Universitat de Girona

LACTIC ACID BACTERIA AS BIOPROTECTIVE AGENTS AGAINST FOODBORNE PATHOGENS AND SPOILAGE MICROORGANISMS IN FRESH FRUITS AND VEGETABLES

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Doctoral Thesis

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Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària
Institut de Tecnologia Agroalimentària

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Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables

Memòria presentada per Rosalia Trias Mansilla, inscrita al programa de doctorat de Ciències Experimentals i de la Salut, itinerari Biotecnologia, per optar al grau de Doctor per la Universitat de Girona

Rosalia Trias Mansilla
2008

Lluís Bañeras Vives, professor titular de l'àrea de Microbiologia del Departament de Biologia, i **Esther Badosa Romañó**, professora de l'àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària, ambdós de la Universitat de Girona

CERTIFIQUEN

Que la llicenciada en Biologia Rosalia Trias Mansilla ha dut a terme, sota la seva direcció, el treball amb el títol "Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables", que presenta en aquesta memòria la qual constitueix la seva Tesi per a optar al grau de Doctor per la Universitat de Girona.

I per a què consti als efectes oportuns, signen la present a Girona, el 14 d'abril del 2008

Vist-i-plau dels directors de la Tesi

Dr. Lluís Bañeras Vives Dra. Esther Badosa Romañó

Emili Montesinos Seguí, catedràtic de l'àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona i director del projecte de recerca "Evaluación de un procedimiento de bioprotección de fruta y hortaliza frescas para el control de podredumbres fúngicas y de patógenos causantes de toxiinfecciones alimentarias" (Ref. CAL03-084),

Rosalia Trias Mansilla, que ha inscrit la tesi doctoral titulada "Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables" amb el número de registre 699 (11 de novembre del 2004),

DECLAREN

Que aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors dels grups d'Ecologia Microbiana Molecular i Patologia Vegetal de la Universitat de Girona (Article 2. apartat 2, RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

I per a que consti als efectes oportuns, signen la present a Girona el 14 d'abril del 2008

Vist-i-plau

Dr. Emili Montesinos Seguí Rosalia Trias Mansilla

Aquesta tesi s'ha realitzat en el marc del projecte de recerca "Evaluación de un procedimiento de bioprotección de fruta y hortaliza frescas para el control de podredumbres fúngicas y de patógenos causantes de toxiinfecciones alimentarias" (Ref. CAL03-084) i ha estat realitzada amb una beca pre-doctoral BR de la Universitat de Girona (BR02/05).

Agraïments

És difícil després d'alguns anys d'esforços poder transmetre en tan sols unes línies l'agraïment a totes les persones que d'alguna manera o una altra han contribuït en aquest treball.

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Summary

There is a growing concern in the microbiological safety of fresh fruit and vegetables. The contamination of these products with plant and human pathogens can cause considerably economic losses for the industry, apart from being the origin of foodborne diseases. Some of the most important contaminants of fresh products can be human pathogens like *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* sp., *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Others, like the fungus *Penicillium expansum,* can cause important postharvest diseases of fruit.

The present thesis focuses on the use of lactic acid bacteria as bioprotective cultures to inhibit pathogenic and spoilage microorganisms as a prevention method. We have made use of *in vitro* and *ex vivo* antagonistic assays to work out this main objective. These results have been complemented with the determination of antimicrobial substances produced by selected strains.

Lactic acid bacteria were isolated from fresh fruit and vegetables and tested *in vitro* against five plant pathogens (*Xanthomonas campestris* pv. *vesicatoria, Erwinia*

carotovora, Botrytis cinerea, Monilinia laxa and *Penicillium expansum)* and five human pathogen test bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Listeria monocytogenes* and *Staphylococcus aureus*). The results showed a low percentage of isolates with high inhibitory abilities against foodborne and spoilage microorganisms. Twenty-three out of 496 strains were selected as good antagonists and further identified. The high number of *Leuconostoc* strains (12) with biocontrol potential was of particular interest. The rest of the isolates were identified as *Lactobacillus plantarum* (6), *Weissella cibaria* (2), *Lactococcus lactis* (2) and *Enterococcus mundtii* (1).

Organic acids, hydrogen peroxide and bacteriocins were detected as the main antimicrobial substances produced by the obtained isolates, being acidification the most common inhibition mechanism. Bacteriocins were produced by *Leuconstoc mesenteroides* strains CM160 and CM135, and a preliminary classification suggested that they were Class IIa bacteriocins.

Lactic acid bacteria strains were tested at *ex vivo* level using two different assays. First, efficacy trials with all the isolates were performed in Golden Delicious apples against the blue mould rot infections, caused by *P. expansum*. The highest effectivity found in this assay was of about 50%, with strain *W. cibaria* TM128. However, the mechanism of inhibition of this strain could not be established, although biofilm formation may be a significant mechanism according to obtained results. Second, selected lactic acid bacteria (CM135, CM160, PM249, TM128 and SE303) were tested as bioprotective agents against *S. typhimurium, E. coli* and *L. monocytogenes* in Iceberg lettuce and Golden Delicious apples. Results indicate that lactic acid bacteria grew well on vegetable cut surfaces, although final population levels were considerably higher in lettuce cuts than in apple wounds. Efficacy trials were performed with high densities of lactic acid bacteria to establish potential inhibition of foodborne pathogens. Lactic acid bacteria interfered efficiently with the growth of two foodborne pathogen bacteria tested, *S. typhimurium*, and *L.*

monocytogenes in apple wounds and lettuce cuts, but showed little effectivity over *E. coli*.

Finally, dose-response assays were done with *Leuconostoc mesenteroides* strains CM135, CM160 and PM249 against *L. monocytogenes*. Among the three strains tested, *L. mesenteroides* strain CM160 showed the highest effectivity at high doses of the pathogen.

In summary, two strains were selected among a total collection of 496 isolates of lactic acid bacteria obtained from fresh fruit and vegetables. Strain *W. cibaria* TM128 was selected for its inhibition of blue mould rot infections *ex vivo*, and strain *L. mesenteroides* CM160 for its inhibition of *L. monocytogenes* in Iceberg lettuce cuts and Golden Delicious apple wounds. The potential application of lactic acid bacteria strains as antagonistic bacteria is reinforced by the fact that no modifications in the general aspect of the fruit and vegetable such as browning, pectinolytic activities or off odours were observed for any of the experiments done.

Resum

L'interès en la seguretat microbiològica de fruites i hortalisses fresques ha augmentat els darrers anys, ja que a la microbiota natural d'aquests productes s'hi poden afegir microorganismes fitopatògens o patògens humans. Aquest fet pot causar considerables pèrdues econòmiques per a la indústria alimentària i, a més a més, ser la causa de toxiinfeccions alimentàries. Alguns dels patògens que poden contaminar els productes vegetals frescos son patògens humans com *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* i *Pseudomonas aeruginosa.* Altres contaminants poden ser microorganismes fitopatògens, com el fong *Penicillium expansum,* causant d'importants pèrdues econòmiques en postcollita.

La present tesi doctoral té com a objectiu l'ús de bacteris de l'àcid làctic com a cultius bioprotectors enfront a microorganismes contaminants de fruites i hortalisses fresques. Per assolir aquest objectiu principal es van realitzar assajos *in*

vitro i *ex vivo.* Aquests resultats es van complementar amb la detecció de substàncies antimicrobianes produïdes per les soques aïllades.

Es va obtenir una col·lecció de 496 soques de bacteris de l'àcid làctic aïllats de fruites i hortalisses fresques, i s'assajà la seva capacitat antagonista *in vitro* contra cinc microorganismes fitopatògens (*Xanthomonas campestris* pv. *vesicatoria, Erwinia carotovora, Botrytis cinerea, Monilinia laxa i Penicillium expansum)* i cinc indicadors de patògens humans (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Listeria monocytogenes* i *Staphylococcus aureus*). Els resultats van mostrar un baix percentatge d'aïllats amb bona capacitat antagonista enfront a patògens causants del deteriorament de fruites i hortalisses fresques i de toxiinfeccions alimentàries. Vint-i-tres soques de les 496 aïllades van ser seleccionades i caracteritzades. Es va considerar d'interès el fet que un elevat nombre de soques amb bon potencial de biocontrol fos identificada com *Leuconostoc spp.* (12). La resta dels aïllats van ser identificats com *Lactobacillus plantarum* (6), *Weissella cibaria* (2), *Lactococcus lactis* (2) i *Enterococcus mundtii* (1).

Els principals compostos antimicrobians detectats produïts pels bacteris de l'àcid làctic eren àcids orgànics, peròxid d'hidrogen i bacteriocines, essent l'acidificació el mecanisme més freqüent entre les soques seleccionades. Les soques *Leuconstoc mesenteroides* CM160 i CM135 van caracteritzar-se com a bacteriocinogèniques, i una caracterització preliminar suggereix que produeixen bacteriocines de la Classe IIa.

Les soques de bacteris de l'àcid làctic es van utilitzar en dos assajos *ex vivo*. En primer lloc, es van realitzar assajos d'eficàcia amb totes les soques en pomes de la varietat Golden Delicious contra infeccions de *P. expansum,* causant de la podridura blava. La soca *W. cibaria* TM128 va ser la més efectiva, reduint les infeccions en un 50%. Els mecanismes d'inhibició d'aquesta soca no es van poder determinar, encara que la producció de biofilms podria ser un dels mecanismes implicats, d'acord amb els resultats obtinguts. En segon lloc, s'assajà la capacitat de bioprotecció de les soques de bacteris làctics seleccionades (CM135, CM160, PM249, TM128 i SE303)

enfront *S. typhimurium, E. coli* i *L. monocytogenes* en enciam Iceberg i pomes Golden Delicious. Els bacteris làctics van mostrar bona capacitat de creixement als dos models, encara que la densitat al final del període d'incubació era major en enciam que en poma. Els assajos d'eficàcia es van realitzar amb altes concentracions de bacteris làctics per determinar el potencial antagonista enfront a patògens causants de toxiinfeccions alimentàries. En el present estudi, els bacteris làctics van interferir eficientment amb el creixement de dos dels tres patògens assajats, *S. typhimurium*, i *L. monocytogenes* en poma i en enciam, i van tenir poca efectivitat enfront *E. coli*.

Finalment, els assajos dosi-resposta es van realitzar amb les soques *Leuconostoc mesenteroides* CM135, CM160 i PM249 enfront *L. monocytogenes*. Entre les tres soques assajades, *L. mesenteroides* CM160 va ser la més efectiva a altes dosis de patogen.

En resum, es van seleccionar dues soques de la col·lecció de 496 aïllats de bacteris de l'àcid làctic procedents de fruites i hortalisses fresques. La soca *W. cibaria* TM128 es va seleccionar per la seva capacitat d'inhibició de la infecció de *P. expansum ex vivo,* i la soca *L. mesenteroides* CM160 per la seva inhibició de *L. monocytogenes* en enciam Iceberg i pomes Golden Delicious. La potencial utilització de bacteris de l'ácid làctic es veu reforçada pel fet que no es van observar modificacions a l'aspecte general del producte, incloent activitat pectinolítica o males olors, en cap dels assajos realitzats.

Resumen

El interés en la seguridad microbiológica de las frutas y hortalizas frescas ha aumentado los últimos años ya que, además de la microbiota natural, estos productos pueden contaminarse con microorganismos fitopatógenos o patógenos humanos. Este hecho puede causar considerables pérdidas económicas para la industria alimentaria y también brotes de toxiinfecciones alimentarias. Algunos de los patógenos que pueden contaminar los productos vegetales frescos son patógenos humanos como *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* y *Pseudomonas aeruginosa.* Otros contaminantes pueden ser hongos fitopatógenos como *Penicillium expansum,* causante de enfermedades de postcosecha.

La presente tesis doctoral tiene como objetivo el uso de bacterias del ácido láctico como organismos bioprotectores para inhibir los microorganismos contaminantes de frutas y hortalizas frescas. Para ello se han utilizado ensayos *in vitro* y *ex vivo.* Los resultados obtenidos se han complementado con la

determinación de las substancias antimicrobianas producidas por las cepas seleccionadas.

Las bacterias del ácido láctico fueron aisladas de frutas y hortalizas frescas. Se obtuvo una colección de 496 cepas, y se ensayó su capacidad antagonista *in vitro* frente a cinco microorganismos fitopatógenos (*Xanthomonas campestris* pv. *vesicatoria, Erwinia carotovora, Botrytis cinerea, Monilinia laxa* y *Penicillium expansum)* y cinco indicadores de patógenos humanos (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Listeria monocytogenes* y *Staphylococcus aureus*). Los resultados mostraron un bajo porcentaje de aislados con buena capacidad antagonista frente a patógenos causantes del deterioro de frutas y hortalizas frescas y de toxiinfecciones alimentarias. Veintitrés de las 496 cepas se seleccionaron como buenos antagonistas y se caracterizaron. Un elevado porcentaje de cepas con potencial de biocontrol fue identificado como *Leuconostoc spp.* (12). Los demás aislados se identificaron como *Lactobacillus plantarum* (6), *Weissella cibaria* (2), *Lactococcus lactis* (2) y *Enterococcus mundtii* (1).

La producción de ácidos orgánicos, peróxido de hidrogeno y bacteriocinas se ha detectado como compuestos antimicrobianos en las cepas seleccionadas, siendo la acidificación el mecanismo más frecuente. Se detectó producción de bacteriocinas en las cepas de *Leuconstoc mesenteroides* CM160 y CM135, y su caracterización preliminar sugiere que se trata de bacteriocinas de la Clase IIa.

Las cepas de bacterias del ácido láctico se utilizaron en dos ensayos *ex vivo*. En primer lugar, se realizaron ensayos de eficacia con todas las cepas en manzanas de la variedad Golden Delicious frente a infecciones de *P. expansum,* causante de la podredumbre azul. La cepa *W. cibaria* TM128 fue la más efectiva, reduciendo las infecciones en un 50%. Los mecanismos de inhibición de esta cepa no se pudieron determinar, aunque la formación de biofilms podría ser uno de los mecanismos implicados. En segundo lugar, se ensayó la capacidad de bioprotección de las cepas de bacterias lácticas seleccionadas (CM135, CM160, PM249, TM128 y SE303) frente a *S. typhimurium, E. coli* y *L. monocytogenes* en lechuga Iceberg y manzana Golden

Delicious. Las bacterias lácticas mostraron buena capacidad de crecimiento en los dos modelos, aunque las poblaciones finales eran mayores en lechuga que en manzana. Los ensayos de eficacia se realizaron con altas concentraciones de bacterias lácticas para determinar el potencial antagonista frente a patógenos causantes de toxiinfecciones alimentarias. En el presente estudio, las bacterias lácticas interfirieron eficientemente con el crecimiento de dos de los tres patógenos probados, *S. typhimurium*, y *L. monocytogenes* en manzana y en lechuga, pero tuvieron poco efecto frente a *E. coli*.

Finalmente, los ensayos dosis-respuesta se realizaron con las cepas *Leuconostoc mesenteroides* CM135, CM160 y PM249 frente a *L. monocytogenes*. Entre las tres cepas probadas, *L. mesenteroides* CM160 fue la más efectiva a altas dosis de patógeno.

En resumen, se seleccionaron dos cepas de entre la colección de 496 aislados de bacterias del ácido láctico aisladas de frutas y hortalizas frescas. La cepa *W. cibaria* TM128 se seleccionó por su capacidad de inhibición de la infección de *P. expansum ex vivo,* y la cepa *L. mesenteroides* CM160 por su inhibición de *L. monocytogenes* en lechuga Iceberg y manzanas Golden Delicious. El potencial de uso de las bacterias del ácido láctico se ve reforzado, ya que no se observaron modificaciones en el aspecto general del producto, incluyendo actividad pectinolítica o malos olores, en ninguno de los ensayos realizados.

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Introduction

1-Microbiology of minimally processed vegetables and fruits

Fresh products like fruit and vegetables, including plant components as leaves, roots, bulbs and tubers, are among the most perishable foods in markets. These products are rich in carbohydrates and poor in proteins with pH values ranging from 7.0 to slightly acidic and are adequate habitats for several bacteria, yeasts and moulds. Fruits exhibit more or less the same characteristics but usually a lower pH.

Microbes inhabiting vegetables and fruits vary considerably depending on the plant component studied, as plants harbour different microbiota in their aerial parts compared with their root system (Francis *et al.*, 1999). Microbial densities are mostly depending on natural variability of the product, ranging from 10^3 to 10^7 cfu g^{-1} (Pla *et al.*, 2005; Badosa *et al.*, 2008). Values can vary according to numerous facts such as harvest conditions, the presence of soil accompanying the product or the postharvest handling procedure. Moreover, another source of variation is the fact

that microorganisms grow faster in wounded, damaged or cut vegetables and fruits than in intact surfaces (Ponce *et al*., 2002).

Lactic acid bacteria (e.g. *Lactobacillus*), *Pseudomonas, Erwinia, Pantoea, Micrococcus, Flavobacterium* and gram-positive spore formers (e.g. *Bacillus, Clostridium*), are usually the dominant bacteria in fresh fruits and vegetables. Besides, different types of moulds such as *Alternaria*, *Penicillium, Fusarium* and *Aspergillus* can also be found at relatively high densities. Finally, yeasts as *Torulopsis*, *Saccharomyces* and *Candida* are part of the dominant microorganisms especially in those fruits that have high sugar content. Fresh fruits and vegetables may be also colonized by spoilage or pathogenic microorganisms such as bacteria, viruses and parasite protozoa (Table 1).

The main characteristics of minimally processed vegetables affecting the probabilities of microbial spoilage and contamination include:

- the presence of cut surfaces and increased moisture content,
- the active metabolism of plant tissue,
- all processing and handling methods, which can not ensure sterility or microbial stability of the product, and can alter the relative presence of the different species by the confinement of the product in a modified atmosphere package.

Microorganisms impact the economic value of fresh-cut products by decreasing product shelf-life through spoilage and by posing a risk to public health (Nguyen-the and Carlin, 1994).

Primary sources for bacterial contamination affecting the bacteriological quality and safety of the final product are present before the crop is even planted. These sources include soil characteristics where pathogenic microorganisms such as *Listeria monocytogenes* can survive for three months or more (Zaho, 2005), wild and domestic animals effects, irrigation with insalubrious water, use of animal manure as fertilizers, inadequate field workers hygiene or rainfall and temperature of the field. During processing and packaging specific sources of contamination are

unsanitary handling during sorting, the use of tidy equipment (ice cooling units, transport vehicles, improper temperature at storage conditions), improper packaging or cross-contamination in storage, display and preparation (Rico *et al.*, 2007) (Figure 1).

Table 1- Main microorganisms contaminating fresh fruit and vegetables (from Pla *et al.*, 2005).

Microorganism	**Type of microorganism**	**Effect**
(a) Spoiling microorganisms		
Penicillium	Fungus	Blue mould rot, mycotoxins
Rhizopus	Fungus	Soft rot
Monilinia	Fungus	Brown rot
Alternaria	Fungus	Black rot, mycotoxins
Botrytis	Fungus	Grey mould rot
Erwinia carotovora	Bacterium	Soft rot
Pseudomonas fluorescens	Bacterium	Soft rot
(b) Human pathogens		
Salmonella	Bacterium	Gastroenteritis
Shigella	Bacterium	Gastroenteritis, shiga toxin
E. coli O157:H7	Bacterium	ETEC, EPEC
Listeria monocytogenes	Bacterium	Gastroenteritis, septicaemia, organ invasion
Campylobacter	Bacterium	Gastroenteritis
Bacillus cereus	Bacterium	Toxin effects
Norwalk virus	Virus	Gastroenteritis
Hepatitis virus	Virus	Liver inflammation
(c) Human parasites		
Cryptosporidium pavum	Protozoa	Gastroenteritis
Giardia lamblia	Protozoa	Gastroenteritis
Cyclospora cayetanensis	Coccidian	Gastroenteritis

Special attention must be paid to ready-to-use (RTU) vegetables, which industry has grown due to a constantly increasing demand for fresh, healthy and convenient foods. The International Fresh-cut Produce Association (IFPA) defines fresh-cut products as fruit or vegetables that have been trimmed and/or peeled and/or cut into 100% usable product that is bagged or pre-packaged to offer the consumers high nutrition, convenient and flavorful products while still maintaining

its freshness (Lamikanra, 2002). Consumers have also become more critical about the use of synthetic additives to preserve food or enhance characteristics such as colour and flavour (Bruhn, 2000). It should be emphasized that minimal processing techniques can promote a faster physiological deterioration, biochemical changes and microbial degradation of the product (Ongeng *et al*., 2006) which may result in degradation of the colour, texture and flavour (Varoquaux and Wiley, 1994).

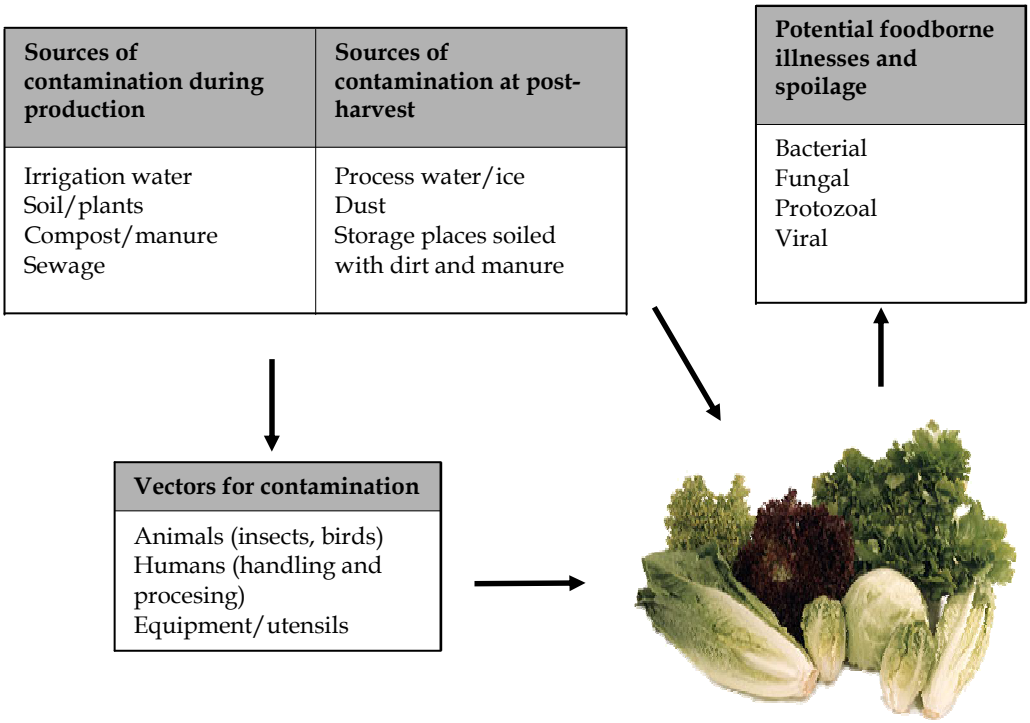

Figure 1- Contamination sources of fresh fruit and vegetables during production and post-harvest process (Adapted from Zaho, 2005).

1.1-Pathogens in fresh fruit and vegetables

Fresh produce and minimally processed fresh-cut products provide a favourable environment for proliferation of spoilage organisms and microorganisms

of public health significance (Francis *et al.*, 1999). A significant increase in the number of fruit and vegetable produce-associated foodborne diseases has been detected in the recent years (NACMCF, 1998). Several pathogens such as the Gram negative bacteria *Aeromonas hydrophyla*, *Escherichia coli, Salmonella* sp., *Shigella* sp., *Plesiomonas shigelloides*, and *Vibrio cholerae*, and the Gram positive *Listeria monocytogenes, Staphylococcus aureus, Clostridium botulinum* and *Bacillus cereus* have been isolated from fresh products and are of significant sanitary importance (Table 2). However, the mere presence of a pathogenic bacterium does not always mean that illness will result. Despite the wide variety of products from which pathogenic bacteria have been isolated, only a few have been confirmed as vehicles for foodborne illnesses (Table 2). The foodborne pathogens *E. coli*, *L. monocytogenes* and *Salmonella* sp. are considered of particular significance because of the large number of outbreaks reported, and are briefly described in the following pages.

1.1.1-*Escherichia coli*

Enterohemorragic *E. coli* O157:H7 is recognized as an important foodborne pathogen and grows rapidly in several types of raw fruits and vegetables, particularly when stored at more than 10 ^{o}C. The infection dose of this pathogen is rather low, from 10 to 700 ingested viable cells (FDA, 2001). Since cattle appear to be a primary reservoir for this bacterium, the majority of outbreaks of illness associated with *E. coli* O157:H7 have been associated with the consumption of undercooked beef and dairy products. However, outbreaks have also been linked to lettuce, unpasteurized apple cider, cantaloupe and sprouts (Zaho, 2005).

Table 2- Examples of products from which bacterial pathogens have been isolated. Bold letters indicate that outbreaks have been related to these products and microorganisms (Beuchat, 1996; NACMCF, 1998; European Commission, 2002).

Product	Pathogen	Product	Pathogen
Alfalfa sprouts	*Areomonas* sp., ***E.coli* O157:H7**, ***Salmonella* sp.**	Endive	*Salmonella* sp.
Apple juice	***E.coli* O157:H7**	Fennel	*Salmonella* sp.
Asparagus	*Aeromonas* sp.	Green onions	***Shigella* sp.**
Bean sprouts	*L.monocytogenes, Salmonella* sp.	Lettuce	*Salmonella* sp., *Staphylococcus* sp., *Aeromonas* sp., ***Shigella*** sp., ***E.coli* O157:H7, *L. monocytogenes***
Brocoli	*Aeromonas* sp.	Mungbean sprouts	*Salmonella* sp.
Cabbage	*E.coli* O157:H7, ***L. monocytogenes***, *V. cholerae, Salmonella* sp.	Mustard cress	*Salmonella* sp.
Cantaloupe	***Salmonella* sp.**	Mustard sprouts	***B. cereus***
Cauliflower	*Aeromonas* sp., *Salmonella* sp., ***L. monocytogenes***	Orange juice	***Salmonella* sp.**
Celery	*Aeromonas* sp., *E.coli* O157:H7	Pepper	*Aeromonas* sp., *Salmonella* sp.
Chilli	*Salmonella* sp.	Potatoes	*L. monocytogenes*
Cilantro	*E.coli* O157:H7	Radish	*Staphylococcus* sp., *L. monocytogenes*
Coconut milk	***V. cholera***	Salad greens	*Salmonella* sp., *S. aureus*
Coriander	*E.coli* O157:H7	Salad vegetables	*Shigella* sp., *S. aureus, L. monocytogenes, Yersinia enterocolitica*
Cress sprouts	*E.coli* O157:H7	Soybean sprouts	*B. cereus*
Cucumber	*L. monocytogenes*	Spinach	*Aeromonas* sp., *Salmonella* sp.
Carrots	***E. coli***	Tomato	*L.monocytogenes,* ***Salmonella* sp.**
Egg plant	*Salmonella* sp.	Watermelon	***Salmonella* sp.**

1.1.2-*Salmonella* spp.

The genus *Salmonella* has over 2,700 serotypes, all of them considered potentially pathogenic (Zaho, 2005). Surveys of fresh produce have revealed the presence of several serotypes causing human infection. Animals and birds are the natural reservoirs of this bacterium, and consequently, meat, dairy products and eggs are the most commonly implicated sources in salmonellosis outbreaks. Although they have been documented, fresh fruit and vegetables are implicated less frequently. Principal incriminated products have been cantaloupe, sprouts and raw tomato (FDA, 2001).

1.1.3-*Listeria monocytogenes*

Listeria monocytogenes causes a mild gastroenteritis in healthy adults, but the illness can be severe in susceptible individuals including pregnant women, neonates and immune-compromised individuals. The infective dose for this bacterium has not been clearly established, since it is very variable depending on the susceptibility degree of every individual (Zaho, 2005). *L. monocytogenes* is widely distributed on raw fruits and vegetables (Beuchat, 1996). Plants and vegetables used as salad vegetables play a role in disseminating the pathogen to human food supply. Moreover, growth of *L. monocytogenes* on fresh cut fruit, lettuce and cauliflower has been reported at refrigeration temperatures (Koseki and Isobe, 2005).

1.2-Spoilage microorganisms in fresh fruit and vegetables

Spoilage of fresh fruits and vegetables is generally due to physiological disorders and microbial spoilage activity caused by fungi and bacteria (Figure 2). Microbial spoilage of fruits and vegetables is known as rot, which consists of changes in colour (black or grey), loss of texture (soft rot), and often off-odour. Wounds present on produce during storage, often as a result of harvesting and transportation, give an easy access to spoiling bacteria and fungi (Spadaro and

Guillino, 2004) and the high water content in the product will facilitate development (Harvey, 1978). Among the most important postharvest fungal pathogens of fruit, *Penicillium expansum*, *Botrytis cinerea*, *Monilinia laxa* and *Rhizopus stolonifer* have been broadly documented (Ogawa, 1995; Pla *et al*., 2005). Soft rot of bacterial origin (*Erwinia carotovora, Xanthomonas campestris* pv. *vesicatoria*) are the most common bacteria derived effects in fresh vegetables (Pla *et al*., 2005). The following descriptions refer to some of the most important spoiling microorganisms of fresh fruits and vegetables.

1.2.1-*Penicillium expansum*

Blue mould is the most important postharvest decay of stored apples. The causative agent, *Penicillium expansum*, not only causes fruit decay but also produces the carcinogenic mycotoxin patulin (Rundberget *et al*., 2004). The rotted areas are soft, watery and light brown in colour. The surface of older lesions may be covered by bluish-green spores that initially are nearly snow white in colour. The lesions are of varying shades of brown, and it is characteristic the musty odour and the formation of conidial tufts or coremia on the surface of well developed lesions. Under cold storage conditions, blue mould lesions caused by *P. expansum* may be expected to be around 3 cm in diameter eight to ten weeks after infection.

1.2.2- *Botrytis cinerea* and *Monilinia laxa* (fungal rot)

Botrytis cinerea is a fungus that affects many plant species, although its most notable hosts may be wine grapes. In horticulture, it is usually called grey mould and it is economically important on soft fruits such as strawberries and bulb crops (Jacometti *et al*., 2006).

Brown rot (*Monilinia laxa*) is a fungal infection that affects stone fruit, commonly affecting peaches, pears, apples and plums (Bonaterra *et al*., 2003). The fruit develops small brown squishy circles, which gradually spread over the surface of the fruit. Once the fruit is entirely infected, it shrivels up and develops a

fuzzy coating of fungus. Brown rot can also infect flowers, leaves and stems of the tree, causing serious damage. Young fruits are not usually susceptible to brown rot unless they are damaged in some way, giving the spores access to the interior of the fruit. Once the fruit ripens and becomes soft, it is more easily infected, especially under warm, moist and humid conditions. In severe infections, an entire crop of fruit can be destroyed in just a few days.

1.2.3- *Xanthomonas vesicatoria* and *Erwinia carotovora* (bacterial rot)

Bacterial spot, caused by the bacterium *Xanthomonas campestris* pv. *vesicatoria*, is one of the most serious diseases of sweet peppers. This bacterial pathogen is also capable of causing disease on tomatoes. The pathogen can cause severe leaf and fruit losses. Infected plants in the seed bed usually have small, irregular, black or water-soaked spots along the edges of the first leaves. Older plants develop small, pale green or water-soaked lesions that are slightly raised on the underside of the leaf (Gürlebeck *et al*., 2006). Spots are often surrounded by a yellow halo and their center may dry and tear.

Erwinia carotovora is a plant pathogen with a wide host range (carrot, potato, tomato, leafy greens, squash and other cucurbits, onion, green peppers, etc.), able to cause disease in almost any plant tissue it invades. It is a very important pathogen in terms of postharvest economical losses, and a common cause of decay in stored fruits and vegetables. Decay caused by *E. carotovora* is often referred to as bacterial soft rot. Most plants or plant parts can resist invasion by the bacteria, unless some type of wound is present. High humidity and temperatures around 30 ^{o}C favour development of decay. Virulence factors include: pectinases, cellulases (which degrade plant cell walls), and also proteases, lipases, xylanases and nucleases (Mijan Hossain *et al*., 2005).

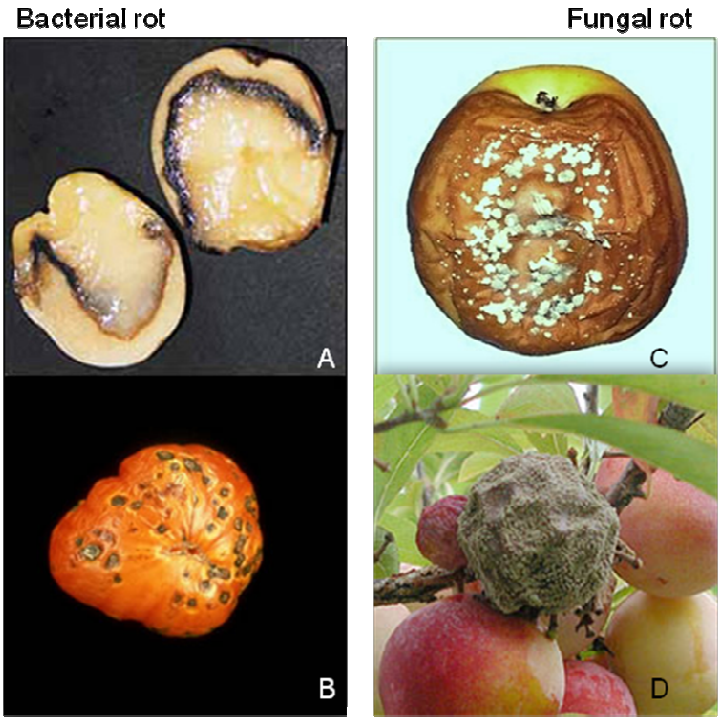

Figure 2 - Examples of fresh fruits and vegetables spoilage due to bacterial and fungal rot. A-*Erwinia carotovora*, B-*Xanthomonas vesicatoria*, C- *Penicillium expansum* and D-*Monilinia laxa* (EPPO, 2007)

2-Techniques to extend the quality of minimally processed fruit and vegetables

Every step the produce undergoes, from cultivation to the shelf, is important for its quality and safety. Specific control methods can be used and integrated into a safe food production and processing system to control pathogens and ensure the microbiological safety. Guidelines for harvesting, storage and packing fresh or minimally processed fruits and vegetables generally specify a washing or sanitising step to remove dirt, pesticide residues, and microorganisms responsible for quality

loss and decay (Sapers, 2003). The current published data suggest that none of the available washing and sanitising methods can guarantee the microbiological quality of minimally processed vegetables without compromising their sensorial quality (Ongeng *et al*., 2006).

2.1- Control methods of plant disease during production and postharvest

During the processing of agricultural products, significant economic losses occur due to the action of pathogenic microorganisms. Physical and biochemical strategies to reduce pathogens growth include accumulation of phytoalexines, modification of cellular walls and synthesis of hidroxylases, chitinases and β-1,3-glucanases as lytic enzymes, which have been proven successful as processes promoted by plants theirselves (Mari *et al.*, 1996). However, these methods are not sufficient to avoid the infection process in practice. Conventional treatments based on the application of bactericides and fungicides are used instead. These methods have been the most used during years because have shown to be effective, persistent on time, with an easy application and prompt results. As a consequence the accumulation of chemical residues on agricultural produce affecting directly the consumer's health and the environment it is a reality we are now facing with. Besides, it has been documented that the continuous use of chemical compounds selects resistances in bacteria and fungi.

Nowadays, there is a trend to rational the use of pesticides, as well as to reduce the number of authorized active substances to those unavoidable, more selective, less toxic and with lower negative impact (Ragsdale and Sisler, 1994; European Comission, 2003). The European Union has established the 91/414/EEC Directive regulating the registration of pesticides. In the case of postharvest, the few permitted chemical products used to control postharvest fruit diseases in Spain are captan, imazalil, iprodione, tiophanate methyl, ortophenylphenol and thiabendazole (MAPA, 2005).

The technology most frequently used to control postharvest diseases is based on storage methods under refrigeration combined or not with modified atmosphere. With such a method transpiration of produces is reduced, biochemical changes of senescence of fruit are restrained, and spore germination and growth of bacteria and fungus are inhibited. In parallel, other technologies widely used are the application of heat treatments as hot water, water vapour and hot air (Lurie, 1998), or the addition of plant extracts and permitted synthetic chemical fungicides to fruit and vegetables before their storage in cool storage chambers (Eckert and Ogawa, 1988; Bautista-Baños *et al*., 2000).

2.2- Control treatments for minimally processed fruit and vegetables

Minimally processed vegetables are mainly treated with chlorine-based washing and decontamination procedures (Seymour, 1999). However, there is a controversy about the formation of carcinogenic chlorinated compounds in water (chloramines and trihalomethanes), calling into question the use of chlorine (Wei *et al*., 1999). The current concern associated with chlorine outlines the need for research on other treatments suitable for use on fresh-cut products. There is a continuous demand for new techniques for maintaining quality while inhibiting undesired microbial growth to be applied in all the steps of the production and distribution chain (Allende and Artes, 2003).

2.2.1- Chemical methods

Chemical methods such as the use of chlorine dioxide, organic acids, hydrogen peroxide, electrolysed water, ozone or calcium based solutions have been developed (Table 3). Most of them are of easy application, and have a strong bactericidal effect. However, many of these methods have some drawbacks. For example, the use of chlorine dioxide has a demonstrated efficiency in reducing bacterial populations of the produce, but affects some of its organoleptical

properties (Rico *et al.,* 2007). Another important fact to consider is that a drastic reduction of microbial population of fresh products can allow the better development of posterior spoilage and pathogenic microorganisms.

2.2.2- Physical treatments

Physical treatments such as irradiation, ultraviolet light or modified atmosphere packaging have been developed (Table 4). These methods can be either bacteriostatic or bactericidal, and have shown high efficiency in the inhibition of microbial contaminants. Non target effects related to these techniques have been also described, making some methods useless for industrial application (Rico *et al.,* 2007). A completely effective technique with the absence of non target effects related has not been developed yet.

Table 3- Chemical treatments used for the preservation of minimally processed vegetables and fruits.

Chemical treatment	Effect	Main Uses	Advantages	Disadvantages	References
Chlorine	Antimicrobial activity related to oxidation capacity.	Liquid chlorine and hypochlorite are used (50-200 ppm) with contact times of less than 5 min.	Easy and economic application.	Corrosion of products. Efficacy questioned. Banned in some European countries. Formation of dangerous compounds such as chloramines.	Francis and O'Beirne, 2002.
Chlorine dioxide (ClO_2)	Antimicrobial activity related to high oxidation capacity, of about 2.5 times greater than chlorine.	Accepted for use in washing fruits and vegetables.	No reaction with nitrogen-containing compounds or ammonia to form dangerous chloramines .	Affects organoleptical properties of the product. Complementary techniques needed.	Benarde *et al.*, 1965 . White, 1992 . FDA, 1998. Singh *et al.* 2002. Lee *et al.*, 2004.
Organic acids	pH reduction, disruption of membrane transport and permeability. Anion accumulation. Reduction in internal cellular pH.	Washes of lactic, citric, acetic, tartaric or ascorbic acid.	Considered GRAS (Generally Recognized As Safe).	Limited spectrum of action.	Priepke *et al.*, 1976. Beuchat, 2000 . Bari *et al.*, 2005.
Hydrogen peroxide	Properties as an oxidant. Generates other cytotoxic oxidising species such as hydroxyl radicals.	Treatment by dipping in H_2O_2 solution.	Efficient reduction of microbial populations.	Variable residual H_2O_2 levels. Causes browning of shredded lettuce.	Juven and Pierson, 1996 . Parish *et al.*, 2003.

Table 3- Continued

Chemical treatment	Effect	Main Uses	Advantages	Disadvantages	References
Calcium-based solutions	Maintain the vegetable cell wall integrity. Inhibit plant tissue senescence. Have antibacterial properties.	Widely used for delicate fruit and products with a high senescence index. Calcium lactate (0.5-2%) has been used as a firming agent for fruit.	The final product can significantly increase the calcium content.	Limited efficacy as antimicrobial. Bitterness and off-flavours associated with calcium chloride.	Smout *et al*., 2005 . Rico *et al*., 2006 . Anino *et al*., 2006. Manganaris *et al*., 2007 .
Ozone	Strong antimicrobial agent. Reduces microbial populations. Oxides ethylene.	Treatment with ozone has a beneficial effect in extending the storage life of fresh non-cut products.	High reactivity, penetrability and spontaneous decomposition to a non-toxic product. Considered GRAS.	Corrosiveness of products and initial capital cost.	FDA, 1997 . Beuchat *et al*.,1998. Smilanick *et al*., 1999. Grass *et al*., 2003. Beltran *et al*., 2005.
Electrolysed water (EW)	Acidic EW (pH 2.1-4.5) has a strong bactericidal effect.	Generated by electrolysis of aqueous sodium chloride to produce oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid, hydrochloric acid, hydrogen gas and sodium hydroxide.	Inactivates several pathogenic and spoilage microorganisms. Neutralises harmful substances such as cyanides, ammonium.	Reduces quality on fresh-cut vegetables.	Kim *et al*., 2000. Hsu, 2003. Bari *et al*., 2003. Wang *et al*., 2004.

Table 4- Physical treatments used for preservation of minimally processed vegetables and fruits.

Physical treatment	Effect	Main Uses	Advantages	Disadvantages	References
Modified atmosphere packaging (MAP)	Low levels of O_2 and high levels of CO_2 reduce the produce respiration rate, with the benefit of delaying senescence.	Extend shelf-life of fresh-cut vegetables by inhibiting spoilage microorganisms.	Fresh-cut products are more tolerant to higher CO_2 concentrations than intact products.	Changes of the gas composition. Fermentation and formation of off-flavours compounds. May allow growth of pathogenic bacteria.	Kader *et al.*, 1989. Kays, 1991. Fonseca *et al.*, 1999. Sivertsvik *et al.*, 2002. Al-Ati and Hotchkiss, 2002. Saltveit, 2003.
Irradiation	Reduces bacterial, parasitic, and protozoan pathogens in raw foods.	Low-dose gamma irradiation applied to fresh vegetables.	Approved by the FDA for use on fruit and vegetables.	Quality may be affected. Texture alteration.	IFT, 1983. Chervin and Boisseau,1994. Niemira *et al.*, 2002. Boynton *et al.*, 2005.
Ultraviolet light	Antimicrobial agent due to DNA damage. Induction of resistance mechanisms in different fruit and vegetables against pathogens.	Ultraviolet light irradiation applied to fresh products.	Relatively inexpensive and easy-to-use equipment.	Can cause damage to the treated tissue, increase stress, respiration rate, and induce a lignification-like process.	Nigro *et al*. 1998. Bintsis *et al.*, 2000. Cantos *et al.*, 2001.
High pressure processing	Microorganisms and enzymes can be inactivated with no degradation in flavour and nutrients.	Usual in meat products.	Not described.	Affects the integrity of porous products disrupting food tissues. Unsuitable for fresh vegetables.	Palou *et al.*, 2000.

2.2.3-Natural preservatives

Concern caused by traditional food preservatives, reporting of occasional allergic reactions in sensitive individuals and the formation of potentially carcinogenic by-products (e.g. nitrosamines from nitrite) among other problems, has increased the interest in antimicrobial compounds found in nature (Roller, 2003). Minimally processed fruits and vegetables industry is aware of consumer trends and aims to avoid the use of chemical preservatives (Meyer *et al*., 2002). The use of natural preservatives may be effective to retain the quality of minimally processed products by having an antimicrobial effect, inhibiting spoilage and avoiding oxidative processes. Moreover, the application of living antagonistic microorganisms can provide a protection against pathogens by means of a combination of mechanisms including the production of antimicrobial compounds. This process is known as bioprotection or biological control.

Natural antimicrobials can be defined as substances produced by living organisms in their competition with other organisms for space and nutrients. The main sources of these compounds are plants (e.g. plant secondary metabolites in essential oils and phytoalexins), microorganisms (e.g. bacteriocins and organic acids) and animals (e.g. lysozyme from eggs and transferrins from milk) (Meyer *et al*., 2002). Other options for obtaining natural preservative sources are by-products from different processing industries, such as whey permeate, which is a by-product of the cheese industry with a strong potential as a sanitising agent. Research and commercial applications have shown natural antimicrobials from these sources could replace traditional sanitising agents (Cherry, 1999). Newer tendency has been reported by Bari *et al*. (2005), who combined the efficacy of chemical disinfectant with the antimicrobial effect of bacteriocins produced by lactic acid bacteria on fresh produce.

2.2.4-Hurdle technologies

Hurdle technology refers to the combination of different of the above mentioned preservation techniques as a preservation strategy. The most important hurdles used in food preservation are based on controlling temperature, water activity, acidity, redox potential combined with the use of preservatives, modified atmosphere and competitive microorganisms (Leistner, 1999). By combining hurdles, the intensity of the individual preservation techniques can be kept comparatively low, minimising the loss of quality, while the overall impact on microbial growth may remain high. Hurdles need to be chosen in function of the quality attributes of a product (Gorris and Tauscher, 1999). Examples of hurdle technologies are the natural preservatives, which are used as hurdles in food deterioration but more work has to be done in more systematic studies on multi-synergistic effects in real food systems, e.g. combining lactoferrin, organic acids and oregano extracts with modified atmosphere packaging and pulsed electric field technology to prevent microbial growth (Rico *et al*., 2007).

3-Biological control and biopreservation

During the past years efforts have been increased to develop alternatives to pesticides such as biological control, consisting of living microorganisms (Biological Control Agents, BCA) with the capacity to avoid or decrease the development of pathogen and consequently its infection (Janisiewicz and Bors, 1995). Biological control and bioprotection fit in well with the concept of sustainable agriculture because they exploit natural cycles with reduced environmental impact (Spadaro and Guillino, 2004).

The National Academy of Sciences of United States of America defines biological control as "the use of natural or modified organisms, genes or gene products to reduce effects of undesirable organisms and to favour desirable organisms such as crops, trees, animals, insects and beneficial microorganisms" (NAS 1987, cited by Thomashow and Weller, 1996).

As it has been commented above, most infections of fruit and vegetables occur through wounds. So, the application of effective antagonists able to colonize wounds inhibiting the development or the growth of the pathogen is necessary. However, colonization is not an easy task since a complex interaction between antagonist, pathogen, host resistance, wound response, and other interacting microorganisms may occur involving a possible variation of biocontrol levels. In case of biological control of postharvest diseases, frigoconservation chambers show stable storage environment with constant temperature, gas composition and humidity, parameters that can be controlled to switch the host-pathogen-antagonist equilibrium towards the antagonist (Wilson *et al.*, 1987, Pusey *et al*., 1988). Moreover, postharvest biocontrol is especially feasible because harvested fruit is easily treated with antagonists and the same facilities used to apply fungicides can be used to treat produce with BCAs.

Summarizing, the following characteristics are desirable for the ideal antagonists (Wilson and Wisniewski, 1994):

- genetic stability,
- efficacy at low concentrations and against a wide range of pathogens on a variety of fruit and vegetables,
- simple nutritional requirements,
- survival in adverse environmental conditions,
- growth on cheap substratum in fermenters,
- lack of pathogenicity for the plant and no production of metabolites potentially toxic to humans,
- resistance to the most frequently used pesticides, and compatibility with other chemical and physical treatments.

The development of microbial antagonists requires many different studies, but usually the first stages include the following steps (Figure 3): to select possible antagonists by means of a screening method able to analyze a high number of

isolates, to develop a mass production method, to find an appropriate formulation which allow to increase biocontrol activity and ensure its stability, to determine the mechanisms of biocontrol to improve its activity, to develop a monitoring system to detect and quantify the BCA in the environment and to make more extensive toxicology tests or environmental impact studies with the aim to register the biopesticide.

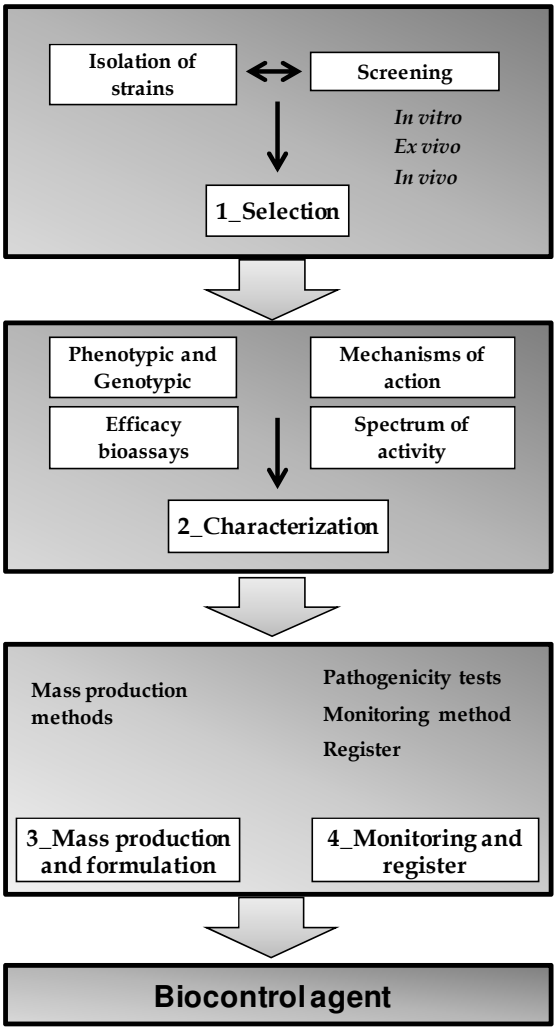

Figure 3.- Scheme of steps required for development of a biological control agent.

3.1-Selection

Selection consists of the isolation and screening of microorganisms able to interfere with the biological cycle of plant pathogens or pests. It is strongly critical to decide aspects such as, nature of samples, media composition and enrichment-isolation techniques to success in the isolation protocol of a BCAs (Montesinos, 2003). For example the use of selective or differencial media can allow the isolation of a microbial group which has been related with good antagonists (e.g. *Pseudomonas* or *Pantoea* species). An interesting site described by some authors to find candidates for BCAs is near of the infection place of the pathogen (Handelsman *et al*., 1990). Finally, microorganisms with bioprotective or biocontrol capacity could be isolated from the same natural environment where will be introduced or applied afterwards, to ensure the ecological adaptation of the BCA. Likewise, the surface and wounds of fruit are places with high probability to find effective postharvest BCAs. As the presence of microorganisms with high antagonistic activity is relatively rare in the environment, the isolation of a high number of candidates is recommended.

The next stage of biocontrol development is to choose a screening method, which needs to be simple and to emulate natural conditions that will occur in the product and postharvest environment. At the moment, screening methods are based on efficacy bioassays that can be performed *in vitro, ex vivo* and *in vivo*, and finally, pilot trials under real conditions of application (field, greenhouse and postharvest) (Montesinos and Bonaterra, 1996). The ideal method to select antagonists consists on direct inhibition of disease in plant, nonetheless it is material and time consuming, becoming an impractical method of screening (Sivan and Chet, 1992). *In vitro* assays are the most used because are relatively easy to perform and prompt results are obtained. However, they only provide information about the production of antimicrobial substances by antagonists, and inhibition by means of other mechanisms can not be detected. In addition, results of the selected BCA obtained *in vitro* do not necessarily correlate with control values obtained using plant material. It might be due to the limited capacity of the antagonist to survive on the new

environment or produce antimicrobial compounds (Mercier and Wilson, 1994). Once antagonists have been isolated from *in vitro* screening, they are analyzed by means of inhibition bioassays on vegetable or fruit material, verifying that they have biocontrol capacity. In this way there is a risk that some effective biocontrol strains on plant or fruit would not be chosen in the *in vitro* screening (Andrews, 1985).

3.2- Characterization of potential antagonists

When a microorganism has been selected for its capacity to control a specific pathogen it is necessary to proceed to its identification and characterization by phenotypic and genotypic analysis which will also allow identifying and monitoring the strain in the application environment. In addition, knowledge of the antagonist-pathogen inoculum density relationships can show what population levels of the antagonist are required to achieve adequate pathogen control (Montesinos and Bonaterra, 1996). Dose-response models have been developed to obtain quantitative parameters that allow to compare control efficacies of antagonist and to know the ratio of cells of BCA needed to inhibit one cell of the pathogen (Smith *et al*., 1997; Bonaterra *et al*., 2003; Francés *et al.,* 2006)

It is extremely important to demonstrate that the biopesticide does not have non-target effects on plants, animals and environment. Rather it is necessary an absence of plant pathogenicity, which is indicated by the absence of reaction of hypersensitivity in solenaceous plants, as well as the lack of acute toxicity on mammals (Montesinos, 2003).

Major limitation in the use of biocontrol is the knowledge of the control mechanisms developed by the antagonist. This knowledge can be crucial for the BCA development and for improving its efficacy. There are several mechanisms by which a microorganism can limit the growth of another. Most biocontrol agents do not strictly use a single control mechanism but a combination of several mechanisms, which all together allow the inhibition of the pathogen. The most important mechanisms operating are production of antimicrobial substances,

nutrient and space competition, induction of host resistance and direct interaction between the antagonist and the pathogen (Whipps, 2001). The production of antimicrobial substances is based on the synthesis of compounds such as antibiotics, bacteriocins or toxins that kill or have a detrimental effect on the target microorganism. This mechanism of action will be further detailed in section 4. Competition for nutrients and space is based on the capacity to competitively exclude other microorganisms (Andrews, 1992).

3.3.-Mass production and formulation

During the development of a biocontrol agent, studies about the production at industrial scale have to be performed to get a stable product through time for delivery. It is a key issue that mass production has to be rapid, efficient and inexpensive. There are several maintenance systems described, for example, refrigeration, freezing adhering cryoprotectant substances of keeping as a dehydrated product (Montesinos, 2003). Methods based on dehydratation such as lyophilization and spray-drying allow optimum conditions of storage, handling and formulation of the microorganism. Moreover, several assays have been performed to test synergistic effect of the BCA combined with different substances of disease control (El Gaouth *et al*., 2000).

3.4-Monitoring and register

The register of plant protection products is regulated by the Directive 91/414/EEC, which was amended specifically for biopesticides by the Directive 2001/36/EC. The difficulties of registration, and the fact that it shows a reduced range of application and supposes an important economical risk for manufacturers is the main cause that most companies are not attracted for this kind of products. Table 5 shows some biopesticides actually registered.

The development of reliable monitoring methods that assure the unequivocal identification and quantification of the biological control agent after

field release is needed for strain register. These methods will provide information about the population dynamics of the biocontrol strain in the target organ. Such knowledge is essential, since the efficacy of the BCA depends on its colonisation and population size. For this purpose, culture or genomic-based methods are used which allow detection at the strain-level (Pujol *et al.*, 2006).

Table 5.- Products registered or under development for biocontrol of plant and postharvest diseases in USA and other countries. (EPA, 2007)

Biocontrol agents for postharvest diseases		
Active ingredient	**Product name**	**Target organisms**
B. subtilis GB03	Kodiak	*Rhizoctonia* sp. *Fusarium* sp. *Aspergillus* sp.
Bacillus turingensis	Several	Insects
Fusarium oxysporum	Biofox C	*F. oxysporum* *F. moniliforme*
P. agglomerans	Bloomtime biological	*E. amylovora*
P. syringae ESC10 and ESC11	Biosave	*B. cinerea* *Penicillium* sp. *Mucor pyroformis*
P. chlororaphis 63-28	*P. chlororaphis* strain 63-28	*Pythium* sp. *Rhizoctonia solani* *Fusarium oxysporum*

Moreover, biological control in other food products, such as meat products has been developed. In this case, different bacterial groups have been described, being the group of lactic acid bacteria one of the most relevant. Strains of lactic acid bacteria have been described as bioprotective agents of several meat products, such as frankfurter sausages (Milani *et al*., 1998), or sliced cooked cured pork (Mataragas *et al*., 2003). Bioprotection of other food products as cheese (Buyong *et al*., 1998) and milk (Roosland *et al*., 2003) have also been described.

4.-Lactic acid bacteria

Lactic acid bacteria (LAB) have been involved for thousands of years in food fermentations and are one of the most ancient preservation techniques. First signs of LAB utilisation date back to 6,000 BC, describing the fermentation of milk, and fermentation of meat (1,500 BC) and vegetable products (300 BC) (Fox, 1993). Obviously, the application of LAB to foodstuff was not carried out being aware of the preservation technique that was being used, but it proved indeed to be effective. Nowadays, the challenge has become the understanding of the preservation mechanisms in order to take advantage of them in the industrial production of foods.

4.1-Classification and physiology of LAB

LAB are gram positive rods and cocci, non-spore forming, anaerobes that require fermentable carbohydrates and mainly produce lactic acid during the fermentation of glucose (Vandamme *et al*., 1996). This definition includes more bacteria than LAB strains historically accepted, and the list is subsequently narrowed by pointing out the positive role that LAB play in the fermentation processes of food products. In spite of the frequent changes in LAB taxonomic classification, the widely accepted genera include: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus*, *Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Vandamme *et al*., 1996; Stiles and Holzapfel, 1997) (Figure 4). Some authors include the genus *Bifidobacterium* because of its probiotic role, although it belongs to a different phylogenetic group (Vandamme *et al*., 1996).

Typically, LAB are mesophyllic bacteria, but they are also able to grow at temperatures ranging from 5 to 45 ^{o}C, and they tolerate both acid and alkaline environments (Wood and Holzapfel, 1996). LAB are weakly proteolytic and lypolitic, which is of interest for the food industry, because many spoilage reactions

are due to these bacterial activities. They require the presence of free aminoacids, nitrogenated bases, and vitamins (Jay, 1996). Their metabolism is based on fermentative processes, homo- or heterofermentative, oxidising carbohydrates in all cases (Axelsson, 1998). They produce a great variety of products, mainly organic acids, alcohol and carbon dioxide (Ray and Deschel, 1992), although they often generate aromatic molecules, vitamins and bioactive peptides (Ross *et al*., 2002).

Among all LAB genera, *Leuconostoc* species have their natural ecological niche in green vegetation and roots, and they are also found in fermented and refrigerated food products (Hemme and Foucaud-Scheunemann, 2004), so they are typical inhabitants of the fresh produce microbiota. The antagonistic activity of *Leuconostoc* strains has been previously reported (Alakomi *et al*., 2000, Condon, 1987, Blom *et al*., 1999). Additionally, despite this genus has been intensively described as good antagonistic bacteria, its application for bioprotection of ready-to-use foods is not enough developed. The prevalence of *Leuconostoc* in fresh fruit and vegetables may be of interest, as bacteriocins produced by these bacteria could act as food preservatives. However, the absence of spoilage reactions on the food product due to the predominance of heterofermentative species has to be tested before the application (Björkroth *et al.,* 1998).

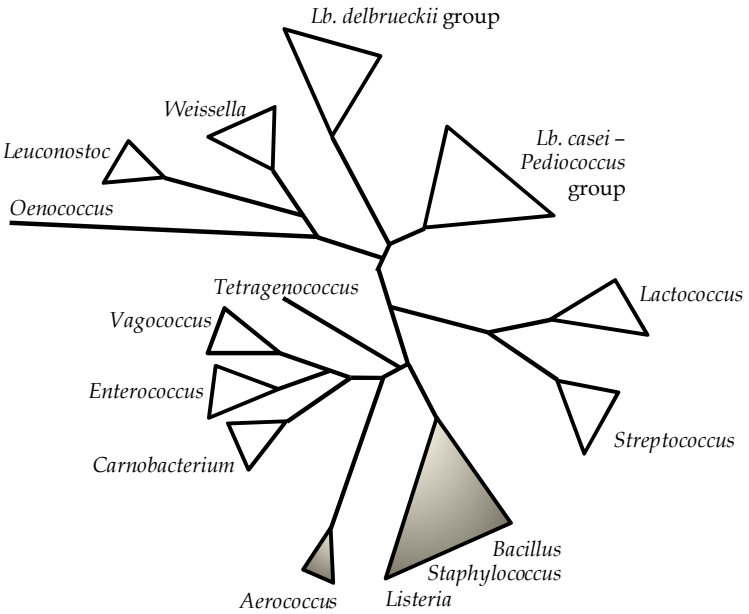

Figure 4.- Schematic, unrooted phylogenetic tree of lactic acid bacteria, including some aerobic and facultatively anaerobic Gram-positives of the low G+C subdivision, such as *Aerococcus, Listeria, Staphylococcus* and *Bacillus* group (in grey) (From Axelsson, 1998).

4.2-Antimicrobial mechanisms of LAB

The antimicrobial mechanisms of LAB that allow the biopreservation of food are due to the combined action of several metabolites produced during the fermentative reactions (Caplice and Fitzgerald, 1999). The production of organic acids (lactic, acetic and propionic acids) has an antagonistic effect on the microbiota thanks to the inhibition of the active transport processes, reactions and the modification of their membrane potential (Cleveland *et al*., 2001). Among them, acetic acid has de most severe antimicrobial effect, and it acts over fungi, yeasts and bacteria (Blom and Morvedt, 1991). Moreover, all organic acids cause a decrease of the pH, thus contributing to biopreservation. LAB's lack of catalase causes an accumulation of hydrogen peroxide (H_2O_2), which is able to inhibit bacteria and

fungi (Caplice and Fitzgerald, 1999). The carbon dioxide produced during heterolactic fermentations create an anaerobic microenvironment which is toxic for some aerobic bacteria, it also contributes to decrease pH (Cleveland *et al*., 2001).

A few LAB strains are able to produce protein compounds with a noteworthy antimicrobial effect, which are known as bacteriocins (Ross, *et al*., 2002). Some of them are highly specific, like lactococcins, and other have a wide antimicrobial spectrum, like nisin.

Bacteriocins

There is a growing interest in the use of bacteriocins for the improvement of food safety, but nisin is the only one being industrially processed so far (Cleveland *et al*., 2001). Bacteriocins are commonly divided into three groups (Table 6). Class I, termed lantibiotics, is mainly represented by nisin (Breukink and Kruijff, 1999). This Class is formed by peptides of 19 to more than 50 amino acids which are characterized by their unusual amino acids, such as lanthionine, methyl-lanthionine,

Table 6- Classification of bacteriocins (Owehand, 1998; Cleveland *et al.,* 2001).

Class	Subclass	Description	Bacteriocins
I		Lantibiotics	
	Ia	Cationic and hydrophobic peptides, flexible compared to Ib	Nisin
	Ib	Globular peptides	Mersacidin
II		Small (<10 kDa), moderate (100 ^{o}C) to high (121 ^{o}C) heat-stable non-lanthionine-containing membrane-active peptides	
	IIa	*Listeria* active peptides with Y-G-N-G-V-X-C near the amino terminus	Pediocin PA-A1, Sakacins A and P, Leucocin A.
	IIb	Two peptide bacteriocins	Lactococcins G and F, Lactacin F
III		Large (>30kDa) heat-labile proteins	Helveticins J and V-1829.

dehydrobutyrine and dehidroalanine. Class I is subdivided into Class Ia and Class Ib. Class Ia bacteriocins, i.e. nisin, consist of cationic and hydrophobic peptides that form pores in target membranes (Figure 5). Class Ib bacteriocins are globular peptides with no net charge or a net negative charge with a more rigid structure, compared with Class Ia (Sahl and Bierbaum, 1998). Class II comprises heat stable non-modified peptides, and can be further subdivided. Class IIa includes Pediocin-like *Listeria* active peptides with a conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val and two cysteines forming a S-S bridge in the N- terminal half of the peptide (Eijsink *et al*., 1998). The two peptide bacteriocins, such as lactococcin and lactacin, form the Class IIb. Less information is available for Class III bacteriocins, which are large and heat labile bacteriocins. Table 7 shows some examples of bacteriocins produced by lactic acid bacteria.

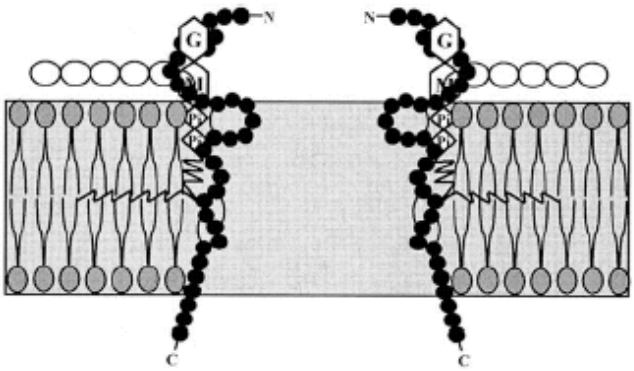

Figure 5_ Formation of pores in target membranes by nisin (Héchard and Sahl, 2002).

4.3-LAB as food preservatives

Several studies have documented the efficacy of lactic acid bacteria as food preservatives. Bioprotection assays have been performed on meat, fermented meat products, fermented vegetables, dairy products and fish. *Lactobacillus* sp. strains inhibiting the growth of *Penicillium* sp. and *Aspergillus* sp. in cereals (Gourama, 1997;

Vanne *et al.,* 2001). The inhibition of *E. coli* O157:H7 by LAB has been described *in vitro* (Reilly and Gilliand, 1996), and in meat (Brashears *et al*., 1996). The studies of the inhibition of *L. monocytogenes* are numerous and have been performed in many food products, such as meat (Schillinger and Lüke, 1989; Amézquita and Brashears, 2002) or cheese (Buyong *et al.,* 1998). Although many efforts have been done to develop bioprotective lactic acid bacteria strains, the application of these strains in fresh fruits and vegetables has not been developed yet. However, some approaches have been recently done. The presence of bacteriocin-producing lactic acid bacteria was reported by Ponce *et al*. (2008). Moreover, Allende *et al.* (2007) have studied the inhibition of *L. monocytogenes* after application of non-purified bacteriocins to fresh-cut lettuce, but have no tested the application of the bacteriocinogenic strains.

4.4-Safety and regulation of lactic acid bacteria

The lactic acid bacteria genera are among the bacteria with the lowest risk to human health. LAB have been used in food products all over the world for many years. Not only have there been no ovbious harmful effects of this enormous exposure to the bacteria, but also LAB are, in fact, an integral part of food safety of many foods. From all genera of LAB in food or probiotic use, only the enterococci and some *Lactobacillus* strains have been documented in opportunistic infections, and a number of virulence determinants have been associated with pathogenic strains of this genus. Lactobacilli have been asociated with isolated cases of infections, usually with bacteremia and endocarditis, but also with localized infections (Vesterlund *et al.,* 2007). Moreover, it has been suggested that the prevalence of infections caused by *Lactobacillus* is increasing (Anthony, 2000), although it has been demonstrated that the increase may not be real (Salminen *et al.,* 2004), and may be due to greater attention and a more active search for these organisms in cases of infection. Also, in most cases of *Lactobacillus* infection, the host's own microbiota is most likely to be the source of infection, rather than the strains provided in the food stuff (MacFie *et al*., 1999).

Table 7- Examples of bacteriocins and antimicrobial activity (From Owehand, 1998).

Class	Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
I	Lactocin S	*Lactobacillus sake* 145	3769 Da	*Lactobacillus, Pediococcus, Lactococcus*
I	Nisin A	*Lactococcus lactis* ssp *lactis*	3488 Da	LAB, *Staphylococcus, Micrococcus, Listeria, Mycobacterium, Clostridium, Bacillus*
II	Acidocin 8912	*Lactobacillus acidophilus*		*Lb. acidophilus, Lb. casei, Lb. plantarum, Lc. lactis*
II	Lacticin 3147	*Lactococcus lactis* DPC3147		LAB, *Clostridium, Staphylococcus*
II	Mesentericin Y105	*Leuconostoc mesenteroides* Y105	3700 Da	"wide"
II	Plantaricin S	*Lactobacillus plantarum*	Two proteins, α and β.	LAB, *Clostridium, Micrococcus, Propionibacterium*
III	Caseicin 80	*Lactobacillus casei* B80	4 kDa	*Lactobacillus casei*
III	Helveticin J	*Lactobacillus helveticus*	37 kDa	*Lb. helveticus, Lb. delbrueckii*

Food grade LAB are regulated in different ways in the different countries. In the USA, LAB are considered as GRAS (generally recognized as safe). A GRAS substance is defined by the USA Federal Food, Drug and Cosmetic Act as "generally recognized, among experts qualified by scientific training end experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case as a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use" (Section 201(s), USA Food and Drug Administration, 1999, cited by Wessels *et al*., 2004). The European Union (EU) has no

single, harmonized legislation that regulates the use of LAB in foods *per se*, whether as traditional starter cultures, as food suplements or as probiotic cultures. However, in anticipation of such regulation, the Scientific Comittee on Animal Nutrition has proposed the creation of a listed microorganisms with a history of safe use (European Comission, 2002), based on a Qualified Presumption of Safety (QPS), defined as "a belief or assumption based on reasonable evidence". This list would contain several species of the LAB group, including 32 species of *Lactobacillus, Lactococcus lactis, Leuconostoc citreun, Ln. lactis, Ln. mesenteroides, Pediococcus acidilactici, P. pentosaceus, P. dextrinicus* and *Streptococcus thermophilus* (Wessels *et al*., 2004).

Objectives

The aim of this work was to evaluate the potential use of lactic acid bacteria as biocontrol and bioprotective agents in fresh fruits and vegetables. Therefore, the work has been developed with the following objectives:

1. The isolation, selection, identification and characterization of potentially antagonistic LAB strains based on *in vitro* assays.
2. The realization of tests against the phytopathogenic microorganisms, especially *Penicillium expansum,* and against foodborne microorganisms.
3. The *in vivo* study of dose-response relationships between bacteriocinogenic strains CM135 and CM160 and non bacteriocinogenic strain PM249 and *Listeria monocytogenes* ATCC 15313.

Results

Although produced in low numbers, the foodborne pathogen outbreaks related to fresh fruits and vegetables are considered of high importance due to the fact that the fruit and vegetable products are consumed raw. Besides, there is a continuous increase of cases in the recent decades. Moreover, contamination by spoilage microorganisms can cause considerable economic losses to the fresh produce industry. Consequently, it is of great importance to avoid the contamination of fresh fruits and vegetables from both spoilage and pathogenic microorganisms. Safe production methods are the most important protection systems, but have shown to be inefficient or insufficient in some cases. Other protection methods, compatible with food industry regulation and procedures are continuously in development. The present thesis purposes the use of bioprotective cultures as a preventive method of fresh products contamination. We have chosen the lactic acid bacteria group as a target for the selection of biocontrol agents for several reasons. First, they are considered as GRAS, and fit all recommendations for

use in food stuff. Second, LAB are part of the natural microbiota of fresh fruits and vegetables. Third, LAB are known as good antagonists and have been successfully used as biocontrol agents of several food products.

The first stage of the study was the isolation of lactic acid bacteria in fresh fruits and vegetables. The collection of strains obtained was tested *in vitro* against five plant pathogens (*Xanthomonas campestris* pv. *vesicatoria, Erwinia carotovora, Botrytis cinerea, Monilinia laxa* and *Penicillium expansum)* and five human pathogen test bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Listeria monocytogenes* and *Staphylococcus aureus*) using two culture media. The second stage consisted in an *ex vivo* assay of the whole collection of isolates against the fungal pathogen *Penicillium expansum* in Golden Delicious apple wounds. The results of both assays were used for the selection of LAB strains as putative bioprotective agents. The selected strains were characterized and identified, and the antimicrobial compounds produced by these strains were analyzed. Some strains considered of interest were tested in an *ex vivo* assay, using three foodborne pathogen test strains, *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* in Golden Delicious apples and iceberg lettuce. Finally, dose-response relationships among the antagonistic LAB strains and the pathogen *L. monocytogenes* were established. The methodology, results and discussion obtained in this work are presented in the following sections as scientific articles which have been submitted for publication in research journals:

- **Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria.**
  Rosalia Trias , Lluís Bañeras , Esther Badosa and Emilio Montesinos.
  International Journal of Food Microbiology. (2008) 123, 50-60.

- **Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi.**
  Rosalia Trias, Lluís Bañeras, Emilio Montesinos and Esther Badosa.
  International Microbiology, submitted April 2008.

- **Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables.**
  Rosalia Trias, Esther Badosa, Emilio Montesinos, Lluís Bañeras.
  International Journal of Food Microbiology, submitted, March 2008.

Rosalia Trias, Lluís Bañeras, Esther Badosa and Emilio Montesinos. “Bioprotection of golden delicious apples and iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International journal of food microbiology*. Vol. 123 (2008) : p. 50-60

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Abstract

Lactic acid bacteria were isolated from fresh vegetables and fruit and its ability to inhibit the growth of foodborne human pathogens (*Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium,* and *Staphylococcus aureus)* was tested using the agar spot assay. Eighteen isolates showed a strong antagonistic capacity and were further characterised and identified using 16S rDNA sequencing and API 50CH. Most of them pertained to *Leuconostoc* spp. and *Lactobacillus plantarum*, and a few corresponded to *Weissella* spp. and *Lactococcus lactis*. Growth and efficacy of control of foodborne pathogen test bacteria by selected strains were tested in wounded Golden Delicious apples and Iceberg lettuce leaf cuts. The strains grew on the substrates and did not cause negative effects on the general aspect of tissues of apple or lettuce. Treatment of apple wounds and lettuce cuts with the antagonistic strains reduced the cell count of *S. typhimurium* and *E .coli* by 1 to 2 log cfu/wound or g, whereas the growth of *L. monocytogenes* was completely inhibited. Results support the potential use of lactic acid bacteria as bioprotective agents against foodborne human pathogens in ready-to-eat fresh fruit and vegetable products.

Keywords: Lactic acid bacteria; Antagonistic activity; Foodborne pathogens; Ready-to-eat vegetables; Fresh fruit

Lactic acid bacteria from fresh fruits and vegetables as biocontrol agents of phytopathogenic bacteria and fungi

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SUMMARY
The aim of the study was to evaluate the efficacy of lactic acid bacteria isolated from fresh fruits and vegetables as biocontrol agents against the phytopathogenic and spoilage bacteria and fungi, *Xanthomonas campestris, Erwinia carotovora, Penicillium expansum, Monilinia laxa,* and *Botrytis cinerea.* The antagonistic activity of 496 lactic acid bacteria strains was tested *in vitro,* and all tested microorganisms except *P. expansum* were inhibited by at least one isolate. The 496 isolates were also tested for inhibition of infection of *P. expansum* in wounds of Golden Delicious apples. Four strains TC97, AC318, TM319 and FF441 reduced the fungal rot diameter of apples by 20%, and only, *Weissella cibaria* strain TM128 decreased infection levels by 50%. Cell-free supernatants of selected antagonistic bacteria were studied to determine the nature of the antimicrobial compounds produced. Organic acids were the preferred inhibition mechanism but hydrogen peroxide was also detected when strains BC48, TM128, PM141 and FF441 were tested against *Erwinia carotovora*.
These results support the potentiality of lactic acid bacteria as biocontrol agents against postharvest rot. Previous works reporting of antifungal activity by lactic acid bacteria are scarce.

Keywords: Lactic Acid Bacteria, *Penicillium expansum*, Biocontrol, Spoilage and phytopathogenic microorganisms, Fresh fruit.

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Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables

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ABSTRACT

Ten *Leuconostoc mesenteroides* and a *Ln. citreum* strains isolated from fresh fruit and vegetables were tested for their antagonistic capacity against *Listeria monocytogenes*. Genetic differences among strains were analyzed by Random Amplified Polymorphic DNA (RAPD). All the isolates clustered together and differed from the type strain *Ln. mesenteroides* ATCC 8293 as well as from *Ln. fallax* and *Ln. citreum*. Organic acids, hydrogen peroxide and bacteriocins were detected as main inhibition mechanisms. Characterization of culture supernatants from the bacteriocinogenic strains, CM135 and CM160 revealed a high resistance of antibacterial activity to temperature and pH, and a bactericidal mode of action against *L. monocytogenes*. The results suggest the production of Class IIa bacteriocins. A study of the effect of the relative dose of pathogen and LAB on control of *L. monocytogenes* in wounds of Golden Delicious apples and Iceberg lettuce leaf cuts was performed. A comparison of the dose of bioprotective strain needed for a ten fold reduction of the viable pathogen concentration (ED_{90}) revealed that strain CM160 was the most effective against *L. monocytogenes*. ED_{90} values varied from $1.3 \cdot 10^4$ to $5.0 \cdot 10^5$ cfu per g or wound, at ranges of pathogen levels from $1.0 \cdot 10^3$ to $5.0 \cdot 10^4$ cfu per g of lettuce or wound of apple. The efficiency of the strains was also calculated as the ratio of the ED_{90} value to the pathogen dose inoculated. The lowest ratio was found for strain CM160 at 5 to 50 cells of LAB per cell of pathogen. The strain offers potential application for prevention of the presence of *L.monocytogenes* in fresh fruit and vegetables.

Keywords: *Leuconostoc*, fresh fruits and vegetables, *Listeria monocytogenes*, bioprotection.

Running title: *Leuconostoc* biocontrols *Listeria* in apple and lettuce

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final dissertation

Fresh vegetable and fruit products are eaten raw without further processing and consequently their microbiological safety is a key issue to ensure quality standards. In the case of minimally processed vegetables and sliced fruit, it is known that there is an increase of the microbial population, due to the characteristic high humidity, and the high number of cut surfaces present (Ongeng, *et al*., 2006). The microbial densities found in fresh fruits and vegetables can range from 10^2 to 10^8 cfu per g being higher in the latter type of products. Differences can be observed among the different products analyzed (Badosa *et al*., 2008) (Figure 6). Some of these microorganisms present in fresh fruit and vegetables can be human pathogens or spoilage microorganisms. This fact has two main consequences. First, these products have been incriminated in outbreaks of foodborne diseases caused by human pathogens (Ackers *et al*., 1998) and second, significant economic losses occur due to the action of deletereous microorganisms causing postharvest rot (Francés *et al*.,

2006). Moreover, the action of spoilage microorganisms may also be important because they promote the development of human pathogens (Wells and Butterfield, 1997; 1999). Thus, the control of spoilage microorganisms can contribute to limit the growth and colonization of pathogenic microorganisms, although this should not be considered as a warranty for product quality.

The use of bioprotective cultures which inhibit these pathogenic and spoilage microorganisms is proposed as a prevention method and has important advantages, such as the temperature-responsive inhibition and the continuous production of antimicrobial compounds, which can circumvent the problem of their decomposition. The main objective of this thesis was the development of biocontrol agents from the lactic acid bacteria group capable to reduce fresh produce spoilage and contamination with pathogens. Lactic acid bacteria have been described as good antagonists and have been traditionally used for the preservation of meat, dairy products, and fermented vegetables (Ruiz-Barba *et al*., 1994; Stiles and Holzapfel, 1997). They are natural inhabitants of fresh fruits and vegetables, and most species are considered as GRAS by the USA Food and Drug Administration (FDA).

All together, the information that was available at the time of starting this experimental work indicated that LAB could be used as good bioprotective agents in fresh fruit and vegetables. However, at that moment we were not fully convinced whether these organisms could be used to fight spoilage and pathogenic microorganisms *in vivo* or not. To address this question successfully we have needed a final screening over a large series of newly LAB isolates.

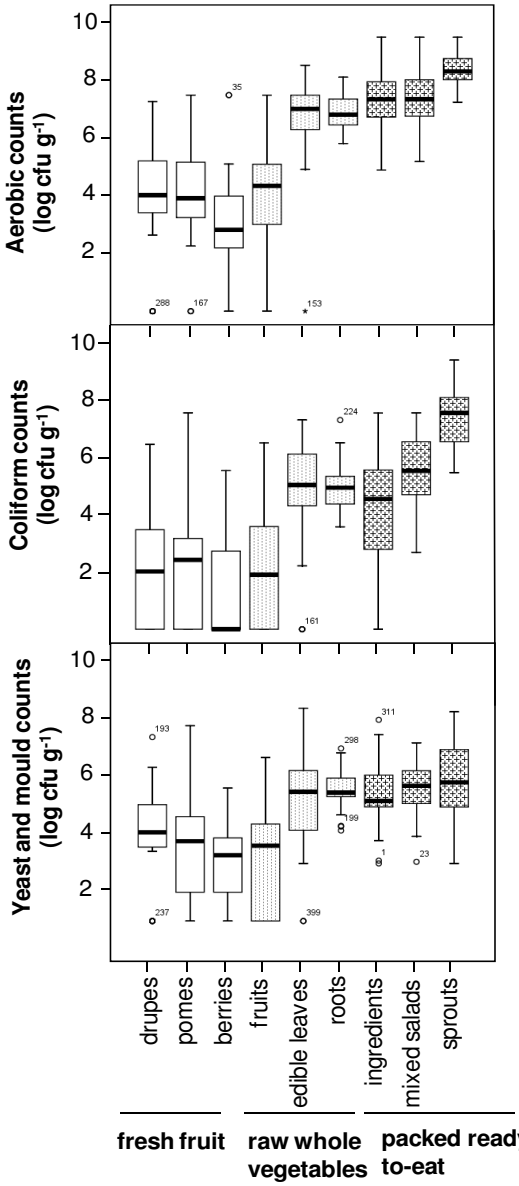

Figure 6- Overall incidence levels of aerobic and coliform bacteria and yeast and mould counts from fresh fruit and vegetable samples from a total amount of 445 samples (from Badosa *et al.,* 2008).

Spoilage microorganisms, both fungi and bacteria seemed to be sensitive to the presence of lactic acid bacteria, and isolates showing a medium to high inhibition were frequently detected (Figure 7). *In vitro* assays clearly indicated that LAB can prevent grey mould rot (*Botrytis cinerea*) and bacterial soft rot (*Erwinia carotovora* and *Xanthomonas campestris* pv. *vesicatoria*, among others) although additional experiments using vegetable models are still needed to assess their potential application. The complete set of results obtained with the blue mould rot causing *P. expansum* allowed us to be reasonably confident about the potential use of lactic acid bacteria as control agents of spoilage microorganisms.

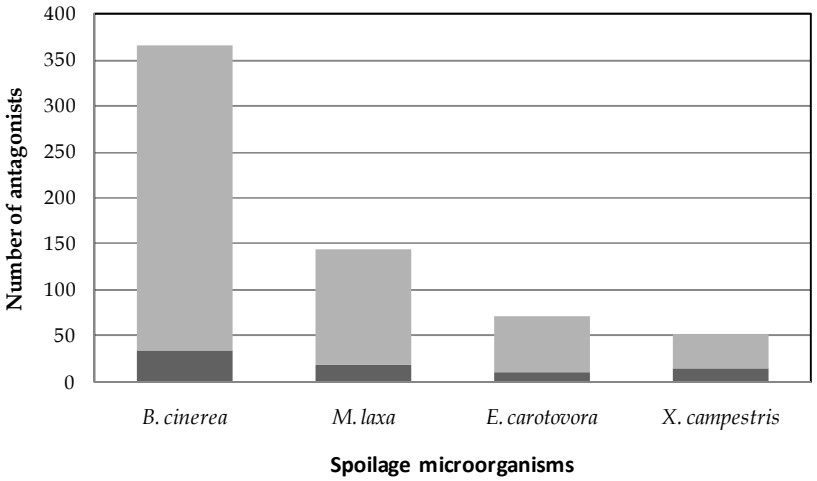

Figure 7- Lactic acid bacteria isolates showing medium to high inhibition of spoilage microorganisms. Dark grey shows moderate to high inhibition and soft grey medium inhibition.

The inhibition of blue mould rot infection was tested in Golden Delicious apple wounds. One strain, *W. cibaria* TM128, was able to reduce infections by a 50%. It can be argued that the attained inhibition effectivity is not good enough to consider strain TM128 as a BCA, providing other microorganisms can reduce the infections by an 80 to 90% (Francés *et al.*, 2006; Yu *et al.*, 2007). From our point of

view, other aspects need to be considered to correctly evaluate the potential use of TM128. Blue mould rot infections develop usually from much lower infective doses than those used here and this could result in the increase of efficiency of the strain TM128. *W. cibaria* TM128 is able to produce hydrogen peroxide and other inhibitory compounds which can be enhanced varying environmental conditions, allowing a better inhibition with lower colonization. Besides, TM128 is presumably a safe organism.

Some reports have related the antifungal activity of LAB to several organic acids, fatty acids and proteinaceous compounds (Schnürer and Magnusson, 2005). Recently, the antifungal activity of a *Pediococcus pentosaceus* strain has been tested in apple-based agar plates, showing inhibition of *P. expansum* due to proteinaceous compounds. Control of fungal spoilage of cucumber by *Lb. plantarum* was studied by Sathe *et al*. (2007) showing a significant delay in spoilage of the product. The above mentioned methods of action open up a full set of possibilities to be exploited using lactic acid bacteria as antifungal BCA.

LAB isolates were also assayed *in vitro* against foodborne pathogens showing significant differences among strains (Figure 8). Although *in vitro* assays are not generally considered as the best choice for screening, they are usually done for time and economic reasons. In our study, *in vitro* assay with foodborne pathogen test bacteria was considered useful, as helped to detect the inhibition of target microorganisms by means of production of antimicrobial substances, which is considered one of the main mechanisms of inhibition used by lactic acid bacteria. *P. aeruginosa* and *S. aureus* were the most sensitive indicators tested. In the most cases, these indicators were inhibited by organic acids. On the contrary, *L. monocytogenes* was the less inhibited indicator. This strain was considerably resistant to acidic pH caused by LAB metabolism.

Bacteriocin production was detected in strains CM135, CM160, and SE303. However, only strains CM135 and CM160 were able to inhibit *L. monocytogenes* in fruit and vegetal tissue. Other non bacteriocinogenic strains, such as PM249 were

almost as effective when tested over vegetable models. The relevant aspect of using bacteriocinogenic strains as BCA is that bacteriocins are highly effective at low concentrations, do not affect sensorial quality and a low colonization of the inhibiting bacteria is needed (Rodgers, 2001). Besides, we have shown that bacteriocin production occur at the final exponential growth phase and is completely dependent on the media composition.

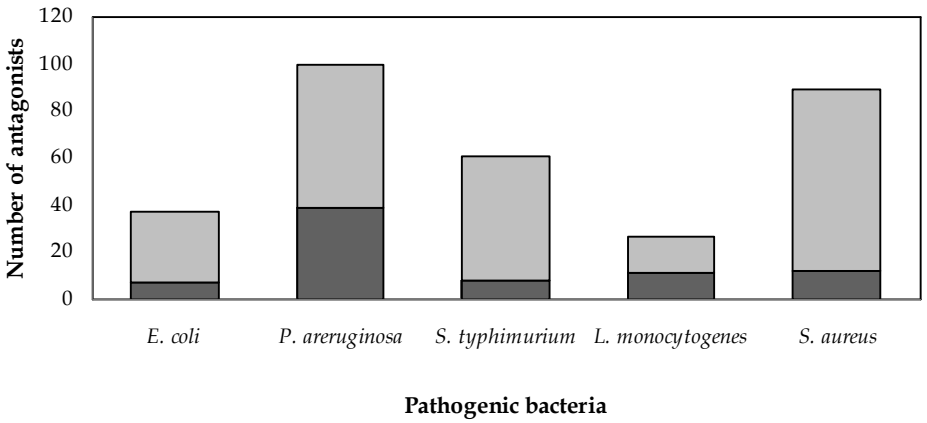

Figure 8- Lactic acid bacteria isolates showing medium to high inhibition of pathogenic microorganisms. Dark grey shows moderate to high inhibition and soft grey medium inhibition.

The comparison of dose-response tests performed with strains CM135, CM160 and PM249 clearly indicated that strain CM160 showed the best capacity of inhibition of *L. monocytogenes*, both in apple and lettuce tissue. Moreover, this strain reduces significantly the growth of *S. typhimurium* and *E. coli* in the two tested models. The results obtained with this strain are encouraging for the development of a bioprotective agent. However, further research is needed to identify molecularly the bacteriocin type produced by this strain.

Characterization of the selected strains showed that none of the selected potential antagonists caused hypersensitive response in tobacco plants. This fact was important, as signifies that no pathogenic or adverse effects would appear after inoculation of LAB in fresh products. In all the assays no modifications in the general aspect of the fruit and vegetable were observed for any of them when inoculated on either apples or lettuce leaves for a period of 5 days.

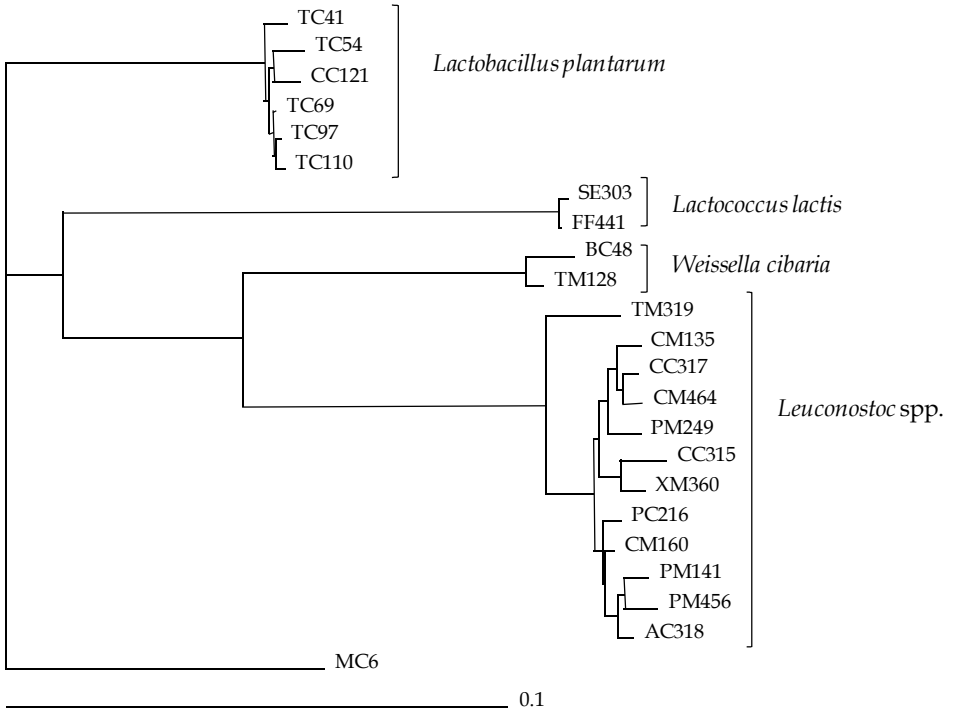

Figure 9- Phylogenetic tree (using the NJ method) showing relationships among almost complete 16S rDNA sequences from selected strains.

Identification of LAB strains with high antagonistic ability showed that *Leuconostoc* spp. were relatively abundant in fresh fruit and vegetables (Figure 9). These species have demonstrated their capacity of biocontrol *in vivo*. Other species

found, such as *Lb. plantarum* and *E. mundtii*, have shown inhibitory activity *in vitro* but have not been considered of interest for the development of a biocontrol agent due to the lack of an efficient activity in vegetable models.

Although it is more recommendable to use selection methods *in vivo*, the use of two *in vitro* media combined with the study of cell-free supernatants and the growth kinetics of the strains on the final sites of application provided valuable information for the appropriate selection of strains. Strain *W. cibaria* TM128 was selected for its partial inhibition of blue mould rot infections, and strains *Ln. mesenteroides* CM160, CM135 and PM249 for their inhibition of *L. monocytogenes* in Iceberg lettuce cuts and Golden Delicious apple wounds.

Results obtained in the persent work are encouraging for the development of lactic acid bacteria as bioptotective cultures of fresh fruits and vegetables.

Conclusions

1- Lactic acid bacteria strains could survive at postharvest conditions (0.5-1.0 ^{o}C, 1.2-1.3% O_2 and 1.2-2.0% CO_2) although reduction of population levels was detected after long periods.

2- The isolated lactic acid bacteria strains did not produce undesirable visible effects on the product, such as browning and off odours.

3- The bacteria *Pseudomonas aeruginosa* and *Xanthomonas campestris* pv. *vesicatoria* were the most sensitive to the inhibition by LAB. *Penicillium expansum* was the most resistant, as it could not be inhibited by any of the isolates *in vitro*.

4- Most of the isolates showing high antagonistic activity were identified as *Leuconostoc* spp. Six isolates were identified as *Lactobacillus plantarum*, two as *Weissella cibaria*, two *Lactococcus lactis* and one *Enterococcus mundtii*.

5- Organic acids were detected as the main antimicrobial substances produced by selected LAB, and were effective against Gram negative bacteria and fungi. Hydrogen peroxide was also detected and inhibited Gram negative and positive bacteria. Finally, bacteriocins were produced by strains CM135, CM160 and SE303, and inhibited Gram positive bacteria.

6- Strain *W. cibaria* TM128 was able to reduce blue mould rot infections at 50 %, and may have potential applicability as a method to prevent blue mould rot. The mechanism of inhibition may be related to biofilm formation or competition for space and substrate.

7- The selected LAB isolates reduced the growth of the test bacteria *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes*. The inhibition was more intense in the assay with *L. monocytogenes*, where the LAB had bactericidal effect.

8- The efficacy of bioprotection of the three selected *Leuconostoc mesenteroides* depended of the vegetable model, the dose of pathogen and the dose of bioprotective agent. Strain CM160 was the most effective strain in both models, and maintained its efficiency at high levels of pathogen.

9- The use of lactic acid bacteria as potential bioprotective strains of fresh fruits and vegetables provided encouraging results, especially for the inhibition of pathogenic *L. monocytogenes* in fresh products.

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