida de firmeza del producto cortado (Oms-Oliu *et al.*, 2010). Existen distintas técnicas de aplicación como la inmersión y la ducha o pulverizado. El tiempo de contacto lo estipula cada productor, según la concentración de los aditivos en la solución y la tecnología de aplicación. En esta etapa también existe la posibilidad de incorporar al tratamiento un compuesto antimicrobiano (por ejemplo aceites esenciales, extractos vegetales, etc.). Existen referencias bibliográficas que evalúan la acción de alguno de estos productos sobre los microorganismos en fruta cortada, observándose resultados favorables. Sin embargo, el uso de estos compuestos no está muy extendido en la industria de las frutas y hortalizas mínimamente procesadas, debido a que algunos confieren un sabor extraño al producto y tienen un elevado precio.

Seguido al tratamiento antioxidante se encuentra la etapa de **escurrido** o **secado**, que tiene el objetivo de eliminar el agua restante, reduciendo así el agua libre para los microorganismos y evitando oxidaciones del producto.

El **envasado** del producto en atmosfera modificada busca proteger el producto del entorno externo a la vez que reducir la concentración de oxígeno del envase. Una menor concentración de oxígeno en el envase, reduce la actividad enzimática que ocasiona el pardeamiento (el oxígeno es un limitante de la reacción) y reduce el posible crecimiento microbiano (Rojas-Grau *et al.*, 2009). Existen dos formas de modificar la atmosfera del interior de un envase, incorporando una mezcla de gases deseada (atmosfera modificada activa, mayoritariamente combinación de CO_2/N_2) o como resultado de la respiración del producto que contiene, que consumirá parte del oxígeno y liberará dióxido de carbono (atmosfera modificada pasiva).

Para la **conservación** de un producto mínimamente procesado es imprescindible la refrigeración del producto por debajo de los 5 °C (Francis *et al.*, 2012). La refrigeración del producto cortado ocasiona que tenga lugar una reducción del ratio respiratorio del producto, una reducción de la actividad enzimática (al no

encontrarse estos en su temperatura óptima de reacción) y una reducción del posible crecimiento microbiano (al conservarse a una temperatura inferior a la óptima de muchos microorganismos alterantes o patógenos). Por lo tanto, es necesario evitar que se rompa la cadena de frío durante la producción y a lo largo de la vida útil del producto ya que esto conllevaría una pérdida de la calidad organoléptica y microbiológica. De todos modos, existen microorganismos que pueden desarrollarse a temperaturas de refrigeración y a bajos niveles de oxígeno (Bennik *et al.*, 1995), uno de ellos es *Listeria monocytogenes*.

La aplicación de la tecnología de barreras u obstáculos ("hurdle technology") que implica el uso de métodos combinados de conservación, se encuentra ampliamente extendido en la industria agroalimentaria a nivel mundial. Esta tecnología busca poner el microorganismo en un ambiente hostil con el objetivo de inhibir su crecimiento, reducir su capacidad de supervivencia o causarle la muerte (Leistner, 2000). En las frutas mínimamente procesadas el uso de múltiples barreras se observa, por ejemplo, con la aplicación de un agente antimicrobiano a la vez que un envasado en atmosfera modificada y, por supuesto, su conservación en condiciones de refrigeración.

2.2. Alteraciones de las frutas mínimamente procesadas

2.2.1. Cambio de color

El procesado mínimo de las frutas puede ocasionar una serie de alteraciones en el producto cortado. La primera de ellas y la más visible es el cambio o pérdida de color debido al pardeamiento enzimático, este se debe a la acción de la enzima polifenoloxidasa (PPO) que reacciona con compuestos fenólicos (liberado de las vacuolas en la etapa de corte) en presencia de oxígeno, generándose unos compuestos coloreados llamados quinonas (Zawistowski *et al.*, 1991). Existen métodos físicos que pueden reducir la aparición de esta alteración, como son el uso de refrigeración y la disminución de la concentración de oxígeno en el envase. Por otro lado, como métodos químicos se emplean los agentes reductores o compuestos acidificantes (el ácido ascórbico, el ácido cítrico y sus sales, 4-hexilresorcinol, glutatión, cisteína, etc.) para reducir el pardeamiento. También se ha extendido el uso de recubrimientos comestibles que reducen la concentración de oxígeno en contacto con el producto cortado, y que a su vez permiten incorporar a su estructura sustancias antipardeantes (Ayala-Zavala y González-Aguilar, 2011; Oms-Oliu *et al.*, 2010). El cambio de color del producto tiene un efecto decisivo en la compra del producto por parte del consumidor. Por este

motivo existe una amplia bibliografía sobre la aplicación de distintos compuestos para reducir el pardeamiento enzimático en distintas frutas.

El Reglamento (CE) nº 1333/2008 junto al Reglamento (CE) nº 1129/2011, el cual modifica el anexo II del primer reglamento, son los documentos que regulan los aditivos que pueden ser adicionados a los alimentos en la Unión Europea. Los aditivos incluidos en la lista positiva para ser utilizados en frutas y hortalizas mínimamente procesadas listas para el consumo son ácido ascórbico (E-300), ascorbato sódico (E-301), ascorbato cálcico (E-302), ácido cítrico (E-330), citratos de sodio (E-331), citratos de potasio (E-332) y citratos de calcio (E-333). Estos aditivos no tienen un límite máximo de aplicación ("quantum satis").

2.2.2. Pérdida de firmeza

Una de las etapas principales del procesado mínimo es el pelado y corte de la pulpa. Éste genera un desgarro de las células y una liberación del contenido intracelular observándose una pérdida de firmeza y una liberación de exudados. Las enzimas pectinmetilesterasa (PME) y poligalacturonasa (PG) tienen un papel importante en la pérdida de firmeza, ya que degradan la pared celular compuesta principalmente por celulosa, hemicelulosa y pectinas (Alandes *et al.*, 2006). La aplicación de sales de calcio (cloruro cálcico (E-509), lactato cálcico (E-327), tartrato cálcico (E-354), gluconato cálcico (E-578), propionato cálcico (E-282) y alginato sódico (E-401)) pueden ayudar a mantener la estructura de la pulpa un mayor tiempo debido a que se forman unos compuestos de pectato de calcio que son insolubles y dan estabilidad a las células (Oms-Oliu *et al.*, 2010). La aplicación de estos aditivos está regulada por el Reglamento (CE) nº 133/2008, con las modificaciones de los Reglamentos (CE) nº 1129/2011 y (CE) nº 969/2014.

2.2.3. Propiedades organolépticas y nutricionales

Para la obtención de un producto mínimamente procesado de calidad es esencial partir de un fruto entero de buena calidad, debido a que el procesado (pelado y corte) ocasionará una serie de desórdenes fisiológicos que llevarán a la degradación de compuestos nutricionales, es decir la calidad del producto no mejora con el procesado mínimo. Dependiendo del tipo de fruto y la variedad, el proceso de corte ocasionará unas pérdidas u otras. Por ejemplo, en el melón "Cantaloupe" cortado se observó una reducción del 25% del contenido de vitamina C después de 6 días conservado a 5 °C, mientras que en fresa, mango y sandía esta pérdida fue inferior al 5% (Gil *et al.*, 2006). Bajo las mismas

condiciones de conservación, en kiwi y sandía no se observó reducción en el contenido de carotenos, mientras que en piña la reducción fue del 25%. En cuanto al contenido de fenoles totales no se observaron diferencia entre la fruta entera y la mínimamente procesada (Gil *et al.*, 2006). Cabe destacar que para todas las frutas evaluadas, la fruta cortada frente al fruto fresco entero presentó una senescencia más temprana. Además, la luz es otro parámetro que puede tener efecto sobre los compuestos nutricionales. En el caso de sandía y mango se observó que cuando la fruta procesada y envasada se expuso a la luz durante su conservación, el contenido de carotenos incrementó (Gil *et al.*, 2006).

Otra problemática en la fruta cortada es la generación de sabores y olores extraños. El bajo nivel de oxígeno y/o elevada concentración de dióxido de carbono en la atmosfera del envase puede inducir que la fruta cortada entre en un metabolismo fermentativo. Esta atmosfera también ocasiona un incremento de la producción de etanol y acetaldehídos que pueden causar olores extraños (Gorny *et al.*, 1999). Este problema puede solucionarse con un adecuado estudio del producto cortado y la elección idónea del material de envasado, junto al mantenimiento de la cadena de frio en todo momento.

2.2.4. Crecimiento microbiano

En la fruta cortada pueden distinguirse dos tipos de crecimiento microbiano, el crecimiento de la población de microorganismos alterantes o de microorganismos patógenos, que han podido llegar a la fruta después de una contaminación. El crecimiento de los microorganismos alterantes, pone bajo amenaza la calidad organoléptica del producto pudiendo llegar a ocasionar pérdidas económicas al productor cuando los niveles iniciales de la fruta cortada son demasiado elevados. Sin embargo, cuando existe el crecimiento de los microorganismos patógenos la calidad organoléptica del producto no se ve afectada pero se ve incrementado el riesgo para la salud del consumidor.

La fruta tiene una barrera protectora natural (la piel) que es efectiva frente a microorganismos alterantes y patógenos. Con el procesado mínimo esta piel se elimina, eliminando así su barrera natural, dejando la pulpa del fruto expuesta a unas condiciones ambientales desfavorables. Así, la posibilidad de ser contaminado por microorganismos durante la manipulación, corte o durante la vida útil incrementa. Al mismo tiempo, la rotura de las células vegetales libera exudados que servirán a estos microorganismos de nutrientes. Si la carga microbiana inicial del producto es elevada, debido a un inadecuado procesado,

pueden sobrevivir y multiplicarse. El crecimiento de estos microorganismos podrá ocasionar un deterioro acelerado del producto. Los agentes alterantes de las frutas y sus derivados pueden ser bacterias, mohos y levaduras. Debido a las características intrínsecas de las frutas, mayoritariamente por su bajo pH, los principales alterantes son levaduras y mohos, siendo las levaduras las que presentan una mayor capacidad de crecimiento sobre frutas cortadas frente a los mohos (Raybaudi-Massilia *et al.*, 2009).

Los microorganismos contaminantes del producto mínimamente procesado serán principalmente los que forman parte de la microbiota superficial de la fruta, junto con la microbiota adquirida a lo largo de la manipulación (manipuladores, utillaje, cajas, cintas, etc.). Las frutas tienen una composición heterogénea y por lo tanto, su microbiota puede variar en función del pH, la disponibilidad de nutrientes y la actividad de agua, entre otros factores (Kalia, Gupta, 2006). Esta microbiota principalmente se compone de bacterias gram-negativas entre las que se encuentran familias de bacterias pectinolíticas (*Pseudomonas* spp. o *Erwinia* spp.), bacterias lácticas (*Leuconostoc* spp. o *Lactobacillus* spp.) y bacterias saprofitas, al mismo tiempo que levaduras (*Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., etc.) y mohos (*Sclerotina*, *Mucor*, *Aspergillus*, *Cladosporium*, etc.) (Beuchat, 1996; Nguyen-The y Carlin, 1994; Raybaudi-Massilia *et al.*, 2009). Algunos autores también han podido aislar de la superficie de frutas y hortalizas comercializadas microorganismos patógenos para los humanos como *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Staphylococcus aureus*, *Clostridium botulinum* y *Bacillus cereus* (Abadias *et al.*, 2008; Nguyen-The y Carlin, 1994).

Antes del procesado, en la etapa de lavado-higienizado es necesario reducir al máximo la carga microbiana del producto entero, debido a que las frutas mínimamente procesadas no van a recibir ningún tratamiento físico que disminuya la carga microbiana del producto. Una vez procesada la fruta, para evitar la proliferación de posibles microorganismos alterantes o patógenos, es necesaria la eliminación de los exudados ocasionados por el corte de las paredes celulares del fruto y es imprescindible eliminar el líquido restante antes de envasar el producto.

No existe regulación sobre los niveles de microorganismos alterantes que debe presentar una fruta mínimamente procesada para poder asegurar su vida útil.

3. Riesgo microbiológico de las frutas mínimamente procesadas

El procesado mínimo de la fruta ocasiona que una mayor superficie de la pulpa quede expuesta al ambiente, incrementando el riesgo a la contaminación por microorganismos alterantes y/o patógenos. En la búsqueda de la obtención de producto mínimamente procesado que perdure por más tiempo en el lineal del supermercado, es decir con una vida útil más larga, se debe tener en cuenta el riesgo que esto puede representar para el consumidor. En el caso que exista contaminación del producto por un microorganismo patógeno damos más tiempo a que este pueda multiplicarse, incrementando la carga microbiana sobre el alimento y a su vez el riesgo para la salud del consumidor.

3.1. Toxiinfecciones alimentarias

En los últimos años se ha observado una tendencia creciente del número de toxiinfecciones alimentarias relacionadas con el consumo de frutas. Una de las primeras que se ha descrito, tuvo como agente causante *Salmonella enterica* serovar Miami y como vehículo de transmisión la sandía (Florida, 1954) (Gayler *et al.*, 1955). Una de las toxiinfecciones más recientes que también tuvo como vehículo de transmisión la fruta, ocurrió en el año 2014 en Estados Unidos, cuando manzanas recubiertas con caramelo contaminadas por *L. monocytogenes* causaron 34 hospitalizaciones y entre ellas 7 defunciones (CDC, 2015a).

Durante el periodo que comprende estos dos casos, las frutas han sido vehículo de toxiinfecciones alimentarias en numerosas ocasiones aunque en menor número que las hortalizas y otros alimentos de origen animal. Según el CDC ("Centers for Disease Control and Prevention") en los últimos años los patógenos responsables de las toxiinfecciones alimentarias transmitidas por frutas han sido mayoritariamente *Escherichia coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., Norovirus y virus de la hepatitis A. Concretamente, del total de toxiinfecciones declaradas en los Estados Unidos 24 (13% del total anual), 16 (8%), 15 (7%) y 10 (5%) tuvieron como vehículo de transmisión la fruta, en los años 2011, 2012, 2013 y 2014, respectivamente (CDC, 2014a, 2014b, 2015b, 2016) (Tabla 1). Entre las frutas que han sido vehículo de intoxicaciones se encuentran: sandia, melón, tomate, mango, uva, fresa o ensalada de frutas (Callejón *et al.*, 2015; Harris *et al.*, 2003).

Cabe destacar que en el año 2011, tuvo lugar una de las toxiinfecciones más graves en la que se ha visto implicada una fruta. En este caso el agente causante fue *L. monocytogenes* en melón "Cantaloupe". Se contabilizaron 147 casos de los que 143 fueron hospitalizados, causando la muerte de 33 personas (CDC, 2012).

Tabla 1. *Agentes etiológicos principales causantes del mayor número de toxiinfecciones relacionadas con frutas en los Estados Unidos durante los años 2011-2014.*

Año	2011	2012 [c]	2013 [d]	2014 [e]
Núm. Total de toxiinfecciones causadas por frutas [a]	24 (13%)	16 (8%)	15 (7%)	10 (5%)
Norovirus	11 (444/0) [b]	4 (210/0) [b]	6 (196/1) [b]	-
Salmonella spp.	-	6 (446/1) [b]	5 (49/1) [b]	-
L. monocytogenes	1 (147/33) [b]	-	-	2 (37/8) [b]
E. coli verotoxigénica	1 (15/2) [b]	-	-	-
Virus de hepatitis A	-	-	2(159/0) [b]	-

(a) Número total de toxiinfecciones que han presentado como vehículo de contagio la fruta (porcentaje de toxiinfecciones con vehículo la fruta del total de toxiinfecciones determinadas (%))

(b) Número de toxiinfecciones causadas por este microorganismo (enfermos/fallecidos)

(c) Frutas implicadas en el año 2012: melón (4 toxiinfecciones), frutas silvestres (2), fruta tropical (1) y no clasificado (9)

(d) Frutas implicadas en el año 2013: no clasificado (6 toxiinfecciones), melón (3), fruta de pepita (3), frutas silvestres (1), fruta sub-tropical (1) y fruta tropical (1)

(e) Frutas implicadas en el año 2014: no clasificado (2 toxiinfecciones.), fruta de pepita (2), frutas silvestres (2), melón (1), fruta de hueso (1), fruta sub-tropical (1) y fruta tropical (1)

3.2. Origen de la contaminación

La contaminación de frutas y hortalizas frescas por microorganismos patógenos puede darse en distintos puntos de la cadena alimentaria. En el campo,

antes de la recolección, puede deberse al riego con agua contaminada, el uso de estiércoles no madurados o por la contaminación directa por animales (insectos, animales domésticos o salvajes, etc.) (Nguyen-The y Carlin, 1994). Para los vegetales de hoja, los tubérculos y los frutos de plantas de tallo rastrero como el melón o la sandía que están en contacto directo con la tierra, éste junto con el agua, los animales salvajes y los insectos, representan las mayores fuentes de contaminación por microorganismos patógenos. Bacterias patógenas para el ser humano como *E. coli*, *L. monocytogenes*, y *Salmonella* spp. se han aislado de distintos entornos (Fernandez-Escartín, 2008). En el caso de *Escherichia coli* O157:H7, esta se encuentra presente en el tracto intestinal de animales destinados al consumo humano como ganado bovino, porcino o aviar, los cuales son portadores (Pruimboom-Brees *et al.*, 2000). Aunque estos no desarrollen la enfermedad, la diseminan al ambiente a través de las heces de donde se ha aislado en distintas ocasiones. *L. monocytogenes* también se encuentra presente en el tracto intestinal de terneros, cerdos y aves, los cuales ejercen de reservorio, sin embargo, esta bacteria se ha aislado ampliamente en el ambiente. Distintos estudios han determinado la presencia de *L. monocytogenes* en muestras de tierra tanto de cultivo como de jardín (Dowe *et al.*, 1997; MacGowan *et al.*, 1994), al igual que en muestras de agua dulce y marina (Colburn *et al.*, 1990). De forma similar a *E. coli*, *Salmonella* al tener el intestino de animales y hombres como hábitat natural podría pensarse que la contaminación por heces debería ser siempre la única vía de contaminación, sin embargo, no es así. Mientras que son pocas las ocasiones en las que se ha aislado *Salmonella* spp. en muestras de tierra, sí se aisló la bacteria de fuentes de agua que no habían estado expuestas a la contaminación por desechos orgánicos (Fair y Morrison, 1967) del mismo modo que en muestras de agua superficial corriente (39% de las muestras positivas) (Dondero *et al.*, 1977).

Para la fruta que se recolecta del árbol, el riesgo de contaminación por microorganismos alterantes y patógenos puede incrementar en el momento de la recolección, cuando una vez cogido el fruto sano del árbol se deposita sobre cajas que están en contacto directo con la tierra. A la vez que es de mayor importancia no recoger los frutos dañados o caídos al suelo debido a que como ya hemos destacado la tierra es un reservorio natural de algunas bacterias patógenas. Una vez en la central procesadora, el no adecuado control del agua de volcado o transporte, puede llevar a una recontaminación de producto no contaminado, al igual que un mal control del agua de lavado-higienizado (bajo nivel del agente desinfectante, pH no óptimo, elevada materia orgánica en suspensión, etc.) (Nguyen-The, 2012; Reij *et al.*, 2004). En el caso de las frutas y hortalizas mínimamente procesadas el pelado y corte del producto también puede ser una

fuente de contaminación cruzada cuando no se siguen unas buenas prácticas de higiene (BPH) adecuadas o buenas prácticas de manipulación (BPM).

3.3. Bacterias patógenas implicadas en intoxicaciones por fruta

En los Estados Unidos y en la Unión Europea, las dos instituciones que se encargan de monitorizar los casos de toxiinfecciones son "Centers for Disease Control and Prevention, CDC" y "European Food Standards Agency, EFSA", respectivamente. Cada uno de estos organismos presenta independientemente de forma anual un informe que permite conocer si algún agente etiológico presenta una mayor o menor incidencia que otro, o si algún alimento presenta un mayor riesgo para la seguridad alimentaria que otro. Cuando no se tiene en cuenta a los virus como agentes causantes de toxiinfecciones, los tres microorganismos con mayor incidencia son *Salmonella* spp., *E. coli* verotoxigénica (VTEC) y *L. monocytogenes*. Tanto a nivel europeo como a nivel americano, la incidencia de casos de *Salmonella* spp. se ha mantenido durante los últimos años mientras que los casos por *L. monocytogenes* han incrementado y los de *E. coli* VTEC se han reducido. Los datos de estos tres patógenos más relevantes de los informes más recientes de CDC y EFSA se presentan en las tablas 2 y 3.

Tabla 2. *Datos relevantes del año 2014 emitidos por el CDC (CDC, 2016).*

	Toxiinfecciones	Enfermos	Hospitalizados	Fallecidos
Salmonella spp.	149	2563 (22%)[a]	400 (58%)[b]	1
E. coli VTEC	24	222 (2%)[a]	62 (9%)[b]	1
L. monocytogenes	9	55 (0%)[a]	51 (7%)[b,c]	13

(a) Porcentaje de enfermos por una misma etiología del total notificado en el 2014 (13.246 enfermos)

(b) Porcentaje de hospitalizados por una misma etiología del total notificado en el 2014 (712 hospitalizados)

(c) Del total de casos de L. monocytogenes*, el 93% de los enfermos necesitaron ser hospitalizados*

Tabla 3. *Datos relevantes del año 2014 emitidos por la EFSA (EFSA, 2015).*

	Enfermos	Hospitalizados	Fallecidos
Salmonella spp.	88.715	9.830 (34,4%)[a]	65 (0,15%)[b]
E.coli VTEC	5.955	930 (39,2%) [a]	7 (0,20%) [b]
L. monocytogenes	2.161	812 (98,9%) [a]	210 (15,0%) [b]

(a) Porcentaje de hospitalizados (%) del total de enfermos por el mismo agente etiológico

(b) Tasa de mortalidad (%)

En el caso de *L. monocytogenes* es preciso destacar que del total de enfermos causados por esta bacteria el 98% y 93%, a nivel europeo y americano, respectivamente, precisaron ser hospitalizados. Este mismo patógeno también presenta la tasa de mortalidad (15%) más elevado de entre las tres bacterias.

Diversos investigadores de zonas distintas del planeta han realizado estudios para determinar la incidencia de estos tres patógenos de transmisión alimentaria en muestras de fruta cortada, cabe destacar que existe un número muy limitado de este tipo de estudios. Concretamente, en Nigeria, Chukwu *et al.* (2010) determinaron la presencia de *Salmonella* spp., *E. coli* y *L. monocytogenes* en piña (50 muestras), sandía (50 muestras) y papaya (50 muestras) cortadas obtenidas de mercados callejeros. Observaron que en las muestras de piña, 7 fueron positivas para *Salmonella* spp. y 26 lo fueron para *E. coli* (en este caso indicador de proceso). En el caso de las 50 muestras de sandía cortada analizadas, 3 presentaron resultado positivo para *Salmonella* spp. y 22 para *E. coli*. Por último en papaya cortada, 3 muestras fueron positivas para *Salmonella* spp. y 21 para *E. coli*. En ninguno de los productos evaluados estaba presente *L. monocytogenes*. Abadias *et al.* (2008), en la zona de Cataluña, evaluaron 21 muestras de fruta cortada que incluían manzana, piña, naranja, mango y melocotón, ninguna de las muestras presentaron *Salmonella* spp., *E. coli* o *L. monocytogenes.* Althaus *et al.* (2012) tampoco observaron muestras positivas para *Salmonella* spp., *L. monocytogenes* y *E. coli* en 64 muestras de fruta cortada obtenida de mercados suizos. Sin embargo, dos de las 64 muestras sí mostraron presencia de *Cronobacter sakazakii*, dado que según indican las plantas pueden ser el hábitat natural de *Cronobacter*.

Badosa *et al.* (2008) determinaron la calidad microbiológica de 151 muestras de frutas frescas enteras de la zona de Cataluña que incluía fruta de hueso

(melocotones, ciruelas, albaricoques y cerezas, 57 muestras), fruta de pepita (manzanas y peras, 57 muestras) y uvas (37 muestras). Se observó que solamente una muestra del grupo de fruta de hueso presentó positivo para *Salmonella*, siendo negativo para *L. monocytogenes* y *E. coli*. Seow *et al.* (2012) analizaron 35 muestras de fruta de Singapur, que incluían manzanas, naranjas y mangos, y ninguna de ellas fue positiva para *Salmonella* spp. y *E. coli* O157:H7.

3.3.1. *Salmonella* spp.

Salmonella es un bacilo gram negativo de la familia *Enterobacteriaceae*. Es aerobia facultativa y generalmente móvil. Este género se divide en un gran número de serovares, teniendo en cuenta la composición antigénica de la bacteria, en función de la presencia de antígenos somáticos (antígenos O) y flagelares (antígenos H). En *Salmonella* se diferencian dos grandes especies, *Salmonella enterica* y *Salmonella bongori*. Los dos serovares de *Salmonella* que representan el mayor número de infecciones de salmonelosis pertenecen a la especie de *Salmonella enterica*, y son *Salmonella enterica* subsp. Enteritidis y Thyphimurium. Concretamente, el año 2014 a nivel europeo, *S. enterica* subsp. Enteritidis representó el 44,4% del total de casos de salmonelosis (31% a nivel americano), mientras que *S. enterica* subsp. Thyphimurium representó un 17,4% del total (11% a nivel americano).

Salmonella es una bacteria patógena para el hombre y muchos animales. Esta bacteria habita principalmente en el intestino de vertebrados superiores, hombres y animales, liberándose al ambiente a través de las heces. Sin embargo, también puede encontrarse en el ambiente, en el agua o la tierra. El humano sin saberlo también puede ser un reservorio de la bacteria cuando este presenta una infección asintomática. Los síntomas de esta infección cursan con dolor abdominal, náuseas y diarrea. El periodo de incubación oscila entre las 3 y 72 horas, pudiendo llegar a ser de una semana o más (Fernandez-Escartín, 2008).

En cuanto a crecimiento, su temperatura óptima se sitúa entre 35 y 37 °C, por debajo de los 5-6 °C la bacteria no se desarrolla. El pH óptimo es de 7,0, mientras que el mínimo y el máximo son 3,8 y 9,0, respectivamente.

Entre los años 2011 y 2014, *Salmonella* spp. en fruta causó 11 brotes de toxiinfección, 495 enfermos y 2 fallecidos (Tabla 1). En el año 2014 se registraron 23,4 casos de salmonelosis por 100.000 habitantes en la comunidad europea. Sin embargo, existen casos de fruta contaminada por *Salmonella* spp. que no llegan al

consumidor gracias a una red de análisis y seguimiento de lotes, que en muchos casos finaliza en una retirada del producto que está a la venta por parte del productor.

3.3.2. *Escherichia coli*

E. coli es un bacilo gram negativo de la familia *Enterobacteriaceae* que abunda en el tracto intestinal de hombre y animales de sangre caliente. Es una bacteria aerobia facultativa. Existen seis grupos de *E. coli* patógena: *E. coli* enteropatógena (EPEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enterotoxigénica (ETEC), *E. coli* enterohemorrágica o verotoxigénica (EHEC, STEC o VTEC), *E. coli* enteroagregantiva (EAggEC) y *E. coli* difusamente adherente (AEEC). *E. coli* O157:H7, causante de la mayoría de toxiinfecciones de *E. coli*, forma parte del grupo de *E. coli* verocitotoxigénicas (VTEC). Una infección por esta bacteria puede ocasionar una meningitis neonatal, infecciones del tracto urinario, sepsis designada SIRS (síndrome de respuesta inflamatoria sistémica) o neumonía (Fernandez-Escartín, 2008). Se ha descrito que la temperatura óptima de *E. coli* se encuentra entre los 35 y 40 °C, no dándose crecimiento por debajo de los 7-8 °C ni por encima de los 44-46 °C. En cuanto a pH, su óptimo se encuentra entre 6-7 (pH mínimo 4,4 y pH máximo 9). En el año 2014 en la comunidad europea se registraron 1,56 casos de toxiinfección por *E. coli* patógena por 100.000 habitantes.

3.3.3. *Listeria monocytogenes*

L. monocytogenes es una bacteria gram positiva, no esporulante y anaerobia facultativa. Se ha observado que según a la temperatura a la que se encuentra la bacteria, esta puede presentar movilidad (entre 22 y 25 °C) o no (a 37 °C). *L. monocytogenes* es una bacteria ubicua del ambiente y principalmente se ha aislado de tierra, agua, a la vez que de áreas de procesamiento de alimentos. *Listeria* spp. crece a temperaturas que oscilan entre -0,4 y 45 °C, siendo su óptimo los 37 °C (Walker y Stringer, 1987). También es capaz de crecer en un rango de pH entre 4,5-8,0.

Los síntomas que ocasiona una gastroenteritis causada por *L. monocytogenes*, principalmente son fiebre, diarrea, dolor articular y cefaleas. En los individuos sanos, esta gastroenteritis es auto-limitante y suele durar unos dos días. Sin embargo, en la población de riesgo como recién nacidos, mujeres embarazadas, ancianos y personas con el sistema inmune comprometido (enfermos del virus de la inmunodeficiencia humana (VIH), pacientes trasplantados, pacientes en

tratamiento de quimioterapia, etc.), es más probable que se pueda contraer una septicemia, meningitis o encefalitis y causar la muerte del paciente (Ooi y Lorber, 2005).

Este microorganismo se puede clasificar en 13 serotipos distintos (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7) (Vazquez-Boland *et al*., 2001), sin embargo, 4b, 1/2a y 1/2b son responsables de más del 90% de los casos de listeriosis humana declarados. *L. monocytogenes* presenta al menos cuatro linajes evolucionados (I, II, III, y IV) que en algunos casos comparten nicho ecológico. La mayoría de los aislados clínicos de *L. monocytogenes* pertenecen a los linajes I (serotipo 1/2a) y II (serotipo 1/2b y 4b). Las cepas del linaje II suelen ser más comunes en los alimentos y en el entorno natural, y acostumbran a aislarse de casos de listeriosis animal y casos esporádicos en humanos. Sin embargo, la mayoría de brotes de toxiinfección humana se asocia con los aislados del linaje I. Mientras que el III y IV, no son tan comunes, y predominan en los aislados de origen animal (Shen *et al*., 2013). En el año 2014 en la comunidad europea se registraron 0,52 casos de listeriosis por 100.000 habitantes, lo que supone un incremento del 30% respeto al año 2013.

3.4. Legislación en productos mínimamente procesados

A nivel Europeo, la normativa vigente que rige los criterios microbiológicos de los alimentos es el Reglamento (CE) nº 2073/2005 modificado por el Reglamento (CE) nº 1441/2007. Es preciso diferenciar entre los criterios de higiene y los criterios de seguridad. *E. coli* en la industria agroalimentaria se emplea como un criterio de higiene en la elaboración de un producto. Concretamente, en la tabla 4 se especifica lo que indica el Reglamento (CE) nº 1441/2007 para las frutas y hortalizas listas para el consumo. Como criterios de seguridad el Reglamento regula los niveles de *L. monocytogenes* y *Salmonella*. En la tabla 5 se especifica lo que indica el Reglamento (CE) nº 1441/2007 para las frutas y hortalizas listas para el consumo en cuanto a niveles de estos dos patógenos.

Tabla 4. *Criterios de higiene para los productos de fruta y hortalizas troceadas (listas para el consumo) descritos en el Reglamento (CE) nº 1441/2007.*

Categorías de alimentos	Microorganismo	[(1)]Plan de muestreo		Límites		Fase en la que se aplica el criterio
		n	c	m	M	
Frutas y hortalizas troceadas (listas para el consumo)	*E. coli*	5	2	100 ufc g^{-1}	1.000 ufc g^{-1}	Proceso de elaboración

(1) n = número de unidades que componen la muestra; c = número de muestras que dan valores entre m y M

El Reglamento incorpora un apartado donde explica cómo interpretar los resultados de las pruebas de la Tabla 4. Concretamente dice:

"Los límites dados se refieren a cada muestra analizada. Los resultados de las pruebas demuestran la calidad microbiológica del proceso analizado."

"*E. coli* en frutas y hortalizas troceadas (listas para el consumo) y en zumos de frutas y hortalizas no pasteurizados (listos para el consumo):

- satisfactorio, si todos los valores observados son inferiores o iguales a m,

- aceptable, si un máximo de c/n valores se encuentran entre m y M y el resto de los valores observados son inferiores o iguales a m,

- insatisfactorio, si uno o varios valores observados son superiores a M o más de c/n valores se encuentran entre m y M."

En el caso de un resultado insatisfactorio el reglamento marca que la acción a tomar es la mejora en la higiene de la producción y en la selección de las materias primas.

Tabla 5. *Criterios de seguridad para los productos de fruta y hortalizas troceadas (listas para el consumo) descritos en el Reglamento (CE) nº 1441/2007.*

Categorías de alimentos	Microorganismo	Plan de muestreo[1]		Límites		Fase en la que se aplica el criterio
		n	c	m	M	
Frutas y hortalizas troceadas (listas para el consumo)	*Salmonella*	5	0	Ausencia en 25 g		Productos comercializados durante su vida útil
Alimentos listos para el consumo que pueden favorecer el desarrollo del microorganismo	*Listeria monocytogenes*	5	0	100 ufc g^{-1}[2]		Productos comercializados durante su vida útil
		5	0	Ausencia en 25 g[3]		Antes que el alimento haya dejado el control inmediato del explotador de la empresa alimentaria que lo ha producido
Alimentos listos para el consumo que no pueden favorecer el desarrollo del microorganismo[4]	*Listeria monocytogenes*	5	0	100 ufc g^{-1}		Productos comercializados durante su vida útil

(1) n = número de unidades que componen la muestra; c = número de muestras que dan valores entre m y M

(2) Este criterio se aplica si el fabricante puede demostrar, a satisfacción de la autoridad competente, que el producto no superará el límite de 100 ufc g^{-1} durante su vida útil. El explotador podrá fijar límites intermedios durante el proceso que deberían ser lo suficientemente bajos para garantizar que no se supere el límite de 100 ufc g^{-1} al final de la vida útil

(3) Este criterio se aplica a los productos antes de que hayan abandonado el control inmediato del explotador de la empresa alimentaria cuando este no pueda demostrar, a satisfacción de la autoridad competente, que el producto no superará el límite de 100 ufc g^{-1} durante la vida útil

(4) Se considera automáticamente que pertenecen a esta categoría los productos con pH ≤ 4.4 o aw ≤ 0.92, productos con pH ≤ 5.0 y aw ≤ 0.94, y los productos con una vida útil inferior a 5 días. Otras categorías de productos también pueden pertenecer a esta categoría, siempre que se justifique científicamente

El Reglamento incorpora un apartado donde explica cómo interpretar los resultados de las pruebas de la Tabla 5. Concretamente dice:

"Los resultados de las pruebas demuestran la calidad microbiológica del lote analizado. *L. monocytogenes* en alimentos listos para el consumo que puedan permitir el desarrollo de *L. monocytogenes* antes de que el alimento haya dejado el control inmediato del explotador de la empresa alimentaria que haya producido, cuando no pueda demostrar que el producto no superará el límite de 100 ufc g^{-1} durante su vida útil:

- satisfactorio, si todos los valores observados indican la ausencia de la bacteria,

- insatisfactorio, si se detecta la presencia de la bacteria en cualquiera de las muestras.

L. monocytogenes en otros alimentos listos para el consumo:

- satisfactorio, si todos los valores observados son inferiores o iguales al límite,

- insatisfactorio, si se detecta la presencia de la bacteria en cualquiera de las muestras.

Salmonella en diferentes categorías de productos alimenticios:

- satisfactorio, si todos los valores observados indican la ausencia de la bacteria,

- insatisfactorio, si se detecta la presencia de la bacteria en cualquiera de las muestras."

4. Crecimiento de microorganismos patógenos sobre fruta mínimamente procesada

La mayoría de las frutas se caracterizan por un pH mucho más bajo (más ácido) que el de las hortalizas. Debido a esta característica, tradicionalmente se había creído que las frutas no favorecían el desarrollo de microorganismos patógenos, sin embargo, no es así. Distintos autores han descrito cómo se desarrollan algunos microorganismos patógenos sobre las frutas mínimamente procesadas conservadas a distintas temperaturas de almacenamiento.

El comportamiento de *Salmonella* spp. en frutas tropicales cortadas como pitahaya, banana, carambola, mango, piña o guayaba se ha estudiado a dos temperaturas distintas (4 y 28 °C) (Ma *et al.*, 2016). Se observó que a temperatura de refrigeración, *Salmonella* spp. no se desarrolló sobre ninguno de los frutos procesados, mientras sí que lo hizo a 28 °C mostrando un gran crecimiento (más de 3 unidades logarítmicas) en todas las frutas, con excepción de la piña (pH 3,2-4,0) donde no tuvo lugar crecimiento y un crecimiento mínimo sobre mango (pH 3,8-4,2) (Ma *et al.*, 2016). Estos dos últimos frutos presentaban un pH más bajo que el resto. Abadias *et al.* (2012) y Feng *et al.* (2015), demostraron que *E. coli* O157:H7 y *L. monocytogenes* sobre piña mínimamente procesadas tampoco presentaron crecimiento cuando se conservaron a 5 °C y 25 °C. Feng *et al.* (2015) demostraron que la población de *L. monocytogenes* sobre mango (pH 4,2), papaya (pH 6,0) y pitahaya (pH 6,1) mínimamente procesados y conservados a 5 °C, mostró un incremento de alrededor de 2 unidades logarítmicas en los dos primeros frutos, mientras que no creció en pitahaya. Sin embargo, creció sobre los tres tipos de fruta cuando las temperaturas de refrigeración fueron de 13 y 25 °C. *Salmonella enterica* sobre mango presentó un comportamiento similar al observado en *L. monocytogenes* (Penteado *et al.*, 2014).

También se han realizado estudios de crecimiento de patógenos en frutas más próximas a nuestra climatología como manzana, melocotón, pera, sandía o melón. Después de 2 días de conservación a 20 °C, en manzana (pH 3,8) y melocotón (pH 3,7-5,1) mínimamente procesados *L. monocytogenes*, *Salmonella enterica* y *E. coli* O157:H7 presentaron en manzana incrementos de población de 4,60, 1,40 y 1,60 unidades logarítmicas, respectivamente, y 2,70, 2,40 y 3,10 unidades logarítmicas, en melocotón, respectivamente (Alegre *et al.*, 2013). Sin embargo, cuando la fruta se conservó a 5 °C durante 10 días, la población de *E. coli* sobre manzana presentó una reducción superior a 0,5 unidades logarítmicas y un incremento pequeño (0,25 unidades logarítmicas) en melocotón. En frutas menos ácidas,

L. monocytogenes en melón "Cantaloupe" mínimamente procesado presentó un incremento leve de población (0,75 unidades logarítmicas) después de 7 días de conservación a 5 °C, mientras que la población de *S. enterica* sobre la misma matriz se mantuvo constante durante el mismo periodo. A temperaturas de conservación incorrectas (> 5 °C), la población de *L. monocytogenes* incrementó 2,86 y 4,17 unidades logarítmicas, a 8 y 12 °C, respectivamente, respecto al control de 5 °C; mientras *S. enterica* presentó un menor incremento, 1,65 y 2,49 unidades logarítmicas, a 8 y 12 °C, respecto al control conservado a la temperatura óptima de refrigeración (Huang *et al.*, 2015).

E. coli O157:H7 sobre melón "Piel de Sapo" (pH 5,94) mínimamente procesado presentó un incremento de población de alrededor de 4 unidades logarítmicas después de un día de conservación a 25 °C. Sin embargo, cuando el melón se conservó a 5 °C, la población del patógeno se mantuvo hasta los 10 días, tiempo en el que se observó una reducción de la población (Abadias *et al.*, 2012). Cuando *E. coli* y *Listeria innocua* se inocularon en sandía mínimamente procesada (pH 4,6-5,9) y se conservó a temperatura de refrigeración (5 °C), ambos patógenos presentaron un incremento de población (2 unidades logarítmicas) después de 15 días de conservación (Ramos-Villarroel *et al.*, 2012). Por lo tanto, existe un gran número de frutas mínimamente procesadas que permiten el desarrollo y crecimiento de microorganismos patógenos.

5. *Listeria monocytogenes*

El creciente interés en productos listos para el consumo y el incremento de la elaboración de este tipo de productos, que no van a recibir ningún tratamiento térmico antes de su consumo, y que a su vez se conservan a temperaturas de refrigeración han ocasionado que las autoridades ejerzan un mayor control para garantizar la inocuidad de estos. La mayor preocupación viene ocasionada por *L. monocytogenes*, microorganismo capaz de desarrollarse a temperaturas de refrigeración, que presentando una menor incidencia de toxiinfecciones que otras bacterias patógenas presenta una mayor tasa de hospitalizaciones y defunciones. De este modo se justifica el creciente interés de incrementar el conocimiento sobre esta bacteria en matrices alimentarias.

L. monocytogenes tiene la capacidad de sobrevivir y de crecer sobre un gran número de alimentos y condiciones, por suerte, no se han notificado alertas alimentarias en todos los alimentos en los que es capaz de crecer. Esto se debe a

que para el desarrollo de la listeriosis no solo el número de células en el alimento (dosis infectiva) es importante sino que también lo son su serotipo, el estado de inmunidad del hospedador y la capacidad de supervivencia a la barrera gástrica de la bacteria (Vazquez-Boland *et al.*, 2001). La composición del alimento que hace de vehículo de transmisión también puede tener un papel importante en el desarrollo de la listeriosis (Lorentzen *et al.*, 2011).

El tracto gastrointestinal es la ruta primaria de la infección por *L. monocytogenes*, mientras que la penetración de la barrera intestinal es la primera etapa del proceso infectivo (Lecuit *et al.*, 2001). Por lo tanto, es clave determinar si *L. monocytogenes* puede verse afectada por su entorno, ocasionando una mayor o menor supervivencia al tracto gastrointestinal y en su efecto, en la adhesión e invasión de la células intestinales.

5.1. Supervivencia de *L. monocytogenes* en la etapa gastrointestinal

El estómago es un órgano que se encuentra entre el esófago y el duodeno, que alberga el bolo alimentario, el cual mediante una acción mecánica y química se degrada permitiendo que los nutrientes sean disponibles para nuestro organismo. Desde el punto de vista de la seguridad alimentaria el estómago cumple otra función, la de inactivar y/o matar a los microorganismos patógenos que hayan podido ser ingeridos con la comida o bebida, antes de pasar al intestino (Smith, 2003). Concretamente, la secreción del ácido clorhídrico es el mayor mecanismo de defensa que presenta el ser humano frente a los microorganismos patógenos (Johnson, 2001).

Existe una gran diferencia en la fisiología del estómago según la edad del hospedador. Mientras que el pH medio del estómago de una persona joven puede ser de 1,7, en el caso de un anciano este pH puede llegar a ser de 4,5 (Russell *et al.*, 1993; Smith, 2003). El uso de antiácidos, los cuales modifican el pH del estómago, puede ocasionar que un hospedador presente una mayor o menor susceptibilidad al desarrollo de una toxiinfección alimentaria (Glupczynski, 1996).

En este apartado se citan varios estudios que evalúan el comportamiento de *L. monocytogenes* durante la simulación de una fase gástrica o la simulación íntegra de todo el tracto gastrointestinal. Del mismo modo que pasa en la realidad, en los estudios se emplean distintos pH y distintas composiciones para simular el estrés gástrico. Junto al serotipo y origen (aislado clínico o alimentario)

de las cepas de *L. monocytogenes* elegidas para cada estudio, el hecho que cada trabajo se realice bajo unas condiciones experimentales distintas (pH fase gástrica, tiempo de permanencia, sistema estático o dinámico, etc.) hace más difícil la comparación entre trabajos. Este es uno de los motivos por los que es necesario especificar bajo qué condiciones de trabajo se realiza cada estudio.

Distintos estudios evalúan el comportamiento de *L. monocytogenes* a la simulación gastrointestinal después de un crecimiento óptimo en un medio de cultivo rico. La conclusión general que puede extraerse de estos estudios es que la capacidad de supervivencia a estos estreses (estrés ácido y osmótico) depende de la cepa estudiada (Barmpalia-Davis *et al.*, 2008a; Cunha *et al.*, 2016; Werbrouck *et al.*, 2008).

5.1.1. Efecto del entorno y la matriz alimentaria en la habilidad de *L. monocytogenes* para superar la barrera gástrica

En la actualidad, las instituciones que se encargan de regular los criterios microbiológicos que deben cumplir los alimentos, en concreto en la regulación de *L. monocytogenes,* no han tenido presente la heterogeneidad que existe entre cepas. Al presentar distintos potenciales patogénicos puede darse que una cepa tenga una mayor probabilidad de causar listeriosis que otra, inclusive si el resto de factores que intervienen en la virulencia se mantienen (dosis infectiva, inmunidad del hospedador, etc.). Además, hace un par de décadas, distintos investigadores (Davis *et al.*, 1996; Ferreira *et al.*, 2003; O'Driscoll *et al.*, 1996) observaron que *L. monocytogenes* presentaba una menor sensibilidad a un estrés ácido fuerte o letal (pH 3,5), si previamente el patógeno se había expuesto un tiempo a unas condiciones de estrés ácido suave o subletal (pH 5,5). Para el estudio de este tipo de estrés ácido suave se utilizaron principalmente ácidos orgánicos comúnmente empleados en la industria agroalimentaria (ácido láctico, ácido acético, etc.) o inorgánicos como el ácido clorhídrico en pequeñas concentraciones. Sin embargo, fue el ácido clorhídrico el que mayoritariamente se utilizó para evaluar el estrés ácido letal, debido a que este es el que está presente en el estómago.

Curiosamente, también se observó que esta adaptación del patógeno podía ocasionar una protección cruzada, es decir, aunque el estrés inicial que inducía este cambio fuera por ejemplo el ácido, posteriormente la bacteria presentaba una mayor resistencia a este mismo estrés, pero también a otros entornos letales, como un estrés salino, de etanol o peróxido de hidrógeno. Concretamente, Lou y Yousef (1997) observaron que después de un estrés ácido (pH 4,5-5,5) *L. monocytogenes*

fue más resistente a una concentración elevada de peróxido de hidrogeno (0,1%), etanol (17,5%) o estrés ácido (pH 3,5).

A lo largo del procesado de alimentos los microorganismos pueden verse expuestos a distintas condiciones de estrés químico y físico, como son distintas temperaturas de conservación, aditivos alimentarios (mayoritariamente sales o ácidos orgánicos), ambientes con bajo oxígeno, ausencia de nutrientes, etc. (Leistner, 2000; Yousef y Courtney, 2003). Cuando *L. monocytogenes* se encuentra en un entorno no óptimo, la bacteria lo percibe y puede modificar su fisiología para combatirlo acumulando solutos (Ko, Smith, 1999), regulando algunas proteínas de membrana (Beales, 2004), cambiando la composición de los ácidos grasos de membrana (a la vez modificando la permeabilidad y la fluidez de la membrana) (Diakogiannis *et al.*, 2013) o sintetizando proteínas de novo (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996). Basándose en este principio, diversas investigaciones se han centrado en cómo el estrés del entorno al que se expone *L. monocytogenes* puede tener un efecto en la posterior supervivencia a un estrés ácido fuerte como el que se encuentra en el estómago humano.

Los estudios iniciales se realizaron modificando un medio de cultivo (adicionando ácidos, sales, fijando pH, etc.) una vez el microorganismo había crecido bajo las condiciones óptimas dejando transcurrir un tiempo de contacto con el nuevo estrés leve. Posteriormente, la bacteria se exponía a un estrés fuerte. De este modo, se determinaba si el hecho de la exposición previa de la bacteria a un estrés leve había desencadenado que *L. monocytogenes* presentara un incremento en la resistencia al posterior estrés letal (Davis *et al.*, 1996; Ferreira *et al.*, 2003; Koutsoumanis *et al.*, 2003; Koutsoumanis y Sofos, 2004; O'Driscoll *et al.*, 1996). También se determinó que en la adaptación de *L. monocytogenes* al estrés ácido, el tiempo de exposición, el tipo de ácido, la temperatura y la fase de crecimiento de la bacteria, tienen un papel importante (Shen *et al.* 2014).

A lo largo de la última década, se ha generado un mayor conocimiento sobre este tema. Sin embargo, la mayoría de estudios se han realizado "in vitro" sin tener en cuenta las condiciones reales a las que se puede ver expuesta *L. monocytogenes*. En un escenario real como un alimento, *L. monocytogenes* puede encontrarse expuesta a varios factores de forma simultánea como la composición del alimento (pH, disponibilidad de agua, etc.) y las condiciones de almacenamiento de éste (temperaturas de refrigeración, envasado en atmosfera modificada, aplicación de compuestos antimicrobianos, etc.).

Matrices alimentarias como salchichas tipo "frankfurt", queso, salami, mortadela o derivados cárnicos de pavo se han empleado para determinar como la presencia de la matriz puede afectar a la supervivencia de *L. monocytogenes* durante la simulación gastrointestinal (Barmpalia-Davis *et al.*, 2008b; Dikici y Calicioglu, 2013; Formato *et al.*, 2007; Peterson *et al.*, 2007; Stopforth *et al.*, 2005). Al revisar qué alimentos se han estudiado, destaca que la mayoría de ellos se han visto implicados en algún brote de infección por *L. monocytogenes*. Peterson *et al.* (2007) determinaron que cuando el patógeno creció sobre embutido de pavo presentó una mayor resistencia a la etapa gástrica (pH 3,5) que cuando había crecido en un medio de cultivo rico como el BHI ("Brain Heart Infusion"). En la salchicha del tipo "Frankfurt" conservada a 10 °C, cuando su composición presentaba aditivos alimentarios como el diacetato de sodio, *L. monocytogenes* resultó ser más resistente a la simulación gástrica (pH 1,0) que en la muestra sin los aditivos (Stopforth *et al.*, 2005). Por otro lado, cuando en la mortadela se emplearon agentes antimicrobianos (lactato de sodio y/o diacetato de sodio) el patógeno decreció y no permitió determinar de forma clara si la matriz ocasionó o no una adaptación al posterior estrés gástrico (Formato *et al.*, 2007). En cambio, en el queso se evaluó el proceso que tendría lugar en una quesería, donde se incluye una maduración del producto a baja temperatura (6 °C) durante un periodo aproximado de tres meses (Dikici y Calicioglu, 2013) y se observó que con el tiempo de conservación, a lo largo del cual el pH y la actividad de agua del producto se reducen, las células bacterianas presentaron un incremento de la supervivencia al estrés gástrico.

En el caso de las frutas mínimamente procesadas, el bajo pH, el tipo de ácido mayoritario presente (málico o cítrico), junto con la conservación en frío y en condiciones de bajo oxígeno y elevado dióxido de carbono (MAP), pueden ser factores de estrés que causen adaptación y cambien la supervivencia de *L. monocytogenes*. Según nuestro conocimiento, no existen estudios sobre el comportamiento de patógenos de transmisión alimentaria en matrices de fruta mínimamente procesadas con una posterior exposición a una simulación gastrointestinal. Sin embargo, sobre zumo de fruta, Yuk y Schneider (2006) observaron que distintos serovares de *Salmonella* expuestos durante 24 h a zumos de distintas frutas (manzana: pH 3,7, naranja: pH 3,9, y tomate: pH 4,4) bajo dos temperaturas de conservación (7 y 20 °C) presentaron una mayor supervivencia a la exposición de un fluido gastrointestinal simulado (pH 1,5) que las células no adaptadas. Destacaron que el incremento de supervivencia al ácido depende del serovar estudiado, el tipo de zumo y la temperatura de adaptación. De forma similar, Yuk *et al.* (2008) estudiaron si este mismo comportamiento se observaba

en *E. coli* O157:H7 cuando se exponía a cuatro tipos de zumo distintos (manzana: pH 3,9, naranja: pH 3,9, tomate: pH 4,0, y zanahoria: pH 4,2), con y sin pulpa, y con y sin lactato cálcico durante aproximadamente 4 días a 7 °C. En este caso no se observó una clara adaptación con el posterior incremento de supervivencia a un fluido gastrointestinal simulado (pH 1,5).

5.2. *L. monocytogenes* frente a la barrera intestinal

La virulencia no es una propiedad estable y puede verse influenciada por su entorno (Pricope-Ciolacu *et al.*, 2013). De este modo, cuando el patógeno modifica su metabolismo para combatir un entorno estresante como el procesado del alimento o el paso a través del tracto gastrointestinal, su virulencia puede verse modificada (Roche *et al.*, 2005).

La virulencia de una bacteria comprende la capacidad de adhesión e invasión, a la vez que la de proliferación en las células eucariotas del hospedador. Los factores de virulencia que cabe destacar en *L. monocytogenes,* son los regulados por los genes de virulencia implicados en la invasión del hospedador (*inlA* y *inlB*), la liberación desde el fagosoma (*hly*, *plcA* y *plcB*), en el crecimiento en el citoplasma (*hpt*) o la diseminación de célula a célula (*actA*, *plcB*, *mpl* y *hly*). La proteína PrfA actúa de regulador de la mayoría de estos genes (Freitag *et al.*, 2009).

5.2.1. Proceso de infección de *L. monocytogenes*

Una vez la bacteria ha recorrido el estómago y ha sobrevivido al estrés gástrico, pasa a través del píloro y se encuentra con el epitelio intestinal. Es entonces cuando *L. monocytogenes* entra en contacto con las células eucariotas que lo forman (1, Figura 2). Estas células presentan en su superficie unas glicoproteínas llamadas E-cadherinas que sirven de receptor para que una célula eucariota se una a otra. *L. monocytogenes* también presenta dos proteínas en su superficie, Internalina A (InlA) y Internalina B (InlB), que son las principales responsables de la invasión de las células eucariotas (Seveau *et al.*, 2007). Concretamente, la bacteria utiliza la InlA para formar una unión covalente con el receptor E-cadherina de la célula eucariota. Esta interacción es crítica para que tenga lugar la invasión del epitelio intestinal.

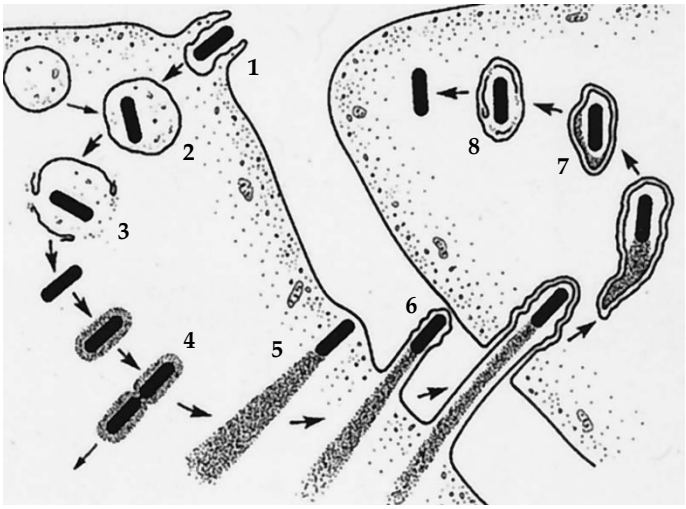


Figura 2. *Ciclo intracelular de* L. monocytogenes *(Vazquez-Boland et al., 2001).*

Una vez en el interior de la célula, la bacteria queda englobada en un fagosoma primario (2, Figura 2) (Freitag *et al.*, 2009). A continuación, la bacteria se libera rápidamente de la vacuola fagocítica que la envuelve (3, Figura 2), gracias a la ayuda de una toxina (hemolisina O o listeriolisina O, LLO) y dos fosfolipasas (fosfatidilinosutol-PLC y fosfatidilcolina-PLC) (Gedde *et al.*, 2000). Esta toxina forma unos poros en la membrana del fagosoma dejando libre a *L. monocytogenes* en el citoplasma. Una vez la bacteria se encuentra en el citoplasma, se multiplica (4, Figura 2) y activa un mecanismo que permite la polimerización de la actina, formando una serie de filamentos que facilitarán el movimiento intracelular (Suarez *et al.*, 2001). Gracias a este movimiento alguna de las bacterias consigue llegar a la periferia de la célula infectada, entrando en contacto con la membrana celular (5, Figura 2). Mediante un proceso de formación de protuberancias hacia la célula colindante (6, Figura 2), la bacteria pasa a la célula vecina pero queda rodeada por un fagosoma de doble membrana (fagosoma secundario) (7, Figura 2), el cual será nuevamente degradado por la acción de la hemolisina O y las fosfolipasas (8, Figura 2). Cabe destacar que al darse en el proceso infectivo el paso de célula a célula de forma directa, la bacteria no entra en contacto con el sistema inmune del hospedador (Cossart y Mengaud, 1989).

5.2.2. Efecto del alimento en el potencial patogénico de *L. monocytogenes*

Para que una infección inicial por *L. monocytogenes* acabe siendo una infección sistémica, es decir una listeriosis, es imprescindible la etapa de invasión de la pared intestinal por parte del patógeno.

Roche *et al.* (2005) observaron que cuando *L. monocytogenes* modificaba su fisiología para combatir un estrés leve, podía ocasionar una modificación en su capacidad para sobrevivir a la barrera gástrica. Se ha observado que una bacteria que modifica su metabolismo en respuesta a un estrés también altera su virulencia (Olesen *et al.*, 2009; Walecka *et al.*, 2011). Del mismo modo, esta mayor expresión de genes de virulencia podía incrementar la capacidad de invasión de *L.* monocytogene (Walecka *et al.*, 2011).

Desde hace años para el estudio de la interacción de los patógenos transmitidos por los alimentos con la barrera intestinal, se han estado utilizando distintos tipos de líneas celulares (Caco-2, T84 y HT-29). Las células Caco-2, son células humanas tumorales que presentan características morfológicas y bioquímicas similares a los enterocitos diferenciados. Concretamente, tienen la capacidad de formar una monocapa polarizada que imita el epitelio intestinal humano, siendo un modelo apropiado para el estudio de la invasión (Pereira *et al.*, 2008; Sambuy *et al.*, 2005).

En varios estudios, *L. monocytogenes* fue sometida a unas condiciones de estrés suave (ácidos orgánicos y/o sales) para determinar si su capacidad de invasión podía verse modificada debido al estrés previo al que se había expuesto (Conte *et al.*, 2000; Garner *et al.*, 2006; Lorentzen *et al.*, 2011; Olesen *et al.*, 2009; Werbrouck *et al.*, 2009). Garner *et al.* (2006) observaron que *L. monocytogenes* mostró una mayor capacidad de invadir las células Caco-2, cuando previamente estuvo expuesta a un medio BHI (pH 7,4, 37 °C) con lactato de sodio (2,5%) o cloruro sódico (2,2%) en comparación con el medio sin los aditivos.

Debido a que se ha relacionado la capacidad de invasión de *L. monocytogenes* con la virulencia de este patógeno (Jaradat y Bhunia, 2003), es importante saber si el hecho de crecer sobre un alimento puede ocasionar un incremento en la capacidad de invasión del patógeno. Cabe destacar que muchos de los estudios realizados utilizan un extracto del alimento o un medio de cultivo que simula las condiciones de estrés y son pocos los que han utilizado una matriz alimentaria como sustrato de crecimiento de *L. monocytogenes* (Duodu *et al.*, 2010; Pang *et al.*, 2007; Pricope-Ciolacu *et al.*, 2013; Rieu *et al.*, 2009).

6. La diferencia entre cepas de un mismo serotipo, visión genética

En el año 2001 se obtuvo la secuencia completa del genoma de *L. monocytogenes*, hecho que permitió que la microbiología junto a la genética pudieran evolucionar más rápidamente para explicar porque existe esta amplia heterogeneidad en el potencial patogénico de distintas cepas.

Como ya se ha comentado anteriormente, existe una serie de genes que tienen un papel importante en la patogenicidad de *L. monocytogenes*. Este es el caso de la proteína InlA, que tiene un papel imprescindible en la invasión de las células epiteliales y que está secuenciada por el gen *inlA*. En la secuencia de *inlA* de diferentes cepas de *L. monocytogenes*, se han observado distintos cambios en algún nucleótido de la secuencia del gen ("single nucleotide polymorphism", SNP). Puede ocurrir que esta mutación ocasione una mutación sin sentido en la secuencia del ADN, es decir que provoque la aparición de un codón de terminación prematuro ("premature stop codon", PMSC), ocasionando que la proteína InlA sintetizada presente alguna alteración o que no se llegue a secretar (Jonquières *et al.*, 1998). Cuando *L. monocytogenes* no presenta la forma completa de la proteína, la capacidad de invasión de esta puede verse comprometida (Olier *et al.*, 2002; Rousseaux *et al.*, 2004). Para tener una visión más global de este hecho, investigadores de todo el mundo han estudiado la distribución y prevalencia de estas mutaciones en aislados de casos clínicos humanos, de alimentos, de zonas de procesado de alimento o incluso de animales sanos (Ciolacu *et al.*, 2015; Cruz *et al.*, 2013; Felicio *et al.*, 2007; Handa-Miya *et al.*, 2007; Kovacevic *et al.*, 2013; Nightingale *et al.*, 2005; Ward *et al.*, 2005). En la mayoría de los casos la presencia de PMSC conllevaba una reducida capacidad de invasión de *L. monocytogenes* sobre células Caco-2.

Otro factor importante es la proteína reguladora PrfA, que es indispensable para la expresión de la virulencia de *L. monocytogenes*. El mecanismo de control mediado por PrfA es uno de los más importantes, debido a que genes de virulencia como los implicados en la invasión del hospedador (*inlA* y *inlB*), la liberación desde el fagosoma (*hly*, *plcA* y *plcB*), en el crecimiento en el citoplasma (*hpt*) o la diseminación de célula a célula (*acta*, *plcB*, *mpl* y *hly*) estan regulados principalmente por este primero (Freitag *et al.*, 2009). También se han observado mutaciones en algunos nucleótidos de la secuencia de *prfA*, sin embargo, estos cambios no han modificado la cadena de aminoácidos, siendo estas mutaciones silenciosas (Roche *et al.*, 2005).

Para que *L. monocytogenes* pueda expresar una mayor o menor capacidad de invasión de las células epiteliales, primero es imprescindible que supere los estreses que se va a encontrar antes de llegar al intestino (como pueden ser el sustrato donde se encuentra y las condiciones en las que va a crecer, o el tracto gastrointestinal del hospedador). *L. monocytogenes* cuenta con un regulador del estrés, el factor σ^B (SigmaB). Este factor contribuye a la supervivencia de la célula bajo condiciones adversas, como pueden ser el agotamiento de la fuente de carbono, exposición al ácido, a un ambiente oxidante, a bajas temperaturas o un estrés osmótico (Huang *et al.*, 2014). Este gen regula bajo condiciones de estrés un gran número de proteínas de estrés (Wemekamp-Kamphuis *et al.*, 2004). Al igual que con *inlA* y *prfA*, se han encontrado distintas variantes de la secuencia de σ^B algunas de las cuales contienen un PMSC (Nightingale *et al.*, 2007).

En el estudio de la adaptación a la tolerancia al ácido de *L. monocytogenes* muchos autores han empleado un entorno de ácido suave (pH 4,5-5,5), generado principalmente por el ácido clorhídrico, acético o láctico (Davis *et al.*, 1996; Ferreira *et al.*, 2003; Koutsoumanis *et al.*, 2003; Koutsoumanis y Sofos, 2004), ocasionando que el patógeno presentara una mayor supervivencia a un estrés ácido fuerte posterior como el que se encuentra en el estómago humano. Sin embargo, no se ha estudiado que efecto pueden tener otros ácidos orgánicos suaves como el málico o el cítrico, en la capacidad de adaptación de *L. monocytogenes*, ocasionando quizás una mejora de la capacidad de supervivencia a un estrés ácido fuerte. Del mismo modo, tampoco se ha determinado que impacto puede tener una matriz vegetal en este mismo escenario.

Por otro lado, otros autores han obviado el paso intermedio entre la contaminación del producto y la invasión del epitelio intestinal, es decir la barrera gástrica, y se han centrado en el efecto directo de algunos estreses (ácido, osmótico, frío, etc.) en la capacidad de virulencia de *L. monocytogenes*. Algunos han podido observar que una etapa previa de adaptación al ácido ocasiona un cambio en la expresión de algunos genes de virulencia, como *inlA*, traduciéndose en algunas ocasiones en una mayor capacidad de invasión de la cepa de *L. monocytogenes* estudiada (Garner *et al.*, 2006; Olesen *et al.*, 2009; Werbrouck *et al.*, 2009).

Por lo tanto, en la presente tesis se desarrollará un producto a base de pera mínimamente procesada a la vez que se evaluará el comportamiento de .. *enterica* subsp. Enteritidis y *L. monocytogenes* bajo distintas condiciones de conservación.

También se abordará el efecto que puede tener una matriz de fruta en el potencial patogénico de *L. monocytogenes*, al existir un vacío de conocimiento sobre este tema. Para evaluar este efecto se utilizará pera mínimamente procesada que presenta un pH entre 4,5-5,0 (siendo el ácido málico el ácido mayoritario). El pH que presenta esta fruta es considerado por diversos autores en estudios "in vitro" como subletal para la bacteria, y puede producir una respuesta adaptativa (Davis *et al.*, 1996; Ferreira *et al.*, 2003; O'Driscoll *et al.*, 1996). También se evaluará el efecto sobre melón mínimamente procesado, ya que presenta un pH superior (su ácido mayoritario es el cítrico), siendo este entorno más óptimo para el desarrollo de *L. monocytogenes.* Estas dos matrices alimentarias nos permitirán evaluar el efecto del alimento en el potencial patogénico de *L. monocytogenes*.

7. Referencias

5aldia. 2016. Frutas y hortalizas frescas, 5 al día. http://www.5aldia.org/#. Consulta: 05/09/16.

Abadias, M., Alegre, I., Oliveira, M., Altisent, R., Vinas, I. 2012. Growth potential of *Escherichia coli* O157:H7 on fresh-cut fruits (melon and pineapple) and vegetables (carrot and escarole) stored under different conditions. *Food Control* 27, 37-44.

Abadias, M., Usall, J., Anguera, M., Solson, C., Vinas, I. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.

Alandes, L., Hernando, I., Quiles, A., Perez-Munuera, I., Lluch, M.A. 2006. Cell wall stability of fresh-cut fuji apples treated with calcium lactate. *Journal of Food Science* 71, S615-S620.

Alegre, I., Viñas, I., Usall, J., Teixidó, N., Figge, M.J., Abadias, M. 2013. Control of foodborne pathogens on fresh-cut fruit by a novel strain of *Pseudomonas graminis*. *Food Microbiology* 34, 390-399.

Althaus, D., Hofer, E., Corti, S., Julmi, A., Stephan, R. 2012. Bacteriological survey of ready-to-eat lettuce, fresh-cut fruit, and sprouts collected from the swiss market. *Journal of Food Protection* 75, 1338-1341.

Altisent, R., Plaza, L., Alegre, I., Viñas, I., Abadias, M. 2014. Comparative study of improved vs. traditional apple cultivars and their aptitude to be minimally

processed as 'ready to eat' apple wedges. *LWT - Food Science and Technology* 58, 541-549.

Amiot, M.J., Tacchini, M., Aubert, S.Y., Oleszek, W. 1995. Influence of cultivar, maturity stage, and storage conditions on phenolic composition and enzymatic browning of pear fruits. *Journal of Agricultural and Food Chemistry* 43, 1132-1137.

Artés, F., Gomez, P., Aguayo, E., Escalona, V., Artes-Hernandez, F. 2009. Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities. *Postharvest Biology and Technology* 51, 287-296.

Ayala-Zavala, J.F., González-Aguilar, G.A. 2011. Use of additives to preserve the quality of fresh-cut fruits and vegetables. En: *Advances in fresh-cut fruits and vegetables processing*, (Ed.) Martín-Belloso, O., Soliva-Fortuny, R. CRC Press (Taylor & Francis Group), Boca Raton, FL.

Badosa, E., Trias, R., Parés, D., Pla, M., Montesinos, E. 2008. Microbiological quality of fresh fruit and vegetable products in Catalonia (Spain) using normalised plate-counting methods and real time polymerase chain reaction (QPCR). *Journal of the Science of Food and Agriculture* 88, 605-611.

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

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N. 2008b. Survival of *Listeria monocytogenes* in a simulated dynamic gastrointestinal model during storage of inoculated bologna and salami slices in vacuum packages. *Journal of Food Protection* 71, 2014-2023.

Barroca, M.J., Guine, R.P.F., Pinto, A., Goncalves, F.M., Ferreira, D.M.S. 2006. Chemical and microbiological characterization of Portuguese varieties of pears. *Food and Bioproducts Processing* 84, 109-113.

Beales. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservation, low pH, and osmotic stress: a review. *Comprehensive Reviews in Food Science and Food Safety* 3, 1-19.

Bennik, M.H.J., Smid, E.J., Rombouts, F.M., Gorris, L.G.M. 1995. Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. *Food Microbiology* 12, 509-519.

Beuchat, L.R. 1996. Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection* 59, 204-216.

Callejón, R.M., Rodríguez-Naranjo, M.I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M.C., Troncoso, A.M. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. *Foodborne Pathogens and Disease* 12, 32-38.

CDC. 2012. Centers for Disease Control and Prevention. Multistate outbreak of listeriosi linked to whole cantaloupes from Jensen Farms, Colorado. http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html. Consulta: 04/08/16.

CDC. 2014a. Centers for Disease Control and Prevention. Surveillance for Foodborne Disease Outbreaks, United States, 2011, Annual Report.

CDC. 2014b. Centers for Disease Control and Prevention. Surveillance for Foodborne Disease Outbreaks, United States, 2012, Annual Report.

CDC. 2015a. Centers for Disease Control and Prevention. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros. Apples http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html. Consulta: 12/08/2016.

CDC. 2015b. Centers for Disease Control and Prevention. Surveillance for Foodborne Disease Outbreaks, United States, 2013, Annual Report.

CDC. 2016. Centers for Disease Control and Prevention. Surveillance for Foodborne Disease Outbreaks, United States, 2014, Annual Report.

Chen, J.L., Wang, Z.F., Wu, J.H., Wang, Q., Hu, X.S. 2007. Chemical compositional characterization of eight pear cultivars grown in China. *Food Chemistry* 104, 268-275.

Chukwu, C.O.C., Chukwu, I.D., Onyimba, I.A., Umoh, E.G., Olarubofin, F., Olabode, A.O. 2010. Microbiological quality of pre-cut fruits on sale in retail outlets in Nigeria. *African Journal of Agricultural Research* 5, 2272-2275.

Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K. 2015. *Listeria monocytogenes* isolated from food samples from a Romanian black market show distinct virulence profiles. *International Journal of Food Microbiology* 209, 44-51.

Colburn, K.G., Kaysner, C.A., Abeyta, C., Wekell, M.M. 1990. *Listeria* species in a California coast estuarine environment. *Applied and Environmental Microbiology* 56, 2007-2011.

Conte, M.P., Petrone, G., Di Biase, A.M., Ammendolia, M.G., Superti, F., Seganti, L. 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. *Microbial Pathogenesis* 29, 137-144.

Cossart, P., Mengaud, J. 1989. *Listeria monocytogenes* - A model system for the molecular study of intracellular parasitism. *Molecular Biology & Medicine* 6, 463-474.

Crozier, A., Jaganath, I.B., Clifford, M.N. 2009. Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports* 26, 1001-1043.

Cruz, C.D., Pitman, A.R., Harrow, S.A., Fletcher, G.C. 2013. *Listeria monocytogenes* associated with New Zealand seafood production and clinical cases: unique sequence types, truncated InlA, and attenuated Invasiveness. *Applied and Environmental Microbiology* 80, 1489-1497.

Cunha, S., Komora, N., Magalhaes, R., Almeida, G., Ferreira, V., Teixeira, P. 2016. Characterization of clinical and food *Listeria monocytogenes* isolates with different antibiotic resistance patterns through simulated gastrointestinal tract conditions and environmental stresses. *Microbial Risk Analysis* 1, 40-46.

DAAM. 2015. Observatori de la Fruita Fresca de Catalunya. http://agricultura.gencat.cat/web/.content/de_departament/de02_estadistiques_observatoris/08_observatoris_sectorials/03_observatori_de_la_fruita_fresca/a2/fitxers_estatics/A2_2015/2015.08-Prev-collita-i-produccio-poma-i-pera-_Cat_EU_.pdf. Consulta: 03/11/15.

Davis, M.J., Coote, P.J., Obyrne, C.P. 1996. Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology-Uk* 142, 2975-2982.

Diakogiannis, I., Berberi, A., Siapi, E., Arkoudi-Vafea, A., Giannopoulou, L., Mastronicolis, S.K. 2013. Growth and membrane fluidity of food-borne pathogen *Listeria monocytogenes* in the presence of weak acid preservatives and hydrochloric acid. *Frontiers in Microbiology* 4, 6.

Dikici, A., Calicioglu, M. 2013. Survival of *Listeria monocytogenes* during production and ripening of traditional turkish savak tulum cheese and in synthetic gastric fluid. *Journal of Food Protection* 76, 1801-1805.

Dondero, N.C., Thomas, C.T., Khare, M., Timoney, J.F., Fukui, G.M. 1977. Salmonella in surface waters of central New-York state. *Applied and Environmental Microbiology* 33, 791-801.

Dowe, M.J., Jackson, E.D., Mori, J.G., Bell, C.R. 1997. *Listeria monocytogenes* survival in soil and incidence in agricultural soils. *Journal of Food Protection* 60, 1201-1207.

Duodu, S., Holst-Jensen, A., Skjerdal, T., Cappelier, J.M., Pilet, M.F., Loncarevic, S. 2010. Influence of storage temperature on gene expression and virulence potential of *Listeria monocytogenes* strains grown in a salmon matrix. *Food Microbiology* 27, 795-801.

Edeogu, I., Feddes, J., Leonard, J. 1997. Comparison between vertical and horizontal air flow for fruit and vegetable precooling. *Canadian Agricultural Engineering* 39, 107-112.

Fair, J.F., Morrison, S.M. 1967. Recovery of bacterial pathogens from high quality surface water. *Water Resources Research* 3, 799-&.

Felicio, M.T.S., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M. 2007. Recurrent and sporadic *Listeria monocytogenes* contamination in alheiras represents considerable diversity, including virulence-attenuated isolates. *Applied and Environmental Microbiology* 73, 3887-3895.

Feng, K., Hu, W.Z., Jiang, A.L., Xu, Y.P., Sarengaowa, Li, X.B., Bai, X. 2015. Growth potential of *Listeria monocytogenes* and *Staphylococcus aureus* on fresh-cut tropical fruits. *Journal of Food Science* 80, M2548-M2554.

Fernandez-Escartín, E. 2008. Microbiología e inocuidad de los alimentos, México.

Ferreira, A., Sue, D., O'Byrne, C.P., Boor, K.J. 2003. Role of *Listeria monocytogenes* sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Applied and Environmental Microbiology* 69, 2692-2698.

Formato, G., Geornaras, I., Barmpalia, I.M., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C., Sofos, J.N. 2007. Effect of acid adaptation on growth during storage at 10 ºC and resistance to simulated gastric fluid of *Listeria monocytogenes* inoculated onto bologna formulated with or without antimicrobials. *Journal of Food Protection* 70, 65-69.

Francis, G.A., Gallone, A., Nychas, G.J., Sofos, J.N., Colelli, G., Amodio, M.L., Spano, G. 2012. Factors affecting quality and safety of fresh-cut produce. *Critical Reviews in Food Science and Nutrition* 52, 595-610.

Freitag, N.E., Port, G.C., Miner, M.D. 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. *Nature Reviews Microbiology* 7, 623-628.

Garner, M.R., James, K.E., Callahan, M.C., Wiedmann, M., Boor, K.J. 2006. 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. *Applied and Environmental Microbiology* 72, 5384-5395.

Gayler, G.E., MacCready, R.A., Reardon, J.P., McKernan, B.F. 1955. An Outbreak of Salmonellosis Traced to Watermelon. *Public Health Reports* 70, 311-313.

Gedde, M.M., Higgins, D.E., Tilney, L.G., Portnoy, D.A. 2000. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infection and Immunity* 68, 999-1003.

Gil, M.I., Aguayo, E., Kader, A.A. 2006. Quality changes and nutrient retention in fresh-cut versus whole fruits during storage. *Jounal of Agricultural Food Chemistry* 54, 4284-4296.

Gil, M.I., Selma, M.V., López-Gálvez, F., Allende, A. 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* 134, 37-45.

Glupczynski, Y. 1996. Prolonged gastric acid suppression therapy: A significant risk factor for *Listeria monocytogenes* infection? *European Journal of Gastroenterology & Hepatology* 8, 1063-1066.

Goodburn, C., Wallace, C.A. 2013. The microbiological efficacy of decontamination methodologies for fresh produce: A review. *Food Control* 32, 418-427.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A. 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. *Journal of Food Science* 65, 541-544.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1998. Effects of fruit ripeness and storage temperature on the deterioration rate of fresh-cut peach and nectarine slices. *Hortscience* 33, 110-113.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1999. Quality changes in fresh-cut peach and nectarine slices as affected by cultivar, storage atmosphere and chemical treatments. *Journal of Food Science* 64, 429-432.

Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., Fujii, T. 2007. Nonsense-mutated *inlA* and *prfA* not widely distributed in

Listeria monocytogenes isolates from ready-to-eat seafood products in Japan. *International Journal of Food Microbiology* 117, 312-318.

Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F. 2003. Outbreaks associated with fresh produce: Incidence, growth and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78-141.

Huang, J.W., Luo, Y.G., Nou, X.W. 2015. Growth of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut cantaloupe under different temperature abuse scenarios. *Journal of Food Protection* 78, 1125-1131.

Huang, Y.N., Ells, T.C., Hansen, L.T. 2014. Role of sigB and osmolytes in desiccation survival of *Listeria monocytogenes* in simulated food soils on the surface of food grade stainless steel. *Food Microbiology* 46, 443-451.

Jaradat, Z.W., Bhunia, A.K. 2003. Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Applied and Environmental Microbiology* 69, 3640-3645.

Johnson, L.R. 2001. Gastric secretion. En: *Gastrointestinal physiology*, (Ed.) Johnson, L.R., 6th ed, Mosby, St. Louise. 75-94.

Jonquières, R., Bierne, H., Mengaud, J., Cossart, P. 1998. The *inlA* gene of *Listeria monocytogenes* L028 harbors a nonsense mutation resulting in release of internalin. *Infection and Immunity* 66, 3420-3422.

Kalia, A., Gupta, R. 2006. Fruit microbiology. En: *Handbook of fruit processing*, (Ed.) Hui, Y., Ames, I. Blackwell Publishing. 3-28.

Ko, R., Smith, L.T. 1999. Identification of an ATP-driven, osmoregulated glycine betaine transport system in *Listeria monocytogenes*. *Applied and Environmental Microbiology* 65, 4040-4048.

Koutsoumanis, K.P., Kendall, P.A., Sofos, J.N. 2003. Effect of food processing-related stresses on acid tolerance of *Listeria monocytogenes*. *Applied and Environmental Microbiology* 69, 7514-7516.

Koutsoumanis, K.P., Sofos, J.N. 2004. Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habituation at different pH conditions. *Letters in Applied Microbiology* 38, 321-326.

Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., Allen, K.J. 2013. Examination of food chain-derived *Listeria monocytogenes* strains of different

serotypes reveals considerable diversity in *inlA* genotypes, mutability, and adaptation to cold temperatures. *Applied and Environmental Microbiology* 79, 1915-1922.

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.

Leistner, L. 2000. Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology* 55, 181-186.

López-Gálvez, F., Allende, A., Truchado, P., Martínez-Sánchez, A., Tudela, J.A., Selma, M.V., Gil, M.I. 2010. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by-product formation. *Postharvest Biology and Technology* 55, 53-60.

Lorentzen, G., Mennen, S., Olsen, R.L., Skjerdal, T. 2011. Invasiveness of *Listeria monocytogenes* strains of Caco-2 cells in response to a period of extreme salt stress reflecting salt-curing and rehydration of cod (*Gadus morhua* L.). *Food Control* 22, 1040-1045.

Lou, Y., Yousef, A. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Applied and Environmental Microbiology* 63, 1252-1255.

Luna, M.C. 2014. Alimentos de IV gama. <https://cienciacebas.wordpress.com/2014/04/02/que-es-la-iv-gama/>. Consulta: 03/08/2016.

Ma, C., Li, J.G., Zhang, Q. 2016. Behavior of *Salmonella* spp. on fresh-cut tropical fruits. *Food Microbiology* 54, 133-141.

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 faces, sewage and soil from urban sources. *International Journal of Food Microbiology* 21, 325-334.

Monje, M. 2016. Informe 2016 del sector IV gama y otros refrigerados hortofrutícolas. *Alimarket*. Abril 2016, 217-229.

Nguyen-The, C. 2012. Biological hazards in processed fruits and vegetables - Risk factors and impact of processing techniques. *LWT - Food Science and Technology* 49, 172-177.

Nguyen-The, C., Carlin, F. 1994. The microbiology of minimally processed fresh fruits and vegetables. *Critical Reviews in Food Science and Nutrition* 34, 371-401.

Nightingale, K., Bovell, L., Grajczyk, A., Wiedmann, M. 2007. Combined sigB allelic typing and multiplex PCR provide improved discriminatory power and reliability for *Listeria monocytogenes* molecular serotyping. *Journal of Microbiological Methods* 68, 52-59.

Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M. 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Applied and Environmental Microbiology* 71, 8764-8772.

O'Driscoll, B., Gahan, C.G.M., Hill, C. 1996. Adaptative acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant wich demonstrates increased virulence. *Applied and Environmental Microbiology* 62, 1693-1698.

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 Pathogens and Disease* 6, 669-680.

Olier, M., Pierre, F., Lemaitre, J.P., Divies, C., Rousset, A., Guzzo, J. 2002. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. *Microbiology-Sgm* 148, 1855-1862.

Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martín-Belloso, O. 2009. Effect of ripeness at processing on fresh-cut 'Flor de Invierno' pears packaged under modified atmosphere conditions. *International Journal of Food Science & Technology* 44, 900-909.

Oms-Oliu, G., Alejandra Rojas-Graue, M., Alandes Gonzalez, L., Varela, P., Soliva-Fortuny, R., Hernando Hernando, M.I., Perez Munuera, I., Fiszman, S., Martin-Belloso, O. 2010. Recent approaches using chemical treatments to preserve quality of fresh-cut fruit: A review. *Postharvest Biology and Technology* 57, 139-148.

Ooi, S.T., Lorber, B. 2005. Gastroenteritis due to *Listeria monocytogenes*. *Clinical Infectious Diseases* 40, 1327-1332.

Pang, H.J., Potenski, C.J., Matthews, K.R. 2007. Exposure of *Listeria monocytogenes* to food and temperature abuse using a dialysis tubing culture method. *Journal of Food Safety* 27, 426-444.

Penteado, A.L., de Castro, M., Rezende, A.C.B. 2014. *Salmonella* enterica serovar Enteritidis and *Listeria monocytogenes* in mango (Mangifera indica L.) pulp: growth, survival and cross-contamination. *Journal of the Science of Food and Agriculture* 94, 2746-2751.

Pereira, A.C.M., Britto, J.D., de Carvalho, J.J., de Luna, M.D., Rosa, A.C.P. 2008. Enteroaggregative *Escherichia coli* (EAEC) strains enter and survive within cultured intestinal epithelial cells. *Microbial Pathogenesis* 45, 310-314.

Peterson, L.D., Faith, N.G., Czuprynski, C.J. 2007. Resistance of *Listeria monocytogenes* F365 cells to synthetic gastric fluid is greater following growth on ready-to-eat deli turkey meat than in brain heart infusion broth. *Journal of Food Protection* 70, 2589-2595.

Pricope-Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K. 2013. The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay. *International Journal of Food Microbiology* 166, 59-64.

Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N.A., Moon, H.W. 2000. Cattle lack vascular receptors for *Escherichia coli* O157 : H7 Shiga toxins. *Proceedings of the National Academy of Sciences of the United States of America* 97, 10325-10329.

Ramos-Villarroel, A.Y., Aron-Maftei, N., Martin-Belloso, O., Soliva-Fortuny, R. 2012. Influence of spectral distribution on bacterial inactivation and quality changes of fresh-cut watermelon treated with intense light pulses. *Postharvest Biology and Technology* 69, 32-39.

Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Soliva-Fortuny, R., Martin-Belloso, O. 2009. Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials. *Comprehensive Reviews in Food Science and Food Safety* 8, 157-180.

Reij, M.W., Den Aantrekker, E.D., ILSI-Europe-Risk-Analysis-in-Microbiology-Task-Force. 2004. Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology* 91, 1-11.

Rieu, A., Guzzo, J., Piveteau, P. 2009. Sensitivity to acetic acid, ability to colonize abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on parsley leaves. *Journal of Applied Microbiology* 108, 560-570.

Roche, S.M., Gracieux, P., Milohanic, E., Albert, I., Virlogeux-Payant, I., Temoin, S., Grepinet, O., Kerouanton, A., Jacquet, C., Cossart, P., Velge, P. 2005. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology* 71, 6039-6048.

Rojas-Grau, M.A., Oms-Oliu, G., Soliva-Fortuny, R., Martin-Belloso, O. 2009. The use of packaging techniques to maintain freshness in fresh-cut fruits and vegetables: a review. *International Journal of Food Science and Technology* 44, 875-889.

Rousseaux, S., Olier, M., Lemaitre, J.P., Piveteau, P., Guzzo, J. 2004. Use of PCR-Restriction fragment length polymorphism of *inlA* for rapid screening of *Listeria monocytogenes* strains deficient in the ability to invade caco-2 cells. *Applied and Environmental Microbiology* 70, 2180-2185.

Russell, T.L., Berardi, R.R., Barnett, J.L., Dermentzoglou, L.C., Jarvenpaa, K.M., Schmaltz, S.P., Dressman, J.B. 1993. Upper gastrointestinal pH in 79 healthy, elderly, north-american men and women. *Pharmaceutical Research* 10, 187-196.

Sambuy, Y., Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., Zucco, F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* 21, 1-26.

Sánchez, M.T. 2016. Intalaciones de prerefrigeración. 10º Curso Internacional de Tecnología Postcosecha y Procesado mínimo. ETSIA-UPCT, Cartagena.

Seow, J., Agoston, R., Phua, L., Yuk, H.G. 2012. Microbiological quality of fresh vegetables and fruits sold in Singapore. *Food Control* 25, 39-44.

Seveau, S., Pizarro-Cerda, J., Cossart, P. 2007. Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. *Microbes and Infection* 9, 1167-1175.

Shen, J.L., Rump, L., Zhang, Y.F., Chen, Y., Wang, X., Meng, J.H. 2013. Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. *Food Microbiology* 35, 58-64.

Sillani, S., Nassivera, F. 2015. Consumer behavior in choice of minimally processed vegetables and implications for marketing strategies. *Trends in Food Science & Technology* 46, 339-345.

Smith, J.L. 2003. The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions. *Journal of Food Protection* 66, 1292-1303.

Soliva-Fortuny, R.C., Oms-Oliu, G., Martín-Belloso, O. 2002. Effects of ripeness stages on the storage atmosphere, color, and textural proprerties of minimally processed apple slices. *Journal of Food Science* 67, 1958-1963.

Stopforth, J.D., Yoon, Y., Barmpalia, I.M., Samelis, J., Skandamis, P.N., Sofos, J.N. 2005. Reduction of *Listeria monocytogenes* populations during exposure to a simulated gastric fluid following storage of inoculated frankfurters formulated and treated with preservatives. *International Journal of Food Microbiology* 99, 309-319.

Suarez, M., Gonzalez-Zorn, B., Vega, Y., Chico-Calero, I., Vazquez-Boland, J.A. 2001. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. *Cellular Microbiology* 3, 853-864.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews* 14, 584-640.

VVA Brussels. 2015. Italian Excellence-the value of fresh-cut products in the italian and European market. "http://www.vva.it/content/Upload/AIIPA_ ENG.pdf". Consulta: 01/08/2016.

Walecka, E., Molenda, J., Karpiskova, R., Bania, J. 2011. Effect of osmotic stress and culture density on invasiveness of *Listeria monocytogenes* strains. *International Journal of Food Microbiology* 144, 440-445.

Walker, S.J., Stringer, M.F. 1987. Growth of *Listeria monocytogenes* and *Aeromonas-hydrophila* at chill temperatures. *Journal of Applied Bacteriology* 63, R20-R20.

Wansink, B., Just, D.R., Hanks, A.S., Smith, L.E. 2013. Pre-Sliced Fruit in School Cafeterias Children's Selection and Intake. *American Journal of Preventive Medicine* 44, 477-480.

Ward, T.J., Evans, P., Wiedmann, M., Usgaard, T., Roof, S.E., Stroika, S.G., Hise, K. 2005. Molecular and phenotypic characterization of *Listeria monocytogenes* from US Department of Agriculture food safety and inspection service

surveillance of ready-to-eat foods and processing facilities. *Journal of Food Protection* 73, 861-869.

Wemekamp-Kamphuis, H.H., Wouters, J.A., de Leeuw, P., Hain, T., Chakraborty, T., Abee, T. 2004. Identification of sigma factor sigma(B)-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogene*s EGD-e. *Applied and Environmental Microbiology* 70, 3457-3466.

Werbrouck, H., Botteldoorn, N., Ceelen, L., Decostere, A., Uyttendaele, M., Herman, L., Van Coillie, E. 2008. Characterization of virulence properties of *Listeria monocytogenes* serotype 4b strains of different origins. *Zoonoses and Public Health* 55, 242-248.

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.

Xue, Z.H., Li, J.M., Yu, W.C., Lu, X.T., Kou, X.H. 2016. Effects of nonthermal preservation technologies on antioxidant activity of fruits and vegetables: A review. *Food Science and Technology International* 22, 440-458.

Yousef, A.E., Courtney, P.D. 2003. Basics of stress adaptation and implications in new-generation foods. En: *Microbial stress adaptation and food safety*, (Ed.) Yousef, A.E., Juneja, V.K. CRC Press, Boca Raton.

Yuk, H.G., Jo, S.C., Seo, H.K., Park, S.M., Lee, S.C. 2008. Effect of storage in juice with or without pulp and/or calcium lactate on the subsequent survival of *Escherichia coli* O157:H7 in simulated gastric fluid. *International Journal of Food Microbiology* 123, 198-203.

Yuk, H.G., Schneider, K.R. 2006. Adaptation of *Salmonella* spp. in juice stored under refrigerated and room temperature enhances acid resistance to simulated gastric fluid. *Food Microbiology* 23, 694-700.

Zawistowski, J., Biliaderis, C.G., Eskin, N.A.M. 1991. Polyphenol oxidases. En: *Oxidative enzymes in Food*, (Ed.) Robinson, D.S., Eskin, N.A.M. Elsevier, London, UK. 217-273.

OBJETIVOS

OBJETIVOS

Los objetivos de esta Tesis Doctoral se detallan a continuación:

1. Optimización del proceso de obtención de pera mínimamente procesada

1.1. Seleccionar la variedad de pera más adecuada para el procesado.
1.2. Determinar el tratamiento antioxidante y estabilizante de la textura para mantener la calidad de la pera cortada durante la vida útil.
1.3. Evaluar la calidad fisicoquímica, nutricional, microbiológica y sensorial de un producto de pera mínimamente procesado elaborado y conservado en condiciones comerciales simuladas (4 °C) y incluyendo una rotura de la cadena de frío.

2. Estudiar el efecto de la temperatura de conservación y el estado de madurez de la pera antes del procesado respecto al comportamiento de microorganismos patógenos de transmisión alimentaria

2.1. Efecto de la temperatura de conservación y la rotura de la cadena de frío en el comportamiento de *Salmonella enterica* y *Listeria monocytogenes* en pera mínimamente procesada y conservada en condiciones comerciales simuladas y simulando una rotura de la cadena de frío.
2.2. Efecto del estado de madurez de la pera en el momento del procesado en el comportamiento de *L. monocytogenes* en pera mínimamente procesada conservada a distintas temperaturas.

3. Determinar el efecto de la matriz alimentaria sobre el potencial patogénico de *L. monocytogenes*

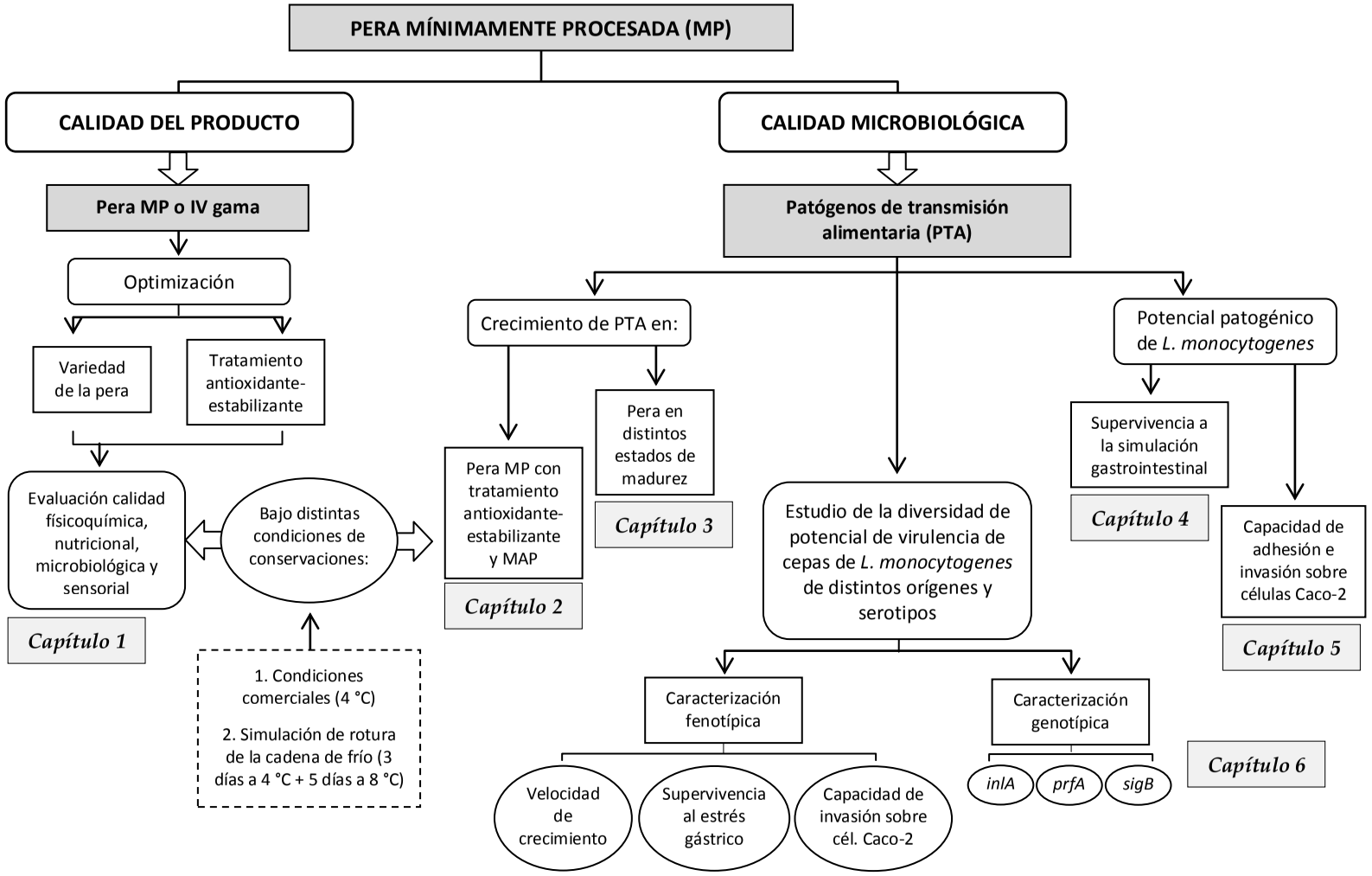
3.1. Supervivencia de *L. monocytogenes* después de la simulación digestiva en función de la matriz en la que ha crecido (pera y melón mínimamente procesados) y temperatura de conservación.
3.2. Efecto del tiempo de conservación y la matriz (pera y melón) en la supervivencia de *L. monocytogenes* después de la simulación digestiva y la posterior habilidad para adherirse e invadir las células epiteliales intestinales (modelo Caco-2).

4. Estudio del potencial de virulencia que presentan cepas de *L. monocytogenes* de distintos orígenes y serotipos, mediante un estudio fenotípico y genotípico

4.1. Caracterización fenotípica de cepas de *L. monocytogenes* de distintos orígenes y serotipos.

4.2. Análisis de secuencia de tres de los genes relacionados con la virulencia y superación al estrés de *L. monocytogenes* de distintos orígenes y serotipos.

PLAN DE TRABAJO


PERA MÍNIMAMENTE PROCESADA (MP)
CALIDAD DEL PRODUCTO
CALIDAD MICROBIOLÓGICA
Pera MP o IV gama
Optimización
Variedad de la pera
Tratamiento antioxidante-estabilizante
Evaluación calidad físicoquímica, nutricional, microbiológica y sensorial
Capítulo 1
Bajo distintas condiciones de conservaciones:
1. Condiciones comerciales (4 °C)
2. Simulación de rotura de la cadena de frío (3 días a 4 °C + 5 días a 8 °C)
Crecimiento de PTA en:
Pera MP con tratamiento antioxidante-estabilizante y MAP
Pera en distintos estados de madurez
Capítulo 2
Capítulo 3
Patógenos de transmisión alimentaria (PTA)
Estudio de la diversidad de potencial de virulencia de cepas de L. monocytogenes de distintos orígenes y serotipos
Caracterización fenotípica
Velocidad de crecimiento
Supervivencia al estrés gástrico
Capacidad de invasión sobre cél. Caco-2
Caracterización genotípica
inlA
prfA
sigB
Capítulo 6
Potencial patogénico de L. monocytogenes
Supervivencia a la simulación gastrointestinal
Capítulo 4
Capacidad de adhesión e invasión sobre células Caco-2
Capítulo 5

METODOLOGÍA

METODOLOGÍA

En este apartado se describirá la metodología utilizada a grandes rasgos, ya que se encuentra con mayor detalle especificada en cada uno de los capítulos que forman esta tesis.

1. Optimización del proceso de obtención de pera mínimamente procesada (*Capítulo 1*)

❖ Seleccionar la variedad de pera más adecuada para el procesado

Se estudiaron las variedades de pera "Conference", "Ercolini", "Flor de Invierno" y "Passa-Crassana" por ser las de mayor producción en nuestra zona y estar además descritas por otros autores por su buena aptitud al procesado mínimo (Figura 1).

Para determinar qué variedad de pera presenta una mejor aptitud al procesado mínimo (desinfección superficie del fruto, pelado, descorazonado, corte en trozos en forma de gajos, baño en solución antioxidante y envasado en atmosfera modificada pasiva), en la etapa del baño antioxidante, se utilizó el producto comercial NatureSeal® AS1 (Agricoat), compuesto por una mezcla de vitaminas y minerales. Este producto es reconocido ampliamente como efectivo para el tratamiento del pardeamiento superficial de frutas mínimamente procesadas.

La fruta se procesó, envasó (120 ± 5 g de fruta en una barqueta de APET (375 ml)) y se selló con un film no pelable APET con perforaciones de 64 μm (permeabilidad al oxígeno de 110 cm^3 m^{-2} d^{-1} atm^{-1}). La fruta se conservó a 5 °C. Después de 7 días, se evaluó la calidad fisicoquímica (composición gaseosa del espacio de cabeza del envase, color y textura de la pulpa, y contenido de sólidos solubles y acidez titulable del zumo preparado a partir de la muestra) de pera mínimamente procesada.

❖ Selección del tratamiento antioxidante y estabilizante de la textura

La finalidad de este estudio fue proporcionar una alternativa al uso de un antioxidante comercial de composición no especificada. En base a los resultados previos, la selección del antioxidante-estabilizante de la textura se realizó en pera "Conference". Se ensayaron tres combinaciones de productos:

- Tratamiento AsAc: 20 g L^{-1} ácido ascórbico + 10 g L^{-1} ácido cítrico + 10 g L^{-1} cloruro de calcio
- Tratamiento CaAs: 20 g L^{-1} ascorbato de calcio + 10 g L^{-1}cloruro de calcio
- Tratamiento NaAs: 20 g L^{-1} ascorbato de sodio + 10 g L^{-1} cloruro de calcio

Su efecto en la calidad de la pera mínimamente procesada se comparó con el tratamiento con NatureSeal® AS1 (Agricoat) y con un tratamiento sin antioxidante-estabilizante de la textura (agua). La fruta se procesó, envasó y conservó a 5 °C, como se había hecho en la selección de la variedad de pera. Después de 7 y 14 días de conservación, se evaluó la calidad fisicoquímica (composición gaseosa del espacio de cabeza del envase, color y textura de la pulpa, y contenido de sólidos solubles y acidez titulable del zumo preparado a partir de la muestra) de pera mínimamente procesada (Figura 1).

- **Evaluación de la calidad fisicoquímica, nutricional, microbiológica y sensorial de un producto de pera mínimamente procesada obtenido en condiciones comerciales simuladas**

De acuerdo a los resultados obtenidos en la selección de variedad de pera y tratamiento antioxidante-estabilizante de la textura, se preparó un producto de pera mínimamente procesada en condiciones comerciales simuladas empleando pera de la variedad "Conference" y aplicando un tratamiento compuesto de ascorbato de calcio (20 g L^{-1}) y cloruro de calcio (10 g L^{-1}), evaluando a continuación su posterior vida útil (Figura 1).

La fruta se procesó, bañó y envasó del mismo modo que se ha descrito anteriormente. En este estudio se evaluaron dos tipos de conservación:

- simulación de condiciones comerciales: las muestras se conservaron a 4 °C durante 8 días.
- simulación de rotura de la cadena de frío durante la conservación: las muestras se conservaron a 4 °C hasta los 3 días, día en que las muestras se pasaron a conservar a 8 °C y se mantuvieron 5 días en estas condiciones, teniendo una conservación total de 8 días.

La determinación de la calidad fisicoquímica (composición gaseosa del espacio de cabeza del envase, color y textura de la pulpa, y contenido de sólidos solubles y acidez titulable del zumo preparado a partir de la muestra), nutricional (contenido

de fenoles totales, actividad antioxidante y acido ascórbico) y microbiológica (recuento población bacterias psicrótrofas, mohos, levaduras, y bacterias lácticas) se realizó en la muestra de pera mínimamente procesada antes y después del tratamiento antioxidante-estabilizante y durante la conservación, a los 3 días y a los 8 días, este último día se evaluó la calidad del producto almacenado en las dos tipologías de conservación. A diferencia de la calidad fisicoquímica, nutricional y microbiológica, la calidad sensorial se evaluó después del tratamiento antioxidante-estabilizante de la pera mínimamente procesada y durante la conservación, a los 3 días y a los 8 días (en condiciones de simulación comercial y de rotura de la cadena de frío).

En el siguiente diagrama se detallan los tratamientos/variedades que se emplearon para la optimización del producto de pera mínimamente procesada, y las posteriores determinaciones que se realizaron en el producto de pera mínimamente procesado (Figura 1).

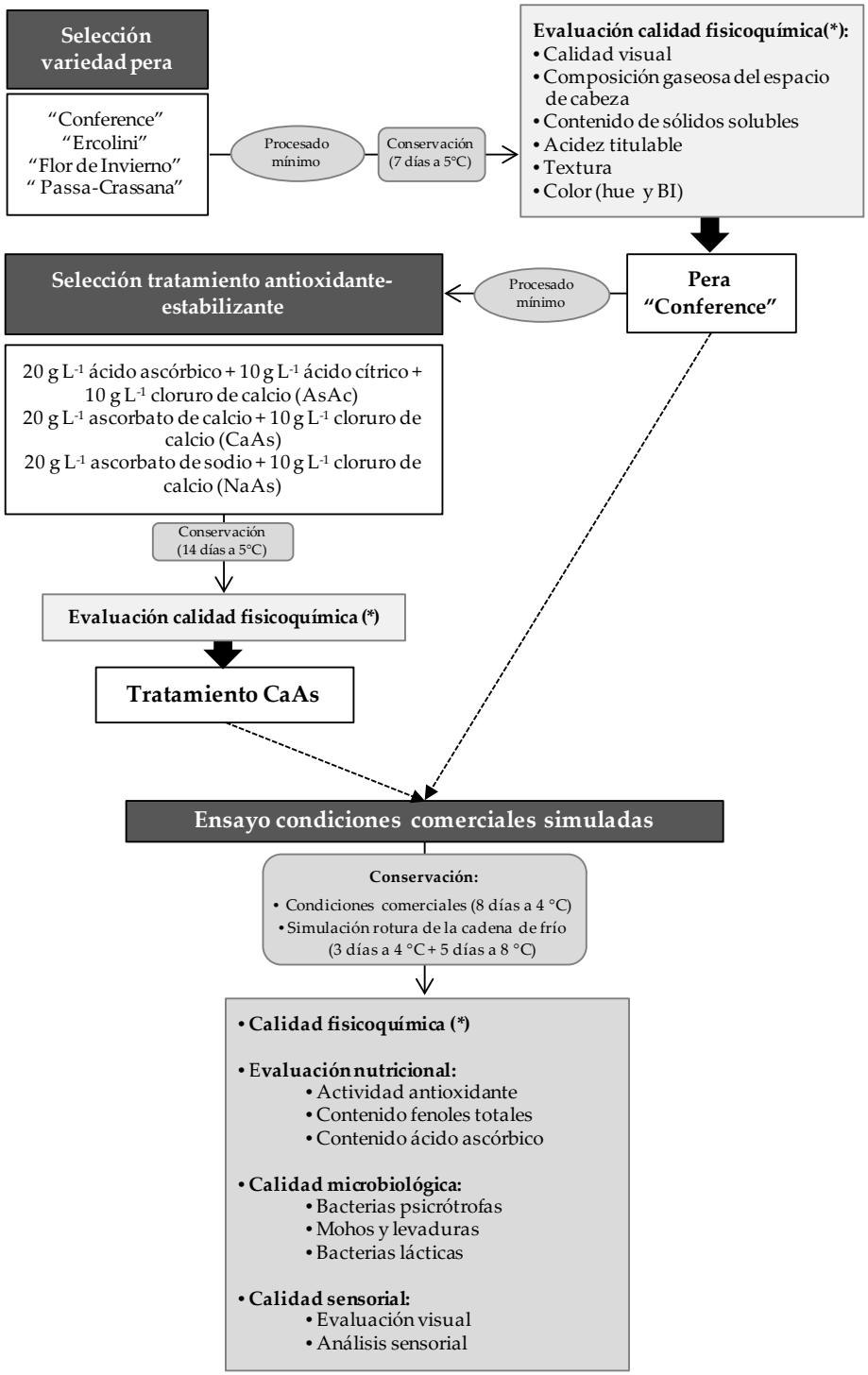


Figura 1. *Proceso de obtención y evaluación de un producto de pera mínimamente procesado.*

2. Estudiar el efecto de la temperatura de conservación y el estado de madurez de la pera antes del procesado en el comportamiento de microorganismos patógenos de transmisión alimentaria

❖ Efecto de la temperatura de conservación y la rotura de la cadena de frío en el comportamiento de *Salmonella enterica* subsp Enteritidis y *Listeria monocytogenes* en pera mínimamente procesada y conservada en condiciones comerciales simuladas (*Capítulo 2*)

Para la determinación del comportamiento de *Salmonella enterica* subsp. Enteritidis y *L. monocytogenes* en pera mínimamente procesada, se empleó un cóctel de 4 cepas de *Salmonella* spp y otro cóctel de 5 cepas de *L. monocytogenes*. Las cepas utilizadas para el cóctel de *Salmonella* fueron, *S. enterica* subsp. Enterica serovar: Agona (BAA-707, ATCC), Michigan (BAA-709, ATCC), Montevideo (BAA-710, ATCC) y Gaminara (BAA-711, ATCC). Las cepas utilizadas para el cóctel de *L. monocytogenes* fueron: *L. monocytogenes* serotipo 1a (CECT 4031), serotipo 3a (CECT 933), serotipo 4d (CECT 940), serotipo 4b (CECT 4032) y serotipo 1/2a (Abadias *et al.*, 2008).

En base a los resultados obtenidos en el *Capítulo 1*, se utilizó pera de la variedad "Conference". De la fruta ya mínimamente procesada (desinfectada, pelada, descorazonada y cortada), se tomaron algunos trozos de pera. Con la ayuda de un sacabocados estéril, se hizo una pequeña hendidura (menor a 5 mm de profundidad) en cada trozo. Toda la fruta, incluyendo los trozos previamente apartados, se bañó en el tratamiento antioxidante-estabilizante compuesto de ascorbato de calcio (20 g L^{-1}) y cloruro de calcio (10 g L^{-1}) durante 2 min a 150 rpm. Después del baño, se dejó escurrir el agua en exceso de la fruta. Se apartaron los trozos de pera que presentaban la hendidura y se inocularon con 15 µL de una suspensión del patógeno (aproximadamente, 10^6 ufc ml^{-1}) de *S. enterica* o *L. monocytogenes*, preparado a partir de un concentrado de las cepas anteriormente descritas. Se dejó que la gota de inóculo se absorbiera y los trozos se envasaron. En cada barqueta se envasó un trozo inoculado más los trozos no inoculados necesarios para completar el peso neto deseado para el envase. El trozo inoculado se dispuso en la parte superior, teniéndolo adecuadamente identificado. La fruta se envasó y selló bajo las mismas condiciones que en el *Capítulo 1*.

El comportamiento de *S. enterica* y *L. monocytogenes* en pera mínimamente procesada se determinó mediante recuento de la población en medios de cultivo

selectivo (Palcam y "Xylose-Lysine-Desoxycholate Agar" (XLD)). El recuento de población se realizó el día de la inoculación y después de 3 y 8 días de almacenamiento, simulando dos tipos de conservación: condiciones comerciales (8 días a 4 °C) y la correspondiente a una rotura de la cadena de frío (3 días a 4 °C más 5 días a 8 °C)).

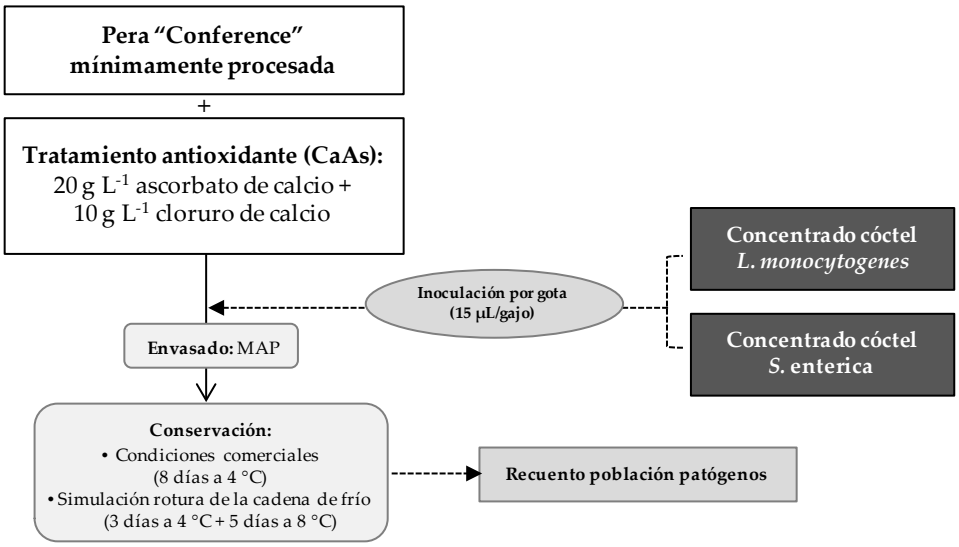


Figura 2. *Efecto de rotura de la cadena de frío en el comportamiento de* S. enterica *y* L. monocytogenes *sobre pera mínimamente procesada y conservada en condiciones comerciales simuladas.*

- **Efecto del estado de madurez de la pera en el momento del procesado en el comportamiento de *L. monocytogenes* en pera mínimamente procesada conservada a distintas temperaturas (***Capítulo 3***)**

Para el estudio del efecto del estado de madurez de la pera en el momento del procesado en relación al comportamiento de *L. monocytogenes* en pera mínimamente procesada conservada a distintas temperaturas, se empleó la cepa de *L. monocytogenes* 230/3 del serotipo 1/2a, aislada de un producto vegetal mínimamente procesado (Abadias *et al.*, 2008).

El estado de madurez de la fruta antes del procesado tiene un efecto sobre la calidad sensorial y la vida útil de la fruta una vez procesada. Sin embargo, no existen estudios sobre el impacto que puede tener este parámetro controlable de procesado en el comportamiento de un patógeno humano como *L. monocytogenes*. Se partió de un mismo lote de peras "Conference", que se acondicionaron a 20 °C hasta obtener las firmezas que se muestran en la tabla 1. El estado de madurez se fijó de acuerdo con la firmeza del fruto, determinado mediante un penetrómetro manual equipado con una sonda de 8 mm de diámetro, en el ecuador de la pera por dos lados opuestos.

Tabla 1. *Estados de madurez de la pera 'Conference' evaluados en el Capítulo 3.*

Estados de madurez	Firmeza (N)
Verde	54-60 N
Parcialmente maduro	43-56 N
Maduro	43-53 N
Sobremaduro	< 31 N

A partir de una suspensión concentrada de *L. monocytogenes* de aproximadamente 10^8 -10^9 ufc ml^{-1}, se preparó una suspensión en agua destilada a la concentración de 10^5 ufc ml^{-1}. La pera "Conference" una vez cortada (desinfectada, pelada, descorazonada y cortada) se bañó en la suspensión de inóculo durante 2 min a 150 rpm, a una proporción 1:2 de fruta/agua. Después del baño, se dejó escurrir el exceso de agua de la fruta y a continuación, se envasó en condiciones de aire y se conservó a tres temperaturas distintas (5, 10 y 20 °C) (Figura 3). Periódicamente, en función de la temperatura de conservación se determinó la población de *L. monocytogenes* mediante siembra en medio selectivo (Palcam).

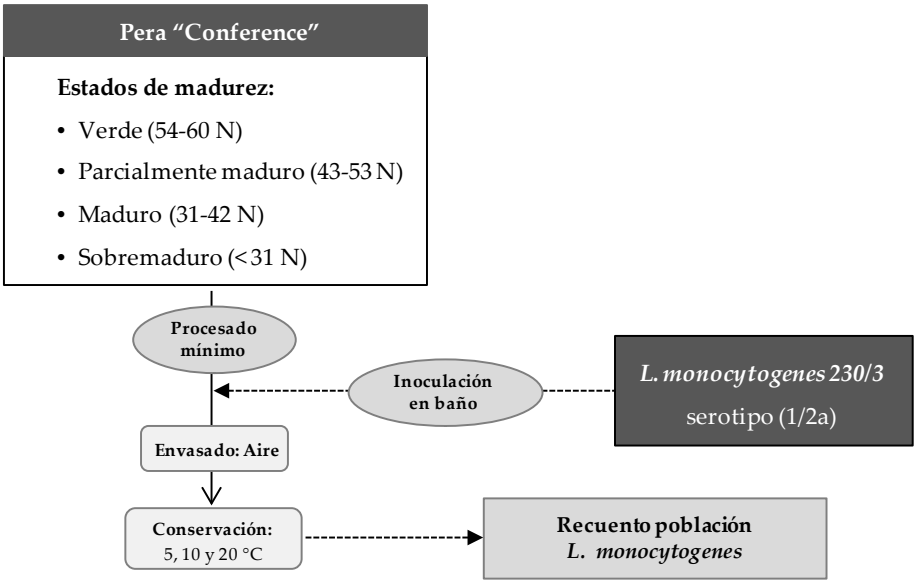


Figura 3. *Efecto del estado de madurez de la pera en el momento del procesado en el comportamiento de* L. monocytogenes *sobre pera mínimamente procesada conservada a distintas temperaturas.*

3. Efecto de la matriz alimentaria sobre el potencial patogénico de *L. monocytogenes*

Tal como se ha comentado en la introducción, *L. monocytogenes* puede presentar una mejor capacidad de supervivencia a la exposición de un estrés ácido fuerte (como el encontrado en el estómago humano), cuando previamente se ha expuesto a un estrés ácido suave (pH 4.5-5.5). La mayoría de estudios se han realizado 'in vitro' en medios de cultivo o bien en matrices alimentarias que no son fruta. Nos interesa evaluar el efecto que puede tener una matriz de fruta, que a la vez presenta el ácido málico como ácido mayoritario y un pH alrededor de 5.0, sobre el potencial patogénico de *L. monocytogenes.* Para estos estudios se empleó otra matriz de fruta mínimamente procesada como es el melón, que contiene como ácido mayoritario el ácido cítrico y presenta un pH cercano a 6.0.

En este capítulo se utilizó pera "Conference" en un estado de madurez parcialmente maduro (43-56 N) y melón de la variedad "Piel de Sapo". La fruta se desinfectó superficialmente, se peló, descorazonó/eliminó las pepitas y se troceó en trozos en forma gajo (pera) o trapecios uniformes (melón). No se aplicó ningún tratamiento antioxidante a la fruta cortada ni se conservó en atmosfera modificada una vez procesada e inoculada, reduciendo así los factores que se estudiaban cada vez (matriz-temperatura).
En este apartado se evaluó el comportamiento de *L. monocytogenes* 230/3 del serotipo 1/2a (aislada de un producto vegetal mínimamente procesado (Abadias *et al.*, 2008)).

- **Evaluación de la supervivencia de *L. monocytogenes* después de la simulación gastrointestinal en función de la matriz (pera y melón mínimamente procesados) y temperatura de conservación (***Capítulo 4***)**

La pera "Conference" y el melón "Piel de Sapo" mínimamente procesados como se ha descrito anteriormente, se inocularon mediante baño, a una concentración de 10^5 ufc ml^{-1} de *L. monocytogenes* 230/3, inoculado a partir de un concentrado preparado el mismo día del ensayo. Después del baño, se dejó escurrir el agua en exceso de la fruta y las muestras se almacenaron a cuatro temperaturas de conservación distintas (1, 5, 10 y 20 °C). Con el mismo concentrado de *L. monocytogenes* empleado para inocular la fruta, se inocularon por triplicado Erlenmeyers con medio de cultivo rico ("triptona soya browth" (TSB)

enriquecido con extracto de levadura, TSBYE) para ser utilizados como control de crecimiento frente a las matrices de fruta.

El comportamiento de *L. monocytogenes* en pera y melón después de la simulación gastrointestinal en las muestras conservadas a 1 y 5 °C, se evaluó el mismo día de la inoculación y después de 1, 2, 6 y 9 días de conservación. Las muestras conservadas a 10 °C, se evaluaron el mismo día de la inoculación y después de 1, 2 y 6 días de conservación. Finalmente, las muestras conservadas a 20 °C, se evaluaron el mismo día de la inoculación y después de 24 y 48 h de conservación.

La simulación gastrointestinal de este capítulo incluye, la simulación de la masticación y el paso por la boca (5 min 37 °C), seguido de la entrada al estómago (pH 3.5 durante 1 h a 37 °C) y por último la llegada al intestino (sales biliares 2 h a 37 °C). La composición de las soluciones se describe de forma detallada en el *Capítulo 4* y *5* de esta tesis. Una vez preparadas se filtraron (0.22 μm) y se ajustó el pH (solución salival (pH 6.5), solución gástrica (pH 2.0), solución duodenal (pH 7.8) y solución biliar (pH 8.0)). Las soluciones contienen enzimas, estos se añadieron una hora antes del ensayo. Antes del inicio del ensayo, las soluciones se atemperaron a 37 °C, temperatura óptima para la actividad de las enzimas.

Brevemente, para simular el paso por el tracto gastrointestinal, se tomaron 10 g de muestra (pera o melón mínimamente procesados) dispuestos en una bolsa estéril con filtro, se añadieron 9 ml de solución salival, se homogenizó (2 min) e incubó durante 5 min 37 °C. A continuación, se añadieron 13.5 ml de solución gástrica, simulando la entrada al estómago (pH 3.5), se homogenizo e incubó durante 1 h a 37 °C. Por último, se añadieron a la bolsa 27 ml de solución duodenal y 9 ml de solución biliar, incubándose a continuación durante 2 h a 37 °C.

A lo largo de todo el proceso, en las distintas etapas se extrajeron alícuotas para el recuento de población de *L. monocytogenes*, a la vez que se midió el pH de las muestras mediante un pH-metro. El pH de la muestra al inicio de la etapa gástrica se ajustó para que la muestra se mantuviese a pH 3.5 durante toda esta etapa.

- **Efecto del tiempo de conservación y la matriz (pera y melón) en la supervivencia de *L. monocytogenes* después de la simulación digestiva y la posterior habilidad para adherirse e invadir las células epiteliales intestinales (modelo Caco-2) (*Capítulo 5*)**

Se ha observado que los mecanismos que confieren la capacidad de superar mejor un estrés ácido fuerte pueden tener también un efecto en la capacidad de virulencia de *L. monocytogenes*. Por este motivo, se evaluó el efecto que podría tener el tiempo de conservación en dos frutas distintas en la capacidad de adhesión e invasión del patógeno sobre un modelo 'in vitro' de epitelio intestinal. Para la simulación del epitelio intestinal, se utilizó la línea celular Caco-2 que son células humanas tumorales que presentan características morfológicas y bioquímicas similares a los enterocitos diferenciados, y que además tienen la capacidad de formar una monocapa polarizada que imita al epitelio intestinal humano. Para el ensayo de adhesión e invasión, estas células se cultivaron sobre placas de cultivo de 12 pocillos, en un medio rico ("Dulbecco's modified Eagle's medium" (DMEM) enriquecido en un 20 % de suero fetal de bovino y 1 % de una combinación de dos antibióticos (Penicilina y estreptomicina)). Cada dos días el medio de los pocillos se cambió, hasta que las células formaron la monocapa (aproximadamente unos 10 días).

La pera "Conference" y el melón "Piel de Sapo" se procesaron e inocularon mediante baño, a una concentración de 10^7 ufc ml^{-1} de *L. monocytogenes* 230/3. En esta ocasión las muestras se conservaron a 10 °C durante 7 días y la capacidad de supervivencia de *L. monocytogenes* al estrés de la simulación gastrointestinal se evaluó el día de la inoculación y después de 2 y 7 días de conservación. Después de la exposición del patógeno crecido en pera o melón mínimamente procesados a la simulación gastrointestinal, se evaluó su capacidad de adhesión e invasión sobre la monocapa de células Caco-2. Previa a la inoculación de la monocapa, la suspensión de *L. monocytogenes* recogida al final de la etapa intestinal se concentró y se resuspendió en medio DMEM.

Para el ensayo de adhesión e invasión, se eliminó y se lavó el medio de cultivo de la placa de células Caco-2 y se substituyó por un medio simple DMEM sin antibiótico. Una vez listas las células, se inocularon seis pocillos con 40 µl de la suspensión de muestra (6 pocillos para cada replica de cada tratamiento). La placa se incubó durante 1 h a 37 °C con el 5 % de CO_2. Pasado este tiempo las células se lavaron, y se lisaron las células de tres de los pocillos. Se recuperó la muestra de los tres pocillos como una sola y se determinó la población de

L. monocytogenes para cada replica del tratamiento, obteniendo a partir de este recuento la cantidad de células patógenas adheridas y invadidas después de 1 h. A las células de los tres pocillos restantes se les añadió medio DMEM enriquecido con antibiótico (gentamicina) y se incubaron 2 h a 37 °C con el 5 % de CO_2. Pasado este tiempo las células se lavaron, para eliminar los restos de antibiótico, y se lisaron las células Caco-2 de los pocillos restantes. Se recuperó la muestra de los tres pocillos como una sola replica del tratamiento y se determinó la población de *L. monocytogenes*, obteniendo a partir de este recuento la cantidad de células patógenas que habían invadido las células Caco-2.

En la parte superior de la siguiente figura se detallan las etapas que componen la simulación gastrointestinal con los parámetros y tiempos que se aplicaron. En la parte inferior, se detalla las etapas del ensayo de adhesión e invasión (Figura 4).

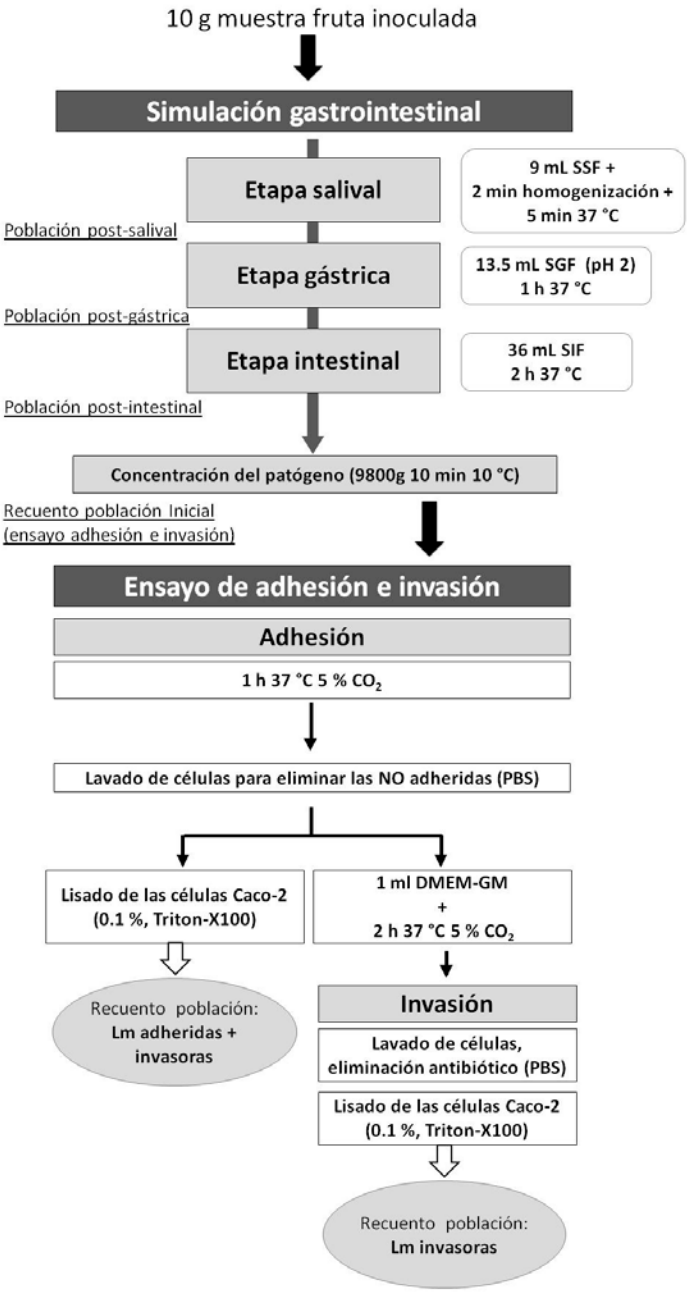


Figura 4. *Proceso completo para la evaluación del potencial de virulencia de* L. monocytogenes.

4. Estudio de la diversidad del potencial de virulencia que presentan cepas de *L. monocytogenes* de distintos orígenes y serotipos (*Capítulo 6*)

No todos los 13 serotipos conocidos de *L. monocytogenes* han sido responsables de toxiinfecciones alimentarias. Sin embargo, los serotipos 1/2a, 1/2b y 4b se han aislados de casos clínicos en un mayor número de ocasiones. Estos serotipos han presentado un mayor potencial de virulencia sobre los seres humanos que otros serotipos, ya que han sido causantes de listeriosis.

Para poder entender esta diferencia de potencial de virulencia entre cepas de *L. monocytogenes* de serotipos y orígenes distintos, se realizó una caracterización a nivel fenotípico y genotípico. Se estudiaron 53 aislados de *L. monocytogenes* de dos orígenes distintos (clínico o alimentario), pertenecientes a tres serotipos concretos (1/2a, 1/2c y 4b), del laboratorio "Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM)" de Bilthoven junto con la *L. monocytogenes* 1/2a que se aisló a partir de un producto vegetal mínimamente procesado en el laboratorio de Microbiología de vegetales del Fruitcentre-IRTA de Lleida (Abadias *et al.* 2008). Esta última cepa es la que se ha estudiado más detalladamente en esta tesis. El número de cepas por origen y serotipo que se utilizaron se muestran en la Tabla 2, pero las cepas se enumeran y describen (origen y serotipo) detalladamente en el *Capítulo 6* de esta tesis.

Tabla 2. *Distribución por origen y serotipo de las 53 cepas de* L. monocytogenes utilizadas.

Serotipo	Número total de cepas	Origen	
		Clínico	Alimentario
1/2a	19	10	9
1/2c	14	11	3
4b	20	11	9
Total	53	32	21

❖ **Caracterización fenotípica de cepas de *L. monocytogenes* de distintos orígenes y serotipos**

Para la caracterización fenotípica de las 53 cepas de *L. monocytogenes*, se determinaron:

- la capacidad de supervivencia a un estrés gástrico (pH 3.5, HCl) de cada aislado.
- la velocidad de crecimiento de la cepa sobre un medio de cultivo líquido rico ("Brain Heart Infusion", BHI) en las condiciones de crecimiento óptimas.
- la capacidad de invasión de cada cepa crecida en condiciones óptimas sobre una monocapa de células Caco-2.

❖ **Caracterización genotípica de cepas de *L. monocytogenes* de distintos orígenes y serotipos**

Para el estudio de la diversidad de secuencias génicas que presentan cepas de *L. monocytogenes* de distintos orígenes y serotipos, nos centramos en el estudio de tres de los genes implicados en la capacidad de virulencia y superación del estrés ambiental de esta bacteria. Concretamente, se secuenciaron los genes *inlA*, *prfA* y *sigB* de las 53 cepas de *L. monocytogenes*. Para realizarlo se siguieron los siguientes pasos:

- secuenciación de los tres genes (*inlA*, *prfA* y *sigB)*
- ensamblaje de las secuencias de nucleótidos obtenidas de cada gen, con ayuda de la bioinformática.
- determinación de los cambios que presentaban las tres secuencias de nucleótidos en comparación con la cepa modelo *L. monocytogenes* EDGe (serotipo 1/2a).

Por último, se estudió la relación entre la presencia de mutaciones en estas tres secuencias con las características fenotípicas de cada cepa de *L. monocytogenes* determinadas anteriormente.

En el siguiente esquema se describen los análisis llevados a cabo para determinar el perfil fenotípico y genotípico de cada cepa de *L. monocytogenes* (Figura 5).

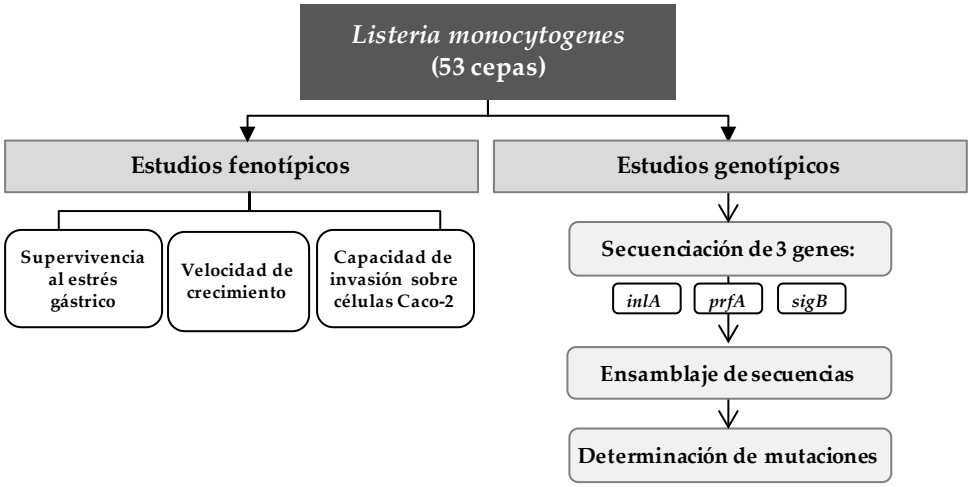


Figura 5. *Estudios realizados para caracterizar las 53 cepas de* L. monocytogenes.

Los estudios descritos en el *Capítulo 6* se realizaron en las instalaciones del "Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM)" durante una estancia de cinco meses en Bilthoven (Holanda).

RESULTADOS

CAPÍTULO 1

Development of a fresh-cut product based on pears and the subsequent evaluation of its shelf life under commercial conditions and after a cold chain break

P. Colás-Medà, M. Abadias, R. Altisent, I. Alegre, L. Plaza, V. Gilabert, R. Lacomba, I. Viñas.

Journal of Food and Nutrition Research (2016), Vol. 4, No. 9, 582-591

Abstract

Processing of pears as a fresh-cut product could offer added value and introduce a product into the market that offers greater convenience and health benefits for consumers. Cultivar selection is one of the most important considerations for fresh-cut fruit processing because characteristics such as flesh texture, skin colour, and browning potential can vary greatly among cultivars. Four pear cultivars ('Flor de invierno', 'Passe-Crassane', 'Ercolini' and 'Conference') and four antioxidant treatments, that is, (NS) 50 g L^{-1} NatureSeal® AS1 (Agricoat) solution, (AsAc) 20 g L^{-1} ascorbic acid + 10 g L^{-1} citric acid + 10 g L^{-1} calcium chloride solution, (CaAs) 20 g L^{-1} calcium ascorbate + 10 g L^{-1} calcium chloride solution and (NaAs) 20 g L^{-1} sodium ascorbate + 10 g L^{-1} calcium chloride solution, were tested to obtain a high-quality fresh-cut pear. For the selected cultivar and treatment, the nutritional changes and physicochemical, microbial and sensorial quality were evaluated under conditions that simulated commercial application followed by storage at 4 °C and a simulated cold chain break at 8 °C. The 'Conference' pear was selected as the best cultivar based on its physicochemical characteristics (high levels of soluble solids content and low acidity), low increase in browning index, and visual acceptance after 7 days of storage. The results demonstrated that CaAs maintained the fresh-cut pear quality after 8 days of storage at 4 °C and also after a cold chain break. Furthermore, application of the selected treatment produced an increase in the ascorbic acid content, total phenolic content and antioxidant activity of minimally processed pear samples. These values were reduced during shelf life, but the total phenolic content at the final sampling point was higher than that of fresh-cut pears after processing without treatment.

Keywords: Minimally processed, variety, anti-browning, total phenolic content, ascorbic acid, antioxidant activity.

1. Introduction

Fruit and vegetables are important components of a healthy diet, and sufficient daily consumption could aid in prevention of major diseases such as cardiovascular diseases and certain cancers. In the last decade, several countries joined to launch international health recommendations that promoted the consumption of 400 to 500 g of fruit and/or vegetables per day, the equivalent of five 80 g servings. This approach to improved consumption was referred to as '5 a day'. Our area (Lleida, Catalonia) of Spain is the country's main producer of pears (176.640 tons produced in 2014), which are primarily commercialized as fresh fruit, and different cultivars such as 'Blanquilla', 'Conference', 'Ercolini', 'Llimonera' and others are grown (DAAM, 2015). Processing of pears as a fresh-cut product could create added value and introduce a product to the market that offers greater convenience and health benefits to consumers. Cultivar selection is one of the most important considerations in fresh-cut fruit processing because characteristics such as flesh texture, skin colour, and browning potential can vary greatly among cultivars (Amiot *et al.*, 1995). The suitability of different cultivars for processing has been previously studied (Amiot *et al.*, 1995; Arias *et al.*, 2008; Gorny *et al.*, 2000; Gorny *et al.*, 1999), but certain pear cultivars in our area have not been studied. Minimal processing operations damage the tissue integrity of fruit, causing an increase in physiological activity and leading to biochemical changes such as browning, off-flavour development and softening (Oms-Oliu *et al.*, 2009). Enzymatic browning occurs when o-diphenol substrates react with oxygen to generate o-quinones, which subsequently polymerize and result in dark melanins. The oxidative reaction is catalysed by polyphenoloxidase (PPO) (Yoruk, Marshall, 2003). To minimize this visual deterioration, treatments that involve dipping of fruit slices into aqueous solutions containing antioxidants and calcium salts are widely practiced to improve the quality of fresh-cut fruit. A great number of studies have been conducted to avoid browning surfaces on fresh-cut pears using selected reducing agents such as ascorbic acid, 4-hexylresorcinol, cysteine, N-acetylcysteine and sodium eritorbate (Arias *et al.*, 2008; Dong *et al.*, 2000; Oms-Oliu *et al.*, 2006; Sapers, Miller, 1998; Soliva-Fortuny *et al.*, 2002). These acidifying additives have a reduction action against quinones, and diphenol prevent browning of minimally processed fruit because it produces only colourless derivates (Arias *et al.*, 2008). Another concern related to extension of shelf life for fresh-cut fruit is softening, which is primarily due to enzymatic degradation of the cell wall, which is mainly composed of cellulose, hemicelluloses and pectins. Calcium salts, and particularly calcium chloride and lactate, are generally used in combination with browning inhibitors as firmness-

maintaining agents in a wide range of cultivars of fresh-cut fruit and vegetables (Alandes *et al.*, 2006). Calcium can interact with the free carboxyl groups liberated by the de-esterification of pectin by pectinmethylesterase (PME) to form insoluble calcium pectates, which strengthen the structure of the cell wall (Oms-Oliu *et al.*, 2010).

To develop a fresh-cut pear product, the main considerations are selection of the most appropriate cultivar, stage of ripeness at cutting, choice of the best antioxidant treatment, and selection of adequate packaging. During storage of the packaged product, certain changes occur in the surrounding atmosphere. These changes depend on the respiratory activity of the product, its storage temperature, the permeability of the packaging films and the ratio of the packaging area to the amount of fruit (Gorny *et al.*, 1998, 1999; Sapers *et al.*, 1998). The low O_2 and/or elevated CO_2 environment generated by modified atmosphere packaging of fresh-cut product can extend the product shelf life by slowing the browning reactions at the cut surfaces, reducing the rates of product transpiration (water loss) and respiration, and reducing ethylene biosynthesis and action (Arias *et al.*, 2008; Gorny, 2003). The aim of this study was to select the best cultivar and antioxidant treatment to obtain a high-quality fresh-cut pear. For the selected cultivar and treatments, the physicochemical quality, nutritional changes, microbial quality and sensorial quality were evaluated at conditions that simulated commercial application at 4 °C and a cold chain break at 8 °C.

2. Materials and methods

2.1. Selection of the most suitable pear cultivar

2.1.1. Fruit and fruit processing

Four pear cultivars ('Flor de invierno', 'Passe-Crassane', 'Ercolini' and 'Conference') were purchased at commercial maturity from commercial orchards in Lleida (Catalonia, Spain). Before processing, the flesh firmness of whole pears from each cultivar was measured on opposite sides of each fruit with a penetrometer (Effegi, Mila, Italy) equipped with a probe 8 mm in diameter. Eight fruits per cultivar were measured, and the results were reported in Newtons (N). Prior to experimental studies, pears were disinfected by immersion in a 0.1 g L^{-1} sodium hypochlorite (NaClO) solution (pH 6.5) for 2 min, rinsed in running tap water and allowed to dry at room temperature. Pears were peeled and cut into 10 wedges using a handheld apple corer and slicer.

2.1.2. Antioxidant treatment

NatureSeal® AS1 was used to select the pear cultivar because its effect was widely studied and is effective in different fresh-cut fruit. Pear wedges were treated by immersion in an antioxidant solution of 50 g L^{-1} NatureSeal® AS1 (NS, Agricoat) (w/v), and distilled water was used as a control (CK). In brief, pear wedges were dipped (1:2 w/v) for 2 min at 150 rpm on an orbital shaker in cold water plus the corresponding treatment. After treatment, the wedges were allowed to dry at room conditions. Fresh-cut pears (120 ± 5 g) were placed in polypropylene terephthalate trays (APET, 375 ml) and sealed with a non-peelable polypropylene terephthalate plastic film (APET-110, ILPRA, Italy) with a thickness of 64 μm and an O_2 permeability of 110 cm^3 m^{-2} d^{-1} atm^{-1} at 23 °C. This packaging was chosen based on a previous short trial. Trays were stored at 5 ± 1 °C, and samples were examined after treatment (0 day) and after 7 days.

2.1.3. Fresh-cut fruit quality evaluation

To determine the most suitable pear cultivar, surface colour, texture, soluble solids content (SSC) and titratable acidity (TA) were assessed after fruit processing. After 7 days of storage at 5 °C, before the quality evaluation, the headspace gas composition was determined using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) and the visual acceptance was evaluated. Surface colour was determined immediately after that trays were opened. Afterwards the rest of determinations were done.

The visual evaluation of pear wedges from different cultivars and treatments (CK and NS, three trays per treatment) was conducted by an untrained panel using a 9-points hedonic scale: 9=excellent; 7=very good; 5=good (limit of marketability); 3=fair (limit of usability); and 1=poor (inedible) (Gil *et al.*, 1998). An average was obtained for each cultivar and treatment after 7 days of storage.

After fruit processing and after 7 days of storage, the surface colour of pear wedges was determined using a chromameter (model CR-200 Minolta, Minolta Inc., Tokyo, Japan). Colour readings were measured on both sides of five pear wedges (n= 10) per cultivar and treatment on the day of processing (0 day), and five wedges per tray were examined in each cultivar and treatment (n= 30) after storage. Data were obtained as CIELab* values but results were expressed as the hue angle (h°= arctan (b*/a*)) and the browning index (BI) value (BI= 100*(x-0.31)/0.172, where x= (a* + 1.75 L*)/ (5.645 L* + a* - 3.012 b*)) according to Buera *et al.* (1986).

Prior the texture evaluation, the pear wedges were cut into 20 x 20 mm pieces. The texture of fresh-cut pears was evaluated after processing and after storage according to Altisent *et al.* (2014) parameters. Five texture measurements per cultivar and treatment were performed after processing (0 day), and three measurements per tray were performed per cultivar and treatment (n= 9) after 7 days of storage.

At each sampling point, the pear wedges were squeezed, and the soluble solids content (SSC) was determined using a handheld refractometer at 20 °C (Atago CO., LTD, Japan). Three measurements were collected per treatment (one measurement per tray), and the results were reported as percentage of soluble solids in fruit juice (%).To measure titratable acidity (TA), triplicate samples of 10 ml of extracted fruit were diluted with 10 ml of distilled water, and 2 drops of phenolphthalein solution 1% RV (Panreac, Barcelona, Spain) were added. The solutions were titrated with sodium hydroxide solution (NaOH, 0.1 mol L^{-1}) until a colour change of the pH indicator occurred. Three measurements were collected per treatment. The results were calculated in terms of g of malic acid per litre of solution.

2.2. Antioxidant selection

2.2.1. Fruit processing

Selection of antioxidant treatment was performed with 'Conference' pears, which were used at their optimum ripeness stage (44 ± 32 N), according to Soliva-Fortuny *et al.* (2004) and our previous experiences. Flesh firmness was measured as described previously. To obtain this ripeness stage, pears were stored at 20 °C until they reached the desired firmness. Pears were subsequently subjected to the processing operations described above.

2.2.2. Antioxidant treatment

In order to evaluate an alternative antioxidant treatment to control browning in fresh-cut pears, the following treatments were tested: (AsAc) 20 g L^{-1} (w/v) ascorbic acid + 10 g L^{-1} (w/v) citric acid + 10 g L^{-1} (w/v) calcium chloride solution, (CaAs) 20 g L^{-1} (w/v) calcium ascorbate + 10 g L^{-1} (w/v) calcium chloride solution and (NaAs) 20 g L^{-1} (w/v) sodium ascorbate + 10 g L^{-1} (w/v) calcium chloride solution. Fresh-cut pears without antioxidant treatment (distilled water, CK) and treated with the commercial NS product (50 g L^{-1} NatureSeal® AS1) were included as controls to evaluate the effectiveness of the proposed antioxidant

combinations. The concentrations of the antioxidant agents were chosen in accordance to current bibliography. All chemical products evaluated in this study are currently approved for use as food additives in minimally processed fruit (EU Commission, 2011). The antioxidant applications in the 'Conference' pear wedges were conducted by immersion, as described above. Subsequently, fresh-cut pears (120 ± 5 g) were placed in the same APET trays and sealed.

Trays were stored at 5 ± 1 °C, and samples were examined on the day of preparation (0 day) and after 7 (three trays) and 14 days of storage (three trays). In addition, headspace gas composition, visual quality, colour, texture, SSC and TA were assessed as previously described. Headspace gas composition and visual quality were only determined after the storage periods.

2.3. Semi-commercial assay

Based on previous results, an assay simulating commercial conditions was performed with the 'Conference' pear and CaAs (20 g L^{-1} (w/v) calcium ascorbate + 10 g L^{-1} (w/v) calcium chloride) as the antioxidant. Water was used as a control (CK). The pears were processed, treated with antioxidant solution, and packaged as described above. Three trays per each treatment were examined at 0 day and after 3 days of storage at 4 ± 1 °C. The remainder of the samples were divided into two lots with one stored at 8 ± 1 °C until 8 days (simulated cold chain break) to simulate more realistic conditions during transport and in the refrigerated display window, and the other was maintained at 4 ± 1 °C until 8 days (realistic cold chain conditions). After 3 days of storage at 4 °C, after 8 days of storage at 4 °C (realistic cold chain conditions) and after 8 days of storage under simulated cold chain break (3 days at 4 °C plus 5 days at 8 °C), the same evaluations were performed as in the previous steps: headspace gas composition, visual quality, colour, texture, SSC and TA. In addition, nutritional analysis, microbial quality and consumer acceptability were evaluated.

2.3.1. Nutritional evaluation: Bioactive compounds and antioxidant activity

Furthermore, the semi-commercial assay ascorbic acid content, total phenolic content and antioxidant activity of samples were determinate at processing day (0 day) and after 3 and 8 days of storage (at 4 or 8 °C). Determination of the ascorbic acid content was performed as described by Altisent *et al.* (2014) with minor modifications. The results were expressed as grams of ascorbic acid per kg of fresh weight. For antioxidant activity and total phenolic content determination,

6 g of frozen sample was homogenized with 20 ml of methanol 70%. The mixture was centrifuged, filtered and adjusted to 25 ml with extraction solution (30 g L^{-1} meta-phosphoric acid + 80 ml L^{-1} acetic acid). With the extracts obtained, the antioxidant activity was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical scavenging assay following the procedure described previously (Altisent *et al.*, 2014) with minor modifications. The total phenolic content was determined by the Folin-Ciocalteu method with certain modifications. The antioxidant activity results and the total phenolic content results were expressed as mmoles of ascorbic acid equivalents per kg of fresh weight and as grams of gallic acid per kg of fresh weight, respectively.

2.3.2. Microbial quality

The microbial quality of minimally processed 'Conference' pears treated with antioxidant solutions was evaluated during the shelf life. At each sampling time, 25 g of each tray were diluted in 225 ml of buffer peptone water (BPW, Oxoid) and homogenized in a stomacher blender (IUL, Masticator, Spain) at 250 impact s^{-1} for 90 s. Serial dilutions of the suspension were conducted in sterile buffer peptone water (BPW, Oxoid) and analysed for psychrotrophic microorganisms (PM), yeasts and moulds (YM), and lactic acid bacteria (LAB) according to standard (ISO) methodologies (ISO 17410:2001, ISO 21527-1:2008, ISO 15214:1998, respectively). In brief, aliquots of serial dilutions were spread onto plates with PCA (plate count agar, Biokar) and DRBC (Dichloran Rose Bengal Chlorotetracycline agar, Biokar) for psychrotrophic microorganism and yeasts and moulds enumeration, respectively, and placed by inclusion in MRS agar (Man-Rogosa-Sharpe, Biokar) for lactic acid bacteria count. PCA plates were incubated at 6.5 ± 1 °C for 10 days, DRBC plates were incubated at 25 ± 1 °C for 5 days, and MRS plates were incubated at 37 ± 1 °C for 7 days. The results were reported as log Colony Forming Units (CFU) per gram of fresh weight. Three determinations per treatment (three trays) were performed in duplicate at each sampling point.

2.3.3. Consumer acceptability

The consumer acceptability test was conducted under controlled conditions (illumination and temperature) with 16 volunteers from the staff of the research centre. The samples were evaluated as described previously by Altisent *et al.* (2014). The overall acceptability was expressed as the percentage of consumers satisfied (scoring 6 or more in a 9-point hedonic scale), the percentage of

consumers who rated that sample as neither liked nor disliked (score=5), and finally, the percentage of consumers that disliked the product (scoring less than 5 in a 9-point hedonic scale).

2.4. Statistical analysis

All data were evaluated using analysis of variance (ANOVA) with JMP®8 statistical software (SAS Institute, Cary, NC, USA). Significant differences between treatments were analysed by Tukey's Honest Significant Difference (HSD) test at a significance level of $P < 0.05$.

3. Results and discussion

3.1. Selection of most suitable pear cultivar

The firmness values of whole pears were determined before processing and displayed ranges of 37.2-53.9 N, 58.8-67.6 N, 49.0-65.6 N and 41.2-52.9 N for 'Flor de invierno', 'Passe-Crassane', 'Ercolini' and 'Conference', respectively (data not shown). Table 1 presents the physicochemical characteristics of the four studied pear cultivars after processing. Significant differences in soluble solids content (SSC) were observed among cultivars.

Table 1. *Physicochemical parameters of fresh-cut pear cultivars after processing.*

	SSC (%)	TA (g malic acid L^{-1})	Hue angle (h°)
'Flor de invierno'	11.5 ± 0.0 c	2.5 ± 0.1 b	103.0 ± 2.2 ab
'Passe-Crassane'	13.8 ± 0.1 b	2.7 ± 0.0 a	102.3 ± 2.2 ab
'Ercolini'	11.3 ± 0.0 d	1.5 ± 0.1 c	104.2 ± 1.6 a
'Conference'	15.0 ± 0.1 a	1.4 ± 0.0 c	101.4 ± 1.9 b

Values are expressed as the mean of three values ± standard deviation for SSC and TA and the mean of ten values ± standard deviation for the hue angle. For each parameter, different lowercase letters (a, b, c and d) in the same column indicate significant differences ($p < 0.05$) among pear cultivars according to Tukey's test

'Conference' had the highest SSC value (15.0%), and 'Ercolini' had the lowest (11.3%). The titratable acidity (TA) ranged from 1.4 and 1.5 g malic acid L^{-1} ('Conference' and 'Ercolini', respectively) to 2.7 g malic acid L^{-1} ('Passe-Crassane'). After dipping pear wedges in NatureSeal® AS1 (Agricoat), unremarkable changes were observed in SSC and TA (data not shown). The hue angle (h°) is an indicator to determine the colour of the flesh. The 'Conference' pears presented a more yellowish colour of the flesh (h° 101.4) than 'Ercolini' pears (h° 104.2) attributed to cultivar differences. After 7 days of storage at 5 °C, samples were analysed again. Untreated (water) and NatureSeal® AS1 (NS) pear wedges did not show significant changes in SSC and TA throughout storage (data not shown). Only untreated 'Conference' pear slices experienced a large decrease in flesh firmness (from 15.61 N to 11.04 N) after storage at 5 °C for 7 days, whereas 'Flor de invierno', 'Passe-Crassane' and 'Ercolini' did not show significant declines in firmness (data not shown). When pear slices were treated with NS, no significant differences in firmness were observed after storage for all cultivars (data not shown).

Table 2. *Variation of the browning index of pear wedges untreated and treated with NatureSeal® AS1 after 7 days of storage at 5 °C.*

	ΔBI	
	Untreated wedges	Treated wedges
'Flor de invierno'	11.4 a *	6.5 a
'Passe-Crassane'	13.4 a *	1.8 b
'Ercolini'	3.0 b	2.0 b
'Conference'	10.8 a *	2.5 b

Values are the mean of thirty values ± standard deviation. Different letters in untreated and treated samples indicate significant differences among cultivars. An asterisk between the untreated and treated columns for each cultivar indicates that significant differences were observed among untreated and treated samples after 7 days of storage according to Tukey's test ($p < 0.05$)

To evaluate the susceptibility of pears to browning during shelf life, the browning index (BI) was evaluated. Browning is one of the majors concerns to fresh-cut processors because it has a direct effect on the consumer's purchase decision. The

browning index after processing was significantly different among the pear cultivars ('Flor de invierno': 8.89, 'Passe-Crassane': 14.23, 'Ercolini': 11.70, and 'Conference': 15.70). It is because each cultivar has a different phenolic concentration which is the polyphenol oxidase (PPO) substrate. The increase in the BI after 7 days of storage compared with the initial values is presented in Table 2. The increase in the BI was higher in untreated pears. In treated pear wedges, 'Flor de invierno' was the cultivar that presented the highest increase in BI (6.51). The 'Flor de invierno' pear would not be an appropriate cultivar to be processed because presented the highest ΔBI even with antioxidant treatment. In contrast, ΔBI in 'Ercolini' was the lowest, and no effect of antioxidant treatment was observed.

After 7 days of storage, the headspace gas composition of the packages was measured. In both samples (CK and NS), a strong decrease in O_2 levels was observed, whereas CO_2 levels increased regardless of cultivar. Untreated pear wedges of 'Flor de invierno', 'Passe-Crassane', 'Ercolini' and 'Conference' reached O_2 values of 9.9, 6.1, 0.8 and 5.6% and CO_2 levels of 8.4, 11.8, 14.2 and 10.8%, respectively. No significant differences in O_2 and CO_2 levels were observed between untreated and treated pear wedges from 'Passe-Crassane', 'Ercolini' and 'Conference' (data not shown). Nevertheless, in 'Flor de invierno', a slight difference of O_2 level was observed between treated and untreated pear wedges after 7 days. Treated wedges showed lower O_2 levels (1.7%), whereas untreated wedges did not (9.9%).

Visual evaluation of the samples after 7 days of storage at 5 °C was conducted. Untreated samples were all below the limit of marketability, but samples treated with NS solution presented excellent visual quality for all tested cultivars (*Figure 1*), with 'Conference' and 'Ercolini' obtaining the highest score (between very good and excellent).

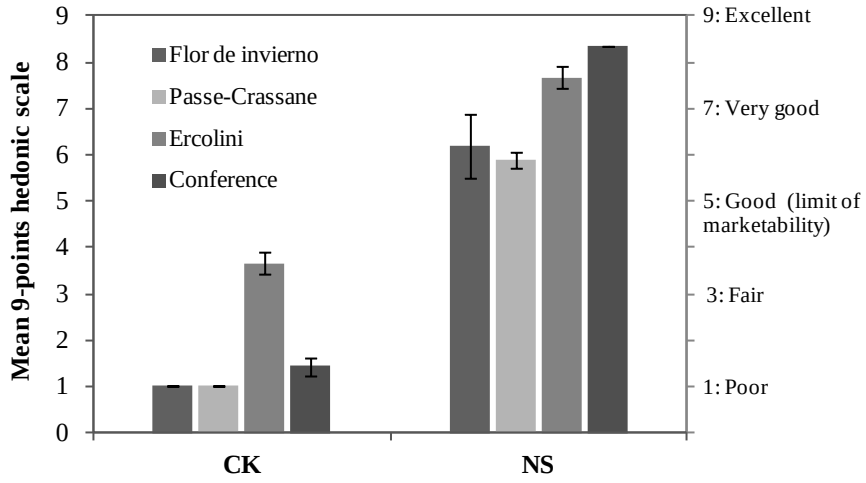


Figure 1. *Overall visual quality of wedges from four pear cultivars after 7 days at 5 °C treated with antioxidant solution (NS; 50 g L^{-1} NatureSeal® AS1) or without treatment (CK; water). The data presented are the means of the visual evaluations of three trays per treatment and cultivar, and bars represent the standard deviation of the mean.*

After evaluation of different pear cultivars, the 'Conference' pear was selected as the best cultivar. This selection was based on physicochemical characteristics, (high levels of soluble solids content and low acidity) and a low increase in BI. 'Conference' also received the best visual acceptance score after 7 days of storage at 5 °C. Although results were also promising for 'Ercolini', the 'Conference' pear can be stored at low temperature in a controlled atmosphere for a long period of time (Nguyen *et al.*, 2007). This property increases the availability of this cultivar throughout the year, and as a result, a fresh-cut pear product could be produced along the all year compared to 'Ercolini'. In addition in 2014, 198277 tons of pears were produced in our area (Catalonia), 'Conference' held the first positions in the pear production (87167 tons) while 'Ercolini' was appeared in the fifth position (6960 tons) (DAAM, 2015). Similarly, Arias *et al.* (2008) found that 'Conference' was the most appropriate cultivar among the three studied varieties ('Conference', 'Williams' and 'Passa-Crassane'). This author observed that 'Conference' was the cultivar best suited for minimal processing.

3.2. Selection of the antioxidant treatment

After processing (0 day), the SSC of 'Conference' pears treated with different antioxidants ranged from 13.9 to 15.0% (Table 3). At the end of evaluation, no significant differences were noted among the SSCs of different treatments (data not shown). For titratable acidity after processing, pears treated with different antioxidants ranged from 1.2 to 1.9 g malic acid L^{-1} (Table 3), and after 14 days, they reached values from 0.9 to 1.2 g malic acid L^{-1} regardless of the treatment applied (data not shown). For the hue angle values, slight differences among treatments were observed initially, and only pear wedges treated with NS were significantly different from the control sample after processing (Table 3). After 14 days of storage at 5 °C, these differences were more significant. When pear wedges were treated with water (CK) or AsAc, the hue angle reached values of 96.6 and 96.0, respectively. Samples treated with CaAs or NaAs had values of 101.3 and 101.5, whereas those samples treated with NS showed the highest value (103.4) (Table 3).

After processing, wedges dipped in different antioxidants reached BI values of CK, 15.70; NS, 12.36; AsAc, 15.47; CaAs, 13.26; and NaAs, 14.00; although only significant differences were observed between samples dipped in water (CK) and NS solution (data not shown). Conversely, after processing (0 day), no significant differences were observed in texture due to the different antioxidants tested (Table 4). Nevertheless, after 14 days, the sample without antioxidant (CK) showed a strong reduction in firmness (from 15.61 to 11.89 N), but firmness was maintained in the remaining samples (14.31 to 16.52 N). After 14 days, the increase in the browning index was higher in untreated and AsAc treated pears (7.50 and 9.01, respectively) than in the other treatments. Treatments that avoided the browning effect in fresh-cut pear surface were NS, CaAs and NaAs which showed browning indexes of 2.58, 1.88 and 3.78, respectively (Table 4).

For the O_2 and CO_2 composition in the headspace, levels of O_2 decreased drastically to 0% after 7 days regardless of the antioxidant treatment (data not shown). The CO_2 levels increased gradually during storage. AsAc-treated pears had the highest value (29.2%), and CO_2 values ranged from 24.8 to 25.1% in untreated and NS- and CaAs-treated pears.

Table 3. *Physicochemical parameters of fresh-cut 'Conference' pears dipped in different antioxidant solutions.*

Treatment	Initial			After storage (5 °C)	
	SSC (%)	TA (g malic acid L^{-1})	Hue angle (h°)	Hue angle (h°) 7 days	Hue angle (h°) 14 days
CK	15.0 ± 0.1 a	1.4 ± 0.0 b	101.4 ± 1.8 b	96.5 ± 2.6 d*	96.6 ± 3.2 c*
NS	14.5 ± 0.1 b	1.2 ± 0.0 c	103.7 ± 0.7 a	103.2 ± 1.8 a	103.4 ± 1.5 a
AsAc	14.4 ± 0.1 b	1.9 ± 0.0 a	101.0 ± 1.6 b	98.2 ± 3.1 c*	96.0 ± 2.8 c*
CaAs	13.9 ± 0.0 c	1.2 ± 0.0 c	102.0 ± 2.1 ab	101.3 ± 1.6 b*	101.3 ± 1.9 b
NaAs	14.4 ± 0.0 b	1.2 ± 0.0 c	103.0 ± 1.9 ab	101.7 ± 1.9 ab	101.5 ± 2.1 b

Values are the mean of three values ± standard deviation for SSC and TA; and the mean of thirty values ± standard deviation for the hue angle. Different letters for the same parameter indicate significant differences among treatments ($p < 0.05$) according to Tukey's test. CK: distilled water; NS: 50 g L^{-1} NatureSeal® AS1; AsAc: 20 g L^{-1} ascorbic acid, 10 g L^{-1} citric acid and 10 g L^{-1} calcium chloride; CaAs: 20 g L^{-1} calcium ascorbate and 10 g L^{-1} calcium chloride; NaAs: 20 g L^{-1} sodium ascorbate and 10 g L^{-1} calcium chloride. An asterisk in the hue angle data at 7 and 14 days of storage means that significant differences were observed with respect to the initial value in each treatment

Table 4. *Evolution of physicochemical parameters of fresh-cut 'Conference' pears dipped in different antioxidant solutions.*

	Storage time	CK	NS	AcAs	CaAs	NaAs
Texture (N)	0 day	15.61 ± 1.48 a A	16.87 ± 3.56 a A	15.97 ± 1.72 a A	15.05 ± 0.97 ab A	17.27 ± 2.65 a A
	7 days	11.04 ± 1.21 b B	12.59 ± 2.20 b AB	13.51 ± 1.59 a A	13.86 ± 0.90 b A	13.67 ± 2.93 b A
	14 days	11.89 ± 1.79 b B	14.31 ± 2.02 ab AB	14.83 ± 1.92 a A	15.74 ± 1.47 a A	16.52 ± 1.84 ab A
Δ BI	7 days	4.02 ± 4.74 A	1.59 ± 3.40 A	2.42 ± 5.23 A	2.37 ± 3.19 A	1.91 ± 3.92 A
	14 days	7.50 ± 6.33 A	1.68 ± 2.79 C	6.80 ± 4.91 AB	3.35 ± 4.28 C	3.78 ± 4.52 BC

Values are the mean of nine values ± standard deviation for texture. Values are the mean of thirty values ± standard deviation for ΔBI. For each parameter, different lowercase letters (a, b and c) in the same column indicate significant differences ($p < 0.05$) among sampling days according to Tukey's test. Different uppercase letters (A, B, C, D and D) in the same row indicate significant differences ($p < 0.05$) among treatments. CK: distilled water; NS: 50 g L^{-1} NatureSeal® AS1; AsAc: 20 g L^{-1} ascorbic acid, 10 g L^{-1} citric acid and 10 g L^{-1} calcium chloride; CaAs: 20 g L^{-1} calcium ascorbate and 10 g L^{-1} calcium chloride; NaAs: 20 g L^{-1} sodium ascorbate and 10 g L^{-1} calcium chloride

Samples treated with NS solution presented an excellent visual quality (*Figure 2*), whereas those samples treated with CaAs and NaAs presented scores near the limit of marketability (good and very good). Untreated and AsAc treated pear wedges received scores below limit of usability (=1). To minimize visual deterioration of fresh-cut pears, certain reducing agents such as ascorbic acid, 4-hexylresorcinol, cysteine, N-acetylcysteine and sodium eritorbate combined with calcium salts such as calcium chloride, calcium lactate have been investigated (Arias *et al.*, 2008; Dong *et al.*, 2000; Gorny *et al.*, 2000; Oms-Oliu *et al.*, 2006; Sapers *et al.*, 1998; Soliva-Fortuny *et al.*, 2002).

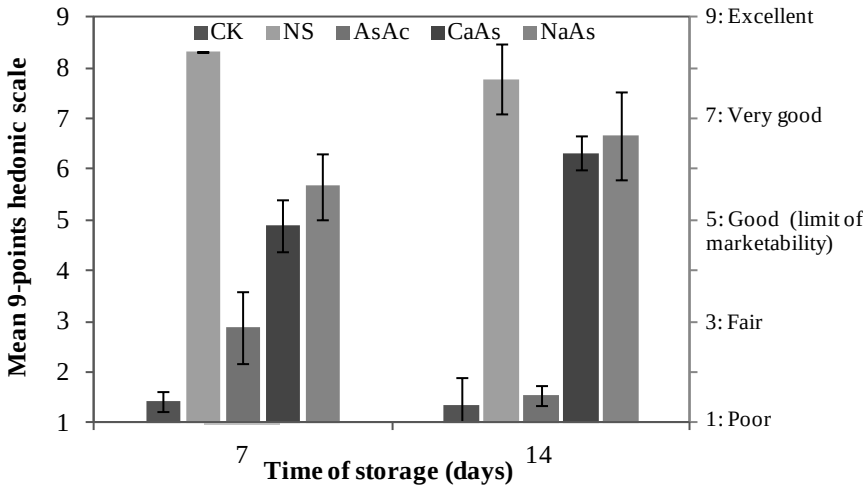


Figure 2. *Overall visual quality of fresh-cut 'Conference' pear after 7 and 14 days at 5 °C treated with different antioxidant solutions (CK: distilled water; NS: 50 g L^{-1} NatureSeal® AS1; AsAc: 20 g L^{-1} (w/v) ascorbic acid, 10 g L^{-1} (w/v) citric acid and 10 g L^{-1} (w/v) calcium chloride solution; CaAs: 20 g L^{-1} (w/v) calcium ascorbate and 10 g L^{-1} (w/v) calcium chloride solution; NaAs: 20 g L^{-1} (w/v) sodium ascorbate and 10 g L^{-1} (w/v) calcium chloride solution). The data presented are the means of the visual evaluations of three trays per treatment, and bars represent the standard deviation of the mean.*

The AsAc treatment composed of 20 g L^{-1} ascorbic acid, 10 g L^{-1} citric acid and 10 g L^{-1} calcium chloride obtained the worst results in our study, which is consistent with the results obtained by Arias *et al.* (2008). Larrigaudiere *et al.* (2008) studied the effects of chemical preservatives on the oxidative behaviour of

fresh-cut 'Fuji' apples and determined the H_2O_2 levels, which are used as a marker for oxidative stress. Ascorbic acid is generally used as antioxidant to prevent oxidation-related processes and to limit the accumulation of H_2O_2. An increase in H_2O_2 levels was observed in fresh-cut apples treated with ascorbic acid. These results might occur because at higher concentration, ascorbic acid might act as a pro-oxidant and therefore tend to have the opposite effect with respect to H_2O_2 accumulation (Kacem *et al.*, 1987; Larrigaudiere *et al.*, 2008), and this is likely what occurred in our treatment. As an alternative anti-browning treatment, other ascorbic salts were evaluated in this study in combination with the most frequently used calcium salt (calcium chloride, $CaCl_2$), which avoided losses in texture. However, these ascorbic salts have not been evaluated previously in fresh-cut pears. We concluded that the use of 20 g L^{-1} calcium ascorbate plus 10 g L^{-1} calcium chloride (CaAs treatment) and 20 g L^{-1} sodium ascorbate plus 10 g L^{-1} calcium chloride (NaAs treatment) as dipping solutions after cutting delivered colour and texture stability and good visual aspects for fresh-cut 'Conference' pears for 14 days of storage at 5 °C. The results confirmed the ability of NS to maintain the freshness of fresh-cut 'Conference' pears, although similar results were obtained with CaAs and NaAs solutions. A similar evaluation of firmness was obtained in samples treated with CaAs and NaAs. However, CaAs was selected for further studies because certain judges found a 'salty' flavour in NaAs-treated pear wedges (data not shown).

3.3. Semi-commercial evaluation

3.3.1. Physicochemical evaluation

Fresh-cut pears before CaAs treatment presented values of 14.3% for SSC, 1.2 g malic acid L^{-1} for TA and 17.35 N for firmness (Table 5). With respect to flesh colour, wedges showed 103.1 and 13.0 values of hue angle and BI, respectively (data not shown). The results revealed that CaAs application did not modify these physicochemical parameters. During shelf life, no remarkable changes were observed in SSC and TA, but flesh firmness significantly increased after 8 days of storage in both realistic (25.17 N) and cold chain break (27.66 N) storage conditions. This phenomenon was also noted by Xiao *et al.* (2011) in minimally processed 'Anjou' pears and could be due to dehydration of the surface pear tissue during storage, which leads to a hardening of the pear wedge that increased the measured resistance and consequently resulted in higher firmness measurements.

Table 5. *Physicochemical parameters of fresh-cut 'Conference' pears during semi-commercial assay.*

Sampling time	SSC (%)	TA (g malic acid L^{-1})	Texture (N)	ΔBI
before CaAs treatment	14.3 ± 0.1 a	1.2 ± 0.1 ab	17.4 ± 0.5 b	-
after treatment (0 day)	14.4 ± 0.0 a	1.3 ± 0.0 a	19.9 ± 2.1 b	1.8 ± 2.0 b
3 days (4 °C)	13.3 ± 0.3 b	1.1 ± 0.0 ab	16.4 ± 1.5 b	2.8 ± 2.6 b
8 days (4 °C)	13.9 ± 0.2 ab	1.0 ± 0.2 ab	25.2 ± 6.6 a	3.6 ±3.6 ab
8 days (3 d 4 °C + 5 d 8 °C)	13.9 ± 0.3 a	0.9 ± 0.1 b	27.7 ± 3.7 a	4.9 ± 2.6 a

Values are the mean of three values ± standard deviation for SSC and TA; the mean of nine values ± standard deviation for firmness; and the mean of ten values ± standard deviation for ΔBI. Different letters in the same parameter indicate significant differences among samples during shelf life ($p < 0.05$) according to Tukey's test

A gradual increase of the BI was observed on pear wedges with increasing storage time. The highest increase was observed in samples stored 8 days under cold chain break conditions (4.9).Change in the package headspace gas composition during shelf life was also observed.

After 3 days of storage, samples showed a reduction of O_2 levels (6.6% O_2) and a strong increase in CO_2 levels (9.3% CO_2). Both samples stored at realistic and cold chain break conditions showed decreased O_2 levels and increased CO_2 levels, although samples stored at 8 °C for 5 days showed the most drastic reduction of O_2 levels and increase of CO_2 levels, e.g., levels of 0.0% O_2 and levels of 21.5% CO_2 (data not shown).

3.3.2. Nutritional evaluation

Nutritional parameters were affected by the CaAs treatment. Before treatment, pear wedges showed values of ascorbic acid content, total phenolic content and antioxidant activity of 0.01 g ascorbic acid kg^{-1} (*Figure 3*), 0.35 g gallic acid kg^{-1} (*Figure 4*) and 0.64 mmoles ascorbic acid kg^{-1} (*Figure 5*), respectively.

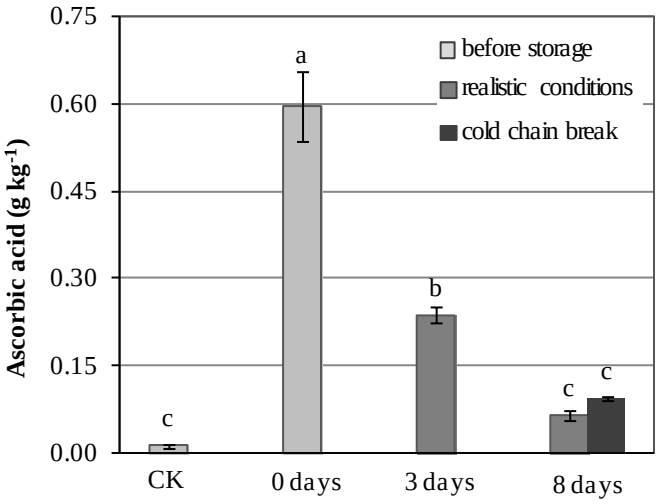


Figure 3. *Ascorbic acid content of fresh-cut pears during storage at ideal and cold chain break conditions (g per kg of fresh weight). The data presented are the means of three values. Different letters indicate significant differences ($p < 0.05$). Vertical bars represent the standard deviation of the means.*

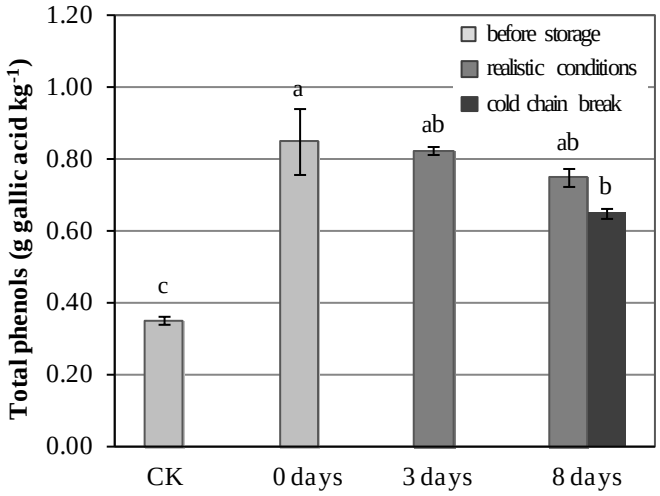


Figure 4. *Total phenolic content of fresh-cut pear during storage at ideal and cold chain break conditions (g gallic acid per kg of fresh weight). The data presented are the means of three values. Different letters indicate significant differences ($p < 0.05$). Vertical bars represent the standard deviation of the means.*

A similar initial phenolic content in fresh-cut 'Conference' pear without treatment was noted by Arias *et al.* (2008) (0.30 g phenols kg^{-1}), and higher content was observed in 'Passe-Crassane' (1.20 g phenols kg^{-1}). Gomes *et al.* (2014) observed that browning development on the tissue surface was affected by pH and the phenolic substrate, and thus polyphenol oxidase (PPO) could develop enzymatic browning in fresh-cut 'Passe-Crassane', which was the likely cause of the increased browning index in this variety.

The ascorbic acid content of untreated fresh-cut pears was 0.01 g ascorbic acid kg^{-1} (*Figure 3*), and as a consequence of CaAs treatment, this content increased by 43.9 times (0.60 g ascorbic acid kg^{-1}), by 2.4 times for total phenolic content (from 0.35 to 0.85 g gallic acid kg^{-1}) and by 5.3 times for antioxidant activity (from 0.64 to 3.41 mmoles ascorbic acid kg^{-1}) (*Figure 4* and *5*). Our results are consistent with others obtained in the 'Conference' pear (Soliva-Fortuny, Martín-Belloso, 2003), which reported that the treatment (10 g L^{-1} ascorbic acid plus 5 g L^{-1} calcium chloride) caused an increase of 60% in the ascorbic acid content after processing of fresh-cut pear (0.05 g kg^{-1}). After sample treatment, the antioxidant activity increased nearly 5 times. This increase could be due to the composition of the treatment chosen, which contains calcium ascorbate with high antioxidant activity. Oms-Oliu *et al.* (2008) optimized an antioxidant treatment for fresh-cut

'Flor de invierno' pears and did not notice an enhancement in antioxidant activity, but their treatment contained no calcium ascorbate.

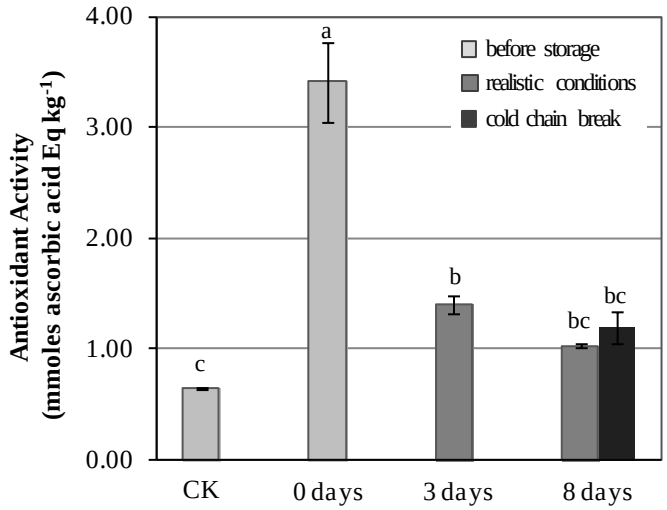


Figure 5. *Antioxidant activity of fresh-cut pears during storage at ideal and cold chain break conditions (mmoles ascorbic acid equivalent per kg of fresh weight). The data presented are the means of three values. Different letters indicate significant differences ($p < 0.05$). Vertical bars represent the standard deviation of the means.*

During fresh-cut pear shelf life, a significant reduction of all nutritional parameters was noted. Gradual reductions of total phenolic content (*Figure 4*) were observed during storage, from 0.85 (0 day) to 0.75 g gallic acid kg^{-1} after 8 days at realistic storage conditions. Nevertheless, the lowest content of total phenol was found at cold chain break storage conditions (0.65 g gallic acid kg^{-1}). In addition, a large significant reduction of ascorbic acid content (*Figure 3*) and antioxidant activity (*Figure 5*) was observed after 3 days of storage at 4 °C, reaching 0.24 g ascorbic acid kg^{-1} and 1.41 mmoles ascorbic acid kg^{-1}, respectively. After 8 days of storage, a weak reduction was observed in ascorbic acid content and antioxidant activity, which was the similar at both storage conditions. Values of ascorbic acid content ranged from 0.07 to 0.09 g ascorbic acid kg^{-1} and those of antioxidant activity ranged from 1.03 to 1.20 mmoles ascorbic acid kg^{-1}. Soliva-Fortuny and Martín-Belloso (2003) also observed that ascorbic acid contents decreased to 0.05 g kg^{-1} after 7 days of storage under MAP conditions.

3.3.3. Microbial quality

Microbial quality changes were not observed between untreated and CaAs-treated fresh-cut 'Conference' pears on the processing day (0 day) (*Figure 6*). The count of psychrotrophic microorganisms (PM) after processing and after dipping of wedges ranged from 2.7 to 2.8 log CFU g^{-1} on untreated and treated pear wedges, respectively. For yeasts and moulds (YM), the majority of samples showed values below the limit of detection (LD, 1.4 log CFU ml^{-1}). The counts of lactic acid bacteria (LAB) were below the detection limit (< 0.5 log CFU ml^{-1}) on both on untreated and treated pear wedges. Oms-Oliu *et al.* (2009) and Soliva-Fortuny and Martín-Belloso (2003) highlighted the importance of evaluating the microbial stability of minimally processed pears and observed that the main native microbiota of 'Conference' fresh-cut pears stored at 4 °C were moulds and yeasts, but MAP inhibited growth of moulds and yeasts, whereas mesophilic bacteria proliferated rapidly.

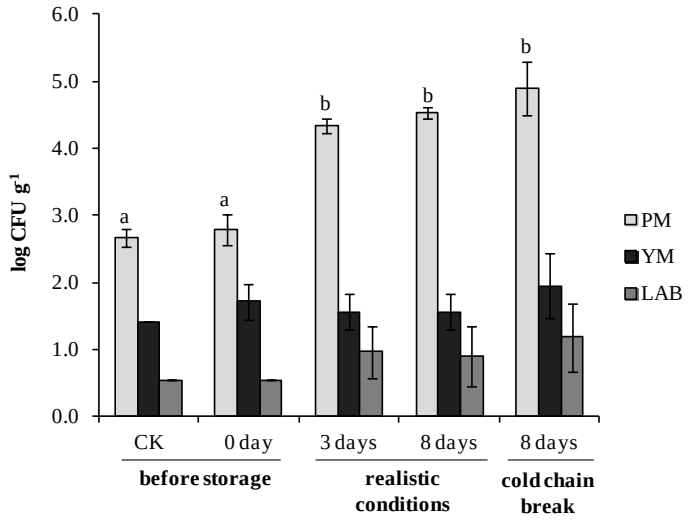


Figure 6. *Population of psychrotrophic microorganisms (PM), yeasts and moulds (YM), and lactic acid bacteria (LAB) (log CFU g^{-1}) in fresh-cut 'Conference' pears during shelf life. Data represent the mean of three determinations, and bars represent the standard deviation of the mean. Different letters indicate significant differences among days ($p < 0.05$).*

After 3 days of storage at 4 °C, PM increased to 4.3 log CFU g^{-1}, whereas yeasts and moulds counts were maintained close to the limit of detection (LOD). LAB counts rise above the LOD although no significant differences were observed compared with the initial count. At the final sampling point (8 day), no differences among storage conditions (realistic and cold chain break storage conditions) were observed in YM and LAB. YM and LAB enumeration ranged from 1.6 to 2.0 log CFU g^{-1} and from 0.8 to 1.2 log CFU g^{-1}, respectively. Oms-Oliu *et al.* (2009) and Soliva-Fortuny and Martín-Belloso (2003) observed that the main microbiota on fresh-cut pear consist of moulds and yeasts, but these could be inhibited because they are sensitive to CO_2. Under MAP storage of fresh-cut pear, CO_2 levels increased during storage and inhibited the proliferation of moulds and yeasts throughout storage, thus facilitating the colonization by populations of bacteria, which were minority microorganisms before processing. These reports support our findings that the moulds and yeasts load was constant during storage, whereas that of psychrotrophic bacteria increased up 5 log CFU g^{-1}. The proliferation of microorganisms on the surface of fresh-cut fruit is currently retarded or inhibited by the use of low storage temperature, modified atmosphere packaging, and antimicrobial substances (Rojas-Graü, Martín-Belloso, 2008). With respect to temperature, we noted that under cold chain beak storage conditions, psychrotrophic bacteria showed a weak increase (4.9 log CFU g^{-1}) compared with storage at realistic conditions (4.4 log CFU g^{-1}), although these values were not significantly different.

3.3.4. Consumer assessment: visual quality and consumer acceptability

Immediately after processing, the samples obtained the highest score (excellent) for visual quality, and after 3 days of storage, acceptance was reduced to very good (*Figure* 7). After 8 days, when samples were stored at constant temperature (4 °C), they received the lowest acceptance (below limit of marketability), whereas samples stored for 3 days at 4 °C plus 5 days at 8 °C reached an acceptance score between good and very good.

After processing, the consumer acceptability was measured, 92% of consumers indicated their satisfaction with the fresh-cut pear (*Figure* 8). This acceptance increased up to 100% after 3 days of storage. After 8 days, under realistic cold chain conditions and cold chain break conditions, 44% of consumers liked the pears. However, fresh-cut pears stored under cold chain break conditions received a greater percentage of unsatisfied consumers (44%) than those maintained at 4 °C over the entire shelf life (25%).

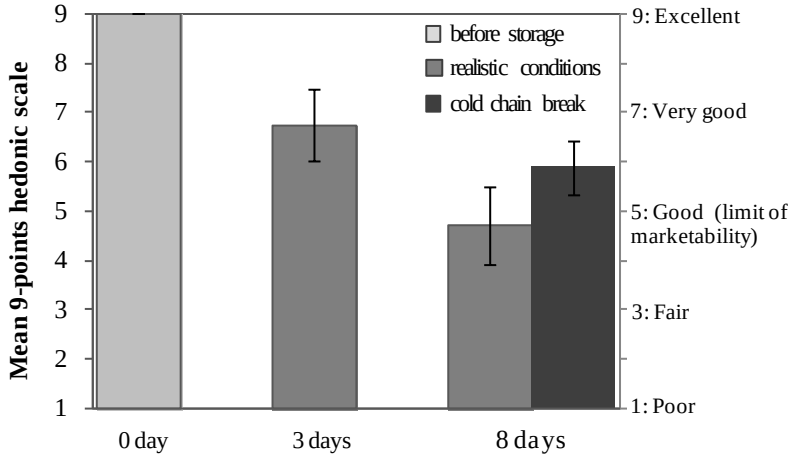


Figure 7. *Overall visual quality of fresh-cut 'Conference' pears during shelf life at ideal and cold chain break storage conditions. The data presented are the means of the visual evaluations of three trays at each sampling time, and bars represent the standard deviation of the mean.*

Overall acceptance (%)			
0 day	Before storage	Like	92
		Neither like or dislike	0
		Dislike	8
3 days	Realistic conditions (4°C)	Like	100
		Neither like or dislike	0
		Dislike	0
8 days	Realistic conditions (4°C)	Like	44
		Neither like or dislike	31
		Dislike	25
	Cold chain break (8°C)	Like	44
		Neither like or dislike	13
		Dislike	44

Figure 8. *Percentage of consumers that liked, neither liked nor disliked, and disliked the fresh-cut pear during the shelf life according to overall acceptance.*

4. Conclusions

In the current study, a minimally processed pear product was optimized using the 'Conference' pear as the fruit cultivar and treatment with a solution consisting of 20 g L^{-1} (w/v) calcium ascorbate and 10 g L^{-1} (w/v) calcium chloride solution. The selected treatment was able to minimize visual deterioration after 8 days of storage at 4 °C and under cold chain break conditions. When our selected treatment was applied, increases in the ascorbic acid content, total phenolic content and antioxidant activity of minimally processed pear samples were observed. These values were reduced during shelf life, but the total phenolic content at the final sampling point was greater than that in samples after processing (without treatment). The microbial stability of our fresh-cut pear had the same tendency as that of the other minimally processed pear products evaluated. The total mesophilic aerobic population exhibited faster growth than yeasts and moulds, which did not increase over the shelf life. Our fresh-cut 'Conference' pear product could offer added value to pear production in our area and introduce to the market a product with higher convenience for consumers. For this product, no more than 8 days of shelf life are recommended to ensure consumer satisfaction.

5. Acknowledgements

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus and Banco Santander for the Pilar Colás Medà PhD grant (UdL-Impuls Program) and to the Spanish Government (Ministerio de Economia y Competitividad, research project AGL-2012-38671) for financial support. L. Plaza thanks the National Institute for Agronomic Research (INIA) for the DOC-INIA research contract.

6. Statement of Competing Interests

The authors have no competing interests.

7. References

Abadias, M., Altisent, R., Usall, J., Torres, R., Oliveira, M., Viñas, I. 2014. Biopreservation of fresh-cut melon using the strain *Pseudomonas graminis* CPA-7. *Postharvest Biology and Technology* 96, 69-77.

Alandes, L., Hernando, I., Quiles, A., Perez-Munuera, I., Lluch, M.A. 2006. Cell wall stability of fresh-cut fuji apples treated with calcium lactate. *Journal of Food Science* 71, 615-620.

Altisent, R., Plaza, L., Alegre, I., Viñas, I., Abadias, M. 2014. Comparative study of improved vs. traditional apple cultivars and their aptitude to be minimally processed as 'ready to eat' apple wedges. *LWT - Food Science and Technology* 58, 541-549.

Amiot, M.J., Tacchini, M., Aubert, S.Y., Oleszek, W. 1995. Influece of cultivar, maturity stage, and storage-conditions on phenolic composition and enzymatic browning of pear fruits. *Journal of Agricultural and Food Chemistry* 43, 1132-1137.

Arias, E., Gonzalez, J., Lopez-Buesa, P., Oria, R. 2008. Optimization of processing of fresh-cut pear. *Journal of the Science of Food and Agriculture* 88, 1755-1763.

Buera, M.P., Lozano, R.D., Petriella, C. 1986. Definition of colour in the non enzymatic browning process. *Die Farbe* 32, 318-322.

DAAM. 2015. Observatori de la Fruita Fresca de Catalunya. http://agricultura.gencat.cat/web/.content/de_departament/de02_estadistiques_observatoris/08_observatoris_sectorials/03_observatori_de_la_fruita_fresca/a2/fitxers_estatics/A2_2015/2015.08-Prev-collita-i-produccio-poma-i-pera-_Cat_EU_.pdf (Accessed: 03/11/15).

Dong, X., Wrolstad, R.E., Sugar, D. 2000. Extending shelf life of fresh-cut pears. *Journal of Food Science* 65, 181-186.

EU Commission. 2011. 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. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:295:0001:0177:en:PDF.

Gil, M.I., Gorny, J.R., Kader, A.A. 1998. Responses of 'Fuji' apple slices to ascorbic acid treatments and low-oxygen atmospheres. *Hortscience* 33, 305-309.

Gomes, M.H., Vieira, T., Fundo, J.F., Almeida, D.P.F. 2014. Polyphenoloxidase activity and browning in fresh-cut 'Rocha' pear as affected by pH, phenolic substrates, and antibrowning additives. *Postharvest Biology and Technology* 91, 32-38.

Gorny, J.R. 2003. A summary of CA and MA requirements and recommendations for fresh-cut (minimally processed) fruits and vegetables. *Acta Horticulturae* 600, 609-614.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A. 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. *Journal of Food Science* 65, 541-544.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1998. Effects of fruit ripeness and storage temperature on the deterioration rate of fresh-cut peach and nectarine slices. *Hortscience* 33, 110-113.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1999. Quality changes in fresh-cut peach and nectarine slices as affected by cultivar, storage atmosphere and chemical treatments. *Journal of Food Science* 64, 429-432.

Kacem, B., Cornell, J.A., Marshall, M.R., Shireman, R.B., Matthews, R.F. 1987. Nonenzymatic browning in aseptically packaged orange drinks: effect of ascorbic acid, aminoacids and oxygen. *Journal of Food Science* 52, 1668-1672.

Larrigaudiere, C., Ubach, D., Soria, Y., Rojas-Graü, M.A., Martín -Belloso, O. 2008. Oxidative behaviour of fresh-cut Fuji apples treated with stabilising substances. *Journal of the Science of Food and Agriculture* 88, 1770-1776.

Nguyen, T.A., Verboven, P., Schenk, A., Nicolai, B.M. 2007. Prediction of water loss from pears (*Pyrus communis* cv.Conference) during controlled atmosphere storage as affected by relative humidity. *Journal of Food Engineering* 83, 149-155.

Oms-Oliu, G., Aguilo-Aguayo, I., Martin-Belloso, O. 2006. Inhibition of browning on fresh-cut pear wedges by natural compounds. *Journal of Food Science* 71, 216-224.

Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martín-Belloso, O. 2009. Effect of ripeness at processing on fresh-cut 'Flor de Invierno' pears packaged under modified atmosphere conditions. *International Journal of Food Science & Technology* 44, 900-909.

Oms-Oliu, G., Rojas-Graü, M.A., González, L., Varela, P., Soliva-Fortuny, R.C., Hernando, I., Munuera, I., Fiszman, S., Martín-Belloso, O. 2010. Recent approaches using chemical treatments to preserve quality of fresh-cut fruit: A review. *Postharvest Biology and Technology* 57, 139-148.

Oms-Oliu, G., Soliva-Fortuny, R., Martín -Belloso, O. 2008. Edible coatings with antibrowning agents to maintain sensory quality and antioxidant properties of fresh-cut pears. *Postharvest Biology and Technology* 50, 87-94.

Rojas-Graü, M.A., Martín-Belloso, O. 2008. Current advances in quality maintenance of fresh-cut fruits. *Stewart Postharvest Review* 4, 1-8.

Sapers, G.M., Miller, R.L. 1998. Browning inhibition in fresh-cut pears. *Journal of Food Science* 63, 342-346.

Soliva-Fortuny, R.C., Alos-Saiz, N., Espachs-Barroso, A., Martin-Belloso, O. 2004. Influence of maturity at processing on quality attributes of fresh-cut conference pears. *Journal of Food Science* 69, 290-294.

Soliva-Fortuny, R.C., Biosca-Biosca, M., Grigelmo-Miguel, N., Martin-Belloso, O. 2002. Browning, polyphenol oxidase activity and headspace gas composition during storage of minimally processed pears using modified atmosphere packaging. *Journal of the Science of Food and Agriculture* 82, 1490-1496.

Soliva-Fortuny, R.C., Martín-Belloso, O. 2003. Microbiological and biochemical changes in minimally processed fresh-cut *Conference* pears. *European Food Research and Technology* 217, 4-9.

Xiao, Z., Luo, Y., Luo, Y., Wang, Q. 2011. Combined effects of sodium chlorite dip treatment and chitosan coatings on the quality of fresh-cut d'Anjou pears. *Postharvest Biology and Technology* 62, 319-326.

Yoruk, R., Marshall, M.R. 2003. Physicochemical properties and function of plant polyphenol oxidase: A review. *Journal of Food Biochemistry* 27, 361-422.

CAPÍTULO 2

The impact of a cold chain break on the survival of *Salmonella enterica* and *Listeria monocytogenes* on minimally processed 'Conference' pear during their shelf life

P. Colás-Medà, I. Viñas, I. Alegre, M. Abadias

Journal of the Science of Food and Agriculture (aceptado)

Abstract

In recent years, improved detection methods and increased fresh-cut processing of produce have led to an increased number of outbreaks associated with fresh fruits and vegetables. During fruit and vegetable processing, natural protective barriers are removed and tissues are cut, causing nutrient rich exudates and providing attachment sites for microbes. Consequently, fresh-cut produce is more susceptible to microbial proliferation than whole produce. The aim of this study was to examine the impact of storage temperature on the growth and survival of *Listeria monocytogenes* and *Salmonella enterica* on a fresh-cut 'Conference' pear over an eight day storage period. Pears were cut, dipped in antioxidant solution, artificially inoculated with *L. monocytogenes* and *S. enterica*, packed under modified atmospheric conditions simulating commercial applications and stored in properly refrigerated conditions (constant storage at 4 °C for 8 days) or in temperature abuse conditions (3 days at 4 °C plus 5 days at 8 °C). After 8 days of storage, both conditions resulted in a significant decrease of *S. enterica* populations on pear wedges. In contrast, when samples were stored at 4 °C for 8 days, *L. monocytogenes* populations increased 1.6 logarithmic units, whereas under the temperature abuse conditions, *L. monocytogenes* populations increased 2.2 logarithmic units. In conclusion, *Listeria monocytogenes* was able to grow on fresh-cut pears processed under the conditions described here, despite low pH, refrigeration and use of modified atmosphere.

Keywords: foodborne pathogen; ready-to-eat; fruit; cold.

1. Introduction

Processed products attractive to consumers could be a way to increase consumption of fruits and vegetables, with a positive impact on consumer health if processing does not alter the nutritional benefits of the raw products (Nguyen-The, 2012). Minimally processed pears could satisfy consumer demand because pears have low protein and lipid contents and are rich in sugars, including fructose, sorbitol, and sucrose, and are low in glucose. Pears also contain micronutrients, including vitamins (vitamin C, vitamin E, and niacin) and minerals (potassium, phosphorous, calcium, and magnesium) (USDA, 2016). However, fruit processing promotes faster deterioration due to tissue damage, which leads to increased physiological activity and major physicochemical changes, including enzymatic browning, softening, and tissue degradation (Sanchis *et al.*, 2016). Several investigators have developed technologies to minimize these processing effects (Amiot *et al.*, 1995; Arias *et al.*, 2008; Dong *et al.*, 2000; Gomes *et al.*, 2014; Gorny *et al.*, 2000; Oms-Oliu *et al.*, 2006; Oms-Oliu *et al.*, 2009; Oms-Oliu *et al.*, 2008; Sapers, Miller, 1998; Soliva-Fortuny *et al.*, 2004; Soliva-Fortuny *et al.*, 2002; Xiao *et al.*, 2011). Use of antioxidant solutions and edible coatings together with a modified atmosphere package (MAP) can reduce surface browning and water loss (Montero-Calderón, Cerdas-Araya, 2010). In addition to improving processing techniques to maintain quality, precautions should be taken to ensure product safety. The potential for microbiological contamination of fruits and vegetables is high because of the wide variety of conditions to which produce is exposed during growth, harvest, processing, and distribution. It is well established that fresh produce may contain high contamination levels after harvest. During processing, spoilage and pathogenic microorganisms can also contaminate the product surface, and the nutrients inside the fruit contribute to their growth (Sanchis *et al.*, 2016). Thus, disinfection is one of the most important processing steps affecting the quality, safety, and shelf life of the end product (Olmez, Kretzschmar, 2009). Safety requirements related to fresh-cut produce include good agricultural practices (GAP) and good processing practices (GMF) that result in the absence of pathogens, mycotoxins, pesticide residues, and any other chemical or physical contamination that might risk consumer health (Montero-Calderón *et al.*, 2010). In the Europe Union, food safety criteria for the presence of microorganisms in food is regulated by EC N° 2073/2005 and subsequent amendments. The food safety criteria for *L. monocytogenes* are of particular interest on ready-to-eat (RTE) foods. In RTE foods that may support the growth of *L. monocytogenes*, the limit is 100 CFU g^{-1} during the shelf life and the absence of *L. monocytogenes* in 25 g of the food just before it has left the immediate

control of the food business operator who produced it. In RTE foods unable to support the growth of *L. monocytogenes*, the limit is also 100 CFU g^{-1} during the shelf life (European, 2005).

In recent years, improved detection methods and increased fresh-cut processing of produce have led to an increased number of outbreaks associated with fresh fruits and vegetables (Tapias, Welti-Chanes, 2002). The storage temperature is an important factor affecting the growth of microorganisms. Thus, effective cold chain management is critical for maintaining the quality and shelf life of the product. The aim of this study was to examine the impact of storage temperature on the growth and survival of *Listeria monocytogenes* and *Salmonella enterica* on a fresh-cut 'Conference' pear during its shelf life.

2. Materials and methods

2.1. Fruit

'Conference' pears (*Pyrus communis* cv. Conference) were obtained from local packing houses in Lleida (Catalonia, Spain). The guide for minimally processing 'Conference' pears used in this paper was based on previous work (Colás-Medà *et al.*, 2016). Prior to processing, the pears were disinfected by immersion in a 0.1 g L^{-1} sodium hypochlorite (NaClO) solution (pH 6.5) for 2 min, rinsed in running tap water and allowed to dry at room temperature. Each pear was peeled and cut into 10 wedges using a handheld apple corer and slicer. In some fruit pieces, a 6 mm diameter well was made at the centre of each wedge for the inoculum. All the 'Conference' pear wedges were treated with an optimum antioxidant solution (20 g L^{-1} calcium ascorbate plus 10 g L^{-1} calcium chloride solution) by immersion for 2 min in the solution (1:2 w/v), which was maintained on a rotating platform at 150 rpm. The treated pear wedges were allowed to dry in a laminar flow biosafety cabinet for a short time. Physicochemical characteristics (soluble solids content and titratable acidity) of the pear wedges were evaluated in triplicate after the antioxidant treatment. The pear wedges were squeezed, and the soluble solids content (SCC) was determined using a handheld refractometer (ATAGO CO., LTD, Japan) at 20 °C. Results were reported as °Brix. To measure titratable acidity (TA), triplicate samples of 10 ml of fruit extract were diluted with 10 ml of distilled water, and 2 drops of phenolphthalein solution, 0.1 ml L^{-1} (Panreac, Barcelona, Spain) were added. The mixture was titrated with sodium hydroxide solution (NaOH, 0.1 N) until the pH indicator changed colour. The results were calculated as g of malic acid per litre of juice.

2.2. Foodborne pathogens

The bacterial strains used in this work included the serovars of *Salmonella enterica* subsp. *enterica:* Agona (ATCC BAA-707), Michigan (ATCC BAA-709), Montevideo (ATCC BAA-710) and Gaminara (ATCC BAA-711) in addition to the *L. monocytogenes* serovar 1a (CECT 4031), serovar 3a (CECT 933), serovar 4d (CECT 940), serovar 4b (CECT 4032) and serovar 1/2a, which was previously isolated in our laboratory from a fresh-cut lettuce sample (Abadias *et al.*, 2008). *Salmonella* strains were grown individually in tryptone soy broth (TSB, Oxoid) for 20-24 h at 37 ± 1 °C. *L. monocytogenes* strains were grown individually in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TSBYE) for 20-24 h at 37 ± 1 °C. Bacterial cells were harvested by centrifugation at 9820 x g for 10 min at 10 °C and then re-suspended in sterile saline solution (SS; 8.5 g L^{-1} NaCl). Equal volumes of the four *S. enterica* concentrated suspensions were mixed to produce a single suspension, and equal volumes of the five *L. monocytogenes* concentrated suspensions were mixed to provide a second suspension. For each inoculum preparation, a volume of the concentrated bacterial suspension was added to saline peptone (SP; 8.5 g L^{-1} and 1 g L^{-1} peptone) to obtain approximately 10^6 CFU ml^{-1}. The inoculum concentration was checked by plating appropriate dilutions on Palcam agar for *L. monocytogenes* (Palcam Agar Base with selective supplement, Biokar Diagnostics) or on XLD (Xylose-Lysine-Desoxycholate Agar, Oxoid) for *S. enterica*. Plates were incubated at 37 °C for 24 h (*S. enterica*) or 48 h (*L. monocytogenes*).

2.3. Inoculation and storage

After the antioxidant treatment, twelve pear wedges were inoculated with *L. monocytogenes* by pipetting 15 μL of the bacterial suspension containing approximately 10^6 CFU ml^{-1} into the well of each wedge. In addition, twelve wedges were inoculated with 15 μL of the *S. enterica* bacterial suspension. The pear wedges (approximately 110 g) treated with the antioxidant without pathogens plus one wedge inoculated with *S. enterica* or *L. monocytogenes* were placed in a polypropylene terephthalate tray, which was sealed with a non-peelable polypropylene terephthalate plastic film (APET-110, ILPRA, Italy) of 64 μm in thickness and O_2 permeability of 110 cm^3 m^{-2} day^{-1} atm^{-1} at 23 °C. There were 3 trays per pathogen for each recovery day.

Initially, samples were stored in conditions simulating a commercial application (constant storage at 4 ± 1 °C). Three trays per pathogen were examined at day 0

and three more after 3 days of storage at 4 ± 1 °C. Then, the rest of the samples were divided into two lots, one was stored at 8 ± 1 °C for 5 days (temperature abuse conditions) simulating more realistic conditions during transport and in a refrigerated display window while the other was maintained at a constant temperature of 4 ± 1 °C for 5 days.

2.4. Bacterial analysis

Recovery of pathogen populations were performed at day 0 and after 3 and 8 days of storage under the two conditions. Before opening the trays, the headspace gas composition was determined using a handheld gas analyser (CheckPoint O2/CO2, PBI Dansensor, Denmark).

To recover the pathogens from the wedges, a plug (1.2 cm in diameter and 1 cm deep, approximately 1 g plug^{-1}) containing the entire well was removed using a sterile cork borer. One plug per repetition was placed in a sterile filter bag (80 ml, BagPage®, Interscience BagSystem, Saint Nom, France) and diluted with 9 ml of buffered peptone water (BPW, Oxoid). The mixture was homogenized in paddle blender for 2 min at high speed (MiniMix, Interscience, France), and aliquots of the mixture were then serially diluted in SP and plated on XLD for enumerating *S. enterica* or on Palcam agar for *L. monocytogenes*. The agar plates were incubated at 37 ± 1 °C for 24 h (*S. enterica*) or 48 h (*L. monocytogenes*). The data were transformed to CFU g^{-1} pear. Three determinations per pathogen were made at each sampling point in duplicate.

2.5. Statistical analysis

All data were checked for significant differences by applying variance analysis (ANOVA) using the JMP8 (SAS, Statistical Analysis System) statistical package. They were subjected to mean separation by least significant differences by Tukey's test ($p< 0.05$).

3. Results and discussion

Several authors have reported that foodborne pathogens, including *L. monocytogenes* and *Salmonella* spp., may often be able to grow on the flesh of some fruits, including apples, peaches, strawberries, melons, watermelons, papayas, persimmons and pears (Alegre *et al.*, 2010a; Alegre *et al.*, 2010b; Colás-Medà *et al.*, 2015; Del Rosario, Beuchat, 1995; Flessa *et al.*, 2005; Penteado, Leitão, 2003; Uchima *et al.*, 2008; Ukuku *et al.*, 2015). The current study evaluated the

behaviour of *L. monocytogenes* and *S. enterica* on minimally processed 'Conference' pears treated with an antioxidant solution and stored under MAP in conditions simulating commercial application (constant storage at 4 °C) and after a simulated cold chain break (temperature abuse conditions). The pear wedges used as a support matrix had SSC values from 14.5 to 14.8 °Brix (SSC average, 14.7 ± 0.1 °Brix, data not shown) and TA values between 2.29 and 2.80 g malic acid L^{-1} (TA average, 2.67 ± 0.22 g malic acid L^{-1}, data not shown). After 3 and 8 days of storage, the headspace gas composition of the packages was measured (*Figure 1*).

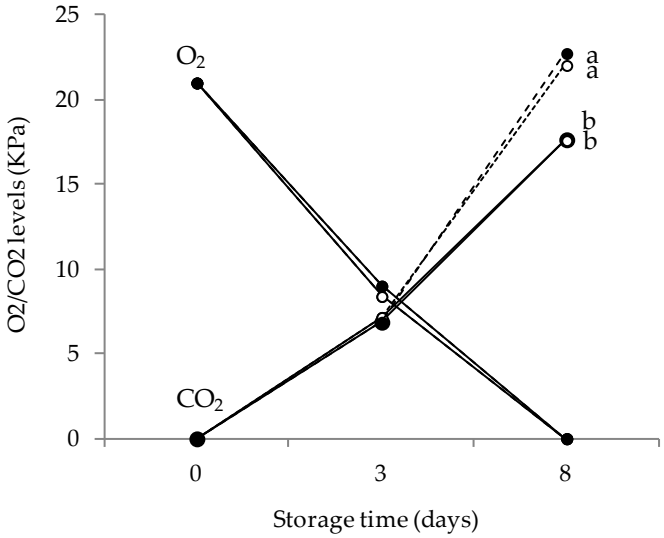


Figure 1. *The headspace gas composition of the packages of fresh-cut 'Conference' pears artificially inoculated with* L. monocytogenes *(open symbols) and* S. enterica *(full symbols) stored under constant storage temperature at 4 °C (continuous line) and under the temperature abuse conditions (dotted line) (n=6). Within each gas, difference ($p < 0.05$) among storage conditions and pathogens in each sampling point is represented by letters (a, b, c and d).*

In both storage conditions and regardless of the pathogen evaluated, a strong decrease in the O_2 levels was observed after 3 days of storage (approximately 9 kPa), reaching O_2 levels of 0 kPa at 8 days of storage. Regardless of the pathogen evaluated, after 3 days of storage a slight increase of CO_2 levels was observed

with values of approximately 7 kPa of CO_2. After 8 days of storage, a significant difference between the storage conditions was observed, regardless of the pathogen evaluated. Samples stored under temperature abuse conditions reached higher concentrations of CO_2 (approximately 22 kPa) than samples under constant storage at 4 °C (approximately 18 kPa).

The initial populations of *L. monocytogenes* and *S. enterica* on fresh-cut pears were 3.2 ± 0.9 and 3.6 ± 0.3 log CFU g^{-1} pear, respectively (*Figure 2*). After 3 days of storage at 4 °C, *L. monocytogenes* and *S. enterica* populations remained at the initial levels.

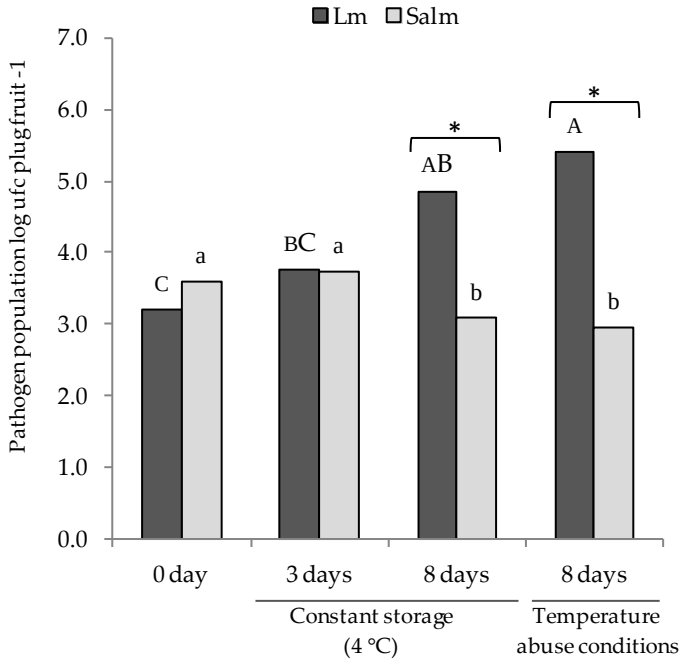


Figure 2. *Populations of* L. monocytogenes *and* S. enterica *on fresh-cut 'Conference' pears stored in conditions simulating a commercial application (constant storage at 4 °C for 8 days) or in temperature abuse conditions (3 days at 4 °C plus 5 days at 8 °C). The data represent the mean of three determinations and two experimental repetitions (n=6). Different letters indicate significant differences ($p < 0.05$). Uppercase letters (A, B, C and D) represent differences among different sampling points in* L. monocytogenes *populations and lowercase letters (a, b, c and d) represent differences among different sampling points in* S. enterica *populations. Difference among pathogens in each sampling point is represented by * ($p < 0.05$).*

After 8 days of storage, both constant storage (4 °C) and temperature abuse conditions caused a significant decrease of *S. enterica* populations on pear wedges, reaching 3.1 ± 0.2 and 3.0 ± 0.3 log CFU g^{-1} pear, respectively. In contrast, *L. monocytogenes* grew in both storage conditions. The values were 4.9 ± 0.5 log CFU g^{-1} pear (1.6 log increase) in samples stored at 4 °C and 5.4 ± 1.0 log CFU g^{-1} pear (2.20 log increase) in the temperature abuse conditions. Thus, the temperature increase for 5 days caused a greater increase in the *L. monocytogenes* populations on the fresh-cut pear product even in the highest CO_2 levels (22 kPa). Other researchers have observed that an active MAP containing 5 kPa O_2 and 30 kPa CO_2 had no inhibitory effect on the growth of *L. monocytogenes* on cactus-pear fruit at 4, 8, 12 and 20 °C,(Corbo *et al.*, 2005) or on coconut packaged under the same conditions and stored at 2, 4, 8 and 10 °C (Sinigaglia *et al.*, 2006). No significant differences between *L. monocytogenes* and *S. enterica* populations on fresh-cut pears were observed at the initial day or after 3 days of storage. However, significant differences among the pathogens were observed at 8 days in both storage conditions; the *L. monocytogenes* population on fresh-cut pears increased, whereas the *S. enterica* populations on fresh-cut pears decreased during same time. In fact, the effect of temperature in the growth rate of foodborne pathogens is well known. Alegre et al. (Alegre *et al.*, 2010a) noticed that *Salmonella* and *L. innocua* were able to grow on fresh-cut apples stored at room temperature and were unaffected by MAP or the addition of antioxidants. Thus, the observed behaviour of *S. enterica* on fresh-cut pears in the present study could be due to the storage temperature (below 10 °C).

Previously, we observed that *L. monocytogenes* was able to grow on fresh-cut pears processed (without antioxidant treatment and without MAP) at different ripeness stages and that the growth rate of *L. monocytogenes* increased with increasing temperature (Colás-Medà *et al.*, 2015). Alegre et al. (Alegre *et al.*, 2010a; Alegre *et al.*, 2010b) determined the survival and growth of *E. coli* O157:H7, *Salmonella* and *Listeria innocua* on some minimally processed fruits, including peaches and apples stored at different temperatures. The influence of fruit cultivar, use of antioxidant solution and passive MAP were also evaluated. Under refrigeration (5 °C), only *L. innocua* could grow on 'Elegant Lady' peach plugs and on 'Golden Delicious' apples. Nevertheless, in both matrices, *E. coli* O157:H7, *Salmonella* and *L. innocua* were able to grow on peach and apple plugs when incubated at 10 °C.

4. Conclusion

This is the first report that evaluates the behaviour of *S. enterica* and *L. monocytogenes* on minimally processed pears under conditions simulating commercial applications and a cold chain break. Although refrigeration at a constant temperature (4 °C) effectively inhibited the growth of *S. enterica* on fresh-cut pears, it did not affect the growth of *L. monocytogenes*. This study demonstrated that *L. monocytogenes* was able to grow on fresh-cut pears processed under the conditions described here, despite low pH, refrigeration and use of modified atmosphere. Thus, minimally processed pears should be protected from contamination by this pathogen during preparation, and food business operators should guarantee the absence of *L. monocytogenes* in 25 g of fruit before the food has left its control area.

5. Acknowledgment

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus and Banco Santander for a PhD grant (UdL-Impuls Program) to Pilar Colás Medà and to the Spanish Government (Ministerio de Economia y Competitividad, research project AGL-2012-38671) for its financial support.

6. References

Abadias, M., Usall, J., Anguera, M., Solsona, C., Vinas, I. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.

Alegre, I., Abadias, M., Anguera, M., Oliveira, M., Vinas, I. 2010a. Factors affecting growth of foodborne pathogens on minimally processed apples. *Food Microbiology* 27, 70-76.

Alegre, I., Abadias, M., Anguera, M., Usall, J., Viñas, I. 2010b. Fate of *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally-processed peaches under different storage conditions. *Food Microbiology* 27, 862-868.

Amiot, M.J., Tacchini, M., Aubert, S.Y., Oleszek, W. 1995. Influece of cultivar, maturity stage, and storage-conditions on phenolic composition and enzymatic browning of pear fruits. *Journal of Agricultural and Food Chemistry* 43, 1132-1137.

Arias, E., Gonzalez, J., Lopez-Buesa, P., Oria, R. 2008. Optimization of processing of fresh-cut pear. *Journal of the Science of Food and Agriculture* 88, 1755-1763.

Colás-Medà, P., Abadias, M., Alegre, I., Usall, J., Viñas, I. 2015. Effect of ripeness stage during processing on *Listeria monocytogenes* growth on fresh-cut 'Conference' pears. *Food Microbiology* 49, 116-122.

Colás-Medà, P., Abadias, M., Altisent, R., Alegre, I., Plaza, L., Gilabert, V., Lacomba, R., Viñas, I. 2016. Development of a fresh-cut product based on pears and the subsequent evaluation of its shelf life under commercial conditions and after a cold chain break. *Journal of Food and Nutrition Research* 4, 582-591.

Corbo, M.R., Campaniello, D., D'Amato, D., Bevilacqua, A., Sinigaglia, M. 2005. Behaviour of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in fresh-sliced cactus-pear fruit. *Journal of Food Safety* 25, 157-172.

Del Rosario, B., Beuchat, L. 1995. Survival and growth of Enterohemorrahagic *Escherichia coli* O157:H7 in cantaloupe and watermelon. *Journal of Food Protection* 58, 105-107.

Dong, X., Wrolstad, R.E., Sugar, D. 2000. Extending shelf life of fresh-cut pears. *Journal of Food Science* 65, 181-186.

European, C. 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs *Official Journal of the European Union* L338, 1-26.

Flessa, S., Lusk, D.M., Harris, L.J. 2005. Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *International Journal of Food Microbiology* 101, 255-262.

Gomes, M.H., Vieira, T., Fundo, J.F., Almeida, D.P.F. 2014. Polyphenoloxidase activity and browning in fresh-cut 'Rocha' pear as affected by pH, phenolic substrates, and antibrowning additives. *Postharvest Biology and Technology* 91, 32-38.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A. 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. *Journal of Food Science* 65, 541-544.

Montero-Calderón, M., Cerdas-Araya, M. 2010. Fruit and vegetables for the fresh-cut processing industry. In: Martín-Belloso, O., Soliva-Fortuny, R., (Eds.), Advances in fresh-cut fruits and vegetables processing. CRC Press. 185-209.

Nguyen-The, C. 2012. Biological hazards in processed fruits and vegetables - Risk factors and impact of processing techniques. *LWT - Food Science and Technology* 49, 172-177.

Olmez, H., Kretzschmar, U. 2009. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *Lwt-Food Science and Technology* 42, 686-693.

Oms-Oliu, G., Aguilo-Aguayo, I., Martin-Belloso, O. 2006. Inhibition of browning on fresh-cut pear wedges by natural compounds. *Journal of Food Science* 71, 216-224.

Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martín-Belloso, O. 2009. Effect of ripeness at processing on fresh-cut 'Flor de Invierno' pears packaged under modified atmosphere conditions. *International Journal of Food Science & Technology* 44, 900-909.

Oms-Oliu, G., Soliva-Fortuny, R., Martín -Belloso, O. 2008. Edible coatings with antibrowning agents to maintain sensory quality and antioxidant properties of fresh-cut pears. *Postharvest Biology and Technology* 50, 87-94.

Penteado, A.L., Leitão, M.F.F. 2003. Growth of *Listeria monocytogenes* in melon, watermelon and papaya pulps. *International Journal of Food Microbiology* 92, 89-94.

Sanchis, E., Gonzalez, S., Ghidelli, C., Sheth, C.C., Mateos, M., Palou, L., Perez-Gago, M.B. 2016. Browning inhibition and microbial control in fresh-cut persimmon (Diospyros kaki Thunb. cv. Rojo Brillante) by apple pectin-based edible coatings. *Postharvest Biology and Technology* 112, 186-193.

Sapers, G.M., Miller, R.L. 1998. Browning inhibition in fresh-cut pears. *Journal of Food Science* 63, 342-346.

Sinigaglia, M., Bevilacqua, A., Campaniello, D., D'Amato, D., Corbo, M.R. 2006. Growth of *Listeria monocytogenes* in fresh-cut coconut as affected by storage conditions and inoculum size. *Journal of Food Protection* 69, 820-825.

Soliva-Fortuny, R.C., Alos-Saiz, N., Espachs-Barroso, A., Martin-Belloso, O. 2004. Influence of maturity at processing on quality attributes of fresh-cut conference pears. *Journal of Food Science* 69, 290-294.

Soliva-Fortuny, R.C., Biosca-Biosca, M., Grigelmo-Miguel, N., Martin-Belloso, O. 2002. Browning, polyphenol oxidase activity and headspace gas composition during storage of minimally processed pears using modified atmosphere packaging. *Journal of the Science of Food and Agriculture* 82, 1490-1496.

Tapias, M.S., Welti-Chanes, J. 2002. Approches for safety assessment of minimally processed fruits and vegetables. In: Welti-Chanes, J., Barbosa-Canovas, G.,

Aguilera, J., (Eds.), Engineering and food for 21st century. CRC Press. 671-695.

Uchima, C.A., de Castro, M.F.P.M., Gallo, C.R., Rezende, A.C.B., Benato, E.R., Penteado, A.L. 2008. Incidence and growth of *Listeria monocytogenes* in persimmon (*Diospyros kaki*) fruit. *International Journal of Food Microbiology* 126, 235-239.

Ukuku, D.O., Huang, L.H., Sommers, C. 2015. Efficacy of sanitizer treatments on survival and growth parameters of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on fresh-cut pieces of cantaloupe during storage. *Journal of Food Protection* 78, 1288-1295.

USDA. 2016. National Nutrient Database for Standard Reference https://ndb.nal.usda.gov/ndb/foods/show/2326?fgcd=&manu=&lfacet=&format=&count=&max=50&offset=&sort=default&order=asc&qlookup=Pear&ds (Accessed: 04/10/16).

Xiao, Z., Luo, Y., Luo, Y., Wang, Q. 2011. Combined effects of sodium chlorite dip treatment and chitosan coatings on the quality of fresh-cut d'Anjou pears. *Postharvest Biology and Technology* 62, 319-326.

CAPÍTULO 3

Effect of ripeness stage during processing on *Listeria monocytogenes* growth on fresh-cut 'Conference' pears

P. Colás-Medà, M. Abadias, I. Alegre, J. Usall, I. Viñas.

Food Microbiology (2015), 49, 116-122

Abstract

There are several factors that affect the shelf life of fresh-cut fruit, including the cultivar, the ripeness stage of the fruit during processing and the fruit's storage atmosphere and temperature. The effect of fruit ripeness during processing on the survival and growth of *Listeria monocytogenes* on fresh-cut 'Conference' pear slices at different temperatures (5, 10 and 20 °C) was studied. The four ripeness stages studied in this work (assessed by a fruit's firmness) were mature-green (54-60 N), partially ripe (43-53 N), ripe (31-42 N) and overripe (< 31 N). In our studies, pH, acidity and soluble solids content did not significantly change during conditioning at 20 °C. *L. monocytogenes* grew under all experimental conditions, showing an increase of approximately 2 log CFU g^{-1} after 8 days of storage at 5 °C. There were significant differences in the *L. monocytogenes* population between different ripeness stages at the end of the experiments at 10 and 20 °C. Regardless of the ripeness stage of a fresh-cut pear, the growth potential of *L. monocytogenes* increased with increasing temperature. A pear's ripeness stage during processing is an important consideration to ensure the quality of a fresh-cut pear, but it is not as important for preventing *L. monocytogenes* growth at common storage temperatures.

Keywords: *Listeria monocytogenes*, fresh-cut pear, ripeness stage, growth.

1. Introduction

In recent years, the consumption of fresh-cut fruits and vegetables has quickly increased. This increase was motivated by fruit's desirable qualities such as its freshness, low-calorie composition, nutritional content and convenience. The average fruit and vegetable consumption in the European Union (EU) and the United States is 6 and 30 kg/year/person, respectively. However, the consumption for each country within the EU varies. For example, in the United Kingdom the average consumption is 20 kg/year/person, followed by France and Italy at 12 and 8 kg/year/person, respectively. Despite the low consumption of fresh-cut fruits and vegetables in Spain (3 kg/year/person, Anonymous, (2014)), the production these fruits and vegetables has continuously increased, from 36700 to 60169 to 129637 ton in 2004, 2007 and 2013, respectively. Some fresh-cut fruits, such as apples, pineapples, melons, mangos and fruit mixes, are being sold in Spanish markets; however, their sales are low compared with vegetable sales (FEPEX, 2013).

Pears (*Pyrus communis L.*) have low protein and lipid contents and are rich in sugars such as fructose, sorbitol, and sucrose and low in glucose. Pears also contain micronutrients, such as vitamins, minerals and antioxidants. Lleida province, located in Catalonia (Spain), is the first province in Spain to produce pears. In 2011, 502434 tons of pears were produced in Spain, 49.8 % of which were produced in Lleida (Magrama, 2012). Moreover, in 2012 175493 ton of common pears produced in Lleida were distributed as follows: 22638 ton of 'Blanquilla', 65810 ton of 'Conference' and 25601 ton of 'Llimonera' (DAAM, 2012). The production of fresh-cut pears can potentially increase profits for companies by serving as an alternative to fresh fruit sold at markets.

Some important factors that affect the shelf life of fresh-cut fruit include the specific variety of the fruit, the fruit's stage of ripeness at the cutting step, and the fruit's storage atmosphere and temperature (Gorny *et al.*, 2000). 'Conference' pears are the most produced variety in Lleida. These pears can be stored at low temperatures in a controlled atmosphere for an extended period of time (Nguyen *et al.*, 2007). Furthermore, among the different varieties of pear, 'Conference' pears are the most suitable for fresh-cut fruit production (Arias *et al.*, 2008; Colás-Medà *et al.*, unpublished data; Soliva-Fortuny *et al.*, 2004) Several studies have been carried out to determine the optimal ripeness stage for pear processing, based on a pear's firmness. Soliva *et al.* (2004) demonstrated that partially ripe (firmness: 44 ± 3.2 N) 'Conference' pears were the most suitable for processing.

Gorny *et al.* (2000) determined that 'Barlett' pears were ideal for processing when they were partially ripe (44 to 58 N). Moreover, Oms-Oliu *et al.* (2009) also studied the effect of the ripeness of 'Flor de Invierno' pears on the growth of indigenous microbiota. They found that rapid microbial growth occurred on ripe pears (36.1 N), and partially ripe pears (43.3 N) were suitable for conservation while gathering desired sensory attributes.

Two key processing steps for preserving fruit are the removal of the peel or rind and the cutting of the fruit. The protective barrier is removed during the processing of fresh-cut fruits and vegetables, which makes the produce especially vulnerable to microbial contamination and colonization and increases the risk of fresh-cut produce becoming a health hazard (Leverentz *et al.*, 2001). In the last few years, there have been several outbreaks linked to the consumption of contaminated fruits and vegetables. *Salmonella* spp. and *Escherichia coli* O157:H7 outbreaks have been linked to the consumption of cantaloupe, watermelon, mango, tomato, papaya and fruit salads (CDC, 2014; Harris *et al.*, 2003). Although *Listeria monocytogenes* outbreaks have only been linked to the consumption of cantaloupes and tomatoes, those incidences resulted in a high mortality rate. *L. monocytogenes* is a gram-positive bacterium, a facultative anaerobic and an important foodborne pathogen. There are 13 serotypes of *L. monocytogenes*, but 90% of human infections are usually associated with three specific serotypes: 1/2a, 1/2b and 4b. This microorganism can grow at temperatures between -0.4 °C and 45 °C, with 37 °C being the optimal growth temperature. In addition, *L. monocytogenes* can grow anywhere between pH 4.4 and pH 7.0, depending on the temperature (Walker, Stringer, 1987). The growth of *L. monocytogenes* under refrigerated and ambient conditions has been evaluated in several studies on fruits, including apples (Alegre *et al.*, 2010a; Conway *et al.*, 2000), peaches (Alegre *et al.*, 2010b), strawberries (Flessa *et al.*, 2005), persimmons (Uchima *et al.*, 2008) and melons, watermelons and papayas (Penteado, Leitão, 2004; Uchima *et al.*, 2008); however, no studies have been carried out on fresh-cut pears.

The objective of the present study was to determine the effect of fruit ripeness during processing on the survival and growth of *L. monocytogenes* on fresh-cut pear slices stored at various temperatures.

2. Materials and Methods

2.1. Fruit

'Conference' pears (*Pyrus communis* L. cv. Conference) were acquired from a local shipper in the city of Lleida (Catalonia, Spain). The fruits were stored at 0 °C until use. The pears were ripened by incubation at 20 °C, for a maximum of 72 h, until the desired ripeness was achieved (Soliva-Fortuny *et al.*, 2004). In this study, the ripeness stage of a pear was determined by its firmness. Flesh firmness was measured on opposite sides of each fruit with a penetrometer (Effegi, Mila, Italy) equipped with a probe 8 mm in diameter. Once the desired firmness values were achieved, the pears were incubated at 0 °C overnight. The ripeness stage categorizes in this work were 54-60 N (mature-green), 43-53 N (partially ripe), 31-42 N (ripe) and < 31 N (overripe) and were determined by sampling 10 fruits for each category.

2.2. Microorganisms and preparation of cell suspensions

For this study, *Listeria monocytogenes* serovar 1/2a was isolated from commercial fresh-cut Iceberg lettuce (Abadias *et al.*, 2008). *L. monocytogenes* was grown overnight at 37 ± 1 °C in tryptone soy broth (TSB, Oxoid, UK) supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB). Bacterial cells were harvested by centrifugation at 9820 x g and 10 °C for 10 min and then resuspended in saline peptone (SP; 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone). The concentration was estimated using a spectrophotometer set at λ = 420 nm and a standard curve. For inoculum preparation, an aliquot of the foodborne pathogen suspension was added to deionized water to obtain approximately 10^5 CFU ml^{-1}. The inoculum concentration was determined by plating dilutions onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics, Beauvais, France) and incubating the plates at 37 ± 1 °C for 48 h.

2.3. Inoculation of fruits and testing of the packaging and storage conditions

Prior to the experiments, pears were washed with running tap water and dried by hand with absorbent paper to eliminate plant debris and pesticide residues. Then the pears' surfaces were disinfected with 70 % ethanol. Pears were peeled and cut into 10 wedges using a handheld apple corer and slicer. Afterwards, pear wedges were inoculated by immersion into an *L. monocytogenes* suspension (1:2 w/v) shaken at 150 rpm for 2 min (Abadias *et al.*, 2014; Alegre *et al.*, 2013). Next, the

liquid was drained off, and the wedges were left to air-dry in a biosafety cabinet. Fresh-cut pears (150 g) were placed on covered polypropylene trays (500 ml) in ambient air (21 % O_2, 0 % CO_2). Once packed, the trays of pears were stored at 20 ± 1 °C, 10 ± 1 °C and 5 ± 1 °C. The pears stored at 5 and 10 °C were examined on the day of inoculation and after 2, 5 and 8 days. The samples stored at 20 °C were examined on the day of inoculation and after 5, 10, 22, 29 and 45 h.

2.4. Physicochemical analyses of fresh-cut pears

Before inoculation, the pH of the fresh-cut pears was measured using a pH meter Model GLP22 (Crison Instruments S.A., Barcelona, Spain) with a penetration electrode (5231 Crison). After the pH reading, the pears were squeezed, and the soluble solids content (SCC) was determined using a handheld refractometer at 20 °C (Atago CO., LTD, Japan). The results were expressed in °Brix. To measure the titratable acidity (TA), 10 ml of pear juice was diluted with 10 ml of deionized water and then titrated with a 0.1 N sodium hydroxide (NaOH) solution to pH 8.1. The results were calculated as g of malic acid per litre of solution.

2.5. Enumeration and detection of *L. monocytogenes*

The population of *L. monocytogenes* was determined for three sample trays for each ripeness stage at each sampling time and temperature. At each sampling time, 10 g of fruit was placed in a sterile plastic bag (400 ml, BagPage, Interscience, BagSystem, St Nom La Breteche, France), and 90 ml of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England) was added. This mixture was homogenized in a stomacher blender at 250 impact s^{-1} for 90 s (IUL, Masticator, Spain). Aliquots of the mixture were serially diluted into SP, the surface was placed onto Palcam agar and the agar plates were incubated at 37 ± 1 °C for 48 h. The results were expressed as colony forming units (CFU) of *L. monocytogenes* per gram of pear. The data were plotted on a decimal logarithm (log) scale. Each experiment was performed in duplicate.

Moreover, the growth potential of *L. monocytogenes* in each ripeness stage was assessed by comparing the difference between the log CFU g^{-1} at the beginning (corresponding to the end of the processing, time 0) and end (at 5 and 10 °C: day 8; and at 20 °C: 45 h) of the assay (Beaufort, 2011). According to Regulation (EC) No. 2073/2005, if the growth potential is higher than 0.5 log CFU g^{-1}, the food is assumed to be capable of facilitating the growth of *L. monocytogenes*.

2.6. Statistical analysis

Data of *L. monocytogenes* growth (log CFU g^{-1}) and quality parameters were analysed using general linear model analysis with JMP8 software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level of $P < 0.05$. When the analysis was statistically significant, the least significance difference (LSD) test for separation of means was used.

3. Results

3.1. Physicochemical parameters of fresh-cut 'Conference' pears

Different pear batches were used for each temperature experiment; therefore, the physicochemical quality parameters are shown for each temperature. At 5 °C, the mean firmness values for 54-60 N (mature-green), 43-53 N (partially ripe), 31-42 N (ripe) and < 31 N (overripe) were 56.3 ± 3.1, 48.1 ± 2.2, 37.4 ± 3.3 and 20.2 ± 3.9 N, respectively (Table 1). The overripe pears had the lowest pH (4.85 ± 0.28), while the highest pH was observed in partially ripe pears (pH 5.17 ± 0.14). SSC values were not significantly different among the ripeness stages studied (14.7-15.1 °Brix). However, there were significant differences (P< 0.05) in TA, with the ripe stage presenting the highest TA (1.60 ± 0.36 g malic acid L^{-1}).

Table 1. *Physicochemical characteristics of the flesh of whole 'Conference' pears prior to use in experiments at 5 °C.*

Fruit ripeness stage	Firmness (N)	pH	Soluble solid content (°Brix)	Titratable acidity (g ac. malic L^{-1})
Mature-green	56.3 ± 3.1 ^{a}	5.05 ± 0.17^{ab}	14.6 ± 0.7^{a}	1.26 ± 0.07^{b}
Partially ripe	48.1 ± 2.2 ^{b}	5.17 ± 0.14^{a}	14.6 ± 0.8^{a}	1.23 ± 0.18^{b}
Ripe	37.4 ± 3.3 ^{c}	5.02 ± 0.22^{ab}	14.7 ± 0.8^{a}	1.60 ± 0.36^{a}
Overripe	20.2 ± 3.9 ^{d}	4.85 ± 0.28^{b}	15.1 ± 1.1^{a}	1.37 ± 0.17^{ab}

Values are expressed as the mean of six values ± standard deviation. For each parameter, different lowercase letters (a, b, c and d) in the same column indicate significant differences (P< 0.05) according to the LSD test

The mean firmness values of mature-green, partially ripe, ripe and overripe pears were 56.8 ± 3.1, 48.2 ± 3.2, 36.1 ± 3.8 and 21.2 ± 4.5 N, respectively, at 10 °C (Table 2). Mature-green pears had the lowest pH (4.70 ± 0.24), and ripe pears had the highest pH (5.17 ± 0.28). The SSC was not affected by the ripeness stage (14.6-15.3 °Brix). Mature-green pears had the highest TA (1.56 ± 0.26 g malic acid L^{-1}), while ripe and overripe pears showed the lowest TAs (1.18 ± 0.18 and 1.21 ± 0.27 g malic acid L^{-1}, respectively).

Table 2. *Physicochemical characteristics of the flesh of whole 'Conference' pears prior to use in experiments at 10 °C.*

Fruit ripeness stage	Firmness (N)	pH	Soluble solid content (°Brix)	Titratable acidity (g ac. malic L^{-1})
Mature-green	56.8 ± 3.1^{a}	4.70 ± 0.24^{b}	15.3 ± 0.2^{a}	1.56 ± 0.26^{a}
Partially ripe	48.2 ± 3.2^{b}	5.03 ± 0.32^{a}	14.9 ± 0.2^{a}	1.24 ± 0.45^{ab}
Ripe	36.1 ± 3.8^{c}	5.17 ± 0.28^{a}	14.9 ± 0.5^{a}	1.18 ± 0.18^{b}
Overripe	21.2 ± 4.5^{d}	4.93 ± 0.19^{ab}	14.7 ± 0.7^{a}	1.21 ± 0.27^{b}

Values are expressed as the mean of six values ± standard deviation. For each parameter, different lowercase letters (a, b, c and d) in the same column indicate significant differences (P< 0.05) according to the LSD test

In the 20 °C experiments, the mean firmness of mature-green, partially ripe, ripe and overripe pears were 58.6 ± 3.7, 50.6 ± 2.6, 36.0 ± 2.6 and 20.6 ± 2.1 N, respectively (Table 3). The pH values were not significantly different (P > 0.05) between the ripeness stages studied (4.91-5.04). Overripe pears had the lowest SSC (14.6 ± 0.5 °Brix), while ripe pears had the highest SSC (15.1 ± 0.5 °Brix). Overripe pears had the highest TA (1.05 ± 0.15 g malic acid L^{-1}), while partially ripe pears showed the lowest TA (0.84 ± 0.15 g malic acid L^{-1}).

Table 3. *Physicochemical characteristics of the flesh of whole 'Conference' pears prior to use in experiments at 20 °C.*

Fruit ripeness stage	Firmness (N)	pH	Soluble solid content (°Brix)	Titratable acidity (g ac. malic L^{-1})
Mature-green	58.6 ± 3.7[a]	4.99 ± 0.19[a]	14.7 ± 0.7 [ab]	1.04 ± 0.07[a]
Partially ripe	50.6 ± 2.6[b]	5.00 ± 0.27 [a]	14.9 ± 0.2 [ab]	0.84 ± 0.15 [b]
Ripe	36.0 ± 2.6[c]	5.04 ± 0.25 [a]	15.1 ± 0.5 [a]	0.99 ± 0.29[ab]
Overripe	20.6 ± 2.1[d]	4.91 ± 0.14 [a]	14.6 ± 0.5[b]	1.05 ± 0.15[a]

Values are expressed as the mean of six values ± standard deviation. For each parameter, different lowercase letters (a, b, c and d) in the same column indicate significant differences (P< 0.05) according to the LSD test

3.2. Effect of the ripeness stage on the growth of *L. monocytogenes* in fresh-cut pears

3.2.1. Survival of *L. monocytogenes* on fresh-cut pears stored at 5 °C

The initial *L. monocytogenes* population in fresh-cut pears was 3.3 ± 0.1 log CFU g^{-1} (*Figure 1*). The overripe fresh-cut pears had significantly lower counts of *L. monocytogenes* than pears of all of the other ripeness stages, with 3.3 ± 0.1 and 4.2 ± 0.3 log CFU g^{-1} after 2 and 5 days of storage at 5 °C, respectively. After 8 days of storage at 5 °C, there were no significant differences among the *L. monocytogenes* population in fresh-cut pears processed at different ripeness stages. All final population counts were between 5.2 ± 0.4 and 5.6 ± 0.4 log CFU g^{-1}.

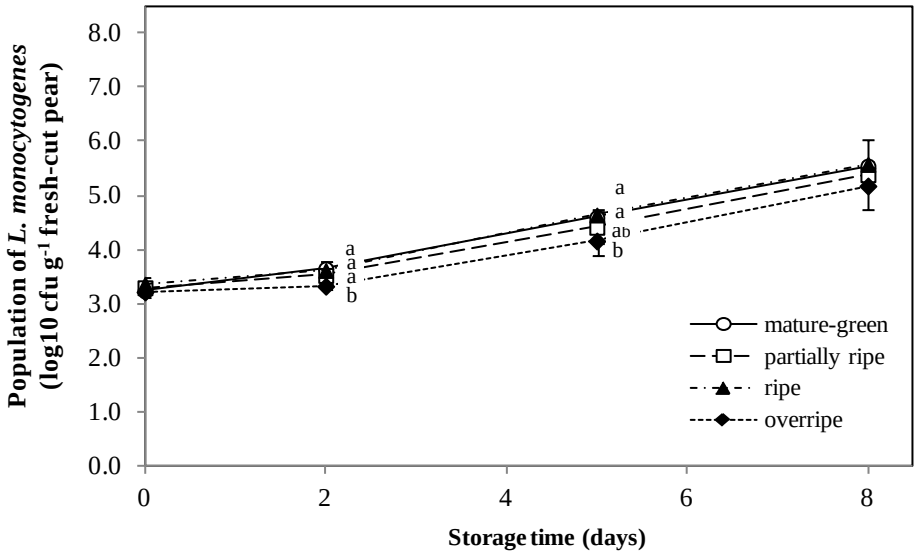


Figure 1. Listeria monocytogenes *population on fresh-cut 'Conference' pears processed at different ripeness stages stored at 5 °C. The values are the average of triplicate samples from two experiments (n=6). The error bars represent the standard deviation of the mean. Different lowercase letters (a, b, c and d) in the same point indicate significant differences (P < 0.05) among firmness states of a pear at each sampling time. There are no letters for points when there were no significant differences.*

3.2.2. Survival of *L. monocytogenes* on pear slices stored at 10 °C

After inoculation, all the fresh-cut pears had an initial *L. monocytogenes* population of 3.4 ± 0.2 log CFU g^{-1}, regardless of the ripeness stage of the pear. After 5 days of storage at 10 °C, the *L. monocytogenes* population was significantly higher in the overripe fresh-cut pears (7.0 ± 0.4 log CFU g^{-1}), while mature-green pears had the lowest pathogen population (6.4 ± 0.4 log CFU g^{-1}). At the end of the experiment (8 days), the populations were 6.8 ± 0.5, 6.9 ± 0.4, 7.3 ± 0.2 and 7.5 ± 0.4 log CFU g^{-1}, in the mature-green, partially ripe, ripe and overripe pears, respectively. Significant differences were observed in the overripe and mature-green pears. The maximum population of *L. monocytogenes* (7.5 ± 0.4 log CFU g^{-1}) was observed in the overripe fresh-cut pears after 8 days of storage (see *Figure 2*).

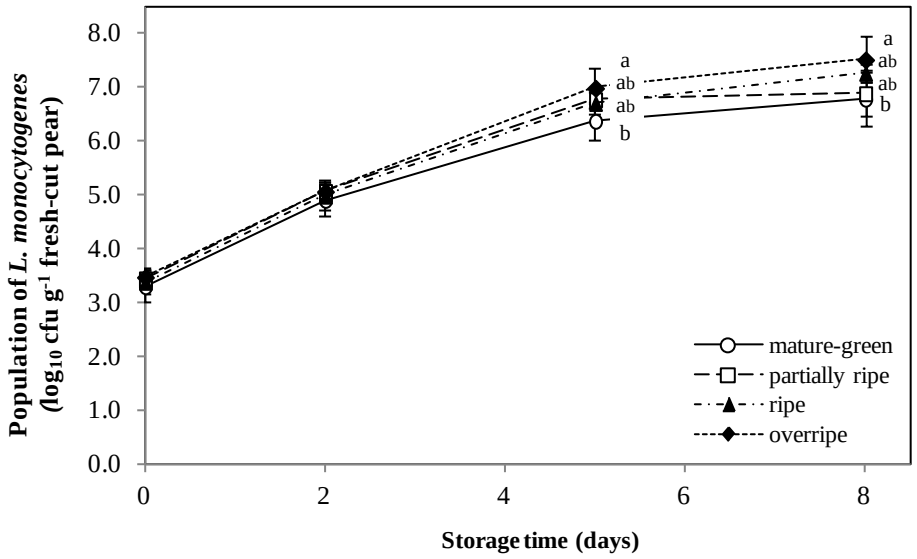


Figure 2. Listeria monocytogenes *population on fresh-cut 'Conference' pears processed at different ripeness stages stored at 10 °C. The values are the average of triplicate samples from two experiments (n=6). The error bars represent the standard deviation of the mean, and different lowercase letters (a, b, c and d) in the same point indicate significant differences (P < 0.05) among firmness states of a pear at each sampling time. There are no letters for points when there were no significant differences.*

3.2.3. Survival of *L. monocytogenes* on pear slices stored at 20 °C

The growth rate of *L. monocytogenes* on fresh-cut pear slices stored at 20 °C was higher than that on slices stored at 5 and 10 °C (*Figure 3*). Moreover, the duration of the 20 °C experiment was shorter than that of the 5 and 10 °C experiments. After inoculation, fresh-cut pears presented an initial *L. monocytogenes* population of 3.3 ± 0.1 log CFU g^{-1}. At the end of the evaluation, around 2 days (exactly 45 h), the population of *L. monocytogenes* was 7.3 ± 0.1, 7.0 ± 0.4, 7.5 ± 0.1 and 7.6 ± 0.1 log CFU g^{-1} in the mature-green, partially ripe, ripe and overripe, respectively; the partially ripe pears had the lowest population (7.0 ± 0.4 log CFU g^{-1}).

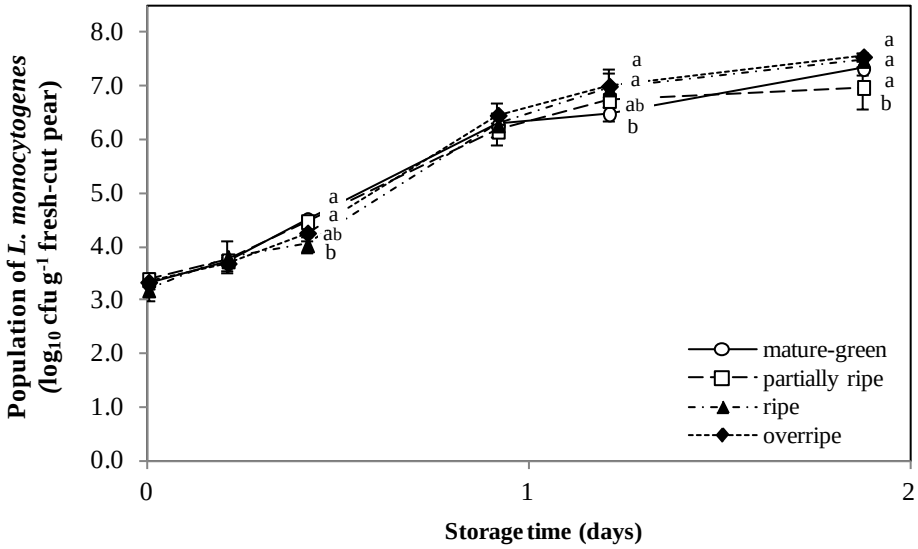


Figure 3. Listeria monocytogenes *population on fresh-cut 'Conference' pears processed at different ripeness stages stored at 20 °C. The values are the average of triplicate samples from two experiments (n=6). The error bars represent the standard deviation of the mean, and different lowercase letters (a, b, c and d) in the same point indicate significant differences (P < 0.05) among firmness states of a pear at each sampling time. There are no letters for points when there were no significant differences.*

3.3. Growth potential (δ) of *L. monocytogenes* on fresh-cut pears

Table 4 shows the growth potential (δ) of *L. monocytogenes* 1/2a at different storage temperatures. As expected, the δ values were higher for higher storage temperatures. After 2 days at 5 °C, the highest δ of *L. monocytogenes* (0.42 log CFU g^{-1}) was observed in mature-green pears, and the lowest δ (0.18 log CFU g^{-1}) was observed in overripe pears. In contrast, at 10 °C the highest δ of *L. monocytogenes* (1.77 log CFU g^{-1}) was found in overripe pears, and the lowest (1.57 log CFU g^{-1}) in mature-green pears.

At 20 °C, the δ of *L. monocytogenes* was similar (4.41 log CFU g^{-1}) in ripe and overripe pears, while partially ripe pears had the lowest δ value (3.84 log CFU g^{-1}) after 2 days. At the end of the 5 °C experiment (8 days) at 5 °C mature-green and ripe pears had the highest δ values (2.41 and 2.44 log CFU g^{-1}, respectively), and partially ripe and overripe had the lowest δ values (2.11 and 2.19 log CFU g^{-1}, respectively).

Table 4. *Results obtained from a growth potential test for* L. monocytogenes *in ready-to-eat pears. The values in table were comparable between 5 and 10 °C because both experiments were carried out for 8 days. Moreover, this table compares all growth potential values after 2 days of storage at 5, 10 and 20 °C.*

	After 2 days of storage (log CFU g^{-1})			After 8 days of storage (log CFU g^{-1})	
	5 °C	10 °C	20 °C	5 °C	10 °C
Mature-green (54-60 N)	0.42	1.57	4.11	2.41	3.48
Partially ripe (43-53 N)	0.36	1.73	3.84	2.11	3.73
Ripe (31-42 N)	0.33	1.70	4.41	2.44	4.00
Overripe (< 31 N)	0.18	1.77	4.41	2.19	4.40

For the 10 °C experiments, the pears with low firmness (overripe) had the highest δ values of *L. monocytogenes* (4.40 log CFU g^{-1}), while the firmest pears (mature-green) had the lowest δ value of 3.48 log CFU g^{-1} after 8 days of storage. The growth potential of *L. monocytogenes* after 8 days of storage (normal shelf life) under reasonable conditions (5 °C) was greater than 0.5 log CFU g^{-1} regardless of the ripeness stage of the pear. These results confirm that fresh-cut 'Conference' pears are able to facilitate the growth of *L. monocytogenes*.

4. Discussion

To our knowledge, this is the first study that assesses the growth of *L. monocytogenes* on fresh-cut 'Conference' pears at different ripeness stages and storage temperatures. The ripeness stage was determined based on the firmness of the pear (Gorny *et al.*, 2000; Oms-Oliu *et al.*, 2009; Soliva-Fortuny *et al.*, 2004).

The 'Conference' pears used in this study had a pH between 4.70 and 5.55, a SSC between 13.8 and 15.8 °Brix and a TA between 0.84 and 1.60 g malic acid L^{-1}. Our studies showed that pH, acidity and soluble solids content did not significantly change during the 20 °C incubation. The slight differences observed were

probably because different batches of pears were used for different sets of experiments. In accordance with our findings, Cano-Salazar *et al.* (2012; Cano-Salazar *et al.*, 2013) did not observe significant changes in the SSC, TA and colour of different peach and nectarine cultivars incubated at 20 °C for 3 days.

Our results confirm that the serovar 1/2a strain of *L. monocytogenes* can grow on fresh-cut 'Conference' pears with a firmness from 59 N to less than 20 N at storage temperatures of 5, 10 and 20 °C. Regardless of the pear's ripeness, *L. monocytogenes* was able to grow on the pears even when they were stored at 5 °C, with an increase in the population of approximately 2 log CFU after 8 days. Notably, for the 5 °C batch, overripe pears had the lowest pH (4.85 ± 0.28), and the growth of *L. monocytogenes* on overripe pears was significantly lower than growth on pears at other ripeness stages after 2 and 5 days of storage. However, these differences were not observed after 8 days. The growth of *L. monocytogenes* on other fresh-cut fruits at refrigeration conditions has been studied, and large differences have been observed for certain fruit varieties. Alegre *et al.* (2010b) found that the *Listeria innocua* population increased by 0.4 log CFU on 'Elegant Lady' peach plugs (pH 3.73 ± 0.28) after 14 days at 5 °C. On the contrary, Alegre *et al.* (2010a) found that the *L. innocua* population steadily declined in 'Granny Smith' apples (pH 3.32 ± 0.13) and exhibited a more drastic decline from 5.1 log CFU plug^{-1} to 1.7 log CFU plug^{-1} in 'Shampion' (pH 4.44 ± 0.26) after 14 days. In 'Golden Delicious' apples (pH 4.16 ± 0.25), there was an initial drop in the *L. innocua* population, and then the population increased to the inoculum's level at the end of the 5 °C experiment. Conway *et al.* (2000) found that the *L. monocytogenes* population on 'Delicious' apples (pH 4.7) did not increase, but the bacteria survived at this temperature throughout the 12-day study. As for strawberries, Flessa *et al.* (2005) observed a reduction of less than 1 log CFU of *L. monocytogenes* (pH 3.7) after 7 days of storage at 4 °C.

At 10 °C, the population of *L. monocytogenes* on fresh-cut pears processed at different ripeness stages increased to values between 3.4 ± 0.2 and 4.0 ± 0.2 log CFU g^{-1} after 8 days of storage. There were significant differences between the growth on mature-green and overripe pears, with lower counts on mature-green pears, which had the lowest pH (4.70 ± 0.24) and highest TA (1.56 ± 0.26 g L^{-1} of malic acid L^{-1}). Similar results have been observed on fresh-cut 'Crimson Sweet' watermelons (pH 5.50) in which *L. monocytogenes* presented an increase of 3.5 log CFU after 7 days at 10 °C (Penteado *et al.*, 2004). Uchima *et al.* (2008) studied two varieties of persimmons, 'Fuyu' and 'Rama Forte', with pH values of 6.3 and 5.5, respectively. In both varieties, there was an increase of 3.5

and 4.0 log CFU in the *L. monocytogenes* population on 'Rama Forte' and 'Fuyu', respectively after 9 days of storage at 10 °C. Higher increases have been observed on fresh-cut melons. Leverentz *et al.* (2003) observed that the population of *L. monocytogenes* in 'honeydew' melon (pH 5.8) increased by 4.5 log CFU after 7 days at 10 °C. Penteado and Leitão (2004) observed an increase of 6 and 7 log CFU in the *L. monocytogenes* population in 'Valenciano amarelo' melons (pH 5.87) after 7 and 2 days, respectively, at 10 and 20 °C. In contrast, on fresh-cut 'Red delicious' apples (pH 4.4) stored at 10 °C, Leverentz *et al.* (2006) observed only a slight increase (0.6 log CFU) in the population of *L. monocytogenes*. Similarly, Conway *et al.* (2000) found that the *L. monocytogenes* population on 'Delicious' apples increased by approximately 1.7 log CFU (pH 4.7) after 12 days.

At 20 °C, the population of *L. monocytogenes* on fresh-cut pears processed at different ripeness stage increased rapidly to between 3.6 and 4.3 log CFU g^{-1} after 2 days of storage. The highest increase was observed in ripe and overripe pears, reaching 7.5 log CFU g^{-1} after 45 h of storage. Similar results were observed in fresh-cut 'Crimson Sweet' watermelons (pH 5.50), in which the *L. monocytogenes* population increased by 4.0 log units after 4 days at 20 °C (Penteado *et al.*, 2004). In previous studies, *L. innocua* grew to 6.9 log CFU $plug^{-1}$ on 'Golden Delicious' apples after 2 days, which corresponded to an increase of approximately 2.2 log CFU (Alegre *et al.*, 2010a). Conway *et al.* (2000) found that the *L. monocytogenes* population increased by approximately 2.7 log CFU after 6 days of storage at 20 °C on 'Delicious' apples (pH 4.7). The population of *L. innocua* on 'Elegant Lady' peaches stored at 20 °C increased by approximately 2.8 log CFU after 6 days. In fresh-cut 'Valenciano amarelo' melons (pH 5.87), the population of *L. monocytogenes* increased by 7 log CFU after 4 days at 20 °C (Penteado *et al.*, 2004). Uchima *et al.* (2008) observed an increase of 6.1 and 5.1 log CFU in the *L. monocytogenes* population after 41 h at 20 °C in 'Fuyu' and 'Rama Forte' persimmons, respectively.

At 10 and 20 °C, the increase in the *L. monocytogenes* population on fresh-cut pears was similar to that of fresh-cut watermelons (pH 5.50) (Penteado *et al.*, 2004). Pears and watermelons have a similar pH values (pH 5.5). The pH values of apples, strawberries and peaches were lower than the pH of pears, with values ranging from 2.9 to 4.5, 3.0 to 3.6 and 3.5 to 5.0, respectively. In contrast, the pH of melons, watermelons and persimmons were greater than or equal to the pH of the pears, with values ranging from 6.2 to 6.7, 5.2 to 5.8 and 5.4 to 5.8, respectively. By comparing growth results, we could link the higher pH of the fresh-cut fruit matrix with greater growth in the *L. monocytogenes* population. However, other

intrinsic factors, such as water activity, redox potential, availability of nutrients, antimicrobial agents, etc. could play an important role in the behaviour of *L. monocytogenes* on minimally processed fruits.

At the end of the experiment, the growth of *L. monocytogenes* was only influenced by the ripeness stage of pears stored at 10 and 20 °C. At 10 °C, the growth of *L. monocytogenes* was significantly higher on overripe pears than on mature-green pears. This difference in growth could be due to the lower TA of the overripe pears (1.21 ± 0.27 g malic acid L^{-1}) compared with the TA of the mature-green pears (1.56 ± 0.26 g malic acid L^{-1}). At 20 °C, a higher increase was observed in the ripe and overripe pears, reaching a population of 7.5 log CFU g^{-1} at the end of the storage experiments, while a smaller increase was observed in partially ripe pears. Oms-Oliu *et al.* (2009) studied the effect of ripeness during processing on the shelf life of fresh-cut 'Flor de Invierno' pears and determined its effect on total mesophilic aerobic bacteria, yeast and mould populations. They observed that counts of aerobic mesophilic microorganisms attained at the stationary phase (*A*) were only significantly different for mature-green (65.2 N) fresh-cut pears. In addition, the maximum growth rate and *A* values increased as the ripeness stage advanced. For example, the *A* value for aerobic mesophilic microorganisms in mature-green (65.2 N) fresh-cut pears was 3.2 log CFU g^{-1}, whereas in ripe (36.1 N) fresh-cut pears the A value was 4.7 log CFU g^{-1}. Alegre *et al.* (2010a) concluded that storage temperature has a major impact on maintaining low levels of foodborne pathogen populations in artificially contaminated fresh-cut apples. Our results confirm that when the storage temperature increases the growth potential of *Listeria monocytogenes* on fresh-cut 'Conference' pears also increases. For example, in partially ripe pears, the growth potential (δ) of *L. monocytogenes* after 2 days of storage at 5, 10 and 20 °C was 0.36, 1.73 and 3.84 log CFU g^{-1}, respectively.

Our study confirms that 'Conference' pears are an ideal substrate for *L. monocytogenes*. Even though fruit ripeness is a very important consideration for maintaining a pear's quality during its shelf life, it is not an important parameter for preventing *L. monocytogenes* growth at 5 °C. In general, no correlation between a pear's ripeness stage and its *L. monocytogenes* growth was found. We observed that at 5 °C, growth of *L. monocytogenes* at different pear ripeness stages was not significantly different. However, when the storage temperature was harsher (10 and 20 °C), *L. monocytogenes* growth was higher for pears of more advanced ripeness stages.

Therefore, it is important for companies to have good harvesting techniques and handling and storage practices to prevent pathogen contamination. Additionally, it is important that the fruit product's temperature is maintained at less than 5 °C until consumption to avoid exceeding the microbiological safety criteria of *L. monocytogenes* (EC No 1441/2007).

5. Acknowledgements

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus, Banco Santander and the Spanish Government (Ministerio de Economia y Competitividad, research project AGL-2012-38671) for its financial support.

6. References

Abadias, M., Altisent, R., Usall, J., Torres, R., Oliveira, M., Vinas, I. 2014. Biopreservation of fresh-cut melon using the strain Pseudomonas graminis CPA-7. *Postharvest Biology and Technology* 96, 69-77.

Abadias, M., Usall, J., Anguera, M., Solson, C., Vinas, I. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.

Alegre, I., Abadias, M., Anguera, M., Oliveira, M., Vinas, I. 2010a. Factors affecting growth of foodborne pathogens on minimally processed apples. *Food Microbiology* 27, 70-76.

Alegre, I., Abadias, M., Anguera, M., Usall, J., Vinas, I. 2010b. Fate of *Escherichia coli O157:H7*, *Salmonella* and *Listeria innocua* on minimally-processed peaches under different storage conditions. *Food Microbiology* 27, 862-868.

Alegre, I., Vinas, I., Usall, J., Anguera, M., Altisent, R., Abadias, M. 2013. Antagonistic effect of *Pseudomonas graminis* CPA-7 against foodborne pathogens in fresh-cut apples under simulated commercial conditions. *Food Microbiology* 33, 139-148.

Anonymous. 2014. IV Gama, innovando para mantener su cuota de mercado. Fruit Logistica. http://www.revistamercados.com/?articulo=iv-gama-innovando-para-mantener-su-cuota-de-mercado (accessed: 28/03/2014).

Arias, E., Gonzalez, J., Lopez-Buesa, P., Oria, R. 2008. Optimization of processing of fresh-cut pear. *Journal of the Science of Food and Agriculture* 88, 1755-1763.

Beaufort, A. 2011. The determination of ready-to-eat foods into *Listeria monocytogenes* growth and no growth categories by challenge tests. *Food Control* 22, 1498-1502.

Cano-Salazar, J., Echeverria, G., Crisosto, C.H., Lopez, L. 2012. Cold-Storage Potential of four yellow-fleshed peach cultivars defined by their volatile compounds emissions, standard quality parameters, and consumer acceptance. *Journal of Agricultural and Food Chemistry* 60, 1266-1282.

Cano-Salazar, J., Lopez, M.L., Echeverria, G. 2013. Relationships between the instrumental and sensory characteristics of four peach and nectarine cultivars stored under air and CA atmospheres. *Postharvest Biology and Technology* 75, 58-67.

CDC. 2014. Centers of Disease Control and Prevention. List of Selected Multistate Foodborne Outbreak Investigations 2006-2014 <http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html> (Accessed: 26/10/2014).

Conway, W.S., Leverentz, B., Saftner, R.A., Janisiewicz, W.J., Sams, C.E., Leblanc, E. 2000. Survival and growth of *Listeria monocytogenes* on fresh-cut apple slices and its interaction with *Glomerella cingulata* and *Penicillium expansum*. *Plant Disease* 84, 177-181.

DAAM. 2012. Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Producció de pera, per varietats. Lleida 2012. <http://agricultura.gencat.cat/web/.content/de_departament/de02_estadistiques_observatoris/02_estructura_i_produccio/02_estadistiques_agricoles/03_fulls_de_previsio_collita/arxius_estatics/fpc_2012/2012_12_lleida_pera_definitius.pdf> (Accessed: 12/08/2014).

FEPEX. 2013. Federación Española de Asociaciones de productores y exportadores de frutas, hortalizas, flores y plantas vivas. Sector frutas y hortalizas preparadas. http://www.fepex.es/ext/sector-frutas-hortalizas-preparadas.aspx (accessed: 20/01/2014).

Flessa, S., Lusk, D.M., Harris, L.J. 2005. Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *International Journal of Food Microbiology* 101, 255-262.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A. 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. *Journal of Food Science* 65, 541-544.

Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F. 2003. Outbreaks associated with fresh produce: Incidence, growth and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78-141.

Leverentz, B., Conway, W.S., Alavidze, Z., Janisiewicz, W.J., Fuchs, Y., Camp, M.J., Chighladze, E., Sulakvelidze, A. 2001. Examination of bacteriophage as

a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *Journal of Food Protection* 64, 1116-1121.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R., Sulakvelidze, A. 2003. Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology* 69, 4519-4526.

Leverentz, B., Conway, W.S., Janisiewicz, W., Abadias, M., Kurtzman, C.P., Camp, M.J. 2006. Biocontrol of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* serovar Poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists. *Applied and Environmental Microbiology* 72, 1135-1140.

Magrama. 2012. Anuario de estadística 2012. http://www.magrama.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2012/default.aspx?parte=3&capitulo=13&grupo=9&seccion=4> (accessed: 11/02/2014).

Nguyen, T.A., Verboven, P., Schenk, A., Nicolai, B.M. 2007. Prediction of water loss from pears (Pyrus communis cv.Conference) during controlled atmosphere storage as affected by relative humidity. *Journal of Food Engineering* 83, 149-155.

Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martín-Belloso, O. 2009. Effect of ripeness at processing on fresh-cut 'Flor de Invierno' pears packaged under modified atmosphere conditions. *International Journal of Food Science & Technology* 44, 900-909.

Penteado, A.L., Leitão, M.F.F. 2004. Growth of *Listeria monocytogenes* in melon, watermelon and papaya pulps. *International Journal of Food Microbiology*.

Soliva-Fortuny, R.C., Alos-Saiz, N., Espachs-Barroso, A., Martin-Belloso, O. 2004. Influence of maturity at processing on quality attributes of fresh-cut conference pears. *Journal of Food Science* 69, S290-S294.

Uchima, C.A., Castro, M.F.P.M., Gallo, C.R., B, R.A.C., Benato, E.R., Penteado, A.L. 2008. Incidence and growth of *Listeria monocytogenes* in persimmon (*Diospyros kaki*) fruit. *International Journal of Food Microbiology* 126, 235-239.

Walker, S.J., Stringer, M.F. 1987. Growth of *Listeria monocytogenes* and *Aeromonas-hydrophila* at chill temperatures. *Journal of Applied Bacteriology* 63, R20-R20.

CAPÍTULO 4

Influence of fruit matrix and storage temperature on the survival of *Listeria monocytogenes* in gastrointestinal simulation

P. Colás-Medà, M. Abadias, M. Oliveira, J. Usall, I. Viñas.

Food Control (2016) doi: 10.1016/j.foodcont.2016.10.18

Abstract

This study aimed to assess the effect of storage temperature and fruit matrix on the survival of *L. monocytogenes* after a gastrointestinal simulation. The growth of *L. monocytogenes* on different matrices (fresh-cut pear and melon and synthetic growth medium as a control) and storage temperature (1, 5, 10 and 20 °C) was evaluated. Subsequently, the ability of the pathogen on different fruit matrices to overcome the gastrointestinal simulation was evaluated. The highest reduction in the population of *L. monocytogenes* on fresh-cut pear and melon subjected to the gastrointestinal simulation was after 6 days of storage at 5 °C (0.84 and 2.12 log reduction on the pear and melon, respectively). Conversely, higher survival ratios of *L. monocytogenes* in both matrices were observed at 1 °C, even with logarithmic increases after the whole gastrointestinal simulation during the experiment. At 20 °C, the survival capacity of *L. monocytogenes* was higher than that under storage at 5 and 10 °C when grown on fresh-cut pear, whereas this was not observed on fresh-cut melon. In general, under the proper storage temperature of fresh-cut fruit (5 °C) and after 1 day of storage, the *L. monocytogenes* on the melon was more sensitive than that on the pear, and this behaviour was maintained for up to 9 days. The ability of *L. monocytogenes* to overcome the gastrointestinal tract was not enhanced when the pathogen grew on fresh-cut pear and melon under the proper storage temperature.

Keywords: foodborne pathogen; melon; pear; gastrointestinal simulation; acid-adaptation; cold.

1. Introduction

The consumption of fruits and vegetables is associated with a healthy lifestyle. Thus, there has been an increasing market demand for minimally processed (MP) fruits and vegetables due to their fresh-like character, convenience, and human health benefits (Anon, 2014). Hurdles technology aims to improve the total quality of foods through the application of an intelligent mix of hurdles. 'Hurdles' are a popular analogy used to describe the concept that minimal food processing introduces sub-lethal stress that bacteria must overcome to survive or thrive in food systems. These cells, which have been 'prepared' or 'trained' to overcome hurdles, would have a significant advantage over wild cells. The most important hurdles used in food preservation are temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrate, sorbate, and sulphite), and competitive microorganisms (e.g., lactic acid bacteria) (Hill *et al.*, 2002; Leistner, 2000). *Listeria (L.) monocytogenes* is capable of surviving and growing in environments where these factors are present, resulting in the long-term adaptation of this pathogen to sub-lethal environmental stress conditions (Farber, Peterkin, 1991). In minimally processed fruit, pathogenic bacteria must overcome different 'hurdles'. First, the intrinsic properties of the fruit are the pH of the tissue, the type of acidity, the sugar content or nutrient availability. Second are external properties linked to their processing (e.g., storage temperature and gas atmosphere) (Ragaert *et al.*, 2011). For successful foodborne infection, *L. monocytogenes* must survive through fresh-cut fruit storage (shelf-life) and the stress conditions encountered during gastrointestinal transit. Some studies have assessed the survival of *L. monocytogenes* inoculated on meat or cheese products after gastrointestinal simulation (Barbosa *et al.*, 2012; Barmpalia-Davis *et al.*, 2008; Dikici, Calicioglu, 2013; Formato *et al.*, 2007; Melo *et al.*, 2013; Peterson *et al.*, 2007; Ramalheira *et al.*, 2010; Stopforth *et al.*, 2005; Tompkins *et al.*, 2011). However, little is known regarding the influence of fresh-cut fruit and storage temperature on the ability of this microorganism to survive gastrointestinal simulation. Thus, the aim of this study was to assess the effect of storage temperature and fruit matrix on *L. monocytogenes* survival along with gastrointestinal simulation.

2. Material and Methods

2.1. Microbial strain

L. monocytogenes serovar 1/2a, which was isolated from commercial fresh-cut iceberg lettuce (Abadias *et al.*, 2008), was used in our study. It was grown for

20-22 h in 50 ml of tryptone soy broth (TSB, Oxoid, UK) supplemented with 6 g L^{-1} of yeast extract (TSBYE) at 37 ± 1 °C and 120 rpm. The bacterial cells were harvested by centrifugation at 9800 × g for 10 min at 10 °C and then resuspended in 25 ml of saline peptone (SP; 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone).

2.2. Fruit

'Conference' pears (*Pyrus communis* L. cv. Conference) were obtained from local packinghouses in Lleida (Catalonia, Spain). 'Piel de sapo' melons (*Cucumis melo* L. var. Piel de sapo) were purchased in a local supermarket. The pears were used when the firmness was between 44 to 58 N, which is an optimum ripeness stage to obtain a better quality of fresh-cut pear (Gorny *et al.*, 2000). Flesh firmness was measured on two opposite sides of each fruit using a penetrometer (Effegi, Mila, Italy) equipped with a probe that was 8 mm in diameter.

2.3. Sample preparation

Prior to cutting, the fruit was washed in running tap water and dried by hand with absorbent paper. Then, the surface was disinfected with 70% ethanol. The pears were peeled and cut into 10 slices using a handheld apple slicer/corer. The melon was cut transversally in 14- to 16-mm slices. The seeds and rind were removed, and each slice was then cut into trapezoidal pieces. Erlenmeyer flasks containing 100 ml of TSBYE medium were used as a control for the growth experiment.

Before inoculation, the pH, soluble solids content (SSC) and titratable acidity (TA) of the fresh-cut pears and melons were determined. The pH of the flesh of the fruits was determined using a pH-meter (Model GLP22, Crison, Instruments S.A., Barcelona, Spain) equipped with a penetration electrode (5231 Crison, Instruments S.A., Barcelona, Spain). Before inoculation, nine determinations were performed per lot of fruit (n=9). After the pH reading, the pears and melons were squeezed separately, and the soluble solids content (SCC) of the extracted juice was determined in triplicate at 20 °C using a hand-held refractometer (Atago CO., Ltd., Tokyo, Japan). The data were expressed in °Brix. To measure the titratable acidity (TA), triplicate samples of 10 ml of the pear or melon juice were diluted with 10 ml of deionized water, and 2 drops of a phenolphthalein solution 1% (Panreac, Barcelona, Spain) were added. The samples were then titrated with a sodium hydroxide solution (NaOH, 0.1 mol l^{-1}) until a specific colour change of

the pH indicator was achieved. The results were calculated as g L^{-1} of malic acid for pears and g L^{-1} of citric acid for melons.

2.4. Inoculation

For the inoculum preparation, a volume of the *L. monocytogenes* suspension was added to deionized water to obtain approximately 10^5 CFU ml^{-1}. Fresh-cut pears and melons were inoculated separately by immersion in the *L. monocytogenes* suspension (1:2 w/v) and shaken at 150 rpm for 2 min. Afterwards, the liquid was drained off and the fruits were left to air-dry in a biosafety cabinet. As a growth control, Erlenmeyer flasks containing 100 ml of TSBYE were inoculated with a specific volume of the *L. monocytogenes* suspension and then homogenized. Previous experiments were performed to establish the correct volume of the pathogen suspension for flask inoculation to obtain similar initial populations for fresh-cut fruit.

2.5. Storage and sampling points

Inoculated fresh-cut fruit samples (100 ± 5 g) were placed into polypropylene trays of a 375 ml volume and sealed with a polypropylene plastic film. To obtain the air conditions, nine 400-μm holes were made in the sealed film using a needle. Once packed, the fruit trays were stored at 20 ± 1 °C, 10 ± 1 °C, 5 ± 1 °C and 1 ± 1 °C. TSBYE flasks were stored at the same temperatures but under shaking conditions (120 rpm). The samples stored at 1 and 5 °C were examined on the day of inoculation and after 1, 2, 6 and 9 days of storage. The samples stored at 10 °C were examined on the day of inoculation and after 1, 2 and 6 days of storage, and the samples stored at 20 °C were examined on the day of inoculation and after 1 and 2 days of storage. The headspace of the gas composition (carbon dioxide and oxygen) on the trays was measured using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) at each sampling point for all of the evaluated temperatures to confirm that the packages remained under aerobic conditions throughout storage. Once the trays were opened, the fruit pH values were measured in two fruit pieces from each tray (n=6) using a pH-meter equipped with a penetration electrode Model GLP22 (Crison Instruments S.A., Barcelona, Spain), and the same trays were used to evaluate the survival of *L. monocytogenes* throughout the gastrointestinal simulation as described below (part 2.6). There were three trays/Erlenmeyer flasks per temperature and the sampling time and experiment was repeated twice. The quality data from each trial were combined after testing to determine if they showed any significant differences.

2.6. Gastrointestinal solutions

Our *in vitro* digestion model procedure was performed according to Oomen *et al.* (2003) and Oliveira *et al.* (2011) with some modifications. These models describe a three-step procedure simulating digestive progress in the mouth, stomach and small intestine. Synthetic saliva fluid (SSF) was prepared with the following composition per litre: 0.90 g potassium chloride (KCl), 0.20 g potassium thiocyanate (KSCN), 1.15 g sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), 0.57 g sodium sulphate (Na_2SO_4), 0.30 g sodium chloride (NaCl), 0.07 g sodium hydroxide (NaOH), 0.20 g urea ($CO(NH_2)_2$), 145 mg α-amylase (Sigma), 15 mg uric acid ($C_5H_4N_4O_3$) and 50 mg mucin (Sigma). The pH was adjusted to 6.5 with hydrochloric acid (HCl, 0.1 N). The synthetic gastric fluid (SGF) was prepared with the following composition per litre: 0.82 g KCl, 0.35 g $NaH_2PO_4 \cdot 2H_2O$, 2.75 g NaCl, 0.40 g calcium chloride dihydrate ($CaCl_2 \cdot 2H2O$), 0.31 g ammonium chloride (NH_4Cl), 0.09 g $CO(NH_2)_2$, 0.65 g glucose ($C_6H_{12}O_6$), 0.02 g glucuronic acid ($C_6H_{10}O_7$), 0.33 g glucosamine hydrochloride ($C_6H_{14}ClNO_5$), 1.00 g bovine serum albumin fraction V (BSA, Sigma), 1.00 g pepsin (Sigma) and 3.00 g mucin (Sigma), and adjusted to pH 2.0 with HCl (6 mol L^{-1}). Two solutions of synthetic intestinal fluid (SIF) were prepared to simulate a duodenal and bile solution. The duodenal solution (DS) was prepared with the following composition per litre: 0.56 g KCl, 7.00 g NaCl, 3.39 g sodium bicarbonate ($NaHCO_3$), 0.08 g potassium dihydrogen phosphate (KH_2PO_4), 0.05 g magnesium chloride ($MgCl_2$), 0.20 g $CaCl_2 \cdot 2H_2O$, 0.10 g $CO(NH_2)_2$, 1.00 g BSA (Sigma), 3.00 g pancreatin (Sigma) and 0.50 g lipase (Sigma). The bile solution (BS) was prepared with the following composition per litre: 0.38 g KCl, 5.26 g NaCl, 5.79 g sodium bicarbonate ($NaHCO_3$), 0.22 g $CaCl_2 \cdot 2H_2O$, 0.25 g $CO(NH_2)_2$, 1.80 g BSA (Sigma) and 6.00 g bile (Sigma). The pH was adjusted to 7.8 and 8.0 in the duodenal and bile solutions, respectively, with NaOH (2 mol L^{-1}).

2.7. *In vitro* gastrointestinal simulation

At each sampling point, 10 g of inoculated fruit sample (pear or melon) were placed into a sterile plastic bag (80 ml, BagPage®, Interscience BagSystem, Saint Nom, France). Three different trays of inoculated pear or melon were used for each sampling point and temperature. Prior to the experiment, the digestive solutions (SSF, SGF, DS and BS) were kept in a water bath (TectronBio-100, J.P. Selecta, Barcelona, Spain) at 37 °C.

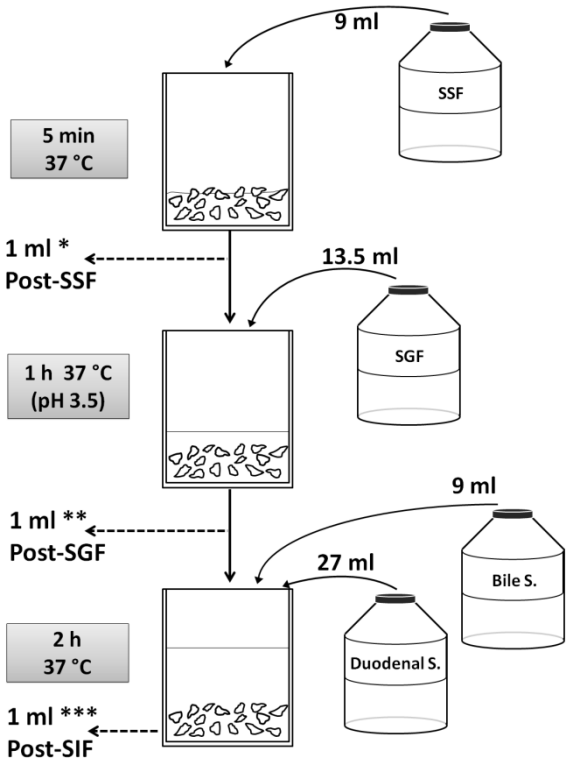


Figure 1. *Schematic overview of the gastrointestinal simulation (* post-saliva* L. monocytogenes *enumeration, ** post-SGF* L. monocytogenes *enumeration and *** post-SIF* L. monocytogenes *enumeration).*

All of the samples underwent the same digestive process (*Figure 1*). Nine millilitres of SSF was added to 10 g of sample and homogenized in a blender for 2 min at high speed (MiniMix, Interscience, Saint Nom, France). The samples were then incubated for 5 min at 37 °C. Afterwards, the pH of the mixture was measured, and duplicate 0.5 ml samples were collected for microbial analysis ('post-saliva count'). Previous experiments showed that the counts of *L. monocytogenes* (CFU g^{-1} fruit) obtained from a 10 g sample plus 90 ml SP or a 10 g sample plus 9 ml of synthetic saliva fluid (SSF) after 5 min of contact were the same. Thus, the 'post-saliva count' was used as the 'initial count'. The same method was used to recover the population of *L. monocytogenes* in TSBYE medium along with the storage at different temperatures to compare with the initial pathogen values on fresh-cut fruit. Then, 13.5 ml of SGF were added to the remaining sample and the mixture was homogenized and the pH measured

again. The pH could be altered by the buffering effect of each type of matrix (pear or melon). Thus, to begin the gastric step with the same values, the pH was adjusted to 3.5 with hydrochloric acid (HCl, 0.1 mol l^{-1}). The samples were incubated for 1 h at 37 °C. After incubation, the pH was measured and duplicate 0.5 ml samples were collected for microbial analysis ('post-SGF' count). The remaining sample (32.5 ml) was mixed with 27 ml of DS and 9 ml of BS, homogenized and the pH of the mixture measured. The sample was incubated for 2 h at 37 °C. Then, duplicate 0.5 ml samples were collected for microbial analysis ('post-SIF' count), and the pH of the final mixture was measured. For microbial analysis, the decimal dilutions were prepared using SP. Enumeration was performed by plating in duplicate on Palcam agar media. The plates were incubated at 37 °C for 48 h.

At each sampling point, all of the cell counts were calculated considering the dilution factors due to the continuous addition of gastrointestinal fluids. To represent the growth of *L. monocytogenes* on minimally processed pear and melon and in TSBYE medium during the storage, all CFU g^{-1} or ml^{-1} data were transformed to log CFU g^{-1} or ml^{-1}. In addition, the data were expressed as initial (population before the gastric step; log CFU g^{-1} of fruit), post-SGF population (after exposure to the gastric step; log CFU g^{-1} of fruit) and post-SIF population (after exposure to the intestinal step; log CFU g^{-1} of fruit). To compare between the fruit matrices, the logarithmic variation of the pathogen population after whole gastrointestinal simulation was calculated as the log N_{SIF}/N_0, where N_{SIF} is the pathogen population count at the end of the gastrointestinal simulation and N_0 is the initial cell density.

2.8. Statistical analysis

L. monocytogenes populations were statistically compared between the gastrointestinal steps at each sampling day for each matrix. Data on the *L. monocytogenes* population and quality parameters were analysed using a general linear model analysis with the JMP8 software (SAS Institute, Cary, NC, USA). The statistical significance was judged at the level of $P < 0.05$. When the analysis was statistically significant, the Tukey's test for the separation of the means was used.

3. Results

3.1. Quality parameters of fresh-cut fruits

The initial pH of the fresh-cut pear was between 4.39 and 5.87 (mean 5.08), and the initial pH of fresh-cut melon was between 5.16 and 6.72 (mean 5.85). The fresh-cut pear had SSC values from 12.5 to 15.7 °Brix (mean 14.5 °Brix) and TA values between 1.27 and 2.14 g malic acid L^{-1} (mean 1.62 g malic acid L^{-1}). The fresh-cut melon had SSC values between 8.4 and 12.4 °Brix (mean 10.5 °Brix) and TA values between 1.07 and 2.87 g citric acid L^{-1} (mean 1.70 g citric acid L^{-1}). The initial pH of the TSBYE medium was between 7.05 and 7.20.

No significant differences in the pH value were observed between the untreated and *L. monocytogenes*-treated fresh-cut pear and melon throughout the study at each storage temperature and sampling day (data not shown). Furthermore, the pH of the treated fresh-cut pear and melon did not change throughout the study under any of the storage conditions (data not shown). In contrast, the pH of the inoculated TSBYE medium exhibited a significant decrease at all of the temperatures throughout storage. At 1, 5, 10 and 20 °C, the inoculated TSBYE medium reached pH values of 6.99, 6.30, 5.94 and 5.61, respectively, at the end of the experiment (data not shown).

The gas composition of the headspace of the trays was measured at every sampling point, and the results demonstrated that air conditions were maintained throughout the experiment in all of the trays (data not shown).

3.2. Population of *L. monocytogenes* on fresh-cut pear and melon during a storage time of 1, 5, 10 and 20 °C

The initial populations of *L. monocytogenes* after inoculation were 3.63 log CFU g^{-1} on pear and 3.62 log CFU g^{-1} on melon (*Figure 2a* and *2b*). When the inoculated fresh-cut pear samples were stored at 1 °C, *L. monocytogenes* reached 3.72, 3.73, 4.03 and 3.81 log CFU g^{-1} and after 1, 2, 6 and 9 days of storage, respectively. For the inoculated fresh-cut melon, *L. monocytogenes* reached 3.70, 3.79, 4.22 and 4.63 log CFU g^{-1} after 1, 2, 6 and 9 days of storage at 1 °C, respectively.

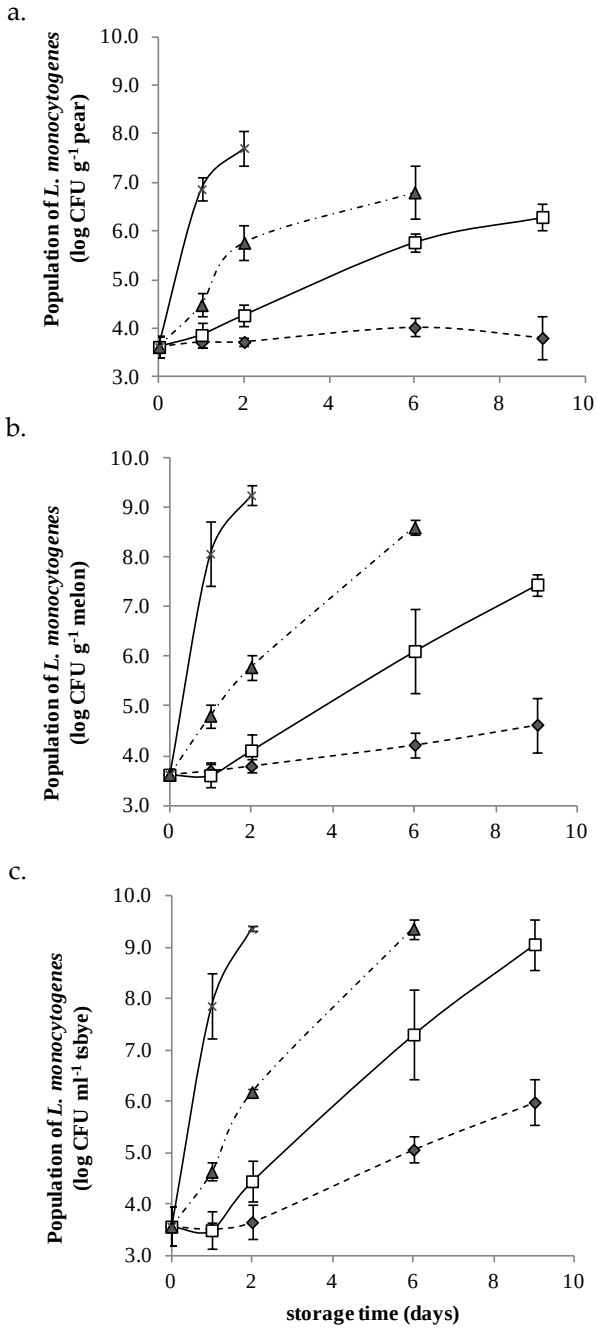


Figure 2. *Population (log CFU g^{-1} or ml^{-1}) of* L. monocytogenes *inoculated onto fresh-cut pear (A), melon (B) and TSBYE medium (C) during storage at 1 °C (diamonds), 5 °C (squares), 10 °C (triangles) and 20 °C (cross). The results are the means of two biological replicates each with three technical replicates (n=6), and the vertical bars indicate the standard deviation of the mean.*

Under the proper storage conditions (5 °C), *L. monocytogenes* on fresh-cut pears showed populations of 3.87, 4.28, 5.78 and 6.30 log CFU g^{-1}, whereas the pathogen on fresh-cut melon reached 3.61, 4.11, 6.11 and 7.46 log CFU g^{-1} after 1, 2, 6 and 9 days of storage, respectively. The higher pathogen population increases were observed under storage at 10 and 20 °C. When inoculated fresh-cut pears were stored at 10 °C, the *L. monocytogenes* population reached 4.49, 5.77 and 6.80 log CFU g^{-1} after 1, 2 and 6 days of storage, respectively. On the fresh-cut melon, *L. monocytogenes* reached populations of 4.81, 5.79 and 8.61 log CFU g^{-1} after 1, 2 and 6 days of storage, respectively. In both food matrices, the storage of samples at 20 °C caused the highest pathogen increase. On fresh-cut pears after 1 day, the *L. monocytogenes* population was 6.87 and increased until it reached 7.71 log CFU g^{-1} after 2 days of storage. On the fresh-cut melon, the *L. monocytogenes* population was 8.07 after 1 day and increased until it reached 9.25 log CFU g^{-1} after 2 days.

The growth of *L. monocytogenes* in an optimum growth media (TSBYE) under storage at 1, 5, 10 and 20 °C is shown in *Figure 2c*. After 1 day of storage at 1 and 5 °C, slight reductions of the pathogen were observed in TSBYE, whereas that pathogen on fresh-cut pear and melon always showed a population increase under the same storage conditions. After 2 days under these cold conditions, the pathogen in TSBYE increased exponentially until 9 days and reached 5.99 and 9.06 log CFU ml^{-1} at 1 °C and 5 °C, respectively. In the experiment at 10 °C, the *L. monocytogenes* count in the TSBYE medium was 6.19 log CFU ml^{-1} after 2 days of storage, whereas after 6 days of storage it was 9.37 log CFU ml^{-1}. After 1 and 2 days of storage at 20 °C, the *L. monocytogenes* population in the TSBYE medium was 7.86 and 9.36 log CFU ml^{-1}, respectively.

3.3. Survival of *L. monocytogenes* throughout the gastrointestinal simulation after different storage temperatures

In matrices that underwent the digestive process immediately after inoculation (0 d), there were no significant reductions in the pathogen throughout the gastric step in both matrices (*Figure 3*); nevertheless, after the intestinal step, population increases were observed on fresh-cut pears and melon. Overall, the logarithmic reduction after the whole gastrointestinal simulation did not cause any effect on *L. monocytogenes* upon the fresh-cut pear, whereas on fresh-cut melon, the growth of the pathogen throughout the gastrointestinal simulation was observed.

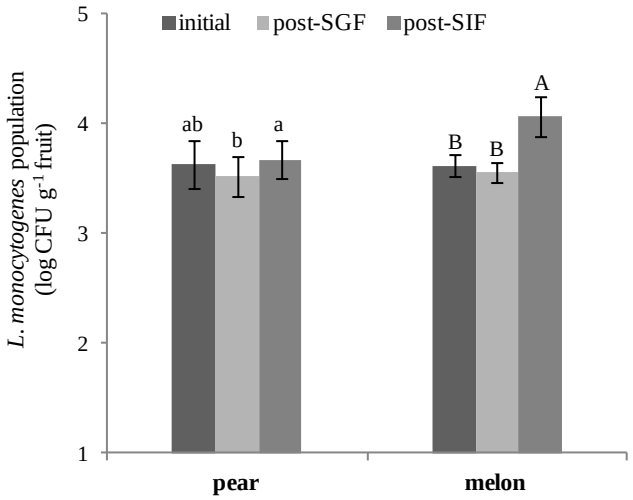


Figure 3. *Survival of* L. monocytogenes *after inoculation onto fresh-cut pear and melon and subsequent exposure to gastric and intestinal steps. The values are the means of two biological replicates and each with three technical replicates (n=6), and the bars represent the standard error. Within each fruit matrix, the values with different letters are significantly different (P < 0.05) among the evaluated gastrointestinal step (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF).*

Considering the logarithmic variation value throughout the entire gastrointestinal simulation, no variation of the *L. monocytogenes* population occurred when the matrix was pear, whereas a 0.43 logarithmic increase was noticed in melon (Table 1). After 1 day of storage at 1 °C and 10 °C (*Figure 4*), *L. monocytogenes* inoculated on fresh-cut pear (*Figure 4a*) showed a reduction throughout gastric step, whereas on fresh-cut melon, the reduction was observed in samples stored at 5 and 10 °C (*Figure 4b*). *L. monocytogenes* on fresh-cut pears showed a significant logarithmic reduction after the entire process of gastrointestinal simulation only in samples stored at 10 °C (0.91 log reduction), whereas on fresh-cut melon, *L. monocytogenes* showed a significant logarithmic reduction after gastrointestinal simulation on samples stored at 5, 10 and 20 °C.

Table 1. *The logarithmic variation of the population of* L. monocytogenes *inoculated onto fresh-cut pear and melon and exposed to the whole gastrointestinal simulation.*

Logarithmic variation of the *L. monocytogenes* population after gastrointestinal simulation																	
Storage time	1 °C					5 °C					10 °C					20 °C				
	pear			melon		pear			melon		pear			melon		pear			melon	
0 day		0.01 b	*	a 0.43			0.01 a	*	a 0.43			0.01 a	*	a 0.43			0.01 a	*	a 0.43	
1 day	AB	-0.16 b	*	a 0.31	X	A	0.02 a	*	b -0.60	Y	B	-0.91 b		b -0.72	Y	A	0.01 a	*	b -0.38	Y
2 days	A	0.09 ab		a 0.36	X	AB	-0.16 ab	*	b -0.59	Y	B	-0.77 b		b -1.16	Z	AB	-0.10 a		b -0.31	Y
6 days	A	-0.08 b	*	a 0.23	X	A	-0.84 c	*	c -2.12	Y	A	-0.25 ab		a 0.10	X					
9 days	A	0.31 a		a -0.05	X	B	-0.56 bc		b -0.55	X										

The values are the mean of gastrointestinal survival, log N_{SIF}/N_0 with N0 being the pathogen population count at the beginning of the gastrointestinal simulation ('initial count', CFU g^{-1}) and N_{SIF} the pathogen population count at the end of the gastrointestinal simulation ('post-SIF' count, CFU g^{-1}). Within each storage temperature (columns) and within each matrix, the values with different lowercase letters are significantly different ($P < 0.05$) among the storage times. Within each storage time (rows) and within each matrix, the values with different uppercase letters (A, B and C for pear and X, Y and Z for melon) are significantly different ($P < 0.05$) among the storage temperatures. Within each storage temperature (columns) and within each storage day, the values with an asterisk are significantly different among the matrices

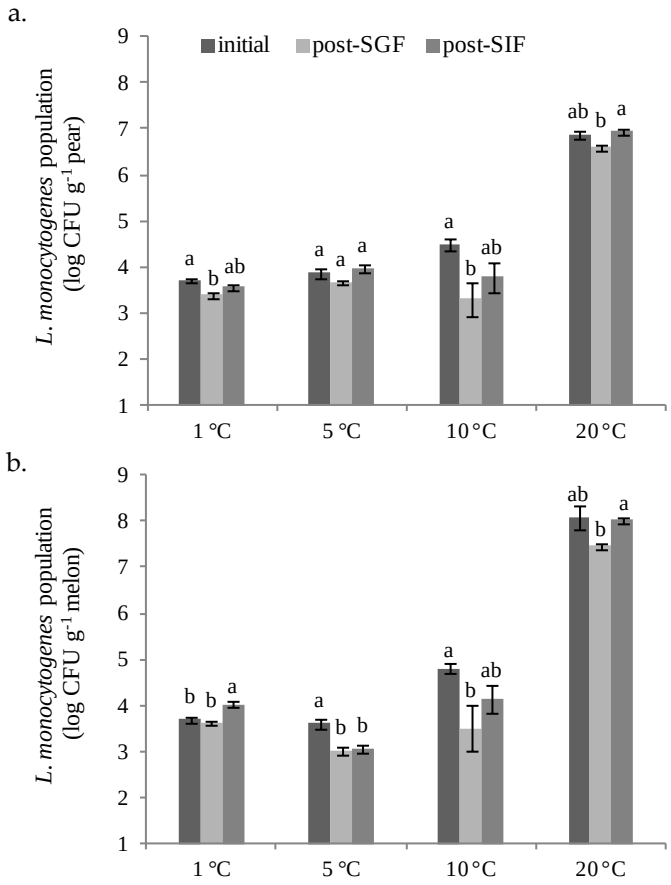


Figure 4. *Survival of* L. monocytogenes *inoculated onto fresh-cut pears (a) and melon (b) after 1 day of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are the means of two biological replicates each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, values with different letters are significantly different (P < 0.05) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF).*

After 2 days of storage at different temperatures, the population of *L. monocytogenes* on fresh-cut pears throughout the gastric step was reduced when the samples were stored at 5 °C (*Figure 5a*), and the same behaviour was observed in the pathogen on fresh-cut melon in samples stored during 2 days at 5 and 10 °C (*Figure 5b*).

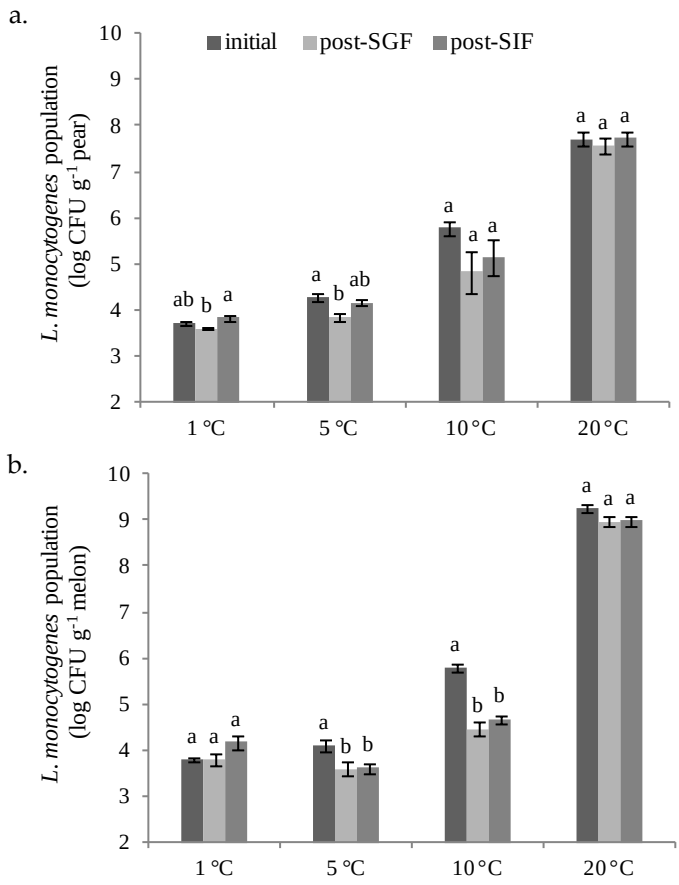


Figure 5. *Survival of* L. monocytogenes *inoculated onto fresh-cut pears (a) and melon (b) after 2 days of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are the means of two biological replicates and each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, values with different letters are significantly different (P < 0.05) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF).*

Regarding to pathogen logarithmic variation after the whole gastrointestinal simulation, *L. monocytogenes* on both fresh-cut fruits was more sensible when the samples were stored at 10 °C for 2 days with 0.77 and 1.16 log reduction. After 6 days, *L. monocytogenes* on fresh-cut melon showed high population reductions after the gastric step in samples stored at 5 and 10 °C (*Figure 6b*). However, at 10 °C, there was an increase of the population during the intestinal step. Thus,

after 6 days of storage, a significant logarithmic reduction was observed only in fresh-cut melon stored at 5 °C.

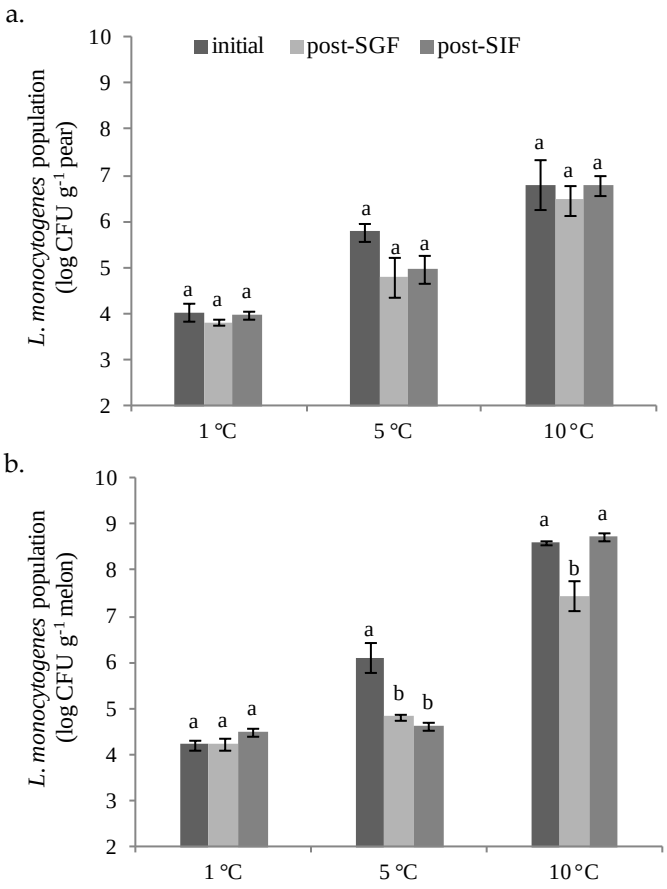


Figure 6. *Survival of* L. monocytogenes *inoculated onto fresh-cut pears (a) and melon (b) after 6 days of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are the means of two biological replicates each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, the values with different letters are significantly different (P < 0.05) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF).*

For the long storage period (9 days), the population of *L. monocytogenes* on both matrices was reduced throughout the gastric step when the samples were stored at 5 °C (*Figure 7*). Conversely, any reduction of the *L. monocytogenes* population in both matrices was observed after the gastric step and when the samples were stored at 1 °C.

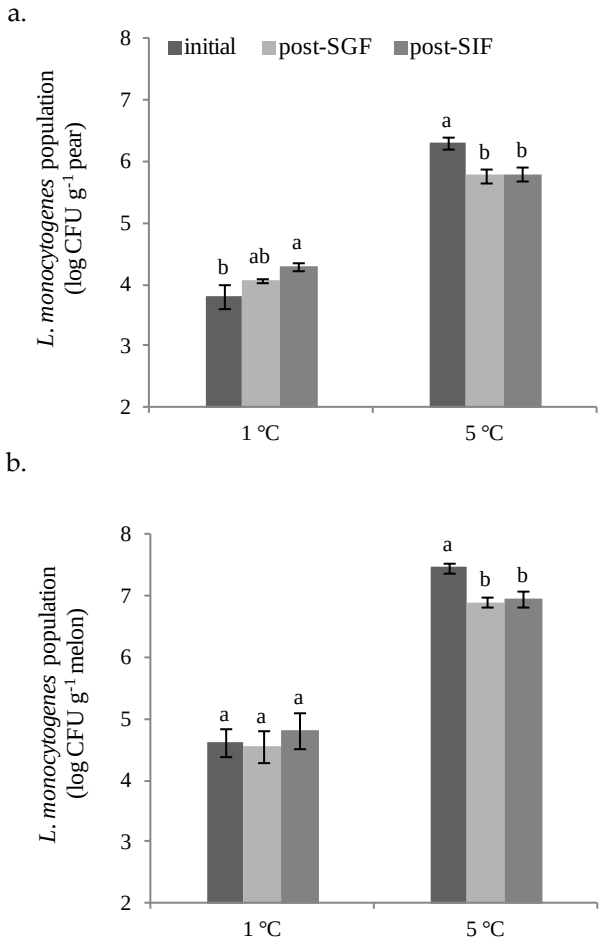


Figure 7. *Survival of* L. monocytogenes *inoculated onto fresh-cut pears (a) and melon (b) after 9 days of storage at 1, 5, 10 and 20 ℃ and exposed to gastric and intestinal steps. The values are the means of two biological replicates each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, the values with different letters are significantly different (P < 0.05) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF).*

Considering the logarithmic variation of *L. monocytogenes* during the whole gastrointestinal simulation (Table 1) and during the storage at 1 °C, similar logarithmic variation values after gastrointestinal simulation were observed for *L. monocytogenes* on fresh-cut pears until 9 days, when it increases up to 0.31 log, and the pathogen on fresh-cut melon had the same logarithmic variations after the gastrointestinal simulation during the study. At 5 °C, a significantly higher

logarithmic reduction after the gastrointestinal simulation was observed in pathogen populations on both matrices after 6 days of storage (0.84 and 2.12 log reductions on pear and melon, respectively). At 10 °C, *L. monocytogenes* showed noteworthy reduction throughout the gastrointestinal simulation regardless of the support matrix and after 1 and 2 days of storage (0.91 and 0.72 log reductions after 1 day of storage and 0.77 and 1.16 log reductions after 2 days of storage, on pear and melon, respectively). Finally, at 20 °C, the survival of *L. monocytogenes* on pear remained constant throughout the gastrointestinal simulation for all of the storage times evaluated, whereas the pathogen grown on melon showed a weak reduction after 1 or 2 days of storage (0.38 and 0.31 log reductions after 1 and 2 days of storage, respectively).

4. Discussion

The first objective of our study was to assess the growth of *L. monocytogenes* (serotype 1/2a) isolated from ready-to-eat lettuce on minimally processed fruits (pear and melon) for their different pH and during storage at 1, 5, 10 and 20 °C. Our study confirms that temperature is not a limiting factor for *L. monocytogenes* growth on fresh-cut mild acid fruit as weak growth was observed in melon even at 1 °C. Under extreme cold conditions (1 and 5 °C), the fresh-cut fruit matrix helped *L. monocytogenes* overcome the cold stress after 1 day of storage but growth in liquid medium was reduced, although a population increase was observed after 2 days under both temperatures.

Second, our hypothesis was that growing *L. monocytogenes* on fresh-cut pears and melon stored at refrigeration temperatures could enhance *L. monocytogenes* survival to subsequent exposure in the gastrointestinal simulation. Several authors confirmed through *in vitro* assays that the optimum range of pH, in which habituation resulted in increased acid resistance, was 5.0-6.0 (Davis *et al.*, 1996; Koutsoumanis, Sofos, 2004; O'Driscoll *et al.*, 1996; Shen *et al.*, 2014). The pH of our evaluated fruit matrices was approximately within this range; nevertheless, it is known that several other factors play critical roles in controlling the induction of acid-stress adaptation in *L. monocytogenes*. Extensive studies determined the influence of sub-lethal acid concentrations, exposure time, the type of acidulant, temperature and bacterial growth stage on acid-stress adaptation in *L. monocytogenes* (Shen *et al.*, 2014). In addition, in the presence of a mild concentration of weak acid preservatives, organisms have been shown to adapt by making changes in their cell membrane permeability and fluidity (Diakogiannis *et al.*, 2013).

Our study evaluated whether the surrounding food environment present during the minimally processed fruit shelf-life (matrix, time and temperature) could affect the survival of *L. monocytogenes* to subsequent exposure to acid stress. Some studies focused on the production chain of minimally processed products because *L. monocytogenes* may encounter various hurdles (stresses) in food processing environments (e.g., acidity, salinity, sanitizers, etc.) (Chorianopoulos *et al.*, 2011). Disinfection is one of the most critical processing steps in fresh-cut vegetable production and affects the quality, safety and shelf-life of the end product (Gil *et al.*, 2009). Pathogens from contaminated produce can be dislodged from the plant surface by the cleaning action of the wash process, and the sanitising agent eliminates them in suspension (Gil *et al.*, 2009; Zhou *et al.*, 2015). Chlorine is the most widely used sanitizer but other alternatives exist such as the use of peroxyacetic acid, chlorine dioxide, hydrogen peroxide, organic acids, electrolyzed water or physical methods such as ultrasound, high pressure, high-intensity electric field pulses, ultraviolet radiation and radio frequency and ionizing radiation (Artes *et al.*, 2009; Gil *et al.*, 2009). Potential acid habituation in a sublethal pH environment of an acid decontaminated food may enhance the survival of pathogens during transit through the stomach and increase the likelihood of intestinal colonization and thus their virulence potential (Samara, Koutsoumanis, 2009). Samara *et al.* (2009) studied the potential adaptation of the phenomena induced by acid decontamination (lactic acid, acetic acid, propionic acid and citric acid at concentrations 0.5 and 1.0%) by studying the behaviour of *L. monocytogenes* during exposure to simulated gastric fluid following storage (48 h at 5 °C and 20 °C) on decontaminated lettuce. The results showed that the tested decontamination treatments did not increase the acid tolerance of *L. monocytogenes*. Moorman *et al.* (2008) observed changes in membrane lipids after exposure of *Listeria innocua* to acid conditions and found that adaptation to acid conditions decreased the pathogen membrane fluidity. The same result was observed as a response to prolonged exposure of *L. monocytogenes* to sublethal levels of benzalkonium chloride (2.5 mg L^{-1}) (Bisbiroulas *et al.*, 2001). This physiological modification may enhance the survival of the pathogen during transit through the stomach. Nevertheless, the survival of *L. monocytogenes* after exposure to simulated gastric fluid was not evaluated after those stresses.

After the disinfection step, Allende *et al.* (2004) observed that shredding, rinsing and centrifugation affect the microbial and sensory quality of fresh processed lettuce and show increased bacterial counts. Thus, every step from production through consumption will influence the microbiology of fresh produce and the

proper use of a good cleaning and disinfection programme should be a major priority of the fresh processed industry (Allende *et al.*, 2004).

In the current study, when the pathogen behaviour was evaluated at 20 °C (without cold stress), we observed that *L. monocytogenes* on fresh-cut pears was more resistant to the gastrointestinal simulation after 1 and 2 days of contact than the pathogen on melon. The mild-low pH of pear flesh could have switched on the acid tolerance response (ATR) of *L. monocytogenes* in this stressful environment and subsequently enhanced the pathogen survival in the gastrointestinal simulation (acid shock, pH 3.5).

For the temperature effect on acid adaptation, Shen *et al.* (2014) showed that the acid-stress adaptation that typically occurs when *L. monocytogenes* cells are pre-exposed to sub-lethal acid at 20 °C or 37 °C was not induced when the cells were pre-exposed to sub-lethal acid at 4 °C. It is likely that between the acid and cold adaptation, cold adaptation becomes the priority task for the bacterium. A cold stress environment may block the penetration of acid into the cytoplasm and therefore fail to trigger the intracellular response to acid stress (Shen *et al.*, 2014). This result might explain our findings at 1 °C, where the lowest pathogen reductions were observed in both matrices and probably due to the hydrochloric acid internalization from the SGF solution to the cytoplasm, which was more difficult due to the cold pre-adaptation of the cells. Al-Nabulsi *et al.* (2015) found that cold (4 °C), acid (5.0) and osmotic (2, 4, 6 and 12% NaCl) stresses increased the resistance of *L. monocytogenes* to nine currently used antibiotics. The increase of the survival of *L. monocytogenes* after the entire gastrointestinal simulation observed after 9 days of storage at 5 °C and 6 days at 10 °C in both fresh-cut matrices could be caused by the pathogen entering into a stationary-phase. After entering into the stationary-phase, cells become naturally resistant because of the activation of a stringent response (mediated by ppGpp) and general stress response (mediated by σ^B) (Shen *et al.*, 2014).

In conclusion, when the samples had not been stored, *L. monocytogenes* on melon was more resistant to the gastrointestinal simulation than the pathogen on the pear. In general, under the proper storage temperature of fresh-cut fruit (5 °C) and in general after 1 day of storage, *L. monocytogenes* on melon was more sensible than on pear and this behaviour was maintained until 9 days when the microorganism in both matrices showed less sensitivity to the gastrointestinal simulation. However, *L. monocytogenes* on pears at 5 °C could seem more hazardous for consumers and it is necessary to consider that the population of

L. monocytogenes on melon was always higher than on pear due to its physicochemical properties. Some authors confirm that the ability of *L. monocytogenes* to survive the acidic conditions of the stomach could contribute to increasing its virulence and thus the likelihood of intestinal colonization. Therefore, more studies should be conducted to determine whether *L. monocytogenes* on fresh-cut fruits during their shelf-life might increase its ability to infect.

5. Acknowledgements

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus and Banco Santander for Pilar Colás Medà PhD grant (UdL-Impuls Program), the Spanish Government (Ministerio de Economia y Competitividad) (research project AGL-2012-38671) and the European Regional Development Fund (FEDER) for their financial support.

6. References

Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. International Journal of Food Microbiology 123, 121-129.

Al-Nabulsi, A.A., Osaili, T.M., Shaker, R.R., Olaimat, A.N., Jaradat, Z.W., Elabedeen, N.A.Z., Holley, R.A., 2015. Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of *Listeria monocytogenes*. Food Microbiology 46, 154-160.

Allende, A., Aguayo, E., Artes, F., 2004. Microbial and sensory quality of commercial fresh processed red lettuce throughout the production chain and shelf life. International Journal of Food Microbiology 91, 109-117.

Anon. 2014. IV gama: Crecimiento lento, pero firme. Available at http://www.alimarket.es/noticia/150889/IV-gama-crecimiento-lento-pero-firme (Accessed: 3/3/2015).

Artés, F., Gomez, P., Aguayo, E., Escalona, V., Artes-Hernandez, F., 2009. Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities. Postharvest Biology and Technology 51, 287-296.

Barbosa, J., Borges, S., Magalhães, R., Ferreira, V., Santos, I., Silva, J., Almeida, G., Gibbs, P., Teixeira, P., 2012. Behaviour of *Listeria monocytogenes* isolates through gastro-intestinal tract passage simulation, before and after two sub-lethal stresses. Food Microbiology 30, 24-28.

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N., 2008. Survival of *Listeria monocytogenes* in a simulated dynamic gastrointestinal model during storage of inoculated bologna and salami slices in vacuum packages. Journal of Food Protection 71, 2014-2023.

Bisbiroulas, P., Psylou, M., Iliopoulou, I., Diakogiannis, I., Berberi, A., Mastronicolis, S.K., 2001. Adaptational changes in cellular phospholipids and fatty acid composition of the food pathogen *Listeria monocytogenes* as a stress response to disinfectant sanitizer benzalkonium chloride. Letters in Applied Microbiology 52, 275-280.

Chorianopoulos, N., Giaouris, E., Grigoraki, I., Skandamis, P., Nychas, G.J., 2011. Effect of acid tolerance response (ATR) on attachment of *Listeria monocytogenes* Scott A to stainless steel under extended exposure to acid or/and salt stress and resistance of sessile cells to subsequent strong acid challenge. International Journal of Food Microbiology 145, 400-406.

Davis, M.J., Coote, P.J., Obyrne, C.P., 1996. Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology 142, 2975-2982.

Diakogiannis, I., Berberi, A., Siapi, E., Arkoudi-Vafea, A., Giannopoulou, L., Mastronicolis, S.K., 2013. Growth and membrane fluidity of food-borne pathogen *Listeria monocytogenes* in the presence of weak acid preservatives and hydrochloric acid. Frontiers in Microbiology 4, 6.

Dikici, A., Calicioglu, M., 2013. Survival of *Listeria monocytogenes* during production and ripening of traditional turkish savak tulum cheese and in synthetic gastric fluid. Journal of Food Protection 76, 1801-1805.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiological Reviews 55, 476-511.

Formato, G., Geornaras, I., Barmpalia, I.M., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C., Sofos, J.N., 2007. Effect of acid adaptation on growth during storage at 10 °C and resistance to simulated gastric fluid of *Listeria monocytogenes* inoculated onto bologna formulated with or without antimicrobials. Journal of Food Protection 70, 65-69.

Gil, M.I., Selma, M.V., Lopez-Galvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. International Journal of Food Microbiology 134, 37-45.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A., 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. Journal of Food Science 65, 541-544.

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

Koutsoumanis, K.P., Sofos, J.N., 2004. Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habituation at different pH conditions. Letters in Applied Microbiology 38, 321-326.

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology 55, 181-186.

Melo, J., Schrama, D., Hussey, S., Andrew, P.W., Faleiro, M.L., 2013. *Listeria monocytogenes* dairy isolates show a different proteome response to sequential exposure to gastric and intestinal fluids. International Journal of Food Microbiology 163, 51-63.

Moorman, M.A., Thelemann, C.A., Zhou, S.Y., Pestka, J.J., Linz, J.E., Ryser, E.T., 2008. Altered Hydrophobicity and membrane composition in stress-adapted *Listeria innocua*. Journal of Food Protection 71, 182-185.

O'Driscoll, B., Gahan, C.G.M., Hill, C., 1996. Adaptative acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Applied and Environmental Microbiology 62, 1693-1698.

Oliveira, M., Wijnands, L., Abadias, M., Aarts, H., Franz, E., 2011. Pathogenic potential of *Salmonella Typhimurium* DT104 following sequential passage through soil, packaged fresh-cut lettuce and a model gastrointestinal tract. International Journal of Food Microbiology 148, 149-155.

Oomen, A.G., Rompelberg, C.J.M., Bruil, M.A., Dobbe, C.J.G., Pereboom, D., Sips, A., 2003. Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. Archives of Environmental Contamination and Toxicology 44, 281-287.

Peterson, L.D., Faith, N.G., Czuprynski, C.J., 2007. Resistance of *Listeria monocytogenes* F365 cells to synthetic gastric fluid is greater following growth on ready-to-eat deli turkey meat than in brain heart infusion broth. Journal of Food Protection 70, 2589-2595.

Ragaert, P., Jacxsens, L., Vandekinderen, I., Baert, L., Devlieghere, F., 2011. Microbiological and safety aspects of fresh-cut fruits and vegetables O. Martín-Belloso and R. Soliva-Fortuny ed. Advances in fresh-cut fruits and vegetables processing. FL: CRC Press, Taylor & Francis INC., Boca Raton.

Ramalheira, R., Almeida, M., Azeredo, J., Brandao, T.R.S., Almeida, G., Silva, J., Teixeira, P., 2010. Survival of clinical and food isolates of *Listeria*

monocytogenes through simulated gastrointestinal tract conditions. Foodborne Pathogens and Disease 7, 121-128.

Samara, A., Koutsoumanis, K.P., 2009. Effect of treating lettuce surfaces with acidulants on the behaviour of *Listeria monocytogenes* during storage at 5 and 20 °C and subsequent exposure to simulated gastric fluid. International Journal of Food Microbiology 129, 1-7.

Shen, Q., Soni, K.A., Nannapaneni, R., 2014. Influence of temperature on acid-stress adaptation in *Listeria monocytogenes*. Foodborne Pathogens and Disease 11, 43-49.

Stopforth, J.D., Yoon, Y., Barmpalia, I.M., Samelis, J., Skandamis, P.N., Sofos, J.N., 2005. Reduction of *Listeria monocytogenes* populations during exposure to a simulated gastric fluid following storage of inoculated frankfurters formulated and treated with preservatives. International Journal of Food Microbiology 99, 309-319.

Tompkins, T.A., Mainville, I., Arcand, Y., 2011. The impact of meals on a probiotic during transit through a model of the human upper gastrointestinal tract. Beneficial Microbes 2, 295-303.

Zhou, B., Luo, Y.G., Nou, X.W., Lyu, S.X., Wang, Q., 2015. Inactivation dynamics of *Salmonella* enterica, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in wash water during simulated chlorine depletion and replenishment processes. Food Microbiology 50, 88-96.

CAPÍTULO 5

Exposure to minimally processed pear and melon during shelf life could modify the pathogenic potential of *Listeria monocytogenes*

P. Colás-Medà, I. Viñas, M. Oliveira, M. Anguera,

J. C. E. Serrano, M. Abadias.

Food Microbiology (2016) doi: 10.1016/j.fm.2016.10.016

Abstract

Survival and virulence of foodborne pathogens can be influenced by environmental factors such as the intrinsic properties of food as well as the extrinsic properties that contribute to food shelf life (e.g., temperature and gas atmosphere). The direct contribution of food matrix characteristics on the survival of *L. monocytogenes* during fresh-cut fruit shelf life is not very well understood. In addition, the gastrointestinal tract is the primary route of listeriosis infection and penetration of the intestinal epithelial cell barrier is the first step in the infection process. Hence, the pathogenic potential of *L. monocytogenes*, measured as the capability for the organism to survive a simulated gastrointestinal tract and the proportion of cells able to subsequently adhere to and invade differentiated Caco-2 cells, subjected to fresh-cut pear and melon shelf life, was investigated. Samples were inoculated, stored at 10 °C for 7 days and evaluated after inoculation and again after 2 and 7 days of storage. A decrease in *L. monocytogenes'* capacity to survive a simulated gastrointestinal tract was observed with increasing storage time, regardless of the fruit matrix evaluated. Furthermore, *L. monocytogenes* placed on fresh-cut pear and melon was subjected to an attachment and invasion assay after crossing the simulated gastrointestinal tract. After inoculation, pathogen on fresh-cut pear showed 5-fold more capacity to adhere to Caco-2 cells than pathogen on fresh-cut melon. After 2 days of storage, *L. monocytogenes* grown on fresh-cut melon showed similar adhesive capacity (1.11%) than cells grown on pear (1.83%), but cells grown on melon had the higher invasive capacity (0.0093%). We can conclude that minimally processed melon could represent a more important hazard than pear under the studied shelf life.

Keywords: fresh-cut fruit, simulated gastrointestinal tract, adhesion, invasion, virulence.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that can cause listeriosis. It has a high mortality rates among infected neonates, elderly, and immunocompromised persons (Walls and Buchanan, 2005). Changes in consumer lifestyles, specifically with significant expansion of the shelf life of foods under refrigerated conditions alongside increased consumer demand for ready-to-eat food, have revealed that *L. monocytogenes* is an important foodborne pathogen causing severe disease (Rantsiou *et al.*, 2012). In recent years, several listeriosis outbreaks have been linked to the consumption of fresh or processed foods such as soft cheeses, ice cream, caramel apples, soy sprouts, dairy products and cantaloupe. The largest listeriosis outbreak in the United States was associated with consumption of cantaloupe, where 147 illnesses, 33 deaths, and 1 miscarriage occurred in 2011 (CDC, 2011). In 2012, economic studies in the USA concluded that fresh-cut cantaloupe had the fifth position in the ranking of minimally processed fruit sales and accounted for 5.4% (from 431.8 million dollars of total sales) while fresh-cut pear did not appear in the ranking (UCDavis, 2015) and has not been linked with any outbreak. Produce outbreaks seem frequently associated with processed produce and often involved storage under suboptimal conditions or environmental cross-contamination after processing (Hoelzer *et al.*, 2012). Human pathogen survival and growth on fresh-cut produce is affected by many factors, including temperature, interaction with the indigenous microbiota, nutrient availability, and use of controlled or modified atmospheres for storage and/or packaging (Sapers *et al.*, 2009). To survive adverse conditions (food processing, gastrointestinal tract, e.g.), bacteria must sense the changes and then respond with appropriate alterations in gene expression and protein activity (Boor, 2006).
Epidemiological evidence shows that the gastrointestinal tract is the primary route of infection and that penetration of the intestinal epithelial cell barrier is the first step in the infection process (Jaradat and Bhunia, 2003; Lecuit and Cossart, 2001). Thus, the serotype, the immune status of the host, the contamination level of the food, and the virulence capacity of the strain all play an important role in the ability to develop listeriosis (Werbrouck *et al.*, 2009). To assess the food safety hazard associated with *L. monocytogenes*, some steps in the infection process, such as gastrointestinal survival or invasiveness, can be measured with an *in vitro* bioassay using a simulated gastrointestinal tract (static or dynamic system) and the intestinal epithelial cell line Caco-2. With these tools, some researchers have been focused on assessing the behaviour of *L. monocytogenes* subjected to stressful environmental conditions to study whether its virulence capacity could be affected. It has been previously reported that environmental conditions can

modulate *in vitro* virulence characteristics such as invasiveness (Garner *et al.*, 2006). Moreover, the ability of *L. monocytogenes* to invade Caco-2 cells is affected by the presence of NaCl, organic acids, pH, growth temperature, and oxygen restriction as well as interactions between these variables (Conte *et al.*, 2000; Garner *et al.*, 2006; Pricope-Ciolacu *et al.*, 2013; Rieu *et al.*, 2009; Werbrouck *et al.*, 2009).

The aim of this work was to study the *in vitro* virulence of *L. monocytogenes* inoculated on two minimally processed fruits. Minimally processed 'Piel de sapo' melon has a pH approximately 6 while minimally processed 'Conference' pear has a pH approximately 5. To mimic a real-life scenario, samples were stored under abuse temperature conditions that resemble some commercial and household practices (10 °C) for 7 days (Marklinder *et al.*, 2004). At each sampling point, the population of *L. monocytogenes* was enumerated and pathogen survival under simulated gastrointestinal tract was studied. Finally, the pathogenic potential of *L. monocytogenes*, measured as the capability for the organism to survive a simulated gastrointestinal tract and the proportion of cells able to subsequently adhere to and invade differentiated Caco-2 cells, subjected to fresh-cut pear and melon shelf life, was investigated.

2. Material and methods

2.1. Fruit

'Conference' pears (*Pyrus communis*) were obtained from local packing-houses in Lleida (Catalonia, Spain). 'Piel de Sapo' melons (Cucumis *melo* L.) were purchased in local supermarkets the day before each experiment. Pears were used in their optimal ripeness stage for processing (44 ± 3.2 N) according to Soliva-Fortuny *et al.* (2004). Pears were stored at 20 °C until they reached the desired firmness. Firmness of whole pears was measured on opposite sides of each fruit with a penetrometer (Effegi, Mila, Italy) equipped with a probe 8 mm in diameter. When values of ripeness fell within the selected range, pears were subjected to processing. Prior to processing, the fruits were washed with water, their surfaces were disinfected with 70% ethanol, and then they were left to dry at room temperature. Pears were peeled and cut into ten slices using a manual fruit slicer/corer. Melons were cut transversally in 14- to 16-mm slices, seeds and rind were removed, and each slice was cut into trapezoidal pieces.

2.2. Fruit quality parameters

Quality analysis of fresh-cut fruits (pH, soluble solid contents and titratable acidity) were performed before each experiment. Fruit flesh pH was measured using a pH meter (Model GLP22, Crison Instruments S.A.) with a penetration electrode (5231 Crison). Soluble solid contents (SSC) were measured at 20 °C with a handheld refractometer (Atago Co. Ltd.) in juice extracted by crushing fruit pieces in a blender. The results were expressed as °Brix. To measure titratable acidity (TA), 10 ml of fruit juice plus 2 drops of phenolphthalein solution 1% RV (Panreac, Barcelona, Spain) were diluted with 10 ml of deionized water and titrated with 0.1 N NaOH until the pH indicator changed colour. The results were calculated as g of citric acid L^{-1} for melon and g of malic acid L^{-1} for pear. There were three determinations of each parameter per fruit.

2.3. Bacterial strain and growth conditions

The *L. monocytogenes* serovar 1/2a strain used in this study was previously isolated from ready-to-eat iceberg lettuce (Abadias *et al.*, 2008). To prepare inoculum for assays, the strain was streaked onto Tryptic Soy Agar (TSA, Biokar Diagnostics) supplemented with 0.6% w/v Yeast Extract (YE, Biokar Diagnostics) (TSAYE) plates and incubated at 37 °C for 24 h. Subsequently, a single colony was inoculated into 50 ml of Tryptic Soy Broth (TSB, Biokar Diagnostics) supplemented with 0.6% w/v YE (TSBYE) and incubated with shaking at 150 rpm for 18-20 h at 37 °C. Cells were harvested by centrifugation at 9800X g for 10 min at 10 °C (Sorvall Legend XTR centrifuge, Thermo Scientific) and re-suspended in 25 ml of saline solution (SS; 8.5 g L^{-1} NaCl) to obtain an approxima-tely 10^9 CFU ml^{-1} suspension.

For the inoculum preparation, a volume of the bacterial concentrated suspension was added to deionized water to obtain approximately 10^7 CFU ml^{-1}. Inoculum concentration was checked by plating appropriate dilutions onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics), followed by incubation at 37 °C for 48 h.

2.4. Inoculation procedures

Separately, pear and melon wedges were dipped (1:2 w/v) for 2 min at 150 rpm in the inoculation suspension and then were allowed to dry in a laminar flow biosafety cabinet. Each type of fruit wedge was packaged (100 ± 5 g) in

polypropylene trays (375-ml) sealed with a non-peel-able polypropylene plastic film (PP-110, ILPRA, Italy). Nine holes of 400 μm were made in the sealed film using a needle to maintain air conditions. Samples were stored at 10 °C.

2.5. Enumeration of *L. monocytogenes* in fruit samples

Samples were examined on the day of inoculation and after 2 and 7 days of storage. *L. monocytogenes* population was determined in three sample trays for each food matrix at each sampling point. For pathogen population enumeration, 10 g of pear or melon from each tray was mixed with 90 ml of buffered peptone water (BPW, Biokar Diagnostics) in a sterile bag (BagPage 400 ml, Interscience BagSystem) and homogenized in a blender for 2 min at high speed (Bagmixer 100, Minimix, Interscience). Additionally, ten-fold dilutions were made with saline peptone (SP; 8.5 g/L NaCl and 1 g/L peptone) and plated, as described previously. These enumerations were used as initial counts in the simulated gastrointestinal tract experiment.

2.6. Survival of *L. monocytogenes* in a simulated gastrointestinal tract

L. monocytogenes from pear and melon samples stored at 10 °C were evaluated for their survival after exposure to a simulated gastrointestinal stress at each sampling time (day of inoculation and after 2 and 7 days). The experimental design is shown in *Figure 1*. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF, composed of duodenal and bile solution) were prepared according to Oomen *et al.* (2003) and Oliveira *et al.* (2011) with some modifications (Table 1).

Table 1. *Composition of synthetic juices comprising the in vitro gastrointestinal simulation.*

	Synthetic saliva fluid (SSF)	Synthetic gastric fluid (SGF)	Synthetic intestinal fluid (SIF)	
			Duodenal solution	Bile solution
Inorganic solutions	0.90 g KCl L^{-1} 0.20 g KSCN L^{-1} 1.15 g $NaH_2PO_4 \cdot 2H_2O$ L^{-1} 0.57 g Na_2SO_4 L^{-1} 0.30 g NaCl L^{-1} 0.07 g NaOH L^{-1}	0.82 g KCl L^{-1} 0.35 g $NaH_2PO_4 \cdot 2H_2O$ L^{-1} 2.75 g NaCl L^{-1} 0.40 g $CaCl_2 \cdot 2H_2O$ L^{-1} 0.31 g NH_4Cl L^{-1}	0.56 g KCl L^{-1} 7.00 g NaCl L^{-1} 3.39 g $NaHCO_3$ L^{-1} 0.08 g KH_2PO_4 L^{-1} 0.05 g $MgCl_2$ L^{-1} 0.20 g $CaCl_2 \cdot 2H_2O$ L^{-1}	0.38 g KCl L^{-1} 5.26 g NaCl L^{-1} 5.79 g $NaHCO_3$ L^{-1} 0.22 g $CaCl_2 \cdot 2H_2O$ L^{-1}
Organic solutions	0.20 g urea L^{-1}	0.09 g urea L^{-1} 0.65 g glucose L^{-1} 0.02 g glucuronic acid L^{-1} 0.33 g glucosamine hydrochloride L^{-1}	0.10 g urea L^{-1}	0.25 g urea L^{-1}
Add to mixture organic + inorganic solutions	145 mg α-amylase L^{-1} 15 mg uric acid L^{-1} 50 mg mucin L^{-1}	1.00 g bovine serum albumin fraction V (BSA) L^{-1} 1.00 g pepsin L^{-1} 3.00 g mucin L^{-1}	1.00 g BSA L^{-1} 3.00 g pancreatin L^{-1} 0.50 g lipase L^{-1}	1.80 g BSA L^{-1} 6.00 g bile L^{-1}
pH	6.5 ± 0.1	2.0 ± 0.1	7.8 ± 0.1	8.0 ± 0.1

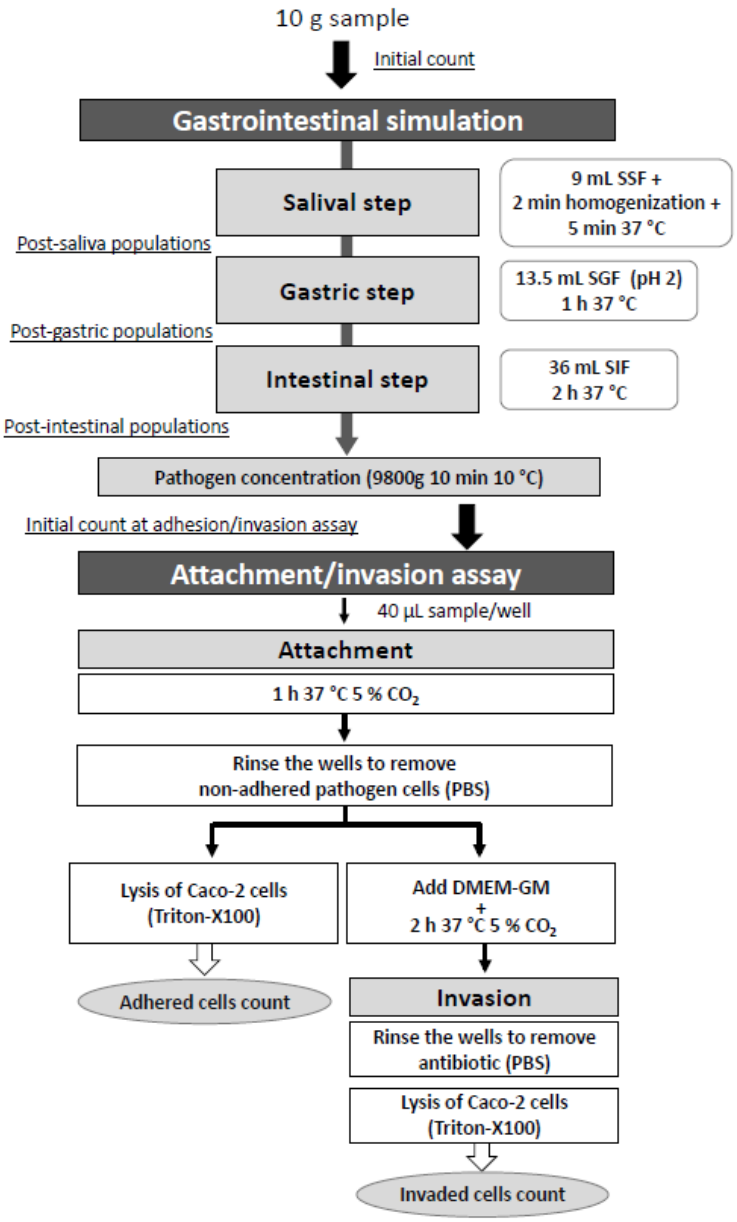


Figure 1. *Schematic overview of the experimental design.*

To simulate mastication, 10 g of each sample was placed into a sterile plastic bag (BagPage 80 ml, Interscience BagSystem) and 9 ml of SSF tempered at 37 °C were added. The mixture was then homogenized in a blender for 2 min at high speed (Bagmixer 100, Minimix, Interscience) and incubated at 37 °C for 5 min. Afterwards, pH was measured and an aliquot (1 ml) was taken out to enumerate *L. monocytogenes*. These enumerations were then used as the post-saliva

population in the simulated gastrointestinal tract experiment. The remaining sample was mixed with 13.5 ml of SGF (pH 2.0 adjusted with HCl 0.1 N). Subsequently, the pH was measured. Due to the different buffering effects of pears and melons, the pH of mixture increased differently between fruits. To avoid these differences, sample pH was normalized to a pH of 3.5 with hydrochloric acid (0.1 N) and incubated at 37 °C for 1 h. Then, the pH was measured and an aliquot (1 ml) was taken out to enumerate *L. monocytogenes*. These enumerations were then used as the post-gastric population counts in the simulated gastrointestinal tract experiment.

The remaining sample was mixed with 36 ml of SIF which was composed of 27 ml of duodenal solution (pH 7.8) and 9 ml of bile solution (pH 8.0). The pH of this mixture was measured and incubated at 37 °C for 2 h. Finally, the pH was measured and a last aliquot (1 ml) was taken out to enumerate *L. monocytogenes*. These enumerations were used as the post-intestinal population counts in the simulated gastrointestinal tract experiment. For *L. monocytogenes* enumeration, appropriate dilutions of aliquots were placed onto Palcam agar and plates were incubated at 37 °C for 48 h. Three samples were analysed for each fruit and sampling time and the experiment was carried out in triplicate.

2.7. Attachment and invasion assay

Human intestinal epithelial Caco-2 cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 20% heat-inactivated FBS (foetal bovine serum, Gibco) and 1% Penicillin/Streptomycin (10,000 units ml^{-1} penicillin and 10,000 $\mu g\ ml^{-1}$ streptomicin, Gibco) in 12-well tissue culture plates (Costar, Corning). The cells were seeded at 2.0 X 10^5 cells per well and incubated until they reached confluence.

Attachment and invasion assays were performed as previously described by Oliveira *et al.* (2011) with minimal modifications. Briefly, prior to the assay, confluent Caco-2 cells were washed twice with pre-warmed sterile phosphate-buffered saline (PBS) to remove traces of antibiotic. After the final washing, 1 ml of pre-warmed DMEM was added to each well. At each sampling point (the day of inoculation and after 2 and 7 days of storage), the experiment was performed with *L. monocytogenes* exposed to the aforementioned simulated gastrointestinal tract. An aliquot (50 ml) of *L. monocytogenes* sample (obtained from the endpoint specimen of the simulated gastrointestinal tract) was removed and centrifuged (9800X g for 10 min at 10 °C) and then was re-suspended in 3 ml of DMEM. This

was carried out to obtain high enough levels of *L. monocytogenes* cells to perform the invasiveness study. Bacterial suspension concentration was checked on Palcam agar plates. These enumerations were used as the initial bacterial count in the attachment and invasion assay. Afterwards, the plates were inoculated with 40 μL of this bacterial suspension per well. The plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 1 h for the attachment assay. After incubation, the medium was aspirated and the monolayers were rinsed three times with PBS to remove non-adhered and loosely adhered bacteria. Cells were lysed (to liberate the bacteria) with using 1 ml of 0.1% (v/v) Triton-X100 (Sigma) in PBS for 5 min at room temperature. Triton lysates from three wells were combined and used for determining the number of *L. monocytogenes* that adhered to the Caco-2 cells.

For the invasion assay, non-adherent bacteria were removed via washing as above and then the Caco-2 cells were treated with DMEM supplemented with 150 μg of gentamicin ml^{-1} (50 mg L^{-1}, Gibco) to quantify invasive bacteria. The plates were incubated for 3 h at 37 °C in 5% CO_2. After incubation, the cells were rinsed three times with PBS to remove excess antibiotic and lysed with Triton-X100 as described above to liberate invaded bacteria. Triton lysate from three wells was combined and used for determining the number of *L. monocytogenes* that invaded the Caco-2 cells. For *L. monocytogenes* enumeration, appropriate dilutions of aliquots were placed onto Palcam agar and plates were incubated at 37 °C for 48 h. The results were expressed as CFU ml^{-1}. The experiment was performed with three independent biological replicates with three technical replicates for each biological replicate.

2.8. Data analysis

All of the data were collected from three independent experiments. To evaluate the survival capacity of *L. monocytogenes* against the gastrointestinal simulation, microbial counts were transformed to logarithmic reduction using the equation: $\log (N/N_0)$, where N is the microbial cell density at the particular sampling time (N_{SGF}, after the gastric step; N_{SIF}, after the intestinal step) and N_0 is the initial cell density. The pathogen capability to adhere to Caco-2 cells (adhesion index) was reported as the number of *L. monocytogenes* (CFU ml^{-1}) recovered after 1 h of contact with Caco-2 cells from each well following Caco-2 cell lysis divided by the number of bacteria (CFU ml^{-1}) that had been used for inoculation, expressed as a percentage. The pathogen invasion capabilities in relation to Caco-2 cells (invasion index) was calculated as the number of bacteria (CFU ml^{-1}) recovered

after 3 h treatment of the Caco-2 cells with 150 μg ml^{-1} gentamicin divided by the total number of inoculated bacteria (CFU ml^{-1}), expressed as a percentage. The data are expressed as the average of three biological replicates with three technical replicates per biological replicate. Each matrix and sampling point was analysed using a one-way analysis of variance (ANOVA) using JMP8 (SAS software). When one-way ANOVA was significant, the Tukey's test was used to locate significant differences.

3. Results and discussion

3.1. Population of *L. monocytogenes* on fresh-cut pear and melon throughout shelf life

The population of *L. monocytogenes* on fresh-cut pear and melon after inoculation was 5.38 and 5.37 log CFU g^{-1}, respectively (*Figure 2*). *L. monocytogenes* grew in fresh-cut pear and melon at 10 °C, reaching a final population of 7.43 and 9.25 log CFU g^{-1} after 7 days of storage, respectively. These results agree with previous studies on fresh-cut pear and melon, which assessed the behaviour of *L. monocytogenes* on minimally processed fruits (Abadias *et al.*, 2014; Colás-Medà *et al.*, 2015; Oliveira *et al.*, 2014).

Initial quality parameters of the fresh-cut pear and melon used in our studies were determined before inoculation. The flesh of pear had a pH 4.99 ± 0.27 while the pH of melon was significantly higher (6.13 ± 0.19). Pear flesh showed a higher SSC (15.1 ± 1.1 °Brix) than melon flesh (11.9 ± 1.0 °Brix). Slight differences were found on titratable acidity between matrices; pear presented 1.59 ± 0.11 g of malic acid ml^{-1} of pear juice while melon had 1.23 ± 0.18 g of citric acid ml^{-1} of melon juice. The major acid present in melon is citric acid whereas in pear flesh it is malic acid. *L. monocytogenes* growth was not inhibited by the citric acid in the melon samples. Nevertheless, other studies carried out with other bacteria such as enterobacteriaceae (Deng *et al.*, 1999) found more of an inhibitory effect by citric acid than malic acid against them. In the current study, the inhibitory effect of citric acid was not observed, which could be due to the low levels of citric acid in the melon flesh. On the other hand, the flesh of pears had higher soluble solid contents than melon and lower *L. monocytogenes* populations were reached on the pear, probably due to its lower pH.

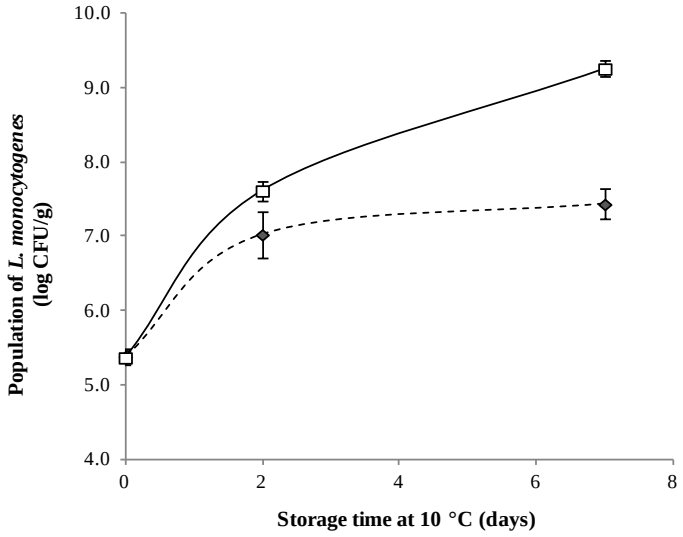


Figure 2. *Population (log CFU g^{-1} or ml^{-1}) of* L. monocytogenes *inoculated onto fresh-cut pear (diamonds) and melon (squares) under storage at 10 °C. Results are the means of three biological replicates each with three technical replicates (n=9), and vertical bars indicate the standard deviation of the mean.*

3.2. Survival of *L. monocytogenes* in a simulated gastrointestinal tract

The *L. monocytogenes* population values that were obtained along the digestive simulation are shown in *Figure 3* (SGF) and *4* (SIF). On the day of inoculation, the same quantity of *L. monocytogenes* entered the simulated gastrointestinal tract regardless of the fresh-cut fruit evaluated. Challenge in SGF revealed that there were no significant differences between 'pear-adapted' (pH 4.9, mainly malic acid) and 'melon-adapted' (pH 6.1, mainly citric acid) bacteria in both fruit matrices throughout the storage period (*Figure 3*). When *L. monocytogenes* on fresh-cut melon grew at 10 °C during 7 days, the log reduction was higher than at inoculation day. At inoculation day, *L. monocytogenes* on fresh-cut pear was able to survive the exposure to the gastric fluid and survive in intestinal fluid, whereas it survived gastric fluid exposure and grew during intestinal step on fresh-cut melon. Similar results were observed after 2 h adaptation in an artificial cheese medium (Melo *et al.*, 2013; Peterson *et al.*, 2007).

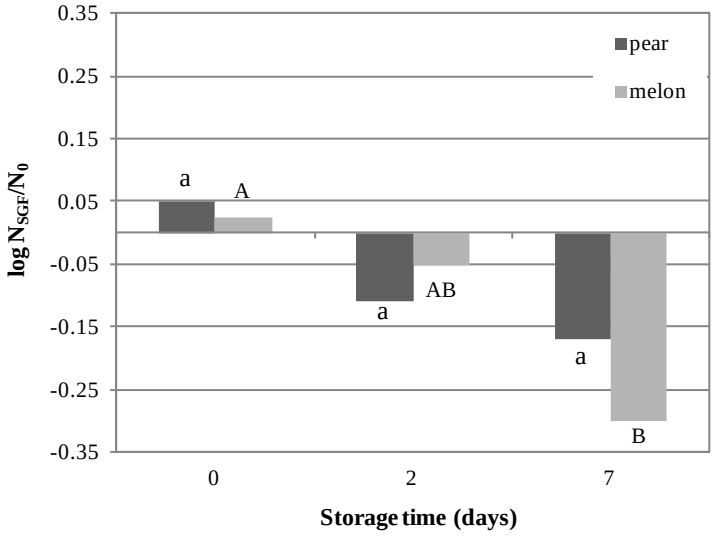


Figure 3. *Logarithmic variation (log* N_{SGF}/N_0*) obtained after the exposure to synthetic saliva fluid (pH 6.5) for 2 min and to synthetic gastric fluid (pH 3.5) for 1 h of* Listeria monocytogenes *inoculated onto fresh-cut pear and melon along of storage at 10 °C. The values are the average of triplicate samples from three independent experiments (n=9). Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences (P < 0.05) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences (P < 0.05) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.*

Furthermore, at inoculation day and after 2 days of storage at 10 °C, *L. monocytogenes* grown on fresh-cut melon better overcame intestinal step (including bile fluid and high osmolality) than that grown on fresh-cut pear and the final population increased about 0.4 log units (*Figure 4*). Peterson et al (2007) found that listerial cells grown on turkey meat were significantly more resitant to SGF than listerial cells grown in brain heart infusion broth (Peterson *et al.*, 2007). Barbosa *et al.* (2012) reported that the osmotic and acidic sub-lethal exposure (modified Buffered Peptone Water) did not confer resistance to the simulated gastrointestinal tract conditions. Nevertheless, they noticed that the resistance of *L. monocytogenes* in a food matrix would be much higher due to the protection conferred by food components.

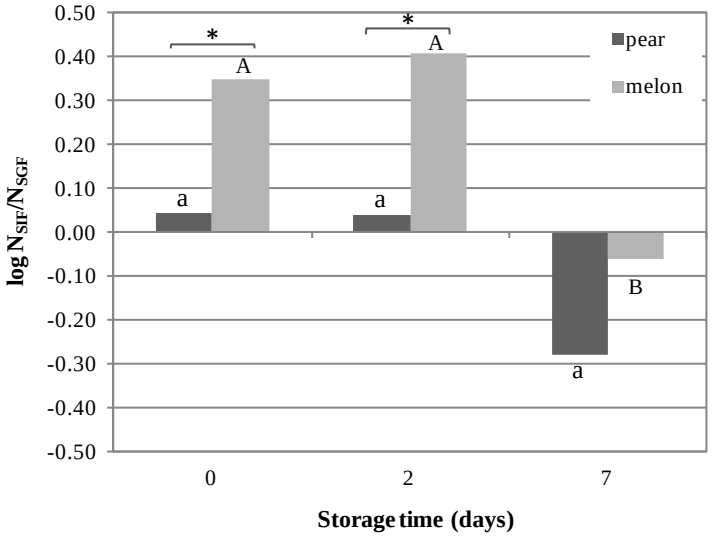


Figure 4. *Logarithmic variation (log* N_{SIF}/N_{SGF}*) obtained after the exposure to synthetic intestinal fluid for 2 h of* Listeria monocytogenes *inoculated onto fresh-cut pear and melon along of storage at 10 °C. The values are the average of triplicate samples from three independent experiments (n=9). Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences (P < 0.05) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences (P < 0.05) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.*

Based on these results, minimally processed melons could represent the more important hazard at inoculation day and after 2 days of storage as compared to pears under the studied shelf life (7 days at 10 °C), because listerial cells better survived and even grew to the exposure to SIF. Moreover, cells survival decreased with storage time, regardless of the fruit matrix evaluated.

After the whole simulated gastrointestinal tract, *L. monocytogenes* on fresh-cut pear reached 5.52 ± 0.23, 7.08 ± 0.32 and 7.17 ± 0.36 log CFU g^{-1} at inoculation day and after 2 and 7 days of storage, respectively. While *L. monocytogenes* on fresh-cut melon reached 5.77 ± 0.11, 8.00 ± 0.15 and 8.99 ± 0.38 log CFU g^{-1} at inoculation day and after 2 and 7 days of storage, respectively (data not shown).

3.3. Attachment and invasion assay

L. monocytogenes was grown on two different support matrices (fresh-cut pear and melon) under the same storage conditions and were subjected to a simulated gastrointestinal tract before subsequently testing for their capacity to adhere to and invade Caco-2 cells. This testing was performed on inoculation day and after 2 and 7 days of storage at 10 °C. On inoculation day, *L. monocytogenes* grown on pear showed the greatest adhesive capacity (6.5%), while it was only 1.4% with pathogen grown on melon (*Figure 5*). In spite of the higher adhesive capacity of pathogen grown on pear, these cells exhibit similar invasive capacity (0.0015%) than cells on melon (0.0047%) (*Figure 6*). After 2 days of storage, similar pathogen adhesive capacity was observed for pathogen grown on both matrices (1.83% vs 1.11% for pear and melon matrices, respectively). Nonetheless, the invasive capacity of pathogen grown on melon (0.0093%) was 3-fold higher than pathogen grown on pear (0.0033%).

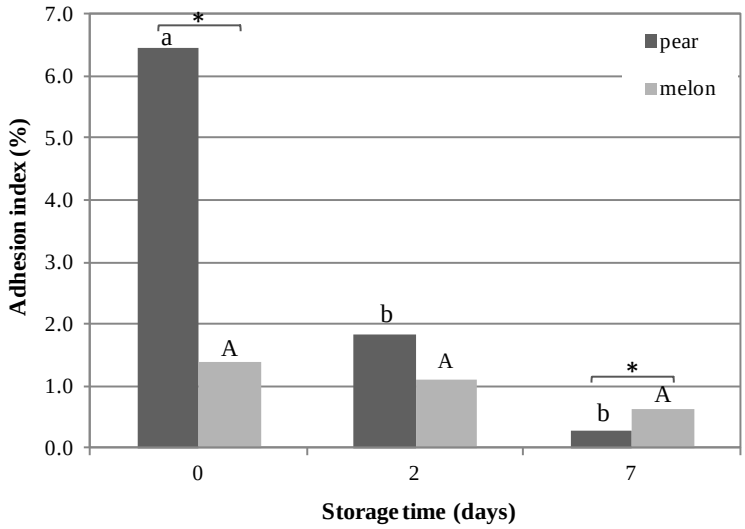


Figure 5. *The adhesion index (the number of bacteria recovered from lysed Caco-2 cells after 1 h of contact divided by the number of bacteria inoculated x 100) to Caco-2 cells of* L. monocytogenes *on fresh-cut pear and melon after the gastrointestinal simulation, along the storage at 10 °C. Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences (P < 0.05) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences (P < 0.05) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.*

L. monocytogenes' ability to adhere to Caco-2 cells showed a weak reduction with increasing storage time in both matrices (0.3% vs 0.6% for pear and melon matrices after 7 days, respectively). Additionally, a reduction in pathogen invasive capacity was observed in both matrices after 7 days (0.0001% vs 0.0007% for pear and melon, respectively). The capacity of *L. monocytogenes* to invade Caco-2 cells was below 1% in all evaluated times. This is in the same, or slightly lower, range than in comparable studies carried out in other food matrices (Lorentzen *et al.*, 2011; Rieu *et al.*, 2009).

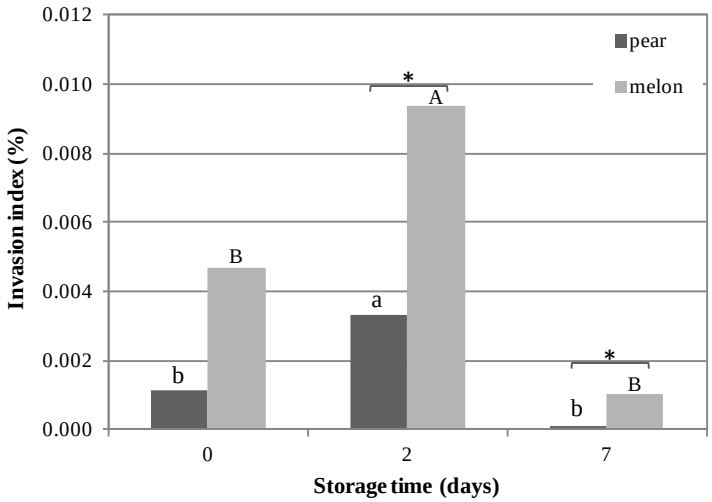


Figure 6. *The invasion index (the number of bacteria recovered from lysed Caco-2 cells after 3 h of contact divided by the number of bacteria inoculated x 100) to Caco-2 cells of* L. monocytogenes *on fresh-cut pear and melon after the gastrointestinal simulation, along the storage at 10 °C. Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences (P < 0.05) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences (P < 0.05) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.*

A general overview of the results obtained (*Figure 7*), demonstrates that just after processing, pathogen grown on fresh-cut pear was 5-fold more adhesive to Caco-2 cells than pathogen grown on fresh-cut melon. Although after 2 days of storage, *L. monocytogenes* showed similar adhesive capacity on both matrices, pathogen grown on melon had the highest invasive capacity. If our contaminated

fresh-cut fruits had been consumed after 2 days of storage (when the same initial load of pathogen in both matrices was observed), the fresh-cut melon could potentially cause a higher number of human infections than the fresh-cut pear. The last sampling point at 7 days post-inoculation demonstrated that pathogen grown on both fresh-cut pear and melon had lower capacity to overcome the simulated gastrointestinal tract and lower capacity to adhere to and invade Caco-2 cells compared to earlier sampling points. It is known that the environmental conditions to which *L. monocytogenes* is exposed prior to ingestion are decisive for determining its *in vivo* pathogenic potential. Unfortunately, the majority of researchers that have evaluated this effect on foodborne pathogens, although having studied both gastrointestinal survival and invasion capacity, have always done it separately. However, in the real infection process *L. monocytogenes* is subjected first to the gastrointestinal tract, followed by subsequent contact to the epithelial cells of the host. In this sense, Oliveira *et al.* (2011) first examined the pathogenic potential of *Salmonella* Thyphimurium, measured as the capability for it to survive a simulated gastrointestinal tract system and the proportion of cells adhering to and invading differentiated Caco-2 cells, after sequential incubations simulating the various production stages of pre-cut, ready-to-eat lettuce. They observed that the sequential incubation of *S.* Thyphimurium in soil and lettuce slightly increased the capability for surviving the simulated gastric fluid and increased the capability to grow in the simulated intestinal fluid, but decreased the capability of epithelial attachment and invasion and decreased the overall probability of surviving the gastrointestinal tract system. In addition, Conte *et al.* (2000) demonstrated that *L. monocytogenes* exposed to a sub-lethal acidic pH (BHI adjusted with lactic acid up to pH 5.1) showed increased invasion of intestinal epithelial Caco-2 cells relative to non-exposed bacteria. Previously, they determined that all of their exposed *L. monocytogenes* were able to readily develop acid tolerance. However, Conte *et al.* (2000) subjected acid-adapted *L. monocytogenes* cells to adhesion and invasion assays, without gastrointestinal tract simulation. To evaluate the effect of some organic acids and temperature on invasiveness, Garner *et al.* (2006) performed an invasion experiment with *L. monocytogenes* grown until stationary phase at 7 or 37 °C. For both temperatures, *L. monocytogenes* cells grown at pH 7.4 were also more invasive than bacteria grown in BHI broth adjusted to pH 5.5 with different combinations of organic acids. We observed that the invasive capacity of *L. monocytogenes* significantly increased from day 0 to day 2 in both matrices, with this increase being more noteworthy on cells grown on melon than on pear. Thus,

we could not attribute this behaviour to the difference in pH between the two food matrices.

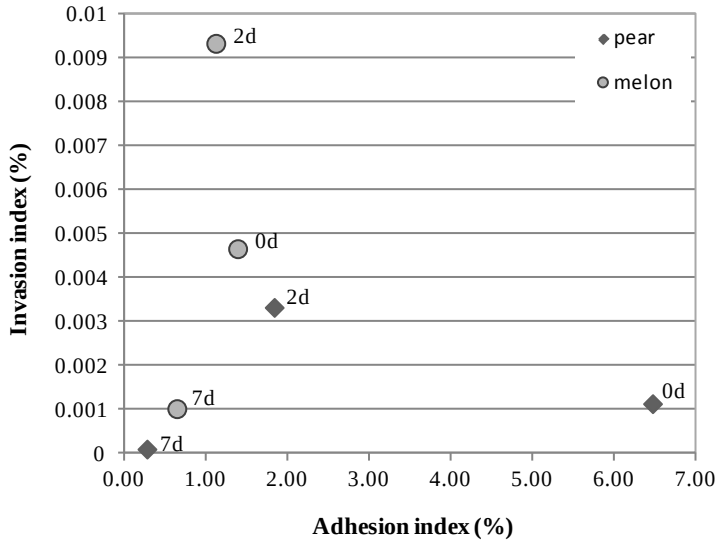


Figure 7. *Overview of pathogenic potential of* L. monocytogenes *with fresh-cut fruit storage. The invasion index (the number of bacteria recovered from lysed Caco-2 cells after 3 h of contact divided by the number of bacteria inoculated x 100) are indicated on the x-axis. The adhesion index (the number of bacteria recovered from lysed Caco-2 cells after 1 h of contact divided by the number of bacteria inoculated x 100) are indicated on the y-axis. The values are the average of triplicate samples from three independent experiments (n = 9).*

In the current study, an increase in *L. monocytogenes* population was observed on both matrices during the experimental shelving time. Furthermore, a significant decrease in the percentage of bacteria associated with the epithelial cells (counts of adherent bacteria plus counts of intracellular bacteria), as well as reduced *L. monocytogenes* invasive capacity, were noted with increasing storage time. Similarly, Pricope-Ciolacu *et al.* (2013) noticed that the period of storage of milk samples, which increased *L. monocytogenes* cell numbers in the food matrix, decreased *in vitro* virulence. Walecka *et al.* (2011) demonstrated that increased density of bacterial culture is accompanied by a stepwise reduction in

invasiveness in all of the tested strains. However, Garner *et al.* (2006) explored whether the number of added bacteria affected the relative invasion efficiencies, and no significant correlation was found. Thus, in our studies the reduction of *L. monocytogenes* invasive capacity with increasing storage can be not only caused by the higher load of pathogen in the longer-stored inoculums.

Moreover, in *in vitro* assays Andersen at al. (2007) noticed that *L. monocytogenes* cultivated under oxygen-restricted conditions were approximately 100-fold more invasive than similar cultures grown without oxygen restriction. Packaging under modified atmosphere conditions is widely established to improve the quality, shelf life as well as some safety aspects of minimally processed fruit. Thus, it could be suggested that *L. monocytogenes* subjected to minimally processed pear or melon stored under modified atmosphere packaging could increase their invasive capacity due to the low oxygen levels presents inside the package, but more research is still required to prove this hypothesis.

In conclusion, these findings suggested that fresh-cut melon is more likely to cause listeriosis if the pathogen has been introduced just before packaging than fresh-cut pear stored under the same conditions. This is supported by the high load of *L. monocytogenes* observed on fresh-cut melon that is a direct consequence of its pH, which is higher than pear pH, allowing for a higher *L. monocytogenes* population, even at 10 °C. In addition, when *L. monocytogenes* grown on fresh-cut melon was subjected to a simulated gastrointestinal tract, it was able to overcome the gastric step and was able to grow during intestinal step on processing day and after 2 days of storage. Finally, an enhancement in invasive capacity of *L. monocytogenes* was observed in this matrix after 2 days of storage at 10 °C. Molecular analyses could be useful to elucidate the genes that might be affected and cause the increase in invasive capacity seen after 2 days of contact with minimally processed pear and melon.

4. Acknowledgments

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus and Banco Santander for the Pilar Colás Medà PhD grant (UdL-Impuls Program), to the Spanish Government (Ministerio de Economía y Competitividad, research project AGL-2012-38671) and to the European Regional Development Fund (FEDER) for its financial support. We also want to thank Anna Cassanyé for her support in the IRB laboratory.

5. References

Abadias, M., Altisent, R., Usall, J., Torres, R., Oliveira, M., Vinas, I., 2014. Biopreservation of fresh-cut melon using the strain *Pseudomonas graminis* CPA-7. *Postharvest Biology and Technology* 96, 69-77.

Abadias, M., Usall, J., Anguera, M., Solsona, C., Vinas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.

Andersen, J.B., Roldgaard, B.B., Christensen, B.B., Licht, T.R., 2007. Oxygen restriction increases the infective potential of *Listeria monocytogenes* in vitro in Caco-2 cells and in vivo in guinea pigs. *Bmc Microbiology* 7, 55.

Barbosa, J., Borges, S., Magalhaes, R., Ferreira, V., Santos, I., Silva, J., Almeida, G., Gibbs, P., Teixeira, P., 2012. Behaviour of *Listeria monocytogenes* isolates through gastro-intestinal tract passage simulation, before and after two sub-lethal stresses. *Food Microbiology* 30, 24-28.

Boor, K.J., 2006. Bacterial stress responses: What doesn't kill them can make them stronger. *Plos Biology* 4, 18-20.

CDC, 2011. Multistate outbreak of Listeriosis associated with Jensen Farms cantaloupe-United States, August-Septermber 2011. MMWR 60, 1357-1358.

Colás-Medà, P., Abadias, M., Alegre, I., Usall, J., Viñas, I., 2015. Effect of ripeness stage during processing on *Listeria monocytogenes* growth on fresh-cut 'Conference' pears. *Food Microbiology* 49, 116-122.

Conte, M.P., Petrone, G., Di Biase, A.M., Ammendolia, M.G., Superti, F., Seganti, L., 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. *Microbial Pathogenesis* 29, 137-144.

Deng, Y., Ryu, J.H., Beuchat, L.R., 1999. Tolerance of acid-adapted and non-adapted *Escherichia coli* O157:H7 cells to reduced pH as affected by type of acidulant. *Journal of Applied Microbiology* 86, 203-210.

Garner, M.R., James, K.E., Callahan, M.C., Wiedmann, M., Boor, K.J., 2006. 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. *Applied and Environmental Microbiology* 72, 5384-5395.

Hoelzer, K., Pouillot, R., Dennis, S., 2012. *Listeria monocytogenes* growth dynamics on produce: a review of the available data for predictive modeling. *Foodborne Pathogens and Disease* 9, 661-673.

Jaradat, Z.W., Bhunia, A.K., 2003. Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Applied and Environmental Microbiology* 69, 3640-3645.

Lecuit, M., Cossart, P., 2001. A transgenic model for listeriosis: role of internalin with E-cadherin in crossing the intestinal barrier. *M S-Medecine Sciences* 17, 1333-1335.

Lorentzen, G., Mennen, S., Olsen, R.L., Skjerdal, T., 2011. Invasiveness of *Listeria monocytogenes* strains of Caco-2 cells in response to a period of extreme salt stress reflecting salt-curing and rehydration of cod (*Gadus morhua* L.). *Food Control* 22, 1040-1045.

Marklinder, I.M., Lindblad, M., Eriksson, L.M., Finnson, A.M., Lindqvist, R., 2004. Home storage temperatures and consumer handling of refrigerated foods in Sweden. *Journal of Food Protection* 67, 2570-2577.

Melo, J., Schrama, D., Hussey, S., Andrew, P.W., Faleiro, M.L., 2013. *Listeria monocytogenes* dairy isolates show a different proteome response to sequential exposure to gastric and intestinal fluids. *International Journal of Food Microbiology* 163, 51-63.

Oliveira, M., Vinas, I., Colas, P., Anguera, M., Usall, J., Abadias, M., 2014. Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. *Food Microbiology* 38, 137-142.

Oliveira, M., Wijnands, L., Abadias, M., Aarts, H., Franz, E., 2011. Pathogenic potential of *Salmonella* Typhimurium DT104 following sequential passage through soil, packaged fresh-cut lettuce and a model gastrointestinal tract. *International Journal of Food Microbiology* 148, 149-155.

Oomen, A.G., Rompelberg, C.J.M., Bruil, M.A., Dobbe, C.J.G., Pereboom, D., Sips, A., 2003. Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. *Archives of Environmental Contamination and Toxicology* 44, 281-287.

Peterson, L.D., Faith, N.G., Czuprynski, C.J., 2007. Resistance of *Listeria monocytogenes* F365 cells to synthetic gastric fluid is greater following growth on ready-to-eat deli turkey meat than in brain heart infusion broth. *Journal of Food Protection* 70, 2589-2595.

Pricope-Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K., 2013. The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay. *International Journal of Food Microbiology* 166, 59-64.

Rantsiou, K., Greppi, A., Garosi, M., Acquadro, A., Mataragas, M., Cocolin, L., 2012. Strain dependent expression of stress response and virulence genes of

Listeria monocytogenes in meat juices as determined by microarray. *International Journal of Food Microbiology* 152, 116-122.

Rieu, A., Guzzo, J., Piveteau, P., 2009. Sensitivity to acetic acid, ability to colonize abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on parsley leaves. *Journal of Applied Microbiology* 108, 560-570.

Sapers, G.M., Doyle, M.P., by, E., Matthews, G.M.S.B.S.R., 2009. Chapter 1 - Scope of the Produce Contamination Problem, *The Produce Contamination Problem*. Academic Press, San Diego, pp. 3-19.

Soliva-Fortuny, R.C., Alos-Saiz, N., Espachs-Barroso, A., Martin-Belloso, O., 2004. Influence of maturity at processing on quality attributes of fresh-cut conference pears. *Journal of Food Science* 69, S290-S294.

UCDavis, 2015. Marketing of fresh produce. Consumption trends. Cook, R.; Available at: http://are.ucdavis.edu/en/people/faculty/roberta-cook/articles-and-presentations/ (accessed 30/09/15).

Walecka, E., Molenda, J., Karpiskova, R., Bania, J., 2011. Effect of osmotic stress and culture density on invasiveness of *Listeria monocytogenes* strains. *International Journal of Food Microbiology* 144, 440-445.

Walls, I., Buchanan, R.L., 2005. Use of food safety objectives as a tool for reducing foodborne listeriosis. *Food Control* 16, 795-799.

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.

CAPÍTULO 6

Variation in phenotypic and genotypic characteristics among *Listeria monocytogenes* serotypes and trade-off between growth and invasion potential

P. Colas-Medà, L. Wijnands, A.H.A.M. van Hoek, M. Abadias,

I. Viñas, E. Franz.

International Journal of Food Microbiology (enviado)

Abstract

For food safety risk assessment, and eventually risk management, it may be important to differentiate between fast growing less virulent strains of pathogenic bacteria and slow growing but highly virulent strains of pathogenic bacteria. The current study aimed to test for a trade-off between maximum growth rate, acid tolerance and virulence potential of *Listeria monocytogenes* and how this relates to different serotypes (1/2a, 1/2c, 4b) and allelic variants of the genes encoding InternalinA (*inlA)*, the transcriptional regulator PrfA (*prfA)* and the stress response factor SigmaB (*sigB).* Within serotype 1/2a and 1/2c, strains from non-human origin showed significant higher growth rates than strains from human origin. Such difference was not observed within serotype 4b. After evaluating the capacity of surviving acid stress (pH 3.5) of all *L. monocytogenes* isolates, no significant differences were observed between serotypes and origin. Although strains of serotype 4b showed significant higher capacity to invade Caco-2 cells compared to strains from serotypes 1/2a and 1/2c, no difference was observed between strains from human and non-human origin. Sequence analysis of *inlA* of the 53 strains analysed revealed that 15 of them contained premature stop codons (PMSCs) in seven different variations. All of these mutations were observed in food-food environment isolates and human clinic ones, belonging to serotypes 1/2a and 1/2c, whereas no PMSC were observed in 4b isolates. A truncated PrfA was observed in one 1/2a strain (food origin) due to a duplication of seven nucleotides in the *prfA* gene, while four amino acid variations were identified in three other strains. No strains were encountered with PMSCs in this gene. Four variations in the deduced amino acid sequence of *sigB* were observed. Three of them represented non-synonymous SNPs while one resulted in a PMSC. Overall, serotype 4b strains were characterised by a relative high invasion capacity but low growth potential. For strains of serotype 1/2c the opposite was observed, while serotype 1/2a strains were characterised by intermediate growth and invasion potential. Strains with a high growth potential and low invasion capacity were strongly associated with PMCSs in *inlA*.

Keywords: *L. monocytogenes*; virulence genes; *inlA*; *prfA*; *sigB*.

1. Introduction

Listeria (L.) monocytogenes is a facultative intracellular pathogen responsible for listeriosis in humans and animals. The main cause of human listeriosis is the consumption of contaminated food products. This disease in healthy adults is generally asymptomatic or develops only mild symptoms with simple gastroenteritis, whereas in immunocompromised or pregnant individuals and newborns an invasive and systemic infection can occur, leading to meningitis, encephalitis and septicemia. Listeriosis is associated with an exceptionally high hospitalization rate and 20 to 30% of cases are fatal (EFSA, 2015). *L. monocytogenes* can be classified into 13 different serotypes, but only a few specific serotypes (1/2a, 1/2b, and 4b) are responsible for the majority (approximately 90%) of human listeriosis cases (Schlech, 2000). *L. monocytogenes* also consists of at least four evolutionary lineages (I, II, III, and IV) with different but overlapping ecological niches. Most *L. monocytogenes* isolates belong to lineages I and II, which include the serotypes more commonly associated with human clinical cases, such as serotypes 1/2a (lineage II), and 1/2b and 4b (lineage I). Lineage II strains are common in foods, are widespread in the natural and farm environments, and are often isolated from animal listeriosis cases and sporadic human clinical cases. Lineage III and IV strains are rare and predominantly isolated from animal sources (Shen *et al.*, 2013).

L. monocytogenes is known for its ability to survive and proliferate in adverse environments, including acid conditions, refrigeration temperatures, and high osmolarity (Beales, 2004). Virulence of *L. monocytogenes* is due to the expression of several genes responsible for the ability of the pathogen to penetrate cells, to proliferate and to spread through them (Braun, Cossart, 2000). Internalin A (*InlA*) plays an important role in the invasion of intestinal epithelial cells by *L. monocytogenes*, a crucial first step to pathogenesis of systemic listeriosis (Lecuit et al., 2001). Various distinct single nucleotide polymorphisms (SNPs)s leading to premature stop codons (PMSCs) in *inlA* have been observed among *L. monocytogenes* isolates collected from multiple countries, including the United States (Nightingale et al., 2005; Orsi et al., 2007), France (Rousseaux et al., 2004), Portugal (Felicio et al., 2007), Japan (Handa-Miya et al., 2007), and New Zealand (Cruz et al., 2013). These PMSC mutations in *inlA* result in a truncated form of the InlA protein that was secreted rather than anchored to the bacterial cell wall, or terminated InlA production (Jonquières et al., 1998; Nightingale et al., 2007; Olier et al., 2002; Orsi et al., 2007; Rousseaux et al., 2004). In a previous study Jacquet et al. (2004) noticed that clinical strains expressed full-length internalin far more frequently than those recovered from food products. In addition, they also found

that all evaluated 4b strains, the most frequently implicated serotype in human listeriosis, expressed full-length internalin. Similar results were observed by Tamburro et al. (2015).

Numerous virulence factors, which play an essential role in the infection pathway of *L. monocytogenes*, are regulated by PrfA (Freitag, 2006; Freitag et al., 2009; Gahan, Hill, 2005). For example, PrfA plays an important part in mediating the *L. monocytogenes* transition from saprophyte to pathogen. It also induces the expression of a bile salt hydrolase (encoded by *bsh*) as well as a bile exclusion system, both of which contribute to bacterial survival in the intestine. Moreover, PrfA induces the expression of a set of genes, the products of which are required for bacterial entry into host cells, escape from the phagosome, growth in the cytosol and cel-to-cell spread. Roche et al. (2005) observed some nonsense mutations in the *prfA* gene.

To cause illness, the pathogen must first survive environmental stresses encountered during food processing, distribution, and preparation, and then it must overcome physiological barriers imposed by the host, such as low pH of the human stomach. In *L. monocytogenes*, the alternative sigma factor B (σ^B) contributes to cellular survival under several adverse conditions, such as carbon depletion, exposure to acid, oxidative or osmotic stress, and low temperature (Huang et al., 2014). The SigmaB gene *sigB* is activated by a diverse range of environmental and energy stresses, to direct the synthesis of more than 100 general stress proteins (Wemekamp-Kamphuis et al., 2004). Several *sigB* allelic variants are reported including PMSCs (Nightingale et al., 2007).

In many countries strict regulations regarding the presence of *L. monocytogenes* have been established. The United States adopted a zero-tolerance policy (Shank et al., 1996) for all ready-to-eat foods, whereas the European Union allows 100 cfu g^{-1} of this pathogen at the best-before date for some classes of foods (European Commission, 2005). These policies have been established based on the hypothesis that all *L. monocytogenes* strains are equal with respect to growth potential and pathogenicity, despite the heterogeneity that has been reported to exist among isolates (Handa-Miya et al., 2007). For food safety risk assessment, and eventually risk management, it may be important to differentiate between fast growing less virulent strains and slow growing but highly virulent ones.

Our main objective was to test for a trade-off between maximum growth rate, acid tolerance and virulence potential with *L. monocytogenes* and how this relates to different serotypes (1/2a, 1/2c and 4b) and allelic variants of *inlA*, *prfA* and *sigB*.

2. Material and method

2.1. Bacterial isolates

The study included 31 human clinical and 22 food *L. monocytogenes* isolates, all belonging to serotypes 1/2a, 1/2c, and 4b (Table 1). These serotypes were selected because they showed the most difference between their prevalence in humans versus food. In food isolates, the most frequent serotype was 1/2a; serotype 4b was the most predominant among human isolates; and serotype 1/2c was more or less equally found in both sources, according to Dutch data (Friesema et al., 2015; Friesema et al., 2016). All isolates had been stored at -80 °C in cryovials.

2.2. Phenotypic studies

The strains were grown individually on tryptic soy agar (TSA (Oxoid, Badhoevedorp, the Netherlands)) at 37 °C for 24 h. One colony of each strain was removed with a sterile loop, transferred into a flask containing 10 ml of brain heart infusion broth (BHI (Becton Dickinson, Breda, the Netherlands)) and incubated at 37 °C 24 h.

2.2.1. Growth rate

The optical density (OD) of the 24 h grown subcultures was determined. Using multiple initial inocula the time to detection (TTD) at a given standard OD was obtained from which the specific growth rate was calculated. The 2-fold dilution (2FD) method which uses TTD and inoculum size variation to obtain values for maximum growth rate (μ_{max}) was used (Biesta-Peters et al., 2010). 96-Well plates were filled with 180 µL BHI per well. Twelve strains were tested per-plate and each strain was tested in four wells in duplicate. Columns 1, 5 and 9 received 20 µL of the appropriate serial dilutions of each strain (with the highest inoculum (2FD)). Using a multichannel-pipette, 20 µL were removed from each well of columns 1, 5 and 9, and transferred to columns 2, 6 and 10, respectively. This was repeated across the plate until columns 4, 8 and 12, respectively, discarding the excess 20 µL in these last wells after mixing. Plates were incubated for 18 h at 37 °C in a BioTek ELx808 Absorbance Microplate Reader (BioSPX B.V., Abcoude, the Netherlands) with shaking every 10 seconds, while the OD_{600} of the wells was being read every 5 min. Data were fitted with Excel® and the slope of TTD versus the natural logarithm (ln) of the concentration was determined. The inverse of the slope was extracted obtaining the maximum growth rate (μ_{max}, h^{-1}).

Table 1. *International* L. monocytogenes *strains used for phenotypic and genotypic study.*

Strain	Lineage	Serotype	Source	Reference
230/3	II	1/2a	RTE Iceberg lettuce	Abadias et al. 2008
317	II	1/2a	Cheese	*L. monocytogenes* 1F; P. Piveteau (Dijon, France, 1990)
318	II	1/2a	Brine	*L. monocytogenes* 2S; P. Piveteau (Dijon, France, 1991)
327	II	1/2a	Meat processing plant	*L. monocytogenes* ATCC 15313
330	II	1/2a	Chicken carcass	*L. monocytogenes* F1; L. Brito
332	II	1/2a	Turkey Frankfurter	*L. monocytogenes* F6854; USDA
340	II	1/2a	Poultry processing environment	*L. monocytogenes* LR2424; M. Pikkemaat RC56, 1995
346	II	1/2a	Minced beef	*L. monocytogenes* NV4; P. Piveteau
349	II	1/2a	Bovine carcass	*L. monocytogenes* NV8; P. Piveteau
256	II	1/2a	Blood	This study
260	II	1/2a	Lumbar fluid	This study
263	II	1/2a	Blood	This study
266	II	1/2a	Synovial fluid knee	This study
272	II	1/2a	Lumbar fluid	This study
285	II	1/2a	Lumbar fluid	This study
288	II	1/2a	Blood	This study
293	II	1/2a	Lumbar fluid	This study
295	II	1/2a	Blood	This study
300	II	1/2a	Lumbar fluid	This study
319	II	1/2c	Rabbit	*L. monocytogenes* ATCC 15313
347	II	1/2c	Minced beef	*L. monocytogenes* NV5; P. Piveteau
348	II	1/2c	Bovine carcass	*L. monocytogenes* NV7; P. Piveteau
264	II	1/2c	Blood	This study
267	II	1/2c	Peritoneal fluid	This study
270	II	1/2c	Blood	This study
275	II	1/2c	Blood	This study

Table 1. *International* L. monocytogenes *strains used for phenotypic and genotypic study (II).*

Strain	Lineage	Serotype	Source	Reference
279	II	1/2c	Blood	This study
284	II	1/2c	Blood	This study
290	II	1/2c	Blood	This study
296	II	1/2c	Blood	This study
299	II	1/2c	Blood	This study
302	II	1/2c	Blood	This study
324	II	1/2c	Unknown	*L. monocytogenes* ATCC 7644
323	I	4b	Unknown	*L. monocytogenes* ATCC 23074
326	I	4b	Smoked meat	*L. monocytogenes* C12; L. brito
328	I	4b	Smoked meat	*L. monocytogenes* C8; L. Brito
331	I	4b	Jalisco cheese	*L. monocytogenes* F2365; USDA
334	I	4b	Hotdog	*L. monocytogenes* H7858; USDA
336	I	4b	Fish equipment	*L. monocytogenes* LR1089, M. Pikkemaat RC3, 1995
339	I	4b	Broiler carcass	*L. monocytogenes* LR2011; M. Pikkemaat RC32, 1995
341	I	4b	Poultry processing environment	*L. monocytogenes* LR2549; M. Pikkemaat RC56, 1995
350	I	4b	Milk	*L. monocytogenes* ScottA; Massachusetts outbreak, 1983
253	I	4b	Cervix	This study
254	I	4b	Blood	This study
268	I	4b	Joint puncture	This study
274	I	4b	Bile	This study
278	I	4b	Blood	This study
280	I	4b	Biopt	This study
283	I	4b	Peritoneal fluid	This study
287	I	4b	Blood	This study
291	I	4b	Blood	This study
297	I	4b	Blood	This study
301	I	4b	Lumbar fluid	This study

2.2.2. Survival to acid stress

To evaluate the acid tolerance, each strain was cultured 24 h in BHI at 37 °C to obtain bacterial counts of approximately 1 x 10^9 cfu ml^{-1}. The initial concentration of *L. monocytogenes* was determined by plating 10-fold serial dilutions onto Palcam agar (Merck, Amsterdam, the Netherlands) before the acid challenge test. A simulated gastric fluid (SGF), mimicking the stomach conditions, was used as acid challenge medium. The SGF was prepared according to the method described by Rotard *et al.* (1995). It contained the following reagents per liter: 175.0 g of sodium chloride, 88.8 g of sodium dihydrogen phosphate, 89.6 g of potassium chloride, 22.2 g of calcium chloride, 30.6 g of ammonium chloride, 65.0 g of glucose, 2.0 g of glucuronic acid, 25.0 g of urea, 33.0 g of glucosamine, 1.0 g of bovine serum albumin fraction V and 3.0 g of mucine (type II from porcine stomach). The solution was adjusted to pH 2.5 with 1M HCl and filter sterilized (0.22 μm). All chemicals were obtained from Merck. A mixture containing 1 ml subculture and 9 ml SGF (pH 2.5) was incubated for 60 min at 37 °C and 5% CO_2. At the start of the assay, the pH was set up to pH 3.5, this was confirmed by measuring the pH of the solution. After the acid challenge, the cell numbers were determined by plating 10-fold serial dilution onto Palcam agar (incubated at 37 °C during 48 h). The survival capacity was reported as the percentage of the number of cells recovered after the acid stress divided by the number of cells that had been used for inoculation. Each strain was assayed twice in independent experiments.

2.2.3. Capacity of invasion

To assess the invasion capacity of each strain a Caco-2 cell assay was used. The Caco-2 cells were routinely grown in plastic tissue flasks containing Dulbecco's modified Eagle's medium (DMEM (Gibco, Landsmeer, the Netherlands)) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% non-essential amino acids, 1% glutamine and 50 μg ml^{-1} gentamicin. Cells were grown to confluence in a humidified atmosphere of 5% CO_2 air for seven days and then passed to new culture flasks mixing 9 ml medium and 1 ml Caco-2 cells at a concentration of approximately 1x10^6 cells ml^{-1}. For the invasion experiments, cells were inoculated into 12-well plates at a concentration of 1.6x10^5 cells per well. Plates were incubated for two weeks. Growth medium was changed every 2-3 days. For invasion experiments Caco-2 cells were used between passages 25 and 45. To perform the invasion assay, *L. monocytogenes* strains were cultured 24 h in BHI at 37 °C to obtain a culture of approximately 1x10^9 cfu ml^{-1}. The cultures

were diluted to achieve cell counts of $1x10^6$ cfu ml^{-1}. The Caco-2 cells in the 12-well plates were washed three times with pre-warmed sterile Phosphate Buffered Saline (PBS). A volume of 1 ml experimental culture medium (ECM: DMEM supplemented with 1% non-essential amino acid (100X), 1% glutamine (200 mM)) and 40 µL *Listeria* sample were added to each well with the Caco-2 monolayer. Per sample three wells were inoculated. Following centrifugation at 175xg for 1 min, the plates were incubated for 30 min at 37 °C and 5% CO_2. After incubation, the medium was removed and the Caco-2 cells were washed three times with 1 ml sterile PBS to remove unattached extracellular *L. monocytogenes* cells. The remaining extracellular *L. monocytogenes* were killed by adding pre-warmed fresh ECM supplemented with 150 µg ml^{-1} gentamicin to the wells and incubation at 37 °C and 5% CO_2 for 1.5 h. This medium was removed, the cells were washed three times with pre-warmed PBS, and Caco-2 cells were detached and lysed by adding 1 ml 1% Triton X100 in PBS. The contents of three wells was combined and from this suspension 1 ml was used to quantify the number of invaded *L. monocytogenes* cells by plating appropriate 10-fold dilutions on Palcam agar plates and incubation for 48 h at 37 °C. The invasion efficiency was reported as the percentage of the number of *L. monocytogenes* recovered after invasion assay divided by the number of bacteria initially used for inoculation. Each strain was assayed twice in independent experiments.

2.3. Genotypic study

2.3.1. DNA extraction

L. monocytogenes strains were grown individually on TSA at 37 °C for 20-24 h. One colony of each isolate was suspended in 300 µL of a 10% Chelex-100 resin suspension (Bio-Rad, Veenendaal, the Netherlands), vortexed, incubated at 99 °C for 15 min and then chilled. Finally, the samples were centrifuged for 5 min at 13,000 rpm at 4 °C and 200 µL of the supernatant was transferred to a new tube and stored at -20 °C.

2.3.2. Sequence analysis of *inlA*, *prfA* and *sigB*

The *inlA*, *prfA* and *sigB* genes of the *L. monocytogenes* strains were amplified by PCR. The PCR reaction mixtures, prepared in 50 µL, contained 5 µL of 10X PCR buffer (Invitrogen, Landsmeer, the Netherlands), 2.5 µL of 50 mM $MgCl_2$ (Invitrogen), 2 µL of 5mM dNTP (Invitrogen), 1 µL of each appropriate forward

and reverse primer (10 μM, Table 2), and 0.25 μL Taq DNA polymerase (Invitrogen).

Table 2. *Description of primers used.*

Name	Oligo sequence (5′-3′)	Length	Reference
inlA -70F	CGGATGCAGGAGAAAATCC	19	Ragon *et al.*, 2008
inlA 727F	GATATAACTCCACTTGGG	18	Ragon *et al.*, 2008
inlA 1510F	GTGGACGGCAAAGAAAC	17	Ragon *et al.*, 2008
inlA 970R	GCTCTAAGTTAGTGAGTGCG	20	Ragon *et al.*, 2008
inlA 1741R	GAGATGTTGTTACACCGTC	19	Ragon *et al.*, 2008
inlA +91R	CTTTCACACTATCCTCTCC	19	Ragon *et al.*, 2008
prfA -96F	CGTGACTTTCTTTCAACAGC	20	This study
prfA +99R	GGTGAAGCAATCGTACGCG	19	This study
sigB -135F	ATGCGGAGATGATGCGTATC	20	This study
sigB 316F	CATGTGCCGCGCCGAATC	18	This study
sigB 638R	ATCTTCTGTTCTCGCTCATC	20	This study
sigB +140R	CCACTTCCAAGTCCATCAG	19	This study

The *inlA* PCR amplification conditions were as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s and finally 72 °C for 10 min. For *prfA* and *sigB* the following PCR conditions were used: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s and a final extension step of 72 °C for 10 min. To remove the unincorporated PCR primers and dNTPs, PCR products were treated with Exo-SAP according to the manufacturers conditions (Sigma-Aldrich, Zwijndrecht, the Netherlands). DNA sequencing for each locus was performed using the same primers as for PCR amplification as well as additional ones to obtain the complete gene sequence (Table 2). The obtained sequences were aligned against the *inlA*, *prfA* and *sigB* genes of *L. monocytogenes* strain EGD by manual editing the alignment in SeaView software (Version 4, (Gouy *et al.*, 2010)) to identify possible mutations.

2.4. Statistical analyse

For the results of phenotypic studies, differences between serotypes were determined by using the Student t test (Excel®). $P < 0.05$ was considered to

indicate a statistically significant difference. For the multivariate analysis, a PCA model was developed. Samples were characterised according to an average value of μmax, % invasion, % survival to acid stress and contain or not PSMCs of the sequence of the three genes studied. SAS studio was used to develop this model.

3. Results and discussion

3.1. Phenotypic studies

The maximum growth rate ranged from 0.75-1.0 h^{-1} (Table 3). Within serotype 1/2a and 1/2c significant higher growth rates were observed among strains of non-human origin (P=0.0002 and 0.0014, respectively). Such a difference was not observed within serotype 4b (P=0.0640) (Table 4).

Table 3. *Growth rate (h^{-1}), survival capacity to gastric stress (%) and capacity of invasion to Caco-2 cells (%) of 53* L. monocytogenes *isolates recovered from food or food environments (non-human origin) and clinical cases (human origin).*

Origin	Strain	Lineage	Serotype	Growth rate (h^{-1})	Survival at acid stress (%)	Capacity of invasion (%)
non-human	230/3	II	1/2a	0.88	64.7	ND*
non-human	317	II	1/2a	0.84	84.8	30.6
non-human	318	II	1/2a	0.91	93.1	30.5
non-human	327	II	1/2a	0.88	61.7	28.8
non-human	330	II	1/2a	0.96	27.2	25.7
non-human	332	II	1/2a	0.82	81.1	41.7
non-human	340	II	1/2a	0.85	81.0	40.2
non-human	346	II	1/2a	0.89	70.2	26.4
non-human	349	II	1/2a	0.90	92.1	24.8
human	256	II	1/2a	0.75	84.7	42.9
human	260	II	1/2a	0.78	66.6	44.8
human	263	II	1/2a	0.75	89.8	42.6
human	266	II	1/2a	0.75	100	45.3
human	272	II	1/2a	0.74	10.8	46.2
human	285	II	1/2a	0.85	73.8	23.6
human	288	II	1/2a	0.82	100	44.0
human	293	II	1/2a	0.85	100	44.3
human	295	II	1/2a	0.78	74.3	26.5
human	300	II	1/2a	0.81	100	39.1
non-human	319	II	1/2c	1.02	78.8	34.2
non-human	347	II	1/2c	0.86	100	42.1

Origin	Strain	Lineage	Serotype	Growth rate (h^{-1})	Survival at acid stress (%)	Capacity of invasion (%)
non-human	348	II	1/2c	1.00	100	26.3
human	264	II	1/2c	0.84	74.2	31.5
human	267	II	1/2c	0.81	96.1	29.5
human	270	II	1/2c	0.76	74.8	29.4
human	275	II	1/2c	0.86	29.3	24.5
human	279	II	1/2c	0.86	100	28.8
human	284	II	1/2c	0.83	100	26.3
human	290	II	1/2c	0.87	100	29.1
human	296	II	1/2c	0.84	58.0	44.9
human	299	II	1/2c	0.88	77.6	39.7
human	302	II	1/2c	0.80	90.7	28.6
human	324	II	1/2c	0.84	70.0	29.1
non-human	323	I	4b	0.79	70.8	44.7
non-human	326	I	4b	0.81	53.1	44.0
non-human	328	I	4b	0.75	76.6	44.1
non-human	331	I	4b	0.79	100	44.2
non-human	334	I	4b	0.76	100	45.9
non-human	336	I	4b	0.80	85.2	45.9
non-human	339	I	4b	0.77	100	45.8
non-human	341	I	4b	0.79	100	38.5
non-human	350	I	4b	0.78	80.8	49.2
human	253	I	4b	0.77	39.9	45.2
human	254	I	4b	0.76	62.9	51.7
human	268	I	4b	0.75	85.5	49.3
human	274	I	4b	0.76	6.5	48.6
human	278	I	4b	0.76	100	46.8
human	280	I	4b	0.76	84.4	49.7
human	283	I	4b	0.75	100	45.3
human	287	I	4b	0.78	95.3	45.6
human	291	I	4b	0.79	100	41.9
human	297	I	4b	0.79	58.8	38.2
human	301	I	4b	0.76	91.5	43.5

** ND, not determined*

The fraction of cells surviving acid stress ranged from 6.5% to 100% (Table 3). No significant differences were observed between serotypes (4b-1/2a; P=0.7102, 1/2a-1/2c; P=0.4968 and 4b-1/2c; P=0.7560) and origins (P=0.6467).

Invasion capacity of the strains ranged from 23.6% to 51.7% (Table 3). Strains of serotype 4b showed significant higher invasion capacity compared to 1/2a (P=0.0002) and 1/2c ($P=2*10^{-9}$). No difference was observed between strains from human and non-human origin (P=0.2764).

Table 4. *Statistic results of phenotypic characterization of 53 strains of* L. monocytogenes *(values average).*

	Survival at acid stres (%)			Growth rate (h^{-1})			Capacity of invasion (%)		
	4b	1/2a	1/2c	4b	1/2a	1/2c	4b	1/2a	1/2c
total	79.6	76.6	82.1	0.77	0.83	0.86	45.4	34.1	31.7
t-tests	4b	1/2a	1/2c	4b	1/2a	1/2c	4b	1/2a	1/2c
(P-values) 4b	-	0.7102	0.7560	-	**0.0003***	**$7.6 \cdot 10^{-6}$***	-	**0.0002***	**$2.1 \cdot 10^{-9}$***
1/2a	-	-	0.4968	-	-	0.2151	-	-	0.4910
	4b	1/2a	1/2c	4b	1/2a	1/2c	4b	1/2a	1/2c
non-human	85.2	72.9	92.9	0.78	0.88	0.96	44.7	27.6	34.2
human	75.0	80.0	79.1	0.77	0.79	0.83	46.0	39.9	31.0
t-test (P-values)	0.3775	0.5324	0.3248	0.0640	**0.0002***	**0.0014***	0.4168	**0.0171***	0.4570
	non-human		human	non-human		human	non-human		human
	78.0		81.0	0.80		0.85	38.9		35.9
t-test (P-values)	0.6467			**0.0024***			0.2764		

**significant difference was observed*

3.2. Genotypic studies

3.2.1. Sequence of *inlA*

The full-length sequence of *inlA* gene was analysed and compared to the deduced amino acid sequences of the reference strain *L. monocytogenes* strain EGDe (serotype 1/2a). Only seven strains had an identical *inlA* sequence as the reference all belonging to lineage II. Among the 53 strains analysed 15 contained PMSCs in seven different variations (Table 5). All *inlA* PMSCs detected in this study were previously observed (Nightingale *et al*., 2005; Olier *et al*., 2002; Ragon *et al*., 2008; Rousseaux *et al*., 2004; Van Stelten, Nightingale, 2008) except the one at position 326. All of these mutations were observed in food-food environment isolates and human clinical isolates, belonging to serotypes 1/2a and 1/2c. The obtained results showed that all strains containing PMSCs belong to lineage II, since no PMSCs were observed in 4b isolates. Shen *et al.* (2013) observed that about half (13/27) of their strains analysed contained PMSCs, leading to a non-functional InlA. This is very similar in the current study while looking at the lineage II strains (42% (8/19) of 1/2a and 50% (7/14) of 1/2c).

In summary, among the 53 isolates evaluated, 28% (15/53) of them contained PMSCs, specifically 19% (6/32) were observed in human clinic isolates and 43% (9/21) in food isolates. Our results are in accordance with Shen *et al.* (2013) and Chen *et al.* (2011) results, whose have been reported similar amounts (30-45%) of PMSC mutations in food isolates. In addition, other sequence types without PMSCs were observed (see Supplementary Table S1). Three sequence types of *InlA* were observed among strains belonging to serotype 4b (20 isolates), six different sequence types in strains of serotype 1/2c (14 isolates), and 15 different sequences types in the strains of serotype 1/2a (19 isolated). Recently, Ciolacu *et al.* (2015) described some nucleotide changes in the InlA deduced amino acid sequence that were observed in 15 *L. monocytogenes* isolates from food products imported from the Republic of Moldavia. Many of these changes were observed in our strains.

Table 5. *Distribution of PMSCs and the 3-codon deletion in* inlA *among the 53 isolates of* L. monocytogenes *see also Supplementary Table S1.*

Strain number	inlA PMSC mutation type*	No. of isolates harbouring inlA PMSC mutations among isolated obtained from:		Total no. of isolates with inlA PMSC mutation type
		Food	Humans	
327	Type 3 (aa 700)	1 (1/2a)	0	1
346	Type 5 (aa 189)	1 (1/2a)	0	1
285, 317, 318, 330, 230/3	Type 6 (aa 492)	4 (1/2a)	1 (1/2a)	5
349	Type 8 (aa 460)	1 (1/2a)	0	1
299, 347	Type 11 (aa 685)	1 (1/2c)	1 (1/2c)	2
348	Type 14 (aa 539)	1 (1/2c)	0	1
267, 279, 284, 290	New PMSC (aa 326) in this study	0	4 (1/2c)	4
	Total	9	6	15

**According to inlA PMSC mutations type described by Van Stelten* et al. *(2010)*

3.2.2. Sequence of *prfA* gene

Opposite to some studies (Ciolacu *et al*., 2015; Handa-Miya *et al*., 2007) amino acid sequence variation of PrfA was observed here as described by Kanki *et al.* (2015) and Roche *et al.* (2005). Kanki *et al.* (2015) observed that seven out of 114 isolates contained a PrfA protein with a 5-nucleotide deletion at nucleotide position 712 to 716 including a stop codon, which was referred to as the stop-codon deletion. Whereas Roche *et al.* (2005) observed that 11 strains assigned to low-virulence group, eight strains had a SNP, PrfAK220T, and the other three had a truncated PrfA, PrfAΔ174-237.

In particular, four variations in the *prfA* deduced amino acid sequence were observed (see Supplementary Table S1). One *prfA* amino acid sequence variation was observed in only one strain (serotype 1/2a, food origin): it had seven nucleotides inserted into its *prfA* gene, introducing an early termination codon that resulted in a truncated protein (PrfAΔ174-240). Roche *et al.* (2005) described that three out of eleven low virulent serotype 1/2a strains with food origin had seven nucleotides inserted after codon 171. This insertion changed the reading frame from codon 174 and introduced an early termination codon at position 184, resulting in a truncated protein (PrfAΔ174-237) as demonstrated by Western blotting. Although only 2% of the strains in the current study harboured this mutation in the Roche study this was around 12% of the evaluated strains. Moreover, all of these failed to enter human adenocarcinoma cells and were either a-virulent or hypo-virulent to mice because of their truncated PrfA protein (Roche *et al.*, 2005).

Deviating *prfA* sequences were observed in three strains of serotype 1/2a and 1/2c, whereas all strains of serotype 4b showed identical PrfA amino acid sequence as *L. monocytogenes* strain EGDe. The amino acid changes were observed previously (Miner *et al.*, 2008; Roche *et al.*, 2005; Rupp *et al.*, 2015). These authors concluded that the mutations lead to a truncated protein, which contributes to a strongly attenuated phenotype. However, the strain which harboured the K197N mutation in the current study showed a high invasion capacity (43%) although in a previous study this mutation caused an attenuated invasion in the strain (Rupp *et al.*, 2015). This could be explained by other (numerous) variations in other virulence genes, such as *actA*. To the authors' knowledge, this is the first report that describes a non-synonymous mutation in *prfA* sequence at amino acid position 184.

3.2.3. Sequence of *sigB* gene

Four variations in the *sigB* deduced amino acid sequence were observed (see Supplementary Table S1). Three of them represented non-synonymous SNPs while one resulted in a PMSC. The substitution of tyrosine to phenylalanine at position 216 was observed in all strains belonging to 4b serotype from both origins. The novel PMSC was observed in a serotype 1/2c isolate with a food origin, where a glycine was substituted to PMSC at position 189. Nightingale *et al*. (2007) observed 29 unique allelic types among 157 *L. monocytogenes* isolates, allowing better characterisation of *L. monocytogenes* based on *sigB* allelic types. The results presented here only demonstrated clear allelic differences between lineage I strains (4b) and lineage II (1/2a and 1/2c).

3.3. Multivariate analysis of phenotypic data

To establish a relation among the phenotypic results, a principal component analysis (PCA) was performed. Principal components 1 (DM1) and 2 (DM2) accounted for 37.5% and 28.3%, respectively, of the total variability (*Figure 1*).

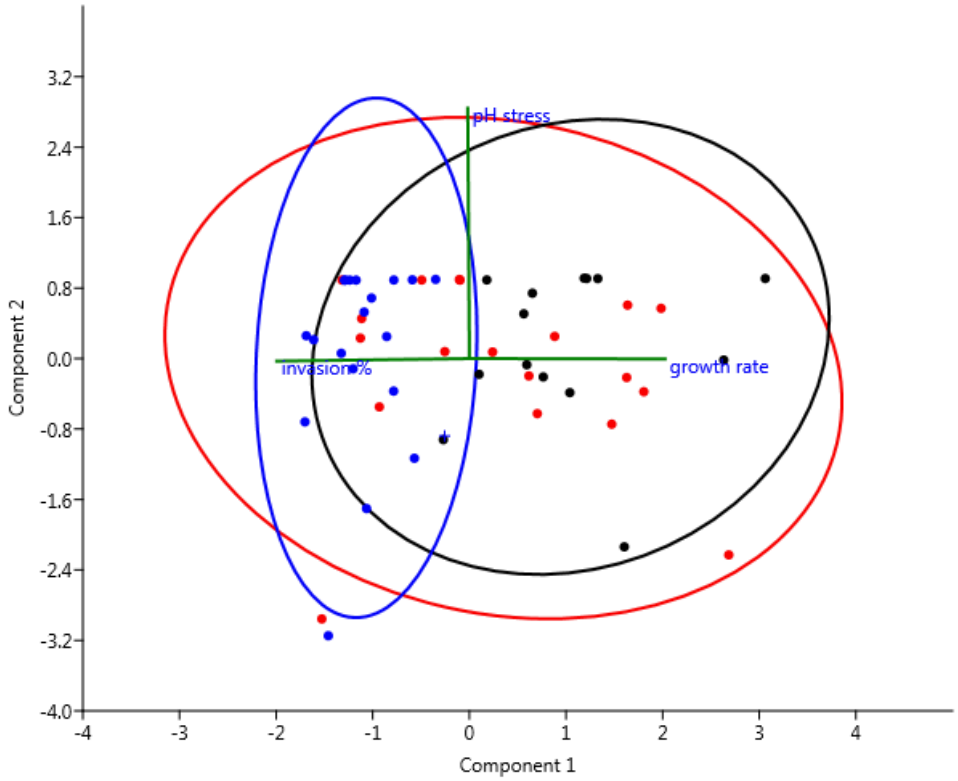


Figure 1. *Principal components analysis. The length of the arrows projected on the horizontal and vertical axis indicate their relative importance in separating the clusters (and thus in explaining observed variation). Variables in identical directions are positively correlated, while variables in opposite direction are negatively correlated. Circles represent 95% confidence intervals at serotype level.*

Clearly, all serotype 4b strains were characterised by a relative high invasion but low growth potential. Although less pronounced, for strains of serotype 1/2c the opposite was observed, while serotype 1/2a strains were characterised by intermediate growth and invasion potential. Invasion capacity and growth rate were clearly negatively correlated, which is confirmed by a correlation test ($r = 0.64$, $P < 0.001$). Variation in acid stress survival explained considerable

variation (almost all variation in the vertical direction of the PCA plot) but did not result in clustering of strains with specific characteristics (serotype, origin, allelic variants). Oliver *et al.* (2013) also did not observe a difference in acid tolerance between one *L. monocytogenes* isolate and its Δ*sigB* mutant.

4. Conclusion

The data presented showed the existence of a negative correlation between growth and virulence potential among *L. monocytogenes* strains. Although the cellular and molecular mechanisms underlying this apparent trade-off between growth and virulence are not elucidated here, the results suggest that food products that test below the maximum allowed number of *L. monocytogenes* colonies are non-random distribution of phenotypic and genotypic characteristics between *L. monocytogenes* serotypes. Especially serotype 4b, which is epidemiologically strongly related to clinical cases, differentiates from serotype 1/2a and 1/2c by a higher epithelial invasion capacity but lower growth potential. The strains with lower invasion capacity were characterised by higher growth rates and a high prevalence of mutations (PMSCs) in *inlA*.

Although all *L. monocytogenes* isolates are considered similarly pathogenic, the current results in agreement with other studies (Tamburro et al., 2015) indicate a significant difference in the pathogenic potential among serotypes. Hence, this information could help food safety risk assessment, and eventually risk management.

5. Acknowledgments

The authors are grateful to the University of Lleida, Grupo Alimentario Citrus and Banco Santander for a PhD grant (UdL-Impuls Program) to Pilar Colás Medà and to the University of Lleida for its financial support for a pre-doctoral stay in the RIVM (Bilthoven, the Netherlands). They also want to thank Wendy van Overbeek for her support in the laboratory.

6. References

Abadias, M., Usall, J., Anguera, M., Solson, C., Vinas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. International Journal of Food Microbiology 123, 121-129.

Beales. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservation, low pH, and osmotic stress: a review. Comprehensive Reviews in Food Science and Food Safety 3, 1-19.

Biesta-Peters, E.G., Reij, M.W., Joosten, H., Gorris, L.G.M., Zwietering, M.H., 2010. Comparison of two optical-density-based methods and a plate count method for estimation of growth parameters of *Bacillus cereus*. Applied and Environmental Microbiology 76, 1399-1405.

Braun, L., Cossart, P., 2000. Interactions between *Listeria monocytogenes* and host mammalian cells. Microbes and Infection 2, 803-811.

Chen, Y.H., Ross, W.H., Whiting, R.C., Van Stelten, A., Nightingale, K.K., Wiedmann, M., Scott, V.N., 2011. Variation in *Listeria monocytogenes* dose responses in relation to subtypes encoding a full-length or truncated Internalin A. Applied and Environmental Microbiology 77, 1171-1180.

Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K., 2015. *Listeria monocytogenes* isolated from food samples from a Romanian black market show distinct virulence profiles. International Journal of Food Microbiology 209, 44-51.

Cruz, C.D., Pitman, A.R., Harrow, S.A., Fletcher, G.C., 2013. *Listeria monocytogenes* associated with New Zealand seafood production and clinical cases: unique cequence types, truncated InlA, and attenuated invasiveness. Applied and Environmental Microbiology 80, 1489-1497.

EFSA. 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA Journal 13, 3991.

European Commission. 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

Official Journal of the European Union L338, 1-26.

Felicio, M.T.S., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M., 2007. Recurrent and sporadic *Listeria monocytogenes* contamination in alheiras represents considerable diversity, including virulence-attenuated isolates. Applied and Environmental Microbiology 73, 3887-3895.

Freitag, N.E., 2006. From hot dogs to host cells: how the bacterial pathogen *Listeria monocytogenes* regulates virulence gene expression. Future Microbiology 1, 89-101.

Freitag, N.E., Port, G.C., Miner, M.D., 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nature Reviews Microbiology 7, 623-628.

Friesema, I.H., Kuiling, S., van der Ende, A., Heck, M.E., Spanjaard, L., van Pelt, W. 2015. Risk factors for sporadic listeriosis in the Netherlands, 2008 to 2013. Eurosurveillance; 20(31); http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=21199.

Friesema, I.H.M., Kuiling, S., Heck, M.E.O.C., Biesta-Peters, E.G., van der Ende, A., Spanjaard, L., van Pelt, W. 2016. Surveillance van *Listeria monocytogenes* in Nederland, 2014, Infectieziekten Bulletin, vol. Jaargang 27. http://rivm.nl/Documenten_en_publicaties/Algemeen_Actueel/Uitgaven/Infectieziekten_Bulletin/Jaargang_27_2016/Februari_2016/Inhoud_februari_2016/Surveillance_van_Listeria_monocytogenes_in_Nederland_2014. 73-80.

Gahan, C.G.M., Hill, C., 2005. Gastrointestinal phase of *Listeria monocytogenes* infection. Journal of Applied Microbiology 98, 1345-1353.

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView Version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27, 221-224.

Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., Fujii, T., 2007. Nonsense-mutated inlA and prfA not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan. International Journal of Food Microbiology 117, 312-318.

Huang, Y.N., Ells, T.C., Hansen, L.T., 2014. Role of sigB and osmolytes in desiccation survival of *Listeria monocytogenes* in simulated food soils on the surface of food grade stainless steel. Food Microbiology 46, 443-451.

Jacquet, C., Doumith, M., Gordon, J.I., Martin, P.M.V., Cossart, P., Lecuit, M., 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. Journal of Infectious Diseases 189, 2094-2100.

Jonquières, R., Bierne, H., Mengaud, J., Cossart, P., 1998. The inlA gene of *Listeria monocytogenes* L028 harbors a nonsense mutation resulting in release of internalin. Infection and Immunity 66, 3420-3422.

Kanki, M., Naruse, H., Taguchi, M., Kumeda, Y., 2015. Characterization of specific alleles in *InlA* and *PrfA* of *Listeria monocytogenes* isolated from foods

in Osaka, Japan and their ability to invade Caco-2 cells. International Journal of Food Microbiology 211, 18-22.

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.

Miner, M.D., Port, G.C., Freitag, N.E., 2008. Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA. Microbiology-Sgm 154, 3579-3589.

Nightingale, K., Bovell, L., Grajczyk, A., Wiedmann, M., 2007. Combined sigB allelic typing and multiplex PCR provide improved discriminatory power and reliability for *Listeria monocytogenes* molecular serotyping. Journal of Microbiological Methods 68, 52-59.

Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M., 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in inlA, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. Applied and Environmental Microbiology 71, 8764-8772.

Olier, M., Pierre, F., Lemaitre, J.P., Divies, C., Rousset, A., Guzzo, J., 2002. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. Microbiology-Sgm 148, 1855-1862.

Oliver, H.F., Orsi, R.H., Wiedmann, M., Boor, K.J., 2013. sigma(B) Plays a Limited Role in the Ability of *Listeria monocytogenes* Strain F2365 To Survive Oxidative and Acid Stress and in Its Virulence Characteristics. Journal of Food Protection 76, 2079-2086.

Orsi, R.H., Ripoll, D.R., Yeung, M., Nightingale, K.K., Wiedmann, M., 2007. Recombination and positive selection contribute to evolution of *Listeria monocytogenes inIA*. Microbiology-Sgm 153, 2666-2678.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S., 2008. A new perspective on *Listeria monocytogenes* evolution. Plos Pathogens 4, 14.

Roche, S.M., Gracieux, P., Milohanic, E., Albert, I., Virlogeux-Payant, I., Temoin, S., Grepinet, O., Kerouanton, A., Jacquet, C., Cossart, P., Velge, P., 2005. Investigation of specific substitutions in virulence genes characterizing

phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. Applied and Environmental Microbiology 71, 6039-6048.

Rotard, W., Christmann, W., Knoth, W., Mailahn, W., 1995. Determination of absorption availability of PCDD/PCDF from "Kieselrot" (red slag) in the digestive tract-Simulation of the digestion of technogenic soil. Bestimmung der resorptionsverfügbaren PCDD/PCDF aus Kieselrot-Simulation der Digestion mit Böden 7, 3-9.

Rousseaux, S., Olier, M., Lemaitre, J.P., Piveteau, P., Guzzo, J., 2004. Use of PCR-Restriction fragment length polymorphism of *inlA* for rapid screening of *Listeria monocytogenes* strains deficient in the ability to invade caco-2 cells. Applied and Environmental Microbiology 70, 2180-2185.

Rupp, S., Aguilar-Bultet, L., Jagannathan, V., Guldimann, C., Drogemuller, C., Pfarrer, C., Vidondo, B., Seuberlich, T., Frey, J., Oevermann, A., 2015. A naturally occurring *prfA* truncation in a *Listeria monocytogenes* field strain contributes to reduced replication and cell-to-cell spread. Veterinary Microbiology 179, 91-101.

Schlech, W.F., 2000. Foodborne listeriosis. Clinical Infectious Diseases 31, 770-775.

Shank, F.R., Elliot, E.L., Wachsmuth, I.K., Losikoff, M.E., 1996. US position on *Listeria monocytogenes* in foods. Food Control 7, 229-234.

Shen, J.L., Rump, L., Zhang, Y.F., Chen, Y., Wang, X., Meng, J.H., 2013. Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. Food Microbiology 35, 58-64.

Tamburro, M., Sammarco, M.L., Ammendolia, M.G., Fanelli, I., Minelli, F., Ripabelli, G., 2015. Evaluation of transcription levels of inlA, inlB, hly, bsh and prfA genes in *Listeria monocytogenes* strains using quantitative reverse-transcription PCR and ability of invasion into human CaCo-2 cells. Fems Microbiology Letters 362, 7.

Van Stelten, A., Nightingale, K.K., 2008. Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the *Listeria monocytogenes* virulence-associated gene *inlA*. Applied and Environmental Microbiology 74, 7365-7375.

Van Stelten, A., Simpson, J.M., Ward, T.J., Nightingale, K.K., 2010. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in *inlA* are common among *Listeria monocytogenes*

isolates from ready-to-eat foods but not human listeriosis cases. Applied and Environmental Microbiology 76, 2783-2790.

Wemekamp-Kamphuis, H.H., Wouters, J.A., de Leeuw, P., Hain, T., Chakraborty, T., Abee, T., 2004. Identification of sigma factor sigma(B)-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogene*s EGD-e. Applied and Environmental Microbiology 70, 3457-3466.

Strain	Origin	Lineage	Serotype	Source	Truncated *inlA*	*inlA* 3	*inlA* 4
EGDe		II	1/2a			K	K
230/3	ANIMAL	II	1/2a	RTE Iceberg lettuce	Yes	–	–
317	ANIMAL	II	1/2a	Cheese	Yes	–	–
318	ANIMAL	II	1/2a	Brine	Yes	–	–
327	ANIMAL	II	1/2a	Meat processing plant	Yes	–	–
330	ANIMAL	II	1/2a	Chicken carcass	Yes	–	–
332	ANIMAL	II	1/2a	Turkey Frankfurter		R	E
340	ANIMAL	II	1/2a	Poultry processing environment		R	–
346	ANIMAL	II	1/2a	Minced beef	Yes	–	–
349	ANIMAL	II	1/2a	Bovine carcass	Yes	–	–
256	HUMAN	II	1/2a	Blood		–	–
260	HUMAN	II	1/2a	Lumbar fluid		–	–
263	HUMAN	II	1/2a	Blood		–	–
266	HUMAN	II	1/2a	Synovial fluid knee		–	–
272	HUMAN	II	1/2a	Lumbar fluid		–	–
285	HUMAN	II	1/2a	Lumbar fluid	Yes	–	–
288	HUMAN	II	1/2a	Blood		–	–
293	HUMAN	II	1/2a	Lumbar fluid		–	–
295	HUMAN	II	1/2a	Blood		–	–
300	HUMAN	II	1/2a	Lumbar fluid		–	–
319	ANIMAL	II	1/2c	Rabbit		–	–
347	ANIMAL	II	1/2c	Minced beef	Yes	–	–
348	ANIMAL	II	1/2c	Bovine carcass	Yes	–	–
264	HUMAN	II	1/2c	Blood		–	–
267	HUMAN	II	1/2c	Peritoneal fluid	Yes	–	–
270	HUMAN	II	1/2c	Blood		–	–
275	HUMAN	II	1/2c	Blood		–	–
279	HUMAN	II	1/2c	Blood	Yes	–	–
284	HUMAN	II	1/2c	Blood	Yes	–	–
290	HUMAN	II	1/2c	Blood	Yes	–	–
296	HUMAN	II	1/2c	Blood		–	–
299	HUMAN	II	1/2c	Blood	Yes	–	–
302	HUMAN	II	1/2c	Blood		–	–
324	HUMAN	II	1/2c	human-Unknown		–	–
323	ANIMAL	I	4b	Unknown		R	–
326	ANIMAL	I	4b	Smoked meat		R	–
328	ANIMAL	I	4b	Smoked meat		R	–
331	ANIMAL	I	4b	Jalisco cheese		R	–
334	ANIMAL	I	4b	Hotdog		R	–
336	ANIMAL	I	4b	Fish equipment		R	–
339	ANIMAL	I	4b	Broiler carcass		R	–
341	ANIMAL	I	4b	Poultry processing environment		R	–
350	ANIMAL	I	4b	Milk		R	–
253	HUMAN	I	4b	Cervix		R	–
254	HUMAN	I	4b	Blood		R	–
268	HUMAN	I	4b	Joint puncture		R	–
274	HUMAN	I	4b	Bile		R	–
278	HUMAN	I	4b	Blood		R	–
280	HUMAN	I	4b	Biopt		R	–
283	HUMAN	I	4b	Peritoneal fluid		R	–
287	HUMAN	I	4b	Blood		R	–
291	HUMAN	I	4b	Blood		R	–
297	HUMAN	I	4b	Blood		R	–
301	HUMAN	I	4b	Lumbar fluid		R	–

A – indicates an identical amino acid as the reference strain EGDe

			inlA												
Strain	**Origin**	**Serotype**	51	94	118	142	157	187	189	192	326	416	420	426	454
EGDe		1/2a	T	V	N	T	I	S	Q	S	E	A	A	A	T
230/3	ANIMAL	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
317	ANIMAL	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
318	ANIMAL	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
327	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
330	ANIMAL	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
332	ANIMAL	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	–
340	ANIMAL	1/2a	–	L	–	–	L	–	–	–	–	–	–	–	A
346	ANIMAL	1/2a	A	–	–	–	L	–	PMSC	–	–	–	–	–	–
349	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
256	HUMAN	1/2a	–	L	D	–	–	N	–	–	–	–	–	–	–
260	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
263	HUMAN	1/2a	A	L	D	–	–	N	–	–	–	–	–	–	A
266	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
272	HUMAN	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
285	HUMAN	1/2a	A		–	–	L	–	–	–	–	–	–	–	A
288	HUMAN	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
293	HUMAN	1/2a	–	L	D	–	–	N	–	–	–	–	–	–	–
295	HUMAN	1/2a	–	L	D	–	–	N	–	F	–	–	–	–	–
300	HUMAN	1/2a	A	–	–	–	L	–	–	–	–	–	–	–	A
319	ANIMAL	1/2c	A	L	D	–	–	N	–	–	–	–	–	–	A
347	ANIMAL	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
348	ANIMAL	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
264	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
267	HUMAN	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
270	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
275	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
279	HUMAN	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
284	HUMAN	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
290	HUMAN	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
296	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
299	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
302	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
324	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
323	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
326	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
328	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
331	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
334	ANIMAL	4b	–	L	D	–	–	–	–	–	–	E	P	V	A
336	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
339	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
341	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
350	ANIMAL	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
253	HUMAN	4b	–	L	D	–	–	–	–	–	–	E	P	V	A
254	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
268	HUMAN	4b	–	L	D	–	–	–	–	–	–	E	P	V	A
274	HUMAN	4b	–	L	D	–	–	–	–	–	–	E	P	V	A
278	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
280	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
283	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
287	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
291	HUMAN	4b	–	L	D	S	–	–	–	–	–	E	P	V	A
297	HUMAN	4b	–	L	D	–	–	N	–	–	–	E	P	V	A
301	HUMAN	4b	–	L	D	–	–	–	–	–	–	E	P	V	A

			inlA										
Strain	**Origin**	**Serotype**	460	474	476	492	500	530	533	539	544	546	558
EGDe		1/2a	W	S	P	Q	V	H	V	Q	K	N	N
230/3	ANIMAL	1/2a	–	N	S	PMSC	A	Y	I	K	–	–	D
317	ANIMAL	1/2a	–	N	S	PMSC	A	Y	I	K	–	–	D
318	ANIMAL	1/2a	–	N	S	PMSC	A	Y	I	K	–	–	D
327	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	–
330	ANIMAL	1/2a	–	N	S	PMSC	A	Y	I	K	–	–	D
332	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	D
340	ANIMAL	1/2a	–	–	–	–	A	–	–	–	–	–	–
346	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	–
349	ANIMAL	1/2a	PMSC	–	–	–	–	–	–	–	–	–	–
256	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–
260	HUMAN	1/2a	–	–	–	–	A	–	–	–	–	–	–
263	HUMAN	1/2a	–	–	–	–	A	–	–	–	–	–	–
266	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–
272	HUMAN	1/2a	–	N	S	–	A	Y	I	K	–	–	D
285	HUMAN	1/2a	–	N	S	PMSC	A	Y	I	K	–	–	D
288	HUMAN	1/2a	–	N	S	–	A	–	–	–	–	–	–
293	HUMAN	1/2a	–	–	–	–	A	–	–	–	–	–	–
295	HUMAN	1/2a	–	–	–	–	A	–	–	–	–	–	–
300	HUMAN	1/2a	–	N	S	–	A	Y	I	K	–	–	D
319	ANIMAL	1/2c	–	–	–	–	A	–	–	–	–	–	–
347	ANIMAL	1/2c	–	–	–	–	–	–	–	–	–	–	–
348	ANIMAL	1/2c	–	–	–	–	–	–	–	PMSC	–	–	–
264	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
267	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
270	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
275	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
279	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
284	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
290	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
296	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	1bp deleted	[redacted]
299	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
302	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	1bp deleted	[redacted]
324	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–
323	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
326	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
328	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
331	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
334	ANIMAL	4b	–	N	S	–	A	Y	I	–	N	–	D
336	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
339	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
341	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
350	ANIMAL	4b	–	N	S	–	A	Y	–	–	–	–	D
253	HUMAN	4b	–	N	S	–	A	Y	I	–	N	–	D
254	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
268	HUMAN	4b	–	N	S	–	A	Y	I	–	N	–	D
274	HUMAN	4b	–	N	S	–	A	Y	I	–	N	–	D
278	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
280	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
283	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
287	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
291	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
297	HUMAN	4b	–	N	S	–	A	Y	–	–	–	–	D
301	HUMAN	4b	–	N	S	–	A	Y	I	–	N	–	D

			inlA												
Strain	**Origin**	**Serotype**	572	573	594	644	648	652	664	671	685	700	725	735	738
EGDe		1/2a	F	D	A	V	S	A	A	T	W	Y	G	P	D
230/3	ANIMAL	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
317	ANIMAL	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
318	ANIMAL	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
327	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	PMSC	–	–	–
330	ANIMAL	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
332	ANIMAL	1/2a	–	E	P	–	–	–	–	–	–	–	A	–	–
340	ANIMAL	1/2a	–	E	P	I	T	T	T	–	–	–	–	–	–
346	ANIMAL	1/2a	–	E	P	–	–	–	–	–	–	–	–	–	–
349	ANIMAL	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
256	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
260	HUMAN	1/2a	–	E	P	I	T	T	–	–	–	–	–	A	–
263	HUMAN	1/2a	–	E	P	–	–	–	–	–	–	–	–	–	–
266	HUMAN	1/2a	–	–	–	–	–	–	–	–	–	–	–	–	–
272	HUMAN	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
285	HUMAN	1/2a	L	–	–	–	T	–	T	–	–	–	–	–	–
288	HUMAN	1/2a	–	E	P	I	T	T	–	–	–	–	–	–	–
293	HUMAN	1/2a	–	–	P	–	T	–	T	–	–	–	–	–	–
295	HUMAN	1/2a	–	–	P	–	T	–	T	–	–	–	–	–	–
300	HUMAN	1/2a	L	–	–	–	T	–	–	–	–	–	–	–	–
319	ANIMAL	1/2c	–	E	P	–	–	–	–	–	–	–	–	–	–
347	ANIMAL	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
348	ANIMAL	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
264	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
267	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
270	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
275	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
279	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
284	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
290	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
296	HUMAN	1/2c													
299	HUMAN	1/2c	–	–	–	–	–	–	–	–	PMSC	–	–	–	–
302	HUMAN	1/2c													
324	HUMAN	1/2c	–	–	–	–	–	–	–	–	–	–	–	–	–
323	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
326	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
328	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
331	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
334	ANIMAL	4b	L	–	–	–	T	–	T	A	–	–	–	–	DEL
336	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
339	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
341	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
350	ANIMAL	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
253	HUMAN	4b	L	–	–	–	T	–	T	A	–	–	–	–	DEL
254	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
268	HUMAN	4b	L	–	–	–	T	–	T	A	–	–	–	–	DEL
274	HUMAN	4b	L	–	–	–	T	–	T	A	–	–	–	–	DEL
278	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
280	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
283	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
287	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
291	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
297	HUMAN	4b	–	–	–	–	T	–	T	–	–	–	–	–	N
301	HUMAN	4b	L	–	–	–	T	–	T	A	–	–	–	–	DEL

			inlA					
Strain	**Origin**	**Serotype**	739	740	764	774	781	790
EGDe		1/2a	T	S	E	D	L	M
230/3	ANIMAL	1/2a	–	–	–	–	–	–
317	ANIMAL	1/2a	–	–	–	–	–	–
318	ANIMAL	1/2a	–	–	–	–	–	–
327	ANIMAL	1/2a	–	–	–	–	–	–
330	ANIMAL	1/2a	–	–	–	–	–	–
332	ANIMAL	1/2a	–	–	D	–	–	–
340	ANIMAL	1/2a	–	–	D	–	–	–
346	ANIMAL	1/2a	–	–	–	–	–	–
349	ANIMAL	1/2a	–	–	–	–	–	–
256	HUMAN	1/2a	–	–	–	–	–	–
260	HUMAN	1/2a	–	–	–	–	–	–
263	HUMAN	1/2a	–	–	–	–	–	–
266	HUMAN	1/2a	–	–	–	–	–	–
272	HUMAN	1/2a	–	–	–	–	–	–
285	HUMAN	1/2a	–	–	–	–	–	–
288	HUMAN	1/2a	–	–	D	–	–	–
293	HUMAN	1/2a	–	–	–	–	–	–
295	HUMAN	1/2a	–	–	–	–	–	–
300	HUMAN	1/2a	–	–	D	–	–	–
319	ANIMAL	1/2c	–	–	–	–	–	–
347	ANIMAL	1/2c	–	–	–	–	–	–
348	ANIMAL	1/2c	–	–	–	–	–	–
264	HUMAN	1/2c	–	–	–	–	–	–
267	HUMAN	1/2c	–	–	–	–	–	–
270	HUMAN	1/2c	–	–	–	–	–	–
275	HUMAN	1/2c	–	–	–	–	–	–
279	HUMAN	1/2c	–	–	–	–	–	–
284	HUMAN	1/2c	–	–	–	–	–	–
290	HUMAN	1/2c	–	–	–	–	–	–
296	HUMAN	1/2c						
299	HUMAN	1/2c	–	–	–	–	–	–
302	HUMAN	1/2c						
324	HUMAN	1/2c	–	–	–	–	–	–
323	ANIMAL	4b	–	–	–	–	I	V
326	ANIMAL	4b	–	–	–	–	I	V
328	ANIMAL	4b	–	–	–	–	I	V
331	ANIMAL	4b	–	–	–	–	I	V
334	ANIMAL	4b	DEL	DEL	–	Y	I	V
336	ANIMAL	4b	–	–	–	–	I	V
339	ANIMAL	4b	–	–	–	–	I	V
341	ANIMAL	4b	–	–	–	–	I	V
350	ANIMAL	4b	–	–	–	–	I	V
253	HUMAN	4b	DEL	DEL	–	Y	I	V
254	HUMAN	4b	–	–	–	–	I	V
268	HUMAN	4b	DEL	DEL	–	Y	I	V
274	HUMAN	4b	DEL	DEL	–	Y	I	V
278	HUMAN	4b	–	–	–	–	I	V
280	HUMAN	4b	–	–	–	–	I	V
283	HUMAN	4b	–	–	–	–	I	V
287	HUMAN	4b	–	–	–	–	I	V
291	HUMAN	4b	–	–	–	–	I	V
297	HUMAN	4b	–	–	–	–	I	V
301	HUMAN	4b	DEL	DEL	–	Y	I	V

			prfA				*sigB*			
Strain	**Origin**	**Serotype**	145	174	184	197	1	27	189	216
EGDe		1/2a	G	L	S	K	M	D	G	Y
230/3	ANIMAL	1/2a	–	–	–	–	I	–	–	–
317	ANIMAL	1/2a	–	–	–	–	–	–	–	–
318	ANIMAL	1/2a	–	–	–	–	–	–	–	–
327	ANIMAL	1/2a	–	–	–	–	–	–	–	–
330	ANIMAL	1/2a	–	–	–	–	–	–	–	–
332	ANIMAL	1/2a	–	–	–	–	–	–	–	–
340	ANIMAL	1/2a	–	–	–	–	–	–	–	–
346	ANIMAL	1/2a	–	7bp inserted			–	–	–	–
349	ANIMAL	1/2a	–	–	–	–	–	–	–	–
256	HUMAN	1/2a	–	–	–	N	–	–	–	–
260	HUMAN	1/2a	–	–	–	–	–	–	–	–
263	HUMAN	1/2a	–	–	–	–	–	–	–	–
266	HUMAN	1/2a	–	–	–	–	–	–	–	–
272	HUMAN	1/2a	–	–	–	–	–	–	–	–
285	HUMAN	1/2a	–	–	–	–	–	–	–	–
288	HUMAN	1/2a	–	–	–	–	–	N	–	–
293	HUMAN	1/2a	–	–	–	–	–	–	–	–
295	HUMAN	1/2a	–	–	–	–	–	–	–	–
300	HUMAN	1/2a	–	–	–	–	–	–	–	–
319	ANIMAL	1/2c	A	–	–	–	–	–	PMSC	–
347	ANIMAL	1/2c	–	–	–	–	–	–	–	–
348	ANIMAL	1/2c	–	–	–	–	–	–	–	–
264	HUMAN	1/2c	–	–	–	–	–	–	–	–
267	HUMAN	1/2c	–	–	–	–	–	–	–	–
270	HUMAN	1/2c	–	–	–	–	–	–	–	–
275	HUMAN	1/2c	–	–	–	–	–	–	–	–
279	HUMAN	1/2c	–	–	–	–	–	–	–	–
284	HUMAN	1/2c	–	–	–	–	–	–	–	–
290	HUMAN	1/2c	–	–	–	–	–	–	–	–
296	HUMAN	1/2c	–	–	–	–	–	–	–	–
299	HUMAN	1/2c	–	–	–	–	–	–	–	–
302	HUMAN	1/2c	–	–	–	–	–	–	–	–
324	HUMAN	1/2c	S	–	P	–	–	–	–	–
323	ANIMAL	4b	–	–	–	–	–	–	–	F
326	ANIMAL	4b	–	–	–	–	–	–	–	F
328	ANIMAL	4b	–	–	–	–	–	–	–	F
331	ANIMAL	4b	–	–	–	–	–	–	–	F
334	ANIMAL	4b	–	–	–	–	–	–	–	F
336	ANIMAL	4b	–	–	–	–	–	–	–	F
339	ANIMAL	4b	–	–	–	–	–	–	–	F
341	ANIMAL	4b	–	–	–	–	–	–	–	F
350	ANIMAL	4b	–	–	–	–	–	–	–	F
253	HUMAN	4b	–	–	–	–	–	–	–	F
254	HUMAN	4b	–	–	–	–	–	–	–	F
268	HUMAN	4b	–	–	–	–	–	–	–	F
274	HUMAN	4b	–	–	–	–	–	–	–	F
278	HUMAN	4b	–	–	–	–	–	–	–	F
280	HUMAN	4b	–	–	–	–	–	–	–	F
283	HUMAN	4b	–	–	–	–	–	–	–	F
287	HUMAN	4b	–	–	–	–	–	–	–	F
291	HUMAN	4b	–	–	–	–	–	–	–	F
297	HUMAN	4b	–	–	–	–	–	–	–	F
301	HUMAN	4b	–	–	–	–	–	–	–	F

A – indicates an identical amino acid as the reference strain EGDe

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

La provincia de Lleida es la principal productora de pera de España y por ello, la valorización de la producción mediante la transformación es de gran interés para el sector primario. El procesado mínimo o de IV gama podría ser una alternativa a la elaboración de zumos, mermeladas, compotas, etc. Actualmente, los consumidores demandan estos productos listos para el consumo porque son nutricionalmente equilibrados, con bajo aporte calórico y alto aporte de compuestos con capacidad antioxidante. La pera mínimamente procesada puede cubrir esta necesidad, bien como monoproducto o como un ingrediente en una ensalada de frutas (macedonia). El procesado mínimo de fruta consta de unas etapas básicas. Sin embargo, existen numerosos factores que pueden afectar a la calidad del producto final. Por ello, es necesaria una etapa previa de desarrollo del producto, para establecer las condiciones óptimas de materia prima, proceso y conservación. En esta tesis, se ha planteado en primer lugar, el desarrollo de un producto a base de pera (*Capítulo 1*).

La adecuada selección de la variedad de pera destaca como uno de los factores de mayor importancia para la posterior evolución del producto procesado una vez cortado. La idoneidad de las variedades de pera para el procesado mínimo se ha estudiado en variedades como "Barlett", "Bosc", "Conference", "d'Anjou", "Passa-Crassana", "Red Anjou" y "Williams" de las cuales "Barlett" y "Conference" han presentado buena aptitud (Arias *et al.*, 2008; Gorny *et al.*, 2000). Para la variedad "Ercolini", otra de las que se produce en mayor cantidad en nuestra región (6.960 toneladas en 2014) (DAAM, 2015), no existe información sobre su aptitud al procesado mínimo. En el caso de "Flor de Invierno", esta variedad tampoco se ha evaluado frente a otras. Sin embargo, sí existen estudios en "Flor de Invierno" mínimamente procesada donde se evaluó la conservación a distintas condiciones de atmosfera modificada, utilización de recubrimientos comestibles, estado de madurez, etc. (Oms-Oliu *et al.*, 2006; Oms-Oliu *et al.*, 2009; Oms-Oliu *et al.*, 2008a; Oms-Oliu *et al.*, 2008b; Oms-Oliu *et al.*, 2008c). El primer objetivo de esta tesis fue determinar, entre las variedades de pera "Conference", "Ercolini", "Flor de Invierno" y "Passa-Crassana", cual presentaba una mejor aptitud al procesado mínimo. Después del estudio realizado en la primera parte del *Capítulo 1*, se concluyó que cuando se aplicó un tratamiento antioxidante comercial (NatureSeal® AS1, 5%), la variedad de pera "Conference" presentó los mejores atributos después de 7 días de conservación a 5 °C frente el resto de variedades.

Como ya se ha comentado, el pardeamiento enzimático es una de las alteraciones fisiológicas que tiene un mayor impacto en la calidad visual de la fruta cortada. El incremento del índice de pardeamiento es una herramienta útil para determinar el comportamiento de distintas variedades de un mismo fruto cortado. El contenido de compuestos fenólicos de las cuatro variedades de pera evaluadas en esta tesis ("Conference", "Ercolini", "Flor de Invierno" y "Passa-Crassana") junto con otras dos variedades ("Abate Fétel" y "Blanquilla") había sido precisamente cuantificado por Falguera *et al.* (2014) en el zumo fresco de las seis variedades. Los resultados obtenidos en esta tesis (*Capítulo 1*) indicaron que la pera cortada de las variedades "Flor de Invierno" y "Passa-Crassana" tratadas con NatureSeal® y conservadas 7 días a 5 °C, presentaron los valores más altos de índice de pardeamiento. Según resultados observados en zumos sin tratar elaborados a partir de la pera fresca (Falguera *et al.*, 2014), las variedades "Flor de Invierno" (350 ± 20 eq. pirogalol g L^{-1}) y "Passa-Crassana" (405 ± 3 eq. pirogalol g L^{-1}) presentaron un mayor contenido de compuestos fenólicos que las variedades "Conference" (169 ± 1 eq. pirogalol g L^{-1}) y "Ercolini" (200 ± 5 eq. pirogalol g L^{-1}) que presentaron un menor índice de pardeamiento en esta tesis. No todos los autores han observado que exista una correlación estrecha entre la cantidad de compuestos fenólicos y el índice de pardeamiento, mientras sí que se ha observado la relación entre la actividad de la enzima polifenoloxidasa (PPO) y el índice de pardeamiento (Soliva-Fortuny *et al.*, 2002).

Para reducir la incidencia del pardeamiento enzimático de la pera cortada, distintos autores han evaluado diferentes tratamientos antioxidantes, algunos de los cuales se han mostrado más eficaces para la inhibición del pardeamiento enzimático que otros (Arias *et al.*, 2008; Dong *et al.*, 2000; Gorny *et al.*, 2002; Oms-Oliu *et al.*, 2006; Sapers y Miller, 1998; Soliva-Fortuny *et al.*, 2004). Sin embargo, no todos los compuestos evaluados en pera se encuentran en la actualidad aprobados para ser empleados en fruta cortada según el Reglamento (CE) nº 1333/2008, como es el caso del 4-hexylresorcinol, la cisteína o el glutatión reducido. Por ejemplo, Arias *et al.* (2008) observaron que el tratamiento antioxidante compuesto por ácido ascórbico al 2%, 4-hexylresorcinol al 0,01% y $CaCl_2$ al 1% permitió que pera "Conference" cortada mantuviera sus características iniciales después de 9 días de conservación a 4 °C. Sin embargo, este tratamiento que es eficaz frente al pardeamiento, no podría emplearse en Europa a nivel industrial debido a que la aplicación del 4-hexylresorcinol no está permitida. Concretamente de los tres tratamientos antioxidantes estudiados en el *Capítulo 1*, el tratamiento CaAs (ascorbato de calcio al 2% y cloruro de calcio al 1%) y NaAs (ascorbato de sodio al 2% y cloruro de calcio al 1%), todos ellos

compuestos por productos aprobados, fueron eficaces para reducir el pardeamiento superficial y la pérdida de firmeza de la pera "Conference" mínimamente procesada, después de 14 días de conservación a 5 °C. Si bien NatureSeal® AS1 mostró los mejores resultados, se pretendía encontrar una alternativa a este producto comercial, que si bien está aceptado en el Reglamento (CE) nº 1333/2008, su composición no viene especificada en la etiqueta del producto. Se optó por seleccionar el tratamiento CaAs (ascorbato de calcio al 2% y cloruro de calcio al 1%) para la elaboración de pera "Conference" mínimamente procesada. Una vez elegida la variedad de pera y el tratamiento que va a recibir para mantener sus propiedades organolépticas, se determinó la evolución de la calidad del producto una vez envasado y conservado en atmosfera modificada pasiva.

Vida útil de la pera mínimamente procesada

Una vez envasada la fruta mínimamente procesada, su calidad ha de mantenerse durante la vida útil. Para poder determinar que la variedad de pera y tratamiento antioxidante elegidos son los adecuados es preciso evaluar la calidad global del producto mínimamente procesado bajo condiciones comerciales simuladas (4 °C). En el *Capítulo 1* se observó que al aplicar el tratamiento antioxidante seleccionado (ascorbato de calcio al 2% y cloruro de calcio al 1%) a la pera "Conference" mínimamente procesada, no se observaron cambios en los parámetros fisicoquímicos (sólidos solubles y acidez titulable). Sin embargo, los parámetros nutricionales sí que se vieron modificados después de la aplicación del tratamiento, presentando un contenido de fenoles totales (2,4 veces superior al valor inicial), ácido ascórbico (43,9 veces superior al valor inicial) y actividad antioxidante (5,3 veces superior al valor inicial) aumentados debido a la aplicación del tratamiento antioxidante-estabilizante de la textura. Sin embargo, el contenido de estos compuestos de interés nutricional en la pera mínimamente procesada se redujo durante la conservación. Del mismo modo, Oms-Oliu *et al.* (2008a) observaron que el contenido de ácido clorogénico, el compuesto fenólico mayoritario en la pera, se redujo significativamente con la conservación en la pera "Flor de Invierno" mínimamente procesada a 4 °C. Cuando la muestra se conservó en una atmosfera más rica en oxígeno, la oxidación de este compuesto fenólico fue más rápida. Esta misma reducción en el contenido de vitamina C y el contenido total de fenoles también se observó en pera "Flor de Invierno" cuando se determinó el efecto de distintos recubrimientos (Oms-Oliu *et al.*, 2008c).

Para la pera "Conference" mínimamente procesada bajo las condiciones estudiadas (tratamiento antioxidante-estabilizante compuesto por ascorbato de calcio al 2% y cloruro de calcio al 1% y envasado en atmosfera modificada pasiva) se determinó una vida útil de 8 días a 4 °C. Tras este tiempo, el contenido de ácido ascórbico y capacidad antioxidante descendieron hasta niveles de la pera sin tratar (día 0). Sin embargo, el contenido de fenoles totales, aunque se redujo en la pera mínimamente procesada conservada 8 días, continuó siendo significativamente superior al observado en la pera sin tratar (día 0).

Calidad microbiológica de un producto de pera mínimamente procesado

En segundo lugar, se estudió la calidad microbiológica del producto de pera obtenido en el *Capítulo 1*, este presentó una estabilidad microbiológica similar a la de otros productos de pera mínimamente procesada (Oms-Oliu *et al.*, 2009; Oms-Oliu *et al.*, 2008c; Sánchez *et al.*, 2015; Soliva-Fortuny y Martín-Belloso, 2003). Concretamente, las poblaciones de mohos y levaduras, y bacterias ácido lácticas, se mantuvieron inferiores a 2 unidades logarítmicas y no incrementaron durante la conservación. Sin embargo, sí que se observó un incremento en la población de bacterias psicrótrofas, siendo de 2,70 log ufc g^{-1} al inicio, aumentó hasta 4,40 log ufc g^{-1} después de 8 días de conservación a 4 °C. Pocos estudios se han centrado en el recuento de las bacterias psicrótrofas en pera cortada, sin embargo, cuando Sánchez *et al.* (2015) determinaron la población de este grupo de bacterias en pera "Rocha" mínimamente procesada observaron niveles mucho más elevados (6,60 log ufc g^{-1}) después del procesado. Con la aplicación de un tratamiento antimicrobiano compuesto por quitosano (0,70 g L^{-1}) en pera "Rocha" procesada, consiguieron que la población de bacterias psicrótrofas se mantuviera alrededor de los niveles iniciales, pasados 10 días a 4 °C. En la pera "Conference" mínimamente procesada y tratada con ascorbato de calcio al 2% y cloruro de calcio al 1%, los niveles de bacterias psicrótrofas incrementaron 1,7 unidades logarítmicas después de 8 días a 4 °C. Destacar que si el procesado de la fruta se realiza de una forma adecuada, con un eficaz tratamiento desinfectante y unas buenas prácticas de elaboración, podemos mantener la calidad microbiológica del producto dentro de unos límites aconsejables sin la necesidad de la aplicación de tratamientos antimicrobianos.

Los productos vegetales mínimamente procesados deben conservarse a una temperatura inferior a 5 °C. Por este motivo, la mayoría de los estudios que evalúan la calidad de un producto de pera mínimamente procesada suelen llevarse a cabo a temperaturas de 4 ± 1 °C. Son pocos los estudios que han evaluado el efecto que puede tener la rotura de la cadena de frío en la calidad fisicoquímica, nutricional, microbiológica y sensorial en fruta mínimamente procesada. La Guía de Buenas Prácticas de Producción de frutas y hortalizas preparadas (FEPEX, 2012) recomienda que se determine la vida útil del producto entre 1-4 °C. Sin embargo, recomiendan que la fecha de caducidad se determine entre 1-4 °C durante el número de días correspondientes al entorno de 1/3 de la vida útil y luego entre 6-8 °C el resto de los días. Una temperatura entre 6-8 °C podría asemejarse más a la temperatura real en los lineales de los supermercados (Marklinder *et al*., 2004).

Al evaluar la calidad microbiológica del producto de pera mínimamente procesada (*Capítulo 1*), no se observaron cambios destacables entre la pera cortada conservada 3 días a 4 °C y 5 días a 8 °C, frente a la que se había conservado los 8 días bajo condiciones comerciales simuladas (4 °C).

Comportamiento de microorganismos patógenos en pera mínimamente procesada

En el caso de los alimentos listos para el consumo, el Reglamento (CE) nº 2075/2005 determina unos requisitos microbiológicos distintos dependiendo si el producto en cuestión puede favorecer el desarrollo de *L. monocytogenes.* Concretamente, si el pH del producto listo para el consumo es inferior o igual a 4,4 se considera que el producto no favorece el desarrollo del patógeno. Debido a que la pera tiene un pH superior a 4,4, se determinó la capacidad de crecimiento de *L. monocytogenes* junto con el de *Salmonella enterica* subsp. Enteritidis en las condiciones optimizadas en el *Capítulo 1*, tratamiento antioxidante (ascorbato de calcio al 2% y cloruro de calcio al 1%) y atmosfera modificada pasiva. El comportamiento de los patógenos se evaluó cuando el producto se conservó bajo condiciones de temperatura constante (4 °C) y en condiciones simuladas de rotura de la cadena de frío (3 días a 4 °C más 5 días a 8 °C). Los resultados obtenidos (*Capítulo 2*) han demostrado que *L. monocytogenes* es capaz de desarrollarse en la pera mínimamente procesada en las condiciones estudiadas. La población de *L. monocytogenes* en pera incrementó 1,6 unidades logarítmicas

después de 8 días a 4 °C. Al incrementar la temperatura de conservación de la pera mínimamente procesada hasta 8 °C durante los últimos 5 días, se favoreció el crecimiento de la bacteria, observándose un aumento de 2,2 unidades logarítmicas después de 8 días de conservación. Al contrario que *L. monocytogenes* bajo las dos condiciones de conservación estudiadas, la población de *S. enterica* se mantuvo constante hasta los 3 días a 4 °C, pasado este tiempo se observó una reducción de 0,5 unidades logarítmicas. Este comportamiento es debido a que *L. monocytogenes* es un microorganismo psicrófilo facultativo que puede crecer a temperaturas de refrigeración mientras que *Salmonella* es un microorganismo mesófilo. Otros autores ya observaron que un abuso de la temperatura de conservación tuvo un efecto directo en la capacidad de crecimiento de *L. monocytogenes* y *S. enterica* en melón "Cantaloupe" (Huang *et al.*, 2015). Estudios previos en manzana "Golden Delicious" (Alegre *et al.*, 2010a) demostraron que el ácido ascórbico (2%) y el NatureSeal® AS1 (6%) no tuvieron un efecto inhibitorio en la población de *L. innocua*. Sin embargo, otros antioxidantes como el N-acetilo-L-cisteína (1%), glutatión (1%), lactato cálcico (1%) y ácido málico (2,5%), sí redujeron la población de *L. monocytogenes* en pera "Flor de Invierno" mínimamente procesada presentando un efecto antioxidante y antimicrobiano (Raybaudi-Massilia *et al.*, 2009).

Debido a que el producto de pera mínimamente procesado evaluado en este trabajo puede ser sustrato de crecimiento de *L. monocytogenes*, el Reglamento (CE) nº 2075/2005 para este producto determinaría que después de analizar cinco muestras de producto en el momento de dejar el control del explotador de la empresa, todas deberían presentar ausencia de la bacteria en 25 g. Sin embargo, una vez el producto estuviera comercializado, al analizar cinco muestras todas deberían presentar niveles de *L. monocytogenes* inferiores a 100 ufc g^{-1}. El hecho que *L. monocytogenes* fuera capaz de crecer en pera mínimamente procesada en condiciones de refrigeración junto al actual incremento de la demanda de productos vegetales listos para el consumo, y el hecho que esta bacteria presente una de las mayores tasas de mortalidad en humanos (15%) (EFSA, 2015), motivaron que en la presente tesis se estudiara el comportamiento de esta bacteria en una matriz de fruta mínimamente procesada bajo distintos escenarios.

El estado de madurez de la fruta antes del procesado afecta tanto a la calidad organoléptica como a la vida útil de la fruta mínimamente procesada (Gorny *et al.*, 1998). Este parámetro suele tener mayor importancia en la calidad final de los frutos climatéricos, y algunos autores ya lo han descrito (Arias *et al.*, 2008; Chen *et al.*, 2003; Dong *et al.*, 2000; Gorny *et al.*, 2000; Soliva-Fortuny *et al.*, 2004). Estos

trabajos demostraron que un fruto procesado con un estado de madurez intermedia presenta una vida útil y calidad organoléptica mayor que un fruto verde o sobremaduro (Gorny *et al.*, 1999). Es preciso destacar que este parámetro también podría afectar al potencial de crecimiento de los microorganismos patógenos. En esta tesis se evaluó (*Capítulo 3*) el efecto del estado de madurez de la pera antes del procesado junto a la posterior temperatura de conservación de la pera mínimamente procesada en la capacidad de crecimiento de *L. monocytogenes* 230/3 (serotipo 1/2a). Cuando el producto mínimamente procesado se conservó a temperaturas entre 10 °C y 20 °C, el estado de madurez de la pera antes del procesado ocasionó diferencias en la capacidad de crecimiento de *L. monocytogenes* 230/3, observándose mayores incrementos de población del patógeno en la pera procesada en un estado maduro (valores de penetromía, 31-42 N) o sobremaduro (< 31 N). Sin embargo, cuando el producto se conservó a la temperatura óptima de almacenamiento (5 °C) no se observaron diferencias significativas entre estados de madurez. Por otro lado, el crecimiento de *L. monocytogenes* 230/3, independientemente del estado de madurez de pera evaluado, incrementó con el aumento de la temperatura de almacenamiento de la pera mínimamente procesada. En pera mínimamente procesada de la variedad "Flor de Invierno", Oms-Oliu *et al.* (2009) observaron que el estado de madurez de la pera antes del procesado y las condiciones de almacenamiento tuvieron un efecto en la calidad microbiológica del producto (microorganismos aerobios mesófilos, mohos y levaduras). La pera que se había procesado en un estado más avanzado de madurez presentó un mayor crecimiento de microorganismos aerobios mesófilos.

Además de la temperatura y el estado de madurez la fruta mínimamente procesada, el tipo de alimento en el que se encuentra *L. monocytogenes* también tiene un impacto directo en el desarrollo del patógeno. Se ha observado que los factores intrínsecos de la fruta como el pH de la pulpa, el tipo de ácido mayoritario, el contenido de azúcares o la disponibilidad de nutrientes, y otros factores extrínsecos como la temperatura de conservación también tienen un efecto en el crecimiento de microorganismos patógenos (Abadias *et al.*, 2012; Alegre *et al.*, 2010a; Alegre *et al.*, 2010b; Penteado y Leitao, 2004). En el *Capítulo 4* se evaluó la capacidad de crecimiento de *L. monocytogenes* 230/3 en pera "Conference" y melón "Piel de Sapo" mínimamente procesados a la vez que en medio de cultivo rico ("Triptona Soya Broth" (TSB) suplementado con extracto de levadura, TSBYE), a distintas temperaturas de conservación (1, 5, 10 y 20 °C). En todas las temperaturas de conservación estudiadas, el patógeno presentó un crecimiento más acelerado en TSBYE que en las frutas mínimamente procesadas.

Sin embargo, la población final de *L. monocytogenes* 230/3 después de crecer 48 h a 20 °C, fue muy similar en el melón mínimamente procesado y el medio de cultivo TSBYE, siendo mucho inferior la población en la pera mínimamente procesada. A la temperatura óptima de almacenamiento, *L. monocytogenes* 230/3 presentó una población de 7, 50 log ufc g^{-1} en el melón mínimamente procesado "Piel de Sapo" después de 9 días a 5 °C, mientras que la misma cepa del patógeno en pera "Conference" mínimamente procesada presentó un población de 6,30 log ufc g^{-1}. Como ya se ha destacado en los *Capítulos 3*, *4* y *5*, estas diferencias son debidas a los factores intrínsecos de la matriz en la que se encuentra la bacteria y los factores extrínsecos que la acompañan. Se observó que cuando la pera "Conference" se inoculó con *L. monocytogenes* 230/3 y se conservó a 1 °C (*Capítulo 4*), esta presentó un incremento de población de 0,2 unidades logarítmicas después de 9 días de conservación, mientras que la misma cepa en el melón mínimamente procesado a 1 °C incrementó 1.0 unidades logarítmicas tras 9 días de conservación. Así el uso de temperaturas extremas de conservación refrigerada (1 °C) tampoco redujo el riesgo de crecimiento de *L. monocytogenes* en pera y melón mínimamente procesados. De nuevo la importancia de evitar la contaminación del producto en cualquiera de las etapas de su elaboración.

Supervivencia de *L. monocytogenes* a la barrera gastrointestinal

El tercer objetivo de esta tesis planteó determinar qué papel podría tener la matriz de fruta mínimamente procesada, en la que se encuentra *L. monocytogenes*, y la temperatura de conservación en la posterior capacidad de la bacteria en superar la barrera gastrointestinal.

Para poder eliminar factores que podrían dificultar el estudio del efecto de la matriz, estos ensayos se realizaron con fruta mínimamente procesada sin ningún tratamiento antioxidante y bajo una conservación en aire. Con el objetivo de evaluar el efecto de la matriz en el crecimiento de *L. monocytogenes* y su posterior capacidad de supervivencia a la simulación gastrointestinal, se trabajó con pera y melón mínimamente procesados que presentaron un pH medio de 5,1 (4,4-5,9) y 5,9 (5,2-6,7), respectivamente.

En el día que se realizó la inoculación, *L. monocytogenes* presentó la misma capacidad de supervivencia a la fase gástrica independientemente de la fruta en la que se había inoculado. Sin embargo, cabe destacar que cuando el patógeno se inoculó en melón mínimamente procesado sobrevivió y multiplicó durante la fase

intestinal, mientras que la bacteria que había sido inoculada en pera durante esta etapa solo sobrevivió. En la pera mínimamente procesada inoculada y conservada a 20 °C (sin estrés por frío) se observó que la capacidad de *L. monocytogenes* de sobrevivir a la simulación gastrointestinal después de 2 días de conservación fue la misma que a tiempo inicial, mientras que la supervivencia de la misma bacteria en melón mínimamente procesado se redujo con el tiempo de conservación. Este hecho podría atribuirse a la capacidad que presenta *L. monocytogenes* de tolerar un entorno ácido (pH 3,5, HCl) cuando previamente se ha expuesto a un estrés ácido leve, como en este caso sería el pH de la pera mínimamente procesada. Cuando *L. monocytogenes* se expuso además a un estrés por frío (1 °C), se observaron las menores reducciones de población después de la exposición a la simulación gastrointestinal en ambas frutas. Los mecanismos que permiten a *L. monocytogenes* sobrevivir a un entorno que presenta un estrés de temperatura (Mastronicolis *et al.*, 2005), pudieron ocasionar que la penetración del ácido clorhídrico al citoplasma de la célula se viera dificultada, reduciendo así la capacidad bactericida del ácido.

En estudios "in vitro", Shen *et al.* (2014) y Ivy *et al.* (2012) observaron que durante la exposición a un estrés ácido subletal (pH 5,0, ácido láctico) a una temperatura de 4 °C, *L. monocytogenes* no fue capaz de desarrollar una adaptación al ácido, mientras que bajo las mismas condiciones pero a 22 y 37 °C sí presentó tolerancia a un estrés ácido letal (pH 3,5). Sin embargo, cuando a 4 °C se dañó levemente la membrana de la bacteria mediante sonicación y perlas de cristal, reduciendo la protección que le confería la membrana modificada por el estrés por frío, se observó que la adaptación al estrés ácido se indujo levemente (Shen *et al.*, 2014).

El tiempo durante el cual se ve expuesta la bacteria en el alimento también puede ocasionar diferencias en su posterior capacidad de supervivencia al tracto gastrointestinal simulado. Al incrementar los días de conservación a 5 y 10 °C de la pera y el melón mínimamente procesados, se observaron mayores reducciones de la población de *L. monocytogenes* después de la exposición a la simulación gastrointestinal, excepto en el último día evaluado para cada temperatura. Al incrementar el tiempo, la fase de crecimiento de la bacteria también cambia presentando un mayor número de células en la fase estacionaria. Se ha demostrado que cuando la bacteria ha crecido en un medio de cultivo en las condiciones óptimas de temperatura, las células de *L. monocytogenes* en la fase estacionaria presentan una mayor tolerancia al ácido que las células en fase exponencial o logarítmica (Davis *et al.*, 1996; Ferreira *et al.*, 2003). Zhang *et al.* (2014) observaron que la capacidad de supervivencia al ácido de *L. monocytogenes*

presentó mayores diferencias entre cepas cuando estas crecieron hasta la fase estacionaria que cuando lo hicieron hasta la fase exponencial antes de ser expuestas al estrés. También se ha observado que *L. monocytogenes* crecida a 5 y 10 °C hasta la fase estacionaria, presentó una menor tolerancia al ácido que cuando la bacteria creció bajo condiciones de temperatura óptima (Ivy *et al.*, 2012; Samelis *et al.*, 2003; Zhang *et al.*, 2014).

En la presente tesis, cuando *L. monocytogenes* se expuso a la simulación gastrointestinal utilizada (HCl, pH 3,5) presentó una elevada capacidad de supervivencia independientemente en la fruta en la que se encontrara (*Capítulos 4* y *5*). En estudios previos (resultados no mostrados) se observó que cuando *L. monocytogenes* 230/3 se mantuvo 6 días a 1 °C en pera, melón mínimamente procesados y medio TSBYE, la bacteria en las frutas presentó una supervivencia después de la exposición a la simulación gástrica de 95% y 100%, respectivamente, mientras que en medio TSBYE sobrevivió el 69% de la población. Cabe destacar que en el día de la inoculación, *L. monocytogenes* 230/3 presentó una supervivencia de 96-99% independientemente de la matriz de fruta evaluada. De forma similar, Melo *et al.* (2013) observaron unos ratios de supervivencia a la simulación gastrointestinal de entre 98% y 100% independientemente si la bacteria se había adaptado o no previamente a un medio de queso simulado (2 h, pH 5,5 y 3,5% NaCl (adaptadas) y pH 7 y 0% NaCl (no adaptadas)). Después de la exposición a la simulación gastrointestinal de ambos tipos de células, adaptadas y no adaptadas, Melo *et al.* (2013) observaron que a nivel proteómico se incrementó la proporción de algunas proteínas de respuesta al estrés. Sin embargo, observaron que mientras las células adaptadas sobreexpresaban un tipo de proteínas relacionadas con el estrés, las no adaptadas expresaban otras distintas. A nivel transcriptómico, después de un proceso largo (18 h) de adaptación a un estrés ácido subletal (BHI pH 5,5 (HCl)), en una de las cepas de *L. monocytogenes* se observó un incremento en la transcripción de genes relacionados con la respuesta al estrés y la invasión (*clpC*, *clpP*, *inlA*, *inlB*, *prfA* y *sigB*) (Olesen *et al.*, 2009). A la vez pudieron determinar que esta adaptación fue capaz de incrementar el potencial de virulencia de la bacteria.

Efecto de la fruta mínimamente procesada en el potencial patogénico de *L. monocytogenes*

Para poder estimar el potencial patogénico de una cepa de *L. monocytogenes* es importante conocer qué capacidad de infección presenta, es decir, una vez ha superado el tracto gastrointestinal y llega a la barrera intestinal, qué número de células van a tener la capacidad de invadir las células epiteliales del intestino y de esta forma proliferar y causar una listeriosis. Por lo tanto, es importante saber si *L. monocytogenes* en pera mínimamente procesada conservada a una temperatura abusiva de 10 °C va a presentar una mayor o menor capacidad de causar la enfermedad que si se encuentra en melón mínimamente procesado. En estudios "in vitro" mediante la línea celular Caco-2, que simula el epitelio intestinal humano, se determinó a diferencia de otros estudios, la capacidad de adhesión e invasión una vez las células se habían expuesto a la simulación gastrointestinal (*Capítulo 5*).

Una vez las células de *L. monocytogenes* 230/3 en pera y melón mínimamente procesados superaron el tracto gastrointestinal se expusieron al epitelio intestinal simulado. El día de inoculación, *L. monocytogenes* 230/3 en pera presentó el mayor porcentaje de adhesión (6,5%) sobre las células Caco-2. Este valor fue el mayor observado durante todo el estudio, siendo mayor que el observado sobre las células Caco-2 cuando la bacteria se encontraba en melón (1,4%). Los porcentajes de invasión fueron similares independientemente de la matriz en la que se inoculó la bacteria (0,0015% y 0,0047%, respectivamente, en pera y melón). Sin embargo, después de 2 días de conservación a 10 °C, *L. monocytogenes* presentó un comportamiento similar en cuanto a la capacidad de adhesión independientemente de la matriz estudiada, mientras que la capacidad de invasión de las células Caco-2 fue tres veces superior cuando la bacteria había crecido en melón mínimamente procesado que en pera. La habilidad de *L. monocytogenes* para adherirse e invadir células Caco-2 se redujo con el incremento del tiempo de conservación de la fruta a 10 °C, observándose los menores índices de adhesión e invasión a los 7 días de conservación en ambas matrices.

Olesen *et al.* (2009) observaron que dos cepas de *L. monocytogenes* que se habían pre-expuesto a condiciones de estrés subletales, ácido (pH 5,5, HCl) o salino (4,5% NaCl), presentaron un incremento en la transcripción de algunos genes de virulencia, por lo que estudiaron si la capacidad de adhesión e invasión se podían ver modificadas. Los resultados demostraron que, aunque no con la misma

intensidad en ambas cepas, la capacidad de adhesión sobre las células Caco-2 se incrementó después de la adaptación a las condiciones de estrés ácido subletal. Por otro lado, la capacidad de invasión para una de las cepas se mantuvo igual, mientras que en la otra esta capacidad de redujo levemente en comparación al control no adaptado.

Pocos estudios han evaluado como puede verse modificada la capacidad de adhesión e invasión de *L. monocytogenes* después de una exposición superior a 24 h. El periodo de tiempo más largo en el que se ha evaluado la capacidad de virulencia de *L. monocytogenes* en una matriz alimentaria ha sido de 3 y 2 semanas, respectivamente, en leche y perejil (Pricope-Ciolacu *et al.*, 2013; Rieu *et al.*, 2009). En el primer caso, al incrementar el tiempo de almacenamiento de la leche a 4 °C, la capacidad de adhesión, invasión y proliferación de *L. monocytogenes* se vio reducida. Sin embargo, todas las cepas después de un largo tiempo de conservación (3 semanas) todavía presentaron capacidad de invasión. En cuanto a la temperatura de conservación, observaron que un corto tiempo de incubación de 2 h en leche a 4 °C hizo que *L. monocytogenes* fuera más virulenta que después del mismo tiempo de conservación a 25 y 30 °C (Pricope-Ciolacu *et al.*, 2013). En el segundo caso, *L. monocytogenes* no encontró un ambiente óptimo de crecimiento en el perejil, ya que con el tiempo de conservación la población llegó a reducirse por debajo de los límites de detección y se observó que después de 5 h de incubación se redujeron los niveles de transcrito de *inlA*, *prfA* y *opuC*, a la vez que la capacidad de invasión en las células Caco-2 (Rieu *et al.*, 2009).

En la presente tesis al comparar de forma general el comportamiento de *L. monocytogenes* 230/3 en pera y melón mínimamente procesados podemos concluir que bajo las mismas condiciones, pasados 2 días de la preparación de la fruta cortada, el riesgo para el consumidor sería mayor si consume melón que pera. Esto se justifica debido a que en melón cortado la bacteria presentó una mayor capacidad de crecimiento, una mayor capacidad para superar el tracto gastrointestinal simulado, y a la vez una mayor capacidad de invasión del epitelio intestinal, incrementando así el riesgo de causar listeriosis.

Para poder complementar este estudio del potencial patogénico de *L. monocytogenes* hubiera sido interesante estudiar la expresión de algunos genes de virulencia. Existe una amplia bibliografía que ya confirma la correlación existente entre la expresión de genes de virulencia como *inlA* y la capacidad de la

bacteria para invadir las células Caco-2 (Nightingale *et al.*, 2005; Rieu *et al.*, 2009; Werbrouck *et al.*, 2009).

Diversidad fenotípica y genotípica de la especie *L. monocytogenes*

La invasión de las células eucariotas por *L. monocytgenes* requiere de la presencia de la proteína Internalina A (InlA) en la membrana de la bacteria, además de otras proteínas, como Internalina B (InlB), hemolisina O (LLO) o ActA. De este modo si una cepa de *L. monocytogenes* presenta una versión alterada de la proteína InlA, puede presentar una alteración en la capacidad de invasión.

En el *Capítulo 6* se estudió el potencial de virulencia que presentaron 53 cepas de *L. monocytogenes* de distintos orígenes y serotipos (origen clínico: 10 (1/2a), 11 (1/2c) y 11 (4b), origen alimentario 9 (1/2a), 3 (1/2c) y 9 (4b)), mediante un estudio fenotípico y genotípico. En el estudio fenotípico se observó que en las cepas de los serotipos 1/2a y 1/2c, los aislados de alimentos o entorno alimentario presentaron mayor capacidad de crecimiento en un medio rico que los aislados clínicos. En cambio, no se observaron diferencias en el serotipo 4b según el origen de los aislados.

Cuando se evaluó la capacidad de supervivencia al estrés gástrico de las distintas cepas de *L. monocytogenes*, no se observaron diferencias entre serotipos y/u orígenes, observando unos resultados heterogéneos entre las 53 cepas. De forma similar, Barmpalia-Davis *et al.* (2008) tampoco observaron diferencias significativas en la capacidad de supervivencia gastrointestinal entre 13 aislados de *L. monocytogenes* de distintos orígenes en fase estacionaria (16 h de incubación).

La capacidad de invasión de *L. monocytogenes* es una de las características de esta bacteria que tiene un impacto directo en la posibilidad de causar listeriosis. En este estudio, las cepas del serotipo 4b presentaron una mayor capacidad para invadir células Caco-2 que las cepas de *L. monocytogenes* pertenecientes al serotipo 1/2a y 1/2c. Concretamente, para esta característica fenotípica se observaron diferencias entre los serotipos aunque no entre los orígenes de las cepas. Werbrouk *et al.* (2009) estudiaron de forma "in vitro" la capacidad de invasión de células Caco-2 de 5 cepas de *L. monocytogenes* del serotipo 4b de distintos orígenes. Del estudio se extrajo que en las 5 cepas estudiadas existía una fuerte correlación entre la capacidad de invasión de estas y los niveles de expresión del gen *inlA*. Destacar que para que se dé la expresión de un gen de forma completa,

es preciso que la secuencia de nucleótidos a partir de la que se transcribe el RNA mensajero esté completa. Por este motivo en esta tesis se determinó si los resultados obtenidos del estudio fenotípico de las 53 cepas podían relacionarse con la presencia de mutaciones en tres de los genes implicados en la capacidad de virulencia y superación al estrés de la bacteria. Al estudiar la secuencia de aminoácidos del gen *inlA*, solo 7 de las cepas del linaje II (1/2a y 1/2c) de las 53 cepas de *L. monocytogenes* estudiadas presentaron la misma secuencia de aminoácidos que la cepa *L. monocytogenes* EDGe (serotipo 1/2a). Esta cepa EDGe se utiliza como modelo en la mayoría de estudios moleculares debido a que a partir de ella se obtuvo el primer genoma completo de *L. monocytogenes*. Entre las 53 cepas secuenciadas, 15 presentaron un codón de terminación prematuro ("premature stop codon", PMSC) en el gen *inlA*. Es decir, la secuencia de aminoácidos contenía una mutación no sinónima que en esta posición codificaba por un aminoácido de parada. Concretamente se observaron 7 variaciones distintas de esta mutación entre las 15 cepas. Seis de estas siete variaciones ya se habían observado con anterioridad en cepas de *L. monocytogenes* (Van Stelten *et al.*, 2010), mientras que la séptima por el momento no se había descrito todavía (PMSC en la posición 326). Este último PMSC se observó en cuatro cepas del serotipo 1/2c de origen clínico. Las mutaciones PMSC en el gen *inlA* ya descritas en otras publicaciones se observaron en aislados de ambos orígenes pero solo de los serotipos 1/2a y 1/2c. En las cepas del serotipo 4b no se observó ningún PMSC. El resto de cepas de *L. monocytogenes*, las cuales su secuencia no albergaba un PMSC y que tampoco presentaron la misma secuencia de aminoácidos para este gen que *L. monocytogenes* EDGe, presentaron 3, 6 y 15 variaciones distintas de la secuencia del gen *inlA*, en los serotipos 4b, 1/2c y 1/2a, respectivamente.

Del total de cepas de *L. monocytogenes* estudiado, 15 de 53 (28%) mostró una mutación PMSC para la secuencia del gen *inlA*. Concretamente, 8 del total de cepas (19) del serotipo 1/2a estudiadas presentó esta mutación (42%), mientras en el serotipo 1/2c, 7 de 14 cepas (50%) la presentaron. Según el origen al que pertenece cada cepa, 6 de 32 cepas (19%) de origen humano presentaron esta mutación, mientras que 9 de 21 (43%) en caso de los aislados de origen alimentario. Una distribución similar (30-45%) de esta mutación que codifica para un PMSC en la secuencia de *inlA* se observó en cepas aisladas de alimento (Chen *et al.*, 2011; Shen *et al.*, 2013). Fueron varios los investigadores que observaron que aislados que presentaban una capacidad de invadir células Caco-2 reducida, también contenían un PMSC en la secuencia del gen *inlA* (Cruz *et al.*, 2013; Felicio *et al.*, 2007; Handa-Miya *et al.*, 2007; Roche *et al.*, 2005). En el presente estudio, se observó que la secuencia del gen *inlA* de la cepa 285 del serotipo 1/2a de origen

clínico contenía un PMSC del tipo 6 (Van Stelten *et al.*, 2010), afectando la posición 492 de la secuencia de aminoácidos. En este caso, esta cepa mostró una capacidad de invasión menor (23,6%) que la de otras cepas que no contenían esta mutación.

Al estudiar la secuencia del gen *prfA* de las 53 cepas de *L. monocytogenes*, se pudieron observar 4 variaciones distintas para esta secuencia frente al modelo *L. monocytogenes* EDGe. Independientemente del origen del aislado, todas las cepas de *L. monocytogenes* del serotipo 4b presentaron la misma secuencia para el gen *prfA* que coincidió ser idéntica que la de *L. monocytogenes* EDGe. Otros autores también observaron que todas las cepas del serotipo 4b que secuenciaron, presentaron una misma variación de la secuencia del gen *prfA* (Miner *et al.*, 2008; Roche *et al.*, 2005; Rupp *et al.*, 2015). En esta tesis, en las cepas de *L. monocytogenes* estudiadas se observaron 4 variaciones distintas de la secuencia del gen *prfA*, todas ellas en los serotipos 1/2a y 1/2c. En tres de estas secuencias la mutación fue sinónima, no observando cambios en la secuencia de aminoácidos, mientras que en la restante se observó una mutación no sinónima en la posición 184 del gen *prfA*, que no se había descrito previamente. Esta mutación se observó en la cepa 324 del serotipo 1/2c de origen clínico, que aunque no presentó ninguna mutación en la secuencia del gen *inlA*, presentó una capacidad de invasión menor (29,1%) que la de otras cepas que sí la presentaron. Roche *et al.* (2005) y Kanki *et al.* (2015) también observaron mutaciones no sinónimas en la secuencias de *prfA*. Sin embargo, Handa-Miya *et al.* (2007) y Ciolacu *et al.* (2015) no observaron ninguna mutación en la secuencia de este gen.

Los dos genes anteriores tienen un papel importante en la capacidad de invasión de la bacteria, sin embargo, el gen *sigB* parece tener un papel destacado en la supervivencia de la bacteria a condiciones de estrés (Ferreira *et al.,* 2003). En el presente estudio se observaron 4 variaciones en la secuencia de nucleótidos de *sigB*, tres de ellas presentaron mutaciones no sinónimas que modificaron la secuencia de aminoácidos, mientras que la cuarta albergó una mutación resultante en un PMSC. De las tres primeras mutaciones no sinónimas, una de ellas se observó en todas las cepas del serotipo 4b, de ambos orígenes. Aquí se demuestra una clara diferencia entre los linajes I (4b) y II (1/2 a y 1/2c). La mutación PMSC en la secuencia del gen *sigB* se observó en la posición 189 de la secuencia de aminoácidos en la cepa 319 del serotipo 1/2a de origen alimentario. Esta cepa presentó la velocidad de crecimiento más elevada (1,02 h^{-1}) de las 53 cepas estudiadas.

Al poner en común los datos del estudio fenotípico mediante un análisis de componentes principales (PCA), se concluyó que las cepas del serotipo 4b se caracterizaron por una mayor capacidad de invasión de las células Caco-2 pero una menor capacidad de crecimiento. Lo contrario que las cepas del serotipo 1/2c, que presentaron una menor capacidad de invasión pero una mayor capacidad de crecimiento en un medio óptimo. En el caso de las cepas del serotipo 1/2a presentaron un valor intermedio para estas dos características fenotípicas. En conclusión, existe una correlación negativa entre la capacidad de invasión y la capacidad de crecimiento, mientras que la capacidad de sobrevivir a un estrés ácido no tiene correlación con el resto de características fenotípicas o genotípicas ensayadas. Las cepas con baja capacidad de invasión y elevada capacidad de crecimiento, se asociaron mayoritariamente con las cepas que presentaron mutaciones en los genes *inlA*.

El serotipo de *L. monocytogenes* 4b es el que se ha aislado con mayor frecuencia en los casos de listeriosis humana que a su vez tiene relación con lo observado en esta tesis, ya que se observó que las cepas del serotipo 4b estudiadas presentaron una elevada capacidad de invadir células Caco-2 y no presentaron ninguna mutación PMSC en la secuencia de su gen *inlA*. Por otro lado, todas las cepas evaluadas, incluyendo las cepas de los serotipos 1/2a y 1/2c, presentaron capacidad de invadir las células Caco-2. En estos últimos serotipos también se observaron mayores velocidades de crecimiento a 37 °C comparando con las cepas del serotipo 4b.

Para que una listeriosis tenga lugar es necesario que se den una serie de factores, algunos de ellos están relacionados con el patógeno y otros con el hospedador. En el caso del hospedador es importante el estado de su sistema inmunológico, ya que si este se encuentra debilitado será más susceptible a sufrir la enfermedad. En el caso del patógeno, la velocidad de crecimiento, que tiene un impacto directo en la densidad de población sobre el alimento en el momento de ser consumido, y la capacidad de la cepa de invadir el epitelio intestinal humano son factores que influirán en que se desarrolle o no la infección de *L. monocytogenes* en el hospedador. En el caso que una cepa menos virulenta pero con una elevada velocidad de crecimiento como podría ser una de las cepas 1/2c del presente estudio contaminase un alimento, podría llegar a presentar un riesgo de causar listeriosis similar al de una cepa más virulenta pero con una menor capacidad para crecer sobre el alimento como pasaría con una de las cepas 4b del presente estudio.

Al concluir esta tesis, y después de observar que el potencial patogénico de *L. monocytogenes* 230/3 en pera mínimamente procesada sin ningún aditivo añadido se vio modificado por las condiciones de conservación, cabe esperar que el hecho de añadir a este entorno ácidos orgánicos y/o sales para incrementar su vida útil, podría ocasionar que la bacteria se viera expuesta a otros estreses subletales a la vez que los ocasionados por la matriz y la temperatura, pudiendo activar más fácilmente el sistema de adaptación al entorno ácido de *L. monocytogenes*. Se podría plantear un menor uso de sustancias que pudieran generar un estrés subletal a la bacteria para no afectar a la capacidad de virulencia de *L. monocytogenes* (Skandamis *et al.*, 2012; Werbrouck *et al.*, 2009), sin embargo, al no poder someter la fruta a un tratamiento físico, el uso de algunos aditivos continúan siendo la mejor opción para reducir el riesgo microbiológico. Por lo tanto, sería interesante evaluar el efecto que pueden tener los nuevos antimicrobianos basados en extractos vegetales en el potencial de virulencia de *L. monocytogenes.* La adición de estos compuestos alargaría el periodo de vida útil aumentando el tiempo de multiplicación del microorganismo, siendo más probable alcanzar la dosis infectiva. En el producto de pera mínimamente procesada estudiado en esta tesis, que no recibe ningún tratamiento posterior antes de su consumo, en el que *L. monocytogenes* puede crecer en atmosfera modificada, en presencia de antioxidantes, a temperaturas de refrigeración (incluso 1 °C), que presenta una elevada supervivencia al paso gastrointestinal y con una capacidad de invasión destacable, se hace indispensable prevenir la contaminación, des del campo hasta el momento de consumo.

Referencias

Abadias, M., Alegre, I., Oliveira, M., Altisent, R., Vinas, I. 2012. Growth potential of *Escherichia coli* O157:H7 on fresh-cut fruits (melon and pineapple) and vegetables (carrot and escarole) stored under different conditions. *Food Control* 27, 37-44.

Alegre, I., Abadias, M., Anguera, M., Oliveira, M., Vinas, I. 2010a. Factors affecting growth of foodborne pathogens on minimally processed apples. *Food Microbiology* 27, 70-76.

Alegre, I., Abadias, M., Anguera, M., Usall, J., Vinas, I. 2010b. Fate of *Escherichia coli O157:H7*, *Salmonella* and *Listeria innocua* on minimally-processed peaches under different storage conditions. *Food Microbiology* 27, 862-868.

Arias, E., González, J., López-Buesa, P., Oria, R. 2008. Optimization of processing of fresh-cut pear. *Journal of the Science of Food and Agriculture* 88, 1755-1763.

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

Chen, P.M., Varga, D.M., Seavert, C.F. 2003. Developing a value-added fresh-cut 'D'Anjou' pear product. *Horttechnology* 13, 314-320.

Chen, Y.H., Ross, W.H., Whiting, R.C., Van Stelten, A., Nightingale, K.K., Wiedmann, M., Scott, V.N. 2011. Variation in *Listeria monocytogenes* dose responses in relation to subtypes encoding a full-length or truncated Internalin A. *Applied and Environmental Microbiology* 77, 1171-1180.

Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K. 2015. *Listeria monocytogenes* isolated from food samples from a Romanian black market show distinct virulence profiles. *International Journal of Food Microbiology* 209, 44-51.

Cruz, C.D., Pitman, A.R., Harrow, S.A., Fletcher, G.C. 2013. *Listeria monocytogenes* associated with New Zealand seafood production and clinical cases: unique sequence types, truncated InlA, and attenuated Invasiveness. *Applied and Environmental Microbiology* 80, 1489-1497.

DAAM. 2015. Observatori de la Fruita Fresca de Catalunya. http://agricultura.gencat.cat/web/.content/de_departament/de02_estadistiques_observatoris/08_observatoris_sectorials/03_observatori_de_la_fruita_fresca/a2/fitxers_estatics/A2_2015/2015.08-Prev-collita-i-produccio-poma-i-pera-_Cat_EU_.pdf. Consulta: 03/11/15.

Davis, M.J., Coote, P.J., Obyrne, C.P. 1996. Acid tolerance in *Listeria monocytogenes*: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology-Uk* 142, 2975-2982.

Dong, X., Wrolstad, R.E., Sugar, D. 2000. Extending shelf life of fresh-cut pears. *Journal of Food Science* 65, 181-186.

EFSA, E.F.S.A. 2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2014. *EFSA Journal* 13, 4329.

Falguera, V., Garvin, A., Garza, S., Pagan, J., Ibarz, A. 2014. Effect of UV-vis photochemical processing on pear juices from six different varieties. *Food and Bioprocess Technology* 7, 84-92.

Felicio, M.T.S., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M. 2007. Recurrent and sporadic *Listeria monocytogenes* contamination in alheiras represents considerable diversity, including virulence-attenuated isolates. *Applied and Environmental Microbiology* 73, 3887-3895.

FEPEX. 2012. Guía de buenas prácticas de producción frutas y hortalizas. http://www2.fepex.es/ficheros/AFHORFRESH/GUIA_IVgama/Guia_FyH-Preparadas_ver3.pdf. Consulta: 10/11/15.

Ferreira, A., Sue, D., O'Byrne, C.P., Boor, K.J. 2003. Role of *Listeria monocytogenes* sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Applied and Environmental Microbiology* 69, 2692-2698.

Gorny, J.R., Cifuentes, R.A., Hess-Pierce, B., Kader, A.A. 2000. Quality changes in fresh-cut pear slices as affected by cultivar, ripeness stage, fruit size, and storage regime. *Journal of Food Science* 65, 541-544.

Gorny, J.R., Hess-Pierce, B., Cifuentes, R.A., Kader, A.A. 2002. Quality changes in fresh-cut pear slices as affected by controlled atmospheres and chemical preservatives. *Postharvest Biology and Technology* 24, 271-278.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1998. Effects of fruit ripeness and storage temperature on the deterioration rate of fresh-cut peach and nectarine slices. *Hortscience* 33, 110-113.

Gorny, J.R., Hess-Pierce, B., Kader, A.A. 1999. Quality changes in fresh-cut peach and nectarine slices as affected by cultivar, storage atmosphere and chemical treatments. *Journal of Food Science* 64, 429-432.

Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., Fujii, T. 2007. Nonsense-mutated *inlA* and *prfA* not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan. *International Journal of Food Microbiology* 117, 312-318.

Huang, J.W., Luo, Y.G., Nou, X.W. 2015. Growth of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut cantaloupe under different temperature abuse scenarios. *Journal of Food Protection* 78, 1125-1131.

Ivy, R.A., Wiedmann, M., Boor, K.J. 2012. *Listeria monocytogenes* grown at 7 ºC shows reduced acid survival and an altered transcriptional response to acid shock compared to *L. monocytogenes* frown at 37 ºC. *Applied and Environmental Microbiology* 78, 3824-3836.

Kanki, M., Naruse, H., Taguchi, M., Kumeda, Y. 2015. Characterization of specific alleles in *InlA* and *PrfA* of *Listeria monocytogenes* isolated from foods in

Osaka, Japan and their ability to invade Caco-2 cells. *International Journal of Food Microbiology* 211, 18-22.

Marklinder, I.M., Lindblad, M., Eriksson, L.M., Finnson, A.M., Lindqvist, R. 2004. Home storage temperatures and consumer handling of refrigerated foods in Sweden. *Journal of Food Protection* 67, 2570-2577.

Mastronicolis, S.K., Arvanitis, N., Karaliota, A., Litos, C., Stavroulakis, G., Moustaka, H., Tsakirakis, A., Heropoulos, G. 2005. Cold dependence of fatty acid profile of different lipid structures of *Listeria monocytogenes*. *Food Microbiology* 22, 213-219.

Melo, J., Schrama, D., Hussey, S., Andrew, P.W., Faleiro, M.L. 2013. *Listeria monocytogenes* dairy isolates show a different proteome response to sequential exposure to gastric and intestinal fluids. *International Journal of Food Microbiology* 163, 51-63.

Miner, M.D., Port, G.C., Freitag, N.E. 2008. Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA. *Microbiology-Sgm* 154, 3579-3589.

Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M. 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Applied and Environmental Microbiology* 71, 8764-8772.

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 Pathogens and Disease* 6, 669-680.

Oms-Oliu, G., Aguilo-Aguayo, I., Martin-Belloso, O. 2006. Inhibition of browning on fresh-cut pear wedges by natural compounds. *Journal of Food Science* 71, S216-S224.

Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martín-Belloso, O. 2009. Effect of ripeness at processing on fresh-cut 'Flor de Invierno' pears packaged under modified atmosphere conditions. *International Journal of Food Science & Technology* 44, 900-909.

Oms-Oliu, G., Odrjozola-Serrano, I., Soliva-Fortuny, R., Martin-Belloso, O. 2008a. Antioxidant content of fresh-cut pears stored in high-O-2 active packages

compared with conventional low-O-2 active and passive modified atmosphere packaging. *Journal of Agricultural and Food Chemistry* 56, 932-940.

Oms-Oliu, G., Soliva-Fortuny, R., Martin-Belloso, O. 2008b. Physiological and microbiological changes in fresh-cut pears stored in high oxygen active packages compared with low oxygen active and passive modified atmosphere packaging. *Postharvest Biology and Technology* 48, 295-301.

Oms-Oliu, G., Soliva-Fortuny, R., Martín -Belloso, O. 2008c. Edible coatings with antibrowning agents to maintain sensory quality and antioxidant properties of fresh-cut pears. *Postharvest Biology and Technology* 50, 87-94.

Penteado, A.L., Leitao, M.F.F. 2004. Growth of *Listeria monocytogenes* in melon, watermelon and papaya pulps. *International Journal of Food Microbiology* 92, 89-94.

Pricope-Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K. 2013. The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay. *International Journal of Food Microbiology* 166, 59-64.

Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Sobrino-Lopez, A., Martin-Belloso, O. 2009. Inactivation of L*isteria monocytogenes*, *Salmonella enteritidis* and *Escherichia coli* O157:H7 and shelf life extension of fresh-cut pears using malic acid and quality stabilizing compounds. *Journal of Food Quality* 32, 539-565.

Rieu, A., Guzzo, J., Piveteau, P. 2009. Sensitivity to acetic acid, ability to colonize abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on parsley leaves. *Journal of Applied Microbiology* 108, 560-570.

Roche, S.M., Gracieux, P., Milohanic, E., Albert, I., Virlogeux-Payant, I., Temoin, S., Grepinet, O., Kerouanton, A., Jacquet, C., Cossart, P., Velge, P. 2005. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology* 71, 6039-6048.

Rupp, S., Aguilar-Bultet, L., Jagannathan, V., Guldimann, C., Drogemuller, C., Pfarrer, C., Vidondo, B., Seuberlich, T., Frey, J., Oevermann, A. 2015. A naturally occurring *prfA* truncation in a *Listeria monocytogenes* field strain contributes to reduced replication and cell-to-cell spread. *Veterinary Microbiology* 179, 91-101.

Samelis, J., Ikeda, J.S., Sofos, J.N. 2003. Evaluation of the pH-dependent, stationary-phase acid tolerance in *Listeria monocytogenes* and *Salmonella Typhimurium* DT104 induced by culturing in media with 1% glucose: a comparative study with *Escherichia coli* O157:H7. *Journal of Applied Microbiology* 95, 563-575.

Sánchez, C., Cebola Lidon, F., Vivas, M., Ramos, P., Santos, M., Graça Barreiro, M. 2015. Effect of chitosan coating on quality and nutritional value of fresh-cut 'Rocha' pear. *Emirates Journal of Food and Agriculture* 27, 206-214.

Sapers, G.M., Miller, R.L. 1998. Browning inhibition in fresh-cut pears. *Journal of Food Science* 63, 342-346.

Shen, J.L., Rump, L., Zhang, Y.F., Chen, Y., Wang, X., Meng, J.H. 2013. Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. *Food Microbiology* 35, 58-64.

Shen, Q., Soni, K.A., Nannapaneni, R. 2014. Influence of temperature on acid-stress adaptation in *Listeria monocytogenes*. *Foodborne Pathogens and Disease* 11, 43-49.

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.

Soliva-Fortuny, R.C., Alos-Saiz, N., Espachs-Barroso, A., Martin-Belloso, O. 2004. Influence of maturity at processing on quality attributes of fresh-cut conference pears. *Journal of Food Science* 69, S290-S294.

Soliva-Fortuny, R.C., Biosca-Biosca, M., Grigelmo-Miguel, N., Martin-Belloso, O. 2002. Browning, polyphenol oxidase activity and headspace gas composition during storage of minimally processed pears using modified atmosphere packaging. *Journal of the Science of Food and Agriculture* 82, 1490-1496.

Soliva-Fortuny, R.C., Martín-Belloso, O. 2003. Microbiological and biochemical changes in minimally processed fresh-cut *Conference* pears. *European Food Research and Technology* 217, 4-9.

Van Stelten, A., Simpson, J.M., Ward, T.J., Nightingale, K.K. 2010. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in *inlA* are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. *Applied and Environmental Microbiology* 76, 2783-2790.

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.

Zhang, Y.M., Carpenter, C.E., Broadbent, J.R., Luo, X. 2014. Habituation to organic acid anions induces resistance to acid and bile in *Listeria monocytogenes*. *Meat Science* 96, 1152-1157.

CONCLUSIONES

CONCLUSIONES

Del estudio para la obtención de un **producto de pera mínimamente procesado**, se concluyó:

1. Para el desarrollo de un producto de pera mínimamente procesado, se seleccionó la variedad "Conference" dado que presentó los mejores atributos frente a "Ercolini", "Flor de Invierno" y "Passa-Crassana".

2. El tratamiento antioxidante-estabilizante de la textura compuesto por ascorbato de calcio al 2% y cloruro de calcio al 1% junto a una conservación en atmosfera modificada pasiva a 5 °C, hasta 14 días, mantuvo el color y la textura de la pera.

3. El tratamiento antioxidante-estabilizante de la textura incrementó significativamente el contenido de fenoles totales, ácido ascórbico y capacidad antioxidante en la pera mínimamente procesada. Cuando esta se sometió a condiciones de rotura de la cadena de frío, la reducción del contenido de fenoles totales, ácido ascórbico y capacidad antioxidante fue similar al almacenamiento a una temperatura constante de 4 °C. Así mismo, no se observó un efecto significativo en la estabilidad microbiológica manteniéndose los recuentos de bacterias psicrótrofas, mohos y levaduras, y bacterias lácticas en los estándares de calidad.

Después del estudio del efecto de la **temperatura de conservación** y el **estado de madurez** de la pera antes del procesado en el comportamiento de *S. enterica* y *L. monocytogenes*, se concluyó:

4. En cuanto al efecto de la temperatura de conservación sobre el crecimiento de las cepas de *S. enterica* estudiadas ambas condiciones evaluadas (temperatura constante (4 °C) y simulación de una rotura de la cadena de frío) inhibieron su crecimiento, mientras que la población de las cepas de *L. monocytogenes* estudiadas incrementó.

5. El efecto del estado de madurez de la pera antes del procesado mínimo tuvo un efecto en la capacidad de crecimiento de *L. monocytogenes* 230/3 (serotipo 1/2a) cuando la pera mínimamente procesada (sin tratamiento antioxidante-

estabilizante) se conservó en aire a 10 y 20 °C. A estas temperaturas, el patógeno presentó un mayor crecimiento en la pera procesada en estado maduro (31-42 N) o sobremaduro (< 31 N). Sin embargo, cuando la pera mínimamente procesada se conservó en aire a 5 °C, *L. monocytogenes* 230/3 presentó la misma capacidad de crecimiento en los cuatro estados de madurez, observándose un incremento de población cercano a 2 unidades logarítmicas después de 8 días de conservación.

Al determinar el efecto de la matriz alimentaria sobre el **potencial patogénico** de *L. monocytogenes* 230/3, se concluyó:

6. *L. monocytogenes* creció en pera y melón mínimamente procesados sin tratamiento antioxidante-estabilizante conservados en aire a 1, 5, 10 y 20 °C, con incrementos a las 48 h entre 0,1 y 5,0 log ufc g^{-1}. La supervivencia de *L. monocytogenes* a la simulación gastrointestinal tras su crecimiento en pera y melón mínimamente procesados conservados en aire a distintas temperaturas, se vio afectada por la temperatura de conservación. Cuando *L. monocytogenes* en melón se conservó a 20 °C, la capacidad de supervivencia de la bacteria a la simulación gastrointestinal se redujo al aumentar el tiempo de conservación, mientras que en pera *L. monocytogenes* presentó una capacidad similar de supervivencia a lo largo del tiempo. La capacidad de *L. monocytogenes* para adaptarse a un estrés ácido leve como el que presenta la pera, pudo conferir mayor capacidad de resistencia al estrés ácido fuerte al que se ve sometida durante la simulación gastrointestinal. En ambas matrices, la capacidad de supervivencia a la simulación gastrointestinal de *L. monocytogenes* conservada a 10 °C, se redujo al aumentar el tiempo de conservación. *L. monocytogenes* en pera y melón mínimamente procesados conservados a 1 °C, después de la simulación gastrointestinal presentó las menores variaciones de población.

7. Tras la inoculación de *L. monocytogenes* en pera y melón mínimamente procesados y la posterior exposición a la simulación gastrointestinal, *L. monocytogenes* en pera presentó una mayor capacidad de adhesión sobre células Caco-2 (6,5%) que la bacteria en melón (1,4%). Sin embargo, tras la conservación de la fruta a 10 °C, la capacidad de invasión de la bacteria fue similar en ambas matrices, siendo de 0,0015% y 0,0047% en pera y melón, respectivamente. La capacidad de adhesión de *L. monocytogenes* tras crecer en ambas matrices y exponerse a la simulación gastrointestinal, se redujo con el

tiempo de conservación. Sin embargo, la capacidad de invasión de *L. monocytogenes* crecida en pera y melón mínimamente procesados y expuesta a la simulación gastrointestinal, presentó una mayor capacidad de invasión a los 2 días de conservación (0,0033% y 0,0093% de índice de invasión en pera y melón, respectivamente) que en el momento de inoculación (0,0015% y 0,0047%) y después de 7 días de conservación (0,0001% y 0,0007%).

Del estudio fenotípico y genotípico del **potencial de virulencia** que presentan las cepas de *L. monocytogenes* de distintos orígenes y serotipos, se concluyó:

8. El estudio del potencial de virulencia realizado en distintas cepas de los serotipos 4b, 1/2a y 1/2c de *L. monocytogenes*, reveló que independientemente de su origen (humano o alimentario), las cepas de serotipo 4b presentaron menor nivel de crecimiento pero mayor capacidad de invasión en células Caco-2; las cepas de serotipo 1/2c mayor crecimiento y menor capacidad de invasión y las cepas de serotipo 1/2a valores intermedios de crecimiento e invasión. El análisis de las secuencias de los genes relacionados con la virulencia, confirmó que todas las cepas del serotipo 4b presentaban la misma secuencia del gen *prf*A siendo idéntica a la del modelo *L. monocytogenes* EDGe, y la misma mutación no sinónima en la secuencia del gen *sig*B, que no se observó en las cepas del serotipo 1/2a y 1/2c.

9. El análisis de las secuencias del gen *inl*A, también relacionado con la virulencia, mostró tres variaciones distintas en la secuencia de aminoácidos en las cepas de serotipo 4b, pero ninguna que codificara para un codón de terminación prematuro ("premature stop codón", PMSC). Dicha mutación PMSC se detectó en el 38% de las cepas de *L. monocytogenes* estudiadas, de las cuales el 32% eran de origen humano y el 45% aisladas de alimentos o entornos alimentarios. La presencia de mutación en los genes *inl*A se pudo relacionar con la menor capacidad de invasión sobre células Caco-2.

Totes les decisions que prenem fan que siguem les persones que som. Per això celebro haver pres les decisions que m'han dut a viure l'experiència d'estudiar un doctorat a la Universitat de Lleida.

Aquesta experiència m'ha enriquit acadèmica i personalment, i durant aquest temps he crescut com a persona, però no ha estat sola. Maribel i Inma, sou les que m'heu donat aquesta oportunitat i m'heu guiat en aquest procés, sempre us ho agrairé. Totes les vostres idees, coneixements i sobretot correccions d'aquests anys, tot m'ho enduc. Al Pep, Neus i Charo també els agraeixo haver creat un enriquidor debat, moltes vegades després d'algun Esmorzar de Ciència o d'una Reunió de Tesi. Aquestes ocasions m'han donat peu a veure les coses des d'una altra perspectiva que molt sovint és necessària. Per tot això i molt més, gràcies per ajudar-me a fer ciència.

M'agrada recordar que vaig començar aquesta tesi treballant al laboratori de Seguretat alimentària de l'IRTA-Lleida en el Campus UdL-ETSEA i l'he finalitzat al laboratori de Microbiologia de productes vegetals del Fruitcentre-IRTA al Parc Tecnològic de Gardeny, diferents noms però la mateixa gent. En aquest laboratori he pogut aprendre i, a la vegada, compartir la meva feina amb persones com Marina, Isabel Alegre, Márcia, Rosa Altisent, Anna Solé, Patri, Jordi Sabata, Belén i Cyrelys, i que per descomptat ha estat un plaer.

Com ja sabeu molts, una tesi no està formada només per la part experimental sinó que de vegades el més dur comença un cop ja es tenen els resultats. Per això haver pogut compartir despatx amb companys d'altres disciplines m'ha enriquit i ajudat. Agraeixo a tots els companys, ja doctorats o encara en procés (ànim a aquests últims!!), que m'heu acompanyat i amb els que he compartit moltes hores de despatx però també riures i converses que recordaré (Carlos, Gemma, Laura, Maria Sisquella, Lulú, Márcia, Maria Bernat, Amparo, Belén, Anna Carbó, Cyrelys, Lorena, Laia Torregrossa i Nuria Baró). A més, aquest últim temps a la sala, les noies m'heu fet sentir encara part d'alguna cosa, per això Gràcies.

Destacar que hi ha molta més gent que forma el grup de Postcollita que no he anomenat, que tot i no haver-hi treballat colze a colze, m'han ensenyat en multitud d'ocasions com moure'm i treballar en el laboratori. N'he de destacar la Cristina i la Cèlia, però a tota la resta del grup, agrair-vos l'interès per aquest llarg procés que ha estat aquesta tesi.

Al llarg d'aquesta tesi he pogut viatjar i veure com treballen altres grups de recerca, donant-me l'oportunitat de treballar i aprendre d'ells. Tant en l'estada al "Centre for Infectious Disease Control, National Institute for Public Health and the Environment (Bilthoven)" com a la "Universidade Católica Portuguesa (Porto)" he tingut la sort d'aprendre coses noves, però sobretot de treballar en condicions molt diferents que m'han fet aprendre que es pot fer ciència de moltes formes. Per descomptat sempre tindré present al Lucas M. Wijnands, que a la vegada de bon investigador és encara millor persona i va demostrar-m'ho amb escreix a l'inici de la meva estada a Utrech. També agraeixo el suport que m'han ofert Eelco Franz, Angela van Hoek, Paula Teixeira, Vania Ferreira i Rui Maghaleas entre d'altres, ajudant-me a desenvolupar part de la meva recerca.

En definitiva, GRÀCIES a tots els que m'heu acompanyat en aquesta etapa de la meva vida.

Lleida, 25 de Novembre de 2016