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Microbial safety of lettuce: foodborne pathogens incidence, their pathogenic potential and biopreservative stratagies

Márcia Patrícia de Sousa Oliveira

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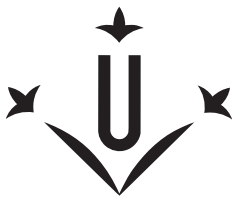

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Universitat de Lleida
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Microbial safety of lettuce: foodborne pathogens incidence, their pathogenic potential and biopreservative strategies

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Aos meus pais, família e amigos,
Pelos braços abertos em todas as idas e voltas.

It always seems impossible until it's done.
Nelson Mandela

Sólo cabe progresar cuando se piensa en grande,
Sólo es posible avanzar cuando se mira lejos.
José Ortega y Gasset

Tot està per fer i tot és possible.
Miquel Martí i Pol

Se estiver tudo errado, comece novamente.
Se estiver tudo certo, continue.
Se sentir saudades, mate-a.
Se perder um amor, não se perca.
Mas, se o achar, segure-o.
Fernando Pessoa

Nine years so full that memories become confused, but some milestones have been unforgettable.

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Nueve años tan llenos que los recuerdos se confunden, pero algunos hitos han sido inolvidables.

Gracias a todos los que caminaron junto a mí hasta llegar al final de esta meta!

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Summary

Fresh-cut vegetables harbor large and diverse populations of microorganisms, such as food-spoiling bacteria, yeasts and fungi. They may also harbor human pathogenic bacteria, such as *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7. Many factors can contribute to the contamination of fresh and fresh-cut produce with human pathogens. Thus, specific measures and interventions should be studied and implemented to prevent and/or minimize the risk of contamination.

The research described in this thesis focuses on determining the influence of field management practices and processing and storage conditions on the microbial quality of fresh-cut lettuce, as well as on studying mitigation strategies to enhance its safety.

Specifically, the effect of the production system (organic or conventional) on the microbiological quality of fresh lettuce was investigated (**Chapter I**). Examination of the samples by principal component analysis revealed a distinct pattern for each production system, with the greatest difference observed for *Enterobacteriaceae* counts. Furthermore, *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* were not detected in any organic or conventional lettuce samples analyzed (n=72).

As a consequence of possible field contamination by foodborne pathogens (FBPs), the transfer and persistence of *L. innocua* and *E. coli* O157:H7 on lettuce leaves and in soil during fall and spring was evaluated using different artificial contaminated irrigation methods (surface and sprinkler) and compost (**Chapter II**). Contamination of *L. innocua* and *E. coli* O157:H7 via soil to the lettuce surface occurred during the growth process, probably through contact with soil, contaminated compost or contaminated surface irrigation water. Both pathogens survived for at least 9 weeks in soil exposed to environmental conditions.

The study described in **Chapter III** assessed the ability of *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 to grow on shredded lettuce packaged in three different atmospheric conditions, created using films of different permeabilities, at two storage temperatures. The composition of the storage atmospheres generated within the packages at refrigeration temperature (5 °C) did not have a significant effect on the survival and growth of any of the three FBPs, while at 25 °C, the growth of *Salmonella* and *E. coli* O157:H7 on lettuce was slightly higher in the less permeable packaging film.

The pathogenic potential of two *S.* Typhimurium DT104 strains (lettuce and carcass isolates) was examined in **Chapter IV**, aiming to measure their ability to survive in a simulated gastrointestinal tract system and to adhere to and invade differentiated Caco-2 cells after sequential incubation into soil, lettuce and cut lettuce stored under modified atmosphere packaging (MAP) conditions. The most important outcome of this study was that sequential incubation of *S.* Typhimurium in soil and lettuce slightly increased the capability to survive the simulated gastric fluid and increased the capability to grow in the simulated intestinal fluid but decreased the capacity for epithelial attachment and invasion.

This thesis also examines the effect of a biopreservation method, consisting in enhancing the native microbiota of lettuce submitted to different pre-conditioning steps, on the survival of *L. monocytogenes* and *E. coli* O157:H7 (**Chapter V**). The study was carried out with conventional and organically produced lettuce. Despite noticeable differences in the initial microbiota load, both pathogens behaved similarly on shredded conventional and organic lettuce during the first days of the study. At the end of the storage period, *L. monocytogenes* seemed to be affected by the native microbiota on organic lettuce, whereas *E. coli* O157:H7 seemed to be affected by the native microbiota on conventional lettuce.

Finally, in **Chapter VI**, the addition of biopreservatives as a method to control or reduce FBPs in fresh-cut lettuce was studied. First, several native microorganisms were isolated and evaluated as putative antagonists against FBPs on lettuce. Next, the best putative antagonist was identified and its effectiveness was compared with *Pseudomonas graminis* CPA-7, nisin and two bacteriophage solutions (Listex P100 and Salmonelex) on fresh-cut lettuce under simulated commercial conditions. The best putative antagonist was identified as *Pseudomonas* sp. (strain M309), which had an antagonistic effect against *S. enterica* and *E. coli* O157:H7 on lettuce discs at refrigeration temperatures. However, this antagonist did not reduce *L. monocytogenes*. The addition of the biopreservative cultures did not significantly reduce the *Salmonella* or *L. monocytogenes* populations under simulated commercial conditions. Treatment with nisin was effective in reducing the initial *L. monocytogenes* population until the end of the storage period. Nisin washing and CPA-7 strain achieved significantly different results to the other treatments.

Overall, the results of this thesis can help to better understand the importance of maintaining the microbial quality of fresh-cut vegetables, which depends on the good hygiene practices during growth and processing from field to consumer, effective washing and decontamination, strict temperature control and appropriate packaging.

Resumen

Las hortalizas mínimamente procesadas contienen grandes y diversas poblaciones de microorganismos, tales como bacterias, levaduras y mohos que pueden llegar a causar su deterioro. Por otra parte, también podrían albergar patógenos humanos, tales como, *Salmonella* spp, *Listeria monocytogenes* y *Escherichia coli* O157:H7. Existen muchos factores que pueden contribuir a la contaminación de los productos frescos y mínimamente procesados con patógenos humanos. Por lo tanto, debe considerarse y aplicarse medidas e intervenciones específicas para prevenir y/o reducir al mínimo el riesgo de contaminación.

La investigación descrita en esta tesis se enfoca en la determinación de la influencia de las prácticas de manejo del cultivo, procesamiento y condiciones de almacenamiento en la calidad microbiológica de la lechuga mínimamente procesada y en el estudio de las estrategias de mitigación para mejorar su seguridad.

En concreto, se ha estudiado el efecto de los sistemas de producción, orgánico o convencional, en la calidad microbiológica de lechuga fresca (**Capítulo I**). De las muestras analizadas por análisis de componentes principales se observó un patrón que diferencia los dos sistemas de producción, siendo la mayor diferencia la concentración de *Enterobacteriaceae*. Además, no se detectó *E. coli* O157:H7, *Salmonella* spp. y *L. monocytogenes* en las muestras de lechuga orgánicas y convencionales analizadas (n=72).

Como consecuencia de la posible contaminación en campo por patógenos de transmisión alimentaria (PTA), se evaluó la transferencia y la persistencia de *L. innocua* y *E. coli* O157:H7 en las hojas de lechuga y en el suelo durante el otoño y la primavera, utilizando diferentes métodos de riego contaminado artificialmente (manta y aspersión) y el compost (**Capítulo II**). La contaminación de *L. innocua* y *E. coli* O157:H7 a través del suelo a las hojas de lechuga se produjo durante el proceso de crecimiento más probablemente a través de contacto con el suelo, el compost contaminado o agua contaminada por el riego a manta. Ambos patógenos fueron capaces de sobrevivir en el suelo expuestos a las condiciones ambientales durante al menos 9 semanas.

El estudio llevado a cabo en el **Capítulo III** fue el de evaluar la capacidad de *L. monocytogenes*, *Salmonella* y *E. coli* O157:H7 para crecer en lechuga mínimamente procesada y envasada en tres diferentes condiciones de atmósfera, creada por la utilización

de películas con diferentes permeabilidades a dos temperaturas de almacenamiento. La composición de la atmósfera de almacenamiento generada dentro de los diferentes envases a temperatura de refrigeración (5 ºC) no tuvo ningún efecto significativo en la supervivencia y crecimiento de los tres PTA, mientras que a 25 ºC, el crecimiento de *Salmonella* y *E. coli* O157:H7 en lechuga fue ligeramente superior (0.50-1.00 log ufc/g) cuando se envasó con la película menos permeable.

Se examinó el potencial patogénico de dos cepas de *S.* Typhimurium DT104 (aislados de lechuga y carcasa) en el **Capítulo IV**, con el objetivo de medir su capacidad para sobrevivir al tracto gastrointestinal simulado y de adherirse e invadir a las células diferenciadas Caco-2, después de la incubación secuencial en el suelo, lechuga y lechuga cortada almacenada en atmosfera modificada (MAP). El resultado más importante de este estudio fue que la incubación secuencial de *S.* Typhimurium en el suelo y lechuga aumentó ligeramente su capacidad de sobrevivir al fluido gástrico simulado y aumentó la capacidad de crecer en el fluido intestinal simulado. Pero disminuyó la capacidad de adhesión e invasión epitelial.

En esta tesis también se evaluó el efecto de aumentar la microbiota de lechuga sometida a las diferentes etapas de pre-acondicionamiento en la supervivencia de *L. monocytogenes* y *E. coli* O157:H7 como método bioconservante (**Capítulo V**). El estudio se llevó a cabo con lechuga convencional y orgánica. Los patógenos se comportaran de manera similar en lechuga convencional y orgánica mínimamente procesada durante los primeros días a pesar de las diferencias en la concentración inicial de la microbiota. Al final del período de almacenamiento, la población de *L. monocytogenes* parecía verse afectada por la microbiota de lechuga orgánica, mientras que la de *E. coli* O157:H7 se vio afectada por la microbiota de lechuga convencional.

Por último, en el **Capítulo VI**, se ha estudiado el uso de la adición de bioconservantes como método para controlar o reducir los PTA en lechuga mínimamente procesada. En primer lugar, se aislaron y se evaluaron varios microorganismos como posibles antagonistas contra PTA en lechuga. En segundo lugar, el mejor antagonista fue identificado y su eficacia fue comparada con el bioconservante *Pseudomonas graminis* CPA-7, nisina y dos soluciones de bacteriófagos (Listex P100 y Salmonelex) en lechuga mínimamente procesada, simulando las condiciones comerciales. El mejor antagonista fue identificado como *Pseudomonas* sp. (cepa M309) y mostró efecto antagonista contra *S. enterica* y *E. coli* O157:H7 en discos de lechuga a temperaturas de refrigeración. Sin embargo, no redujo *L. monocytogenes*. La adición de los bioconservantes no produjo una reducción significativa de la población de *Salmonella* y *L. monocytogenes* en condiciones que simulan la aplicación comercial. El tratamiento con nisina fue eficaz en la reducción de la población inicial de *L. monocytogenes* hasta el final del período de almacenamiento. El tratamiento con nisina y la cepa CPA-7 fue significativamente diferente de los otros tratamientos.

Los resultados generales obtenidos en esta tesis nos pueden ayudar a comprender mejor la importancia de mantener la calidad microbiológica de las hortalizas mínimamente procesadas, ya que se precisa unas buenas prácticas de higiene durante el crecimiento y el procesamiento desde el campo al consumidor para garantizar la calidad del producto final.

Resum

Les hortalisses mínimament processades contenen grans i diverses poblacions de microorganismes, com bacteris, llevats i floridures que causen el deteriorament. D'altra banda, també podrien albergar bacteris patògens humans, com *Salmonella* spp., *Listeria monocytogenes* i *Escherichia coli* O157:H7. Hi ha molts factors que poden contribuir a la contaminació dels productes frescs i mínimament processats amb patògens humans. Per això, s'ha de considerar i aplicar les mesures i intervencions específiques per prevenir i/o reduir al mínim el risc de contaminació.

La investigació descrita en aquesta tesi es centra en la determinació de la influència de les pràctiques de maneig del cultiu, processament i les condicions d'emmagatzematge en la qualitat microbiològica de l'enciam mínimament processat i en l'estudi de les estratègies de mitigació per millorar la seva seguretat.

En concret, s'ha estudiat l'efecte del sistema de producció, orgànica o convencional, de la qualitat microbiològica d'enciam fresc (**Capítol I**). De les mostres analitzades per anàlisi de components principals es va observar un patró amb els dos sistemes de producció, sent la major diferència la concentració de *Enterobacteriaceae*. A més, *E. coli* O157:H7, *Salmonella* spp. i *L. monocytogenes* no es van detectar en les mostres d'enciam orgànics i convencionals analitzades (n=72).

Com a conseqüència de la possible contaminació al camp per patògens transmesos pels aliments (PTA), es va avaluar la transferència i la persistència de *L. innocua* i *E. coli* O157:H7 en els fulls d'enciam i en el sòl durant la tardor i la primavera utilitzant diferents mètodes de reg contaminats artificialment (superfície i aspersió) i el compost (**Capítol II**). La contaminació de *L. innocua* i *E. coli* O157:H7 a través del sòl a les fulles de l'enciam es va produir durant el procés de creixement, més probablement, a través de contacte amb el sòl, el compost contaminat o l'aigua contaminada per el reg de superfície. Tots dos patògens van ser capaços de sobreviure al sòl exposats a les condicions ambientals durant almenys 9 setmanes.

L'estudi dut a terme en el **Capítol III** va ser el d'avaluar la capacitat de *L. monocytogenes*, *Salmonella* i *E. coli* O157:H7 per créixer en enciam envasat en tres condicions d'atmosfera diferents creades per mitjà de la utilització de pel·lícules de diferents permeabilitats a dues temperatures d'emmagatzematge. La composició de l'atmosfera d'emmagatzematge generada dins de diferents envasos a temperatura

de refrigeració (5 ºC) no va tenir cap efecte significatiu sobre la supervivència i el creixement dels tres PTA, mentre que a 25 ºC, el creixement de *Salmonella* i *E. coli* O157:H7 en l'enciam va ser lleugerament superior (0.50-1.00 log ufc/g) quan se'n va envasar amb la pel·lícula menys permeable.

El potencial patogènic de dues soques de *S.* Typhimurium DT104 (aïllats d'enciam i carcassa) es va examinar en el **Capítol IV**, amb l'objectiu de mesurar la seva capacitat per sobreviure al tracte gastrointestinal simulat i d'adherir i envair cèl·lules diferenciades Caco-2, després d'una incubació seqüencial al sòl, l'enciam i enciam tallat emmagatzemat en condicions de atmosfera modificada (MAP). El resultat més important d'aquest estudi va ser que la incubació seqüencial de *S.* Typhimurium al sòl i l'enciam va augmentar lleugerament la seva capacitat de sobreviure el fluid gàstric simulat y va augmentar la capacitat de créixer en el fluid intestinal simulat. Però va disminuir la capacitat d'adhesió i invasió epitelial.

En aquesta tesi l'efecte de millorar la microbiota d'enciam sotmesa a les diferents etapes de pre-condicionament en la supervivència de *L. monocytogenes* i *E. coli* O157:H7 com a mètode bioconservant també va ser avaluat (**Capítol V**). L'estudi es va dur a terme amb enciam produït de manera convencional i orgànica. Els patògens es van comportar de manera similar en enciam convencional i orgànica durant els primers dies tot i les diferències en la concentració inicial de la microbiota. Al final del període d'emmagatzematge, la població de *L. monocytogenes* sembla estar afectada per la microbiota de l'enciam orgànica, mentre que la de *E. coli* O157:H7 sembla estar afectada per la microbiota de l'enciam convencional.

Finalment, en el **Capítol VI**, s'ha estudiat l'ús de l'addició de bioconservants com a mètode per a controlar o reduir PTA en enciam mínimament processada. En primer lloc, es van aïllar i es van avaluar diversos microorganismes com possibles antagonistes contra PTA en enciam. En segon lloc, el millor antagonista va ser identificat i la seva eficàcia comparada amb el bioconservant *Pseudomonas graminis* CPA-7, nisina i dues solucions de bacteriòfags (Listex P100 i Salmonelex) en enciam mínimament processada simulant les condicions comercials. El millor antagonista va ser identificat com *Pseudomonas* sp. (soca M309) i va presentar efecte antagonista contra *S. enterica* i *E. coli* O157:H7 en discos d'enciam a temperatures de refrigeració. No obstant, no va reduir *L. monocytogenes*. L'addició dels bioconservants no va produir una reducció significativa de la població de *Salmonella* i *L. monocytogenes* en condicions que simulen la aplicació comercial. Es va trobar que el tractament amb nisina va ser eficaç en la reducció de la població inicial de *L. monocytogenes* fins al final del període d'emmagatzematge. Els tractaments amb la nisina i la soca CPA-7 va ser significativament diferent dels altres tractaments.

Els resultats generals obtinguts en aquesta tesi ens poden ajudar a comprendre millor la importància de mantenir la qualitat microbiològica de les hortalisses mínimament processades, ja que fa falta unes bones pràctiques d'higiene durant el creixement i el processat des del camp al consumidor per garantir la qualitat del producte final.

Resumo

As hortaliças minimamente processadas contêm grandes e diversificadas populações de microrganismos, como bactérias, leveduras e fungos que podem causar deterioração. Além disso, também podem conter bactérias patogénicas, como *Salmonella* spp., *Listeria monocytogenes* e *Escherichia coli* O157:H7. Muitos factores podem contribuir para a contaminação do produto fresco e minimamente processado com agentes patogénicos humanos. Assim sendo, devem ser analisadas e aplicadas medidas e intervenções específicas para prevenir e/ou minimizar o risco de contaminação.

O estudo descrito nesta tese está focado na determinação da influência de práticas de gestão de campo, processamento e condições de armazenamento na qualidade microbiológica da alface minimamente processada e no estudo de estratégias de mitigação para aumentar a sua segurança.

Especificamente, o efeito do sistema de produção, orgânica ou convencional, na qualidade microbiológica da alface fresca foi estudado (**Capítulo I**). Das amostras analisadas por análise de componentes principais foi observado um padrão com dois sistemas de produção, sendo que a maior diferença estava na concentração de *Enterobacteriaceae*. Além disso, patogénicos como *E. coli* O157:H7, *Salmonella* spp. e *L. monocytogenes* não foram detectados em nenhuma das amostras de alface orgânicas e convencionais analisadas (n=72).

Como consequência da possível contaminação em campo por patogénicos de origem alimentar (POA), foi avaliada a transferência e persistência de *L. innocua* e *E. coli* O157:H7 em folhas de alface e no solo durante o outono e primavera, utilizando diferentes métodos de irrigação (superfície e aspersão) e compostagem contaminados artificialmente (**Capítulo II**). A contaminação das folhas da alface com *L. innocua* e *E. coli* O157:H7, via solo, ocorreu durante o processo de crescimento, muito provavelmente, através do contato com o solo, compostagem contaminada ou água contaminada com irrigação por superfície. Ambos os patógenicos foram capazes de sobreviver pelo menos 9 semanas em solo exposto às condições ambientais.

O estudo conduzido no **Capítulo III** foi avaliar a capacidade de *L. monocytogenes*, *Salmonella* e *E. coli* O157:H7 para crescer em alface minimamente processada e embalada em três condições diferentes de atmosfera criada por meio da utilização de películas de diferentes permeabilidades a duas temperaturas de armazenamento. A composição da atmosfera de armazenamento gerada dentro das diferentes em-

balagens à temperatura de refrigeração (5 ºC) não teve nenhum efeito significativo na sobrevivência e crescimento dos três POA, no entanto, a 25 ºC, o crescimento de *Salmonella* e *E. coli* O157:H7 em alface foi ligeiramente superior (0.50-1.00 log ufc/g), quando embalado com o filme menos permeável.

O potencial patogénico de duas estirpes de *S.* Typhimurium DT104 (isolados de alface e carcaça) foi examinado no **Capítulo IV**, com o objetivo de medir a sua capacidade de sobreviver a um sistema de trato gastrointestinal simulado e em aderir e invadir células Caco-2 diferenciadas, após incubação sequencial no solo, alface e alface cortada armazenada em condições de atmosfera modificada. O resultado mais importante deste estudo foi que a incubação sequencial de *S.* Typhimurium no solo e na alface aumentou ligeiramente a capacidade de sobreviver ao fluido gástrico simulado, aumentou a capacidade de crescer em fluido intestinal simulado, mas diminuiu a capacidade de adesão e invasão epitelial.

Nesta tese, foi também avaliado o efeito de aumentar a microbiota da alface, submetida a diferentes etapas de pré-condicionamento na sobrevivência de *L. monocytogenes* e *E. coli* O157:H7, como um método bioconservante (**Capítulo V**). O estudo foi realizado com alfaces produzidas de maneira convencional e orgânica. Os patógenicos comportaram-se de forma semelhante em alface convencional e orgânica minimamente processada durante os primeiros dias, apesar das diferenças na concentração inicial da microbiota. No final do período de armazenamento, *L. monocytogenes* aparentou ser afetada pela microbiota da alface orgânica, enquanto que *E. coli* O157:H7 mostrou ser afetada pela microbiota da alface convencional.

Finalmente, no **Capítulo VI**, foi estudado o uso da adição de bioconservantes, como um método para controlar ou reduzir POA em alface minimamente processada. Em primeiro lugar, vários microrganismos foram isolados e avaliados como possiveis antagonistas contra POA na alface. Em segundo lugar, o melhor antagonista foi identificado e a sua eficácia comparada com *Pseudomonas graminis* CPA-7, nisina e duas soluções de bacteriófagos (Listex P100 e Salmonelex) sobre alface minimamente processada simulando condições comerciais. O melhor antagonista foi identificado como *Pseudomonas* sp. (estirpe M309) e apresentou efeito antagônico contra *S. enterica* e *E. coli* O157:H7 em discos de alface a temperaturas de refrigeração. No entanto, não reduziu *L. monocytogenes*. A adição dos bioconservantes não resultou numa redução significativa da população de *Salmonella* e *L. monocytogenes* em condições simulando a aplicação comercial. O tratamento com nisina foi eficaz na redução da população inicial de *L. monocytogenes* até ao final do período de armazenamento. Os tratamentos de nisina e da estirpe CPA-7 foram significativamente diferentes dos demais tratamentos.

Os resultados gerais obtidos nesta tese podem ajudar-nos a compreender melhor a importância de manter a qualidade microbiológica de hortaliças minimamente processadas, que ainda depende de boas práticas de higiene durante o crescimento

e processamento desde o campo ao consumidor, limpeza e descontaminação eficaz, temperatura rigorosa e embalagem apropriada.

Introduction

1. Fresh-cut produce

The International Fresh-cut Produce Association (IFPA) defines fresh-cut products as fruits or vegetables that have been trimmed, peeled and/or cut into 100 % usable product that is bagged or pre-packaged to offer consumers high nutrition, convenience, and flavour while still maintaining freshness. Fresh-cut products are minimally, or lightly, processed fruits and vegetables, which should be in a raw state, not frozen or thermally processed, and ready to eat or cook (Anonymous, 1998a, b; Rajkowski and Baldwin, 2003).

A basic idea on which all nutritional scientists can agree is that the increased consumption of diets rich in a variety of fruit and vegetables will improve the health of almost any human population. This diet (of which the Mediterranean diet is the best sample) is known to be beneficial for health, especially with regard to the development of chronic degenerative disease (Corpet and Gerber, 1997; Gerber and Corpet, 1997).

Minimal processing of raw fruits and vegetables has two purposes (Huxsoll and Bolin, 1989):

– keeping the produce fresh, without losing its nutritional quality
– ensuring a product shelf-life sufficient to make distribution feasible within a region of consumption

As a result of peeling, cutting and shredding, produce will change from a relatively stable commodity with a shelf-life of several weeks or months to a perishable state with a limited shelf-life. During processing, many cells are broken and intracellular products are released. Minimally processed produce deteriorates owing to physiological ageing, biochemical changes and microbial spoilage, which may result in degradation of the colour, texture and flavor (Kabir, 1994; Varoquaux and Wiley, 1994). While conventional food-processing methods extend the shelf-life of fruit and vegetables, the minimal processing to which fresh-cut fruit and vegetables are subjected renders products highly perishable, requiring chilled storage to ensure a reasonable shelf-life.

1.1. Consumption of fresh-cut vegetables

During recent years, the market for freshly prepared fruit and vegetable products increased explosively. The main driving force for this market growth is the increasing consumer demand for fresh, healthy, convenient and additive-free prepared products.

The United Kingdom (UK) is the largest fresh-cut fruits and vegetables market in the European Union (EU), accounting for around a third of total EU consumption. In Spain, Germany and Netherlands this sector is still in an early stage of development. Although in Spain the impact of the economic recession in the first decade of the twenty-first century has been quite severe, the market for fresh-cut fruits and vegetables has shown continuous growth. Sales of ready-to-eat vegetables showed an annual increase of 5-6 %, with 70,600 and 74,064 tons in 2010 and 2011, respectively (Anonymous, 2013). After this period, the Spanish market stabilized to approximately 77,000 tons sales in 2013 (Anonymous, 2014).

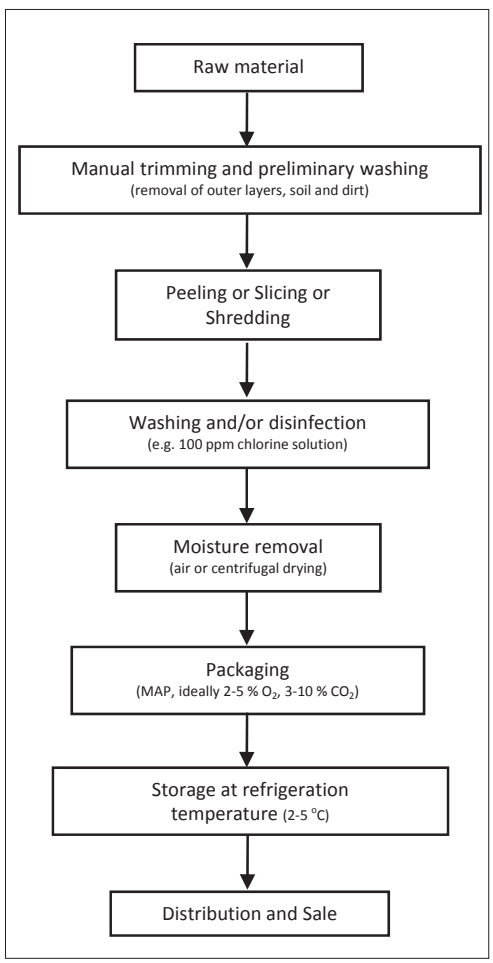

Figure 1. A flow diagram for the production of minimally processed vegetables. (Adapted from Francis *et al.*, 1999).

1.2. Fresh-cut produce production

Various steps are included in the preparation of fresh-cut products, each of which are specific separate operations (Figure 1). Each separate operation must be performed properly to ensure that quality, shelf-life, and food safety of the finished products are satisfactory (Gorny, 1996).

The first step to ensure food safety of raw vegetable is removal of outer layers or surface dirt. After removal of contaminated external layers, the vegetables are sliced/shredded. Fresh-cut vegetables are thoroughly washed and are often dipped in antimicrobial solutions. The washing agent can be water alone, but efficacy of washing is

improved by including antimicrobials, typically chlorine (100 ppm) or citric and ascorbic acid (1 %) in the wash water. After washing, the excess of water added during washing is removed, usually by centrifugation (Bolin *et al.*, 1977; Reyes, 1996). The final step in minimal processing is packaging and storage. Minimally processed vegetables are usually sealed in semi-permeable packages under modified atmosphere and stored at refrigeration temperatures (2-5 ºC) (Francis *et al.*, 1999).

1.3. Lettuce

Lettuce (*Lactuca sativa* L.) is grown commercially worldwide and is the most common fresh-cut vegetable in the marketplace. In 2012, the world production of lettuce was more than 24 million tons on about 1 million ha in 2012. About two-thirds of the production area in the world is in Asia. China is the largest lettuce producing country followed by United States with 14,000,000 and approximately 4,000,000 tons, respectively in 2012. Western Europe claims about 13 % of the total lettuce production and area due to its higher yield. Spain is the largest lettuce producer in Europe with 870,200 tons and approximately 34,000 ha of harvest area in 2012 (FAOSTAT, 2012).

1.3.1. Lettuce cultivars

There is a great diversity of shape, size, and colour among lettuce cultivars, which are classified into types mainly based on leaf shape, size, texture and head formation. Four principal types of lettuce (Figure 2) are recognized: crisphead, butter head, romaine and loose-leaf (Rubatzky and Yamaguchi, 1997; Splittstoesser, 1990):

– Crisphead (*L. sativa* var. capitata), more commonly known as iceberg or head lettuce. Leaves are thin and crisp and frequently have curled and serrated edges.
– Butterhead (*L. sativa* var. capitata), is a head type in which the leaves are loosely folded. The inner leaves are cream or yellow and the outer leaves are darker green or brownish.
– Romaine or Cos *(L. sativa* var. longifolia), is an upright plant that has long narrow leaves that are coarse and tender. Outer leaves are usually light to dark green and inner leaves are yellowish.
– Loose-leaf or Leaf *(L. sativa* var. crispa), they form a bunch or rosette of leaves that may have broad, elongated, or lobed shape like oak leaves, smooth or friled margins, and yellow, green or red colours in varying shades.

Crisphead (*L. sativa* var. capitata)

Butterhead (*L. sativa* var. capitata)

Romaine or Cos *(L. sativa* var. longifolia)

Loose-leaf or Leaf *(L. sativa* var. crispa)

Figure 2. Lettuce varieties.

1.3.2. Cultivation practices

Lettuce seed is used directly for field sowing or for the production of seedlings. Germination is optimum between 16 to 23 ºC but the rate of germination diminishes with lower temperatures, down to about 2 ºC. Between 25-30 ºC germination is not reliable because of high-temperature induced dormancy. Using seedlings is a common practice for many lettuce producers. Lettuce is well adapted to growing in a cool season, with optimum growing temperatures at 13 to 18 ºC. The optimum growing conditions for lettuce are warm days (14 to 18 ºC) and cool nights (5 to 8 ºC) because lettuce grows better with distinct differences in temperature between day and night (Rubatzky and Yamaguchi, 1997).

Warm sandy soils are preferred for the early harvest, loam to clay loam or peat for late production. Good drainage, good soil moisture holding capacity and high organic matter content are essential for a good quality lettuce crop. High humidity and excess water close to the time of harvest can be destructive to the yield and quality of the crop (Rubatzky and Yamaguchi, 1997).

1.4. Organic and conventional production

Conventional and organic agricultural practices include combinations of farming practices that vary greatly depending upon region, climate, soils, pests and disease pressures, and the economic and physiological factors guiding the particular management practices used on the farm. Nonetheless, fundamental differences do exist between organic and conventional production systems, particularly in soil fertilization and pest management (Mitchell *et al.*, 2008).

Traditional organic farms practise polyculture and rely less on off-farm inputs than conventional farms. In comparison, conventional agricultural practices generally utilize high-yield crop cultivars, monoculture, chemical fertilizers and pesticides, irrigation and mechanization. Despite the high yield of conventional agriculture, there is concern regarding the negative biological and environmental consequences (Mitchell *et al.*, 2008).

In general, organic production methods promote biodiversity, reduce dependence on off-farm inputs and emphasize soil and water conservation.

Organic production has been considered to represent an increased risk to public health compared to conventional production, due to the method of cultivation and processing. Organic production implies the use of natural fertilizers such as animal manure; no chemicals are employed to reduce the microbiological load of the raw product. However, there is little scientific evidence to support this suggestion (McMahon and Wilson, 2001). Nevertheless, some reports have promoted the idea that organic produce poses a greater risk of transmitting foodborne diseases than conventional produce (Avery, 2002; Stossel, 2000).

1.4.1. Organic agriculture in Spain

In Spain, from 1996 to 2003, the number of growers and land under organic management increased substantially, partly due to the agri-environmental economic support scheme for organic farming. The number of organic farms increased by more than 15 %, from 15,609 in 2001 to 18,226 in 2007. The area of organically managed land increased from 485,079 to 988,323 ha, and thus nearly doubled. Although the number of the organic farms in Spain is still small in comparison to the number of conventional farms, it grew continuously in the last years at an annual rate of 10 to 20 %. In 2012 the organic farms numbers were 30,462 with a total production area of 1,756,548 ha (MAGRAMA, 2013).

In Spain, organic farming was officially regulated in 1989 with the National regulation of Generic Denomination 'Organic Agriculture'. This regulation was applied until the EU Regulation Nº 2092/91 on Organic Agriculture came into force. In 1993, a Spanish regulation, RD Nº 1852/1993, established a new regulation for organic farming, based on the EU Regulation. At the same time the Spanish regions

assumed official responsibility in the monitoring of organic production. Under the same law (RD Nº 1852/1993) the Advisory Group of the Regulatory Commission for Organic Farming (CRAE) of the National Ministry of Agriculture, Food and the Environment (MAGRAMA) was created. This group includes organic stakeholders, regional and central authorities, and the directors of the regional public certification bodies. On January 1, 2009, EU Regulation Nº 2092/91 was substituted by Regulation Nº 834/2007.

2. Microbiology of fresh-cut vegetables

The unique physical and biochemical qualities of each plant surface, as a result of the plant genotype and of responses to environmental stimuli (light, temperature, humidity, atmosphere, pH, soil), are major determinants of the plant microbial community. Large differences in types and numbers of microorganisms can occur on different plants and leaf-to-leaf, seasonally and even daily (Hirano *et al.*, 1982; Hirano and Upper, 2000; Kinkel *et al.*, 2000). Microorganisms usually colonize and/or are present in those areas of a leaf, which retain water and are protected from UV light (Lund, 1992).

2.1. Native microbiota

Fresh-cut vegetables harbour large and diverse populations of microorganisms, such as bacteria, yeasts and fungi that may cause spoilage (Abadias *et al.*, 2008). Counts of 10^5-10^7 cfu/g are frequently present. Eighty to 90 % of bacteria are Gram-negative, predominantly *Pseudomonas* and *Enterobacteriaceae* species (Magnuson *et al.*, 1990; Nguyen-the and Prunier, 1989). Lactic acid bacteria (LAB) belong to the native microbiota of vegetables and are associated with spoilage organisms, causing unpleasant odours due to the production of ethanol, organic acids, esters and CO_2 (Babic *et al.*, 1992; Fleet, 1992). Yeasts and moulds (YM) are present in smaller numbers than bacteria, but, when present in high numbers, can contribute to spoilage of fermented vegetable products and the development of soft rot (Fleet, 1992).

2.2. Pathogenic microorganisms

Vegetables, and in particular leafy greens that are consumed raw, are increasingly recognized as important vehicles for human pathogens that were traditionally associated with foods of animal origin (Beuchat, 1996b; Francis *et al.*, 1999; Nguyen-the and Carlin, 1994). Vegetables contaminated with human pathogens have been implicated in an increasing number of outbreaks of foodborne illness (Long *et al.*, 2002). The pathogens most frequently associated with fresh-cut vegetables are *Lis-*

teria monocytogenes, *Salmonella* spp., *Escherichia coli* O157:H7, some viruses and parasites.

2.2.1. *Listeria* spp.

All members of this genus are Gram-positive, non-sporeforming rods, facultative anaerobic, catalase positive and oxidase negative. These microorganisms are psychrotrophic and grow over a temperature range of around 0 to 45 ºC, with an optimum around 37 ºC (Hoffman *et al.*, 2003). *Listeria* spp. are ubiquitous in the environment and can be isolated from soil, water, vegetation, the faeces of livestock and vegetation irrigated with contaminated water (Heaton and Jones, 2008).

The genus *Listeria* comprises fifteen species: *L. monocytogenes* (Pirie, 1940), *L. grayi* (Errebo and Seeliger, 1966), *L. innocua* (Seeliger, 1981), *L. welshimeri* (Rocourt and Grimont, 1983), *L. seeligeri* (Rocourt and Grimont, 1983), *L. ivanovii* (Seeliger *et al.*, 1984), *L. marthii* (Graves *et al.*, 2010), *L. rocourtiae* (Leclercq *et al.*, 2010), *L. fleischmannii* (Bertsch *et al.*, 2013), *L. weihenstephanensis* (Lang Halter *et al.*, 2013) and five new species that have recently been isolated: *L. floridensis* sp. nov, *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov. and *L. grandensis* sp. nov. (den Bakker *et al.*, 2014). Only *L. monocytogenes* and *L. ivanovii* are considered pathogens. While both are haemolytic, facultative intracellular pathogens, *L. ivanovii* is predominantly associated with disease (specifically abortions) in sheep. On the other hand, *L. monocytogenes* causes human and animal disease and is the only *Listeria* species that represents a human public health concern. From that perspective, the foodborne pathogen *L. monocytogenes* is the most important species of this genus. Infection with *L. monocytogenes* can cause several different forms of listeriosis in pregnant women, neonates, the immunosuppressed, and elderly individuals. Nevertheless, healthy people can also be infected by this pathogen. Symptoms of listeriosis include diarrhoea, fever, and muscle aches. More serious complications associated with human listeriosis include stillbirth, septicaemia, and infections of the central nervous system (meningoencephalitis, encephalitis) (Rocourt *et al.*, 2000).

Data from the Foodborne Diseases Active Surveillance Network (FoodNet) show that, although human listeriosis is less common that many other foodborne diseases, it is by far the most severe. Exposure to rather high doses is required for infection and disease. Differences in virulence of strains and differences in host susceptibility may also contribute to the fact that exposure to *L. monocytogenes* from contaminated foods rarely appears to cause disease (Notermans and Hoornstra, 2000).

In several countries, criteria or recommendations for tolerable levels of *L. monocytogenes* in ready-to-eat (RTE) foods have been established. Some countries, such as the USA, require absence of *L. monocytogenes* in 25 g of foods (zero tolerance). Food safety criteria in Spain for fresh-cut fruits and vegetables are regulated by the Commission Regulation EU Nº 1441/2007 (OJEU L322/12-29, 7 December 2007)

as a follow up of Regulation EU Nº 2073/2005 (OJEU L338/1-26, 22 December 2005). In ready-to-eat foods able to support growth of *L. monocytogenes*, absence of *L. monocytogenes* is demanded in 25 g before the food has left the immediate control of the producing food business operator and <100 cfu/g in products placed on the market during their shelf life (Table 1).

L. monocytogenes has been isolated from a wide range of intact vegetables (Heisick *et al.*, 1989; Steinbruegge *et al.*, 1988) and from bagged fresh-cut iceberg lettuce (Abadias *et al.*, 2008). It has been demonstrated that *L. monocytogenes* can grow on a variety of vegetables even at refrigeration temperatures (Brackett, 1999; Carrasco *et al.*, 2008; Francis and O'Beirne, 1997; Jacxsens *et al.*, 2002).

Table 1. Food safety criteria for *L. monocytogenes* and *Salmonella* in vegetables and fruits.

Food category	**Microorganisms**	**Sampling plan** [1]		**Limits**		**Stage where the criteria applies**
		n	c	m	M	
Ready-to-eat foods able to support microrganism growth	*Listeria monocytogenes*	5	0	100 cfu/g		Products placed on the market during their shelf-life
		5	0	Absence in 25 g		Before the food has left the immediate control of the food business operator, who has produced it
Ready-to-eat foods unable to support microrganism growth	*Listeria monocytogenes*	5	0	100 cfu/g		Products placed on the market during their shelf-life
Pre-cut fruit and vegetables (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		Products placed on the market during their shelf-life

n = number of units comprising the sample.
c = number of sample units giving values between m and M.

Although *Listeria* contamination of fresh produce and survival up to point-of-sale seems likely, outbreaks linked to fresh produce are infrequent and tend to be lim-

ited to vulnerable groups. The two documented outbreaks that have occurred, in 1979 and 1981 respectively, were attributed to cabbage (in coleslaw) and salad items (celery, lettuce and tomatoes) (Farber and Peterkin, 1991). More recently (October 2011), an outbreak of *L. monocytogenes* due to contaminated melon affected a total of 147 persons and caused 33 deaths in the United States (CDC, 2011a).

2.2.2. *Escherichia coli*

Escherichia coli is a Gram-negative, non-sporulating straight rod, facultative anaerobic, oxidase negative, catalase positive and belongs to the family of *Enterobacteriaceae*. This microorganism is mesophilic, with optimum temperatures for growth of 35-40 ºC and is considered part of the normal microbiota of the intestinal tract of humans and most other warm-blooded animals. Hence, it is generally present in faeces. Most strains of *E. coli* are harmless, but a small proportion has evolved into pathogens that can cause serious clinical symptoms in humans (Doyle and Cliver, 1990).

There are several types of *E. coli* strains that may cause gastrointestinal illness in humans. That can be divided into six groups or pathotypes:

- Enteropathogenic *E. coli* (EPEC)
- Attaching and effacing *E. coli* (A/EEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enteroinvasive *E. coli* (EIEC)
- Enterohaemorrhagic *E. coli* (EHEC)
- Enteroaggregative *E. coli* (EAggEC)

Despite the commensal status of the majority of strains, pathogenic strains, particularly EHEC *E. coli* O157:H7 is recognized as an emerging foodborne pathogen. It was first identified in 1982 as the causative agent of bloody diarrhoea and haemolytic uremic syndrome in humans, and was associated with the consumption of undercooked beef (Riley *et al.*, 1983). Three principal syndromes are to *E. coli* O157:H7: firstly, hemorrhagic colitis (HC), in which the stools contain frank (red) blood; secondly, haemolytic uremic syndrome (HUS), the leading cause of renal failure in children, and thirdly, thrombotic thrombocytopenic purpura (TTP). TTP is similar to HUS but involves brain damage too, causing a high mortality rate. TTP, however, is a very infrequent disease syndrome (Doyle and Cliver, 1990). One of the key virulence factors of EHEC is the ability to produce Shiga-toxins (Stx) which consists of two types: Stx1 and Stx2, which play a crucial role in causing HC and HUS (Kaper *et al.*, 2004).

E. coli O157:H7 is generally considered to be more virulent than other EHEC, although the reason for this is unclear (Vanselow *et al.*, 2005). The high virulence of EHEC strains is not only determined by genes coding for toxins and adherence fac-

tors but possibly also by its ability to survive environmental stresses. Their capacity to colonize the human gut is for a large part due to their resistance to low pH levels like encountered in the human stomach, resulting in a relatively low infectious dose, which has been estimated to be occasionally as low as 50-100 cells (Armstrong *et al.*, 1996; Tilden *et al.*, 1996; Tuttle *et al.*, 1999).

Pathogenic *E. coli* are not included in safety criteria in the EU, although *E. coli* is included as process hygiene criterion. Process hygiene criteria in Spain for fresh-cut fruits and vegetables are based on the Commission Regulation EU Nº 1441/2007 (OJEU L322/12-29, 7 December 2007). These criteria for *E. coli* are shown on the table 2.

Table 2. Process hygiene criteria in vegetables and fruits for *E. coli*.

Food category	Microorganism	Sampling plan [1]		Limits		Stage where the criteria applies	Action in case of unsatisfactory results
		n	c	m	M		
Pre-cut fruit and vegetables (ready-to-eat)	*Escherichia coli*	5	2	100 cfu/g	1000 cfu/g	Manufacturing process	Improvements in production hygiene, selection of raw materials

n = number of units comprising the sample;
c = number of sample units giving values between m and M.

The limits refer to each sample unit tested. The results for the presence of *E. coli* are satisfactory if all the values observed are ≤ m; acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are ≤ m; unsatisfactory, if one or more of the values observed are > M or more than c/n values are between m and M.

Lettuce has been associated to several outbreaks of *E. coli* O157:H7. In July 1998, an outbreak involving 40 Montana residents was associated with contaminated leaf lettuce (Ackers *et al.*, 1998). Following this outbreak, several additional outbreaks of *E. coli* O157:H7 infection were linked lettuce (Buck *et al.*, 2003; CDC, 2011b; Ethelberg *et al.*, 2010; Friesema *et al.*, 2007, 2008). In 2006, a major outbreak of foodborne illness associated with the consumption of spinach tainted with EHEC occurred in the United States. This episode was linked to contamination of a spinach field by EHEC-infected wild pigs roaming in the Salinas Valley in California (FDA, 2007).

In May 2011, Germany endured one of the largest outbreaks of haemolytic uremic syndrome and bloody diarrhoea caused by EHEC *E. coli*, also referred to as Shiga toxin-producing *E. coli* (STEC) serotype O104:H4. Figures updated by the Robert

Koch-Institute (RKI) on 9 September 2011, reported a total of 855 cases of HUS and 2,987 cases of EHEC gastroenteritis (without development of HUS) hence a total of 3,842 cases are attributable to the outbreak. Death was reported for 35 (4.1 %) of the patients identified with HUS and 18 (0.6 %) of the patients with EHEC gastroenteritis (RKI, 2011). German officials initially suspected cucumbers from Spain as the source of contamination, but further tests showed that those vegetables did not contain the *E. coli* O104:H4 strain. Epidemiological evidence suggested that STEC-contaminated fenugreek seeds imported from Egypt were the vehicle of infection (EFSA, 2011).

2.2.3 *Salmonella* spp.

Salmonella species are Gram-negative, non-sporeforming, rod shaped, facultative anaerobic, oxidase negative, catalase positive, motile bacteria which belong to the family of *Enterobacteriaceae*. The microorganisms are mesophilic, with optimum growth temperatures of 35-43 ºC. The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals and is usually transmitted by ingestion of food or water contaminated by infected faeces.

The taxonomic classification of *Salmonella* has been continually revised over the years. According to the CDC system, the genus *Salmonella* contains two species, each of which contains multiple serotypes. The two species are *S. bongori* and *S. enterica*. The latter includes six subspecies, *S. enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*, also known as subspecies I, II, IIIa, IIIb, IV and VI, respectively (Popoff and Le Minor, 1997; Reeves *et al.*, 1989). *S. enterica* subspecies are differentiated biochemically and by genomic relatedness (Brenner and McWhorter-Murlin, 1998; Reeves *et al.*, 1989). Over 2500 serovars of *Salmonella* have been classified based on reactivity of antisera to somatic lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens (EFSA, 2010; Popoff and Le Minor, 1997).

Salmonella enterica serotypes Enteritidis and Typhimurium are encountered most frequently worldwide and are the two most important serotypes for salmonellosis transmitted from animals to humans (EFSA, 2010). The two main human types of salmonellosis are typhoid or typhoid-like fever and gastroenteritis. Gastroenteritis is by far the most common manifestation of disease caused by *Salmonella*. The incubation period is typically 6 to 48 h and is followed by fever, abdominal pain, nausea, and sometimes vomiting. However, in some patients, particularly in the very young and in the elderly, the infection may be more serious and the associated dehydration can be life threatening (Darwin and Miller, 1999). It is estimated that more than 10^5 cells are required to initiate an infection (Blaser and Newman, 1982). However, in some outbreaks the infectious dose was reported to be between 10-100 cells. The exact amount needed for infection depends on the type of food, type of strain, the physiological state of bacteria and characteristics of the host (Darwin and Miller, 1999). The establishment of a human *Salmonella* infection depends on the ability

to survive the environment outside the host, the ability to survive the gastric acid of the human stomach and the ability of the pathogen to attach and invade intestinal cells (Franz and van Bruggen, 2008).

The microbiological criterion for *Salmonella* spp. in fresh-cut fruits and vegetables (Table 1) is absence of *Salmonella* in 25 g of foods (zero tolerance, Commission Regulation EU Nº 1441/2007, OJEU L322/12-29, 7 December 2007).

Salmonella spp. have been isolated from several fresh intact vegetables (Doyle, 1990; Tauxe, 1991). In addition, this pathogen is detected regularly on fresh leafy-green vegetables during surveys at retail level (Abadias *et al.*, 2008; Elviss *et al.*, 2009; Garcia-Villanova Ruiz *et al.*, 1987; Pielaat *et al.*, 2008; Sagoo *et al.*, 2003). A range of fresh fruit and vegetable products have been implicated in *Salmonella* infection, including tomatoes (CDC, 1993; Hedberg and Osterholm, 1993; Wood *et al.*, 1991), bean sprouts (Mahon *et al.*, 1997; O'Mahony *et al.*, 1990; Werner *et al.*, 2007) and melons (Blostein, 1991; Munnoch *et al.*, 2009; Ries *et al.*, 1990).

Salmonella was implicated in 18 % of the lettuce associated outbreaks and 10 % of the produce-related *Salmonella* outbreaks were associated with lettuce in United States between 1973 and 1997 (Sivapalasingam *et al.*, 2004). In Europe, between 2000 and 2005, several outbreaks were linked to *Salmonella* and lettuce (Anonymous, 2005; Crook *et al.*, 2003; Horby *et al.*, 2003; Takkinen, 2005).

Survival of stomach passage allows *Salmonella* to enter the small intestine. Bacteria that survive the stomach barrier can subsequently adhere and colonize the intestine and invade the intestinal cells. Invasion is necessary to cause a systemic infection. After *Salmonella* has penetrated the epithelial cells of the small intestine it enters the sub epithelial space. In this sub epithelial space, cells from the human immune system are present. These are mainly white blood cells. Macrophages are white blood cells that can also remain in the tissue and are important in clearing extracellular bacteria and parasites. In the sub epithelial space, macrophages absorb *Salmonella* by phagocytosis. Although macrophages are designed to kill bacteria, *Salmonella* can survive and replicate in the macrophage (Groisman and Saier, 1990). Residing in macrophages protect some *Salmonella* strains cells from other cells of the immune system. Moreover, macrophages also transport the *Salmonella* through the bloodstream to other organs (*e.g.* liver and spleen), so *Salmonella* can cause a systemic infection through the host (Figure 3) (Alpuche-Aranda *et al.*, 1995; Fields *et al.*, 1986; Libby *et al.*, 1994; Miller and Mekalanos, 1990).

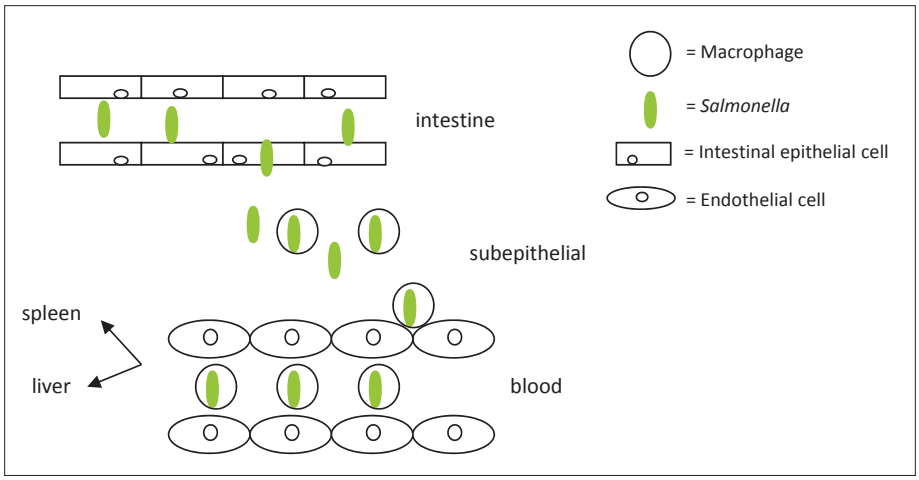

Figure 2. Schematic drawing of the invasion and translocation of *Salmonella* across the intestinal wall and transfer to secondary organs, like liver and spleen (Adapted from Berk, 2008).

Several human intestinal cell lines are available to study interactions of *Salmonella* with epithelial cells. The human adenocarcinoma cell lines, Caco-2, HT-29 and T84, have been shown to form polarized monolayers and well-defined brush borders, mimicking the human intestinal epithelium. The Caco-2 cell line is known for its spontaneous differentiation for formation of such a polarized cell layer (García Véscovi *et al.*, 1994). The Caco-2 cell line is a good *in vitro* system for the analysis of *Salmonella* virulence characteristics (Finlay and Falkow, 1990).

3. Sources of contamination

Vegetables can become contaminated with foodborne pathogens at various stages of their production: during growth, harvesting, processing, distribution and preparation at home. Therefore, prevention is the most important measure and potential sources of contamination from the environment to the table should be identified.

3.1. Preharvest contamination

Primary sources of preharvest contamination include soil amended with untreated or improperly composted manure, contaminated irrigation water, the presence of wild and domestic animals, infected workers, and unclean containers and tools used in harvesting (FDA, 2008). However, the most important sources of contamination are the use of manure or compost as fertilizer to fields where crops are grown and

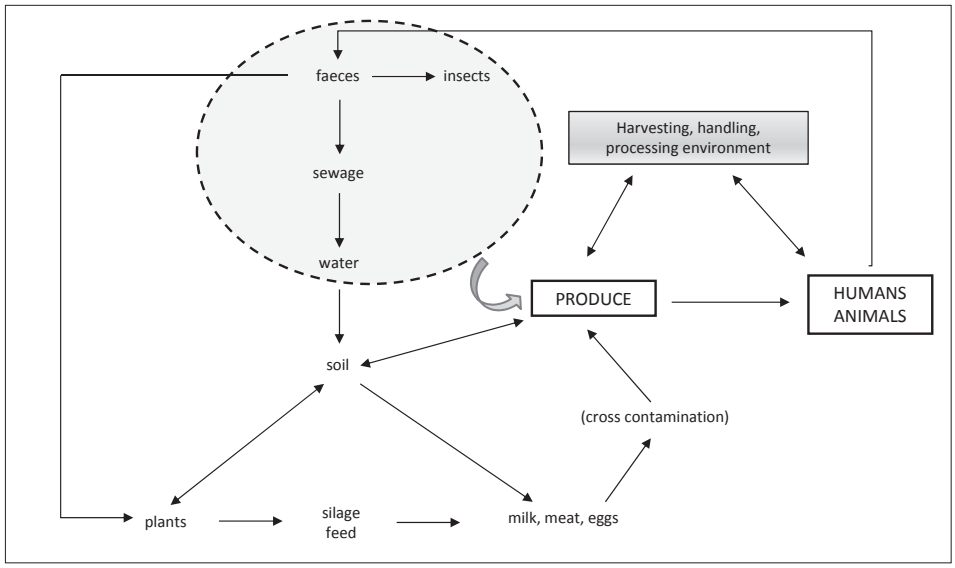

Figure 3. Mechanisms by which raw fruits and vegetables may become contaminated with pathogenic microorganisms (Adapted from Beuchat, 1996a).

the use of faecally contaminated irrigation water (Franz and van Bruggen, 2008). As mentioned before, organic production is considered to represent an increased risk to public health over conventional production, due to the method of cultivation and processing, such as using natural fertilizers like animal manure and refraining from the use of chemical treatments.

A schematic representation of potential sources of contamination in production systems is provided in Figure 3.

3.1.1. Animal wastes

Human pathogens can be introduced to production systems through animal faeces from wildlife or livestock or by the application of manures or sewage sludges as fertilizers in agricultural systems (Beuchat, 1996a, b). Contaminated manure can come into contact with the produce directly through its use as soil fertilizer or indirectly through irrigation water (Doyle, 2000). Some reports demonstrate that pathogens like *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* are able to survive for extended periods in manure (Franz *et al.*, 2005; Kudva *et al.*, 1998; Scott *et al.*, 2006), manure-amended soil (Franz *et al.*, 2008; Gagliardi *et al.*, 2002; Jiang *et al.*, 2004) and sewage sludge (Garrec *et al.*, 2003). Proper manure and waste treatments and timing of application are needed to ensure the microbial quality of produce at harvest.

3.1.2. Irrigation water

Irrigation water is a potential point of pathogen entry into the food chain as many bacteria, viruses and protozoa of faecal origin can be found in water, which is used in the primary production of food crops (Ethelberg *et al.*, 2010; Nygard *et al.*, 2008; Soderstrom *et al.*, 2008).

Besides the quality of irrigation water, the type of irrigation system also influences the microbial safety of fresh produce (Aruscavage *et al.*, 2006; Brackett, 1999). Overhead irrigation is often highlighted as carrying a higher risk for ready-to-eat crops than sub-surface or drip systems because contaminated water is deposited directly onto the edible leaves of produce.

Several studies demonstrated the association of pathogens with surfaces of vegetables when grown in soils irrigated with contaminated water (Islam *et al.*, 2004a, b; Solomon *et al.*, 2002, 2003).

The interval between irrigation and harvest could affect the likelihood of survival of pathogenic bacteria and thus the likelihood of reaching the consumer. Environmental factors such as UV radiation and temperature could also reduce the pathogen load (FSA, 2007).

3.1.3. Soil

Pathogens may be naturally present in soil or may be incorporated through the use of organic wastes added as fertilizers or through contaminated irrigation water. Contamination of vegetable can occur through splashing of rain or water irrigation onto the leaves.

The ability of pathogens to survive in soil depends on abiotic (temperature, pH, soil moisture, soil type) and biotic (composition and diversity of the microbial community) factors (Van Veen *et al.*, 1997). It has been reported that *E. coli* O157:H7, *Salmonella* and *Listeria* may survive in soil for several weeks depending on the soil type, moisture level, temperature and source of contamination (Erickson *et al.*, 2010; Guo *et al.*, 2002; Jiang *et al.*, 2004; Lang and Smith, 2007; Zhang *et al.*, 2009).

The use of good agricultural practices and strict hygiene practice programs specific for the pathogen(s) of concern is essential to minimise the contamination of produce during production.

3.2. Harvest and Postharvest contamination

Harvest provides many opportunities for the introduction to or dissemination of human pathogens in the fruit and vegetable crops. Leafy vegetables are harvested by hand or mechanically. In the field, some minor processing takes place such as remov-

al of outer leaves or coring, washing or spraying. Also, produce comes into contact with bins or conveyors belts. These processes involve many points of contact with people, surfaces, water and the environment (*e.g.* soil, dust) and represent potential risks for contamination with foodborne pathogens.

Persons, who come into contact with fresh produce directly, must follow hygiene and health requirements. Prevention strategies based on physical barriers such as gloves or disinfection methods can effectively reduce such risks in the food-processing or food-service environment (Michaels *et al.*, 2003, 2004; Montville *et al.*, 2002). Handling practices in distribution facilities as well as during transport and marketing, and improper temperature conditions may result in an additional increase in microbial load of fresh produce.

As mentioned before, harvesting and processing may influence the microbiological safety of fresh and fresh-cut produce. These activities include human and mechanical contact, immersion in water, and cutting or slicing, which not only have the potential to contaminate produce, but also can enhance bacterial growth (Brackett, 1999).

Both, hygiene of personnel and technical equipment cannot be underestimated and HACCP plans are also needed during the processing chain.

Fresh-cut produce is not subjected to any 'lethal' treatment that kills all pathogens prior to consumption. Therefore, it is essential to prevent contamination and bacterial growth in order to maintain the quality of ready-to-eat vegetables. Such can only be achieved by applying good hygiene practices during growth and processing from field to consumer, effective washing and decontamination, strict temperature control and appropriate packaging.

4. Intervention methods to enhance the safety of minimally processed fruits and vegetables

The detection of human pathogens in fresh produce and occurrence of outbreaks of foodborne illness associated with contaminated produce, as documented previously, represent serious public health problems. Consequently, produce industry has to develop interventions to reduce and prevent the risk of microbial contamination. Although preharvest (good agricultural practices, GAPs), postharvest (good manufacturing practices, GMPs) and supply-chain (good handling practices, GHPs) controls can help to reduce the risk, they have not been able to prevent contamination.

A variety of intervention methods is used as mild preservation and/or disinfection techniques to enhance safety of minimally processed produce. Traditional methods of reducing microbial populations on produce involve chemical and physical treat-

ments. Washing sanitation is an important measure to reduce pathogen contamination; however, not all washing methods are equally effective.

The application of multiple intervention methods (hurdle technology), where two or more preservation technologies are used to reduce and prevent growth of microorganisms could have additive, synergistic, or antagonistic effects. It is widely accepted within the fresh produce industry that a decontamination step, the use of refrigeration and modified atmosphere packaging can inhibit or slow down growth and reduce bacterial count (WHO, 2008).

4.1. Chemical treatments

A variety of antimicrobial wash solutions to diminish populations of microorganisms on fresh produce is in use. There are many factors that will affect disinfection, such as initial bacterial load and type of microorganisms on the surface of produce, treatment type, the surface to be treated, the type of disinfectant, possible internalization of pathogens and the time and temperature of exposure to the disinfectant (Beuchat *et al.*, 2004, 2005).

Chlorine added to water as calcium hypochlorite, sodium hypochlorite and chlorine gas is the most frequently used disinfectant for fresh fruits and vegetables. The use of chlorine washing is under discussion. Considerations have included public health concerns with chlorine and its by-products (Hrudey, 2009; Parish *et al.*, 2003) and an increasing awareness of the negative environmental impact of chlorine (Beuchat, 1998). Trihalomethanes (THM) have been the main disinfection by-products of concern when chlorine is in contact with organic matter and have been classified by the WHO's International Agency for Research on Cancer as possibly carcinogenic to humans (IARC, 1999a, b). Water is the classical exposure route of concern but some authors describe that vegetables can absorb THMs from the washing water (Huang and Batterman, 2010). However, under specific experimental conditions, Gómez-López *et al.* (2013) observed by using chlorine-based sanitizers in baby spinach very low concentrations of THMs were obtained after washing, and no THMs were detected in the rinsed leaves after rinsing.

Moreover, some studies indicate that the traditionally used chlorine concentrations (50-200 ppm) are not effective in reducing pathogen load on fresh-cut produce (Behrsing *et al.*, 2000; Delaquis *et al.*, 2002; Lee and Baek, 2008). For this reason, the need to develop better, safer and environmentally friendlier methods has become evident.

There is a wide variety of other disinfectants that have also been evaluated, including electrolyzed water, peroxyacetic acid, chlorine dioxide, acidified sodium chlorite, hydrogen peroxide, organic acids, trisodium phosphate, bromine, iodine, quaternary

ammonium compounds and ozone, and its legal use differs from country to country (Ramos *et al.*, 2013).

4.2. Physical treatments

Some physical methods are available for reducing the microbiological load of produce and enhance their shelf-life. Efforts to improve the overall effectiveness of the washing step by the use of classical physical treatments, such as mild heat or ethanol vapours, may enhance pathogen destruction. Various studies describe and discuss the use of novel decontamination techniques, including ultra violet (UV) irradiation, high-pressure treatment, pulsed electric fields, microwave, ultrasound, cold plasma, high intensity pulsed light and thermal destruction using condensing steam. (Ignat *et al.*, 2014; James, 2007; Srey *et al.*, 2014). Modified atmosphere packaging (MAP), in combination with refrigeration, is successfully and widely used as a packaging strategy for whole and minimally processed fruits and vegetables, in order to maintain product safety, to extend the shelf-life of these products, and to maintain organoleptic properties (Werner and Hotchkiss, 2006).

4.2.1. Temperature

Refrigeration is the most convenient and effective means to maintain the organoleptic properties, to reduce the spoilage, and to extend the shelf life of fresh produce. IFPA recommends to store minimally processed produce at 1 to 4 ºC to maintain quality and safety during the whole chain until consumption.

Besides the characteristics of the packaging film, temperature control is very important in order for a MAP system to work effectively. Temperature strongly affects respiration rate and permeability of gases through the packaging films. As a result of these characteristics, atmosphere changes within the packaging can occur (Hertog *et al.*, 1998; Jacxsens *et al.*, 2000). Furthermore, storage temperature is one of the most important factors that affect survival and growth of pathogens on fresh-cut produce. Maintaining produce temperature at or below 4 ºC throughout the entire processing chain is essential for microbial safety. For example, the concentration of *L. monocytogenes*, a psychrotrophic microorganism, remained constant or decreased on packaged vegetables stored at 4 ºC; at 8 ºC, on the other hand, the pathogen grew on all vegetables, with the exception of coleslaw mix (Francis and O'Beirne, 2001).

Mesophilic pathogens, such as *Salmonella* and *E. coli* O157:H7, are unable to grow at storage temperatures below or equal to 4 ºC. However, if temperature abuse occurs, they may grow. On a range of vegetables, *Salmonella* is unable to grow, but it consistently survived for more than 28 days at 2-4 ºC (ICMSF, 1996). *E. coli* O157:H7 populations survived on produce stored at 4 ºC, and proliferated rapidly when stored at 15 ºC (Richert *et al.*, 2000).

Because of difficulties in maintaining low temperatures in the cold-chain, additional barriers to control the growth of pathogens will be useful. Combining beneficial intrinsic, processing and extrinsic factors will result in considerable improvement in the microbial safety of the product.

4.2.2. Modified Atmosphere Packaging

Modified atmosphere packaging of fresh produce relies on modification of the atmosphere inside the package. This can be achieved by using active or passive MAP. Active MAP uses displacement or replacement of gases in the package, or gas scavengers or absorbers to establish a desired mixture of gases. Passive MAP occurs when the product is packaged using a selected film type, and a desired atmosphere develops naturally as a consequence of the products' respiration and the diffusion of gases through the film (Lee *et al.*, 1996; Moleyar and Narasimham 1994).

Oxygen, CO_2 and N_2, are the gases most often used in MAP. During product storage, O_2 is consumed and CO_2 is generated as a result of produce respiration. Nitrogen is an inert gas, which is used as filler gas in MAP to balance the volume decrease due to CO_2 absorption and to prevent package collapse (Sandhya, 2010). Generally, an atmosphere of 3 to 6 % O_2 and 2 to 10 % CO_2 achieves microbial control and extension of shelf life for a wide variety of fresh-cut produce (Werner and Hotchkiss, 2006).

Of the major gases used in MAP, CO_2 is the only that has significant and direct antimicrobial effect. Broadly, dissolved CO_2 inhibits microbial growth, affecting the lag phase, maximum growth rate, and maximum population densities that can be reached (Daniels *et al.*, 1985; Devlieghere and Debevere, 2000). The inhibitory effect of CO_2 is not universal but is dependent on the microorganism and growth phase, temperature, water activity and produce characteristics.

MAP studies describe a considerable variability in behavior of pathogens, mainly due to commodity differences and extrinsic factors. For example, Abdul-Raouf *et al.* (1993) reported declining cell populations on fresh-cut lettuce at 5 ºC, while Koseki and Isobe (2005) found no change at this temperature. Posada-Izquierdo *et al.* (2013) reported high variability of growth data between replicates on iceberg lettuce stored at 8 ºC under passive MAP with very low O_2 levels (<0.5 kPa). However, Francis and O'Beirne (2001) observed an increase of *E. coli* O157:H7 populations on iceberg lettuce and dry coleslaw under passive MAP at the same temperature. Oliveira *et al.* (submitted) reviewed the effect of MAP on foodborne pathogens in fresh-cut produce (Annex).

Other gases such as helium, argon and xenon (inert gases) and nitrous oxide (N_2O) are in use in MAP applications to reduce microbial growth and maintain the quality of produce (Meng *et al.*, 2012; Rocculi *et al.*, 2004; Tomás-Callejas *et al.*, 2011;

Zhang *et al.*, 2008). Also, high O_2 at superatmospheric levels is able to inhibit enzymatic discoloration, to prevent anaerobic fermentation reactions, and to influence aerobic and anaerobic microbial growth (Van der Steen *et al.*, 2002).

MAP in combination with refrigerated temperatures can be used as a mild preservation technique for safety of minimally processed produce. However, the effect of MAP on microorganisms can vary, mainly depending on the storage conditions and the type of produce. Furthermore, a potential consumer safety risk may occur due to MAP inhibition of the aerobic microorganisms that usually warn consumers for spoilage. This inhibition may result in reduction in growth competition, and may create an altered environment, allowing enhanced or unrestricted growth of anaerobic or facultative anaerobic pathogens capable of growing under MAP conditions at refrigeration temperatures. These include *L. monocytogenes*, *Clostridium botulinum*, *Yersinia enterocolitica* and *Aeromonas hydrophila*.

4.3. Biopreservation

Among alternative food preservation technologies, particular attention should be paid to biopreservation, or biological control, to extend the shelf-life and to enhance the hygienic quality of perishable food products. By biopreservation, storage life is extended and food safety improved through the use of native microbiota and/or their metabolites (Stiles, 1996). Different mechanisms, like production of inhibitory compounds, competition for nutrients, space or even colonization sites, are responsible for pathogen inhibition by biocontrol agents, (Whipps *et al.*, 2008).

Biopreservation presents a number of advantages over other control systems (Deacon, 1983):

- the use of antagonist microorganisms is safer than the chemicals mainly used at the moment because these microorganisms do not accumulate in the food;
- it may be more persistent over the time than chemical treatments because it is difficult for pathogens to develop resistance;
- it has a negligible effect on the ecological balance and does not affect the native microbiota as with the chemical treatments;
- it may be compatible with other control systems and therefore can be applied together (hurdle technologies);

Microorganisms used as protective agents should possess high antagonistic activity towards target species, be safe for human health by themselves and have no adverse effect on the product's sensory and nutritional quality (Rodov, 2007).

Biopreservation could be achieved by directly adding the biopreserving culture (epiphytic microorganisms, lactic acid bacteria, bacteriophages,...) or by applying the purified antimicrobial metabolites they produce.

4.3.1. Biopreservative cultures

Packaged produce harbors large populations of microorganisms including pseudomonads, lactic acid bacteria (LAB) and *Enterobacteriaceae*. Various studies describe that the presence of competing microorganisms on the surfaces of fresh produce contributes to the reduction of pathogens (Janisiewicz *et al.*, 1999; Liao and Fett, 2001; Parish *et al.*, 2003).

The use of native microbiota as biopreservative culture can be affected by disinfection processes, as they reduce their population and may favor the growth of foodborne pathogens. Carlin *et al.* (1996) showed that reducing the native microbiota on broad-leaved endive leaves by a chemical disinfection permitted a better growth of *L. monocytogenes*. Similar results were observed on fresh-cut lettuce dipped with chlorine or citric acid, where *L. innocua* counts increased compared to undipped samples (Francis and O'Beirne, 1997). On the other hand, addition of 2 selected strains, representatives of the native microbiota of endive, significantly limited the growth of *L. monocytogenes*. Babic *et al.* (1997) also reported that native microbiota in spinach inhibited the growth of *L. monocytogenes*.

Some authors hypothesize that a reduction in the population of the native microbiota as a result of the antimicrobial dip treatment may give foodborne pathogens a competitive advantage. In this sense, Francis and O'Beirne (1998) investigated the effect of inoculating sliced lettuce leaves with a mixture of the lettuce native microbiota at three different initial levels, on the growth of *L. innocua*. Results showed that the survival and growth of *L. innocua* was not affected by the numbers of the native microbiota population. The results suggested that the behavior of the human pathogens on produce is not simply related to the concentration of the native microbiota but also to the removal or reduction of key competitive subpopulations of the microbiota present.

L. monocytogenes and *Salmonella* Chester populations on green pepper disks stored at 20 ºC were not markedly affected by the presence of higher concentrations of the native microbiota. However, coinoculation with a strain of *Pseudomonas fluorescens* and a yeast, isolated from these green peppers, reduced the growth of both pathogens (Liao and Fett, 2001). Furthermore, several studies using biocontrol agents against foodborne pathogens in fresh-cut fruits were carried out by our research group. *Pseudomonas graminis* strain CPA-7 was effective in reducing *Salmonella* and *L. monocytogenes* on fresh-cut apples, peaches and melon (Abadias *et al.*, 2014; Alegre *et al.*, 2013a, b). This strain has been patented (Viñas *et al.*, 2010, 2011) and several studies are carried out for commercialization purposes. *Enterobacteriaceae* strain CPA-6 isolated from apples, reduced *E. coli* O157:H7, *Salmonella* and *L. innocua* populations on fresh-cut apples and peaches (Alegre *et al.*, 2012), and the probiotic strain *Lactobacillus rhamnosus* GG was able to reduce *L. monocytogenes* growth on fresh-cut apples (Alegre *et al.*, 2011).

Therefore, the potential of manipulation of the microbial ecology of produce to control human pathogens exists, and it has been suggested that understanding the microbial communities of produce is the key to pathogen control (Beuchat, 2002).

4.3.2. Bacteriocins

Bacteriocins are, low-molecular-mass peptides or proteins (usually 30-60 amino acids), which are ribosomally synthesized and excreted, and which have a bactericidal or bacteriostatic effect on other bacteria (Klaenhammer, 1988; Tagg *et al.*, 1976). Bacteriocins comprise a very heterogeneous group with respect to their primary structure, composition and physicochemical properties.

Most of them are effective against many gram-positive bacteria, which are either pathogenic or cause spoilage. In contrast, gram-negative bacteria are intrinsically resistant due to the protection offered by the cell wall. However, the effectiveness of bacteriocins can be improved by combining them with EDTA or technologies that affect the cell wall of the bacteria.

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation (Galvez *et al.*, 2007):

- they are generally recognized as safe (GRAS) substances;
- they are not active against or toxic to eukaryotic cells;
- they are inactivated by digestive proteases, thus having little influence on the gut microbiota;
- they are usually pH and heat-tolerant;
- they have a relatively broad antimicrobial spectrum, against many foodborne pathogenic and spoilage bacteria;
- they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane:
- they show no cross resistance with antibiotics;
- their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation;

Of the many bacteriocins produced by bacteria, nisin is the only commercially available one that is recognized as a safe and legal biological food preservative by FAO, WHO and the FDA. In the EU it is an approved preservative additive for use in certain foods (E-234).

Nisin is a small, heat-stable antimicrobial peptide of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis* (Davidson and Zivanovic, 2003). It shows a narrow antimicrobial spectrum, inhibiting only gram-positive bacteria and it does not generally inhibit gram-negative bacteria, yeasts or moulds.

The use of nisin as a biopreservative has been widely investigated in a large variety of fresh and processed foods. With respect to vegetables, Allende *et al.* (2007) evaluated the effect of bacteriocin-containing washing solutions on survival of *L. monocytogenes* on fresh-cut lettuce. The viability of *L. monocytogenes* decreased by more than 1 log-unit immediately after treatment. However, subsequent growth was not prevented. Randazzo *et al*. (2009) found that washing fresh-cut iceberg lettuce with nisin resulted in a decrease of about 1 log-unit of *L. monocytogenes* after 7 days of storage at 4 ºC. Moreover, washing with bacteriocin solutions did not significantly affect survival and proliferation of the native microbiota populations.

Bari *et al.* (2005) showed that washing fresh-cut cabbage and broccoli, inoculated with *L. monocytogenes*, with nisin alone or in combination with EDTA, sodium lactate, potassium sorbate or phytic acid resulted in approximately 3.0 log cfu/g reduction compared to unwashed controls.

Because bacteriocins are proteins and 'natural', there is great interest in their use as a novel method for ensuring the safety of minimally processed refrigerated foods.

4.3.3. Bacteriophages

Bacteriophages or phages are bacterial viruses that invade specific bacterial cells, disrupt bacterial metabolism, and cause the bacterium to lyse without compromising the viability of other flora in the habitat. They are the most abundant microorganisms in our environment, with estimations of 10^{31} phage particles in the world (Bergh *et al.*, 1989; Brüssow and Hendrix, 2002) and are present in high numbers in water and foods, (Hsu *et al.*, 2002; Kennedy *et al.*, 1986). Phages are also part of the gastrointestinal system (Greer, 2005), and may provide a natural, non-toxic, feasible approach for controlling several human pathogens.

Phages were first described and their viral nature appreciated by Felix d'Herelle in 1917, although their antibacterial activity had been independently recognized by Hankin in 1896, Gamaleya in 1898 and Twort in 1915 (Sulakvelidze *et al.*, 2001). Phages were used therapeutically in humans in the 1930s and 1940s, and, although their use in the United States and Western Europe was curtailed after the use of antibiotics became widespread, phages were continuously been used in the former Soviet Union and Eastern Europe (Sulakvelidze *et al.*, 2001). Their results, together with the study by Bruttin and Brüssow (2005), indicate that phages are safe for oral administration.

All phages require a bacterial host for replication, and virulent phages kill host cells by cell lysis. Host-phage interaction is mediated by the specificity of the phage, and the interaction can thus be specific at genus, species, or even strain level. Desirable characteristics of phages are the increase in numbers through lytic infection and the

lack of interference with the growth of the native competitive microbiota (Hudson *et al.*, 2009).

Promising results of phage biocontrol in food have been reported for several pathogens, including *Salmonella* (Guenther *et al.*, 2012; Kocharunchitt *et al.*, 2009; Leverentz *et al.*, 2001), *Campylobacter* (Goode *et al.*, 2003), *L. monocytogenes* (Carlton *et al.*, 2005; Dykes and Moorhead, 2002; Guenther *et al.*, 2009; Leverentz *et al.*, 2003; Oliveira *et al.*, 2014) and *E. coli* O157:H7 (Abuladze *et al.*, 2008; Sharma *et al.*, 2009; Viazis *et al.*, 2011). However, some studies reported that the effectiveness of many phages seems to decline at acidic pH (Leverentz *et al.*, 2001, 2003; Oliveira *et al.*, 2014). Moreover, the efficacy of phages in food also depends on the structure and chemical composition of the different food items. For efficient action of phages, homogeneous distribution and sufficient diffusion ability of the particles are necessary (Guenther *et al.*, 2009).

There are several phage preparations commercially available for food safety applications, such as ListShield™ and EcoShield™ (Intralytix, Inc., USA), Agriphage™ (Omnilytics, Inc., USA), Listex™ P100 and Salmonelex™ (Micreos Food Safety, formerly EBI Food Safety, The Netherlands). The approval of using phage preparations in food products by FDA and USDA provides the impetus for further investigation of phage applications.

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Objectives

The incidence of foodborne illness associated with the consumption of minimally processed produce has risen consistently, so consumers must be reassured that the fresh products they purchase are safe. To ensure the safety of minimally processed produce, their microbiological quality is a critical issue given that these produce do not receive any 'lethal' treatment that would guarantee the total elimination of pathogens prior to consumption.

The general objective of this thesis is to determine the influence of field management practices, processing and storage conditions on the microbial quality of fresh-cut lettuce, as well as to study intervention methods to enhance its safety.

The specific objectives can be divided into five sections:

1. Microbiological quality of fresh lettuce from the field

   1.1 To determine the microbiota present in lettuces at the time of harvest and to identify major groups of microorganisms (aerobic mesophilic, psychrotrophic, *Enterobacteriaceae*, lactic acid bacteria, yeasts and molds, *Pseudomonas*, *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*)

   1.2 To compare the microbiota of lettuces from conventional and organic production systems

2. Influence of pre-harvest management (irrigation water and compost) on the presence of FBPs on lettuce and in soil during two growing seasons

   2.1 To study the transfer of FBPs to lettuce leaves through contaminated water and compost

   2.2 To assess the effect of different types of irrigation water contaminated with FBPs: surface and sprinkler irrigation

   2.3 To evaluate the persistence of FBPs in soil and on lettuce leaves throughout the production cycle

3. Ability of FBPs to grow on minimally processed lettuce under different storage conditions

   3.1 To evaluate the survival and growth of FBPs on minimally processed lettuce packaged in films with different permeabilities at various storage temperatures

3.2 To determine the growth of psychrotrophic and mesophilic microorganisms on minimally processed lettuce stored under MAP conditions

4. Influence of various production stages of lettuce on the pathogenic potential of *Salmonella* Typhimurium DT104

4.1 To evaluate pathogen survival and growth after sequential incubation into soil, lettuce and cut lettuce stored under MAP conditions

4.2 To measure the capability of FBPs to survive a simulated gastrointestinal tract system and the proportion of bacterial cells adhering to and invading differentiated Caco-2 cells

5. The importance of biopreservation on the development of FBP populations in minimally processed lettuce

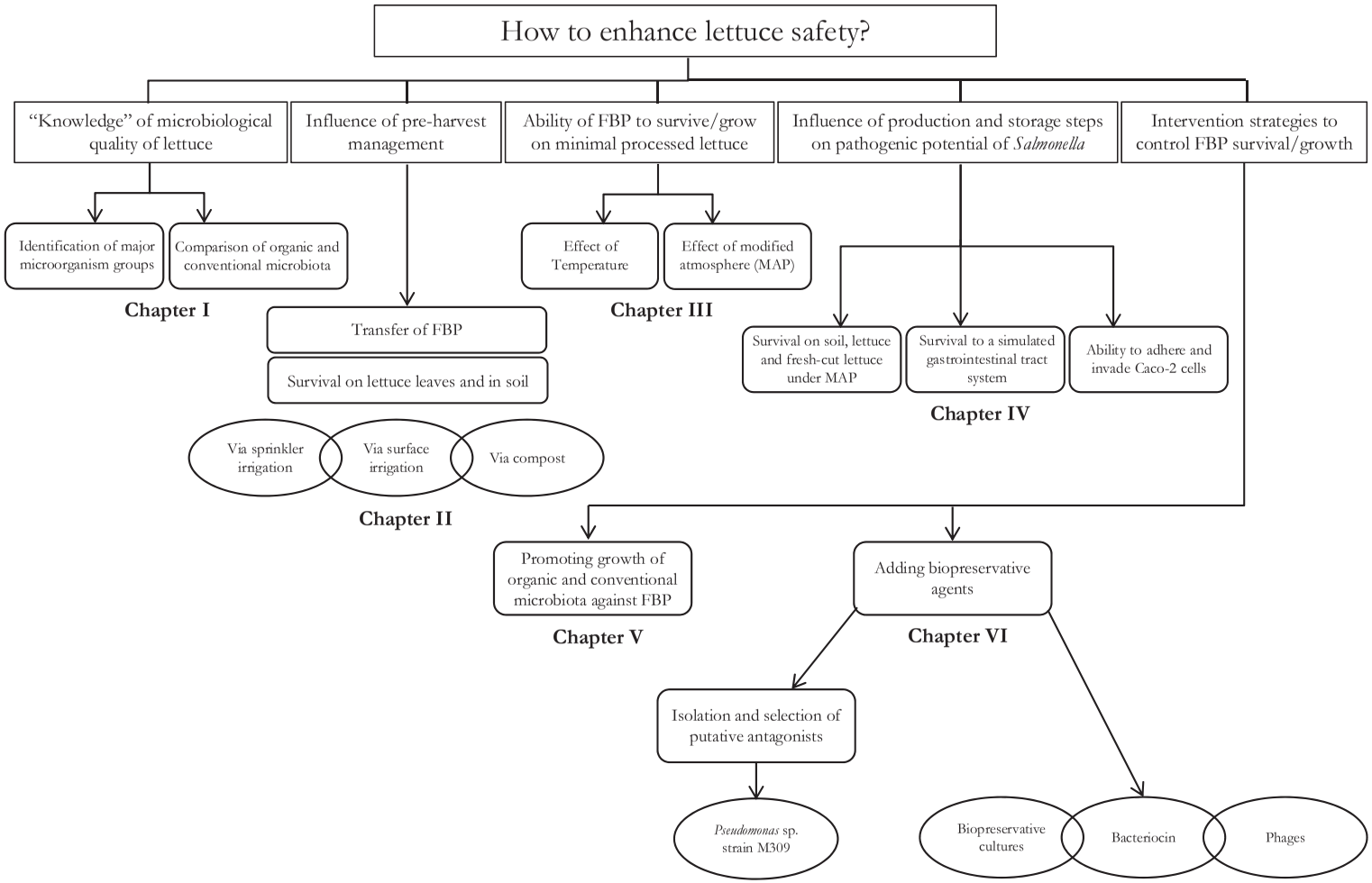
5.1 To study the interactions between native microbiota from conventional and organic lettuce and *L. monocytogenes* or *E. coli* O157:H7

5.2 To investigate the use of adding biopreservatives as a method to control and/or reduce FBP populations in minimally processed lettuce

5.2.1 To isolate native microorganisms from fresh and minimally processed produce and to assess their potential inhibitory effect on FBPs on lettuce

5.2.2 To compare the effect of the best putative bioprotective cultures, nisin and two bacteriophages on FBPs on fresh-cut lettuce under simulated commercial conditions.

Scheme


How to enhance lettuce safety?
"Knowledge" of microbiological quality of lettuce
Influence of pre-harvest management
Ability of FBP to survive/grow on minimal processed lettuce
Influence of production and storage steps on pathogenic potential of *Salmonella*
Intervention strategies to control FBP survival/growth
Identification of major microorganism groups
Comparison of organic and conventional microbiota
Chapter I
Transfer of FBP
Survival on lettuce leaves and in soil
Via sprinkler irrigation
Via surface irrigation
Via compost
Chapter II
Effect of Temperature
Effect of modified atmosphere (MAP)
Chapter III
Survival on soil, lettuce and fresh-cut lettuce under MAP
Survival to a simulated gastrointestinal tract system
Ability to adhere and invade Caco-2 cells
Chapter IV
Promoting growth of organic and conventional microbiota against FBP
Chapter V
Adding biopreservative agents
Chapter VI
Isolation and selection of putative antagonists
Pseudomonas sp. strain M309
Biopreservative cultures
Bacteriocin
Phages

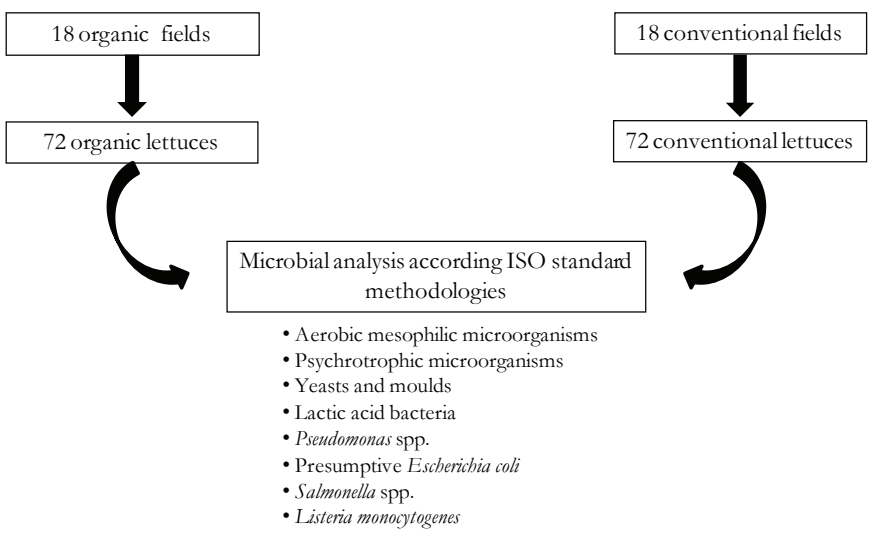
Methodology

In this section, a brief description of each chapter methodology will be explained:

Determination of the microbiological quality of fresh lettuce from the field (Chapter I)

Lettuce samples were obtained from organic and conventional farms that were located in the northeast of Spain. Organic lettuces were produced according the EU Regulation (CEE) 2092/91.

A total of 72 lettuce (*Lactuca sativa* L.) samples for each type of management practices were collected directly from the farm fields and were transported to the laboratory without being washed and immediately analyzed (Scheme 1). For each type of production system, 18 farms were analyzed and four samples of each were collected. The lettuce samples were analyzed for the presence of aerobic mesophilic and psychrotrophic microorganisms, yeasts and moulds, *Enterobacteriaceae*, mesophilic lactic acid bacteria, *Pseudomonas* spp. and presumptive *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*. Microbial analyses were carried out according to ISO standard methodologies for each group of microorganisms (Table 1).



Scheme 1. Determination of the microbiological quality of fresh lettuce from organic and conventional fields (**Chapter I**).

Table 1. List of methodologies used to determine microbial quality

Determination	Methodology	Description
Aerobic mesophilic count (AM)	ISO 4833:2003	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of microorganisms. Colony-count technique at 30 ºC.
Psychrotrophic microorganisms	ISO 17410:2001	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of psychrotrophic microorganisms.
Yeasts and moulds	ISO 7954:1987	Microbiology – General guidance for enumeration of yeasts and moulds – Colony count technique at 25 ºC.
Lactic acid bacteria	ISO 15214:1998	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of mesophilic lactic acid bacteria. Colony-count technique at 30 ºC.
Enterobacteriaceae	ISO 21528-2:2004	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Enterobacteriaceae* – Part 2: Colony-count method.
Pseudomonas spp.	ISO 13720:1995	Meat and meat products – Enumeration of *Pseudomonas* spp. (used for vegetable products).
Presumptive *E. coli*[a]	ISO 7251:2005	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique.
Salmonella spp.	ISO 6579:2002	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection of *Salmonella* spp.
L. monocytogenes	ISO 11290-2:1998	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Listeria monocytogenes*. Part 2: Enumeration method.

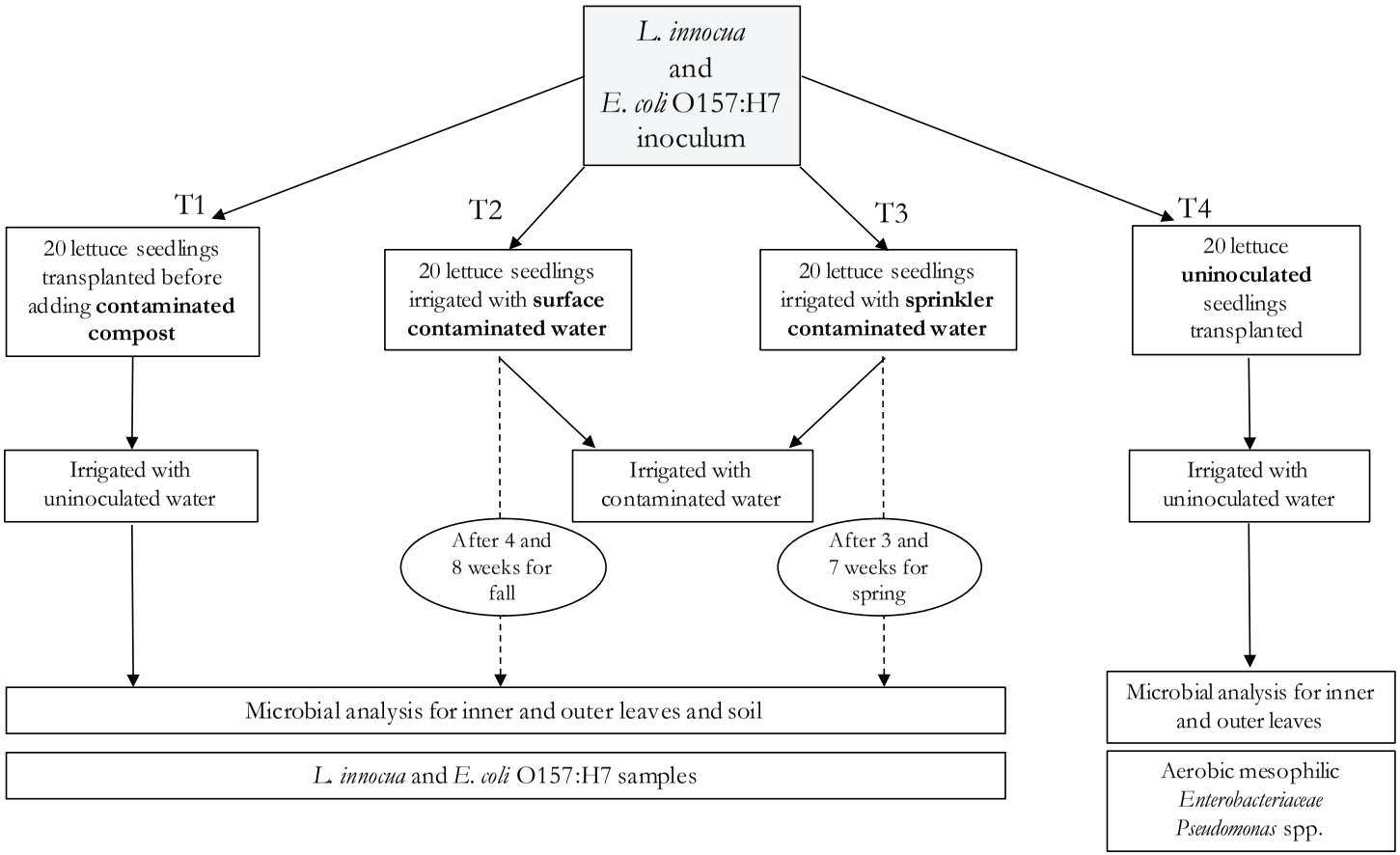
[a]Presumptive *E. coli* strains isolated were subsequently plated in Tergitol BCIG agar and Sorbitol MacConkey Agar and incubated at 44 ± 1 ºC for the detection of β-glucuronidase and sorbitol positive strains, respectively.

Influence of pre-harvest management (irrigation water and compost) in the presence of *L. innocua* and *E. coli* O157:H7 on lettuce and soil at harvest time during fall and spring (Chapter II)

For each pathogen, three treatments were performed and in the case of the *E. coli* O157:H7 assay, another treatment (uninoculated lettuce) was added. The experiment was carried out under outdoor conditions in two different seasons, first from early October to December 2008 (fall) and second from early April to early June 2009 (spring), in Lleida (Catalonia). A design plan was done according the following treatments (Scheme 2); T1: seedlings of lettuce (var. 'Romaine') were transplanted onto pots containing soil amended with contaminated compost; T2: each pot containing uninoculated compost was manually surface irrigated with contaminated water after the seedlings were transplanted, after 4 and 8 weeks for fall and 3 and 7 weeks, for spring; T3: contaminated irrigation water was hand-sprayed onto the lettuce leaves after the seedlings were transplanted, after 4 and 8 weeks for fall and 3 and 7 weeks, for spring; T4: seedlings were transplanted to uninoculated pots (control treatment). Twenty replicate lettuce pots were used for each treatment. Pots were grouped by treatments and separated 75 cm each other to avoid cross contamination.

As experiments were carried out outdoor, the strain *L. innocua* (CECT-910) was used as a microbial surrogate of *L. monocytogenes*. In the other assay, the non-pathogenic strain of *E. coli* O157:H7 (NCTC 12900) was used. In the uninoculated treatment, aerobic mesophilic, *Enterobacteriaceae* and *Pseudomonas* spp. populations were determined in lettuce leaves. At each sampling time, 5 g of the inner and outer lettuce leaves were obtained and 15 g of soil were collected from around the plant. There were four replications (4 pots) for each treatment at each sample time.

Daily measurements of environmental temperature (T), rainfall (mm) and percent relative humidity (RH) were collected by automated weather-monitoring equipment located close to the lettuce plants. Average T, rainfall and RH were calculated for each treatment period (from October to December and from April to June).



Scheme 2. Pre-harvest management (irrigation water and compost) in the presence of *L. innocua* and *E. coli* O157:H7 on lettuce and soil during fall and spring (**Chapter II**).

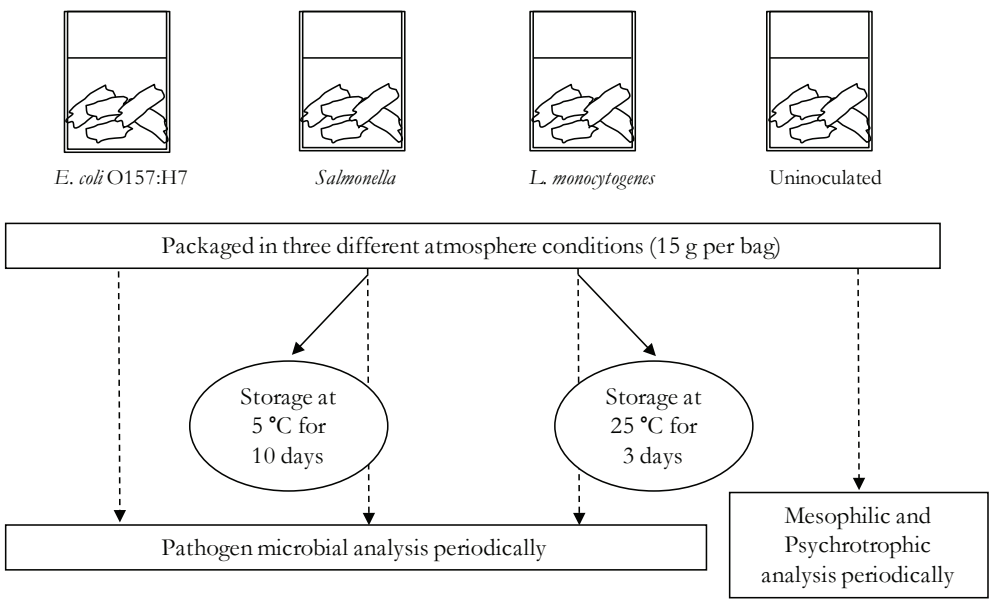
Study of FBP growth on minimally processed lettuce under different storage conditions (Chapter III)

Fresh 'Romaine' lettuce heads were used and obtained from a local supermarket in Lleida. The lettuce leaves were cut into pieces, washed in cold tap water and the excess of water was removed by a manual kitchen centrifuge. The shredded lettuce was divided in four batches (Scheme 3). Three of them were inoculated by dipping in a solution containing each of the pathogens (*E. coli* O157:H7, *Salmonella* and *L. monocytogenes*). The other batch was left uninoculated (control) and mesophilic and psychrotrophic microorganisms growth were determined.

Three different atmosphere conditions were studied: two different passive modified atmospheres and air condition. Uninoculated and inoculated samples were weighted (15 g) in different film bags (12 cm x 20 cm). Bags were sealed and one half were stored at 5 ºC for 10 days and the other half were stored at 25 ºC for 3 days for subsequent evaluation of microbial growth.

Throughout the experiment, CO_2 and O_2 concentrations and the pH of all samples were measured.

All analysis were carried out in triplicate packages for each microorganism and for each temperature/gas condition and the experiment was repeated twice.

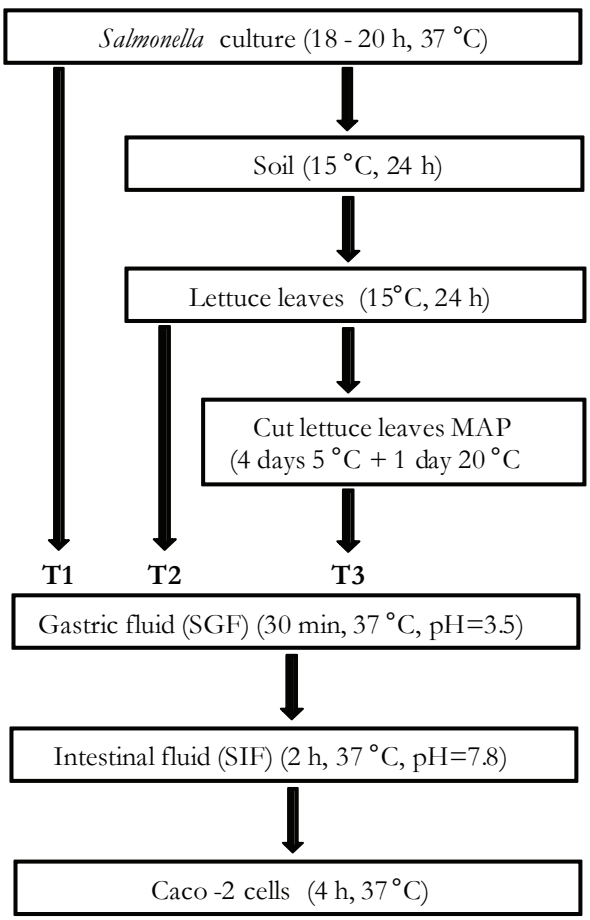


Scheme 3. Foodborne growth on fresh-cut lettuce under different storage conditions (**Chapter III**).

Influence of the various production stages of lettuce on pathogenic potential of Salmonella Typhimurium DT104 (Chapter IV)

Two *S.* Typhimurium DT104 strains were used, one isolate from a pig carcass and one isolate from lettuce. Both strains were subjected individually to the different steps studied. Soil was obtained from a local agricultural field (Zeist, The Netherlands) with 14 % of humidity and neutral pH. 'Butterhead' lettuce was obtained from a local supermarket in Zeist.

The study consisted of three treatments to evaluate the influence of different stages as encountered in the lettuce production chain on the pathogenic potential of *S.* Typhimurium. Initially, *S.* Typhimurium was inoculated into soil and transferred to lettuce which was subsequently cut and stored under MAP conditions (Scheme 4). From dif-



Scheme 4. Influence of production and storage stages of lettuce on pathogenic potential of *Salmonella* Typhimurium (**Chapter IV**).

ferent steps in this chain (T1, T2 and T3) *S.* Typhimurium was transferred into a static simulated gastrointestinal tract system including gastric fluid (SGF) and intestinal fluid (SIF). Finally differentiated Caco-2 cells were exposed to SIF samples containing *S.* Typhimurium to study the level of adhesion and invasion. Microbiological analysis was determined during all production and storage steps for both *S.* Typhimurium strains. All treatments were carried out in duplicate with four replications each.

Biopreservation as intervention strategy to control FBP growth in minimally processed lettuce

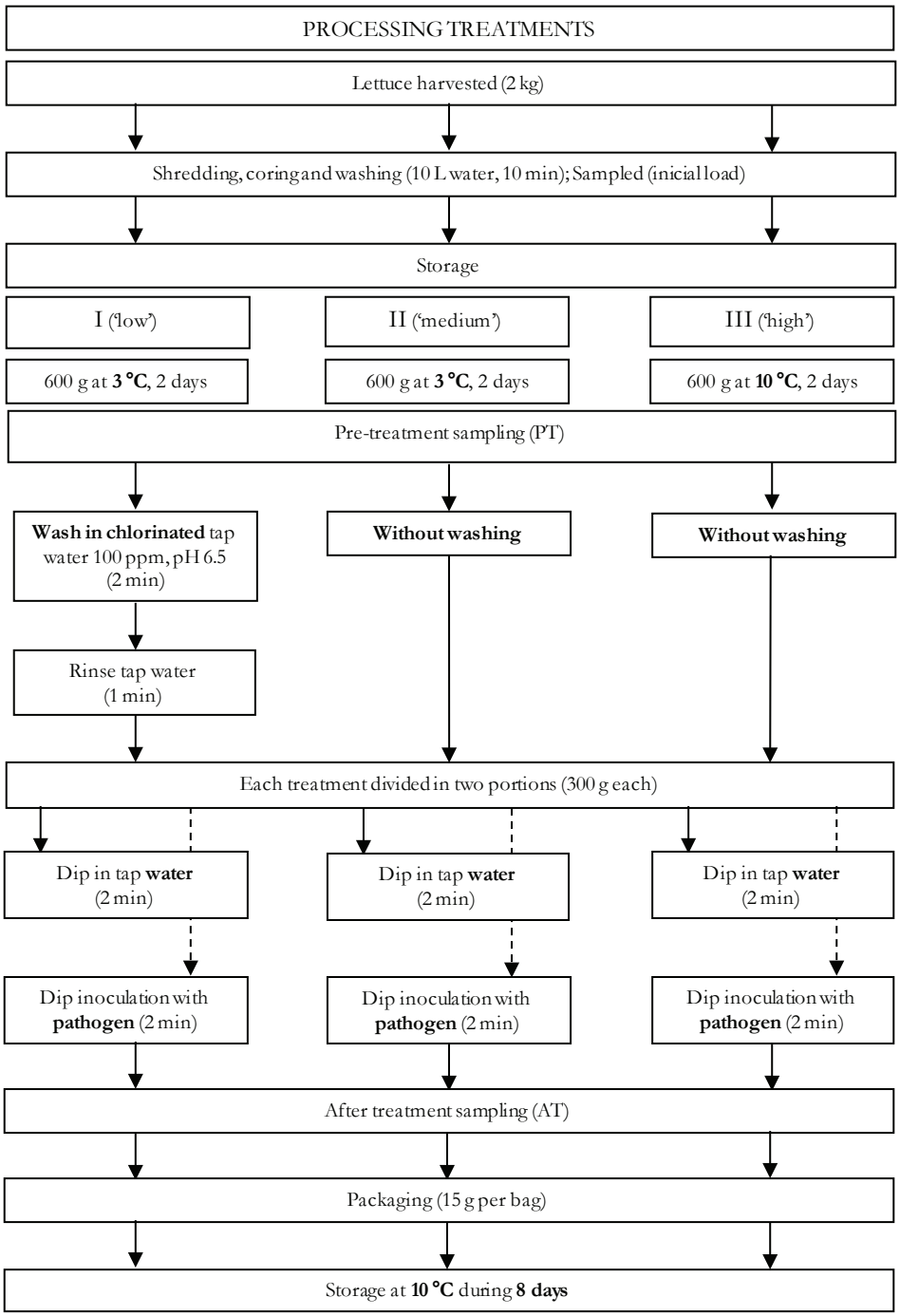
Effect of native microbiota from conventional and organic lettuce on the growth of L. monocytogenes *and* E. coli *O157:H7 (Chapter V)*

Organic and conventional 'Romaine' lettuce samples were obtained from a local supermarket in Lleida on the day of the experiment. Lettuce leaves were cut into pieces and were washed with tap water. Afterwards, the leaves were subjected to three different processing procedures designed to achieve different initial concentration levels of native microbiota (Scheme 5).

A non-pathogenic strain of green fluorescent protein (GFP)- expressing and ampicillin resistant *E. coli* O157:H7 and five strains of *L. monocytogenes* were used. Uninoculated and inoculated lettuce samples (15 g) were packaged individually in polypropylene plastic film bags (12 cm x 20 cm). Packages were sealed and stored at 10 ºC for 8 days for subsequent evaluation of microbial growth.

The native microbiota was enumerated on the day of arrival (initial load), after storage at different temperature (pretreatment, PT) and immediately after washing treatments (after treatments, AT). To achieve 'low' initial microbial load, one batch of fresh-cut lettuce was stored at 3 ºC for 2 days and then washed in chlorinated tap water to reduce it. The second batch of fresh-cut lettuce was stored at 3 ºC for 2 days without wash to obtain a 'medium' initial microbial load. Finally, the third batch was stored at 10 ºC for 2 days to promote microorganism growth. After processing, packages (uninoculated and inoculated) were sampled on the day of inoculation (AT) and after 2, 5 and 8 days to determine the effects of washing and temperature on the microbial load.

All experiments were replicated twice with three different lettuce bags per treatment/day for each type of lettuce origin (conventional or organic). Reported populations therefore represent the means of six values.



Scheme 5. Flow diagram used to obtain fresh-cut lettuce with different initial mesophilic load, pathogen contamination and storage of samples (**Chapter V**).

Isolation, screening for putative biopreservation cultures and comparison of different biopreservation products (Chapter VI)

Isolation and evaluation of several native microorganisms from fresh and minimally processed produce for potential inhibitory effect against FBP on lettuce

At first, a number of epiphytic microorganisms from whole and fresh-cut produce were isolated to study whether these microorganisms were antagonistic towards FBP (Scheme 6). A total of 112 putative antagonist isolates were screened for their ability to inhibit the growth of *S. enterica* on 'Romaine' lettuce leaf discs at 20 ºC for 3 days. When the pathogen population size in the presence of the putative antagonist was reduced by more than 1-log unit, the assay was repeated again to study the consistency of the results. On the contrary, when the reduction of the pathogen was less than 1-log unit, the microorganism was rejected. Moreover, when a microorganism reduced the size of the *S. enterica* population by more than 1.0-log unit in two consecutive screenings, it was tested twice against *L. monocytogenes* 230/3. Five different genera, belonging to *Pantoea*, *Pseudomonas*, *Enterobacter*, *Hafnia* and *Serratia,* were identified as putative antagonists to reduce *S. enterica* and *L. monocytogenes* 230/3 growth. Secondly, *Pseudomonas* sp. M309 strain was selected to be tested on lettuce discs at 10 ºC during 9 days against *S. enterica*, *E. coli* O157:H7 and a cocktail of *L. monocytogenes*.

To achieve this goal, 25 μL of a mixture of the putative antagonist and pathogen suspension were pipetted onto lettuce discs in individual small drops, and placed in plastic bowls before storage. The control treatments consisted of the pathogen suspension without antagonist. To recover the pathogen, five lettuce discs were used. To evaluate the antagonistic activity, the population sizes of the pathogen inoculated alone or in the presence of the possible antagonist were compared. Three replicas were assessed per treatment and sampling time.

Comparison the effect of the best putative bioprotective cultures, nisin and two bacteriophages solutions against FBP on fresh-cut lettuce under semi-commercial conditions

In this section, different biopreservation products, including *Pseudomonas* sp. strain M309, *Pseudomonas graminis* CPA-7, bacteriophages (Listex™ P100 and Salmonelex™) and nisin together with MAP and refrigerated temperature were used to inhibit a cocktail of *Salmonella* and *L. monocytogenes* on fresh-cut lettuce.

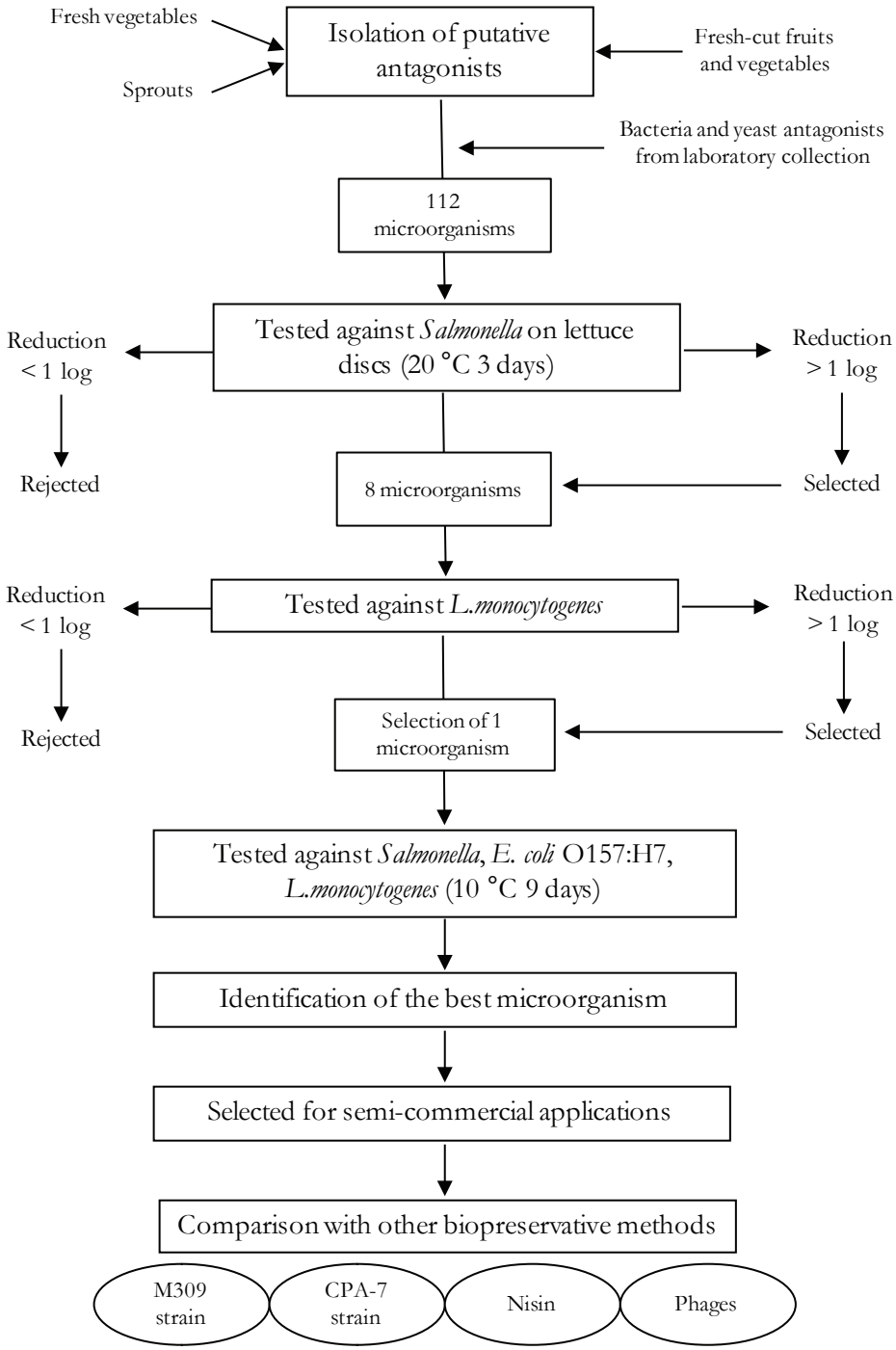
'Romaine' lettuce was hand-cut into pieces before inoculation. Three strains of *L. monocytogenes* and six strains of *Salmonella* were used in this assay. The strain M309 previously isolated in this study and *P. graminis* CPA-7 which was isolated from apple surface in our laboratory were used as putative antagonist microorgan-

isms. Both strains were selected because they have demonstrated antagonistic effect against FBP.

The shredded lettuce was inoculated with *Salmonella* and *L. monocytogenes* (both at 10^3 cfu/mL) separately by dipping and was stored at 10 ºC for 24 h. Samples of inoculated lettuce were separately immersed in the biopreservative solutions: phage (Listex P100 or Salmonelex, 10^8 pfu/mL), nisin (400 IU/mL), *Pseudomonas* sp. strain M309 and *P. graminis* (both at 10^7 cfu/mL). Sodium hypochlorite (SH, 100 ppm) and deionized water (DW) were used as a control treatments. Nisin was only used to wash lettuce contaminated with *L. monocytogenes*. The concentration of each pathogen on lettuce was determined both before and after the treatment.

Treated samples (15 g) were packaged individually in MAP in polypropylene plastic film bags (12 cm x 12 cm). Packages were sealed and stored at 10 ºC for 6 days for subsequent microbial evaluation. Due to the low pathogen concentration, microbial evaluation was carried out by enumeration and by detection after enrichment. Throughout the experiment and prior to all analysis, O_2 and CO_2 contents of all lettuce packages were measured.

The experiment was replicated twice with three different replications per treatment/day. Therefore, reported populations represent the mean of six values.



Scheme 6. Isolation, screening for putative biopreservation cultures and comparison of different biopreservation methods (**Chapter VI**).

Results

Chapter I

Microbiological quality of fresh lettuce from organic and conventional production

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Abstract

Previously there was no available information on the levels of indicator bacteria and the prevalence of pathogens in fresh lettuce grown in organic and conventional farms in Spain. A total of 72 lettuce samples (18 farms for 4 repetitions each) for each type of the agriculture were examined in order to assess the bacteriological quality of the lettuces, in particular the prevalence of selected pathogens. The lettuce samples were analyzed for the presence of aerobic mesophilic, psychrotrophic microorganisms, yeasts and moulds, *Enterobacteriaceae*, mesophilic lactic acid bacteria, *Pseudomonas* spp. and presumptive *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*. The mean aerobic mesophilic counts (AM) were 6.35 ± 0.69 log cfu/g and 5.67 ± 0.80 log cfu/g from organic and conventional lettuce, respectively. The mean counts of psychrotrophic microorganisms were 5.82 ± 1.01 log cfu/g and 5.41 ± 0.92 log cfu/g from organic and conventional lettuce, respectively. Yeasts and moulds (YM) mean counts were 4.74 ± 0.83 log cfu/g and 4.21 ± 0.96 log cfu/g from organic and conventional lettuce, respectively. Lactic acid bacteria (LAB) were present in low numbers and the mean counts were 2.41 ± 1.10 log cfu/g and 1.99 ± 0.91 log cfu/g from organic and conventional lettuce, respectively. *Pseudomonas* spp. mean counts were 5.49 ± 1.37 log cfu/g and 4.98 ± 1.26 log cfu/g in organic and conventional lettuce, respectively. The mean counts for *Enterobacteriaceae* were 5.16 ± 1.01 log cfu/g and 3.80 ± 1.53 log cfu/g in organic and conventional lettuce, respectively. *E. coli* was detected in 22.2 % (16 samples) of organic lettuce and in 12.5 % (9 samples) of conventional lettuce. None of the lettuce samples was positive for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. From the samples analyzed by principal component analysis (PCA) a pattern with two different groups (conventional and organic) can be observed, being the highest difference between both kinds of samples the *Enterobacteriaceae* count.

Keywords

Organic and conventional lettuce; Prevalence; Foodborne pathogens; Microbiological quality.

Introduction

In recent years, an increasing number of gastrointestinal disease outbreaks have been linked to the consumption of fresh fruits and vegetables. Most reports indicated that raw vegetables may harbor potential foodborne pathogens. Some outbreaks associated with consumption of lettuce contaminated with pathogens such as *Listeria monocytogenes* (Francis *et al.*, 1999; Sagoo *et al.*, 2003), *Salmonella* (Ercolani, 1976; FDA, 2001; Garcia-Villanova *et al.*, 1987; Sagoo *et al.*, 2003) and *Escherichia coli* O157:H7 (Ackers *et al.*, 1998; Friesema, 2007; Hilborn *et al.*, 1999) have been reported. Vegetables can become contaminated with such pathogenic organisms while growing or during harvesting, postharvest handling, or during distribution. Pre-harvest contamination of vegetables can occur directly or indirectly via animals, insects, water, soil, dirty equipment, and human handling. However, the most important considerations are the application of manure or compost as fertilizer to fields where crops are grown and the fecal contamination of irrigation water.

Manure and other animal wastes are widely used in agriculture, both organic and conventional. The use of manure as fertilizer, whether in organic or conventional agriculture, gives rise to concern about the possible contamination of produce with microbial pathogens (IFST, 1999). Some reports demonstrated that pathogens like *E. coli* O157, *Salmonella enterica* and *L. monocytogenes* are able to survive for extended periods (up to months) in manure (Franz *et al.*, 2005; Scott *et al.*, 2006) and manure-amended soil (Franz, 2008; Watkins and Sleath, 1981).

Organic production has been considered to represent an increased risk to public health than conventional production, due to the method of cultivation and processing, where natural fertilizers such as animal manure are used, and where no chemical treatments are employed to reduce the microbiological loading of the raw product, but there is little scientific evidence to support this suggestion (McMahon and Wilson, 2001).

In Spain, from 1996 to 2003 a substantial increase in the number of growers and land under organic management took place, partly due to the agri-environmental economic support scheme for organic farming. The number of organic farms increased by more than 15 % from the year 2001, from 15609 to 18226 organic farms in 2007. The area of organically managed land increased from 485,079 to 988,323 ha and thus nearly doubled. Although the size of the organic processing industry in Spain is still small, it has been growing continuously in the last five years at an annual rate of 10 % to 22 % (MAPA, 2007).

Organic farming was officially regulated in Spain in 1989, with the National regulation of Generic Denomination 'Organic Agriculture', which was applied until the EU Regulation 2092/91 on Organic Agriculture came into force. A Spanish regulation, RD 1852/1993 established a new regulation for organic farming, based on EU Regulation (CEE) 2092/91, and at the same time the Spanish regions assumed official responsibility in the monitoring of organic production. Under the same law

(RD 1852/1993) the Advisory Group CRAE of the National Ministry for Agriculture, Fisheries and Food (MAPA) was created. This group includes organic stakeholders, regional and central authorities as well as the directors of the regional public certification bodies (Gonzálvez, 2007). The EC regulation Nº 2092/91 was repealed in the 1 January 2009 by the Council Regulation (EC) Nº 834/2007 of 28 June 2007.

The aim of this study was to determine the effect of production system (conventional or organic) on the microbiological quality of fresh lettuce produced in Spain.

Material and methods

Origin of samples

Farmers that grew lettuces were invited by telephone or personal contact to participate in this study. The samples were obtained from organic and conventional farms that were located in the northeast of Spain. Organic lettuces were produced according the EU Regulation (CEE) 2092/91, as samples were analyzed before the 1 January 2009. All organic fields were certified by competent national authorities. Farmers were asked about the use of organic and inorganic fertilizers and chemical treatments. Livestock manure (sheep or cattle) were only applied in four organic fields. The others were fertilized with composted farmyard or plant manure. Conventional producers did not report the use of livestock manure.

Sampling and preparation of lettuce

A total of 72 lettuce (*Lactuca sativa L.*) samples for each type of the agriculture were collected directly from the farm fields and were transported to the laboratory without being washed and immediately analyzed. For each type of the agriculture, 18 farms were analyzed and four samples of each were collected. Different lettuce cultivars belonging to two different groups were used: *L. sativa* var. longifolia (Romaine lettuce) and *L. sativa* var. capitata (Batavia, 'Trocadero', Iceberg and 'Maravella' lettuce). The outer leaves and core of the lettuce were removed and discarded. The remaining leaves were hand cut on pieces with a disinfected sharp knife.

Microbiological analyses

Microbial analyses were carried out using the standard methodologies described in Table 1. Twenty-five grams of lettuce were transferred in 225 mL of saline peptone solution (SP, 8.5 g/L NaCl and 1 g/L peptone), in sterile stomacher bags. The samples were homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Further decimal dilutions were made with the same diluent and analyzed for aerobic mesophilic, psychrotrophic microorganisms, yeasts and moulds,

Enterobacteriaceae, mesophilic lactic acid bacteria, *Pseudomonas* spp. and presumptive *E. coli*. Another 25 g were diluted in 225 mL of buffered peptone water (Oxoid, CM1049) for the enumeration of *L. monocytogenes* and detection of *Salmonella*.

Table 1. List of methodologies used to determine microbial quality

Determination	Methodology	Description
Aerobic mesophilic count (AM)	ISO 4833:2003	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of microorganisms. Colony-count technique at 30 ºC.
Psychrotrophic microorganisms	ISO 17410:2001	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of psychrotrophic microorganisms.
Yeasts and moulds	ISO 7954:1987	Microbiology – General guidance for enumeration of yeasts and moulds – Colony count technique at 25 ºC.
Lactic acid bacteria	ISO 15214:1998	Microbiology of food and animal feeding stuffs – Horizontal methods for the enumeration of mesophilic lactic acid bacteria. Colony-count technique at 30 ºC.
Enterobacteriaceae	ISO 21528-2:2004	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Enterobacteriaceae* – Part 2: Colony-count method.
Pseudomonas spp.	ISO 13720:1995	Meat and meat products – Enumeration of *Pseudomonas* spp. (used for vegetable products).
Presumptive *E. coli* [a]	ISO 7251:2005	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique.
Salmonella spp.	ISO 6579:2002	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection of *Salmonella* spp.
L. monocytogenes	ISO 11290-2:1998	Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Listeria monocytogenes*. Part 2: Enumeration method.

[a] Presumptive *E. coli* strains isolated were subsequently plated in Tergitol BCIG agar and Sorbitol MacConkey Agar and incubated at 44 ± 1 ºC for the detection of β-glucuronidase and sorbitol positive strains, respectively.

The pathogenicity of *E. coli* strains was analyzed by the "Servicio de Bacteriología, Centro Nacional Microbiología, Instituto de Salud Carlos III" (Majadahonda, Madrid, Spain). The following tests were carried out: verotoxin gene Type 1; verotoxin gene Type 2; Intimin (gene eae); Enterohemolysin gene; adhesin (gene bfp); CVD432 plasmid; ipaH gene; heat stable toxins (st gene) and heat-labil toxin (lt gene).

Statistical analyses

To provide a general overview of the samples, a principal component analysis (PCA) was developed. Samples, coded as ORG (organically produce lettuce) and CON (conventional produce lettuce), were characterized by the microbial content, labelled as AM, PSI, YM, LAB, ENT and PSE, referring to aerobic mesophilic, psychrotrophic, yeast and moulds, lactic acid bacteria, *Enterobacteriaceae* and *Pseudomonas* spp., respectively. Raw data was used in the PCA calculation since variables show similar values of the respective standard deviations.

Results and Discussion

The present study was intended to provide some assessment on the microbiological quality of organic and conventional lettuce in Spain. To our knowledge, this is the first report that compares the microbiological quality of lettuce in two types of agriculture. By sampling during the preharvest stage, the counts of microorganisms were only influenced by farm practices and the source of those bacteria was likely the farm environment. Organic farmers used various types of animal wastes such as sheep, cattle and composted farmyard or plant manure. However, these differences of fertilizers' application did not have any significant effect on the contamination levels in the lettuce samples (data not show).

The aerobic mesophilic count (AM) ranged from <3 to 7 log cfu/g in conventional and from 5 to >7 log cfu/g in organic lettuce (Table 2). The highest percentage of the samples (76.4 and 86.1 % from organic and conventional farms, respectively) was found between 5 to 7 log cfu/g. In organic lettuce, 23.6 % of the samples had AM counts >7 log cfu/g, while only 1.4 % of conventionally-produced lettuce had such counts. The mean AM counts were 6.4 and 5.7 log cfu/g for organic and conventional samples, respectively (Table 3).

Psychrotrophic microorganisms counts were similar to those of mesophilic microorganisms, with ranges between 3 and >7 log cfu/g in organic lettuce and between <3 and 7 log cfu/g in conventional lettuce (Table 2). The highest percentage of the samples (68.0 and 72.3 % from organic and conventional farms, respective-

ly) was found in the range between 5 and 7 log cfu/g. Populations >7 log cfu/g only were found in 13.9 % of organic samples. The mean counts of these microorganisms were 5.8 and 5.4 log cfu/g from organic and conventional samples, respectively (Table 3). The number of AM present in this study was similar to that psychrotrophic counts and therefore most of the microorganisms present were able to grow at storage temperatures. The mean counts of these two groups of microorganisms were very similar to those obtained by Abadias *et al.* (2008) in Romaine lettuce purchased from supermarket. Aerobic colony count is useful for indicating the overall microbial quality of food product: generally it does not relate to food safety hazards but acts as an indicator for food quality and shelf-life duration (Pianetti *et al.*, 2008).

Yeasts and moulds (YM) were present in smaller numbers than bacteria, with ranges between <3 and >6 log cfu/g for both origins (Table 2). The highest percentage of the samples (51.4 and 52.8 % from organic and conventional farms, respectively) was found in the range between 4 and 5 log cfu/g. The YM mean was 4.7 and 4.2 log cfu/g from organic and conventional samples, respectively (Table 3). Abadias *et al.* (2008) and Tournas (2005) obtained similar results with samples of fresh and minimally-processed vegetables. Yeasts and moulds, when present in high numbers, can contribute to spoilage of fermented vegetable products and the development of soft rot (Fleet, 1992). Spoilage as a result of mould growth does not appear to be a major problem in ready-to-eat salads (Heard, 1999). However, some authors (Tournas, 2005; Tournas and Katsoudas, 2005) referred the possible health problems associated with the presence of moulds in vegetables, as some may produce mycotoxins and others are known to cause allergies when they are able to produce large numbers of conidia.

Lactic acid bacteria (LAB) were also present in low numbers and the range for LAB was <3 to 5 log cfu/g for both samples (Table 2). The highest percentage of the samples (73.6 and 87.4 % from organic and conventional farms, respectively) was found at values <3 log cfu/g. At concentrations >3 log cfu/g organic lettuce had higher numbers of samples than the conventional lettuce. There were no much differences between LAB mean count in organic (2.4 log cfu/g) and conventional (2.0 log cfu/g) lettuces (Table 3). These results agree with those obtained by Abadias *et al.* (2008) in shredded and whole lettuce. LAB is normal flora of vegetables and associated with spoilage organisms, causing unpleasant odors due to the production of ethanol, organic acids, esters and CO_2 (Babic *et al.*, 1992; Fleet, 1992).

Table 2. Prevalence of microorganisms counts on conventional and organic farms analyzed in each interval

Microorganisms	Lettuce samples	Percentage of samples in the indicated interval					
		$<10^3$ [a]	10^3-10^4	10^4-10^5	10^5-10^6	10^6-10^7	$>10^7$
Aerobic mesophilic count (AM)	Conventional	1.4	2.8	8.3	45.8	40.3	1.4
	Organic	0	0	0	38.9	37.5	23.6
Psychrotrophic microorganisms	Conventional	2.8	4.2	20.8	43.1	29.2	0
	Organic	0	2.8	15.4	37.5	30.5	13.9
Yeasts and moulds	Conventional	9.7	22.2	52.8	13.9	1.4	0
	Organic	2.8	15.3	51.4	20.8	9.7	0
Lactic acid bacteria	Conventional	87.4	6.9	5.6	0	0	0
	Organic	73.6	13.9	12.5	0	0	0
Pseudomonas spp.	Conventional	5.5	13.9	23.6	34.7	22.2	0
	Organic	4.2	5.5	22.2	30.5	23.6	13.9
Enterobacteriaceae	Conventional	29.2	20.8	23.6	22.2	4.2	0
	Organic	2.8	5.5	41.7	29.2	19.4	1.4

[a]: Range in cfu/g.

Table 3. Microorganism counts for conventional and organic lettuce samples

		Mean (log cfu/g)	Standard deviation	Minim (log cfu/g)	Maximum (log cfu/g)
Aerobic mesophilic count (AM)	Conventional	5.67	0.80	2.85	7.04
	Organic	6.35	0.69	5.19	7.81
Psychrotrophic microorganisms	Conventional	5.41	0.92	2.30	6.86
	Organic	5.82	1.01	3.38	7.85
Yeasts and moulds	Conventional	4.21	0.96	1.41	6.97
	Organic	4.74	0.83	2.70	6.45
Lactic acid bacteria	Conventional	1.99	0.91	0.54	4.90
	Organic	2.41	1.10	0.54	5.03
Enterobacteriaceae	Conventional	3.80	1.53	0.54	6.34
	Organic	5.16	1.01	2.50	7.81
Pseudomonas spp.	Conventional	4.98	1.26	1.41	6.93
	Organic	5.49	1.37	1.41	7.63

Pseudomonas spp. counts were similar in both origins, with ranges between <3 and >7 log cfu/g in organic lettuce and between <3 and 7 log cfu/g in conventional lettuce (Table 2). The highest percentage of the samples (30.5 and 34.7 % from organic and conventional farms, respectively) was found between 5 and 6 log cfu/g. Only organic lettuce samples (13.9 %) had *Pseudomonas* spp. counts >7 log cfu/g. *Pseudomonas* spp. mean counts were 5.5 and 5.0 log cfu/g in organic and conventional samples, respectively (Table 3). As LAB, pseudomonads are normal microbiota of vegetables, whereas coliforms, yeasts and moulds may arise from the raw material or from contamination during harvest and processing (Nguyen-the and Carlin, 1994). High numbers of pseudomonads are undesirable because they are often responsible for spoilage of fresh vegetables due to the production of pectinolytic enzymes which cause breakdown of the peptic polymers in plant cells (Membré and Burlot, 1994) and they grow at refrigeration conditions after processing (Brackett, 1994; Nguyen-The and Carlin, 1994). These results were also obtained for Pianetti *et al.* (2008) in ready-to-eat vegetables salads with concentrations of *Pseudomonas* spp. between 5 and 7 log cfu/g.

The microflora of vegetables is diverse but consists predominantly of gram negative bacteria such as *Enterobacteriaceae*. The range for these group in both samples was <3 to 7 log cfu/g (Table 2). About 92 % of the organic samples had counts of >4 log cfu/g, while the conventional samples were distributed all over the range. Only 4.2 % of the conventional samples had counts of >6 log cfu/g while 20.8 % of organic samples were found in this range. The mean counts were 5.2 and 3.8 log cfu/g from organic and conventional samples, respectively (Table 3). The levels of *Enterobacteriaceae* found in this study are common in raw vegetables and are not necessarily associated with fecal contamination, because the majority of the genera are part of the endogenous microflora of the product (Brackett and Splittstoesser, 2001), such as *Erwinia*, *Pantoea* or *Enterobacter*.

Levels of fecal organisms, such as *E. coli* are a better indicator of contamination (Nguyen-the and Carlin, 1994) and this could explain why this organism has been included as a hygienic criterion in the EU regulation (Nº 2073/2005). *E. coli* were detected in 22.2 % (16 samples) of organic lettuce. These samples came from 9 different fields. Nine lettuces (12.5 %) belonging to 3 different conventional fields harbored *E. coli* (Table 4). The highest incidence of *E. coli* in organic lettuce was found between 30 and 99 MPN/100 g (13.9 %) and it was not detected in counts of >1000 MPN/100 g. In conventional lettuce the highest number of samples (8.4 %) had counts of >1000 MPN/100 g. This fact could be due to inadequate hygienic practices during preharvest or sporadic contamination through irrigation water. However, no data concerning the quality of the water used was obtained from the producers. About 8.3 % of organic lettuce samples exceeded the hygienic criteria established by the 2073/2005 guidelines, while this limit was exceeded in 11.2 % of conventional lettuce samples. Mukherjee *et al.* (2004) reported that 22.4 % of organic lettuce was

positive for *E. coli*, whereas in conventional lettuce the prevalence was 16.7 %. These results are consistent with the levels we found. However, Loncarevic *et al.* (2005) reported lower *E. coli* prevalence (8.9 %) in samples of Norwegian organically grown lettuce. Mukherjee *et al.* (2006) reported an average *E. coli* count ranging from 2 to 2.4 log MPN/g for organic and conventional fresh produce samples. Bohaychuk *et al.* (2009) found that no statistical difference in the percentage of produce samples containing this bacterium grown by either organic or conventional practices. A total of 16 colonies of *E. coli* were isolated in our study. None of them had the virulence genes that are pathogenic for humans. The *E. coli* counts in this study were lower than the counts observed by others reports.

Table 4. Presence and enumeration of *Escherichia coli* in lettuce from organic and conventional farms.

	Escherichia coli prevalence					
	No. of samples <30 MPN/100 g	**No. of positive samples (%)**	**No. fields**	**30-99 MPN/100 g (%)**	**100-999 MPN/100 g (%)**	**>1000 MPN/100 g (%)**
Conventional	63 (87.5)	9 (12.5)	3	1.4	2.8	8.4
Organic	56 (77.8)	16 (22.2)	9	13.9	8.3	0

%: number of positive samples/number of samples

Salmonella spp. and *L. monocytogenes* were not detected in any organic and conventional lettuce samples. In other recent study in Norway, *Salmonella* and *E. coli* O157:H7 were not detected in 179 organically grown lettuce samples (Loncarevic *et al.*, 2005). Mukherjee *et al.* (2004) reported no *E. coli* O157:H7 contamination in any of the organic and conventional produce analyzed; however, one lettuce sample had *Salmonella* contamination. *Salmonella* species have been isolated from a range of vegetables in Spain (Garcia-Villanova Ruiz *et al.*, 1987) but in other recent studies, a variety of organic fresh produce samples from United Kingdom producers were free of *Salmonella* (McMahon and Wilson, 2001; Sagoo *et al.*, 2001). Authors concluded that the use of contaminated irrigation water or animal manure as fertilizer can be an important factor in such outbreaks.

The absence of *L. monocytogenes* in this study is comparable to that found by Sagoo *et al.* (2001; 2003) and McMahon and Wilson (2001) in organic vegetables. However, Wilson (1995) showed that 2.0 % of conventional farmed ready-to-eat vegetable products in Northern Ireland contained *Listeria* spp. *Listeria* is environmental microorganism that is found in soil and water. Therefore, vegetables may easily become contaminated with this bacterium. Furthermore, the background microbiota

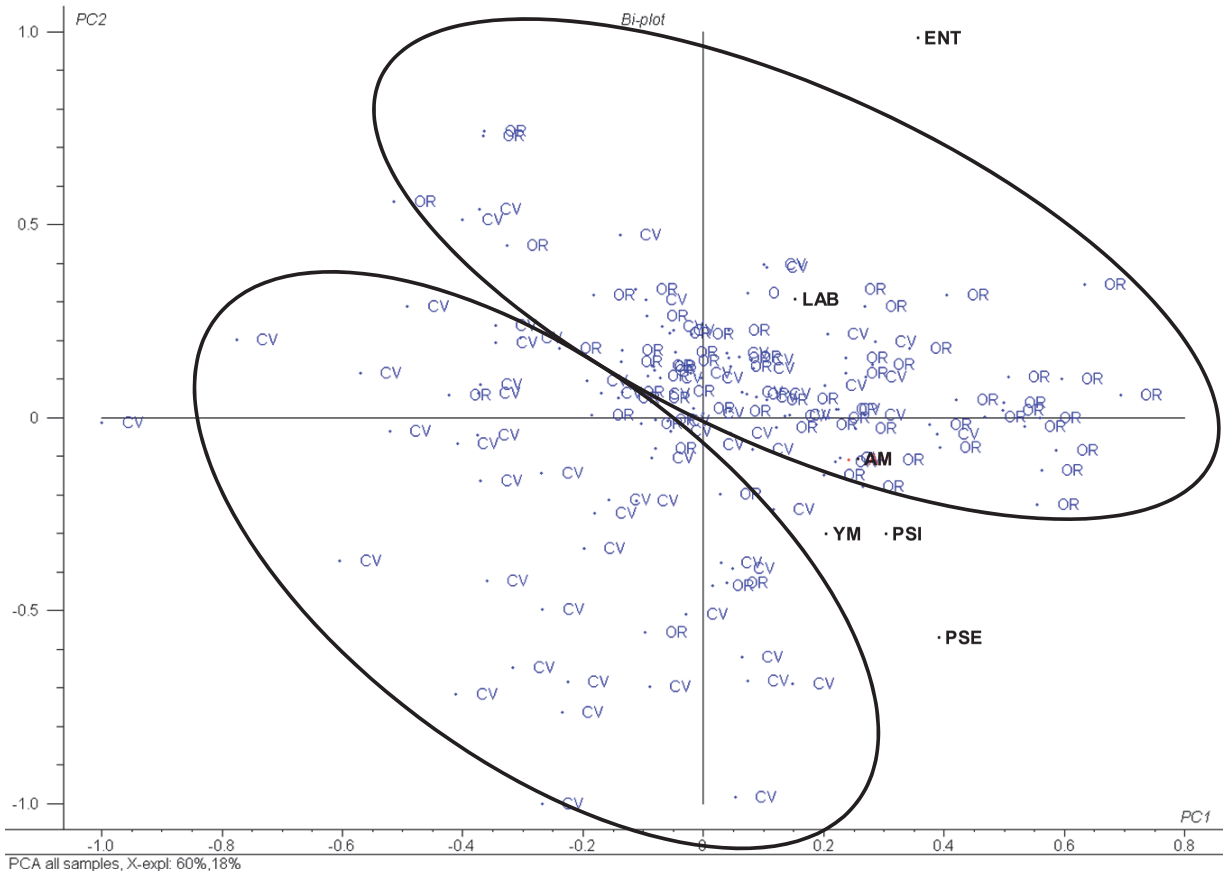

Figure 1. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model for organic and conventional farms. Codes of variables are defined in material and methods section.

present on the vegetables might have an inhibitory effect on *L. monocytogenes* (Babic *et al.*, 1997; Carlin *et al.*, 1996; Liao and Fett, 2001).

A global overview of all these differences between conventional and organic lettuce samples can be seen in Figure 1 which depicts the scores and loadings according to principal components 1 (PC1) and 2 (PC2). These components accounted for 60.0 and 18.0 % of the total variance, respectively. Even though there are some mixed samples, a pattern with two different groups can be observed in Figure 1: organic samples tend to be located at higher values of PC1 and PC2 while conventional lettuce tend to show lower scores on PC1 and PC2. Thus, organic samples can be associated to higher values of all the microbial analyzed since all of them show positive loading with respect to PC1. The highest difference between both kinds of samples arises in the *Enterobacteriaceae* count since this characteristics show the highest score on both PC1 and PC2. Thus, figure 1 summarizes the detailed behavior depicted in Table 2 for organic and conventional lettuce samples.

Even the number of samples was not too high; the absence of pathogens associated with these organic and conventional lettuce samples indicates that overall agricultural, hygiene, harvesting and practices carried out by farmers were good. A number of reports have promoted the idea that organic produce poses a greater risk of transmitting foodborne diseases than does conventional produce (Avery, 2002; Stossel, 2000). In contrast, the results obtained showed that the consumption of organically produced lettuce does not represent an increasing risk of a foodborne disease by consumers.

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Chapter II

Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves

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Abstract

Many foodborne outbreaks of some pathogens such as *Escherichia coli* O157:H7, *Salmonella* or *Listeria* have been associated with the consumption of contaminated vegetables. Contaminated manure and polluted irrigation water are probable vehicles for the pathogens. The aim of this study was to determine the potential transfer of *Listeria innocua* from soil fertilized with contaminated compost or irrigated with contaminated water to the edible parts of lettuce grown on these soils together with its survival in lettuce and in soil under field conditions during two different seasons. Moreover, its survival on lettuce sprinkled with contaminated irrigation water was evaluated. *L. innocua* survived in soil samples for 9 weeks at high concentrations, 10^5 cfu/gdw in fall and 10^3 cfu/gdw in spring. Pathogen survived better in fall, indicating an important influence of temperature and humidity. *L. innocua* population in lettuce leaves was very high on lettuce leaves after sprinkling, but decreased to undetectable levels at field conditions. There was also transfer of *L. innocua* from soil contaminated with compost or irrigated with contaminated water to lettuce leaves, mainly to the outer ones. Survival profiles of *L. innocua* on lettuce and soil samples contaminated either by application of contaminated compost or surface irrigation water was similar. Our results indicated that contaminated compost and contaminated irrigation water can play an important role in the presence of foodborne pathogens on vegetables.

Keywords

Lettuce; Soil; Compost; Irrigation water; *Listeria innocua*; Transfer.

Introduction

Minimally processed vegetables, such as lettuce, have recently undergone an increase in consumer demand because of their healthy image and convenience. Some of these products can also contain potential pathogens (Beuchat, 1996b; Francis *et al.*, 1999; Nguyen-The and Carlin, 1994), and some have been implicated in an increasing number of outbreaks of foodborne illnesses (Long *et al.*, 2002). Bacterial pathogens such as *Salmonella enterica* (Guo *et al.*, 2001), *Escherichia coli* O157:H7 (Solomon *et al.*, 2002), *Bacillus cereus* (Beuchat and Ryu, 1997), *Listeria monocytogenes* (Robertson *et al.*, 2002), *Campylobacter jejuni* (Beuchat and Ryu, 1997), and *Pseudomonas* spp. (Hamilton-Miller and Shah, 2001; Viswanathan and Kaur, 2001) are especially of major concern due to the environmental occurrence of these bacteria.

L. monocytogenes has been linked to potentially severe foodborne diseases characterized by meningitis, encephalitis or septicaemia in immunocompromised patients, pregnant women, infants and the old people (Farber and Peterkin, 1991). *L. monocytogenes* is found in diverse environments, therefore the research have focused attention on cross-contamination of processed vegetables by this pathogen, and explain the difficulty in producing minimally processed vegetables free of pathogenic bacteria (Gravani, 1999).

Some outbreaks of listeriosis have been associated with the consumption of lettuce and cabbage (Farber and Peterkin, 1991) and it has been demonstrated that *L. monocytogenes* can grow on a variety of vegetables even at refrigeration temperatures (Brackett, 1999; Oliveira *et al.*, 2010).

Fresh produce can become contaminated at any point during the primary production, processing, distribution and preparation. Primary sources of preharvest contamination include soil amended with untreated or improperly composted manure, contaminated irrigation water, the presence of wild and domestic animals, infected workers, and unclean containers and tools used in harvesting (FDA, 2008). The research has demonstrated that many human pathogens are able to survive for extended periods in soils (Davies and Wray, 1995), manure (Kudva *et al.*, 1998), and water (Chalmers *et al.*, 2000; Wang and Doyle, 1998), and thereby provide potential inocula for contamination.

Some authors have reported that survival times of *E. coli* O157:H7 in manure-amended soil range from several weeks to more than 6 months (Franz *et al.*, 2008; Islam *et al.*, 2004a; Jiang *et al.*, 2002; Johannessen *et al.*, 2005;). Furthermore, studies have demonstrated the ability of this pathogen to survive for extended periods in water (Chalmers *et al.*, 2000; Wang and Doyle, 1998). The presence of *L. monocytogenes* has been reported in sewage sludge (Al-Ghazali and Al-Azawi, 1986; De Luca *et al.*, 1998; Garrec *et al.*, 2003), in soil (Welshimer, 1960; Welshimer and Donker-Voet, 1971), on plants (Weis and Seeliger, 1975; Welshimer and Donker-Voet, 1971), including vegetables (Beuchat, 1996a) and on the surface of plants cultivated in a

gnotobiotic system (Jablasone *et al.*, 2005). Survival of the pathogens into the soil habitat, depends of the abiotic (temperature, pH, soil moisture, soil type) and biotic (composition and diversity of the microbial community) factors (Van Veen *et al.*, 1997); however, competition for limited water and nutrients makes it difficult to survive on leaf surfaces (Mercier and Lindow, 2000). Moreover, *L. monocytogenes* is able to survive and multiply under various stress conditions, including those likely encountered on plant surfaces (Brandl, 2006; Garner *et al.*, 2006; Vermeulen *et al.*, 2007).

The aim of this study was to investigate the potential for an avirulent strain of *Listeria* (*Listeria innocua*) to be transferred from soil fertilized with contaminated compost and contaminated irrigation water (surface or sprinkler) to the edible parts of lettuce, when seedlings were transplanted into soil pots under natural environmental conditions. Survival of *L. innocua* on the leaves and in the soil was also studied.

Material and methods

Experimental design

A design plan was followed with three treatments; T1: seedlings of lettuce were transplanted onto pots containing soil amended with contaminated compost; T2: each pot containing uninoculated compost was manually surface irrigated with contaminated water after the seedlings were transplanted, after 4 and 8 weeks for fall and 3 and 7 weeks, for spring; T3: contaminated irrigation water was hand-sprayed onto the lettuce leaves after the seedlings were transplanted, after 4 and 8 weeks for fall and 3 and 7 weeks, for spring. Twenty replicate lettuce pots were produced for each treatment. The experiment was repeated in two seasons as explained before.

Preparation of inocula

A strain of *L. innocua*, CECT-910, was used 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 tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) supplemented with 6 g/L yeast extract (TYSEB) at 37 ºC for 20-24 h. Bacterial cells were harvested by centrifugation at 9820 x g for 10 min at 10 ºC and resuspended in sterile saline peptone (SP, 8.5 g/L NaCl and 1 g/L peptone). The concentration of *L. innocua* was estimated using a spectrophotometer set at λ=420 nm according to the standard curves and it was checked by plating duplicate serial suspension dilutions on Palcam medium (Biokar Diagnostics) followed by incubation at 37 ºC for 48 h.

Lettuce production conditions

Seedlings with four or five true leaves of 'Romaine' lettuce (*Lactuca sativa* var. longifolia) were purchased from a local producer in Lleida (Spain).

The lettuce plants were grown in plastic pots (23 cm diameter) containing commercial autoclaved (30 min at 121 ºC) soil (1200 g per pot, Terraplant®[1], manufactured by COMPO GmbH & Co. KG, Münster, Germany) amended with inoculated or uninoculated organic compost (150 g per pot) (S1, Compost Segria, Lleida, Spain). Each pot was punctured at the bottom and placed inside another unpunctured pot for collection of drainage solution potentially contaminated by the inoculums and hence preventing contamination of the environment. Plants were watered when the soil surface began to dry.

The experiment was carried out under outdoor conditions in two different seasons, first from early October to December 2008 (fall) and second from early April to early June 2009 (spring), in Lleida (Catalonia, Spain). The climate of the area in which the experiment was carried out is dry continental. Daily measurements of environmental temperature (T), rainfall (mm) and percent relative humidity (RH) were collected by automated weather-monitoring equipment located around the lettuce plants. Average T, rainfall and RH were calculated for each treatment period (from October to December and from April to June).

Inoculation of compost and irrigation water

Compost (3000 g) was sprayed with 300 mL of a suspension containing 10^7 cfu/mL and mixed thoroughly before addition to soil. Soil and contaminated compost were mixed vigorously to ensure homogeneous distribution of the pathogen into each pot before planting the seedlings.

Contaminated irrigation water with *L. innocua* at 10^7 cfu/mL was prepared. Each pot was manually surface irrigated with 200 mL of contaminated water after planting the lettuce seedlings. The water was applied carefully to prevent splashing of the inoculum onto the edible portion of the lettuce plant. In another treatment, a hand sprayer was used, at the same time of surface irrigation, to sprinkle 500 mL of contaminated water onto the lettuce leaves of all twenty pots.

Soil and lettuce sample collection

All samples were collected using alcohol-sanitized gloves and disinfected spoon, tweezers and scalpel. At each sampling time, approximately 5 g of the inner and outer lettuce leaves were obtained aseptically and placed in a sterile Stomacher bag separately. Approximately 15 g of soil was aseptically collected in a plastic bag

from around the plant, from each pot. There were four replications (4 pots) for each treatment at each sample time.

Microbiological analysis

Soil and lettuce samples from each treatment were collected randomly on the day of transplanting (d0) and after 4, 6, 8 and 9 weeks for fall, and 3, 5, 7 and 9 weeks for spring. Moreover, in irrigation treatments (T2, T3), soil samples were collected before and after irrigation in the days of re-inoculation. In the treatment by sprinkler (T3), leaves samples were also collected before and after irrigation.

For the analysis, 5 g of lettuce leaves (inner and outer) were mixed with 45 mL of buffered peptone water (Oxoid, CM1049) in a sterile Stomacher bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Ten grams of soil were added to 90 mL of buffered peptone water in an Erlenmeyer flask and homogenized by shaking at 150 rpm for 10 min. Serial dilutions (1:10) of each sample were prepared using SP and enumerated by plating on Palcam agar media and incubated at 37 ºC for 48 h. Moisture content of soil amended with compost was determined by drying approximately 3 g of soil at 105 ± 1 ºC for 24 h in a drying oven and then weighing the residual. The colony forming units in the soil were calculated per dry weight (dw). When the pathogen was not detected by direct plating (*L. innocua* detection limit was 50 cfu/g), soil or lettuce samples were incubated for 24 h at 37 ºC for non-selective pre-enrichment. Afterwards, two consecutive selective enrichment steps in Half-Fraser and Fraser were done based on ISO 11290-1:1996/FDAM 1:2004. In the primary enrichment, 9 mL of half-concentrated Fraser broth (Biokar Diagnostics) was inoculated with 1 mL of the non-selective pre-enriched sample material and incubated for 24 h at 30 ºC. From the first enrichment step, 0.1 mL was inoculated on to 10 mL of the secondary enrichment media Fraser broth (Biokar Diagnostics) and incubated at 37 ºC for 48 h. After each 24 h period of incubation, cultures were plated out on the selective media (Palcam) for 48 h at 37 ºC, for confirmation the presence or absence of *L. innocua* colonies. In samples that were *L. innocua* positive after enrichment, an arbitrary value of 1.40 log cfu/g (half of detection limit) was assigned for data representation. Data were transformed to log cfu/gdw for soil samples and to log cfu/g for lettuce samples.

Results

Environmental data

Weekly average temperatures, precipitation and relative humidity for the area are presented in Tables 1 and 2. In fall, the average weekly temperature varied from a maximum of 27.5 ºC in week 1 to a minimum of -0.5 ºC in week 8 (Table 1). Average relative humidity varied from a minimum of 39 % in week 1 to a maximum of 100 % in week 6. A maximum rainfall of 55 mm was in week 4.

Table 1. Average weekly temperature, relative humidity and rainfall for fall (October-December)

week	Average temperature (ºC)			Average relative humidity (%)			Total rainfall (mm)
	Maximum	Minimum	Mean	Maximum	Minimum	Mean	
1	27.5	8.8	17.7	88	39	69	0
2	25.9	12	17.7	91	53	79	1
3	20.9	5.8	12.2	92	51	77	0
4	16	2.3	10.0	90	56	81	55
5	17.8	2.5	9.0	92	53	82	1
6	19.4	2.3	7.9	100	46	92	0
7	14.4	3	7.5	99	70	91	0
8	10.9	-0.5	4.5	95	47	83	0
9	13.1	1	7.5	98	53	88	3

In spring, the average weekly temperature varied from a minimum of 4.3 ºC in week 1 to a maximum of 32.5 ºC in week 8 (Table 2). Average relative humidity varied from a maximum of 91 % in weeks 1 and 2 to a minimum of 27 % in week 9. A maximum rainfall of 85 mm was observed in week 2.

Table 2. Average weekly temperature, relative humidity and rainfall for spring (April-June)

week	Average temperature (ºC)			Average relative humidity (%)			Total rainfall (mm)
	Maximum	Minimum	Mean	Maximum	Minimum	Mean	
1	20.3	4.3	11.1	91	50	76	9
2	18.9	4.9	11.2	91	49	77	85
3	21.2	5.7	13.0	87	41	66	3
4	25.5	7.1	15.4	89	35	61	9
5	24.5	7.5	16.2	83	35	59	0
6	29.2	8.4	19.6	85	30	58	0
7	27.7	8.5	19.0	88	30	58	0
8	32.5	12.6	23.3	79	32	55	0
9	31.6	12.0	21.6	81	27	53	3

Survival of L. innocua *in soil*

Regardless of the studied season, all soil samples of soil amended with contaminated compost, surface or sprinkle irrigated with contaminated water were positive for *L. innocua* after inoculation during 9 weeks (Figure 1).

Population of *L. innocua* in soil amended with contaminated compost in fall (Figure 1A), dropped after inoculation by approximately 1.00 log cfu/gdw during the first 4 weeks, followed by a period of 5 weeks when it was stabilized or decreased, reaching final population of 5.17 log cfu/gdw.

In spring (Figure 1B), the average density in soil amended with contaminated compost of *L. innocua* was 6.14 log cfu/gdw after inoculation. Thereafter, the pathogen declined continuously, reaching final population of 3.70 log cfu/gdw.

For the pots surface watered with contaminated water, *L. innocua* cell numbers in soil at the day of transplanting (d0) were 6.98 and 6.04 log cfu/gdw for fall and spring, respectively. In fall, before re-inoculation, *L. innocua* population decreased approximately 1.00 log cfu/gdw (Figure 1A). After re-inoculation, density of the pathogen was 6.06 log cfu/gdw and decreased approximately 0.70 log cfu/gdw until the next re-inoculation. At second re-inoculation (8 weeks), population of pathogen increased to 5.91 log cfu/gdw and declined to a final population of 5.19 log cfu/gdw after 9 weeks.

In spring, population of *L. innocua* decreased 0.40 log cfu/gdw and after re-inoculation was 6.17 log cfu/gdw (Figure 1B). Thereafter, the pathogen declined approxi-

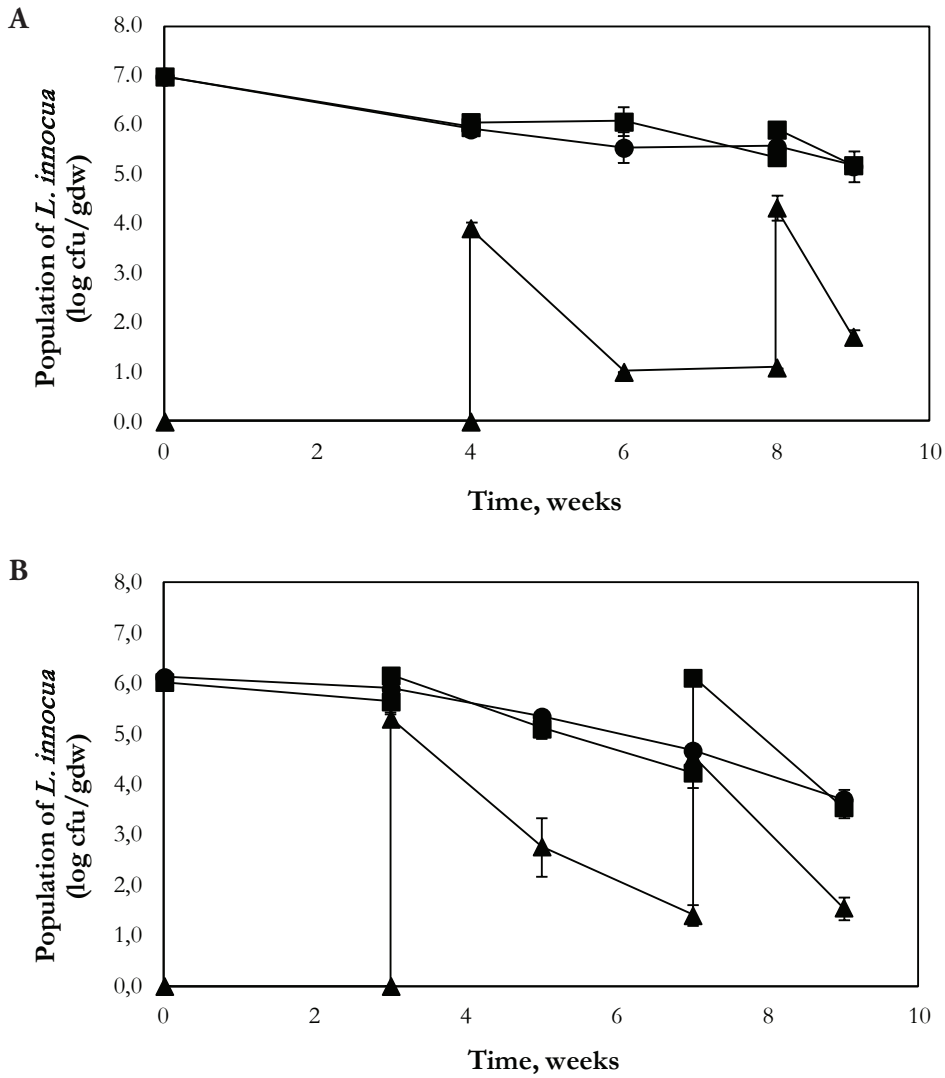

Figure 1. Survival of *Listeria innocua* in soil samples amended with inoculated compost (10^7 cfu/g) or irrigated with inoculated water (10^7 cfu/g) in fall (A) and spring (B). Treatments included contaminated compost (●), surface irrigation (■), and sprinkle irrigation (▲). In fall, surface irrigation with contaminated water was done after transplanting and after 4 and 8 weeks and sprinkle irrigation with contaminated water were done 4 and 8 weeks after transplanting the seedlings. In spring, surface irrigation with contaminated water was done after transplanting and after 3 and 7 weeks and sprinkle irrigation with contaminated water were done 3 and 7 weeks after transplanting the seedlings. Data represent the mean of four determinations. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

mately 2.00 log cfu/gdw before the second re-inoculation (7 weeks). After re-inoculation, population of *L. innocua* was 6.13 log cfu/gdw and reaching at final population of 3.55 log cfu/gdw.

In fall, population of *L. innocua* in soil sprinkle irrigated (4 weeks after transplanting) was 3.91 log cfu/gdw and declined approximately 2.80 log cfu/gdw before re-inoculation (8 weeks) (Figure 1A). After re-inoculation, population of pathogen was 4.33 log cfu/gdw and decreased continuously reaching final population of 1.72 log cfu/gdw.

In spring, initial count of pathogen in soil that was sprinkle irrigated was 5.31 log cfu/gdw and declined continuously before re-inoculation approximately 3.90 log cfu/gdw (Figure 1B). Followed the next re-inoculation (7 weeks), population of *L. innocua* decreased from 4.55 to 1.56 log cfu/gdw at final of 9 weeks.

Population of *L. innocua* in the soil amended with inoculated compost was similar to that observed in soil contaminated by surface irrigation, whereas in sprinkle irrigated soil, the survival of the pathogen was the lowest. When comparing the two seasons, the survival of *L. innocua* in soil was in general, lower in spring (warm and dry) than in fall (cold and wet).

Transfer of L. innocua *from contaminated compost to lettuce leaves*

In fall, all outer leaves from lettuce transplanted in soil with contaminated compost analyzed, were positive for *L. innocua* during the 9 weeks. However, in the inner leaves, only 5 samples were positive in total of 16 samples analyzed (Figure 2A). After 4 weeks of transplanting, population of *L. innocua* on outer leaves was 2.69 log cfu/g and slowly decreased until 9 weeks to 1.40 log cfu/g. On inner leaves, population of the pathogen was 1.78 log cfu/g on week 4. At week 6, it was only detected in one sample after enrichment and was not present after 8 and 9 weeks after transplanting.

In spring, *L. innocua* was not detected by direct plating. On outer leaves, the pathogen was recovered in all samples on weeks 3, 5 and 7 after enrichment but on week 9 was recovered only from two of four samples (Figure 2B). On weeks 3 and 7, all inner leaves samples were positive for *L. innocua* after enrichment. However, on weeks 5 and 9, only one of four samples was positive. In general, population of *L. innocua* in the lettuce leaves was higher in fall than in spring.

Transfer of L. innocua *from soil contaminated with surface irrigation water to lettuce leaves*

Lettuce plants were surface irrigated three times (after transplanting and twice pos-transplanting) with water containing 10^7 cfu/mL of *L. innocua*.

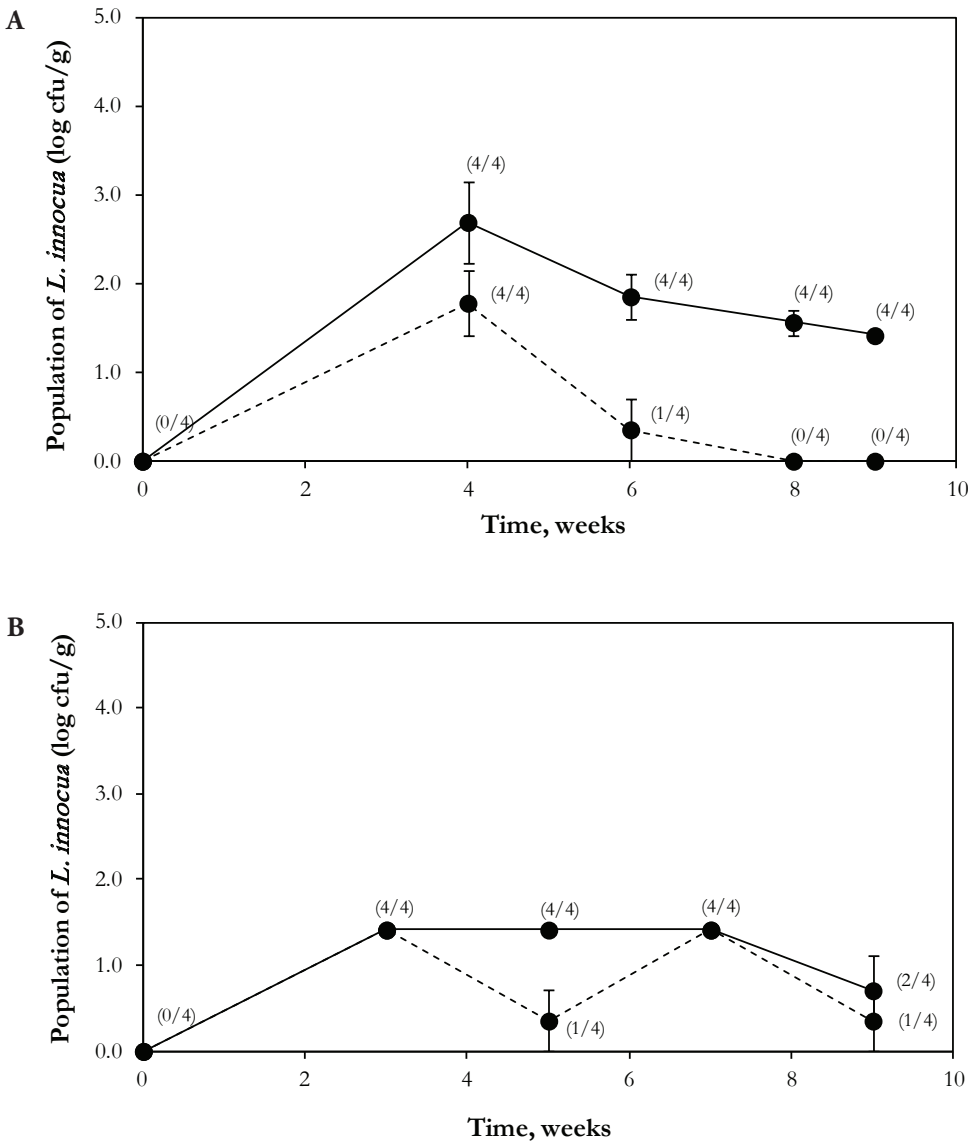

Figure 2. Survival of *Listeria innocua* on lettuce leaves grown in soil amended with contaminated compost (10^7 cfu/g) in fall (A) and spring (B). Treatments included outer leaves (continuous line) and inner leaves (dotted line). Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

In fall, after 4 weeks of watering with contaminated water, *L. innocua* was detected in 4 of 4 samples of lettuce on outer and inner leaves (Figure 3A). On outer leaves, the pathogen was then detected in 4 of 4, 3 of 4 and 2 of 4 samples of lettuce at 6, 8 and 9 weeks after transplanting, respectively. On the inner leaves, the pathogen was detected in 1 of 4 and 2 of 4 samples of lettuce at 6 and 8 weeks after transplanting, respectively. Samples collected 9 weeks after transplanting were negative for the pathogen.

In spring, *L. innocua* was only detected on lettuce leaves after enrichment because no colonies were found by direct plating (Figure 3B). The higher number of contaminated samples was on week 3 in which all inner and outer lettuce samples were positive. During the following weeks, the number of contaminated samples was lower. The pathogen was not detected on leaves after 5 weeks and was detected in 2 of 4 samples 7 weeks after the first irrigation. On week 9, the pathogen was detected in 2 of 4 and 1 of 4 samples of outer and inner leaves, respectively. In general, the lettuce leaves were more contaminated in fall than in spring.

Survival of **L. innocua** *on lettuce leaves irrigated by sprinkling with contaminated water*

Lettuce plants were sprayed twice with water containing 10^7 cfu/mL of *L. innocua*, the first irrigation was done after 4 and 3 weeks post-transplanting in fall and in spring, respectively.

In fall, after first irrigation (4 weeks) initial population of *L. innocua* was 3.76 and 4.89 log cfu/g for outer and inner leaves, respectively (Figure 4A). During the following 4 weeks and before re-inoculation, population of the pathogen in all samples decreased to undetectable numbers by direct plating and was detected only by enrichment at week 8. After the second irrigation, population of *L. innocua* on leaves was around 5.00 log cfu/g and continuously decreased to undetectable numbers in just one week. After 9 weeks, *L. innocua* was only detected by direct plating in one sample of outer leaves and in the other samples it was only detected after enrichment.

In spring, survival of *L. innocua* was lower than in fall and was detected after irrigation (week 3) at populations of 4.14 and 5.74 log cfu/g for outer and inner leaves, respectively (Figure 4B). Thereafter, population of the pathogen decreased continuously and all leaf samples were negative for the pathogen after 4 weeks of irrigation (week 7). After re-irrigation at week 7, the pathogen was detected at concentrations of 4.81 and 5.63 log cfu/g for outer and inner leaves, respectively. During the following 2 weeks, *L. innocua* population decreased in all samples to concentrations of 2.25 and 0.94 for outer and inner leaves, respectively. However, on inner leaves only one sample was positive after enrichment.

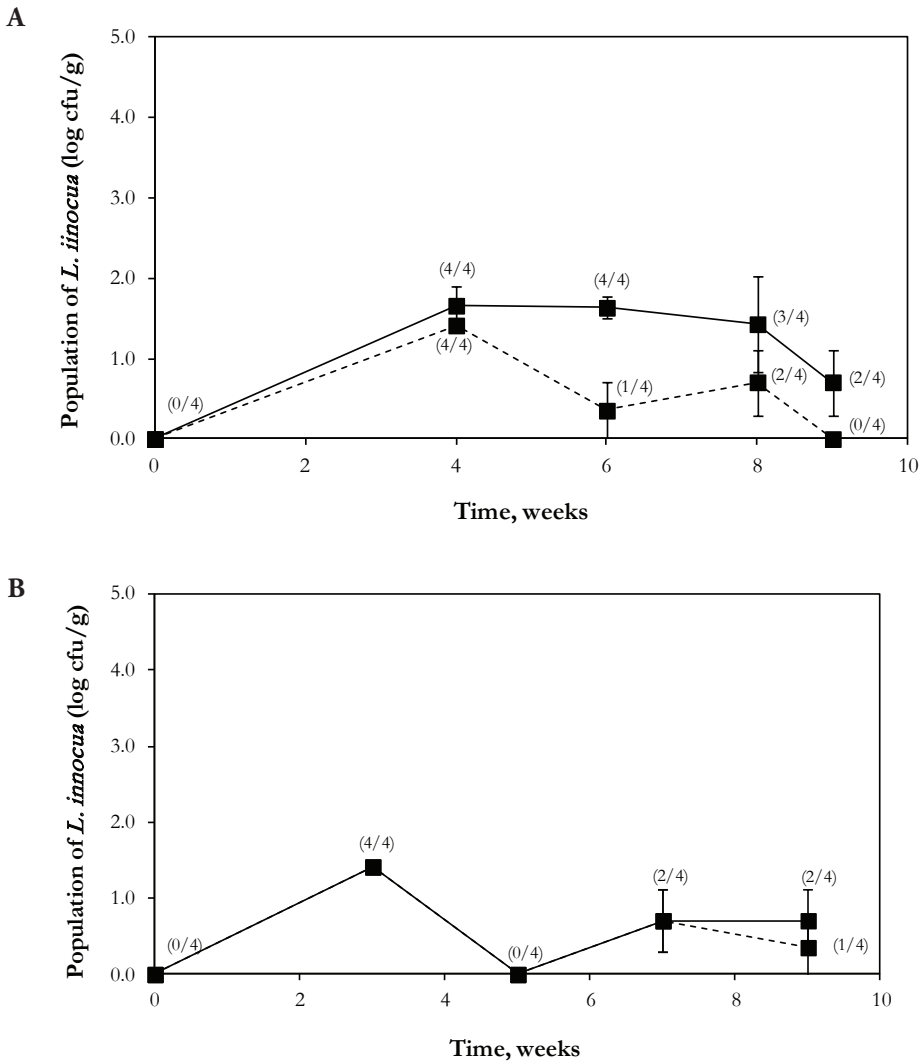

Figure 3. Survival of *Listeria innocua* on lettuce leaves grown in soil contaminated (10^7 cfu/g) with surface irrigation in fall (A) and in spring (B). Treatments included outer leaves (continuous line) and inner leaves (dotted line). In fall, surface irrigation with contaminated water was done after transplanting and after 4 and 8 weeks. In spring, surface irrigation with contaminated water was done after transplanting and after 3 and 7 weeks. Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

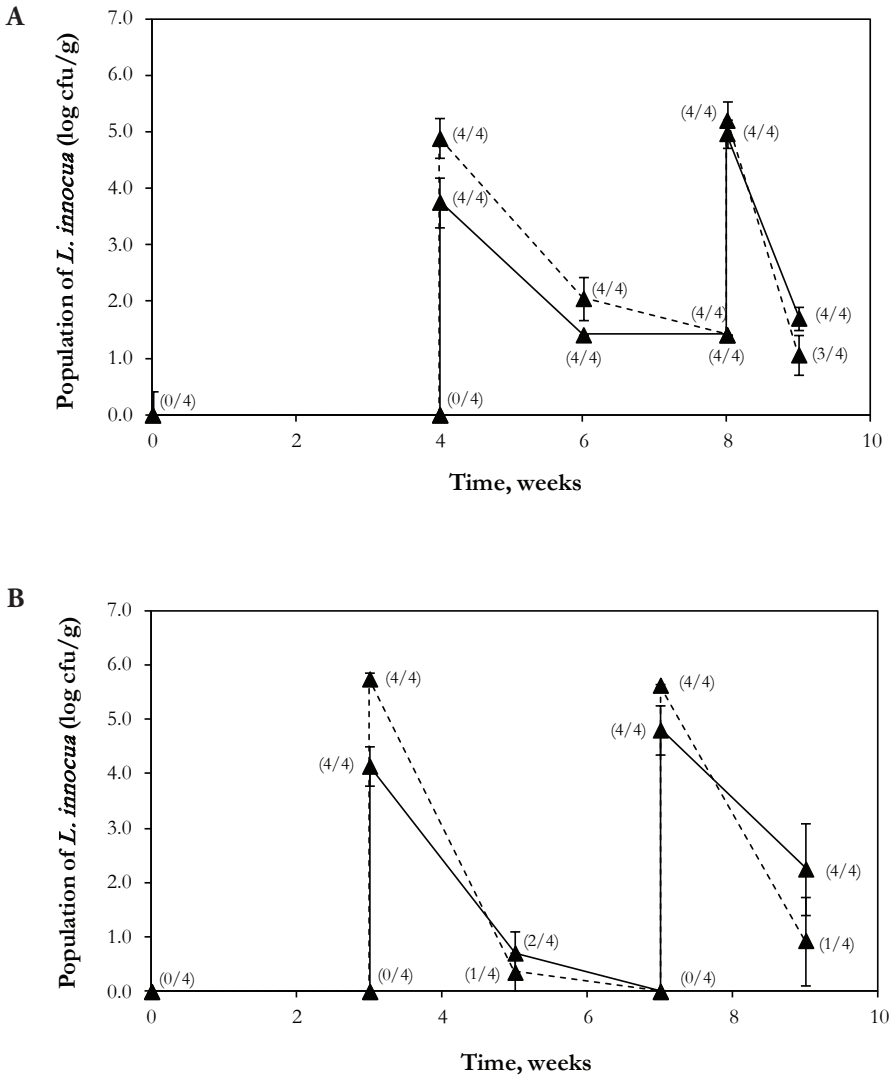

Figure 4. Survival of *Listeria innocua* on lettuce leaves inoculated with sprinkle irrigation water (10^7 cfu/g) in fall (A) and in spring (B). Treatments included outer leaves (continuous line) and inner leaves (dotted line). In fall, sprinkle irrigation with contaminated water was applied 4 and 8 weeks after transplanting de seedlings. In spring, sprinkle irrigation with contaminated water was applied 3 and 7 weeks after transplanting de seedlings. Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

Discussion

L. innocua and *L. monocytogenes* are both ubiquitous bacteria widely distributed in the environment and are frequently associated, suggesting that these two species have similar ecological requirements (Aguado *et al.*, 2004; MacGowan *et al.*, 1994; Welshimer and Donker-Voet, 1971). In this study, we assume that the behaviour of the surrogate *L. innocua* is similar to that of the pathogenic species *L. monocytogenes*. This is supported by results of Girardin *et al.* (2005) in soil microcosms that suggest the ability of *L. innocua* to survive in soil is similar or slightly higher than that of *L. monocytogenes*.

In the present study, contamination of *L. innocua* on lettuce leaves via soil to the lettuce surface ocurred during the growth process as they probably come in contact with contaminated soil. Also, when the lettuce samples were irrigated by sprinkling with contaminated water, the pathogen survived on surface leaves during some days. Another important finding of this work was that *L. innocua* can survive for >9 weeks in soil exposed to environmental conditions.

Spring (April to June) conditions were clearly more detrimental to *L. innocua* survival in soil than fall (October to December) conditions. Factors affecting the survival of pathogenic bacteria in soil include the soil composition, pH, water activity, oxidation-reduction potential, presence of rhizosphere, and microbial interactions (Fenlon *et al.*, 2000; Tamasi, 1981). Under field conditions, other variables, such as solar radiation, temperature and dryness, may also affect the survival of human pathogens. In our study, autoclaved soil was used as a control to minimize the influence of indigenous soil microorganisms on the survival of *L. innocua*.

Our results demonstrated that *L. innocua* survived in soil at the same rate when inoculated in compost or by surface irrigation. It is remarkable that one-time application of contaminated irrigation water or compost can result in pathogen contamination of vegetables. However, we observed some differences between the seasons, in fall reached at final populations about 5.20 log cfu/gdw and in spring about 3.50 log cfu/gdw for contaminated irrigation water and compost treatments. This difference can be a result of the effect of temperature and humidity. The average of temperature and humidity in fall were 10 ºC and 82 %, respectively. Whilst in spring the average were 17 ºC and 62 %. Generally, pathogen survival is favored in aqueous environments but temperature is also an important consideration, with higher temperatures reducing pathogen survival (Sorber and Moore, 1987). Higher temperature values in spring may contribute to a greater stress and energy expenditure for the bacterium than the lower temperature values observed in fall. Temperature presumably had an important role on *Listeria* survival in soil. These results are consistent with previous work done in soil microcosms which showed better survival of *L. monocytogenes* at ≤5 ºC than at 15-20 ºC (Jiang *et al.*, 2004; Picard-Bonnaud *et al.*, 1989). Girardin *et al.* (2005) and Welshimer (1960) observed the same results for *Listeria* in soil during

the winter and spring. The faster decline rate of the pathogen observed in soil by sprinkling irrigation when compared with surface irrigation can be explained for the low amount of the water that arrives to the soil surface without runoff through the soil and the exposure to UV and wind-mediated drying of the soil surface. The soil surface is more susceptible to be affected by daily temperature differences and UV radiations than the soil below the top.

L. innocua inoculated with sprinkle irrigation on the surface of lettuce leaves survived for at least 5 weeks in fall. However, in spring, no positive samples were found after 4 weeks of the first inoculation. This finding could be due to the high temperature and low relative humidity of the week before. Solomon *et al.* (2002) reported that *E. coli* O157:H7 persisted on leaf surfaces plants for 20 days after sprinkle irrigation with contaminated water. In another study, *E. coli* O157:H7 survived 30 days on lettuce plants after sprinkle irrigation, and repeated irrigation with contaminated water increased the pathogen level on the plant (Solomon *et al.*, 2003). Milillo *et al.* (2008) showed that *L. monocytogenes* deposited on mature plant model *Arabidopsis thaliana* leaves could rapidly attach, grew, and persisted over time, suggesting that application of contaminated irrigation water few days before harvest represents another risk factor of vegetable contamination.

We observed *L. innocua* contamination on the leaves in the treatments with surface irrigation with contaminated water and contaminated compost. Several experiments have indicated that pathogens can be transferred from contaminated soil and water to the surface of vegetables (Islam *et al.*, 2004a, b; Solomon *et al.*, 2002). In a study of vegetables grown in inoculated soil under greenhouse conditions, Van Renterghem *et al.* (1991) found that *L. monocytogenes*, previously added to soil at a level of 5 log cfu/g, was detected by enrichment on three of six radish samples 3 months after inoculation. Jablasone *et al.* (2005) found that *L. monocytogenes* did not internalize within seedlings, but did persist on the surface of plants throughout the cultivation period. Girardin *et al.* (2005) detected *L. innocua* on parsley leaves during 30 days of cultivation on inoculated soil under field conditions. However, *L. innocua* was not detected in internal tissues of parsley, in contrast to observations of *E. coli* in lettuce seedlings (Solomon *et al.*, 2002) and *Salmonella* in tomato plants (Guo *et al.*, 2002).

The presence of *L. innocua* was higher on leaves grown during fall than spring. Perhaps factors such as light intensity, moisture, irradiation and high temperature have high influence on the survival of the pathogen. In generally, we observed more contamination on the outer leaves than on the inner ones. The outer leaves are more exposed to environmental conditions and will be more favorable to be contaminated by direct contact with the soil. The survival on the surface of leaves can also be explained by the not homogeneously distribution of nutrients, and thus, motility of the microorganism is important for survival (Liao and Fett, 2001). Nutrient pools can also differ on individual leaves on the same plant. For example, more leached

nutrients are available on the surface of older leaves than young leaves (Tukey and Morgan, 1963).

The results from our study as well as results from other studies demonstrated that *L. innocua* can survive for extended periods in the soil and indicated that lettuce grown in soil containing contaminated compost or irrigated with contaminated water results in contamination of the edible portion of the lettuce plant. The levels of *L. innocua* used in this study are far greater than what may be found on a field; therefore, they might only indicate the worst-case scenario. However, high numbers of bacteria were used to be readily detected.

We observed that there is a transfer of pathogen from contaminated soil to edible parts of lettuce leaves. However, we could not demonstrate if there was internalization via root system, or by direct contact to soil, insects or other vectors. Some authors reported the internalization via root systems by some pathogens, but different conclusions were found. More studies should be carried out to determine if *Listeria* is able to internalize via root system.

Our results indicated that contaminated irrigation water can play an important role in contaminating vegetables mainly in sprinkle irrigation done close to harvest date. Long-term survival of *L. innocua* in soil amended with artificially contaminated compost illustrates the need for appropriate farm waste management and manure should be treated to eliminate pathogenic microorganisms.

Acknowledgments

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Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water

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Abstract

Escherichia coli O157:H7 outbreaks associated with produce consumption have brought attention to contaminated compost manure, and polluted irrigation water as potential sources of pathogens for the contamination of these crops. The aim of this study was to determine the potential transfer of *E. coli* O157:H7 from soil fertilized with contaminated compost or irrigated with contaminated water to edible parts of lettuce together with its persistence in soil under field conditions in two different seasons (fall and spring). Moreover, its survival on lettuce sprinkled with contaminated irrigation water was evaluated, as well as the prevalence of aerobic mesophilic, *Enterobacteriaceae* and *Pseudomonadaceae* in control lettuce samples. Four treatments, contaminated compost, surface and sprinkle irrigation with contaminated water and uninoculated pots, were used in this work. Contaminated compost was applied to soil in the pots before lettuce was transplanted and contaminated irrigation water was applied twice and three times on the plants after the seedlings were transplanted, for sprinkle and surface irrigation, respectively. *E. coli* O157:H7 survived in soil samples for 9 weeks at levels, 4.50 log cfu/gdw (dw, dry weight) in fall and 1.50 log cfu/gdw in spring. The pathogen survives better in fall, indicating an important influence of environmental factors. *E. coli* O157:H7 population in lettuce leaves after sprinkle irrigation was very high (between 10^3 and 10^6 cfu/g), but decreased to undetectable levels at field conditions. There was also transfer of *E. coli* O157:H7 from soil contaminated with compost or irrigated with contaminated water to lettuce leaves, mainly to the outer ones. The mean counts for aerobic mesophilic, *Enterobacteriaceae* and *Pseudomonadaceae* populations were also influenced by environmental conditions; higher levels were observed under fall conditions than in spring conditions. Contamination of lettuce plants in the field can occur through both contaminated composted manure and irrigation water and persist for several months.

Keywords

Lettuce; Soil; Drip and sprinkle irrigation; Compost; *Escherichia coli* O157:H7; Transfer.

Introduction

Vegetables and fruits are generally colonized by a wide variety of microorganisms, such as bacteria, yeasts and fungi that could cause spoilage (Abadias *et al.*, 2008; Lindow and Brandl, 2003). Contamination of lettuce is predominantly by gram-negative bacteria, in particular, members of the *Pseudomonadaceae* and *Enterobacteriaceae* (Oliveira *et al.*, 2010). In addition, it is well established that some products can also contain potential pathogens (Beuchat, 1996; Francis *et al.*, 1999; Nguyen-the and Carlin, 1994), and some of these have been implicated in an increasing number of outbreaks of foodborne illness (Long *et al.*, 2002). One pathogenic enteric bacterium of high interest is *Escherichia coli* O157:H7. Enterohemorrhagic *E. coli* (EHEC) is a major cause of enteric disease, ranging from mild or bloody diarrhea (hemorrhagic colitis) to the deadly hemolytic uremic syndrome (HUS). The emerging and deadly infectious disease caused by this organism affects humans of all ages, but the young and old are the most susceptible to HUS (Paton and Paton, 1998). Lettuce, in particular, has been connected to several outbreaks of *E. coli* O157:H7. In July 1998, an outbreak of *E. coli* O157:H7 infection involving 40 Montana residents was associated with contaminated leaf lettuce (Ackers *et al.*, 1998). Following this outbreak, at least four additional outbreaks of *E. coli* O157:H7 infection have been implicated lettuce (Buck *et al.*, 2003). In 2006, a major outbreak of foodborne illness associated with the consumption of spinach tainted with EHEC occurred in the United States. This episode was linked to contamination of a spinach field by EHEC-infected wild pigs roaming in the Salinas Valley in California (FDA, 2007). These outbreaks highlight the increasing importance of fresh produce as a vehicle for foodborne illness.

The increasing association between fresh vegetables and foodborne disease outbreaks has led to concerns about contamination of vegetables with faecal pathogenic bacteria in the agricultural environment (Tauxe *et al.*, 1997). Potential pre-harvest sources of contamination include soil, feces, green or inadequately composted manure, irrigation water, water used to apply fungicides and insecticides, dust, insects, wild and domestic animals, and human handling (Beuchat, 1996). Non-composted or improperly composted manure can contaminate fruits and vegetables when used as a fertilizer or soil amendment. Runoff water from cattle feedlots and application of contaminated irrigation water to soil also represent possible sources of contamination (Buck *et al.*, 2003). Evidence to support these as sources of contamination is largely based on controlled experimental studies in the laboratory and field. For example, *Salmonella enterica* serovar Typhimurium was detected on roots and leaves of lettuce and parsley for up to 63 days and 231 days in soil contaminated with irrigation water and manure compost, respectively (Islam *et al.*, 2004b; Semenov *et al.*, 2009). Similarly, *E. coli* O157:H7 was detected on roots and leaves of lettuce and parsley for 77 and 177 days, respectively, when soil had been contaminated by irrigation water or manure compost (Islam *et al.*, 2004a; Semenov *et al.*, 2009).

Once pathogens are introduced into fields, soil can become a pathogen reservoir. For example, *S. enterica* persisted for 161 and up to 231 days in soils amended with contaminated composts on which lettuce and parsley were grown (Islam *et al.*, 2004b), whereas *E. coli* O157:H7 survived in soil samples for 154 to 217 days in lettuce and parsley fields (Islam *et al.*, 2004a). Hence, direct contact of aerial tissue with the ground or through rain or irrigated water splashes of soil onto the aerial tissue is likely a significant contributing factor for continued contamination events. The survival of enteric microorganisms is influenced by soil and environmental variables, including soil texture and organic matter content, the extent of eutrophication and availability of substrate, pH value, temperature and moisture content (Semenov *et al.*, 2008). Temperature was determined to be the most important factor influencing pathogen survival in manure and in manure- or sludge-amended soils, with increasing survival times being a function of decreasing temperature (Semenov *et al.*, 2007, 2010; Sorber and Moore, 1987). The objective of this study was to determine the fate of *E. coli* O157:H7 on lettuce leaves and in soil when compost or different types of irrigation water contaminated with *E. coli* O157:H7 were applied under environmental conditions during two different seasons.

Material and methods

Experimental design

A design plan was followed with four treatments; T1: seedlings of lettuce were transplanted onto pots containing soil amended with contaminated compost; T2: each pot containing uninoculated compost was manually surface irrigated with contaminated water after the seedlings were transplanted and after 4 and 8 weeks for fall and 3 and 7 weeks, for spring; T3: contaminated irrigation water was hand-sprayed onto the lettuce leaves after the seedlings were transplanted and after 4 and 8 weeks for fall and 3 and 7 weeks, for spring; T4: seedlings were transplanted to uninoculated pots (control treatment). Twenty replicate lettuce pots were used for each treatment. The experiment was repeated in two seasons as explained before.

Pots were grouped by treatments and separated 75 cm each other to avoid cross contamination.

Preparation of inoculum

The non-pathogenic strain of *E. coli* O157:H7 (NCTC 12900) was used as inoculum. This strain is devoid of the ability to produce verotoxins (stx1 and stx2) and is a biosafety level 1 (BSL1) strain. *E. coli* O157:H7 was grown on Tryptone Soy Agar (TSA, Oxoid) at 37 ºC for 20-24 h. A single colony was transferred into a flask with 50 mL of Tryptone Soy Broth (TSB, Oxoid) at 150 rpm for 20-24 h at 37 ºC.

Bacterial cells were harvest by centrifugation at 9820 x g for 10 min at 10 ºC and resuspended in sterile saline peptone (SP, 8.5 g/L NaCl and 1 g/L peptone). The concentration of *E. coli* O157:H7 was estimated using a spectrophotometer set at λ=420 nm according to standard curves previously determined and it was checked by plating duplicate serial suspension dilutions on Sorbitol MacConkey agar supplemented with Cefixime and Tellurite (CT-SMac, Biokar Diagnostics) followed by incubation at 37 ºC for 20-24 h.

Lettuce production conditions

Seedlings with four or five true leaves of 'Romaine' lettuce (*Lactuca sativa* var. longifolia*)* were purchased from a local producer in Lleida (Spain).

Lettuce plants were grown in plastic pots (23 cm diameter) containing 1200 g of commercial autoclaved (30 min at 121 ºC) soil (Terraplant®[1], manufactured by COMPO GmbH & Co. KG, Münster, Germany) amended with 150 g of inoculated or uninoculated organic compost (S1, Compost Segrià, Lleida, Spain). Each pot was punctured at the bottom and placed inside another unpunctured pot for collection of drainage solution potentially contaminated by the inoculum and hence preventing contamination of the environment. Plants were regularly watered when the soil surface began to dry.

The experiment was carried out under outdoor conditions in two different seasons, fall and spring, in Lleida (Catalonia, Spain). The climate of the area in which the experiment was carried out is dry continental. Daily measurements of environmental temperature (T), rainfall (mm) and percent relative humidity (RH) were collected by an automated weather-monitoring equipment located around the lettuce plants.

Inoculation of compost and irrigation water

Compost (3000 g) was sprayed with 300 mL of a suspension containing 10^7 cfu/mL and mixed thoroughly before addition to soil. Soil and contaminated compost were mixed vigorously to ensure homogeneous distribution of the pathogen into each pot before planting the seedlings.

Contaminated irrigation water with *E. coli* O157:H7 at 10^7 cfu/mL was prepared. Each pot was manually surface irrigated with 200 mL of contaminated water after planting the lettuce seedlings. The water was applied carefully to prevent splashing of the inoculum onto the edible portion of the lettuce plant. For sprinkle irrigation, a hand sprayer was used to sprinkle 25 mL of contaminated water onto the lettuce leaves of each pot. Additionally, these pots were surface irrigated with non-contaminated water.

Soil and lettuce sample collection

All samples were collected using alcohol-sanitized gloves and disinfected spoons, tweezers and scalpels. Samples from control pots were collected first to minimize cross contamination. At each sample time, approximately 5 g of the inner and outer lettuce leaves was obtained aseptically and placed in a sterile Stomacher bag separately. Approximately 15 g of soil from each pot was aseptically collected in a plastic bag from around the plant. There were four replications (4 pots) for each treatment at each sampling time.

Microbiological analysis

Soil and lettuce samples from each treatment and the control were collected randomly on the day of transplanting (d0) and after 4, 6, 8 and 9 weeks (fall), and 3, 5, 7 and 9 weeks (spring). Moreover, in those treatments involving irrigation (T2, T3), soil samples were collected before and after irrigation in the days of re-inoculation. In the treatment by sprinkler (T3), leave samples were also collected before and after irrigation.

For the analysis, 5 g of lettuce leaves (inner and outer) was mixed with 45 mL of buffered peptone water (BPW, Oxoid, CM1049) in a sterile Stomacher bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Ten grams of soil was added to 90 mL of BPW in an Erlenmeyer flask and homogenized by shaking at 150 rpm for 10 min. Serial dilutions (1:10) of each sample were prepared using SP and enumerated by plating on CT-SMac and incubated at 37 ºC for 24 h for inoculated samples. Homogenized samples in BPW were incubated for 24 h at 37 ºC for non-selective pre-enrichment in case that *E. coli* O157:H7 was not detected by direct plating (detection limit, dL, 50 cfu/g). After pre-enrichment step, 1 mL of samples was transferred on to 9 mL of modified Tryptone Soy Broth (mTSB, Biokar Diagnostics), a selective medium for *E. coli*, and incubated at 41.5 ± 1 ºC for 24 h. One loopful of mTSB enrichment culture was then streaked onto CT-SMac agar and incubated at 37 ºC for 24 h. Presumptive *E. coli* O157:H7 colonies grown on selective medium were confirmed using the *E. coli* O157:H7 latex agglutination test (Oxoid). In samples were *E. coli* O157:H7 was not detected by direct plating (< 50 cfu/g) but positive after enrichment, an arbitrary value of 1.40 log cfu/g (half of detection limit) was assigned for data representation.

Moisture content of soil amended with compost was determined by drying approximately 3 g of soil at 105 ± 1 ºC for 24 h in a drying oven and then weighing the residual. The colony forming units in the soil were calculated per dry weight (dw).

In the uninoculated treatment (control), aerobic mesophilic, *Enterobacteriaceae* and *Pseudomonas* spp. populations were determined in lettuce leaves by enumerating colonies on plates with PCA (Plate Count Agar, Biokar Diagnostics), VRBG (Violet

Red Bile Glucose Agar, Biokar Diagnostics) and CFC (Cetrimide Fucidin Cephalosporin, Biokar Diagnostics) according to ISO 4833:2003, ISO 21528-2:2004, ISO 13720:1995, respectively.

Results

Environmental data

Daily temperatures, rainfall and relative humidity for the area are presented in Figure 1. In fall (Figure 1A), the temperature varied from a maximum of 27.5 °C in the first week to a minimum of -0.5 °C in week 8. The relative humidity varied from a maximum of 100 % in week 6 to a minimum of 39 % in week 1. During this season, a total rainfall of 55 mm was observed between the 3rd and 4th weeks. In spring (Figure 1B), the temperature varied from a minimum of 4.3 °C in week 1 to a maximum of 32.5 °C in week 8. The relative humidity varied from a maximum of 91 % in weeks 1 and 2 to a minimum of 27 % in week 9. During the first 2 weeks, 88 mm of rainfall was observed.

Survival of E. coli *O157:H7 in soil*

All soil samples of soil amended with contaminated compost, surface irrigation and sprinkle irrigation with contaminated water were positive for *E. coli* O157:H7 after inoculation and until the end of the experiment (Figure 2).

Population of *E. coli* O157:H7 in soil amended with contaminated compost in fall (Figure 2A), dropped after inoculation from 6.76 to 4.88 log cfu/gdw during the first 4 weeks, followed by a period of 5 weeks when it was stabilized or slightly decreased, reaching a final population of 4.43 log cfu/gdw.

In soil contaminated by surface watering, *E. coli* O157:H7 cell numbers at the day of transplanting (d0) were 6.57 log cfu/gdw (Figure 2A). During the following 4 weeks and before re-inoculation, the pathogen population decreased approximately 1.40 log cfu/gdw. After re-inoculation, the density of the pathogen was 5.68 log cfu/gdw and decreased approximately 1.00 log cfu/gdw until the next re-inoculation. At second re-inoculation (8 weeks), the population of *E. coli* O157:H7 increased to 5.68 log cfu/gdw and declined to a final population of 4.72 log cfu/gdw after 9 weeks.

The population of *E. coli* O157:H7 in soil where lettuce was sprinkle irrigated (4 weeks after transplanting) was 3.83 log cfu/gdw and declined approximately 2.10 log cfu/gdw before re-inoculation (8 weeks) (Figure 2A). After re-inoculation, the population of the pathogen was 4.14 log cfu/gdw and stabilized.

In spring (Figure 2B), the average density in soil amended with contaminated compost of *E. coli* O157:H7 was 6.11 log cfu/gdw after inoculation. The pathogen population

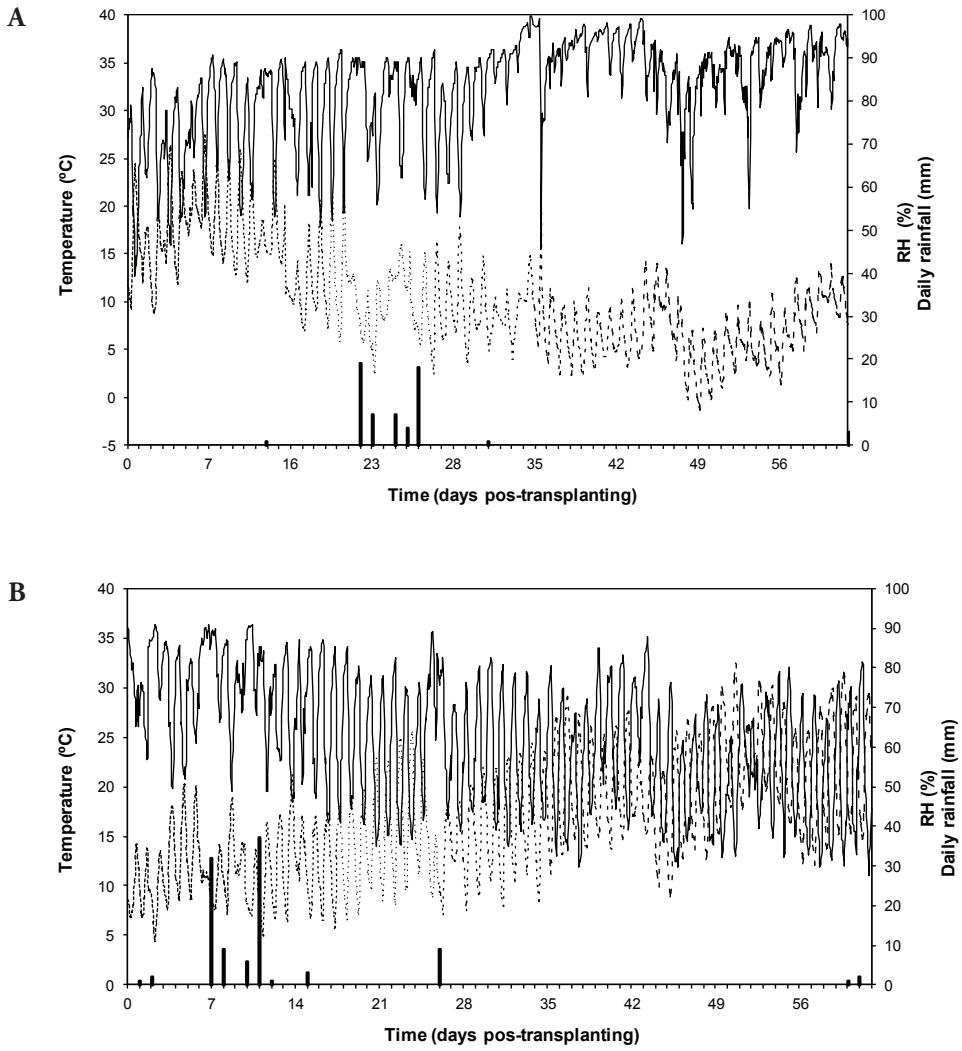

Figure 1. Daily temperature (dotted line), relative humidity (continuous line) and rainfall (bars) during fall (A) and spring (B).

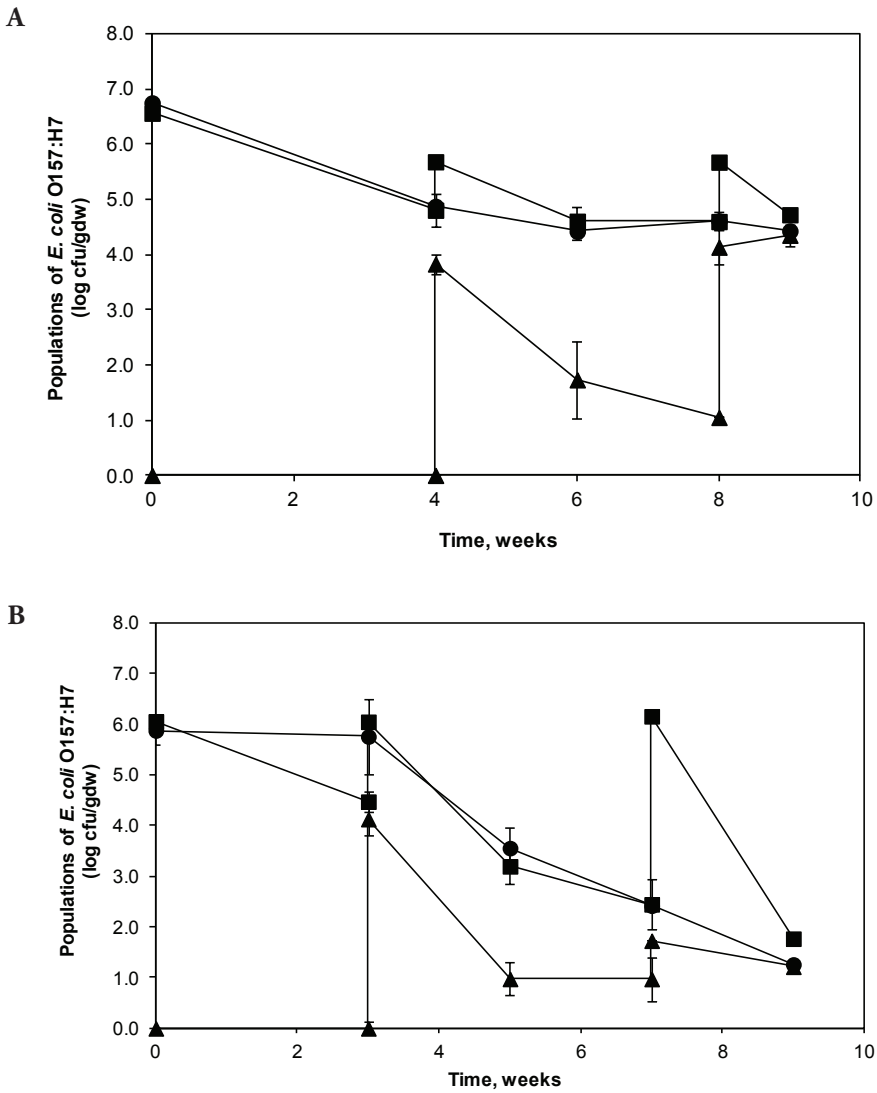

Figure 2. Survival of *Escherichia coli* O157:H7 in soil samples amended with inoculated compost (10^7 cfu/g) or irrigated with inoculated water (10^7 cfu/g) in fall (A) and spring (B). Treatments included contaminated compost (●), surface irrigation (■), and sprinkle irrigation (▲). In fall, surface irrigation with contaminated water was done after transplanting and after 4 and 8 weeks and sprinkle irrigation with contaminated water were done 4 and 8 weeks after transplanting the seedlings. In spring, surface irrigation with contaminated water was done after transplanting and after 3 and 7 weeks and sprinkle irrigation with contaminated water were done 3 and 7 weeks after transplanting the seedlings. Data represent the mean of four determinations. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

slightly decreased during the first 3 weeks. Thereafter, the pathogen declined continuously during the following 6 weeks, reaching a final population of 1.26 log cfu/gdw.

Initial counts of *E. coli* O157:H7 in soil contaminated by surface irrigation at d0 were 6.34 log cfu/gdw (Figure 2B). After 3 weeks of inoculation, the population of *E. coli* O157:H7 decreased 1.85 log cfu/gdw and after re-inoculation was 6.06 log cfu/gdw. Thereafter, the pathogen declined approximately 3.60 log cfu/gdw before the second re-inoculation (7 weeks). After re-inoculation, the population of *E. coli* O157:H7 was 6.16 log cfu/gdw and decreased, reaching a final population of 1.77 log cfu/gdw.

The population of the pathogen in soil that was sprinkle irrigated was 4.13 log cfu/gdw and declined continuously during the following 4 weeks before re-inoculation approximately 3.15 log cfu/gdw (Figure 2B). Following the next re-inoculation (7 weeks), the population of *E. coli* O157:H7 decreased from 1.73 to 1.20 log cfu/gdw a final of 9 weeks.

Transfer of E. coli *O157:H7 from contaminated compost to lettuce leaves*

In fall, 10 of 16 samples analyzed after inoculation of outer leaves, were positive for *E. coli* O157:H7, whilst in the inner leaves, only 3 of 16 samples were positive during the 9 weeks (Figure 3A). Four weeks after transplanting, the population of *E. coli* O157:H7 on outer leaves was 1.56 log cfu/g and slowly decreased until 9 weeks. On inner leaves, *E. coli* O157:H7 was only detected in 3 samples after enrichment.

In spring, *E. coli* O157:H7 was not detected by direct plating in any of the samples. On outer leaves, the pathogen was recovered on weeks 3 and 7 by positive enrichment of 2 samples, on week 5 was recovered from one of four samples and on week 9 all samples were negative (Figure 3B). On weeks 3 and 5, only one sample was *E. coli* O157:H7 positive after enrichment on inner leaves. On weeks 7 and 9, all samples were negative. In general, the population of *E. coli* O157:H7 on lettuce leaves was higher in fall than in spring.

Transfer of E. coli *O157:H7 from soil contaminated with surface irrigation water to lettuce leaves*

Lettuce plants were surface irrigated three times (after transplanting and twice post-transplanting) with water containing 10^7 cfu/mL of *E. coli* O157:H7.

In fall, after 4 weeks of watering with contaminated water, *E. coli* O157:H7 was detected in 3 of 4 samples of lettuce on outer leaves and in 1 of 4 samples on inner leaves (Figure 4A). On outer leaves, the pathogen was then detected in 2 of 4 samples of lettuce at 6, 8 and 9 weeks after transplanting. On the inner leaves, the contamination was lower than on the outer leaves. The pathogen was detected in 1

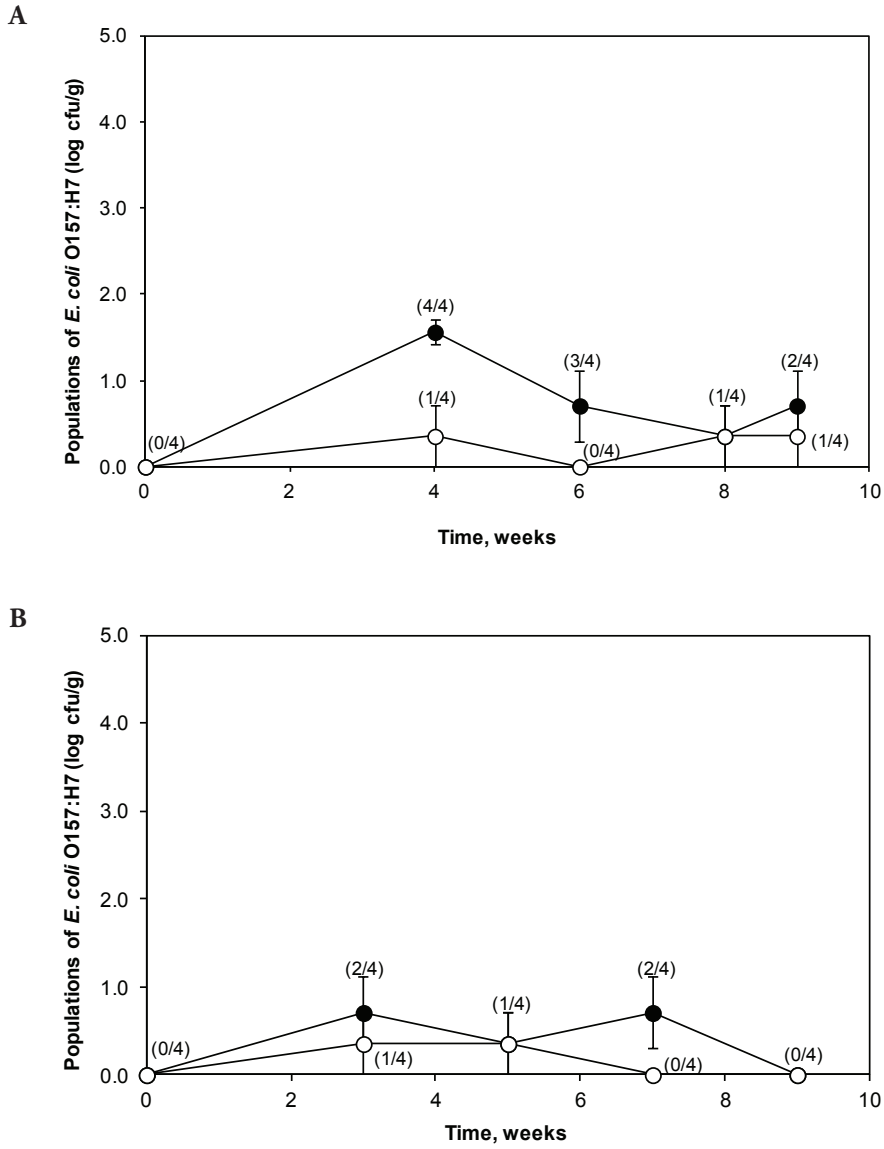

Figure 3. Survival of *Escherichia coli* O157:H7 on lettuce leaves grown in soil amended with contaminated compost (10^7 cfu/g) in fall (A) and spring (B). Treatments included outer leaves (full symbol) and inner leaves (open symbol). Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

of 4 samples of lettuce at 4 and 6 weeks after the first irrigation. Samples collected after 8 and 9 weeks from the first irrigation were negative for the pathogen.

In spring, *E. coli* O157:H7 was only detected on lettuce leaves after enrichment and no colonies were found by direct plating (Figure 4B). The pathogen was detected on outer leaves in 1 of 4 samples of lettuce at 3 and 5 weeks after the first irrigation. At week 7, 2 of 4 samples were positive and at week 9 all samples were negative. *E. coli* O157:H7 was not detected on inner leaves at 3, 7 and 9 weeks and only one sample was positive at week 5 after the first irrigation. In general, lettuce leaves were more contaminated in fall than in spring.

Survival of E. coli *O157:H7 on lettuce leaves irrigated by sprinkling with contaminated water*

Lettuce plants were sprinkled twice with water containing 10^7 cfu/mL of *E. coli* O157:H7, the first irrigation was done after 4 and 3 weeks post-transplanting in fall and spring, respectively.

In fall, after the first irrigation (week 4), the initial population of *E. coli* O157:H7 was 2.93 and 4.99 log cfu/g for outer and inner leaves, respectively (Figure 5A). During the following 4 weeks and before re-inoculation, the population of the pathogen in all samples decreased to undetectable numbers by direct plating and was detected only by enrichment at week 8. After the second irrigation, the population of *E. coli* O157:H7 on leaves was 5.10 and 5.88 log cfu/g for outer and inner leaves, respectively, and continuously decreased to populations of 1.61 and 2.93 log cfu/g for outer and inner leaves, respectively.

In spring, the *E. coli* O157:H7 population after irrigation (week 3) was 2.94 and 5.11 log cfu/g in the outer and inner leaves, respectively (Figure 5B). Thereafter, the population of the pathogen decreased continuously and all leaf samples were negative for the pathogen after 4 weeks of irrigation (week 7). After re-irrigation at week 7, the pathogen was found at concentrations of 5.11 and 4.56 log cfu/g for outer and inner leaves, respectively. During the following 2 weeks, *E. coli* O157:H7 population decreased in all samples and was detected only by enrichment in 3 of 4 samples. The survival of *E. coli* O157:H7 in fall was lower than in spring.

Counts of epiphytic microorganisms on control lettuce samples

Control (uninoculated) lettuce leaves were analyzed for the presence of total aerobic mesophilic (AM), *Enterobacteriaceae* and *Pseudomonas* spp. The AM counts in fall ranged from 4.78 to 5.89 log cfu/g in outer leaves and from 4.83 to 6.43 log cfu/g in inner leaves (Table 1). The initial population of AM in outer leaves was 5.65 log cfu/g and after 9 weeks was 5.05 log cfu/g. For inner leaves, initial AM

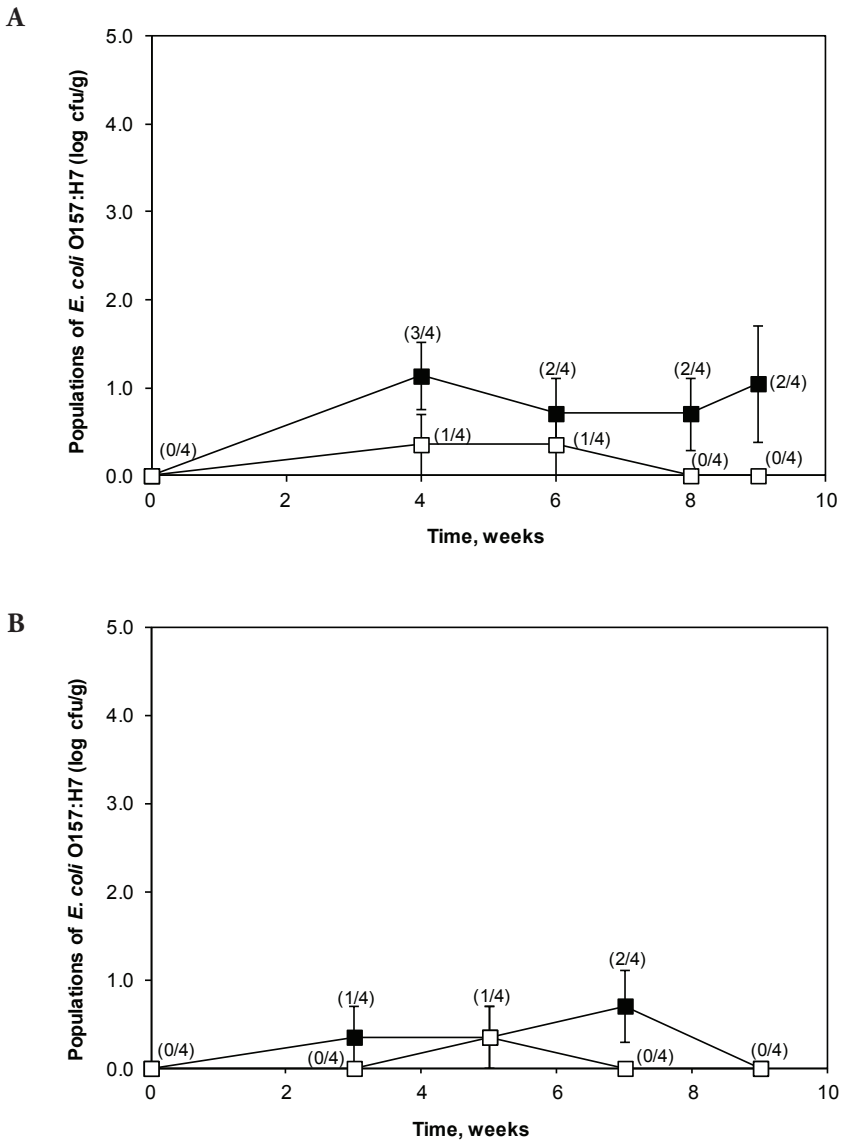

Figure 4. Survival of *Escherichia coli* O157:H7 on lettuce leaves grown in soil contaminated (10^7 cfu/g) with surface irrigation in fall (A) and in spring (B). Treatments included outer leaves (full symbol) and inner leaves (open symbol). In fall, surface irrigation with contaminated water was done after transplanting and after 4 and 8 weeks. In spring, surface irrigation with contaminated water was done after transplanting and after 3 and 7 weeks. Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

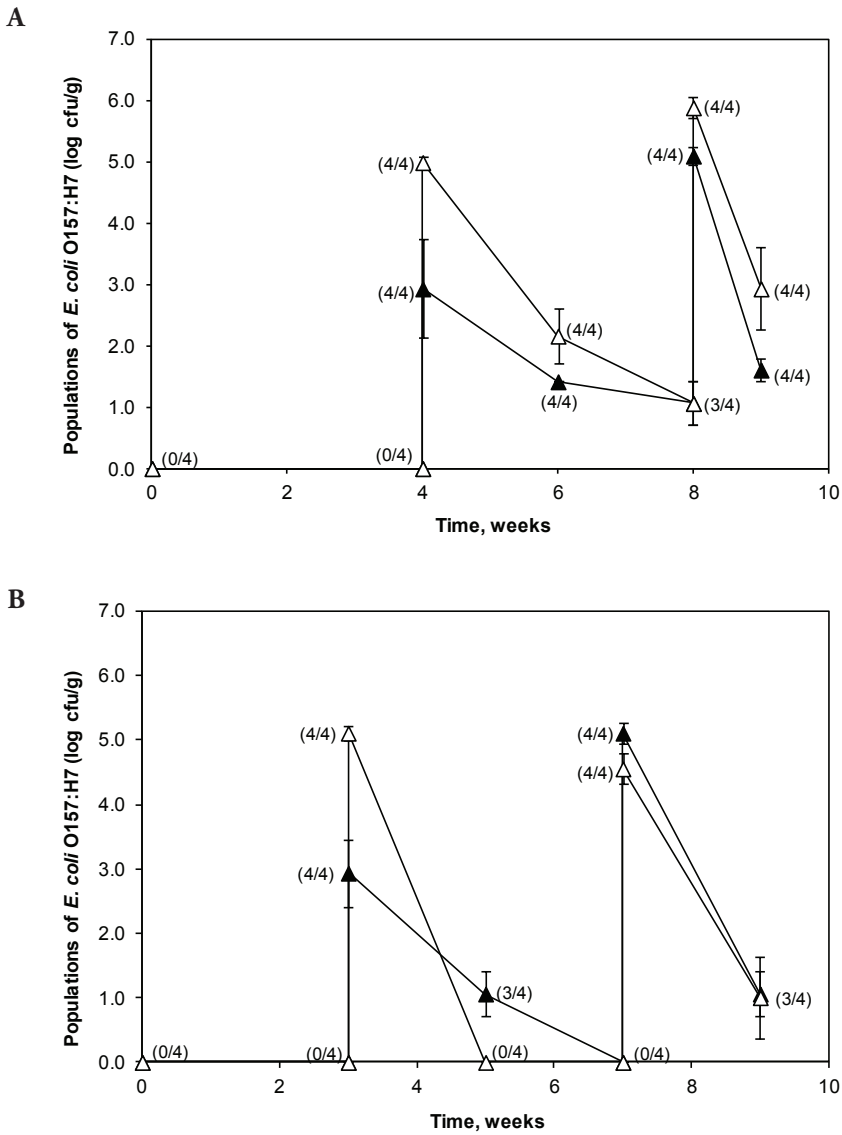

Figure 5. Survival of *Escherichia coli* O157:H7 on lettuce leaves inoculated with sprinkle irrigation water (10^7 cfu/g) in fall (A) and in spring (B). Treatments included outer leaves (full symbol) and inner leaves (open symbol). In fall, sprinkle irrigation with contaminated water was applied 4 and 8 weeks after transplanting de seedlings. In spring, sprinkle irrigation with contaminated water was applied 3 and 7 weeks after transplanting de seedlings. Data represent the mean of four determinations. Data in parentheses represent the number of positive samples/number of samples analyzed. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

count was 4.83 log cfu/g and at the end of experiment was 5.48 log cfu/g. The mean counts during the whole period were 5.34 and 5.58 log cfu/g for outer and inner leaves, respectively.

In spring, the AM levels were lower than in fall. The AM counts ranged from 3.96 to 4.44 log cfu/g in outer leaves and from 2.80 to 3.93 log cfu/g in inner leaves (Table 2). The initial count was 4.44 log cfu/g in outer leaves and after 9 weeks was 4.17 log cfu/g. For inner leaves, the initial count was 3.60 log cfu/g and at week 9 was 3.93 log cfu/g. The mean counts for AM were 4.20 and 3.32 log cfu/g for outer and inner leaves, respectively.

The range for *Enterobacteriaceae* was very broad, from 2.62 to 6.00 log cfu/g and from 0.91 to 6.00 log cfu/g for outer and inner leaves in fall, respectively (Table 1). Initial populations were 4.23 and 2.85 log cfu/g and at the end of the production time were 2.79 and 0.91 log cfu/g for outer and inner leaves, respectively. The mean counts were 3.91 log cfu/g for outer leaves and 2.98 log cfu/g for inner leaves.

In spring, *Enterobacteriaceae* were present at low levels. Counts were between 0.75 and 1.86 log cfu/g for outer leaves and between 0.45 and 1.37 log cfu/g for inner leaves (Table 2). The initial population was 0.75 log cfu/g and at the end was 1.86 log cfu/g for outer leaves. For inner leaves, counts were 1.11 and 0.84 log cfu/g for initial and final populations, respectively. Population means were 1.15 and 0.94 log cfu/g for outer and inner leaves, respectively.

In fall, *Pseudomonas* spp. was present between 3.53 and 4.93 log cfu/g for outer leaves and between 4.46 and 6.59 log cfu/g for inner leaves (Table 1). Initial counts were 4.93 and 4.46 log cfu/g for outer and inner leaves and reached values 3.53 and 4.54 log cfu/g for outer and inner leaves at the end of the production time, respectively. The mean counts were 4.32 and 5.24 log cfu/g for outer and inner leaves, respectively.

In spring, *Pseudomonas* spp. counts ranged from 0.58 to 2.35 log cfu/g for outer leaves and from < detection limit (dL) to 2.80 log cfu/g for inner leaves (Table 2). Initial populations were 1.43 and 2.07 log cfu/g for outer and inner leaves, respectively. At the end of 9 weeks, counts were 0.58 and 2.80 log cfu/g for outer and inner leaves, respectively. The mean populations of *Pseudomonas* spp. were 1.41 log cfu/g for outer leaves and 1.22 log cfu/g for inner leaves.

Table 1. Counts of epiphytic microorganisms during fall season on control lettuce samples

Microorganisms	Lettuce leaves	Initial counts (log cfu/g)	Final counts (log cfu/g)	Range (log cfu/g)	Mean (log cfu/g)
Aerobic mesophilic count	Outer	5.65	5.05	4.78-5.89	5.34
	Inner	4.83	5.48	4.83-6.43	5.58
Enterobacteriaceae	Outer	4.23	2.79	2.62-6.00	3.91
	Inner	2.85	0.91	0.91-6.00	2.98
Pseudomonas spp.	Outer	4.93	3.53	3.53-4.93	4.32
	Inner	4.46	4.54	4.46-6.59	5.24

Table 2. Counts of epiphytic microorganisms during spring season on control lettuce samples

Microorganisms	Lettuce leaves	Initial counts (log cfu/g)	Final counts (log cfu/g)	Range (log cfu/g)	Mean (log cfu/g)
Aerobic mesophilic count	Outer	4.44	4.17	3.96-4.44	4.20
	Inner	3.60	3.93	2.80-3.93	3.32
Enterobacteriaceae	Outer	0.75	1.86	0.75-1.86	1.15
	Inner	1.11	0.84	0.45-1.37	0.94
Pseudomonas spp.	Outer	1.43	0.58	0.58-2.35	1.41
	Inner	2.07	2.80	<dL-2.80	1.22

<dL: below detection limit (50 cfu/g)

Discussion

The potential presence of human pathogens such as *E. coli* O157:H7 in vegetables grown in soils amended with manure and irrigated with contaminated water is of growing concern. Cross-contamination of the produce with fecal waste, improperly composted manure and contaminated irrigated water used on the farm is suggested as a possible source of this pathogen during pre-harvest. In this study, results revealed that *E. coli* O157:H7 from inoculated compost and surface irrigation exposed to spring and fall environmental conditions can survive in soil for 9 weeks. In

lettuce, it could be found on inner or outer leaves during 9 weeks in fall and until 7 weeks in spring. Moreover, we observed a remarkable decrease of *E. coli* O157:H7 when sprinkle irrigation was used.

Properties of soil influencing the survival of pathogenic bacteria include soil composition, pH, water activity, oxidation-reduction potential, presence of rhizosphere, and microbial interactions (Fenlon *et al.*, 2000; Tamasi, 1981). Under field conditions, other variables, such as solar radiation, temperature and dryness, may also affect the survival of human pathogens. In fall, *E. coli* O157:H7 reached final populations of about 4.50 log cfu/gdw and in spring about 1.50 log cfu/gdw for contaminated irrigation water and compost treatments. This difference probably was a result of the effect of temperature and humidity. The average of temperature and humidity in fall was 10 ºC and 82 %, respectively, whereas in spring the average was 17 ºC and 62 %. Temperature has an important influence on survival of pathogens, because high temperatures reduced the survival of pathogen (Sorber and Moore, 1987). Higher temperatures may contribute greater stress and energy expenditure for the pathogenic bacteria than lower temperatures. These results are consistent with previous work done in sludge-amended soils inoculated with *Salmonella* spp., with increasing survival times being a function of decreasing temperature (Sorber and Moore, 1987). Girardin *et al.* (2005) and Welshimer (1960) observed the same results for *Listeria* in soil during the winter and spring. Zhang *et al.* (2009) reported that *E. coli* O157:H7 survived in soil for at least 60 days in an environmental chamber. Islam *et al.* (2005) observed that *E. coli* O157:H7 from inoculated animal manure compost can survive for more than 6 months in the soil of vegetable field. The low level of the pathogen present in soil after 9 weeks when lettuce was sprinkle irrigated could be due to the small amount of inoculated water reaching the soil and probably remaining in the first layers. This fact makes *E. coli* O157:H7 more exposed to UV and wind-mediated drying of the soil surface. The soil surface is more available to be affected by daily temperature differences and UV radiations than the soil below the top.

On the surface of lettuce, *E. coli* O157:H7 inoculated by sprinkle irrigation survived for at least 5 weeks (week 4 to week 9) in fall. However, in spring and after 7 weeks, no positive samples were found before re-contamination. This finding could be due to the high temperatures and low relative humidity of the week before. Perhaps factors such as light intensity, humidity, irradiation and high temperature have high influence on the survival of the pathogen. Solomon *et al.* (2002) reported that *E. coli* O157:H7 persisted on leaf surface for 20 days after spray irrigation with contaminated water. In another study, *E. coli* O157:H7 survived 30 days on lettuce plants after spray irrigation, and repeated irrigation with contaminated water increased the pathogen level on the plant (Solomon *et al.*, 2003). In a previous work, *L. innocua* inoculated with sprinkle irrigation on the surface of lettuce leaves survived for at

least 5 weeks in fall. However, in spring, no positive samples were found after 4 weeks of the first inoculation (Oliveira *et al.*, 2011).

Contamination of *E. coli* O157:H7 on lettuce leaves in treatments with surface irrigation and contaminated compost was diverse. The presence of *E. coli* O157:H7 was higher on leaves grown during fall than spring which correspond to higher soil contamination. In generally, we observed more contamination on the outer leaves than on the inner leaves. The outer leaves are more exposed to environmental conditions and will be more favorable to be contaminated through direct contact with the soil. Islam *et al.* (2004a) reported that *E. coli* O157:H7 can be transferred from contaminated soil and water to the surface of lettuce and parsley leaves. In another study, lettuce and parsley leaves were also contaminated with *Salmonella* Typhimurium from contaminated soil and water (Islam *et al.*, 2004b). It is remarkable that a one-time application of contaminated irrigation water or compost can result in pathogen contamination of vegetables.

It is known that the leaf surface is generally colonized by a wide variety of microorganisms. The natural microbiota in plants is considered as one of the main factors that influences the fitness of enteric pathogens on leafy crops in the pre-harvest environment. For this reason, we studied the behavior of aerobic mesophilic bacteria, *Enterobacteriaceae* and *Pseudomonas* spp. on lettuce leaves during the 9 weeks under environmental conditions. We observed that the levels of these microorganisms were lower than the results obtained for other studies under field conditions in other lettuce cultivars (Oliveira *et al.*, 2010). We could also observe the influence of the season conditions. In fall, indigenous microbiota counts were higher than in spring, similar to what happened with *E. coli* O157:H7. The same results were observed by Ailes *et al.* (2008) in cilantro and parsley.

In natural conditions, environmental factors are never static. Our results showed that survival of *E. coli* O157:H7 in soil and lettuce leaves under fluctuating conditions is different. Therefore, the predicted survival time and risk assessment can be overestimated if based under static conditions, such as in laboratory.

We observed that there was transfer of pathogen from contaminated soil or irrigation water to edible parts of lettuce leaves. However, we could not demonstrate if this transfer took place by internalization via root system or by direct contact to soil, insects or other vectors. Some authors reported the internalization via root systems by some pathogens, but different conclusions were found. Hora *et al.* (2005) and Sharma *et al.* (2009) did not detect internalization of *E. coli* O157:H7 into the aerial tissue of spinach. Franz *et al.* (2005) and Jablasone *et al.* (2005) reported that this pathogen was not detected in edible parts of the lettuce. However, there are reports that *E. coli* O157:H7 and *Salmonella* colonized interior tissues of lettuce plants (Franz *et al.*, 2007; Klerks *et al.*, 2007a, b). Because different plant cultivars and types, surface sterilize methods and pathogen strains were used in these exper-

iments, it is difficult to directly compare the results from different studies. More studies should be carried out to determine if *E. coli* O157:H7 is able to internalize to lettuce leaves via root system.

In our study, results demonstrated that contaminated irrigation water or manure compost used as a soil amendment could play an important role in contaminating lettuce and the soil in which it grows and could be a human health hazard. The origin and distribution of irrigation water, as well as the history of the land and the compost, should be known to limit the introduction of pathogens to produce fields.

Acknowledgments

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Chapter III

Effects of packaging type and storage temperature on the growth of foodborne pathogens on shredded 'Romaine' lettuce

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Abstract

Fresh produce can be a vehicle for the transmission of pathogens capable of causing human illnesses and some of them can grow on fresh-cut vegetables. The survival and growth of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* inoculated onto shredded lettuce was determined under modified atmosphere packaging conditions, at various storage temperatures. We also monitored changes in pH and gas atmospheres within the packages and the growth of psychrotrophic and mesophilic microorganisms. After pathogen inoculation, shredded lettuce was packaged in films of different permeability and stored at 5 and 25 ºC. After 10 days at 5 ºC populations of *E. coli* O157:H7 and *Salmonella* decreased approximately 1.00 log unit while *L. monocytogenes* increased about 1.00 log unit, in all package films. Moreover, the pathogens level increased between 2.44 and 4.19 log units after 3 days at 25 ºC. Psychrotrophic and mesophilic bacteria had similar growth at both temperatures with higher populations in air than in the other atmospheres. The composition of the storage atmosphere within the packaging of lettuce had no significant effect on the survival and growth of the pathogens used in this study at refrigeration temperatures. The results obtained can be considered as a warning indicator, which reinforces the necessity for corrective measures to avoid contamination of vegetables.

Keywords

Salmonella; *Escherichia coli* O157:H7; *Listeria monocytogenes*; Fresh-cut vegetables; Modified atmosphere packaging.

Introduction

Production and consumption of minimally processed (ready-to-eat) lettuce has increased dramatically in many countries in recent years. The convenience of cut, prewashed, packaged lettuce benefits consumers and has created a demand for high quality products (Brecht, 1995). In Spain, the consumption per capita in 2006 of fresh-cut fruits and vegetables was still low (1-1.5 Kg) compared with the rest of Europe and USA (Anonymous, 2007). With the increase of consumption, the incidence of foodborne outbreaks caused by contaminated fresh fruits and vegetables has increased in recent years (Mukherjee *et al.*, 2006). Fresh produce can be a vehicle for the transmission of bacterial, parasitic and viral pathogens capable of causing human illnesses and a number of reports refer to raw vegetables harbouring potential foodborne pathogens (Beuchat, 1996; Nguyen-The and Carlin, 1994). *Listeria monocytogenes* (Schlech *et al.*, 1983), *Salmonella* (Doyle, 1990), and *Escherichia coli* (Nguyen-The and Carlin, 1994) have been isolated from raw vegetables, which can become contaminated while growing or during harvesting, postharvest handling, or distribution. Food safety criteria for fresh-cut fruits and vegetables is regulated by the Commission Regulation EC Nº 2073/2005 (OJEU L338/1-26, 22 December 2005) which has been modified by EC Nº 1441/2007 (OJEU L322/12-29, 7 December 2007). Those criteria are absence of *Salmonella* in products placed on the market during their shelf life and absence of *L. monocytogenes* in 25 g before the food has left the immediate control of the food business operator who has produced it and <100 cfu/g in products placed on the market during their shelf life.

The preparation of fresh-cut products causes damage to plant tissues, reducing shelf-life of the more perishable products, compared to the intact fruits and vegetables (Guerzoni *et al.*, 1996; Watada *et al.*, 1996). This problem is primarily due to a higher respiration rate and the significant damage resulting from cutting (Pirovani *et al.*, 1997). Modified atmosphere packaging (MAP) is the alteration of the gaseous environment resulting from produce respiration (passive MAP) or from the addition and removal of gases from food packages (active MAP) to manipulate the levels of oxygen (O_2) and carbon dioxide (CO_2). MAP has been successfully used to maintain the quality of fresh-cut fruit and vegetables. It can affect the type and growth rates of microorganisms present on the produce (Day, 1992) e.g. it may enhance the growth of *L. monocytogenes* (Francis and O'Beirne, 1998). Fresh-cut produce can modify the atmosphere in their packages as a result of O_2 consumption and CO_2 production (Pirovani *et al.*, 1998). In general, gas compositions inside an MA package depend primarily on temperature, product fill weight, respiration rate, O_2 and CO_2 transmission rates of the package film, and the total respiring surface area (Bolin and Husxoll, 1991; Heimdal *et al.*, 1995; López-Gálvez *et al.*, 1997; Willox, 1995; Zagory and Kader, 1988). Different films have been used to create modified atmospheres, with polyvinylchloride (PVC) being one of the most commercially used (Robertson, 1993). The marketing temperature recommended

for the MAP of vegetables is 3 ºC, but these products are often stored at 10 ºC, an abusive temperature (Day, 1992). The aim of this study was to evaluate the potential of *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 to grow in shredded lettuce packaged in two types of modified atmosphere packaging (MAP) and in air, as well as the growth of mesophilic and psychrotrophic microorganisms at 5 ºC and 25 ºC.

Material and methods

Preparation of inocula

The non-pathogenic strain of *E. coli* O157:H7 (NCTC 12900), and the strain BAA-709 (ATCC) of *Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Michigan were used. Both strains were adapted to grow on Tryptone Soy Agar (TSA, Oxoid) supplemented with 100 µg/mL of Streptomycin (St, Sigma). Cultures grown on TSA-St at 37 ºC for 20-24 h were inoculated into a flask with 50 mL of Tryptone Soy Broth (Oxoid) supplemented with Streptomycin (TSB-St) at 150 rpm for 20-24 h at 37 ºC.

The strain of *L. monocytogenes* serotype 1/2a was isolated from bagged fresh-cut iceberg lettuce in our laboratory (Abadias *et al.*, 2008) and was identified by the "Servicio de Bacteriologia, Centro Nacional Microbiologia, Instituto de Salud Carlos III" (Majadahonda, Madrid, Spain). *L. monocytogenes* was grown in TYSEB (TSB amended with 6.0 g/L of yeast extract) for 20-24 h at 37 ºC.

The cultures were harvested by centrifugation at 9820 x g for 10 min at 10 ºC and resuspended in sterile saline peptone (SP, 8.5 g/L NaCl and 1.0 g/L peptone). Concentrations were determined with a spectrophotometer set at λ=420 nm according to the standard curves.

Sample preparation

'Romaine' lettuce (*Lactuca sativa* var. longifolia) was obtained from a local supermarket in Lleida (Spain). The outer leaves and core of the lettuce were removed and discarded. The remaining leaves were cut into pieces with a sharp knife. All the shredded leaves were washed in cold tap water for approximately 1 min. The excess surface water remaining on the leaves was removed by a manual kitchen centrifuge.

Inoculation of samples

The shredded lettuce was divided in four batches of approximately 1000 g. Three of them were inoculated by dipping into a 3 L tank containing each of the pathogens studied at 10^5 cfu/mL for 2 min. The other batch was left uninoculated (control). In addition, a dip in 10^3 cfu/mL of *L. monocytogenes* was made. These concentrations of

cells were estimated to be necessary to allow accurate enumeration by direct plating. The batch that served as control was dipped into 3 L deionized water for 2 min. Inoculated lettuce was let to dry for 30 min in a laminar flow biosafety cabinet.

The actual concentration of each pathogen in the dip tank was determined by plating dip suspension on the selective media. *E. coli* O157:H7 and *Salmonella* Michigan were plated on TSA-St followed by incubation at 37 ºC for 20-24 h. *L. monocytogenes* was enumerated on Palcam (Biokar Diagnostics) agar media after incubation at 37 ºC for 48 h. All analysis was carried out in triplicate packages for each microorganism and for each temperature/gas condition and the experiment was repeated twice.

Packaging of samples

Three different atmosphere conditions were studied: two different passive modified atmospheres and air condition. For this, samples were packaged in two different polypropylene plastic films (Amcor Flexibles, Ledbury, Herefordshire UK). Film I (35 μ in thickness) had O_2 and CO_2 permeability of 3500 cm^3/m^2/day/atm at 23 ºC and water steam permeability of 0.9 g/m^2/day at 25 ºC and 75 % relative humidity. Film II (35 μ in thickness) had O_2 and CO_2 permeability of 1100 cm^3/m^2/day/atm at 23 ºC and water steam permeability of 0.9 g/m^2/day at 25 ºC and 75 % relative humidity. To create air conditions, film was manually perforated with five holes of 400 μm each (Film III).

Uninoculated and inoculated samples were weighted (15 ± 1 g) in different film bags (12 cm x 20 cm). Bags were sealed and one-half were stored at 5 ºC for 10 days and the other half were stored at 25 ºC for 3 days for subsequent evaluation of microbial growth.

Microbiological analyses

Populations of *E. coli* O157:H7, *Salmonella*, *L. monocytogenes*, mesophilic and psychrotrophic bacteria were determined in three sample bags at each time. The samples from each pathogen and the control treatments were examined on the day of inoculation (d0) and after 1, 2 and 3 days for samples stored at 25 ºC, and 2, 6, 8 and 10 days for samples stored at 5 ºC. For the analysis, 10 g of lettuce from each bag were mixed with 90 mL of SP in sterile Stomacher bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Further ten-fold dilutions were made with the same diluent. For the inoculated samples, colonies of *E. coli* O157:H7 and *Salmonella* were enumerated by plating onto TSA-St and the plates were incubated at 37 ºC for 20-24 h. *L. monocytogenes* colonies were enumerated on Palcam medium incubated at 37 ºC for 48 h. In the uninoculated samples, mesophilic and psychrotrophic microorganisms were determined by enumerating

colonies on plates with Nutrient Agar (NA, Biokar Diagnostics) incubated at 30 ± 1 ºC for 3 days and at 6.5 ± 1 ºC for 10 days, respectively.

Gas analysis and measurement of pH

Throughout the experiment, CO_2 and O_2 concentrations in the bags were analyzed using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark). The pH of all samples (10 g lettuce + 90 mL SP) was measured after homogenization using a pH-meter (Model GLP22, Crison).

Results

Survival of E. coli *O157:H7 in modified atmosphere at various temperatures*

The initial populations of *E. coli* on shredded lettuce after inoculation and drying were 4.48, 4.53 and 3.87 log cfu/g for Film I, Film II and Film III, respectively (Figure 1).

Populations of *E. coli* O157:H7 on lettuce slightly decreased during the 10-day storage period at 5 ºC, whereas they increased after 3 days at 25 ºC. Significant increases in populations (2.12, 2.92 and 2.62 log cycles, for Film I, Film II and Film III, respectively) were detected in all package films during the first 24 h on lettuce kept at 25 ºC. The populations in Film II were approximately 0.5 log units greater than in the other two package films. The increase in this package film was also more rapid than in the other packages.

At 5 ºC, the populations declined by approximately 1.00 log unit over the 10 day storage period. Packaging film had no significant effects on *E. coli* O157:H7 populations at this temperature.

Survival of Salmonella *in modified atmosphere at various temperatures*

Initial populations of *Salmonella* on shredded lettuce, were 4.43, 4.45 and 4.24 log cfu/g for Film I, Film II and Film III, respectively (Figure 2).

Salmonella populations increased 2.41, 3.30 and 2.01 log units during the first 24 h at 25 ºC on lettuce packaged in Film I, Film II and Film III, respectively. At 5 ºC, population decreased by 0.49-0.96 log units over the storage period.

Populations of *Salmonella* inoculated on lettuce and stored at 25 ºC into Film II, increased by approximately 1.00 log unit more when compared with the other packages. Populations were lower on lettuce with Air atmosphere (Film III) than in all other MAP at both temperatures.

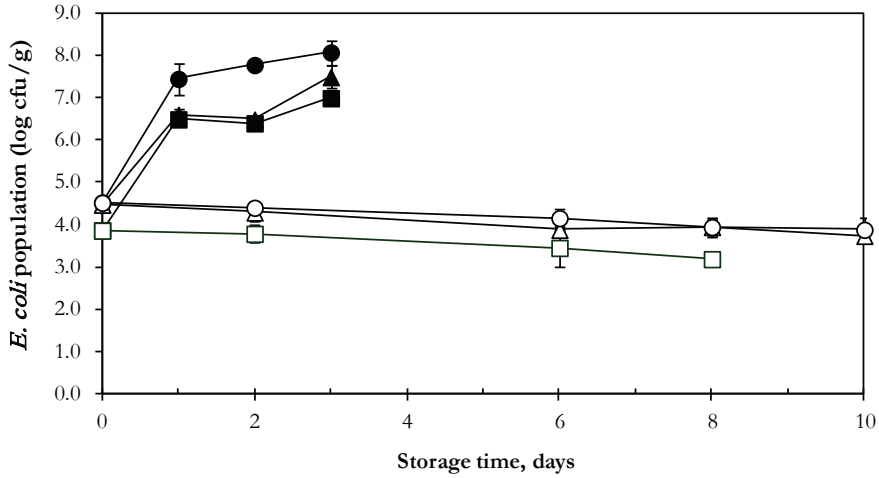

Figure 1. Effects of film type on survival of *Escherichia coli* O157:H7 on shredded lettuce stored at 25 ºC (full symbols) and 5 ºC (open symbols) in passive modified atmosphere: Film I (triangles), Film II (circles) and Film III (squares). Data represent the mean of three determinations and two experiment repetitions. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

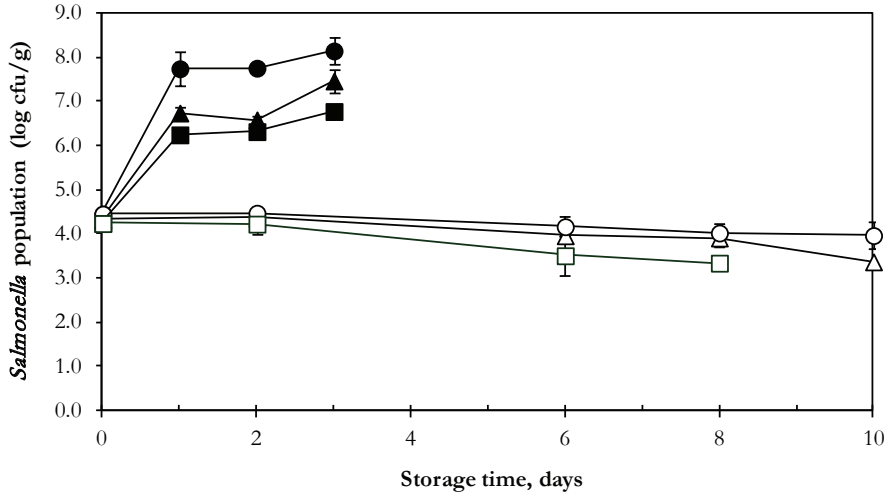

Figure 2. Effects of film type on survival of *Salmonella choleraesuis* on shredded lettuce stored at 25 ºC (full symbols) and 5 ºC (open symbols) in passive modified atmosphere: Film I (triangles), Film II (circles) and Film III (squares). Data represent the mean of three determinations and two experiment repetitions. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

Survival of L. monocytogenes *in modified atmosphere at various temperatures*

Growth of *L. monocytogenes* on shredded lettuce packaged in different films and stored at 5 and 25 ºC was studied at two different initial concentrations.

The initial population of *L. monocytogenes* on shredded lettuce with low inoculum (10^3 cfu/mL) was 1.83 log cfu/g for all package films (Figure 3A). *L. monocytogenes* was able to grow at 5 ºC, approximately 1.00 log unit in all package films regardless of initial concentration. At 25 ºC, the bacterium grew rapidly and increased about 3.00 log units during 3 days.

L. monocytogenes population raised approximately 2.50 log units during the first 24 h at 25 ºC for all package films. At the end of storage period, populations increased 3.40, 4.19 and 3.23 log units for Film I, Film II and Film III, respectively. Populations of this microorganism, stored at 25 ºC into Film II, increased by approximately 1.00 log unit more than in the other packages. At 5 ºC, the populations within all packages increased almost 1.00 log unit during the 10 days of storage, reaching the final population of 3.00 log cfu/g.

In the case of high inoculum dose (10^5 cfu/mL), the initial populations were 4.00 log cfu/g of lettuce for all package films and increased by approximately 2.00 log cycles during the first 24 h, and at the end of storage period, increased between 2.50-2.90 log units for all package films at 25 ºC (Figure 3B). At 5 ºC, populations within all packages types increased approximately 1.00 log unit during the 10 days of storage, reaching final population around 5.00 log cfu/g.

Effects of modified atmosphere and storage temperature on survival of Mesophilic and Psychrotrophic microorganisms

Populations of mesophiles and psychrotrophs on shredded lettuce under storage conditions of all Films were similar. The initial populations of mesophiles on shredded lettuce after washing were 4.90, 4.31 and 4.13 log cfu/g for Film I, Film II and Film III, respectively (Figure 4A). The initial populations of psychrotrophs were 4.90, 4.21 and 3.87 log cfu/g for Film I, Film II and Film III, respectively (Figure 4B).

Mesophiles and psychrotrophs populations increased significantly over the storage period in all packaging types, but growth rate was faster at 25 ºC than at 5 ºC.

Populations of mesophiles and psychrotrophs increased by 2.50-3.42 log units during the first 24 h at 25 ºC. At the end of storage period increases of 3.67-5.52 log units for the two groups of microorganisms were observed, with the final populations of approximately 8.60, 8.50 and 9.40 log cfu/g for Film I, Film II and Film III, respectively.

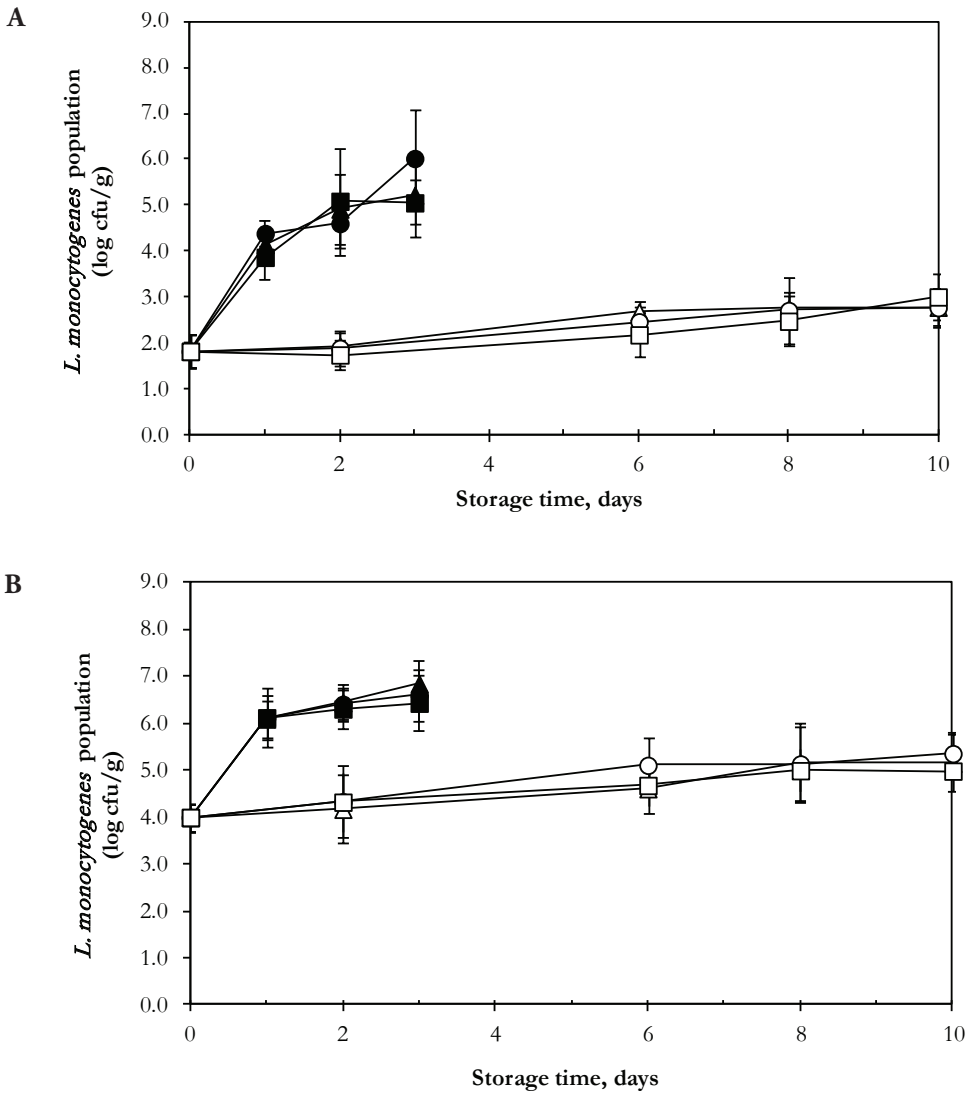

Figure 3. Effects of film type on survival of *Listeria monocytogenes* 10^3 cfu/g (A) and 10^5 cfu/g (B) on shredded lettuce stored at 25 ºC (full symbols) and 5 ºC (open symbols) in passive modified atmosphere: Film I (triangles), Film II (circles) and Film III (squares). Data represent the mean of three determinations and two experiment repetitions. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

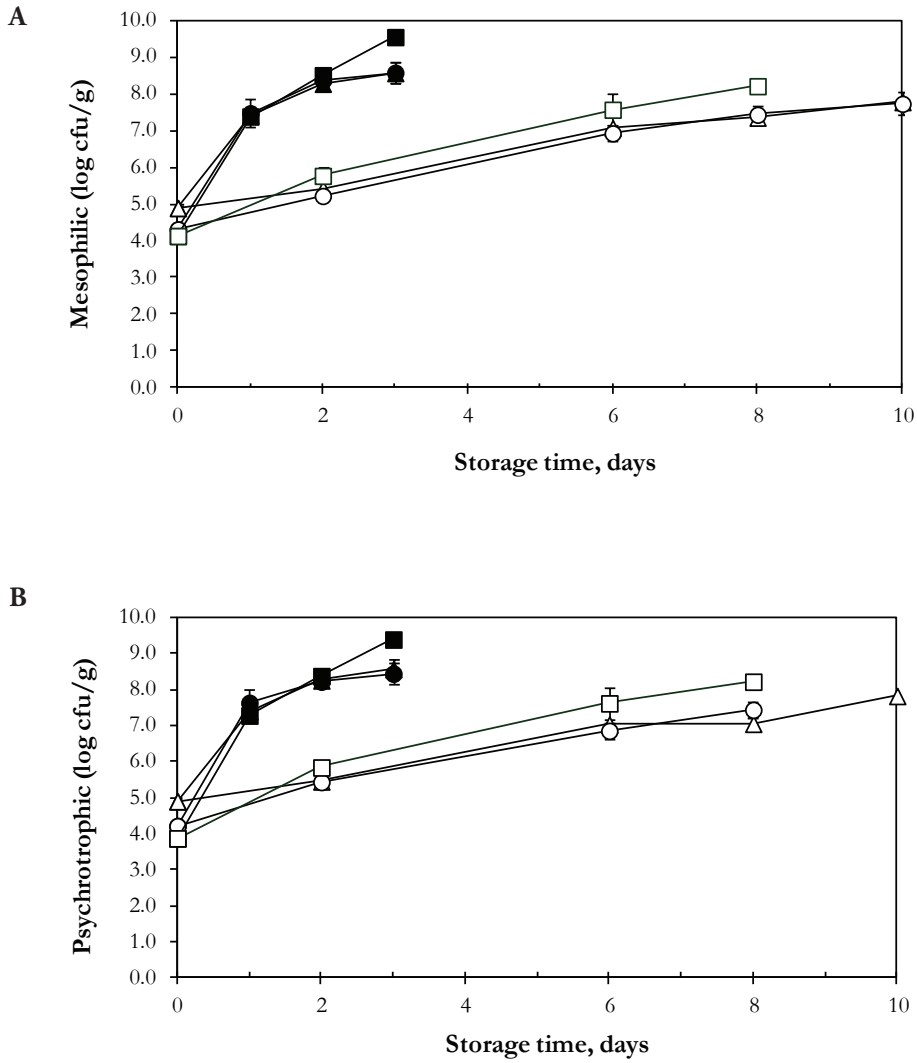

Figure 4. Effects of film type on survival of Mesophiles (A) and Psychrotrophs (B) on shredded lettuce stored at 25 ºC (full symbols) and 5 ºC (open symbols) in passive modified atmosphere: Film I (triangles), Film II (circles) and Film III (squares). Data represent the mean of three determinations and two experiments repetitions. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

At 5 ºC, populations of two groups of microorganisms increased 2.15-4.35 log cycles by day 8 within all packaging films, reaching final population of 7.80, 7.50 and 8.20 log cfu/g for Film I, Film II and Film III, respectively. Some effects of type of packaging were observed at 5 ºC; increases were lower in Film I and Film II than in Film III (air) by day eight. A similar trend was observed at 25 ºC where populations in Film I and Film II were lower than in Film III from day 3.

Evolution of package atmospheres and pH of lettuce

The evolution of the O_2 and CO_2 concentration within the packages depended on the film permeability and temperature, but there were no significant differences between inoculated and uninoculated samples. Thus data were pooled for all inoculation treatments.

The atmosphere within the Film I and Film II at 25 ºC was modified more rapidly than within the packages stored at 5 ºC (Figure 5).

The O_2 concentration within the Film I lettuce bags stored at 25 ºC (Figure 5A), dropped rapidly from 20.0 % to approximately 10.0 % during the first 24 h and after day 3 it was 0.8 %. At 5 ºC, the O_2 concentration decreased slowly to 14.0 % after 6 days and reached 8.0 % at the end of storage. The CO_2 concentration (Figure 5B) increased to more than 8.0 % during the first day at 25 ºC and reached approximately 11.0 % after 3 days. At 5 ºC, this increase was slower and reached levels around 6.0 % after 10 days of storage.

On lettuce samples stored under Film II at 25 ºC the O_2 concentration (Figure 5A) dropped sharply from 20 % to approximately 7.0 % after 1 day, and at the end of the storage period was 0.8 %. At 5 ºC, the O_2 concentration decreased slowly to 9.0 % after 6 days and reached 6.0 % after 10 days. The CO_2 concentration increased rapidly to 12.0 % during the first 24 h at 25 ºC and reached 13.3 % after 3 days of storage. At 5 ºC, the CO_2 concentration increased slowly to about 8.0 % after 6 days and maintained the same level until the end of storage period (Figure 5B).

In Film III (air), the CO_2 level was maintained between 0.4 and 1.1 % at 25 ºC and between 0.4 and 0.5 % at 5 ºC. The O_2 level remained around 20.5 % (data not shown).

There were no significant effects of modified atmosphere on pH values in all experiments. They remained at around pH 6.0-6.6 at 25 ºC and pH 6.3-6.5 at 5 ºC (data not shown).

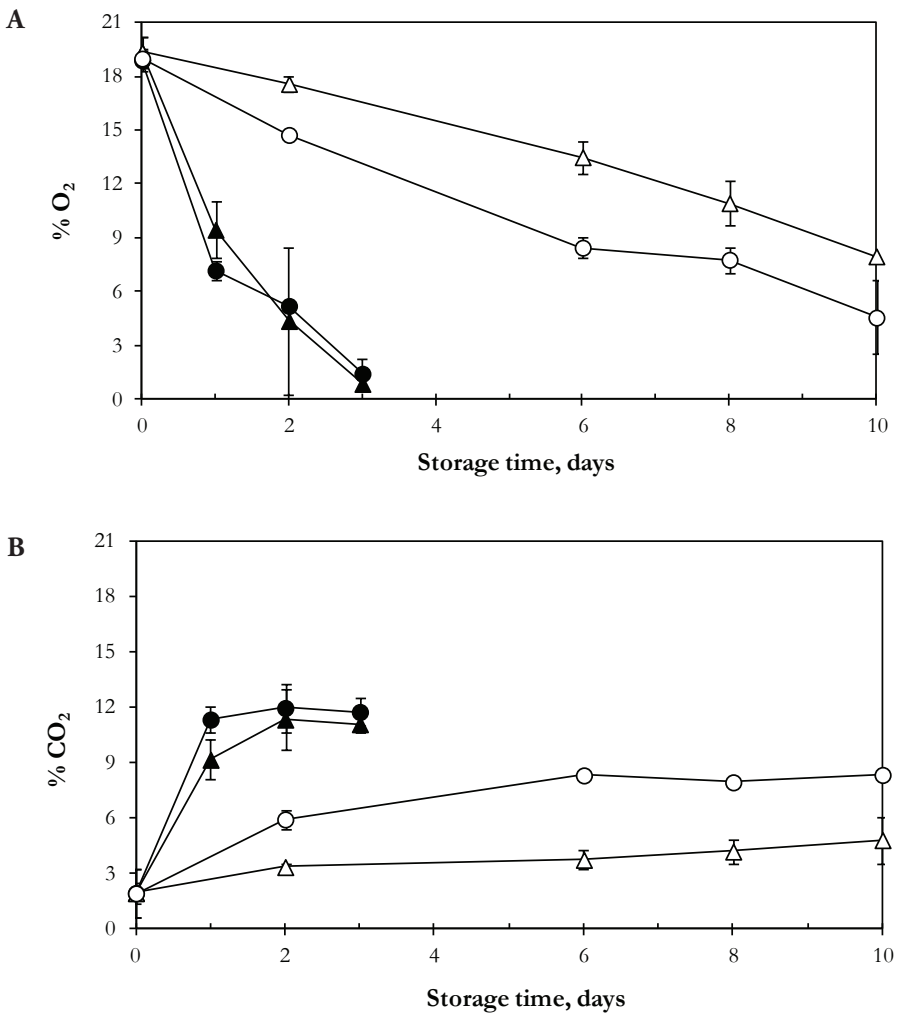

Figure 5. Effects of film type and storage temperature on % O_2 (A) and % CO_2 (B) within packages of Film I (triangles) and Film II (circles) stored at 25 ºC (full symbols) and 5 ºC (open symbols). Data represent the mean of all treatments determinations for each package film. Bars represent standard deviation of the mean. When vertical bars are not visible, they are smaller than symbol size.

Discussion

The composition of the storage atmosphere generated within different packages during their preservation at 5 ºC (usual storage temperature) had no significant effect on the survival and growth of the three foodborne pathogens on romaine lettuce. At 25 ºC, growth of *Salmonella* and *E. coli* O157:H7 on lettuce was slightly higher when packaged in Film II (the least permeable) than in the other two films.

We have shown that *E. coli* O157:H7 inoculated on lettuce increased around 3.00 log cycles after 24 h at 25 ºC and decreased slightly approximately 1.00 log cycle after 10 days storage at 5 ºC for all package films. The O_2 and CO_2 levels that developed in the different types of films did not affect the survival and growth of *E. coli* O157:H7. Similar results were found by Abdul-Raouf *et al.* (1993) and Diaz and Hotchkiss (1996), who demonstrated that iceberg lettuce packaged in low O_2 atmosphere, had no apparent effect on survival and growth of *E. coli* O157:H7. The behaviour of the strain of *Salmonella* Michigan studied in this work under all storage conditions was similar to *E. coli* O157:H7. It achieved significant increment levels at 25 ºC during the first 24 h of storage regardless of the package film studied. In the air storage conditions, we observed a decrease approximately 1.00 log cycle at 25 ºC when compared with the other two package films. At 5 ºC, populations decrease approximately 1.00 log cycle for all package films over the 10 day storage period. Kakiomenou *et al.* (1998) observed similar decrease in shredded lettuce inoculated with *Salmonella* Enteritidis stored under air and active MAP conditions at 4 ºC.

When *L. monocytogenes* was inoculated at the same concentration of *E. coli* and *Salmonella*, we observed an increase of 1.00 log cycle at 5 ºC during the 10-days of storage for all package film. Thus, as *L. monocytogenes* count should not be greater than 100 cfu/g during their shelf-life, we determined if this increase also would happen at low initial population (<100 cfu/g). In this study, we observed that even starting with low inoculum dose this microorganism reached concentrations above 5.00 log cfu/g at 25 ºC. At 5 ºC, the increase in population was lower but after 6 days, *L. monocytogenes* concentration was above the EC regulation (>100 cfu/g). At 25 ºC, the growth of this pathogen was much more rapid than at 5 ºC at both concentrations. Previous studies demonstrated that when *L. monocytogenes* is inoculated on fruits and vegetables, it is able to survive/grow at low O_2 percentage and at refrigeration temperatures (Berrang *et al.*, 1989; Beuchat and Brackett, 1990) because it is a facultative anaerobic and psychrotrophic microorganism (Francis and O'Beirne, 1997, 1998). Carrasco *et al.* (2008) obtained an increase of 2.66 cfu/g on iceberg lettuce packaged at 5 ºC but other studies have reported lower increments at the same temperature. Published data on the effects of gas atmospheres on survival and growth of *L. monocytogenes* on refrigerated fresh-cut vegetables are contradictory. Francis and O'Beirne (1997, 1998) observed that low O_2 atmospheres that develop in MAP vegetables may actually increase the growth of *L. monocytogenes*. In contrary,

our results indicate that the passive MAP conditions reported here did not influence the growth of this pathogen on lettuce at 5 ºC. Discrepancies over the behaviour of *L. monocytogenes* at low temperatures may be explained by the type of vegetable, the competitors, strain variation and the type of package film (Carlin and Nguygen, 1994; Varnam and Evans, 1996).

In this study, the samples of shredded lettuce presented similar initial concentration of mesophiles and psychrotroph microorganisms, approximately 5.00 log cfu/g. Accordingly, other authors have reported microbial densities between 10^4 and 10^6 cfu/g (Barriga *et al.*, 1991; García-Gimeno and Zurera-Cosano, 1997; Garg *et al.*, 1990; King *et al.*, 1991; Valero *et al.*, 2006). Counts of these two groups of microorganisms increased significantly over the storage period in all packaging types and reaching final populations between 10^7 and 10^{10} cfu/g but, the rate of growth was more rapid at high temperatures than at refrigerated temperatures. These final populations are consistent with reported by Gleeson and O'Beirne (2005) and Carrasco *et al.* (2008) on iceberg lettuce storage at 8 and 5 ºC respectively. Some effects of MAP were observed at both temperatures, counts were higher in air conditions (Film III) than in the others two package films over the storage period. Barriga *et al.* (1991) observed the similar results, mesophilic aerobes in cut iceberg lettuce increased under air at 4 ºC. Abdul-Raouf *et al.* (1993) observed similar increases in mesophiles (21 ºC) and psychrotrophs (5 and 12 ºC) in air. In fact, these high populations of mesophiles and psychrotrophs microorganisms are not unusual. In a recent study published by Abadias *et al.* (2008) similar counts were obtained in samples of fresh-cut vegetables from supermarkets.

The increase of CO_2 level and decrease O_2 at both temperatures in the Film I was slower than the changes in the gases within the packages of Film II. The differences between these two treatments were attributed to their film permeabilities being Film I more permeable than Film II. The atmosphere within the package films at 25 ºC was modified more rapidly than within the packages stored at 5 ºC; these results indicated the importance of the temperature in determining the respiration rate of fresh vegetables.

Dissolved CO_2 has been found to inhibit microbial growth (Daniels *et al.*, 1985; Devlieghere and Debevere, 2000; Devlieghere *et al.*, 1998) although not all microorganism strains or species are sensitive to the antimicrobial effect of CO_2. The CO_2 concentration generated in our study was not high enough to cause foodborne pathogens inhibition as all of them were able to grow at 25 ºC. Possibly, the modification of CO_2 and O_2 concentration inside the bags was not fast enough to prevent growth of foodborne pathogens.

In conclusion, our results indicated the ability of these pathogens to grow at abuse temperatures on romaine lettuce stored in air or in passive MAP. Notably, if minimally processed lettuce is contaminated with these pathogens during processing,

they will be able to grow and survive if the product is not kept at refrigerated conditions. Moreover, *L. monocytogenes* inoculated at low dose reached populations >100 cfu/g at 5 ºC. Packaging, storage, and handling conditions not unlike those used in commercial practice will not prevent growth or survival of these pathogens on lettuce. These and other observations emphasize the importance of strict hygiene during production, processing and packaging to avoid contamination of lettuce before and after washing or sanitizing and to maintained the cold storage chain until consumption, as it is recommended by producers.

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Chapter IV

Pathogenic potential of *Salmonella* Typhimurium DT104 following sequential passage through soil, packaged fresh-cut lettuce and a model gastrointestinal tract

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Abstract

From a quantitative microbial risk assessment perspective it is important to know whether certain food environments influence the pathogenic potential of pathogens and to what extent. The purpose of the present study was to examine the pathogenic potential of *Salmonella* Typhimurium DT104, measured as the capability to survive a simulated gastrointestinal tract system and the capability of adhering to and invading differentiated Caco-2 cells, after sequential incubation (without intermediate culturing) into soil, lettuce and cut lettuce stored under modified atmosphere (MAP) conditions. Two *S.* Typhimurium DT104 strains were used, one isolated from a pig carcass and one isolated from lettuce. The most important result of the present study is that the sequential incubation of *S.* Typhimurium in soil and lettuce slightly increased the capability of surviving the simulated gastric fluid, increased the capability to grow in the simulated intestinal fluid but decreased the capability of epithelial attachment and invasion and decreased the overall survival probability of the gastrointestinal tract system. Some variation in responses between the strains was observed, with the lettuce strain maintaining higher epithelial attachment capability and the carcass strains maintaining higher epithelial invasion capability. This study provided quantitative data on the effect of environmental and food matrices on the pathogenic potential of *S.* Typhimurium DT104 using a realistic system of sequential incubations in environmental and food matrices, followed by simulated gastrointestinal tract passage without intermediate culturing. These results could aid the development of more realistic quantitative microbial risk assessments.

Keywords

Salmonella Typhimurium; Virulence; Food; Gastrointestional system; Caco-2.

Introduction

Outbreaks of salmonellosis are traditionally associated with consumption of food of animal origin. However, in recent years, outbreaks have increasingly been linked to raw and minimally processed fruits and vegetables (Doyle and Erickson, 2008; Hanning *et al.*, 2009; Sivapalasingam *et al.*, 2004). In Europe, several large-scale salmonellosis outbreaks associated with leafy green vegetables have been reported (Horby *et al.*, 2003; Irvine *et al.*, 2009; Nygard *et al.*, 2008; Sagoo *et al.*, 2003; Takkinen, 2005). In addition, *Salmonella* has been detected regularly on fresh leafy-green vegetables during surveys at retail level (Abadias *et al.*, 2008; Elviss *et al.*, 2009; RIVM, 2008; Sagoo *et al.*, 2003). Leafy-green vegetables can become contaminated with *Salmonella* throughout the production chain, but contamination by irrigation water or organic fertilizer during primary production is considered the most likely sources (Franz and van Bruggen, 2008). Generally, under field conditions, population sizes of *Salmonella* associated with vegetable surfaces decline over time and no proliferation occurs (Islam *et al.*, 2004; Natvig *et al.*, 2002). However, after harvest, enteric pathogens have opportunities for growth depending on temperature, water availability, tissue damage, nutrients, native microflora and atmospheric composition of packaging (Aruscavage *et al.*, 2008; Cooley *et al.*, 2006; Koseki and Isobe, 2005; Oliveira *et al.*, 2010). Shredding and packaging under modified atmospheric conditions in combination with an abusive time-temperature profile in the supply chain may subsequently lead to spread and proliferation of pathogens (Doyle and Erickson, 2008; Tromp *et al.*, 2010).

Current microbial risk assessments generally only consider the frequency and level of contamination, the potential for growth and the amount consumed. However, there is concern about the fact that adaptation to the conditions encountered in the food supply chain could alter the pathogenic potential of pathogens by inducing (cross-)resistance mechanisms and/or inducing the expression of virulence factors, thereby increasing the likelihood of infection (Wesche *et al.*, 2009). Likely, not only the number of pathogens and the genetic characteristics of the strain but also the conditions encountered during processing and storage of the food product play a role in determining the likelihood and severity of infection. From a quantitative microbial risk assessment perspective it is important to know whether certain food environments influence the pathogenic potential of pathogens and to what extent. Although there is a large body of literature describing how environmental conditions affect the pathogens physiological and virulence characteristics (Mekalanos, 1992; Rowan, 1999; Wesche *et al.*, 2009), relatively little is known about the effect of environmental and food matrices on the pathogens ability to breach the gastric stomach barrier (Chua *et al.*, 2008; Peterson *et al.*, 2007) and to colonize the intestine (Barmpalia-Davis *et al.*, 2008; Baron *et al.*, 2004; Larsen *et al.*, 2010).

The purpose of the present study was therefore to examine the pathogenic potential of *S.* Typhimurium DT104, measured as the capability to survive a simulated gastrointestinal tract system and the proportion of cells adhering to and invading differentiated Caco-2 cells, after sequential incubations simulating various production stages of pre-cut ready-to-eat lettuce. *S.* Typhimurium was inoculated into soil, transferred (without culturing) to lettuce which was subsequently cut and stored under modified atmosphere (MAP) conditions. Subsequently, *S.* Typhimurium containing samples of the MAP stored cut lettuce were introduced into a static simulated gastro-intestinal tract system including gastric fluid (SGF) and intestinal fluid (SIF). Finally differentiated Caco-2 cells were exposed to SIF samples containing *S.* Typhimurium to study the level of adhesion and invasion. By carrying out appropriate control-experiments the influence of the simulated production chain on the pathogenic potential of *S.* Typhimurium (defined by the relative number of viable cells surviving the gastrointestinal system and invading Caco-2 cells) was determined.

Materials and methods

Experimental design

The study consisted of three treatments to evaluate the influence of different stages as encountered in the lettuce production chain on the pathogenic potential of *S.* Typhimurium. Initially, *S.* Typhimurium was inoculated into soil and transferred to lettuce which was subsequently cut and stored under modified atmosphere (MAP) conditions. From different steps in this chain *S.* Typhimurium was transferred (without culturing) into a static simulated gastro-intestinal tract system including gastric fluid (SGF), intestinal fluid (SIF) and Caco-2 cells to study the level of attachment and invasion. Throughout the simulated chain the relative population density was assessed. See Figure 1 for an overview of the experimental design. T1 (control): overnight culture of *S.* Typhimurium was added to SGF, followed by SIF and exposure to Caco-2 cells for adhesion and invasion; T2: overnight culture of *S.* Typhimurium was added to soil, incubated for 24 h at 15 °C, transferred to intact lettuce, and incubated for 24 h at 15 °C. Subsequently, half of the samples, followed simulated gastrointestinal passage and inoculation on Caco-2 cells; the other half continued to T3: the intact lettuce leaves were cut and incubated under modified atmosphere (MAP) conditions for 4 days at 5 °C to simulate cold storage, and 24 h at 20 °C to simulate consumer temperature abuse. Subsequently, simulated gastrointestinal passage and inoculation on the Caco-2 cells were carried out. All treatments were carried out in duplicate with 4 replications each.

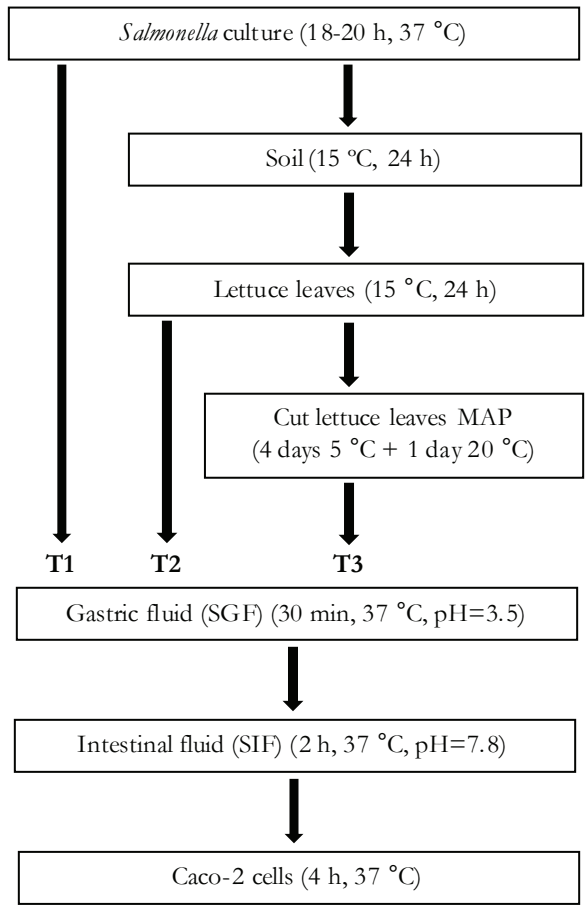

Figure 1. Schematic overview of the experimental setup.

Bacterial strains, soil and lettuce

Two strains of *Salmonella enterica* serovar Typhimurium DT104 were used in this study: *S.* Typhimurium 1638 isolate isolated from lettuce and *S.* Typhimurium 301a-1 isolated from pig carcass (RIVM collection). Bacteria were grown in Brain Heart Infusion (BHI, Oxoid) broth under shaking conditions (150 rpm) at 37 ºC for 18-20 h after transferring a glass bead from the -70 ºC stock. The soil was obtained from a local agricultural field (Zeist, The Netherlands) with 14 % of humidity and neutral pH. 'Butterhead' lettuce (*Lactuca sativa* var. capitata) was obtained from a local supermarket in Zeist. The outer leaves and core of two

lettuce heads were removed and discarded; the remaining leaves were separated for further inoculation.

Simulated gastrointestinal fluids

The composition and preparation of simulated SGF and SIF was based on a formulation by Rotard *et al.* (1995). SGF consisted of sodium chloride (175.0 g/L), sodium dihydrogen phosphate (88.8 g/L), potassium chloride (89.6 g/L), calcium chloride (22.2 g/L), ammonium chloride (30.6 g/L), glucose (65.0 g/L), glucuronic acid (2.0 g/L), urea (25.0 g/L), glucosamine (33.0 g/L), bovine serum albumin fraction V (1.0 g/L) and mucine (Type II from porcine stomach) (3.0 g/L). The pH was set at 3.5 with hydrochloric acid (1.0 mol/L). SIF consisted of sodium chloride (175.3 g/L), sodium bicarbonate (84.7 g/L), potassium dihydrogen phosphate (8.0 g/L), potassium chloride (89.6 g/L), magnesium chloride (5.0 g/L), urea (25.0 g/L), calcium chloride dehydrate (29.8 g/L), bovine serum albumin fraction V (1.0 g/L), lipase (0.5 g/L) and pancreatin (3.0 g/L). SIF also has bile solution with some reagents: sodium chloride (175.3 g/L), sodium bicarbonate (84.7 g/L), potassium chloride (89.6 g/L), urea (25.0 g/L), calcium chloride dehydrate (29.8 g/L), bovine serum albumin fraction V (1.8 g/L) and bile (6.0 g/L). The pH was set at 7.8 ± 0.2 with hydrochloric acid (1.0 mol/L). All reagents were from Merck (Darmstadt, Germany) except, ammonium chloride from MP Biomedicals (Solon, USA) and calcium chloride dehydrate, glucuronic acid, lipase and bile (Sigma, St Louis, USA).

Caco-2 cell culture

Differentiated Caco-2 cells were used as a simulation of the small intestinal epithelium (Pinto *et al.*, 1983). Gentamycin does not affect differentiated Caco-2 cells but will inactivate adhered cells (Berk, 2008). Caco-2 cells were obtained from the American Type Culture Collection (Caco-2, ATCC HTB-37) and cells of passage 27-43 were used in this study. At late confluency, these cells express both structural and functional characteristics of enterocytes present in the small intestine (Hendricks *et al.*, 1996). Cells were routinely cultured in Dubleco's Modified Eagle's Medium (DMEM, Gibco, Scotland) supplemented with 10.0 % heat-inactivated fetal bovine serum (FBS, Integro, The Netherlands), 1.0 % non-essential amino acids (Gibco), 1.0 % glutamine 100X (Gibco) and 0.1 % gentamicin (50.0 mg/mL, Gibco) in 75 cm^2 flasks (Corning Incorporated, NY, USA). The cells were grown to confluence (ca. 1.0 x 10^6 cells/mL, 7 days) at 37 ºC in a humidified atmosphere of 95.0 % air and 5.0 % CO_2. For the infection assays, monolayers were cultured in 12-well tissue culture plates (Corning). Caco-2 cells were seeded at a density of 1.6 x 10^5 cells/mL, and growth medium was changed

every 2 or 3 days. These cells are known to be fully differentiated after being cultured for 14 days.

Transferring procedures and microbiological analysis

A total of 500 g of soil was sprayed with 50 mL of an overnight culture of *Salmonella* (10^9 cfu/mL) and mixed thoroughly to ensure homogeneous distribution of the pathogen. Soil was stored at 15 ºC for 24 h. After storage, an extract from the soil was prepared by mixing 30 g of soil + 270 mL of sterile saline-peptone (SP, Bio Trading, The Netherlands). After settling of the solid particles the lettuce leaves were sprayed with 200 mL of this extract. Inoculated leaves were left to dry for 2 h in a laminar flow biosafety cabinet and stored for 24 h at 15 ºC. For treatment 3 lettuce leaves were cut into pieces with a sharp knife and stored at 5 ºC during 4 days plus 1 day at 20 ºC in MAP conditions (3.0 % O_2; 7.0 % CO_2, Anoxomat, Mart Microbiology, The Netherlands).

The inoculum for the gastro-intestinal system passage was obtained by shredding 5 g of lettuce leaves with an ultra-Turrax homogenator and mixing with 5 mL of SGF. The pH of the lettuce – SGF mixture was set at 3.5 using 6 N hydrochloric acid. The samples were incubated in a water bath at 37 ºC during 30 min. After incubation, 1 mL of the sample was taken for microbiological analysis. The remaining sample (9 mL) was mixed with 90 mL of SIF and incubated a 37 ºC for 2 h under microaerophilic conditions (6.0 % O_2; 94.0 % N_2, Anoxomat, Mart Microbiology, The Netherlands). The control treatment was realized by subjecting the overnight culture to the gastro-intestinal system passage. Afterwards, the samples were used for attachment and invasion assays with Caco-2 cells as describe bellow.

At the start and end of each discrete step samples were taken to assess the population density. Soil samples were analyzed at 0 and 24 h. Ten grams of soil were added to 90 mL of SP in an Erlenmeyer flask and homogenized by shaking at 150 rpm for 20 min and 10 min in an ultrasonic bath. Lettuce samples were examined on the day of inoculation (d0) and after 1, 4 and 5 days of storage. Five grams of lettuce leaves were mixed with 45 mL of SP and homogenized with an ultra-Turrax homogenator (IKA, Germany). Decimal dilutions of each sample from the 3 treatments were prepared using SP. Enumeration was carried out by plating in duplicate on XLD (Xylose Lysine Deoxycholate agar, Oxoid, UK) agar media. Plates were incubated at 37 ºC for 24 h.

Attachment and invasion assay

The method used for studying the rate of epithelial attachment and invasion was based on that described previously (Berk, 2008). Prior to attachment and inva-

sion assays, Caco-2 cells were washed three times with sterile phosphate-buffered saline (PBS, Bio Trading) to remove traces of antibiotic. After the final washing 1 mL prewarmed DMEM without FBS and gentamycin (ECM, experimental culture medium) was added to each well. Afterwards, the plates were inoculated with 40 µL bacterial suspension per well. The plates were incubated at 37 ºC in a humidified atmosphere of 95.0 % air and 5.0 % CO_2 during 1 h for attachment assay. After incubation, the medium was aspirated and the monolayers were rinsed three times with PBS in order to remove non-adhered and loosely adhered bacteria. Cells were lysed (in order to liberate the bacteria) with 1 mL 1.0 % (v/v) Triton-X100 (Merck) in PBS, for 5 min at room temperature. Triton lysate from three wells was combined and used for determining the number of *Salmonella* that adhered to the Caco-2 cells. After non-adherent bacteria had been removed by washing, Caco-2 cells were treated with ECM supplemented with gentamicin (50 mg/mL, Gibco) in order to quantify invasive bacteria. Gentamicin does not diffuse into Caco-2 cells, so any externally adhered bacteria are rapidly killed but the viability of any invaded bacteria is not affected. The plates were incubated for 3 h at 37 ºC and 5.0 % CO_2. After incubation, the cells were washed three times with PBS to remove excess antibiotic and lysed in order to liberate invaded bacteria. Triton lysate was used for determining the number of *Salmonella* that invaded the Caco-2 cells.

Data analysis

Cell counts were 10 log transformed before statistical analysis. Subsequently, the *S.* Typhimurium total population sizes present in the experimental unit at the end of each step of the gastrointestinal tract system (SGF, SIF, ATT, INV) was expressed as the relatively percentage of the population at the beginning of the gastrointestinal system. These expressions were compared statistically between treatments (within and between strains) by testing for equality of means by Student T-tests (with significance level $P<0.05$). The decrease in population size at discrete steps in the gastrointestinal system was calculated in the form of survival probabilities belonging to each step of the gastrointestinal system (P_{sgf}, P_{sif}, P_{att}, P_{inv}). These survival probabilities were summarized in a simple Fermi model describing the fate of *S.* Typhimurium DT104 cells during gastrointestinal passage: $N = M \cdot P_{sgf} \cdot P_{sif} \cdot P_{att} \cdot P_{inv}$, with N is expected number of invaded cells and M is the initial number of cells entering the gastrointestinal system. It should be noted that the P can represent a survival probability ($0<P\leq1$) as well as factor accounting for growth ($P\geq1$).

Results

General population dynamics of S. Typhimurium DT104 in soil and on lettuce

S. Typhimurium DT104 populations in soil (approximately log 7.5 cfu/g) remained stable over 24 h at 15 ºC (data not shown). *S.* Typhimurium DT104 populations transferred from the soil on to lettuce leaves increased approximately 1.0 log during the first 24 h at 15 ºC from approximately 5.5 log cfu/g to 6.5 log cfu/g and remained sat this level during storage for 5 days under MAP conditions.

Survival of S. Typhimurium DT104 during simulated gastrointestinal passage

Simulated gastric fluid (SGF)

After SGF (pH=3.5) passage, the relative population size (relative to the population at the beginning of the model gastrointestinal tract) of the lettuce isolate differed significantly between T2 (85.0 % ± 6) and T3 (92.0 % ± 5) (P=0.027) but both treatment did not differ significantly from the control treatment T1 (90.0 % ± 4) (Figure 2A). In contrast, the relative population size of the carcass isolate differed significantly between T1 (91.0 % ± 1) and T2 (97.0 % ± 1) (P<0.001), and between T1 and T3 (97.0 % ± 3) (P=0.001) (Figure 2B).

When comparing both strains, the carcass isolate showed higher relative population sizes and higher survival probabilities (Table 1) in T2 and T3 compared with the lettuce isolate (P<0.001, P<0.001, P=0.048 and P=0.0049).

Simulated intestinal fluid (SIF)

After passage of the simulated intestinal fluid (SIF) both strains subjected to the treatments T2 and T3 increased in relative population size, while the population size of the control treatment T1 remained constant (Figure 2A and B). With both strains the relative population size after SIF was significantly higher for the treatments (T2 and T3) compared to the control (T1) (all P<0.05). In addition, with the lettuce isolate the relative population size with T3 (110.0 % ± 2) was significantly higher compared to T2 (99.0 % ± 6) (P<0.001) (Figure 2A). The relative population size of the carcass strain after SIF did not differ between both treatments (113.0 % ± 1 and 113.0 % ± 3, respectively). For both strains, the SIF growth factor (survival probability >1) was significantly higher for T2 and T3 compared to the control treatment T1 (all P<0.001) (Table 1). The difference in growth factor between T2 and T3 was not statistically different (P=0.103).

When comparing both strains the relative population size in T2 after SIF passage (Figure 2) was significantly higher for the carcass strain (P<0.001). However, SIF

growth factors (Table 1) did not differ significantly between both strains within each treatment.

Table 1. *S.* Typhimurium DT104 survival probabilities of four different steps in a simulated gastrointestinal tract system (SGF = simulated gastric fluid, SIF = simulated intestinal fluid, ATT = attachment to Caco-2 cells, INV = invasion of caco-2 cell layer) for different growth histories (T1 = control, T2 = 24 h soil, 24 h lettuce, T3 = 24 h soil, 24 h lettuce, 5 days cut-lettuce under MAP storage)

	***S.* Typhimurium lettuce isolate**			***S.* Typhimurium carcass isolate**		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**
SGF	0.90±0.04 [ab]	0.84±0.06 [a]	0.92±0.05 [b]	0.91±0.01 [a]	0.97±0.01 [b]	0.97±0.03 [b]
SIF	1.02±0.03 [a]	1.15±0.05 [b]	1.19±0.04 [b]	1.00±0.02 [a]	1.15±0.01 [b]	1.17±0.01 [b]
ATT	0.65±0.05 [a]	0.65±0.10 [a]	0.59±0.07 [a]	0.59±0.02 [a]	0.49±0.04 [b]	0.44±0.06 [b]
INV	0.89±0.03 [a]	0.65±0.03 [b]	0.66±0.03 [b]	0.94±0.02 [a]	0.90±0.06 [a]	0.82±0.03 [b]
Overall	0.53±0.06 [a]	0.40±0.07 [b]	0.43±0.06 [b]	0.51±0.03 [a]	0.50±0.05 [a]	0.41±0.06 [b]

[a] Survival ratio's of the strain does not differ between treatments when sharing the identical letter

Attachment to Caco-2 cells (ATT)

With both strains, the relative population size that attached to the Caco-2 cells (relative to the population at the beginning of the model gastrointestinal tract) was significantly higher with both treatments (T2 and T3) as compared to the control treatment (T1) (Figure 2A and B) (all $P<0.01$). The differences between the treatments T2 and T3 were not significant.

With the lettuce strain the ATT survival probability was not significantly different between both treatments and between the treatments and the control (Table 1). In contrast, with the carcass strain the ATT survival probability was significantly lower for both T2 and T3 compared to the control T1 (both $P<0.001$) (Table 1).

When comparing both strains, for T1 (control) and T3 the relative population size (relative to the population at the beginning of the model gastrointestinal tract) attached to the Caco-2 cells was higher with the lettuce strain compared to the carcass strain ($P=0.002$ and $P<0.001$ respectively) for T1 (control) and T3 (Figure 2). For all 3 treatments, the ATT survival probability was significantly higher for the lettuce strain ($P=0.021$, $P<0.001$ and $P<0.001$ for T1, T2 and T3 respectively).

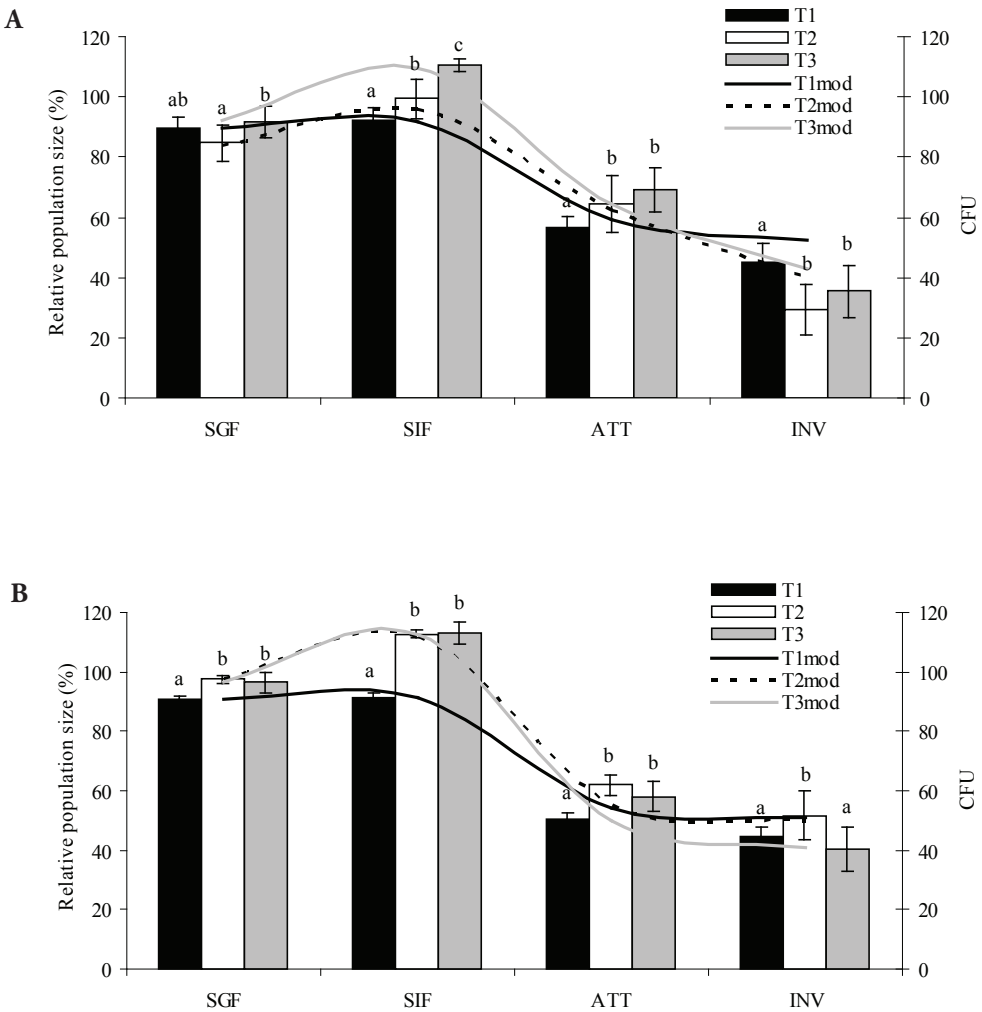

Figure 2. Relative population size (bars) (N/N0*100, with No being the population size at beginning of the model gastrointestinal tract) of *S.* Typhimurium DT104 1638 (lettuce isolate) (A) and *S.* Typhimurium DT 104 301a-1 (carcass isolate) (B) after passage of the four different phases of the gastrointestinal model (SGF = simulated gastric fluid, SIF = simulated intestinal fluid, ATT = attachment to Caco-2 cells, INV = invasion into the Caco-2 layer). T1, T2 and T3 represent the different treatments (T1: control, T2: 24 h soil, 24 h lettuce, T3: 24 h soil, 24 h lettuce, 5 days cut-lettuce under MAP storage). The lines represent the modeled population size when starting with 100 cells at the start of the gastrointestinal system according to the calculated survival probabilities of each step.

Invasion of Caco-2 cells (INV)

With the lettuce strain the relative population size that invaded Caco-2 cells (relative to the population at the beginning of the model gastrointestinal tract) of T2 (29.0 % ± 8) and T3 (35.0 % ± 9) were significantly lower than that of T1 (45.0 % ± 6) ($P<0.001$ and $P=0.010$) (Figure 2A). The difference between T2 and T3 was not significant. In contrast, with the carcass strain the final relative population size that invaded Caco-2 cells was significantly higher in T2 (52.0 % ± 7) compare to T1 (44.0 % ± 3) ($P=0.044$) and T3 (40.0 % ± 8) ($P=0.001$) (Figure 2B). With the carcass strain no difference was observed between T1 and T3.

With the lettuce strain the INV survival probability was significantly higher for both treatments T2 and T3 compared to the control T1 (both $P<0.001$), but did not differ between both treatments T2 and T3 ($P=0.643$) (Table 1). With the carcass strain, the INV survival probability was significantly higher for T3 compared to the control T1 ($P<0.001$) and compared to T2 and T3 ($P<0.001$) (Table 1). For the carcass strain, no difference was observed between T2 and the control T1.

When comparing both strains, the relative population size (Figure 2) in T2 (relative to the population at the beginning of the model gastrointestinal tract) that invaded the Caco-2 cells was significantly higher for the carcass strain compared to the lettuce strain ($P<0.001$). In addition, the INV survival probability (Table 1) was significantly higher in all treatments for carcass strain ($P<0.001$).

Discussion

With the aim of investigating how growth conditions influence the ability of *S.* Typhimurium DT104 to survive gastrointestinal conditions and to attach to and invade human epithelial cells, we determined these capabilities after sequential incubations in soil, lettuce and fresh-cut lettuce under modified atmosphere conditions. In contrast to the more traditional approach of single introductions, this sequential experimental setup allowed the pathogen to experience different environments without any intermediate isolation steps that might otherwise lead to the loss of certain physiological adaptations acquired during the growth history. Using this approach, the effects of different stages of a food production chain on the pathogenic potential can be assessed in a more realistic way since pathogens are displaced between different substrates and/or environmental conditions when cycling through agricultural ecosystems, food production chains and hosts. The application of such a sequential incubation method in order to study pathogen dynamics in food production chains is rather limited (Franz *et al.*, 2005; Kupriyanov *et al.*, 2009a, b). In addition, following the sequential incubation in soil, lettuce and fresh-cut lettuce under MAP conditions, *Salmonella* was exposed to sequential passage (again without culturing) of simulated gastric fluid, intestinal fluid and interaction with epithelial cells. This

allowed a more complete and more realistic assessment of pathogen behavior in the digestive tract compared to studies only considering single isolated parts of the digestive tract.

The most important result of the present study is that the sequential incubation of *S.* Typhimurium in soil and lettuce slightly increased the capability of surviving the simulated gastric fluid, increased the capability to grow in the simulated intestinal fluid but decreased the capability of epithelial attachment and invasion and decreased the overall survival probability of the gastrointestinal tract system. The results indicate that the magnitude of these effects might be strain dependent. However, more strains should be studied in order to draw inference on strain dependency.

Survival in simulated gastric fluid

The importance of gastric fluid to humans and other animals as the first defense against enteric pathogens has been well documented (Smith, 2003). With the present experiment the population density of *S.* Typhimurium DT104 decreased on average only 8.0 % after 30 min. incubation in simulated gastric fluid (SGF) with a pH of 3.5. Comparable results were previously obtained for vegetative cells of *Bacillus cereus* where no relevant inactivation took place in simulated gastric fluid (Wijnands *et al.*, 2009). In other studies, the *S.* Typhimurium DT104 population was reduced by two third after nearly 2 min exposure to SGF with pH 1.5 (Perez *et al.*, 2010), and survived only for 5 min in SGF with pH 1.5 with concentrations decreasing at least 5.5 log during this time (Roering *et al.*, 1999). However, the relevance of such extremely fast decrease in SGF with pH 1.5 for food safety is debatable since gastric pH rises fast after ingesting of food. In the fasting state the gastric pH is around 2, but during and after consumption of a meal the pH increases to 4-7 in a very short time due to the buffering effect of the foodstuff (Takumi *et al.*, 2000). The pH of 3.5 that *S.* Typhimurium DT104 experiences in the present study is therefore thought to be more realistic with respect to studying gastrointestinal tract survival and have been used in previous studies (Mainville *et al.*, 2005; Radcliffe *et al.*, 1998). In addition, the exposure time (30 min.) of *S.* Typhimurium DT104 present in lettuce extract exposed to SGF relates to measured stomach emptying times of 50.0 % food passage to the intestines for semi-liquid substrates (Clarkston *et al.*, 1997).

Sequential incubation in soil, lettuce and cut lettuce stored under MAP conditions resulted in an increased survival probability of the simulated gastric fluid of the carcass isolate, which is indicative of increased acid adaptation. Moderate acidic conditions have been reported to render strains of *Salmonella* more resistant to gastric fluid (Perez *et al.*, 2010; Yuk and Schneider, 2006). In addition, surface contact was shown to mediate acid protection in *S.* Typhimurium (Gawande and Bhagwat, 2002a, b; Kroupitski *et al.*, 2009). Therefore, the attachment to MAP stored fresh-

cut lettuce and the associated light acidic conditions might be responsible for the observed increased acid resistance.

Survival in simulated intestinal fluid

Within the intestine, *Salmonella* must be able to resist the action of pancreatic enzymes and bile salts which are detergents made by the liver and secreted in bile into the intestines from the gallbladder to aid in the dispersion and degradation of fats. A major result obtained with the present study is the significant growth of both strains in the intestinal fluid following a growth history in soil and lettuce compared with the control treatment. Pathogen growth in under intestinal conditions is not uncommon and in many cases even necessary in order increase population densities. For example, *Bacillus cereus* has to grow in the intestines in order produce enterotoxins (Wijnands *et al.*, 2006). The tolerance of *S.* Typhimurium to bile (Merritt and Donaldson, 2009) and the protective effects of food components (Kos *et al.*, 2000) have been well documented. Bile tolerance involves a virulence regulator that includes genes necessary for protection against the action of antimicrobials (Prouty *et al.*, 2002; Van Velkinburgh and Gunn, 1999). In addition, several studies identified bacteria from lettuce displaying antagonistic effects against enteric pathogens like *S.* Typhimurium (Cooley *et al.*, 2006; Johnston *et al.*, 2009; Schuenzel and Harrison, 2002) and recently coliform strains producing non-lethal antimicrobial substances were isolated from salad samples (Fleming *et al.*, 2010). Possibly, activated mechanisms involved in protection against antimicrobials produced by native bacteria in the soil and on the lettuce resulted in cross-protection against bile salts and thereby increased the ability to grow in this medium.

Adhesive and invasive capacity of S. Typhimurium DT104 to differentiated Caco-2 cells

In order to colonize the intestine *S.* Typhimurium must attach to the epithelial surface layer. Subsequently, a key aspect of *Salmonella* pathogenesis is the ability to penetrate and pass the intestinal epithelial monolayer. We used fully differentiated intestinal Caco-2 cells, which are expected to mimic the *in vivo* infection pathway more closely because of the higher degree of similarity of Caco-2 cells with epithelial cells of the ileum (*i.e.* the site of *Salmonella* invasion) as compared to undifferentiated intestinal cells like Int-407. In addition, *in vitro* obtained data with Caco-2 cell lines correlated well with results obtained from animal infection models for *S.* Typhimurium (Bolton *et al.*, 2000; Takeuchi, 1967).

Both the adhesive and invasive capacities of the *S.* Typhimurium DT104 strains used in the present study decreased as a result of sequential incubations in soil, lettuce, MAP stored cut lettuce, gastric fluid and intestinal fluid. The process of cell

invasion requires the production and transport of secreted effector proteins by a type III secretion apparatus encoded in *Salmonella* pathogenicity island SPI-1. In broth, *S.* Typhimurium induces SPI-1 genes in response to carbon starvation, exposure to excess short-chain fatty acids and oxygen limitation (Lucas *et al.*, 2000; Song *et al.*, 2004). Possibly, the excess availability of carbon sources as a result of shredding the lettuce overruled the effect of oxygen limitation in activating the SPI-1 genes. Moreover, since the major regulator of *Salmonella* invasion (HilA) is activated at neutral pH (Bajaj *et al.*, 1996), the slightly acidic conditions in the MAP stored cut lettuce might have impaired the invasive capability. Previously, it was reported that acid adaptation resulted in a lower level of invasion in Int-407 cell lines (Fratamico, 2003). Gene expression profiles of key virulence factors involved in attachment and invasion of Caco-2 cells could provide answers to verify this hypothesis.

Interestingly, where the lettuce strain showed the maintenance of a higher attachment ratio after the applied growth history, the carcass strain showed maintenance of a higher invasion ratio compared to lettuce. This might be related to genetic differences between the strains, with the lettuce strain exhibiting a stronger attachment mechanism at the expense of efficient epithelial invasion. The genetic material of bacteria encodes for a variety of adherence mechanisms including fimbrial adhesions, flagella and biofilm formation (Holden *et al.*, 2009) and these genes have been reported to be involved in both attachment to plant surfaces and the animal intestinal tract (Barak *et al.*, 2005; Baumler *et al.*, 1996). However, more strains should be evaluated (phenotypically and genetically) in order to investigate this.

Implications for food safety

When bacteria are confronted with a non-favorable environment, the adapted microorganisms are often more resistant to a range of stresses and more difficult to eradicate than would be predicted from experimental data obtained under laboratory growth conditions, thereby increasing the likelihood of human infection (Wesche *et al.*, 2009). This study showed that sequential incubations in soil, lettuce, MAP stored cut lettuce increased the ability of *S.* Typhimurium to survive the gastric fluid and to grow in the intestinal fluid increased, but decreased the ability to attach to and invade the epithelial layer. The observation that several *Salmonella* virulence factors are involved in the attachment to plant surfaces (Barak *et al.*, 2005) seem, according to our result, not to result in an increased adhesive capacity of Caco-2 cells by for example priming of adhesion genes after incubation at fresh-cut lettuce stored under MAP conditions. Similarly to our results, the adaptation of *Salmonella* to an unfavorable environment that mimicked egg white conditions did not result in increase virulence, and acid adaptation as well as sublethal heat stress resulted in impaired epithelial invasion (Baron *et al.*, 2004; Fratamico, 2003; Karatzas *et al.*, 2008; Sirsat *et al.*, 2011).

As mentioned, the effects of the growth history in soil and lettuce on the pathogenic potential of *S.* Typhimurium DT104 are not unambiguous. Although the capability of epithelial attachment and invasion was reduced, the results showed that *S.* Typhimurium present in pre-packed fresh-cut lettuce still seems capable to efficiently survive the gastrointestinal system and invade the epithelial layer. Moreover, the pathogenesis of salmonellosis is multifactorial and *Salmonella enterica* serovars primarily cause gastroenteritis with diarrhea as most important symptom. *S.* Typhimurium induced diarrhea is mainly is the result of an inflammatory reaction triggered by injection of effector proteins (Sop and Sip family) encoded by the *Salmonella* pathogenicity island I into the host epithelial cells leading to the recruitment of polymorphonuclear neutrophils (PMNs) and subsequently to tissue injury, fluid accumulation and diarrhea (Berk, 2008). In addition, the *Salmonella* enterotoxin, encoded by the *stn* gene is a major virulence factor involved gastroenteritis symptoms (Chopra *et al.*, 1999; Xu *et al.*, 2010). Determination of the transcriptional expression of these genes might shed light on how virulence (*i.e.* severity of gastroenteritis symptoms) is affected by the sequential incubations as applied in the present study. Complementary, the production of chemokines like IL-8 in the infected epithelial tissue can be determined as a measure for virulence since IL-8 production promotes PMN migration through the matrix resulting in diarrhea (Berk, 2008).

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Chapter V

Fate of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in the presence of natural background microbiota on conventional and organic lettuce

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Abstract

Foodborne illnesses due to the consumption of contaminated raw vegetables is a continuing food safety concern. The limited efficacy of chlorine products to disinfect in fresh-cut industries, has led to study other methods or strategies to improve the safety of processed fresh-cut products. It has been reported that the presence of competing microorganisms on the surfaces of fresh produce can contribute to the reduction of pathogens. The aim of this study was to evaluate the interactions between the natural background microbiota of shredded conventional and organic lettuce and *Listeria monocytogenes* and *Escherichia coli* O157:H7. The effect of different initial load of background microbiota ('low', 'medium' and 'high') was tested for its ability to reduce *L. monocytogenes* and *E. coli* O157:H7 populations on shredded lettuce during storage at 10 ± 1 ºC for 8 days. After the different pre-conditioning steps in order to obtain different initial loads of microbiota, in general, we observed that varying its size had no effect on *L. monocytogenes* and *E. coli* O157:H7 survival/growth during the storage period. Only differences on the survival/growth of *L. monocytogenes* and *E. coli* O157:H7 inoculated onto organic and conventional lettuce, respectively at the end of storage period at 10 ºC were found. These results highlight the necessity for corrective measures to avoid contamination of fresh-cut vegetables with foodborne pathogens.

Keywords

Lettuce; Background microbiota; *Escherichia coli* O157:H7; *Listeria monocytogenes.*

Introduction

The increasing consumption of fresh produce has been attributed to the associated nutritional and health benefits. In Spain, sales of ready-to-eat vegetables showed an annual increase of 6.0 %, with 69100 tons in 2010 (Anonymous, 2011). With the increasing consumption of fresh vegetables there has been a corresponding rise in the number of foodborne outbreaks linked to produce (Beuchat, 1996; Tauxe *et al.*, 1997). Vegetables are generally colonized by a wide variety of microorganisms (Abadias *et al.*, 2008), predominantly by gram-negative bacteria, in particular, members of the *Pseudomonadaceae* and *Enterobacteriaceae* (Oliveira *et al.*, 2010b). Pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 may also be present on vegetables, and a number of outbreaks associated with consumption of lettuce contaminated with *L. monocytogenes* (Francis *et al.*, 1999; Sagoo *et al.*, 2003) and *E. coli* O157:H7 (Ethelberg *et al.*, 2010; Friesema *et al.*, 2007, 2008) have been reported. Many factors can contribute to the contamination of fresh and fresh-cut produce with human pathogens. Potential sources of contamination at the preharvest level are soil, irrigation water, manure, wild and domestic animals, harvest workers, and harvest equipment. At the postharvest level, wash water, workers, packing materials, process equipment, and transportation vehicles are potential sources of contamination (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire *et al.*, 2006). After processing, pathogens can survive and even grow on lettuce, depending on temperature, water availability, level of tissue damage, available nutrients and native microbiota (Abdul-Raouf *et al.*, 1993; Aruscavage *et al.*, 2006; Brandl, 2006; Cooley *et al.*, 2006). In a previous study, Oliveira *et al.* (2010a) demonstrated that *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* could grow in fresh-cut lettuce under certain conditions. The control of pathogenic microorganisms on fresh vegetables plays an important role in maintaining its quality and safety for human consumption. A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce initial bacterial populations on minimally processed vegetables (Beuchat, 1998). Chlorine is probably the most widely used sanitizer in the fresh-produce industry. However, studies indicate that chlorine concentrations traditionally used (50-200 ppm) are not effective in reducing pathogen load on vegetables (Behrsing *et al.*, 2000; Delaquis *et al.*, 2002; Lee and Baek, 2008). Modified atmosphere packaging (MAP) in combination with refrigerated temperatures is used as a mild preservation technique for minimally processed vegetables. The alteration of the gaseous environment inside of packages slows product respiration, delays physiological ageing, inhibits enzymatic browning and maybe affects the

type and growth rates of microorganisms present on the produce (Day, 1992). It has also been reported that the presence of competing microorganisms on the surfaces of fresh produce contributes to the reduction of pathogens. The typical microbiota present on fresh vegetables is composed of many species and compete with 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). Babic *et al.* (1997) reported that background microbiota in spinach inhibited the growth of *L. monocytogenes*, which can grow on 'Romaine' lettuce stored at 5 ºC at MAP conditions (Oliveira *et al.*, 2010a). In a previous study, organic lettuce was associated to higher values of all the microbial groups analyzed. The highest difference between both kinds of samples (organic and conventional lettuce) arises in the *Enterobacteriaceae* counts (Oliveira *et al.*, 2010b).

The objective of this study was to evaluate the effect of the natural background microbiota of lettuce submitted to different pre-conditioning steps on survival of *L. monocytogenes* and *E. coli* O157:H7. The study was carried out with conventional and organic produced lettuce.

Material and methods

Bacteria and preparation of inocula

A non-pathogenic strain of green fluorescent protein (GFP)-expressing and ampicilin resistant *E. coli* O157:H7 (B6-914 GFP-91) (Fratamico, 1997) and five strains of *L. monocytogenes*: serovar 1a (CECT 4031), serovar 3a (CECT 933), serovar 4a (CECT 940), serovar 4b (CECT 4032) and serovar 1/2a (LM230/3), were used.

E. coli O157:H7 was grown on Sorbitol MacConkey agar supplemented with Cefixime and Tellurite (CT-Smac, Biokar Diagnostic) and 50 µg/mL ampicilin (ampicillin sodium salt, Sigma, St. Louis, USA) (CT-Smac + Amp) at 37 ± 1 ºC for 20-24 h. A single colony was transferred into a flask with 50 mL of tryptone soy broth supplemented with 50 µg/mL ampicilin (TSB + Amp, Oxoid) at 150 rpm for 20-24 h at 37 ± 1 ºC. *L. monocytogenes* strains were grown individually on tryptone soy agar (TSA, Oxoid) supplemented with 6.0 g/L yeast extract, 2.5 g/L glucose and 2.5 g/L dipotassium hydrogen phosphate (TYSEA) at 37 ± 1 ºC for 20-24 h. An individual colony of each strain was transferred into a flask with 50 mL of TSB supplemented with 6.0 g/L yeast extract (TYSEB) at 37 ± 1 ºC for 20-24 h. Bacterial cells were harvested by centrifugation at 9820 x g for 10 min at 10 ± 1 ºC and resuspended in sterile saline peptone (SP, 8.5 g/L NaCl and 1.0 g/L peptone). Equal volumes of each strain were combined to obtain the five strain cocktail of *L. monocytogenes*. The concentration of *E. coli* O157:H7 and *L. monocytogenes* were estimated using a spectrophotometer set at λ=420 nm according to the standard curves and were checked by plating duplicate serial suspension dilutions on CT-Smac + Amp and on

Palcam medium (Biokar Diagnostics) followed by incubation at 37 ± 1 ºC for 20-24 h and 48 ± 4 h, for *E. coli* O157:H7 and *L. monocytogenes* respectively.

Origin of samples

Organic and conventional 'Romaine' lettuce (*Lactuca sativa* var. longifolia) samples were obtained from a local supermarket in Lleida (Spain) on the day of the experiment. Organic lettuces were produced according the EU Regulation (CEE) 2092/91 and all organic fields were certified by competent national authorities.

Lettuce processing and storage

The outer leaves and core were removed and the remaining leaves were cut into pieces with a sharp knife. Afterwards, lettuce leaves were washed in water (10 min) and then were subjected to three different processing procedures designed to achieve different initial concentration levels of background microbiota (Figure 1):

I. Storage at 3 ± 1 ºC for 2 days and subsequent washing in tap water containing 100 ppm free chlorine (pH 6.5, 2 min) ('low' initial counts). Two lots were separated, one was inoculated by dipping in the pathogen suspension at 10^5 cfu/mL for 2 min and the other was left uninoculated (dip in tap water for 2 min) used as a control.

II. Storage at 3 ± 1 ºC for 2 days ('medium' initial counts). Two lots were separated, one was inoculated by dipping in the pathogen suspension at 10^5 cfu/mL for 2 min and the other was left uninoculated (dip in tap water for 2 min) used as a control.

III. Storage at 10 ± 1 ºC for 2 days ('high' initial counts). Two lots were separated, one was inoculated by dipping in the pathogen suspension at 10^5 cfu/mL for 2 min and the other was left uninoculated (dip in tap water for 2 min) used as a control.

Uninoculated and inoculated lettuce samples (15 ± 1 g) were packaged individually in polypropylene plastic film bags (12 cm x 20 cm) (Amcor Flexibles, Ledbury, Herefordshire UK; 35 µ in thickness, O_2 and CO_2 permeability of 1100 $cm^3/m^2/day/atm$ at 23 ± 1 ºC and water steam permeability of 0.9 $g/m^2/day$ at 25 ± 1 ºC and 75.0 % relative humidity). Packages were sealed with a sealing machine (Bag sealer, SK-410, Lovero) and stored at 10 ± 1 ºC for 8 days for subsequent evaluation of microbial growth.

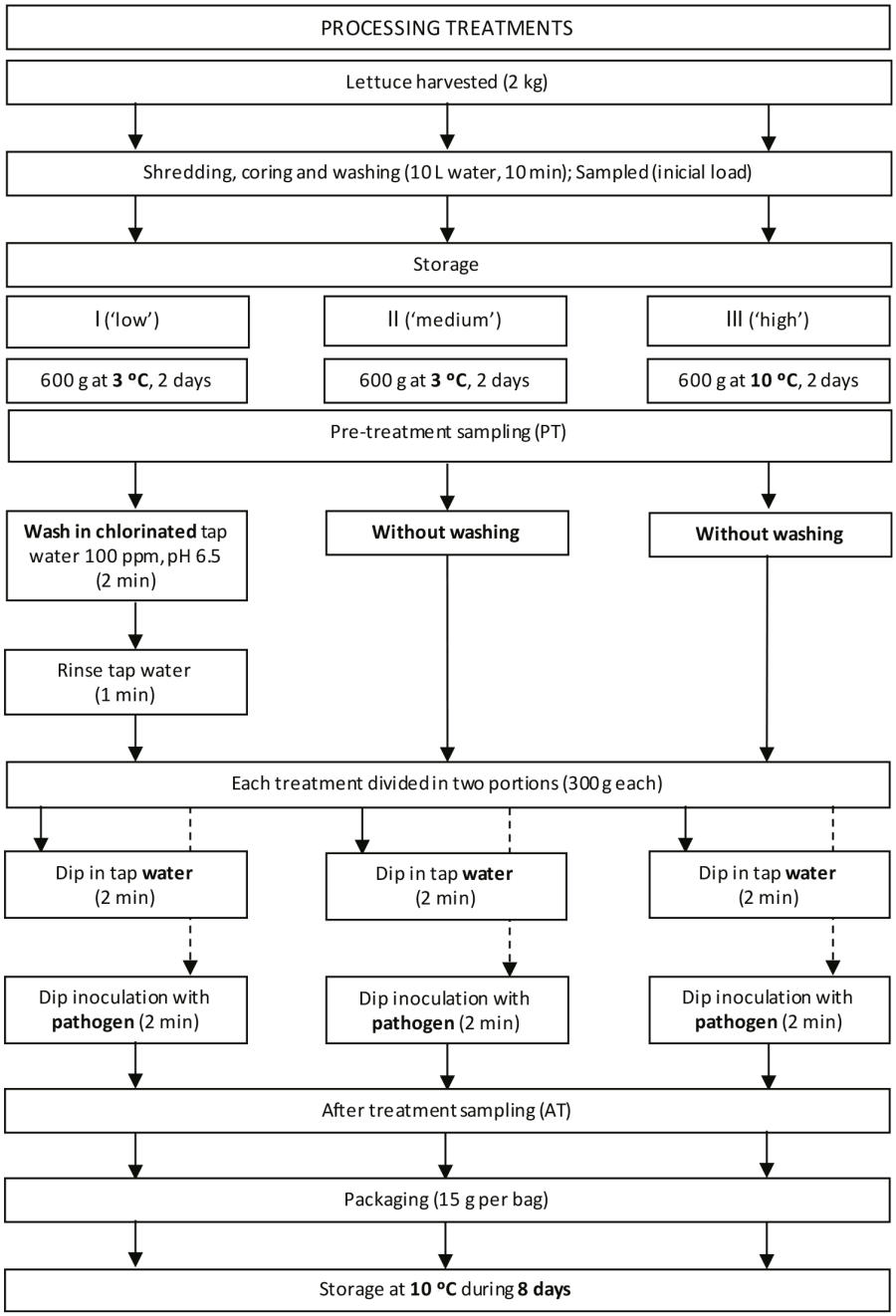

Figure 1. Flow diagram used in our study to obtain fresh-cut lettuce with different initial load.

Microbiological analysis

The background microbiota was enumerated on the day of arrival (initial load), after storage at different temperature (pre-treatment, PT) and immediately after washing treatments (after treatments, AT). After processing, packages (uninoculated and inoculated) were sampled on the day of inoculation (AT) and after 2, 5 and 8 days to determine the effects of washing and temperature on the microbial load. For sampling, 10 g of lettuce were mixed with 90 mL of buffered peptone water (Oxoid, CM1049) in a sterile stomacher bag and homogenized in a Stomacher 400 (Seward, London, UK) set at 230 rpm for 2 min. Further ten-fold dilutions were made using SP. For the inoculated samples, colonies of *E. coli* O157:H7 were enumerated by plating on CT-Smac + Amp and incubated at 37 ± 1 ºC for 24 ± 2 h. After incubation, fluorescent colonies were enumerated by examination with a UV light at 365 nm (CN-15 cabinet, Vilber Lourmat, France). *L. monocytogenes* colonies were enumerated on Palcam medium incubated at 37 ± 1 ºC for 48 ± 4 h. In the uninoculated samples, mesophilic microorganisms were determined by enumerating colonies on plates with PCA (Plate Count Agar, Biokar Diagnostics) and incubated at 30 ± 1 ºC for 3 days.

All analysis were carried out in triplicate packages for each microorganism and processing treatments.

Gas analysis inside lettuce bags

Throughout the experiment and prior to all analysis, O_2 and CO_2 contents of all lettuce packages were measured using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI-Dansensor, Denmark).

Statistical analysis

All experiments were replicated twice with three different lettuce bags per treatment/day for each type of lettuce origin (conventional or organic). Reported populations therefore represent the means of six values. Data were transformed to log cfu/g. The General Linear Model (GLM) procedure of the Statistical Analysis System (SAS Institute, version 9.2, Cary, NC, USA) was applied. Significant differences between treatments were analyzed by least significance difference (LSD) test at a significance level of $P<0.05$.

Results

Effects of background microbiota on L. monocytogenes *growth*

The initial load of mesophilic bacteria on conventional lettuce immediately after shredding without pre-washing was 5.3 log cfu/g (Figure 2A) and 6.2 log cfu/g on organic lettuce (Figure 2B). After storage at different temperature for 2 days (PT), significant differences were observed on growth of mesophilic bacteria ($P<0.05$). An increase of 1.6 log cfu/g at 10 ± 1 ºC and 0.8 log cfu/g at 3 ± 1 ºC was observed on conventional lettuce. Increases on organic lettuce were lower, 1.0 log cfu/g at 10 ± 1 ºC and 0.4 log cfu/g at 3 ± 1 ºC. After treatments, three different concentrations were observed for mesophilic bacteria ($P<0.05$). On conventional lettuce levels of 6.5, 5.5 and 4.7 log cfu/g for 'high', 'medium' and 'low' population, respectively, were observed. On organic lettuce, levels of 6.7, 6.1 and 5.3 log cfu/g for 'high', 'medium' and 'low' population, respectively, were observed. Populations of mesophilic bacteria increased during the storage period in both lettuce origins, reaching levels of about 8.0-8.4 log cfu/g after 8 days of storage. At that time, there were no significant differences on mesophilic population ($P>0.05$) between treatments.

From day 0 and throughout storage there were no significant differences ($P>0.05$) in *L. monocytogenes* populations on conventional lettuce with 'low', 'medium' or 'high' counts of background microbiota (Figure 2A). *L. monocytogenes* population increased from around 3.5 log cfu/g and reach at levels of 5.7-6.0 log cfu/g at the end of storage. Thus, varying the initial load of background microbiota had no effect on growth of *L. monocytogenes* on conventional shredded lettuce. However, there were significant differences on the behaviour of *L. monocytogenes* on shredded organic lettuce throughout storage period ($P<0.05$) depending on the initial load of background microbiota (Figure 2B). Populations of *L. monocytogenes* on the 'high' counts increased slightly from 3.8 to 5.0 log cfu/g after 8 days. Numbers on the 'medium' mesophilic counts were similar with levels ranging from 3.8 to 5.3 log cfu/g. On the contrary, when the initial mesophilic count was 'low', populations of *L. monocytogenes* increased rapidly with significant differences between the other treatments during storage period, reaching final population of 6.1 log cfu/g.

Effects of background microbiota on E. coli *O157:H7 growth*

The initial load of mesophilic bacteria on conventional lettuce as determined immediately after shredding without pre-washing was 5.3 log cfu/g (Figure 3A) and 6.0 log cfu/g on organic lettuce (Figure 3B). After storage at different temperature for 2 days (PT), significant differences were observed on growth of mesophilic bacteria ($P<0.05$). An increase of 1.3 log cfu/g at 10 ± 1 ºC and 0.3 log cfu/g at 3 ± 1 ºC was observed for mesophilic bacteria on conventional lettuce. The increase on organic lettuce was similar, 1.0 log cfu/g at 10 ± 1 ºC and 0.4 log cfu/g at

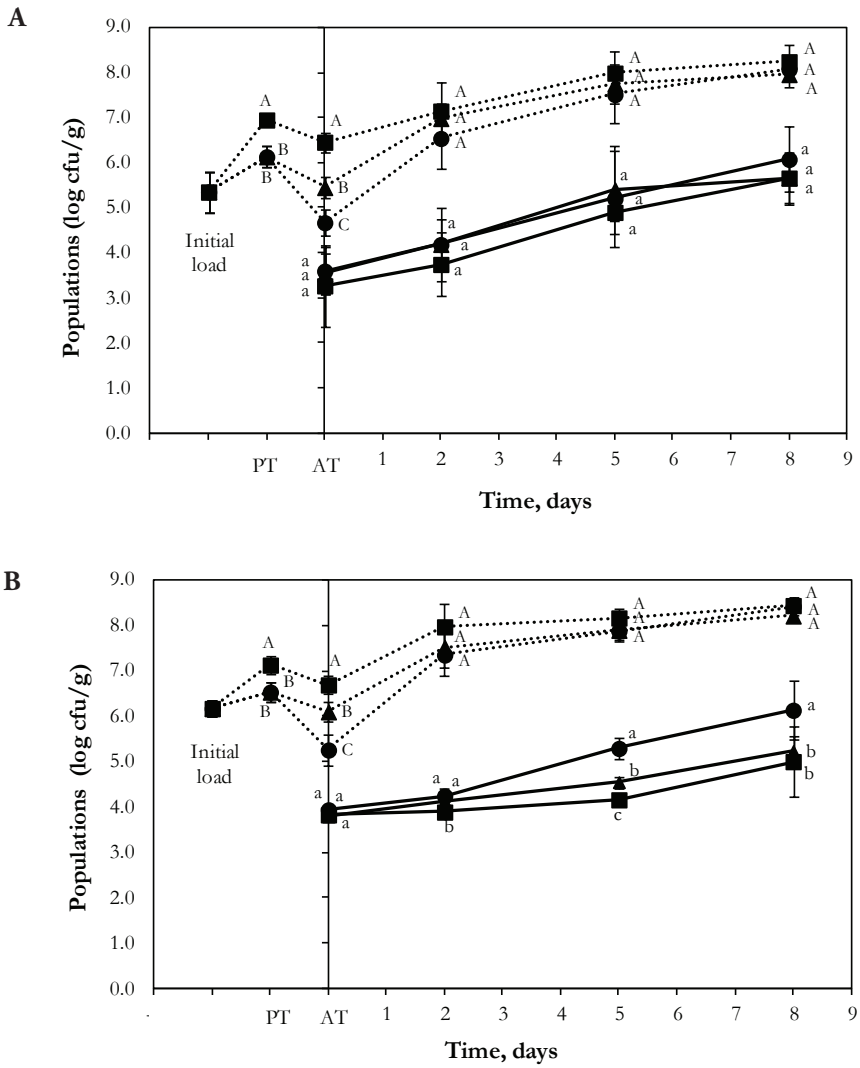

Figure 2. Populations of mesophilic bacteria (dotted line) and *L. monocytogenes* (continuous line) before treatment (BT), after treatment (AT) and its survival during storage at 10 ºC on shredded conventional (A) and organic (B) lettuce with 'high' (■), 'medium' (▲) or 'low' (●) initial counts of background microbiota. Data represent the mean of three determinations and two experiment repetitions. Bars represent standard deviation of the mean and where are not visible, they are smaller than symbol size. For each time, different letters indicate significant differences (P<0.05) among treatments.

3 ± 1 ºC. After treatments, three different concentrations were observed for mesophilic bacteria (P<0.05). On conventional lettuce levels of 6.6, 5.4 and 4.5 log cfu/g for 'high', 'medium' and 'low' population, respectively, were observed. On organic lettuce levels of 6.9, 5.8 and 5.5 log cfu/g for 'high', 'medium' and 'low' population were observed, respectively, with no significant differences between 'low' and 'medium' counts. Regardless the production system, the populations of mesophilic bacteria increased during storage period with significant differences reaching final populations of 8.2-8.5 log cfu/g on day 8 (P<0.05).

In the case of *E. coli* O157:H7 inoculated in conventionally produced lettuce, there were not significant differences in its counts among the treatments (P>0.05) after 5 days of storage. However, significant differences were observed at day 8 between the levels of the pathogen. *E. coli* O157:H7 population in the treatments with the 'medium' and 'low' initial mesophilic counts were higher than that observed with 'high' counts (P<0.05). The pathogen reached levels of 5.2 log cfu/g and 4.0 log cfu/g, respectively.

The behaviour of *E. coli* O157:H7 on shredded organic lettuce had no differences between treatments throughout storage period (P>0.05). Populations of *E. coli* O157:H7 remained around 4.0 log cfu/g among treatments until the end of storage period.

Evolution of package atmosphere

Concerning gases concentration within packages, lettuce samples exhibited a similar response to different lettuce production system and the inoculated pathogen. Therefore, data presented in Table 1 show an example to explain the gases behaviour inside lettuce packages during the storage period at 10 ± 1 ºC. In general, (for all samples) there was a rapid increase in CO_2 within the first 2 days and remained relatively stable until the end of the storage period, reaching values around 10.0 %. The O_2 concentration inside lettuce bags dropped rapidly during the first 5 days and more slowly until the end of storage, with final values around 1.0 %.

Table 1. Gas atmosphere concentration inside organic lettuce packages inoculated with *L. monocytogenes* during storage time

Gas	**Percentage (%) of gas at each storage day**			
	0	2	5	8
O_2	20.5-20.7	9.2-11.6	2.5-5.9	0.4-1.6
CO_2	0.5-0.7	6.9-8.1	7.8-9.7	8.6-9.9

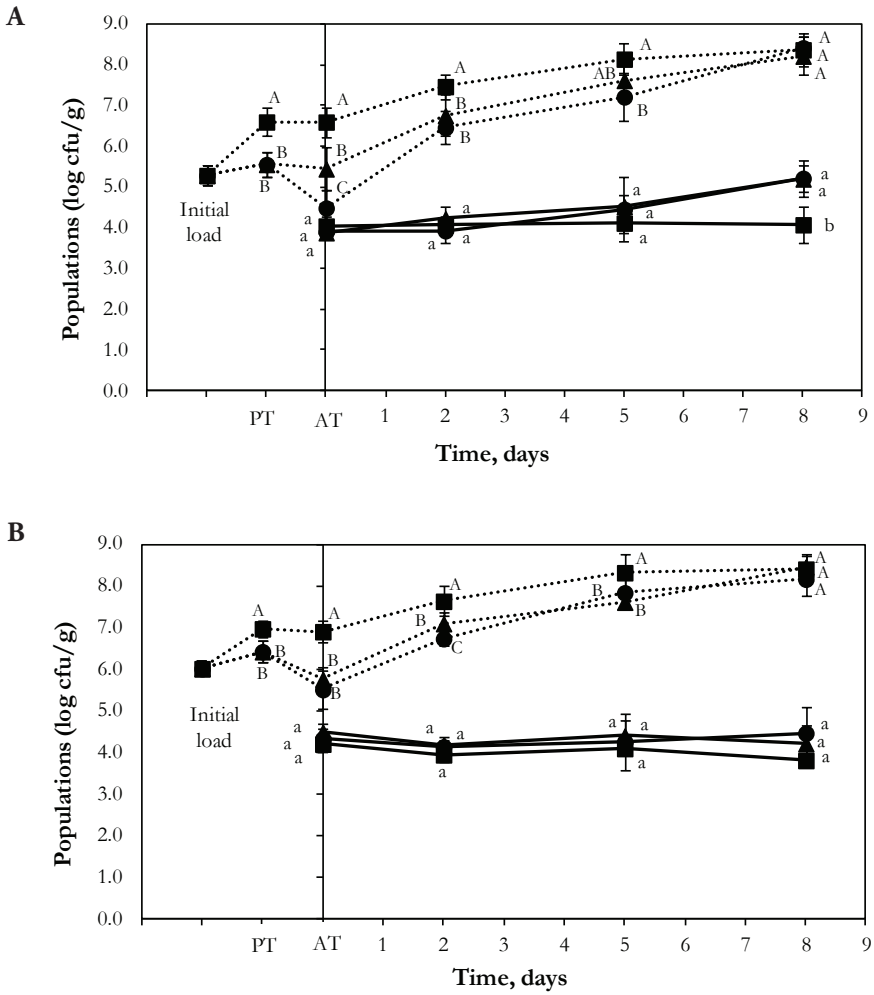

Figure 3. Populations of mesophilic bacteria (dashed line) and *E. coli* O157:H7 (continuous line) before treatment (BT), after treatment (AT) and its survival during storage at 10 ºC on shredded conventional (A) and organic (B) lettuce with 'high' (■), 'medium' (▲) or 'low' (●) initial counts of background microbiota. Data represent the mean of three determinations and two experiment repetitions. Bars represent standard deviation of the mean and where are not visible, they are smaller than symbol size. For each time, different letters indicate significant differences (P<0.05) among treatments.

Discussion

Various studies have shown that *L. monocytogenes* and *E. coli* O157:H7 can grow on lettuce and other leafy green vegetables (Francis and O'Beirne, 2001; Oliveira *et al.*, 2010a). However, the fate of pathogens in presence of different populations of natural microorganisms it is not well known. With the aim of investigating how natural background microbiota could influence pathogens survival, we determined the interactions between natural background microbiota of shredded conventional and organic 'Romaine' lettuce and *L. monocytogenes* and *E. coli* O157:H7. Different levels of indigenous microbiota were achieved by carrying out different pre-conditioning steps.

Our work reported here, shows that the pathogens behaved similarly on shredded conventional and organic lettuce during the first days despite the differences on the initial microbial load of lettuce. At the end of storage period *L. monocytogenes* seemed to be affected by the background microbiota only on organic lettuce and *E. coli* O157:H7 seemed to be affected only by the background microbiota in the conventional lettuce. A previous study demonstrated that the main difference between organic and conventional produced lettuce on the microbiological population was the *Enterobacteriaceae* counts (Oliveira *et al.*, 2010b). However, in this work, only total aerobic mesophilic counts were determined.

Regardless of initial mesophilic counts, *L. monocytogenes* grew on shredded lettuce at 10 ± 1 ºC while *E. coli* O157:H7 presented little increase at same temperature during storage period. Similar results were observed by Oliveira *et al.* (2010a) at 5 ºC on minimal processed lettuce. Conflicting reports exist in the literature about the relationship between human pathogens and background microbiota. Accordingly, Francis and O'Beirne (1998) observed that even with different size of the indigenous populations on lettuce, the survival and growth of *L. innocua* was not affected. On the contrary, previous work showed that *L. innocua* grew better on chlorine disinfected lettuce, and thus had a reduced background microbiota, than on lettuce washed in water (Francis and O'Beirne, 1997). Ready-to-use lettuce harbour a large and diverse populations of microorganisms and therefore it is not surprising to find some variability in results. This variability may also explain the results obtained in our study (Figure 2 and 3). The results presented here suggested that the behaviour of the human pathogens on lettuce is not simply related to the size of the background microbiota. Thus, genera of microorganisms, species or even particular strains present on lettuce may be more important than the overall initial population size of the background microbiota. These differences in species composition might explain the observed differences between organic and conventional lettuce for both pathogens. Cooley *et al.* (2006) investigated the interaction between *E. coli* O157:H7 and epiphytic bacteria in lettuce seedlings and reported that coinoculation with *Enterobacter asburiae* reduced survival while coinoculation with *Wausteria paucula* enhanced

survival of the pathogen. Francis and O'Beirne (1998) found that the growth of *L. innocua* was not affected by coinoculation with three different pseudomonads isolates in lettuce medium. However, in the same work, they found that pathogen survival and growth was reduced by two lactic acid bacteria and *Enterobacter* spp. The enhanced growth of pathogens on chlorinated washed lettuce presented in some works is possibly explained by removal or reduction of key competitive populations of the microbiota, for example lactic acid bacteria or *Enterobacter* spp. Nguyen-The and Carlin (1994) reported that faecal coliforms seemed to be sensitive to chlorine, they were not detected in 90.0 % of salad samples treated with 50 ppm free chlorine, whereas 70.0 % of untreated samples were positive.

Gas atmosphere within packages was modified, mainly as a result of the respiration of the vegetables, during which O_2 is consumed to aid in the breakdown of complex stored organic materials to organic acids and then to simpler compounds such as carbon dioxide and water. Our results showed a similar response to different pre-conditioning treatments and production system with respect to O_2 consumed and CO_2 produced inside lettuce packages during storage at 10 ºC. It is assumed that the gas changes inside bags are more likely caused by processing, temperature and packaging than individual treatments. The package material used in this work was tested in previous work (Oliveira *et al.*, 2010a) and showed that pathogens growth was not inhibited by gas mixtures at 5 ºC. Thus, the low O_2 and high CO_2 levels seemed not to be a barrier to pathogens growth on our lettuce packages.

Pre-treatments used in this study changed the initial load of natural background microbiota on conventional and organic lettuce, but only affected the survival/growth of *L. monocytogenes* and *E. coli* O157:H7 inoculated onto organic and conventional lettuce, respectively at the end of storage period at 10 ºC. According the results presented in this study, it is essential to prevent contamination and bacterial growth in order to maintain the quality of ready-to-eat vegetables that still depends on the good hygiene practices during growth and processing from field to consumer, effective washing and decontamination, strict temperature and appropriate packaging. Moreover, the effect of individual/key competitive strains should be evaluated for controlling foodborne pathogens growth.

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Chapter VI

Biopreservation as a technology to enhance microbial safety on fresh-cut lettuce

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Abstract

Fruits and vegetables can become contaminated by foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes*, and it has been demonstrated that current industrial sanitizing treatments do not eliminate the pathogens when present. Chemical control is widely used, but biological control appears to be a better solution, mainly using the native microbiota present on fresh produce.

The first objective of this study was to isolate native microbiota from whole and fresh-cut produce and to determine whether these bacteria were antagonistic towards foodborne pathogens. A total of 112 putative antagonist isolates were screened for their ability to inhibit the growth of *S. enterica* on lettuce discs. Fifth different genera reduced *S. enterica* growth more than 1-log unit at 20 ºC for 3 days. When tested against *L. monocytogenes* 230/3, only *Pseudomonas* sp. strain M309 (M309) was able to reduce pathogen counts more than 1-log unit. Therefore, M309 strain was selected to be tested on lettuce discs at 10 ºC against *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes*. M309 strain was only able to reduce *S. enterica* and *E. coli* O157:H7 populations.

The second objective was to test different biopreservative methods including M309 strain, *P. graminis* CPA-7 (CPA-7), bacteriophages (Listex P100 and Salmonelex) and nisin at conditions simulating commercial applications against *Salmonella* and *L. monocytogenes* on fresh-cut lettuce. The addition of the biopreservative agents did not result in a significant reduction of *Salmonella* population. However, CPA-7 strain together with nisin reduced *L. monocytogenes* population after 6 days of storage at 10 ºC. The cocktail of *Salmonella* and *L. monocytogenes* was not markedly inactivated by the respective bacteriophage solutions. This study highlighted the potential of biocontrol but the combination with other technologies may be required to improve their application on fresh-cut lettuce.

Keywords

Foodborne pathogens; Native microbiota; Biopreservative agents; Lettuce.

Introduction

The increase in fresh fruit and vegetable consumption has led to an increase in foodborne outbreaks associated with their consumption. A number of outbreaks associated with consumption of lettuce contaminated with *L. monocytogenes* (Francis *et al.*, 1999; Sagoo *et al.*, 2003), *Salmonella* spp. (Crook *et al.*, 2003; Horby *et al.*, 2003; Takkinen *et al.*, 2005) and *E. coli* O157:H7 (Ethelberg *et al.*, 2010; Friesema *et al.*, 2007, 2008) have been reported.

Minimally processed vegetables can be contaminated by pathogens and there is no kill step involved in the processing of these vegetables, therefore the need for intervention methods to maintain the safety of minimally processed produce is very important. A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce initial bacterial populations on minimally processed produce (Beuchat, 1998). Chlorine is the most widely used sanitizer in the fresh produce industry. However, studies indicate that chlorine concentrations traditionally used (50-200 ppm) are not effective in reducing pathogen load on fresh-cut produce (Behrsing *et al.*, 2000; Delaquis *et al.*, 2002; Lee and Baek, 2008). Moreover, a prolonged exposure to chlorine vapor may cause adverse effects to the workers, may affect the quality of foods and also adversely affect the environment (Beuchat, 1998). Thus, there is a need for better, safer and more environmental friendly methods to reduce the contamination. Therefore, it is desirable to preserve foods by natural means.

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 microbiota present on the surface of fresh produce can play an important role as they compete with the 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). Several antagonistic microorganisms have been used to inhibit the growth of foodborne pathogens (FBP) (Janisiewicz *et al.*, 1999; Leverentz *et al.*, 2006). Recently, a strain of *Pseudomonas graminis* CPA-7 has been used to prevent the growth of FBP in fresh-cut apples (Alegre *et al.*, 2013a, b) and melon (Abadias *et al.*, 2014).

The bacteriocin nisin actually has GRAS (generally recognized as safe) status by FAO (Food and Agriculture Organization of the United Nations), WHO (World Health Organization) as well as the FDA (USA Food and Drug Administration). In the European Union (EU) it is an approved preservative additive for use in certain foods (E-234). Nisin and other bacteriocins produced by lactic acid bacteria (LAB) have received a great deal of attention because they are produced by bacteria largely considered beneficial to human health and to food production. The use of nisin as a biopreservative has been widely investigated in a large variety of fresh and processed foods. Concerning vegetables, Allende *et al.* (2007) and Randazzo *et al.* (2009) eval-

uated the effect of bacteriocin-containing washing solutions on survival of *L. monocytogenes* in fresh-cut lettuce at refrigerated temperatures.

Bacteriophage (phage) prophylaxis is also a possible natural method to be used as a biopreservative. Phages are bacterial viruses that invade specific bacterial cells, disrupt bacterial metabolism, and cause the bacterium to lyse without compromising the viability of other flora in the habitat. They are the most abundant microorganisms in our environment (Brussow and Hendrix, 2002) and are present in high numbers in water and foods (Hsu *et al.*, 2002; Kennedy *et al.*, 1986). Promising results using phage biocontrol have been reported for several pathogens, including *Salmonella* spp. (Guenther *et al.*, 2012; Kocharunchitt *et al.*, 2009; Leverentz *et al.*, 2001), *L. monocytogenes* (Carlton *et al.*, 2005; Dykes and Moorhead, 2002; Guenther *et al.*, 2009; Leverentz *et al.*, 2003; Oliveira *et al.*, 2014) and *E. coli* O157:H7 (Abuladze *et al.*, 2008; Sharma *et al.*, 2009; Viazis *et al.*, 2011). There are several commercialized phage preparations, such as ListShield™ and EcoShield™ (Intralytix, Inc., USA), Agriphage™ (Omnilytics, Inc., USA), Listex™ P100 and Salmonellex™ (Micreos Food Safety, The Netherlands).

The aim of this study was to evaluate native microorganisms from fresh and fresh-cut fruits and vegetables for potential inhibitory effect against FBP on lettuce. The best antagonist together with *P. graminis* CPA-7, nisin and two commercial phage preparations (Listex P100 and Salmonellex) were tested as potential biopreservative agents on minimally processed lettuce under simulated commercial conditions.

Material and methods

Isolation of putative antagonists

Putative antagonists were isolated from whole vegetables, fresh-cut fruit and vegetables and sprouts. Samples of whole vegetables, fresh-cut vegetables and sprouts were purchased from different supermarkets in Lleida (Spain). Fresh-cut fruit samples were obtained from vending machines. For whole products, the 3-4 outer leaves were discarded and the inner portion was cut up in the laboratory.

Twenty-five grams of each sample were diluted in 225 mL of saline peptone solution (SP, 8.5 g/L NaCl and 1.0 g/L peptone) and homogenized for 2 min at normal speed in a Stomacher (Model 400 Circulator, Seward). Serial dilutions of the suspension were made in SP and plated on the following different media: Man, Rogosa and Sharpe medium (MRS, Biokar Diagnostics, Beauvais, France) for LAB isolation and Nutrient Agar (NA, Biokar Diagnostics) to isolate psychrotrophic microorganisms. Plates were incubated at 30 ºC for 3 days and at 7 ºC for 10 days, respectively. Colonies of different morphologies were selected and isolated.

A collection of antagonists (bacteria and yeasts) belonging to the Pathology Laboratory collection (IRTA, Lleida), which have demonstrated efficacy in reducing fungal postharvest diseases, was also tested.

In vivo assay of antagonistic activity

Lettuce preparation

'Romaine' lettuce (*Lactuca sativa* var. longifolia) was obtained from a local supermarket. The outer or damaged leaves of lettuce were removed and discarded. Leaf discs (2.3 cm in diameter) were cut using an aseptic cork borer and placed in commercial 500 mL food plastic bowls. The covers on the plastic bowls allowed sufficient air exchange to prevent modified atmosphere creation.

Microorganisms and inoculum preparation

A total of one hundred and twelve microorganisms were tested for its potential antagonistic activity. *Salmonella enterica* subsp. *enterica* (Smith) Weldin serotype Michigan (BAA-709, ATCC) was adapted to grow on Tryptone Soy Agar (TSA, Oxoid, UK) supplemented with 100 µg/mL of streptomycin sulphate salt (TSA-St), thereby enabling their detection on selective medium in the presence of the antagonists and the native microbiota associated with lettuce. *S. enterica* was grown in Tryptone Soy Broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) for 20-24 h at 37 ºC. Five strains of *L. monocytogenes*: serovar 1a (CECT 4031), serovar 3a (CECT 933), serovar 4a (CECT 940), serovar 4b (CECT 4032) and serovar 1/2a (LM230/3); and a non-pathogenic strain of green fluorescent protein (GFP)-expressing and ampicillin resistant *Escherichia coli* O157:H7 (B6-914 GFP-91) (Fratamico *et al.*, 1997) were also used. *L. monocytogenes* strains were grown individually on TSA supplemented with 6.0 g/L yeast extract, 2.5 g/L glucose and 2.5 g/L dipotassium hydrogen phosphate (TYSEA) at 37 ºC for 20-24 h. Each strain was transferred to TSB supplemented with 6.0 g/L yeast extract (TYSEB) at 37 ºC for 20-24 h. *E. coli* O157:H7 was grown on Sorbitol MacConkey agar supplemented with Cefixime and Tellurite (CT-Smac, Biokar Diagnostic) and 50 mg/mL Ampicillin (ampicillin sodium salt, Sigma, St. Louis, USA) (CT-Smac + Amp) at 37 ºC for 20-24 h. A single colony was transferred into a flask with TSB supplemented with 50 mg/mL Ampicillin (TSB + Amp) at 37 ºC for 20-24 h.

The cultures were harvested by centrifugation at 9820 x g for 10 min at 10 ºC and resuspended in a sterile 8.5 g/L NaCl solution (SS).

The antagonists were grown in plates as previously described and the colonies were scraped from the medium, and a suspension of 30 ± 5 % transmittance (λ=420 nm) was prepared in 5 mL of sterile deionized water. For the inocula preparation, a volume of the FBP concentrated suspension was added to the 30 %-transmit-

tance antagonist suspension to obtain a pathogen concentration of approximately 1 x 10^7 cfu/mL.

Inoculation and microbiological analyses

Twenty five microliters of antagonist and pathogen suspension were pipetted onto lettuce discs in individual small drops, and the plastic bowls were stored at 20 ºC for 3 days. The control treatments consisted of the pathogen suspension without the antagonist. To recover the pathogen, five lettuce discs were placed into a sterile plastic bag (Bagpage 80 mL, Interscience BagSystem, St Nom La Breteche, France), and 9 mL of SP were added. The sample was homogenized in a stomacher and blended for 120 s at a high speed (Bagmixer 100 Minimix, Interscience). Aliquots of this mixture were serially diluted and spread onto the specific media for each pathogen. The plates were incubated at 37 ºC for 20-24 h. Three replicates were assessed per treatment and sampling time.

Experimental design

To evaluate the antagonistic activity, the population sizes of the pathogen inoculated alone or in the presence of the putative antagonist after storage were compared. The reduction in the numbers of the studied FBP was calculated as follows:

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

where N_{FBP} is the size of the FBP population in the control treatment (FBP alone, cfu/g) after the storage period and $N_{FBP+Ant}$ is the size of the FBP population after the storage period in the presence of the antagonist (cfu/g).

All the putative antagonists were first tested against *S. enterica* and when the pathogen population size in the presence of the putative antagonist was reduced by more than 1-log unit, the assay was repeated again to study the consistency of the results. On the contrary, when the reduction of the pathogen was less than 1-log unit, the microorganism was rejected. Moreover, when a microorganism reduced the size of the *S. enterica* population by more than 1.0-log unit in two consecutive screenings, it was tested twice against *L. monocytogenes* 230/3.

Of all tested isolates, one inhibited both FBP growth; therefore, it was selected to be tested under refrigeration conditions against *E. coli* O157:H7, *S. enterica* and the cocktail of *L. monocytogenes*. In these assays, the lettuce discs were stored at 10 ºC, and FBP were recovered after 3, 6 and 9 days.

Identification of putative antagonist microorganisms

The microorganisms that reduced *S. enterica* and *L. monocytogenes* population more than 1-log unit (Table 1) were identified by the sequence of the 16S rDNA. The M309 strain that presented the best antagonistic activity was also phenotypically characterized.

Table 1. *Salmonella* and *L. monocytogenes* reductions on lettuce discs after 3 days at 20 °C (log cfu/g)

	Putative antagonists strains							
	M247	**CPA-2**	**M230**	**M290**	**M201**	**M309**	**PN6**	**M308**
Salmonella	1.0	1.2	1.4	1.2	1.1	3.3	1.3	1.1
L. monocytogenes	0.5	0.4	0.6	0.7	0.5	1.8	0.4	0.6

Phenotypic characterization of *Pseudomonas* sp. strain M309

Gram stain, catalase, oxidase and kligler iron agar test (for glucose and lactose fermentation and hydrogen sulfide production) were performed before the API 20NE (bioMérieux, Marcy-l'Etoile, France) was used. Catalase activity was determined by assessing the bubble production of the bacteria in 3.0 % v/v H_2O_2, and oxidase activity was tested using oxidase reagent (bioMérieux) according to the manufacturer instructions.

Biopreservation of Salmonella spp. *and* L. monocytogenes *on minimally processed lettuce under simulated commercial conditions*

Lettuce preparation

'Romaine' lettuce (*Lactuca sativa* var. longifolia) was obtained from a local supermarket. The outer or damaged leaves as well as the core of the lettuce were removed and discarded. Inner leaves were hand-cut into pieces using a sharp knife.

Pathogens and preparation of inoculum

Three strains of *L. monocytogenes*: serovar 1a (CECT 4031), serovar 4a (CECT 940) and serovar 4b (CECT 4032) and six strains of *Salmonella*: *S. enterica* Michigan (ATCC BAA-709), *S. enterica* Agona (ATCC BAA-707), *S. enterica* Montevideo (ATCC BAA-710), *S. enterica* Gaminara (ATCC BAA-711), *S. enterica* Typhi-

murium DT104 and *S. enterica* Enteritidis (CECT 4300) were used in this assay. *L. monocytogenes* and *Salmonella* strains were grown individually in TYSEB and TSB at 37 ºC, respectively as described previously. Equal volumes of each strain were combined to obtain the three strain cocktail for *L. monocytogenes* and six strain cocktail for *Salmonella*. Subsequently, the cells were harvested by centrifugation and the concentrated suspensions were obtained as described previously. The concentration of the pathogens was confirmed by plating duplicate serial suspension dilutions on Palcam medium (Palcam Agar Base with selective supplement, Biokar Diagnostics) and incubated at 37 ºC for 48 h for *L. monocytogenes* and on XLD agar medium (Xylose Lysine Deoxycholate Agar, Oxoid) followed by incubation at 37 ºC for 24 h for *Salmonella* spp.

Antagonists

The strain M309 previously isolated in this study and the *P. graminis* CPA-7 that was isolated from apple surface in our laboratory (Alegre *et al.*, 2013b) were used as putative antagonist microorganisms. Both strains were selected because they demonstrated an antagonistic effect against FBP. The selected microorganisms were grown in TSB for 20-24 h at 30 ºC. Bacterial cells were harvested by centrifugation and resuspended in sterile distilled water.

Phages

The bacteriophages Listex P100 and Salmonelex (Micreos Food Safety, The Netherlands), characterized by its broad spectrum toward *L. monocytogenes* and *Salmonella* strains, respectively, were used in this assay. The concentration of the phage preparations was approximately 10^{11} plaque forming units (pfu)/mL in buffered saline and were maintained at 5 ºC.

Nisin

The bacteriocin nisin was obtained from Sigma (Sigma-Aldrich, St. Louis, Mo.). A solution containing 400 IU/mL of nisin was prepared immediately before the experiment.

Application of the treatments

The shredded lettuce (approximately 1000 g) was inoculated by dipping into a 10 L pathogen suspension at 10^3 cfu/mL for 2 min at 150 rpm. Inoculated lettuce was left to dry for 30 min and was stored at 10 ºC for one day. The actual concentration of each pathogen in the dip tank was determined by plating on the selective media.

The inoculated lettuce leaves were divided into 120-g samples and transferred to 5-L beakers before the application of the different treatments. Samples of inoculated lettuce were separately immersed in 3 L of biopreservative solutions: phage (Listex or Salmonelex, 10^8 pfu/mL), nisin (400 IU/mL), M309 and CPA-7 strains (10^7 cfu/mL) for 1 min at 150 rpm. Sodium hypochlorite (SH, 100 ppm) and deionized water (DW) were used as a control treatments. Nisin was only used to wash lettuce contaminated with *L. monocytogenes*. After treatment, the lettuce samples were drained off and left to dry for 30 min. The concentration of each pathogen on lettuce was determined both before and after the treatment.

Treated samples (15 ± 1 g) were packaged individually in polypropylene plastic film bags (12 cm x 12 cm) (PP110, ILPRA Systems Espanya SL, Mataró, Espanya; O_2 and CO_2 permeability of 110 cm^3/m^2/day/bar and 500 cm^3/m^2/day/bar respectively, at 23 ºC and 0 % relative humidity). Bags were sealed with a sealing machine (Bag sealer, SK-410, Lovero) and stored at 10 ºC for 6 days for subsequent microbial evaluation.

Microbiological analyses

Populations of *L. monocytogenes* and *Salmonella* spp. were determined in three sample bags at each time. The lettuce samples from each treatment were examined on the day of treatment (d0) and after 6 days at 10 ºC. For the analysis, 10 g of lettuce from each bag were mixed with 90 or 45 mL of buffered peptone water (depending on the sampling day) in sterile Stomacher bag and homogenized in a Stomacher 400 (Seward, London, UK) set at normal speed for 2 min. Serial dilutions (1:10) of each sample were prepared using SP and enumerated by plating on Palcam medium and incubated at 37 ºC for 48 h for *L. monocytogenes*; *Salmonella* spp. samples were enumerated on XLD and incubated at 37 ºC for 24 h.

When the pathogen was not detected by direct plating, lettuce samples were incubated for 24 h at 37 ºC for non-selective pre-enrichment. Afterwards, selective enrichment steps were done according to ISO 11290-1:1996/A1:2004 for *L. monocytogenes* detection and ISO 6579:2002/A1:2007 for *Salmonella* spp. detection. In samples that were positive after enrichment, an arbitrary value of 0.38 log cfu/g (half of detection limit) was assigned for data representation. Data were transformed to log cfu/g of lettuce.

Gas analysis inside lettuce bags

Throughout the experiment and prior to all analysis, O_2 and CO_2 contents of all lettuce packages were measured using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI-Dansensor, Denmark).

Statistical analysis

All experiments were replicated twice with three different replications per treatment/day. Therefore, reported populations represent the mean of six values. The bacterial data were transformed to log units. Pathogen population recovered from lettuce treated with different solutions was subjected to Statistical software JMP (SAS Institute, version 9.2, Cary, NC, USA). Significant differences between treatments were analyzed by Tukey test at a significance level of $P < 0.05$.

Results

In vivo assay of antagonistic activity

The inhibitory potential of 112 microorganisms was tested against *S. enterica* on lettuce discs after storage at 20 ºC for 3 days. *S. enterica* population increased approximately 1.2-1.8 log cfu/g when inoculated alone after 3 days at 20 ºC (data not shown). Of the 112 microorganisms tested, 104 (92.9 %) did not have any effect on the numbers of *S. enterica* (reduction <1.0-log units compared with the control, *S. enterica* inoculated alone, data not shown), and therefore they were rejected. Eight isolates designated as strain M247, CPA-2, M230, M290, M201, M309, PN6 and M308, reduced the number of *S. enterica* on lettuce discs at least 1.0-log units (Table 1). These eight isolates with demonstrated inhibitory effect were identified as members of the genera *Pantoea*, *Pseudomonas*, *Serratia, Enterobacter* and *Hafnia* (Table 2). Among them, *Pseudomonas* sp. strain M309 (M309) reduced the number of *S. enterica* by 3.3-log units. Those microorganisms that were effective in reducing the size of the *S. enterica* population were selected to be tested against *L. monocytogenes* 230/3 at 20 ºC. The reduction in *L. monocytogenes* population by the antagonists was, in general, lower than that of *Salmonella* (Table 1). Of the eight microorganisms tested, only M309 strain had effect against *L. monocytogenes* 230/3, which reduced approximately 1.8-log units *L. monocytogenes* population. This antagonist was selected to continue the studies under refrigeration temperature (10 ºC) and was tested against *S. enterica*, *E. coli* O157:H7 and cocktail of *L. monocytogenes* on lettuce discs (Figure 1).

Table 2. Identification results of the best microorganisms that reduced *S. enterica* and *L. monocytogenes* population by the sequence of the 16S rDNA

Genus/species	Strain	Source of isolation
Pantoea sp.	M247	Lettuce
Pantoea agglomerans	CPA-2	Apple
Pseudomonas trivialis	M230	Fresh-cut Iceberg lettuce
Serratia sp.	M290	Iceberg lettuce
Hafnia alvei	M201	Lettuce
Pseudomonas sp.	M309	'Fulla Roure' lettuce
Pantoea agglomerans	PN6	'Golden' Apple
Enterobacter sp.	M308	Fresh-cut Iceberg lettuce

Initial *S. enterica* population was 6.9 log cfu/g and increased up to 7.5 log cfu/g after 9 days of storage when inoculated alone (data not shown). When it was co-inoculated with M309 strain, *S. enterica* population was increasingly reduced during the experiment and the reduction was more than 3.5-log units when compared to the pathogen inoculated alone after 9 days of storage (Figure 1).

Initial *E. coli* O157:H7 population was 6.5 log cfu/g and it also slowly increased (7.3 log cfu/g) when inoculated alone until the end of storage (data not shown). When it was co-inoculated with the antagonistic strain, a large population reduction was observed (3.7-log units) after 9 days of storage (Figure 1).

Initial *L. monocytogenes* population was 6.7 log cfu/g and did not grow during storage period and reached at levels of about 6.6 log cfu/g at the end of 9 days (data not shown). Co-inoculation with M309 strain did not have significant effect on *L. monocytogenes* counts on lettuce discs stored at 10 ºC and only a 0.9-log units reduction was observed at the end of storage (Figure 1).

Of all the isolates tested, M309 strain presented the highest inhibitory activity against FBP and was selected for further studies together with other preservative treatments in fresh-cut lettuce.

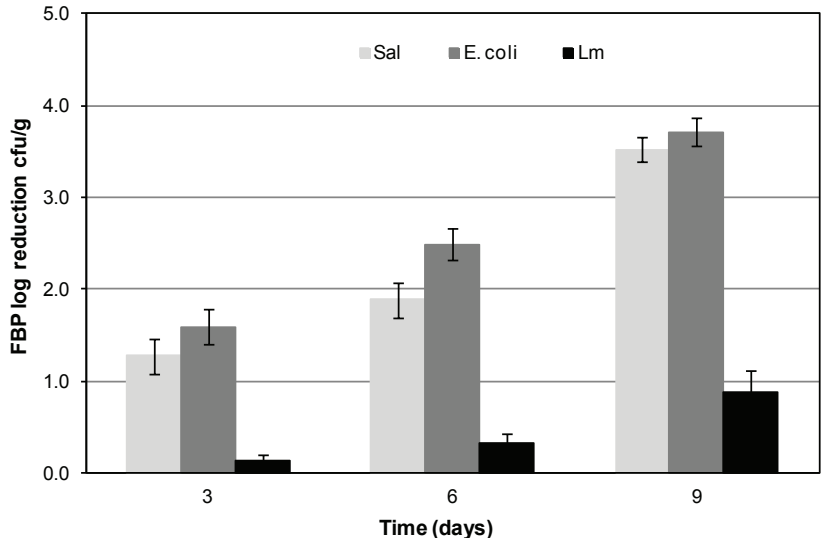

Figure 1. Population reductions (log cfu/g) of *Salmonella* (Sal), *E. coli* O157:H7 (*E. coli*) and *L. monocytogenes* (Lm) co-inocolated with *Pseudomonas* sp. strain M309 on lettuce discs at 10 ºC during 9 days. Data represent the mean of six values and vertical bars indicate the standard deviation of the mean.

Biopreservation of Salmonella spp. *and* L. monocytogenes *on minimally processed lettuce under simulated commercial conditions*

Survival and growth of FBP on fresh-cut lettuce treated with DW, SH, phages (Listex or Salmonelex), nisin, strain M309 or CPA-7 and then stored at 10 ºC up to 6 days were compared.

In this assay and regardless of the treatment, *Salmonella* spp. populations on fresh-cut lettuce tended to decrease while *L. monocytogenes* populations tended to increase in all treatments tested throughout storage at 10 ºC (Figure 2).

DW wash did not significantly reduced FBP population when compared with the initial load. However, SH treatment significantly reduced initial *Salmonella* spp. populations approximately 1.0-log unit (Figure 2A) and *L. monocytogenes* about 1.8-log units (Figure 2B). Nevertheless, after storage both pathogens behaved as in water-treated lettuce, where the final population was approximately 0.5 and 3.7 log cfu/g for *Salmonella* spp. and *L. monocytogenes*, respectively.

Initial *Salmonella* spp. population was also significantly reduced by phage Salmonelex treatment. Population reduction with Salmonelex treatment was similar to the

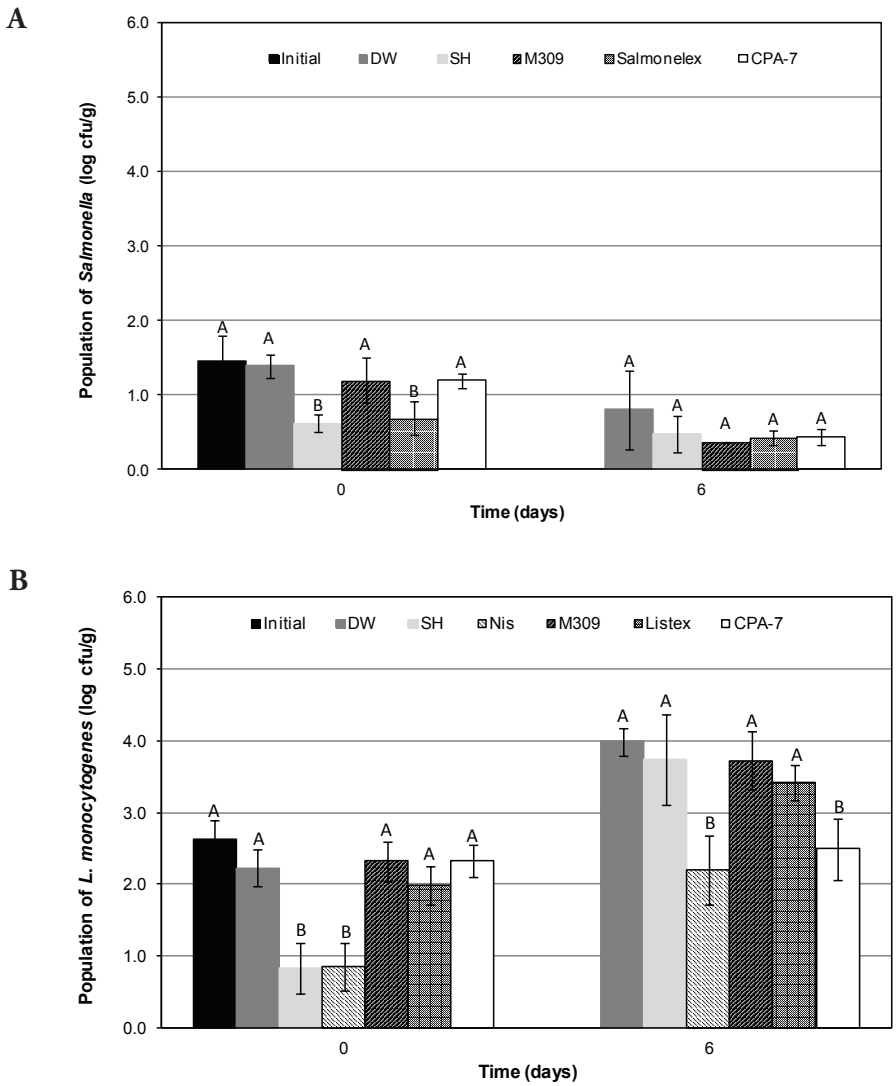

Figure 2. *Salmonella* (A) and *L. monocytogenes* (B) populations (log cfu/g) on fresh-cut lettuce stored under MAP conditions at 10 °C for 6 days. Columns represent the growth of pathogen before treatments (initial) and after the different treatments applied, water (DW), sodium hypochlorite (SH), *Pseudomonas* sp. strain M309 (M309), bacteriophage (Listex or Salmonelex), *P. graminis* CPA-7 (CPA-7) and nisin (nis). Data represent the mean of six values and vertical bars indicate the standard deviation of the mean. For each time, different letters indicate significant differences ($P < 0.05$) among treatments.

reduction obtained by SH and significantly higher than reductions obtained with DW. M309 and CPA-7 strains did not significantly reduced *Salmonella* spp. initial population. *Salmonella* spp. populations decreased on lettuce treated regardless of washing treatment throughout storage at 10 ºC and, although the highest final population (about 0.8 log cfu/g) was observed on DW-treated lettuce, it was not significantly different from the other treated lettuce samples.

Initial *L. monocytogenes* population was significantly reduced by nisin wash at the same levels as the SH treatment. Nisin treatment caused approximately 1.8-log units reduction on pathogen populations. No significant reductions were observed with the other treatments (phage Listex, M309 and CPA-7 strains). After storage period, *L. monocytogenes* population increased regardless of the treatment applied. Bacterial populations were significantly lower on lettuce treated with nisin and CPA-7 strain than those observed with the other treatments. The reduction of pathogen compared with DW was between 1.8 and 1.5-log units, reaching populations close to 2.2 and 2.5 log cfu/g after 6 days at 10 ºC, respectively. There was no significant effect in *L. monocytogenes* populations on lettuce treated with phage Listex and M309 strain during storage period and counts were around 3.4 and 3.7 log cfu/g, respectively.

Evolution of package atmosphere

Concerning gases concentration within packages, lettuce samples exhibited a similar response to different treatments applied and the inoculated pathogen (data not shown). In general, (for all samples) there was an increase in CO_2 levels until the end of the storage period, reaching values around 15.0 %. The O_2 concentration dropped rapidly with final values between 2.0 to 4.0 %.

Discussion

Naturally occurring microorganisms on foods have been recognized as having inhibitory activity against some foodborne pathogens (Alegre *et al.*, 2013a, b; Jablasone *et al.*, 2005; Liao and Fett 2001; Schuenzel and Harrison 2002). However, conflicting reports exist in the literature about the relationship between human pathogens and native microbiota. Cooley *et al.* (2006) studied the interaction between *E. coli* O157:H7 and epiphytic bacteria in lettuce seedlings and reported that co-inoculation with *Enterobacter asburiae* reduced survival while co-inoculation with *Wausteria paucula* enhanced survival of the pathogen. Francis and O'Beirne (1998) found that the growth of *L. innocua* was not affected by co-inoculation with three different pseudomonads isolates in lettuce medium. However, Liao and Fett (2001) identified a number of native microorganisms that exhibited antagonistic effect against *E. coli* O157:H7.

Accordingly, we also observed different levels of efficacy on the antagonists tested. In this study, 112 microorganisms were tested against *S. enterica* on lettuce discs stored at 20 ºC but only eight isolates reduced the number of the pathogen at least 1.0-log units. Of the 8 microorganisms tested, only one had effect against *L. monocytogenes*. This microorganism that was identified as *Pseudomonas* sp. (strain M309) presented different antagonistic effect against different pathogens at both 20 and 10 ºC. When M309 strain was tested under refrigerated temperature (10 ºC) also showed high efficacy to control the *S. enterica* and *E. coli* O157:H7 growth on lettuce discs. However, M309 strain did not have significant effect on the growth of *L. monocytogenes* during storage period.

Previous studies have also reported the effectiveness of microorganisms as bioprotective agents. Wei *et al.* (2006) demonstrated that the 'Romaine' lettuce isolate *Pseudomonas putida* LTH 5878, reduced cell counts of *Salmonella* Typhimurium and *L. innocua* on shredded lettuce stored at 4 ºC for 8 days. They also observed that the antagonistic activity of *Pseudomonas* was stronger against *Salmonella* than *L. innocua*. Trias *et al.* (2008) found five LAB strains that shown efficacy in inhibiting *L. monocytogenes* and *S.* Thyphimurium on lettuce cuts and 'Golden Delicious' apple wounds stored at 25 ºC for 96 h. All LAB strains tested showed little effect over *E. coli* on both lettuce and apple samples.

Due to high effectiveness observed on lettuce discs, M309 strain was selected for further assays on fresh-cut lettuce stored under MAP at 10 ºC and was compared with other biopreservative agents such as nisin, bacteriophages and *P. graminis* CPA-7.

The addition of the biopreservative agents did not result in a significant *Salmonella* and *L. monocytogenes* population reduction at conditions simulating commercial application. We have observed that efficacy of M309 strain decreased when tested under simulated commercial conditions. This could be attributed to the inoculation method (spot or dip inoculation) and the decrease in O_2 and increase in CO_2 concentration observed inside the packages as the *Pseudomonas* spp. are strict aerobic microorganisms.

P. graminis CPA-7 strain, which was isolated from 'Golden Delicious' apples, reduced around 2.0 log cfu/g *L. monocytogenes* population on apples plugs after 7 days at 10 ºC (Alegre *et al.*, 2013a). Similarly, efficacy decreased when it was tested at conditions simulating commercial application in apples (Alegre *et al.*, 2013a) and in melon (Abadias *et al.*, 2014).

Leverentz *et al.* (2006) found that seven promising microorganisms reduced the size of *L. monocytogenes* and *Salmonella* on apple plugs stored at 25 ºC, but none of them reduced the size of the *Salmonella* population at 10 ºC. Carlin *et al.* (1996) found that high numbers (10^6-10^7 cfu/g) of *Pseudomonas* spp. reduced the growth of *L. monocytogenes* on endive leaves. However, they observed that the effect of an individual isolate varied from experiment to experiment.

In this study, the mode of action of the putative antagonists was not determined, however, the production of antimicrobial substances by M309 strain (data not shown) as well as by CPA-7 strain (Alegre *et al.*, 2013b) was analyzed and the *in vitro* assays demonstrated that antagonists cell-free supernatant did not have an effect against the FBP studied. Hence, the production of antimicrobial substances is unlikely and competition could be the reason for the inhibition. In numerous studies, the antimicrobial effect of *Pseudomonas* spp. has been demonstrated, however, the mode of action is unclear. Janisiewicz *et al.* (1999) reported that the mode of action of *P. syringae* seemed to be competition for nutrients and space. Nevertheless, Liao and Sapers (1999) and Bender and Rangaswamy (1999) described the production of secondary metabolites, such as iron-chelating siderophores or antimicrobial substances mainly of the polyketide type.

The bacterial cocktail of *Salmonella* and *L. monocytogenes* was not markedly inactivated by the respective bacteriophage solutions on fresh-cut lettuce under MAP conditions. As mentioned before, *Salmonella* populations were very low during all storage period despite the treatments used. Thereby, the inability of Salmonelex to reduce *Salmonella* counts may be due to a low probability of pathogen contacting common binding sites with the phage. Listex P100 was just able to slightly reduce *L. monocytogenes* populations and no significant differences from M309 strain, hypochlorite and water treatments were observed after 6 days of storage. Other researchers have observed that phages were effective to reduce bacterial pathogens when attached to fresh produce such as honey melon and apples (Leverentz *et al.*, 2001, 2003), tomatoes (Abuladze *et al.*, 2008; Ye *et al.*, 2009), spinach and broccoli (Abuladze *et al.*, 2008), lettuce (Guenther *et al.*, 2009; Sharma *et al.*, 2009), and others.

Oliveira *et al.* (2014) reported high efficacy of Listex P100 in reduction the same *L. monocytogenes* strains in melon and pear juices as well as on fresh-cut melon and pear. However, the inoculation method and the pathogen concentration were completely different and the dip inoculation used in this study could be the reason for the reduced effect on lettuce. Additionally, phages effectiveness is influenced by pH and other physiochemical properties of foods, activity on a solid substrate or biofilm, the emergence of resistant bacteria mutants, and the relative numbers of phages and host required to allow replication (Hudson *et al.*, 2005). Another reason to the reduced effectiveness of phages could be the limited diffusion through the solid surface after addition to the lettuce leaves and therefore could not come into contact with the surviving bacteria (Guenther *et al.*, 2009).

Nisin solution was not tested against *Salmonella* because it has shown a narrow antimicrobial spectrum, inhibiting only Gram-positive bacteria. We found that the treatment with nisin was effective in reducing initial *L. monocytogenes* population until the end of storage period. Nisin wash together with CPA-7 strain was significantly different from the other treatments with reductions more than 1.5-log units after 6 days of storage. Although nisin was initially very effective in reducing path-

ogen counts it started losing effectiveness probably due to the loss of activity (Leverentz *et al.*, 2003). It was previously reported that in fresh-cut 'Honeydew' melons, *L. monocytogenes* populations treated with nisin were 2.8 to 3.2-log units lower than when treated with water after 7 days of storage at 10 ºC and the treatment of 'Red Delicious' apple slices reduced *L. monocytogenes* populations by approximately 0.9-2.0-log units when compared with water (Leverentz *et al.*, 2003). Allende *et al.* (2007) observed that in the case of fresh-cut iceberg lettuce inoculated with *L. monocytogenes*, nisin wash decreased initial pathogen population more than 3.0 log cfu/g. However, its effectiveness decreased during storage at 4 ºC. Randazzo *et al.* (2009) found that nisin wash of fresh-cut iceberg lettuce resulted in a decrease of *L. monocytogenes* population less than 1.0 log unit after 7 days at 4 ºC.

Compared to other studies, the inoculation method as well as the incubation of contaminated lettuce for 24 h before the application of treatments could be the reason for the low effectiveness of all biopreservative agents. Furthermore, the possibility of biofilms creation, the internalization and the low concentration of pathogens could influence the accessibility of the biopreservative treatments to be effective in reducing pathogen populations.

Previous works have proven the effectiveness of microorganisms as biopreservative agents (Abadias *et al.*, 2014; Alegre *et al.*, 2013a, b; Janisiewicz *et al.*, 1999; Leverentz *et al.*, 2006), and moreover, a combination of inoculated lytic phages with nisin has also proven useful for the inhibition of *L. monocytogenes* in fruit slices (Leverentz *et al.*, 2003).

Future experiments will focus on the mechanism of biocontrol as well as the benefits of combining these antagonists with other biopreservatives. It was demonstrated that nisin has an instantaneous inhibitory effect and the antagonists controlling the pathogen populations over the time. This makes them suitable for combining their application.

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Discussion

Fresh vegetables (especially fresh-cut leafy greens) are increasingly recognized as important vehicles for transmission of human pathogens. For this thesis we chose lettuce as a leafy green sample of study because it is implicated in several outbreaks with foodborne pathogens (FBP) such as *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7 and because it is the major worldwide produced. Extensive research was carried out to show that FBP can contaminate produce at various stages of their production: during growth, harvest, processing, distribution and final preparation. Although preharvest (good agricultural practices, GAPs), postharvest (good manufacturing practices, GMPs; hazard analysis and critical control points, HACCP) and supply-chain (good handling practices, GHPs) controls can help to reduce the risk, they have not been able to prevent foodborne outbreaks.

To minimize the risk of infection associated with fresh and fresh-cut vegetables, potential sources of contamination from the field to consumer should be identified and specific measures and interventions to prevent and/or minimize the risk of contamination should be considered and correctly implemented. Where the possibility of contamination cannot be excluded, the application of the most effective decontamination process should be contemplated.

The research described in this thesis focuses therefore on determining the influence of field management practices, processing and storage conditions on the microbial quality of fresh-cut lettuce and to study intervention (mitigation) methods (strategies) to enhance its safety.

For a better understanding, this general discussion has been divided into three subsections that address the three global topics of interest.

Preharvest microbial contamination

Leafy greens like lettuce are identified as the fresh produce commodity group of highest concern from a microbiological safety perspective (WHO, 2008), because they are often grown in the open field and vulnerable to contamination from water, manure, soil and contact with animals and workers. Moreover, leafy greens are likely to be consumed raw, without any form of microbiologically lethal processing.

A better understanding of the microbiological quality of the surface of raw produce would be extremely useful to develop interventions to minimize the risk of contamination, to prevent the growth of pathogens, and to remove them at various stages of production, processing, marketing and preparation for consumption (Beuchat, 2002). This microbiological quality is extremely diverse and complex. The presence and numbers of microorganisms depends on the type of produce, agronomic practices, geographical area of production, and the weather conditions prior to harvest (Brackett, 1999; Nguyen-The and Carlin, 1994). Among them, organic production has been considered to represent an increased risk to public health compared to conventional production, due to fertilization with natural fertilizers like animal manure instead of chemical fertilizers. However, there is little scientific evidence to support this suggestion (McMahon and Wilson, 2001).

Therefore, the effect of production system, organic or conventional, on the microbiological quality of fresh lettuce produced in the northeast of Spain was studied. These investigations are described in **Chapter I**.

The lettuce samples from both organic and conventional production system were analyzed for the presence of total aerobic mesophilic (AM) and psychrotrophic microorganisms, yeasts and moulds (YM), *Enterobacteriaceae*, mesophilic lactic acid bacteria (LAB), *Pseudomonas* spp. and presumptive *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*.

Psychrotrophic and AM counts were similar to those found by Abadias *et al.* (2008) for these two groups of microorganisms in 'Romaine' lettuce purchased from supermarkets in Lleida (Spain). However, Khalil and Gomaa (2014) found lower AM counts in whole conventional lettuce samples collected from retail local markets in Alexandria (Egypt). On the other hand, Ryu *et al.* (2014) found no significant differences between AM counts in organic and conventional romaine lettuce in Korea. Aerobic mesophilic microorganisms are useful for indicating the overall microbial quality of food product: generally it does not relate to food safety hazards but acts as an indicator for food quality and shelf-life duration (Pianetti *et al.*, 2008).

Yeasts and moulds were present in smaller numbers than bacteria and no differences were found between organic and conventional samples. Abadias *et al.* (2008) and Tournas (2005) obtained similar results with samples of fresh and minimally-processed vegetables. Maffei *et al.* (2013) reported higher YM counts in organic and conventional vegetables. When present in high numbers, yeasts and moulds can contribute to spoilage of fermented vegetable products and the development of soft rot (Fleet, 1992). Spoilage as a result of mould growth does not appear to be a major problem in ready-to-eat salads (Heard, 1999). However, some authors (Tournas, 2005; Tournas and Katsoudas, 2005) refer to possible

health problems associated with the production of mycotoxins. Others moulds are known to cause allergies when able to produce large numbers of conidia.

Lactic acid bacteria were also present in low numbers. These results are also in agreement with a previous study in shredded and whole lettuce from supermarkets in the same area (Abadias *et al.*, 2008). Lactic acid bacteria are part of the native microbiota of vegetables and associated with spoilage organisms, causing unpleasant odors due to the production of ethanol, organic acids, esters and CO_2 (Babic *et al.*, 1992; Fleet, 1992).

Pseudomonas spp. counts were similar in both production practices and the same mean counts were also obtained by Pianetti *et al.* (2008) in ready-to-eat vegetable salads. Pseudomonads as well as LAB are native microbiota of vegetables, whereas coliforms and YM may arise from the raw material or from contamination during harvest and processing (Nguyen-The and Carlin, 1994).

The microbiota of vegetables is diverse but consists mainly of gram-negative bacteria such as *Enterobacteriaceae*. In our study, these microorganisms showed the major differences between both production systems analyzed by principal component analysis. The levels of *Enterobacteriaceae* found are not unusual in raw vegetables and are not necessarily associated with fecal contamination, because the majority of the genera are part of the native microbiota of the product (Bracket and Splittstoesser, 2001).

E. coli is a fecal organism, and both *E. coli* and *Listeria* spp. are common environmental microorganisms found in soil and water. Therefore, vegetables may easily become contaminated with these bacteria. About 8.3 % of organic lettuce samples exceeded the hygienic criteria established for *E. coli* by EU regulation Nº 1441/2007, while this limit was exceeded in 11.2 % of conventional lettuce samples. Mukherjee *et al.* (2004) reported that 22.4 % of organic lettuce was positive for *E. coli*, whereas in conventional lettuce the prevalence was 16.7 %. However, Loncarevic *et al.* (2005) reported lower *E. coli* prevalence (8.9 %) in samples of Norwegian organically grown lettuce. Low incidence of *E. coli* was also reported by Ryu *et al.* (2014) in 'Romaine' lettuce and no significant differences were found between the two production systems. A total of 16 colonies of *E. coli* were isolated in our study. None of them had the virulence genes that are pathogenic for humans.

As mentioned before, vegetables may easily become contaminated with *Listeria* spp. However, in this study *L. monocytogenes* was not detected in any organic and conventional lettuce samples analyzed. Our results are comparable to that found by Sagoo *et al.* (2001, 2003) and McMahon and Wilson (2001) in organic vegetables. However, Wilson (1995) showed that 2 % of conventionally farmed ready-to-eat vegetable products in Northern Ireland contained *Listeria* spp.

Salmonella spp. and *E. coli* O157:H7 were not detected in our samples. Leaf lettuce samples (179) collected directly from Norwegian farms with organic production practices that included the use of manure fertilizers were free of *E. coli* O157:H7 and *Salmonella* (Loncarevic *et al.*, 2005). Similarly, no *E. coli* O157:H7 was reported for an expanded investigation that included farms with varied agronomic practices in both Minnesota and Wisconsin (Mukherjee *et al.*, 2006). Mukherjee *et al.* (2004) reported no *E. coli* O157:H7 contamination in any of the organic and conventional produce analyzed; however, one lettuce sample was *Salmonella* positive. Ceuppens *et al.* (2014) found 5 salmonellae and 3 *E. coli* O157:H7 from 260 organic samples, of which only one was lettuce and the other samples were manure, soil and water. In other studies, a variety of organic fresh produce samples from producers from the United Kingdom were free of *Salmonella* (McMahon and Wilson, 2001; Sagoo *et al.*, 2001). Recently, Holvoet *et al.* (2014) and Ryu *et al.* (2014) published that neither *E. coli* O157:H7 nor *Salmonella* spp. were detected in their vegetable samples.

For the study described in this thesis, 72 lettuce samples from each type of production were investigated. The results appear to support the claim that organic farming holds increased microbiological risks, since we observed that the prevalence of *E. coli* was significantly higher in organic than in conventional produce. This finding also supports the idea that organic produce is more susceptible to fecal contamination. However, the absence of pathogens associated with these organic and conventional lettuce samples indicates that overall agricultural, hygiene, harvesting and practices carried out by farmers were good in our study. Our results do not support allegations that organic produce poses a substantially greater risk of pathogen contamination than does conventional produce.

Some authors have found FBP contamination on produce during the production management. Contamination of fresh produce with FBP could be rare but has been linked to some outbreaks. The sources of preharvest contamination include soil amended with untreated or improperly composted manure, contaminated irrigation water, the presence of wild and domestic animals, infected workers, and unclean containers and tools used in harvesting (FDA, 2008).

As described before, a great variety of sources potentially harboring FBP are used in the production of fresh produce. Examples are contaminated manure and water. The public health risk associated with the introduction of FBP by these contaminated sources is determined by i) the prevalence and concentration of FBP in the manure and water used, ii) the amount of manure and water and consequently the number of bacteria transferred and adhering to the edible parts of the plants, and iii) the persistence of FBP in the soil and on produce until its consumption.

The transfer to and the persistence of *L. innocua* and *E. coli* O157:H7 on lettuce leaves and in soil during fall and spring was studied and discussed in **Chapter II**. The studies suggest that the presence of the FBP is influenced by the use of different contaminated irrigation methods (surface and sprinkler) and compost. Also seasonal influence was observed.

In both studies, contamination of *L. innocua* and *E. coli* O157:H7 via soil to the lettuce surface occurred during the growth process most probably through contact with soil, contaminated either through contaminated compost or contaminated surface irrigation water. Also we found that, when the lettuce samples were irrigated by sprinkling with contaminated water, the pathogen survived on the surface of leaves during some days. Several studies report long-term persistence of pathogens on plant leaves after irrigation, suggesting that the application of contaminated irrigation water shortly before harvest represents a risk factor of vegetable contamination (Milillo *et al.*, 2008; Solomon *et al.*, 2002, 2003). Additionally, Islam *et al.* (2004a, b) reported that *E. coli* O157:H7 and *S.* Typhimurium can be transferred from contaminated soil and water to the surface of lettuce and parsley leaves.

Bacterial contamination of green leaves occurs mostly on the surface of the tissues of the plants but also due to internalization. We observed that there was transfer of pathogen to edible parts of lettuce leaves. However, we could not demonstrate that this transfer took place through internalization via the root system or by direct contact to soil, insects or other vectors. Although plant contamination by pathogens is mainly on the plant surface, several recent reviews have summarized studies conducted over the past decade that have revealed the potential for pathogens to be internalized through several routes (Deering *et al.*, 2012; Erickson, 2012; Hirneisen *et al.*, 2012). In this sense, we carried out several studies on the internalization of a green fluorescent protein (GFP)-labeled *E. coli* O157:H7. One of these studies was to determine possible internalization from contaminated lettuce seeds to seedlings (Figure 1) but inconclusive results were found (results not published). Some authors reported the internalization via root systems by some pathogens, but different conclusions were found. From the collected data it is clear that several factors greatly influence the likelihood of internalization of a human pathogenic bacterium within a plant. These factors are type of plant, strain and/or serovar of bacteria, route of contamination, and age of the plant.

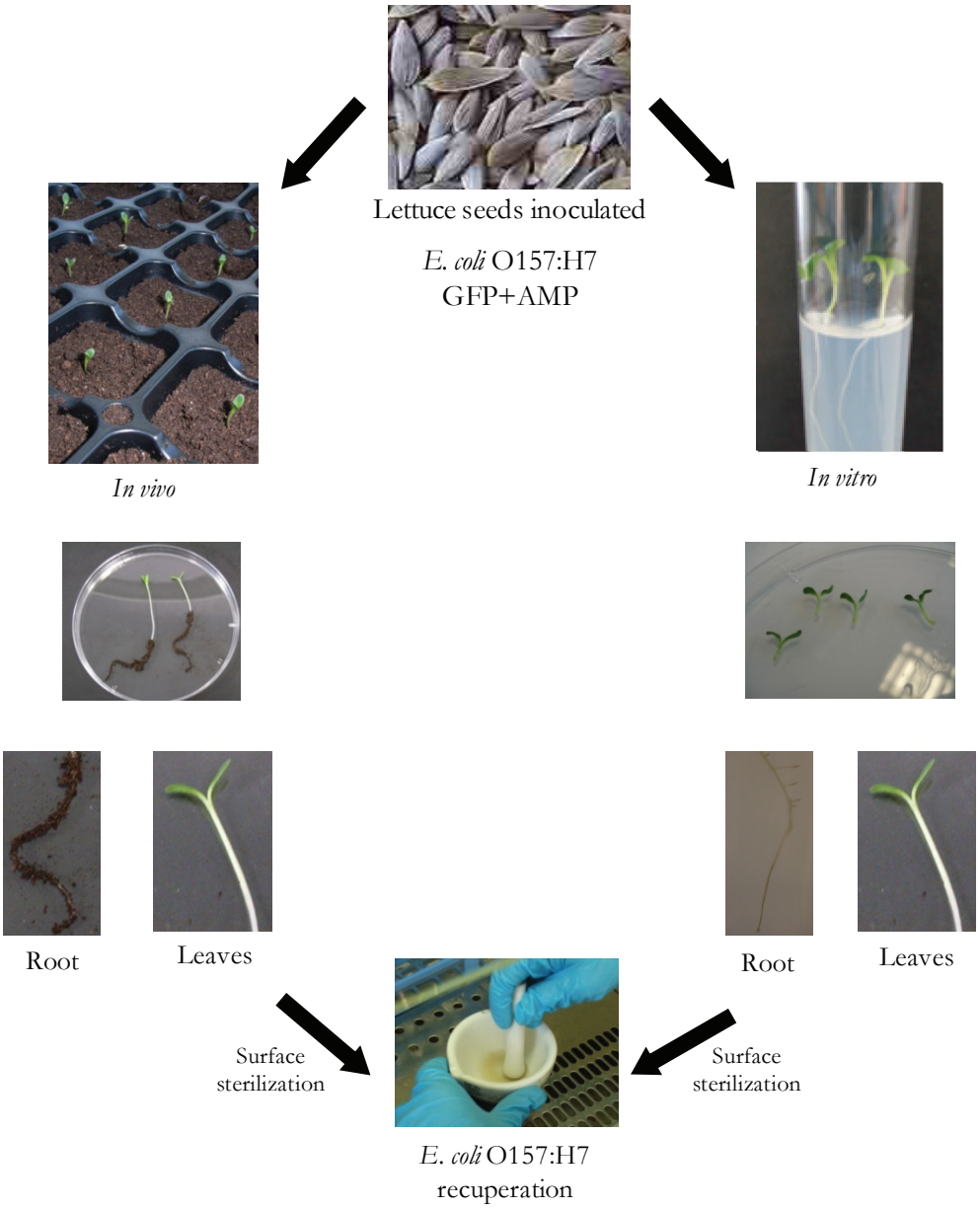

Figure 1. Example of internalization studies from contaminated seeds to lettuce seedlings.

Another important finding was that *L. innocua* and *E. coli* were able to survive for at least 9 weeks in soil exposed to environmental conditions. We also observed seasonal influence, as the pathogens survived better during fall than spring. Variables, such as solar radiation, temperature and dryness, may also affect the survival of human pathogens under field conditions. Other factors including soil composition, pH, water activity, oxidation reduction potential, presence of rhizosphere, and microbial interactions also affect the survival of pathogenic bacteria (Fenlon *et al.*, 2000; Tamasi, 1981). Numerous studies have investigated the survival of human pathogens in manure and soil. Several studies demonstrate that *S. enterica* and *E. coli* O157:H7 were able to survive in soil amended with contaminated compost for over 200 days (Franz *et al.*, 2011; Islam *et al.*, 2004a, b; You *et al.*, 2006). In contrast, one other study reports shorter survival for these pathogens in soil (Bolton *et al.*, 2011). These discrepancies can be due to different experimental conditions, inoculation methods and microbial quantification strategies.

Although many environmental factors can affect the persistence of FBP under field environment conditions, these pathogens can survive long enough to contaminate plant roots and leaves and gain access to consumer. If present at harvest, contaminated lettuce will enter in the production chain.

Processing and storage conditions

Fresh-cut commodities are raw fruits and vegetables that have been washed, peeled, sliced, chopped or shredded prior to packaging for consumption. In the processing plants, there are several critical points to be considered. The water applied in food processing as well as in preharvest management may act as a vehicle for pathogens. Cutting or slicing is also a source of contamination and also is considered to increase the bacterial growth, as they not only offer at the cut edges additional sites for adhesion to and penetration into the leaves, but also provide the bacteria with nutrients in the form of exudates. Modified atmosphere packaging (MAP) has been used successfully and widely in combination with refrigeration for these minimally processed fruits and vegetables as a strategy to maintain product safety during storage. Moreover, MAP may extend the shelf-life of these products (Werner and Hotchkiss, 2006).

Survival and growth of pathogens on produce is influenced by a number of factors. These are mainly storage temperature, minimal processing operations, mild technologies, package atmosphere and competition with the native microbiota present on the product. Moreover, pathogen growth varies significantly with product type, since each vegetable has an exclusive combination of compositional and physical properties (Austin *et al.*, 1998; Jacxsens *et al.*, 1999).

The study conducted in the **Chapter III** evaluates the ability of *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 to grow in shredded lettuce packaged in two types of packaging film used for making different MAP along storage and in atmosphere conditions (Air). Also various storage temperatures were used, and growth of psychrotrophic and mesophilic microorganisms under the same conditions were monitored.

Non-inoculated shredded lettuce samples presented similar initial concentrations of mesophilic and psychrotrophic microorganisms (5.0 log cfu/g). This is in accordance with other authors who report microbial densities between 10^4 and 10^6 cfu/g (Barriga *et al.*, 1991; García-Gimeno and Zurera-Cosano, 1997; Garg *et al.*, 1990; King *et al.*, 1991; Valero *et al.*, 2006). Counts of these two groups of microorganisms increased significantly over the storage period in all packaging types and reached final concentrations between 10^7 and 10^{10} cfu/g. However, the growth rate was faster at ambient temperatures than at refrigerated ones. These final populations are consistent with those reported by Gleeson and O'Beirne (2005) and Carrasco *et al.* (2008) on iceberg lettuce stored at 8 and 5 ºC, respectively. One effect of MAP were observed at both temperatures, namely that counts were higher in lettuce stored at Air conditions than in the other two package films.

The composition of the storage atmosphere generated within different packaging films during their preservation at refrigerated temperature had no significant effect on the survival and growth of the three foodborne pathogens on 'Romaine' lettuce.

At 25 ºC, growth of *Salmonella* and *E. coli* O157:H7 on lettuce was slightly higher when packaged with less permeable film than in the other two films.

The behavior of *Salmonella* Michigan studied in this work under all storage conditions was similar to *E. coli* O157:H7. We have shown that both pathogens significantly increase in concentration at 25 ºC and slightly decrease (approximately 1.0 log unit after 10 days) at 5 ºC for all package films. However, when *L. monocytogenes* was inoculated at the same concentration, we observed an increase of 1.0 log unit at 5 ºC during 10 days of storage. Thus, as *L. monocytogenes* numbers should not be greater than 100 cfu/g during their shelf-life, we determined if this increase would also happen at low initial population (<100 cfu/g). We observed that, even at low starting dose, this microorganism was able to grow reaching to significant population numbers.

We observed that storage of fresh-cut produce at temperatures ≤ 8 ºC led to different outcomes, ranging from slight growth to no change or to measurable loss in viability. For example, Abdul-Raouf *et al.* (1993) reported declines in *E. coli* O157:H7 populations on fresh-cut lettuce at 5 ºC while Koseki and Isobe (2005) found no changes at this temperature. Posada-Izquierdo *et al.* (2013) reported high variability in growth between replicates on iceberg lettuce stored at 8 ºC under passive MAP with very low O_2 levels (<0.5 kPa). However, Francis and O'Beirne (2001) observed

an increase of *E. coli* O157:H7 populations on iceberg lettuce and dry coleslaw under passive MAP at 8 ºC.

In carrots and lettuce, *S.* Enteritidis survived or decreased during storage at 4 ºC (Kakiomenou *et al.*, 1998; Tassou and Boziaris, 2002), regardless of the MAP condition studied. Although, *Salmonella* spp. are unable to grow under refrigerated temperature, the pathogen survives for a long time at refrigerated temperatures on produce (ICMSF, 1996). *S.* Typhimurium concentrations on lettuce were higher in active MAP than in Air and passive MAP at 8 and 20 ºC (Horev *et al.*, 2012). In this study CO_2-enriched atmosphere resulting from produce respiration did not reduced pathogen populations.

As mentioned before, *L. monocytogenes* is able to grow over a refrigerated temperature range and some studies have reported that survival and growth of *L. monocytogenes* on produce is not reduced or affected by MAP atmosphere (Beuchat and Brackett, 1991; Jacxsens *et al.*, 1999). Francis and O'Beirne (1997) observed that nitrogen flushing combined with storage at 3 ºC extended *L. monocytogenes* and *L. innocua* survival on lettuce and at 8 ºC enhanced their growth. Jacxsens *et al.* (2002) examined the behavior of *L. monocytogenes* on lettuce subjected to a simulated distribution chain for 7 days under an active MAP (3 % O_2/5 % CO_2) and found that the pathogen was able to survive. However, in another study, *L. monocytogenes* concentrations decreased in lettuce packaged under two active MAP atmospheres stored at 4 ºC (Kakiomenou *et al.*, 1998). Carrasco *et al.* (2008) obtained an increase of *L. monocytogenes* on iceberg lettuce packaged under active MAP (4.6-6.2 % CO_2/2.1-4.3 % O_2) stored at 5 and 13 ºC. It is well known that CO_2 can have an antimicrobial effect. However, the high levels of CO_2, as used in different studies, were not effective to inhibit foodborne pathogens growth.

Due to commodity differences and extrinsic factors applied in the individual works, a wide variability of pathogens behavior was observed on MAP studies. Therefore, characterization of the behavior of *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. on fresh-cut vegetables under MAP conditions is very difficult. Although some authors recommend percentages of the gases CO_2 and O_2 for fresh-cut vegetables for prevention of microbial growth and maintaining quality, these gas-combinations may not ensure microbiological safety as unique hurdle technology.

During the production chain, human pathogens encounter several unfavorable circumstances such as environmental conditions, disinfectants, MAP, refrigeration temperatures, low pH, additives, which can change their genetic or physical fitness. Current microbial risk assessments generally only consider the frequency and level of contamination, the potential for growth and the amount consumed. However, there is concern about the fact that adaptation to the conditions found in the food supply chain can alter the pathogenic potential of pathogens by inducing (cross-) resistance mechanisms and/or influencing the expression of virulence factors, thereby increas-

ing the probability of infection (Wesche *et al.*, 2009). Therefore, it is likely that not only the number of pathogens and the genetic characteristics of the strain, but also the conditions encountered during processing and storage of the food product, play a role in determining the likelihood and severity of infection.

For that purpose, in **Chapter IV** investigations are described that examine the pathogenic potential of two *S.* Typhimurium DT104 strains (lettuce and carcass isolates, respectively). This pathogenic potential is measured as the capability to survive a simulated gastrointestinal tract system and the capability to adhere to and invade differentiated Caco-2 cells (mimicking human small intestinal epithelial cells), after sequential incubation into soil, lettuce and cut lettuce stored under MAP conditions. This approach allowed a more complete and more realistic assessment of pathogen behavior in the digestive tract compared to studies only considering single isolated parts of the digestive tract.

Initially, *S.* Typhimurium strains were inoculated in the soil, simulating contamination in the field, and concentrations remained stable over the 24 h at 15 °C. When both strains were transferred from the soil to lettuce leaves, pathogen concentrations increased about 1.0 log-unit after 24 h at 15 °C and remained at the same level (6.5 log cfu/g) during storage for 5 days under active MAP. This last finding is in accordance with studies mentioned before, were MAP conditions did not influence *Salmonella* survival (Kakiomenou *et al.*, 1998; Tassou and Boziaris, 2002).

The most important result of the present study is that the sequential incubation of *S.* Typhimurium in soil and on lettuce slightly increased the capability of surviving the simulated gastric fluid (SGF), increased the capability for growth in the simulated intestinal fluid (SIF), but decreased the capability for epithelial attachment (ATT) and invasion (INV), and decreased the overall survival probability in the gastrointestinal tract system. The results indicate that the magnitude of these effects may be strain dependent. However, more strains should be studied in order to draw conclusions on strain dependency.

As gastric fluid is the first line of defense against microbial foodborne diseases, the ability of bacteria to survive in acidic environments could play a crucial role in pathogen virulence. In the present study, we found slight variations in the level of acid resistance between the *S.* Typhimurium DT104 strains. Interestingly, the carcass isolate presented more acid resistance, which is indicative of increased acid adaptation. Moderate acidic conditions have been reported to render strains of *Salmonella* more resistant to gastric fluid (Perez *et al.*, 2010; Yuk and Schneider, 2006). Moreover, resistance in *Salmonella* against various stress-types are linked, since Shah *et al.* (2013) reported that the preadaptation of *S.* Typhimurium to cold stress (5 °C) significantly increased its survival during subsequent acid stress exposure (pH 4.0, 90 min).

As mentioned before, *Salmonella* spp. are constantly faced with different stress conditions, both in their environment as well as in the gastrointestinal tract of their hosts. Following the stomach barrier, these microorganisms are also exposed to bile salts, high osmolarity and low oxygen tension in the intestine. However, a major result obtained with the present study is the significant growth of both strains in the SIF after a growth history in soil and lettuce compared with control treatment. One reason to support this result could be the tolerance of *S.* Typhimurium to bile salts (Merrit and Donaldson, 2009) and the protective effect of food (Kos *et al.*, 2000). Bile tolerance involves a virulence regulator that includes genes necessary for protection against the action of antimicrobials (Prouty *et al.*, 2002; Van Velkinburgh and Gunn, 1999).

Another important trait of *S.* Typhimurium is the ability to adhere and invade host cells (Figure 2). Therefore, initial adherence helps to bring *S.* Typhimurium in close contact with the host cells. In the present study, adhesion and invasion experiments were performed in order to compare the pathogenicity of adapted and non-adapted *S.* Typhimurium to processing steps. Both the adhesive and invasive capacities of the

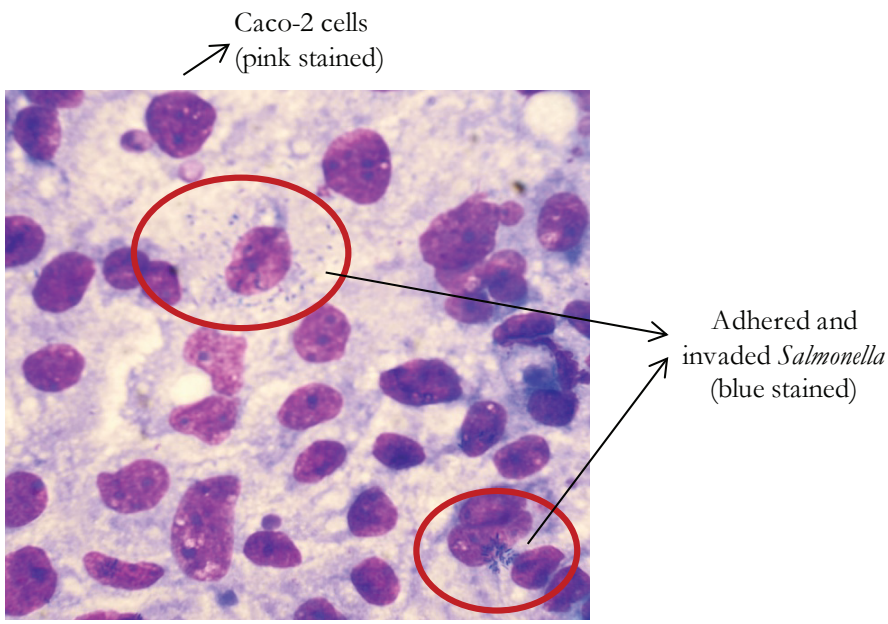

Figure 2. Giemsa staining of adhesion and invasion of *Salmonella* Typhimurium DT104 (lettuce isolate) on Caco-2 cells.

S. Typhimurium strains used in the present study decreased as a result of sequential incubations in soil, lettuce, MAP stored cut lettuce, gastric fluid and intestinal fluid.

It was reported that the major regulator of *Salmonella* invasion (HilA) is activated at neutral pH (Bajaj *et al.*, 1996) so the slightly acidic conditions in the MAP storage of the cut lettuce might have impaired the invasive capability. Previously, it was reported that acid adaptation results in a lower level of invasion in Int407 cell lines (Fratamico, 2003). On the other hand, our results suggest that the carcass strain, which was more acid resistant, was also more invasive to Caco-2 cells, while the lettuce strain was less acid resistant but had more capability to attach and less to invade. Genetic material of bacteria, encoding for a variety of adherence mechanisms including fimbrial adhesions, flagella and biofilm formation (Holden *et al.*, 2009), has been reported to be involved in both attachment to plant surfaces and the animal intestinal tract (Barak *et al.*, 2005; Baumler *et al.*, 1996). Gene expression profiles of key virulence factors involved in attachment and invasion of Caco-2 cells of both strains could provide answers to verify this hypothesis.

In addition, the exposure of the foodborne pathogens to sublethal stresses may increase the virulence of pathogens, because the expression of many virulence factors is dependent upon environmental conditions such as low pH and low temperatures (Mekalanos, 1992). Therefore, determination of the transcriptional expression of such genes could clarify how virulence is affected by the sequential incubations as applied in our study.

The findings reported in chapters III and IV suggest that foodborne pathogens are able to survive and grow in fresh-cut lettuce and capable to attach and invade the epithelium cells of the host. Therefore, the implementation of good sanitation measures is needed to enhance food safety.

Intervention strategies to reduce and control FBP growth

Over the last years, a number of strategies to minimize the microbial load of raw products have been explored. A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) has been used to reduce initial bacterial populations on minimally processed produce (Beuchat, 1998). There are many factors that will affect disinfection, such as initial bacterial load and type of microorganisms on the surface of produce, treatment type, the surface to be treated, the type of disinfectant, whether or not there is any internalization of pathogens and the time and temperature of exposure to the disinfectant (Beuchat *et al.*, 2004, 2005). Chlorine is the most widely used sanitizer in the fresh produce industry. However, studies indicate that the traditionally used chlorine concentrations (50-200 ppm) are not effective in reducing pathogen load on fresh-cut produce (Behrsing *et al.*, 2000; Delaquis *et al.*, 2002; Lee and Baek, 2008). Moreover, a prolonged exposure

to chlorine vapor may cause irritation to the skin and respiratory tract of the workers, may affect the quality of foods, and may also adversely affect the environment (Beuchat, 1998). It is also known that the reaction of chlorine with organic matter results in the formation of carcinogenic products (trihalomethanes) for consumers (Nieuwenhuijsen *et al.*, 2000). Trihalomethanes (THM) have been the main disinfection by-products of concern and have been classified by the WHO's International Agency for Research on Cancer as possibly carcinogenic to humans (IARC, 1999a, b). Water is the classical exposure route of concern but some authors have described that vegetables can absorb THMs from the washing water (Huang and Batterman, 2010). However, Gómez-López *et al.* (2013) observed in their experiments that the use of chlorine-based sanitizers in baby spinach resulted in very low concentrations of THMs after washing. After subsequent rinsing, no THMs were detected in the leaves. They concluded that chlorine based sanitizers do not represent a risk for THMs formation during baby spinach leaf processing. Due to the great controversy about the use of chlorine, there is a need for better, safer and more environmental friendly methods to reduce the contamination.

There are several other chemical and physical environmental friendly methods to use as good strategies to control and reduce FBP on fresh-cut produce, but in this thesis we only focused on biopreservative methods. It has been reported that the presence of competing microorganisms on the surfaces of fresh produce contributes to the reduction of pathogens growth (Janisiewicz *et al.*, 1999; Liao and Fett, 2001; Parish *et al.*, 2003).

The effect of enhanced native microbiota of lettuce submitted to different pre-conditioning steps on survival of *L. monocytogenes* and *E. coli* O157:H7 has been evaluated in **Chapter V**. The study was carried out with lettuce of conventional and organic origin.

With the aim of investigating how different native microbiota concentrations could influence pathogen survival, we submitted the shredded conventional and organic 'Romaine' lettuce to different temperatures and washes. Our results show that the pathogens behaved similarly on shredded conventional and organic lettuce during the first days despite the differences in initial microbiota load of lettuce. At the end of storage period *L. monocytogenes* seemed to be affected by the native microbiota only on organic lettuce. In contrast, *E. coli* O157:H7 seemed to be affected only by the native microbiota from conventional lettuce. However, regardless of the production system affecting each pathogen, the concentrations of mesophilic bacteria increased during storage period reaching final populations of about 8.0-8.5 log cfu/g while no differences between the initial loads were observed. Similarly, some authors demonstrated the efficiency of the washing solution in reducing the initial microbial loads (Bennik *et al.*, 1996; Beuchat *et al.*, 2004; Inatsu *et al.*, 2005; Ukuku *et al.*, 2005). These authors also demonstrated that disinfected and non-disinfected

fresh-cut produce have different concentrations of epiphytes at day 0, while these differences disappear after storage.

In this work, regardless of initial mesophilic counts, *L. monocytogenes* grew on shredded lettuce at 10 ºC while *E. coli* O157:H7 showed little increase at the same temperature during the storage period. Similarly, Francis and O'Beirne (1998) investigated the effect of inoculating sliced lettuce leaves with a mixture of the lettuce native microbiota at three different initial levels, on growth of *L. innocua*. The results show that *L. innocua* survival and growth was not affected by the native microbiota concentrations. Our results suggest that the behavior of the human pathogens on produce is not simply related to the size of the native microbiota but also to the removal or reduction of key competitive subpopulations of the microbiota.

Conflicting reports exist in the literature about the relationship between human pathogens and native microbiota. Carlin *et al.* (1996) showed that reducing the native microbiota on broad-leaved endive leaves by chemical disinfection permitted better growth of *L. monocytogenes*. Similar results were observed on fresh-cut lettuce dipped with chlorine or citric acid, where *L. innocua* counts increased compared to undipped samples (Francis and O'Beirne, 1997). The addition of two selected key competitive microorganisms to endive samples limited the growth of *L. monocytogenes* significantly (Carlin *et al.*, 1996).

Fresh-cut vegetables harbor a large and diverse population of microorganisms. Therefore, it is not surprising to find some variability in effectiveness of results. The mechanisms to suppress growth of FBPs could be related to the interaction for competition of nutrients, sites or colonization. Thus, it is likely that the lower fitness of human pathogens compared to that of microorganisms of the native microbiota is partly rooted in the low abundance and restricted range of nutrients that they can assimilate on plant surfaces (Brandl *et al.*, 2013). Moreover, plant surfaces are not homogenous and contain various microsites with available nutrients which may support growth of human pathogens after contamination (Leveau and Lindow, 2001). Because these sites are also attractive to native microbiota microorganisms, cells of FBP must interact and compete with the plant-associated bacteria in order to occupy such preferred sites (Brandl, 2006). Human pathogens on leaves have been shown a preference to move towards stomata and colonize the vein areas, the bases of trichomes and lesions, or other surface irregularities (Aruscavage *et al.*, 2008; Barak *et al.*, 2011; Brandl, 2008; Kroupitski *et al.*, 2009, 2011). These microenvironments may provide increased nutrient and water availability, and hence, may offer conditions that are conducive to survival and growth of human pathogens that can behave like many epiphytic bacteria.

It was well reported that the presence of plant pathogens can promote survival and growth of FBP on plants, and that the competition for nutrients with members of *Enterobacteriaceae* can reduce the fitness of *Salmonella* spp. and *E. coli* O157:H7 on

plant niches (Aruscavage *et al.*, 2008; Cooley *et al.*, 2003, 2006; López-Velasco *et al.*, 2012). Taking this into consideration, competition for nutrients is unlikely to be the unique mechanism by which human pathogens can be excluded from plant communities (López-Velasco *et al.*, 2012). They can also be exposed to phages and antibiotic-producing epiphytic bacteria.

Due to the results obtained, the next step was to test the effect of individual/key competitive strains for controlling FBP survival and growth (**Chapter VI**).

Firstly, several native microorganisms were isolated and evaluated as putative antagonists against FBP on lettuce. Secondly, the best putative antagonist was identified and compared with *Pseudomonas graminis* CPA-7, nisin and two bacteriophage solutions (Listex P100 and Salmonelex) on fresh-cut lettuce simulating commercial conditions.

In the first studies, 112 putative antagonists were tested on lettuce discs and only one had effect against *S. enterica* and *L. monocytogenes* at 20 ºC. It was identified as *Pseudomonas* sp. (strain M309) by partial 16S rDNA sequencing and was tested under refrigerated temperature (10 ºC). It showed high efficacy to control the *E. coli* O157:H7 growth on lettuce discs. However, M309 strain did not have significant effect on the growth of *L. monocytogenes* during storage. Previous studies have also reported the effectiveness of *Pseudomonas* as bioprotective agent against *Escherichia coli* O157:H7 (Janisiewicz *et al.*, 1999; Johannessen *et al.*, 2005; Schuenzel and Harrison, 2002), *Listeria monocytogenes* (Buchanan and Bagi, 1999; Carlin *et al.*, 1996; Freedman *et al.*, 1989; Liao and Fett, 2001) and *Salmonella* (Fett, 2006; Liao and Fett, 2001; Matos and Garland, 2005).

Due to its high effectiveness observed on lettuce discs, M309 strain was selected for further assays on fresh-cut lettuce stored under MAP at 10 ºC and was compared with the other, afore mentioned, biopreservative agents.

The addition of biopreservative agents did not result in a significant reduction of the *Salmonella* and *L. monocytogenes* concentrations under conditions simulating commercial application. Concerning the use of biopreservative cultures, we have observed that efficacy of M309 strain decreased when tested under simulated commercial conditions. Similarly, *P. graminis* CPA-7 strain reduced the *L. monocytogenes* concentration on apple plugs around 2.0 log cfu/g but its efficacy decreased when it was tested under conditions simulating commercial application in fresh-cut apples (Alegre *et al.*, 2013a) and melon (Abadias *et al.*, 2014). This could be attributed to the inoculation method (spot or dip) and the decrease in O_2 and increase in CO_2 concentration observed inside the packages, which could affect M309 and CPA-7 efficacy as *Pseudomonas* spp. are strict aerobic microorganisms. Therefore, it could be necessary to optimize packaging permeability or MAP atmosphere in order to increase effectiveness.

Although *Pseudomonas* spp. are effective antagonists for many foodborne pathogens, the mechanism of their effect is quite varied and poorly understood. In this study, the mode of action of the putative antagonists was not determined. However, the production of antimicrobial substances by M309 strain as well as by CPA-7 strain was analyzed and the *in vitro* assays demonstrated that antagonists cell-free supernatant did not have an effect against the FBP studied. Hence, the production of antimicrobial substances is unlikely and competition for nutrients and space could be the reason for the inhibition (Carlin *et al.*, 1996; Janisiewicz *et al.*, 1999; Matos and Garland, 2005; Schuenzel and Harrison, 2002). Nevertheless, Bender and Rangaswamy (1999) and Liao and Sapers (1999) described the production of secondary metabolites in *Pseudomonas* spp, such as iron-chelating siderophores or antimicrobial substances mainly of the polyketide type.

The bacterial cocktail of *Salmonella* and *L. monocytogenes* was not markedly inactivated by their respective bacteriophage solutions on fresh-cut lettuce. *Salmonella* populations were very low during all storage period despite the various treatments. Thereby, the inability of Salmonelex to reduce *Salmonella* counts may be due to the low probability of pathogen to contact common binding sites of the phage. Listex P100 slightly reduced the *L. monocytogenes* concentrations. No significant differences between M309 strain, hypochlorite and water treatments were observed after 6 days of storage. Oliveira *et al.* (2014) reported high efficacy of Listex P100 in reducing the same *L.monocytogenes* strains in melon and pear juices as well as on fresh-cut melon and pear. However, the inoculation method and the pathogen concentration were completely different and the dip inoculation used in this study could be the reason for the reduced effect on lettuce. Additionally, phages effectiveness is influenced by a variety of physico-chemical factors (Hudson *et al.*, 2005).

Another reason for the reduced effectiveness of phages could be the limited diffusion through the solid surface after addition to the lettuce leaves and therefore the absence of contact with the surviving bacteria (Guenther *et al.*, 2009).

We found that the treatment with nisin was effective in reducing initial *L. monocytogenes* population until the end of storage period. Nisin wash together with CPA-7 strain were significantly different from the other treatments after 6 days of storage. Although nisin was initially very effective in reducing pathogen counts after washing, it started losing effectiveness probably due to the loss of activity (Leverentz *et al.*, 2003). Accordingly, Allende *et al.* (2007) observed that in the case of fresh-cut iceberg lettuce inoculated with *L. monocytogenes*, nisin wash decreased the initial pathogen concentration more than 3.0 log cfu/g. However, its effectiveness decreased during storage.

Previous works have proven the effectiveness of microorganisms as bioprotective agents (Abadias *et al.*, 2014; Alegre *et al.*, 2013a, b; Janisiewicz *et al.*, 1999; Leverentz *et al.*, 2006), and moreover, a combination of inoculated lytic bacteriophages

with nisin has also proven useful for the inhibition of *L. monocytogenes* in both apples and honeydew melon slices (Leverentz *et al.*, 2003).

A variety of factors, such as water temperature, contact time, and concentration of the active compound, can impact the efficacy of produce washes (Parish *et al.*, 2003). Produce washes are generally less effective on damaged or cut produce than on intact produce. Contributing factors likely include limited accessibility of bacteria to washes in damaged areas, changes in produce surface characteristics that may favor bacterial adherence, and increased protein loads due to the liberation of proteins from the damaged produce cells. Moreover, produce washes are likely to be ineffective if bacteria have been internalized into produce or if they have formed biofilms (Han *et al.*, 2001; Sapers, 2001).

Different *L. monocytogenes* strains are known to have variable resistance to disinfectants (Nascimento *et al.*, 2003) and this may lead to better survival of certain strains during the washing of the lettuce. Gorski *et al.* (2004) observed that strain-specific differences exist in the ability of *L. monocytogenes* to attach to the surface of alfalfa sprouts: the poorest colonizing strain was unable to attach to the sprout. In our study, a cocktail of different *L. monocytogenes* and *Salmonella* strains was used and some attachment differences among strains could be present.

It is likely that attachment and penetration of bacteria into cut surfaces of lettuce provides some protection against antimicrobial dipping treatments. The availability of nutrients and the moisture content of samples are important factors for bacterial attachment and growth (Akbas and Ölmez, 2007). Takeuchi *et al.* (2000) demonstrated that *L. monocytogenes* and *E. coli* O157:H7 showed preferential attachment to cut edges of iceberg lettuce compared to intact leaf tissues. In contrast, *S.* Typhimurium attached similarly to both types of surfaces. Since the study by Ells and Hansen (2006) clearly demonstrates that *L. monocytogenes* can attach rapidly at refrigeration temperature and initiate biofilm formation, conditions may become permissive to allow the psychrotrophic *L. monocytogenes* to multiply to dangerous levels during processing and storage.

Using combined treatments is consistent with the hurdle concept by Leistner (1992), who described the effective control of foodborne pathogens by using a combination of compatible control measures to ensure the safety of food. In our study, it was demonstrated that nisin has an instantaneous 'killing' effect meanwhile the antagonists controlling the pathogen populations over the time. This makes suitable for combining their application.

From a practical point of view, a major effort is needed to enhance the effectiveness of these biopreservatives in the food matrix as their antimicrobial activity may be diminished *in situ*.

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Concluding remarks

Are conventional produced lettuces microbiologically safer than organic ones?

A total of 72 lettuce samples for each type of agriculture (organic and conventional) were examined in order to assess the microbial quality of the lettuces, in particular the prevalence of selected foodborne pathogens. None of the lettuce samples was positive for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp.

The mean aerobic mesophilic counts were about 6.35 log cfu/g and 5.67 log cfu/g in organic and conventional lettuce, respectively. Psychrotrophic microorganisms counts were 5.82 log cfu/g and 5.41 log cfu/g in organic and conventional lettuce, respectively. Yeasts and moulds mean counts were 4.74 log cfu/g and 4.21 log cfu/g from organic and conventional lettuce, respectively. Lactic acid bacteria were present in low numbers and the mean counts were 2.41 log cfu/g and 1.99 log cfu/g from organic and conventional lettuce, respectively. *Pseudomonas* spp. mean counts were 5.49 log cfu/g and 4.98 log cfu/g in organic and conventional lettuce, respectively. *Enterobacteriaceae* counts were 5.16 log cfu/g and 3.80 log cfu/g in organic and conventional lettuce, respectively. *E. coli* was detected in 22.2 % (16 samples) of organic lettuce and in 12.5 % (9 samples) of conventional lettuce.

From the samples analyzed by principal component analysis (PCA) a pattern with two different groups (conventional and organic) were observed, being the highest difference between both kinds of samples the *Enterobacteriaceae* counts.

The absence of pathogens associated with these organic and conventional lettuce samples indicates that overall agricultural, hygiene, harvesting and practices carried out by farmers were good. The results obtained in this study showed that the consumption of organically produced lettuce does not represent an increasing risk of a foodborne disease by consumers.

How lettuce leaves can be contaminated?

L. innocua and *E. coli* O157:H7 populations in lettuce leaves after sprinkle irrigation were very high, but decreased to undetectable levels at field conditions. There was also transfer of *L. innocua* and *E. coli* O157:H7 from soil contaminated with com-

post or irrigated with contaminated water to lettuce leaves, mainly to the outer ones. Survival profiles of both pathogens on lettuce and soil samples contaminated either by application of contaminated compost or surface irrigation water was similar.

L. innocua survived in soil samples for 9 weeks at high concentrations, 10^5 cfu/gdw in fall and 10^3 cfu/gdw in spring. *E. coli* O157:H7 survived in soil samples also for 9 weeks at lower levels, 4.50 log cfu/gdw in fall and 1.50 log cfu/gdw in spring. Both pathogens survived better in fall, indicating an important influence of temperature and humidity conditions.

The results indicated that contamination of lettuce plants in the field can occur through both contaminated composted manure and irrigation water and persist for several months.

If there is contamination, could foodborne pathogens survive and grow on fresh-cut lettuce?

The composition of the storage atmosphere generated within different packages during their preservation at refrigerated temperature had no significant effect on the survival and growth of the three foodborne pathogens studied on 'Romaine' lettuce. At 25 ºC, growth of *Salmonella* and *E. coli* O157:H7 on lettuce was slightly higher when packaged with less permeable film than in the other two films. The population of both pathogens significantly increased their population at 25 ºC and slightly decreased (approximately 1.0 log unit) after 10 days at 5 ºC for all package films.

When *L. monocytogenes* was inoculated at the same concentration of the other pathogens, we observed an increase of 1.0 log unit at 5 ºC during the 10-days of storage. Moreover, when *L. monocytogenes* started with low inoculum dose this microorganism was able to grow reaching to significant population numbers at both temperatures.

The composition of the storage atmosphere within the packaging of lettuce had no significant effect on the survival and growth of the pathogens used in this study at refrigeration temperatures. Thus the results obtained emphasized that MAP cannot be considered as a unique hurdle technology, which reinforces the necessity for corrective measures to avoid contamination of vegetables.

Can the production stages of lettuce influence the pathogenic potential of *Salmonella*?

S. Typhimurium strains (carcass and lettuce isolates) were inoculated in the soil and populations remained stable over the 24 h at 15 ºC. When both strain inocula were transferred from the soil to lettuce leaves, the pathogen populations increased about

1.0 log-unit during 24 h and remained at the same level (6.5 log cfu/g) during storage for 5 days under MAP conditions.

The sequential incubation of *S.* Typhimurium in soil and lettuce slightly increased the capability of surviving the simulated gastric fluid, increased the capability to grow in the simulated intestinal fluid but decreased the capability of epithelial attachment and invasion. The results indicate that the magnitude of these effects might be strain dependent.

The carcass isolate presented more acidic resistance than the lettuce one. However, a major result obtained with the present study is the significant growth of both strains in the simulated intestinal fluid following a growth history in soil and lettuce compared with control treatment.

Another important trait of *S.* Typhimurium is the ability to adhere and invade host cells. Both the adhesive and invasive capacities of the *S.* Typhimurium strains used in the present study decreased as a result of sequential incubations in soil, lettuce, MAP stored cut lettuce, gastric and intestinal fluid.

The results suggested that carcass strain, which was more acid resistant, was also more invasive to Caco-2 cells, while lettuce strain was less acid resistant but with more capability to attach and less to invade.

The findings reported in this study suggested that foodborne pathogens are able to survive in soil and fresh-cut lettuce and capable to attach and invade the epithelium cells of the host.

Is biopreservation a good mitigation strategy to improve lettuce safety?

With the aim of investigating how different native microbiota concentrations could influence pathogens survival, our results showed that the pathogens behaved similarly on shredded conventional and organic lettuce during the first days despite the differences on the initial microbiota load of lettuce. At the end of storage period *L. monocytogenes* seemed to be affected by the native microbiota only on organic lettuce while *E. coli* O157:H7 seemed to be affected only by the native microbiota from conventional lettuce.

Regardless the production system for both pathogens, populations of mesophilic bacteria increased during storage period reaching final populations of about 8.0 log cfu/g and no differences between the three initial loads were observed.

Thus, the behavior of the human pathogens on produce is not simply related to the size of the native microbiota but also to the removal or reduction of key competitive subpopulations of the microbiota present.

Eight out of one hundred and twelve putative antagonists were effective against *S. enterica* on lettuce discs at 20 ºC and were identified into five different genera. When tested against *L. monocytogenes* 230/3, only *Pseudomonas* sp. strain M309 (M309) was able to reduce pathogen counts more than 1.0-log unit at the same conditions. When tested at 10 ºC this microorganism showed high efficacy to control *S. enterica* and *E. coli* O157:H7 growth on lettuce discs. However, M309 strain did not have significant effect on the growth of *L. monocytogenes* during the same storage period.

M309 strain was selected for further assays on fresh-cut lettuce stored under MAP at 10 ºC and was compared with other biopreservative agents such as nisin, bacteriophages (Salmonelex or Listex) and *Pseudomonas graminis* CPA-7. The addition of the biopreservative agents did not result in a significant reduction of *Salmonella* population. However, CPA-7 strain together with nisin reduced *L. monocytogenes* population after 6 days of storage at 10 ºC. The cocktail of *Salmonella* and *L. monocytogenes* was not markedly inactivated by the respective bacteriophage solutions.

This study highlighted the potential of biocontrol but the combination with other technologies may be required to improve their application on fresh-cut lettuce.

Future perspectives

The results obtained in this thesis allowed us to open new range of possibilities, such as:

- More studies are needed in order to determine if foodborne pathogens are able to internalize to lettuce leaves via root system.
- To study the effect of different active modified atmospheres on the growth of foodborne pathogens on minimal processed lettuce.
- To determine the transcriptional expression of virulence genes that can be affected by the sequential incubations as applied in the present study.
- To study possible applications of M309 and CPA-7 strains as biopreservatives in other vegetables.
- To optimize the application of bacteriophages in order to improve their efficacy on minimal processed vegetables.
- Under the concept of hurdle technologies, to investigate the possibility of combine the application of different biopreservatives.
- To analyze the overall quality and the shelf-life of minimal processed lettuce treated with different biopreservation methods.

ANNEX

Application of Modified Atmosphere Packaging as a safety approach to fresh-cut produce – A review

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Abstract

This review provides an overview of the effect of modified atmosphere packaging (MAP) on survival and growth of foodborne pathogens on fresh-cut fruits and vegetables. There has been a substantial technological advance in this area, mainly in improvement of the quality and shelf-life of minimal processed products. Nevertheless, attention must be paid on the survival and growth of pathogenic microorganisms that can be present in produce. MAP in combination with refrigerated temperatures could be used as a mild preservation technique for safety of minimally processed produce. However, the effect of MAP on microorganisms can vary depending mainly on the storage conditions and the type of produce packaged.

Keywords

Modified atmosphere packaging; Foodborne pathogens; Fresh-cut fruits and vegetables.

Fresh-cut fruits and vegetables

The International Fresh-cut Produce Association (IFPA) defines fresh-cut products as fruits or vegetables that have been trimmed, peeled, or cut into 100 % usable product that is bagged or pre-packaged to offer consumers high nutrition, convenience, and flavour while still maintaining freshness. Fresh-cuts are one category of minimally, or lightly, processed fruits and vegetables, which should be in a raw state, not frozen or thermally processed, and ready to eat or cook. The terms used to refer to these kind of products are 'fresh-cut' (the more commonly used in USA), 'minimally processed' and 'ready to eat' (more common in Europe), 'pre-prepared' and 'ready to use', and also '4e Gamme' (more common in France, Italy and Spain).

During the last decade there has been a growth in the market for fresh prepared fruit and vegetable products. The main driving force for this market growth is the increasing consumer demand for fresh, healthy, convenient and additive-free prepared product items. Moreover, different organizations (WHO, FAO, USDA, EFSA) recommend the increasing fruit and vegetable consumption to decrease the risk of cardiovascular diseases and cancer.

The United Kingdom (UK) is the largest fresh-cut fruits and vegetables market in the European Union (EU), accounting for around a third of total EU consumption. In Germany and Spain, this sector is still in an early stage of development. However, despite the economic crisis, the fresh-cut fruits and vegetables market has shown continuous growth during the recent years. Sales of ready-to-eat vegetables showed an annual increase of 5-6 %, with 70,600 and 74,064 tons in 2010 and 2011, respectively (Anonymous, 2013). Nevertheless, after this period, the Spanish market stabilized and showed approximately 77,000 tons sales in 2013 (Anonymous, 2014).

Various steps are included in the preparation of fresh-cut products, each of which are specific unit operations (Figure 1). Each unit operation must be performed properly to ensure that finished product quality, shelf-life, and food safety are satisfactory (Gorny, 1996). Minimally processed produce deteriorates owing to physiological ageing, biochemical changes and microbial spoilage, which may result in degradation of the colour, texture and flavor (Varoquaux and Wiley, 1994). While conventional food-processing methods extend the shelf-life of fruit and vegetables, the minimal processing to which fresh-cut fruit and vegetables are subjected renders products highly perishable, requiring chilled storage to ensure a reasonable shelf-life and safety. Modified atmosphere packaging (MAP) has been successfully and widely used in combination with refrigeration for whole and minimally processed fruits and vegetables as a packaging strategy to maintain product safety and to extend the shelf-life of these products (Werner and Hotchkiss, 2006).

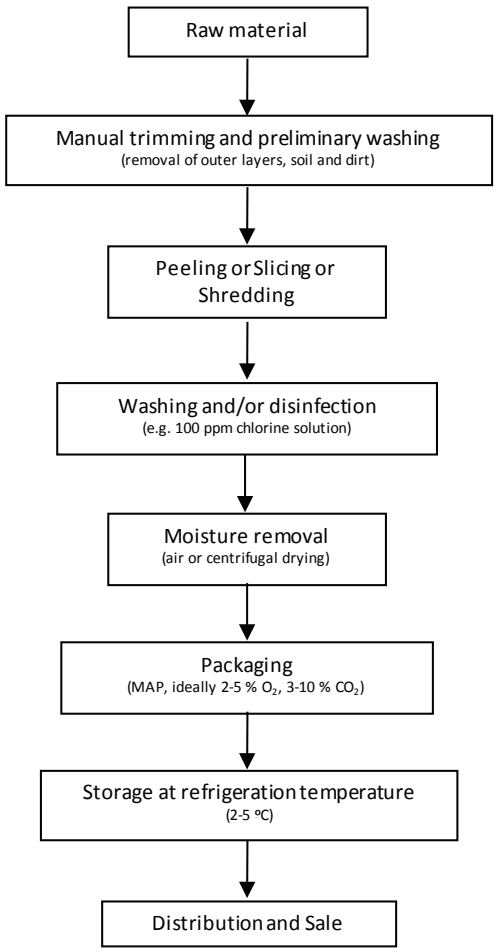

Figure 1. A flow diagram for the production of minimally processed fruits and vegetables.

Pathogens associated with fruits and vegetables

Fresh-cut fruits and vegetables harbour large and diverse populations of microorganisms, such as bacteria, yeasts and fungi that cause spoilage (Abadias *et al.*, 2008). Eighty to ninety percent of bacteria are Gram-negative, predominantly *Pseudomonas* and *Enterobacteriaceae* species (Nguyen-The and Prunier, 1989). Lactic acid bacteria (LAB) is normal flora of fruits and vegetables and associated with spoilage organisms, causing unpleasant odours due to the production of ethanol, organic acids, esters and CO_2 (Fleet, 1992). Yeasts and moulds (YM) are present in smaller numbers than bacteria and when present in high numbers, can contribute to spoilage of fermented products and the development of soft rot (Fleet, 1992).

Epidemiological surveys have demonstrated that fresh-cut produce could also harbour pathogenic bacteria capable of causing human infections, such as *Salmo-*

nella spp., *L. monocytogenes* and *E. coli* O157:H7 (Abadias *et al.*, 2008; Beuchat, 1996; Francis *et al.*, 1999; Nguyen-The and Carlin, 1994; Sagoo *et al.*, 2003). Many factors can contribute to the contamination of fresh and fresh-cut produce with human pathogens. Preharvest contamination of produce can occur directly or indirectly via animals, insects, water, soil, dirty equipment, and human handling. At the postharvest level, wash water, workers, packing materials, process equipment, and transportation vehicles are potential sources of contamination (Beuchat and Ryu, 1997).

Due to the occasional presence of foodborne pathogens on produce, several outbreaks associated to fruits and vegetables consumption have been reported. For example, melons, tomatoes, pears, watermelons, strawberries, mangoes, grapes, spinach and lettuce have been implicated in outbreaks caused by *Salmonella* spp. and *E. coli* O157:H7 (CDC, 2007; Ethelberg *et al.*, 2010; Friesema *et al.*, 2007, 2008; Harris *et al.*, 2003). *L. monocytogenes* has been implicated in outbreaks linked to contaminated lettuce, broad-leaved endive, broccoli, radishes, cabbages, potatoes, cucumbers and melons (Beuchat, 1996; Carlin and Nguyen-The, 1994; CDC, 2011; Farber and Peterkin, 1991; Little and Gillespie, 2008).

During processing, many vegetable cells are broken and their released intracellular products may enhance bacterial growth. To minimize the risk of infection or intoxication associated with the consumption of contaminated fresh-cut fruits and vegetables, specific measures and interventions to prevent and/or minimize the risk of contamination should be considered and implemented.

A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce initial bacterial populations on minimally processed produce (Beuchat, 1998). Chlorine is the most widely used sanitizer in the fresh produce industry. However, studies indicate that chlorine concentrations traditionally used (50-200 ppm) are not effective in reducing pathogen load on fresh cut produce (Behrsing *et al.*, 2000; Delaquis *et al.*, 2002; Lee and Baek, 2008). Moreover, a prolonged exposure to chlorine vapour may cause irritation to the skin and respiratory tract of the workers and also adversely affect the environment (Beuchat, 1998). In addition, chlorinated organic compounds, such as trihalomethanes, can be produced when the chlorine contacts the organic matter.

MAP in combination with refrigerated temperatures could be used as a mild preservation technique to enhance safety of minimally processed produce. However, the effect of MAP on microorganisms can vary depending mainly on the storage conditions and the type of produce packaged.

Packaging Technology

Modified atmosphere packaging of fresh produce relies on modification of the atmosphere inside the package, achieved by the natural interaction between the respiration rate of the produce and the transfer of gases through the packaging material. This can be achieved by using active or passive MAP. Active MAP occurs by the displacement or replacement of gases in the package, or the use of gas scavengers or absorbers to establish a desired mixture of gases, while passive MAP occurs when the product is packaged using a selected film type, and a desired atmosphere developed naturally as a consequence of the products' respiration and the diffusion of gases through the film (Lee *et al.*, 1996).

MAP is used with various types of products, where the mixture of gases in the package depends on product type, packaging materials and storage temperature. If the permeability (for O_2 and CO_2) of the packaging film is adapted to the product respiration, an equilibrium modified atmosphere will establish in the package and the shelf-life of the product will increase.

Oxygen, CO_2 and N_2, are the gases most often used in MAP. During product storage, O_2 is consumed and CO_2 is generated as a result of produce respiration. Nitrogen is an inert gas which is used as filler gas in MAP to balance the volume decrease due to CO_2 absorption and to prevent package collapse (Sandhya, 2010). Generally, an atmosphere of 3 to 6 % O_2 and 2 to 10 % CO_2 achieves microbial control and extension of shelf life for a wide variety of fresh-cut produce (Gorny, 1997; Table1). Moreover, other gases such as helium, argon and xenon (noble gases) and nitrous oxide (N_2O) have been reported to be used in MAP applications to reduce microbial growth and maintain the quality of produce (Meng *et al.*, 2012; Rocculi *et al.*, 2004; Tomás-Callejas *et al.*, 2011; Zhang *et al.*, 2008). However, the effect of these gases on foodborne pathogens has not been studied. The application of high O_2 or superatmospheric levels was also reported to inhibit enzymatic discoloration, preventing anaerobic fermentation reactions, and influencing aerobic and anaerobic microbial growth (Van der Steen *et al.*, 2002). As with most MAP gases, an atmosphere with high O_2 concentration has different effects depending on the commodity.

Of the major gases used in MAP, CO_2 is the only that has significant and direct antimicrobial effect. Broadly, dissolved CO_2 inhibit microbial growth, affecting the lag phase, maximum growth rate and maximum population densities reached (Devlieghere and Debevere, 2000). The inhibitory effect of CO_2 is not universal and this is dependent on the microorganism and growth phase, temperature, water activity and produce characteristics. The mode of action on bacteria is thought to be due to a number of effects, including changes in intracellular pH, alteration of microbial protein and enzyme structure and function, and alteration of cell membrane function and fluidity (Davies, 1995).

Table 1. Modified atmosphere storage recommendations for selected fresh-cut fruits and vegetables.

Product	Temperature (°C)	Atmosphere	
		O_2 (%)	CO_2 (%)
Fresh-cut Vegetables			
Broccoli	0-5	2-3	6-7
Shredded cabbage	0-5	5-7.5	15
Shredded, sticks or sliced carrots	0-5	2-5	15-20
Sliced leek	0-5	5	5
Chopped butterhead lettuce	0-5	1-3	5-10
Chopped green leaf lettuce	0-5	0.5-3	5-10
Chopped or shredded iceberg lettuce	0-5	0.5-3	10-15
Chopped red leaf lettuce	0-5	0.5-3	10-15
Chopped romaine lettuce	0-5	0.5-3	5-10
Sliced mushrooms	0-5	3	10
Sliced or diced onion	0-5	2-5	10-15
Diced peppers	0-5	3	5-10
Sliced or whole-peeled potato	0-5	1-3	6-9
Sliced rutabaga	0-5	5	5
Cleaned spinach	0-5	0.8-3	8-10
Sliced tomato	0-5	3	3
Sliced zucchini	5	0.25-1	-
Fresh-cut Fruits			
Sliced apple	0-5	<1	-
Cubed cantaloupe	0-5	3-5	6-15
Cubed honeydew	0-5	2	10
Sliced kiwifruit	0-5	2-4	5-10
Sliced orange	0-5	14-21	7-10
Sliced peach	0	1-2	5-12
Sliced pear	0-5	0.5	<10
Sliced persimmon	0-5	2	12
Arils (seed coating) pomegranate	0-5	-	15-20
Sliced strawberry	0-5	1-2	5-10

Reproduced from Gorny, 1997.

As mentioned previously, the use of MAP for fresh-cut produce implies a careful selection of the film and package type for each product. In addition, several film characteristics are important to select the appropriate packaging, such as the protection provided, the strength, sealability and clarity, ability to label, and the gas gradient formed by the closed film (Zagory, 1995).

Besides film characteristics, temperature control is very important in order for a MAP system to work effectively. Temperature strongly affects respiration rate, permeability of gases through packaging films and therefore, atmosphere changes within the packaging could occur (Hertog *et al.*, 1998; Jacxsens *et al.*, 2000). Furthermore, storage temperature is one of the most important factors that affect survival and growth of pathogens on fresh-cut produce. Maintaining produce temperature at or below 4 ºC throughout the cold-chain is essential for microbial safety.

Influence of packaging atmosphere on growth and survival of pathogens on fresh-cut produce

The growth and survival of microorganisms in fresh-cut fruits and vegetables is significantly influenced by the intrinsic properties of the produce, as well as by extrinsic factors. Produce vary in their intrinsic factors, for instance, pH, nutrient composition, water activity, natural microbiota, presence of natural antimicrobial compounds, among others. Due to commodity differences and extrinsic factors applied in the individual works, a wide variability of pathogens behaviour was observed on MAP studies. To characterize the behaviour of *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. on fresh-cut fruits and vegetables under MAP conditions, we reviewed the scientific literature and summarized the available data (Table 2 to 4).

E. coli O157:H7

E. coli O157:H7 is an ecologically fit microorganism with superior ability to survive and grow in extraintestinal environments and has been connected to several outbreaks in fresh-cut fruits and vegetables. These observations have led to concern about the survival of faecal pathogenic bacteria on produce.

Fresh-cut produce stored at temperatures ≤ 8 ºC led to different outcomes, ranging from slight growth to no change or to measurable losses in viability (Table 2). For example, Abdul-Raouf *et al.* (1993) and Oliveira *et al.* (2010) reported declines in cell populations on fresh-cut lettuce at 5 ºC while Koseki and Isobe (2005) found no change at this temperature. Posada-Izquierdo *et al.* (2013) reported high variability of growth data between replicates on iceberg lettuce stored at 8 ºC under passive MAP with very low O_2 levels (< 0.5 kPa). However, Francis and O'Beirne (2001)

observed an increase of *E. coli* O157:H7 populations on iceberg lettuce and dry coleslaw under passive MAP at the same temperature.

Sharma *et al.* (2011) measured a more considerably decrease of *E. coli* O157:H7 populations on iceberg lettuce stored at 4 ºC in Air than in high CO_2/low O_2 atmosphere. On baby spinach stored at 4 and 7 ºC, Brown *et al.* (2011) observed an inhibitory effect of Air packaging and active MAP (high O_2 level) compared with high nitrogen atmosphere. However, Bae *et al.* (2011) reported that active MAP with 100 % of CO_2 and 100 % of N_2 were effective in delaying growth of the pathogen on white cabbage stored at 10 ºC.

Oliveira *et al.* (2010) observed that the O_2 and CO_2 levels developed in the different types of films did not affect the survival and growth of *E. coli* O157:H7 on 'Romaine' lettuce stored at 5 and 25 ºC. Similar results were found by Abdul-Raouf *et al.* (1993) on iceberg lettuce, cucumber and carrots, and Abadias *et al.* (2012) on curly endive and carrot, who demonstrated that low O_2 atmosphere, had no apparent effect on survival and growth of *E. coli* O157:H7 at different temperatures.

A similar fluctuation of pathogen behaviour at refrigerated temperatures was also observed on fresh-cut fruits. Raybaudi-Massilia *et al.* (2009a, b) observed reduced pathogen counts on fresh-cut apples and pears under passive MAP conditions at 5 ºC for 30 days. However, Alegre *et al.* (2010a) found that *E. coli* O157:H7 populations fluctuated over time on fresh-cut apples under passive MAP conditions at the same temperature but no differences between MAP and Air were found.

On fresh-cut strawberries stored at 7 ºC, *E. coli* O157:H7 was unable to grow in equilibrium-modified atmosphere (EMA, 3 % O_2/5 % CO_2) and high-oxygen atmosphere (HOA, 95 % O_2/5 % N_2) but it slightly grew until day 5 under Air conditions (Siro *et al.*, 2006).

E. coli O157:H7 grew on fresh-cut fruits at room temperature regardless the MAP conditions. However, there is insufficient information available on whether gas atmospheres inhibit or enhance its growth. CO_2 had little or no inhibitory effect on pathogen growth on fresh-cut melon stored at 25 ºC under high CO_2 levels of 11, 25 and 39 % after 1, 2 and 3 days of storage (Abadias *et al.*, 2012).

A significant effect of the commodity, more than the effect of MAP or temperature, was observed on pineapple and on cactus-pear fruit. On fresh-cut pineapple, the pathogen was unable to grow at 5 and 25 ºC regardless the packaging atmospheres (Abadias *et al.*, 2012). Furthermore, *E. coli* O157:H7 was able to grow on cactus-pear fruit stored at 4 and 8 ºC but not at 20 ºC in Air and active MAP (5 % O_2/ 30 % CO_2/65 % N_2) conditions (Corbo *et al.*, 2005).

Table 2. Overview of the impact of MAP storage on growth and survival of *E. coli* on fresh-cut vegetables and fruits

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7	Romaine lettuce	Lettuce samples were packaged under three different atmospheric conditions: air and two passive MAP. Two films of O_2 permeability of 3500 cm^3/m^2/day/atm (Film I) and 1100 cm^3/m^2/day/atm (Film I) were used. Bags were stored at 5 ºC for 10 days and 25 ºC for 3 days.	Populations of *E. coli* on lettuce slightly decreased during the 10-day storage period at 5 ºC, whereas they increased after 3 days at 25 ºC. Populations in Film II (the least permeable) were greater than in the other two package films at 25 ºC. No differences were observed on samples stored at 5 ºC. Differences in gases levels between films were observed only at 5 ºC, where O_2 levels decreased slowly reaching values of 8.0 and 6.0 % approximately for film I and film II, respectively. The CO_2 increased slowly and reached levels around 6.0 % and 8.0 % for film I and film II, respectively. At 25 ºC, gases changed very fast and reached O_2 values around 0.8 % and around 12 % for CO_2.	Oliveira *et al.*, 2010
E. coli O157:H7	Iceberg lettuce	Three different MAP conditions were applied: (1) film permeability of 110 cc O_2/100 in^2/24 h (cm^3/m^2/24 h) and initial flush with N_2 to reach O_2 level of 2 %; (2) same film permeability with microperforations, near-ambient air atmospheric conditions; (3) high CO_2 and low O_2 levels in gas-impermeable film. All samples were stored for 10 d at 4 and 15 ºC.	A decrease in population counts was observed in all treatments at 4 ºC, but more considerably (1.70 log cfu/g) under treatment (2). In treatment 1, the O_2 decreased quickly to 1.5 % and maintained throughout storage. In treatment 3 O_2 declined rapidly reaching to 0 % on day 3. CO_2 increased gradually in treatment 1, reaching 8.9 % on day 10. For treatment 3, a rapidly accumulation of CO_2 (34 %) was observed after 10 days. At 15 ºC, *E. coli* populations increased by at least 2.7 log cfu/g under all storage conditions. Same results of gases levels were observed but were achieved more rapidly.	Sharma *et al.*, 2011

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7 cocktail	Iceberg lettuce	Lettuce was packaged in a film with O_2 permeability of 504 mL/m^2/day atm. Samples packaged in passive MAP were stored at 4, 8, 13 and 16 °C.	Storage at 4 °C produced a slight decrease of *E. coli* O157:H7. *E. coli* O157:H7 was able to grow at 8, 13 and 16 °C under very low O_2 levels (< 0.5 kPa). CO_2 increased during storage up to 14 kPa approximately for all temperatures. Samples stored at 8 °C, presented a high variability of growth data between replicates. This can be explained by the fact that 8 °C might be the temperature defining growth/non growth for this pathogen.	Posada-Izquierdo *et al.*, 2013
E. coli O157:H7 cocktail	Iceberg lettuce, cucumber and carrots	Lettuce was packaged in a film with O_2 permeability of 3000 cm^3/m^2/day. Cucumbers and carrots were packaged in a film with O_2 permeability of 7000 cm^3/m^2/day. Bags were sealed under air conditions or active MAP containing 3 % O_2 and 97 % N_2 and stored at 5 or 12 °C for 14 days or at 21 °C for 7 days.	Populations of *E. coli* declined on vegetables stored at 5 °C and increased on vegetables stored at 12 and 21 °C. Packaging under active MAP had no apparent effect on *E. coli* O157:H7 counts.	Abdul-Raouf *et al.*, 1993
E. coli O157:H7 (two strains)	Iceberg lettuce, swedes (rutabaga), dry coleslaw mix (80 % shredded cabbage, 20 % shredded carrot), raw soybean sprouts	Vegetables were packaged in bags with O_2 permeability of 1200 mL/m^2/day/atm and stored at 4 and 8 °C for 12 days.	*E. coli* populations increased during storage on lettuce at 8 °C. The pathogen also increased on coleslaw and soybean sprouts but up to day 5, then declined until the end of storage. Differences were observed between strains on packaged swedes at 8 °C. In lettuce bags, CO_2 increased to 10-12 % and O_2 fell to 3-4 %. O_2 fell to 2 % and CO_2 rose to 8-10 % in bags of sweeds. In dry coleslaw mix and soybean sprouts bags, CO_2 rose to 25-27 % and O_2 fell to 0-1 %. At 4 °C, reduced growth of *E. coli* was observed.	Francis & O'Beirne 2001

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli	Sliced carrots, butterhead and iceberg lettuce	Samples were packaged in 35 μm oriented polypropylene film and butterhead samples were flushed with 100 % N_2 before sealing. Film permeability not mentioned. All samples were stored at 8 °C for 9 days.	*E. coli* populations declined on carrots and both lettuce varieties. In packages of sliced carrots, butterhead and iceberg lettuce, gas concentration was: O_2 0-5 %, 0-1 % and 5-11 % and CO_2 approximately 8-18 %, 4-8 % and 5-7 %, respectively. Gas levels observed were unlikely to have had adverse effects on the growth patterns of *E. coli*.	Gleeson & O'Beirne 2005
E. coli O157:H7	Curly endive and carrot	Samples of fresh-cut carrot or endive were packaged under three different atmospheric conditions: air and two passive MAP. Two films of O_2 permeability of 3500 $cm^3/m^2/day/atm$ (Film I) and 1100 $cm^3/m^2/day/atm$ (Film II) were used. Bags were stored at 5 °C for 10 days.	The pathogen population significantly declined in endive during storage. O_2 in Film I decreased from 20.7 % to 9.4 % after day 8 and remained unchanged and progressively decreased in Film II. O_2 in Film II were lower than in Film I, with a final value of 3.7 %. CO_2 gradually increased but never exceeded 10 %. *E. coli* populations decreased in carrots. O_2 in Film I decreased during the first day and then no differences were observed. In Film II, O_2 decreased, reaching 0.2 % at the end of the storage. CO_2 increased in Film II throughout storage and reached 18 %. The concentrations in Film I bags were significantly lower at each tested time.	Abadias *et al.*, 2012
E. coli O157:H7 cocktail	Spinach	After treated with 100-ppm chlorine dioxide or 100-ppm sodium hypochlorite, spinach was packaged in bags with oxygen permeability of 55 cc μm/kPa d m^2. Four different packaging methods were applied: (1) air; (2) vacuum; (3) CO_2 (100 %); (4) N_2 (100 %). All samples were stored for 7 d at 7 °C.	The pathogen grew during storage in samples packaged in air. Vacuum and active MAP atmospheres were effective on maintaining *E. coli* levels.	Lee & Baek 2008

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7 cocktail	Baby spinach	Commercially packaged baby spinach was inoculated and resealed (passive MAP). Film permeability was not mentioned. Samples were stored at 1, 5, 8 and 12 ºC for 12 days.	The pathogen grew at 12 ºC and at 8 ºC. *E. coli* populations on samples stored at 5 and 1 ºC declined over time. There was a rapid increase in CO_2 and decrease in O_2 within the first 3 days after sealing bags at all temperatures. After that, package atmosphere remained relatively stable until the end of storage. The gases levels inside packages depended on temperature.	Luo *et al.*, 2009
E. coli O157:H7	Spinach	Samples were packaged in bags with O_2 permeability of 450 cc/100 sq. in. Bags were sealed under 30 % vacuum and stored at 4 ºC or 10 ºC for 15 days.	A slight decrease in *E. coli* populations was observed at 4 ºC, while at 10 ºC resulted in a small increase of the pathogen. Gases concentration depended on temperature and treatments applied before packaging. Generally, O_2 dropped to a maximum of 14 % and CO_2 increased no more than 2 % during storage in all treatments.	Lopez-Velasco *et al.*, 2010
E. coli O157:H7 cocktail	Baby spinach	Two active MAP conditions containing 80 % O_2, 20 % CO_2 (high oxygen) and 80 % N_2, 20 % CO_2 (nitrogen) and air were created. For high oxygen or nitrogen atmospheres, samples were packaged with O_2 permeability of <20 cc/m^2/24 h. In air conditions, samples were packaged with O_2 permeability of 1450 cc/100 sq. in/24 h. Packages were stored for 9 days at 4 to 7 ºC.	Packaging with air and high oxygen reduced *E. coli* O157:H7 populations by 0.26 and 0.15 logs, compared to nitrogen.	Brown *et al.*, 2011

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7	White cabbage	After treated with 2 % lactic acid, cabbage was packaged in bags with permeability of 55 mL-μm/kPa-s-m^2. Four different packaging methods were applied: (1) air; (2) vacuum; (3) CO_2 (100 %); (4) N_2 (100 %). All samples were stored for 7 d at 10 °C.	*E. coli* increased in cabbage after vacuum packaging after 7 days at 10 °C. Air, N_2 and CO_2 gas packaging were effective in delaying growth of the pathogen after lactic acid treatment.	Bae *et al.*, 2011
E. coli O157:H7	Cabbage	Two films of O_2 permeability of 1,277±159 mL/m^2/day/atm (Film I) and 54.8±0.7 mL/m^2/day/atm (Film II) were used. Six packaging treatments were made with modified atmospheres and films. Two active MAP with a gas mixture of 70 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP1); 5 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP2); a moderate vacuum packaging (MVP) with a vacuum degree of 10.1 kPa. Samples were stored at 5 °C for 10 days.	The populations were kept lower in MAP1 with Film I or Film II than in the other treatments. The population of *E. coli* increased in the MVP treatment with Film II which maintained low O_2 partial pressure during storage. However, no proliferation of this pathogen was observed in MVP with Film I. High levels of O_2 (>40 kPa) were only observed in samples packaged under MAP1 regardless the film used. However, in MAP2 O_2 were less than 10 kPa and in Film II achieved 0 kPa after 5 days. CO_2 concentration increased to more than 20 kPa for MAP1 regardless the film used. Conversely, in MAP2 CO_2 decreased reaching 2 kPa at the end of storage for both films. Gas composition within the packages depended on initial gas conditions and was greatly affected by film permeability.	Lee *et al.*, 2011
E. coli O157:H7	Cactus-pear fruit	Samples were packaged with O_2 permeability of $9.23x10^{-19}$ mol m/m^2/s/Pa. Two different packaging conditions were applied: (1) Air and (2) active MAP 5 % O_2, 30 % CO_2, and 65 % N_2. Samples were stored at 4, 8, 12 and 20 °C for 14 days.	*E. coli* was able to grow only in samples stored at 4 and 8 °C in both package atmospheres. Pathogen was suppressed after 7 days at 20 °C. Differences on the behaviour of *E. coli* between package atmospheres only were observed at 12 °C.	Corbo *et al.*, 2005

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli cocktail	Strawberries	Samples were packaged in a film with O_2 permeability of 4679 mL/m²/day/atm. Three different packaging conditions were applied: (1) Air; (2) active MAP 3 % O_2, 5 % CO_2, balance N_2 (EMA); (3) active MAP, 95 % O_2, 5 % N_2 (HOA). Samples were stored at 7 ºC for 14 days.	The pathogen was unable to grow on EMA and HOA, but it survived. A slight growth until day 5 was observed in Air and then started to decrease. In EMA bags, O_2 presented little variation during storage (1.0-6.4 %) and CO_2 also remained constant (5.0-8 %) after 14 days. In HOA bags, O_2 rapidly decreased from 95 to 10.9 % in 7 days and reached a minimum value of 4.7 % on day 12. CO_2 were around 5-8 %.	Siro *et al.*, 2006
E. coli cocktail	Raspberries	Samples were packaged in a film with O_2 permeability of 3200 mL/m²/day/atm. Three different packaging conditions were applied: (1) Air; (2) active MAP 3 % O_2, 5 % CO_2, balance N_2 (EMA); (3) active MAP, 95 % O_2, 5 % N_2 (HOA). Samples were stored at 7 ºC for 14 days.	The pathogen survived during storage in all atmosphere packages. In HOA packages, *E. coli* presented higher counts than in EMA and Air. In EMA bags, O_2 remains stable around 9.5-12.8 % and CO_2 decreased slightly to 3.2-4.8 % after 14 days. In HOA bags, O_2 rapidly decreased from 95 to 10.4 % in 7 days and remained at 7.5 % until day 14. CO_2 were around 5-8 %.	Siro *et al.*, 2006
E. coli O157:H7	Apples	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) pear samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m²/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	*E. coli* populations were undetectable from the day 3 when treated with CGLW+MA. A decrease of pathogen counts was observed on apples dipped with CGLW or W during storage. The MAP in the packages did not affect in a direct way the survival or decrease of the pathogen.	Raybaudi-Massilia *et al.*, 2009a

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7	Apples	Apple trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*E. coli* populations increased at 25 ºC and at 5 ºC it fluctuated over time, but the final population was reduced. Differences in growth of the pathogen in MAP and in Air were not observed. O_2 decreased continuously throughout storage. At 25 ºC declined to 17.0 % and at 5 ºC 19.6 %. In contrast, CO_2 increased during storage. The rise was more significant in 3 days at 25 ºC (6.8 %) than in 14 days at 5 ºC (2.8 %).	Alegre *et al.*, 2010a
E. coli O157:H7	Pears	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) pear samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m^2/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	*E. coli* populations were undetectable from the day 14 when treated with CGLW+MA. A slight decrease of pathogen counts was observed on pears dipped with CGLW or W. The MAP in the packages did not affect in a direct way the survival or decrease of the pathogen.	Raybaudi-Massilia *et al.*, 2009b
E. coli O157:H7	Peaches	Peach trays were sealed in a film with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*E. coli* populations increased at 25 ºC and at 5 ºC it decreased throughout storage time. Differences of pathogen growth between MAP and Air were not observed. At 25 ºC, O_2 decreased sharply after 24 h to 13.2 % and then stabilized. The same happened to CO_2 that increased exponentially to 24.6 % after two days and then remain stable. At 5 ºC, O_2 only reduced by 2.5 % and CO_2 increased to 3.9 % after 14 days.	Alegre *et al.*, 2010b

Microorganism	Vegetables and Fruits	MAP Conditions/Temperature*	Outcomes	References
E. coli O157:H7	Melon	Melon trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*E. coli* grew very well at 25 ºC regardless of the packaging atmosphere, with an increase of 4-log units after one day of storage. No growth was observed at 5 ºC, but cells survived throughout the storage and decreased significantly between 10 and 14 days in MAP. No significant gases changes were observed at 5 ºC, values were closed to those of atmospheric conditions. On the contrary, CO_2 rose to 39.4 % after 3 days at 25 ºC, respectively. O_2 decreased by 2.2 % during the same time period.	Abadias *et al.*, 2012
E. coli O157:H7	Pineapple	Samples were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 8 days. Samples were packaged in passive MAP and in Air.	*E. coli* was unable to grow on fresh-cut pineapple at both studied temperatures. At 5 ºC, *E. coli* survived during the whole experiment, with a significant population decrease after 8 days in MAP. CO_2 and O_2 remained almost constant, with values close to those of atmospheric conditions. At 25 ºC, the CO_2 increased rapidly, with values higher than 38 % after 2 days and a final value of 50.3 % at the end of the experiment. The packages reached anaerobic conditions after 2 days of storage.	Abadias *et al.*, 2012

*Permeability units are shown as indicated in the original paper.

Salmonella spp.

Outbreaks of salmonellosis are traditionally associated with consumption of food of animal origin. However, in recent years, a range of raw and minimally processed fruits and vegetables have been implicated in *Salmonella* spp. infections (Doyle and Erickson, 2008; Hanning *et al.*, 2009; Sivapalasingam *et al.*, 2004) and thus, becoming a foodborne pathogen of particular concern.

Salmonella spp. presented a similar behaviour to *E. coli* O157:H7 under storage temperatures in the different studies (Table 3). Growth is usually evident at storage temperatures of ≥ 8 ºC but growth rates and maximum population densities are variable (Alegre *et al.*, 2010a, b; Bae *et al.*, 2011; Oliveira *et al.*, 2010). In carrots and lettuce, *S.* Enteritidis survived or decreased during storage at 4 ºC (Kakiomenou *et al.*, 1998; Tassou and Boziaris, 2002), regardless of the MAP conditions studied. Although, *Salmonella* spp. is unable to grow where storage temperature is adequate, the pathogen survives for a long time at refrigerated temperatures on produce (ICMSF, 1996).

S. Typhimurium populations on lettuce were higher in active MAP than in Air and passive MAP at 8 and 20 ºC (Horev *et al.*, 2012). In this study CO_2-enriched atmosphere did not result in reduced pathogen populations. Similar results were obtained by Sant'Ana *et al.* (2013) who observed higher *Salmonella* spp. populations under active MAP with high CO_2 levels than in Air conditions at 7 ºC in lettuce salad and collard greens. On the other hand, Bae *et al.* (2011) observed that active MAP with high N_2 and CO_2 levels were effective in delaying *S.* Typhimurium growth on white cabbage at 10 ºC. However, under vacuum packaging the pathogen slightly increased in cabbage at 5 and 10 ºC (Bae *et al.*, 2011; Lee *et al.*, 2011).

Although the behaviour of *Salmonella* spp. on fresh-cut vegetables stored under MAP conditions has been well studied, data for fresh-cut fruits are scarce.

In general, when stored at refrigeration conditions, *Salmonella* spp. on fresh-cut fruits decreased during storage, regardless of the packaging atmosphere. *Salmonella* spp. was unable to grow and showed a pronounced decrease in equilibrium-modified atmosphere (EMA, 3 % O_2/5 % CO_2) and high-oxygen atmosphere (HOA, 95 % O_2/5 % N_2) compared with Air conditions on fresh-cut raspberries at 7 ºC (Siro *et al.*, 2006). Under passive MAP, Raybaudi-Massilia *et al.* (2009a, b) reported a slight decrease of the *S.* Enteritidis populations on fresh-cut apples and pears at 5 ºC. Similarly, Alegre *et al.* (2010a, b) found that *S.* Michigan populations decreased over time on fresh-cut apples and peaches at the same temperature but no differences between MAP and Air were found. On the other hand, the pathogen increased on fresh-cut peaches when samples were stored at 25 ºC, where the CO_2 level was more than 20 % (Alegre *et al.*, 2010b).

Table 3. Overview of the impact of MAP storage on growth and survival of *Salmonella* spp. on fresh-cut vegetables and fruits

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
S. Michigan	Romaine lettuce	Lettuce samples were packaged under three different atmospheric conditions: air and two passive MAP. Two films of O_2 permeability of 3500 $cm^3/m^2/day/atm$ (Film I) and 1100 $cm^3/m^2/day/atm$ (Film II) were used. Bags were stored at 5 °C for 10 days and 25 °C for 3 days.	At 5 °C, populations decreased almost 1-log unit over the storage period. Differences in gases levels between films were observed only at 5 °C, where O_2 decreased slowly reaching values of 8.0 and 6.0 % approximately for film I and film II, respectively. CO_2 increased slowly and reached levels around 6.0 % and 8.0 % for film I and film II, respectively. Populations of *Salmonella* stored at 25 °C into Film II (the least permeable) increased by approximately 1-log unit more when compared with the other packages. Gases changed very fast and reached O_2 values around 0.8 % and around 12 % for CO_2. Populations were lower on lettuce with Air atmosphere than in all other MAP at both temperatures.	Oliveira *et al.*, 2010
S. Typhimurium	Romaine lettuce	Lettuce samples were packaged with O_2 permeability of 5700 $cm^3/m^2/day/atm$. Three different packaging conditions were applied: (1) Air; (2) passive MAP; (3) active MAP, 80 % N_2, 10 % O_2, 10 % CO_2. Samples were stored at 8 °C for 7 days and at 20 °C for 3 days.	Lettuce in active MAP at both temperatures was continuously exposed to lower concentrations of O_2 and higher concentrations of CO_2 than the samples in passive MAP. *S.* Typhimurium populations were higher in the active MAP treatment than in the others.	Horev *et al.*, 2012

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
Salmonella spp. cocktail: *S.* Typhimurium, *S.* Enteritidis	Lettuce salad: Iceberg and Crisp Collard greens	Samples were packaged in MAP with O_2 permeability of 1375 m^3/m^2/day and in perforated film (Air). An active MAP containing 5 % O_2, 15 % CO_2 and 80 % N_2 was created and samples were stored at four different conditions: (1) 6 days at 7 ºC; (2) 4.2 days at 7 ºC and 1.8 days at 15 ºC; (3) 1.8 days at 7 ºC and 4.2 days at 15 ºC; (4) 6 days at 15 ºC.	Higher *Salmonella* populations were observed in lettuce than in collard greens. In both vegetables, *Salmonella* was inhibited when perforated film (Air) packaging was used.	Sant'Ana *et al.*, 2013
Salmonella spp. cocktail: *S.* Typhimurium, *S.* Typhi., *S. enterica*, *S.* Infantis, *S.* Concord	Green salad (crisp, romaine, butter lettuce), cabbage, escarole, collard green, spinach, watercress, arugula, grated carrot, mix for yakisoba (broccoli, cabbage, cauliflower, leek, carrots, chard)	Samples were packaged in bags with O_2 permeability of 1375 m^3/m^2/day. An active MAP containing 5 % O_2, 15 % CO_2 and 80 % N_2 was created and samples were stored at three different conditions: (1) 6 days at 7 ºC; (2) 1.8 days at 7 ºC and 4.2 days at 15 ºC; (3) 6 days at 15 ºC.	At storage condition (1), the pathogen only was able to grow in escarole (approximately 1.0 log) and arugula (approximately 2 log). In cabbage and carrots, the pathogen population decreased. At storage condition (2), Salmonella was able to grow in all vegetables except cabbage and carrots. At storage condition (3), the pathogen was not able to grow in cabbage.	Sant'Ana *et al.*, 2012
S. Enteritidis	Carrots and lettuce	Vegetable samples were packaged in a film with O_2 permeability of 3841 ± 434 mL/m^2/day/atm. Two different active MAP conditions were applied: (1) 4.9 % CO_2, 2.1 % O_2, 93 % N_2; (2) 5 % CO_2, 5.2 % O_2, 89.8 % N_2 and also air conditions. The samples were stored at 4 ºC for 14 days.	In both vegetables numbers of *S.* Enteritidis decreased during storage in all packages systems. The atmosphere in the packages did not affect the pathogen behaviour.	Kakiomenou *et al.*, 1998

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
S. Enteritidis	Carrots	Carrots were packaged in a film with O_2 permeability of 1000 ml/m^2/day/atm and sealed in aerobic conditions or flushed with 4.9 % CO_2, 2.1 % O_2 and 93 % N_2. The samples were stored at 4 ºC for at least 8 days.	*Salmonella* survived in carrots despite the atmosphere conditions and inoculums size.	Tassou & Boziaris, 2002
S. Typhimurium	White cabbage	After treated with 2 % lactic acid, cabbage was packaged in bags with O_2 permeability of 55 mL-µm/KPa-s-m^2. Four different packaging methods were applied: (1) Air; (2) Vacuum; (3) CO_2 (100 %); (4) N_2 (100 %) and stored for 7 d at 10 ºC.	*Salmonella* increased in cabbage after vacuum packaging after 7 days at 10 ºC. Air, N_2 and CO_2 gas packaging were effective in delaying growth of the pathogen.	Bae *et al.*, 2011
S. Typhimurium	Cabbage	Six packaging treatments were made with modified atmospheres and films. Two films of O_2 permeability of 1,277±159 mL/m^2/day/atm (Film I) and 54.8±0.7 mL/m^2/day/atm (Film II) were used. Two active MAP with a gas mixture of 70 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP1); 5 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP2); a moderate vacuum packaging (MVP) with a vacuum degree of 10.1 kPa. Samples were stored at 5 ºC for 10 days.	Little influence in *Salmonella* counts were observed during storage in MAP1 and MAP2. However, the inoculated bacteria in MVP with Film II significantly increased or levelled off. High levels of O_2 (>40 kPa) were only observed in samples packaged under MAP1 regardless the film used. However, in MAP2 O_2 were less than 10 kPa and in Film II achieved 0 kPa after 5 days. CO_2 concentration increased to more than 20 kPa for MAP1 regardless the film used. Conversely, in MAP2 CO_2 decreased reaching 2 kPa at the end of storage for both films. Gas composition within the packages depended on initial gas conditions and was greatly affected by film permeability.	Lee *et al.*, 2011

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
Salmonella spp. cocktail: *S.* Typhimurium, *S.* Enteritidis	Raspberries	Samples were packaged in a film with O_2 permeability of 3200 mL/m^2/day/atm. Three different packages conditions were applied: (1) perforated packages (Air); (2) active MAP 3 % O_2, 5 % CO_2, balance N_2 (EMA); (3) active MAP, 95 % O_2, 5 % N_2 (HOA). Samples were stored at 7 ºC for 14 days.	*Salmonella* was unable to grow and showed a pronounced decrease mainly in HOA and EMA packages. In EMA bags, O_2 remains stable around 9.5-12.8 % and CO_2 decreased slightly to 3.2-4.8 % after 14 days. In HOA bags, O_2 rapidly decreased from 95 to 10.4 % in 7 days and remained at 7.5 % until day 14. CO_2 were around 5-8 %.	Siro *et al.*, 2006
S. Enteritidis	Apples	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) apple samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m^2/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	The CGLW+MA treatment reduced almost totally the population of *Salmonella*. A slight decrease of pathogen counts was observed on apples dipped with CGLW or W. The MAP in the packages did not affect in a direct way the survival or decrease of the pathogen.	Raybaudi-Massilia *et al.*, 2009a
S. Michigan	Apples	The apple trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*Salmonella* populations increased at 25 ºC and at 5 ºC it was reduced. Differences in growth of the pathogen in MAP and in Air were not observed. O_2 decreased continuously throughout storage. At 25 ºC declined to 17.0 % and at 5 ºC 19.6 %. In contrast, CO_2 increased during storage. The rise was more significant in 3 days at 25 ºC (6.8 %) than in 14 days at 5 ºC (2.8 %).	Alegre *et al.*, 2010a

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
S. Enteritidis	Pears	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) pear samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m^2/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	The CGLW+MA treatment reduced almost totally the population of *Salmonella*. A slight decrease of pathogen counts was observed on pears dipped with CGLW or W. The MAP in the packages did not affect in a direct way the survival or decrease of the pathogen.	Raybaudi-Massilia *et al.*, 2009b
S. Michigan	Peaches	The peach trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*Salmonella* populations increased at 25 ºC and at 5 ºC, the population of the pathogen reduced throughout storage time. Differences of pathogen growth between MAP and Air were not observed. At 25 ºC, O_2 decreased sharply after 24 h to 13.2 % and then stabilized. The same happened to CO_2 that increased exponentially to 24.6 % after two days and then remain stable. At 5 ºC, O_2 only reduced by 2.5 % and CO_2 increased to 3.9 % after 14 days.	Alegre *et al.*, 2010b

*Permeability units are shown as indicated in the original paper.

***Listeria* spp.**

L. monocytogenes and *L. innocua* are ubiquitous in the environment and can be isolated from soil, water, vegetation, the faeces of livestock and vegetation irrigated with contaminated water (Heaton and Jones, 2008). These microorganisms are psychrotrophic and can grow over a refrigerated temperature range. Due to its facultative anaerobic and psychrotrophic nature, and the outbreaks linked to produce, concerns about the pathogenic species (*L. monocytogenes*) contamination in MAP produce have been raised. Some studies used *L. innocua* as surrogate of *L. monocytogenes* (Alegre *et al.*, 2010a, b; Francis and O'Beirne, 1997; Gleeson and O'Beirne, 2005).

Numerous studies have reported that survival and growth of *L. monocytogenes* on produce is not reduced or affected by MAP atmosphere (Beuchat and Brackett, 1991; Jacxsens *et al.*, 1999). However, the behaviour of *L. monocytogenes* on fresh-cut produce under MAP and refrigerated temperatures is conflicting (Table 4).

Francis and O'Beirne (1997) observed that nitrogen flushing combined with storage at 3 ºC extended *L. monocytogenes* and *L. innocua* survival on lettuce and at 8 ºC enhanced their growth. Jacxsens *et al.* (2002) examined the behaviour of *L. monocytogenes* on lettuce salad, bell peppers and cucumber subjected to a simulated distribution chain for 7 days under an active MAP (3 % O_2/5 % CO_2) and found that the pathogen was able to grow on cucumber, survived on lettuce salad and decreased on bell peppers. However, in other study, *L. monocytogenes* populations decreased in lettuce and carrots packaged under two active MAP atmospheres stored at 4 ºC (Kakiomenou *et al.*, 1998). Carrasco *et al.* (2008) obtained an increase of *L. monocytogenes* on iceberg lettuce packaged under active MAP (4.6-6.2 % CO_2/2.1-4.3 % O_2) stored at 5 and 13 ºC.

Oliveira *et al.* (2010) found that *L. monocytogenes* grew on lettuce at 5 ºC and the passive modified atmosphere developed in their study did not influence its growth. Niemira *et al.* (2005) reported that passive MAP conditions at 4 ºC, did not affect the growth of *L. monocytogenes* on endive. Contrarily, the same author found that endive packaged under active MAP (5 % O_2/5 % CO_2; 10 % O_2/10 % CO_2) did not support the growth of the pathogen. However, the combination of the gases in active MAP prevented the regrowth of *L. monocytogenes* on the irradiated endive.

High variability of *L. monocytogenes* and *L. innocua* behaviour on fresh-cut fruits under refrigerated temperatures were also observed in the different studies. An active MAP containing 5 % O_2 and 30 % CO_2 had no effect on the growth of *L. monocytogenes* on cactus-pear fruit, the pathogen not only survived but was also able to grow at 4, 8, 12 and 20 ºC (Corbo *et al.*, 2005). Similarly, Sinigaglia *et al.* (2006) observed that *L. monocytogenes* grew at 2, 4, 8 and 10 ºC on coconut packaged under an active MAP containing high CO_2 levels (30 %).

Table 4. Overview of the impact of MAP storage on growth and survival of *Listeria* spp. on fresh-cut vegetables and fruits

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes *L. innocua*	Iceberg lettuce	Three different packages conditions were applied: (1) Air; (2) passive MAP; (3) active MAP, 96 % N_2, 4 % O_2. Samples were stored at 3 and 8 °C for 14 days.	At 3 °C, flushing with N_2 extended *Listeria* spp. survival, while counts in passive MAP and Air were not different. At 8 °C, passive MAP also enabled *Listeria* spp. to survive, but N_2 flushing resulted in higher growth. *L. innocua* and *L. monocytogenes* presented a similar behaviour at this storage conditions. For samples in passive MAP, O_2 fell to 8-10 % (3 °C) and 3-4 % (8 °C) after 10 days. CO_2 increased to 5-7 % (3 °C) and 9-10 % (8 °C). In the active MAP samples, O_2 decreased to 1-2 % and CO_2 rose to 4 %.	Francis & O'Beirne 1997
L. monocytogenes	Iceberg lettuce	Lettuce samples were packaged under active MAP (4.65-6.2 % CO_2, 2.1-4.3 % O_2, balanced with N_2) with O_2 permeability of 1200 cc/m^2/day. Bags were stored at 5 and 13 °C for 14 days.	*L. monocytogenes* grew on lettuce held at 5 and 13 °C with increments of 2.66 and 4.85 log cfu/g, respectively after 14 days. At 13 °C, O_2 decreased rapidly to approximately 0-1 % and CO_2 increased to approximately 25 % at the end of storage. At 5 °C, O_2 ranged between 5 to 2 % and CO_2 increased to approximately 15 %.	Carrasco *et al.*, 2008
L. monocytogenes	Romaine lettuce	Lettuce samples were packaged under three different atmospheric conditions: air and two passive MAP. Two films of O_2 permeability of 3500 cm^3/m^2/day/atm (Film I) and 1100 cm^3/m^2/day/atm (Film II) were used. Bags were stored at 5 °C for 10 days and 25 °C for 3 days.	*L. monocytogenes* was able to grow at 5 °C, approximately 1-log unit in all package films. Passive MAP conditions did not influence its growth. Differences in gases levels between films were observed only at 5 °C, where O_2 decreased slowly reaching values of 8.0 and 6.0 % approximately for film I and film II, respectively. CO_2 increased slowly and reached levels around 6.0 % and 8.0 % for film I and film II, respectively. At 25 °C, gases changed very fast and reached O_2 values around 0.8 % and around 12 % for CO_2.	Oliveira *et al.*, 2010

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. innocua	Butterhead and iceberg lettuce	Samples were packaged in 35 µm oriented polypropylene film and butterhead samples were flushed with 100 % N_2 before sealing. Film permeability not mentioned. All samples were stored at 8 ºC for 9 days.	*L. innocua* grew well on both butterhead lettuce and iceberg lettuce. In packages of sliced butterhead and iceberg lettuce O_2 decreased to approximately 0-1 % and 5-11 % and CO_2 increased to approximately 4-8 % and 5-7 %, respectively. The gas levels observed are unlikely to have had adverse effects on the growth patterns of *L. innocua*.	Gleeson & O'Beirne 2005
L. monocytogenes cocktail	Green salad (crisp, romaine, butter lettuce), cabbage; escarole, collard green, spinach, watercress, arugula, grated carrot, mix for yakisoba (broccoli, cabbage, cauliflower, leek, carrots, chard)	Samples were packaged in bags with O_2 permeability of 1375 m^3/m^2/day. An active MAP containing 5 % O_2, 15 % CO_2 and 80 % N_2 was created and samples were stored at three different conditions: (1) 6 days at 7 ºC; (2) 1.8 days at 7 ºC and 4.2 days at 15 ºC; (3) 6 days at 15 ºC.	At storage condition (1), the pathogen was not able to grow in escarole, collard green, watercress, yakisoba and was complete inhibited in carrots. At storage condition (2) and (3), *L. monocytogenes* was able to grow in all vegetables except carrots. The highest growth was observed in collard greens and arugula, while the lowest was in cabbage.	Sant'Ana *et al.*, 2012
L. monocytogenes cocktail	Mixed lettuce salad: endive, lollo rosso, lollo bionta, radicchio Mixture bell peppers: green, red and yellow Cucumber	An active MAP containing 3 % O_2, 5 % CO_2 balanced with N_2 was created for each vegetable. Lettuce samples were packaged with film permeability of 1945 ± 229 mL $O_2/(m^2$ 24 h atm), bell papers with 2897 ± 471 mL $O_2/(m^2$ 24 h atm) and cucumber with 2543 ± 123 mL $O_2/(m^2$ 24 h atm). Samples were subjected to a simulated distribution chain for 7 days.	*L. monocytogenes* was able to grow on cucumber, survived on mixed lettuce and decreased on bell peppers with no influence of packaging MAP.	Jacxsens *et al.*, 2002

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes (two strains)	Iceberg lettuce, swedes (rutabaga), dry coleslaw mix (80 % shredded cabbage, 20 % shredded carrot), soybean sprouts	Vegetable samples were packaged in bags with O_2 permeability of 1200 mL/m^2/day/atm. Samples packaged in passive MAP were stored at 4 and 8 ºC for 12 days.	*L. monocytogenes* populations increased during storage on lettuce and swedes, maintaining on soybean sprouts and decreased on coleslaw mix at 8 ºC. In lettuce bags, CO_2 increased to 10-12 % and O_2 fell to 3-4 %. O_2 fell to 2 % and CO_2 had risen to 8-10 % in bags of sweeds. In dry coleslaw mix and soybean sprouts bags, CO_2 rose to 25-27 % and O_2 fell to 0-1 %. At 4 ºC, growth of the pathogen was reduced.	Francis & O'Beirne 2001
L. monocytogenes (fifteen strains)	Irish Iceberg lettuce Dry coleslaw mix (80 % cabbage, 20 % carrot)	Vegetable samples were packaged in bags with O_2 permeability of 1200 mL/m^2/day/atm. Samples packaged in passive MAP were stored at 8 ºC for 10 days.	Survival and growth patterns on vegetables were dependent on strain, vegetable type and package atmosphere. Most of the strains examined grew well on lettuce. Differences among strains were especially evident on coleslaw. Populations of most strains decreased during storage but to different extents. In lettuce packages O_2 fell to 2-4 % and CO_2 increased to 10-12 %. Higher CO_2 were reached in packs of coleslaw; CO_2 rose to 25-30 % and O_2 decreased to 0-1 %.	Francis & O'Beirne 2005
L. monocytogenes cocktail	Lettuce salad: Iceberg and Crisp Collard greens	Samples were packaged in MAP with O_2 permeability of 1375 m^3/m^2/day and in perforated film. An active MAP containing 5 % O_2, 15 % CO_2 and 80 % N_2 was created and samples were stored at four different conditions: (1) 6 days at 7 ºC; (2) 4.2 days at 7 ºC and 1.8 days at 15 ºC; (3) 1.8 days at 7 ºC and 4.2 days at 15 ºC; (4) 6 days at 15 ºC.	*L. monocytogenes* grew better in collard greens than in lettuce. Active MAP enhanced the growth of the pathogen at these storage conditions.	Sant'Ana *et al.*, 2013

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes	Lettuce and carrots	Vegetable samples were packaged in a film with O_2 permeability of 3841 ± 434 mL/m^2/day/atm. Two different active MAP conditions were applied: (1) 4.9 % CO_2, 2.1 % O_2, 93 % N_2; (2) 5 % CO_2, 5.2 % O_2, 89.8 % N_2 and also Air conditions. The samples were stored at 4 ºC for 14 days.	*L. monocytogenes* counts decreased in vegetable samples under all storage conditions, although the inhibition was most pronounced in carrots than in lettuce. The atmosphere in the packages did not affect the pathogen behaviour.	Kakiomenou *et al.*, 1998
L. monocytogenes cocktail	Endive	Three different packages conditions were applied: (1) passive MAP; (2) active MAP 5 % O_2, 5 % CO_2, 90 % N_2 (5/5); (3) active MAP, 10 % O_2, 10 % CO_2, 80 % N_2 (10/10). Film permeability data is not mentioned. Different irradiation treatments were applied after packaging and samples were stored at 4 ºC for 19 days.	The non-irradiated passive samples showed increased counts of *L. monocytogenes* after 2 days. In contrast, on non-irradiated 5/5 and 10/10 active samples the populations tended not to increase during storage. In contrast to the results obtained in passive samples, the pathogen did not regrow following irradiation on endive packages in 5/5 or 10/10 atmosphere conditions. By day 5, CO_2 in the passive samples rose to 8-13 % and O_2 fell to 4-8 % and supported the regrowth of the pathogen. However, the combination of elevated CO_2 and very low O_2 levels in the 5/5 and 10/10 samples during storage prevented the regrowth of *L. monocytogenes* on the irradiated endive.	Niemira *et al.*, 2005
L. monocytogenes	White cabbage	After treated with 2 % lactic acid, cabbage was packaged in bags with permeability of 55 mL-µm/KPa-s-m^2. Four different packaging methods were applied: (1) Air; (2) vacuum; (3) CO_2 (100 %); (4) N_2 (100 %). All samples were stored for 7 d at 10 ºC.	*L. monocytogenes* increased in cabbage after vacuum packaging after 7 days at 10 ºC. Air, N_2 and CO_2 gas packaging were effective in delaying growth of the pathogen.	Bae *et al.*, 2011

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes	Cabbage	Six packaging treatments were made with modified atmospheres and films. Two films of O_2 permeability of 1,277±159 mL/m²/day/atm (Film I) and 54.8±0.7 mL/m²/day/atm (Film II) were used. Two active MAP with a gas mixture of 70 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP1); 5 kPa O_2 and 15 kPa CO_2 balanced with N_2 (MAP2); a moderate vacuum packaging (MVP) with a vacuum degree of 10.1 kPa. Samples were stored at 5 ºC for 10 days.	Populations were kept lower in MAP1 with Film I or Film II than in the other treatments. Population of *L. monocytogenes* increased in the MVP treatment with Film II which maintained low O_2 partial pressure during storage. However, no proliferation of this pathogen was observed in MVP with Film I. High levels of O_2 (>40 kPa) were only observed in samples packaged under MAP1 regardless the film used. However, in MAP2 O_2 were less than 10 kPa and in Film II achieved 0 kPa after 5 days. CO_2 concentration increased to more than 20 kPa for MAP1 regardless the film used. Conversely, in MAP2 CO_2 decreased reaching 2 kPa at the end of storage for both films. Gas composition within the packages depended on initial gas conditions and was greatly affected by film permeability.	Lee *et al.*, 2011
L. monocytogenes (two strains)	Tomatoes	Samples were packaged in a film with O_2 permeability of 8750 mL/m²/day. Two different packaging conditions were applied: (1) Air and (2) active MAP 3 % O_2, 97 % N_2 and stored at 10 ºC for 10 days and at 21 ºC for 8 days.	A significant decrease of the strains counts was observed in chopped tomatoes at both temperatures. Changes in populations were not affected by MAP regardless of storage temperature.	Beuchat & Brackett 1991
L. monocytogenes	Cactus-pear fruit	Samples were packaged in a film with O_2 permeability of $9.23x10^{-19}$ mol m/m²/s/Pa. Two different packaging conditions were applied: (1) Air and (2) active MAP 5 % O_2, 30 % CO_2, and 65 % N_2 and stored at 4, 8, 12 and 20 ºC for 14 days.	*L. monocytogenes* not only survived but was able to grow in cactus-pear stored in Air and MAP at all temperatures. A rise in storage temperature resulted in an increase in the growth rate. No differences were observed between air and active MAP.	Corbo *et al.*, 2005

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes cocktail	Raspberries	Samples were packaged in a film with O_2 permeability of 3200 mL/m²/day/atm. Three different packaging conditions were applied: (1) perforated packages (Air); (2) active MAP 3 % O_2, 5 % CO_2, balance N_2 (EMA); (3) active MAP, 95 % O_2, 5 % N_2 (HOA). Samples were stored at 7 ºC for 14 days.	A slight inactivation of the pathogen was observed in samples packaged in EMA and Air, but was able to survive in HOA packages. In EMA bags, O_2 remains stable around 9.5-12.8 % and CO_2 decreased slightly to 3.2-4.8 % after 14 days. In HOA bags, O_2 rapidly decreased from 95 to 10.4 % in 7 days and remained at 7.5 % until day 14. CO_2 were around 5-8 %.	Siro *et al.*, 2006
L. monocytogenes cocktail	Strawberries	Samples were packaged in a film with O_2 permeability of 4679 mL/m²/day/atm. Three different packaging conditions were applied: (1) perforated packages (Air); (2) active MAP 3 % O_2, 5 % CO_2, balance N_2 (EMA); (3) active MAP, 95 % O_2, 5 % N_2 (HOA). Samples were stored at 7 ºC for 14 days.	*L. monocytogenes* could only survive until day 5 and then started to grow in HOA and in Air atmospheres whereas in EMA started to decrease after day 5. In EMA bags, O_2 presented little variation during storage (1.0-6.4 %) and CO_2 also remained constant (5.0-8 %) after 14 days. In HOA bags, O_2 rapidly decreased from 95 to 10.9 % in 7 days and reached a minimum value of 4.7 % on day 12. CO_2 were around 5-8 %.	Siro *et al.*, 2006
L. monocytogenes	Apples	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) apple samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m²/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	The CGLW+MA treatment reduced almost totally the population of *L. monocytogenes*. No changes were observed in pathogen survival when dipped with water and a small decrease was observed when treated with CGLW. The MAP in the packages did not affect in a direct way the growth/survival/decrease of the pathogen.	Raybaudi-Massilia, *et al.*, 2009a

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. innocua	Apples	Apple trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*L. innocua* populations increased at 25 ºC. Pathogen population fluctuated over time when stored at 5 ºC. Although, it increased slowly over the time. O_2 decreased continuously throughout storage. At 25 ºC declined to 17.0 % and at 5 ºC 19.6 %. In contrast, CO_2 increased during storage. The rise was more significant in 3 days at 25 ºC (6.8 %) than in 14 days at 5 ºC (2.8 %).	Alegre *et al.*, 2010a
L. monocytogenes	Pears	After treated with (1) N-acetyl-L-cysteine, glutathione, calcium lactate pent-hydrate (CGLW) and (2) CGLW plus malic acid (CGLW+MA) pear samples were packaged in trays with O_2 permeability of 52.38 fmol/s/m^2/ kPa. Sterile water (W) was used as a control treatment. Trays were sealed under passive MAP and stored at 5 ºC for 30 days.	The CGLW+MA treatment reduced almost totally the population of *L. monocytogenes*. The pathogen survived very well when dipped with the other treatments. The MAP in the packages did not affect in a direct way the growth/survival/decrease of the pathogen.	Raybaudi-Massilia, *et al.*, 2009b
L. innocua	Peaches	Peach trays were sealed with O_2 permeability of 40000 cc/m^2/day and stored at 25 ºC for 3 days and at 5 ºC for 14 days. Samples were packaged in passive MAP and in Air.	*L. innocua* populations increased at 25 ºC. The population of the pathogen increased slowly over the time at 5 ºC. Differences of pathogen growth between MAP and Air were not observed. At 25 ºC, O_2 decreased sharply after 24 h to 13.2 % and then stabilized. The same happened to CO_2 that increased exponentially to 24.6 % after two days and then remain stable. At 5 ºC, O_2 only reduced by 2.5 % and CO_2 increased to 3.9 % after 14 days.	Alegre *et al.*, 2010b

Microorganism	Vegetables and fruits	MAP Conditions/Temperature*	Outcomes	References
L. monocytogenes	Coconut	Samples were packaged in a film with O_2 permeability of $9.23x10^{-19}$ mol m/m^2/s/Pa. Two different packages conditions were applied: (1) Air and (2) active MAP 5 % O_2, 30 % CO_2, and 65 % N_2. Samples were stored at 2, 4, 8 and 10 ºC for 12 days.	*L. monocytogenes* not only survived but was able to grow on coconut stored in Air and MAP at all temperatures. A rise in storage temperature resulted in an increase in the growth rate. No differences were observed between Air and active MAP.	Sinigaglia *et al.*, 2006

*Permeability units are shown as indicated in the original paper.

L. monocytogenes was slightly inactivated on raspberries stored at 7 ºC and packaged under Air and equilibrium-modified atmosphere (EMA, 3 % O_2/5 % CO_2) while was able to survive under high-oxygen atmosphere (HOA, 95 % O_2/5 % N_2). Moreover, the same high-oxygen atmosphere and Air was favourable for survival and growth of the pathogen on strawberries (Siro *et al.*, 2006).

Beuchat and Brackett (1991) observed that two strains of *L. monocytogenes* decreased in chopped tomatoes under Air and active MAP with 3 % O_2 at 10 and 21 ºC, and changes in populations were not affected by atmosphere conditions. Also, Alegre *et al.* (2010a, b) did not observe influence of MAP on the *L. innocua* behaviour on fresh-cut apples and peaches at 5 ºC.

Final remarks

A wide variety of studies have been conducted about foodborne pathogens behaviour on minimally-processed fruits and vegetables. These studies have different experimental design, tested strains, raw materials and packaging conditions, thus it was difficult to find data under identical or even similar conditions.

It is well known that CO_2 can have an antimicrobial effect on microbial growth. However, high levels of CO_2 used in the different studies were not effective to inhibit foodborne pathogens growth. Although, Bae *et al.* (2011) observed that CO_2 gas packaging was effective in delaying *E. coli* O157:H7 and *S.* Typhimurium growth on white cabbage.

As discussed earlier, although some authors recommended a percentage of gases (Table 1) for fresh-cut fruits and vegetables, for both microbial growth and quality, it may not ensure microbiological safety. Therefore, an additional inhibitory step to MAP packaging, in the form of a hurdle technology, is necessary to enhance food safety. The hurdle technology may improve microbiological and sensory quality of foods as a result of synergistic effects of combined preservative treatments. This concept has been studied to control foodborne pathogens in various fresh-cut fruits and vegetables. For example, Gomes *et al.* (2011) demonstrated that the combination of high O_2 levels and e-beam radiation was effective in reducing or eliminating *Listeria* spp. populations on baby spinach. Similarly, Caillet *et al.* (2006) observed that the combination of treatments (coating and irradiation) was more effective in reducing *L. innocua* in active MAP than in Air conditions in baby carrot. Niemira and Boyd (2013) demonstrated that the behaviour of the pathogen to the irradiation treatment varies depending on the atmosphere used. The lowest oxygen MAP systems showed the highest irradiation dose to reduce *Salmonella* spp. on tomatoes.

Overall, the results from the studies discussed above demonstrate that MAP packaging when applied as a unique technology is not effective to control foodborne pathogens growth. Hurdle technology may be the most effective approach for food safety. Therefore, there is a need to conduct more investigations using MAP packaging in combination with other treatments.

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