Universitat de Lleida

Capacidad de crecimiento de patógenos de transmisión alimentaria en manzana y melocotón mínimamente procesados y nuevas estrategias de intervención para mejorar su seguridad microbiológica

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Memoria presentada por
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Jamás se descubriría nada si nos consideráramos satisfechos con las cosas descubiertas

(Séneca)

La ciencia no es perfecta, con frecuencia se utiliza mal, no es más que una herramienta, pero es la mejor herramienta que tenemos, se corrige a sí misma, esta siempre evolucionando y se puede aplicar a todo. Con esta herramienta conquistamos lo imposible.

(Carl Sagan)

ÍNDICE

ABREVIATURAS

AA	Ácido ascórbico
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
AM	Atmósfera modificada
APPCC	Análisis de Peligros y Puntos de Control Crítico
BAL	Bacterias ácido-lácticas
BPA	Buenas Prácticas Agrícolas
BPP	Buenas Prácticas de Producción
BPW	'Buffered Peptone Water'
cfu	'Colony forming units'
DW	'Deionized water'
ECEP	*Escherichia coli* enteropatógeno
ECEI	*Escherichia coli* enteroinvasivo
ECET	*Escherichia coli* toxigénico
ECEH	*Escherichia coli* enterohemorrágico
ECEAg	*Escherichia coli* enteroagregantes
ECDA	*Escherichia coli* difusamente adherentes
EFSA	'European Food Safety Authority'
EPA	'Environmental Protection Agency'
EUA	Estados Unidos de América
FAO	'Food and Agricultural Organization'
FDA	'Food and Drug Administration'
GLM	'General Linear Models'
GRAS	'Generally recognized as safe'
MA	'Modified atmosphere'
MAP	'Modified atmosphere packaging'
MP	'Minimally processed'
NAC	N-acetilo-L-cisteína

NS	NatureSeal® AS1
OMS	Organización Mundial de la Salud
ORP	Potencial de óxido-reducción
OTR	‘Oxygen transmission rate’
PAA	Ácido peroxiacético o peracético
ppm	partes por millón
QPS	‘Qualified Presumption of Safety’
SAS	‘Statistical Analysis System’
SH	‘Sodium hypochlorite’
Tm, t	Toneladas
ufc	Unidades formadoras de colonia
USDA	‘United States Department of Agriculture’
WHO	‘World Health Organization’

RESÚMENES

RESUMEN

En los últimos años se ha popularizado el consumo de frutas y hortalizas mínimamente procesadas o de IV gama ya que ofrecen al consumidor un producto fresco y saludable con una presentación cómoda y atractiva que se adapta al estilo de vida actual. Aunque tradicionalmente las frutas se han considerado seguras debido a su bajo pH, este aumento de consumo ha provocado un incremento de los casos de toxiinfecciones alimentarias asociados con frutas y hortalizas mínimamente procesadas. Durante las operaciones llevadas a cabo para su producción se incrementa su vulnerabilidad a la contaminación microbiana y el riesgo de que se conviertan en vehículos de patógenos de transmisión alimentaria. Además, las frutas y hortalizas mínimamente procesadas no reciben ningún tratamiento capaz de eliminar todos los patógenos antes de su consumo. Todos estos factores ponen de relevancia la necesidad de utilizar métodos de producción seguros y procedimientos de desinfección adecuados. Actualmente, el hipoclorito sódico es el desinfectante habitual en la industria de IV gama. Sin embargo, las reducciones de microorganismos en frutas y hortalizas lavadas con agua clorada no superan los 2 logaritmos y en algunos países se ha prohibido su uso. Además, en los últimos años ha incrementado la presión de los consumidores para reducir y/o eliminar los aditivos de los alimentos, por lo que se buscan alternativas más seguras para los humanos y más respetuosas con el medio ambiente.

En este escenario se planteó la realización de esta tesis con dos objetivos principales. En primer lugar se determinó la supervivencia y crecimiento de tres patógenos de transmisión alimentaria, *Escherichia coli* O157:H7, *Salmonella enterica* y *Listeria innocua*, en manzanas y melocotones mínimamente procesados en función de la temperatura de conservación, la variedad de la fruta, el uso de antioxidantes y la atmósfera de envasado (Capítulos I y II). A continuación se estudiaron posibles estrategias de intervención, como sustancias desinfectantes alternativas al hipoclorito sódico en manzana mínimamente procesada (Capítulo III) y el control biológico o bioconservación (Capítulos IV, V y VI). Por último, se evaluó la posibilidad de aplicar un microorganismo probiótico, *Lactobacillus rhamnosus* GG en manzana mínimamente procesada y su efecto en la dinámica poblacional de *Salmonella* spp. y *Listeria monocytogenes* (Capítulo VII).

Los resultados obtenidos han demostrado que las cepas de *E. coli* O157:H7, *Salmonella* y *L. innocua* utilizadas pueden crecer en manzana y melocotón mínimamente procesado conservado a temperaturas a partir de 10 °C. El crecimiento de los tres patógenos no se vio afectado por el uso de antioxidantes (ácido ascórbico 2 % y NatureSeal® AS1 6 % en manzana y ácido ascórbico en melocotón) o por el envasado en atmósfera modificada pasiva. La variedad de manzana no influyó en el crecimiento de los patógenos. En cambio se observaron

diferencias de crecimiento entre las variedades de melocotón, debidas principalmente a las diferencias de pH.

El ácido peroxiacético (80 y 120 mg L^{-1}), el peróxido de hidrógeno (5, 10 y 20 mL L^{-1}) y el N-acetilo-L-cisteína (10 g L^{-1}) podrían ser tratamientos alternativos al hipoclorito sódico para manzana mínimamente procesada ya que no sólo redujeron la población de patógenos tras el tratamiento sino que evitaron su crecimiento a lo largo de la conservación a 10 °C. Además, el ácido peroxiacético, el peróxido de hidrógeno y el producto comercial Citrox podrían evitar la contaminación cruzada en la industria de IV gama ya que redujeron la concentración de patógenos en el agua de lavado por debajo del límite de detección. Sin embargo, antes de la utilización de estas sustancias son necesarios estudios sobre su efecto en la calidad de la fruta cortada.

A continuación, y como una estrategia complementaria a la desinfección de fruta mínimamente procesada, se estudió la posibilidad de aplicar microorganismos antagonistas o cultivos bioconservantes. De los 97 y 107 microorganismos testados en manzana y melocotón cortados, respectivamente, dos, CPA-6 y CPA-7, mostraron una gran capacidad antagonista reduciendo la población de *E. coli* O157:H7, *Salmonella* y *L. innocua* incluso por debajo del nivel inoculado tras 2 días a 20 °C. El antagonista CPA-7 se identificó como una cepa de *Pseudomonas graminis* y CPA-6 como una nueva especie perteneciente a la familia *Enterobacteriaceae*. En manzana cortada, la mínima dosis inhibitoria de la cepa CPA-6 para reducir la población de patógenos cuando éstos fueron inoculados a 10^7 ufc mL^{-1} fue de 10^6 ufc mL^{-1}, mientras que la cepa CPA-7 necesitó estar a la misma concentración para ser efectiva. Para determinar si las cepas CPA-6 y CPA-7 eran fitopatógenas se estudió su capacidad de producir reacción de hipersensibilidad en la planta del tabaco. Ninguna de las dos cepas causó necrosis en la planta de tabaco, sin embargo, se observó que la cepa CPA-6 causó daños visibles en fruta mínimamente procesada durante su conservación y, por tanto, se descartó. El siguiente paso consistió en testar la efectividad de la cepa CPA-7 en manzana cortada en condiciones semi-comerciales. CPA-7 fue compatible con la aplicación de NatureSeal® AS1 y con el envasado en atmósfera modificada pasiva, sin embargo para ser efectiva en estas condiciones su concentración tuvo que ser 2 unidades logarítmicas superiores a la de los patógenos. La aplicación de la cepa antagonista no afectó a los parámetros de calidad fisicoquímica (color, sólidos solubles, acidez titulable y firmeza) de la manzana cortada. La cepa CPA-7 no redujo la población de patógeno pero evitó su crecimiento y por tanto podría aplicarse como un obstáculo adicional en la conservación de frutas mínimamente procesadas.

En el último capítulo de la tesis se demostró que la cepa probiótica *Lactobacillus rhamnosus* GG puede ser una cepa adecuada para la producción de manzana probiótica ya que su concentración se mantuvo por encima de 10^6 ufc g^{-1} durante 28 días tanto a 5 como a 10 °C sin afectar a la calidad de la manzana tratada.

Además, su aplicación redujo el crecimiento de *L. monocytogenes* 1 unidad logarítmica a ambas temperaturas.

Los resultados de esta tesis han demostrado la necesidad de mantener una adecuada higiene en la producción de fruta mínimamente procesada, así como una temperatura de conservación adecuada (inferior a 10 °C). Además, se han encontrado algunas alternativas al uso del hipoclorito sódico y se ha aislado una cepa, *P. graminis* CPA-7, capaz de controlar el crecimiento de patógenos de transmisión alimentaria en manzana y melocotón. La aplicación de dicho microorganismo no ha afectado a la calidad físicoquímica de la fruta, con lo que el control biológico o bioconservación puede utilizarse como un obstáculo adicional para garantizar la seguridad microbiológica de estos productos. Finalmente, se ha demostrado que la cepa probiótica *L. rhamnosus* GG podría ser usada para producir manzana mínimamente procesada probiótica.

RESUM

En els últims anys s'ha popularitzat el consum de fruites i hortalisses mínimament processades o de IV gama ja que ofereixen al consumidor un producte fresc i saludable amb una presentació còmoda i atractiva que s'adapta a l'estil de vida actual. Tot i que, tradicionalment, les fruites s'han considerat segures gràcies al seu baix pH, aquest augment de consum ha comportat un increment de les intoxicacions alimentaries associades a fruites i hortalisses mínimament processades. Durant les operacions dutes a terme en la seva producció s'incrementa la vulnerabilitat a la contaminació microbiana i el risc de ser vehicles de patògens de transmissió alimentaria. A més, les fruites i hortalisses mínimament processades no reben cap tractament capaç d'eliminar tots els patògens abans del seu consum. Tots aquests factors demostren la necessitat d'utilitzar mètodes de producció segurs i procediments de desinfecció adequats. Actualment, el hipoclorit sòdic és el desinfectant habitual en la indústria de IV gama. No obstant, les reduccions de microorganismes en fruites i hortalisses rentades amb aigua clorada no superen els 2 logaritmes i en alguns països se n'ha prohibit l'ús. A més, en els últims anys ha augmentat la pressió dels consumidors per reduir i/o eliminar els additius dels aliments, raó per la qual es busquen alternatives més segures pels humans i més respectuoses amb el medi ambient.

En aquest escenari es va plantejar la realització d'aquesta tesis amb dos objectius principals. En primer lloc, es va determinar la supervivència i creixement de tres patògens de transmissió alimentaria, *Escherichia coli* O157:H7, *Salmonella enterica* i *Listeria innocua,* en pomes i préssecs mínimament processats en funció de la temperatura de conservació, la varietat de la fruita, l'ús d'antioxidants i l'atmosfera d'envasat (Capítols I i II). A continuació es van estudiar possibles estratègies d'intervenció, com productes desinfectants alternatius a l'hipoclorit sòdic en pomes mínimament processades (Capítol III) i el control biològic o bioconservació (Capítols IV, V i VI). Per acabar, es va avaluar la possibilitat d'aplicar un microorganisme probiòtic, *Lactobacillus rhamnosus* GG, en poma mínimament processada i el seu efecte en la dinàmica poblacional de *Salmonella* spp. i *Listeria monocytogenes* (Capítol VII).

Els resultats obtinguts han demostrat que les soques de *E. coli* O157:H7, *Salmonella* i *L. innocua* utilitzades poden créixer en poma i préssec mínimament processats conservats a temperatures a partir de 10 °C. El creixement dels patògens no es va veure afectat per l'ús d'antioxidants (àcid ascòrbic 2 % i NatureSeal® AS1 6 % en poma i àcid ascòrbic en préssec) ni per l'envasat en atmosfera modificada passiva. La varietat de poma no va afectar al creixement dels patògens. En canvi, es van observar diferències de creixement entre les varietats de préssec, degudes, principalment, a les diferències de pH.

L'àcid peroxiacètic (80 i 120 mg L^{-1}), el peròxid d'hidrogen (5, 10 i 20 mL L^{-1}) i la N-acetil-L-cisteïna (10 g L^{-1}) podrien ser tractaments alternatius a l'hipoclorit sòdic en poma mínimament processada ja que, no només van reduir la població de patògens després del tractament, sinó que van evitar el seu creixement durant la conservació a 10 °C. A més, l'àcid peroxiacètic, el peròxid d'hidrogen i el producte comercial Citrox podrien evitar la contaminació creuada en la industria de IV gama ja que van reduir la població de patògens en l'aigua de rentat per sota del límit de detecció. No obstant, abans d'utilitzar aquestes substàncies cal estudiar el seu efecte en la qualitat de la fruita tallada.

A continuació, i com una estratègia complementària a la desinfecció de fruita mínimament processada, es va estudiar la possibilitat d'aplicar microorganismes antagonistes o cultius bioconservants. Dels 97 i 107 microorganismes avaluats en poma i préssec tallats, respectivament, dos, CPA-6 i CPA-7 van mostrar una gran capacitat antagonista reduint la població de *E. coli* O157:H7, *Salmonella* i *L. innocua* fins i tot per sota del nivell inoculat després de 2 dies a 20 °C. L'antagonista CPA-7 es va identificar com una soca de *Pseudomonas graminis* i la CPA-6 com una nova espècie de la família *Enterobacteriaceae*. La mínima dosi inhibitòria de la soca CPA-6 per reduir la població de patògens inoculats a 10^7 ufc mL^{-1} va ser de 10^6 ufc mL^{-1}, mentre que la soca CPA-7 va necessitar estar a la mateixa concentració per a ser efectiva. Per determinar si les soques CPA-6 i CPA-7 eren fitopatògenes es va estudiar si produïen reacció d'hipersensibilitat en la planta del tabac. Cap de les dues soques va causar necrosi en la planta de tabac, però es va observar que la soca CPA-6 va causar danys visibles en fruita mínimament processada durant la conservació i, per tant, es va descartar. El següent pas va consistir en determinar l'efectivitat de la soca CPA-7 en condicions semi-comercials en poma tallada. La soca CPA-7 va ser compatible amb l'aplicació de NatureSeal® AS1 i amb l'envasat en atmosfera modificada passiva, però, en aquestes condicions, per a ser efectiva la seva concentració va haver de ser 2 unitats logarítmiques superiors a la dels patògens. L'aplicació de la soca antagonista no va afectar els paràmetres de qualitat fisicoquímica (color, sòlids solubles, acidesa titulable i fermesa) de la poma tallada. La soca CPA-7 no va reduir la població de patogen però va evitar el seu creixement i, per tant, podria aplicar-se com un obstacle addicional en la conservació de fruites mínimament processades.

En l'últim capítol de la tesi es va demostrar que la soca probiòtica *Lactobacillus rhamnosus* GG pot ser una soca adient per a la producció de poma probiótica ja que la seva concentració es va mantenir per sobre de 10^6 ufc g^{-1} durant 28 dies tant a 5 com a 10 ° C sense afectar la qualitat de la poma. A més, la seva aplicació va reduir el creixement de *L. monocytogenes* 1 unitat logarítmica a ambdues temperatures.

Els resultats d'aquesta tesi han demostrat la necessitat de mantenir una adequada higiene en la producció de fruita mínimament processada, així com una

temperatura de conservació adequada (al menys inferior a 10 °C). A més, s'han trobat algunes alternatives a l'ús de l'hipoclorit sòdic i s'ha aïllat una soca, *P. graminis* CPA-7, capaç de controlar el creixement de patògens de transmissió alimentària en poma i préssec. L'aplicació d'aquest microorganisme no va afectar la qualitat fisicoquímica de la fruita, de manera que el control biològic o bioconservació pot utilitzar-se con un obstacle addicional per garantir la seguretat microbiològica d'aquests productes. Finalment, s'ha demostrat que la soca probiòtica *L. rhamnosus* GG podria ser utilitzada per produir poma mínimament processada probiótica.

SUMMARY

Recently, consumption of minimally processed or fresh-cut fruits and vegetables has risen significantly because they offer a fresh and healthy product with a convenient and attractive presentation that fits perfectly on today's lifestyle. Although fruits have been traditionally considered as safe due to its low pH, this increase in consumption has resulted in increased frequency of outbreaks of illness associated with minimally processed fruits and vegetables. During production of minimally processed fruit and vegetables, their susceptibility to microbial contamination and the risk of becoming vehicles of foodborne pathogens increases. In addition, these products do not receive any treatment able to completely eliminate all foodborne pathogens before consumption. Therefore, safe production methods and proper disinfection/decontamination procedures are required. Currently, chlorine is the most commonly disinfectant used to wash produce in the fresh-cut industry. However, the reduction of microorganisms in fruits and vegetables washed in chlorinated water do not exceed 2 log units and in some countries its use has been forbidden. Moreover, in recent years consumer's pressure to reduce and/or eliminate food additives has increased, so researchers are looking for safer and more environmentally friendly alternatives.

In this scenario, the realization of this thesis raised two main objectives. First, we determined the survival and growth of three foodborne pathogens, *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria innocua* on minimally processed apples and peaches depending on the storage temperature, variety of fruit, the use of antioxidants and atmosphere packaging (Chapters I and II). Then, we studied possible intervention strategies as alternatives to chlorine disinfection in minimally processed apple (Chapter III) and biological control or biopreservation (Chapters IV, V and VI). Finally, we evaluated the possibility of applying a probiotic microorganism, *Lactobacillus rhamnosus* GG, to minimally processed apple and its effect on the population dynamics of *Salmonella* spp. and *Listeria monocytogenes* (Chapter VII).

The results obtained demonstrated that the strains of *E. coli* O157:H7, *Salmonella* and *L. innocua* used were able to grow in minimally processed apples and peaches stored at temperatures of 10 °C and above. The growth of these pathogens was not affected by the use of antioxidants (ascorbic acid 2 % and 6 % NatureSeal® AS1 in apple and ascorbic acid in peach) or the passive modified atmosphere packaging. The apple variety did not influence the pathogens growth, but growth differences were observed among peach varieties mainly due to pH differences among fruits.

Peroxiacetic acid (80 and 120 mg L^{-1}), hydrogen peroxide (5, 10 and 20 mL L^{-1}) and N-acetyl-L-cysteine (10 g L^{-1}) could be alternative treatments to chlorine disinfection in minimally processed apple production as they not only reduced the pathogen population after treatment but also avoided their growth throughout storage at 10 °C. Furthermore, peroxiacetic acid, hydrogen peroxide and the

commercial product Citrox could prevent cross-contamination in the fresh-cut industry as they reduced the pathogens population in wash water below the detection limit. However, before the use of these substances, studies on their effect on the quality of the cut fruit are required.

Then, as a complementary strategy to disinfection of minimally processed fruit, we studied the possibility of applying antagonistic microorganisms. Two of the 97 and 107 microorganisms tested on fresh-cut apple and peach, respectively, CPA-6 and CPA-7, showed a great antagonistic capacity reducing the population of *E. coli* O157:H7, *Salmonella* and *L. innocua* below the inoculated level after 2 days at 20 °C. The antagonist CPA-7 was identified as a strain of *Pseudomonas graminis* and CPA-6 as a new species belonging to the family *Enterobacteriaceae*. The minimum inhibitory dose of the strain CPA-6 to reduce the pathogen population when inoculated at 10^7 cfu mL^{-1} was 10^6 cfu mL^{-1}, whereas strain CPA-7 needed to be at the same concentration to be effective. To ascertain if CPA-6 and CPA-7 strains are phytopathogens, their capacity to produce hypersensitive reaction in leaf mesophyll tissue of tobacco plants was determined. None of the two strains caused necrosis in tobacco plants. However, CPA-6 strain caused visible damage on minimally processed fruit during product storage and it was discarded. The next step was to test the effectiveness of the strain CPA-7 at semi-commercial conditions. CPA-7 was compatible with the application of NatureSeal® AS1 and the passive modified atmosphere packaging, but at these conditions, its concentration needed to be 2 log units higher than pathogens concentration to be effective. The application of the antagonistic strain did not affect the physicochemical quality parameters (color, soluble solids, titratable acidity and firmness) of the fresh-cut apple. The strain CPA-7 did not reduce the pathogen population but avoided its growth therefore it could be applied as an additional barrier in the conservation of minimally processed fruits.

In the last chapter of the thesis we have demonstrated that the probiotic strain *Lactobacillus rhamnosus* GG could be a suitable strain for the production of probiotic apple as its concentration was maintained above 10^6 cfu g^{-1} for 28 days at 5 and 10 °C without affecting the quality of the treated apple. Furthermore, its application reduced *L. monocytogenes* growth 1 log unit at both temperatures.

The results of this study have demonstrated the need to maintain an adequate hygiene in minimally processed fruit production, as well as an appropriate storage temperature (at least below 10 °C). In addition, we have found some alternatives to chlorine and we have isolated a strain, *P. graminis* CPA-7, capable of reducing the growth of foodborne pathogens on minimally processed apples and peaches. The application of CPA-7 did not affect the physicochemical quality of the fruit, so that biological control or biopreservation could be an additional hurdle to ensure the microbiological safety of these products. Finally, it has been shown that the probiotic strain *L. rhamnosus* GG may be used to produce probiotic minimally processed apples.

I. INTRODUCCIÓN

1. FRUTA DE IV GAMA O MÍNIMAMENTE PROCESADA

Las frutas y hortalizas forman parte de una dieta equilibrada ya que, no sólo contribuyen a prevenir trastornos ocasionados por la falta de nutrientes, sino que también reducen el peligro de padecer enfermedades cardiovasculares y distintos tipos de cáncer. Un informe de expertos publicado por la OMS y la FAO, titulado *Dieta, nutrición y prevención de las enfermedades crónicas*, establece como meta poblacional una ingesta de, al menos, 400 g diarios de frutas y verduras. Sin embargo, en España y en la mayoría de los países industrializados el consumo per cápita es muy inferior. Por eso, diferentes organizaciones de todo el mundo (OMS, FAO, USDA, EFSA) han promocionado campañas para lograr un consumo regular de, al menos, 5 raciones de frutas y hortalizas al día.

La provincia de Lleida es la principal productora de manzana del país, con 187.344 Tm el año 2009, que suponen el 31.1 % de la producción nacional. La provincia de Lleida es, además, una de las principales zonas productoras de melocotón, con un total de 143.072 Tm en el 2009, que representa un 18.3 % de la producción estatal (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010). Actualmente, la mayoría de esta fruta se destina al consumo en fresco tanto para mercado interior como para la exportación. La aparición de industrias dedicadas a la producción de fruta de IV gama podría dar a productores y centrales hortofrutícolas un nuevo mercado a su producto.

1.1. Definición y consumo

Las frutas y hortalizas de IV Gama o mínimamente procesadas son aquellas obtenidas mediante la aplicación de una o varias operaciones unitarias de preparación, tales como pelado, cortado, reducción de tamaño y envasado, incluyendo tratamientos químicos, cuya combinación puede tener un efecto sinérgico (Wiley, 1994).

El consumo de este tipo de productos es más elevado en los países desarrollados con alto poder adquisitivo y especialmente en el medio urbano. El consumo medio europeo de productos de IV Gama es de 3 kg por persona y año. Sin embargo, en el Reino Unido se llega a los 12 kg por habitante y año, Francia ocupa la segunda posición con 6 kg por cápita e Italia ocupa el tercer lugar con 4 kg por persona y año. Otros países donde la IV Gama está bien implantada, aunque distanciados de los anteriores son Bélgica, Holanda y Alemania. En España el consumo está entre 1 y 1.5 kg por persona y año.

En España, la IV Gama fue introducida en Navarra hacia los años 80, y ha ido adquiriendo cada vez más importancia, extendiéndose a otras zonas típicas de producción hortofrutícola como Murcia, Comunidad Valenciana, Andalucía y

Cataluña (http://www.fepex.es/publico/presentacion/IVGama.aspx). En la actualidad se consumen productos de IV gama casi en el 60 % de los hogares españoles. En 2010 el volumen de frutas y hortalizas de IV Gama comercializadas en España fue de 70.6 millones de kilos, de los que 69.1 millones de kilos correspondieron a hortalizas y 1.5 millones de kilos a frutas. El incremento con respecto a 2009 fue del 6 %. Asimismo, del total comercializado en España, el 81 % aproximadamente se dirigió a la distribución y el 19 % restante a la hostelería y restauración (FEPEX, 2011).

1.2. Procesado

El procesado mínimo de frutas y hortalizas pretende mantener el producto fresco, ofrecer comodidad sin perder calidad y conseguir una vida útil de, al menos, 7 días. Para conseguir estos objetivos es importante utilizar una materia prima de buena calidad, tener una higiene estricta y seguir buenas prácticas de fabricación. En la Figura 1 se observa el esquema general de preparación de frutas frescas cortadas. No obstante, según el tipo de fruta que se elabore, se deberá aplicar un tratamiento específico para las distintas operaciones y métodos de conservación (Wiley, 1994). Cada etapa del proceso de elaboración juega un papel importante en el control de los mecanismos de alteración de las frutas y hortalizas frescas, así como en la presencia de microorganismos en el producto.

La elaboración de frutas y hortalizas mínimamente procesadas comienza por una buena selección de la materia prima siendo la primera operación el control del grado de madurez, de la presencia de residuos de pesticidas, de elevadas cargas microbianas, de metales tóxicos, de compuestos indeseables naturalmente presentes y de reguladores del crecimiento de plantas.

A continuación, el lavado y desinfección de las frutas y hortalizas permite eliminar en gran medida residuos de pesticidas, restos vegetales y otros posibles contaminantes como los microorganismos alterantes.

En el caso de las frutas mínimamente procesadas, las operaciones mecánicas realizadas durante su procesado, como el pelado y cortado, dañan los tejidos y son responsables de la alteración microbiana, cambios de textura, deshidratación, pardeamiento y producción de malos sabores y olores. Por consiguiente, se debe tener en cuenta la influencia de las operaciones de corte en la calidad.

El lavado y desinfección realizados tras el pelado y/o cortado reducen la carga microbiana y eliminan los fluidos celulares. Estas operaciones juntamente con los tratamientos antimicrobianos y antioxidantes reducen la oxidación enzimática y el crecimiento de microorganismos durante la conservación. Antes del envasado, las piezas de fruta cortada deben escurrirse ya que el exceso de agua o zumo puede ser un medio excelente para el crecimiento de microorganismos y algunas reacciones enzimáticas pueden acelerarse.

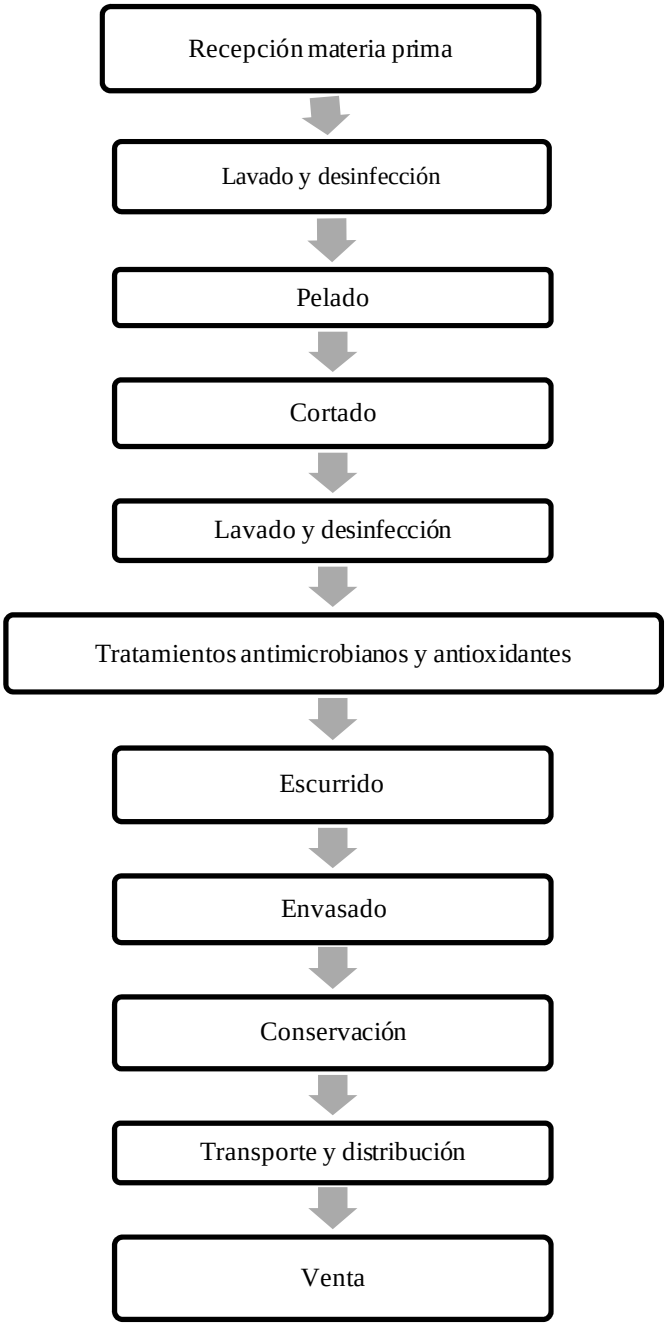

Fig. 1 Diagrama de flujo de elaboración de fruta mínimamente procesada.

El envasado en atmósfera modificada proporciona una barrera que mantiene alta la humedad relativa en el ambiente y evita la deshidratación de las superficies cortadas. La modificación de la atmósfera es también muy importante para controlar la alteración microbiana de la fruta mínimamente procesada.
La vida útil comercial de las frutas mínimamente procesadas viene determinada principalmente por la temperatura de conservación. Para obtener la frescura, calidad y seguridad óptima, la cadena de frío (entre 0 y 4 °C) debe mantenerse a lo largo de toda la vida útil, ya que son productos frescos que continúan respirando y son altamente susceptibles a la alteración microbiana. Se debe prestar especial atención a la temperatura de las neveras en los comercios ya que las estanterías demasiado llenas, el flujo de aire bloqueado o incluso la posición del producto en la estantería pueden influir significativamente en la temperatura del producto.

2. MICROBIOLOGÍA DE PRODUCTOS VEGETALES

Las frutas y hortalizas tienen una composición heterogénea y por lo tanto su microbiota puede variar en función de su pH, disponibilidad de nutrientes y actividad de agua, entre otros factores (Kalia y Gupta, 2006). La mayoría de las bacterias presentes en la superficie de las plantas son gram-negativas y pertenecen a la familia *Enterobacteriaceae* o al género *Pseudomonas* (Lund, 1992). La mayoría de estas bacterias no son patógenas para los humanos y su concentración (10^4-10^8 ufc g^{-1}) depende de las variaciones estacionales y climáticas. Es posible encontrar altas concentraciones de microorganismos sin que los productos presenten signos de alteración (Nguyen-The y Carlin, 1994). Normalmente, los tejidos internos de frutas y hortalizas se consideran estériles.

Las frutas difieren principalmente de las hortalizas en que normalmente contienen mayores cantidades de azúcar y tienen un pH más ácido. Este bajo pH, combinado con la presencia de ácidos orgánicos previene el crecimiento de bacterias a excepción de las bacterias ácido-lácticas (BAL). Por consiguiente los hongos son los microorganismos predominantes de las frutas, siendo los mohos los responsables de dos terceras partes de las alteraciones de frutas y hortalizas (ICMSF, 1998). Los géneros más comunes son *Penicillium*, *Aspergillus*, *Sclerotinia*, *Botrytis* y *Rhizopus*. La alteración se asocia normalmente con actividad celulolítica y pectinolítica, que causa ablandamiento y debilitación de las estructuras vegetales, importantes para prevenir el crecimiento de microorganismos.

Por tanto, tradicionalmente las frutas se han considerado generalmente seguras desde el punto de vista microbiológico gracias a su alto contenido en ácido y a que el número de intoxicaciones alimentarias relacionadas con el consumo de frutas es bajo en comparación con otros alimentos (Beuchat, 1996). Sin embargo, los cambios en las prácticas agrícolas, el aumento del comercio internacional y la producción de nuevos productos, como la fruta mínimamente procesada han

ocasionado un aumento en la frecuencia de intoxicaciones alimentarias asociadas a frutas (Beuchat, 2002). Durante la elaboración de las frutas mínimamente procesadas se elimina la piel que es su protección natural y se produce una salida de zumo y azúcares de los tejidos dañados. Por tanto, son más susceptibles a la contaminación microbiana y al crecimiento de microorganismos.

2.1. Fuentes de contaminación

Las frutas y hortalizas mínimamente procesadas se consumen frecuentemente crudas. Además, no existe ningún paso en el procesado capaz de eliminar totalmente la contaminación microbiana. Consecuentemente, la prevención de la contaminación con patógenos humanos es el único método efectivo de garantizar que son seguros para el consumo. Para minimizar el riesgo de contaminación es necesario identificar las posibles fuentes de contaminación en cada punto de la cadena de producción:

Materia prima

La presencia de patógenos en las frutas y hortalizas frescas procedentes de campo es un riesgo para la industria de IV gama, ya que si no son eliminados antes del procesado pueden contaminar las partes internas de los productos (Brackett, 1999). La mejor manera de prevenir la presencia de patógenos en frutas y hortalizas mínimamente procesadas es asegurar la buena calidad del producto fresco aplicando Buenas Prácticas Agrícolas (BPA) durante la precosecha y cosecha (Raybaudi-Massilia y Mosqueda-Melgar, 2009). Las principales fuentes de contaminación de la materia prima son:

1. Los animales salvajes y domésticos y los insectos (Beuchat, 2006; NACMCF, 1999).
2. El agua de riego puede ser la fuente principal de contaminación del material vegetal durante la producción. La calidad microbiológica del agua de riego depende del origen del agua (Steele y Odumeru, 2004). Las aguas superficiales procedentes de estanques, ríos, lagos y arroyos tienen una calidad microbiológica variable y la calidad microbiológica de las aguas residuales es muy pobre y requiere tratamientos extensos antes de que pueda ser utilizada como agua de riego. Por otro lado, el método de riego puede afectar a la transmisión de patógenos. Es preferible el riego por goteo al riego por aspersión ya que minimiza el contacto del cultivo con los contaminantes presentes en el agua.
3. Los abonos no tratados adecuadamente pueden ser una fuente potencial de patógenos de transmisión alimentaria (Beuchat, 2002). El abono de origen animal puede contener patógenos entéricos como *E. coli* O157:H7 y *Salmonella* spp. que, en caso de no eliminarse, pueden persistir hasta 3 meses en los suelos abonados (Natvig *et al.*, 2002).

4. Los recolectores y los manipuladores pueden ser vectores de intoxicaciones alimentarias en caso de ser portadores de patógenos y tener una higiene personal deficiente (Gomes da Cruz *et al.*, 2006).
5. Los utensilios utilizados para la cosecha (por ejemplo, cuchillos, tijeras) y contenedores (bolsas, cajas, camiones y cámaras de almacenamiento) pueden contaminar la materia prima ya que si las frutas y hortalizas, el suelo o el polvo están previamente contaminados con patógenos, éstos se transfieren a los utensilios y contenedores hasta el próximo saneamiento.

Agua

Las frutas y hortalizas frescas se lavan a la llegada del campo para eliminar residuos químicos, físicos y biológicos de la superficie antes de entrar en la línea de producción (Balla y Farkas, 2006). Sin embargo, el elevado precio del agua y del tratamiento de aguas residuales ha favorecido que la industria reutilice el agua de lavado (Allende *et al.*, 2008). Las sucesivas utilizaciones del agua de lavado pueden, en lugar de reducir, aumentar la contaminación microbiológica de los productos y por tanto representar un riesgo de salud pública. Para evitar la contaminación se necesitan técnicas sanitarias capaces de inactivar los microorganismos en el agua de proceso.

Manipuladores

El contacto humano durante el procesado de frutas y hortalizas mínimamente procesadas es uno de los factores más importantes involucrados en la transferencia de microorganismos patógenos en caso de falta de prácticas higiénicas por parte de los trabajadores. Es necesario que los manipuladores utilicen guantes, gorros y mascarillas durante el procesado para evitar la transferencia de patógenos a las frutas y hortalizas mínimamente procesadas, a las superficies y al agua (USFDA, 2008).

Instalaciones, equipos de proceso y utensilios

Las instalaciones, equipos y utensilios pueden ser puntos de contaminación microbiana en caso de no existir una adecuada desinfección (USFDA, 2008).

Plagas

Las plagas de roedores, pájaros, reptiles, anfibios e insectos pueden ser importantes vectores para una gran variedad de patógenos en las plantas de procesado (Reij *et al.*, 2004).

2.2. Incidencia de patógenos de transmisión alimentaria en fruta mínimamente procesada e intoxicaciones asociadas con su consumo

La mayoría de los estudios existentes evalúan la incidencia de patógenos en la superficie de fruta entera (Badosa *et al.*, 2008; Branquinho Bordini *et al.*, 2007; Harris *et al.*, 2003; Uchima *et al.*, 2008). Sin embargo, existen pocos estudios sobre la incidencia de patógenos en fruta mínimamente procesada. Abadias *et al.* (2008) analizaron 21 muestras de fruta mínimamente procesada (manzana, piña, naranja, mango y melocotón) sin detectar *Salmonella*, *Listeria monocytogenes* ni *Escherichia coli* en ninguna de las muestras.

A continuación se muestra una relación de intoxicaciones alimentarias asociadas con el consumo de fruta (Tabla 1).

Tabla 1 Intoxicaciones alimentarias causadas por *E. coli* O157:H7, *L. monocytogenes* y *Salmonella* asociadas con el consumo de fruta

Patógeno	**Año**	**Producto**	**Casos (muertos)**	**Lugar**
E. coli O157:H7	1993	Melón Cantalupo	9	Restaurante
E. coli O157:H7	1997	Melón	9	Hogar
E. coli O157:H7	1998	Ensalada de frutas	45	Hogar
E. coli O157:H7	1998	Ensalada de frutas	47	Restaurante
E. coli O157:H7	2000	Uva	14	Verdulería
E. coli O157:H7	2000	Sandía	736 (1)	Restaurante
E. coli O157:H7	2001	Pera	14	Escuela
E. coli O157:H7	2005	Ensalada de frutas	18 (1)	Múltiple
Listeria monocytogenes	1979	Tomate	20 (5)	Hospitales
Listeria monocytogenes	2011	Melón Cantalupo	146 (13)	Hogar
Salmonella Baildon	1998	Tomate	83 (3)	Múltiple
Salmonella Berta	2002	Uvas, melón cantalupo, sandía	19	Iglesia
Salmonella Berta	2006	Tomate	16	Múltiple
Salmonella Braenderup	2004	Tomate Roma	137	Restaurante; hogar
Salmonella Chester	1990	Melón cantalupo	25000 (2)	Desconocido
Salmonella Enteritidis	1999	Melón Honeydew, sandía	82	Escuela
Salmonella Enteritidis	1999	Fruta	13	Restaurante
Salmonella Enteritidis	2000	Ensalada de frutas	4	Restaurante
Salmonella Enteritidis	2005	Melón cantalupo	126	Hogar
Salmonella Enteritidis	2005	Tomate	20	Restaurante; hogar

Salmonella Heidelberg	2000	Melón	4	Restaurante
Salmonella Javiana	1990	Tomate	174	Múltiple
Salmonella Javiana	1991	Sandía	39	Desconocido
Salmonella Javiana	1999	Fruta mezclada	11	Hogar
Salmonella Javiana	2002	Tomate	3	Restaurante
Salmonella Javiana	2002	Tomate	159	Restaurante
Salmonella Litchfield	2007	Melón cantalupo	11	Hogar
Salmonella Litchfield	2007	Melón cantalupo, melón Honeydew, uva	30	Restaurante
Salmonella Miami	1954	Sandía	17 (1)	Supermercado
Salmonella Montevideo	1993	Tomate	84	Múltiple
Salmonella Muenchen	2003	Melón cantalupo, melón Honeydew	58	Hogar
Salmonella multiserotipos	2004	Tomate Roma	429	Restaurante
Salmonella Newport	1999	Mango	79	Múltiple
Salmonella Newport	2002	Ensalada de frutas	51	Restaurante
Salmonella Newport	2002	Tomate	510	Desconocido
Salmonella Newport	2003	Melón Honeydew	68 (2)	Múltiple
Salmonella Newport	2005	Melón cantalupo	24	Desconocido
Salmonella Newport	2005	Tomate	52	Restaurante
Salmonella Newport	2006	Melón Honeydew	12	Múltiple
Salmonella Newport	2006	Tomate	115	Restaurante
Salmonella Newport	2006	Sandía	20	Restaurante
Salmonella Newport	2007	Tomate	10 (1)	Desconocido
Salmonella Newport	2007	Tomate	65	Restaurante, hogar
Salmonella Newport	2007	Tomate, aguacate	46	Restaurante, hogar
Salmonella Oranienburg	1979	Sandía	18	Supermercado
Salmonella Oranienburg	1998	Melón cantalupo	22	Múltiple
Salmonella Oranienburg	1998	Mango	9	Hogar
Salmonella Oranienburg	2006	Ensalada de frutas	41	Múltiple
Salmonella Poona	1991	Melón cantalupo	>400	Desconocido
Salmonella Poona	2000	Melón cantalupo	46	Desconocido
Salmonella Poona	2001	Melón cantalupo	50 (2)	Hogar
Salmonella Poona	2001	Melón Honeydew, sandía	23	Múltiple
Salmonella Poona	2002	Melón cantalupo	26	Múltiple
Salmonella Saintpaul	2001	Mango	26	Hogar
Salmonella Saintpaul	2003	Mango, tomate	17	Restaurante, hogar

Salmonella Saintpaul	2003	Tomate	33	Restaurante
Salmonella Saphra	1997	Melón cantalupo	24	Múltiple
Salmonella Senftenberg	2001	Uva	40	Hogar
Salmonella spp.	2003	Fresas	13	Desconocido
Salmonella Thompson	2000	Tomate	43	Hogar
Salmonella Typhimurium	2006	Tomate	18	Restaurante, hogar
Salmonella Typhimurium	2006	Tomate	8	Desconocido
Salmonella Typhimurium	2006	Tomate	192	Restaurante, hogar
Salmonella Typhimurium	2007	Tomate	23	Restaurante
Salmonella Typhimurium var Cope	2006	Sandía	7	Restaurante
Salmonella Virchow	2003	Tomate	11	Desconocido

Adaptada de CDC, 2007; CDC, 2011 y Harris *et al.*, 2003.

El número de intoxicaciones alimentarias asociadas con frutas y hortalizas mínimamente procesadas ha aumentado en los últimos años por varias razones. En primer lugar, las mejoras en los diagnósticos e inspecciones han estimulado el estudio de los casos de intoxicaciones, de manera que han incrementado los casos asociados a todos los alimentos. Además, los cambios en las prácticas industriales y los cambios demográficos han influido en la epidemiología de estas intoxicaciones. Por ejemplo, una mayor producción y el aumento de vida útil pueden permitir el aumento de la concentración de patógenos y su distribución en áreas dispersadas geográficamente. El aumento del comercio global acerca al consumidor alimentos producidos en otras zonas y permite la desaparición de la estacionalidad. No obstante, este comercio puede exponer a los consumidores a microbiota exótica. La demanda de comodidad ha aumentado el consumo de frutas y hortalizas mínimamente procesadas y zumos recién exprimidos. Tras esta manipulación, una incorrecta combinación de tiempo y temperatura durante la conservación, puede permitir la supervivencia y crecimiento de los patógenos y por tanto aumentar el riesgo de intoxicación. Además, el cambio en la demografía social, ha provocado un aumento de población de edad avanzada, inmunocomprometida o con enfermedades crónicas que tienen un mayor riesgo de sufrir una intoxicación alimentaria. Los cambios en las preferencias alimentarias de los consumidores han ocasionado un aumento de consumo de frutas y hortalizas frescas y, por consiguiente, ha aumentado el número de personas expuestas a los patógenos asociados a este tipo de productos. Al mismo tiempo muchos consumidores prefieren productos cultivados orgánicamente, que conllevan el uso de abono orgánico en lugar de fertilizantes químicos. Desgraciadamente, un abono orgánico tratado inadecuadamente puede contener patógenos entéricos como *Salmonella* spp. y *E. coli* O157:H7 (De Roever, 1999).

2.3. Principales bacterias patógenas relacionadas con frutas y hortalizas mínimamente procesadas

2.3.1. *Salmonella*

Salmonella es un género de bacterias gram-negativas de la familia *Enterobacteriaceae.* Entre los más de 2700 serotipos pertenecientes a este género, *Salmonella* Enteritidis y *Salmonella* Typhimurium son los que se han asociado con más frecuencia a intoxicaciones alimentarias. Sin embargo, una gran variedad de serotipos se han asociado con intoxicaciones alimentarias causadas por frutas y hortalizas frescas (EU Scientific Committee on Food, 2002). Los síntomas de la salmonelosis son diarrea, fiebre, calambres abdominales y vómito durante 4-7 días.

Las bacterias del género *Salmonella* son mesófilas. Las temperaturas y pH óptimos para el crecimiento se muestran en la Tabla 2. Asimismo, *Salmonella* es un anaerobio facultativo capaz de sobrevivir en atmósferas con baja concentración de oxígeno.

Se puede encontrar *Salmonella* en materia fecal y aguas residuales, donde pueden permanecer viables durante meses, y, por lo tanto, pueden contaminar el suelo y los cultivos con los que estén en contacto. Este patógeno se asocia habitualmente con animales, siendo las aves y otros productos cárnicos, huevos y lácteos las fuentes más comúnmente implicadas en salmonelosis. Varios estudios han demostrado la presencia de *Salmonella* en melón cantalupo entero (Harris *et al*., 2003), pero no en naranjas (Pao *et al.*, 1998). En la Tabla 1 se pueden ver algunos ejemplos de intoxicaciones alimentarias causadas por este patógeno en productos como melones y tomate.

Tabla 2 Condiciones mínimas, óptimas y máximas de temperatura (°C) y pH para el crecimiento de *Salmonella*, *E. coli* y *L. monocytogenes*.

		Mínimo	**Óptimo**	**Máximo**
Salmonella	Temperatura	5.2*	35-43	46.2
	pH	3.8	7-7.5	9.5
E. coli	Temperatura	7-8	35-40	44-46
	pH	4.4	6-7	9
L. monocytogenes	Temperatura	-0.4	37	45
	pH	4.4	7	9.4

*La mayoría de los serotipos no crecen a pH inferiores a 7.

Aunque el pH no se encuentre en el óptimo de crecimiento, varios autores han descrito el crecimiento de diferentes cepas de *Salmonella* en gran variedad de frutas. Por ejemplo, varias cepas de *Salmonella* crecieron en tomates cortados (pH 3.4-4.7) y en naranjas 'Hamlin' peladas a temperaturas superiores a 20 °C, pero no a temperaturas inferiores a 10 °C (Asplund y Nurmi, 1991; Pao *et al.*, 1998; Zhuang *et al.*, 1995). En cambio, una mezcla de 6 cepas diferentes de *Salmonella* fue incapaz de crecer en fresas (pH 3.2-4.1) cortadas y conservadas tanto a 24 como a 4 °C (Knudsen *et al.*, 2001). Leverentz *et al.* (2001) observaron que *Salmonella* Enteritidis sobrevivía a 5 °C y crecía a 10 y 20 °C en manzanas 'Red Delicious' (pH 4.2) y en melón 'Honeydew' (pH 5.8) mínimamente procesados. Sin embargo, el crecimiento observado fue mayor en melones (más de 5 unidades logarítmicas tras 7 días) que en manzanas (aproximadamente 2 unidades logarítmicas). Los resultados obtenidos por Penteado y Leitao (2004b) demuestran que las pulpas de melón, sandía y papaya son substratos adecuados para el crecimiento de *Salmonella* Enteritidis tanto a 10 como a 20 °C. Posteriormente, Leverentz *et al.* (2006) no observaron crecimiento de *Salmonella* Poona en cilindros de manzana 'Golden Delicious' conservados a 10 °C, en cambio creció aproximadamente 3 unidades logarítmicas en 2 días cuando se conservó a 25 °C. La población de la cepa de *Salmonella* Typhimurium LT2 ATCC15277 aumentó en 2 unidades logarítmicas tras 24 h de incubación a 25 °C en heridas de manzana 'Golden Delicious' (Trias *et al.*, 2008b). La población de *Salmonella* Enteritidis en peras 'Flor de Invierno' se mantuvo o disminuyó a lo largo de la conservación a 5 °C (Raybaudi-Massilia *et al.*, 2009a). También se ha descrito crecimiento de *Salmonella* en mango 'Tommy Atkins' (pH 4.2) y papaya 'Red Lady' (5.7) a 12 y 23 °C (Strawn y Danyluk, 2010).

2.3.2. *Escherichia coli*

Escherichia coli es una especie gram-negativa que pertenece la familia *Enterobacteriaceae.* Es un habitante común del tracto intestinal de los animales donde juega un papel importante manteniendo la fisiología intestinal. La clasificación da lugar a una subdivisión en seis grupos: cepas enteropatógenas (ECEP), cepas enteroinvasivas (ECEI), cepas enterotoxigénicas (ECET), cepas verotoxigénicas o enterohemorrágicas (ECEH), cepas enteroagregantes (ECEAg) y cepas difusamente adherentes (ECDA). Las cepas causantes de intoxicaciones alimentarias se diferencian en función de las propiedades virulentas, los mecanismos de patogenicidad, los síntomas y las características antigénicas. La cepa enterohemorrágica, *E. coli* O157:H7 está reconocida como un importante patógeno alimentario. La dosis infectiva es muy baja y las secuelas de la gastroenteritis pueden incluir diarrea sanguinolenta (colitis hemorrágica) y síndrome urémico hemolítico. Este último es común en niños inferiores a 5 años y ancianos.

La especie *E. coli* es mesófila y anaerobia facultativa. Las temperaturas y pH óptimos para el crecimiento pueden verse en la Tabla 2.

El principal reservorio de *E. coli* O157:H7 es el tracto intestinal del ganado bovino. La contaminación del agua y supervivencia del patógeno en ella, hace que el agua sea una fuente importante de distribución de la infección, particularmente si se consume el agua no tratada o se utiliza para lavar alimentos crudos. Existen pocos estudios sobre la presencia de *E. coli* O157:H7 en fruta entera o mínimamente procesada. No obstante, existen casos de intoxicaciones alimentarias debidas a *E. coli* O157:H7 en ensaladas de frutas, melón, uvas, pera y sandía (Tabla 1).

Experimentalmente, se ha observado el aumento de la población de *E. coli* O157:H7 en naranjas 'Hamlin' peladas a lo largo de la conservación a 24 °C (Pao *et al.*, 1998). De forma similar a lo observado con *Salmonella*, una mezcla de 5 cepas de *E. coli* O157:H7 tampoco fue capaz de crecer en fresas cortadas conservadas a 4 y 24 °C (Knudsen *et al.*, 2001). La población de diferentes cepas de *E. coli* aumentó en heridas de manzanas 'Golden Delicious', 'Macoun', 'Melrose' y 'Red Delicious' a temperatura ambiente (Dingman, 2000; Janisiewicz *et al.*, 1999a; Trias *et al.*, 2008b). En cambio, el tejido dañado de manzanas 'McIntosh' tuvo un efecto inhibitorio en el crecimiento del patógeno aunque los valores de pH y sólidos solubles de esta variedad no fueron significativamente diferentes a las otras cuatro. Por lo tanto, Dingman (2000) apuntó a la existencia de otro factor causante de la inhibición como algún compuesto no presente en las otras variedades. Según Gunes y Hotchkiss (2002) la población de *E. coli* O157:H7 en manzana 'Delicious se mantuvo constante durante la conservación a 15 °C en atmósfera modificada con 1 % de O_2 y 0, 15 y 30 % de CO_2 mientras que aumentó en las manzanas envasadas en aire. A 20 °C, la población de *E. coli* O157:H7 también se mantuvo constante a lo largo de 9 días de conservación con 30 % de CO_2 y 21 % de O_2, mientras que aumentó más de una unidad logarítmica en los envases en aire. En peras 'Flor de Invierno' (pH 4.31), la población de *E. coli* O157:H7 disminuyó ligeramente a lo largo de la conservación a 5 °C (Raybaudi-Massilia *et al.*, 2009a). En melón cantalupo, la población de *E. coli* O157:H7 disminuyó a lo largo de la conservación a 4 °C, sin embargo aumentó más de 3.5 unidades logarítmicas cuando se conservó a 20 °C (Sharma *et al.*, 2009). Strawn y Danyluk (2010) observaron crecimiento de una mezcla de 4 cepas de *E. coli* O157:H7 en mango cortado (pH 4.2) conservado a 23 °C, pero no a 4 y 12 °C. En cambio, este patógeno creció rápidamente en papaya cortada (pH 5.7) conservada a 12 y 23 °C y sobrevivió a 4 °C. Las diferencias en crecimiento fueron atribuidas a las diferencias intrínsecas entre las frutas, incluyendo la diferencia de pH. Recientemente, Abadias *et al.* (2012) observaron que *E. coli* O157:H7 fue capaz de crecer en melón cortado conservado a 25 °C (pH 5.94) pero no en piña (pH 3.59).

2.3.3. *Listeria monocytogenes*

Listeria monocytogenes es una bacteria gram-positiva y un importante patógeno de transmisión alimentaria. Aunque existen 12 serotipos de *L. monocytogenes*, los serotipos 1/2a, 1/2b y 4b son los responsables del 90 % de las infecciones humanas. Es particularmente importante su capacidad de crecimiento a temperaturas de refrigeración (Walker y Stringer, 1987). Sus temperaturas y pH de crecimiento óptimos se muestran en la Tabla 2. Además, *L. monocytogenes* es un anaerobio facultativo. Se considera un patógeno ubicuo, que ha sido aislado del suelo, excrementos, aguas residuales, abono, agua, barro, heno, alimentos para animales, polvo, pájaros y otros animales y humanos. Se ha asociado también con material vegetal como arbustos, hierbas silvestres, cereales y vegetación en descomposición (Francis *et al.*, 1999). *L. monocytogenes* puede, por lo tanto, encontrarse de forma natural en muchas frutas y hortalizas contaminadas a través de prácticas agrícolas como el riego con agua contaminada o la fertilización con abono contaminado (Nguyen-The y Carlin, 1994). Sin embargo, la incidencia de *L. monocytogenes* en frutas frescas es muy baja y sólo se conocen dos casos de intoxicaciones alimentarias (Tabla 1).

La listeriosis puede causar gastroenteritis, sin embargo, los síntomas más comunes son la fiebre, dolor muscular y náuseas. Los grupos de riesgo en caso de listeriosis son las mujeres embarazadas, los ancianos y los inmunocomprometidos. En el caso de las mujeres embarazadas, la enfermedad cursa similar a la gripe y la infección puede producir la muerte del feto o un parto prematuro. En el caso de ancianos e inmunocomprometidos la enfermedad puede cursar como bacteremia y meningitis. La duración de la enfermedad es variable y tiene una tasa de mortalidad del 30 % en países industrializados.

En cuanto al crecimiento/supervivencia de *L. monocytogenes* en fruta, Beuchat y Brackett (1991) observaron reducciones significantes en la población de *L. monocytogenes* inoculada en tomate picado conservado a 10 y 21 °C, sin embargo, la reducción fue más lenta a 10 que a 21 °C. En naranjas 'Hamlin' peladas, la población de *L. monocytogenes* se mantuvo constante durante la conservación a 4 y 8 °C mientras que aumentó ligeramente a 24 °C (Pao *et al.*, 1998). La población de *L. monocytogenes* aumentó en manzanas 'Red Delicious' (pH 4.4) y melón 'Honeydew' (pH 5.8) mínimamente procesados conservados a 10 °C durante 7 días (Leverentz *et al.*, 2003a). Penteado y Leitao (2004a) demostraron que las pulpas de melón (pH 5.87±0.13), sandía (pH 5.50±0.06) y papaya (pH 4.87±0.01) son sustratos adecuados para el crecimiento de *L. monocytogenes* a 10, 20 y 30 °C. Los autores observaron además que la velocidad de crecimiento del patógeno era proporcional al pH de la pulpa y, por tanto, se observaron mayores incrementos en melón seguido de sandía y por último en papaya. La población de *L. monocytogenes* inoculada en fresas cortadas (pH 3.6-3.8) disminuyó tras 48 h a 24 °C y 7 días a 4 °C, respectivamente (Flessa *et al.*, 2005). Leverentz *et al.* (2006) observaron crecimiento de la población de

L. monocytogenes en trozos de manzana 'Golden Delicious' tras 5 días a 10 °C y 2 días a 25 °C. La población de *L. monocytogenes* aumentó aproximadamente 2 unidades logarítmicas en heridas de manzana 'Golden Delicious'tras 2 días a 25 °C (Trias *et al.*, 2008b). A diferencia de lo descrito para *Salmonella* y *E. coli* O157:H7, la población de *L. monocytogenes* aumentó en peras 'Flor de Invierno' cortadas tras 7 días de conservación a 5 °C (Raybaudi-Massilia *et al.*, 2009a).

2.4. Legislación

El Reglamento (CE) nº 1441/2007 que modifica el Reglamento (CE) nº 2073/2005 relativo a los criterios microbiológicos aplicables a los productos alimenticios, establece los criterios microbiológicos para las frutas y hortalizas mínimamente procesadas. Este reglamento fija el recuento de *E. coli* como un índice de la higiene del proceso. En el caso de "frutas y hortalizas troceadas (listas para el consumo)" el recuento de *E. coli* debe ser inferior a 100 ufc g^{-1} en la fase de elaboración (n=5, c=2, M=10^3 ufc g^{-1}; m=10^2 ufc g^{-1}, donde n=número de unidades que componen la muestra y c=número de muestras que pueden dar entre m y M). Este reglamento marca, además, los criterios de seguridad de los alimentos. Según las referencias existentes, y aunque habría que estudiar cada caso en particular, podemos considerar que las frutas y hortalizas mínimamente procesadas son "Alimentos listos para el consumo que pueden favorecer el desarrollo de *L. monocytogenes*, que no sean los destinados a los lactantes ni para usos médicos especiales" y, por tanto, *L. monocytogenes* debe estar ausente en 25 g de alimento en el lugar de elaboración y debe ser inferior a 100 ufc g^{-1} a lo largo de su vida útil (n=5, c=0). En el caso de *Salmonella*, en "frutas y hortalizas troceadas (listas para el consumo)" debe estar ausente en 25 g de producto a lo largo de su vida útil (n=5, c=0).

3. ESTRATEGIAS DE INTERVENCIÓN PARA REDUCIR MICROORGANISMOS PATÓGENOS Y ALTERANTES EN FRUTAS Y HORTALIZAS

La presencia de patógenos de transmisión alimentaria en frutas y hortalizas frescas así como las intoxicaciones alimentarias asociadas a este tipo de productos representan un serio problema de salud pública. La contaminación de frutas y hortalizas con patógenos humanos tiene también importantes consecuencias económicas. Por lo tanto, a la industria le interesa desarrollar intervenciones para reducir el riesgo de contaminación microbiana. La intervención más eficaz es reducir el riesgo mediante la implementación de planes como los de Buenas Prácticas Agrícolas, Buenas Prácticas de Producción (BPA y BPP) y el programa de Análisis de Peligros y Puntos de Control Crítico (APPCC). Sin embargo no

siempre es posible evitar la contaminación microbiana y son necesarias intervenciones de control, ya sean de desinfección o de conservación.

La eficacia de los métodos de desinfección ha de evaluarse desde diferentes puntos de vista. En primer lugar debe evaluarse la reducción microbiana obtenida e, incluso más importante, el mantenimiento de esta reducción a lo largo de la vida útil del producto. Por lo tanto, la efectividad dependerá de la sensibilidad del microorganismo y la accesibilidad del agente antimicrobiano o tratamiento a los microorganismos. Otro factor que determinará el éxito de las técnicas de desinfección y/o conservación es la aparición de efectos indeseables como el ablandamiento de tejidos o decoloración. Además, el daño fisiológico producido por los tratamientos puede aumentar la actividad microbiana, ya que la fracción de microorganismos supervivientes podrá crecer más rápidamente gracias al aumento en la disponibilidad de nutrientes. Otros factores relacionados son el efecto de estas técnicas en la calidad nutricional del producto y la posible formación de subproductos que puedan tener implicaciones en la salud humana, como en el caso de la desinfección con cloro y la formación de subproductos como los trihalometanos y ácidos haloacéticos (Chang *et al.*, 2000; Nieuwenhuijsen *et al.*, 2000). Por último, el éxito de una técnica de conservación está también relacionado con factores económicos como los gastos relacionados a los equipos necesarios para la aplicación de dicha técnica (Ragaert *et al.*, 2007).

En el control de microorganismos en alimentos es interesante la aplicación de tecnologías combinadas (tecnología de barreras u obstáculos o *hurdle technology*). Esta tecnología está basada en la aplicación de combinaciones adecuadas de factores limitantes para el crecimiento de microorganismos como la actividad de agua, pH, temperatura, acidez, potencial redox, microorganismos competitivos, atmósfera modificada y conservantes (Wiley, 1994). Mediante la combinación de estos obstáculos, la intensidad de cada una de las técnicas puede ser relativamente baja, minimizando la pérdida de calidad, mientras que el impacto total producido en los microorganismos es alto (Rico *et al.*, 2007).

3.1. Estrategias de intervención físicas

La vida útil de las frutas mínimamente procesadas viene determinada principalmente por la temperatura de conservación y el envasado en atmósfera modificada. El uso de ambas tecnologías ayuda a reducir los procesos de degradación que ocurren a lo largo de la conservación, frenando la respiración y deshidratación de los productos. Su uso es también muy importante para controlar la alteración microbiana de la fruta mínimamente procesada.

3.1.1. Temperatura de conservación

El control de la temperatura de conservación (entre 0 y 4 °C) es el factor más importante para mantener la calidad y seguridad de las frutas y hortalizas mínimamente procesadas. La temperatura óptima de conservación depende de cada producto, sin embargo, el crecimiento microbiano, la tasa de respiración y el deterioro de la calidad de la mayoría de productos se reduce drásticamente utilizando temperaturas de conservación bajas (2-5 °C) (Luo, 2007).

La conservación en frío es crítica para el control de *Salmonella* y *E. coli* O157:H7 en frutas y hortalizas mínimamente procesadas. Zhuang *et al.* (1995) observaron que *Salmonella* puede crecer en tomates conservados a 20 y 30 °C pero no a 10 °C. La población de *E. coli* O157:H7 aumentó en melón mínimamente procesado a temperaturas entre 12 y 25 °C, pero no a 5 °C (Del Rosario y Beuchat, 1995). Sin embargo, la población de *L. monocytogenes*, microorganismo psicrótrofo, aumentó en frutas y hortalizas frescas y cortadas a temperaturas de refrigeración (Farber *et al.*, 1998).

El aumento en la temperatura de conservación ocurre normalmente durante la distribución y venta. Debido a las dificultades de mantener la cadena de frío, se necesitan barreras adicionales para controlar el crecimiento de patógenos, especialmente los psicrótrofos. La combinación de factores inhibitorios intrínsecos, extrínsecos y de proceso pueden mejorar considerablemente la seguridad de los productos.

3.1.2. Envasado en atmósfera modificada

El envasado en atmósfera modificada se utiliza comercialmente, tanto en frutas y hortalizas frescas como mínimamente procesadas, como estrategia para mantener la seguridad del producto y aumentar su vida útil. Esta estrategia utiliza, generalmente, una atmósfera en el interior del envase diferente al aire (el aire es, aproximadamente, <0.1 % CO_2, 21 % O_2, 78 % N_2). Aunque se han estudiado muchos gases diferentes, normalmente se usan combinaciones de O_2, CO_2 y N_2, bajando los niveles de O_2 e incrementando los niveles de CO_2 por encima de los valores atmosféricos. Los microorganismos pueden verse afectados por la actividad antimicrobiana del CO_2.

El envasado en atmósfera modificada es un proceso dinámico donde las características ambientales y de envasado y el producto interaccionan para crear una atmósfera interna equilibrada. Esta atmósfera se consigue cuando la tasa de consumo de O_2 y la tasa de generación de CO_2, como resultado de la respiración del producto envasado, igualan a la tasa de transmisión de los gases a través del material de envasado. La atmósfera de equilibrio puede crearse activamente, cuando la atmósfera interna se establece inicialmente mediante la inserción de la atmósfera deseada, o bien pasivamente, cuando la atmósfera de envasado se crea por la propia respiración del producto. La atmósfera interna depende de factores

extrínsecos e intrínsecos, incluyendo la tasa de respiración, la permeabilidad del film a los gases y vapor de agua, las dimensiones del envase y la cantidad de producto. La actividad respiratoria del producto depende, así mismo, del producto en cuestión, la variedad, el estado de madurez, el tipo de tejido y el peso. La temperatura es el factor extrínseco más importante ya que afecta tanto a la respiración del producto como a la permeabilidad del film (Werner y Hotchkiss, 2006). Por tanto, la permeabilidad del film al O_2 y CO_2 debe estar relacionada con la respiración del producto envasado. Hay que tener en cuenta que las frutas se envasan normalmente en contenedores rígidos para protegerlas de los daños mecánicos producidos durante el transporte y manipulación y, por tanto, la transmisión de los gases tiene lugar únicamente por las tapas de los contenedores.

El envasado en atmósfera modificada ayuda a ofrecer una vida útil adecuada para los productos mínimamente procesados, sobretodo si se usan en combinación con otras barreras o estrategias de control. Las estrategias de envasado deben definirse para cada producto y método de preparación, ya que las características del producto y los efectos indirectos del procesado pueden influir en la atmósfera de envasado, el crecimiento microbiano y la vida útil.

El CO_2 inhibe el crecimiento microbiano (Devlieghere y Debevere, 2000), afectando a la fase de latencia, a la tasa de crecimiento y a las densidades máximas de población alcanzables. Los niveles de CO_2 superiores al 5 % tienen un efecto bacteriostático (Hotchkiss y Banco, 1992). Aunque no se conoce al 100 % el modo de acción del CO_2, parece ser que produce una serie de cambios como variaciones del pH intracelular, la alteración de la estructura y función de proteínas y enzimas microbianos y la alteración de la función de la membrana celular. El efecto antimicrobiano del CO_2 se incrementa al disminuir la temperatura ya que aumenta la solubilidad del CO_2.

Sin embargo, el uso del envasado en atmósferas modificadas podría ser motivo de preocupación para la salud pública. En primer lugar, las atmósferas gaseosas y las temperaturas de conservación utilizadas pueden inhibir el desarrollo de algunos microorganismos alterantes aerobios (Farber, 1991). Algunos de estos microorganismos pueden ser competidores naturales de los patógenos y su supresión puede facilitar la supervivencia y el crecimiento de patógenos anaerobios facultativos sin que el producto muestre signos de alteración. En segundo lugar, el envasado en atmósfera modificada incrementa la vida útil de los productos y por tanto aumenta el tiempo para que los patógenos puedan crecer. En tercer lugar, aunque los bajos niveles de oxígeno en los envases (2-5 %) deberían inhibir el crecimiento de anaerobios estrictos, como *Clostridium botulinum*, la temperatura de conservación excesiva, facilita que los envases puedan llegar a tener condiciones de anaerobiosis debido al aumento de respiración del producto y podría permitir el crecimiento de *C. botulinum* y la producción de la toxina botulínica.

3.2. Estrategias de intervención químicas

El lavado y desinfección de los productos vegetales es el único método existente para eliminar la contaminación microbiana de los productos mínimamente procesados. Antes del procesado, la fruta puede ser lavada, al igual que tras el pelado y cortado, con agua a la que se ha añadido algún tipo de desinfectante como el cloro, dióxido de cloro, fosfato trisódico, peróxido de hidrógeno, ácidos orgánicos u ozono. Aunque existe una gran variedad de agentes desinfectantes, su eficacia es variable y ninguno de ellos es capaz de asegurar la completa eliminación de patógenos. Por lo tanto, no es posible depender únicamente de la desinfección para controlar la contaminación y debe tenerse en cuenta la posibilidad de contaminación cruzada en el caso que el agua no esté suficientemente desinfectada. Asimismo, debe considerarse la seguridad de estas sustancias, así como sus requerimientos legales (EU Scientific Committee on Food, 2002).

Un lavado adecuado de los productos ya cortados debe implicar un pre-lavado en forma de ducha para eliminar la suciedad y los exudados celulares de las superficies, seguido por la inmersión del producto en un tanque de lavado que contenga el agente desinfectante. El aclarado del producto es opcional en función del desinfectante. Es recomendable que el agua fluya en la dirección contraria al movimiento del producto a través de las diferentes operaciones unitarias, así, el agua utilizada en el tanque de lavado puede ser utilizada en el prelavado y el agua del aclarado puede incorporarse al tanque desinfectante. Los productores de frutas y hortalizas mínimamente procesadas deben incluir en el proceso sistemas de monitorización de la calidad del agua. Los parámetros a monitorizar son el caudal de agua, el nivel de oxidantes libres, el pH, la temperatura, el potencial de óxido-reducción, la conductividad y la detección microbiana por métodos rápidos (Gil *et al.*, 2009).

La legislación sobre las sustancias utilizadas para reducir la carga microbiana de frutas y hortalizas es compleja y, en muchos casos, ambigua. En cada país el estatus de los diferentes desinfectantes es diferente. En EUA, los desinfectantes utilizados en el agua de lavado de frutas y hortalizas están regulados por la FDA como un aditivo indirecto a menos que estén considerados como sustancias GRAS (*Generally recognized as safe*). En caso de productos agrícolas que se lavan en una planta de procesado de alimentos, como es el caso de una planta de productos mínimamente procesados, tanto la EPA (*Environmental Protection Agency*) como la FDA tienen jurisdicción regulatoria y el desinfectante debe registrarse como pesticida por la EPA. En el *Code of Federal Regulations* 21 CFR *Sections* 173.315 y 178.1010, la FDA ha listado los desinfectantes para agua y soluciones desinfectantes aprobadas (21CFR173.315, 2007; 21CFR178.1010, 2003). En Europa, las sustancias utilizadas en la desinfección de frutas y hortalizas se consideran coadyuvantes de producción, es decir, “cualquier sustancia no consumida como alimento, utilizada intencionadamente en el procesado de los alimentos o sus ingredientes para cumplir ciertos propósitos tecnológicos durante el

procesado y que puede resultar en la no intencionada pero inevitable presencia de residuos de dicha sustancia o sus derivados en el producto final siempre que estos residuos no presenten ningún riesgo para la salud y no tengan ningún efecto tecnológico en el producto final" (AESAN). El cloro y el dióxido de cloro utilizados en el lavado y desinfección de frutas y hortalizas son considerados coadyuvantes de proceso (Gil *et al.*, 2009). La legislación europea relativa a los coadyuvantes de proceso no está armonizada y por tanto algunas sustancias que pueden ser legales en algunos países pueden no estar permitidas en otros países miembros.

Los posibles agentes desinfectantes en la industria de IV gama son el hipoclorito sódico, el dióxido de cloro, el ozono, el agua electrolizada, algunos ácidos orgánicos, el clorito de sodio acidificado y el ácido peracético, entre otros. A continuación se detallan los agentes desinfectantes evaluados en el transcurso de la tesis.

3.2.1. Hipoclorito sódico

El hipoclorito sódico se ha utilizado durante muchos años para potabilizar el agua y tratar las aguas residuales, así como para desinfectar los equipos de procesado de alimentos y las superficies. Asimismo, es el desinfectante más utilizado en la industria de frutas y hortalizas frescas a unas concentraciones de 50-200 ppm con un tiempo de contacto de 1-2 minutos (Beuchat, 1998).

La actividad letal o inhibitoria del hipoclorito sódico depende de la cantidad de cloro libre (como ácido hipocloroso o HOCl) disponible en el agua que entra en contacto con las células microbianas. La disociación del hipoclorito depende del pH, favoreciéndose el HOCl a medida que se reduce el pH de la solución. A 20 °C, los porcentajes de hipoclorito en forma HOCl a pH de 6.0 y 8.0 son del 97 % y 23 %, respectivamente. Los valores de pH de 6.0 a 7.5 son los más apropiados para evitar la posible corrosión de contenedores metálicos y equipos del proceso. Asimismo, hay que tener en cuenta que por debajo de pH 4 se forma cloro en gas, que es tóxico. A un mismo pH, a medida que la temperatura disminuye, el equilibrio favorece al ácido hipocloroso ya que el cloro se vaporiza a medida que la temperatura del agua aumenta. El hipoclorito pierde su actividad rápidamente en contacto con la materia orgánica o la exposición al aire, luz o metales. Además, una exposición prolongada a los vapores del cloro pueden causar irritaciones en la piel y el tracto respiratorio de los trabajadores y, en contacto con la materia orgánica, se pueden formar compuestos organoclorados (trihalometanos y ácidos haloacéticos) potencialmente peligrosos. La máxima solubilidad del cloro en agua es a 4 °C. Sin embargo, la temperatura del agua clorada para el tratamiento de frutas y hortalizas frescas debería de ser al menos 10 °C superior a la de los productos, de manera que exista un diferencial de temperatura positivo y se minimice la internalización de agua en los tejidos (Zhuang *et al.*, 1995). La internalización del agua, que puede contener microorganismos, incluyendo patógenos, es un punto de control crítico en la manipulación, procesado y

desinfección de las frutas y hortalizas crudas. La eficacia del hipoclorito depende, además, del tipo de producto y la diversidad de microorganismos.

La FDA especifica que el hipoclorito sódico puede utilizarse para lavar frutas y hortalizas siempre que esté seguido por un aclarado (21CFR173.315, 2007). El lavado con cloro es efectivo eliminando células bacterianas, levaduras, mohos y virus, sin embargo las esporas bacterianas y fúngicas son más resistentes (Dychdala, 2001). No obstante, el lavado con cloro es poco efectivo para inactivar las bacterias adheridas o cuando forman biofilms en las superficies de frutas y hortalizas. En general, las reducciones de microbiota nativa o patógenos humanos en frutas y hortalizas lavadas con agua clorada no superan los 2 logaritmos debido a la inaccesibilidad a los microorganismos adheridos, la resistencia de los microorganismos en biofilms y a la rápida degradación del cloro. Por ejemplo, el tratamiento de lechuga y col cortada con 200 ppm de hipoclorito durante 10 min redujo la concentración de *L. monocytogenes* 1.7 y 1.2 unidades logarítmicas, respectivamente (Zhang y Farber, 1996). Asimismo, Beuchat *et al.* (1998) demostraron que la inmersión de manzanas, tomates y lechuga en una solución de 2000 ppm de hipoclorito durante 1 min alcanzaba una reducción máxima de patógenos humanos de 2.3 unidades logarítmicas por cm^2. Aunque estas reducciones son suficientes para reducir el deterioro de los productos, no lo son para asegurar su seguridad en caso de contaminación con patógenos. La efectividad del cloro puede mejorarse con la adición de agentes humectantes y surfactantes de manera que pueda penetrar en las grietas y poros de las superficies de los productos en que los microorganismos pueden introducirse y evitar el contacto con el desinfectante (Beuchat, 1998).

La concentración de cloro total o libre debe controlarse mediante el uso de kits basados en la colorimetría o midiendo el potencial de óxido-reducción (ORP) del agua de proceso y se debe utilizar este valor para controlar la adición de hipoclorito y el pH. El valor de ORP recomendado es de 650 mV (Suslow *et al.*, 2000).

Las ventajas de la desinfección con cloro son su amplio espectro antimicrobiano, su facilidad de aplicación y su bajo coste. No obstante es muy corrosivo y puede dañar los equipos de acero inoxidable tras una exposición prolongada, así como puede dar lugar a manchas en los productos. Otro inconveniente es su rápida descomposición y la formación de productos potencialmente carcinogénicos y mutagénicos en contacto con materia orgánica. Además, el uso del cloro se asocia a la producción de grandes cantidades de aguas residuales con niveles muy altos de demanda biológica de oxígeno. Por los riesgos ambientales y de salud, Europa ha prohibido su uso en la producción orgánica y en algunos países como Alemania, los Países Bajos, Dinamarca, Bélgica y Suiza su uso se ha prohibido incluso en productos convencionales. Por tanto, existe la tendencia de eliminar el cloro del proceso de desinfección. Deben evaluarse otros productos desinfectantes para utilizarlos como alternativa.

3.2.2. Ácido peroxiacético

El ácido peroxiacético o ácido peracético (PAA) es el resultado del equilibrio cuaternario de la mezcla de ácido acético y peróxido de hidrógeno (Vandekinderen *et al.*, 2009). Los únicos residuos de descomposición son el ácido acético, el agua y el oxígeno. Se utiliza para la desinfección de superficies. En EUA la concentración máxima autorizada en la desinfección de frutas y hortalizas es de 80 ppm (21CFR173.315, 2007). Sin embargo existen estudios que demuestran que esta concentración de PAA en el agua de lavado no es suficiente para obtener reducciones sustanciales de carga microbiana en frutas y hortalizas mínimamente procesadas (Wisniewski *et al.*, 2000).

El ácido peroxiacético es un fuerte oxidante y su manipulación a altas concentraciones puede ser peligrosa. No obstante, las concentraciones de los productos comerciales no causan problemas. Entre los productos comerciales se encuentran Tsunami®, comercializado por Ecolab, Inc., VigorOx® comercializado por FMC Corp. y Proxitane® comercializado por Solvay Interox.

3.2.3. Peróxido de hidrógeno

El peróxido de hidrógeno (H_2O_2) tiene actividad bacteriostática y bactericida gracias a su fuerte poder oxidante y a la producción de especies citotóxicas (Juven y Pierson, 1996). Aunque está considerado como una sustancia segura por la FDA (GRAS), su uso en la industria alimentaria está limitado a la leche, huevo en polvo, almidón, té y vino a concentraciones entre 0.04 y 1.25 %. Además, el peróxido de hidrógeno residual en los alimentos debe eliminarse con métodos físicos y químicos apropiados durante el procesado. Sin embargo, los desinfectantes a base de ácido peracético, que contienen bajos niveles de peróxido de hidrógeno están aprobados por la FDA para ser utilizados en el lavado de frutas y hortalizas (21CFR173.315, 2007). En este caso la presencia residual de peróxido de hidrógeno no representa un obstáculo ya que la mayoría de frutas y hortalizas contienen suficiente cantidad de catalasa que puede descomponer rápidamente los residuos en agua y oxígeno. Un importante inconveniente es que es fitotóxico para algunos productos como lechuga y bayas.

3.2.4. Agentes de desinfección experimentales

En los últimos años se ha incrementado la presión por parte de los consumidores en reducir y/o eliminar los aditivos sintetizados químicamente de los alimentos. Por ello, se están realizando esfuerzos para encontrar alternativas más seguras para los humanos y más respetuosas con el medio ambiente.

Las sales de los ácidos carbónicos, como el carbonato sódico y el bicarbonato sódico son aditivos alimentarios que están permitidos sin restricciones en muchas aplicaciones (Lindsay, 1985; Multon, 1988). Recientemente se ha estudiado el uso

del carbonato sódico como potencial alternativa a los fungicidas sintéticos en el control de enfermedades de poscosecha de cítricos ya que es barato y puede usarse sin riesgo a dañar la fruta (Palou *et al.*, 2001; Smilanick *et al.*, 1999).

Los aceites esenciales son aceites aromáticos obtenidos de material vegetal (flores, yemas, semillas, ramas, cortezas, hierbas, madera, frutas y raíces). La acción antimicrobiana de los aceites esenciales en modelos alimentarios está bien documentada (Koutsoumanis *et al.*, 1998; Skandamis y Nychas, 2000; Tsigarida *et al.*, 2000). La mayoría de los aceites esenciales están clasificados como sustancias GRAS (Kabara, 1991), sin embargo, su uso como conservantes está limitado ya que las dosis efectivas pueden exceder los niveles organolépticos aceptables. El carvacrol ($C_{10}H_{14}O$) es el principal componente de los aceites esenciales del orégano y tomillo (Arrebola *et al.*, 1994; Lagouri *et al.*, 1993). Los aceites esenciales que contienen carvacrol son bioestáticos y/o biocidas frente muchas bacterias, mohos y levaduras (Burt, 2004). La vainillina (3-metox-4-hidroxibenzaldehído) es el fitoquímico predominante en las vainas de vainilla. Se le conoce efecto antimicótico (Beuchat y Golden, 1989) y bacterioestático (Fitzgerald *et al.*, 2004).

El desinfectante comercial Citrox está compuesto por flavonoides extraídos de cítricos y combinados con ácidos naturales de origen vegetal. Citrox 14 WP está diseñado específicamente para desinfectar frutas y hortalizas mínimamente procesadas. Sus distribuidores afirman que tiene un amplio espectro de actividad frente a microorganismos patógenos, hongos y parásitos, no es tóxico a las dosis recomendadas, no contaminante y con acción rápida con largo efecto residual.

El compuesto N-acetilo-L-cisteína (NAC) es un químico natural con propiedades antioxidantes sugerido como inhibidor del pardeamiento enzimático en manzana, patata y pera (Molnar-Perl y Friedman, 1990; Oms-Oliu *et al.*, 2006; Rojas-Grau *et al.*, 2006) al que también se le ha atribuido actividad antimicrobiana (Raybaudi-Massilia *et al.*, 2009b).

El quitosano, un derivado N-acetilado del polisacárido quitina, es un biopolímero de interés para su aplicación en agricultura, biomedicina, biotecnología e industria alimentaria gracias a su compatibilidad, biodegradabilidad y bioactividad (Wu *et al.*, 2005). El quitosano alarga la vida útil y controla la alteración de diversas frutas (Bautista-Baños *et al.*, 2006; Romanazzi *et al.*, 2002, 2006). El biopolímero tiene un mecanismo doble, por un lado inhibe a los mohos alterantes (El Ghaouth *et al.*, 1992) y por otro induce la respuesta de defensa del huésped (Amborabe *et al.*, 2008).

3.3. Bioconservación

Como se ha visto hasta ahora, existen muy pocos obstáculos para prevenir el crecimiento de microorganismos en frutas y hortalizas mínimamente procesadas. Los productos se lavan para eliminar la contaminación excesiva, pero tras el procesado, los únicos controles utilizados son la temperatura de refrigeración y el envasado en atmósferas modificadas. Los enfoques actuales de la conservación de alimentos abogan por el concepto de barreras, en que se utilizan múltiples factores para prevenir el crecimiento microbiano (Leistner, 1995). Sin embargo, en frutas y hortalizas mínimamente procesadas no pueden utilizarse operaciones de procesado tradicionales como la cocción y, además, los consumidores demandan productos libres de conservantes químicos. Por lo tanto la aplicación del concepto de bioconservación puede ser útil para crear obstáculos 'extras' en los productos mínimamente procesados.

Un "cultivo bioconservante" o "agente de biocontrol" es aquel cultivo que protege la vida útil de los alimentos por su capacidad para suprimir gérmenes indeseables sin alterar las propiedades organolépticas de los productos en que se aplican (Baker y Cook, 1974).

El control biológico o bioconservación presenta una serie de ventajas frente a otros sistemas de control (Deacon, 1983): el uso de antagonistas es más seguro en comparación a los principales productos químicos utilizados actualmente ya que no se acumulan en los alimentos; pueden ser más persistentes en el tiempo que los tratamientos químicos ya que es difícil que los patógenos puedan desarrollar resistencia a ellos; tienen un efecto insignificante en el balance ecológico ya que no destruyen los enemigos naturales de las especies patógenas como ocurre con los tratamientos químicos y, además, pueden ser compatibles con otros sistemas de control y por tanto se pueden aplicar juntos.

Según Wisniewski y Wilson (1992), un agente de biocontrol ideal debe ser genéticamente estable, efectivo a bajas concentraciones, poco exigente nutricionalmente, capaz de sobrevivir en condiciones adversas, efectivo contra un gran número de patógenos en diversas frutas y vegetales, capaz de reproducirse en medios de crecimiento económicos, fácil de aplicar, no tóxico para la salud humana, resistente a los productos químicos que se puedan utilizar, compatible con procedimientos comerciales y con posibilidad de formulación con larga vida útil. No obstante, aunque un antagonista tenga todas las características deseables, el factor económico decide si se comercializará o no. Asimismo, si no existe un mercado potencial no podrá comercializarse.

Un aspecto muy importante en la comercialización de los agentes de biocontrol es la aceptación por parte de la sociedad de la aplicación de microorganismos 'vivos' en los alimentos. Esta idea no es nueva, ya que desde tiempos muy antiguos, las fermentaciones han sido un método importante para preservar los alimentos, como la adición de microorganismos en la preparación del pan y en productos derivados

de la leche. Por tanto, parece que la sociedad aceptaría los antagonistas microbianos si éstos llegasen a ser una alternativa segura y efectiva a los productos químicos. En una encuesta nacional canadiense sobre la percepción pública del control biológico de enfermedades poscosecha, en general los canadienses consideraron los agentes de biocontrol como una alternativa aceptable y segura: un 70 % de los encuestados preferirían comprar alimentos producidos utilizando agentes de biocontrol que utilizando productos fitosanitarios (McNeil *et al.*, 2010).

Existen básicamente dos alternativas para usar microorganismos antagónicos como agentes de biocontrol. La primera es utilizar, estimular y manejar los microorganismos existentes en los productos. La segunda se basa en la introducción de microorganismos artificialmente para que controlen los patógenos (Leverentz *et al.*, 2003b).

3.3.1. Microorganismos epifitos

La flora microbiana presente de forma natural en las frutas y hortalizas frescas puede jugar un papel importante para mantener la seguridad alimentaria de las frutas y hortalizas mínimamente procesadas (Nguyen-The y Carlin, 1994), compitiendo con los patógenos por espacio físico y nutrientes y/o produciendo compuestos antimicrobianos que afecten de forma negativa la viabilidad de los patógenos (Liao y Fett, 2001). Existen pocas publicaciones referentes al uso de agentes de biocontrol en la prevención del crecimiento de patógenos de transmisión alimentaria en fruta mínimamente procesada. La primera referencia es un estudio llevado a cabo por Janisiewicz *et al.* (1999b) en la que se estudió la efectividad de la cepa L-59-66 de *Pseudomonas syringae* (comercializada bajo el nombre de BioSave11 o BioSave110 para el control de enfermedades de poscosecha de manzanas y peras) sobre el crecimiento de *E. coli* O157:H7 en heridas de manzana 'Golden Delicious'. Los autores no observaron crecimiento de *E. coli* O157:H7 cuando se coinoculó con el agente de biocontrol e hipotetizaron que el mecanismo antagonista era la competición por nutrientes y espacio, lo que es ventajoso ya que es muy difícil que el patógeno pueda desarrollar mecanismos de resistencia a este mecanismo. Posteriormente, Leverentz *et al.* (2006) seleccionaron 7 antagonistas de la superficie de manzanas 'Golden Delicious' que fueron ensayados frente a *L. monocytogenes* y *Salmonella enterica* serovar Poona en cilindros de manzana. De estos 7 antagonistas, *Gluconobacter asaii*, *Candida* sp., *Dicosphaerina fagi* y *Metschinikowia pulcherrima* tuvieron actividad antagonista frente uno o ambos patógenos. La población de *L. monocytogenes* se vio inhibida tanto a 10 como 25 °C, no obstante, la reducción fue mayor a 25 °C. La diferencia entre temperaturas fue debida a la mayor velocidad de crecimiento de patógeno y antagonista a 25 °C. En el caso de *Salmonella*, sólo 3 de los antagonistas fueron capaces de reducir la población de patógeno a 25 °C y ninguno de ellos fue efectivo a 10 °C ya que el patógeno sólo creció ligeramente a 10 °C. Recientemente, Abadias *et al.* (2009) probaron la eficacia del agente de biocontrol *Candida sake* CPA-1 frente a una mezcla de 5 cepas de

E. coli en manzana 'Golden Delicious'. En este caso el agente de biocontrol redujo, en aproximadamente 1 unidad logarítmica, la población de *E. coli* en heridas de manzana durante la conservación a 25 °C, sin embargo no se observó ningún efecto en cilindros de manzana.

3.3.2. Bacterias ácido-lácticas

Durante siglos se han utilizado bacterias ácido-lácticas para fermentar alimentos y así obtener alimentos estables. El consumo masivo de productos con altas concentraciones de BAL sin producir efectos negativos en la salud de los consumidores ha facilitado que estén clasificadas como GRAS por la FDA o como QPS (*Qualified Presumption of Safety*) por la EFSA (*European Food Safety Authority*). Las BAL pueden inhibir o eliminar el crecimiento de gran variedad de microorganismos como bacterias, levaduras y mohos mediante la producción de ácidos orgánicos, diacetilo, peróxido de hidrógeno, enzimas, agentes líticos y péptidos antimicrobianos o bacteriocinas. Existen diferencias considerables en el espectro antimicrobiano de los compuestos producidos por las BAL y no todas ellas pueden producir estas sustancias en la misma cantidad. El control biológico utilizando BAL puede conseguirse mediante la aplicación de dicho microorganismo como cultivo protector o bien añadiendo sus compuestos antimicrobianos al alimento.

Aplicación de BAL como cultivos protectores

Senaratne y Gilliland (2003) ensayaron la eficacia de dos cepas de BAL, *Lactobacillus delbrueckii* ssp. *lactis* RM 2-5 y *Pediococcus acidilactici* D3 frente a *Salmonella choleraesuis* en trozos de melón cantalupo. *L. delbrueckii* ssp. *lactis* RM 2-5 no tuvo ningún efecto en la población del *S. choleraesius* y, aunque *P. acidilactici* redujo significativamente la población de patógeno, la reducción observada fue tan pequeña que no tuvo importancia práctica. Posteriormente, Trias *et al.* (2008b) seleccionaron 6 BAL aisladas de frutas y hortalizas frescas para ser testadas como agentes de biocontrol frente a *E. coli*, *S.* Typhimurium y *L. monocytogenes* en heridas de manzana 'Golden Delicious'. La población de *L. monocytogenes* y *Salmonella* se redujo significativamente con 5 de las 6 BAL testadas. En cambio, ninguna de las cepas redujo la población de *E. coli*. De las 5 cepas efectivas, 3 de ellas fueron cepas de *Leuconostoc mesenteroides*, una fue *Weissella cibaria* y, la última, la cepa productora de nisina *Lactococcus lactis* subsp. *lactis* ATCC 11454. En un segundo estudio llevado a cabo por Trias *et al.* (2008a) se caracterizó la actividad antimicrobiana de 2 de las cepas de *Leuconostoc* que fue similar a las bacteriocinas de Clase II, a la que pertenece la pediocina.

Aplicación de compuestos antimicrobianos: bacteriocinas

En la mayoría de los casos, la eficacia de los cultivos de bacterias ácido lácticas protectoras se debe a los diversos antimicrobianos que producen. Las bacteriocinas son péptidos antimicrobianos sintetizados por una gran variedad de bacterias (Tagg *et al.*, 1976). Las primeras bacteriocinas descritas fueron las colicinas, producidas por *E. coli*. La mayoría de las colicinas son proteínas relativamente grandes (hasta 80 kDa) y son efectivas frente bacterias estrechamente relacionadas mediante la unión a la membrana interior (Cascales *et al.*, 2007). Actualmente el término bacteriocina se utiliza para describir péptidos catiónicos pequeños y estables al calor sintetizados por bacterias ácido-lácticas con un espectro de inhibición más amplio (Cotter *et al.*, 2005). La biopreservación se ha centrado en las bacteriocinas producidas por las bacterias ácido-lácticas ya que están asociadas de forma tradicional a los alimentos y están consideradas como seguras.

Las bacteriocinas están compuestas por un grupo de sustancias muy heterogéneas en cuanto a su estructura primaria, composición y propiedades físico-químicas. En la Tabla 3 se puede ver una clasificación propuesta por Heng y Tagg (2006).

La mayoría de las bacteriocinas son efectivas frente muchos microorganismos gram-positivos, ya sean patógenos de transmisión alimentaria o alterantes. En cambio, las bacterias gram–negativas son intrínsecamente más resistentes gracias a la protección ofrecida por la pared celular. Sin embargo, algunas pueden ser efectivas en combinación con agentes desestabilizantes de la membrana (ej. EDTA).

Un caso especial de BAL: los cultivos probióticos

Los probióticos se definen como ‘microorganismos vivos que cuando se administran en cantidades adecuadas confieren beneficios para la salud del huésped’ (WHO, 2001). La mayoría de probióticos son lactobacilos o bifidobacterias, representantes de la microbiota del intestino humano. Los probióticos han demostrado eficacia en el tratamiento de desórdenes gastrointestinales, infecciones respiratorias y síntomas alérgicos.

Los efectos de los probióticos pueden clasificarse en tres mecanismos de acción (Oelschlaeger, 2010):

1. Modulación de las defensas del huésped, tanto en el sistema inmunitario innato como en el adquirido.
2. Pueden tener un efecto directo en otros microorganismos, comensales y/o patógenos permitiendo la prevención y terapia de infecciones y en la restauración del equilibrio microbiano en el intestino.

3. Pueden basarse en acciones que afecten a los productos microbianos como las toxinas o a productos del huésped como las sales biliares o los ingredientes alimentarios de modo que pueden inactivar toxinas y desintoxicar al huésped.

Tabla 3 Clasificación de las bacteriocinas según Heng y Tagg (2006).

Clase	**Características generales**	**Ejemplos**
I. Lantibióticos	Modificadas, estables al calor, <15 kDa	
Ia. Lineal	Forman poros, catiónicas	Nisina, Lactacina 481, Plantaricina C
Ib. Globular	Inhibidor de enzimas, no catiónicas	Ninguna
Ic.Multi-componente	Dos péptidos	Lct3147, Plantaricina W
II. Péptidos no modificados	Estables al calor, <15 kDa	
IIa. Pediocinas	Anti-listeria	Pediocina PA1/AcH, Enterocina A, Sakacina A
IIb. Miscelaneo	Diferentes a las pediocinas	Enterocina B, L50, Carnobacteriocina A
IIc.Multi-componente	Dos péptidos	Lactoccina G, Plantaricina S, Lactacina F
III. Proteínas grandes	Lábil al calor, > 30 kDa	
IIIa. Bacteriolíticas	Degradan la pared celular	Enterolisina A, Lcn972
IIIb. No líticas	Atacan el citoplasma	Colicinas E2-E9
IV. Péptidos circulares	Estables al calor, enlace péptido entre cabeza y cola	AS-48, Gassericina a, Acidocina B

Las bacterias probióticas, algunas de las cuales son bacterias ácido-lácticas, no sólo mejoran la salud con su consumo, si no que pueden tener un papel protector frente a patógenos de transmisión alimentaria en el alimento a lo largo de su conservación, actuando como bioconservantes (Rydlo *et al.*, 2006).

La aplicación de los cultivos probióticos se ha realizado principalmente en productos lácteos, sin embargo, el consumo de lácteos presenta inconvenientes como la intolerancia a la lactosa, el contenido en colesterol y la presencia de proteínas lácteas alérgenas. Por lo tanto, es esencial el desarrollo de alimentos

probióticos no lácteos (Granato *et al.*, 2010). Los zumos de frutas, postres y productos a base de cereales podrían ser medios adecuados para la distribución de los probióticos (Cargill, 2008) así como las frutas y hortalizas, ya que contienen nutrientes beneficiosos como minerales, vitaminas, fibras y antioxidantes, además de estar exentos de alérgenos lácticos (Sheehan *et al.*, 2007).

La viabilidad del probiótico en la matriz alimentaria depende de factores como el pH, la temperatura de conservación, niveles de oxígeno y presencia de microorganismos competidores e inhibidores. Por tanto, la aplicación de cultivos probióticos en productos no lácteos representa un gran reto ya que la actividad y viabilidad del probiótico debe mantenerse por periodos extensos de tiempo y llegar al consumidor de forma viable para poder ejercer sus propiedades funcionales (Shah, 2007). Sin embargo, no existe un acuerdo general sobre los niveles de probiótico recomendados y se han sugerido desde 10^6 ufc g^{-1} (Kurman y Rasic, 1991) hasta 10^7 y 10^8 ufc g^{-1} (Lourens-Hattingh y Viljoen, 2001). No obstante, incluso las células no viables pueden ejercer ciertas propiedades funcionales como la inmunomodulación (Ouwehand *et al.*, 1999). La viabilidad y actividad del probiótico son consideraciones de gran importancia, ya que estas bacterias deben sobrevivir en el alimento durante su vida útil, durante el tránsito a través de las condiciones ácidas del estómago y resistir la degradación de enzimas hidrolíticos y sales biliares en el intestino delgado.

Existen pocos estudios sobre la viabilidad de cultivos probióticos en frutas y zumos de frutas. Por ejemplo, Sheenan *et al.* (2007) observaron que la cepa probiótica *Bifidobacterium animalis* ssp. *lactis* Bb-12 mantuvo su viabilidad por encima de 10^6 ufc mL^{-1} hasta 6 semanas en zumo de naranja y hasta 4 semanas en zumo de piña conservados a 4 °C. En cambio la cepa comercial *Lactobacillus rhamnosus* GG se mantuvo viable a niveles aceptables en ambos zumos durante 12 semanas. Sin embargo, la concentración fue superior en zumo de naranja. Por tanto, el bajo pH del zumo de piña (3.40) comparado con el de naranja (3.65) causó un descenso más rápido en la población del probiótico. No obstante, ninguna de las cepas probióticas testadas sobrevivió en zumo de arándanos. Tapia *et al.* (2007) observaron que la viabilidad de *B. lactis* Bb-12 en recubrimientos comestibles a base de alginato y goma gellan en manzana y papaya mínimamente procesadas se mantuvo a lo largo de 10 días de conservación a 2 °C. Recientemente, Rößle *et al.* (2010) estudió la viabilidad de *L. rhamnosus* GG en trozos de manzana 'Breaburn' conservadas a 2-4 °C durante 10 días. Este probiótico mantuvo su concentración a valores superiores a 10^8 ufc g^{-1} a lo largo de la conservación con evaluaciones físico-químicas y sensoriales aceptables.

Además de la viabilidad en los alimentos, las bacterias probióticas deben resistir al tracto intestinal. En primer lugar, los cultivos deben tolerar la presencia de pepsina y el bajo pH del estómago. Aunque el pH del estómago puede aumentar hasta 6.0 después de la ingesta de comida (Johnson, 1977), normalmente está entre 2.5 y 3.5 (Holzapfel *et al.*, 1988), pudiendo llegar a un pH de 1.5 (Lankaputhra y Shah,

1995). Tras el paso por el estómago, el intestino delgado es la segunda barrera del tracto intestinal. Aunque su pH (7.0-8.5) es más favorable, la presencia de pancreatinina y sales biliares pueden tener efectos adversos. La bilis secretada en elintestino delgado reduce la viabilidad de las bacterias destruyendo sus membranas celulares, compuestas principalmente por lípidos y ácidos grasos. Estas modificaciones pueden no sólo afectar a la permeabilidad celular y viabilidad, sinó también a las interacciones entre la membrana y el ambiente (Gilliland *et al.*, 1984). Cepas de *L. rhamnosus* aisladas de queso Parmigiano Reggiano evidenciaron buena supervivencia en presencia de 1.0, 4.5 y 2.0 % de sales biliares (Succi *et al.*, 2005).

La literatura muestra que un bajo pH durante el crecimiento bacteriano puede inducir una respuesta de tolerancia al ácido (adaptación al estrés ácido o habituación). La inducción de esta respuesta puede proteger la bacteria probiótica no sólo del pH sinó también de otros estreses como los choques térmicos, osmóticos u oxidativos (Van de Guchte *et al.*, 2002). Champagne y Gardner (2008) sugirieron que la conservación de bacterias en una bebida de fruta ácida podría mejorar la resistencia al subsiguiente estrés gastrointestinal. Sin embargo, observaron lo contrario con 4 cepas de probióticos que habían estado conservadas durante 35 días a 4 °C en una bebida de frutas.

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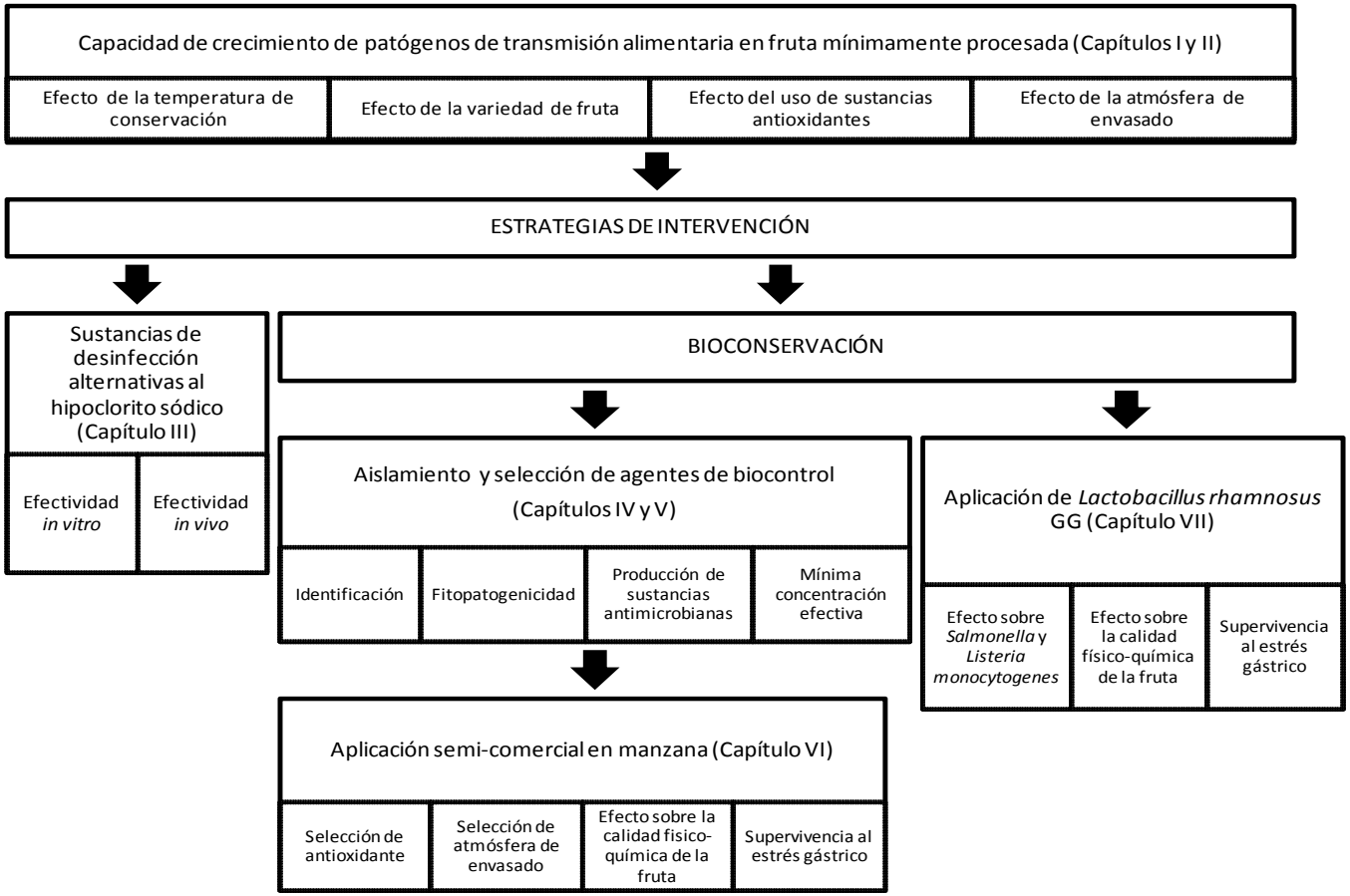
II. OBJETIVOS

El aumento de popularidad y consumo de los productos de IV gama entre la población representa un nuevo reto en el ámbito de la seguridad alimentaria. Por ello, con la realización de esta tesis se persiguen dos objetivos principales. En primer lugar, determinar si los patógenos de transmisión alimentaria, *E. coli* O157:H7, *Salmonella* spp. y *Listeria* spp. son capaces de sobrevivir y/o multiplicarse en frutas ácidas mínimamente procesadas que, debido a su bajo pH tradicionalmente se han considerado seguras, y, si es así, estudiar nuevas estrategias de intervención.

Para la consecución de estos objetivos generales se plantean los siguientes objetivos específicos:

1. Determinar la supervivencia y crecimiento de tres patógenos de transmisión alimentaria, *E. coli* O157:H7, *Salmonella* y *Listeria innocua*, en manzanas y melocotones mínimamente procesados en función de la temperatura de conservación, la variedad de fruta, la presencia de sustancias antioxidantes y la atmósfera de envasado.
2. Evaluación de distintas sustancias antimicrobianas como alternativa al hipoclorito sódico para reducir la población de *E. coli* O157:H7, *Salmonella* spp. y *Listeria* spp. en manzana 'Golden Delicious' mínimamente procesada y su evolución durante la posterior conservación a 10 °C.
3. Evaluación de la capacidad antagonista de microorganismos aislados de fruta fresca y mínimamente procesada frente a *E. coli* O157:H7, *Salmonella* y *L. innocua* en manzanas y melocotones mínimamente procesados.
4. Seleccionar los mejores antagonistas, identificarlos, testar su fitopatogenicidad, la producción de sustancias antimicrobianas y determinar la mínima concentración efectiva.
5. Evaluar la compatibilidad de la cepa antagonista *Pseudomonas graminis* CPA-7 con tratamientos antioxidantes utilizados habitualmente en la industria de IV gama, su efectividad frente *Salmonella* spp. y *L. monocytogenes* en manzanas 'Golden Delicious' en condiciones que simulan su aplicación comercial y el efecto de su aplicación sobre la calidad de la fruta.
6. Estudio del efecto de la aplicación de la cepa probiótica *L. rhamnosus* GG en manzana 'Golden Delicious' mínimamente procesada conservada en condiciones comerciales simuladas en la calidad de la fruta y en la población de *Salmonella* spp. y *L. monocytogenes*.
7. Evaluación de la supervivencia de los cultivos de *P. graminis* CPA-7, *L. rhamnosus* GG, *Salmonella* spp. y *L. monocytogenes* a condiciones de estrés gástrico simuladas tras su cultivo en manzana mínimamente procesada.

III. PLAN DE TRABAJO


Capacidad de crecimiento de patógenos de transmisión alimentaria en fruta mínimamente procesada (Capítulos I y II)
Efecto de la temperatura de conservación
Efecto de la variedad de fruta
Efecto del uso de sustancias antioxidantes
Efecto de la atmósfera de envasado
ESTRATEGIAS DE INTERVENCIÓN
Sustancias de desinfección alternativas al hipoclorito sódico (Capítulo III)
Efectividad *in vitro*
Efectividad *in vivo*
BIOCONSERVACIÓN
Aislamiento y selección de agentes de biocontrol (Capítulos IV y V)
Identificación
Fitopatogenicidad
Producción de sustancias antimicrobianas
Mínima concentración efectiva
Aplicación de *Lactobacillus rhamnosus* GG (Capítulo VII)
Efecto sobre *Salmonella* y *Listeria monocytogenes*
Efecto sobre la calidad físico-química de la fruta
Supervivencia al estrés gástrico
Aplicación semi-comercial en manzana (Capítulo VI)
Selección de antioxidante
Selección de atmósfera de envasado
Efecto sobre la calidad fisico-química de la fruta
Supervivencia al estrés gástrico

IV. PUBLICACIONES

CAPÍTULO I

Factors affecting growth of foodborne pathogens on minimally processed apples

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ABSTRACT

Escherichia coli O157:H7, *Salmonella* and *Listeria innocua* increased by more than 2 $\log_{10}$ units over a 24 h period on fresh-cut 'Golden Delicious' apple plugs stored at 25 and 20 °C. *L. innocua* reached the same final population level at 10 °C meanwhile *E. coli* and *Salmonella* only increased 1.3 $\log_{10}$ units after 6 days. Only *L. innocua* was able to grow at 5 °C. No significant differences were observed between the growth of foodborne pathogens on fresh-cut 'Golden Delicious', 'Granny Smith' and 'Shampion' apples stored at 25 and 5 ºC. The treatment of 'Golden Delicious' and 'Granny Smith' apple plugs with the antioxidants, ascorbic acid (2 %) and NatureSeal® (6 %), did not affect pathogen growth. The effect of passive modified atmosphere packaging (MAP) on the growth of *E. coli*, *Salmonella* and *L. innocua* on 'Golden Delicious' apple slices was also tested. There were no significant differences in growth of pathogens in MAP conditions compared with air packaging of 'Golden Delicious' apple plugs, but the growth of mesophilic and psychrotrophic microorganisms was inhibited. These results highlight the importance of avoiding contamination of fresh-cut fruit with foodborne pathogens and the maintenance of the cold chain during storage until consumption.

Keywords: temperature; variety; antioxidant substances; modified atmosphere packaging, *Escherichia coli*; *Salmonella*; *Listeria innocua*

1. INTRODUCTION

During the last decade pre-prepared minimally processed fruit and vegetables have become popular among European consumers due to increased interest in healthy and nutritious diets and changes in consumer lifestyles. In Spain, sales of ready-to-eat fruit and vegetables increased by 13.4 % between 2006 and 2007 (Anonymous, 2008). However, only 0.9 % of total sales in 2007 (55,156 Tm) was fresh-cut fruit (Anonymous, 2008). Thus, a high potential for fresh-cut fruit industry exists, especially, in our area (Lleida, Catalonia) which is the main apple and peach producer in Spain. Unfortunately, the rise in consumption of fresh-cut produce has resulted in increased frequency of outbreaks of illness associated with raw fruits and vegetables. Outbreaks of illness caused by the consumption of bacteria-contaminated intact fruits and vegetables occurs less frequently than those caused by the consumption of other foods (Beuchat, 1996; CDC 1990; Drosinos *et al.*, 2000). The difference is believed to be due, in part, to the protective barriers (physical and chemical) provided by the peel or rind. However, these protective barriers are removed during the processing of fresh-cut fruits and vegetables potentially increasing their vulnerability to microbial contamination and colonization thereby increasing the risk of fresh-cut produce becoming a health hazard (Leverentz *et al.*, 2001).

Minimally processed produce has been implicated in outbreaks caused by foodborne pathogens (FBP) such as *Escherichia coli* O157:H7 (Ackers *et al.*, 1998), *Salmonella* spp. (Lin *et al.*, 1996; Salleh *et al.*, 2003) and *Listeria monocytogenes* (Beuchat, 1996). Fresh apple products, especially juices, have been associated with outbreaks of illness caused by *E. coli* O157:H7 (Burnett and Beuchat, 2000; Dingman, 2000) and several studies have shown that *E. coli* O157:H7 can survive and grow on fresh apple tissues stored in air (Dingman, 2000; Fisher and Golden, 1998; Gunes and Hotchkiss, 2002; Janisiewicz *et al.*, 1999). *Salmonella* has been implicated in human illnesses that have been associated with consumption of apple cider and unpasteurized orange juices (CDC, 1975, 1995, 1999; Krause *et al.*, 2001). Furthermore it was demonstrated that *Salmonella* Enteritidis was able to survive at 5 °C and grow at 10 and 20 °C on 'Red Delicious' apple flesh (pH 4.2) (Leverentz *et al.*, 2001). Although *L. monocytogenes* has been isolated from a variety of raw vegetables, its association with fruits or acidic vegetables is less well documented. The growth of *L. monocytogenes* under refrigerated and ambient conditions was demonstrated for several vegetables (Harris *et al.*, 2003) and non-acidic fruits (Ukuku and Fett, 2002). Growth has also been demonstrated on the outer surface of acidic fruits such as tomatoes (Beuchat and Brackett, 1991) and peeled Hamlin oranges (Pao *et al.*, 1998) when stored at temperatures greater than 20 °C. *L. monocytogenes* growth has also been demonstrated in 'Golden Delicious' apple slices stored at 20 and 10 °C (Conway *et al.*, 2000).

The shelf life of minimally processed products can be extended by using modified atmosphere packaging (MAP) with reduced-O_2 and/or elevated CO_2 modified atmosphere (MA), the use of anti-browning agents (such as ascorbic acid and NatureSeal®), and refrigeration (Soliva-Fortuny *et al.*, 2001). Atmospheres with low O_2 inhibit the growth of most aerobic spoilage microorganisms, while the growth of pathogens, especially the anaerobic psychrotrophic, non-proteolytic clostridia, may occur or even become stimulated (Farber, 1991). Passive MAP, where the atmosphere is derived from the produce respiration rate and gas permeability of the packaging film, induces a passively established steady state after a long transient period. In contrast, active MAP involves the introduction of a gas scavenging system within the package and is used to accelerate gas composition modification in order to avoid product exposure to high concentrations of unsuitable gases (Vermeiren *et al.*, 1999).

Within framework of an Integrated EU Project 'Increasing fruit consumption through a trans-disciplinary approach delivering high quality produce from environmentally friendly, sustainable production methods' (www.isafruit.org) we aim to determine the effect of apple variety and temperature on the growth of *E. coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally processed apples. We also investigated the effect of the addition of antioxidant substances and the use of modified atmosphere packaging on the survival and growth of all three FBP.

2. MATERIALS AND METHODS

2.1. Fruit

'Golden Delicious', an example of a sweet eating variety, and 'Granny Smith', a variety that is very acidic, apples were obtained from packinghouses in Lleida, Catalonia. 'Shampion' apples were received from the Research Institute of Pomology and Floriculture (RIPF, Skierniewice, Poland, ISAFRUIT Partner). Prior to the experimental studies, all apples were washed in running tap water then surface disinfected with ethanol 70 %.

2.2. Preparation of bacterial inoculum

A non-pathogenic strain of *E. coli* O157:H7 (NCTC 12900), and a pathogenic strain of *Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Michigan (BAA-709 ATCC) were used. Both strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin sulphate salt (St, Sigma, Germany), thereby enabling detection on a selective medium (TSA-St) in the presence of the natural microbial flora associated with apples. The strains were grown in tryptone soy broth medium (TSB, Oxoid,

UK) supplemented with St (TSB-St) for 20-24 h at 37 °C. A strain of *Listeria innocua,* CECT-910, was used as a microbial surrogate of *L. monocytogenes* as previous studies have demonstrated that this is a valid model for *L. monocytogenes* (Francis and O'Beirne 1997). *L. innocua* was grown in TSB supplemented with 6 g L^{-1} yeast extract (tryptone yeast extract soy broth, TYSEB) at 37 °C for 20-24 h.

Bacterial cells were harvested by centrifugation at 9820 × g, 10 min at 10 °C 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 according to standard curves. For the inoculum preparation, a volume of the FBP concentrated suspension was added to 5 mL of SP in order to obtain approximately 1×10^{7} cfu mL^{-1}. Concentration of inoculum was checked by spread plating appropriate dilutions on TSA-St for *E. coli* O157:H7 and *Salmonella* or on Palcam agar (Palcam Agar Base with Palcam selective supplement, Biokar Diagnostics) for *L. innocua*. The agar plates were incubated overnight at 37±1 °C.

2.3. Population dynamics in ambient conditions

Apples were cut in half and plugs 1.2 cm diameter, 1 cm long were taken using a cork borer. For the antioxidant assay, 'Golden Delicious' apple plugs were suspended in a 2 % (w/v) solution of ascorbic acid (AA) for 2 min at 150 rpm. In the case of 'Granny Smith' apples, the antioxidant used was AS1 NatureSeal® (NS, AgriCoat Ltd, UK). To test the effect of NS, 'Granny Smith' apple plugs were suspended in a 6 % (w/v) solution of the antioxidant. After the treatment, apple plugs were let to dry in a laminar flow biosafety cabinet. Apple plugs (with and without antioxidant) were placed into sterile glass test tubes.

The prepared *E. coli* O157:H7, *Salmonella* or *L. innocua* inoculum was pipetted (15 µL) onto the top surface of fruit tissue plugs. Apple plugs were incubated at 25±1 °C, 20±1 °C, 10±1 °C and 5±1 °C. The incubation time was as long as apple pieces had satisfactory appearance (no visible symptoms of decay).

At each sample time, fruit plugs were each placed into a sterile plastic bag (BagPage 80 mL, Interscience BagSystem, St Nom La Breteche, France) and 9 mL of SP were added. It was homogenised in a stomacher blender for 120 s at high speed (Bagmixer 100 Minimix, Interscience). Aliquots of the mixture were then serially diluted and surfaced plated onto the selective agars for determination of bacterial counts. There were three replicate apple plugs for each pathogen and sampling time and each assay was repeated twice.

2.4. Population dynamics in modified atmosphere conditions

'Golden Delicious' apples were cut into 2 cm slices to simulate real conditions. Groups of four slices were placed in polypropylene (10×15×6.67 cm) trays and a 6 mm diameter well was made on each slice apple tissue using a cork borer. The *E. coli* O157:H7, *Salmonella* or *L. innocua* suspension was pipetted (15 µL) into the tissue well (each FPB inoculated in an apple slice in the same tray, one slice was left uninoculated). The apple trays were then sealed with a 40,000 cc m^{-2} 24 h^{-1} oxygen transmission rate (OTR) polypropylene film and incubated at 25±1 °C for 1, 2 and 3 days and at 5±1 °C for 2, 6, 8, 10 and 14 days.

To recover the pathogen from the apple slices, a tissue plug containing the entire well (1.2 cm diameter and 1 cm long) was removed aseptically with a sterile cork borer and processed for bacterial counts. In addition, mesophilic and psychrotrophic microorganisms were determined in the modified atmosphere assays. From the uninoculated apple slice in each tray, an apple plug was removed and after blending it with SP, aliquots of the mixture were diluted, plated on Nutrient Agar (NA, Biokar Diagnostics) and incubated at 30±1 °C for 3 days and 6.5±1 °C for 10 days for mesophilic and psychrotrophic microorganisms, respectively. Each pathogen and mesophilic and psychrotrophic determination was replicated three times for each storage temperature and sampling time and the assay was repeated once.

2.5. Fruit quality parameters

On the day of each assay, an apple sample was tested for pH with a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). After pH determination, the apples were crushed and soluble solids content determined at 20 °C using a handheld refractometer (Atago CO., LTD. Japan). To measure titratable acidity, 10 mL of apple juice were diluted with 10 mL of distilled water then titrated with 0.1 N sodium hydroxide (NaOH) up to pH 8.1. The results were then calculated as g of malic acid per litre.

2.6. Determination of pH and atmospheric conditions

At each sampling date and pathogen, the pH of the apple plug plus 9 mL of SP was measured using a pH-meter (GLP22 Crison).

Carbon dioxide and oxygen content in single trays were determined using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark).

2.7. Statistical analysis

Prior to ANOVA, all cfu mL^{-1} data were transformed to log_{10} cfu $plug^{-1}$.The General Linear Models (GLM) procedure of the Statistical Analysis System (SAS) was applied (v.8; SAS Institute, Cary, NC, USA). Significant differences between treatments were analyzed by Duncan's Multiple Range test at a significance level of $P< 0.05$.

3. RESULTS

3.1. Fruit quality parameters

'Granny Smith' apples had the lowest pH (3.32±0.13) followed by 'Golden Delicious' apples (4.16±0.25). The highest pH (4.44±0.26) was measured in the 'Shampion' apples (Table 1). 'Shampion' and 'Granny Smith' apples had the highest soluble solids content (13.9 and 13.5±0.1 °Brix respectively) and the lowest was observed in 'Golden Delicious' apples (12.7±0.4 °Brix). 'Granny Smith' had the highest titratable acidity (8.2±0.3 g malic acid L^{-1}), followed by 'Golden Delicious' (3.6±1.1 g malic acid L^{-1}) and 'Shampion' apples (2.16 g malic acid L^{-1}).

In all the assays, pH modifications during storage time were not significant (±0.14, data not shown).

Table 1 Determination of initial pH, soluble solids (°Brix) and titratable acidity (g malic acid L^{-1}) of 'Golden Delicious', 'Granny Smith' and 'Shampion' apples used in the assays

Apple variety	'Golden Delicious'	'Granny Smith'	'Shampion'
pH	4.16±0.25[a]	3.32±0.13	4.44±0.26
Soluble solids	12.7±0.4	13.5±0.1	13.9
Titratable acidity	3.6±1.1	8.2±0.3	2.16

[a] Results expressed as mean plus and minus the standard deviation for each analysis.

3.2. Population dynamics in ambient conditions

3.2.1. Effect of storage temperature on bacterial population dynamics in 'Golden Delicious' apples

The survival and growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* applied to 'Golden Delicious' apple plugs then stored at 25, 20, 10 and 5 °C are shown in Fig. 1.

The population of all three foodborne pathogens on apple plugs increased exponentially after inoculation when they were stored at 25 and 20 °C. Maximum growth of *E. coli* occurred after 2 days of incubation at both temperatures with the population increasing by 2.7 $\log_{10}$ units (final population of 8.0 $\log_{10}$ cfu plug^{-1}). *Salmonella* reached its maximum growth (7.6 $\log_{10}$ cfu plug^{-1}) after 24 h when the apple plugs were incubated at 25 °C and reached maximum growth after 3 days when stored at 20 °C. *L. innocua* growth peaked at 6.9 $\log_{10}$ cfu plug^{-1} after 2 days.

Greater differences between the three bacteria were detected when apple plugs were stored at lower temperatures. At 10 °C, *E. coli* and *Salmonella* increased by 1.3 $\log_{10}$ units to 6.5 $\log_{10}$ cfu plug^{-1}. In contrast, *L. innocua* increased by 2.4 $\log_{10}$ units after 6 days of storage at 10 °C to 7.2 $\log_{10}$ cfu plug^{-1}. At 5 °C, *E. coli* and *Salmonella* populations remained stable from inoculation at day zero to day 9 and then slowly declined. In contrast, *L. innocua* was able to grow at 5 °C.

3.2.2. Effect of apple variety on bacterial population dynamics

The effect of apple variety ('Golden Delicious', 'Granny Smith' and 'Shampion') on population dynamics of FBP was measured at 25 and 5 °C (Fig. 2).

E. coli O157:H7 populations sharply increased after 24 h of incubation at 25 °C in all three varieties tested (Fig. 2A). Higher population counts occurred on 'Golden Delicious' apple plugs (8.0 $\log_{10}$ cfu plug^{-1}), followed by 'Granny Smith' then 'Shampion'. At 5 °C, the *E. coli* population gradually declined in all three varieties.

Growth of *Salmonella* populations at 25 °C was almost the same in the three varieties (Fig. 2B). Higher population counts (8.0 $\log_{10}$ cfu plug^{-1}) occurred on 'Shampion' apples after 3 days. The population of *Salmonella* at 5 °C declined in all three varieties.

L. innocua population followed similar pattern when it was inoculated in the different apple varieties and stored at 25 °C. Higher population counts (6.7 $\log_{10}$ cfu plug^{-1}) occurred after 24 h. At 5 °C population in 'Granny Smith' apples declined steadily, but in 'Shampion' the population decline was more dramatic, decreasing from 5.1 $\log_{10}$ cfu plug^{-1} at day zero to 1.7 $\log_{10}$ cfu plug^{-1} after 14 days (a decline of approximately 3.4 $\log_{10}$ cfu plug^{-1}). In 'Golden

Delicious' there was an initial drop and then population rose to the inoculum's level at the end of the experiment (Fig. 2C).

3.2.3. Effect of ascorbic acid on FBP in 'Golden Delicious' apples

The effect of ascorbic acid, used to prevent browning in fresh-cut apples, on the growth of FBP's applied to fresh-cut 'Golden Delicious' apples is summarized in Table 2.

No significant differences were observed in any of the bacteria when apple plugs were stored at 25 °C. At 5 °C storage, the three bacteria showed the same pattern, populations were lower when AA was used but populations after six days were the same in both treatments. Experiments were finished after 6 days due to spoilage of samples.

3.2.4. Effect of NatureSeal® on FBP in 'Granny Smith' apples

The effect of NatureSeal® on the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* applied to fresh-cut 'Granny Smith' apples stored at 25 and 5 °C was determined (Table 3).

The growth of *E. coli* at 25 °C was not influenced by the presence of NS until day 6. At 5 °C, the population of *E. coli* declined gradually and there were no significant differences between treated and untreated apple plugs until day 14.

At 25 °C storage, there were no significant differences due to the NS treatment in the growth of *Salmonella* until day 3. At 5 °C, the *Salmonella* population was not significantly affected by NS throughout the duration of this experiment.

No significant differences in *L. innocua* growth were observed between apples plugs treated or not with NS at 25 °C. However, when apple plug were stored at 5 °C significant differences were observed after day 9 and day 14.

Experiments with NS were extended until the first visible symptoms of decay appeared.

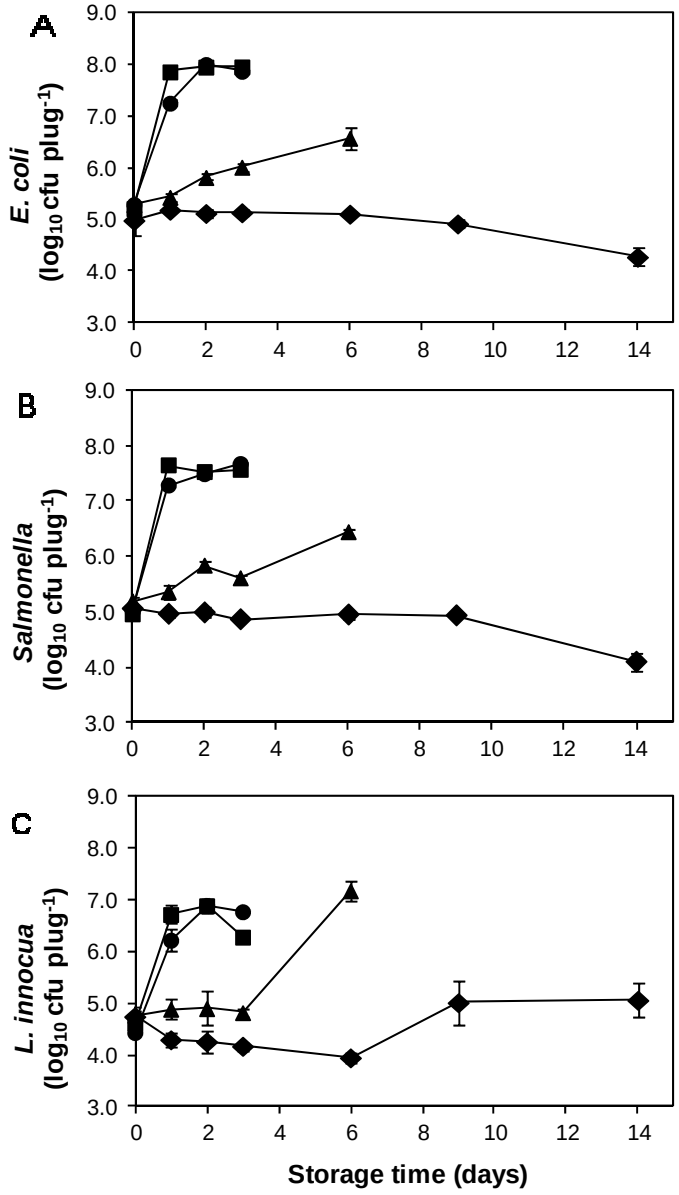

Fig. 1 Survival and growth of *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) on 'Golden Delicious' apple plugs stored at 25 (squares), 20 (circles), 10 (triangles) and 5 (diamonds) °C without antioxidant. Inoculum 10^7 cfu mL^{-1}, 15 µL (n=6, bars are standard erros of the mean. When the standard error are not visible, they ares maller than the size of the symbol).

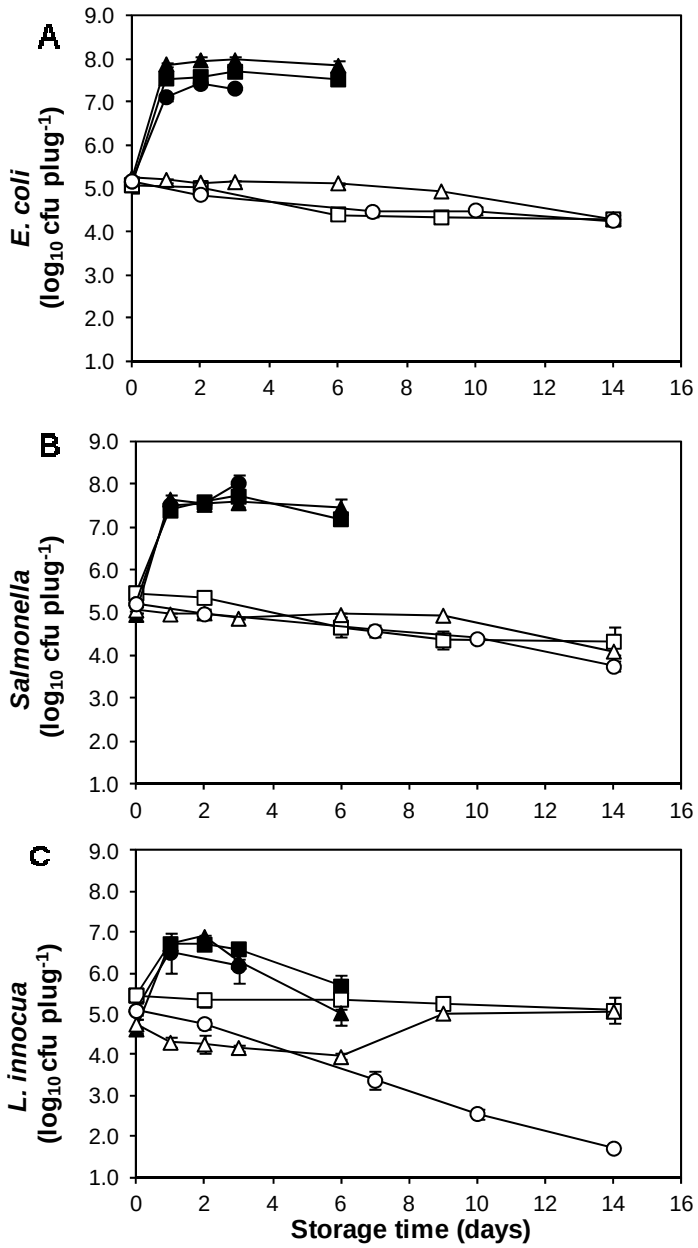

Fig. 2 Survival and growth of *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) on 'Golden Delicious' (triangles), 'Granny Smith' (squares) and 'Shampion' (circles) apple plugs stored at 25 °C (shadded symbols) and at 5 °C (open symbols) without antioxidant. Inoculum 10^7 cfu mL^{-1}, 15 μL (n=6, bars are standard erros of the mean. When the standard error are not visible, they ares maller than the size of the symbol)

Table 2 Effect of the addition of ascorbic acid applied to fresh-cut 'Golden Delicious' apples then stored at 25 and 5 °C on the growth of three foodborne pathogens.

Pathogen	Temperature	AA[a]	Days				
			0	1	2	3	6
E. coli O157:H7	25 °C	-	5.23±0.06a[b]	7.85±0.17a	7.88±0.14a	7.97±0.34a	nd
		+	5.19±0.01a	7.89±0.03a	8.11±0.35a	7.94±0.07a	nd
	5 °C	-	5.25±0.06a	5.24±0.06a	5.22±0.08a	5.22±0.06a	5.11±0.09a
		+	5.23±0.06a	5.14±0.10a	4.83±0.22b	4.97±0.02b	5.20±0.06a
Salmonella	25 °C	-	5.00±0.14a	7.47±0.29a	7.56±0.29a	7.64±0.31a	nd
		+	4.86±0.06a	7.98±0.13a	7.44±0.09a	7.41±0.25a	nd
	5 °C	-	5.12±0.22a	5.03±0.08a	5.07±0.23a	4.92±0.04a	4.95±0.17a
		+	5.10±0.27[a]	5.06±0.25a	4.75±0.07b	4.77±0.08b	4.96±0.11a
L. innocua	25 °C	-	4.65±0.25a	6.79±0.44a	6.92±0.14a	6.35±0.21a	nd
		+	4.56±0.04a	6.58±0.64a	6.82±0.22a	6.16±0.15a	nd
	5 °C	-	4.80±0.38a	4.51±0.26a	4.62±0.44a	4.31±0.13a	3.95±0.22a
		+	4.69±0.19a	4.45±0.66a	3.40±0.35b	3.89±0.04b	3.55±0.40a

Values are the average $\log_{10}$ cfu $plug^{-1}$ plus and minus the standard deviation of triplicate samples from two experiments (n=6).
nd: not determined

[a] Ascorbic acid treatment: -: control treatment (no AA added); +: AA treatment (2 % w/v for 2 min)

[b] Mean values for each pathogen, time and temperature that are followed by the same letter are not significantly different ($P<0.05$) based on Duncan's multiple range test.

Table 3 Effect of the addtion of AS1 NatureSeal® applied to fresh-cut 'Granny Smith' apples stored at 25 and 5 °C on the growth of three foodborne pathogens.

Pathogen	Temperature	NS[a]	Days						
			0	**1**	**2**	**3**	**6**	**9**	**14**
E. coli O157:H7	25 °C	-	5.07±0.46a[b]	7.54±0.16a	7.57±0.37a	7.69±0.32a	7.51±0.23a	nd	nd
		+	5.17±0.39a	7.61±0.29a	7.52±0.15a	7.56±0.27a	6.47±0.27b	nd	nd
	5 °C	-	5.07±0.46a	nd	5.00±0.47a	nd	4.39±0.17a	4.32±0.29a	4.28±0.31a
		+	5.17±0.39a	nd	4.78±0.60a	nd	4.10±0.50a	3.85±1.38a	3.40±0.55b
Salmonella	25 °C	-	5.46±0.27a	7.39±0.21a	7.58±0.22a	7.71±0.18a	7.18±0.32a	nd	nd
		+	5.47±0.37a	7.44±0.18a	7.41±0.25a	7.17±0.10b	5.67±0.41b	nd	nd
	5 °C	-	5.46±0.27a	nd	5.35±0.27a	nd	4.66±0.61a	4.36±0.55a	4.33±0.90a
		+	5.47±0.37a	nd	5.09±0.27a	nd	4.90±0.24a	4.67±0.26a	3.08±0.96a
L. innocua	25 °C	-	5.46±0.12a	6.72±0.12a	6.70±0.17a	6.59±0.13a	5.68±0.81a	nd	nd
		+	5.59±0.11a	6.69±0.23a	6.78±0.42a	6.37±0.49a	5.71±0.10a	nd	nd
	5 °C	-	5.46±0.12a	nd	5.34±0.50a	nd	5.35±0.48a	5.25±0.27a	5.08±0.84a
		+	5.59±0.11a	nd	5.59±0.21a	nd	5.36±0.31a	4.28±0.46b	3.75±0.55b

Values are the average $\log_{10}$ cfu $plug^{-1}$ plus and minus the standard deviation of triplicate samples from two experiments (n=6).
nd: not determined

[a] AS1 NatureSeal® treatment: -: control treatment (no NS added); +: NS treatment (6 % w/v for 2 min)

[b] Mean values for each pathogen, time and temperature that are followed by the same letter are not significantly different ($P<0.05$) based on Duncan's multiple range test.

3.3. Population dynamics in modified atmosphere conditions

The growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* in 'Golden Delicious' apple tissue at 25 and 5 °C, without antioxidant and stored in a passive modified atmosphere is shown in Fig. 3.

E. coli, *Salmonella* and *L. innocua* populations increased to 7.0 $\log_{10}$ cfu plug^{-1} in the first 24 h at 25 °C. Over the next 48 h, *E. coli* and *Salmonella* populations stabilized meanwhile *L.innocua* population reduced from 7.0 $\log_{10}$ cfu plug^{-1} to 6.0 $\log_{10}$ cfu plug^{-1}. The population of the three bacteria fluctuated over time when the apple plugs were stored at 5 °C. Although final populations of *E. coli* and *Salmonella* were reduced, *L. innocua* population increased, relatively slowly over the time, from 4.2 to 5.8 $\log_{10}$ cfu plug^{-1} after 14 days.

The number of mesophilic microorganisms was stable over the time in apple tissue stored at 5 °C, from 1.7 to 2.1 $\log_{10}$ cfu plug^{-1}, and it fluctuated when storage was at 25 °C, from below the level of detection (1.7 $\log_{10}$ cfu plug^{-1}) to 2.1 $\log_{10}$ cfu plug^{-1}. Psychrotrophic microorganisms were below the level of detection (1.7 $\log_{10}$ cfu plug^{-1}) at both temperatures (data not shown).

The concentration of O_2 and CO_2 in the package headspace of fresh-cut apples is shown in Fig. 4. Oxygen levels decreased continuously throughout storage. The decline was more pronounced at 25 °C (21.0 % to 17.0 %) compared with 5 °C (21.0 % to 19.6 %). In contrast, carbon dioxide production increased during storage. Again, the rise was more significant in 3 days at 25 °C (6.8 %) than in 14 days at 5 °C (2.8 %).

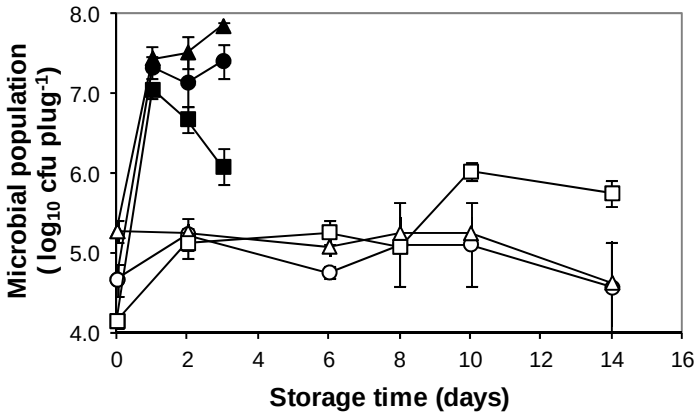

Fig. 3 Growth of *E. coli* O157:H7 (circles), *Salmonella* (triangles) and *L. innocua* (squares) in 'Golden Delicious' apple tissue without antioxidant stored at 25 °C (shaded symbols) and at 5 °C (open symbols) in passive modified atmosphere. Inoculum 10^7 cfu mL^{-1}, 15 μL (n=3, bars are standard erros of the mean. When the standard error are not visible, they ares maller than the size of the symbol).

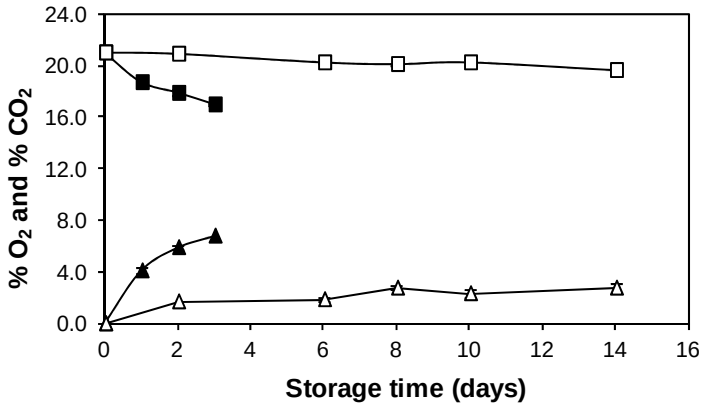

Fig. 4 Evolution of O_2 (squares) and CO_2 (triangles) in apple trays stored at 25 °C (shadded symbols) and at 5 °C (open symbols) (n=3, bars are standard erros of the mean. When the standard error are not visible, they ares maller than the size of the symbol).

4. DISCUSSION

To our knowledge, this is the first study that compares the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* on different varieties of apple flesh treated and not treated with antioxidant with or without the use of modified atmosphere packaging, under different temperature regimes. Importantly, the three bacteria studied were able to grow on fresh-cut apples stored at room temperature and were unaffected by modified atmosphere packaging or the addition of antioxidants.

Storage temperature had a major impact on keeping the FBP population at low levels in artificially contaminated fresh-cut apples. *E. coli* O157:H7 and *Salmonella* populations increased by more than 2 $\log_{10}$ units in 'Golden Delicious' apple plugs incubated at 25 and 20 °C and more than 1 $\log_{10}$ unit at 10 °C after 24 h and 6 days of incubation, respectively. Similar findings were reported for *E. coli* O157:H7 by Janisiewicz *et al.* (1999) and Dingman (2000); while Gunes and Hotchkiss (2002) and Fisher and Golden (1998) reported lower population increases in the same apple variety. In the case of *Salmonella*, our results agree with previous findings in 'Red Delicious' (pH 4.2) and 'Golden Delicious' apples (Leverentz *et al.*, 2001; 2006). *L. innocua* population increased by more than 2 $\log_{10}$ units after two days of incubation at 25 and 20 °C. However, it required 6 days to reach a similar level of population increase when the incubation temperature was reduced to 10 °C. These results are similar to those obtained by Leverentz *et al.* (2006) and Conway *et al.* (2000) but different to those reported by Leverentz *et al.* (2003) who described a lower population increase (0.6 log units after 7 days at 10 °C) in fresh-cut 'Red Delicious' apples (pH 4.4).

The growth of these foodborne pathogens in highly acidic fruit (pH 3.5) was unexpected because bacteria, in general, colonize fruit and vegetables with higher pH than usually found in apple flesh. Interestingly, *Salmonella* Montevideo grew at pH levels that would not be expected to support growth of the bacterium in tomatoes (Zhuang *et al.*, 1995). Furthermore, the survival of *L. monocytogenes* was enhanced on peeled Hamlin oranges incubated at 4 and 8 °C (Pao *et al.*, 1998) and on the surface of whole tomatoes stored at 10 °C (Beuchat and Brackett, 1991). Therefore, the growth of *E. coli* and other foodborne pathogenic bacteria on apple tissue may result from the ability of these bacteria to modify the adjacent microenvironment (Leverentz *et al.*, 2001).

All the cultivars tested, 'Golden Delicious', 'Granny Smith' and 'Shampion', promoted growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* in fresh-cut apple plugs incubated at 25 °C. Despite differences observed in pH, soluble solids and titratable acidity between these varieties, slight differences in the growth of the FBPs were measured. In other studies, growth of *E. coli* was also promoted in 'Red Delicious', 'Golden Delicious', 'Rome', 'Winesap', 'Macoun' and 'Melrose' apples but was inhibited in damaged 'McIntosh' apple tissue (Dingman, 2000; Fisher and Golden, 1998). 'McIntosh' apples had the lowest pH values, so this variable may account for the difference in growth of *E. coli*.

The effect of ascorbic acid in the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* inoculated onto 'Golden Delicious' apple plugs was also tested. At 25 °C, no significant differences were detected in the growth of any of the foodborne pathogens in the presence and absence of AA. At 5 °C significant differences were measured only at day 2 and 3. Similarly, in the case of the addition of NatureSeal® to fresh-cut 'Granny Smith' apples, no significant differences were observed until the last days of storage, when higher populations of FBP's were detected in the apple plugs that were not treated with antioxidant. Previous research reported that the application of NatureSeal® immediately after slicing and storage of 'Empire' and 'Crispin' apple slices completely inhibited enzymatic browning and maintained the original white flesh colour for up to 21 days at 4 °C, but there was no reduction in the microbial growth compared with untreated apple slices (Rupasinghe *et al.*, 2005). Therefore, ascorbic acid and NatureSeal® do not have bioactivity against FBP's and may not be useful in reducing FBP growth on fresh-cut apples and there is the potential for human pathogenic bacterial populations to increase to very high levels during storage, despite the visual quality of the fruit being acceptable.

Population dynamics of the FBP inoculated onto apple slices then stored under passive MAP conditions at 25 and 5 °C was similar to that obtained to the apple plugs stored in air but MAP prevented the growth of mesophilic and psychrotrophic microorganisms. At 25 °C the oxygen concentration in the package headspace reduced from 21.0 % to 17.0 % and carbon dioxide concentration increased to 6.8 %. At 5 °C, MAP had no effect on initial atmosphere. Previous

research reported that low oxygen storage atmosphere had no effect on the populations of *L. monocytogenes* recovered from apple slices, but the visual quality of the slices was improved in controlled-atmosphere conditions compared with air storage (Conway *et al.*, 2000). In contrast, the growth of *E. coli* O157:H7 was inhibited on fresh apple slices stored under low O_2 (1 %) and/or high CO_2 (0, 15, 30 %) atmospheres in comparison to air (Gunes and Hotchkiss, 2002). These atmospheres reduced the growth of yeast and moulds so the shelf life of apple slices was increased. MAP inhibits polyphenol oxidase activity and decreases the total colour change of cut apples. Therefore, together with the use of antioxidants and low temperature, MAP is beneficial in extending the shelf life of various fruits and vegetables.

This study has demonstrated that three important foodborne pathogens, *E. coli* O157:H7, *Salmonella* and *L. innocua*, used as a microbial surrogate of *L. monocytogenes*, can grow on three varieties of fresh-cut apple at temperatures of and above 10 °C regardless of whether antioxidants are applied or modified atmosphere packaging is used. Therefore, it is more important to avoid any fruit contamination during processing and manipulation and as soon as the product has been processed it will be necessary to maintain the cold chain until consumption. The addition of antioxidant substances or the use of modified atmosphere packaging prolongs the shelf-life of fresh-cut produce slowing-down the chemical deterioration and growth of spoilage microorganisms. However, extending the shelf life of this product raises food safety concerns since there is more time for FBP to multiply in the product without organoleptic rejection especially if it is not maintained at refrigeration conditions.

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CAPÍTULO II

Fate of *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally-processed peaches under different storage conditions

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ABSTRACT

Consumption of fresh-cut produce has sharply increased recently causing an increase of foodborne illnesses associated with these products. As generally, acidic fruits are considered 'safe' from a microbiological point of view, the aim of this work was to study the growth and survival of *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally-processed peaches. The three foodborne pathogens population increased more than 2 $\log_{10}$ units on fresh-cut peach when stored at 20 and 25 °C after 48 h. At 10 °C only *L. innocua* grew more than 1 $\log_{10}$ unit and it was the only pathogen able to grow at 5 °C. Differences in growth occurred between different peach varieties tested, with higher population increases in those varieties with higher pH ('Royal Glory' 4.73±0.25 and 'Diana' 4.12±0.18). The use of common strategies on extending shelf-life of fresh-cut produce, as modified atmosphere packaging and the use of the antioxidant substance, ascorbic acid (2 % w/v), did not affect pathogens' growth at any of the temperatures tested (5 and 25 °C). Minimally-processed peaches have shown to be a good substrate for foodborne pathogens' growth regardless use of modified atmosphere and ascorbic acid. Therefore, maintaining cold chain and avoiding contamination is highly necessary.

Keywords: temperature, variety, modified atmosphere packaging, ascorbic acid, foodborne pathogen**.**

1. INTRODUCTION

Recently, consumption of fresh produce has risen significantly due to health benefits associated with their consumption. Changes in lifestyles and major shifts in consumption trends have produced a demand for a wider range of products, and have led people to spend less time cooking at home and to eat out more often. Such trends have been reflected in an increased popularity of salad bars and have prompted the appearance of minimally-processed convenience foods that are ready-to-eat. Among them, the consumption of fresh-cut or minimally-processed fruit and vegetables has undergone a sharp increase (Abadias *et al.*, 2008). In Catalonia (Spain) consumption of fresh-cut fruit increased from 12,697 t in 2004 to 20,143 t in 2008 (Departament d'Agricultura). Catalonia is one of the main peach producers in Spain, with a production of 212,898 t, or 25.4 % of total Spanish peach production in 2007, and 79.7 % of Catalan production took place in Lleida province (MARM, 2009) .

Fruits and vegetables contain nutrients necessary for the rapid growth of foodborne pathogens, yet outbreaks of illness caused by ingestion of fruits and vegetables are less frequent than outbreaks from other food. This is due, in part, to external barriers such as the peel and rind which prevent microorganisms from entering and subsequently growing in the interiors of fruits and vegetables. However, in some cases, such as on fresh-cut fruit, this external barrier is broken, thus creating and opportunity for bacterial colonization (Janisiewicz *et al.*, 1999). Fresh produce, and in particular fruit, does not receive any 'lethal' treatment that kills all pathogens prior to consumption. Hence, pathogens introduced at any point of the production chain may be present when the produce is consumed. Safe production methods and proper disinfection/decontamination procedures are therefore critical steps in ensuring the safety of ready-to-eat fresh fruits and vegetables (Abadias *et al.*, 2006). Fresh fruit and fruit juices have been incriminated in outbreaks of foodborne illnesses caused by human pathogens like *Escherichia coli* O157:H7 and *Salmonella* spp. (CDC, 2007; Harris *et al.*, 2003; Powell and Luedtke, 2000).

Although the growth of human pathogens on acidic fresh produce is thought to be limited because of the acidity, recent studies have documented the exponential growth of *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* on a variety of produce. Several studies have shown that *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* can survive and grow on fresh apple tissues stored in air (Abadias *et al.*, 2009; Alegre *et al.*, 2010; Dingman, 2000; Fisher and Golden, 1998; Gunes and Hotchkiss, 2002; Janisiewicz *et al.*, 1999). In fresh-cut strawberries *E. coli* O157:H7 survived when stored at 4 °C (Knudsen *et al.*, 2001), *Salmonella* populations decreased over a 7-day storage period at 5 °C (Knudsen *et al.*, 2001) and *L. monocytogenes* population was maintained when stored for 48 h or 7 days at 24 or 4 °C, respectively (Flessa *et al.*, 2005). Meanwhile *Salmonella*

not only survived, but also grew on chopped tomatoes at ambient temperature despite the relative low pH (4.0-4.5) (Zhuang *et al.*, 1995), significant decreases in *L. monocytogenes* populations occurred in chopped tomatoes stored at 10 and 21 °C (Beuchat and Brackett, 1991). It has also been shown the ability of *Escherichia coli* O157:H7 and *Salmonella* to grow on temperature-abused fresh-cut mangoes (pH 4.2) and to survive on refrigerated mangoes (Strawn and Danyluk, 2010). Minimally-processed orange fruits have also shown to be an adequate substrate for *L. monocytogenes* growth (Caggia *et al.*, 2009; Pao *et al.*, 1998). Pathogen's growth has also been demonstrated in non-acidic fruits as melon, watermelon, papaya and persimmon (Penteado and Leitao, 2004; Rezende *et al.*, 2009; Strawn and Danyluk, 2010; Uchima *et al.*, 2008; Ukuku and Sapers, 2007).

Development of fresh-cut fruit has been hampered by the rapid oxidative browning of fruit flesh, the risk of microbial development, and physiological deterioration during transport and storage (Abbot *et al.*, 2004). Enzymatic browning of fruit slices can be essentially eliminated by the use of anti-browning agents such as ascorbic acid and modified atmosphere packaging (MAP) (Gorny, 1997). Meanwhile, passive MAP is an alteration of the gaseous environment produced as a result of respiration, active MAP is produced by the addition and removal of gases from food packages to manipulate the levels of O_2 and CO_2. Atmospheres with low O_2 levels inhibit the growth of most aerobic microorganisms, whose growth usually warns consumers about spoilage, while the growth of pathogens may be allowed or even stimulated (Farber, 1991).

The objective of this study was to determine the survival and growth of *E. coli* O157:H7, *Salmonella* and *L. innocua*, on minimally-processed peach stored at different temperatures. The influence of peach variety, the use of ascorbic acid and passive modified atmosphere packaging were also determined.

2. MATERIALS AND METHODS

2.1. Fruit

Peaches (*Prunus persica* L. Bastch) were obtained from packinghouses in Lleida, Catalonia and from the IRTA Experimental Station located in Lleida. The varieties used were 'Diana', 'Royal Glory', 'Elegant Lady' and 'Plácido' peaches. Prior to the experimental studies, peaches were washed in running tap water and surface disinfected with ethanol 70 % and let to dry at room temperature.

2.2. Preparation of bacterial inoculum

A non-pathogenic strain of *E. coli* O157:H7 (NCTC 12900), and a pathogenic strain of *Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Michigan (BAA-709 ATCC) were used. Both strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin sulphate salt (St, Sigma, Germay) thereby enabling detection on a selective medium (TSA-St) in the presence of the natural microbial flora associated with peaches. The strains were grown in tryptone soy broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) medium for 20-24 h at 37 °C. The strain of *Listeria innocua* CECT-910 was used as a microbial surrogate of *L. monocytogenes* as previous studies have demonstrated that this is a valid model for *L. monocytogenes* (Francis and O'Beirne, 1997). *L. innocua* was grown overnight in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) at 37±1 °C.

Bacterial cells were harvested by centrifugation at 9820 × g, 10 min at 10 °C 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 according to standard curves. For the inoculum preparation, a volume of the foodborne pathogen concentrated suspension was added to 5 mL of SP to obtain approximately 1×10^{7} cfu mL^{-1}. Concentration of inoculum was checked by spread plating appropriate dilutions on TSA-St for *E. coli* O157:H7 and *Salmonella* or on Palcam agar (Palcam Agar Base with Palcam selective supplement, Biokar Diagnostics) for *L. innocua*. The agar plates were incubated overnight at 37±1 °C.

2.3. Population dynamics in ambient conditions

Peaches were cut in half and plugs of 1.2 cm of diameter and 1 cm long were taken using a cork borer. For the antioxidant assay, peach plugs were suspended in a 2 % (w/v) solution of ascorbic acid (AA) for 2 min at 150 rpm. After the treatment, peach plugs were let to dry in a laminar flow biosafety cabinet. Peach plugs (with and without antioxidant) were placed into sterile glass test tubes.

The bacterial suspension was pipetted (15 µL) onto fruit tissue plugs. Peach plugs were stored at 5±1 °C, 10±1 °C, 20±1 °C and 25±1 °C. Time storage was as long as no visible symptoms of decay were observed.

At each sample time, fruit plugs were each placed into a sterile plastic bag (BagPage 80 mL, Interscience BagSystem, St Nom La Breteche, France) and 9 mL of SP were added. It was homogenised in a stomacher blender for 120 s at high speed (Bagmixer 100 Minimix, Interscience). Aliquots of the mixture were then serially diluted and surface plated as described previously.

There were three replicate peach plugs for each pathogen and sampling time and each assay was repeated twice.

2.4. Population dynamics in modified atmosphere conditions

'Elegant Lady' peaches were cut into 2 cm slices. Groups of 4 slices were placed in polypropylene (10 × 15 × 6.67 cm) trays and a 6 mm well was made on each peach slice tissue using a cork borer. Each bacterium was pipetted (15 µL) in a tissue well and one slice was left uninoculated. Peach trays were sealed with a 40,000 cc m^2 24 h^{-1} oxygen transmission rate polypropylene film and incubated at 5±1 °C for 2, 6, 8, 10 and 14 days and at 25±1 °C for 1, 2 and 3 days.

To recover the pathogen from the peach slices, a tissue plug containing the entire well (1.2 cm of diameter and 1 cm long) was removed with a sterile cork borer and processed for bacterial counts.

In addition, mesophilic and psychrotrophic microorganisms were determined in the modified atmosphere conditions assays. From the uninoculated slice of each tray, a peach plug was removed and after blending it with SP, aliquots of the mixture were diluted, plated on Nutrient Agar (NA, Biokar Diagnostics) and incubated at 6.5±1 °C for 10 days or at 30±1 °C for 3 days for psychrotrophic and mesophilic microorganisms, respectively. Each pathogen and psychrotrophic and mesophilic determination was replicated three times for each storage temperature and sampling time and the assay was repeated once.

2.5. Peach quality parameters

On the day of the assay, a sample of each of the peaches used was tested for pH with a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). After pH determination, the peaches were crushed and soluble solids content was determined at 20 °C using a handheld refractometer (Atago CO., LTD. Japan). To measure titratable acidity, 10 mL of peach juice were diluted with 10 mL of distilled water and it was titrated with 0.1 N NaOH up to pH 8.1. The results were calculated as g of malic acid per litre.

2.6. Determination of pH and headspace gas composition

At each sampling date and pathogen, the pH of the peach plug plus 9 mL of SP was measured using a pH-meter (GLP22 Crison).

Carbon dioxide and oxygen content in single trays were determined using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark).

2.7. Statistical analysis

Prior to ANOVA, all cfu mL^{-1} data were transformed to log_{10} cfu $plug^{-1}$. The General Linear Models (GLM) procedure of the Statistical Analysis System (SAS) was applied (v.8; SAS Institute, Cary, NC, USA). Significant differences between treatments were analyzed by Duncan's Multiple Range test at a significance level of $P<0.05$.

3. RESULTS

3.1. Peach quality parameters

The pH, soluble solids, titratable acidity and maturity index of peaches used in the assays are summarized in Table 1. The pH of the peaches ranged from 3.49±0.18 ('Plácido' peaches) to 4.73±0.25 ('Royal Glory' peaches). 'Plácido' peaches had the highest soluble solids content (14.7 °Brix) and 'Royal Glory' had the lowest (8.9 °Brix). The highest titratable acidity (8.6±0.7 g acid malic L^{-1}) was determined in 'Placido' peaches, followed by 'Elegant Lady' then 'Royal Glory' (7.1±0.9 and 4.1±0.5 g acid malic L^{-1}, respectively). Maturity index (soluble solids/titratable acidity) ranged from 1.7±0.2 ('Elegant Lady' and 'Plácido' peaches) to 2.3±0.5 ('Royal Glory').

Table 1 Determination of initial pH, soluble solids (ºBrix), titratable acidity (g malic acid L^{-1}) and maturity index (soluble solids/titratable acidity) of all peach varieties used in the assays.

Peach variety	pH	Soluble solids	Titratable acidity	Maturity index
'Diana'	4.12±0.18 b[a]	-[b]	-	-
'Elegant Lady'	3.73±0.28 c	11.6±0.5 b	7.1±0.9 b	1.7±0.2 b
'Plácido'	3.49±0.18 d	14.7±0.5 a	8.6±0.7 a	1.7±0.2 b
'Royal Glory'	4.73±0.25 a	8.9±1.3 c	4.1±0.5 c	2.2±0.5 a

[a] Results expressed as mean plus and minus standard desviation for each analysis. Mean values within columns followed by the same letter are not significantly different based on Duncan's multiple range test ($P \leq 0.05$).

[b] not determined

3.2. Population dynamics in ambient conditions

3.2.1. Effect of storage temperature on bacterial population dynamics on fresh-cut 'Elegant Lady' peaches

The effect of storage temperature on the population dynamics of *E. coli* O157:H7, *Salmonella* and *L. innocua* inoculated to minimally-processed 'Elegant Lady' peaches then stored at 5, 10, 20, 25 °C was determined (Fig. 1).

Initial population of *E. coli* on peach plugs was 5.2 $\log_{10}$ cfu plug^{-1}. It grew exponentially on peach plugs stored at 20 and 25 °C. While maximum growth (8.0 log cfu plug^{-1}) occurred after 24 h incubation at 25 °C; it occurred after two days at 20 °C with population increases of 2.8 and 3.0 $\log_{10}$ units, respectively (final population higher than 8.0 $\log_{10}$ cfu plug^{-1}). When storage temperature was reduced, *E. coli* growth peaked at 6.2 $\log_{10}$ cfu plug^{-1} after three days at 10 °C and it was reduced by more than 1 $\log_{10}$ units after 14 days at 5 °C.

Salmonella population increased by 2.3 and 2.9 $\log_{10}$ units, reaching a final population of 7.7 and 8.2 $\log_{10}$ cfu plug^{-1} after three days at 20 and 25 °C, respectively. At 5 and 10 °C, *Salmonella* population dynamics were similar to *E. coli*.

L. innocua initial population on peach plugs was 5.3 $\log_{10}$ cfu plug^{-1}. Maximum growth occurred after six days of incubation at 25 °C (8.1 $\log_{10}$ cfu plug^{-1}) and after three days at 20 °C (7.9 $\log_{10}$ cfu plug^{-1}). At 10 °C, the highest population increases were detected on the psychrotrophic *L. innocua* (1.6 $\log_{10}$ units). In addition, *L. innocua* was the only microorganism able to grow on peach plugs incubated at 5 °C, with a population increase of more than 1 $\log_{10}$ unit.

3.2.2. Effect of peach variety on bacterial population dynamics

The effect of peach variety on the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* was measured at 5 and 25 °C (Fig. 2).

E. coli grew exponentially in all peach varieties tested incubated at 25 °C. However, maximum population increases (approximately 4 $\log_{10}$ units) occurred on 'Diana' and 'Royal Glory', followed by 'Elegant Lady' (approximately 3 $\log_{10}$ units) then 'Placido' with pathogen population increasing by less than 2 $\log_{10}$ units. At 5 °C, no significant differences between *E. coli* growths on different peach varieties were determined.

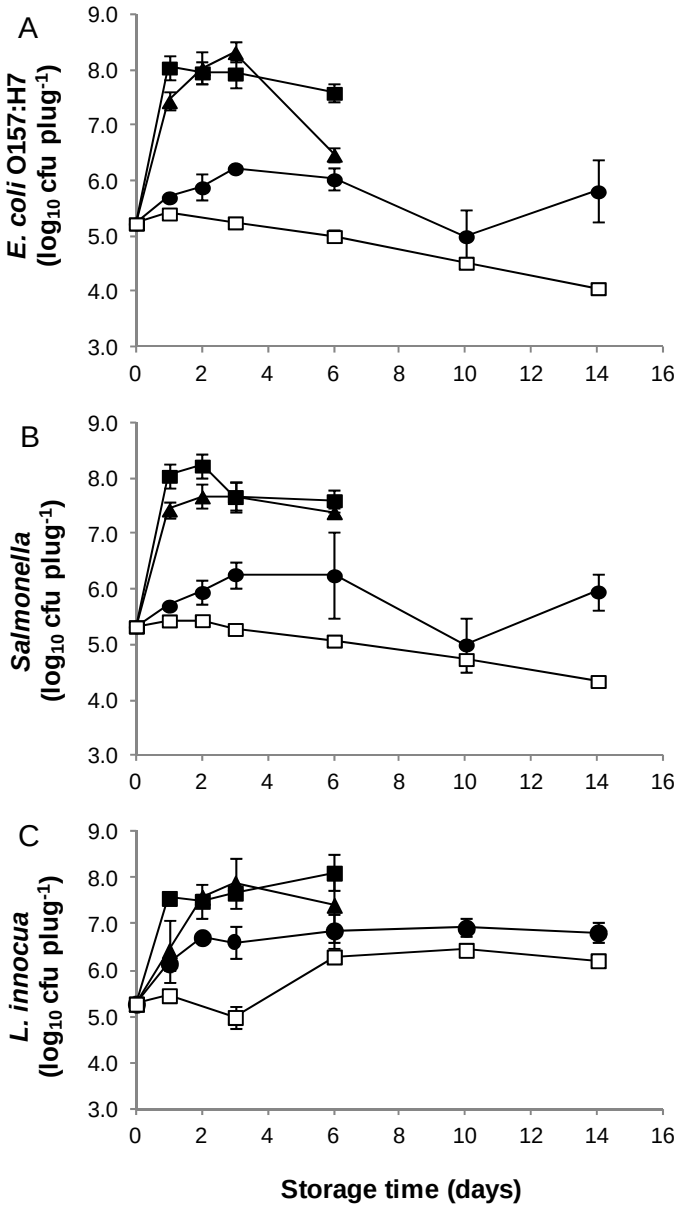

Fig. 1 Survival and growth of *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) on 'Elegant Lady' peach plugs stored at 25 (■), 20(▲), 10 (●) and 5 (□) ºC without antioxidant. Inoculum 10^7 cfu mL^{-1}, 15µL (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

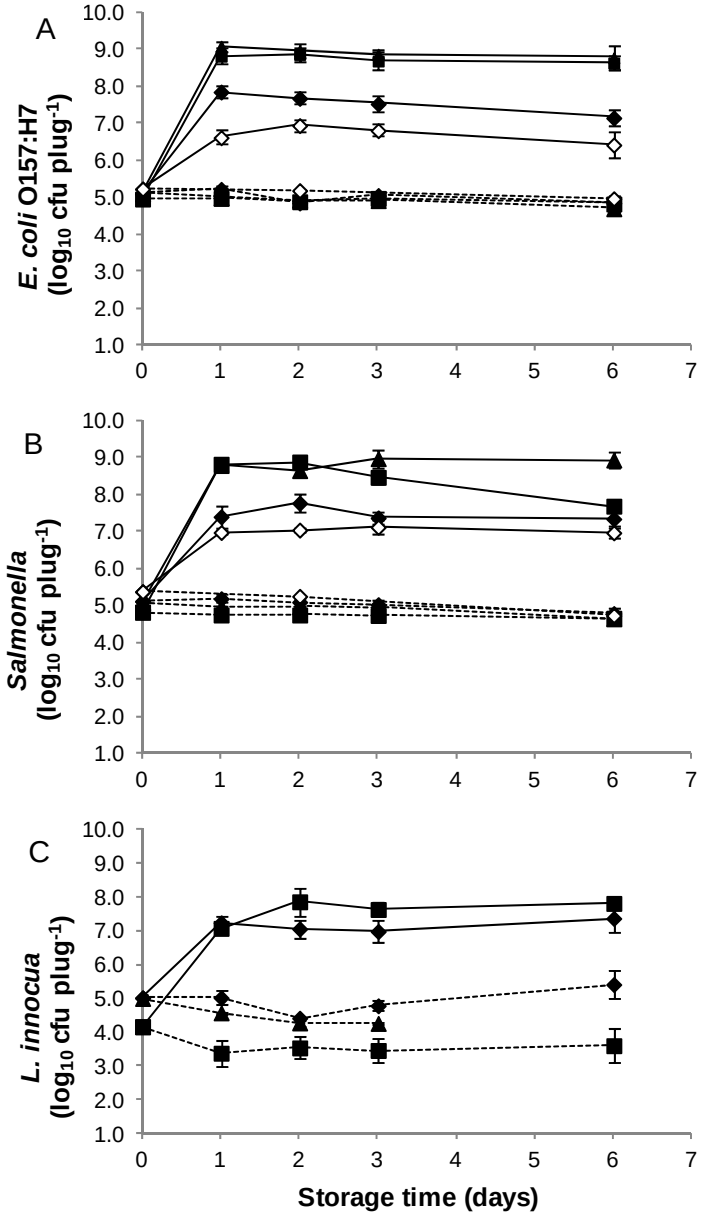

Fig. 2 Survival and growth of *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) on 'Royal Glory' (▲), 'Diana' (■), 'Elegant Lady' (◆) and 'Placido' (◇) peach plugs stored at 25 (continuous line) and at 5 ºC (dotted line) without antioxidant. Inoculum 10^7 cfu mL^{-1}, 15 µL (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

Similarly to *E. coli* population dynamics, *Salmonella* grew exponentially in the four peach varieties tested when incubated at 25 °C. Again, the highest population increases (approximately 4 $\log_{10}$ units) occurred on 'Diana' and 'Royal Glory' peaches, followed by 'Elegant Lady' then 'Plácido' with pathogen population increasing by less than 2 $\log_{10}$ units. At 5 °C, as in population dynamics of *E. coli*, no differences between peach varieties were found.

L. innocua grew exponentially in the two peach varieties tested at 25 °C. Maximum population increases (approximately 3.5 $\log_{10}$ units) occurred on 'Diana' and then 'Elegant Lady' with 2 $\log_{10}$ units of population increase. At 5 °C, although *L. innocua* population increased by 0.4 $\log_{10}$ units on 'Elegant Lady' peach plugs, it reduced 0.6 and 0.8 $\log_{10}$ units on 'Diana' and 'Royal Glory' peaches respectively.

3.2.3. Effect of ascorbic acid on FBP on fresh-cut 'Elegant Lady' peaches

The effect of ascorbic acid on the growth of *E. coli*, *Salmonella* and *L. innocua* applied to fresh-cut 'Elegant Lady' peaches is summarized in Table 2.

Growth of the studied bacteria was not affected by the addition of ascorbic acid, as no significant differences were observed except to *E. coli* population after six days of storage at 5 °C and to *L. innocua* population after one and six days of storage at 25 °C in which the population was lower in those peaches treated with AA.

3.3. Population dynamics of FBP on fresh-cut 'Elegant Lady' peaches stored in modified atmosphere conditions

Population dynamics of *E. coli*, *Salmonella* and *L. innocua* on 'Elegant Lady' peach tissue, without antioxidant, stored in a passive modified atmosphere and in air conditions at 5 °C and 25 °C was determined (Fig. 3).

E. coli, *Salmonella* and *L. innocua* populations grew exponentially on fresh-cut 'Elegant Lady' peaches when incubated at 25 °C regardless atmosphere conditions. At 5 °C, whereas *E. coli* and *Salmonella* populations reduced throughout storage time, *L. innocua* population increased between 0.7 and 1 $\log_{10}$ units at MAP and air conditions. No differences between atmospheric conditions were observed to either temperature for any of the pathogens.

Population of mesophilic and psychrotrophic microorganisms on uninoculated peach slices remained stable from below the level of detection (1.4 $\log_{10}$ cfu plug^{-1}) to 2.5 $\log_{10}$ cfu plug^{-1} on peach tissue incubated at 5 and 25 °C at both, MAP and air, atmosphere conditions (data not shown).

Modifications of O_2 and CO_2 in the package headspace of fresh-cut peaches are shown in Figure 4. At 25 °C, O_2 levels decreased sharply after 24 h to 13.2 % and then stabilized. The same happened to CO_2 levels that increased exponentially to 24.6 % after two days and then remain stable. At 5 °C, O_2 concentration only reduced by 2.5 % to 18.5 % and CO_2 increased to 3.9 % after 14 days.

Table 2 Effect of the addition of ascorbic acid applied in fresh-cut 'Elegant Lady' peaches stored at 5 and 25 ºC on the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua.*

Pathogen	Temperature	AA[a]	Days				
			0	**1**	**2**	**3**	**6**
E. coli O157:H7	25ºC	-	4.96±0.09a[b]	7.55±0.01a	7.39±0.14a	7.11±0.16a	6.70±0.25a
		+	4.97±0.09a	7.50±0.31a	7.34±0.41a	7.47±0.89a	6.94±0.43a
	5ºC	-	4.96±0.09a	5.02±0.06a	4.85±0.04a	4.84±0.91a	4.68±0.01a
		+	4.97±.09[a]	4.94±0.01a	4.85±0.05a	4.89±0.03a	4.39±0.09b
Salmonella	25ºC	-	4.90±0.07a	6.76±0.30a	7.31±0.23a	7.06±0.06a	6.95±0.35a
		+	4.91±0.05a	7.39±0.28a	7.58±0.46a	7.40±0.36a	6.88±0.05a
	5ºC	-	4.90±0.07a	4.93±0.02a	4.74±0.16a	4.76±0.04a	4.49±0.61a
		+	4.91±0.05[a]	4.83±0.12a	4.84±0.09a	4.86±0.05a	4.71±0.21a
L. innocua	25ºC	-	4.75±0.16a	6.90±0.29a	6.60±0.48a	6.27±0.35a	6.61±0.15a
		+	4.81±0.10a	5.69±0.23b	6.46±0.17a	6.29±0.28a	5.71±0.25b
	5ºC	-	4.75±0.16a	4.57±0.07a	4.41±0.01a	4.56±0.16a	4.52±0.03a
		+	4.81±0.10a	4.54±0.18a	4.28±0.07a	4.35±0.20a	3.98±0.33a

Values are the average $\log_{10}$ cfu $plug^{-1}$ plus and minus standard deviation of triplicate samples (n=3).

[a] Ascorbic acid treatment, -: control treatment (no AA added); +: AA treatment (2 % w/v for 2 min)

[b] Mean values for each pathogen, time and temperature that are followed by the same letter are not significantly different based on Duncan's multiple range test ($P \leq 0.05$).

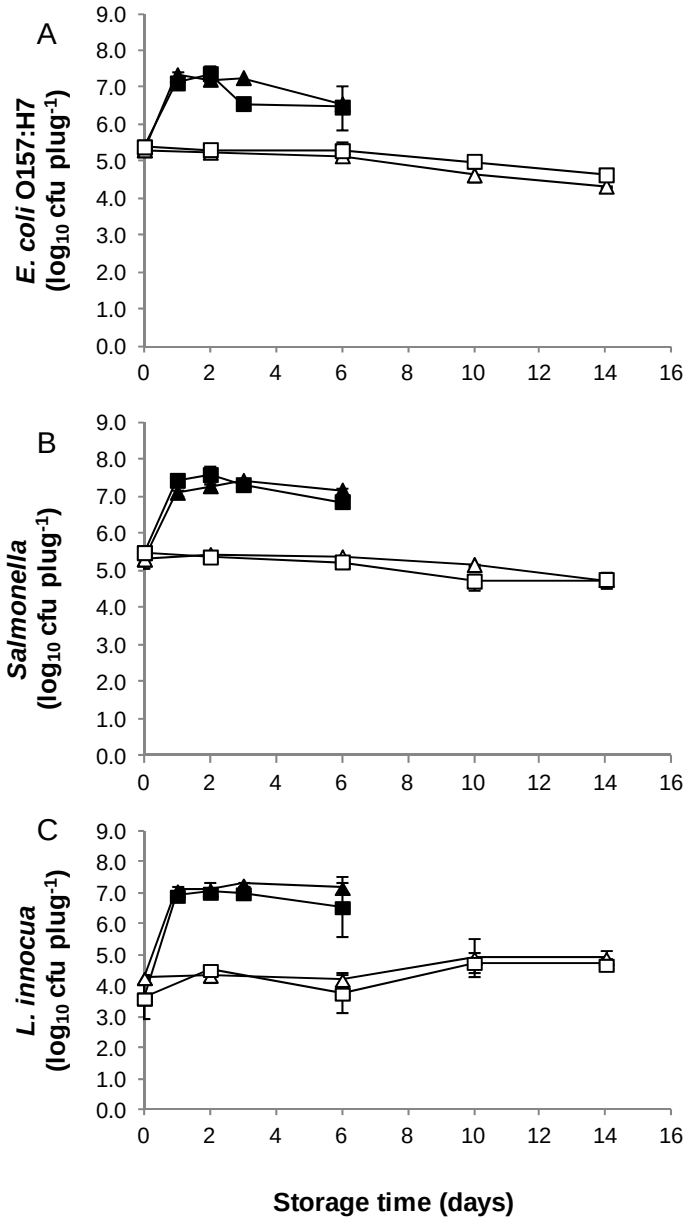

Fig. 3 Growth of *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) in 'Elegant Lady' peach tissue without antioxidant stored at 25 (shaded symbols) and at 5 ºC (open symbols) in passive modified atmosphere (■, □) or in air (▲, △). Inoculum 10^7 cfu mL^{-1}, 15 µL (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

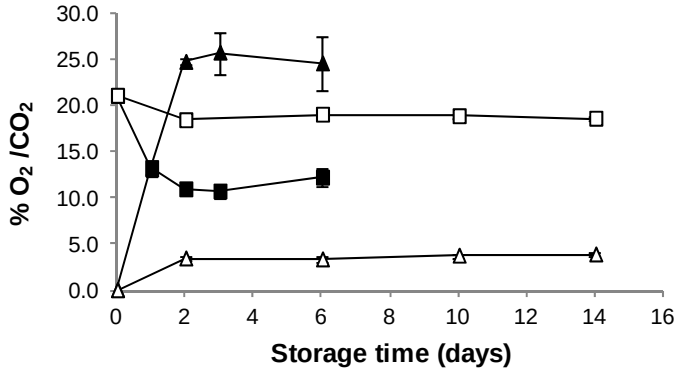

Fig. 4 Concentration of O_2 (■, □) and CO_2 (▲, Δ) in peach trays stored at 25 (shaded symbols) and at 5 ºC (open symbols) (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

4. DISCUSSION

To our knowledge this is the first report of the survival and growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* (used as a surrogate of *L. monocytogenes*) on minimally-processed peaches. Results have demonstrated that the three pathogens were able to grow on peach plugs when incubated at 10 °C or above. Under refrigeration conditions (5 °C), only *L. innocua* could grow on 'Elegant Lady' peach plugs. Pathogens' growth was unaffected by antioxidant treatment or modified atmosphere packaging.

E. coli O157:H7, *Salmonella* and *L. innocua* multiplied rapidly (a population increase higher than 2.0 $\log_{10}$ units in 24 h) on 'Elegant Lady' peach plugs stored at 20 and 25 °C even the low pH (3.73±0.28). At 10 °C, *E. coli* and *Salmonella* population increased by 1 $\log_{10}$ units, meanwhile *L. innocua* increased more than 1.5 $\log_{10}$ units. At refrigeration temperature (5 °C), population increases were only appreciated for *L. innocua*. These results showed the adequacy of minimally-processed peach as a substrate for the three FBP at temperature of 10 °C and above, hence storage temperature contributes to microbial safety of fresh-cut fruits. Along with storage temperature, pH has been cited as the principal determinant for bacteria growth on fresh fruit; however FBP growth has been widely demonstrated in highly acidic produce. For example, *E. coli* O157:H7, *Salmonella* and *L. innocua* grew exponentially on fresh-cut 'Golden Delicious' apples when incubated at 10 °C or above (Alegre *et al.*, 2010). Similar *E. coli* behavior was reported by Abadias *et al.* (2009), Janisiewicz *et al.* (1999), Dingman (2000) and Gunes and Hotchkiss (2002) on apple flesh. *Salmonella* population increased on fresh-cut 'Red Delicious' and 'Golden Delicious' apple (Leverentz *et*

al., 2001; Leverentz *et al.*, 2006) and on chopped ripe tomatoes (Zhuang *et al.*, 1995) at temperatures higher than 10 °C. Growth of *L. monocytogenes* has been reported on fresh-cut 'Red Delicious' and 'Golden Delicious' apples (Leverentz *et al.*, 2003, 2006) but *L. monocytogenes* population reduced on chopped tomatoes stored at 10 and 20 °C (Beuchat and Brackett, 1991). *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* counts increased on freshly peeled 'Hamlin' oranges incubated at 24 °C, but their counts decreased or remained constant when incubated at 4 or 8 °C (Pao *et al.*, 1998). However, neither *E. coli* nor *Salmonella* nor *L. monocytogenes* were able to grow on fresh-cut strawberries (pH 3.6-3.8) incubated at 24 °C (Flessa *et al.*, 2005; Knudsen *et al.*, 2001). This differences among products may be because the effectiveness of pH on the inhibition of microorganisms is affected by the type of organic acid (Wiley, 1994). For example, Deng *et al.* (1999) found that in acidified TSA medium the order of inhibition of *E. coli* O157:H7 at a given pH was acetic acid>citric acid>malic acid. Similarly Ryu *et al.* (1999) reported that acetic acid was the most lethal acid to *E. coli* O157:H7, followed by lactic, citric, and malic acids, when tested over a range of pH values. At a given pH, Jung and Beuchat (2000) found that, in the case of *Salmonella typhimurium*, acetic acid was the most inhibitory acidulant in acidified TSB, followed by lactic acid and citric and malic acids were the least inhibitory. Antilisterial activity of acetic, lactic, citric and hydrochloric acids has been reported by several authors to follow the same pattern (Conner *et al.*, 1990; Sorrells *et al.*, 1989; Vasseur *et al.*, 1999). Although malic acid is the main organic acid in peaches composition, fruits may contain different types of organic acids (Wiley, 1994), therefore, information on minimum pH for growth, given any of the number of organic acid combinations, could not be found (Basset and McClure, 2008).

Differences in *E. coli*, *Salmonella* and *L. innocua* growth occurred in the different peach varieties tested incubated at 25 °C. Highest population increases occurred on 'Royal Glory' and 'Diana' peaches, which had the highest pH values. The lowest *E. coli* and *Salmonella* population increase occurred on fresh-cut 'Placido' peaches (lowest pH values, 3.49±0.18); meanwhile *L. innocua* lowest population was on 'Elegant Lady' peaches. At 5 °C differences were only observed for *L. innocua* since growth was only observed on 'Elegant Lady' peaches. In a previous report, no differences in growth on three varieties of fresh-cut apples occurred for any of the FBP (Alegre *et al.*, 2010). Similar findings with *E. coli* on apples were reported by Fisher and Golden (1998). In contrast, Dingman (2000) found that bruised tissue of 'McIntosh' apples (that had the lowest pH values, 3.33-3.84, among other varieties tested) had an inhibitory effect on the growth of *E. coli* O157:H7. Thus differences among varieties found in growth of pathogens tested could be due to differences in pH and titratable acidity.

The effect of ascorbic acid in the growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* inoculated onto 'Elegant Lady' fresh-cut peaches was also tested. As previously reported on 'Golden Delicious' apple plugs (Alegre *et al.*, 2010), no

significant differences were observed between pathogen growths on treated or untreated fruit. Therefore, ascorbic acid does not have bioactivity against FBP on neither fresh-cut apple nor peach and its use is only devoted to prevent browning.

No significant differences between *E. coli* O157:H7, *Salmonella*, *L. innocua* and mesophilic and psychrotrophic microorganisms growth on 'Elegant Lady' peaches occurred between passive MAP and air atmospheric conditions. Modifications of atmospheric gases were more important at 25 °C than at 5 °C. Similar results with these bacteria were obtained on 'Golden Delicious' apples (Alegre *et al.*, 2010) and with *L. monocytogenes* (Conway *et al.*, 2000). However *E. coli* population was inhibited on fresh apple slices stored under low O_2 (1 %) and/or high CO_2 (0, 15, 30 %) atmospheres in comparison to air (Gunes and Hotchkiss, 2002). Although pathogen growth is not always inhibited by MAP, it can improve quality by reducing the growth of yeast and molds and polyphenol oxidase activity, so MAP is beneficial in extending shelf life of fresh-cut fruit and vegetable.

Our studies have demonstrated the ability of *E. coli* O157:H7, *Salmonella* and *L. innocua* to grow on different varieties of fresh-cut peaches at temperatures of 10 °C and above. In addition, we have demonstrated that growth is not affected by the treatment with ascorbic acid or the use of passive MAP. Although refrigeration (5 °C) effectively inhibited growth of *E. coli* O157:H7 and *Salmonella*, it did not affect the growth of *L. innocua*. Thus, fresh-cut peaches should be protected from contamination by these pathogens during preparation so adequate processing sanitation, quality control, and HACCP practices are necessary to prevent contamination of fresh-cut fruit with pathogenic bacteria during preparation and marketing (Pao *et al.*, 1998). Refrigeration is also required for this product since exposure to abusive temperatures can cause considerably growth of FBP within 24 h. In addition, the control of FBP during storage would be desirable, with, for example the use of bioprotective cultures such as lactic acid bacteria (Trias *et al.*, 2008a, 2008b) and other microorganisms (Abadias *et al.*, 2009; Leverentz *et al.*, 2006; Ukuku *et al.*, 2004).

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DISCLAIMER

The views and opinions expressed in this publication are purely those of the writers and may not in any circumstances be regarded on stating an official position of the European Commission.

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CAPÍTULO III

Evaluation of alternative sanitizers to chlorine disinfection for reducing foodborne pathogens in fresh-cut apple

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ABSTRACT

The risk of undesirable by-products of chlorine disinfection on fresh-cut industries, together with its limited efficacy has led to a search for other alternative agents. The aim of this study was to test several alternative putative antimicrobial substances to reduce *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria* spp. populations on fresh-cut apple. Carvacrol, vanillin, peroxyacetic acid, hydrogen peroxide, N-acetyl-L-cysteine and Citrox were selected for their results in *in vitro* assays against *E. coli* O157:H7 and *Listeria* spp. to be tested on fresh-cut apple plugs. Apple flesh was inoculated by dipping in a suspension of a mix of the studied pathogens at 10^6 cfu mL^{-1}, and then treated with the antimicrobial substances. All treatments were compared to deionized water and a standard sodium hypochlorite treatment (SH, 100 mg L^{-1}, pH 6.5). Pathogen population on apple plugs was monitored up to 6 days at 10 ºC. Bacterial reductions obtained by peroxyacetic acid (80 and 120 mg L^{-1}), vanillin (12 g L^{-1}), hydrogen peroxide (5, 10, 20 mL L^{-1}) and N-acetyl-L-cysteine (5 and 10 g L^{-1}) were similar or higher than reduction obtained by SH. In addition, bacterial population was maintained at low levels throughout storage. No cells of any of the pathogens were detected in the peroxyacetic acid, hydrogen peroxide, Citrox and SH washing solutions after apple treatment. Peroxyacetic acid, hydrogen peroxide and N-acetyl-L-cysteine could be potential disinfectants to be used in fresh-cut industry as an alternative to chlorine disinfection. However, their effect on sensory quality and their effectiveness under commercial processing should be evaluated.

Keywords: *Escherichia coli* O157:H7; *Salmonella*; *Listeria*; fruit disinfection; antimicrobial substances

1. INTRODUCTION

Fruit and vegetables are frequently in contact with soil, animals, insects, or humans during growing or harvesting (Shewfelt, 1987), therefore, by the time they reach the packinghouse or industry, most fresh produce retain populations of 10^4-10^6 cfu g^{-1} (Beuchat, 1996). Minimally processed (MP) fruit go through preparation steps such as peeling, cutting or slicing, and during processing, spoilage and pathogenic microorganisms can gain access to the nutrients inside fruit and multiply. Growth of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* has been demonstrated previously on fresh-cut apples (Abadias *et al.*, 2009; Alegre *et al.*, 2010; Conway *et al.*, 2000; Gunes and Hotchkiss, 2002; Janisiewicz *et al.*, 1999). Moreover, fresh fruit and fruit juices have been incriminated in outbreaks of foodborne illnesses caused by human pathogens like *E. coli* O157:H7 and *Salmonella* spp. (CDC, 2007; Harris *et al.*, 2003; Powell and Luedtke, 2000).

Fresh produce does not receive any 'lethal' treatment that kills all pathogens prior to consumption. Hence, pathogens introduced at any point of the production chain may be present when the produce is consumed. Safe production methods and proper disinfection/decontamination procedures are therefore critical steps in ensuring the safety of ready-to-eat fresh fruit and vegetables (Abadias *et al.*, 2006). In the fresh-cut industry, chlorine is commonly used to disinfect produce at a concentration of 50-200 mg L^{-1}, with a contact time of 1-2 min (Beuchat, 1998). However, inhibitory or lethal activity depends on the amount of free available chlorine (in the form of hypochlorous acid, HOCl) and it loses its activity in contact with organic matter and with exposure to air, light and metals. Furthermore, a prolonged exposure to chlorine vapour may cause irritation to the skin and respiratory tract (Beuchat, 1998). Therefore, there is much interest in developing safer and more effective sanitizers for fruit and vegetable. Several alternative disinfectants (including hydrogen peroxide, organic acids and ozone) have been tested to reduce bacterial populations mainly on vegetables (Allende *et al.*, 2008; Beuchat, 1998; López-Gálvez *et al.*, 2009; Vandekinderen *et al.*, 2009). The efficacy of decontamination methods is reflected in the microbiological reduction obtained and, even more important, in the maintenance of this reduction during storage.

Carbonic acid salts, such as sodium carbonate and sodium bicarbonate are food additives widely used in the food industry that have demonstrated to be a potential alternative to reduce citrus postharvest disease (Palou *et al.*, 2001; Smilanick *et al.*, 1997). Essential oils have also demonstrated good antimicrobial action in model food systems (Skandamis and Nychas, 2000; Tsigarida *et al.*, 2000) and the majority of them are classified as Generally Recognised As Safe (GRAS) (Kabara, 1991). Among them, carvacrol ($C_{10}H_{14}O$) and vanillin have demonstrated biostatic

and/or biocidal effect on produce matrices (Burt, 2004; Fitzgerald *et al.*, 2004; Kisko and Roller, 2005).

Peroxyacetic acid (PAA) is a strong oxidant that has demonstrated to be effective against spoilage and pathogenic bacteria on fresh-cut produce (Allende *et al.*, 2008; Rodgers *et al.*, 2004). Hydrogen peroxide is considered as environmentally friendly because water and oxygen are its sole reaction products (Koivunen and Heinonen-Tanski, 2005) and it has demonstrated efficacy to reduce native and pathogenic microorganism on whole produce (Artés *et al.*, 2007; Sapers and Simmons, 1998). Other studies have shown that the thiol-containing compound N-acetyl-L-cysteine (NAC), a natural chemical suggested as browning inhibitor to enzymatic browning on fresh-cut apple, potato and pear (Molnar-Perl and Friedman, 1990; Oms-Oliu *et al.*, 2006; Raybaudi-Massilia *et al.*, 2007) has also antimicrobial activity (Raybaudi-Massilia *et al.*, 2009).

Other natural extracts are chitosan and citrus flavonoids. Chitosan has shown to prolong storage life and control decay of several fruit (Bautista-Baños *et al.*, 2006). Citrox 14WP is a commercial sanitizer which active compounds are flavonoids extracted from citrus fruit and combined with a number of natural acids (from fruit and vegetable origins).

Currently, investigations have been focused on the search of alternative sanitizers to chlorine based on assuring the quality and safety of the produce. Some of the mentioned substances have been tested against natural microbiota and/or some foodborne pathogens (FBP). However, none of them has been previously tested on fresh-cut apple against three FBP, *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. Therefore, the main objective of this study was to evaluate the efficacy of alternative sanitizers to sodium hypochlorite in reducing *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. on artificially contaminated 'Golden Delicious' apple plugs, as an experimental form of fresh-cut apple. Their capacity to maintain pathogen population at low levels throughout 6 days of storage at abusive temperatures (10 °C) was also determined. The effectiveness was always compared with deionized water and with chlorinated water at a standard chlorine concentration (100 mg L^{-1}).

2. MATERIALS AND METHODS

2.1. Bacterial strains

A non-pathogenic strain of *Escherichia coli* O157:H7 (*E. coli*, NCTC 12900), two pathogenic strains *Salmonella choleraesuis* subsp. *cholerasuis* (Smith) Weldin serotype Michigan (ATCC BAA-709) and Montevideo (ATCC BAA-710), a strain

of *Listeria innocua* Seeliger (CECT 910) and the strains CECT 4031 and CECT 940 of *Listeria monocytogenes* serovar 1a and 4d, respectively, were used.

Salmonella and *E. coli* strains were grown individually in tryptone soy broth (TSB, Oxoid, UK) for 20-24 h at 37 °C. *Listeria* spp. strains were grown individually in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) for 20-24 h at 37 °C. Bacterial cells were harvested by centrifugation at 9820 × g, 10 min at 10 °C and then resuspended in saline peptone (SP; 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone). For the inoculum preparation, bacterial concentration was estimated using a spectrophotometer set at λ=420 nm according to standard curves.

2.2. Antimicrobial substances preparation

The following antimicrobial substances were prepared in sterile deionized water: 10, 50 and 100 g L^{-1} sodium carbonate (Na_2CO_3, Qemical, Quality Chemicals, Esparraguera, Spain), 10, 50 and 100 g L^{-1} sodium bicarbonate ($NaHCO_3$, Sigma, Madrid, Spain), 10, 50 and 100 g L^{-1} potassium carbonate (K_2CO_3, Panreac, Barcelona, Spain), 10, 50 and 100 g L^{-1} potassium bicarbonate ($KHCO_3$, Panreac), 250, 875 and 1500 mg L^{-1} carvacrol (Fluka, Madrid, Spain, plus 10 g L^{-1} Tween 80, Prolabo, Fontenay s/Bois, France), 3, 6 and 12 g L^{-1} vanillin (Sigma, dissolved by heating up to 70 ºC), 0.1, 1, 10, 100 and 1000 mg L^{-1} bromelain (Sigma-Aldrich, Madrid, Spain), 20, 40 and 80 mg L^{-1} peroxyacetic acid (PAA, Fluka), 5, 10 and 20 mL L^{-1} hydrogen peroxide (H_2O_2, Panreac), 2.5, 5 and 10 g L^{-1} N-acetyl-L-cysteine (NAC, Panreac), 5 mL L^{-1} Citrox 14WP (using provider instructions, Citrox Limited, Middlesbrough, UK) and 0.1, 0.5, 1, 5 and 10 g L^{-1} Chitosan (Sigma, from a 20 g L^{-1} stock solution prepared in acetic acid 1 M). In addition, a standard sodium hypochlorite (SH) treatment of approximately 100 mg L^{-1} of free chorine (pH 6.5, adjusted with citric acid 5 M) was prepared by combining sodium hypochlorite solution (10 %, w/v, Panreac) and deionized water.

2.3. *In vitro* assays

All putative antimicrobial substances were tested first in *in vitro* assays against *E. coli* as example of Gram-negative bacteria and against *L. innocua* as example of Gram-positive bacteria. A suspension containing 10^8 cfu mL^{-1} of both, *E. coli* and *L. innocua* strains, was prepared, and 1 mL of this suspension was added to 9 mL of each of the antimicrobial substances, obtaining a pathogen population of 10^7 cfu mL^{-1}. Samples were taken for each treatment after 1, 3 and 5 min of exposure to the antimicrobial substance (only after 5 min in the case of chitosan) and bacterial population was determined by 10 –fold diluting on SP and plating (20 µL) onto Sorbitol MacConkey Agar (SMAC, Biokar Diagnostics, Beauvais, France) supplemented with Cefixime-Tellurite (CT-SMAC, Biokar) for *E. coli* or

onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics, Beauvais, France) for *L. innocua*. Plates were incubated at 37±1 °C for 24±2 h (*E. coli*) and 48±2 h (*L. innocua*). There were two replicates for each substance and concentration and the assay was repeated once. Antimicrobial substances that achieved better results were selected for *in vivo* assays.

2.4. *In vivo* assays

To prepare the bacterial inoculum, the necessary quantity of each pathogenic strain concentrated suspension was added to 1 L of deionized water to obtain a mixed inoculum of *E. coli*, *Salmonella* spp. and *Listeria* spp. at a concentration of 10^6 cfu mL^{-1} each. Inoculum concentration was checked by plating onto CT-SMAC for *E. coli*, onto Xylose Lysine Deoxycholate agar (XLD, Oxoid, Cambridge, UK) for *Salmonella* spp. or onto Palcam agar for *Listeria* spp. and incubated at 37±1 °C for 24±2 h (*E. coli* and *Salmonella*) and 48±2 h (*Listeria* spp.).

'Golden Delicious' apples were obtained from local packinghouses in Lleida, Catalonia. Apples were washed in running tap water, disinfected with ethanol 70 % and let to dry at room temperature. Then, apples were cut in halves and plugs of 1.2 cm of diameter, 1 cm long (1 g approx.) were taken using a cork borer. Apple plugs were used as an experimental form of fresh-cut apple. Apple plugs were inoculated by immersion in the mixed bacterial inoculum for 2 min at 150 rpm and then were allowed to dry for 20 min in a biosafety cabinet. Inoculated plugs were then divided into 12-plug samples and separately washed with 120 mL (1:10, w:v) of the selected antimicrobial substances at different concentrations for 1 min at 150 rpm (5 min wash for Citrox, following provider instructions). Antimicrobial washes were compared with deionized water (DW) and a standard hypochlorite treatment (SH, 100 mg L^{-1} free chlorine, pH 6.5). After all treatments, the apple plugs were drained off. In the case of apple plugs washed with SH, they were rinsed, to remove chemical residue, with DW for 1 min at 150 rpm and drained. After draining, apple plugs were left to dry in a flow cabinet, placed into sterile test tubes and stored at 10 °C. All the experiment was carried out at room temperature.

The concentration of each pathogen on apple plugs was determined before (BT) and after (AT) the treatment and after 3 and 6 days at 10 °C. At each sample time, one apple plug was placed into a sterile plastic bag (BagPage 80 mL, Interscience BagSystem, St Nom La Breteche, France) and 9 mL of SP were added. It was homogenised in a stomacher blender for 120 s at high speed (Bagmixer 100 Minimix, Interscience). Aliquots of the mixture were then serially diluted and surface plated onto the selective agars for determination of bacterial counts. There were three replicate apple plugs for each pathogen and sampling time and each assay was repeated twice.
For all washing solutions, pH and oxidation-reduction potential (ORP) were determined before and after apple treatment. Free chlorine was determined for SH

solution using a free and total photometer (HI 93734, Hanna Instruments, Eibar, Spain). ORP and pH were determined using a pH/ion/conductivity meter (Model GLP-22, Crison), with a pH electrode (Crison, 52-01) or an ORP electrode (Crison, platinum Ag/AgCl electrode 52-61). In addition, the microbial load of each washing solution after treatment was evaluated. To carry out such determinations, 1 mL of each washing solution was added to 9 mL of buffered peptone water (BPW, Oxoid) or to 9 mL of sodium thiosulphate 5-hydrate (0.5 %, w/v, Panreac), in the case of SH to neutralize chlorine, and 0.1 mL were spread-plated as described previously. Additionally, an enrichment step was carried out by adding 1 mL of the BPW or sodium thiosulphate plus washing solution to 10 mL of TSB. After incubation at 37 °C for 24 h, turbidity of TSB was evaluated and presence of *E. coli*, *Salmonella* spp. and *Listeria* spp. was checked on their respective selective media. There was one sample for each washing treatment and each assay was repeated twice.

2.5. Statistical analysis

All cfu mL^{-1} data were transformed to $\log_{10}$ cfu mL^{-1} or $\log_{10}$ cfu $plug^{-1}$. The reductions in bacteria were calculated by subtracting the initial mean bacteria population from the bacteria population after each treatment. The General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) was applied (v.8; SAS Institute, Cary, NC, USA). Significant differences between treatments were analyzed by Duncan's Multiple Range test at a significance level of $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *In vitro* assays

No reduction in *E. coli* O157:H7 and *L. innocua* pure cultures was achieved in $NaHCO_3$ (10, 50 and 100 g L^{-1}), $KHCO_3$ (10, 50 and 100 g L^{-1}), vanillin (3, 6 and 12 g L^{-1}) and bromelain (0.1, 1, 10, 100, 1000 mg L^{-1}) solutions. Therefore $NaHCO_3$, $KHCO_3$ and bromelain were rejected for *in vivo* assays. However, vanillin solutions were tested *in vivo* as its effect has been reported to be bacteriostatic (Fitzgerald *et al.*, 2004) and good results were obtained in apple juice (Moon *et al.*, 2006).

E. coli population reductions obtained with Na_2CO_3, K_2CO_3, H_2O_2 and NAC increased with increases in doses and all of them achieved a population reduction higher than 4.0 log units (*E. coli* population below detection limit, 2.5×10^3 cfu mL^{-1}) at highest doses (Table 1). Carvacrol at 875 and 1500 mg L^{-1} reduced *E. coli* population below detection limit from the first minute of exposure and PAA and Citrox reduced *E. coli* population at all doses tested. In contrast, only

highest chitosan concentration (10 g L^{-1}) reduced *E. coli* population. In general, *L. innocua* population reductions observed with the antimicrobial substances tested were lower than those observed for *E. coli*. No bacterial reductions were observed with Na_2CO_3 and chitosan and *L. innocua* was only reduced by K_2CO_3 at 100 g L^{-1} solution after 3 and 5 min of exposure. Pathogen population reductions were dose dependent for H_2O_2, NAC and Citrox solutions.

Na_2CO_3 and K_2CO_3 were rejected because of the possibility of causing problems in industrial applications due to carbonate fouling at the effective dose. Chitosan was also rejected, due to economical reasons, as it was only effective against *E. coli* at the highest concentration tested (10 g L^{-1}). Therefore, carvacrol, vanillin, PAA, H_2O_2, NAC and Citrox were selected for *in vivo* assays.

3.2. *In vivo* assays

In general, *E. coli* population on 'Golden Delicious' apple plugs tended to decrease throughout storage at 10 °C while *Salmonella* spp. populations tended to increase. *Listeria* spp. increased on the first three days of storage and then was maintained or reduced. SH treatment reduced *E. coli* and *Salmonella* spp. population approximately 1.0-log units and *Listeria* spp. between 0.4 and 1.9 log units. After treatment the three FBP behaved as in water-treated apple plugs due to removal of chemical residue by rinse.

3.2.1. Apple plugs disinfection with carvacrol

Survival and growth of foodborne pathogens on 'Golden Delicious' apple plugs treated with DW, SH and carvacrol (500, 875 and 1000 mg L^{-1}) and then stored at 10 °C up to 6 days were compared. Population reductions obtained washing with all carvacrol solutions were similar to the reduction values obtained by DW and significantly lower compared to reductions obtained by SH (1.0 log units, data not shown). After 6 days of storage at 10 °C, pathogen populations on carvacrol treated apple plugs were at least 1.0 log units higher than bacterial populations on DW treated apple plugs. In contrast, Roller and Seedhar (2002) reported that microbial flora was inhibited on kiwifruit treated with 750, 1500 and 2250 mg L^{-1} of carvacrol along 21 days of storage at 4 °C. However, at the higher concentration tested, browning of fruit wedges was observed. Recently, *E. coli* O157:H7 was inactivated in apple juice by the addition of 187 mg L^{-1} of carvacrol (Kisko and Roller, 2005).

Table 1 Reduction (log cfu mL^{-1}) of pure cultures of *Escherichia coli* O157:H7 and *Listeria innocua* by different exposure times to antibacterial substances.

Substance	Concentration	*E. coli* O157:H7			*L. innocua*		
		1'	3'	5'	1'	3'	5'
Na_2CO_3	10 g L^{-1}	N[a]	1.2	>4.0[b]	N	N	N
	50 g L^{-1}	N	2.7	>4.0	N	N	N
	100 g L^{-1}	N	>4.0	>4.0	N	N	N
K_2CO_3	10 g L^{-1}	1.4	>4.0	>4.0	N	N	N
	50 g L^{-1}	1.7	>4.0	>4.0	N	N	N
	100 g L^{-1}	3.1	>4.0	>4.0	N	1.7	2.5
Carvacrol	250 mg L^{-1}	N	N	N	N	N	N
	875 mg L^{-1}	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
	1500 mg L^{-1}	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
PAA	20 mg L^{-1}	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
	40 mg L^{-1}	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
	80 mg L^{-1}	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
H_2O_2	5 mL L^{-1}	1.7	2.9	>4.0	N	N	1.5
	10 mL L^{-1}	2.3	3.7	>4.0	1.3	1.5	1.6
	20 mL L^{-1}	>4.0	>4.0	>4.0	1.4	2.9	>4.0
NAC	2.5 g L^{-1}	N	N	1.6	N	N	N
	5 g L^{-1}	2.0	3.5	>4.0	N	N	N
	10 g L^{-1}	>4.0	>4.0	>4.0	1.0	1.9	>4.0
Citrox	5 mL L^{-1}	>4.0	>4.0	>4.0	N	1.4	2.2
Chitosan	10 g L^{-1}	-[c]	-	3.2	-	-	N

Values for reduction are present as mean of two replicates.
[a] No reduction
[b] Bacterial populations after treatment under detection limit (2.5×10^3 cfu mL^{-1})
[c] Not determined

3.2.2. Apple plugs disinfection with vanillin

Only highest vanillin concentration caused an *E. coli* population reduction value similar to that obtained by SH (between 0.7 and 1.0-log units, Fig. 1A) and no differences between populations on apple plugs treated with SH and vanillin 12 g L^{-1} (1.8 and 2.6 log cfu $plug^{-1}$, respectively) were observed after 6 days of storage at 10 °C. Although *Salmonella* spp. population reduction obtained with vanillin solutions after treatments did not equal SH reductions (Fig. 1B), at the end

of storage period the *Salmonella* spp. population on apple plugs treated with vanillin 12 g L^{-1} was the lowest (3.1 log cfu $plug^{-1}$) and significantly different from all other treatments. All treatments caused the same initial *Listeria* spp. population reductions, between 1.2 and 1.8 log units (Fig. 1C). However, the pathogen population was only maintained at low levels (below 2.0 log cfu $plug^{-1}$) when treated with vanillin 6 and 12 g L^{-1} and SH.
Greater effectiveness has been reported previously, when a lower vanillin concentration (3 g L^{-1}) was lethal to *E. coli* O157:H7 and *L. monocytogenes* in apple juice (Moon *et al.*, 2006) and, even lower concentrations (1.8 g L^{-1}), were reported to inhibit pathogenic and spoilage microbial growth on apple slices (Rupasinghe *et al.*, 2006). However, Rupashinghe *et al.* (2006) observed that vanillin concentration beyond 1.8 g L^{-1} could produce unacceptable flavours and aromas for fresh-cut apples, therefore it would not be a suitable sanitizer for fresh-cut apple at the effective concentrations found it this assay.

3.2.3. Apple plugs disinfection with peroxyacetic acid (PAA)

E. coli and *Salmonella* spp. reductions obtained by SH and PAA 40 mg L^{-1} were the same (around 1.0 log unit) and the highest reductions were caused by PAA 80 and 120 mg L^{-1} treatments (2.0 log units, Fig. 2A and B). The *E. coli* population on apple plugs treated with PAA 80 and 120 mg L^{-1} after 6 days of storage at 10 °C was 1.8 and 2.3 log units lower than the initial population. However, no significant differences between treatments were detected. *Salmonella* spp. populations increased on all apple plugs regardless of washing treatment throughout storage at 10 °C and, although the lowest final populations were observed on PAA-treated apple plugs, it was not significantly different from SH treated apple plugs. *Listeria* spp. population reductions obtained after all treatments were the same (0.2-0.8 log units, Fig. 2C). Throughout storage, the *Listeria* spp. population was maintained at low levels on apple plugs treated with PAA and, after 6 days at 10 °C, *Listeria* spp. population on apple plugs treated with PAA was significantly lower (from 1.7 to 2.3 log units) than those treated with SH.
PAA 80 mg L^{-1} has previously been reported to be effective in controlling *E. coli* and *L. monocytogenes* in MP apples and lettuce during 9 days of storage at 4 °C (Rodgers *et al.*, 2004) and PAA 68 mg L^{-1} reduced psychrotrophic and mesophilic counts in MP 'Galia' melon (Silveira *et al.*, 2008). The commercial disinfectant Tsunami (PAA, 300 mL L^{-1}) has been shown to be effective in reducing total bacterial counts on rocket leaves throughout storage at 4 °C (Martínez-Sánchez *et al.*, 2006). However, lower concentrations (80 µL L^{-1}) did not improve fresh-cut escarole disinfection (Allende *et al.*, 2008).

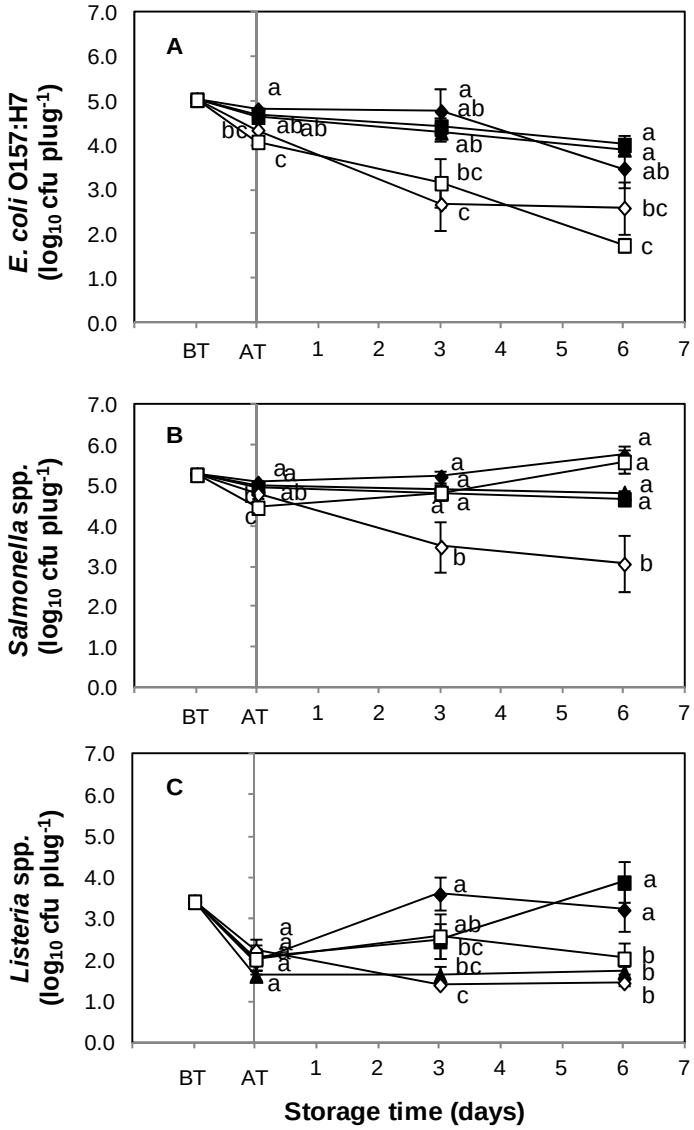

Fig. 1 Population of *Escherichia coli* O157:H7 (A), *Salmonella* spp. (B) and *Listeria* spp. (C) on minimally processed 'Golden Delicious' apple plugs before (BT) and after (AT) treatment with DW (◆), SH (□), vanillin 3 g L^{-1} (▲), vanillin 6 g L^{-1} (■) and vanillin 12 g L^{-1} (◇) and its survival during storage at 10 °C (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol). For each time, different letters indicate significant differences (P<0.05) among treatments.

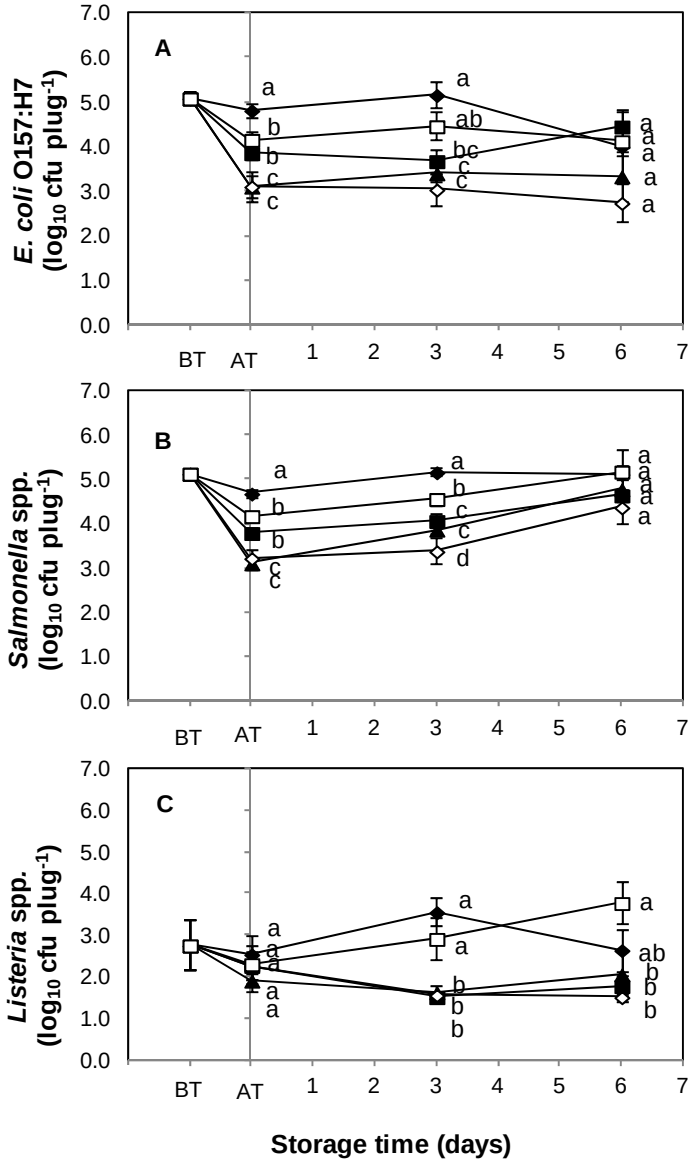

Fig. 2 Population of *Escherichia coli* O157:H7 (A), *Salmonella* spp. (B) and *Listeria* spp. (C) on minimally processed 'Golden Delicious' apple plugs before (BT) and after (AT) treatment with DW (◆), SH (□), peroxyacetic acid 40 mg L^{-1} (▲), peroxyacetic acid 80 mg L^{-1} (■) peroxyacetic acid 120 mg L^{-1} (◇) and its survival during storage at 10 ºC (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol). For each time, different letters indicate significant differences (P<0.05) among treatments.

3.2.4. Apple plugs disinfection with hydrogen peroxide (H_2O_2)

Important *E. coli* and *Salmonella* spp. population reductions were detected when apple plugs were treated with all of H_2O_2 solutions (above 2.5 log units, Fig. 3A and B). After 6 days of storage at 10 °C, *E. coli* populations on apple plugs treated with H_2O_2 solutions were significantly lower (below 2.3 log cfu plug^{-1}) than populations on apple plugs treated with SH. In the case of *Salmonella* spp., the population was significantly lower in H_2O_2 treated apple plugs than in SH treatments throughout all storage period and H_2O_2 20 mL L^{-1} treatment was the most effective treatment in maintaining *Salmonella* spp. populations at low levels (around 3.1 log cfu plug^{-1}). SH and H_2O_2 treatments reduced *Listeria* spp. populations to the same extent (around 2 log units, Fig. 3C). During storage at 10 °C, *Listeria* spp. grew on apple plugs treated with SH, meanwhile they remained low on apple plugs treated with H_2O_2, with final populations below 1.5 log cfu plug^{-1}, not significantly different from DW treated apple plugs.
The efficacy of H_2O_2 washing has previously been demonstrated to extend shelf life and reduce native microbial and pathogen populations including *E. coli*, in whole fruit and vegetables and in MP cucumber, zucchini, bell peppers, and melons (Artés *et al.*, 2007; Sapers, 2003). The use of H_2O_2 (5 mL L^{-1}) resulted in a reduction on total microbial counts on fresh-cut 'Galia' melon during 10 days of storage at 5 °C (Silveira *et al.*, 2008) and on fresh-cut cantaloupe stored at 4 °C (Sapers *et al.*, 2001).

3.2.5. Apple plugs disinfection with N-acetyl-L-cyteine (NAC)

NAC 5 g L^{-1} treatment reduced *E. coli* population by approximately 1.0 log unit which was similar to the reduction obtained by SH (Fig. 4A) and NAC 10 g L^{-1} reduced it 2.0 log units. Although the *E. coli* population decreased for all treatments throughout storage, the lowest population was observed in SH and NAC 10 g L^{-1} treated apple plugs (below 2.0 log cfu plug^{-1}). Similar *Salmonella* spp. population reduction values were observed after all treatments (Fig. 4C). However, *Salmonella* spp. population increased for all treatments, with the exception of NAC 10 g L^{-1} treated apple plugs, where *Salmonella* populations decreased to 2. log cfu plug^{-1}. Lower reduction values were observed with the Gram-positive *Listeria* spp. However NAC 10 g L^{-1} gave lowest *Listeria* spp. population after 6 days of storage at 10 °C (below detection limit 50 cfu g^{-1}). Therefore, we have demonstrated that NAC has not only an antioxidant effect (Rojas-Grau *et al.*, 2006) but also an antimicrobial effect. Previously, the dip of minimally processed 'Fuji' apples in aqueous solution containing 10 g L^{-1} NAC, 10 g L^{-1} glutathione and 10 g L^{-1} calcium lactate and subsequent contamination with *L. monocytogenes*, *Salmonella* Enteritidis and *E. coli* prevented pathogens growth throughout 30 days of storage at 5 °C. The greatest effectiveness was observed against *E. coli* and *Salmonella* (Raybaudi-Massilia *et al.*, 2009).

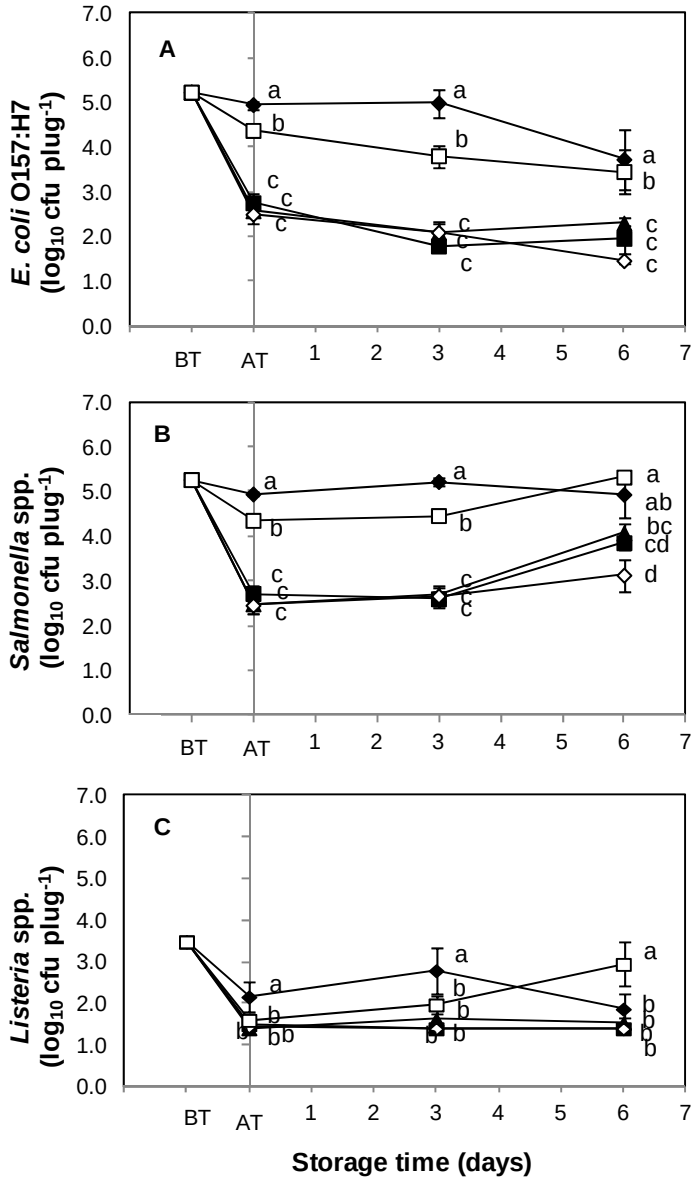

Fig. 3 Population of *Escherichia coli* O157:H7 (A), *Salmonella* spp. (B) and *Listeria* spp. (C) on minimally processed 'Golden Delicious' apple plugs before (BT) and after (AT) treatment with DW (◆), SH (□), hydrogen peroxide 5 mL L^{-1} (▲), hydrogen peroxide 10 mL L^{-1} (■) and hydrogen peroxide 20 mL L^{-1} (◇) and its survival during storage at 10 ºC (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol). For each time, different letters indicate significant differences (P<0.05) among treatments.

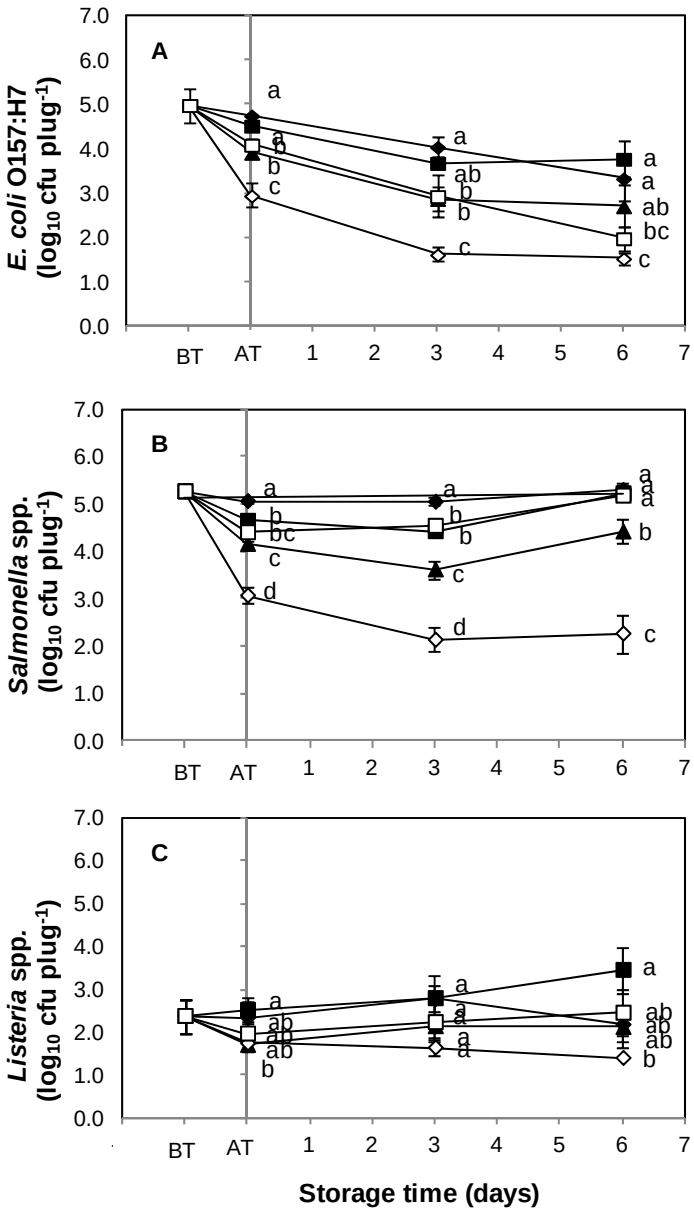

Fig. 4 Population of *Escherichia coli* O157:H7 (A), *Salmonella* spp. (B) and *Listeria* spp. (C) on minimally processed 'Golden Delicious' apple plugs before (BT) and after (AT) treatment with DW (◆), SH (□), NAC 2.5 g L^{-1} (▲), NAC 5 g L^{-1} (■) and NAC 10 g L^{-1} (◇) and its survival during storage at 10 ºC (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol). For each time, different letters indicate significant differences (P<0.05) among treatments.

3.2.6. Apple plugs disinfection with Citrox

Treatment of apple plugs with SH or Citrox caused, approximately, 1.0 log reduction on *E. coli* and *Salmonella* spp. populations (Fig. 5A and B). However, no significant differences were observed after 6 days of storage among treatments. Although the Citrox treatment reduced initial *Listeria* spp. populations approximately 1 log more than DW and SH, no significant differences among treatments were observed at the end of the storage period. In addition, apple plugs were stained slightly brown due to the brown colour of the Citrox solution. Citrox has previously been found to be effective in reducing microorganisms on fresh-cut escarole after washing, however, microbial loads after 8 days of storage at temperatures lower than 10 °C were significantly higher than in SH treatment (Allende *et al.*, 2008).

3.2.7. Comparison of antimicrobial substances efficacy

The reduction obtained by each antimicrobial substance for each pathogen compared to SH has been calculated by subtracting log cfu mL^{-1} population recovered from each treatment minus pathogen population recovered from SH treated apple plugs (Table 2). *E. coli* highest reductions were observed on apples plugs washed with all H_2O_2 solutions, NAC 10 g L^{-1} and PAA 80 and 120 mg L^{-1} after treatment and throughout storage, with reductions between 0.8±0.6 and 2.0±0.1 log units higher than SH. Antimicrobial treatments which reduced *Salmonella* spp. the most after treatment when compared to SH disinfection, were H_2O_2 treatments and NAC 10 g L^{-1}. Throughout storage at 10 °C, vanillin 12 g L^{-1}, H_2O_2 and NAC 20 g L^{-1} were the best antimicrobials with reduction values between 1.3±0.2 and 2.9±0.4 log units higher than SH. Initial reductions of *Listeria* spp. populations were similar to SH for all treatments. However, throughout storage PAA and H_2O_2 treatments were the best, avoiding pathogens growth.

3.2.8. Physicochemical properties of tested solutions

NAC solutions were the most acidic (Table 3), with pH ranging from 2.12 to 2.44, followed by PAA (3.58-3.86) solutions and Citrox (3.74). Carvacrol and vanillin solutions had pH values below 5.0 and H_2O_2 solutions pH ranged from 4.41 to 5.47. DW and SH solutions had pH values higher than 6.0. Differences in pH among concentrations tested were detected for PAA and NAC solutions. In both cases, higher pHs were determined for less concentrated solutions. Significant differences between pH before and after treatment were only observed for PAA, with higher pH after treatment (data not shown).

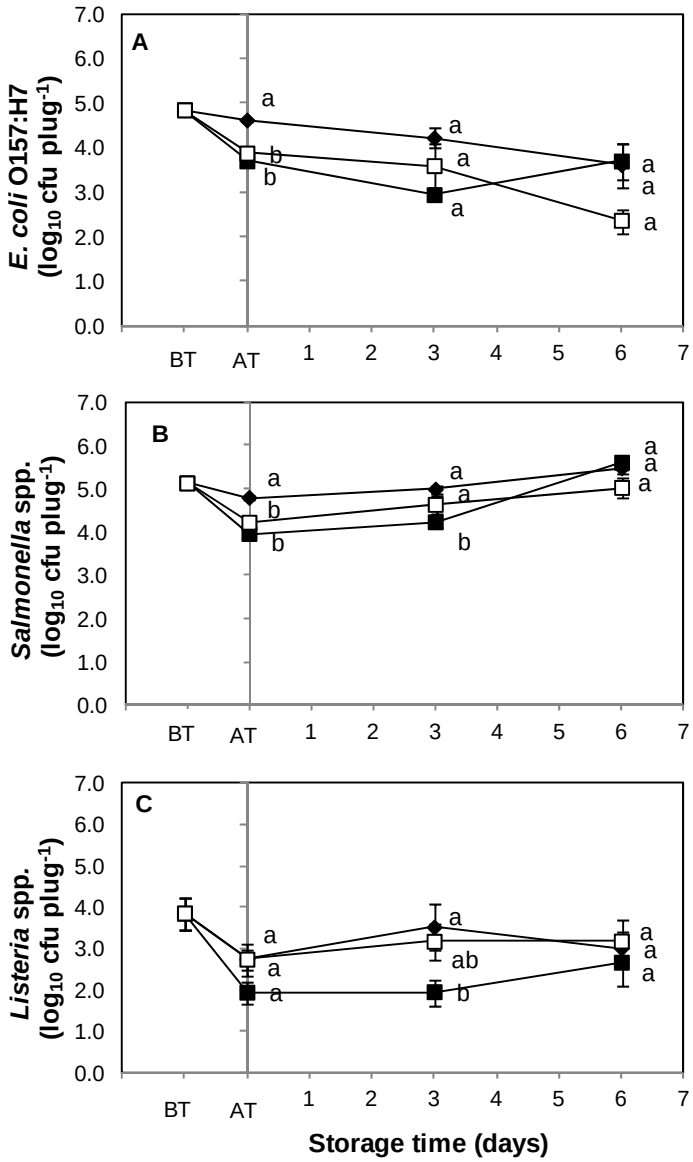

Fig. 5 Population of *Escherichia coli* O157:H7 (A), *Salmonella* spp. (B) and *Listeria* spp. (C) on minimally processed 'Golden Delicious' apple plugs before (BT) and after (AT) treatment with DW (◆), SH (□) and Citrox 5 mL L^{-1} (■) and its survival during storage at 10 ºC (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol). For each time, different letters indicate significant differences (P<0.05) among treatments.

Table 2 *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria* spp. population reductions compared to SH solution on apple plugs throughout storage at 10 °C.

Washing solution	**Storage time at 10 °C (days)**					
	***E. coli* O157:H7**		***Salmonella* spp.**		***Listeria* spp.**	
	0	**6**	**0**	**6**	**0**	**6**
Vanillin 3 g L^{-1}	-0.6±0.1 A	-2.3±0.2 A	-0.5±0.0 A	0.9±0.0 BCD	-0.0±0.3 ABC	-1.9±0.5 A
Vanillin 6 g L^{-1}	-0.6±0.1 A	-2.2±0.1 A	-0.6±0.0 A	0.8±0.1 BCD	0.4±0.1 BCD	0.3±0.1 B
Vanillin 12 g L^{-1}	-0.3±0.3 ABC	-0.8±0.6 BC	-0.3±0.1 A	2.5±0.7 F	-0.2±0.3 AB	0.6±0.1 BC
H_2O_2 5 mL L^{-1}	1.6±0.2 DE	1.5±0.3 EF	1.6±0.2 DE	1.5±0.1 DE	0.1±0.1 ABCD	1.6±0.0 CDE
H_2O_2 10 mL L^{-1}	1.8±0.2 E	1.2±0.3 EF	1.9±0.2 E	1.3±0.2 DC	0.2±0.0 ABCD	1.4±0.1 CDE
H_2O_2 20 mL L^{-1}	1.9±0.2 E	2.0±0.1 F	1.9±0.2 E	2.2±0.4 EF	0.2±0.1 ABCD	1.6±0.0 CDE
NAC 2.5 g L^{-1}	-0.4±0.0 AB	-1.8±0.4 AB	-0.2±0.1 A	-0.1±0.2 AB	-0.6±0.3 A	-1.0±0.6 A
NAC 5 g L^{-1}	0.2±0.1 BC	-0.7±0.4 BC	0.3±0.1 B	0.8±0.2 BCD	0.3±0.1 ABCD	0.3±0.5 B
NAC 10 g L^{-1}	1.1±0.3 D	0.5±0.1 DE	1.3±0.2 CD	2.9±0.4 F	0.2±0.2 ABCD	1.1±0.0 BCD
Citrox	0.2±0.2 BC	-1.3±0.4 ABC	0.3±0.1 B	-0.6±0.3 A	0.8±0.3 D	0.5±0.6 BC
PAA 40 mg L^{-1}	0.3±0.2 C	-0.3±0.4CD	0.4±0.1 B	0.5±0.1 BCD	-0.0±0.3ABCD	2.0±0.2 DE
PAA 80 mg L^{-1}	1.1±0.3 D	0.8±0.6 E	1.1±0.2 C	0.4±0.2 BC	0.7±0.4 CD	1.7±0.5 DE
PAA 120 mg L^{-1}	1.0±0.2 D	1.4±0.4 EF	1.0±0.2 C	0.8±0.4 BCD	0.0±0.3 BCD	2.3±0.1 E

Pathogen reduction was obtained by subtracting log cfu mL^{-1} population recovered from each treatment minus pathogen population recovered from SH treated apple plugs. Values are the mean of six values ±standard error of the mean. A positive value represented that bacterial reduction obtained by the treatment was higher than reduction obtained by SH. For each column, different letters indicate significant differences (P<0.05) among treatments.

The lowest ORP values were determined in NAC solutions (from 280 to 297 mV), followed by H_2O_2, Citrox, DW and vanillin (below 400), then carvacrol (from 407 to 416 mV), PAA (from 588 to 610 mV) and finally SH (higher than 850 mV). ORP did not change significantly after treatment for any of the solutions tested (data not shown).

In SH solution, changes in free chlorine were also determined. Initial free chlorine was 114±11 mg L^{-1} and it decreased to 66±5 mg L^{-1} after the apple plug treatment.

Table 3 Determination of pH and ORP (mV) of tested antimicrobial solutions before apple treatment.

Treatment		**pH**	**ORP (mV)**
		Initial	**Initial**
DW		6.19±0.16	327±21
Carvacrol	500 mg L^{-1}	4.60±0.04	407±3
	875 mg L^{-1}	4.59±0.00	416±1
	1000 mg L^{-1}	4.66±0.08	411±3
Vanillin	3 g L^{-1}	4.81±0.04	377±19
	6 g L^{-1}	4.59±0.04	395±27
	12 g L^{-1}	4.43±0.14	396±24
PAA	40 mg L^{-1}	3.86±0.00	589±0
	80 mg L^{-1}	3.76±0.09	588±3
	120 mg L^{-1}	3.58±0.01	610±24
H_2O_2	5 mL L^{-1}	5.47±0.18	306±1
	10 mL L^{-1}	5.15±0.12	335±0.00
	20 mL L^{-1}	4.41±0.07	377±21
NAC	2.5 g L^{-1}	2.44±0.00	297±32
	5 g L^{-1}	2.26±0.01	291±34
	10 g L^{-1}	2.12±0.00	280±22
Citrox	5 mL L^{-1}	3.74±0.03	330±14
SH		6.52±0.03	859±11

Values are expressed as mean of two replicates ± standard error of the mean.

3.2.9. Microbial load of washing treatments

Pathogen populations were not recovered from PAA, H_2O_2, Citrox and SH solutions (Table 4). However, *Salmonella* was detected in one of two samples of H_2O_2 5 mL L^{-1} washing solution after enrichment. *E. coli* and *Salmonella* spp. populations were recovered from the rest of washing solutions, however, *Listeria* spp. was not detected by direct plating in carvacrol 875 and 1000 mg L^{-1}, but it was possible to detect it after enrichment.
Processing wash water, if not properly sanitized, can become a source of microbiological contamination for every piece of product that passes through (Zagory, 1999). It has been shown previously that the use of PAA based sanitizers, such as Tsunami, are a good alternative for the disinfection of processing water, as it was as effective as chlorine in avoid cross-contamination, however, Citrox did not completely avoid it (López-Gálvez *et al.*, 2009). A contact has to occur between the microorganisms and the sanitizer in order to kill them (Gómez-López *et al.*, 2008), therefore differences between sanitizer efficacy on washing solution and on apple flesh could be explained as contact is likely to be much difficult on apple flesh.

4. CONCLUSIONS

Our results have demonstrated that alternative sanitizers, such as PAA (80 and 120 mg L^{-1}), H_2O_2 (5, 10 and 20 mL L^{-1}) and NAC (10 g L^{-1}), could be used instead of chlorinated water in MP apple productions as they did not only reduce pathogen population after treatment but also maintained them at low levels throughout storage, even at an abusive temperature (10 °C). However, PAA, H_2O_2 and NAC preservative action against *Salmonella* spp. and *Listeria* spp. should be improved by combining with other antibacterial substances or by adding additional hurdles. For example, maintaining a correct storage temperature could prevent *Salmonella* growth. In addition, and based on our results, PAA, H_2O_2 and Citrox solutions could prevent cross-contamination of fresh produce in the fresh-cut industry. In this study the effect of antimicrobial treatments on the sensory quality of the MP apple was not tested, hence further studies should be carried out to simulate typical commercial conditions and evaluate the sensory quality of the product.

Table 4 Population of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria* spp. recovered from wash solutions after treatment.

Treatment		***E. coli* O157:H7**	***Salmonella* spp.**	***Listeria* spp.**
DW		3.9±0.1[a]	3.8±0.1	2.5±0.3
Carvacrol	500 mg L^{-1}	3.7	3.3	1.7
	875 mg L^{-1}	3.5	3.3	1/1[b]
	1000 mg L^{-1}	3.6	3.3	1/1
Vanillin	3 g L^{-1}	3.8±0.2	3.6±0.2	2.6±0.2
	6 g L^{-1}	3.8±0.1	3.6±0.1	1.4±1.4
	12 g L^{-1}	3.8±0.1	3.4±0.1	1.1±1.1
PAA	40 mg L^{-1}	0	0	0
	80 mg L^{-1}	0	0	0
	120 mg L^{-1}	0	0	0
H_2O_2	5 mL L^{-1}	0	1/2[c]	0
	10 mL L^{-1}	0	0	0
	20 mL L^{-1}	0	0	0
NAC	2.5 g L^{-1}	3.6±0.1	3.7±0.1	1.9±0.5
	5 g L^{-1}	3.4±0.1	3.6±0.1	2.0±0.6
	10 g L^{-1}	2.4±1.0	2.1±0.6	1.2±1.2
Citrox	5 mL L^{-1}	0	0	0
SH		0	0	0

[a]Values are the mean of two determinations ±standard error of the mean. The detection limit was 1.7 log cfu mL^{-1}. If pathogen was not detected by both direct plating and enrichment, the recovery was 0; if the pathogen was only detected by enrichment an arbitrary value of 1.4 log cfu mL^{-1} (half of detection limit) was assigned for statistical reasons.

[b]Not detected by direct plating, but one of one samples was positive by enrichment.

[c]Not detected by direct plating, but one of two samples was positive by enrichment.

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CAPÍTULO IV

New species of *Enterobacteriaceace* to control foodborne pathogens on fresh-cut apples and peaches

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ABSTRACT

Currently, chlorine is the most widely decontamination method used in minimally processed (MP) industry, however, it does not achieve more than 1-2-log reduction of the bacterial population. An efficient decontamination of MP produce could create a less competitive environment where pathogens can unrestrictedly multiply. Thus, our objective was to test the effect of the biopreservative bacterial strain CPA-6, isolated from MP apple, to control *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on MP apples and peaches. Apple and peach plugs were co-inoculated with a suspension containing one of the pathogens (10^5 cfu plug^{-1}) and CPA-6 (10^6 cfu plug^{-1}) and stored at 20 ºC or 5 ºC. CPA-6 effectively inhibited growth or even reduced, in some cases below detection level, pathogen population on both fruits stored 2 d at 20 ºC and *E. coli* on both fruits stored at 5 ºC, when compared to the pathogen inoculated alone. The minimal effective dose able to inhibit any of pathogens tested on both fruits and both temperatures was 10^4 cfu plug^{-1} and it did not cause hypersensitive reaction on tobacco plants. Finally, CPA-6 was identified as new specie belonging to family *Enterobacteriaceae*. Therefore; it could be a suitable microorganism to use as biopreservative culture to control growth of foodborne pathogens on MP fruit.

Keywords: *Escherichia coli* O157:H7; *Salmonella*; *Listeria innocua*; biopreservation; minimally processed fruit; antagonist

1. INTRODUCTION

Fruit and vegetables are part of a balanced diet and there is considerable evidence of the health and nutritional benefits associated with their consumption. Different organizations (WHO, FAO, USDA, EFSA) recommend the regular consumption of at least five portions of fruit and vegetables a day (a minimum of 400 g). Besides the health aspect, other social and demographic factors are influencing positively the popularity of fresh-cut produce.

Although consumption of fresh produce is beneficial for optimal health, these foods may be associated with risks of foodborne illness. Reportable outbreaks of foodborne illness related to the consumption of fresh and minimally processed (MP) fruits, mainly due to *Escherichia coli* O157:H7 and *Salmonella*, have increased dramatically since the 1970s (CDC, 2007; Harris *et al.*, 2003). Contamination of fresh fruit with human pathogens can occur at several points during growing, harvesting, processing and handling and although pH is thought to be a limiting factor, growth of *E. coli*, *Salmonella*, *Listeria innocua* and *Listeria monocytogenes* has been previously reported on, for example, fresh-cut apples and peaches (Abadias *et al.*, 2009; Alegre *et al.*, 2010a, 2010b; Conway *et al.*, 2000; Dingman, 2000; Janisiewicz *et al.*, 1999b; Leverentz *et al.*, 2003, 2006).

There are limited tools to prolong the shelf life of MP produce. Modified atmosphere packaging and refrigeration can be applied in order to slow down a faster physiological degradation (King *et al.*, 1991). The use of a decontamination method is another tool to prolong the shelf life of MP produce, but it should be mild enough to not impair the fresh or fresh-like attributes of MP produce (Gómez-López *et al.*, 2005). Currently, chlorine is the most widely used among the washing and sanitizing agents available for fresh produce. However, published data indicate that the most that can be expected at permitted concentrations is a 1-2-log reduction of the bacterial population (Abadias *et al.*, 2008; Beuchat, 1998; Brackett, 1999). Therefore, there is still a need to achieve an efficient and sustaining decontamination of ready-to-eat produce, which takes into account that an efficient decontamination of MP produce creates a less competitive environment in which pathogens can unrestrictedly multiply (Carlin *et al.*, 1996; Li *et al.*, 2002).

The use of protective cultures, bacteriophages and bacteriocins could be an alternative to chemical treatments to reduce foodborne pathogens on fresh and fresh-cut fruits (Janisiewicz *et al.*, 1999a; Leverentz *et al.*, 2001, 2003, 2006). The native microbial association naturally present on the surfaces of fresh produce is assumed to play an important role in maintaining the health supporting status of MP produce (Nguyen-The and Carlin, 1994) by out-competing pathogens for physical space and nutrients and/or producing antagonistic compounds that negatively affect the viability of pathogens (Liao and Fett, 2001; Parish *et al.*, 2003). These organisms have the advantage of being part of the natural microbial

community already established on the target produce, which may facilitate their colonization of and survival on the produce when applied in appropriate numbers (Leverentz *et al.*, 2006). Thus, there is a potential for the use of native microflora to reduce pathogen growth and survival on fruits and vegetables.

It has been demonstrated that the antagonist *Pseudomonas syringae* L-59-66, used for controlling portharvest decay of pome fruit and commercialized as BioSave by EcoScience Corp. (Orlando, Fla.) could also prevent the growth of *E. coli* O157:H7 on wounded apple tissue (Janisiewicz *et al.*, 1999a). The strains *Gluconobacter asaii* (T1-D1), *Candida* spp. (T4-E4), *Dicosphaerina fagi* (ST1-C9) and *Metschnikowia pulcherrima* (T1-E2) inhibited the growth or reduced the populations of either or both, *L. monocytogenes* and *Salmonella enterica* serovar Poona inoculated on 'Golden Delicious' apple plugs and stored at 10 and 25 ºC (Leverentz *et al.*, 2006). Although *Candida sake* CPA-1 reduced, approximately, 1-log unit *E. coli* population on 'Golden Delicious' apple wounds stored at 25 ºC, it did not affect the survival of *E. coli* on fresh-cut apples (Abadias *et al.*, 2009). Lactic acid bacteria (LAB) are considered as food-grade microorganisms and generally recognized as safe (GRAS) that have historically been used to preserve meat and dairy products and to bioprotect fermented vegetables (Ruiz-Barba *et al.*, 1994; Stiles and Holzapfel, 1997). Trias *et al.* (2008) tested six LAB strains as bioprotective agents against *E. coli*, *Salmonella typhimurium* and *L. monocytogenes* in apple wounds. LAB interfered with the growth of *S. typhimurium* and *L. monocytogenes* but showed little effect over *E. coli*. The inhibition had a bactericidal effect against *L. monocytogenes* that could be related to bacteriocin production. Recently, application of the probiotic strain *Lactobacillus rhamnosus* GG reduced growth of *L. monocytogenes* on fresh-cut apple (Alegre *et al.*, 2011).

The objective of this study was to evaluate the effectiveness of an antagonistic bacterial strain, CPA-6, isolated from fresh-cut apple, to avoid *E. coli* O157:H7, *Salmonella* and *L. innocua* growth on fresh-cut apples and peaches. In addition, minimal effective dose and phytopathogenicity were determined. Finally, CPA-6 strain was identified.

2. MATERIALS AND METHODS

2.1. Fruit

'Golden Delicious' apples and 'Royal Glory', 'Elegant Lady' and 'Merry O'Henry', peaches were used in the experiments. Different varieties of peaches were used due to the high seasonality and low storage capability of these fruits. Fruit that had not received any postharvest treatment was obtained from the IRTA Experimental Station and from packinghouses in Lleida (Catalonia).

Fruit was washed in running tap water and surface disinfected with ethanol 70 %. Then it was cut in half and plugs of 1.2 cm of diameter, 1 cm long were taken using a cork borer. Plugs were placed into sterile glass test tubes.

2.2. Bacterial strains

The antagonistic strain CPA-6 used in this assay was isolated from minimally processed 'Golden Delicious' apples and was selected as it demonstrated antagonistic effect against *E. coli* O157:H7 in previous studies (data not shown).

A non pathogenic strain of *E. coli* O157:H7 (NCTC 12900) and a pathogenic strain of *Salmonella enterica* subsp. *enterica* (Smith) Weldin serotype Michigan (BAA-709 ATCC) were used. Both strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin sulphate salt (St, Sigma, Madrid, Spain) thereby enabling detection on selective medium (TSA-St) in the presence of CPA-6 and the natural microbial flora associated with apples and peaches. The strains were grown in tryptone soy broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) for 20-24 h at 37 ºC. The strain of *L. innocua,* CECT-910 was used as a microbial surrogate of *L. monocytogenes* as previous studies have demonstrated that it is a valid model for *L. monocytogenes* behaviour (Francis and O'Beirne, 1997). *L. innocua* was grown overnight in TSB supplemented with 6 g L^{-1} of yeast extract (Biokar Diagnostics, Beauvais, France, Tryptone soy broth yeast extract, TSBYE) at 37 ºC.

E. coli O157:H7, *Salmonella* and *L. innocua* cells were harvested by centrifugation at 9820 × g for 10 min at 10 ºC and then resuspended in a sterile 8.5 g L^{-1} NaCl solution (SS) obtaining a concentrated suspension. The concentration was estimated using a spectrophotometer set at λ=420 nm according to previously determined standard curves.

2.3. Antagonistic effect of CPA-6 on minimally processed apples and peaches

CPA-6 was grown on nutrient yeast dextrose agar (NYDA, 8 g L^{-1} nutrient broth, Biokar Diagnostics, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose, VWR International Eurolab S. L. Spain, and 15 g L^{-1} agar, Industrias Roko S.A. Spain) plates overnight at 25±1 ºC. Colonies were scraped from the medium and a suspension of 30±5 % transmittance (λ=420 nm), which corresponded to approximately 1×10^{8} cfu mL^{-1}, was prepared in 5 mL of sterile deionised water. Then, a volume of the *E. coli* O157:H7, *Salmonella* or *L. innocua* concentrated suspension was added to the 30%-transmittance antagonist suspension to obtain a pathogen concentration of, approximately, 1×10^{7} cfu mL^{-1}. The antagonist and pathogen suspension was pipetted (15 µL) onto apple and peach tissue plugs and then fruit plugs were stored at 20±1 ºC for 2 days and at 5 ºC up to 10 days (only for *E. coli* O157:H7). Control

treatment consisted of a pathogen suspension without antagonist. For the pathogen recovery, each fruit plug was placed into a sterile plastic bag (Bagpage 80 mL, Interscience BagSystem, St Nom La Breteche, France) and 9 mL of saline peptone (SP, 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) was added. It was homogenised in a stomacher blended for 120 s at high speed (Bagmixer 100 Minimix, Interscience, Weymouth, Mass.). Aliquots of the mixture were then serially diluted and spread plated on TSA-St for *E. coli* O157:H7 and *Salmonella* or on Palcam agar (Palcam Agar Base with Palcam selective supplement, Biokar Diagnostics) for *L. innocua*. The agar plates were incubated overnight at 37±1 ºC. Initial pathogen population on apple and peach plugs was also determined following the same methodology. There were three replicates fruit plugs per treatment and sampling time.

To evaluate the results obtained, population of the pathogen inoculated alone or in presence of the antagonist was compared. Reduction of the foodborne pathogens (FBP) was calculated as follows:

$$\text{Reduction} = \log N_{FBP} - \log N_{FBP+CPA\text{-}6}$$

being N_{FBP}: FBP population in the control treatment (FBP alone, cfu $plug^{-1}$) after storage period and $N_{FBP+CPA\text{-}6}$: FBP population (cfu $plug^{-1}$) after storage period in the presence of antagonist.

2.4. Determination of lowest effective antagonist dose

CPA-6 was grown in TSB for 20-24 h at 30 ºC. Then, cells were harvested by centrifugation at 15344 × g for 15 min at 10 ºC and resuspended in sterile SS. The concentration was estimated using a spectrophotometer set at λ=420 nm according to a curve previously determined. For inoculum preparation, a volume of the CPA-6 concentrated suspension was added to 5 mL of deionized water to obtain 10^8 cfu mL^{-1} and then it was serially diluted to obtain 10^7, 10^6, 10^5 and 10^4 cfu mL^{-1}. The necessary volume of *E. coli* O157:H7 concentrated suspension was added to each of the CPA-6 suspension to obtain a pathogen concentration of 10^7 cfu mL^{-1}. Apple plugs were inoculated as described previously and stored at 20 ºC for 2 days. Minimum CPA-6 concentration able to reduce *E. coli* O157:H7 population more than 2-log units was chosen to be tested against *Salmonella* and *L. innocua* at 20 ºC and against *E. coli* O157:H7 at 5 ºC on apple plugs.

In addition, growth of CPA-6, when inoculated at different concentrations, on apple flesh stored at 20 ºC was also monitored. The growth of the minimum effective dose was determined on apple plugs stored at 5 ºC and on minimally processed peach stored at 20 and 5 ºC.

There were three replications for each determination and all the experiments were repeated twice.

2.5. Hypersensitive reaction on tobacco plants

The hypersensitive reaction of CPA-6 in leaf mesophyll tissue of tobacco plants was determined to ascertain phytopathogenicity of the strain.

Pantoea ananatis (CPA-3) was used as positive control. Antagonist and *P. ananatis* were grown on TSB at 30 ºC overnight and centrifuged. The supernatant was removed and cells were washed with 25 mL of sterile deionised water twice. Inoculums were prepared at 10^9 cfu mL^{-1} for both microorganisms. Inoculums were injected between the veins of tobacco leaves using an insulin syringe (Noval, 1991). Sterile deionised water was inoculated as negative control. For each microorganism four leaves were inoculated. Inoculated plants were maintained at room temperature and they were observed for typical symptoms of hypersensitivity response in the form of necrosis, yellowing of the infiltrated area and leaf dead on the following days. The experiment was conducted twice.

2.6. Phenotypic characterization

The Gram-reaction was determined by two tests: lysis in 3% KOH (Ryu, 1938) and the presence of L-alanine aminopeptidase using Bactident tests strips (Merck). Catalase activity was determined by assessing bubble production in 3% v/v H_2O_2 and oxidase activity was tested using oxidase reagent (bioMérieux) according manufacturer's instructions. Motility was tested microscopically from cells grown overnight as well as a thick suspension of cells grown for 4 h in TSB at 25 °C. Because motility was negative microscopically we stab inoculated a tube containing 0.7% TSA. The temperature range (6, 9, 15, 18, 25, 30, 33, 36, 37 and 42 ºC) for growth was tested on the base of colony formation on TSA plates.

Anaerobic and microaerophilic growth was tested by growing the isolate in anaerobic jars (Anoxomat). Oxidation and fermentation of glucose and production of gas was tested in tubes with glucose O/F medium (10 g glucose, 2 g peptone, 5 g NaCl, 0.3 g K_2HPO_4, 0.080 g bromothymol blue and 2.5 g agar L^{-1} of demi water). To create anaerobic conditions for fermentation after inoculation, a 5 cm layer of melted paraffin (Merck) was added with an additional layer of liquid paraffine (Brocacef) on top. Ability to oxidise various carbon sources was tested by using Biolog GN plates (Biolog Inc., Hayward, USA Release 4.2). In addition nitrate reduction, production of indole and H_2S, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, gelatinase activity, and Vogel Proskauer test were assayed using an API20E kit (bioMérieux).

2.7. rRNA sequencing and sequencing analysis

Almost full 16S rRNA analysis was performed to identify CPA-6. A 5 µL aliquot of a colony resuspended in Tris/EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA

pH 8.0) was spotted onto CloneSaverCards (Whatman Bioscience) according to the manufacturer's recommendations. PCR was performed on 1.2 mm punches of the card with the following primers (sequences in 5'→3' direction) 16S1500F (ccgaattcgtcgacaacagagtttgatcctggctcag) and 16S1500R (cccgggatccaagcttacggctaccttgttacgactt).

PCR products were sequenced with 16S500F (tggagagtttgatcctggctcag), BSF349/17 (aggcagcagtggggaat), 16S500R (taccgcggctgctggcac), BSF 784 (rggattagataccc), BSR798 (ggggtatctaatccc), BSF 1099 (gyaacgagcgcaaccc), BSR1114/16 (gggttgcgctcgttrc), and 16S1500R (cccgggatccaagcttacggctaccttgttacgactt) primers by using DYE-ET terminator cycle sequencing (Amersham Biosciences). Prior to separation on an ABI 3700 system (Applied Biosystems), sequencing products were purified by using Sephadex G-50 Superfine. Contigs were built with the DNASTAR package

Phylogenetic analysis was performed by using Bionumerics software version 4.6 (Applied Maths). A tree was constructed by the UPGMA method.

3. RESULTS

3.1. Antagonistic effect of CPA-6 on minimally processed apples and peaches

Effectiveness of CPA-6 strain on reducing *E. coli* O157:H7, *Salmonella* and *L. innocua* populations on minimally processed apples and peaches stored 2 d at 20 ºC is shown in Fig. 1.

E. coli O157:H7 initial population on apple plugs was 5.4±0.0 log cfu plug^{-1} and it increased 1.3±0.3-log units after storage at 20 ºC on apple plugs when inoculated alone. Co-inoculation with the strain CPA-6 reduced *E. coli* O157:H7 population 5.3±0.9-log units when compared to pathogen population inoculated alone (Fig 1A). *Salmonella* behaviour on apple plug was similar to behaviour observed for *E. coli* O157:H7. However, CPA-6 caused lower population reduction (2.6±0.3-log units). *L. innocua* was the pathogen with highest population increases on apple plugs, from the initial 4.4±0.2 log cfu plug^{-1} to 7.1±0.1 log cfu plug^{-1} after to 2 d at 20 ºC when it was inoculated alone. Co-inoculation with CPA-6 caused a reduction of 7.1-log units, with a final population below detection limit (50 cfu plug^{-1}).

Higher pathogen increases were observed when they were inoculated on peach plugs. *E. coli* O157:H7 population increased approximately 3.7±0.3-log units, with a final population over 8.0 log cfu plug^{-1}. *Salmonella* was the pathogen with the lowest increase, 2.4±0.3-log units and *L. innocua* population increased 3.1±0.1-log units. Reduction values of the three FBP by CPA-6 were similar to those observed on apple flesh. CPA-6 reduced *E. coli* O157:H7 population 4.8±0.8-log units, *Salmonella* population 2.4±0.3-log units, and again, *L. innocua* was the most reduced, with a reduction value of 7.4±0.0-log units (final population below detection limit, 50 cfu plug^{-1}).

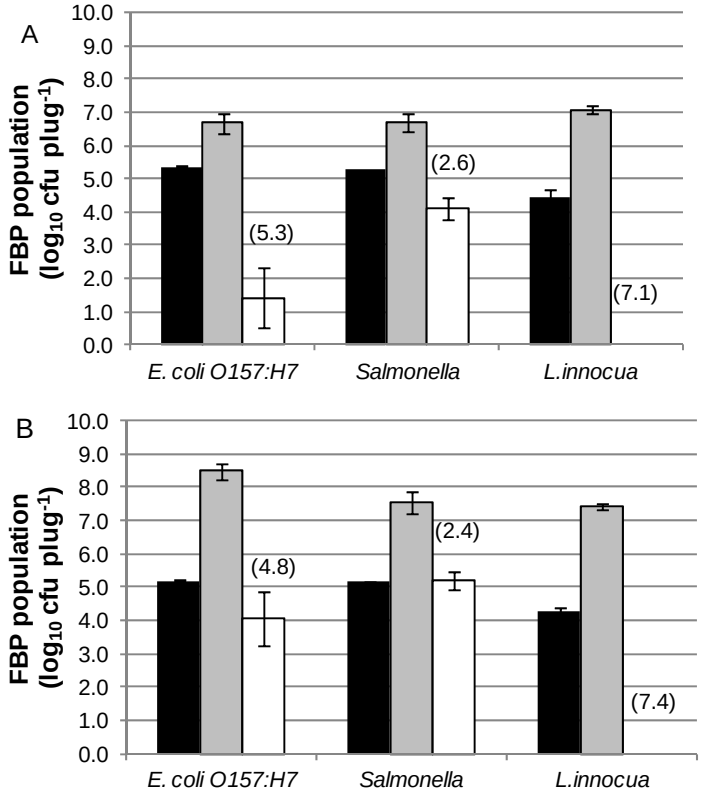

Fig. 1 *E. coli* O157:H7, *Salmonella* and *L. innocua* population on 'Golden Delicious' apple (A) and peach (B) plugs after inoculation (■) and after 2 days of incubation at 20 ºC alone (□) or together with CPA-6 strain inoculated at 10^8 cfu mL^{-1} (□). Values are the mean of 6 values (2 assays with 3 replicates each) and bars in the columns represent standard error of the mean. The numbers in brackets show the average value of the log-reduction achieved by the antagonist.

Ability of the strain CPA-6 in reducing *E. coli* O157:H7 at refrigeration conditions was tested in both apple and peach plugs (Fig. 2). *E. coli* O157:H7 population on apple plugs was slightly reduced throughout storage, with a final population 0.6±0.1-log units lower than the initial one. Co-inoculation with the strain CPA-6 did not cause important reductions on *E. coli* O157:H7 population and maximum reduction was observed after 10 days of storage (0.5±0.2-log units). In contrast, *E. coli* O157:H7 population on peach plugs maintained until day 6 and then increased slightly, with a final population 0.3±0.3 higher than initial. In peach plugs, co-inoculation with CPA-6 resulted in a drastic reduction of *E. coli* O157:H7 population, being lower to detection limit (50 cfu $plug^{-1}$) after 6 days of storage (more than 5.0-log units reduction).

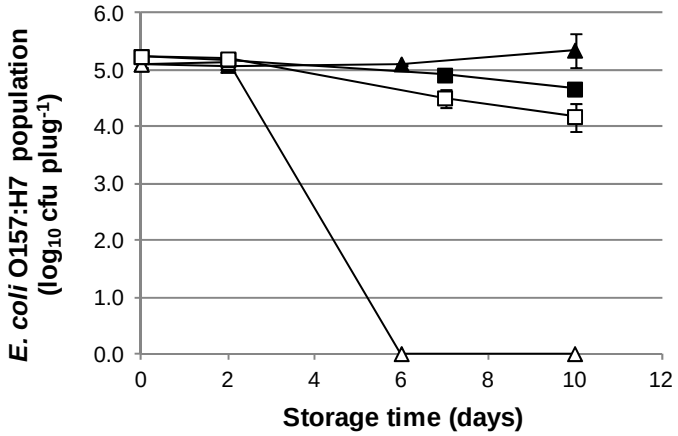

Fig. 2 *E. coli* O157:H7 population on apple (squares) and peach (triangle) plugs throughout conservation at 5 ºC alone (full symbols) or with CPA-6 strain inoculated at 10^8 cfu mL^{-1} (open symbols). Values are the mean of 6 values (2 assays with 3 replicates each) and bars represent standard error of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol.

3.2. Determination of lowest effective antagonist dose

Lower inoculums of CPA-6 were tested against *E. coli* O157:H7 population on apple plugs stored 2 d at 20 ºC in order to determine the lowest effective dose (data not shown). Initial *E. coli* O157:H7 population was 5.2±0.0 log cfu $plug^{-1}$. Throughout storage it increased 1.8±0.1-log units, giving a final population of 7.0±0.1 log cfu $plug^{-1}$. CPA-6 inoculum levels of 10^6, 10^7 and 10^8 cfu mL^{-1}, reduced *E. coli* O157:H7 population 5.0-log units or more. However, when CPA-6 inoculum was reduced to 10^5 cfu mL^{-1}, *E. coli* O157:H7 population reduction was lower than 2.0-log units. Therefore, 10^6 cfu mL^{-1} was chosen as the minimal effective dose of the antagonistic strain CPA-6 and was tested against *Salmonella* and *L. innocua* on apple plugs (Table 1). CPA-6 reduced both pathogens population 4.5-log units or more.

Then, 10^6 cfu mL^{-1} CPA-6 concentration was tested against *E. coli* O157:H7, *Salmonella* and *L. innocua* on peach plugs stored at 20 ºC and reduction values were 2.8±0.9, 2.9±0.6 and 4.4±0.7-log units, respectively (Table 1).

Finally, effectiveness of selected concentration (10^6 cfu mL^{-1}) was tested against *E. coli* O157:H7 inoculated on both, apple and peach plugs stored at 5 ºC (Fig. 3). *E. coli* O157:H7 population on apple plugs was reduced more than 1-log units after 10 days storage. In peach plugs, *E. coli* O157:H7 population reduction of 2.0-log units was observed after 7 days of storage and it continued decreasing in co-inoculated with CPA-6 peach plugs until end of storage, giving a final population of 1.5±0.0 log cfu $plug^{-1}$ (more than 3.0-log units reduction).

Table 1 *E. coli* O157:H7, *Salmonella* and *L. innocua* population (log cfu plug^{-1}) on apple and peach plugs after inoculation and after 2 d of storage at 20 ºC together with 0 and 10^6 cfu mL^{-1} of the antagonistic strain CPA-6

		Initial	**CPA-6 concentration (cfu mL^{-1})**	
			0	**10^6**
Apple	*E. coli* O157:H7	5.2±0.0[a]	7.0±0.1	2.0±0.4
	Salmonella	4.9±0.0	7.1±0.2	2.4±0.5
	L. innocua	3.2±0.4	5.9±0.4	1.4±0.0
Peach	*E. coli* O157:H7	5.1±0.0	7.4±0.2	4.7±0.9
	Salmonella	5.0±0.1	8.4±0.1	5.5±0.6
	L. innocua	4.8±0.1	7.9±0.2	3.5±0.7

[a] Results are expressed as mean plus and minus standard error of the mean

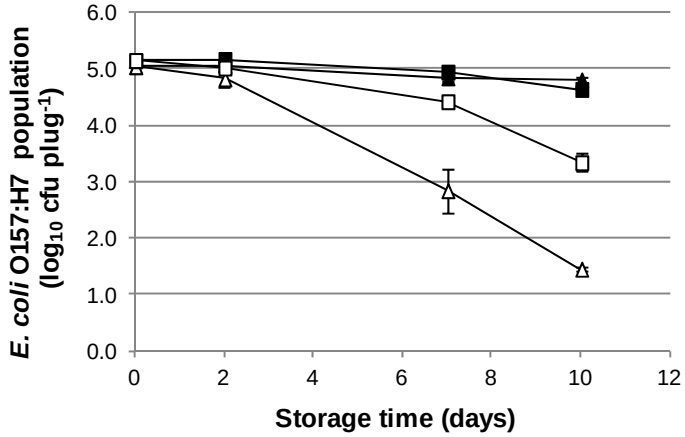

Fig. 3 *E. coli* O157:H7 population on apple (squares) and peach (triangle) plugs throughout conservation at 5 ºC alone (full symbols) or with CPA-6 strain inoculated at 10^6 cfu mL^{-1} (open symbols). Values are the mean of 6 values (2 assays with 3 replicates each) and bars represent standard error of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol.

Initial CPA-6 concentrations on apple plugs were 1.9±0.2, 3.2±0.0, 4.6±0.1, 5.6±0.1 and 6.6±0.1 log cfu plug^{-1} when inoculated at different concentrations (Table 2). However, after 2 d of storage at 20 ºC, all CPA-6 populations were between 7.3±0.2 and 8.2±0.1 log cfu plug^{-1}, which represented growths between 1.4 and 5.4-log units. Then, as 10^6 cfu mL^{-1} was chosen as the minimum effective dose, growth of CPA-6 on peach plugs stored at 20 ºC and on apple and peach plugs stored at 5 ºC was only tested for this inoculum. Initial CPA-6 population on peach plugs was 4.5±0.1 log cfu plug^{-1} and it increased more than 2-log units, giving a final population of 6.9±0.8 log cfu plug^{-1} after storage at 20 ºC (data not shown). When CPA-6 was inoculated on apple and peach plugs and stored at 5 ºC up to 10 d (Fig. 4) highest population increases were observed on peach plugs (more than 4.0-log units), meanwhile it increased by approximately 3.0-log units on apple plugs.

Table 2 CPA-6 population (log cfu plug^{-1}) on apple plugs after inoculation and after 2 d of storage at 20 ºC when inoculated at different levels (cfu mL^{-1}).

CPA-6 inoculum concentration (cfu mL^{-1})	**CPA-6 population on apple plugs (log cfu plug^{-1})**	
	Initial	**2 d 20 ºC**
10^4	1.9±0.2[a]	7.3±0.2
10^5	3.2±0.0	7.8±0.1
10^6	4.6±0.1	7.9±0.1
10^7	5.6±0.1	8.2±0.1
10^8	6.6±0.1	8.0±0.2

[a] Results are expressed as mean plus and minus standard error of the mean

There were three replications for each determination. Data was combined from two experiments.

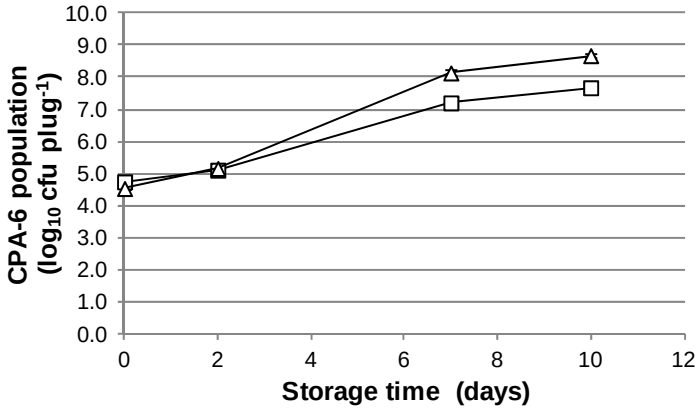

Fig. 4 CPA-6 population on apple (squares) and peach (triangles) plugs troughout storage at 5 ºC inoculated at 10^6 cfu mL^{-1}. Values are he mean of 6 values (2 assays with 3 replicates each) and bars represent standard error of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol.

3.3. Hypersensitive reaction on tobacco plants

CPA-6 was examined for its capability to produce a hypersensitive reaction in leaf mesophyll tissue of tobacco plants. CPA-6 did not caused any reaction on tobacco leaves when compared with the positive control *P. ananatis* CPA-3.

3.4. CPA-6 identification

CPA-6 cells are Gram-negative, facultative anaerobic, non-spore-forming rods. Colonies on TSA are circular and non-pigmented. Cells are oxidase negative, catalase positive and non-motile at 25 °C in TSB. Fermentation of glucose is positive but no production of gas. Growth occurred at 6-33 °C, but not at 37 °C or higher temperatures. Growth at 36 ºC is delayed and weak. Nitrate is reduced but there is no formation of nitrite which indicates the possibility of gas production. Phenotypic properties of strain CPA-6 and other close related genera of CPA-6 are shown in Table 3. Strain CPA-6 differs from other genera within the Enterobacteriaceae family in its disability to grow at 37 °C and utilisation of inositol.

The almost full 16SrRNA sequence of strain CPA-6 revealed that the strain belongs to the family *Enterobacteriaceae* but it is not close related to any known genera. Closest related species were *Obesumbacterium proteus* (biogroup 1) (98.2 %), *Hafnia alvei* (98.0 %), *Serratia grimesii* (98.0 %), *Serratia proteomaculans* (98.0 %), *Serratia plymuthica* (97.7 %), *Serratia liquefaciens* (97.6 %) and *Rahnella aquatilis* (96.7 %). In Fig. 5 an UPGMA dendrogram is shown.

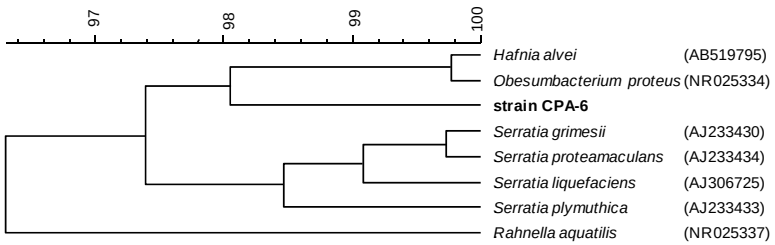

Fig. 5 UPGMA phylogenetic tree based on 16S rRNA gene sequences showing the position of strain CPA-6 among different genera of the family *Enterobacteriaceae*. The horizontal bar joining two isolates or clusters indicating the level of similarity.

4. DISCUSSION

Currently, preservation techniques in the fresh-cut industry include sanitation techniques that reduce background microflora, which has been widely demonstrated to inhibit pathogen's growth. The use of competitive microflora may be a useful tool to enhance the safety of fresh fruits and vegetables. In this study, we have demonstrated that the strain CPA-6, isolated from minimally processed apple, effectively inhibited growth or even reduced initial population of *E. coli* O157:H7, *Salmonella* and *L. innocua* on fresh-cut apple and peaches. In addition, CPA-6 was not phytopathogen to tobacco plant. Finally, it was identified as new specie belonging to genera *Enterobacteriaceae*.

CPA-6 reduced *E. coli* O157:H7, *Salmonella* and *L. innocua* populations inoculated on both apple and peach plugs stored 2 d at 20 ºC. On both fruits, *L. innocua* was the pathogen that was reduced most (below limit of detection) and *Salmonella* the pathogen that was reduced least. At refrigeration conditions (5 ºC), *E. coli* O157:H7 population on apple plugs co-inoculated with CPA-6 was slightly lower than *E. coli* O157:H7 inoculated alone from 7 days of storage and above and it reduced significantly on peach plugs, with final population below level of detection. At both temperatures tested, CPA-6 antagonistic action was not instantaneous and it was only noticeable after storage period. Other authors have previously reported efficacy of microbial antagonist in reducing foodborne pathogens on apples. For example, Janisiewicz *et al.* (1999a) showed that co-inoculation of apple wounds with fresh cells of the antagonist, *P. syringae* L-59-66 and *E. coli* O157:H7 prevented the growth of the pathogen after 48 h of incubation at 24 ºC. Leverentz *et al.* (2006) found that four microorganisms isolated from apple inhibited growth or reduced (between 2.1 and 2.8-log units) the populations of *L. monocytogenes* on 'Golden Delicious' apples slices stored at 10 ºC. However, at 25 ºC reduction values increased (from 5.7 to 6.0-log units) due to more rapidly growth of both, the antagonists and the pathogen. Nevertheless,

only three of the isolates reduced the gram-negative *S. enterica* population on apple slices stored at 25 ºC and none were effective at 10 ºC. The biocontrol yeast *Candida sake* CPA-1 reduced an *E. coli* mixture population on 'Golden Delicious' apple wounds throughout storage at 25 ºC but not in fresh-cut apples neither at 5 or 25 ºC (Abadias *et al.*, 2009). Trias *et al.* (2008) found five LAB strains that reduced *L. monocytogenes* population below detection level in wounded apples after 2 d at 25 ºC, meanwhile *Salmonella* was only significantly reduced by 4 of them and none of them reduced *E. coli* population.

Table 3 Phenotypic characteristics that distinguish strain CPA-6 from other species.

Characteristic	**1**[a]	**2**	**3**	**4**	**5**	**6**
Oxidase	-[b]	-	-	-	-	-
Colonies > 1mm on TSA 30 ˚C	+	-	+	+	+	+
Growth at 37 °C	-	+	+	+	+	+
Growth at 6 °C[c]	+	nd	+	nd	nd	+
Motility	-	-	+	+	+	-
Fermentation of glucose	+	+	+	+	+	+
D-glucose, acid production	+	+	+	+	+	+
D-glucose, gas production	-	-	+	+	+	+
Ornithine decarboxylase	-	+	+	+	+	-
Lysine decarboxylase	-	+	+	+	+	-
Citrate	+	-	-	+	+	+
Acid production of mannitol	+	-	+	+	+	+
Acid production of inositol	+	-	-	nd	nd	-
Acid production of sorbitol	-	-	-	+	+	+
Acid production of rhamnose	+	-	+	-	v	+
Acid production of sucrose	+	-	-	+	+	+
Acid production of melibiose	+	-	-	+	+	+
Acid production of arabinose	+	-	+	+	+	+
Hydrolysis of Gelatin	-	-	-	+	+	-
Nitrate reduction	+	+	+	+	+	+

[a]Species: 1, strain CPA-6; 2, *Obesumbacterium proteus* (biogroup 1); 3, *Hafnia alvei*; 4, *Serratia grimesii;* 5, *Serratia proteomaculans;* 6, *Rahnella aquatilis.* Data for reference species were taken from Bergey's Manual of Determinative Bacteriology-ninth edition and Ridell *et al*. (1997).
[b] +, positive; -, negative; nd, no data available.

[c] Ridell *et al*. (1997)

Effectiveness of antagonist against FBP population was expected to be dose dependent. CPA-6 inoculum at 10^6 cfu mL^{-1} was selected as minimal effective dose. However, levels of FBP that may exist in the environment or on fresh produce are assumed to be much lower than concentration tested (10^7 cfu mL^{-1}), thus, the effectiveness of the antagonist is expected to be even greater.

CPA-6 strain did not cause hypersensitive reaction on tobacco leaves, hence it was not phytopathogen. This is an important characteristic as it would be impractical to inoculate large numbers of competitive bacteria onto the product, especially if the microorganism of interest was a potential spoilage agent. Any benefit gained from the inhibition of foodborne pathogen would be lost if the product spoils more rapidly (Johnston *et al.*, 2009).

From the molecular data we can conclude that strain CPA-6 is phylogenetically close related to the genera *Serratia*, *Obesumbacterium* and *Hafnia*. However, it has several unique biochemical properties which discriminate the strain from the earlier mentioned related genera. Therefore, this strain is likely to belong to a new genus within the family *Enterobacteriaceae*, but further extensive taxonomic study is necessary to confirm this.

In spite of promising results found with this study, several hurdles may be overcome before antagonistic microorganisms can be used in a biocontrol strategy with fresh or fresh-cut produce. Once identified, the safety of CPA-6 would be of foremost concern and practical application methods also must be developed. In addition, the inhibitory effect of the CPA-6 on FBP was not instantaneous as it was just detected after storage time; therefore, combination with other strategies would be desirable.

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CAPÍTULO V

Control of foodborne pathogens on fresh-cut fruit by a novel strain of *Pseudomonas graminis*

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Enviado a: Food Microbiology

ABSTRACT

Consumption of fresh-cut fruits has substantially risen over the last few years, which has led to an increase in the number of outbreaks associated with fruit. Moreover, consumers are currently demanding wholesome, fresh-like and safe foods without addition of chemicals. As a response, the aim of this study was to determine if naturally occurring microorganisms on fruit are 'competitive with' or 'antagonistic to' potentially encountered pathogens. From all the isolates tested, seven showed a strong antagonistic capacity against *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on fresh-cut apples and peaches stored at 20 ºC. One of them, CPA-7, achieved the best reduction values and was the only able to reduce *E. coli* O157:H7 at refrigeration temperatures on both fruit. Hence, it was selected for further assays. Dose-response assays showed that CPA-7 should be at least at the same load that pathogen to give good reductions. Hypersensitive reaction test on tobacco plants showed that it was not phytopathogenic and from results obtained in *in vitro* assays, competition seemed to be its mode of action. The strain was identified as *Pseudomonas graminis*. Thus, results support the potential use of CPA-7 as a bioprotective agent against foodborne pathogens in minimally processed fruit.

Keywords: *Escherichia coli* O157:H7; *Salmonella*; *Listeria innocua*; biopreservation; minimally processed fruit; antagonist

1. INTRODUCTION

The consumption of minimally processed fruits and vegetables has increased continuously during the last decades due to a change in the consumer tendencies like the lack of time to buy and cook, the public consciousness of the health benefits associated with the consumption of produce, the year-round availability of vegetable products, and an increase in the variety of commodities offered.

Spoilage bacteria, yeast and moulds dominate the microflora on raw fruits and vegetables; however, the occasional presence of pathogenic bacteria, parasites, and viruses capable of causing human infections, and, therefore outbreaks of foodborne diseases linked to fresh fruits and vegetables consumption have been reported (Beuchat, 2002). For example, *Salmonella* and *Escherichia coli* O157:H7 have been implicated in outbreaks from cantaloupes and honeydew melons, tomatoes, pears, watermelons, strawberries, mangoes and grapes (CDC, 2007; Harris *et al.*, 2003). *Listeria monocytogenes* has been shown to contaminate vegetables such as lettuce, broad-leaved endives, broccoli, radishes, cabbages, potatoes and cucumbers (Beuchat, 1996; Carlin and Nguyen-The, 1994; Little and Gillespie, 2008).

During processing of fresh-cut produce, cutting, slicing, skinning and shredding remove or damage the protective surfaces of the plant or fruit so nutrients become more available, and pathogens can be spread from contaminated to uncontaminated parts (EU Scientific Committee on Food, 2002). Moreover, a treatment to guarantee the total elimination of microorganisms from fresh-cut fruits and vegetables does not exist so they are particularly susceptible to the growth of spoilage bacteria and pathogens. Previous studies have demonstrated the capability of *Salmonella* Michigan, *E. coli* O157:H7 and *Listeria innocua* to grow on fresh-cut apples (Abadias *et al.*, 2009; Alegre *et al.*, 2010a; Conway *et al.*, 2000; Dingman, 2000; Gunes and Hotchkiss, 2002; Janisiewicz *et al.*, 1999b; Leverentz *et al.*, 2006) and peaches (Alegre *et al.*, 2010b).

In the fresh-cut industry, chlorine is commonly used to disinfect produce. However chlorine does not ensure elimination or even an efficient reduction in pathogen levels (Beuchat, 1998). A prolonged exposure to chlorine vapor may cause irritation to the skin and respiratory tract of handlers. In addition, chlorinated organic compounds, such as trihalomethanes, can be produced in contact with organic matter. In addition, there is an increasing demand for “natural” and “additive-free” products. Therefore, it is desirable to preserve foods by natural means (Kim, 1993). Biological control fits well with this new tendency, and several bacteria and yeasts have been identified as bioprotective agents (Vermeiren *et al.*, 2004). The native microflora established on food may have inhibitory properties against contaminating foodborne pathogens and therefore, via competition or antibiosis, function as a hurdle to pathogen growth and survival (Leistner and Gorris, 1995; Schuenzel and Harrison, 2002). So that, it seems promising to find specific organisms among the natural microflora that are responsible

for exhibiting these pathogenic features. For example, the strain *Pseudomonas syringae* L-59-66, commercialized as BioSave 110 (EcoScience Corp. Orlando, Fla.) for controlling postharvest decays on apples and pears, prevented the growth of *E. coli* O157:H7 on wounded apple tissue (Janisiewicz *et al.*, 1999a). The growth of *L. monocytogenes* and *S. enterica* in fresh-cut apples was prevented using fungal antagonists (Leverentz *et al.*, 2006). Trias *et al.* (2008) found five strains of lactic acid bacteria able to inhibit *L. monocytogenes* and *Salmonella typhimurium* in apple wounds which were not effective in reducing *E. coli*. Recently, Abadias *et al.* (2009) found that the application of the fungal postharvest antagonist *Candida sake* CPA-1 reduced growth of a mixture of *E. coli* strains in apple wounds at 25 ºC.

The objective of this study was to evaluate native microorganisms from fresh and fresh-cut fruit that showed inhibitory potential against the foodborne pathogens (FBP) *E. coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally processed apples and peaches. The best antagonist was selected to be tested for phytopathogenicity, antimicrobial substances production, and minimum inhibitory concentration. Finally, it was identified.

2. MATERIALS AND METHODS

2.1. Fruit

'Golden Delicious' apples, and 'Royal Glory', 'Elegant Lady', 'Merry O'Henry', 'Tardibelle', 'Placido' and 'Roig d'Albesa' peaches were used in the experiments. Different varieties of peaches were used due to the high seasonality and low storage capability of these fruits. Fruits, which had not received any postharvest treatment, were obtained from the IRTA Experimental Station and from packinghouses in Lleida (Catalonia, Spain).

Fruits were washed in running tap water and surface disinfected with ethanol 70 %. They were cut in half and plugs of 1.2 cm of diameter, 1 cm long were taken using a cork borer. Plugs were placed into sterile glass test tubes.

On the day of the assay some quality parameters were determined. A sample of the apples and the peaches used was tested for pH with a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). After pH determination, fruits were crushed and soluble solids content was determined at 20 ºC using a handheld refractometer (Atago CO., LTD. Japan). To measure titratable acidity, 10 mL of fruit juice were diluted with 10 mL of distilled water and it was titrated with 0.1 N NaOH up to pH 8.1. The results were calculated as g of malic acid L^{-1}.

2.2. Antagonists

Bacteria and yeasts to be tested as putative antagonists were isolated from fresh-cut apples, peaches and pineapples and from the surface of fresh apples, peaches and nectarines. Whole fruits were rinsed with sterile deionised water, submerged in saline peptone (SP, 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and sonicated for 10 min. To isolate microorganisms from fresh-cut fruits, 25 g of the products were mixed with 225 mL of SP in a stomacher blender for 2 min at 150 rpm (Stomacher 400 Circulator, Seward). Several dilutions, from either whole or fresh-cut fruits, were plated on different media: nutrient yeast dextrose agar (NYDA, 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 15 g L^{-1} agar), NYDA supplemented with imazalil (20 ppm, Sigma, Madrid, Spain) for bacteria isolation or with streptomycin sulphate salt (500 ppm, St, Sigma) for yeast and moulds isolation and de Man, Rogosa and Sharpe medium (MRS, Biokar Diagnostics, Beauvais, France) for lactic acid bacteria isolation. Plates were incubated at 25±1 ºC for 3 days. Colonies of different morphology were selected and isolated.

A collection of fungal antagonists belonging to the Pathology Laboratory collection, which had demonstrated efficacy in reducing fungal postharvest diseases, was also tested.

Antagonists were grown on NYDA plates at 25±1 ºC for 2-3 days. Colonies were scraped from the medium and a suspension of 30±5 % transmittance (λ=420 nm), which corresponded to a concentration between 10^6 and 10^8 cfu mL^{-1}, was prepared in 5 mL of sterile deionised water.

2.3. Biological control in in vivo tests on fresh-cut apples and peaches

A non pathogenic strain of *E. coli* O157:H7 (NCTC 12900) and a pathogenic strain of *Salmonella enterica* subsp. *enterica* (Smith) Weldin serotype Michigan (BAA-709, ATCC) were used. Both strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin thereby enabling detection on selective medium (TSA-St) in the presence of antagonists and the natural microbial flora associated with apples and peaches. The strains were grown in tryptone soy broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) for 20-24 h at 37 ºC. The strain of *L. innocua*, CECT-910 was used as a microbial surrogate of *L. monocytogenes* as previous studies have demonstrated that it is a valid model for *L. monocytogenes* behaviour (Francis and O'Beirne, 1997). *L. innocua* was grown overnight in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) at 37 ºC.

E. coli O157:H7, *Salmonella* and *L. innocua* cells were harvested by centrifugation at 9820 × g for 10 min at 10 ºC and then resuspended in a sterile 8.5 g L^{-1} NaCl solution (SS). The concentration was estimated using a spectrophotometer set at λ=420 nm according to previously determined standard curves.

For the inoculum preparation, a volume of the FBP concentrated suspension was added to the 30 %-transmittance antagonist suspension to obtain approximately 1×10^{7} cfu mL^{-1}. The antagonist and pathogen suspension was pipetted (15 µL) onto fruit tissue plugs and then fruit plugs were stored at 20±1 ºC for 2 days. Control treatment consisted on a pathogen suspension without antagonist. For the pathogen recovery, each fruit plug was placed into a sterile plastic bag (Bagpage 80 mL, Interscience BagSystem, St Nom La Breteche, France) and 9 mL of SP was added. It was homogenised in a stomacher blended for 120 s at high speed (Bagmixer 100 Minimix, Interscience). Aliquots of the mixture were then serially diluted and spread plated on TSA-St for *E. coli* O157:H7 and *Salmonella* or on Palcam agar (Palcam Agar Base with Palcam selective supplement, Biokar Diagnostics) for *L. innocua*. The agar plates were incubated overnight at 37±1 ºC. Initial pathogen population on apple and peach plugs was determined. Each fruit plug was a replicate and there were three replicates per treatment and sampling time.

To evaluate the results obtained, populations of the pathogen inoculated alone or in presence of the possible antagonist were compared. Reduction of the FBP was calculated as follows:

$$\text{Reduction} = \log N_{FBP} - \log N_{FBP+Ant}$$

being N_{FBP}: FBP populations in the control treatment (FBP alone, cfu $plug^{-1}$) after storage period and $N_{FBP+Ant}$: FBP population (cfu $plug^{-1}$) after storage period in the presence of antagonist. Higher values indicate better antagonists. Negative values indicate that the antagonist favoured FBP growth.

All the putative antagonists were first tested against *E. coli* O157:H7. When the populations of *E. coli* O157:H7 in presence of the possible antagonist were reduced by more than 1-log unit, biocontrol assay was repeated to study the consistency of the results in two screening tests. On the contrary, when microorganisms showed a reduction of FBP development lower than 1-log unit, they were rejected.

When a microorganism reduced *E. coli* O157:H7 populations for more than 1-log unit in two consecutive screenings, it was tested twice against *L. innocua* and *Salmonella*. When its efficacy was good against both FBP, it was tested against *E. coli* O157:H7 at refrigeration conditions. In these assays, fruit plugs were stored at 5 ºC and pathogen was recovered after 2, 6 and 10 days.

2.4. Dose-response relationships in the biocontrol of *E. coli* O157:H7, *Salmonella* and *L. innocua* on fresh-cut apples

From all the microorganisms tested, one achieved the best results in reducing the three foodborne pathogens; therefore it was selected to be used in dose-response assays on fresh-cut apples.

The selected antagonist was grown in TSB at 30 ºC overnight. Then, it was centrifuged at 9820 × g for 10 min at 10 ºC and resuspended in sterile deionised water. Pathogen's concentrated suspensions were obtained as described previously. The concentrations of antagonist used were 10^5, 10^6, 10^7 and 10^8 cfu mL^{-1} and for the FBP, 10^5, 10^6 and 10^7 cfu mL^{-1}. Apple plugs were prepared and inoculated as described previously. Both, pathogen and antagonist concentrations, when inoculated alone or in combination, were determined after inoculation and after storage at 20 ºC for 2 days. For antagonist recovery, nutrient agar plates (NA, Biokar Diagnostics) were incubated at 25±1 ºC for 2 days.

There were three replicate fruit plugs per treatment, and sampling time and the assay was repeated twice.

2.5. Hypersensitive reaction on tobacco plants

The hypersensitive reaction of the best isolate in leaf mesophyll tissue of tobacco plants was determined (Noval, 1991) to ascertain the phytopathogenicity of the strain.

Pantoea ananatis (CPA-3) was used as positive control. Antagonist and *P. ananatis* were grown on TSB at 30 ºC overnight and centrifuged. The supernatant was removed and cells were washed with 25 mL of sterile deionised water twice. Inocula were prepared at 10^9 cfu mL^{-1} for both microorganisms. Inocula were injected between the veins of tobacco leaves using an insulin syringe (Noval, 1991). Sterile deionised water was inoculated as negative control. For each microorganism four leaves were inoculated. Inoculated plants were maintained at room temperature and they were observed for typical symptoms of hypersensitivity response in the form of necrosis, yellowing of the infiltrated area and leaf dead on the following days. The experiment was conducted twice.

2.6. In vitro determination of antagonistic activity

The isolate that achieved the best reduction values in the above experiment was tested for antagonistic *in vitro* activity. The selected antagonist was grown in TSB at 30 ºC overnight. From the culture obtained, a fraction was reserved to be tested as 'culture'. The rest was centrifuged at 9820 × g for 10 min at 10 ºC. The supernatant was adjusted to pH=6.5 and sterilized by membrane filtration (0.22 µm) obtaining a neutralized cell-free supernatant (NCFS). Cells were resuspended in sterile deionised water, centrifuged and washed twice obtaining an antagonist cell suspension.

In *in vitro* assays, in addition to *E. coli* O157:H7, *Salmonella* and *L. innocua* strains, *Listeria monocytogenes* CECT-4031, *Aeromonas hydrophila* ATCC 7966 and *Pseudomonas marginalis* CECT-229 were also used as indicator strains.

Salmonella and *E. coli* O157:H7 were grown overnight in TSB-St and *L. innocua* and *L. monocytogenes* in TYSEB at 37 ºC. *A. hydrophila* and *P. marginalis* were grown overnight in TSB at 30 ºC without and with agitation respectively.

All purpose agar plates (meat extract 20 g L^{-1}, glucose 20 g L^{-1} and agar 15 g L^{-1}) were overlaid with 5-6 mL of TSA or TSA supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy agar, TYSEA) soft agar (7.5 g L^{-1} agar) after inoculation with 50 µL of the correspondent indicator culture. Drops (5 µL) of antagonist 'culture', NCFS or cell suspension were spotted on the lawns of pathogens and incubated overnight at 30 ºC. Inhibition was recorded as positive if a translucent halo zone was observed around the spot. There were three replications for each indicator strain and the experiment was repeated twice.

2.7. Identification of antagonistic strains

Gram stain, catalase, and oxidase tests were performed for the isolates with the best inhibitory results. The API 20E, API 20NE and API C Aux Systems (bioMerieux, Marcy-l'Etoile, France) were used. Some of them were also identified using partial 16S rRNA.

3. RESULTS

3.1. Biological control in in vivo tests on fresh-cut apples

Two batches of 'Golden Delicious' apples were used. The initial pH of both batches was, approximately, 3.8, soluble solids ranged from 12.2 to 15.6 ºBrix and titratable acidity ranged from 1.37 to 2.91 g malic acid L^{-1}.

The inhibitory potential of 97 microorganisms was tested against *E. coli* O157:H7 on fresh-cut apples after storage at 20 ºC for 2 days. Twelve of them belonged to the Pathology Laboratory Collection, 40 were isolated from whole 'Golden Delicious' and 'Granny Smith' apples surface, 19 were isolated from commercial fresh-cut apples and peaches, 25 were isolated from whole peaches and nectarines surface and one was isolated from fresh-cut pineapple.

Initial *E. coli* O157:H7 population on apple plugs was around 5.2 log cfu $plug^{-1}$ After 2 days of storage at 20 ºC it reached a final population of 6.8 log cfu $plug^{-1}$ (Fig. 1A). From the 97 microorganisms tested, 87 (89.7 %) did not have any effect on *E. coli* O157:H7 population (reduction <1.0-log unit compared with the control, *E. coli* O157:H7 inoculated alone, data not shown), and therefore they were rejected. Ten isolates reduced *E. coli* O157:H7 population on fresh-cut apple at least 1.0-log units (Fig. 1A). The strains CPA-1, CPA-2, CPA-5, 128-M and C9P21 belonged to the Pathology Laboratory Collection, PN5, PN6, EL8 and CPA-7 were

some of the microorganisms isolated from whole fruits and M174BAL2 was isolated from fresh-cut apple. One microorganism, CPA-7 reduced *E. coli* O157:H7 population 4.5-log units, hence not only reduced growth of the pathogen but also had a bactericidal effect.

Those microorganisms that were effective in reducing *E. coli* O157:H7 population were selected to be tested against *Salmonella* and *L. innocua* at 20 ºC. Initial *Salmonella* populations on apple plugs were approximately 5.3 log cfu plug^{-1} and reached a final population around 6.7 log cfu plug^{-1} after 2 days of storage at 20 ºC (Fig. 1B). *Salmonella* population reductions were, in general, lower than *E. coli* O157:H7 reductions. From the ten microorganisms tested, 8 did not have any effect on *Salmonella* population, 128-M reduced it 1.0-log units and CPA-7 reduced it 4.7-log units.

In general, the highest pathogen reductions were found when the ten selected microorganisms were tested against *L. innocua*. Initial *L. innocua* populations were around 4.4 log cfu plug^{-1} and it augmented approximately 2.6-log units after 2 days of storage at 20 ºC (Fig. 1C). Population of *L. innocua* was reduced between 1.0 and 2.0-log units by five of the microorganisms tested and the other five reduced it more than 2.0-log units. Again, CPA-7 achieved the highest reduction value, 5.9-log units.

Finally, efficacy of the ten selected microorganisms was tested against *E. coli* O157:H7 at refrigeration conditions: apple plugs were stored at 5 ºC and pathogen population was determined after 2, 6 and 10 days of storage (Table 1). Populations of *E. coli* O157:H7 inoculated alone decreased 0.6-log units throughout storage. From the ten microorganisms tested, only CPA-7 significantly reduced *E. coli* O157:H7 population (approximately 3.5-log units reduction). However, the reduction was not observed until day 6.

3.2. Biological control in in vivo tests on fresh-cut peaches

Due to fruit seasonality, different peach varieties were used for the experiments. The pH of peaches used ranged from 3.69±0.06 to 5.11±0.24 according to the variety used. The most acidic variety was 'Elegant Lady' (3.69±0.06) and the least was 'Roig d'Albesa' (5.11±0.24). Soluble solids ranged from 11.0±1.0 ºBrix ('Royal Glory' peaches) to 13.6 ºBrix ('Roig d'Albesa' peaches). 'Royal Glory' peaches had the lowest acid content (3.65±0.87 g malic acid L^{-1}) and 'Elegant Lady' peaches the highest (7.20±0.60 g malic acid L^{-1}).

A total of 107 microorganisms were tested for antagonistic properties against *E. coli* O157:H7 on peach plugs at 20 ºC. Twenty belonged to the Pathology Laboratory collection, 54 were isolated from peaches and nectarines surface, 24 from apples surface, 8 from commercial fresh-cut apples and peaches and one isolated from fresh-cut pineapple.

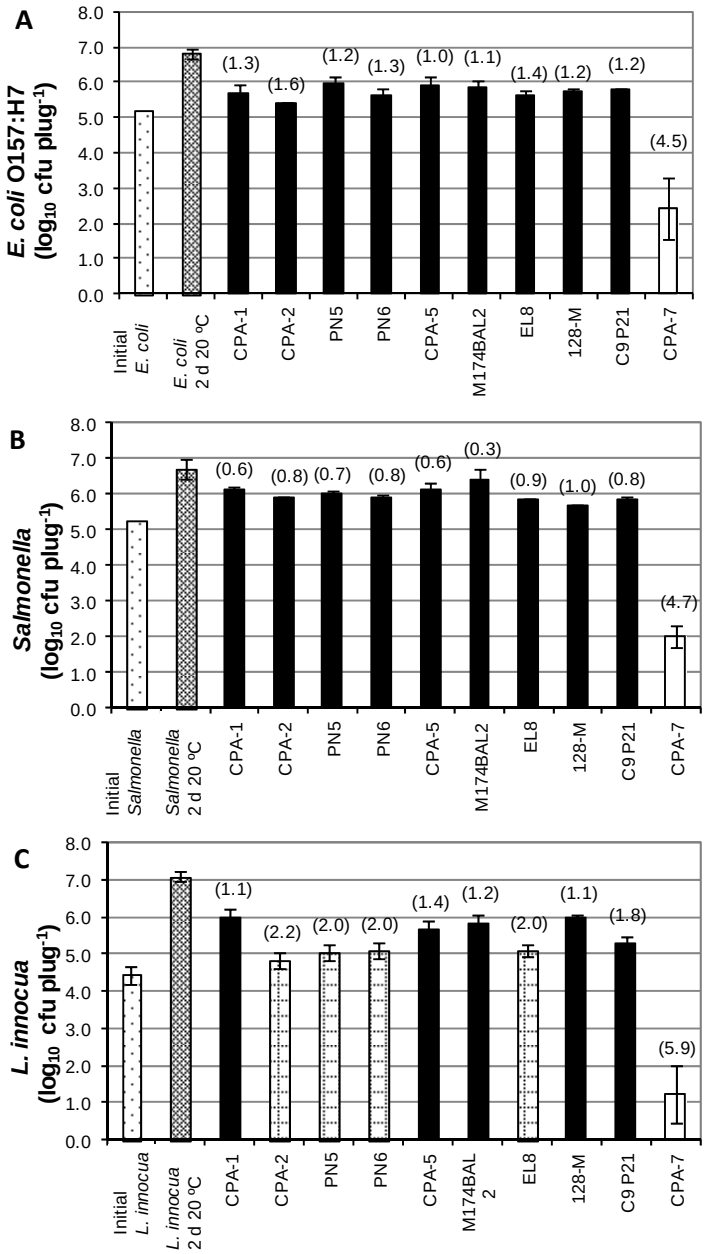

Fig. 1 *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) population on apple plugs after inoculation (□) and after 2 days of incubation at 20 ºC alone (▨) or with each of the 10 selected microorganisms as putative antagonists. Values are the mean of 6 values (2 assays with 3 replicates each) and bars in the columns represent standard error of the mean. ■: treatment with microorganisms that reduced pathogen population < 2-log units; □: treatment with microorganisms that reduced pathogen population ≥ 2-log units. The numbers in brackets show the average value of the reduction achieved by the microorganisms.

Table 1 Population of *E. coli* O157:H7 on 'Golden Delicious' apple plugs in the presence of different antagonists and stored at 5 ºC over 10 days.

Antagonist	**Population (log cfu plug^{-1}) after indicated storage time (days) at 5 ºC[a]**			
	0	2	6	10
None	5.23±0.04[b] a	5.17±0.01 a	4.90±0.01 a	4.65±0.08 a
CPA-1	5.23±0.04 a	5.15±0.02 a	4.97±0.01 a	4.79±0.05a
CPA-2	5.23±0.04 a	4.97±0.02 b	4.76±0.02 a	4.56±0.02 a
PN5	5.23±0.04 a	5.12±0.02 a	4.98±0.05 a	4.84±0.03 a
PN6	5.23±0.04 a	5.13±0.04 a	4.94±0.03 a	4.84±0.03 a
CPA-5	5.23±0.04 a	5.17±0.03 a	4.82±0.02 a	4.81±0.06 a
M174BAL2	5.23±0.04 a	5.14±0.01 a	4.63±0.29 a	4.80±0.05 a
EL8	5.23±0.04 a	5.17±0.02 a	5.00±0.03 a	4.83±0.02 a
128-M	5.23±0.04 a	5.10±0.02 a	4.88±0.02 a	4.78±4.78 a
C9P21	5.23±0.04 a	5.14±0.02 a	4.79±0.17 a	4.83±0.04 a
CPA-7	5.23±0.04 a	5.14±0.01 a	1.34±0.69 b	1.14±0.57 b

[a] Plugs were inoculated with *E. coli* O157:H7 at 10^7 cfu mL^{-1}.

[b] Results expressed as mean plus and minus standard deviation for each analysis (n=3). Means within columns with different letters (a, b) are different at the 0.05 significance level.

E. coli O157:H7 initial population on peach plugs after inoculation was approximately 4.8 log cfu plug^{-1} and it reached a final population around 7.9 log cfu plug^{-1} after 2 days at 20 ºC (Fig. 2A). Although *E. coli* O157:H7 populations were not affected by 84 of the microorganisms tested, 23 reduced it more than 1.0-log units. Only results from the 10 with highest reductions are shown. RG4 and EL8 were isolated from whole peaches, PN6 and CPA-7 from whole apples and CPA-5, CPA-2, CPA-3, C9P21, 128-M and F-10 belonged to the Pathology Laboratory collection. One microorganism, CPA-7, that achieved the best results on apple, was also the best in reducing *E. coli* O157:H7 on peach with a reduction of 4.3-log units.

The efficacy of the ten selected microorganisms was tested against *Salmonella* on peach plugs. Initial pathogen populations on peach plugs were 5.1 log cfu plug^{-1} and it increased approximately 2.4-log units after 2 days of storage at 20 ºC (Fig. 2B). Once more, the most effective microorganism was CPA-7 which reduced *Salmonella*'s growth 2.8-log units. In general, for each antagonist, reductions obtained with *Salmonella* were lower than those obtained with *E. coli* O157:H7.

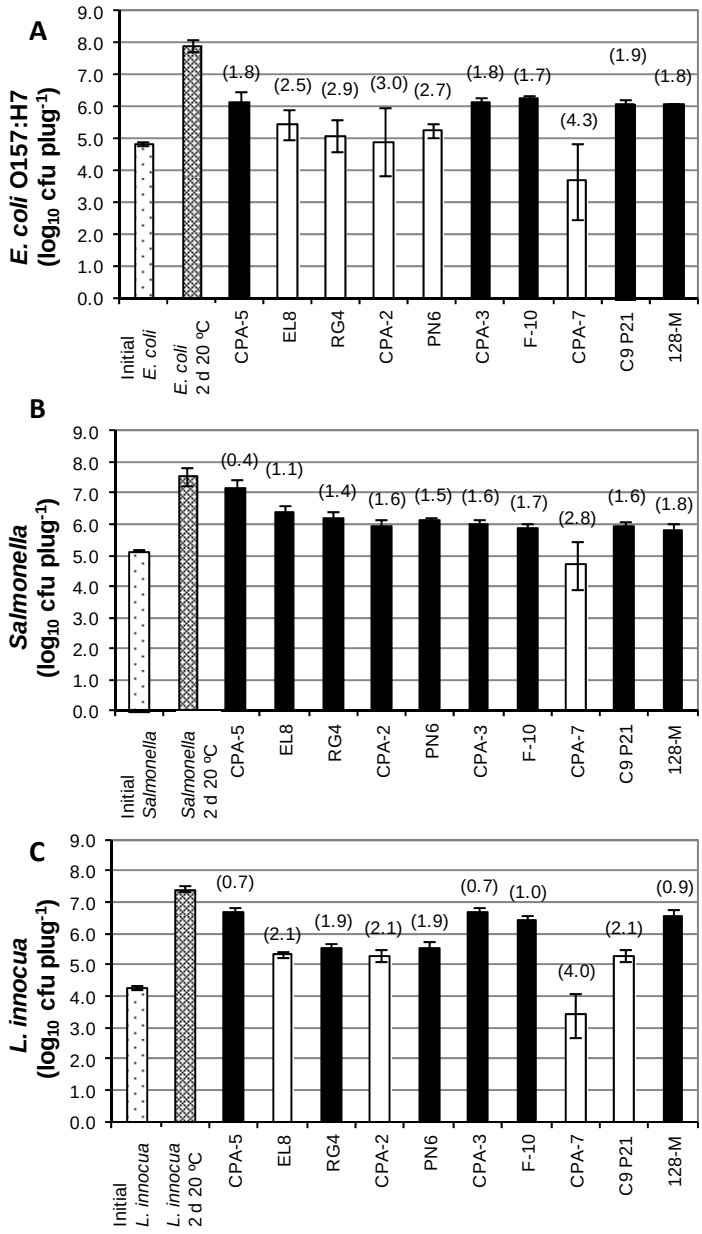

Fig. 2 *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) population on peach plugs after inoculation (⊡) and after 2 days of incubation at 20 ºC alone (▨) or with each of the 10 selected microorganisms as possible antagonists. Values are the mean of 6 values (2 assays with 3 replicates each) or 9 values (3 assays with 3 replicates each) for *Salmonella* and bars in the columns represent standard error of the mean. ■: treatment with microorganisms that reduced pathogen population < 2-log units; □: treatment with microorganisms that reduced pathogen population ≥ 2-log units. The numbers in brackets show the average value of the reduction achieved by the microorganisms.

L. innocua initial populations on peach plugs were around 4.3 log cfu plug^{-1} and population after 2 days of storage at 20 ºC were approximately 7.4 log cfu plug^{-1} (Fig. 2C). From the ten microorganisms tested against *L. innocua*, four of them (EL8, CPA-2, CPA-7 and C9P21 reduced its growth more than 2.0-log units. Again, CPA-7 was the most effective with a reduction value of 4.0-log units.

E. coli O157:H7 population increased by 0.3-log units after 10 days at 5 ºC (Table 2). When the efficacy of the ten selected microorganisms was tested against *E. coli* on peach plugs at refrigeration temperatures, CPA-7 reduced *E. coli* O157:H7 populations below detectable level (50 cfu plug^{-1}) after 6 days of storage.

From all the isolates tested, CPA-7 was selected for further studies as it exhibited the strongest inhibitory action against *E. coli* O157:H7, *Salmonella* and *L. innocua* on fresh-cut apples and peaches.

Table 2 Population of *E. coli* O157:H7 on 'Elegant Lady' peach plugs in the presence of different antagonists and stored at 5 ºC over 10 days.

Antagonist	**Population (log cfu plug^{-1}) after indicated storage time (days) at 5 ºC[a]**			
	0	2	6	10
None	5.09±0.07 a	5.07±0.06 ab	5.09±0.02 a	5.34±0.29 a
CPA-5	5.09±0.07 a	5.08±0.11 ab	5.09±0.01 a	4.88±0.11 b
EL8	5.09±0.07 a	5.16±0.03 a	5.01±0.04 ab	4.89±0.06 b
RG4	5.09±0.07 a	5.12±0.03 a	4.94±0.03 ab	4.60±0.15 b
CPA-2	5.09±0.07 a	4.71±0.33 b	4.85±0.10 b	4.68±0.02 b
PN6	5.09±0.07 a	5.13±0.02 a	5.03±0.02 ab	4.76±0.14 b
CPA-3	5.09±0.07 a	5.06±0.03 ab	4.92±0.10 ab	4.61±0.12 b
F-10	5.09±0.07 a	5.05±0.01 ab	5.05±0.03 ab	4.68±0.02 b
CPA-7	5.09±0.07 a	5.19±0.01 a	<1.7 c	<1.7 c
C9P21	5.09±0.07 a	4.90±0.14 ab	4.92±0.10 ab	4.80±0.11 b
128-M	5.09±0.07 a	4.90±0.04 ab	4.95±0.06 ab	4.78±0.08 b

[a] Plugs were inoculated with *E. coli* O157:H7 at 10^7 cfu mL^{-1}.

[b] Results expressed as mean plus and minus standard deviation for each analysis (n=3). Means within columns with different letters (a, b, c) are different at the 0.05 significance level.

3.3. Dose-response relationships in the biocontrol of *E. coli* O157:H7, *Salmonella* and *L. innocua* on fresh-cut apples

E. coli O157:H7 inoculum was prepared at 1.3×10^5, 1.0×10^6 and 1.4×10^7 cfu mL^{-1} and initial population on apple plugs after inoculation was 3.2, 4.1 and 5.1 log cfu $plug^{-1}$, respectively (Fig. 3A). *E. coli* populations augmented 2.4, 3.1 and 2.1-log units, respectively, after 2 days of storage at 20 ºC when inoculated alone. Application of CPA-7 at a concentration of 10^8 cfu mL^{-1}, resulted in great reductions (more than 3.5 log units) in the three pathogenic population levels. Reducing CPA-7 inoculum caused a decrease in antagonistic effect.

Salmonella initial populations were 3.1, 4.0 and 5.1 log cfu $plug^{-1}$ (Fig. 3B). Populations increased between 2.1 and 3.5-log units, and although highest growth was observed with the lowest inoculum level, final populations were the lowest (below 7.0-log cfu $plug^{-1}$). Application of CPA-7 at concentrations of 10^7 and 10^8 cfu mL^{-1}, resulted in reductions of *Salmonella* populations higher than 3.3-log units. When CPA-7 inoculum was reduced to 10^5 and 10^6 cfu mL^{-1}, reductions obtained were strongly dependent to *Salmonella* populations. Reductions of 1.9-log units were achieved when antagonist and pathogen were inoculated at the same level.

Initial *L. innocua* populations on apple plugs were 2.4, 3.9 and 4.7 log cfu $plug^{-1}$ (Fig. 3C) and they augmented 4.0, 2.5 and 1.5-log units during storage at 20 ºC, respectively. Application of CPA-7 at 10^7 and 10^8 cfu mL^{-1}, caused a pathogen reduction higher than 2.0-log units and, similarly to *Salmonella*, the same proportion of antagonist: pathogen was needed to achieve a pathogen reduction higher than 2.0 log units.

Growth of CPA-7, when inoculated at different levels, on fresh-cut apples was also determined (Fig. 4). Initial antagonist populations on fresh-cut apple ranged from 2.9 to 5.9 log cfu $plug^{-1}$ and regardless initial concentration, after 2 d of storage at 20 ºC, all antagonist populations were over 7.0-log units, which represented population increases among 1.4 and 4.2-log units.

3.4. Hypersensitive reaction on tobacco plants

CPA-7 capacity to produce a hypersensitive reaction in leaf mesophyll tissue of tobacco plants was examined and no reaction was observed, even at 10^9 cfu mL^{-1}, when compared with the positive control *P. ananatis* CPA-3.

3.5. *In vitro* determination of antagonistic activity

Neither the culture nor the neutralized cell-free supernatant or the cells produced inhibition zones in the growth of any of the pathogens tested (data not shown).

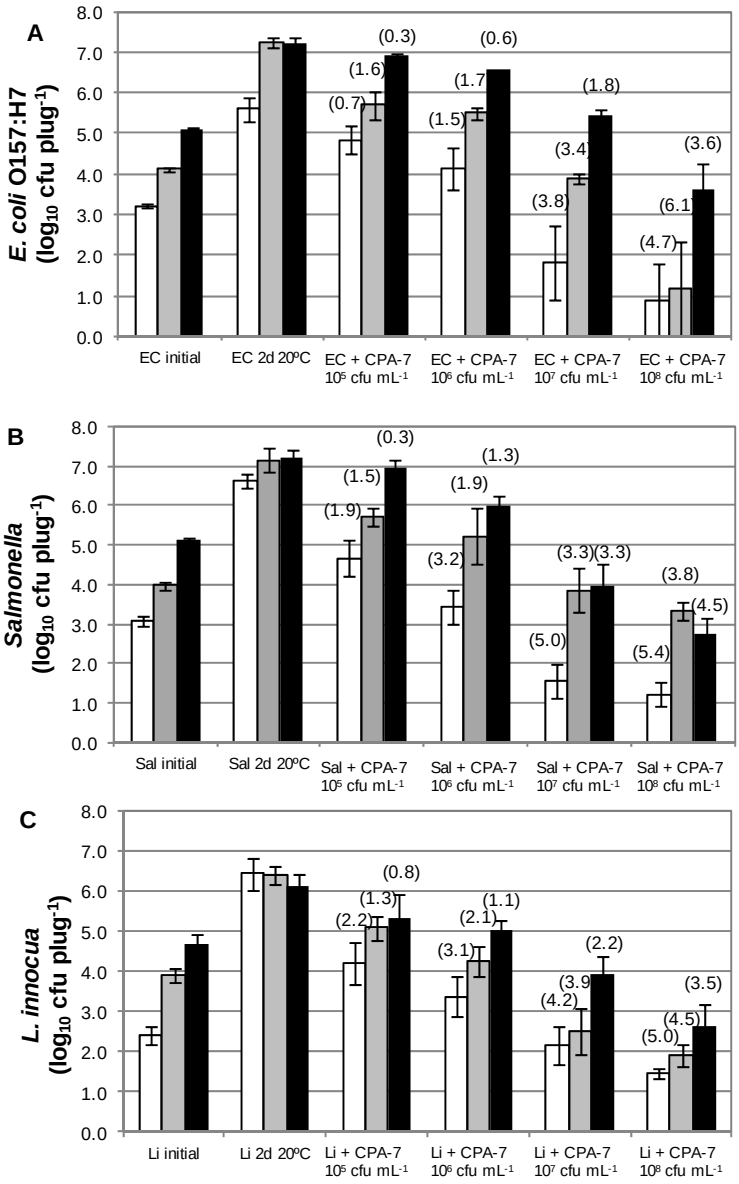

Fig. 3 *E. coli* O157:H7 (A), *Salmonella* (B) and *L. innocua* (C) population on apple plugs after inoculation at three different inoculum levels 10^7 (■), 10^6 (▣) and 10^5 (□) cfu mL^{-1} (initial) and after 2 days of incubation at 20 ºC alone (2 d 20 ºC) or with CPA-7 at different inoculum levels 10^8, 10^7, 10^6 and 10^5 cfu mL^{-1}. Values are the mean of 6 values (2 assays with 3 replicates each) and bars in the columns represent standard error of the mean. The numbers in brackets show the average value of the reduction achieved by CPA-7.

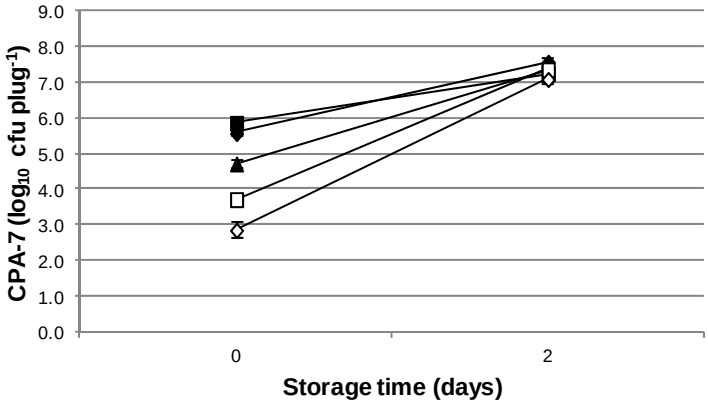

Fig. 4 Growth of CPA-7 on apple plugs throughout storage at 20 ºC when inoculated at different inoculums: 10^8 (■), 10^7 (◆), 10^6 (▲), 10^5 (□) and 10^4 (◇) cfu mL^{-1}. Values are the mean of 6 values (2 assays with 3 replicates each) and bars are standard error of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol.

3.6. Identification of antagonistic strains

The taxonomic status of 13 strains was determined by physiological, biochemical or morphological testing. As shown in Table 3, in some cases the identification by biochemical testing did not agree with that of partial 16S rDNA sequencing. Two of them were yeasts, CPA-1 was *Candida sake* and M174BAL2 was *Candida famata*. All the other strains were gram negative bacilli and oxidase and catalase reaction were negative and positive respectively. Most of them (6 out of 13) belonged to *Pantoea* spp. and 3 belonged to *Pseudomonas* spp.

3.7. CPA-7 identification

Cells of CPA-7 strain are Gram-negative, strict aerobic, non-spore-forming, motile rods. Colonies on TSA are circular and yellow pigmented. Cells are oxidase negative and catalase positive. Growth occurred at 6-30 °C, but not at 33 °C or higher temperatures. Growth below 6 °C was not tested in media. Phenotypic properties of strain CPA-7 and other close relatives of the genus *Pseudomonas* are shown in Table 4.

The partial 16S rRNA sequence of strain CPA-7 revealed that the strain belonged to the genus *Pseudomonas* with 99.9 % sequence identity to the type strain of *Pseudomonas graminis*. Other close relatives were *Pseudomonas lutea* (99.3%) and *Pseudomonas rhizosphaerae* (98.8%). In Fig. 5 an UPGMA dendrogram is shown.

The strain was identified as *Pseudomonas graminis* (Behrendt *et al.*, 1999) based on almost full 16S rRNA analysis and phenotypic tests.

Table 3 Identification results obtained with the aid of API 20E and API 20NE systems and 16S rDNA sequence analysis.

Microorganism	API20E/API20NE/API CAux/Biolog	16S rDNA
CPA-7	*Chryseomonas luteola*	*Pseudomonas graminis*
128-M	*Pantoea* spp.	*Pantoea ananatis*
C9P21	*Pantoea* spp.	*Pantoea agglomerans*
PN6	*Pantoea* spp.	*Pantoea agglomerans*
CPA-5	*Pseudomonas syringae*	-
EL8	*Pantoea* spp. 3	-
RG4	*Rahnella aquatilis*	-
CPA-2	*Pantoea* spp.	*Pantoea agglomerans*
CPA-3	*Pantoea ananatis*	-
F-10	*Pseudomonas syringae*	-
CPA-1	*Candida sake*	-
PN5	*Flavimonas oryzihabitans*	-
M174BAL2	*Candida famata*	-

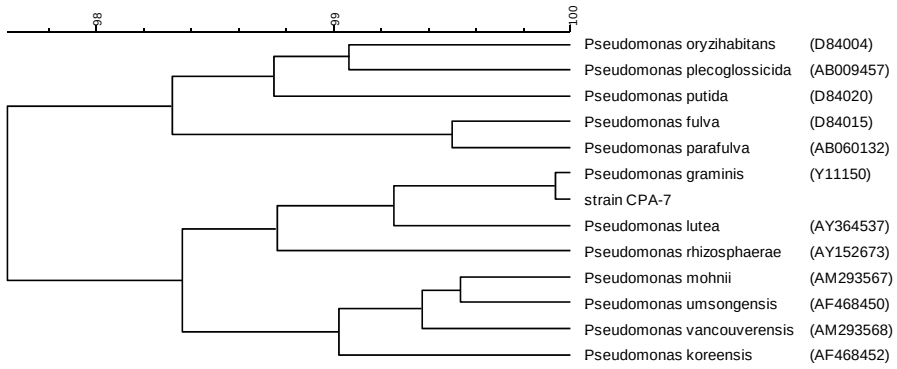

Fig. 5 UPGMA phylogenetic tree based on 16S rRNA gene sequences showing the position of strain CPA-7 among species of the genus *Pseudomonas.* The horizontal bar joining two isolates or clusters indicating the level of similarity.

Table 4 Phenotypic characteristics that distinguish strain CPA-7 from other species.

Characteristic	**1**[a]	**2**	**3**	**4**
Oxidase	-[b]	-	-	-
Growth at 6 ˚C	+	+	+	nd
Acid from glucose	-	-	-	-
Utilization of erythritol	-	-	-	+
Utilization of sorbitol	w	+	-	+
Utilization of xylitol	-	v	+	-
Utilization of melibiose	-	-	+	-
Utilization of rhamnose	-	-	-	+
Hydrolysis of aesculin	+	+	+	-
Hydrolysis of gelatin	-	v	-	-

[a]Species: 1: strain CPA-7; 2: *P. graminis;* 3: *P. lutea;* 4: *P. rhizosphaerae*. Data for reference species were taken from Peix *et al.* (2004), Peix *et al.* (2003) and Behrendt *et al.* (1999).

[b]+, positive; -, negative; w, weak; v, different reactions between strains; nd, no data available

4. DISCUSSION

In this work, we have focused on finding indigenous microorganisms from fresh fruit to control survival and growth of *E. coli* O157:H7, *Salmonella* and *L. innocua* on fresh-cut apples and peaches. Our results have shown that some of these isolated microorganisms could reduce the growth or even eliminate the FBP tested so that, they could be used as an alternative or additional tool to chemical or physical interventions to control the survival and growth of FBP on fresh-cut fruit.

From all bacteria and yeasts tested, *E. coli* O157:H7 population was reduced at least 1-log by 10.3 % and 21.5 % of isolates on apple and peach plugs incubated at 20 ºC, respectively, when compared to the control (the pathogen inoculated alone). Seven of these microorganisms were the same for both fruits: PN6, CPA-5, EL8, 128-M, C9P21, CPA-2 and CPA-7. PN6, 128-M, EL8 and C9P21 belonged to *Pantoea* spp.; CPA-5 and CPA-7 were *Pseudomonas* spp. In general, the smallest reduction values were detected when the antagonists were tested against *Salmonella*, and the greatest against *L. innocua* in both fruits. With some exceptions, higher reductions were attained by the same microorganisms when they were tested against *E. coli* O157:H7 and *Salmonella* on peach plugs than on apple plugs. This might be due to the differences in growth of the pathogens on both fruits as population increases were lower on apple plugs than in peach plugs. In contrast, similar population increases and, therefore similar reductions values, were

measured with *L. innocua*. Despite differences between pathogens and food matrix, one microorganism, CPA-7 achieved the best reductions values at 20 ºC (from 2.8 to 5.9-log units) in all assays. In addition, only CPA-7 reduced *E. coli* O157:H7 population at refrigeration conditions (5 ºC). Even though, inhibition was not significant until 6 days of storage due to the lack of *E. coli* O157:H7 population increase during storage at 5 ºC. Efficacy trials were performed with high densities of putative antagonists (30 % transmittance which corresponded to a concentration between 10^6 and 10^8 cfu mL^{-1}) to establish potential inhibition of foodborne pathogens (at an inoculum level of, approximately, 10^7 cfu mL^{-1}). Under natural conditions, concentrations of pathogens will be much lower than the concentration used in our study. Thus, the effectiveness of the antagonist CPA-7 in preventing colonization of apple flesh by foodborne pathogens at different inoculum levels was tested. In order to obtain a reduction value higher than 1.0-log unit for the three FBP tested, the same proportion of CPA-7:FBP was needed. Hence, dose of CPA-7 could be reduced under natural conditions.

Previous works have also proven the effectiveness of microorganisms as bioprotective agents. Janisiewicz *et al.* (1999a) demonstrated that fresh cells of the commercialized antagonist *P. syringae* L-59-66, used for controlling postharvest decay of pome fruits, can also prevent the growth of the foodborne pathogen *E. coli* O157:H7 on wounded apple tissue stored at 24 ºC for 48 h. Leverentz *et al.* (2006) found seven promising microorganisms that reduced *L. monocytogenes* and *Salmonella* populations on 'Golden Delicious' apple plugs. The greatest reductions (from 5.7 to 6.0-log units after 7 days) were measured against *L. monocytogenes* at 25 ºC with a low pathogen inoculum by strains of *Gluconobacter assai*, *Candida* spp., *Dicosphaerina fagi* and *Metschnikowia pulcherrima*. Only three of them reduced *L. monocytogenes* populations at 10 ºC and *Salmonella* populations at 25 ºC storage, but none of them reduced *Salmonella* populations at 10 ºC as it only grew slightly at this temperature and there were less competition. At high pathogen inoculum levels (10^8 cfu mL^{-1}) only *G. assai* and *Candida* spp. reduced *L. monocytogenes* populations to non-detectable levels. Trias *et al.* (2008) selected six strains of lactic acid bacteria (LAB) and tested their effect as bioprotective agents against *E. coli*, *S. thyphimurium* and *L. monocytogenes* on apple wounds. Results showed that, meanwhile five of them reduced significantly *L. monocytogenes* populations below detection limit, lower reductions were observed in *Salmonella* and none against *E. coli*. Recently, the populations of a five strain mixture of *E. coli* were reduced by the postharvest biocontrol agent *C. sake* CPA-1 on 'Golden Delicious' apple wounds at 25 ºC regardless initial pathogen level (Abadias *et al.*, 2009). However, in contrast to the results obtained with CPA-7, no effect of the antagonist *C. sake* on *E. coli* population was observed at 5 ºC on apple wounds and on fresh-cut apples.

From all isolates tested on apples and peaches, CPA-7 was selected to further assays as it achieved the greatest pathogen reduction values on both fruits. Good candidates to biocontrol agents may be presumed to be safe for humans and should

cause not troublesome sensory effects or a lessening of the shelf life of the product. Hence, CPA-7 was tested for hypersensitive reaction on tobacco plants. CPA-7 did not cause any reaction, so it seems not to be a phytopathogenic microorganism although more specific studies on fresh-cut apples and peaches should be conducted.

The aim of this study was not to determine the mode of action of the isolates. However, from the results obtained in *in vitro* assays, CPA-7 cell-free supernatant did not have effect against the studied FBPs thus antimicrobial substances production in the studied medium is unlikely and competition could be the inhibitory cause. Similarly, in the assays carried out by Janisiewicz *et al.* (1999a) the mechanism of action of *P. syringae* seemed to be competition for nutrients and space. This mode of action is advantageous because it is very unlikely that the pathogens will develop resistance to a biocontrol agent whose mechanism of action is based on competitive exclusion. In the case of biological control with LAB, mainly *Leuconostoc* spp., the inhibition had a bactericidal effect against *L. monocytogenes* but not against Gram negative bacteria. This specificity of LAB for Gram positive bacteria may be related to bacteriocin production (Trias *et al.*, 2008).

CPA-7 strain, isolated from whole 'Golden Delicious' apples, was identified as *P. graminis*. Little is known about this specie that was first described by Behrendt *et al.* (1999) and isolated from grasses as a non-fluorescent pseudomonads. It has been previously reported that *Pseudomonas* species have antagonistic effect on pathogens such as *E. coli* O157:H7 (Janisiewicz *et al.*, 1999a; Schuenzel and Harrison, 2002), *L. monocytogenes* (Carlin *et al.*, 1996; Liao and Fett, 2001) and *Salmonella* (Fett, 2006; Matos and Garland, 2005). However, none other study has described *P. graminis* as biological control agent. This strain has neither been described as harmful to human health. A patent application for this strain has been submitted (Viñas *et al.*, 2010).

The results have shown that biocontrol agents, specifically CPA-7, could reduce or slowdown FBP's development on minimally processed fruit and even eliminate them. This strain has demonstrated very good efficacy in reducing foodborne pathogens on fresh-cut apple and peach. In addition, no effect on visual quality of the fruit was observed. It could be a good biological control agent as it does not grow at 37 ºC and seems that it does not produce antimicrobial substances against the FBP tested. However, as the inhibitory effect of the antagonists on foodborne pathogens was not instantaneous and it only became apparent after 6 days at 5 ºC, biological control agents should be used in combination after other disinfection treatments.

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CAPÍTULO VI

Antagonistic effect of *Pseudomonas graminis* CPA-7 against foodborne pathogens in fresh-cut apples under commercial conditions

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Enviado a: Food Microbiology

ABSTRACT

Recently, we reported that the application of the strain CPA-7 of *Pseudomonas graminis*, previously isolated from apple, could reduce the population of foodborne pathogens on minimally processed (MP) apples and peaches under laboratory conditions. Therefore, the objective of the present work was to find an antioxidant treatment and a packaging atmosphere condition to improve CPA-7 efficacy in reducing a cocktail of four *Salmonella* and five *Listeria monocytogenes* strains on MP apples under simulated commercial processing. The effect of CPA-7 application on apple quality and its survival to simulated gastric stress were also evaluated. Ascorbic acid (2 %, w/v) and N-acetyl-L-cysteine (1 %, w/v) as antioxidant treatments reduced *Salmonella*, *L. monocytogenes* and CPA-7 recovery, meanwhile no reduction was observed with NatureSeal® AS1 (NS, 6 %, w/v). The antagonistic strain was effective on NS-treated apple wedges stored at 10 °C with or without modified atmosphere packaging (MAP). Then, in a semi-commercial assay, efficacy of CPA-7 inoculated at 10^5 and 10^7 cfu mL^{-1} against *Salmonella* and *L. monocytogenes* strains on MP apples with NS and MAP and stored at 5 and 10 °C was evaluated. Although high CPA-7 concentrations/populations avoided *Salmonella* growth at 10 °C and lowered *L. monocytogenes* population increases were observed at both temperatures, the effect was not instantaneous. No effect on apple quality was detected and CPA-7 did not survived to simulated gastric stress throughout storage. Therefore, CPA-7 could avoid pathogens growth on MP apples during storage when use as part of a hurdle technology in combination with disinfection techniques, low storage temperature and MAP.

Keywords: *Escherichia coli* O157:H7; *Salmonella*; *Listeria monocytogenes*; biopreservation; minimally processed apples; biocontrol

1. INTRODUCTION

Recently, 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, and, in particular, fresh-cut apples have recently emerged as popular snacks in food service establishments, school lunch programs, and for family consumption (Gorny, 2003a).

In spite of the low pH of many fruits, including apples and peaches, foodborne pathogens (FBP) such as *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes* could be present and cause public health problems. The incidence and/or survival/growth of these FBP in MP apples and peaches has been demonstrated (Abadias *et al.*, 2006, 2008, 2009; Alegre *et al.*, 2010a, 2010b; Harris *et al.*, 2003; Liao and Sapers, 2000). In addition, outbreaks linked to fresh-cut fruit have been reported (CDC, 2007; Harris *et al.*, 2003).

There are several processing steps in the fresh-cut produce production chain and many points for potential microbial contamination exist in each of these steps (Nguyen-The and Carlin, 1994). The only step for reducing microorganisms during processing is washing. A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce bacterial populations on fruit and vegetables (Beuchat, 1998; EU Scientific Committee on Food, 2002). However, besides their potential toxicity, they have proved incapable of completely removing or inactivating microorganisms on fresh produce (Koseki and Itoh, 2001; Park *et al.*, 2001). Washing raw fruit and vegetables removes only a portion of pathogenic and spoilage microorganisms as some of them may escape contact with washing or sanitizing agents attaching to the surface of fruit and vegetables and tending to locate in protected binding sites (Allende *et al.*, 2008; Sapers *et al.*, 2001; Takeuchi and Frank, 2001).

In addition, reducing/controlling the native microbial populations by washing and sanitizing can allow human pathogens to flourish on produce surface (Brackett, 1992) as it reduces competition for space and nutrients thereby providing growth potential for pathogenic contaminants. Chemical synthetic additives can reduce decay rate, but consumers are concerned about chemical residues in the product, which could affect their health and cause environmental pollution (Ayala-Zavala *et al.*, 2008; Roller and Lusengo, 1997). Therefore alternative methods for controlling fresh-cut fruit decay are required.

Biological control fits well with this new tendency. Some bioprotective microorganisms have already shown its potential for application in MP apples. For example, the strain L-59-66 of *Pseudomonas syringae* prevented the growth of *E. coli* on apple wounds (Janisiewicz *et al.*, 1999). Growth of *L. monocytogenes* and *Salmonella* on fresh-cut apple was reduced by strains of *Gluconobacter asaii*,

Candida spp., *Dicosphaerina fagi* and *Metschnikowia pulcherrima* (Leverentz *et al.*, 2006). The postharvest biocontrol agent *Candida sake* CPA-1 reduced *E. coli* growth on apple wounds, but not in MP apples (Abadias *et al.*, 2009). Lactic acid bacteria were also reported to be inhibitory of *L. monocytogenes* on wounded apples (Trias *et al.*, 2008). Recently, we have demonstrated the ability of *Pseudomonas graminis* CPA-7, isolated from whole apple surface, to reduce *E. coli* O157:H7, *Salmonella* and *L. innocua* on MP apples and peaches (Alegre *et al.*, previous work). However, none of these studies were performed under realistic conditions for MP apples.

Beyond microbiological contamination, development of fresh-cut apple slices has been hampered by the rapid oxidative browning of apple flesh. Browning can be delayed by reducing agents. For example, ascorbic acid has long been applied in combination with organic acids and calcium salts to prevent enzymatic browning of fruits (Gorny *et al.*, 1998; 2002; Pizzocaro *et al.*, 1993; Sapers *et al.*, 1989; Soliva-Fortuny *et al.*, 2001; 2002). Several studies have shown that NatureSeal® products can reduce browning in fresh-cut fruit slices (Abbott *et al.*, 2004; Bhagwat *et al.*, 2004; Rößle *et al.*, 2009; Rupasinghe *et al.*, 2005; Toivonen, 2008) and some natural thiol-containing compounds, such as N-acetylcysteine, have also been investigated as an alternative method to control enzymatic browning (Gorny *et al.*, 2002; Molnar-Perl and Friedman, 1990; Oms-Oliu *et al.*, 2006; Rojas-Grau *et al.*, 2006; Son *et al.*, 2001).

Enzymatic browning of apple slices can also been delayed by the use of modified atmosphere packaging (MAP) with very low oxygen levels (Gorny, 2003b); but extremely low O_2 levels pose the risk of anaerobic respiration and consequent off-flavors (Luo and Barbosa-Canovas, 1996) and, potentially, the growth of microaerophilic human pathogens, such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Buck *et al.*, 2003; Gunes and Hotchkiss, 2002).

The objective of this study was to test the efficacy of the antagonistic strain *P. graminis* CPA-7 against a cocktail of four *Salmonella* strains and five *L. monocytogenes* strains on MP 'Golden Delicious' apples under simulated commercial conditions throughout storage at 5 and 10 °C. Different antioxidant treatments and modified atmospheres were tested. The effect of antagonist application on quality of MP apple was also evaluated. In addition, the ability of *Salmonella*, *L. monocytogenes* and *P. graminis* CPA-7 to survive to simulated gastric stress conditions following storage was studied.

2. MATERIALS AND METHODS

2.1. Fruit

'Golden Delicious' apples were obtained from local packinghouses in Lleida (Catalonia, Spain). Prior to the experimental studies, apples were washed in running tap water and let to dry at room temperature. Apples were cut in 10 skin-on wedges using an apple slicer/corer.

2.2. Bacterial strains

The bacterial strains used in this work are listed in Table 1. The antagonistic strain of *P. graminis* CPA-7 was isolated from apple surface in our laboratory (Alegre *et al.*, previous work). CPA-7 strain was grown in tryptone soy broth (TSB, Oxoid, UK) for 20-24 h at 30 °C. *Salmonella* strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin sulphate salt (St, Sigma, Germany) thereby enabling detection on a selective medium (TSA-St) in the presence of the antagonist and the natural microbial flora associated with apples. The strains were grown individually in TSB supplemented with streptomycin (TSB-St) medium for 20-24 h at 37 °C. *L. monocytogenes* strains were grown individually in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) for 20-24 h at 37 °C. Bacterial cells were harvested by centrifugation at 9820 × g for 10 min at 10 °C and then resuspended in sterile distilled water (CPA-7) or saline solution (SS; 8.5 g L^{-1} NaCl, *Salmonella* and *L. monocytogenes*). The four *Salmonella* concentrated suspensions were mixed, as well as the five *L. monocytogenes* concentrated suspensions.

For the inoculum preparation, bacterial concentration was estimated using a spectrophotometer set at λ=420 nm according to standard curves, and a volume of each of the bacterial concentrated suspensions was added to deionized water with or without antioxidant to obtain approximately 10^5 cfu mL^{-1} or 10^7 cfu mL^{-1}. Inoculum concentration was checked by plating appropriate dilutions onto TSA-St for *Salmonella*, onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics, Beauvais, France) for *L. monocytogenes* and onto Nutrient Agar (NA, Biokar Diagnostics) for CPA-7. Plates were incubated at 37±1 °C, for *Salmonella* and *L. monocytogenes*, or at 30±1 °C for CPA-7.

2.3. Selection of best antioxidant treatment

In order to choose an antioxidant treatment for minimally processed apples that not affect effectiveness of the antagonistic strain CPA-7, ascorbic acid (AA, 2 % w/v, Prolabo, Mollet del Vallès, Spain), NatureSeal® AS1 (NS, 6 % w/v; AgriCoat Ltd., Great

Shefford, UK) and N-acetyl-L-cysteine (NAC, 1 % w/v, Panreac, Barcelona, Spain) were tested. Solutions were prepared and inoculated with *Salmonella*, *L. monocytogenes* or CPA-7 (10^7 cfu mL^{-1}). Afterwards, apple wedges were suspended (1:2 w/v) for 2 min at 150 rpm in 1 L of each of the inoculated antioxidant treatments (or deionized water as control treatment) then were allowed to dry in a laminar flow biosafety cabinet. Approximately 10 apple wedges (200±5 g) were placed in polypropylene (192×137×55 mm) trays and sealed with a polypropylene plastic film (Amcor Flexibles, Ledbury, Hereforshire UK) of 35 µ in thickness with an O_2 and CO_2 permeability of 3500 cm^3 m^{-2} day^{-1} atm^{-1} at 23 ºC and a water steam permeability of 0.9 g m^{-2} day^{-1} at 25 ºC and 75 % relative humidity. Apple trays were stored at 10 ºC.

Table 1 Bacterial strains used in this study

Number	Microorganism	Serovar	Source
CPA-7	*Pseudomonas graminis*		Apple surface (isolated in our laboratory, Alegre *et al.*, previous work, Viñas *et al.* 2010)
ATCC BAA-707	*Salmonella enterica* subsp. *enterica*	Agona	Alfalfa sprouts
ATCC BAA-709	*Salmonella enterica* subsp. *enterica*	Michigan	Cantaloupe
ATCC BAA-710	*Salmonella enterica* subsp. *enterica*	Montevideo	Clinical (patient with salmonellosis associated with tomatoes)
ATCC BAA-711	*Salmonella enterica* subsp. *enterica*	Gaminara	Orange juice
CETC 4031/ ATCC 15313	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	1a	Rabbit
CECT 933/ ATCC 19113	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	3a	Human
CECT 940/ ATCC 19117	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	4d	Sheep
CECT 4032	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	4b	Cheese
LM230/3	*Listeria monocytogenes*	1/2a	Fresh-cut iceberg lettuce (isolated in our laboratory, (Abadias *et al.* 2008))

Samples were examined on the day of inoculation and after 2 days. Populations of *Salmonella*, *L. monocytogenes* and CPA-7 were determined in three sample trays for each treatment. For the analysis, 10 g of apple from each tray were mixed with 90 mL of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England) in a sterile bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Further ten-fold dilutions were made with saline peptone (SP; 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and plated as described previously.

The antioxidant product that had less influence in CPA-7 population on apple wedges was selected to corroborate the effectiveness of CPA-7 in the presence of the antioxidant treatment. Apple wedges were suspended in the antioxidant treatment (or deionized water as control treatment), drained and dip inoculated with (a) *Salmonella* and *L. monocytogenes* (10^5 cfu mL^{-1}, each), (b) CPA-7 (10^7 cfu mL^{-1}) or (c) *Salmonella* and *L. monocytogenes* and CPA-7. Then, apple wedges were allowed to dry in a laminar flow biosafety cabinet and packed as described previously. Apple trays were stored at 10 °C up to 7 days. Microbial populations were determined in three sample trays for each treatment at each sampling time (0, 2, 5 and 7 days) as described previously.

2.4. Influence of packaging atmosphere on antagonistic effect

Apple wedges were treated with antioxidant (NatureSeal®) and inoculated as described previously. At the moment of packaging, two different atmosphere conditions were studied, a passive modified atmosphere (MAP, using the polypropylene plastic film described previously) and air conditions (Air, using the same film manually perforated with 9 holes of 400 µm each). Once packed, apple trays were stored at 10 °C. Microbial populations were determined in three sample trays for each treatment after inoculation and after 7 days.

2.5. Semi-commercial trials

Apple wedges treated with NatureSeal®, drained and then suspended (1:2 w/v) for 2 min at 150 rpm in one of the following treatments (a) control: water, (b) Sal+Lm: *Salmonella* and *L. monocytogenes* inoculum, (c) CPA-7: CPA-7 inoculum or (d) Sal+Lm+CPA-7: *Salmonella* and *L. monocytogenes* and CPA-7 inoculum; and then were allowed to dry in a laminar flow biosafety cabinet. Then apple wedges were packed in trays in modified atmosphere as described previously and stored at 5 and 10 °C up to 14 days. *Salmonella* and *L. monocytogenes* inocula concentration was 10^5 cfu mL^{-1}. Low (10^5 cfu mL^{-1}) and high (10^7 cfu mL^{-1}) inocula of *P. graminis* CPA-7 were tested.

Populations of *Salmonella*, *L. monocytogenes* and CPA-7 were determined in three sample trays for each treatment (b, c and d) at each sampling time and temperature.

The samples were examined on the day of inoculation and after 4, 7 and 14 days. Mesophilic microorganisms were determined in control treatment (a) by enumerating colonies on plates with plate count agar (PCA, Biokar Diagnostics) and incubated at 30±1 °C for 3 days.

Each tray was a replicate and there were three replicates for each treatment at each sample date and temperature.

2.6. Survival in simulated gastric conditions

Salmonella, *L. monocytogenes* and CPA-7 from apple samples stored at 5 °C (treatments (b) Sal+Lm; (c) CPA-7 and (d) Sal+Lm+CPA-7) were evaluated for their survival to the exposure to simulated gastric stress at each sampling time. To simulate mastication 10 g of each sample was placed into a sterile plastic bag (80 mL, IUL Instruments, Barcelona, Spain) and 10 mL of artificial saliva solution (6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$ and 1.2 g L^{-1} $NaHCO_3$) tempered at 37 °C were added. It was homogenized in a stomacher blender for 120 s at high speed (Bagmixer 100 Minimix, Interscience). The mix was transferred to an Erlenmeyer flask containing 80 mL of gastric fluid (pH 2.0; 2M HCL containing 0.3 g L^{-1} of pepsin and tempered at 37 °C) and was incubated in an incubator shaker at 37 °C and 150 rpm for 2 h. Populations of *Salmonella*, *L. monocytogenes* and CPA-7 after being incubated with simulated gastric acid for 2 h were compared with those counts in BPW.

2.7. Quality analysis of apple wedges

Quality analysis of apple wedges was performed for each single tray. Three trays were used for each treatment at each sample time and temperature. Visual quality, headspace composition and pH were determined for all treatments, meanwhile colour, texture, soluble solid content and titratable acidity were only determined for the treatments without pathogens as analysis took place outside the Biosafety Laboratory.

2.7.1. Visual quality

Visual evaluations of apple wedges from each tray were made by an untrained panel composed by the personnel working in the laboratory (n=3), which judged the apple by overall quality by using a 9 point hedonic scale, being 1 inedible (0 % edible), 5 fair (75 % edible) and 9 excellent (100 % edible). Mean values were calculated.

2.7.2. Headspace gas composition

Carbon dioxide and oxygen content in single trays were determined using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) at each sampling time.

2.7.3. pH

Apple flesh pH was determined using a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). There were three determinations per tray (9 determinations per treatment at each sample time and temperature).

2.7.4. Colour

Fresh-cut apple surface colour was directly measured with a CR-400 Minolta chroma meter (Minolta, INC., Tokyo, Japan). Colour was measured using CIE L^*, a^*, b^* coordinates. Illuminant D65 and 10° observer angle were used. The instrument was calibrated using a standard white reflector plate (Y=94.00, x=0.3158, y=0.3322). Five apple wedges were evaluated for each tray. Two readings were made in each replicate by changing the position of the apple wedge (n=30). A decrease in L^* value indicates a loss of whiteness, a more positive a^* value means progressive browning and a more positive b^* value indicates more yellowing.

2.7.5. Soluble solids content

Percent of soluble solids (°Brix) was measured at 20 °C with a handheld refractometer (Atago Co. Ltd., Tokio, Japan) in juice extracted by crushing apple wedges in a blender. There was one measurement per tray.

2.7.6. Titratable acidity

To measure titratable acidity, 10 mL of apple juice (obtained by crushing apple wedges of each tray) were diluted with 10 mL of distilled water and it was titrated with 0.1 N NaOH up to 8.1. The results were calculated as g of malic acid L^{-1}. There was one measurement for each tray.

2.7.7. Texture

Apple firmness evaluation was performed using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., England, UK) by measuring the maximum penetration

force required for a 8 mm diameter probe to penetrate into an apple cube of 20×20×20 mm to a depth of 8 mm. Three apple pieces from each tray were measured.

2.8. Statistical analysis

Prior to ANOVA, all cfu mL^{-1} data were transformed to $\log_{10}$ cfu g^{-1}. Other data were not transformed. The General Linear Models (GLM) procedure of the SAS Enterprise Guide was applied (v.4.1; SAS Institute, Cary, NC, USA). Significant differences between treatments were analyzed by Duncan's Multiple Range test at a significance level of $P<0.05$.

3. RESULTS

3.1. Selection of best antioxidant treatment

Initial *Salmonella* populations on apple wedges without antioxidant treatment (control) were 4.0 log cfu g^{-1}. Similar populations were detected on NS-treated apple wedges (Table 2). However, significantly lower population were found in AA treated apple pieces and the lowest were in NAC-treated apple wedges, with pathogen populations below detection limit (50 cfu g^{-1}). After 2 days of storage at 10 °C, *Salmonella* populations on control and NS-treated apple wedges increased slightly meanwhile it maintained at initial level on AA and NAC-treated apple wedges.

L. monocytogenes populations were the same in control and AA-treated apple wedges (around 1.8-1.9 log cfu g^{-1}), meanwhile the highest initial population was observed in NS-treated apple wedges. Initial pathogen population on NAC-treated apple wedges was below detection limit. As in the case of *Salmonella*, although *L. monocytogenes* population increased on control and NS-treated apple wedges stored at 10 °C, its population maintained at initial level on AA and NAC-treated apple wedges.

Initial CPA-7 population was similar in control and NS-treated apple wedges (between 6.0 and 6.2 log cfu g^{-1}), however, it was below detection limit (250 cfu g^{-1}) on AA and NAC-treated wedges. Throughout storage at 10 °C, antagonist population increased in both, control and NS-treated apple wedges, but it maintained or slightly increased on NAC and AA-treated apple wedges.

Table 2 Population of *Salmonella (Sal)*, *L. monocytogenes* (*Lm*) and CPA-7 (log cfu g^{-1}) on apple flesh after inoculation (0 d) and after 2 d of storage at 10 °C in presence of different antioxidants (CK: without antioxidant; AA: ascorbic acid 2 %; NS: NatureSeal® AS1 6 %; NAC: N-acetyl-L-cysteine 1 %).

	Initial				**2 d 10 ºC**			
	CK	**AA**	**NS**	**NAC**	**CK**	**AA**	**NS**	**NAC**
Sal.	4.0±0.0 x	3.7±0.1 y	4.1±0.0 x	<dl[a] z	4.4±0.1 x	3.6±0.1 y	4.6±0.1 x	<dl[a] z
Lm	1.9±0.2 y	1.8±0.2 yz	3.5±.1.0 x	<dl[a] z	2.9±0.2 y	1.7±0.2 z	4.2±0.1 x	<dl[a] z
CPA-7	6.0±0.1 y	<dl[b] z	6.2±0.1 x	<dl[b]z	7.4±0.0 x	3.1±0.3 y	7.5±0.0 x	<dl[b] z

Values are expressed as mean of three values plus and minus standard error of the mean. Different lowercase letters (x, y, z) in the same line indicate significant differences ($P<0.05$) among treatments at each sampling time.

[a] Below detection limit (50 cfu g^{-1})

[b] Below detection limit (250 cfu g^{-1})

The antioxidant treatment chosen was NS 6 % as it affected microbial population the least. Therefore, effectiveness of antagonistic strain against *Salmonella* and *L. monocytogenes* on NS-treated MP apple wedges stored at 10 °C was evaluated (Fig. 1).

Initial *Salmonella* populations on apple wedges were between 4.4 and 4.5 log cfu g^{-1} (Fig. 1A), and they were maintained during the first two days of storage at 10 °C. Then, *Salmonella* populations started to increase on apple wedges where they were inoculated alone, reaching final populations of approximately 6.0 log cfu g^{-1}. In contrast, *Salmonella* populations on apple plugs co-inoculated with CPA-7 reduced significantly, being below detection limit (50 cfu g^{-1}) after 7 days of storage. No differences due to antioxidant treatment were observed.

L. monocytogenes populations increased slightly during first 2 days of storage in all treatments. Then, *L. monocytogenes* populations on apple wedges inoculated alone continued increasing and it decreased when co-inoculated with CPA-7 (Fig. 1B). After 5 days of storage, higher pathogen reductions were observed in absence of NS, however no differences were observed between both treatments after 7 days of storage.

P. graminis CPA-7 growth on NS treated apple wedges was also monitored. Initial populations were around 6.5 log cfu g^{-1} and they increased slightly throughout storage with final populations of 8.4 log cfu g^{-1}. No effect of antioxidant treatment was observed (data not shown).

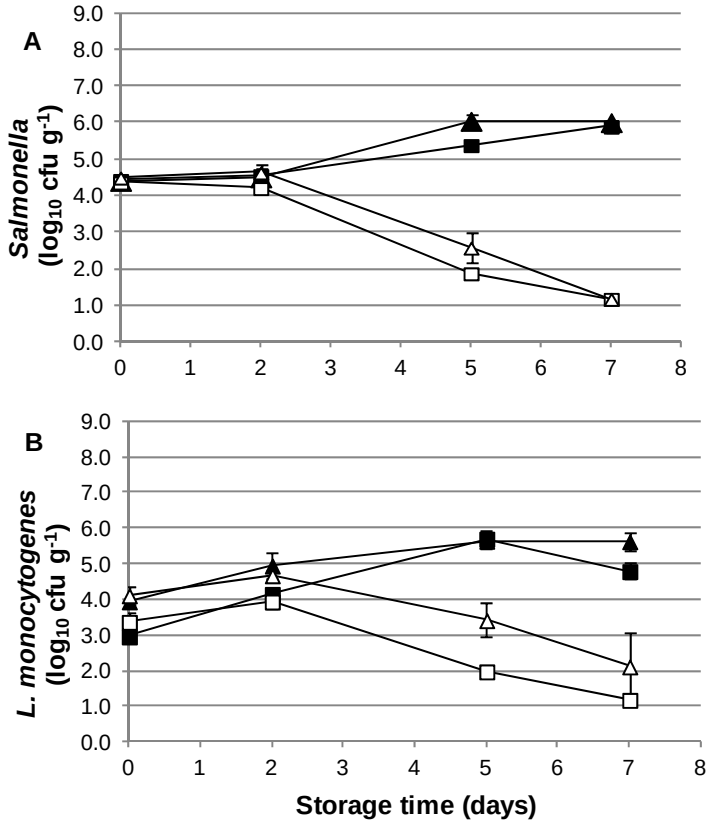

Fig. 1 *Salmonella* (A) and *L. monocytogenes* (B) population on 'Golden Delicious' apple wedges treated (triangles) or untreated (squares) with NatureSeal® AS1 6 %, inoculated alone (full symbols) or in with CPA-7 (open symbols) and stored at 10 °C (n=3, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

3.2. Influence of packaging atmosphere on antagonistic effect

Salmonella initial populations were 4.2 log cfu g^{-1} and they increased up to 5.6 log cfu g^{-1} after 7 days of storage when inoculated alone at both atmosphere conditions (Table 3). When it was co-inoculated with CPA-7, *Salmonella* population after 7 d at 10 °C was reduced more than 3-log units regardless atmosphere conditions when compared to the pathogen inoculated alone.

Initial *L. monocytogenes* population was 4.1 log cfu g^{-1} and it increased more than 2.5-log units when inoculated alone at both atmosphere conditions. When it was co-inoculated with the antagonistic strain, the highest population reduction was observed in air (3.8-log units), meanwhile in MAP the reduction observed was 2.5-log units.

CPA-7 initial population was 6.4 log cfu g^{-1} and it increased more than 1.5-log units after 7 days of storage at 10 °C regardless atmosphere conditions.

Oxygen and carbon dioxide concentration in Air apple trays maintained over 20.0 % and below 1.0 %, respectively, until the end of storage. In contrast, O_2 concentration decreased to 0 % and CO_2 increased to 16 % in MAP trays.

Table 3 *Salmonella*, *L. monocytogenes* and CPA-7 population (log cfu g^{-1}) on NS-treated apple wedges (inoculated alone or in combination) after inoculation (0 d o initial) and after 7 d at 10 °C stored in air and MAP.

	0 d	**7 d 10 °C**	
	AIR /MAP	**AIR**	**MAP**
Salmonella	4.2±0.0	5.6±0.2	5.6±0.0
Salmonella + CPA-7	4.2±0.0	2.2±0.1	2.3±0.1
L. monocytogenes	4.1±0.0	6.7±0.1	6.8±0.1
L. monocytogenes + CPA-7	4.1±0.0	2.9±0.4	4.3±0.1
CPA-7	6.4±0.0	8.2±0.1	7.9±0.2

Values are expressed as mean plus and minus standard error of the mean.

3.3. Semi-commercial trials

Populations of *Salmonella* and *L. monocytogenes* on NS-treated 'Golden Delicious' apple wedges in MAP with or without CPA-7 (at a low and high inoculum) along 14 days of storage at 5 and 10 °C are shown in Fig. 2.

Salmonella initial populations on apple wedges were around 4.0 log cfu g^{-1} (Fig. 2A and 2B). *Salmonella* populations increased slightly on apple wedges stored at 10 °C (between 1.2 and 1.9-log units), but it decreased, between 0.4 and 0.8-log units, after 14 days at 5 °C. When *Salmonella* was co-inoculated with the antagonistic strain CPA-7 at low inoculum (10^5 cfu mL^{-1}, Fig. 2A) no differences with the control (*Salmonella* population on apple wedges inoculated alone) were observed at any temperature. In contrast, *Salmonella* did not increased at 10 °C when it was co-inoculated with the antagonistic strain CPA-7 at high inoculum level (10^7 cfu mL^{-1}), although no differences on *Salmonella* population were observed at 5 °C (Fig. 2B).

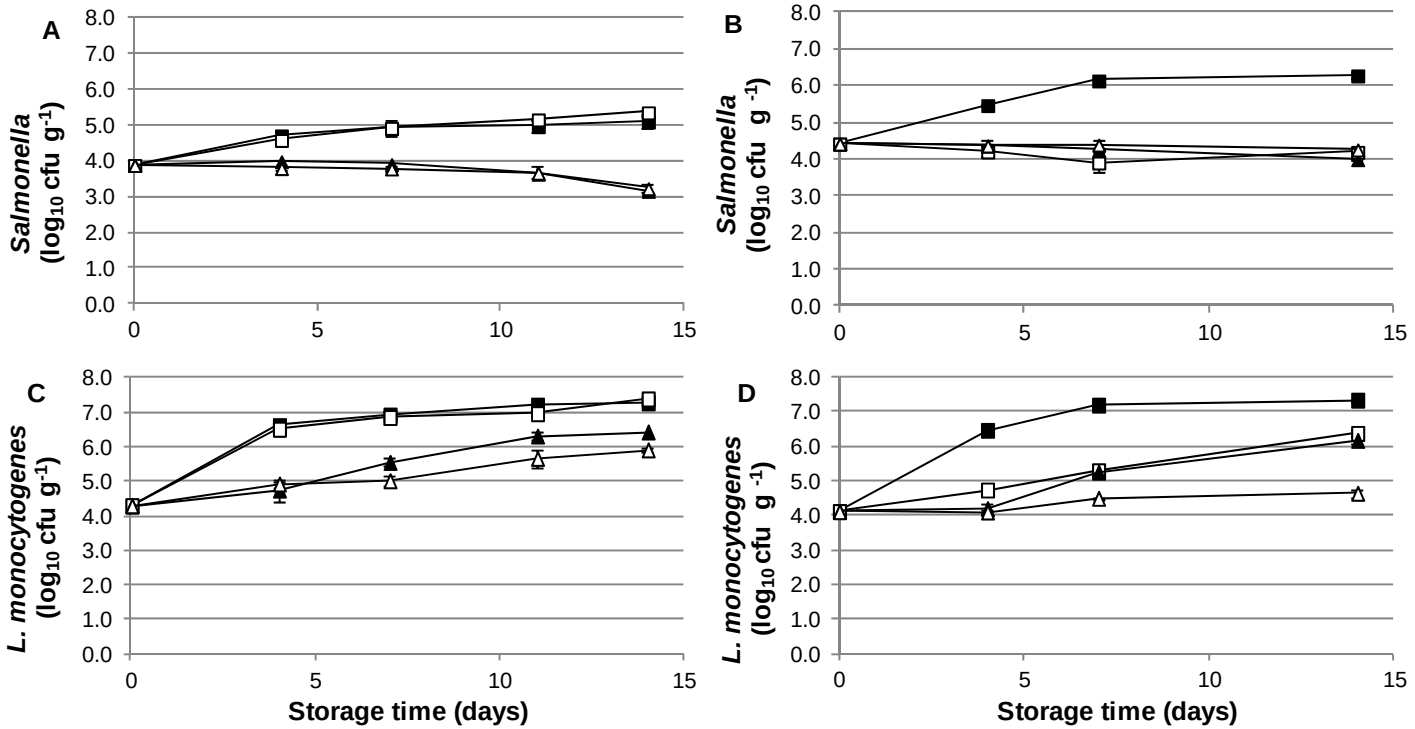

Fig. 2 *Salmonella* (A, B) and *L. monocytogenes* (C, D) population on NS-treated 'Golden Delicious' apple wedges inoculated alone (full symbols) or together with *P. graminis* CPA-7 (open symbols) at low inoculum (10^5 cfu mL^{-1}, A, C) or high inoculum (10^7 cfu mL^{-1}) and stored in MAP at 5 °C (triangles) and 10 °C (squares) (n=3, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

Regarding to *L. monocytogenes* alone, initial population was around 4.0 log cfu g^{-1} (Fig. 2C and 2D) and increased rapidly on the first 7 days at 10 °C reaching a population of approximately 7.0 log cfu g^{-1} and then continued increasing slowly. At 5 °C, *L. monocytogenes* increased more slowly, reaching a population over 6.0 log cfu g^{-1} after 7 days of storage. Co-inoculation with CPA-7 at low inoculum did not have any effect on *L. monocytogenes* population on apple wedges stored at 10 °C and only a 0.5-log reduction was observed at 5 °C. When it was inoculated with CPA-7 at high inoculum, *L. monocytogenes* was between 1 and 2-log units lower than the control throughout all storage at 10 °C. At 5 °C, no differences in pathogen population were detected until 7 days of storage. Then, *L. monocytogenes* population on apple wedges co-inoculated with CPA-7 was maintained meanwhile it increased when inoculated alone.

Population dynamics of *P. graminis* CPA-7 on apple wedges stored at 5 and 10 °C was also determined (Fig. 3). Initial populations on apple wedges inoculated with low inoculum were 4.1±0.2 log cfu g^{-1} and increased rapidly during the first 7 days of storage at 10 °C (more than 2.0-log units). Afterwards, CPA-7 populations decreased to 5.1 log cfu g^{-1} at the end of the experiment. At 5 °C, CPA-7 population increase was more gradual, it reached its maximum after 11 days (5.5 log cfu g^{-1}) and then it started to decrease. Initial CPA-7 populations when it was inoculated at high inoculum were 6.3±0.1 log cfu g^{-1}. In this case, maximum population increase on apple wedges stored at 10 °C was after 4 days of storage (8.0±0.1 log cfu g^{-1}) and then it decreased slightly. At 5 °C, it increased slightly throughout all storage period.

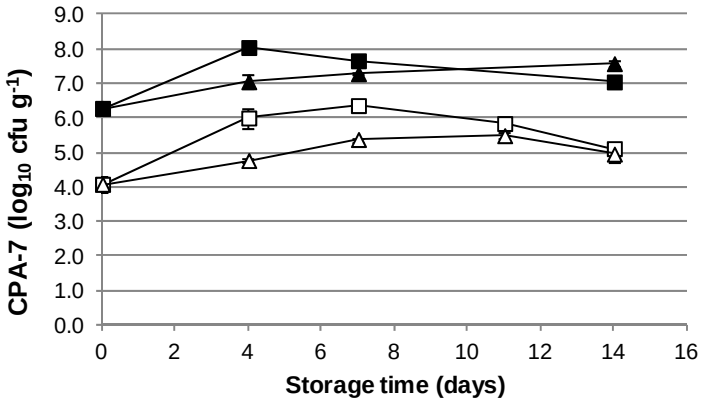

Fig. 3 *P. graminis* CPA-7 population on NS-treated 'Golden Delicious' apple wedges stored in MAP at 5 °C (triangles) and 10 °C (squares) when inoculated at low inoculum (10^5 cfu mL^{-1}, open symbols) or at high inoculum (10^7 cfu mL^{-1}, full symbols) (n=3, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

Mesophilic population on apple wedges was only determined in uninoculated samples (treatment (a), control, data not shown). Initial populations were 2.4±0.4 log cfu g^{-1}. At 10 °C, it augmented exponentially (approximately 4-log units) until day 7, and then continued increasing slightly, reaching final populations of 6.8±0.3 log cfu g^{-1}. When apple wedges were stored at 5 °C, mesophilic populations increased slightly reaching 5.4±0.2 log cfu g^{-1}. No symptoms of microbial decay were observed.

3.4. Survival in simulated gastric conditions

Salmonella populations recovered after exposure to gastric stress the day of inoculation (time 0 h) were 3.6±0.1 (Fig. 4A) and 3.4±0.2 log cfu g^{-1} (Fig. 4B) when inoculated alone or in combination with CPA-7, respectively, meanwhile populations before exposure were over 4.0 log cfu g^{-1}. Storage period of apple wedges caused a sharp reduction of tolerance to gastric acid conditions, and viability was reduced below detection limit (50 cfu g^{-1}) from day 4 to the end of storage in both treatments.

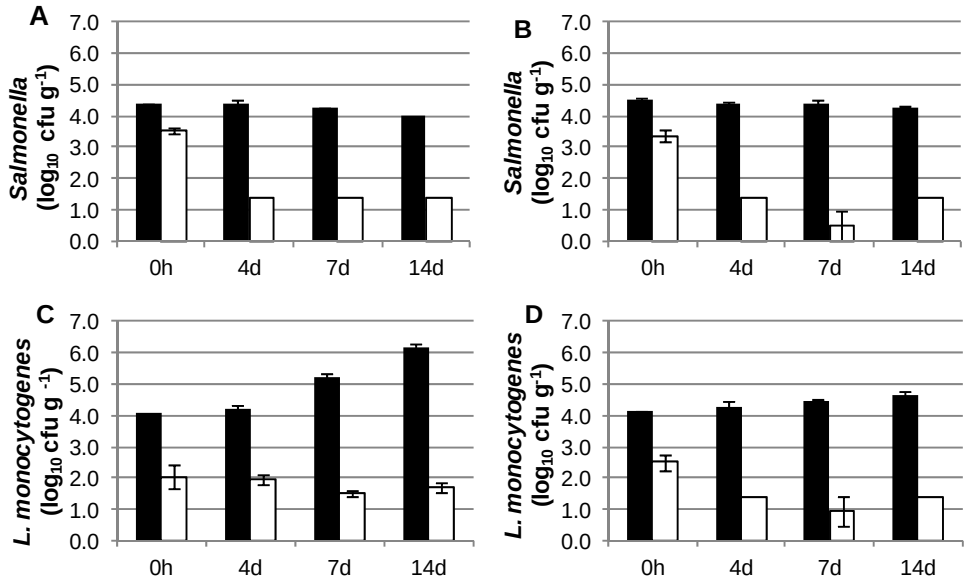

Fig. 4 *Salmonella* (A, B) and *L. monocytogenes* (C, D) population recovered from NS-treated 'Golden Delicious' apple wedges before (■) and after (□) exposure to simulated gastric fluid. Apples were inoculated with *Salmonella* and *L. monocytogenes* alone (A, C) or in combination with CPA-7 (B, D) and stored in MAP at 5 °C up to 14 days (n=3, bars are standard deviation of the mean).

Lower initial survival to gastric stress was observed for *L. monocytogenes* (2.1±0.4 and 2.5±0.3 log cfu g^{-1} when inoculated alone or in combination, Fig. 4C and 4D, respectively) compared to population before treatment (over 4.0 log cfu g^{-1}). Although *L. monocytogenes* inoculated alone on apple wedges tended to increase throughout storage at 5 °C, its viability to gastric stress was maintained below 2.0 log cfu g^{-1}. Lower survival rates were observed when this pathogen was co-inoculated with the antagonistic strain, *P. graminis* CPA-7.
Regarding the antagonistic strain, no viable cells were recovered after exposure to simulated gastric stress at any sampling time (data not shown).

3.5. Quality analysis of apple wedges

3.5.1. Visual quality

No significant differences among treatments on visual quality were observed at any temperature (data not shown). Although visual quality changed significantly (P<0.05) over the 14 days storage period at both temperatures, all samples were around 75 % edible (5 in the hedonic scale) until 7 days storage.

3.5.2. Headspace gas composition

Significant differences among treatments were observed on O_2 and CO_2 concentrations at both temperatures (Table 4). Initial O_2 concentration was 21.0 %. It significantly reduced throughout storage at both temperatures. However, it reduced more rapidly in CPA-7 and Sal+Lm+CPA-7 treated apple trays than in control and Sal+Lm treated apple trays. Absence of O_2 was detected after 14 days of storage at 5 °C and after 7 days of storage at 10 °C. CO_2 concentration significantly increased along storage at both temperatures. Similar to observations with O_2, CO_2 increased more rapidly in CPA-7 and Sal+Lm+CPA-7 apple trays. Maximum CO_2 concentration on apple trays stored at 5 °C was 19.7±0.1, meanwhile it was 33.2±0.8 at 10 °C.

3.5.3. pH

pH values of apple flesh varied between 3.94±0.03 and 4.48±0.08 over the 14 days storage period at both temperatures (data not shown). However, no significant effects of treatment or storage time on pH sample variability were observed.

Table 4 Oxygen and carbon dioxide concentration (%) in apple trays stored at 5 and 10 °C.

	Temperature	Treatment	Days			
			0	4	7	14
O_2	5 °C	Control	21.0±0.0 Aw	9.1±0.3 ABx	5.5±0.6 Ay	1.7±1.1 Az
		CPA-7	21.0±0.0 Aw	7.3±0.4 Cx	2.4±1.3 By	0.0±0.0 Az
		Sal+Lm	21.0±0.0 Aw	9.4±0.3 Ax	4.0±0.7 ABy	0.0±0.0 Az
		Sal+Lm+CPA-7	21.0±0.0 Aw	8.1±0.3 BCx	1.3±0.3 By	0.0±0.0 Az
	10°C	Control	21.0±0.0 Aw	3.4±0.3 Ax	0.0±0.0 Ay	0.0±0.0 Ay
		CPA-7	21.0±0.0 Aw	0.6±0.2 Bx	0.0±0.0 Ay	0.0±0.0 Ay
		Sal+Lm	21.0±0.0 Aw	3.7±0.3 Ax	0.0±0.0 Ay	0.0±0.0 Ay
		Sal+Lm+CPA-7	21.0±0.0 Aw	0.7±0.1 Bx	0.0±0.0 Ay	0.0±0.0 Ay
CO_2	5 °C	Control	0.0±0.0 Aw	6.6±0.2 Bx	9.2±0.1 By	12.7±0.7 Cz
		CPA-7	0.0±0.0 Aw	7.8±0.2 Ax	11.0±0.6 Ay	18.6±0.6 Az
		Sal+Lm	0.0±0.0 Aw	6.5±0.3 Bx	10.0±0.4 ABy	15.9±0.3 Bz
		Sal+Lm+CPA-7	0.0±0.0 Aw	7.5±0.2 Ax	11.1±0.2 Ay	19.7±0.1 Az
	10 °C	Control	0.0±0.0 Aw	11.0±0.1 Bx	17.3±0.0 By	29.0±0.6 Bz
		CPA-7	0.0±0.0 Aw	13.4±0.4 Ax	20.2±1.0 Ay	33.2±0.8 Az
		Sal+Lm	0.0±0.0 Aw	10.9±0.2 Bx	17.5±0.3 By	28.9±0.6 Bz
		Sal+Lm+CPA-7	0.0±0.0 Aw	12.8±0.2 Ax	20.2±0.1 Ay	32.0±0.3 Az

Each value is the mean ± standard error of the mean of three replicates with three evaluations each.

Different lowercase letters (w, x, y, z) in the same line indicate significant differences ($P<0.05$) among storage days for each treatment whereas different capital letters (A, B, C) in the same column indicate significant differences ($P<0.05$) between treatments at the same time and temperature.

3.5.4. Colour

In general, at each storage time, no significant differences in L*, a* and b* values were observed between treatments at both temperatures (Table 5). Along storage, Hunter L* values were maintained or slightly increased. In contrast, no significant differences were observed in a* and b* values, and in the case of some differences they did not follow any trend.

Table 5 Changes in the color parameters (L*, a*, b*) of uninoculated (control) or inoculated with CPA-7 'Golden Delicious' apple wedges during storage at 5 and 10 °C.

	Temperature	Treatment	Days			
			0	4	7	14
L*	5 °C	Control	77.5±0.4 Ay	77.1±0.6 Ay	79.8±0.4 Ax	77.8±0.5 Ay
		CPA-7	77.5±0.4 Axy	76.0±0.8 Ay	79.2±0.5 Ax	77.9±0.6 Ax
	10 °C	Control	77.5±0.4 Ax	78.1±0.7 Ax	78.3±0.7 Bx	78.3±0.5 Ax
		CPA-7	77.5±0.4 Ay	78.0±0.9 Ay	80.6±0.4 Ax	78.3±0.7 Ay
a*	5 °C	Control	-4.1±0.1 Ax	-4.3±0.1 Axy	-4.4±0.1 Axy	-4.5±0.1 Ay
		CPA-7	-4.1±0.1 Ax	-4.1±0.1 Ax	-4.4±0.1 Ax	-4.2±0.1 Ax
	10 °C	Control	-4.1±0.1 Ax	-4.4±0.1 Ax	-4.3±0.1 Ax	-4.3±0.1 Ax
		CPA-7	-4.1±0.1 Ax	-4.5±0.1 Ax	-4.4±0.1 Ax	-4.5±0.1 Ax
b*	5 °C	Control	23.3±0.4 Ax	24.2±0.4 Ax	23.0±0.5 Ax	23.6±0.4 Ax
		CPA-7	23.3±0.4 Ax	23.2±0.1 Ax	23.5±0.6 Ax	22.2±0.5 Bx
	10 °C	Control	23.3±0.4 Ax	22.5±0.3 Ax	22.9±0.5 Ax	22.8±0.5 Ax
		CPA-7	23.3±0.4 Axy	22.9±0.4 Ayz	21.5±0.4 Bz	23.5±0.6 Ax

Each value is the mean ± standard error of the mean of three replicates with ten evaluations each. Different lowercase letters (x, y, z) in the same line indicate significant differences ($P<0.05$) among storage days for each treatment whereas different capital letters (A, B) in the same column indicate significant differences ($P<0.05$) between treatments at the same time and temperature.

3.5.5. Soluble solids content, titratable acidity and texture

Although significant differences were observed in SSC among treatments and throughout storage, they did not follow any trend (data not shown). Soluble solids content ranged from 10.6 ° Brix to 12.2 ° Brix.

In the case of TTA, no significant differences between treatments were observed at any temperature (data not shown). Along storage, significant differences were only detected in CPA-7 treated apple wedges, however no trend was observed. TTA values ranged from 2.2 to 2.7 g malic acid L^{-1}.

No significant differences in texture between untreated and CPA-7 treatments were noted at any of the storage temperatures (data not shown). Throughout storage, significant differences in firmness values were observed in all treatments and in all of them the tendency was to increase. Initial firmness value was 13.2±0.9 N and maximum value observed was 19.7±0.9 N.

4. DISCUSSION

To our knowledge this is the first report on the efficacy of an antagonistic strain against two foodborne pathogens on MP apples under conditions simulating commercial application. Our results have shown the suitability of the antagonistic strain, *P. graminis* CPA-7, to control the growth of a cocktail of *Salmonella* and *L. monocytogenes* strains on 'Golden Delicious' apple wedges treated with NatureSeal® AS1 (NS), packaged in modified atmosphere and stored at refrigeration and abusive temperatures (5 and 10 °C). In addition, its application did not affect apple quality.

Ascorbic acid (2 %) and N-acetyl-L-cysteine (NAC, 1 %) antioxidant treatments resulted in reduction of microbial populations recovered from apple tissue. This is of importance because the viability of the antagonistic strain was greatly affected by the antioxidant; therefore they could not be used in the same dip treatment as CPA-7. On the other hand, treatment with these antioxidants reduced pathogen populations, acting as antimicrobials. In contrast, treatment of apple wedges with NatureSeal® AS1 at 6 % did not affect microbial population, so it was selected for semi-commercial trials. NS treatment of apple wedges did also not affect behaviour of *Salmonella*, *L. monocytogenes* and CPA-7 throughout 7 d of storage at 10 °C. Although, ascorbic acid treatment had not previously affected *E. coli* O157:H7, *Salmonella* and *L. innocua* survival and/or growth on MP apples and peaches (Alegre *et al.*, 2010a, 2010b), NAC has previously been reported to inhibit FBP on fresh-cut 'Golden Delicious', 'Granny Smith' and 'Fuji' apples (Abadias *et al.*, 2011; Bhagwat *et al.*, 2004; Raybaudi-Massilia *et al.*, 2009). However, the effect of NAC on the viability of CPA-7 has not been determined previously. Similar to the results obtained, NS treatment did neither affect pathogenic population on apple plugs in a previous study (Alegre *et al.*, 2010a).

Salmonella, *L. monocytogenes* and *P. graminis* CPA-7 growth on MP apple wedges stored in air and MAP conditions was the same. Although the same reduction values were observed for *Salmonella*, higher reduction values of *L. monocytogenes* were detected in air conditions. Several studies have previously found that MAP technologies have very little effect on the survival and growth of FBP on fresh-cut produce (Alegre *et al.*, 2010a; 2010b; Beuchat and Brackett, 1990; Jacxsens *et al.*, 1999). It is known that atmospheres with low O_2 levels inhibit the growth of most aerobic microorganisms, whose growth usually warns consumers about spoilage, while the growth of pathogens, especially the anaerobic psychrotrophic, may be allowed or even stimulated (Farber, 1991). In this study, growth of the aerobic antagonistic strain was affected by MAP when O_2 concentration reached 0 % (7 d at 10 °C with the film used), and antagonistic effect against *L. monocytogenes* was slightly reduced, probably due to higher pathogen growth rate at MAP storage conditions in comparison to CPA-7 growth rate.

Two inoculums levels of the antagonistic strain were tested in semi-commercial assays. *P. graminis* CPA-7 was not effective in reducing *Salmonella* and *L. monocytogenes* populations on apple wedges treated with NS at 6 % and stored under MAP at 5 and 10 °C up to 14 days when they were co-inoculated at the same level (10^5 cfu mL^{-1}). However, increasing CPA-7 inoculum to 10^7 cfu mL^{-1} resulted in reduction of both pathogens. In a previous study, the antagonistic strain CPA-7 needed to be at least at the same inoculum level than FBP to reduce their population (Alegre *et al.*, previous work) on apple plugs stored at 20 °C. The difference observed between both assays may be due to the different storage temperature, the presence of an antioxidant substance and storage atmosphere conditions.

The antagonistic strain grew on apple wedges stored with MAP at both temperatures. It increased faster on apple wedges stored at 10 °C than at 5 °C. However, it started to decrease first on apple wedges stored at 10 °C than at 5 °C. This could be due to the faster depletion of O_2 in the package at 10 °C, since CPA-7 is strict aerobic and/or to the faster microbial growth rate at that temperature.

It is thought that microbial adaptation to sublethal acidic environments could increase resistance to extreme pH conditions, and therefore, enhance survival of pathogens during transit through stomach, and increase the likelihood of intestinal colonization and, thus, their virulence potential. However, survival and/or growth of *Salmonella*, *L. monocytogenes* and *P. graminis* CPA-7 on apple wedges (pH 3.94-4.48) throughout storage at 5 °C did not result in an increased microbial survival to simulated gastric stress. *Salmonella* and *L. monocytogenes* survival reduced dramatically on the first 4 days of storage and CPA-7 did not survive gastric acid simulation from the inoculation day. Similar *Salmonella* and *L. monocytogenes* behaviour on apple wedges has been described previously (Alegre *et al.*, 2011). Several studies have shown that the pH of habituation or growth environment is the most important factor affecting tolerance during subsequent exposure to extreme acidic conditions (Koutsoumanis *et al.*, 2003; Koutsoumanis and Sofos, 2004). *L. monocytogenes* has been reported to exhibit increased acid resistance after habituation at pH 5.0 or 5.5 (Gahan *et al.*, 1996; Koutsoumanis and Sofos, 2004; Lou and Yousef, 1997; Phan-Thanh *et al.*, 2000). Regarding *Salmonella*, Koutsoumanis and Sofos (2004) and Lee *et al.* (1995) reported an increased acid resistance of stationary phase *Salmonella* Typhimurium after exposure to pH 4.3. In addition, it has been shown that adaptation for a short period (a few hours) at a moderate sublethal acidic pH helps the bacteria to better resist posterior lethal acidic pH. For example, Phan-Thanh and Montagne (1998) showed that treatment for an extended period (overnight) at a moderate acidic pH rendered *Listeria* more sensitive to subsequent acid kill. Growth temperature is also an important factor affecting the bacterial acid resistance. Álvarez-Ordóñez *et al.* (2010) and Samelis *et al.* (2003) demonstrated that *Salmonella* Typhimurium cells grown at 10 °C showed a reduced acid resistance in comparison to cells obtained at

30 °C. In our case, the reduction of survival to gastric stress as a result of the lack of habituation to low pH could be due to the low pH of apple (3.9-4.5), the long storage period and low storage temperature.

Effect of *P. graminis* CPA-7 application on quality of MP apple wedges was analysed. No differences in visual quality, pH, colour, soluble solid content, titratable acidity and firmness were observed between apple treatments. However headspace gas composition within the apple trays changed quicker when apple wedges have been treated with *P. graminis* CPA-7. Throughout storage and regardless of the treatment, visual quality of apple wedges was reduced significantly and it was only over 5 in the hedonic scale, which represented it was 75 % edible until 7 days of storage. Regarding colour, Hunter L* values, which indicate the darkness of apple surface, were maintained or slightly increased showing a whitening effect. Similar results on NS treated MP apple have been reported previously (Alegre *et al.*, 2011; Rößle *et al.*, 2009; Rupasinghe *et al.*, 2005; Toivonen, 2008). Hunter a* values (indicating redness) and b* values (indicating yellowness) did not significantly change. Firmness of apple wedges increased along storage at both temperatures. Other studies have also reported an increase in firmness in apple pieces treated with NS due to the cross-linking of both cell wall and middle-lamella pectin by calcium ions present on NS formulation (Rico *et al.*, 2007).

Therefore, results from this study have shown the compatibility of the antagonistic strain *P. graminis* CPA-7 with MP apple commercial processing procedures such as use of NatureSeal® AS1 and MAP without losing effectiveness. However, only one MAP condition was tested in this assay; hence different MAP conditions should be tested to ensure the antagonist efficacy. In addition, survival and/or growth of CPA-7 on apple tissue throughout storage at 5 °C did not render it more resistance to acid stress. A good antagonist should not be harmful to human health. Although *P. graminis* is not referred as human pathogen its inability to survive to gastric stress is a good characteristic for a potential antagonist. Another characteristic of an ideal antagonist for biological control is to show a remarkable antimicrobial activity without negatively affecting the organoleptic characteristics of the product. In this study, no effect on MP apple quality was observed. Sensorial quality was not evaluated as the antagonist is not yet registered for food applications. The effect of CPA-7 at semi-commercial conditions consisted in preventing the growth of *Salmonella* and *L. monocytogenes*, but it did not eliminate existing cells, it did not have a bactericidal effect. Moreover, its effect was not instantaneous and, consequently, it should be used in combination with current disinfection techniques and other microbial growth-limiting factors such as low temperatures and MAP, as part of hurdle technology. In this sense, application of CPA-7 could avoid growth of remaining FBP in case of temperature abuse and, therefore increase safety of fresh-cut fruit.

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CAPÍTULO VII

Microbiological and physicochemical quality of fresh-cut apple enriched with the probiotic strain *Lactobacillus rhamnosus* GG

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ABSTRACT

The effectiveness as protective culture of the probiotic *Lactobacillus rhamonosus* GG (*L. rham*. GG) against *Salmonella* and *Listeria monocytogenes* on minimally-processed apples throughout storage as well as its effect on apple quality and natural microflora was evaluated. Survival to subsequent exposure to gastric stress was also reported. Apples were cut into wedges and dipped in a solution containing *Salmonella* and *L. monocytogenes* (10^5 cfu mL^{-1}) and/or *L. rham*. GG (10^8 cfu mL^{-1}). Apple wedges were packed and stored at 5 and 10 °C. Periodically, microbial population, bacterial survival to gastric stress and quality of apple wedges were evaluated. Although *Salmonella* was not affected by co-inoculation with *L. rham*. GG, *L. monocytogenes* population was 1-log units lower in the presence of *L. rham*. GG. *L. rham*. GG population maintained over recommended levels for probiotic action (10^6 cfu g^{-1}) along storage, however, viable cells after gastric stress were only above this level during the first 14 days. Pathogen survival after gastric stress was < 1 % after 7 days at 5 °C. Moreover, apple wedges quality was not affected by *L. rham*. GG addition. Thus, *L. rham*. GG could be a suitable probiotic for minimally-processed apples capable to reduce *L. monocytogenes* growth; nevertheless shelf life should not be higher to 14 days to guarantee the probiotic effect.

Keywords: *Salmonella*; *Listeria monocytogenes*; gastric acid survival; protective cultures; biocontrol

1. INTRODUCTION

Fresh fruit and vegetables are essential components of the human diet and there is considerable evidence of the health and nutritional benefits associated with the consumption of fresh fruits and vegetables (Abadias *et al.*, 2008). Consumer trends with respect to food choice are changing due to the increasing awareness of the link between diet and health, consequently consumption of fresh produce, as well as functional foods, has increased significantly over the past years. In Europe, the largest segment of functional foods market comprises foods fortified with probiotics, prebiotics or synbiotics (Sheehan *et al.*, 2007). Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Current industrial probiotic foods are basically dairy products (yogurt and other fermented dairy products) which may represent inconveniences due to their lactose and cholesterol content (Heenan *et al.*, 2004). Therefore there is an increasing demand for non-dairy based probiotic products (Rivera-Espinoza and Gallardo-Navarro, 2010).

The probiotic microorganisms consist mostly of strains of the genera *Lactobacillus* and *Bifidobacterium* which are types of lactic acid bacteria (LAB) (Prado *et al.*, 2008). *Lactobacillus rhamnosus* GG (*L. rham.* GG) is one of the extensively studied strains with well-documented probiotic properties: it is known to colonize the intestine and to be active against organisms causing traveler's diarrhea and rotavirus infection (Ouwehand and Salminen, 1998; Salminen *et al.*, 1998; Saxelin, 2008).

The efficacy of added probiotic bacteria depends on inoculum level and their viability must be maintained throughout storage of the product's shelf life (Kailasapathy and Chin, 2000). There is no clear agreement on the minimum concentration of probiotic intake to achieve beneficial effect on the host. While some researchers suggest that concentrations higher than 10^6 cfu mL^{-1} (Dave and Shah, 1997; Kurman and Rasic, 1991) are required, others suggest a concentration of at least 10^7 and 10^8 cfu mL^{-1} (Kailasapathy and Rybka, 1997; Lourens-Hattingh and Viljoen, 2001). In a recent study, *L. rham*. GG concentration on apple wedges was maintained at 10^8 cfu g^{-1} over a 10 days storage period at 2-4 °C with acceptable quality of apple (Rößle *et al.*, 2010). Probiotics should not only survive in the food product but cells should also be able to reach the small intestine alive. It has been demonstrated that the food matrix affects survival to the gastric environment (Saarela *et al.*, 2006; Stanton *et al.*, 1998). Although it was hypothesized that cells which were stored in an acid fruit drink could have enhanced resistance to the subsequent acid challenge of a simulated gastrointestinal stress, Champagne and Gardner (2008) observed higher viability losses for probiotic cultures after having been stored for 35 days at 4 °C in fruit drinks than for fresh cultures.

Although consumption of fresh produce is beneficial for optimal health, these foods may be associated with risks of foodborne illness. Thus, the rise in consumption of minimally-processed (MP) produce has resulted in increased frequency of outbreaks of illness associated with raw fruits and vegetables as protective barriers (physical and chemical) are removed during processing of MP fruits and vegetables and that increases vulnerability to microbial contamination and colonization (Leverentz *et al.*, 2001). Fresh fruit and fruit juices have been incriminated in outbreaks of foodborne illnesses caused by human pathogens like *Salmonella* (CDC, 2007; Harris *et al.*, 2003; Powell and Luedtke, 2000). Although low pH of apples the growth of *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* on MP apple tissue stored in air has been demonstrated (Abadias *et al.*, 2009; Alegre *et al.*, 2010; Dingman, 2000; Fisher and Golden, 1998; Janisiewicz *et al.*, 1999b).

Lactic acid bacteria (LAB) not only improve health when consumed, but they can also play a protective role against pathogens in the product itself during storage by competing with pathogens for nutrients (vitamins, minerals, trace elements and peptides), producing organic acids and bacteriocins (antimicrobial peptides) (Rydlo *et al.*, 2006). The presence of cultures with inhibitory properties could improve the shelf life and safety of vegetable products while reducing the need to use increasing levels of chemical additives (Schuenzel and Harrison, 2002). Growth of foodborne pathogens on fruit flesh has been prevented using epiphytic microorganisms previously (Abadias *et al.*, 2009; Janisiewicz *et al.*, 1999a; Leverentz *et al.*, 2006; Trias *et al.*, 2008).

The acidity of the stomach is considered a major defense barrier against foodborne infection (Smith, 2003). Recent studies have indicated the ability of foodborne bacteria, as *Salmonella* and *L. monocytogenes*, to increase resistance to extreme pH conditions after adaptation to sublethal acidic environments (acid habituation or acid tolerance) (Bearson *et al.*, 1997; Foster, 1995; Lou and Yousef, 1997; O'Driscoll *et al.*, 1996). This ability may have significant implications for food safety, in particular in acidic food as minimally-processed apples.

The objective of this work was to apply a probiotic microorganism, *L. rhamnosus* GG, to fresh-cut apple wedges to report its effect on growth of foodbone pathogens (*L. monocytogenes* and *Salmonella*), the natural microflora, and on apple wedges quality along conservation. The ability of the cultures to survive simulated gastric stress conditions following storage was also studied.

2. MATERIALS AND METHODS

2.1. Fruit

'Golden Delicious' apples were obtained from local packinghouses in Lleida, Catalonia. Prior to the experimental studies, apples were washed with water, surface disinfected with ethanol 70 % and let to dry at room temperature. Apples were cut in 10 skin-on wedges using an apple slicer/corer.

2.2. Bacterial strains and inoculum preparation

The bacterial strains used in this work are listed in Table 1. The commercial strain *L. rhamnosus* GG (*L. rham.* GG) was obtained from Ashtown Food Research Centre (Teagasc; Ashtown, Dublin, Ireland). The probiotic strain was grown in de Man, Rogosa and Sharpe (MRS, Biokar Diagnostics, Beauvais, France) broth for 15±2 h at 37 °C. The cells were obtained by centrifugation at 15344 × g for 15 min at 10 °C. The broth was decanted and the cells were suspended in sterile distilled water.

Salmonella strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 µg mL^{-1} of streptomycin sulphate salt (St, Sigma, Germany) thereby enabling detection on a selective medium (TSA-St) in the presence of the natural microbial flora associated with apples. The strains were grown individually in tryptone soy broth (TSB, Oxoid) supplemented with streptomycin (TSB-St) medium for 20-24 h at 37 °C. *L. monocytogenes* strains were grown individually in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) for 20-24 h at 37 °C. Bacterial cells were harvested by centrifugation at 9820 × g, 10 min at 10 °C and then resuspended in saline solution (SS; 8.5 g L^{-1} NaCL). The four *Salmonella* concentrated suspensions were mixed, as well as the five *L. monocytogenes* concentrated suspensions.

For the inoculum preparation, bacterial concentration was estimated using a spectrophotometer (SP-2000UV, Optic Ivymen System, Barcelona, Spain) set at λ=420 nm according to previously determined standard curves. A volume of each of the bacterial concentrated suspensions was added to 4 L of water with 6 % NatureSeal® AS1 (w/v; AgriCoat Ltd., Great Shefford, UK) to obtain approximately 10^5 cfu mL^{-1} in the case of *Salmonella* and *L. monocytogenes* or 10^8 cfu mL^{-1} for *L. rham*. GG. Inoculum concentration was checked by plating appropriate dilutions onto TSA-St for *Salmonella*, onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics, Beauvais, France) for *L. monocytogenes* and onto MRS agar for *L. rham*. GG.

Table 1 Bacterial strains used in this study

Number	Microorganism	Serovar	Source
ATCC 53103	*Lactobacillus rhamnosus* GG (*L. rham*. GG)		Human feces
ATCC BAA-707	*Salmonella choleraesuis* subsp. *cholerasuis* (Smith) Weldin	Agona	Alfalfa sprouts
ATCC BAA-709	*Salmonella choleraesuis* subsp. *cholerasuis* (Smith) Weldin	Michigan	Cantaloupe
ATCC BAA-710	*Salmonella choleraesuis* subsp. *cholerasuis* (Smith) Weldin	Montevideo	Clinical (patient with salmonellosis associated with tomatoes)
ATCC BAA-711	*Salmonella choleraesuis* subsp. *cholerasuis* (Smith) Weldin	Gaminara	Orange juice
CETC 4031/ATCC 15313	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	1a	Rabbit
CECT 933/ ATCC 19113	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	3a	Human
CECT 940/ ATCC 19117	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	4d	Sheep
CECT 4032	*Listeria monocytogenes* (Murray *et al.* 1926) Pirie 1940	4b	Cheese
LM230/3	*Listeria monocytogenes*	1/2a	Fresh-cut iceberg lettuce (isolated in our laboratory, (Abadias *et al.* 2008))

2.3. Apple treatment

Apple wedges were suspended (1:2 w/v) for 2 min at 150 rpm in one of the following treatments (a) control: 6 % NatureSeal® AS1 (w/v) solution, (b) Sal+Lm: 6 % NatureSeal® AS1 (w/v) solution inoculated with *Salmonella* and *L. monocytogenes*, (c) *L. rham*. GG: 6 % NatureSeal® AS1 (w/v) solution inoculated with *L. rham*. GG or (d) Sal+Lm+*L. rham* GG: 6 % NatureSeal® AS1 (w/v) solution inoculated with *Salmonella* and *L. monocytogenes* and *L. rham*. GG; and then were allowed to dry in a laminar flow biosafety cabinet.

Approximately 10 apple wedges (200±5 g) were placed in polypropylene trays (192×137×55 mm, CL1000TPP, Alphacel) and sealed with a polypropylene plastic film (Amcor Flexibles, Ledbury, Hereforshire UK) of 35 µ in thickness with an O_2 and CO_2 permeability of 3500 cm^3 m^{-2} day^{-1} atm^{-1} at 23 °C and a water steam permeability of 0.9 g m^{-2} day^{-1} at 25 °C and 75 % relative humidity. Apple trays were stored at 5 and 10 °C for 28 days.

2.4. Enumeration of bacterial concentration on apple wedges

Populations of *Salmonella*, *L. monocytogenes* and *L. rham*. GG were determined in three sample trays for each treatment (b, c and d) at each sampling time and temperature. The samples were examined on the day of inoculation and after 4, 7, 14, 21 and 28 days. For the analysis, 10 g of apple from each tray were mixed with 90 mL of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England) in a sterile bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Further ten-fold dilutions were made with saline peptone (SP; 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and plated as described previously. The agar plates were incubated at 37±1 °C for 24 h for *Salmonella* and *L. rham* GG and for 48 h for *L. monocytogenes*.

Mesophilic microorganisms were determined in control treatment (a) and yeast and moulds in control and *L. rham*. GG treatment (a and c) by enumerating colonies on plates with plate count agar (PCA, Biokar Diagnostics, Beauvais, France) or chloramphenicol glucose agar (GCA, Biokar Diagnostics, Beauvais, France) and incubated at 30±1 °C or 25±1 °C for 3 and 5 days, respectively.

Three replicate determinations (one tray each) were carried out for each treatment at each sampling date and temperature. The assay was repeated twice.

2.5. Survival in simulated gastric conditions

Salmonella, *L. monocytogenes* and *L. rham.* GG from apple samples stored at 5 ºC (Sal+Lm; *L. rham*. GG and Sal+Lm+*L. rham*. GG treatments) were evaluated for their survival to the exposure to simulated gastric stress at each sampling time. To simulate mastication, 10 g of each sample was placed into a 80 mL sterile plastic bag (IUL Instruments, Barcelona, Spain) and 10 mL of artificial saliva solution (6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$ and 1.2 g L^{-1} $NaHCO_3$) tempered at 37 °C were added. It was homogenized in a stomacher blender for 120 s at high speed (Bagmixer 100 Minimix, Interscience). The mix was transferred to an Erlenmeier flask containing 80 mL of gastric fluid (pH 2.0; 2M HCL containing 0.3 g L^{-1} of pepsin and tempered at 37 °C) and was incubated in an incubator shaker at 37 °C and 150 rpm for 2 h. Populations of *Salmonella*, *L. monocytogenes* and *L. rham*. GG after being incubated with simulated gastric acid for 2 h were compared with those counts in BPW. Cell survival was calculated as percentage of viable cfu g^{-1} after simulated gastric challenge over total counts in BPW.

2.6. Quality analysis of apple wedges

Quality analysis of apple wedges was performed for each single tray. Three trays were used for each treatment at each sample time and temperature. Headspace composition and pH were determined for all treatments, meanwhile color, texture,

soluble solid content and titratable acidity were only determined for the treatments without pathogens as analysis took place outside the Biosafety Laboratory.

2.6.1. Headspace gas composition

Carbon dioxide and oxygen content in single trays were determined using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) at each sampling time.

2.6.2. pH

Apple flesh pH was determined using a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). Three measurements were carried out for each tray (9 determinations per treatment at each sample time and temperature).

2.6.3. Color

Fresh-cut apple surface color was measured with a CR-400 Minolta chroma meter (Minolta, INC., Tokyo, Japan). Color was measured using CIE L^*, a^*, b^* coordinates. Illuminant D65 and 10° observer angle were used. The instrument was calibrated using a standard white reflector plate (Y=94.00, x=0.3158, y=0.3322). Five apple wedges were evaluated for each tray. The surface on both sides of the apple wedges was measured for each replicate (n=30). A decrease in L^* value indicates a loss of whiteness, a more positive a^* value means progressive browning and a more positive b^* value indicates more yellowing.

2.6.4. Soluble solids content

Percent of soluble solids (°Brix) was measured at 20 °C with a handheld refractometer (Atago Co. Ltd., Tokio, Japan) in juice extracted by crushing apple wedges. There was one measurement for each tray.

2.6.5. Titratable acidity

To measure titratable acidity, 10 mL of apple juice (obtained by crushing apple wedges of each tray) were diluted with 10 mL of distilled water and it was titrated with 0.1 N NaOH up to 8.1. The results were calculated as g of malic acid L^{-1}. There was one measurement for each tray.

2.6.6. Texture

Apple firmness evaluation was performed using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., England, UK) by measuring the maximum penetration force required for a 8 mm diameter probe to penetrate into an apple cube of 20×20×20 mm to a depth of 8 mm. Three apple pieces from each tray were measured.

2.7. Statistical analysis

Prior to ANOVA, all cfu mL^{-1} data were transformed to log_{10} cfu g^{-1}. The General Linear Models (GLM) procedure of the SAS Enterprise Guide was applied (v.4.1; SAS Institute, Cary, NC, USA). Significant differences between treatments were analyzed by Duncan's Multiple Range test at a significance level of $P<0.05$.

3. RESULTS

3.1. Bacterial concentration on apple wedges.

Populations of *Salmonella* and *L. monocytogenes* on 'Golden Delicious' apple wedges with or without *L. rham*. GG along 28 days of storage at 5 and 10 °C are shown in Fig. 1.

Salmonella initial population on apple wedges was 3.9±0.0 log_{10} cfu g^{-1} (Fig. 1A). Meanwhile apple wedges stored at 10 °C showed an increase in *Salmonella*'s population (up to 5.4±0.3 log_{10} cfu g^{-1} after 28 days), a decrease of approximately 0.5-log units was observed at 5 °C. Only slight differences were observed between *Salmonella* population on apple wedges with or without *L. rham*. GG throughout storage period at both temperatures.

L. monocytogenes (Fig. 1B) initial population was 3.7±0.0 log_{10} cfu g^{-1}. Although at 10 °C *L. monocytogenes* increased rapidly on the first 7 days (more than 2.4-log units) and then continued increasing slowly, reaching a population of 7.0±0.1 log_{10} cfu g^{-1} after 28 days of storage, at 5 °C, population growth was not noticeable until 14 days of storage, and final population was 6.4±0.1 log_{10} cfu g^{-1}. When it was co-inoculated with *L. rham*. GG, pathogen population was approximately 1-log unit lower when compared with Sal+Lm treatment from 7 to 14 days at 10 and 5 °C, respectively.

Initial *L. rham*. GG population on MP apple wedges was approximately 7.0 log_{10} cfu g^{-1} and it fluctuated between 6.8 and 7.4 log_{10} cfu g^{-1} throughout storage at both temperatures regardless it was inoculated alone or in combination with *Salmonella* and *L. monocytogenes* (Fig. 2).

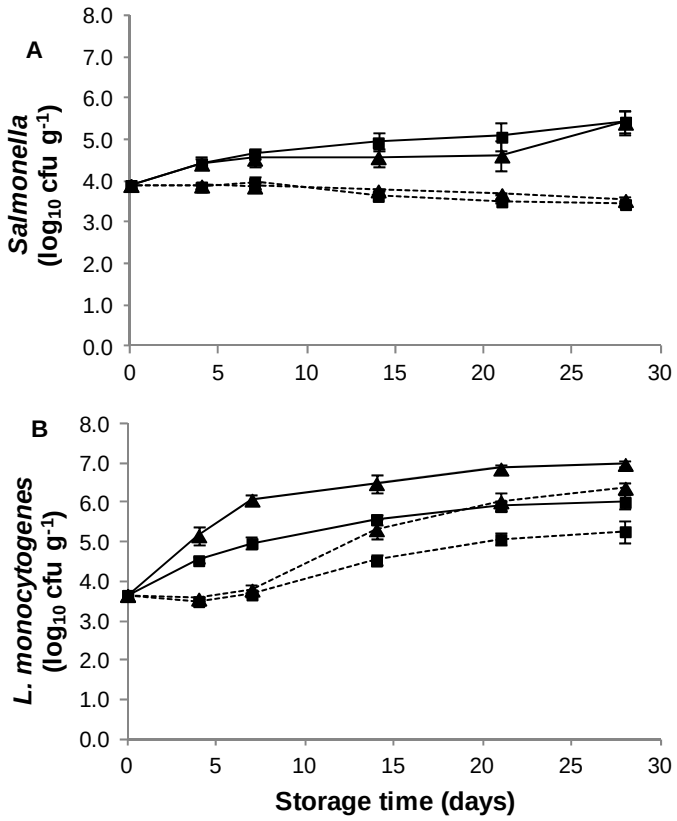

Fig. 1 *Salmonella* (A) and *L. monocytogenes* (B) population on 'Golden Delicious' apple wedges inoculated alone (▲) or together with *L. rham*. GG (■) and stored at 5 °C (dotted line) or 10 °C (continuous line) (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

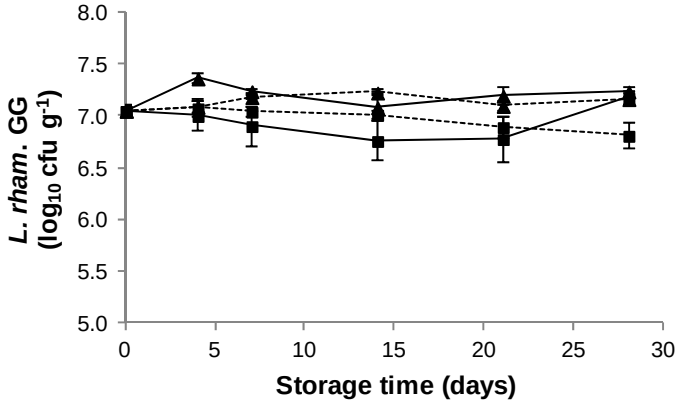

Fig. 2 *L. rham*. GG population on 'Golden Delicious' apple wedges inoculated alone (▲) or together with *Salmonella* and *L. monocytogenes* (■) and stored at 5 °C (dotted line) or 10 °C (continuous line) (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

Initial mesophilic population on uninoculated apple wedges (Fig. 3A) was 2.6±0.1 $\log_{10}$ cfu g^{-1}. At 10 °C, it augmented exponentially (more than 3-log units) until day 7, and then it continued increasing slightly, reaching a final population of 6.5±0.4 $\log_{10}$ cfu g^{-1}. Although no mesophilic increase was observed during the first 4 days at 5 °C, then it augmented exponentially reaching a final population of 6.1±0.4 $\log_{10}$ cfu g^{-1}.

Yeast and moulds were evaluated on control and *L. rham*. GG treatments at both storage temperatures (Fig. 3B). Initial population was 2.2±0.4 $\log_{10}$ cfu g^{-1} and it augmented slightly during storage with maximums populations between 3.7±0.4 and 4.7±0.14 $\log_{10}$ cfu g^{-1}. No differences were observed among yeast and moulds growth at any temperature and treatment.

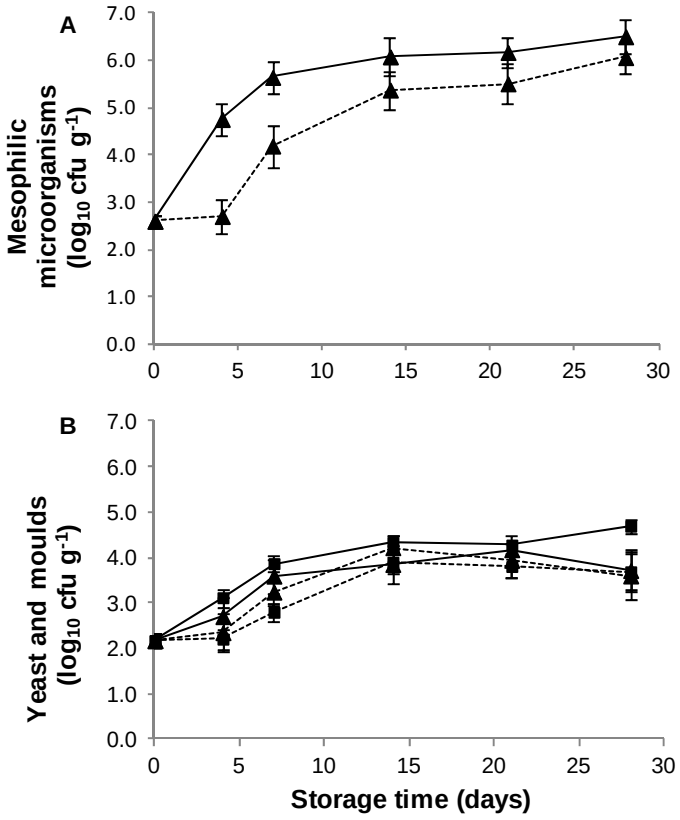

Fig. 3 Population of mesophilic microorganisms (A) and fungi (B) on 'Golden Delicious' apple wedges inoculated alone (▲) or together with *L. rham*. GG (■) and stored at 5 °C (dotted line) or 10 °C (continuous line) (n=6, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

3.2. Survival in simulated gastric conditions

The tolerance to gastric stress of *Salmonella*, *L. monocytogenes* and *L. rham*. GG prior and after storage at 5 °C up to 28 days on 'Golden Delicious' apple wedges when inoculated alone or in combination was investigated (Table 2). Viability of any of the bacteria was not affected by cultivation alone (Sal+Lm or *L. rham*. GG) or in combination (Sal+Lm+*L. rham*. GG).

Salmonella cell survival in simulated gastric conditions after inoculation of apple wedges (t=0) was around 30 % for both treatments. Increasing storage times caused a sharp reduction of *Salmonella* viability after gastric acid incubation, lowering to 4 % after 4 days of storage at 5 °C and being lower than 1 % from day 7 until the end of storage (<1.7-log units g^{-1}).

Table 2 *Salmonella*, *L. monocytogenes* and *L. rham*. GG survival (%) after exposure to simulated gastric fluid. Apples were inoculated with *Salmonella*, *L. monocytogenes* or *L. rham*. GG alone (Sal+Lm or *L. rham*. GG) or in combination (Sal+Lm+*L. rham*. GG) and stored at 5 °C up to 28 days.

Day	*Salmonella*		*L. monocytogenes*		*L. rham* GG	
	Sal+Lm	Sal+Lm+ *L. rham*. GG	Sal+Lm	Sal+Lm+ *L. rham*. GG	*L. rham*. GG	Sal+Lm+ *L. rham*. GG
0	35.6±8.1	27.7±5.7	15.0±4.3	14.3±6.1	78.6±10.2	82.3±15.7
4	4.5±0.6	4.0±3.3	1.7±1.1	1.1±0.5	82.0±46.3	66.7±22.8
7	0.3±0.0	0.9±0.3	0.1±0.0	0.8±0.3	30.5±9.5	11.3±3.0
14	0.6±0.1	0.6±0.1	0.0	0.1±0.0	22.3±3.4[b]	7.3±1.8[b]
21	0.3±0.3	0.6±0.3	0.0	0.0	6.7±5.4	19.5±5.1
28	0.0[a]	0.5±0.5	0.0	0.0	2.9±1.2	1.6±0.2

Each value is the mean ± standard error of the mean of three replicates.
[a]not detected after incubating 10 g of digested sample in 90 mL of BPW 24 h at 37 ºC

[b]Means that bacterial survival from apple wedges when inoculated alone differs from survival from apple wedges when inoculated together ($P<0.05$) according to Duncan's Multiple range test.

Higher reductions in viability during the gastric acid challenge were observed for *L. monocytogenes*. Initial cell survival (t=0) was 15 % approximately, it was reduced to around 1 % on the first 4 days of storage and below 1 % from day 7.

L. rham. GG initial cell survival was around 80 %. Although, increasing storage time of apple wedges caused a progressive reduction of *L. rham*. GG viability, it was higher to 10^7 cfu g^{-1} up to 4 days of storage in both treatments. From day 4 to day 7 a sharp decrease of viability was detected. However, *L. rham*. GG viability was maintained up to 10^6 cfu g^{-1} until 14 days of storage at 5 °C on apple wedges.

3.3. Quality analysis of apple wedges

3.3.1. Headspace gas composition

The O_2 and CO_2 concentration data were pooled for all treatments as the evolution on the trays was not different among them (Fig. 4). The O_2 concentration within MP apple trays stored at 10 °C dropped rapidly from 21.0 % to approximately 10.0 % during the first 4 days of storage and after 14 days it was 0.0 %. At 5 °C, it needed 21 days to reach 0.3 %. The CO_2 concentration increased to more than 10.0 % during the first 4 days of storage at 10 °C and it reached a concentration higher than 30.0 % after 28 days. At 5 °C, it was not over 10.0 % until 14 days of storage.

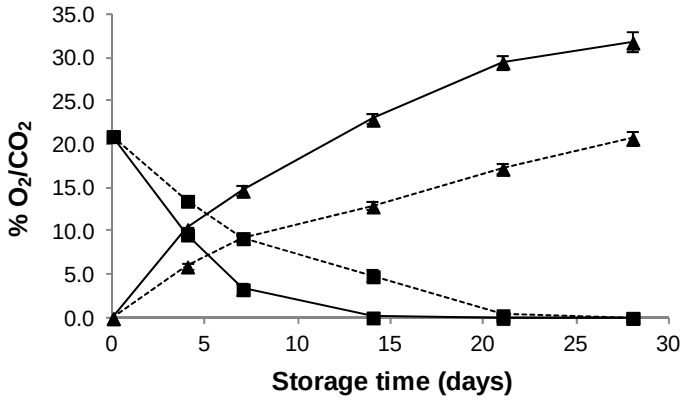

Fig. 4 Concentration of O_2 (■) and CO_2 (▲) in apple trays stored at 5 °C (dotted line) and at 10 °C (continuous line) (n=12, bars are standard deviation of the mean. When the standard error bars are not visible, they are smaller than the size of the symbol).

3.3.2. pH

Initial pH values of apple flesh were 3.93±0.20 and they varied between 3.94±0.16 and 4.26±0.10 over the 28 days storage period (data not shown). However, no significant effects of treatment or storage time on pH sample variability were observed.

3.3.3. Color

Table 3 shows the L*, a* and b* values for both the control and *L. rham*. GG inoculated apple wedges stored at 5 and 10 °C over 28 days of storage. Although some significant differences were observed between treatments for instrumental colour values (L*, a* and b*) at both temperatures, no clear tendency could be observed. However, during storage Hunter L* values increased for all treatments from 72.74 to approximately 80 in the first 4 days of storage. Then, they oscillated from 80.51±0.43 to 82.92±0.44. Regarding a* values, they decreased for both treatments at both storage temperatures on the first 4 days and then they maintained around -3.00. In terms of yellowness, b* values oscillated from 23.82±0.43 (initial value) to 27.75±0.61 in all treatments along storage.

Table 3 Changes in the color parameters (L*, a*, b*) of uninoculated (control) or inoculated with *L. rham*. GG 'Golden Delicious' apple wedges during storage at 5 and 10 °C.

	Temperature	**Treatment**	**Days**					
			0	**4**	**7**	**14**	**21**	**28**
L*	5 °C	Control	72.74±0.39 Az	79.75±0.42 Ay	80.98±0.39 Ax	81.29±0.28 Awx	82.24±0.20 Aw	81.27±0.49 Awx
		L. rham. GG	72.74±0.39 Az	79.67±0.38 Ay	81.28±0.31 Ax	81.19±0.31 Ax	80.51±0.43 Bxy	82.31±0.28 Aw
	10 °C	Control	72.74±0.39 Az	80.67±0.33 Ay	81.97±0.32 Awx	81.07±0.35 Axy	80.87±0.36 Bxy	82.92±0.44 Aw
		L. rham. GG	72.74±0.39 Az	79.75±0.42 Ay	80.98±0.39 Ax	81.28±0.28 Awx	82.24±0.20 Aw	81.28±0.49 Bwx
a*	5 °C	Control	-2.48±0.11 Aw	-3.03±0.08 Ax	-3.31±0.16 Axy	-3.19±0.10 Ax	-3.05±0.11 Ax	-3.54±0.12 Ay
		L. rham. GG	-2.48±0.11 Aw	-3.55±0.11 Bxy	-3.08±0.09 Ax	-3.55±0.13 Byz	-3.25±0.18 Axy	-3.76±0.16 Az
	10 °C	Control	-2.48±0.11 Aw	-3.26±0.18 Ax	-3.11±0.15 Ax	-2.89±0.12 Ax	-2.88±0.11 Ax	-3.22±0.09 Ax
		L. rham. GG	-2.48±0.11 Aw	-3.03±0.08 Ax	-3.31±0.16 Axy	-3.19±0.10 Ax	-3.05±0.11 Ax	-3.54±0.12 By
b*	5 °C	Control	23.82±0.43 Ax	23.83±0.41 Bx	26.43±0.39 Aw	25.97±0.53 Aw	23.90±0.36 Bx	25.95±0.68 Aw
		L. rham. GG	23.82±0.43 Ay	25.37±0.48 Axy	24.08±0.41 Bxy	25.53±0.53 Awx	25.48±0.52 Awx	26.31±0.61 Aw
	10 °C	Control	23.82±0.43 Ay	25.91±0.49 Awx	27.75±0.61 Axy	25.78±0.47 Awx	26.73±0.58 Awx	24.40±0.50 Aw
		L. rham. GG	23.82±0.43 Ax	23.83±0.41 Bx	26.43±0.39 Aw	25.97±0.53 Aw	23.89±0.36 Bx	25.95±0.68 Aw

Each value is the mean ± standard error of the mean of three replicates with ten evaluations each. Different lowercase letters (w, x, y, z) in the same line indicate significant differences ($P<0.05$) among storage days for each treatment whereas different capital letters (A, B) in the same column indicate significant differences ($P<0.05$) between treatments at the same time and temperature.

3.3.4. Soluble solids content, titratable acidity and texture

Apple wedges solid soluble content (SSC), titratable acidity (TTA) and firmness values were determined in control and *L. rham*. GG treatments along storage at 5 and 10 °C (Table 4).

SSC was not influenced by *L. rham*. GG treatment at any temperature ($P<0.05$). However, significant differences were observed along storage within some treatments and the tendency was to decrease in all treatments. Soluble solids content ranged from 14.4 °Brix to 13.1 °Brix.

Significant differences in titratable acidity between control and *L. rham*. GG inoculated samples were only observed at 7 days of storage at 10 °C. Along storage period, no significant differences were observed for *L. rham*. GG treatments at any temperature; and even though significant differences could be noted on control treatments, they were very small in practical terms. TTA ranged from 2.15 to 2.70 g malic acid L^{-1}.

No significant differences in texture between untreated and *L. rham*. GG treatments was noted at any of the storage temperatures and, also the storage period had very little effect on the firmness of apple wedges as significant differences were only observed for *L. rham.* GG treatment at 10 °C. Firmness values ranged from 17.61±1.00 to 23.31±1.74 N for all treatments.

4. DISCUSSION

Nowadays there is an increasing demand for non-dairy based probiotic products and the development of fruits and vegetables with probiotic content is a topic of high interest for consumers. However, the available information is very limited (Puente *et al.*, 2009). The effect of probiotic bacteria against foodborne pathogens on fresh-cut fruit has not been previously evaluated. Thus, the possibility to produce minimally-processed apples enriched with the probiotic *L. rhamnosus* GG and its effect against pathogenic bacteria was investigated. *L. rham*. GG population maintained viable at a concentration of around 10^7 cfu g^{-1} when added to apple wedges without quality rejection for 28 days. Moreover, addition of *L. rham*. GG resulted in a 1-log unit reduction of *L. monocytogenes* population on apple wedges at both temperatures tested (5 and 10 °C) but it did not affect *Salmonella* population. *L. rham*. GG, *Salmonella* and *L. monocytogenes* survival decreased when exposed to gastric stress throughout apple wedges storage.

Table 4 Changes in soluble solids content (SSC, ° Brix), titratable acidity (TTA, g malic acid L^{-1}) and firmness (N) of uninoculated (control) or inoculated with *L. rham*. GG 'Golden Delicious' apple wedges during storage at 5 and 10 °C.

	Temperature	**Treatment**	**Days**					
			0	**4**	**7**	**14**	**21**	**28**
SSC	5 °C	Control	14.4±0.4 Ax	14.1±0.1 Ax	14.2±0.2 Ax	13.9±0.1 Ax	13.7±0.2 Ax	13.7±0.1 Ax
		L. rham. GG	14.4±0.4 Ax	14.1±0.1 Ax	14.2±0.1 Ax	14.1±0.1 Ax	13.8±0.1 Axy	13.4±0.2 Ay
	10 °C	Control	14.4±0.4 Ax	14.2±0.2 Ax	14.2±0.2 Ax	13.8±0.2 Ax	14.0±0.3 Axy	13.1±0.0 Ay
		L. rham. GG	14.4±0.4 Ax	13.8±0.2 Axyz	14.1±0.1 Axy	14.2±0.1 Ax	13.5±0.1 Ayz	13.3±0.1 Az
TTA	5 °C	Control	2.67±0.14 Ax	2.16±0.06 Ay	2.22±0.05 Ay	2.26±0.18 Axy	2.34±0.21 Axy	2.15±0.01 By
		L. rham. GG	2.70±0.15 Ax	2.53±0.20 Ax	2.47±0.09 Ax	2.57±0.09 Ax	2.30±0.1 Ax	2.70±0.00 Ax
	10 °C	Control	2.67±0.14 Ax	2.34±0.24 Axy	2.23±0.06 Bxy	2.38±0.11 Axy	2.12±0.14 Ay	2.17±0.09 Axy
		L. rham. GG	2.70±0.15 Ax	2.70±0.25 Ax	2.63±0.09 Ax	2.43±0.14 Ax	2.63±0.23 Ax	2.27±0.14 Ax
Firmness	5 °C	Control	19.98±1.68 Ax	20.34±1.80 Ax	20.53±1.33 Ax	20.04±1.09 Ax	21.28±1.33 Ax	23.15±1.51 Ax
		L. rham. GG	19.98±1.68 Ax	20.77±1.00 Ax	22.80±1.48 Ax	23.31±1.74 Ax	20.60±1.91 Ax	22.65±2.25 Ax
	10 °C	Control	19.98±1.68 Ax	18.42±1.35 Ax	20.52±1.37 Ax	19.80±1.43 Ax	20.94±4.23 Ax	18.37±1.40 Ax
		L. rham. GG	19.98±1.68 Axy	19.98±1.29 Axy	17.61±1.00 Ay	20.58±1.21 Axy	21.92±0.78 Ax	21.71±1.22 Ax

Each value is the mean ± standard error of the mean of three replicates with ten evaluations each. Different lowercase letters (x, y, z) in the same line indicate significant differences ($P<0.05$) among storage days for each treatment whereas different capital letters (A, B) in the same column indicate significant differences ($P<0.05$) between treatments at the same time and temperature.

Salmonella population on apple wedges stored at 10 °C increased slightly and decreased when incubated at 5 °C. Nevertheless, *L. monocytogenes* population increased on apple flesh at both temperatures. Similar *Salmonella* and *L. monocytogenes* behaviour on apple flesh has been reported previously (Alegre *et al.*, 2010). Pathogens growth was unaffected by headspace gas composition as both, *Salmonella* and *L. monocytogenes*, survived and/or grew even the high CO_2 content (over 10 % in the first four days at 10 °C). Dissolved CO_2 has been found to inhibit microbial growth (Daniels *et al.*, 1985; Devlieghere and Debevere, 2000; Devlieghere *et al.*, 1998), affecting the lag phase, maximum growth rate and/or maximum population densities reached. However, generally it has been found that CO_2 does not affect or in some cases promotes growth of *L. monocytogenes* (Cutter, 2002).

Meanwhile *Salmonella* population was not affected by co-inoculation with *L. rham.* GG at any of the temperatures tested; *L. monocytogenes* growth was significantly ($P<0.05$) affected by co-inoculation with *L. rham.* GG, being 1-log unit lower. It is known that lactobacilli are able to produce antimicrobial substances when grown in specific media. Silva *et al.* (1987) described a low molecular weight, broad-spectrum inhibitory substance produced by *Lactobacillus* spp. strain GG. Recently, Lee *et al.* (2008) found that *L. rhamnosus* GG was a good antagonistic probiotic candidate against four strains of *E. coli* O157:H7 in solid media and broth and they hypothesized antagonistic metabolites to be responsible.

L. rham. GG population on apple wedges maintained around the initial population of 10^7 cfu g^{-1} during all storage period (28 d) at both temperatures, 5 and 10 °C. Previously, *L. rham*. GG has also been reported to maintain inoculum level on apple wedges over 10 days of storage period at 2-4 °C (Rößle *et al.*, 2010). *L. rham*. GG also remained viable in orange juice (pH 3.65) and pineapple juice (pH 3.40) over 12 weeks of storage at 4 °C at levels greater than 10^7 and 10^6 cfu mL^{-1}, respectively (Sheehan *et al.*, 2007). Another probiotic strain, *Bifidobacterium lactis* Bb-12, maintained between 6 and 7 $\log_{10}$ cfu g^{-1} in alginate- or gellan-based edible coatings on fresh-cut papaya and apples during 10 days storage period at 2 °C (Tapia *et al.*, 2007). Although the minimum recommended level of viable probiotics which should be present in foods for any health benefits to be achieved can vary, in general the food industry has adopted the recommended level of 10^6 cfu g^{-1} at the time of consumption (Kailasapathy and Chin, 2000; Kurman and Rasic, 1991). Thus, probiotics have a promising potential for exploitation as functional supplements in fruit products due to their impressive tolerance to acidic environments (Rivera-Espinoza and Gallardo-Navarro, 2010).

Moreover, another aspect that should be considered is the survival of foodborne pathogens and the probiotic strain *L. rham*. GG to gastric conditions. Thus, *Salmonella*, *L. monocytogenes* and *L. rham.* GG cell survival to gastric acid stress after incubation on apple wedges stored at 5 °C up to 28 days was evaluated. *Salmonella* and *L. monocytogenes* cell survival decreased quickly (to less than 5 %,

<2.5 $\log_{10}$ units cfu g^{-1}) within the first 4 days of storage. In the case of *L. rham*. GG, cell survival decrease was slower and more progressive and viable cells remained over 10^6 cfu g^{-1} until 14 days of storage. Several studies have shown that the pH of habituation or growth environment is the most important factor affecting tolerance during subsequent exposure to extreme acidic conditions (Koutsoumanis *et al.*, 2003; Koutsoumanis and Sofos, 2004). Koutsoumanis and Sofos (2004) and Lee *et al.* (1995) reported an increased acid resistance of *Salmonella* Typhimurium after exposure to pH between 4.3 and 4.5. Regarding to *L. monocytogenes*, Koutsoumanis and Sofos (2004) reported that the pH range within which habituation resulted in increased acid resistance was 5.0-6.0, while no acid tolerance was induced after habituation at pH<5. In addition, it has been demonstrated that adaptation for a short period (few hours) at a moderate sublethal acidic pH helps the bacteria to better resist posterior lethal acidic pH. However, treatment for extended period at a moderate acidic pH rendered *Listeria* more sensitive to subsequent acid kill (Phan-Thanh and Montagne, 1998). Growth temperature is another important factor affecting the bacterial acid resistance. Álvarez-Ordóñez *et al.* (2010) and Samelis *et al.* (2003) demonstrated that *Salmonella typhimurium* cells grown at 10 °C showed a reduced acid resistance in comparison to cells obtained at 30 °C. Thus, the decrease in *Salmonella, L. monocytogenes* and *L. rham*. GG cell survival along storage period on MP apple wedges at 5 °C could be explained. Champagne *et al.* (2008) also reported strong viability losses following a 2h/37 °C incubation at pH 2.0 for *L. rhamnosus* LB11 after 35 days storage in a fruit juice blend at 4 °C.

Mesophilic population on uninoculated apple wedges increased over 6.0 $\log_{10}$ cfu g^{-1} after the 28 days storage period at both temperatures. Yeast and moulds populations were not affected by the presence of *L. rham*. GG on the apple wedges and they reached 4.0 $\log_{10}$ cfu g^{-1} after 14 d of storage regardless storage temperature. However, symptoms of decay were not observed throughout storage period.

No significant differences in instrumental color values between uninoculated and *L.rham*. GG treatment apple wedges was noted. Nevertheless, L* values increased throughout storage period in all treatments, indicating a whitening effect. Hunter a* values decreased significantly between day 0 and 4 indicating no development of redness, meanwhile b* augmented. A decrease of the L* values and the rise in the a* value have commonly been used as indicators of browning (Mastrocola and Lerice, 1991; Monsalve-González *et al.*, 1993; Rojas-Graü *et al.*, 2006). Other researchers have also obtained good results using NatureSeal® as an apple antioxidant (Rößle *et al.*, 2009, 2010; Rupasinghe *et al.*, 2005; Toivonen, 2008).

Soluble solids content and titratable acidity were not influenced by apple treatment with *L. rham.* GG or storage period supporting results obtained by Rößle *et al.* (2010).

Firmness of apple wedges was not influenced by the treatment with *L. rham*. GG, as values were not different from untreated samples. Storage period had very little

effect on storage as differences were only noted in one treatment. However, Rößle *et al.* (2010) found that shear values for apples wedges treated or not with *L. rham*. GG increased during the first days of storage. This increase was attributed to the NatureSeal® AS1 browning inhibitor as it has a high content in calcium so the firming effect could be due to cross-linking of both cell wall and middle lamella pectin by calcium ions (Rico *et al.*, 2007).

In conclusion, our results have shown that *L. rhamnosus* GG could be a suitable probiotic strain to be added to apple wedges as it survived at concentrations higher than 10^6 cfu g^{-1} throughout 28 days of storage at 5 and 10 °C without any quality rejection. Moreover, it had a protective action against *L. monocytogenes* population, reducing its growth by 1-log units at both temperatures. Neither *Salmonella* nor *L. monocytogenes* nor *L. rham*. GG were acid adapted while surviving/growing on apple wedges so that subsequent exposure to simulated gastric fluid resulted in high viability losses. Thus, the probability to *Salmonella* and *L. monocytogenes* causing infection after ingestion would be reduced. In the case of *L. rham*. GG, cell viability was only higher to 10^6 cfu g^{-1} until 14 days of storage at both temperatures; hence apple wedges shelf life should not overcome this period to claim for probiotic effects.

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V. DISCUSIÓN

En esta tesis se pretendía determinar, en primer lugar, la capacidad de supervivencia y/o crecimiento de los principales patógenos de transmisión alimentaria en frutas mínimamente procesadas ácidas (manzana y melocotón) en diferentes condiciones (temperatura, atmósfera de envasado, etc.). Una vez demostrada la capacidad de crecimiento de los patógenos de transmisión alimentaria utilizados en manzana y melocotón mínimamente procesados y debido a sus implicaciones en la seguridad alimentaria, se estudiaron nuevas estrategias de intervención (eliminación y control de crecimiento) como sustancias antimicrobianas alternativas al hipoclorito sódico (ácido peroxiacético, peróxido de hidrógeno, y aceites esenciales, entre otros) y la bioconservación. Todo ello con el objetivo final de conseguir un producto más seguro para el consumidor utilizando tecnologías más respetuosas con el medio ambiente.

Las frutas seleccionadas para la realización de esta tesis fueron manzana y melocotón ya que la provincia de Lleida es la principal productora de manzana del país (31.1 % del total en 2009) y una de principales zonas productoras de melocotón (18.3 % del total en 2009). En cuanto a los patógenos de transmisión alimentaria estudiados, se seleccionaron *Salmonella* y *L. monocytogenes* ya que están incluidos dentro de los criterios de seguridad de los alimentos (incluyendo fruta mínimamente procesada) del Reglamento (CE) nº 1441/2007. El tercer patógeno seleccionado, *E. coli* O157:H7, se incluyó en los estudios por su creciente importancia en la seguridad alimentaria de frutas y hortalizas. Las cepas de *Salmonella* utilizadas en esta tesis pertenecen a *Salmonella enterica* subsp. *enterica* (antiguamente *Salmonella choleraesuis* subsp. *choleraesuis*), siendo sus serotipos Agona, Michigan, Montevideo y Gaminara. En algunos de los estudios realizados se utilizó una cepa de *L. innocua* en sustitución de *L. monocytogenes* ya que *L. innocua* ha demostrado ser un modelo válido para *L. monocytogenes* (Francis y O'Beirne, 1997).

1. SUPERVIVENCIA Y CRECIMIENTO DE PATÓGENOS DE TRANSMISIÓN ALIMENTARIA EN MANZANA Y MELOCOTÓN MÍNIMAMENTE PROCESADOS

En los últimos años han aumentado el número de intoxicaciones alimentarias debidas al consumo de frutas y hortalizas mínimamente procesadas. La ecología microbiana de los patógenos en alimentos de origen animal es muy conocida pero en alimentos de origen vegetal está menos definida (Beuchat, 2002). Las frutas mínimamente procesadas pueden contaminarse con microorganismos capaces de causar intoxicaciones alimentarias en el campo, durante la cosecha, el transporte y el procesado. Esta contaminación puede representar un riesgo importante ya que el procesado necesario en la elaboración de productos mínimamente procesados

omite cualquier operación capaz de eliminar efectivamente la carga microbiana del producto. Una vez en el producto, y según las condiciones de conservación, los microorganismos pueden ser capaces de multiplicarse, incrementando el riesgo de producir intoxicaciones alimentarias. En esta tesis se ha estudiado el efecto de la temperatura de conservación, la variedad de fruta, la presencia de sustancias antioxidantes y el envasado en atmósfera modificada en el crecimiento de los tres principales patógenos de transmisión alimentaria.

1.1. Efecto de la temperatura de conservación

En los estudios llevados a cabo en la realización de esta tesis, las cepas de los tres patógenos de transmisión alimentaria utilizados, *E. coli* O157:H7, *Salmonella* y *L. innocua* crecieron de forma exponencial (más de 2 unidades logarítmicas en 24-48 h) en manzanas 'Golden Delicious' (pH 4.16±0.25) y melocotones 'Elegant Lady' (pH 3.73±0.28) mínimamente procesados conservados a 20 y 25 °C. Al reducir la temperatura de conservación a 10 °C, la velocidad de crecimiento de los patógenos se redujo considerablemente, siendo los incrementos de población aproximadamente de 1.3 y 1.0 unidades logarítmicas tras 6 y 14 días para ambos patógenos gram-negativos en manzanas y melocotones, respectivamente y de 2.4 y 1.6 unidades logarítmicas para *L. innocua*, siendo el patógeno que alcanzó poblaciones más elevadas. A temperatura de refrigeración (5 °C), no se observó crecimiento ni de *E. coli* O157:H7 ni de *Salmonella*. En cambio, *L. innocua* creció en ambas frutas (hasta 1 unidad logarítmica en melocotón). El crecimiento de cada uno de los patógenos fue similar en ambas frutas a pesar de las diferencias de pH. Los resultados obtenidos han demostrado que las manzanas y los melocotones mínimamente procesados pueden ser sustratos adecuados para el crecimiento de los tres patógenos de transmisión alimentaria ensayados a temperaturas de 10 °C y superiores. Varios autores han demostrado la capacidad de *E. coli* O157:H7 para crecer tanto en manzanas mínimamente procesadas como en heridas de manzanas (Abadias *et al.*, 2009; Dingman, 2000; Gunes y Hotchkiss, 2002; Janisiewicz *et al.*, 1999a; Trias *et al.*, 2008) conservadas a temperaturas superiores a 15 °C. La población de *Salmonella* también aumentó exponencialmente en trozos de manzanas 'Red Delicious' conservados a 10 y 20 °C (Leverentz *et al.*, 2001) y en heridas de manzana 'Golden Delicious' a 25 °C (Trias *et al.*, 2008). En lo que respecta a *L. monocytogenes*, su población aumentó en manzanas 'Red Delicious' y 'Golden Delicious' conservadas a temperaturas de 10 °C y superiores (Leverentz *et al.*, 2003, 2006; Trias *et al.*, 2008). En un ensayo realizado por Zhuang *et al.* (1995), la población de *Salmonella* aumentó significativamente en tomate picado (3.99-4.37) conservado a 20 y 30 °C, pero no a 5 °C. En cambio, la población de *L. monocytogenes* disminuyó en tomate picado conservado a 10 y 21 °C (Beuchat y Brackett, 1991).

De los datos indicados se observa que el control de la temperatura es un factor crítico para prevenir el crecimiento microbiano. Con frecuencia, los

microorganismos pueden sobrevivir a temperaturas de refrigeración, aunque su capacidad de multiplicarse desaparece o se reduce, a excepción de los microorganismos psicrótrofos como *L. monocytogenes* (Harris *et al.*, 2003). Por ello, tiene que garantizarse que durante toda la vida útil del producto la temperatura de conservación no excede los 5 °C. El control de la temperatura de conservación es especialmente importante en los lineales de los supermercados ya que, en muchos casos, las temperaturas son superiores a las recomendadas. Por ejemplo, en un estudio llevado a cabo por Morelli *et al.* (2012), los lineales evaluados superaron los 7 °C el 70 % del tiempo.

1.2. Efecto de la variedad de fruta

A continuación se estudió el efecto de la variedad de fruta utilizada en la dinámica poblacional de los tres patógenos a temperatura de refrigeración (5 °C) y a temperatura ambiente (25 °C). En el caso de manzana mínimamente procesada, la cepa de *E. coli* O157:H7 aumentó 2 unidades logarítmicas en las primeras 24 h a 25 °C en las tres variedades ensayadas, 'Golden Delicious' (pH 4.16±0.25), 'Granny Smith' (pH 3.32±0.13) y 'Shampion' (pH 4.44±0.26). El mayor incremento se observó en manzanas 'Golden Delicious', seguido de 'Granny Smith' y por último en 'Shampion'. A 5 °C la población de *E. coli* O157:H7 se redujo en las tres variedades. El crecimiento de *Salmonella* a 25 °C fue prácticamente igual en las tres variedades estudiadas. De forma similar a lo ocurrido con *E. coli* O157:H7, la población de *Salmonella* se redujo en las tres variedades a 5 °C. En el caso de *L. innocua* no se observaron diferencias entre las variedades a 25 °C pero sí a 5 °C, cuando su población se vio drásticamente reducida en manzanas 'Shampion' (más de 3 unidades logarítmicas tras 14 días de conservación). Las manzanas de la variedad 'Shampion' son las que presentaron mayor pH (4.44±0.26), mayor cantidad de sólidos solubles (13.9) y menor acidez titulable (2.16 g de ácido málico L^{-1}). En los estudios llevados a cabo por Fisher y Golden (1998) no hubo diferencias en el crecimiento de *E. coli* en diferentes variedades de manzana, sin embargo Dingman (2000) observó crecimiento en todas las variedades de manzana dañadas utilizadas ('Golden Delicious', 'Red Delicious', 'Macoun' y 'Melrose') a excepción de la variedad 'McIntosh', variedad con el menor valor de pH, aunque no estadísticamente diferente.

En melocotones mínimamente procesados se observaron mayores diferencias entre variedades. Los mayores incrementos de población *E. coli* O157:H7 y *Salmonella* se observaron en las variedades 'Royal Glory' y 'Diana' (aproximadamente 4 unidades logarítmicas) que fueron las que presentaron un pH más elevado ('Royal Glory' 4.73±0.25 y 'Diana' 4.12±0.18), seguidas de las variedades 'Elegant Lady' (incremento inferior a 3 unidades logarítmicas) y 'Plácido' (incremento inferior a 2 unidades logarítmicas). A 5 °C, las poblaciones de *E. coli* O157:H7 y *Salmonella* se redujeron en todas las variedades utilizadas. *L. innocua* creció exponencialmente en las dos variedades ensayadas a 25 °C. El

mayor incremento de población se observó en melocotones 'Diana' (aproximadamente 3.5 unidades logarítmicas) y a continuación en melocotones 'Elegant Lady' con un incremento de 2 unidades logarítmicas. A 5 °C, la población de *L. innocua* aumentó en melocotones 'Elegant Lady' (0.4 unidades logarítmicas) y se redujo en melocotones 'Royal Glory' y 'Diana'. No existe ningún trabajo que haya reportado el crecimiento de patógenos de transmisión alimentaria en melocotón ni en otras frutas de hueso.

Además de la temperatura, el bajo pH de las frutas es el principal factor que influye en el crecimiento de los patógenos de transmisión alimentaria en fruta mínimamente procesada. Sin embargo, la efectividad del pH en la inhibición de los microorganismos depende del tipo de ácido orgánico del alimento, ya que existen ácidos más efectivos que otros (Basset y McClure, 2008). No obstante, los alimentos pueden contener más de un tipo de ácido diferente (Wiley, 1994), por lo que es imposible hallar la información sobre el pH mínimo de crecimiento. Por ejemplo, los ácidos orgánicos débiles tienen un efecto más perjudicial en *L. monocytogenes* que el ácido clorhídrico y, el ácido acético es más tóxico que otros ácidos orgánicos volátiles (Phan-Thanh y Montagne, 1998). Los ácidos fuertes se disocian completamente en solución y los protones pasan a través de la membrana celular interaccionando con los mecanismos que controlan el flujo de protones. En cambio, los ácidos orgánicos débiles permeabilizan la membrana celular como moléculas no disociadas y una vez disociadas en el interior de la célula no pueden salir al exterior y disminuyen el pH intracelular a valores dramáticos que desestabilizan el metabolismo de la célula. Además, al mismo pH extracelular, los ácidos orgánicos causan una disminución mayor del pH intracelular que el ácido clorhídrico (Phan-Thanh y Montagne, 1998). Varios estudios con diferentes cepas de *L. monocytogenes* han demostrado que el ácido acético y el láctico eran más inhibitorios que los ácidos cítrico y clorhídrico (Conner *et al.*, 1990; Sorrells *et al.*, 1989; Vasseur *et al.*, 1999). En el caso de *E. coli* O157:H7, el orden de inhibición es de ácido acético> cítrico>málico (Deng *et al.*, 1999). En un estudio reciente, Abadias *et al.* (2012) observaron que la cepa de *E. coli* O157:H7 utilizada en esta tesis fue incapaz de crecer en piña mínimamente procesada conservada a 25 °C (pH 3.59), mientras que, como se ha visto anteriormente, ha sido capaz de crecer en manzanas 'Granny Smith' con un pH más bajo (pH 3.32±0.13). Esta diferencia puede deberse a que el principal ácido de la piña es el cítrico, mientras que el de manzana es el málico. De forma similar, a un determinado pH, el ácido acético fue el más inhibitorio para *Salmonella*, seguido del ácido láctico, el cítrico y el málico (Jung y Beuchat, 2000). Álvarez-Ordóñez *et al.* (2010) encontraron un orden de inhibición similar. Janisiewicz *et al.* (1999a) apuntaron que el crecimiento de bacterias en productos ácidos puede ser el resultado de la modificación del pH en el microambiente adyacente.

La inhibición en el crecimiento de patógenos de transmisión alimentaria no se ha dado específicamente en las variedades más ácidas Por tanto, se deduce que podría existir algún otro factor, a parte del pH, o algún componente de la fruta (polifenoles u otros compuestos con actividad antimicrobiana) que puede causar la inhibición

del crecimiento de patógenos en algunas variedades de fruta. Estas variedades con efecto inhibidor podrían ser más adecuadas para la producción de fruta mínimamente procesada desde el punto de vista microbiológico, siempre que cumplan posteriormente con las necesidades de conservación manteniendo las características organolépticas en valores adecuados.

1.3. Efecto del uso de antioxidantes

El pardeamiento enzimático es uno de los principales problemas en la producción de algunas frutas mínimamente procesadas, entre ellas manzana y melocotón, por tanto, es necesaria la utilización de sustancias antioxidantes para prevenirlo. En esta tesis se evaluó si la aplicación de estos antioxidantes presentaba, además, un efecto antimicrobiano. Los antioxidantes estudiados han sido el ácido ascórbico al 2 % y el producto comercial NatureSeal® AS1 al 6 % en manzana 'Golden Delicious' mínimamente procesada y el ácido ascórbico al 2 % en melocotón 'Elegant Lady' mínimamente procesado. El uso de estas sustancias antioxidantes no tuvo ningún efecto en la población de ninguno de los patógenos ni a 5 ni 25 °C en ninguna de las dos frutas. Por tanto, el uso de estas sustancias antioxidantes a las dosis ensayadas sólo es efectivo mejorando la calidad visual de la fruta mínimamente procesada y, en consecuencia alargando la vida útil del producto. Este aumento de vida útil puede tener connotaciones negativas ya que, en caso de darse las condiciones necesarias para su desarrollo, los patógenos de transmisión alimentaria podrían crecer durante un mayor periodo de tiempo sin influir en la calidad visual de los productos, pudiendo llegar a ser un problema de seguridad alimentaria. Sin embargo, otros autores (Raybaudi-Massilia *et al.*, 2009) demostraron que una mezcla antioxidante compuesta por N-acetilo-L-cisteína (1 %), glutatión (1 %), láctato cálcico (1 %) y ácido málico (2.5 %) redujo la población de *L. monocytogenes*, *Salmonella* Enteritidis y *E. coli* O157:H7 en manzanas mínimamente procesadas.

1.4. Efecto de la atmósfera modificada

El envasado en atmósfera modificada pasiva ha sido el último factor estudiado en los ensayos de dinámica poblacional en manzanas 'Golden Delicious' y melocotones 'Elegant Lady' mínimamente procesados y conservados a 5 y 25 °C. La fruta, cortada en trozos e inoculada se envasó en barquetas selladas con un film adecuado (Figura 1). La variación de gases en el interior tuvo lugar por la propia respiración del fruto. En ambas frutas, la supervivencia y crecimiento de los tres patógenos ensayados y envasados en atmósfera modificada fue equiparable al observado en aire. Los niveles de O_2 y CO_2 alcanzados en manzana fueron de 17.0 % y 6.8 % tras 3 días a 25 °C y de 19.6 % y 2.8 % tras 14 días a 5 °C, respectivamente. En melocotón se observó un mayor cambio de la atmósfera gaseosa llegando a unas concentraciones finales de 13.2 % de O_2 y 24.6 % de CO_2

tras 6 días a 25 °C y de 18.5 % de O_2 y 3.9 % de CO_2 tras 14 días a 5 °C. La atmósfera interior fue diferente en función de la fruta y de la temperatura de conservación debido a la diferente tasa de respiración de cada fruta y a su variación con la temperatura (a mayor temperatura, mayor tasa de respiración).

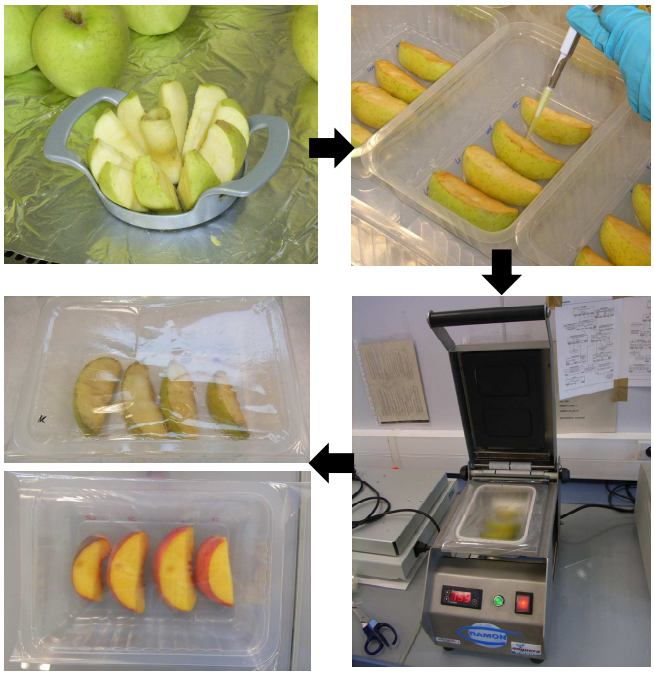

Fig. 1 Preparación de las barquetas de manzana y melocotón para estudiar el efecto de la atmósfera de conservación en el crecimiento y supervivencia de *E. coli* O157:H7, *Salmonella* y *L. innocua*.

Algunos investigadores han estudiado el efecto del envasado en atmósfera modificada activa. Conway *et al.* (2000) observaron que *L. monocytogenes* creció de forma parecida en trozos de manzana 'Delicious' conservados a 5, 10 y 20 °C en atmósfera controlada (0.5 % O_2 y 15 % CO_2) y aire, mejorando notablemente la calidad visual de los trozos de manzana conservados en atmósfera modificada. Sin embargo, Gunes y Hotchkiss (2002) observaron que el envasado en atmósfera modificada (1 % O_2 y 0, 15, 30 % CO_2 o 30 % CO_2 y 21 % O_2) inhibió significativamente el crecimiento de *E. coli* O157:H7 en trozos de manzana 'Delicious' conservados a 15 y 20 °C.

La vida útil de frutas y hortalizas mínimamente procesadas se puede alargar mediante el uso de atmósferas con bajo O_2 y alto CO_2 ya que éstas actúan disminuyendo el deterioro natural de los productos. Sin embargo, los plásticos de

envasado disponibles actualmente para frutas y hortalizas mínimamente procesadas, frecuentemente no tienen una suficiente tasa de transmisión de O_2 y CO_2 para evitar la presencia de condiciones anaerobias o la acumulación de niveles excesivos de CO_2, especialmente durante condiciones de temperaturas superiores a las recomendables (inferiores a 5 °C). En el caso que el nivel de O_2 disminuya por debajo del límite de fermentación, se puede producir respiración anaerobia por parte del producto y dar lugar a malos sabores y estimular el crecimiento de patógenos anaerobios (Soliva-Fortuny y Martín-Belloso, 2003). Los niveles altos de humedad generados en el interior de los envases de atmósfera modificada evitan la pérdida de humedad por parte del producto, pero crean condiciones favorables para el crecimiento microbiano, especialmente si existen fluctuaciones de temperatura (Hertog, 2003). El envasado en atmósfera modificada ayuda además a mantener la integridad de los tejidos, reduciendo la cantidad de exudados ricos en nutrientes que facilitan el crecimiento de microorganismos. Sin embargo, su efecto en el crecimiento microbiano no es consistente y, normalmente, la temperatura de conservación es la responsable del control (Zagory, 1999). Además, cuando las concentraciones de CO_2 alcanzan valores bacteriostáticos en el interior de los envases con atmósfera modificada pasiva, la población de patógeno ya podría haber aumentado considerablemente. Por ello, en muchas ocasiones se utilizan atmósferas modificadas activas. En la Tabla 1 se pueden ver los valores de atmósfera modificada activa recomendados para manzanas y melocotones mínimamente procesados desde el punto de vista de calidad.

Tabla 1 Recomendaciones de atmósfera de envasado para manzanas y melocotones mínimamente procesados

Producto	**Temperatura**	**% O_2**	**% CO_2**
Manzana	0-5	<1	4-12
Melocotón	0	1-2	5-12

Adaptada de Gorny (2003).

Los resultados obtenidos han demostrado la capacidad de crecimiento de los patógenos de transmisión alimentaria utilizados en manzana y melocotón mínimamente procesados cuando se conservan a temperaturas iguales o superiores a 10 °C. Durante la distribución y comercialización de los productos es muy difícil mantener valores de temperatura adecuados y, por tanto, es posible que en ocasiones se superen las temperaturas óptimas de conservación capaces de reducir el crecimiento de patógenos, por ejemplo en los lineales de los supermercados, como muestran diferentes estudios (Marklinder *et al.*, 2004; Morelli *et al.*, 2012). Así pues, es muy importante evitar la contaminación de los productos mediante la aplicación de Buenas Prácticas Agrícolas y Buenas Prácticas de Producción,

además del sistema de APPCC. Sin embargo, es imposible asegurar que no existirá contaminación con patógenos y es por tanto de vital importancia establecer estrategias de intervención para eliminar, reducir y controlar estos patógenos en fruta mínimamente procesada.

2. NUEVAS ESTRATEGIAS DE INTERVENCIÓN FRENTE A PATÓGENOS DE TRANSMISIÓN ALIMENTARIA EN FRUTA MÍNIMAMENTE PROCESADA

Para obtener frutas y hortalizas mínimamente procesadas seguras, de calidad y alto valor nutricional, la industria necesita implementar nuevas estrategias introduciendo y/o combinando técnicas sostenibles, especialmente procedimientos estándar de desinfección. Las técnicas de conservación más importantes para prevenir o retrasar la alteración son la refrigeración y el envasado en atmósferas modificadas combinadas con tratamientos químicos (soluciones antimicrobianas, acidulantes, antioxidantes, etc.) (Leistner y Gould, 2002). Los puntos clave para la producción de productos vegetales mínimamente procesados seguros incluyen el control de los productos que entran en la cadena productiva, la supresión del crecimiento microbiano, la reducción de la carga microbiana durante el procesado y la prevención de la contaminación post-procesado (Artés y Allende, 2005).

2.1. Sustancias antimicrobianas alternativas al hipoclorito sódico

Durante el procesado de frutas y hortalizas mínimamente procesadas, la desinfección es una de las operaciones más importantes que afectan a la calidad, seguridad y vida útil del producto final. Debido a los riesgos de salud y ambientales asociados con el uso del hipoclorito sódico, actualmente existe una tendencia a eliminarlo del proceso productivo. Por lo tanto es necesario buscar alternativas que garanticen la seguridad de los productos manteniendo la calidad y la vida útil, a la vez que se reduce el consumo de agua.

En base a referencias bibliográficas, se seleccionaron una serie de sustancias que se testaron en primer lugar *in vitro* frente a *E. coli* O157:H7 y *L. innocua* mediante contacto directo durante 1, 3 y 5 min. Se utilizaron estos dos microorganismos como modelos de patógenos gram-negativo y gram-positivo, respectivamente, para comprobar si existían diferencias de inhibición atribuibles a las diferencias en la composición de la pared celular. Ninguna de las concentraciones ensayadas de bicarbonato sódico (10, 50 y 100 g L^{-1}), bicarbonato potásico (10, 50 y 100 g L^{-1}), vainillina (3, 6 y 12 g L^{-1}) y bromelaína (0.1, 1, 10, 100 y 1000 mg L^{-1}) redujo las poblaciones de ninguno de los patógenos. A excepción del carvacrol (875 y 1500 mg L^{-1}) y el ácido peroxiacético (20, 80 y 120 mg L^{-1}), que redujeron ambas poblaciones por debajo del límite de detección (2.5×10^3 ufc mL^{-1}), las reducciones

obtenidas con el resto de las sustancias utilizadas (carbonato sódico, 10, 50 y 100 g L^{-1}; carbonato potásico, 10, 50 y 100 g L^{-1}; peróxido de hidrógeno, 5, 10 y 20 mL L^{-1}; N-acetilo-L-cisteína, 2.5, 5 y 10 g L^{-1}; Citrox, 5 mL L^{-1} y quitosano, 10 g L^{-1}) fueron dependientes de la dosis utilizada, observándose mayores reducciones en *E. coli* O157:H7 que en *L. innocua*. A la vista de los resultados, los dos bicarbonatos y la bromelaína fueron descartados para los ensayos posteriores. Sin embargo, la vainillina se testó posteriormente *in vivo* ya que se ha visto que tiene efecto bacteriostático (Fitzgerald *et al.*, 2004), difícil de observar en ensayos *in vitro*, y, además, ha dado buenos resultado en zumo de manzana (Yuk *et al.*, 2006). También se descartaron los carbonatos, por la posibilidad de causar incrustaciones en la maquinaria industrial a las dosis estudiadas, y el quitosano, por ser económicamente inviable a la dosis efectiva.

A continuación se ensayó la efectividad del carvacrol, vainillina, ácido peroxiacético, peróxido de hidrógeno, N-acetilo-L-cisteína y Citrox para reducir y mantener a niveles bajos la población de *E. coli*, *Salmonella* spp. y *Listeria* spp. en manzanas 'Golden Delicious' mínimamente procesadas inoculadas artificialmente y conservadas 6 días a 10 °C. Se utilizó una temperatura de conservación de 10 °C para poder observar el crecimiento de patógenos de transmisión alimentaria en los trozos de manzana ya que a 5 °C no hubiésemos detectado crecimiento ni para *E. coli* ni para *Salmonella*. Además, 10 °C es una temperatura que se da frecuentemente en los establecimientos de venta. La efectividad de estas sustancias se comparó con agua desionizada y una solución de hipoclorito sódico (SH) de 100 mg L^{-1} de cloro libre (pH 6.5).

En el caso del carvacrol (500, 875 y 1500 mg L^{-1}), su efectividad fue inferior a la del agua, siendo la población de los patógenos al menos 1 unidad logarítmica superior que en el tratamiento con agua tras 6 días a 10 °C. Sin embargo, otros autores han descrito su efectividad en kiwi y zumo de manzana (Kisko y Roller, 2005; Roller y Seedhar, 2002). La desinfección de manzana mínimamente procesada con vainillina a la mayor concentración ensayada, 12 g L^{-1}, causó reducciones en las poblaciones de *E. coli* O157:H7 y *Listeria* spp. similares a las obtenidas con SH (aproximadamente 1 unidad logarítmica), tanto inicialmente como a lo largo de la conservación a 10 °C. En el caso de *Salmonella* las reducciones observadas fueron más de 1 unidad logarítmica, superiores a las del hipoclorito a partir del tercer día de conservación Otros autores han demostrado mayores efectividades de la vainillina utilizando concentraciones inferiores, 3 g L^{-1} y 1.8 g L^{-1}, en zumo y trozos de manzana, respectivamente (Rupasinghe *et al.*, 2006; Yuk *et al.*, 2006). Aunque la mayoría de aceites esenciales son considerados GRAS, su uso en los alimentos como conservantes tiene limitaciones ya que, frecuentemente las dosis antimicrobianas efectivas pueden exceder las dosis organolépticamente aceptables. En el caso de la vainillina, Rupasinghe *et al.* (2006) detectaron que dosis superiores a 1.8 g L^{-1} producían sabores y aromas inaceptables en manzanas mínimamente procesadas. Por tanto, y a pesar de su efectividad, la vainillina no sería una alternativa viable al hipoclorito.

El tratamiento de la manzana mínimamente procesada con el producto comercial Citrox (5 mL L^{-1}) fue tan efectivo como el tratamiento con hipoclorito sódico reduciendo y manteniendo las poblaciones de patógenos a niveles bajos. El tratamiento redujo la población de *E. coli* O157:H7 y *Salmonella* 1 unidad logarítmica, igual que el tratamiento con SH y la población de *Listeria* spp. 1 unidad logarítmica más que el SH. No obstante, no se observaron diferencias entre tratamientos tras los 6 días de conservación a 10 °C para ninguno de los patógenos. Los cilindros de manzana tratados con Citrox se tiñeron ligeramente de marrón debido al propio color de la solución y, por tanto, tampoco sería un desinfectante adecuado para ser utilizado en la desinfección de manzana mínimamente procesada.

Los resultados *in vivo* demostraron que el ácido peroxiacético (PAA, 80 y 120 mg L^{-1}) fue igual o más efectivo que el hipoclorito sódico en la desinfección de manzana mínimamente procesada. Las poblaciones de *E. coli* O157:H7 y *Salmonella* en las manzanas tratadas con PAA tras 6 días a 10 °C no fueron significativamente distintas de las poblaciones en las manzanas tratadas con SH. En cambio, la población de *Listeria* spp. fue entre 1.7 y 2.3 unidades inferior a la población en manzana tratada con SH. Ensayos anteriores han demostrado su eficacia en el control de *E. coli* y *L. monocytogenes* en manzanas y lechuga mínimamente procesadas durante 9 días de conservación a 4 °C (Rodgers *et al.*, 2004). Además, este desinfectante ha demostrado eficacia reduciendo los recuentos totales en melón 'Galia' y en rúcula mínimamente procesada (Martínez-Sánchez *et al.*, 2006; Silveira *et al.*, 2008).

Las diferentes concentraciones de peróxido de hidrógeno ensayadas (5, 10 y 20 mL L^{-1}) fueron eficaces en la reducción de los patógenos y manteniendo baja su concentración a lo largo de los 6 días de conservación a 10 °C. La población de *E. coli* O157:H7 y *Salmonella* fue aproximadamente 2 unidades logarítmicas inferior al tratamiento con SH tras la desinfección, manteniéndose esta diferencia a lo largo de la conservación. En el caso de *Listeria* spp. la reducción inicial fue similar a la conseguida por el tratamiento con SH (2 unidades logarítmicas), sin embargo, la población de *Listeria* spp. fue 1.5 unidades logarítmicas inferior a la de SH tras la conservación. El peróxido de hidrógeno había demostrado con anterioridad su eficacia reduciendo los microorganismos epifitos y patógenos en frutas y hortalizas enteras y mínimamente procesadas como pepino, calabacín, pimientos y melones (Artés *et al.*, 2007; Sapers, *et al.*, 2001; Sapers, 2003; Silveira *et al.*, 2008).

El tratamiento con N-acetilo-L-cisteína a una concentración de 10 g L^{-1} fue más efectivo que el hipoclorito sódico reduciendo a los tres patógenos en manzana mínimamente procesada. La reducción inicial de *E. coli* O157:H7 y *Salmonella* fue de 1 unidad logarítmica superior a la del tratamiento con SH, manteniéndose la población baja a lo largo de la conservación. Aunque *Listeria* spp. fue el patógeno más resistente, su población tras los 6 días de conservación fue la más baja (por debajo del nivel de detección). Así pues, queda demostrado que no sólo tiene efecto antioxidante, sinó que también tiene efecto antimicrobiano. Raybaudi-

Massilia *et al.* (2009) demostraron que la inmersión de manzanas 'Fuji' mínimamente procesadas en una solución acuosa de 1 % N-acetilo-L-cisteína, 1 % glutatión y 1 % lactato cálcico y subsiguiente inoculación artificial con *L. monocytogenes*, *Salmonella* Enteritidis y *E. coli* inhibió el crecimiento de los patógenos durante 30 días a 5 °C.

En el procesado de frutas y hortalizas mínimamente procesadas es muy importante mantener la calidad del agua ya que si no está desinfectada correctamente puede ser una fuente de contaminación microbiana (Zagory, 1999). Así pues, el uso de sustancias desinfectantes ayuda a prevenir la contaminación cruzada. En la realización de los ensayos, no se encontraron células viables de ninguno de los patógenos estudiados en las soluciones de ácido peroxiacético, peróxido de hidrógeno, Citrox e hipoclorito sódico tras la desinfección de los trozos de manzana mínimamente procesada. Estudios previos han demostrado que el uso de desinfectantes a base de ácido peracético, como Tsunami, son una buena alternativa para la desinfección del agua de proceso ya que fue tan efectivo como el hipoclorito sódico evitando la contaminación cruzada, a diferencia del Citrox que no pudo evitarla (López-Gálvez *et al.*, 2009).

La efectividad de los desinfectantes ha sido mayor en solución (contacto directo) que sobre la fruta. Esto es debido a que para poder eliminar a los microorganismos debe existir un contacto directo entre el desinfectante y el microorganismo. Esto es mucho más fácil en solución que en los alimentos, donde pueden estar internalizados o localizados en zonas inaccesibles como en estomas, en las irregularidades de la superficie o formando biofilms. En el caso de productos mínimamente procesados, los daños ocasionados durante el procesado pueden ofrecer zonas en que el contacto directo es difícil, permitiendo que los microorganismos puedan sobrevivir y crecer.

A la vista de los resultados obtenidos, el ácido peroxiacético (80 y 120 mg L^{-1}), el peróxido de hidrógeno (5, 10 y 20 mL L^{-1}) y N-acetilo-L-cisteína (10 g L^{-1}) podrían ser posibles alternativas al hipoclorito sódico en la desinfección de frutas y hortalizas mínimamente procesadas ya que no sólo redujeron la población inicial de patógenos, sinó que la mantuvieron a niveles bajos a lo largo de la conservación. Además, el uso del ácido peroxiacético y del peróxido de hidrógeno está autorizado por la FDA. Sin embargo, en este estudio no se tuvo en cuenta la influencia de las diferentes sustancias en la calidad organoléptica de la manzana mínimamente procesada, como cambios de textura, aroma, sabor y color y, por tanto, éste debería ser el siguiente paso antes de utilizar a nivel comercial estas sustancias. Hay que destacar que ninguna de las sustancias eliminó totalmente la población de patógeno y por tanto sería deseable mejorar su efectividad mediante la combinación con otras sustancias y/o otras barreras adicionales.

2.2. Bioconservación

2.2.1. Microorganismos epifitos de fruta

Selección de agentes de biocontrol

El uso de cultivos protectores puede ser una alternativa a los tratamientos químicos o bien un obstáculo adicional al crecimiento de patógenos de transmisión alimentaria en frutas y hortalizas mínimamente procesadas. La flora nativa presente de forma natural en la superficie de frutas y hortalizas puede jugar un importante papel en la seguridad de estos productos (Nguyen-The y Carlin, 1994) mediante la competición con los patógenos de transmisión alimentaria por espacio físico y nutrientes y/o produciendo compuestos antagonistas que afecten negativamente a la viabilidad de los patógenos (Liao y Fett, 2001; Parish *et al.*, 2003). Por ello, se aislaron bacterias y levaduras de frutas enteras y mínimamente procesadas y, juntamente con una colección de antagonistas fúngicos del Laboratorio de Patología del IRTA de Lleida, se testó su capacidad para inhibir tres patógenos de transmisión alimentaria, *E. coli* O157:H7, *Salmonella* y *L. innocua* en manzana y melocotón mínimamente procesados. Se ensayaron un total de 98 y 108 microorganismos frente a *E. coli* O157:H7, utilizado como microorganismo diana, en manzana y melocotón mínimamente procesado, respectivamente, mediante co-inoculación y conservación a 20 °C durante 2 días. El 88.8 % de los microorganismos testados en manzana no tuvo ningún efecto en la población de *E. coli* O157:H7 en comparación con la población de patógeno inoculado solo, sin embargo, 11 microorganismos (CPA-1, CPA-2, CPA-5, 128-M, C9P21, PN5, PN6, EL8, CPA-6, CPA-7 y M174BAL2) redujeron la población del patógeno en, al menos, 1 unidad logarítmica y fueron seleccionados para ensayos posteriores. En el caso de melocotón, el 21.3 % de los microorganismos testados redujo la población de *E. coli* O157:H7 una unidad logarítmica o más. En este caso, los microorganismos seleccionados fueron aquellos 11 que mostraron los mayores valores de reducción (RG4, EL8, PN6, CPA-2, CPA-3, CPA-5, CPA-6, CPA-7, C9P21, 128-M y F-10). Los microorganismos seleccionados de ambas frutas fueron testados frente a *Salmonella* y *L. innocua* en condiciones idénticas (2 días a 20 °C) y frente a *E. coli* O157:H7 a temperaturas de refrigeración (10 días a 5 °C). Ocho de los microorganismos seleccionados en manzana fueron los mismos que los seleccionados en melocotón: PN6, CPA-5, EL8, 128-M, C9P21, CPA-2, CPA-6 y CPA-7. PN6, 128-M, EL-8, C9P21 y CPA-2 pertenecen al género *Pantoea* spp., CPA-5 y CPA-7 son *Pseudomonas* spp. y CPA-6 pertenece a la familia *Enterobacteriaceae*. De los 11 microorganismos seleccionados en manzana, sólo tres redujeron la población de *Salmonella* (128-M, CPA-6 y CPA-7) en, al menos, 1 unidad logarítmica. En cambio, todos ellos redujeron la población de *L. innocua* en más de 1 unidad logarítmica. En melocotón, 10 de los microorganismos seleccionados redujeron la población de *Salmonella* 1 unidad logarítmica o más y sólo ocho redujeron la población de *L. innocua* en las mismas magnitudes. En general, se observaron mayores reducciones de *E. coli* O157:H7 y *Salmonella* en

melocotón que en manzana que pueden atribuirse al mayor crecimiento de los patógenos en melocotón (3.1 y 2.4 unidades logarítmicas, respectivamente) que en manzana (1.6 y 1.4 unidades logarítmicas, respectivamente). En el caso de *L. innocua* no se observaron grandes diferencias entre las frutas ya que su crecimiento fue similar en ambas (3.1 y 2.6 unidades logarítmicas en melocotón y manzana respectivamente). A 5 °C, sólo dos antagonistas, CPA-6 y CPA-7, redujeron la población de *E. coli* O157:H7.

De todos los antagonistas ensayados, dos (CPA-6 y CPA-7) destacaron por su gran capacidad de inhibición de los tres patógenos a 20 °C, con reducciones entre 2.8 y 7.4 unidades logarítmicas, y de *E. coli* O157:H7 a 5 °C no sólo inhibiendo su crecimiento, sino reduciendo su población por debajo del nivel inoculado y, en algún caso, por debajo del nivel de detección del ensayo. Trabajos anteriores han demostrado la efectividad de microorganismos antagonistas frente a patógenos de transmisión alimentaria en manzana. Por ejemplo, Janisiewicz *et al.* (1999b) demostraron que las células del agente de biocontrol *P. syringae* L-59-66, usado para controlar podredumbres causadas por mohos en poscosecha de frutas de pepita, prevenía el crecimiento de *E. coli* O157:H7 en manzanas con heridas conservadas a 24 °C durante 2 días. Posteriormente, Leverentz *et al.* (2006) encontraron siete microorganismos con potencial para reducir *L. monocytogenes* y *Salmonella* en cilindros de manzana 'Golden Delicious'. Las mayores reducciones fueron observadas para *L. monocytogenes* utilizando cepas de *Gluconobacter assai*, *Candida* spp., *Dicosphaerina fagi* y *M. pulcherrima.* Recientemente, el agente de control biológico en poscosecha *C. sake* CPA-1 redujo la población de una mezcla de cinco cepas de *E. coli* en heridas de manzanas 'Golden Delicious' (Abadias *et al.*, 2009).

En condiciones naturales, las concentraciones de patógenos de transmisión alimentaria que se puedan dar en fruta mínimamente procesada serán mucho más bajas a las utilizadas en estos ensayos (10^7 ufc mL^{-1}), por tanto, la efectividad de los agentes de biocontrol puede verse incrementada. El siguiente paso fue la determinación de la dosis mínima inhibitoria de los dos mejores antagonistas frente a los tres patógenos de transmisión alimentaria durante la conservación a 20 °C. En el caso de la cepa antagonista CPA-6, se determinó la concentración mínima para reducir la población de patógenos de transmisión alimentaria inoculados a 10^7 ufc mL^{-1}, que fue de 10^6 ufc mL^{-1}. Para la cepa CPA-7 se realizaron ensayos dosis-respuesta con diferentes concentraciones de patógeno (10^5, 10^6 y 10^7 ufc mL^{-1}) y de antagonista (10^5, 10^6, 10^7 y 10^8 ufc mL^{-1}). Éste presentó una efectividad menor que el CPA-6 ya que necesitaba estar, como mínimo, a la misma concentración del patógeno para ser efectivo (reducción superior a 1.5 unidades logarítmicas), es decir que para una concentración de patógeno de 10^7 ufc mL^{-1}, la concentración de antagonista necesaria sería de 10^7 ufc mL^{-1}.

Aunque en la realización de esta tesis no se pretendía conocer el modo de acción de los antagonistas seleccionados, se realizó una prueba *in vitro* para evaluar si producían sustancias antimicrobianas. Para ello se evaluó la efectividad del cultivo

(medio en el que ha crecido el microorganismo con las células), las células solas y el sobrenadante libre de células de ambos antagonistas frente a los patógenos *E. coli* O157:H7, *Salmonella*, *L. innocua* y *L. monocytogenes* y los alterantes *Pseudomonas marginalis* y *Aeromonas hydrophila*. El cultivo y las células de CPA-6 causaron halos de inhibición para todos los microorganismos indicadores, mientras que en el caso de la cepa CPA-7 no se observó inhibición ni con el cultivo, ni con las células ni con el sobrenadante. Por tanto, aunque únicamente con este estudio no podemos extraer ninguna conclusión del modo de acción de la cepa CPA-6, podemos inferir que es poco probable que la cepa CPA-7 produzca sustancias antimicrobianas en las condiciones ensayadas y, por tanto, la causa de la inhibición podría ser la competición por espacio y nutrientes. Este modo de acción es el atribuido a otros agentes de biocontrol como *P. syringae* (Janisiewicz *et al.*, 1999b) y puede ser ventajoso ya que evita la creación de resistencias por parte de los patógenos y, además, es menos probable que cause problemas en la salud humana. Sin embargo, para conocer exactamente el mecanismo de acción de ambos antagonistas se deberían realizar ensayos adicionales.

Para poder utilizar un agente de biocontrol, éste no debe ser fitopatógeno. Una primera aproximación que permite conocer la fitopatogenicidad de un microorganismo es estudiar su capacidad de producir reacción de hipersensibilidad en la planta del tabaco. Para la realización de esta prueba se inocularon las cepas antagonistas en hojas de tabaco a una concentración de 10^9 ufc mL^{-1}. Las plantas inoculadas se mantuvieron a temperatura ambiente. Asimismo, se utilizó agua destilada como control negativo y *Pantoea ananatis* CPA-3 como control positivo. Ninguna de las dos cepas seleccionadas causó tal reacción a diferencia del control positivos (*P. ananatis*) que causó necrosis en las hojas (Figura 2). Por tanto, las cepas seleccionadas no son fitopatógenas.

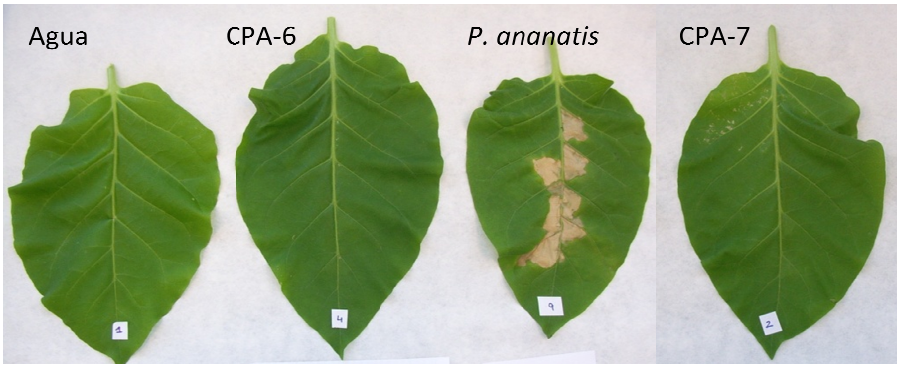

Fig. 2 Reacción de hipersensibilidad producida en planta de tabaco por CPA-6 (10^9 ufc mL^{-1}) y CPA-7 (10^9 ufc mL^{-1}) en comparación del agua y del control positivo *P. ananatis* (10^9 ufc mL^{-1}).

Una vez seleccionados los mejores antagonistas, se procedió a su identificación mediante pruebas bioquímicas realizadas en laboratorio y mediante secuenciación parcial y total de la región RNA 16S. La cepa CPA-6, pertenece a un nuevo género dentro de la familia *Enterobacteriaceae*, próxima a *Obesumbacterium proteus*, *Hafnia alvei*, *Serratia liquefaciens*, *Serratia grimesii*, *Serratia proteomaculans* y *Rhanella aquatilis*.
La cepa CPA-7 se identificó como *Pseudomonas graminis*. Esta especie fue descrita por primera vez por Behrendt *et al.* (1999) como una pseudomonas no fluorescente. Estudios recientes han demostrado también la efectividad de CPA-7 en melón mínimamente procesado frente a una mezcla de cepas de *Salmonella* y una mezcla de cepas de *L. monocytogenes* a 5, 10 y 20 °C (datos no mostrados). Por todo ello, el uso de la cepa CPA-7 ha sido patentada a nivel nacional (Viñas *et al.*, 2010) y se ha solicitado su extensión internacional (Viñas *et al.*, 2011).

Paralelamente se determinó si las cepas antagonistas producían daños en fruta cortada. Para ello se inocularon diferentes concentraciones de ambas cepas (10^6, 10^7 y 10^8 ufc mL^{-1}) en rodajas de melocotón y manzana usando agua destilada como control. Las rodajas inoculadas se conservaron a 10 y 20 °C. Tal y como se se puede observar en la Figura 3, la cepa CPA-6 causó daños visibles en ambas frutas, que además fueron dosis-dependientes. Debido a estos daños, la cepa CPA-6 fue descartada.

A partir de aquí se continuó únicamente con la cepa CPA-7, realizando ensayos semi-comerciales, es decir, ensayos a nivel de laboratorio simulando el procesado industrial. Para ello, se pelaron las manzanas y se cortaron en 10 trozos, se trataron con sustancias antioxidantes, se envasaron en atmósfera modificada pasiva y se conservaron a 5 y 10 °C. La elección de estas temperaturas fue para simular una correcta temperatura de conservación (5 °C) y para simular una temperatura de conservación excesiva y que permitiera el crecimiento de todos los patógenos de transmisión alimentaria. El objetivo de estos ensayos fue comprobar la efectividad del agente de biocontrol seleccionado en presencia de sustancias antioxidantes y el envasado en atmósfera modificada.

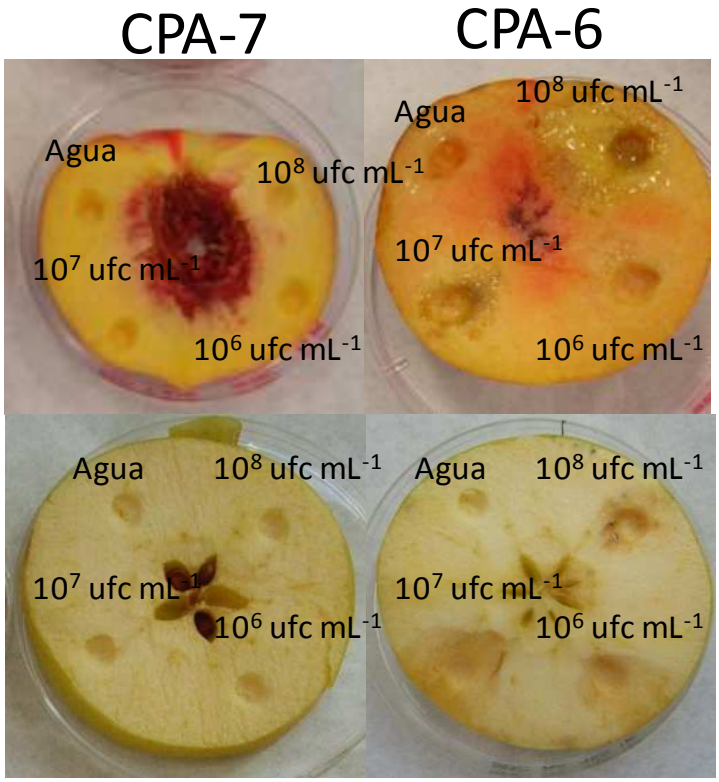

Fig. 3 Aspecto de los pozos producidos en rodajas de melocotón (3 días 20 °C) y de manzana (6 días 20 °C) inoculadas con las cepas CPA-6 (derecha) y CPA-7 (izquierda) a 10^8, 10^7, y 10^6 ufc mL^{-1} en comparación con agua (tratamiento control).

Selección de antioxidante y atmósfera de envasado para la aplicación semi-comercial de CPA-7

Para la aplicación de la cepa antagonista *P. graminis* CPA-7 en condiciones semi-comerciales simuladas, se buscó un tratamiento antioxidante compatible con su aplicación, es decir un tratamiento que no afectara ni a su viabilidad ni a su efectividad. Para ello, los trozos de manzana se trataron con soluciones de ácido ascórbico (2 %), N-acetilo-L-cisteína (NAC, 1 %) o NatureSeal® AS1 (6 %) inoculadas con una mezcla de cuatro cepas de *Salmonella* a 10^5 ufc mL^{-1}, cinco cepas de *L. monocytogenes* a 10^5 ufc mL^{-1} y la cepa antagonista CPA-7 a 10^7 ufc mL^{-1} y se conservaron 2 días a 10 °C. En el caso del ácido ascórbico, las concentraciones de *Salmonella*, *L. monocytogenes* y CPA-7 tras la conservación fueron 0.8, 1.2 y 4.3 unidades logarítmicas inferiores al tratamiento con agua, respectivamente. En cuanto al tratamiento con NAC, la concentración de los tres microorganismos estuvo por debajo del nivel de detección tras la conservación. Sin

embargo, en los ensayos de dinámica poblacional que se habían realizado en la etapa anterior, el tratamiento de manzanas y melocotones mínimamente procesados con ácido ascórbico no tuvo ningún efecto en las poblaciones de *E. coli* O157:H7, *Salmonella* y *L. innocua*. Esta diferencia puede ser debida a la diferente aplicación del patógeno, ya que en los ensayos de dinámica poblacional el patógeno se aplicó con pipeta tras el tratamiento de la fruta con el antioxidante y en estos ensayos se realizó una inoculación mediante inmersión de los trozos de manzana en el inóculo y la sustancia antioxidante. El tratamiento con N-acetilo-L-cisteína ha demostrado con anterioridad capacidad de inhibir patógenos de transmisión alimentaria en diferentes variedades de manzana (Bhagwat *et al.*, 2004; Raybaudi-Massilia *et al.*, 2009). En cambio, el tratamiento con el antioxidante comercial NatureSeal® AS1 no tuvo ningún efecto en la viabilidad microbiana. Por tanto, a continuación se evaluó la efectividad del antagonista frente a *Salmonella* y *L. monocytogenes* en manzanas mínimamente procesadas tratadas con NatureSeal® AS1 conservadas 7 días a 10 °C. Este tratamiento antioxidante no influyó en la efectividad del antagonista frente a ninguno de los dos patógenos, obteniéndose reducciones superiores a 3 unidades logarítmicas al final de la conservación. El antioxidante NatureSeal® AS1 fue seleccionado para los ensayos posteriores.

A continuación se determinó la efectividad de CPA-7 en manzana mínimamente procesada envasada en atmósfera modificada conservada a 10 °C ya que es un aerobio estricto y podría verse afectado por la reducción de O_2 y el aumento de CO_2. Se observó que el envasado en atmósfera modificada no afectó la efectividad de la cepa CPA-7 frente a *Salmonella* (reducción superior a 3 unidades logarítmicas), sin embargo, la reducción de la población de *L. monocytogenes* en atmósfera modificada (2.5 unidades logarítmicas) fue inferior a la reducción en aire (3.8 unidades logarítmicas). Esto puede deberse a que aunque el crecimiento del antagonista no se vio afectado por la atmósfera de envasado hasta que las concentraciones de O_2 alcanzaron valores del 0 % (aproximadamente tras 7 días a 10 °C), el crecimiento de *L. monocytogenes* se vio favorecido gracias a las bajas temperaturas y la baja concentración de oxígeno.

Control biológico en condiciones semi-comerciales

Para la realización de los ensayos semi-comerciales se utilizaron manzanas 'Golden Delicious' cortadas en 10 trozos y tratadas con una solución de NatureSeal® AS1 al 6 % (Figura 4). A continuación se inocularon por inmersión en una solución con cuatro cepas de *Salmonella* y cinco cepas de *L. monocytogenes* conjuntamente o no con la cepa antagonista CPA-7. La concentración de patógenos se redujo de 10^7 ufc mL^{-1} (en los ensayos anteriores) a 10^5 ufc mL^{-1} (que equivale aproximadamente a 10^3 ufc g^{-1}) para simular en lo posible condiciones reales. Aunque la concentración utilizada puede considerarse elevada, no se redujo más para poder llevar a cabo el recuento del patógeno y cuantificar la reducción obtenida por el antagonista. En el caso de la cepa antagonista CPA-7 se ensayaron

dos concentraciones, la mínima concentración efectiva obtenida en ensayos anteriores, es decir la misma concentración que los patógenos (10^5 ufc mL^{-1}) y una concentración superior, 10^7 ufc mL^{-1}. Tras la inoculación, los trozos de manzana se envasaron y se conservaron a 5 y 10 °C.

Cuando el patógeno y antagonista se inocularon al mismo nivel, el antagonista no tuvo ningún efecto sobre las poblaciones de *Salmonella* y *L. monocytogenes* en manzana mínimamente procesada conservada a 5 y 10 °C. Esta diferencia con los resultados anteriores puede atribuirse al cambio de temperatura de conservación, ya que aquí se disminuyó de 20 °C a 5 y 10 °C y, por tanto, la velocidad de crecimiento del antagonista se vio reducida significativamente y su actividad antagonista se vio dificultada. Además, se han incluido otros factores que han podido reducir la efectividad del antagonista, como el uso de sustancias antioxidantes (NatureSeal® AS1) y el envasado en atmósfera modificada.

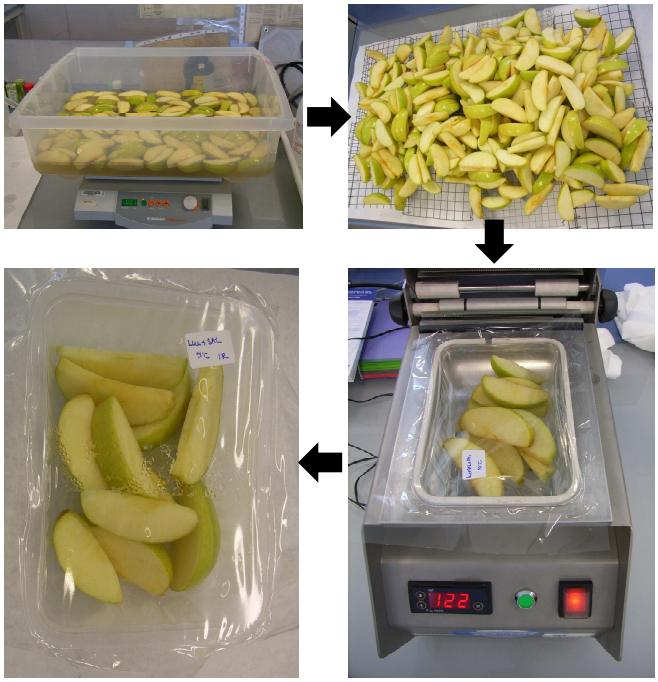

Fig. 4 Inoculación de los trozos de manzana, escurrido y envasado.

Al aumentar la concentración de antagonista hasta 10^7 ufc mL^{-1}, se aumentó su efectividad, obteniendo reducciones significativas de ambos patógenos. La efectividad frente a *Salmonella* fue superior en manzana mínimamente procesada conservada a 10 °C, evitando su crecimiento a lo largo de toda la conservación siendo la población de *Salmonella* 2 unidades logarítmicas inferior respecto a

Salmonella inoculada sola tras 14 días, mientras que a 5 °C no pudo observarse su efecto ya que la población de *Salmonella* se mantuvo constante. En el caso de *L. monocytogenes*, el antagonista fue efectivo a ambas temperaturas de conservación. A 10 °C la reducción del patógeno fue entre 1 y 2 unidades logarítmicas a lo largo del periodo de conservación, mientras que a 5 °C, no se observó reducción del patógeno hasta el séptimo día de conservación. En vista de los resultados, la aplicación de la cepa antagonista CPA-7 en manzana mínimamente procesada podría representar un obstáculo adicional al desarrollo de los patógenos en frutas mínimamente procesadas. Su aplicación tiene un efecto protector evitando el crecimiento de los patógenos de transmisión alimentaria que pudieran llegar a estar presentes en los productos en caso de que la temperatura de conservación aumentara a valores favorables para su crecimiento.

La población de la cepa antagonista CPA-7 aumentó en manzana mínimamente procesada conservada tanto a 5 como a 10 °C en atmósfera modificada aunque el crecimiento más rápido se observó a 10 °C (más de 1.7 unidades logarítmicas). Sin embargo, la población se estabilizó o empezó a disminuir tras 4-7 días a ambas temperaturas, probablemente al alcanzar su población máxima y al agotarse el O_2 en el interior de los envases, ya que la cepa *P. graminis* CPA-7 es aerobia estricta.

Posteriormente, se han realizado ensayos similares en una fruta no ácida como es el melón, donde el agente de biocontrol CPA-7 también ha reducido el crecimiento de *Salmonella* y *L. monocytogenes* a 10 y 20 °C (datos no mostrados). En la bibliografía consultada no se han encontrado trabajos con agentes de biocontrol en condiciones semi-comerciales en fruta mínimamente procesada.

Efecto de la aplicación del agente de biocontrol CPA-7 en la calidad de manzana mínimamente procesada

La aplicación de agentes de biocontrol en fruta mínimamente procesada no debe ocasionar ningún efecto negativo en la calidad del producto a lo largo de la conservación. Por tanto, paralelamente a los ensayos semi-comerciales se evaluó el efecto de la aplicación del antagonista CPA-7 en diferentes parámetros de calidad de la fruta.

La aplicación de la cepa CPA-7 no causó efectos significativos en la calidad de la fruta, siendo todos los parámetros evaluados (calidad visual, pH, contenido en sólidos solubles, acidez titulable, color y firmeza) iguales en muestras inoculadas respecto a las no inoculadas. En la determinación del color, se observó un incremento en los valores de L* a lo largo de la conservación independientemente de la adición del cultivo antagonista, lo que indica un aumento en la luminosidad de los trozos de manzana. El pardeamiento enzimático viene normalmente indicado por una disminución de los valores de L* y un aumento de los valores de a* (Rojas-Grau *et al.*, 2006). Este efecto blanqueante atribuido al antioxidante utilizado, NatureSeal® AS1, ha sido previamente observado por otros autores

(Rößle *et al.*, 2009, 2010; Rupasinghe *et al.*, 2005; Toivonen, 2008). En cuanto a los valores de firmeza, éstos se mantuvieron o incluso aumentaron a lo largo de la conservación tanto en muestras inoculadas como en muestras no inoculadas. Este efecto es también atribuible al uso de NatureSeal® AS1, ya que contiene una alta concentración de calcio (Rico *et al.*, 2007; Rößle *et al.*, 2009).

La composición de la atmósfera en el interior de los envases de manzana mínimamente procesada inoculada con *P. graminis* CPA-7 cambió más rápidamente que en los envases no inoculados a ambas temperaturas. Este efecto podría ser debido a que la aplicación del antagonista CPA-7 provocara un aumento en la respiración del producto. Existen estudios que investigan las respuestas de plantas y/o frutas a la presencia de microorganismos, ya sean patógenos o agentes de biocontrol. Estos estudios muestran el efecto de los microorganismos aumentando la producción de especies reactivas de oxígeno (Heller y Tudzynski, 2011) o incluso, el efecto de los microorganismos en la síntesis de proteínas implicadas en la ruta energía y en el metabolismo de los azúcares en la mitocondria (Chan *et al.*, 2007).

Supervivencia del agente de biocontrol al estrés gástrico simulado

A continuación, se estudió la viabilidad de la cepa CPA-7 a un estrés gástrico simulado a lo largo de la conservación en manzana mínimamente procesada a 5 °C. Si un microorganismo es incapaz de pasar la barrera del estómago es menos probable que cause algún efecto nocivo en la salud humana. En este estudio, *P. graminis* CPA-7 no sobrevivió al estrés gástrico desde el día de inoculación. Asimismo, es interesante remarcar que no existe ninguna referencia respecto a la patogenicidad humana de la especie *P. graminis* y que no crece a partir de 33 °C.

2.2.2. Bacterias ácido-lácticas. Probióticos

Las bacterias ácido-lácticas son microorganismos clasificados como GRAS por la FDA que se han utilizado históricamente para preservar carnes y productos lácteos así como vegetales fermentados. Por tanto, ya que están autorizados en alimentos, su uso como agentes de biocontrol en fruta mínimamente procesada puede ser muy interesante. Así pues, paralelamente a la selección de microorganismos epifitos, se evaluó la efectividad *in vitro* de 123 bacterias ácido-lácticas aisladas de vegetales mínimamente procesados y de productos lácteos utilizando el método de la gota (agar spot test) y observando los halos de inhibición resultantes. Además se determinó el efecto de 31 de estos aislados sobre la población de una mezcla de 5 cepas de *L. monocytogenes* y una mezcla de 4 cepas de *Salmonella* mediante co-inoculación de cilindros de manzana 'Golden Delicious' conservados 2 días a 20 °C. Ninguna de las cepas testadas tuvo ningún efecto, ni *in vitro* ni *in vivo*, por lo tanto se descartaron (datos no mostrados). Otros autores, como Trias *et al.*

(2008), encontraron cepas de BAL efectivas frente a patógenos de transmisión alimentaria *in vitro*.

Las bacterias probióticas, algunas de las cuales son BAL, pueden tener un efecto directo en otros microorganismos, por ejemplo patógenos, ya sea por la producción de sustancias antibacterianas o por competición de recursos limitados. Por tanto, no sólo pueden reforzar la salud, sinó además pueden tener un papel protector frente a patógenos de transmisión alimentaria en los alimentos, actuando como agentes de biocontrol. La aplicación de cultivos probióticos se ha realizado principalmente en productos lácteos, sin embargo su consumo presenta problemas a los individuos con intolerancia a la lactosa o niveles altos de colesterol. Por tanto se están buscando nuevas matrices para la aplicación de estos cultivos beneficiosos. La fruta, en este caso manzana, puede ser un sustrato adecuado por su valor nutricional. Sin embargo, la aplicación de estos cultivos en manzana mínimamente procesada no es tan sencilla ya que el probiótico debe mantener su viabilidad a concentraciones elevadas a lo largo de todo el periodo de conservación. Por tanto, en la realización de esta tesis se estudió la viabilidad de tres cepas probióticas *Lactobacillus acidophilus* LA-5® (CHR HANSEN), *Bifidobacterium animalis* subsp. *lactis* BB-12® (CHR HANSEN) y *Lactobacillus rhamnosus* GG LGG® (Valio) en manzana mínimamente procesada y envasada en atmósfera modificada 2 días a 20 °C. De las tres cepas, únicamente *L. rhamnosus* GG mantuvo su concentración al nivel inoculado coicidiendo con resultados de otros autores (Rößle *et al.*, 2010) y fue, por ello, la cepa seleccionada para los ensayos semi-comerciales.

Ensayos en condiciones semi-comerciales con L. rhamnosus *GG*

Se realizaron ensayos semi-comerciales de forma análoga a los realizados con el agente de biocontrol CPA-7. En este caso, los trozos de manzana se inocularon con *Salmonella* y *L. monocytogenes* conjuntamente o no con *L. rhamnosus* GG a la vez que se aplicó el tratamiento antioxidante con NatureSeal® AS1 al 6 %. La población de patógenos aplicada fue de 10^5 ufc mL^{-1} y la concentración de *L. rhamnosus* GG de 10^8 ufc mL^{-1}. Una vez inoculados y tratados con antioxidante, los trozos de manzana se envasaron en atmósfera modificada y se conservaron a 5 y 10 °C. *L. monocytogenes* fue el patógeno más afectado por la aplicación de la cepa probiótica con 1 unidad logarítmica de reducción respecto al control no tratado con el probiótico a ambas temperaturas. Sin embargo, *L. rhamnosus* GG no fue eficaz contra *Salmonella* a ninguna de las temperaturas. Esta especificidad hacia bacterias gram-positivas podría deberse a la producción de algún tipo de sustancias antimicrobianas similares a las bacteriocinas ya que es conocido que los lactobacilos son capaces de producir sustancias antimicrobianas cuando crecen en medios específicos. No obstante, Silva *et al.* (1987) describieron una sustancia inhibitoria producida por *L. rhamnosus* GG, de bajo peso molecular y activa frente a un gran espectro de bacterias gram-positivas y gram-negativas, pero no frente a

otros lactobacilos y por tanto sería una sustancia diferente a las bacteriocinas. Recientemente, Lee *et al.* (2008) observaron que *L. rhamnosus* GG era un buen antagonista de cuatro cepas diferentes de *E. coli* O157:H7 en medio sólido y líquido debido a la producción de metabolitos antagonistas.

La eficacia del efecto saludable de un probiótico depende del nivel de bacteria inoculada y de su viabilidad a lo largo de la vida útil del producto, por lo tanto el cultivo probiótico *L. rhamnosus* GG fue aplicado en manzana mínimamente procesada a una alta concentración, obteniendo una población inicial de, aproximadamente 10^7 ufc g^{-1}. Además, esta población se mantuvo al nivel inoculado a lo largo de los 28 días de conservación tanto a 5 como a 10 °C. Estudios anteriores han demostrado la supervivencia de este probiótico en manzana 'Breaburn' mínimamente procesada conservada a 2-4 °C (Rößle *et al.*, 2010) y en zumos de naranja y piña conservados a 4 °C (Sheehan *et al.*, 2007) a niveles superiores al mínimo recomendado para ofrecer los beneficios de salud en el momento de consumo (10^6 ufc g^{-1}) (Rivera-Espinoza y Gallardo-Navarro, 2010).

Efecto de la aplicación de L. rhamnosus *GG en la calidad de la manzana mínimamente procesada*

Como se ha comentado anteriormente, la aplicación de microorganismos en fruta mínimamente procesada, ya sea su finalidad principal, el control de patógenos de transmisión alimentaria o bien el efecto probiótico, no debe ocasionar ningún efecto negativo en la calidad del producto a lo largo de la conservación.

La aplicación del probiótico tampoco causó efectos significativos en la calidad de la fruta en ninguno de los parámetros evaluados (calidad visual, pH, contenido en sólidos solubles, acidez titulable, color y firmeza) siendo iguales a las muestras no inoculadas. En la investigación llevada a cabo por Rößle *et al.* (2010) se realizó una evaluación sensorial de manzana mínimamente procesada enriquecida con *L. rhamnosus* GG. En este estudio la aceptabilidad de las muestras inoculadas fue igual a las no inoculadas, sin embargo, las muestras inoculadas con el probiótico presentaron una textura menos firme y un ligero olor láctico.

Supervivencia de microorganismos al estrés gástrico

La literatura muestra que un bajo pH durante el crecimiento bacteriano puede inducir una respuesta de adaptación o habituación al ácido (Champagne y Gardner, 2008). Esta adaptación o habituación microbiana a ambientes subletalmente acídicos podría aumentar su resistencia a condiciones de pH extremos y, por tanto, incrementar la supervivencia de los microorganismos al tránsito a través del estómago. Por ello se estudió si la supervivencia y/o crecimiento de los patógenos y los antagonistas en manzana mínimamente procesada durante su conservación mejoraría su resistencia al estrés gástrico. En el caso de los patógenos una mayor resistencia podría aumentar la probabilidad de colonización intestinal y, por tanto,

su virulencia. De forma contraria, *L. rhamnosus* GG debe ser capaz de sobrevivir al estrés gástrico y llegar al intestino a altas concentraciones para poder llevar a cabo su efecto beneficioso.

La viabilidad de *Salmonella* y *L. monocytogenes* tras el estrés gástrico se redujo drásticamente a lo largo de la conservación de las manzanas mínimamente procesadas. En el caso de *Salmonella* la viabilidad pasó de, aproximadamente, un 30 % a menos del 5 % y de un 15 % a inferior del 2 % para *L. monocytogenes* a partir del cuarto día de conservación. En cambio, la viabilidad de *L. rhamnosus* GG tras la exposición al estrés gástrico disminuyó de forma progresiva, manteniéndose por encima de 10^6 ufc g^{-1} hasta los 14 días de conservación de la manzana mínimamente procesada. Los resultados demuestran que no ha existido adaptación al ácido por parte de los patógenos en las condiciones estudiadas.

Uno de los factores influyentes en la adaptación de las bacterias al ácido es el tiempo de exposición. La adaptación por un corto periodo (unas horas) ayuda a resistir valores de pH letales posteriores, en cambio, si la adaptación es por un periodo más largo (12 h) puede provocar mayor sensibilidad por parte de las bacterias (Phan-Thanh y Montagne,1998). Otro parámetro en la adaptación al ácido es el valor de pH del ambiente de habituación, aunque los rangos de pH que pueden conducir a la incrementada resistencia al ácido o el 'óptimo pH de adaptación' aún no se han estudiado extensamente. En el caso de *L. monocytogenes*, diversos investigadores han observado que tiene una mayor resistencia tras la habituación a pH entre 5.0 y 6.0 (Gahan *et al.*, 1996; Koutsoumanis y Sofos, 2004; Lou y Yousef, 1997; Phan-Thanh *et al.*, 2000) pero no a pH de 4.5 o inferior (Koutsoumanis y Sofos, 2004). Para *Salmonella*, los valores de pH que han inducido a habituación se encuentran entre 4.0 y 5.0, mientras que a 5.5 o valores superiores ya no existe habituación (Koutsoumanis y Sofos, 2004; Lee *et al.*, 1995). Por último, otro factor importante en el fenómeno de habituación al ácido es la temperatura. Álvarez-Ordóñez *et al.* (2010) y Samelis *et al.* (2003) demostraron que las células de *Salmonella* Typhimurium que habían crecido a 10 °C tenían una resistencia al ácido inferior que aquellas que habían crecido a 30 °C. Así pues, los datos de la bibliografía existente respaldan nuestros resultados.

En el caso de los probióticos, la resistencia al tránsito gastrointestinal es un criterio importante de selección. En este caso la incubación de *L. rhamnosus* GG en un medio ácido, manzana mínimamente procesada, conservada a 5 °C, no aumentó su viabilidad a un estrés gástrico simulado, aunque mantuvo una alta viabilidad hasta los 14 días. Champagne y Gardner (2008) observaron grandes reducciones de viabilidad en varias cepas probióticas tras su incubación durante 2 h a 37 °C a pH 2.0.

3. CONSIDERACIONES FINALES

Esta tesis pretende conseguir un producto alimentario de consumo creciente, como es la fruta mínimamente procesada, más seguro para el consumidor, de mayor calidad microbiológica y utilizando tecnologías más respetuosas con el medio ambiente. Los estudios realizados se han centrado en manzanas y melocotones ya que Lleida es una de las zonas de mayor producción de España.

¿Son las manzanas y melocotones mínimamente procesados un sustrato adecuado para el crecimiento de patógenos de transmisión alimentaria?

Tal y como se ha visto, los tres patógenos de transmisión alimentaria utilizados en los ensayos han sido capaces de crecer tanto en manzana como en melocotón cortados a lo largo de la conservación a temperaturas de 10 °C y superiores, siendo el control de temperatura de conservación una intervención eficaz para evitar el crecimiento. Por tanto, si estos patógenos llegaran de forma accidental al producto, podrían crecer exponencialmente si la temperatura de almacenaje fuera superior a la adecuada para su conservación.

¿Existe alguna posible alternativa al hipoclorito sódico en la desinfección de fruta mínimamente procesada?

El ácido peroxiacético, el peróxido de hidrógeno y el N-acetilo-L-cisteína podrían ser algunas de las sustancias alternativas al hipoclorito. No obstante, son necesarios más estudios y existen impedimentos de índole legislativo a superar antes de su aplicación industrial.

¿Es la bioconservación un posible método de control de patógenos en fruta mínimamente procesada?

En esta tesis, se ha estudiado con éxito la posible aplicación de agentes de biocontrol para reducir el crecimiento de las cepas de patógenos de transmisión alimentaria utilizadas en los ensayos como técnica complementaria al proceso de desinfección. La utilización de microorganismos antagónicos ha demostrado que puede ser una alternativa viable para el control de patógenos de transmisión alimentaria en fruta mínimamente procesada. Gracias a los resultados prometedores, incluso en ensayos semi-comerciales, una de las cepas estudiadas, *P. graminis* CPA-7, ha sido patentada. Sin embargo, para que la bioconservación sea exitosa es necesario, en primer lugar, su aprobación a nivel legislativo. En segundo lugar, los consumidores deben aceptar su aplicación en los productos. Esta condición podría no ser problemática ya que existen gran variedad de alimentos que utilizan microorganismos en su elaboración y conservación.

La manzana mínimamente procesada, ¿alimento probiótico?

Los resultados obtenidos en esta tesis avalan la posible aplicación de una bacteria probiótica, *L. rhamnosus* GG en manzana mínimamente procesada. De este modo, se podría incrementar el valor añadido de la fruta mínimamente procesada al mismo tiempo que se daría una alternativa de consumo de probióticos a las personas con intolerancia a la lactosa.

En definitiva…

En la producción de fruta mínimamente procesada es importante reducir el riesgo de intoxicaciones alimentarias mediante la implementación de planes como los de Buenas Prácticas Agrícolas, Buenas Prácticas de Producción (BPA y BPP) y el programa de Análisis de Peligros y Puntos de Control Crítico (APPCC), así como el mantenimiento de la cadena de frío a temperaturas de refrigeración. Sin embargo, con la realización de esta tesis se han demostrado que existen nuevas estrategias de intervención altamente eficaces para mejorar la calidad microbiológica de la fruta mínimamente procesada. Para lograr la mayor eficacia de estas estrategias deberían aplicarse de forma combinada, la desinfección en primer lugar para reducir la población microbiana inicial en el producto y, en segundo lugar la aplicación de un agente de biocontrol (CPA-7) para reducir el crecimiento de los patógenos de transmisión alimentaria que pudieran haber llegado al producto accidentalmente.

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V. CONCLUSIONES

En base a los resultados obtenidos y a su interpretación se pueden extraer las siguientes conclusiones:

Respecto a la supervivencia y crecimiento de patógenos de transmisión alimentaria en manzana y melocotón mínimamente procesados:

1. *E. coli* O157:H7, *Salmonella* y *Listeria innocua* crecieron más de 2 unidades logarítmicas en manzanas 'Golden Delicious' (pH 4.16±0.25) y melocotones 'Elegant Lady' (pH 3.73±0.28) mínimamente procesados conservados a 20 y 25 °C. Al reducir la temperatura a 10 °C, la población de *E. coli* O157:H7 y *Salmonella* aumentó aproximadamente 1 unidad logarítmica y la de *L. innocua* más de 1.5 unidades logarítmicas. A 5°C sólo *L. innocua* fue capaz de crecer tanto en manzana como en melocotón.
2. Los tres patógenos estudiados crecieron de forma similar en tres variedades de manzana ('Golden Delicious', 'Granny Smith' y 'Shampion') conservadas a 25 °C y 5 °C. Sin embargo, la población de *L. innocua* redujo su crecimiento en manzanas 'Shampion' conservadas a 5 °C.
3. Los tres patógenos estudiados crecieron exponencialmente en todas las variedades de melocotón utilizadas conservadas a 25 °C. Los mayores incrementos se observaron en melocotones 'Royal Glory' y 'Diana', seguidos de los 'Elegant Lady' y por último 'Plácido'. A 5 °C, las poblaciones de *E. coli* y *Salmonella* se redujeron en todas las variedades utilizadas y la población de *L. innocua* aumentó en melocotones 'Elegant Lady' pero se redujo en melocotones 'Royal Glory' y 'Diana'.
4. Los tratamientos antioxidantes estudiados (ácido ascórbico al 2 % y el producto comercial NatureSeal® AS1 al 6 %) y el envasado con la atmósfera modificada pasiva ensayada no afectó a la supervivencia y/o crecimiento de los patógenos de transmisión alimentaria en manzanas y melocotones de IV gama.

Las manzanas y melocotones mínimamente procesados, a pesar de tratarse de productos ácidos, son un medio adecuado para el crecimiento de los patógenos de transmisión alimentaria siendo la temperatura de conservación el factor más importante para prevenir su crecimiento, por lo que estos productos deben conservarse a temperaturas inferiores a 10 °C. Asimismo es necesario centrar los esfuerzos en encontrar variedades que posean compuestos que inhiban el crecimiento de patógenos o en las que su crecimiento no sea tan acusado y en hallar nuevas estrategias de intervención para el control de patógenos y así obtener productos seguros.

Respecto a las sustancias antimicrobianas alternativas al hipoclorito sódico:

1. El bicarbonato sódico (10, 50 y 100 g L^{-1}), el bicarbonato potásico (10, 50 y 100 g L^{-1}), la vainillina (3, 6 y 12 g L^{-1}) y la bromelaína (0.1, 1, 10, 100 y 1000 mg L^{-1}) no redujeron las poblaciones de *E. coli* O157:H7 y *L. innocua* en ensayos *in vitro*. El carvacrol (875 y 1500 mg L^{-1}) y el ácido peroxiacético (20, 80 y 120 mg L^{-1}), redujeron ambas poblaciones por debajo del límite de detección (2.5 x 10^3 ufc mL^{-1}), y las reducciones obtenidas con el carbonato sódico, 10, 50 y 100 g L^{-1}; el carbonato potásico, 10, 50 y 100 g L^{-1}; el peróxido de hidrógeno, 5, 10 y 20 mL L^{-1}; el N-acetilo-L-cisteína, 2.5, 5 y 10 g L^{-1}; el Citrox, 5 mL L^{-1} y el quitosano, 10 g L^{-1} fueron dependientes de la dosis utilizada.

2. En los ensayos en manzana mínimamente procesada la efectividad del carvacrol fue inferior a la desinfección con agua. La desinfección con vainillina a una concentración de 12 g L^{-1} fue tan o más efectiva que la desinfección con hipoclorito sódico (100 mg L^{-1} de cloro libre, pH 6.5) reduciendo la población de *E. coli* O157:H7, *Salmonella* y *Listeria* spp, tanto inicialmente como a lo largo de la conservación a 10 °C. Sin embargo, se descartó la posibilidad de usar la vainillina como desinfectante por los sabores y aromas que puede provocar en el producto a dicha concentración.

3. El tratamiento con el producto comercial Citrox fue tan efectivo como el tratamiento con hipoclorito sódico reduciendo y manteniendo las poblaciones de *E. coli* O157:H7, *Salmonella* y *Listeria* spp. a niveles bajos. No obstante, este desinfectante alteró el color de la manzana tratada, por tanto, no es un producto apto para manzana mínimamente procesada.

4. Los tratamientos de ácido peroxiacético (80 y 120 mg L^{-1}), peróxido de hidrógeno (5, 10 y 20 mL L^{-1}) y N-acetilo-L-cisteína (10 g L^{-1}) fueron tan o más efectivos que la desinfección con hipoclorito sódico reduciendo la población y posterior crecimiento de los tres patógenos estudiados.

5. No se encontraron células viables de ninguno de los patógenos estudiados en las soluciones de ácido peroxiacético, peróxido de hidrógeno, Citrox e hipoclorito sódico tras la desinfección de los trozos de manzana mínimamente procesada.

El peróxido de hidrógeno (5, 10 y 20 mL L^{-1}), el ácido peroxiacético (80 y 120 mg L^{-1}) y el N-acetilo-L-cisteína (10 g L^{-1}) podrían utilizarse como alternativas al cloro en la desinfección de frutas y hortalizas, aunque no son capaces de eliminar totalmente los patógenos. Sin embargo, el tratamiento con N-acetilo-L-cisteína no previene la contaminación cruzada. Antes de su utilización, se debe estudiar el efecto de estas sustancias en la calidad del producto. El

tratamiento antimicrobiano con estas sustancias debería realizarse en combinación con otros métodos de control para evitar el crecimiento de los posibles patógenos que hayan resistido a la desinfección.

Respecto a la bioconservación de fruta mínimamente procesada con microorganismos epifitos de fruta:

1. El 11.2 % y el 21.3 % de los microorganismos testados frente a *E. coli* O157:H7 en manzana y melocotón mínimamente procesados, respectivamente, redujeron la población del patógeno en, al menos, 1 unidad logarítmica respecto al control tras 2 días de conservación a 20 °C.
2. Dos microorganismos, las cepas CPA-6 y CPA-7, aisladas de manzana mínimamente procesada y de la superficie de manzana entera, respectivamente, mostraron gran efecto antagonista contra *E. coli* O157:H7, *Salmonella* y *L. innocua* tanto en manzana como en melocotón mínimamente procesados conservados 2 días a 20 °C y contra *E. coli* O157:H7 a temperaturas de refrigeración (5 °C).
3. A 20 °C, la dosis mínima efectiva de la cepa CPA-6 fue de 10^6 ufc mL^{-1} para una concentración de patógeno de 10^7 ufc mL^{-1}. En cambio, la cepa CPA-7 necesitó estar a la misma concentración de patógeno para ser efectiva.
4. La cepa CPA-6 fue identificada como una especie nueva dentro de la familia de las *Enterobacteriaceae* y la cepa CPA-7 pertenece a la especie *P. graminis.*
5. Ninguna de las dos cepas causó reacción de fitotoxicidad en la planta del tabaco. No obstante, la cepa CPA-6 causó daños visibles cuando se aplicó en fruta cortada y por tanto se desestimó para futuras investigaciones.
6. El tratamiento de manzana mínimamente procesada con ácido ascórbico (2 %) y N-acetilo-L-cisteína (1 %) afectó negativamente a la viabilidad del antagonista *P. graminis* CPA-7. Mientras que el antioxidante comercial NatureSeal® AS1 no afectó ni a su viabilidad ni a su efectividad, por tanto es un antioxidante compatible con la aplicación del antagonista.
7. El envasado en atmósfera modificada pasiva no afectó a la viabilidad de la cepa antagonista *P. graminis* CPA-7. Si embargo, su efectividad frente a *L. monocytogenes* fue inferior respecto al envasado en aire.
8. La aplicación semi-comercial de la cepa CPA-7 en manzana mínimamente procesada tratada con NatureSeal® AS1, envasada en atmósfera modificada pasiva y conservada a 5 y 10 °C no fue efectiva cuando se inoculó a la

misma concentración que el patógeno (una mezcla de 4 cepas de *Salmonella* y de 5 cepas de *L. monocytogenes*), pero sí aplicado a una concentración superior en dos unidades logarítmicas. La efectividad frente a *Salmonella* fue mayor a 10 °C evitando su crecimiento, con una diferencia de 2 unidades logarítmicas respecto al patógeno inoculado solo tras 14 d. En el caso de *L. monocytogenes*, el antagonista fue efectivo a ambas temperaturas de conservación, reduciendo la población del patógeno entre 1 y 2 unidades logarítmicas a lo largo del periodo de conservación.

9. La aplicación del agente de biocontrol, *P. graminis* CPA-7 no afectó al aspecto visual ni a los parámetros de calidad fisicoquímica (color, pH, firmeza, sólidos sobles y acidez titulable) evaluados en manzana mínimamente procesada tratada con NatureSeal® AS1 y envasada en atmósfera modificada a lo largo de la conservación a 5 y 10 °C.
10. El agente de biocontrol *P. graminis* CPA-7 no sobrevivió tras 2 h de incubación en jugo gástrico simulado a 37 °C.

Por tanto, el control biológico podría ser un método de control novedosa. La aplicación del antagonista CPA-7 en manzanas mínimamente procesadas tuvo un efecto protector evitando el crecimiento de los patógenos de transmisión alimentaria cuando aumentó la temperatura de conservación. Por ello, podría ser útil para ser aplicado como una barrera adicional en la conservación de este tipo de producto.

Respecto a la bioconservación de fruta mínimamente procesada con bacterias ácido lácticas y la aplicación de bacterias probióticas:

1. De las 123 bacterias ácido lácticas aisladas ninguna mostró capacidad antagonista frente a patógenos de transmisión alimentaria ni *in vitro* ni *in vivo*.
2. De tres cepas probióticas, *L. acidophilus* LA-5® (CHR HANSEN), *B. animalis* subsp. *lactis* BB-12® (CHR HANSEN) y *L. rhamnosus* GG, inoculadas en manzana mínimamente procesada conservada 2 días a 20 °C, únicamente *L. rhamnosus* GG mantuvo su concentración al nivel inoculado, y fue, por tanto, la cepa seleccionada para los ensayos semi-comerciales.
3. La aplicación de *L. rhamnosus* GG en manzana mínimamente procesada tratada con NatureSeal® AS1 y conservada en atmósfera modificada pasiva a 5 y 10 °C no tuvo ningún efecto en la población de *Salmonella*, en cambio redujo la población de *L. monocytogenes* 1 unidad logarítmica.
4. La población de *L. rhamnosus* GG se mantuvo al nivel inoculado en manzana mínimamente procesada, por encima del valor mínimo de

10^6 ufc g^{-1}, necesario para reivindicar su efecto probiótico, durante 28 días de conservación a 5 y 10 °C.

5. La aplicación de la bacteria probiótica *L. rhamnosus* GG no afectó a los parámetros de calidad evaluados en manzana mínimamente procesada.

Por tanto, *L. rhamnosus* GG podría ser una cepa probiótica apta para ser utilizada en manzanas mínimamente procesadas ya que mantuvo su viabilidad a lo largo de la conservación y no produjo defectos de calidad. Además, este probiótico presentó un papel protector evitando el crecimiento de *L. monocytogenes*.

Respecto a la supervivencia de microorganismos al estrés gástrico

1. La viabilidad al estrés gástrico de *Salmonella* pasó de, aproximadamente, un 30 % inicial a menos del 5 % a partir del cuarto día de conservación en manzana mínimamente procesada a 5 °C.
2. La viabilidad inicial al estrés gástrico de *L. monocytogenes* fue de un 15 %. Esta viabilidad se redujo drásticamente (por debajo del 2 %) a partir del cuarto día de conservación a 5°C.
3. La viabilidad de *L. rhamnosus* GG fue decreciendo paulatinamente manteniéndose por encima de 10^6 ufc g^{-1} hasta los 14 días de conservación de manzana mínimamente procesada.

Salmonella y *L. monocytogenes* no se adaptaron al estrés ácido tras inocularse y mantenerse en manzana mínimamente procesada tratada con antioxidante y envasada en atmósfera modificada pasiva conservada a 5 °C. La cepa probiótica *L. rhamnosus* GG mantuvo su viabilidad tras el estrés gástrico hasta los 14 días de conservación a 5 °C.

VI. PERSPECTIVAS DE FUTURO

A partir de los estudios llevados a cabo y los resultados obtenidos en esta tesis pueden derivarse varios estudios:

1. Seleccionar aquellas variedades de fruta que muestren un efecto inhibitorio al crecimiento de patógenos de transmisión alimentaria, averiguar los componentes responsables y optimizar el uso de estas variedades para la industria de IV gama.
2. Estudiar el efecto de diferentes atmósferas modificadas activas en el crecimiento de patógenos de transmisión alimentaria en manzana y melocotón mínimamente procesados.
3. Evaluar el efecto de los agentes desinfectantes peróxido de hidrógeno, ácido peroxiacético y N-acetilo-L-cisteína en la calidad de la fruta mínimamente procesada, incluyendo la evaluación por un panel sensorial.
4. Estudiar posibles aplicaciones de la cepa antagonista *Pseudomonas graminis* CPA-7 en otras frutas y hallar las mejores condiciones para su aplicación (método de aplicación, tratamiento antioxidante y atmósfera de envasado).
5. Analizar la calidad organoléptica de la fruta tratada con la cepa antagonista, incluyendo la evaluación por un panel de cata, así como calidad nutricional.
6. Realizar estudios toxicológicos de la cepa CPA-7.
7. Optimizar la producción y formulación de *P. graminis* CPA-7 para su aplicación comercial.
8. Dentro del concepto de tecnología de barreras, investigar la posibilidad de aplicar la cepa antagonista en fruta tratada con las sustancias desinfectantes propuestas en esta tesis.