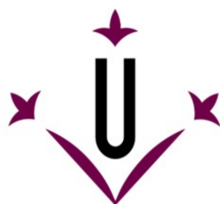

Novel preservation strategies for microbial decontamination of fresh-cut fruit and vegetables

Cyrelys Collazo Cordero

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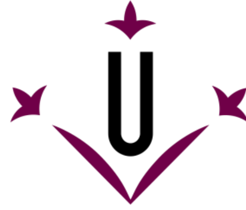

Novel preservation strategies for microbial decontamination of fresh-cut fruit and vegetables

by

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Thesis submitted for the degree of Doctor in Agricultural and Food Science and Technology

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Every mistake is a lesson if you can discover its cause and come up with a solution...Next time you´ll be wiser for not to repeat it.

Every result, as insignificant it may seem, makes a piece of your career if it leads you to a conclusion...The time spent in getting it makes you an expert.

Every person has something to teach you… Never underestimate anyone… Dealing with different personalities is a skill as useful as the 'pull and push' door signs

Bad times makes you tougher, good times makes you… wake up in the morning and keep going, even when they only exist in your mind...hold to them.

(My learning)

Life is a journey,
Not a destination,
There are no mistakes,
Just chances we've taken…
It's the dawn of a new day
New hopes, new dreams, new ways
(‘A beautiful day’ by India Arie)

Thank you so much to the people who have given me the great opportunity of obtaining this degree, of growing as a researcher and as a person, and acquiring experience, confidence and organizational skills. To those who directly participated in obtaining the results and in the preparation of the final report. To those with whom I shared laughs, jokes and good moments and that have patiently endured my complaints and concerns. To those from whom I gained valuable experiences and knowledge, that were by my side at work, helped me in some way or, at least, that did not trip me up while I was running from one side to another in IRTA. To who gave me life and made me who I am. To who has supported me in my successes and failures. To those who are always with me 'though being far away. All of you are implicit in this book, and you will also be forever included in this chapter of my life.

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ABBREVIATIONS

$^1O_2$	Singlet oxygen
AA	Ascorbic acid
ACC	1-amicyclopropane-1-carbocylic acid
ACD	ACC dehydrogenase
ACO	ACC oxidase
ACS	ACC synthase
AHL	N-acyl-homoserine lactones
AIP	Auto-inducing oligopeptides
AS1	NatureSeal® AS1
BCA	Biocontrol agent
BHI	Brain heart infusion
C	Carbon
C_2H_4	Ethylene
CA	Controlled atmosphere
CAA	Casaminoacids
CAS	Chrome azurol S
CAT	Catalase
CFS	Cell-free supernatant
CFU	Colony Forming Units
CHS	Chalcone synthase
DAPG	2,4-diacetylphloroglucinol
DNA	Deoxyribonucleic acid
EC	European Union
EPS	Extracellular polymeric matrix
FDA	United States Food and Drug Administration
GN	Gram negative
GP	Gram positive
GSH	Glutathione
GSR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HACCP	Hazard Analysis of Critical Control Points
HCN	Hydrogen cyanide
HR	Hypersensitive response
ISR	Induced Systemic Resistance
LAB	Lactic acid bacteria
LLO	Listeriolysin O
LP	Lipopeptide
M63	Minimal salts medium
MAMPs	Microbe–activated molecular patterns
MAP	Modified atmosphere packaging
MDA	Malondialdehyde
MGA	Meat extract-glucose-agar
MPP	Minimally processed produce
N	Nitrogen
NA	Nutrient agar
NH_4	Ammonia

O_2	Oxygen
O_3	Ozone
OD	Optical density
PAL	Phenylalanine-ammonia lyase
PAMPs	Pathogen activated molecular patterns
PCA	Plate count agar
PGPR-	plant-growth promoting rhizobacteria
PL	Pulsed light
POX	Peroxidase
PPA	Peroxyacetic acid
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PRRs	Plant pattern recognition receptors
QPS	Qualified presumption of safety
QS	Quorum-Sensing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	salicylic acid
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
SSC-	Soluble solids content
STEC	Shigatoxin-producing *E. coli*
TAC	Total antioxidant capacity
THM	Trihalomethanes
TPC	Total phenolic compounds
TSA	Tryptone soy agar
TSB	Tryptone soy broth
T3SS	Type 3 secretion system
UV	Ultraviolet light
UV-C	Short-wave Ultraviolet light
WUV	Water-assisted UV-C irradiation

ABSTRACT

The commercialization of minimally processed produce implies the application of preservation strategies for maintaining their freshness as well as preventing the cross-contamination with human pathogens while avoiding the generation of harmful byproducts. In the present work, various preservation strategies based on biological, chemical and physical methods and combinations thereof, were assayed to control foodborne pathogens in fresh-cut fruit and vegetables. A great part of this thesis focused on the study of the mode of action of biopreservation using the bacterium *Pseudomonas graminis* (CPA-7) against *L. monocytogenes, S. enterica* ser. Enteritidis and *E. coli* O157:H7 both *in vitro* and on fresh-cut fruit. *In vitro* experiments did not reveal any direct antimicrobial effect via the secretion of secondary metabolites during growth in liquid media or solid medium, melon juice or melon pieces. Neither did they reveal the ability of CPA-7 to form biofilms on inert surfaces or to produce molecules with binding or surfactant properties. However, the high nutritional overlapping indices (NOI) for the *in vitro* use of single nitrogen/carbon sources, suggested that CPA-7 could restrict the metabolic activity of *E. coli* O157:H7 and *S. enterica* subsp. *enterica* (NOIs of 0.92 and 0.83, respectively) through the competition for carboxylic acids. Furthermore, CPA-7 attenuated the adherence of *S. enterica* subsp. *enterica* ser. Enteritidis to a human epithelium model (Caco-2 cells) by 0.8 log_{10} regardless of the pre-adaptation on fresh-cut pear and reduced the invasiveness of both *L. monocytogenes* (by 1.3 log_{10}) and *S. enterica* subsp. *enterica* ser. Enteritidis (to < 5 CFU g^{-1}) after co-incubation on the fruit. CPA-7's ability to adhere to intestinal epithelium enabled it for competition, but at a very low probability due to the drastic reduction of its populations (by ≥ 6 log_{10}) after gastric digestion and the decrease of its recovery during the intestinal phase and its adhesiveness (by 0.8 log_{10}) after habituation on the fruit. CPA-7 could also potentially act indirectly against foodborne pathogens through the induction of

the plant host's defense response, since it enhanced the activities of antioxidant enzymes and delayed the decline in non-enzymatic antioxidants in fresh-cut apple according to the storage conditions. According to *in vitro* tests, the activation of the plant defense response did not seem to occur through the induction of ethylene biosynthetic pathways because CPA-7 reduced C_2H_4 levels in MAP by 29 to 43%, the lowest reduction occurring at the highest maturity level. Nevertheless, the reduction of C_2H_4 levels could have been associated to the induction of the enzyme ACC-deaminase, as suggested by the presence of this gene in the bacterial genome.

A water-assisted alternative of short-wave ultraviolet light C (UV-C) irradiation (WUV) was developed as a non-thermal physical preservation alternative and was evaluated for the decontamination of baby spinach leaves and fresh-cut 'Iceberg' lettuce as well as of conventional and organic broccoli. A reduction by 2 $\log_{10}$ of natural total mesophilic aerobic microbiota (MAM) of conventional broccoli was obtained upon processing with 0.5 kJ m^{-2} WUV, without compromising the product's physical quality. WUV (at doses ranging from 0.1 to 0.5 kJ m^{-2}) did not reduce MAM in baby spinach (0.3 kJ m^{-2}), 'Iceberg' lettuce (0.1 kJ m^{-2}) or organic broccoli (0.5 kJ m^{-2}). However, the combination of at least 0.3 kJ m^{-2} WUV and 50 mg L^{-1} peroxyacetic acid (PAA) equaled in organic broccoli the result obtained in conventional one. Treatment with 0.5 kJ m^{-2} WUV also increased total antioxidant capacity (TAC) compared to the water-control by 90 and 22%, in conventional and organic broccoli, respectively, 24 h post-treatment. It also increased the content in sulforaphane in conventional broccoli by 1.5-fold in respect of the water-washed control. Regarding the effect on foodborne pathogens, 0.3 and 0.5 kJ m^{-2} WUV reduced *L. innocua* populations in organic broccoli by 1.7 and 2.4 $\log_{10}$, respectively. No further improvement was obtained by combining 0.3 kJ m^{-2} WUV with PAA. The inhibitory effect of 0.5 kJ m^{-2} WUV was maintained throughout 8 d of refrigerated MAP storage, showing no population recovery regardless of exposure to daylight. In fresh-cut 'Iceberg'

lettuce and baby spinach leaves, WUV at doses ranging from 0.1 to 0.3 kJ m^{-2} allowed the effective decontamination and growth inhibition of both *Salmonella* subsp. *enterica* and *L. monocytogenes* by up to 2.1 log_{10} without significantly impacting respiration or overall quality of the products. Combining WUV and PAA showed no synergistic reduction of pathogens' populations in respect of the individual control treatments, but improved the effectiveness of PAA alone by completely inactivating both pathogens in the single-used sanitation solutions.

On the other hand, the application of wide-spectrum pulsed light (PL) (5 to 20 kJ m^{-2}, containing 10 % of UV-C) applied in air was ineffective for the control of MAM and *L. innocua* in fresh-cut broccoli beyond water-washing, without diminishing physical quality during 14 d of aerobic refrigerated storage. However, 24 h after 3-pulse treatment (15 kJ m^{-2}) total phenolic content increased by 25 % compared to the chlorine control, and TAC was enhanced by 12 and 18 %, compared to water and chlorine-washed controls, respectively. Finally, the integration of pretreatment with PAA + WUV and the subsequent inoculation with CPA-7 inhibited *L. monocytogenes* after 2 d of refrigerated storage in fresh-cut 'Iceberg' lettuce but it was inconsistent for controlling this pathogen in baby spinach leaves. However, in samples pretreated with PAA, the antagonistic activity of CPA-7 inhibited the growth of *L. monocytogenes* after 6 d of refrigerated MAP storage in baby spinach leaves and of *S. enterica* subsp. *enterica* in 'Iceberg' lettuce upon a cold-chain breakage. In summary, biopreservation and water-assisted UV are promising alternative-to-chlorine technologies which, acting through the concomitant activation of several mechanisms, can be implemented single or combined to improve the microbiological quality and bioactive properties of fresh-cut produce.

RESUMEN

La comercialización de frutas y verduras mínimamente procesadas implica la necesidad de estrategias de preservación que mantengan su apariencia fresca, previniendo la contaminación cruzada y el establecimiento de patógenos humanos de transmisión alimentaria así como la formación de subproductos dañinos. En el presente trabajo se evaluaron varias estrategias de preservación, basadas en métodos biológicos, químicos y físicos, para el control de patógenos de transmisión alimentaria en frutas y verduras mínimamente procesadas. Una gran parte de este trabajo se centró en estudio del modo de acción de la biopreservación empleando la bacteria *Pseudomonas graminis* CPA-7 contra los patógenos *Listeria monocytogenes, Salmonella enterica* subsp. *enterica* and *Escherichia coli* O157:H7, en condiciones *in vitro* y en fruta mínimamente procesada. Los experimentos *in vitro* no mostraron actividad antimicrobiana directa a través de la secreción de metabolitos secundarios durante el crecimiento en medios de cultivo líquido o sólido, ni en zumo o trozos de melón. Tampoco revelaron la capacidad de CPA-7 de formar biofilms en superficies inertes o de producir moléculas con propiedades adherentes o tensioactivas. No obstante, los altos índices de solapamiento nutricional (NOI) en el uso *in vitro* de fuentes individuales de carbono y nitrógeno, sugirieron que CPA-7 podría restringir la actividad metabólica de *E. coli* O157:H7 y de *S. enterica* subsp. *enterica* (NOIs de 0.92 y 0.83, respectivamente) a través de la competencia por ácidos carboxílicos. Por otro lado, CPA-7 atenuó en 0.8 $\log_{10}$ la adherencia de *S. enterica* subsp. *enterica* ser. Enteritidis al modelo celular de epitelio humano Caco-2, independientemente de la pre-adaptación en pera cortada. Además, redujo la invasividad tanto de *L. monocytogenes* (en 1.3 $\log_{10}$) como de *Salmonella* ser. Enteritidis (a niveles indetectables: < 5 UFC g^{-1} fruto) después de la co-incubación en la fruta. La capacidad de CPA-7 de adherirse a la superficie del epitelio intestinal indicó la posibilidad de que al ser ingerida ésta pudiera

establecer competencia con los patógenos por sitios de adhesión en el epitelio. Sin embargo, la drástica reducción de las poblaciones de CPA-7 (> 6 $\log_{10}$) durante la digestión gástrica y la reducción tanto de su capacidad recuperación durante la fase intestinal como la disminución de su adherencia (en 0.8 $\log_{10}$) después de la pre-incubación en el fruto, mostraron que la probabilidad de establecer competencia es muy baja. También se observó que CPA-7 es potencialmente capaz de actuar indirectamente contra los patógenos de transmisión alimentaria a través de la activación de la respuesta defensiva de la planta, ya que indujo la actividad de las enzimas antioxidantes y retardó la reducción del contenido en compuestos antioxidantes en manzana 'Golden' mínimamente procesada, en dependencia de las condiciones de conservación. Según los experimentos i*n vitro*, la señalización durante la respuesta defensiva de la planta inducida por CPA-7 no parece ser mediada por la activación de la síntesis de etileno ya la acumulación de esta molécula disminuyó de un 29 a un 43 % durante el almacenamiento en atmósfera modificada. La mayor reducción correspondió a una mayor madurez del fruto. Sin embargo, la reducción de los niveles de C_2H_4 pudo estar asociada a la inducción de la actividad de la enzima ACC-deaminasa en CPA-7 como sugirió la presencia del gen que codifica para esta enzima en el genoma de la bacteria.

Como otra alternativa a la descontaminación con cloro, se desarrolló una estrategia de preservación física no térmica que combina la aplicación de luz ultravioleta de onda corta (UV-C) y la inmersión del producto vegetal (WUV). Esta tecnología, a dosis de 0.1 a 0.5 kJ m^{-2}, se probó para la higienización de brotes de espinaca, lechuga 'Iceberg' y brócoli de cuarta gama, tanto orgánico como convencional. Los resultados mostraron una reducción de 2 $\log_{10}$ de la microbiota aeróbica mesofílica total (MAM) nativa del brócoli convencional (0.5 kJ m^{-2}). WUV no fue efectiva para la reducción de MAM en lechuga (0.1 kJ m^{-2}), brotes de espinaca (0.3 kJ m^{-2}) o en el brócoli orgánico (0.5 kJ m^{-2}). Sin embargo combinando UV-C y ácido peroxiacético (PAA) (al menos 0.3 kJ m^{-2} de WUV y 50

mg L^{-1} PAA) si se logró igualar en brócoli orgánico el resultado obtenido en brócoli convencional. El tratamiento con 0.5 kJ m^{-2} de WUV también incrementó la capacidad antioxidante total (TAC) en brócoli convencional en un 90% respecto al control lavado con agua, 24 h tras el procesado. El tratamiento con WUV (0.5 kJ m^{-2}) incrementó en un 22% la capacidad antioxidante en brócoli orgánico y en 1.5 veces el contenido en sulforafano en brócoli convencional, respecto al control lavado con agua.

En relación a la eficacia de la WUV para el control de los patógenos de transmisión alimentaria, el tratamiento de brócoli orgánico con 0.5 kJ m^{-2} de WUV redujo las poblaciones de *L. innocua* en 2.4 log_{10}. La inhibición de *L. innocua* se mantuvo durante 8 d de almacenamiento refrigerado en MAP sin mostrar ninguna recuperación poblacional, independientemente de su exposición a la luz blanca. En brotes de espinaca y lechuga 'Iceberg' mínimamente procesada, el tratamiento con WUV a dosis entre 0.1 y 0.3 kJ m^{-2}, en agua o en PAA (40 mg L^{-}1), redujo las poblaciones iniciales de *S. enterica* subsp. *enterica* y de *L. monocytogenes* hasta 2.1 log_{10} aunque no tuvo efecto sobre las poblaciones de MAM. La inhibición del crecimiento de los patógenos se mantuvo durante 6 d de almacenamiento en MAP a 5 °C sin afectar significativamente la calidad de los productos. La aplicación de la UV-C en ácido peroxiacético no mostró efecto sinérgico sobre las poblaciones de los patógenos en lechuga o espinaca respecto a los controles, químico o físico por separado, pero mejoró la efectividad del tratamiento individual con PAA inactivando ambos patógenos en las soluciones de higienización después de un solo uso.

Por otro lado, también evaluó la efectividad de los pulsos de luz de amplio espectro (PL) para la descontaminación de brócoli de cuarta gama ya que permite controlar las poblaciones microbianas en tiempos de tratamiento muy cortos, debido principalmente a los efectos directo y hormético de la fracción UV-C. Se ensayaron dosis de 5 a 20 kJ m^{-2} conteniendo un 10% de UV-C) pero ninguna de ellas mostró efecto sobre las poblaciones de MAM o de *L. innocua*

del brócoli respecto al control lavado con agua, sin afectar negativamente la calidad del producto, por lo que esta tecnología no es efectiva para la descontaminación de brócoli de cuarta gama. Sin embargo, el contenido de fenoles totales se incrementó en un 25 % respecto al control lavado con cloro, y la capacidad antioxidante total aumentó en un 12 y un 18 %, comparado con los controles lavados con agua o cloro, respectivamente, 24 h después de la aplicación de 3 pulsos de luz (15 kJ m^{-2}).

Finalmente, la integración del pretratamiento con WUV y PAA y la subsecuente inoculación con CPA-7, inhibió el crecimiento de *L. monocytogenes* después de 2 d de almacenamiento en refrigerado en MAP en lechuga 'Iceberg' mínimamente procesada. Sin embargo, la inhibición del crecimiento de este patógeno fue inconsistente en brotes de espinaca y no observó efecto sobre las poblaciones de *S. enterica* ser. Enteritidis en ninguna de las dos matrices evaluadas. No obstante, en muestras pre-tratadas con PAA, la actividad antagónica de CPA-7 inhibió el crecimiento de *L. monocytogenes* en brotes de espinacas a los 6 d de almacenamiento a 5 °C. También inhibió el crecimiento de *S. enterica* ser. Enteritidis lechuga 'Iceberg' ante una ruptura de la cadena de frío. En resumen, la biopreservación y la luz ultravioleta combinada con inmersión (WUV) son tecnologías alternativas al cloro que actúan simultáneamente a través de varios mecanismos y que son recomendables para ser implementadas, de manera independiente o combinada, para mantener o mejorar la calidad microbiológica y las propiedades bioactivas de productos vegetales de cuarta gama.

RESUM

La comercialització de fruites i verdures mínimament processades implica la necessitat d'estratègies de preservació que mantinguin la seva aparença fresca i previnguin la contaminació creuada amb patògens de transmissió alimentària evitant la formació de subproductes nocius. En el present treball es van avaluar diverses estratègies de preservació basades en mètodes biològics, químics i físics i en les seves combinacions, per al control de patògens de transmissió alimentària en fruites i verdures mínimament processades. Primerament es va investigar la biopreservació i el seu mode d'acció contra els patògens *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* i *Escherichia coli* O157: H7, a través de l'ús del bacteri *Pseudomonas graminis* CPA-7. Per a això es van avaluar mecanismes directes i indirectes tant *in vitro* com en fruita mínimament processada. Els experiments *in vitro* no van mostrar cap activitat antimicrobiana directa a través de la secreció de metabòlits secundaris en medi líquid o sòlid, o en suc o trossos de meló. Tampoc van revelar la capacitat de CPA-7 de formar biofilms en superfícies inerts o de produir molècules amb propietats aglutinants o tensioactives com l'alginat o els biosurfactants. No obstant això, els alts índexs de solapament nutricional (NOI) de l'ús *in vitro* de fonts de carboni i nitrogen van mostrar que CPA-7 podria restringir l'activitat metabòlica d'*E coli* O157: H7 i de *S. enterica* subsp. *enterica*, a través de la competència per àcids carboxílics (NOIs de 0.92 i 0.83, respectivament). A més, CPA-7 va disminuir l'adherència de *S. enterica* ser. Enteritidis al model d'epiteli humà (cèl·lules Caco-2) en 0.8 $\log_{10}$ independentment de la pre-adaptació en pera mínimament processada i va reduir la invasivitat de *L. monocytogenes* (en 1.3 $\log_{10}$) i de *S. enterica* ser. Enteritidis a nivells per sota del límit de detecció (5 UFC g-1 fruit) després de la pre-incubació en el fruit. CPA-7 va mostrar la capacitat de adherir-se a la superfície de l'epiteli intestinal, la qual cosa va indicar la possibilitat d'establiment de competència encara que a nivells molt limitades degut a la

dràstica reducció de les seves poblacions (≥ 6 log_{10}) després de la digestió gastrointestinal i la disminució de seva adherència en 0.8 log_{10} després de la pre-incubació en el fruit. CPA-7 també podria actuar indirectament contra els patògens de transmissió alimentària a través de l'activació de la resposta defensiva de la planta, ja que es va observar que aquest antagonista indueix els mecanismes antioxidants enzimàtics i va retardar la disminució dels capacitat antioxidant en poma 'Golden' mínimament processada en dependència de les condicions de conservació. Segons els experiments *in vitro*, l'activació de la resposta defensiva de la planta no sembla que es degut a l'inducció de la síntesi d'etilè com a molècula senyalitzadora ja que l'antagonista va reduir els nivells d'aquesta molècula en MAP d'un 29 a un 43% en funció de l'estat de maduresa del fruit. Pèro, va poder estar associat a la inducció de l'enzim ACC-deaminasa, com va suggerir la presència del gen que codifica per aquest enzim en el genoma del bacteri.

Com un altra alternativa a la descontaminació amb clor, es van provar mètodes de preservació físics no tèrmics basats en l'aplicació de llum ultraviolada d'ona curta (UV-C) a causa de la no generació de residus tòxics així com del seu efecte dual, antimicrobià directe i a l'activació dels mecanismes de defensa de la planta (efecte hormetic). Com a resultat d'aquest últim efecte, amés de reduir els poblacions microbianes s'incrementen les propietats bioactives del vegetal. Es va desenvolupar una tecnologia que combina la irradiació amb UV-C i la immersió del producte vegetal (WUV), el que millora l'accessibilitat de la llum a tots els costats del producte respecte a les càmeres convencionals d'UV-C alhora que permet la descontaminació de la solució de rentat. Es va avaluar la WUV per a la descontaminació de brots d'espinacs, enciam 'Iceberg' mínimament processat i bròquil de quarta gamma, aquest últim, de producció tant orgànica com convencional. Aquest assaig va mostrar una reducció de 2 log_{10} de la microbiota aeròbica mesòfila total (MAM) nativa del bròquil convencional en emprar dosi de 0.5 kJ m^{-2}, sense conseqüències negatives en la paràmetres físics de producte.

No obstant això, aquesta dosi no va ser suficient per reduir les poblacions de MAM en el bròquil orgànic més enllà del que es van reduir amb el rentat amb aigua. Per això, va ser necessari emprar la combinació d'almenys 0.3 kJ m^{-2} d'WUV i 50 mg L^{-1} de PAA per aconseguir el mateix efecte sobre les poblacions de MAM en bròquil orgànic. Després de 24 h de l'aplicació de 0.5 kJ m^{-2} de WUV la capacitat antioxidant total es va incrementar en un 90% en bròquil convencional i en un 22% en bròquil orgànic respecte al control rentat amb aigua. A més, aquesta dosi va incrementar en 1.5-vegades el contingut de sulforafà respecte a l'obtingut en tallar i rentar amb aigua. Pel que fa a l'eficàcia de la WUV per al control dels patògens de transmissió alimentària, dosis de 0.3 i 0.5 kJ m^{-2} de WUV van reduir les poblacions de *L. innocua* en bròquil orgànic en 1.7 o 2.4 log_{10}, respectivament. Aquest efecte es va mantenir durant 8 d d'emmagatzematge refrigerat en MAP, sense mostrar cap recuperació poblacional de *L. innòcua* en presència de llum blanca respecte al creixement observat a la foscor. En enciam 'Iceberg' i brots de espinacs, el tractament amb WUV as dosis de 0.1 a 0.3 kJ m^{-2}, emprant aigua o una solució de 40 mg L^{-1} de PAA, va controlar tant les poblacions de *S. enterica* ser. Enteritidis com les de *L. monocytogenes* fins a 2.1 log_{10} encara que no va reduir la microbiota aeròbia mesòfila total. La inhibició del creixement del patògens es va mantenir fins a 6 d d'emmagatzematge en MAP a 5 °C, sense afectar negativament la respiració o l'aparença general dels productes. L'aplicació de la llum ultraviolada en àcid peroxiacètic (40 mg L-1) no va donar com a resultat una reducció sinèrgica de les poblacions dels patògens en enciam "Iceberg" mínimament processat o en brots de espinacs respecte als controls físic o químic, però va millorar la efectivitat del tractament químic individual mitjançant la inactivació dels dos patògens en les solucions higienització.

L'aplicació de polsos de llum d'ampli espectre (PL) (5 a 20 kJ m^{-2}, contenint un 10% d'UV-C) va mostrar ser inefectiva per a controlar la població de MAM o de *L. innòcua* en bròquil mínimament processat durant 15 d d'emmagatzematge

refrigerat més enllà del que s'ha obtingut mitjançant el rentat amb aigua, sense causar efectes negatius en la qualitat física del producte. No obstant, l'aplicació de 3 polsos de llum (15 kJ m^{-2}) va incrementar el contingut de fenols totals en un 25% comparat amb el control rentat amb clor, 24 h després del processament. A més, va incrementar la capacitat antioxidant total en un 12 i un 18%, comparat amb els controls rentats amb aigua o clor, respectivament.

Finalment, es va avaluar la integració dels mètodes físics, químics i biològics per al control de patògens transmesos pels aliments en verdures IV gamma. L'efecte sinèrgic del pre-tractament amb llum ultraviolada C en àcid peroxoacètic i la posterior inoculació amb *P. graminis* CPA-7 va permetre una major inhibició de *L. monocytogenes* en enciam 'Iceberg' de quarta gamma als 2 d en MAP a 5 °C respecte als tractaments controls per separat, però va ser inconsistent en brots de espinacs. Davant un trencament de la cadena de fred, l'activitat antagònica de CPA-7 va controlar les poblacions de *S. enterica* en enciam 'Iceberg' de quarta gamma, però no va mostrar efecte en aquest patogen en brots de espinacs o en *L. monocytogenes* en ninguna de les dos matrius alimentaries avaluades. En resum, la biopreservació i la llum ultraviolada combinada amb immersió (WUV) són tecnologies alternatives al clor que actuen a través de diversos mecanismes simultàniament i que són recomanables per a ser implementades, independentment o combinades, per mantenir o millorar la qualitat microbiològica i nutricional dels productes vegetals de quarta gamma.

CHAPTER I

BACKGROUND

1.1 MINIMALLY PROCESSED PRODUCE

Minimally processed or fresh-cut produce is defined as 'fresh-cut fruits and vegetables that have been minimally processed (e.g., no lethal kill step), and altered in form, by peeling, slicing, chopping, shredding, coring, or trimming, with or without washing or other treatment, prior to being packaged for use by the consumer or a retail establishment' (US Food and Drug Administration, 2008). Fresh-cut produce includes those products that do not require additional preparation and are consumed raw and those that are ready to be cooked before consumption. Since the level of alteration is kept to a minimum except for the removal of inedible and damaged parts, final products preserve most of the nutritional properties of whole fruits and vegetables including a wide range of nutrients, phytochemicals, and fiber that promote good health and reduce the risk of suffering from cancer and chronic illnesses (Boeing et al., 2012; Cisneros-Zevallos, 2003).

Fresh-cut produce meets both the 'healthy' properties of fresh produce and the convenience and functionality demanded in current society. Those features have shuttled fresh-cut industry to vertiginous growth and development. In Spain, since its introduction in the market in 1989, the industry of fresh-cut produce has continuously grown reaching its higher rate in the period of 2005 – 2009 when sales increased from 45,000 to almost 67,000 tons per year. Afterwards, it has continued to grow although at a slower rate reaching sales of 670 million of euros for 92,000 tons in 2015. For 2020, the retailing of minimally processed produce is estimated to grow by 8 to 10% reaching 320,000 million of euros in

sales (Valencia Fruits, 2017). Currently, Spanish people eat about 3 kg per capita per year, very close to the 3.5 kg per capita per year that are consumed in average in Europe. However, consumption greatly varies within the European Union (EU) countries with a reduction by a half from the first consumer country, United Kingdom, with 12 kg per capita per year to the second, France, with 6 kg per capita per year (Baselice *et al.*, 2014).

1.1.1 Processing workflow

The processing of fresh-cut produce consists of a sequence of postharvest operations after transportation of commodities -usually refrigerated at 10 °C- from the field (Fig **I.**1). These include general and specific steps according to the type of commodity (i.e. leafy vegetables, sprouts, inflorescences, tubercles or fruits). When fresh produce arrives to the processing facilities it immediately enters pre-cooling and refrigerated storage in a low-temperature controlled area (5 °C) or a cold room until processing.

Afterwards, fresh produce undergoes **manual selection** and **classification** to discard visually damaged, spoiled, or potentially hazardous products and keep those ones with similar caliber or physiological stage (Center for Food Safety and Applied Nutrition, 2017). Then intact agricultural commodities are **washed** in chilled tap chlorinated water (5 °C) to eliminate residues of chemicals, plant debris, soil, insects, and foreign matter from the field.

This process is followed by **trimming, peeling** and separating fresh produce into pieces of various shapes and sizes through **cutting**, grating, chopping, shredding, slicing or chipping. Cutting is a critical step in fruit and vegetables processing. Tools and equipment used in this process must be cleaned, disinfected, and sharpened regularly during every working day keeping organic residues to lowest levels and procuring to inflict the least physical damage to the product since the way of cutting has a direct effect on the nutritional value,

overall quality, safety, and shelf life of minimally processed produce (Toivonen & Dell, 2002).

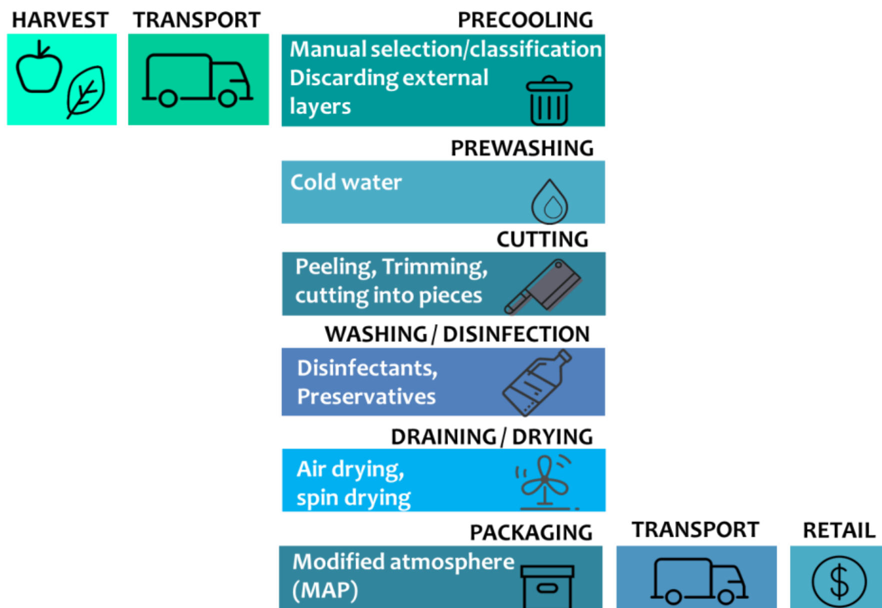


Figure I.1. General workflow for minimally processing of fresh produce: separate areas for incoming, in process and finished products, and a one-direction process, without looping, are strictly necessary.

Final steps of processing include washing/disinfection (5 °C) of processed products, draining/drying (10 °C) and packaging (Artés and Allende, 2005). **Washing and disinfection** of prepared products is one of the key steps in the processing workflow as it helps to remove some of the cellular fluids that could serve as nutrients for microbial growth, and reduce microbial load from the surface of products. Therefore, it prevents the build-up of organic material in the water and reduces or prevents cross-contamination of processed produce. Usually chlorinated water or other sanitizing compounds as well as preservatives including antioxidant and stabilizing substances which help to enlarge final products shelf-life, are included in this step. In order to assure its efficacy, the

contact time, and specific characteristics of the sanitizing solution such as pH, acidity, hardness and temperature as well as amount and rate of product throughput, type of product, water to product ratio, amount of organic material, and the resistance of pathogens to the particularly used sanitizer, should be thoroughly controlled and monitored (US Food and Drug Administration, 2008).

After washing, **draining/ drying** reduces the products moisture content, and removes cell leakage contributing to hamper microbial growth. This step should be performed carefully to avoid damaging plant tissues. Fresh-cut produce is usually stored under modified atmosphere packaging and refrigerated conditions to achieve the required commercial shelf life. Well-designed facilities for processing fresh-cut fruit or vegetables should keep separate areas for raw incoming products, those in processing, and stocked finished product without looping during the workflow, in order to reduce the probabilities for microbial cross-contamination.

The whole process must strictly follow a Hazard Analysis Critical Control Point (HACCP) program. HACCP is a structured approach to the identification, assessment of risk (likelihood of occurrence and severity), and control of hazards associated with a food production process or practice (US Food and Drug Administration, 2008). Since there is no single lethal step in the processing operation of the fresh-cut industries, HACCP must be built around a set of preservative methods (which would act as hurdles) to control the growth of spoilage and pathogenic microbiota, which in turn should help to maintain the physicochemical quality of the product.

1.1.2 Impact of processing on microbiological quality

Processing of fresh produce increases the risk of bacterial growth and contamination by removing or disrupting the integrity of most of the constitutive barriers of plants. Peeling and cutting result in the exposure of

normally un-accessible produce surfaces and in the leakage of plant cellular fluids providing a nutritive environment in which spoilage microorganisms as well as pathogens, either present on the produce surface or in the processing facilities, can survive or grow (Gil *et al.*, 2009). Furthermore, the degree of handling and product mixing add opportunities for cross-contamination and for spreading this contamination through a large volume of product (European Comission for Health and Consummer Protection, 2002). The probability for pathogens to survive or grow is also increased by lack of a lethal process (i.e. heating) during MPP production or before consumption and by the potential for temperature abuse during processing, storage, transport, and retailing. In addition, prevalence and persistence of pathogens in processing facilities in spite of disinfection measures, poses a serious challenge to the maintenance of fresh-cut products safety (Ibusquiza, Herrera, & Cabo, 2011; Ilic, Odomeru, & LeJeune, 2008; Yang et al., 2016).

The **incidence of foodborne outbreaks** caused by contaminated fresh fruit and vegetables has increased in the last decade (CDC - Center for Disease Control and Prevention, 2016; European Food Safety Authority (EFSA), 2016). *Listeria monocytogenes*, pathogenic *Escherichia coli* O157:H7, and *Salmonella* spp. are among the human pathogens of greatest concern in fresh-cut produce (Ramos *et al.*, 2013). *Salmonella enterica* was the bacterial etiological agent of most outbreaks associated to fresh produce in the US and EU during the period 2004–2012 (Callejón *et al.*, 2015). *L. monocytogenes* has become another focus of attention as a causal agent of foodborne diseases associated to fresh-cut produce, not for its high incidence but for its high mortality and morbidity rates (Zhu, Gooneratne and Hussain, 2017). In addition, *E. coli* O157:H7- associated diseases are also of great concern in the fresh-cut produce industry although they have lower incidence than salmonellosis, because they show much higher hospitalization and fatality rates.

Current regulations of the European Commission regarding the microbiological safety of food fresh produce establish a maximum limit for *E. coli* of 10-1000 CFU g^{-1} detected in 2 from 5 samples during sanitizing of ready-to-eat food (EC-European Comission for Health and Consumer Protection, 2007). Regarding *L. monocytogenes*, no presence is allowed during the production process and a limit of 100 CFU g^{-1} is established as the maximum during the shelf-life of products once placed on the market (EC-European Comission for Health and Consumer Protection, 2007). Regarding *S. enterica,* no presence is permitted in 25 g of sample.

FOODBORNE PATHOGENS

LISTERIA MONOCYTOGENES

Listeria spp. are flagellated, non-spore-forming, facultative anaerobic Gram-positive bacilli. *Listeria* spp are considered as **ubiquitous** bacteria since they can be isolated from plants, animals, soil, water, agricultural, and production environments (Zhu, Gooneratne and Hussain, 2017). *Listeria* genus belongs to the Firmicutes phylum characterized by DNAs with low GC contents (≈ 38%). It consists of 17 distinct species including two pathogenic species: *L. monocytogenes,* which cause disease to humans and other animals, and *Listeria ivanovii,* which mainly affects ruminants (Camejo *et al.*, 2011)*.* The serotyping scheme of *L. monocytogenes* strain, based on somatic (O) and flagellar (H) antigens, discriminates 13 serotypes (Cossart, 2011). From them, four **serotypes** (1/2a, 1/2b, 1/2c and 4b) are responsible for the 95% of human infection cases (Kathariou, 2002). For the research and development of control strategies in food-related experiments carried out in industrial facilities, *Listeria innocua***,** which is a non-pathogenic species, is usually used as a **surrogate for** *L. monocytogenes* (Friedly *et al.*, 2008; Gómez *et al.*, 2014; Tremonte *et al.*, 2016; Costa *et al.*, 2018**.** Genomic analysis have shown that *L. innocua* evolved to non-host environments such as industrial facilities and consequently lost specific

genes associated with infection (Buchrieser, 2007). Such genes include the main virulence gene cluster, the genes encoding InlA and InlB and the bile salt hydrolase (bsh) gene as well as genes associated with the MEP pathway for isoprenoid biosynthesis which is involved in the growth within the mammalian host (Buchrieser, 2007). *L. innocua* has shown similar biofilm-forming abilities and susceptibility to antimicrobials than *L. monocytogenes* which suggests it as a suitable surrogate for evaluating the susceptibility to biological and physical methods in food matrices.

Although *L. monocytogenes* is a facultative intracellular pathogen of homoeothermic animals, it also can **grow** and survive outside a host at a wide pH range (4.4 to 9.2) and high salt concentrations (100 g L^{-1} NaCl) (Koutsoumanis, Kendall and Sofos, 2004). *L. monocytogenes* is also a psychrotrophic microorganism which can grow from refrigeration to corporal temperatures (-1.3 to 45 °C), being 37 °C its optimal (Hudson & Mott, 1993; Gandhi & Chikindas, 2007). Furthermore, sub-lethal **adaptation** to a mildly acidic environment (pH 5.0 to 6.0) and high concentrations of salt and carbohydrates such as glucose, induce mechanisms that allow this pathogen to survive upon subsequent exposure to more extreme conditions (pH 3.5) (Koutsoumanis, Kendall and Sofos, 2003). This adaptive response is highly dependent on the simultaneous influence of several stress factors such as low temperature, salts concentration and oxygen availability (Buchanan, Stahl, & Whiting, 1989).

The plasticity of the growth range of *L. monocytogenes* allows it to subsist in inert surfaces at food processing facilities, proliferate in the complex food matrix environment, overcome hurdles such as decontamination, refrigeration and packaging, and subsequently invade human body in spite of its protective barriers causing invasive and non-invasive human syndromes called **listeriosis**. In invasive listeriosis the microorganism is able to trespass the intestinal, blood-brain and placental barriers to infect liver, spleen, cerebral spinal fluid, blood, placenta, and fetus (Zhu, Gooneratne and Hussain, 2017). In

immunocompromised hosts invasive listeriosis can cause septicemia and meningoencephalitis (Camejo *et al.*, 2011). Perinatal listeriosis increases the probability of abortion and stillbirth, and can provoke pneumonia, sepsis or meningitis to the fetus. More rare localized infections in the heart, the skin, or the joints have also been reported (Zhu, Gooneratne and Hussain, 2017). Non-invasive listeriosis can affect also immunocompetent individuals causing febrile gastroenteritis symptoms (Lomonaco, Nucera and Filipello, 2015). Foodborne listeriosis is a not very frequent but serious disease with a high fatality rate ranging from 20 to 40% (Harris *et al.*, 2003). Contaminated lettuce, apples, and melon are some of the commodities that have served as vehicles for listeriosis **outbreaks** affecting hundreds of people and resulting in several casualties in the USA, from 2011 to 2016 (CDC - Center for Disease Control and Prevention, 2016; Buchanan *et al.*, 2017; Zhu, Gooneratne and Hussain, 2017) In the EU, listeriosis incidence increased during the period 2008-2015 reaching a maximum of about 2,200 affected people with a 17.7% of fatality in 2015. The proportion of cases in the over 64 age group, and particularly in people over 84 years old, steadily increased from 56% in 2008 to 64% in 2015. The most common source of infection in this region is ready-to-eat food (European Food Safety Authority (EFSA), 2016).

For causing infection, *L. monocytogenes* enters the organism mainly through the uptake of contaminated food and crosses the intestinal barrier and spreads through sanguineous flow to the liver and spleen, which are major sites for replication (Ireton, 2007). I**nfection cycle** begins with bacterial adhesion to enteric epithelium, which involves several molecules and triggers host signaling pathways leading to bacterium internalization (Fig. **I.**2).

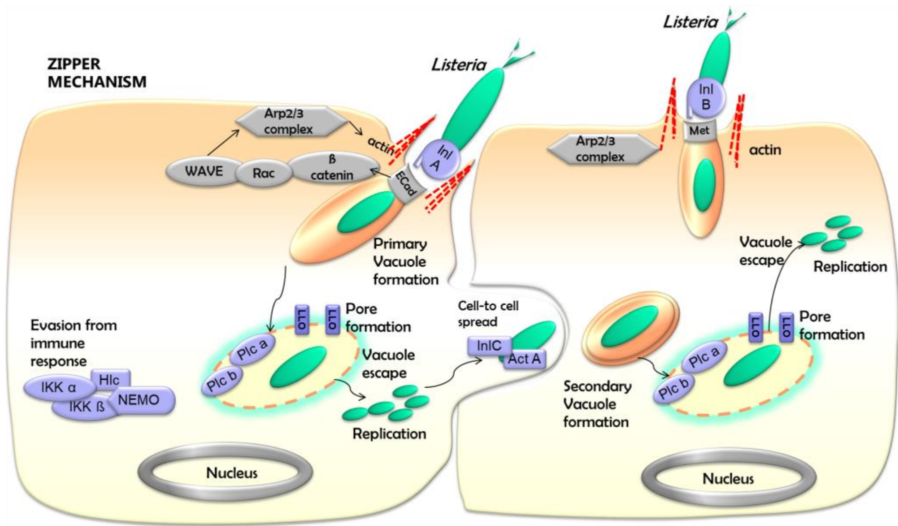


Figure I.2. Internalization mechanisms of *L. monocytogenes* into a human host. Adapted from Cossart, 2011 PNAS108:19484-19491). **Adhesion** is mediated by the union of bacterial internalin (InlA) and the host receptor (E-cadherin), this latter usualy located in the basolateral surface of polarized enterocytes. This union activates a cascade of events including the rearrangement of cellular citoesqueleton leading to **internalization** through endocytosis and formation of the primary vacuole. Then, bacterial pore-forming toxin listeriolysin O (LLO) and two phospolipases, PlcA and PlcB, release the microorganism from the vacuole into the cytoplasm where it can **replicate**. Once in the cytoplasm, by means of the activation of the Arp2/3 complex, the actin of the host cell is mobilized and in conjunction with bacterial Actin Polymerization Factor (ActA), the microorganism can move through protruding pseudopods into the adjacent cells. Then, bacteria **colonize** nearby cells upon escape the secondary double-membrane–bound vacuole by means of LLO and the phospholipase PlcB completing and repeating the cycle (Cossart, 2011). During the colonization of non-phagocytic cells such as hepatocytes, InlB -another protein belonging to the internalin family (lnB) act as recognition molecule binding the host receptor (tyrosine kinase Met), induces Met ubiquitination and bacterial internalization via clathrin-mediated endocytosis mechanism (Camejo *et al.*, 2011). It has been shown that both InlA and InlB are required for placental invasion.

SALMONELLA ENTERICA SUBSP. *ENTERICA*

Salmonella spp. is a Gram-negative, facultative anaerobic bacillus (2–5 µm x 0.5–1.5 µm), motile by peritrichous flagella, belonging to *Enterobacteriaceae* family (Bonifield and Hughes, 2003). There is only two described species within the *Salmonella* genus: *S. bongori* and *S. enterica.* The last mentioned species comprises six **subspecies** *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica.* Although more than 2,500 serovars have been described, more than

99.5% of isolated *Salmonella* strains belong to the subspecies *enterica* (Grimont and Weill, 2007). *Salmonella* spp. are considered to be mesophilic microorganisms with some strains being able to survive at extremely low or high temperatures (2 ºC to 54 ºC) (Andino and Hanning, 2015). *Salmonella* spp. are facultative intracellular microorganism whose main hosts are humans and farm animals although they can also inhabit in wild birds, reptiles, and insects (Andino and Hanning, 2015).

Clinically, **salmonellosis** associated to serovars Typhi and Paratyphi may be manifested as gastroenteritis, septicemia, or enteric fever. Non typhoidal infections, associated to serovars Enteritidis and Typhimurium, usually provoke gastroenteritis or bacteraemia, and main symptoms are nausea, vomiting, and diarrhea (Andino and Hanning, 2015).

The most frequent **food vehicles** associated with this *S. enterica* serovars are eggs, meat-derived (poultry and pork), and dairy products (Andino and Hanning, 2015). However, fresh fruit and vegetables have also been associated to salmonellosis transmission. Indeed, in the US, an **outbreak** traced to frozen shredded coconut affected 25 people early in 2018 (CDC- Center for Disease Control and Prevention, 2018). In 2017, *S. enterica* was identified as etiological agents for four outbreaks associated to the consumption of infected 'Maradol' papayas which affected more than 250 people, causing two deaths (CDC- Center for Disease Control and Prevention, 2018). Moreover, in 2016 over 900 people from which 4 died, were affected by imported cucumber contaminated with *S. enterica* (CDC- Center for Disease Control and Prevention, 2018). Fresh fruit have also been associated to salmonellosis affecting hundreds of people (261 cases were traced to Cantaloupes and 127 were traced to mangoes) in 2012. In the EU, salmonellosis was the second more frequent zoonosis in 2015 with more than 94,600 cases and 126 deaths (0.24 % fatality) reported in that year (European Food Safety Authority (EFSA), 2016). According to that report, serovar Enteritidis was responsible for the 45.7 % of the infections.

For causing infection *S. enterica* **invade,** non-phagocytic cells such as enterocytes and M cells through two mechanisms called 'trigger' and 'zipper' (Fig. **I.**3).

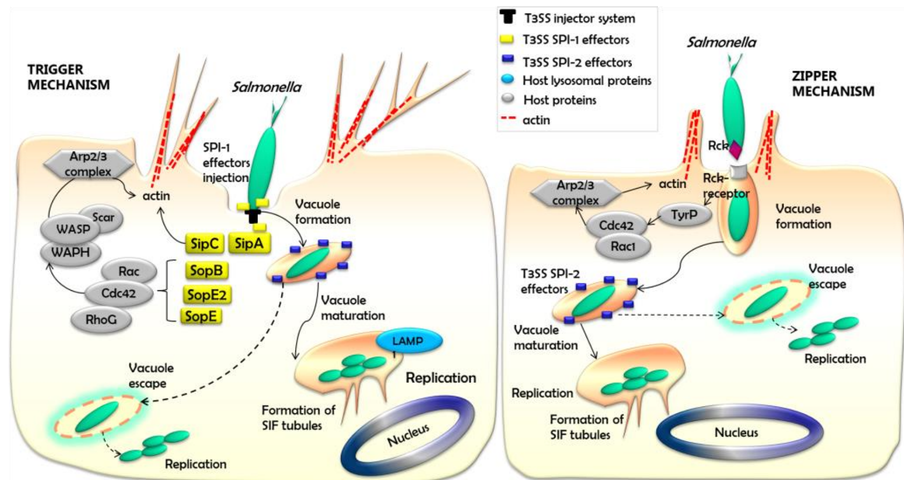


Figure I.3. *Salmonella* spp. adhesion-invasion mechanisms: Trigger mechanism mediated by the type III secretion system (T3SS) which inject more than 13 effectors including SipA, SipC, SopB, SopE and SopE2, encoded in 'Salmonella Pathogenicity Island' (SPI-1), into the host cell. SopE, SopE2, and SopB activate a signal transduction cascade including RhoGTPases (Rac1/Cdc42/RhoG) which promote massive rearrangement of host actin cytoskeleton to form 'membrane ruffles' with the involvement of Arp2/3 complex and the recruitment of the exocyst (Agbor & Mccormick, 2011). In the Zipper mechanism **adhesion** is mediated by the union of bacterial membrane protein invasin (Rck) and its host-receptor which through a signaling cascade involving the GTPases Rac1 and Cdc42, downstream of Akt/PI 3-kinase, promotes actin polymerization via the Arp2/3 complex (Reviewed by Boumart, Velge, & Wiedemann, 2014). Following **internalization**, *S. enterica* stays in a membrane bound compartment called 'Salmonella Containing Vacuole' (SCV) adapted for its survival and replication through the activities of T3SS effectors. Some effectors such as SteC contribute masking bacterial virulence and other effectors block the production of bactericidal compounds that would kill intracellular bacteria. The acquisition of host proteins such as lysosomal-associated membrane protein-1 (LAMP1) allows bacterial **replication**, its migration toward a perinuclear position and the formation of dynamic membrane tubules (SIF) that extend from the SCV ensuring the delivery of nutrients to the SCV (Boumart, Velge and Wiedemann, 2014). This process is called maturation and is promoted by effectors from SPI-2. A proportion of internalized bacteria can escape from the SCV and replicate in the cytoplasm of epithelial cells (Malik-Kale, Winfree and Steele-Mortimer, 2012).

ESCHERICHIA COLI O157:H7

E. coli is a Gram-negative, facultative anaerobic bacillus belonging to Proteobacteria class and *Enterobacteriaceae* family which is a majoritarian

member of mammals' normal intestinal microbiota. It can be non-motile or motile by peritrichous flagella. Pathogenic *E coli* are a subgroup of strains that are called 'enterotoxigenic', 'enterohemorrhagic', 'shigatoxin-producing' or 'verocytotoxin-producing'. They contain bacteriophages with the genes *stx 1* and *stx2* encoding for **toxins** that inhibit protein synthesis causing intestinal and endothelial cells injury and death (Lim, Yoon, & Hovde, 2010; Riemann & Cliver, 1998; Tarr & Neill, 2001).

Shigatoxin-producing *E. coli* (STEC) are resident commensal bacteria commonly inhabiting cattle and small ruminants that are also etiological agents of severe syndromes in humans (Riemann and Cliver, 1998). *E. coli* 0157:H7 belongs to this pathogenic group and it has been reported that cause infections with acute symptoms such as bloody diarrhea, and in the worst cases, hemolytic uremic syndrome (HUS) and thrombocytopenic purpura syndrome (TTP) (Tarr and Neill, 2001). HUS/TTP syndromes may lead to kidney failure, hemolytic anemia, and thrombocytopenia as well as neurological symptoms such as seizures, paralysis, and coma. Complications of a HUS/TTP include rectal prolapse and infections in the colon and the appendix.

The development of STEC O157:H7 infection is the result of the direct action of shigatoxins, the intracellular invasion of enterocytes mediated by the LEE pathogenicity island (encoding for the Type 3 Secretion System: T3SS), and the several **virulence factors** encoded in pO157 plasmid. The production of the latter mentioned molecules enables this bacterium with the ability of altering the lipid composition of its membrane and producing exopolysaccharides (EPS) to form biofilms. Therefore, STEC O157:H7 can survive in extreme conditions, adapt to wide variations of the environmental factors (temperature, pH, and osmolarity), and live in inert and living surfaces including soil, water, food and animal hosts (Lim, Yoon and Hovde, 2010).

The **adhesion and invasion** of the epithelial cells of the distal ileum and the large bowel by STEC are the primary steps for **establishing infection** after entering to the host with the food. This process is mediated by T3SS-mediated **trigger mechanism** (Fig. **I.**4) (Reviewed by Lim et al., 2010; Clements, Young, Constantinou, & Frankel, 2012). The **toxigenic mechanism** of STEC relays on the secretion of Shigatoxins (Stx), formed by two types of subunits, A and B, in a ratio 1:5. Stx is secreted to the extracellular medium where the B subunits bind host receptors Gb3 or Gb4 and internalize into the cytoplasm. Then the A subunit separates from the rest of the subunits and can provoke changes in the structure of the 28S rRNA preventing protein synthesis, or activate programmed cell death in the endoplasmic reticulum, all of which lead to host cell death (Reviewed by Ho, Aleah C, Kathene, & Sherman, 2012).

The principal vehicles for ***E. coli*** O157 transmission are meat products, mainly beef, although fruits, vegetables and drinking water can eventually get contaminated with ruminant feces and represent also a cause of infection (Rangel *et al.*, 2005). STEC outbreaks are attributed to its low infectious dose (< 100 CFU) and high transmissibility. In the US, *E. coli* O157 accounts for approximately 75% of all STEC infections (Harris *et al.*, 2003). Several **outbreaks** associated to fresh produce have been reported recently. For example, fresh spinach contaminated with *E. coli* O157:H7 were associated to the infection of 183 persons with one fatality in 2006 (CDC-Center for Disease Control and Prevention, 2006). Another outbreak traced to fresh-press apple cider affected 18 persons in 1991 (Harris *et al.*, 2003).

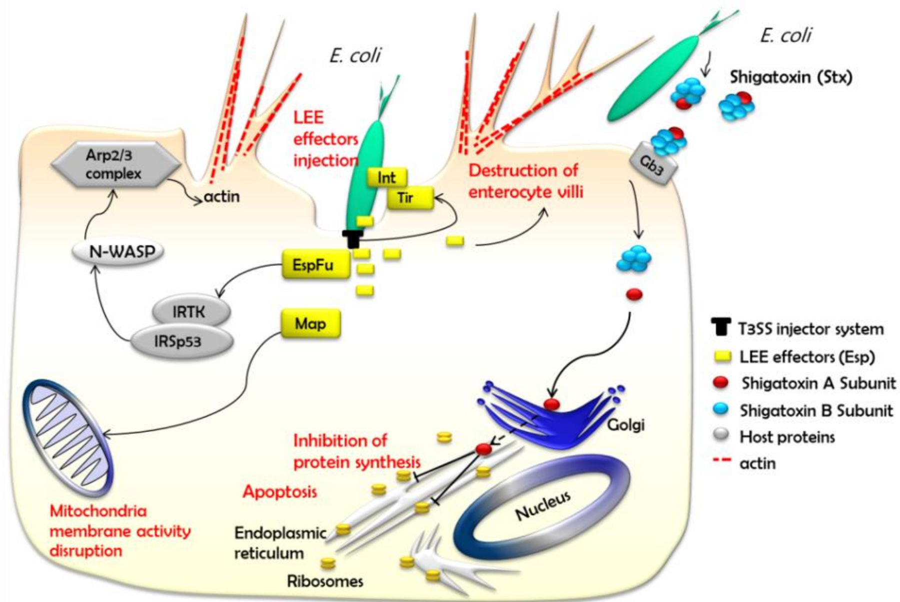


Figure I.4. Shigatoxin-producing *E coli* adhesion/toxigenic mechanism. Type 3 secretion system (T3SS) LEE injects more than 50 effector proteins into the host cell cytoplasm one of which is Translocated intimin receptor (Tir) that is able to anchor onto the surface of the host cell membrane acting as a receptor for the bacterial adhesin: intimin. The binding of these two molecules (Intimin – Tir) triggers the recruitment of the effector EspFu, which by means of the host adaptor proteins IRTKS and IRSp53 binds the protein N-WASP, activating the host Arp2/3 complex which results in actin polymerization and membrane ruffles formation (Reviewed by Ho, Aleah C, Kathene, & Sherman, 2012).

Spoilage microorganisms

The predominant spoilage microbiota of fresh-cut vegetables and fruits with pH close to neutrality such as melons, are mesophilic psychrotrophic aerobic microorganisms. Their growth is favored by the high water activity, nutrients leakage and the refrigerated temperatures during processing, storage and retailing. These microorganisms are usually non-pathogenic to humans and their populations vary from 10^3 – 10^6 CFU g^{-1} immediately after processing to up to 10^9 CFU g^{-1} during retailing (Ragaert, Devlieghere and Debevere, 2007). Variations in the population levels and community composition are conditioned by

seasonality, type of commodity, and temperature fluctuations during the chain of transport-commercialization (Barth *et al.*, 2009). Among the most common Gram-negative bacteria causing **spoilage to vegetables** and **neutral fruit** are from *Pseudomonadaceae* and *Enterobacteriaceae* families, namely *Pseudomonas fluorescens* and *Erwinia herbicola*, or belonging to the group of lactic acid bacteria (LABs) including *Leuconostoc mesenteroides* (Siroli, Patrignani, Serrazanetti, Gardini, *et al.*, 2015; Söderqvist *et al.*, 2017). Several yeast genera (*Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Trichosporon* spp., *Pichia* spp., and *Torulaspora* spp.) have also been found in fresh-cut vegetables (Ragaert, Devlieghere and Debevere, 2007).

In **more acidic** minimally processed **fruit**, as most of them, molds are considered as the main spoilage agents because their growth is favored by the low pH (Raybaudi-Massilia *et al.*, 2009). Molds such as *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp., *Alternaria* spp., *Cladosporium* spp., *Paecilomyces* spp., and *Botrytis* spp. are common to fruit-based ready-to-eat products (Farber *et al.*, 2003). However, in addition to vegetables, *Erwinia* spp., *Enterobacter* spp., *Alicyclobacillus* spp., *Propionibacterium* spp., *Pseudomonas* spp., and LABs are also natural acid-tolerant and osmophilic microbiota that have been reported as deteriorative of fresh-cut fruit (Belletti *et al.*, 2008). Yeasts such as *Saccharomyces* spp. *Cryptococcus* spp., *Candida* spp. and *Rhodotorula* spp. are also important spoilage microbiota in fresh-cut fruit since they are more favored that LABs by the high sugar content and carbon/nitrogen ratio of fruits (Siroli, Patrignani, Serrazanetti, Gardini, *et al.*, 2015) and because they grow faster than molds.

The effects of microbial spoilage on fresh-cut produce include the generation of off-flavor and off-odor, slimy surface, wetness and soft rot, discoloration, and presence of microbial colonies. Pseudomonads are able to transform a wide variety of organic compounds including sugars such as glucose and/or maltose as carbon sources which, in turn, modify the nutritional composition of fresh-cut

products. Pectinolytic activity, common to this bacterial group, affects texture causing soft-rot. Off-flavors and off-odors in stored fresh-cut produce are provoked by changes in volatile and non-volatile compounds due to microbial activities such as alcoholic or acid-lactic fermentation with the production of ethanol, acetaldehyde among others. Yeasts are responsible for the fermented taste and carbon dioxide production while acid lactic bacteria (LAB) can produce a buttermilk off-flavor (Ragaert, Devlieghere and Debevere, 2007). Molds can contribute to spoilage by their surface growth as well as by causing translucency and loss of texture due to the secreted pectinolytic enzymes (cutinases and lipases) (van Kan, 2006).

1.1.3 Impact of processing on physicochemical and nutritional quality

Relevant **quality parameters** to fruit and vegetables are general appearance (lack of visual damage and deformities, shape, size, gloss, and vibrant color), flavor (sweetness, sourness, acidity, astringency, bitterness, and aroma), texture (moistness and juiciness, firmness and crispiness), and nutritional value (carbohydrates, lipids, proteins, vitamins, fiber, minerals, antioxidants and other bioactive compounds) (Bansal and Siddiqui, 2015). These parameters are thought to be evaluated in that order by consumers since a bad appearance would prevent them from buying a product and tasting it (Barrett, Beaulieu and Shewfelt, 2010). The susceptibility of quality parameters to the physical and chemical factors involved in processing is an important issue to take into account to design a proper production workflow and extend fresh-cut products shelf-life.

Fresh-cut produce essentially behave as wounded tissues, since peeling, cutting, shredding chopping, and other cutting styles during processing disrupt cells walls and membranes. Wounding has numerous effects comprising physical,

chemical and physiological changes in the plant tissues (Fig. **I.**5). The rupture of membranes allows the **leakage of cellular components** with the subsequent relocation of water and loss of turgor (Toivonen and Dell, 2002). Moreover, to expose surfaces without protective cuticles makes them susceptible to **evaporation,** changes the pattern of gas permeation, and can lead to a dehydration process such as 'white blush' in carrots (Garcia and Barrett, 2002). Cutting compromises plant cell compartmentalization allowing enzymes to interact with their substrates, thereby activating metabolic reactions. Among enzymatic reactions closely related to quality is **'browning'**, which occurs when the enzyme polyphenol oxidase (PPO) catalyzes the hydroxylation of mono-phenols to di-phenols and oxidize the latter compounds to O-quinones, which eventually polymerize non-enzymatically to brown-colored melanin (Holderbaum *et al.*, 2010). Pear PPO has also been reported to catalyze the oxidation of ascorbic acid, which modulates fruit nutritional content (Espin, Veltman and Wichers, 2000). Peroxidases (POX) have also shown to participate in browning through the oxidation of di-phenols in the presence of electron acceptor compounds such as superoxide radical, hydrogen peroxide, and lipid peroxides (Chisari, Barbagallo and Spagna, 2008).

Wounding also **triggers** a complex **signaling network** that comprises the production and release of several chemical (volatile, diffusible) and physical signals (hydraulic, electrical) which induce the expression of defense and stress-related genes, resulting in the **activation of several plant responses** (Saltveit, 2016). For example, the cells adjacent to the injury are stimulated via the induction of phenylalanine-ammonia lyase (PAL) to produce more phenolic compounds in an attempt to initiate repair processes such as lignification (Valentines *et al.*, 2005). The accumulation of secondary metabolites such as phenolic compounds increases with wounding intensity and with higher storage temperature as observed for several fruit and vegetables (Han *et al.*, 2016; Li *et al.*, 2017; Torres-Contreras *et al.*, 2017; Van de Velde *et al.*, 2018) In broccoli,

cutting also induces the biosynthesis or some glucosinolates and releases myrosinase enzyme activating their hydrolysis into isothiocyanates and other hydrolysis products (Mahn and Reyes, 2012; Torres-Contreras *et al.*, 2017).

Furthermore, around the site of wounding phospholipases and other lipases are activated, leading to the release of poly-unsaturated fatty acids from the cell membranes which are as substrates for **lipoxygenase.** This enzymes catalyzes the production of hydroperoxy-poly-unsaturated fatty acids (lipid hydroperoxide) activating jasmonic acid (JA)-mediated defense response (Prasad *et al.*, 2017). Lipoxygenase can also oxidize lipidic pigments such as carotenoids and chlorophylls leading to **discoloration** (Karakurt and Huber, 2004). Wound-induced polygalacturonase releases oligogalacturonic acid from cell walls which is part of the signaling cascade that induces an accelerated production of reactive oxygen species (ROS) known as **'oxidative burst'**. Among ROS are included molecules such as superoxide (O^-), singlet oxygen ($^1O_2$) and hydrogen peroxide H_2O_2 (Roach *et al.*, 2015). The immediate oxidative burst upon wounding may range from 10 to 20 min to several hours (Minibayeva, Beckett and Kranner, 2015).

ROS play a **dual role** in plants depending on their amount. At high concentrations, these compounds can cause damage to biomolecules, whereas at low concentrations, they act as secondary messengers in intracellular signaling cascades leading to defense responses (Baxter, Mittler and Suzuki, 2014). Thus, ROS production as well as enzymatic and non-enzymatic scavenging mechanisms are concomitantly activated (Fig. **I**.5) (Cheong *et al.*, 2002; Roach *et al.*, 2015). Elevated ROS levels also enhance lipid peroxidation. This takes place in both cellular and organelle membranes, which, in turn, affect normal cellular functioning, and leads to the production of lipid-derived radicals that can damage proteins and DNA. The level of products of lipid peroxidation (i.e. transformation of unsaturated fatty acids in phospholipids) such as

malondialdehyde (MDA) is a marker for cell membrane damage (Sharma *et al.*, 2012).

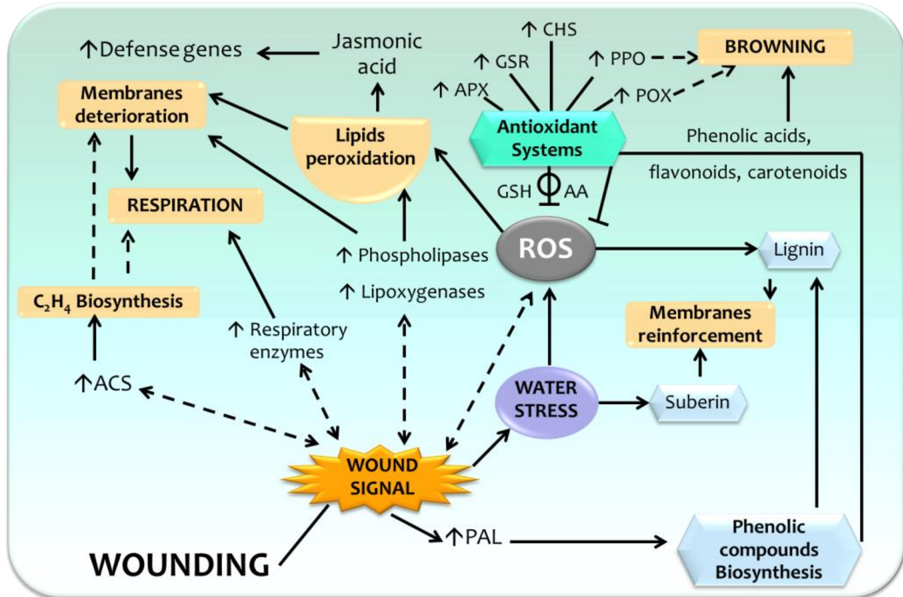


Figure I.5. Schematic representation of the some mechanisms involved in the plant response network to wounding, (adapted from Reyes, Villarreal and Cisneros-Zevallos, 2006). sharp-end arrows represent induction, flat-end arrows represent inhibition.

Enzymatic ROS scavenging mechanisms comprise the induction of the biosynthesis and the increase in the activities of enzymes with high affinity to specific ROS. Plant's antioxidant enzymes include superoxide dismutase (SOD), which catalyze the dismutation of superoxide to H_2O_2 and O_2; catalases (CAT), which transform H_2O_2 to H_2O and O_2; POX, which decompose H_2O_2 during the oxidation of phenolic and enodiolic co-substrates hydroxylated compounds (R/HOOH) to H_2 and R-OH; peroxiredoxins, which transform ROOH to ROH, (Demidchik, 2015). Concomitantly, the enzymes of the glutathione (GSH)-ascorbate cycle also act as enzymatic scavengers; ascorbate peroxidase (APX) uses ascorbate as an electron source to transform H_2O_2 to H_2O and O_2, and glutathione peroxidase oxidizes GSH to glutathione disulfide and H_2O (reviewed by Gill and Tuteja, 2010; Pandey *et al.*, 2017).

Non-enzymatic ROS scavenging mechanisms include the action of cellular redox buffers ascorbate and glutathione as well as secondary metabolites such as tocopherol, carotenoids, and phenolic compounds. They can act by interacting with cellular components and as enzyme cofactors (Sharma *et al.*, 2012). In addition of its involvement in the ascorbate-glutathione cycle, ascorbic acid (AA) provides membrane protection by directly reacting with O_2^- and H_2O_2, regenerating α-tocopherol from tocopheroxyl radical (Akram, Shafiq and Ashraf, 2017).

Another of the several effects of wounding is the activation of enzymes of the ethylene biosynthetic pathway, such as 1-aminociclopropane-1-carboxylic acid (AAC) synthase, increasing ethylene accumulation (Yu and Yang, 1980; Alexander, 2002). **High ethylene levels** induce the expression and activities of several enzymes including those related to cell-wall and membrane disassembly such as cell wall hydrolase, polygalacturonase, and β-galactosidase, increasing the rate of softening in fruit. This leads to **accelerated senescence and ripening** in clicmateric fruit and may even precipitate the onset of ripening in pre-climacteric fruit (Liu *et al.*, 2015). Wounding also results in **increased respiration rates**, which could lead to anaerobic respiration when the cut product is submitted to high CO_2 levels during modified atmosphere packaging (MAP). The removal of the peel can potentially increase the surface per unit of volume for the diffusion of O_2 (Saltveit, 2016). Wound-induced respiration has been associated with an enhanced synthesis of the enzymes involved in the respiratory pathway and to increased aerobic respiration rates in fresh-cut carrots (Surjadinata and Cisneros-Zevallos, 2003).

Internal and external **factors that can affect the wound response** include species, cultivar, maturity stage, storage/processing temperature, cutting styles, sharpness of cutting tools, CO_2 and O_2 levels, and water vapor pressure (Reyes, Villarreal and Cisneros-Zevallos, 2006; Saltveit, 2016). Increased wounding intensity and higher storage temperature led to a sharp rise in respiration rates,

and loss of pungency, soluble solids content and ascorbic acid in onions, shortening their shelf life (slices < pieces < shreds) (Yoruk and Marshall, 2003). Cut cylinders of melon preserved firmness better than slices or trapezoidal sections during storage for up to 10 d but became more translucent; thus trapezoidal cutting was suggested as the preferred protocol (Aguayo, Escalona and Artes, 2004). Similarly, papaya slices had longer shelf-life than cubes when stored at 5 or 10 °C (Rivera-López *et al.*, 2005). Cutting styles also influence nutritional and bioactive content (phenolic compounds and glucosinolates) in broccoli (Torres-Contreras *et al.*, 2017).

1.2 PRESERVATION METHODS FOR MINIMALLY PROCESSED PRODUCE

Limiting the extent of microbiological and physiological effects of processing requires the inhibition of the plant's metabolic activity, the reduction of pre-existing microbial levels on the plant product, the inhibition of growth and activity of the remaining microbiota after the decontamination step, as well as the prevention of cross-contamination with foodborne pathogens. With those goals, several physical, chemical and physical methods are applied, usually in combined manner.

1.2.1 CHEMICAL METHODS

CHLORINE

The most widely used decontamination method in the industry of minimally processed produce is chlorine sanitation. Chlorine-based products are mainly obtained in the form of gaseous chlorine [Cl_2] or of sodium [NaOCl] or calcium hypochlorites [$Ca(OCl)_2$] (Hua and Reckhow, 2007). The antimicrobial activity of chlorine is exerted by the antioxidant properties of hypochlorous acid (HOCl)

and hypochlorite ions (OCl^-) (Fukuzaki, 2006). HOCl is formed when chlorine dissolves in water but, as a weak acid in aqueous solution, it readily dissociates into OCl^- at pH > 7.5 or into Cl_2 at pH < 6.0 (Sapers *et al.*, 2001). **Bactericidal effect** of HOCl is based on the loss of physiological functions as a result of the oxidation of sulfhydryl groups of essential enzymes, the formation of chlorinated derivatives of nucleotide bases and disruption of oxidative phosphorylation during ATP utilization or generation (Albrich, McCarthy and Hurst, 1981). OCl^- is also a strong oxidizing agent which decomposes proteins into low molecular weight fragments and reacts with proteins such as bovine serum albumin to give chloramines (Fukuzaki, 2006).

Chlorine **efficacy** as a sanitizer is tightly linked to the concentrations of un-dissociated OCl^- and HOCl which **depends on** pH, temperature, and amount of organic material present in the solution (Shen, Norris, Williams, Hagan, & Li, 2016; Artés and Allende 2014). Typical **industrial application** of free chlorine ranges from concentrations of 50 to 200 mg L^{-1}, with contact times of 1 to 2 min, keeping pH values between 6.0 and 7.5 in order to stabilize HOCl (Banach *et al.*, 2015).

The most prevalent groups of **disinfection byproducts** produced during water sanitation with chlorine are trihalomethanes (THMs) and haloacetic acids. Their maximum levels have been restricted in several countries (Hua and Reckhow, 2007). Efforts to reduce chlorine doses for the sanitation of fresh-cut produce showed that keeping free chlorine residuals at 7 mg L^{-1} during processing was able to completely eliminate *E. coli* O157:H7 cells (reduction by 5 log_{10}) in the process wash water of inoculated fresh-cut spinach (Gómez-López *et al.*, 2014). However, since more than 1000 µg L^{-1} of THMs were still generated, great concern arises about the elevated amount of disinfection byproducts that must be generated at the higher chlorine doses that are commonly applied at industry. For this reason, the use of chlorine as a sanitizer in the production of minimally processed vegetables has been banned in several European countries

such as Germany, The Netherlands, Switzerland, and Belgium (Artés and Allende, 2005) or restricted to up to 100 ppm total chlorine at pH 6-7 for maximum 2 min in United Kingdom and up to 80 ppm for 2 min in France (EC-European Comission for Health and Consummer Protection, 2005; Chilled Food Association, 2010; Mortureux, 2012). The formation of toxic disinfection byproducts in addition to foreseeable most restrictive future regulations has motivated scientists and processors to investigate alternative disinfection methods.

CHLORINE-ALTERNATIVE CHEMICAL SANITIZERS

Other chemical compounds with antimicrobial activity that are been investigated for fresh-cut produce sanitation include organics acids, quaternary ammonium compounds and oxidizing agents such as ozone, chlorine dioxide, hydrogen peroxide, and electrolyzed water (Ölmez and Kretzschmar, 2009). Oxidizing agents act through the oxidation of cellular components, but their efficacy varies according to the type of microorganism and their sensitivity to pH, temperature and organic matter (Finnegan *et al.*, 2010). **Ozone** is effective against bacteria, molds and yeast even at very low concentrations 1 to 5 mg L^{-1} for short exposure times (1–5 min). It is residue-free and its activity is not affected by pH, but it is corrosive and toxic if inhaled (Selma *et al.*, 2008; Renumarn *et al.*, 2014). **Chlorine dioxide** has stronger oxidizing activity than chlorine, is effective in a wider range of pH and is less corrosive than both chlorine and ozone. However, its use is banned for fresh produce sanitation at concentrations over 3 mg L^{-1} (FDA - Food and Drugs Administration, 2018). It is explosive and it can react with iodine forming iodinated halogenated byproducts (Ölmez and Kretzschmar, 2009). **Hydrogen peroxide** has a wide action-range that includes spores and it decomposes into water and oxygen which are not harmful. However, it may damage the plant products through phytotoxicity or the induction of browning (Joshi *et al.*, 2013; Lin *et al.*, 2017). Acidic and neutral

electrolyzed water are generated by electrolysis of a NaCl solution. They have high bactericidal effect on *E. coli, S. enterica* and *L. monocytogenes*, achieved due to the reduction of pH, the direct oxidative action or the action of their derivative oxidant molecules like HOCl (Graça *et al.*, 2017). The production of chlorine gas, metal corrosion, synthetic resin degradation, and potential negative effects on products quality are the main disadvantages of electrolyzed water (Huang *et al.*, 2008).

Quaternary ammonium compounds are cationic surfactants, non-corrosive, non-irritating to skin, and able to penetrate food contact surfaces (Parish *et al.*, 2003). They act by disrupting cell membranes and cell wall structures but show reduced effectiveness against Gram-negative bacteria compared to fungi and Gram-positive bacteria. **Organic acids** such as citric, lactic, and acetic acid restrict microbial growth due to pH reduction. They do not produce harmful byproducts but require longer exposure times and generally are less effective than chlorine (Ölmez and Kretzschmar, 2009). Moreover, they can also prevent browning but they can provoke changes in organoleptic properties of the plant product.

Peroxyacetic acid (PAA), or peracetic acid, is an organic peracid formed by a quaternary equilibrium combination of peracetic acid, acetic acid and H_2O_2 in water solution (Luukkonen *et al.*, 2015). PAA breaks down to acetic acid, O_2, CO_2, and water, which are not considered as harmful to the environment (Dell'Erba *et al.*, 2007). Microbicide activity of PAA combines the oxidative action (by reacting with lipoproteins of bacterial cell membranes and by the production of ROS), which affect membranes and proteins, with the reduction of pH after decomposing in acetic acid (McFadden *et al.*, 2017). Although the efficacy of PAA is influenced by pH (higher at pH 6.5), it is less affected than chlorine by suspended organic matter (Ruiz-Cruz *et al.*, 2007; McFadden *et al.*, 2017). PAA has been approved by the EU for the disinfection of drinking water and food areas and as a preservative for liquid-cooling and processing systems (EC-European Comission for Health and Consummer Protection, 2016). It is also an

FDA-approved chemical for the sanitation of fruit and vegetables at a maximum concentration of 80 mg L^{-1} (FDA - Food and Drugs Administration, 2018) while its approval for this purpose in Europe is still under review (Banach *et al.*, 2015). Concentrations ranging from 40 to 80 mg L^{-1} PAA have shown variable effectiveness for food surface decontamination. For example, in fresh-cut apple, 80 mg L^{-1} PAA achieved reductions of *S. enterica, E. coli* O157:H7 and *L. monocytogenes* by 1 to 4.4 log_{10}, while 40 mg L^{-1} PAA was ineffective (Wisniewsky *et al.*, 2000; Rodgers *et al.*, 2004; Abadias *et al.*, 2011). However, in fresh-cut carrot, 40 mg L^{-1} PAA reduced *S. enterica, E. coli* O157:H7 and *L. monocytogenes* populations by 2.1, 1.2 and 0.8 log_{10}, respectively (Ruiz-Cruz *et al.*, 2007). Since there is no chemical antimicrobial compound without drawbacks, selection of the appropriate product and dose must be adjusted in function of its performance in each commodity according to the specific storage conditions.

Antibrowning and texture-preservative compounds

Several types of chemicals are used in the control of browning which may act by inhibiting PPO, hampering the browning reaction, or reversing it by reacting with intermediate products, thereby preventing the formation of brown-colored compounds (Arpita *et al.*, 2010). Organic acids such as citric acid, tartaric acid, malic acid and lactic acid **reduce pH**, lowering PPO activity, thus hampering the browning reaction (Suttirak and Manurakchinakorn, 2010). Ascorbic acid acts as a **reducing agent**, and like sulfhydryl compounds such as L-cysteine, reacts with o-quinones to form colorless compounds (Ali *et al.*, 2015). **Chelating agents** such as EDTA and anions such as chloride interact with the cofactor Cu^{2+} preventing PPO function (Remorini *et al.*, 2015). Ions such as calcium, mostly used as 5–30 g L^{-1} solutions of calcium chloride ($CaCl_2$), lactate, or ascorbate have effectively been used as a **texture preservative** in several fresh-cut fruit and vegetables such as apples, pears, carrots and, lettuces (Gorny *et al.*, 2002; Soliva-Fortuny, Oms-Oliu and Martìn-Belloso, 2002; Martín-Diana *et al.*, 2006; Rico *et al.*, 2007).

Probable mechanisms for Ca^{2+} to preserve texture are the formation of pectin polymer which improves cell- adhesion and thus mechanical strength, the delay of membrane lipid changes by lowering down lipase activity (Toivonen and Brummell, 2008). Ascorbate, in the other hand, can quench lipid alkoxyl and peroxyl radicals involved in membrane deterioration (Espin, Veltman and Wichers, 2000). Thus, formulations containing calcium and ascorbate can simultaneously prevent cell and membrane breakdown and modulate PPO activity in fresh-cut produce.

1.2.2 Biological methods

Biological methods used for decontamination of fresh produce are based on the application of use of antagonistic microorganisms and/or the metabolites they produce, and bacteriophages (Cotter, Hill and Ross, 2005; Gálvez *et al.*, 2010; Siroli, Patrignani, Serrazanetti, Tabanelli, *et al.*, 2015). Antagonistic microorganisms including plant epiphytic bacteria and yeasts, and lactic acid bacteria have been effectively used for controlling plant and human pathogens in several commodities (Leverentz, Janisiewicz and Conway, 2002; N.Teixidó *et al.*, 2011; Oliveira *et al.*, 2014). Biopreservation mediated by bacterial biocontrol agents was one of the methods evaluated in the present work and will be therefore, more extensively described hereafter.

Bacterial biocontrol agents: focusing on *Pseudomonas* spp.

The use of microbial biocontrol agents (BCA) to control post-harvest decay of fresh produce has arisen as a ‘biofriendly’ alternative to the use of synthetic fungicides (Spadaro and Gullino, 2004). A desirable ‘microbial biocontrol agent’ should have genetic stability, high effectiveness at low concentrations, activity against a wide range of pathogens on several plant hosts or food matrices, simple nutritional requirements and the ability to grow in the low-cost culture

media usually used for industrial production. It also should also be able to survive and adapt to adverse environmental conditions and show no pathogenicity to the plant or the human hosts (Wilson and Wisniewski 1989). Additionally, in order to be used in ready-eat-food, a BCA should also preserve the plant product quality for a reasonable period of time, keeping metabolic changes at low levels to avoid the formation of off-odors or off-flavors and the loss of texture and negative visual changes of the final product. Alternatively, it should be compatible with the preservative methods used for that purpose.

Bacterial BCAs have been mainly **registered and commercialized** for the pre-harvest treatment or prevention of plant infections caused by pathogenic fungi. Currently commercialized BCA-based products include several strains of *Bacillus subtilis* (Serenade Max®, Bayer, Germany; Avogreen®, University of Pretoria, South Africa; and Botokira Wettable Powder®, Idemitsu Kosan Inc., Japan) and other *Bacillus* species including *B. amyloliquefaciens* (Amylo-X® WG, Certis, Spain) and *B. megaterium* (Bio Arc®, Sphere Bio-Arc PVTLtd, India), which are applied as broad spectrum fungicides against pathogens belonging to the genera *Penicillium* spp. *Fusarium* spp., *Botrytis* spp., *Oydium* spp. *Sclerotinia*, spp., *Monilinia* spp., and *Cercospora* spp. among others, in a wide number of fruit (stone fruits, pome fruits, grapevine, berries, mango, avocado) and vegetables (pepper, tomato, eggplant, lettuce, cucurbits) (Taguchi *et al.*, 2003; El-zawahry *et al.*, 2015; Bayer-Crop Science-España, 2018) (Demoz and Korsten, 2006).

There is much less examples of antagonistic bacteria that are currently commercialized as BCAs for the post-harvest treatment of plant diseases. *Pseudomonas syringae* Van Hall (BioSave® 10LP and 11LP, EcoScience, Jet Harvest Longwood, FL, US) are commercialized as a post-harvest biological preventive treatment for potatoes, sweet potatoes, citrus, and some fruits, to control the fungal decay associated to *Fusarium sambucinum, Helminthosporium solani*, *Botrytis* spp.*, Penicillium* spp.*, Mucor* spp., and *Geotrichum* spp. infections (Janisiewicz and Jeffers, 1997). *Pantoea agglomerans* (Pantovital®, Udl, IRTA,

Spain) was isolated and developed in Spain to be applied for the postharvest control of molds of the genera *Penicilium* spp., *Botrytis* spp. *and Rhizopus* spp., in citrus and pome fruits, but up to date it has not been registered (Teixidó *et al.*, 2001; Nunes *et al.*, 2002).

The limited use of bacterial BCA-based products in post-harvest is due to inconsistent efficacy under commercial conditions or business and marketing-related shortcomings. In addition, BCA registration in the Quality Presumption of Safety (QPS) list of the EU in order to achieve their commercial application in minimally processed produce for the control of foodborne pathogens is still unachieved because they don't meet current legislation requirements. The postharvest application of microbial BCAs to prevent or limit microbial infection and/or growth has been investigated much extensively for plant pathogens and spoilage organisms than for human pathogens. Thus, there is an evident lack of information about BCA effectiveness on fresh-cut produce. Therefore, deepening in the knowledge of the mechanisms of action for biocontrol is essential to allow a closer approach to microbial BCAs commercial application in the minimally processed industry. This it will contribute to not only establishing the harmlessness of BCA-based preservatives but to developing appropriate formulations and application procedures enhancing their efficacy while preserving or extending plant product's shelf-life in optimal quality. The **mode of action** of bacterial biocontrol agents has been associated to multiple mechanisms acting directly by restricting the pathogens' life, growth and activity through the competition for ecological niche or acting indirectly, via the activation of host defenses. However, it is generally recognized BCAs mode of action is due to the combined effect of several mechanisms influenced by the extrinsic and intrinsic factors involved in the tripartite interaction antagonist-pathogen-food matrix (reviewed by Jamalizadeh et al. 2011).

Biopreservation: Direct action mechanisms

Interference with pathogen's growth and activity

Adherence & colonization: biofilms & biosurfactants

Competition for ecological niche is a suitable biocontrol mechanism for microbial antagonists that are endemic of fruit surfaces because they are already adapted and established in the plant host, and therefore they can **use the available resources** more **efficiently** than the newly coming pathogen (reviewed by Sharma, Singh, and Singh 2009). *Pseudomonas* spp. and several yeasts have the ability to **rapidly grow**, dominate, and **colonize** a new niche where resources are temporarily abundant. This mode of action can be compared to that of a fungicide which is applied before the arrival of the pathogen or prior the beginning of the infection cycle (Janisiewicz and Korsten 2002).

The ability to form **biofilms** is seen as an important colonization strategy aiding antagonistic bacteria to out-compete other naturally present or emergent microorganisms. Biofilms are structured communities of bacterial cells which act coordinately for certain functions and develop in a self-excreted extracellular polymeric matrix (EPS). They allow embedded microorganisms to adhere to an inert or a living surface and contributes to their protection and survival in unfavorable environments (Prakash, Veeregowda and Krishnappa, 2003). **EPS matrices** are primarily composed of polysaccharides but also contains proteins, lipids, and extracellular DNA, which can self-hydrate its structure by hydrogen-bonding to water (Zhao, 2017). The formation of biofilms is a common trait among Pseudomonads including *P. asplenii, P. corrugata , P. fluorescens , P. marginalis , P. putida, P. savastanoi,* and *P. syringae* (Ude *et al.*, 2006). It usually starts with protein synthesis and is influenced by environmental factors such as temperature and pH (O'Toole and Kolter 1998; Zhao 2017). EPS chemical composition varies among microorganisms, e.g. in *P. aeruginosa's* EPS is mainly formed by alginate which is also considered as a virulence factor (Boyd and

Chakrabarty 1995; May et al. 1991). Alginate is a polysaccharide that forms temperature-resistant water-insoluble hydrogels in the presence of multivalent cations such as Ca^{2+} which protects embedded bacterial and attaches them to surfaces (Draget and Taylor, 2011). EPS production increases with the age of the biofilm and is affected by the composition of the growth medium, being stimulated by excess carbon and limited nitrogen, as well as by the presence of iron, potassium, and phosphate (Claessen et al. 2014; O'Toole and Kolter 1998; Monds et al. 2007; Prakash, Veeregowda, and Krishnappa 2003).

Biofilm development mainly includes four **stages**: bacterial attachment, formation of micro-colonies, maturation, and bacterial dispersion (Claessen et al. 2014; Zhao 2017). The initial attachment of bacteria to surfaces is mediated by non-specific electrical attraction or hydrophobic interactions and involves bacterial structures such as flagellum, pili, curli, and fimbriae (Zhao, 2017). Adhesion of enteropathogenic *E. coli* and *S. enterica* to vegetable surfaces is mediated by curli and T3SS (Berger *et al.*, 2010). *L. monocytogenes* is also able to adhere and colonize inert and biotic surfaces through several molecular determinants, such as flagella, biofilm-associated proteins (Bap), SecA2, and cell-cell communication systems (Guilbaud *et al.*, 2015). Attachment to plant surfaces depends on pathogenic and nutritional conditions of the plants, hydrophobicity, cellular surface charge and roughness, injured or intact surface, waxy materials on the surface, electron donation and acceptance with bacteria, and inter-bacteria interactions (Ukuku and Fett, 2002). Overall, waxy materials of uncut surfaces repress attachment while hydrophilic and injured surfaces promote adherence (Jahid and Ha, 2012).

In advanced stages, biofilms contain canals allowing the circulation of nutrients, oxygen, and even antimicrobial agents (Xavier and Foster, 2007). In different zones of the mature biofilm bacterial cells express different genes, as in a specialized community functioning similarly to a multicellular organism showing increased adhesiveness; resistance to antibiotics, UV light, and chemical

biocides; increased rates of genetic exchange; altered biodegradability, and increased secondary metabolite production (Claessen *et al.*, 2014).

All stages of biofilm development are regulated by **quorum sensing** (QS). This is a cell-to-cell communication process mediated by signaling molecules which are secreted in response to changes in the cell density of the surrounding microbial community. QS can activate certain genes in the bacteria leading to the production of EPS, proteins, and toxins during biofilm formation. In plant growth-promoting rhizobacterium and pathogenic *Pseudomonas* spp., **acyl homoserine lactones** (AHLs) are examples of QS signaling molecules which stimulate the expression of genes for the production of polysaccharides, rhamnolipids, lipodepsipeptides, and other virulence factors (Licciardello *et al.*, 2012).

Accessing biofilm formation on inert surfaces is usually carried out through **methods** involving static systems. The microtiter plate biofilm assay is relatively quick and simple method allowing the screening of large numbers of bacterial strains or species by measuring the staining with crystal violet of the biomass adhering to a 96-well plate (Martín-Espada *et al.*, 2014). The study of biofilms on produce surfaces involves techniques such as epifluorescence, atomic force, confocal laser, or scanning electron microscopy (Jahid and Ha, 2012).

Biosurfactants are low-molecular weight amphiphilic molecules which have shown to be produced by a wide number of bacteria such as *Pseudomonas* spp. and *Bacillus* spp. that reduce the surface tension at the air/water interfaces and the interfacial tension at oil/ water interfaces (Banat *et al.*, 2010). There are different types of biosurfactant based on their physico-chemical properties including glycolipids, lipopeptides, neutral lipids, phospholipids, rhamnolipids, fatty acids, polysaccharides, proteins and lipoproteins, or mixtures thereof (Raaijmakers *et al.*, 2010). *Pseudomonas* spp. can produce a varied group of **lipopeptides** (LPs) including viscosin, amphisin, tolaasin, massetolides,

syringomycin, orfamide, arthrofactin, putisolvins, pseudodesmins, and entolysins (reviewed by Raaijmakers et al. 2010). The regulation of **LPs** production in *Pseudomonas* is mastered by the regulatory system GacA/GacS (Dubern *et al.*, 2005) and quorum sensing. The latter is involved in viscosin and putisolvin biosynthesis in plant pathogenic *P. fluorescens* strain 5064 and saprophytic *P. putida* PCL1445, respectively (Dubern, Lugtenberg and Bloemberg, 2006).

LPs play an important role in surface attachment and biofilm formation in *Pseudomonas*, although the effect can be opposed sometimes due to the diverse molecular structure and hydrophobicity of LPs. The latter factor depends not only on the charge of the LP itself but also on charge of the cell surface of the producing bacterium and on the charge of the substratum or the inert surface (Raaijmakers *et al.*, 2010). For example, arthrofactin and putisolvin reduce biofilm formation on plastic surfaces by *Pseudomonas* spp. strain MIS38, *P. putida,* respectively, while viscosin and massetolide induce initiation of biofilms by *P. fluorescens* strains SBW25 and SS101, respectively (Kuiper et al. 2004; Roongsawang et al. 2003). In the same way, **rhamnolipids** are essential for the initiation of biofilm formation by *P. aeruginosa* as well as in maintaining water channels and motility within mature biofilms (Pamp and Tolker-Nielsen, 2007). LPs produced by Pseudomonads, such as massetolid and amphisin, also play a role in bacterial motility, contributing to most efficient colonization of plant surfaces (Jacobsen *et al.*, 1999; Pamp and Tolker-Nielsen, 2007). Additionally, biosurfactants such LPs and rhamnolipids also have direct antibacterial and antifungal and anti-oomycete activities, particularly on *Pythium* and *Phytophthora* species, since they cause the lysis of zoospores (Raaijmakers et al. 2010; Vatsa et al. 2010). On the other hand, rhamnolipids produced by *P. aeruginosa*, as well as viscosin and massetolide A produced *P. fluorescens*, enhance resistance to grapevine, broccoli, and tomato against fungal infections (Braun et al. 2001; Vatsa et al. 2010).

INTAKE AND USE OF NUTRIENTS

The level of **ecological similarity** between a BCA and pathogenic microbiota can be used for estimating the effectiveness of the BCA. An approximate quantification can be made through **niche overlap index** (NOI) which are calculated based on *in vitro* utilization patterns of single carbon and nitrogen sources. NOI values greater than 0.9 indicate possible exclusive co-existence among species in an ecological niche while scores under 0.9 indicate separate niches (Wilson and Lindow 1994). Nutritional profiles of carbon source utilization using Biolog GN microplates, have been applied as a tool for approaching niche overlap in several heterotrophic bacterial communities and antagonist-pathogen systems (Garland and Mills, 1991; Preston-mafham, Lynne and Randerson, 2002; Dianese, Ji and Wilson, 2003; Cabrefiga, Bonaterra and Montesinos, 2007). This redox colorimetric technology is based on tetrazolium dye reduction as an indicator of single carbon and nitrogen sources oxidation.

Iron is an essential micronutrient for bacterial metabolism, acting as a cofactor for enzymes involved in oxidative metabolism (catalase, peroxidase, superoxide dismutase, and oxygenase), tricarboxylic acid cycle (aconitase); and DNA biosynthesis (ribonucleotide reductase B_2 subunit). Iron is also a structural component of iron-containing proteins (cytochromes, ferredoxin, hydrogenase, and succinate dehydrogenase) (Djibaoui and Bensoltane, 2005). Bacteria with the abilities for a more efficient uptake of iron have advantages for competition in Fe limiting conditions. Several *Pseudomonas* spp. have the ability to produce and secrete iron-chelating transport molecules called **siderophores** that sequester iron from the surrounding medium depriving the pathogen from Fe nutrition.

Most of fluorescent pseudomonads produce complex fluorescent peptidic with high Fe- affinity siderophores called **pyoverdines** or pseudobactins. These are very efficient iron scavengers and are responsible for the fluorescence of those

bacteria's colonies (Cornelis and Matthijs, 2002). Non-fluorescent *Pseudomonas* spp. strains also produce non-fluorescent alternatives of pyoverdines with similar Fe- affinities to the fluorescent homologues (Bultreys *et al.*, 2001). Several **other siderophores** with less Fe-affinity have been isolated from Pseudomonads such as pyochelin, pseudomonine, pyridine-2, 6-dithiocarboxylic acid and quinolobactin which have inhibitory effects on microbial growth (Cornelis and Matthijs, 2002; Matthijs *et al.*, 2004).

Growth **conditions** such as pH as well as the availability and accessibility of nitrogen and carbon sources and iron, **influence** the stimulation and yield of **siderophores production**. The solubility of iron is highly influenced by pH (increases at alkaline levels) which in turn determines its availability to the growing organism in the medium. In aerobic conditions and at physiological pH, the reduced ferrous (Fe^{2+}) form is unstable and is oxidized to the ferric (Fe^{3+}) form, normally forming low soluble iron hydroxide which is unavailable to biological systems. For instance, the best yield in siderophores production by *P. fluorescens* and *P. putida* was attained in Fe-free succinate medium (4 g L^{-1} succinic acid) prepared with neutral-pH water for an iron threshold level of 30 µM ($FeCl_3$) (Sayyed *et al.*, 2005). In that case, siderophores production started after 12 h of incubation and reached the maximum yield at 24-30 h, declining thereafter (Sayyed *et al.*, 2005).

Production of antimicrobial metabolites and lytic enzymes

The production of low-molecular weight secondary metabolites, deleterious at low concentrations (less than 10 mg L^{-1}) to the growth or metabolic activities of other microorganisms, is a direct mode of action shared by several antagonistic bacteria (Jamalizadeh *et al.*, 2011). Pseudomonads produce a broad chemically varied battery of antimicrobial compounds. They include N-containing **heterocycle diffusible antibiotics** such as phenazines, pyoluteorin, 2,4-diacetylphloroglucinol (DAPG) and pyrrolnitrin as well as lipids (e.g. LP and

glicolipids), volatile compounds (e.g. hydrogen cyanide), proteins (e.g. bacteriocins), and lytic enzymes. Their action range includes fungi, bacteria, viruses and helminthes (reviewed by Dwivedi and Johri 2003). In general, secondary metabolites accumulate until the beginning of the stationary phase and are affected by physical (temperature, moisture, pH) and chemical factors (nutrients concentration such as carbon, inorganic phosphate, and minerals) (Fernando, Nakkeeran and Zhang, 2006; Park *et al.*, 2011; Kremmydas, Tampakaki and Georgakopoulos, 2013).

Pseudomonads heterocycle diffusible antibiotics have been associated mainly to antifungal activities and each one of them shows a different target spectrum. For example, pyrrolnitrin, produced by *P. fluorescens, P. chlororaphis, P. aureofaciens,* and *P. cepacia,* antagonize *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Fernando, Nakkeeran and Zhang, 2006). Phenazines and their more than 50 derivative compounds, including pyocyanin, phenazine-1-carboxyclic acid, 2-hydroxyphenazines and phenazine-1-carboxamide, are widely distributed within the Pseudomonads group and are effective against *Sclerotinia sclerotium, Gaeumannomyces graminis* var. *tritici* and *Fusarium oxysporum* (Haas, Blumer and Keel, 2000; Fernando, Nakkeeran and Zhang, 2006; Pierson and Pierson, 2010; Wallace, Hirkala and Nelson, 2017). Pyoluteorin has been isolated from *P. aeruginosa, P. fluorescens* (Pf-5 and CHAO), and *P. protegens,* and shows bactericidal, herbicidal, and fungicidal properties (Ramette et al. 2011; Upadhyay and Srivastava 2008). DAPG is a polyketide antibiotic with antiviral, antibacterial, antifungal, and antihelminthic effect which is only produced by the *P. fluorescens* group (Redondo-Nieto *et al.*, 2013).

Bacterial **lytic enzymes** such as peptidases, chitinases and glucanases, produced by several Pseudomonads, degrade key structural cell wall components such as peptidoglycan of bacteria as well as chitin and β-1,3-glucan of fungi. Alkaline proteases, active against Gram negative bacteria, have been isolated from several *Pseudomonas* spp. (Kadurugamuwa and Beveridge, 1996; Shastry and

Prasad, 2002; Nicodème *et al.*, 2005; Zambare, Nilegaonkar and Kanekar, 2011). Laminariase and chitinase are involved in the control of *Fusarium solani* by *P. stutzeri* VPL1 (Lim, Kim, and Kim 1991). Furthermore, *P. fluorescens* antagonized *R. solani* and *Pythium ultimum* through the action of endochitinase, chitobiosidase and β-1,3-glucanase (Nielsen and Sørensen, 1999; Nagarajkumar, Bhaskaran and Velazhahan, 2004).

On the other hand, **bacteriocins** are another kind of lytic proteins produced by bacteria which have DNAse, RNAse or membrane pore-forming activity, and are generally effective against closely related bacterial species (Cleveland *et al.*, 2001). Bacteriocins from Pseudomonads are called pyocins. Several of them were characterized from strains such as *P. putida* BW11P2 and *P. aeruginosa* PAO1, and up to four different molecules were found produced by the latter species (Parret and De Mot, 2000; Michel-Briand and Baysse, 2002; Parret and Mot, 2002).

The **regulation** of antimicrobial metabolites in Pseudomonads is carried out at different levels (Hass and Keel, 2003). Global post-transcriptional regulation is controlled by the two component positive regulatory system (*Gac*S/*Gac*A) which induce the biosynthesis of extracellular diffusible and volatile metabolites and exo-enzymes, and QS molecules (Dwivedi and Johri, 2003; Hass and Keel, 2003). *rpo*S and *rpo*N—alternative sigma factors are involved in the transcriptional regulation of the biosynthesis phenazine, pyrrolnitrin and DAPG (Sarniguet *et al.*, 1995; Whistler *et al.*, 1998; Oh *et al.*, 2013). *Phz*I and *phz*R and the five-gene operon *phl*ACBDE are pathway-specific regulators of phenazine biosynthesis, DAPG and QS (Maddula, 2008). Among negative regulators are the two component (RpeA/RpeB); *rsm*Z, *rsm*Y, and *rsm*X—small untranslated regulatory RNAs that modulate activity of translational repressors RsmA and RsmE (Wang *et al.*, 2012); and *phl*F and *plt*R as repressors of DAPG and pyoluteorin production, respectively (Weller *et al.*, 2007).

Impairment of pathogens' virulence

The interference with pathogen's virulence is another direct action mechanism for BCAs to prevent pathogens infection and reduce disease incidence and severity. Some BCAs can block pathogens' cell-to-cell communication or **quorum sensing**, thereby preventing the expression of virulence genes (Dong et al., 2007; Kalia, 2013). The most representative QS signals are N-acyl-homoserine lactones (AHLs), produced by Gram-negative bacteria, small auto-inducing oligopeptides (AIPs), produced by Gram-positive bacteria and the LuxS or auto-inducer 2 (AI-2) system produced by both Gram-negative and Gram-positive species (Zhang and Dong, 2004). QS participates in the regulation of genes involved in invasion and spread, production of antibiotics and virulence factors, formation of biofilms as well as in many other microbial activities that allow the survival of microorganisms within the plant or human hosts (Jordan *et al.*, 2014). For example, the *agr* QS system, which has been characterized in *L. monocytogenes* and *S. aureus*, regulates the timeline production of virulence factors i.e. the early expression of adhesins and the late production of toxins and tissue-degrading enzymes (Autret *et al.*, 2003; Zetzmann *et al.*, 2016).

Interference with QS, also known as '**quorum-quenching**', can be achieved through the production of enzymes (lactonases, amidohydrolases, oxidoreductases) that degrade or inactivate pathogen's AHLs, or by producing small molecules (halogenated furanones, cyclic dipeptides, and small peptides) that prevent QS signals biosynthesis or interaction with their receptors (Dong *et al.*, 2000, 2004; Dong, Wang and Zhang, 2007; LaSarre and Federle, 2013). Reduction of virulence of human pathogens through quorum quenching and other mechanisms has been mainly investigated for probiotics (Abdel-Daim et al., 2013; Medellin-Peña et al., 2007). It has been observed that the probiotics *Bifidobacterium longum* NCC2705 and *Lactobacillus acidophilus* La-5 down-regulated the expression of the virulence genes flaA and ciaB in *Campylobacter jejuni* 81-176 by mechanisms involving interference with *lux*S function (Mundi *et*

al., 2013). Secreted molecules by *L. acidophilus* La-5 can also alter *E. coli* O157:H7 QS, *in vitro* and *in vivo*, reducing its virulence (Medellin-Peña *et al.*, 2007). In addition, some rhizosphere *Pseudomonas* spp. isolates can degrade AHLs or produce negative-signals capable of blocking quorum sensing of other bacteria, thereby preventing antibiotics production (Uroz *et al.*, 2003; Morello, Pierson and Pierson, 2004).

Interference with virulence can also be achieved through the knockout or down-regulation of the gene encoding for **virulence factors** or the **impairment of their activity** during the several steps involved in stablishing infection: adhesion, invasion, multiplication, translocation, and cytotoxicity (Cossart and Sansonetti, 2004; Fleckenstein *et al.*, 2010; Ribet and Cossart, 2015). The probiotics ability for the impairment of virulence has been related to the secretion of bacteriocins and organic acids, the competition for adhesion sites or for molecules involved in the invasion process, and the activation of host defense mechanisms such as the secretion of mucins (Servin, 2004).

Extensive research has been carried out to elucidate probiotics mechanisms for antagonizing human pathogens through *in vitro* assays. Most of *in vitro* experiments have been carried out using the human colon adenocarcinoma cell lines Caco-2 and HT29, which reproduce the morphology and function of the brush border intestinal epithelium (Sambuy *et al.*, 2005). Those experiments have shown that *B. thermophilum* subsp. *infantis* RBL67 and *B. thermacidophilum* subsp. *suis* (RBL68 and RBL70) reduced the adhesion and invasion of *L. monocytogenes,* probably through competition for adhesion sites (Moroni *et al.*, 2006). Moreover, LAB belonging to the genus *Bifidobacterium* and *Lactobacillus* can reduce *E. coli* IC2 virulence by antagonizing its adhesiveness and biofilm formation capacity (Abdelhamid, Esaam and Hazaa, 2018). Competitive exclusion assays using HT-29 cell line pre-exposed for 3 h to *L. plantarum* LB95 showed a significant reduction of both the adhesive and the invasive abilities of *S. enterica* subsp. *enterica* serovar *Enteritidis* as well as the

invasiveness of *L. monocytogenes* (Dutra *et al.*, 2016). Similarly, the probiotic *Pediococcus acidilactici* P9 showed a weak effect on *L. monocytogenes* adhesion but it blocked the invasion of HT-29 cell line by this pathogen, via bacteriocin secretion (Wang *et al.*, 2014). In non-LAB such as the probiotic *E. coli* Nissle 1917, a secreted non-bacteriocin unidentified molecule interfered with the invasion of *S. enterica* serovar *Typhimurium* into INT407 cell line (Altenhoefer *et al.*, 2004).

The effect of Pseudomonads on the virulence determinants of pathogens has been rather studied in plant or other animal hosts. The BCA *P. fluorescens* Pf29Arp affected the expression of PR-genes (laccase-encoding gene *Lac2* and lytic enzyme EXO-encoding gene) in the take-all fungus *G. graminis* var. *tritici* in wheat seedlings (Daval *et al.*, 2011). There is a report of *in vitro* and *in vivo* antagonistic activities of *Pseudomonas* spp. M162 against *Flavobacterium psychrophilum,* a pathogen of rainbow trout, through siderophore production and the enhancement of immunological responses (i.e. stimulated peripheral blood leucocyte counts, serum lysozyme activity and total serum immunoglobulin levels) (Korkea-aho *et al.*, 2012). In that study, *Pseudomonas* spp. M162 was considered as a probiotic since it was able to colonize the gastrointestinal tract.

Biopreservation: Indirect action mechanisms

Induction of the plant host's defense response

BCAs may also indirectly inhibit pathogens' growth and activity by priming plant defense mechanisms. **Inducible plant defense mechanisms** include Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). They are both acquired states of defense in the whole plant, but SAR is usually associated to plant-pathogen interactions while ISR has been linked to the ability of some plant-growth promoting rhizobacteria (PGPR) of priming the plant to respond more quickly and intensely to a subsequent pathogenic attack. PGPR-induced

ISR have been proved to reduce incidence and severity of both pre-harvest and post-harvest plant diseases in several fruit and vegetables (Vidhyasekaran *et al.*, 2001; Audenaert *et al.*, 2002; Anand *et al.*, 2010; Sangeetha *et al.*, 2010; Shi *et al.*, 2013; Lamia *et al.*, 2017).

Triggering plant defense response begins when surface molecules of pathogenic or non-pathogenic microorganisms called pathogen–activated molecular patterns (PAMPs) or microbe–activated molecular patterns (MAMPs) which include components of the outer membrane and the extracellular matrix, pili, flagella, and fimbriae, are recognized by the plant pattern recognition receptors (PRRs) (Van Wees, Van der Ent and Pieterse, 2008; Beneduzi, Ambrosini and Passaglia, 2012). In addition to surface molecules, ISR activation by Pseudomonads may involve the secretion of several molecules with antimicrobial and/or siderophore- activity (DAPG, pyocyanin, pyochelin pyoverdin, and salicylic acid) as well as by biosurfactants, AHLs, N-alkylated benzylamines, and volatile compounds (Audenaert *et al.*, 2002; Iavicoli *et al.*, 2003).

After the initial recognition of PAMPs or MAMPs, plant signaling pathways involving salicylic acid (SA), jasmonic acid (JA), and ethylene (C_2H_4) are activated (Choudhary et al., 2007). Those **downstream signaling pathways** differ for SAR and ISR: although both responses require a functional NPR1 protein (non-expressor of PR genes), SAR is characterized by the activation of SA-mediated cascades while ISR is mediated by JA and C_2H_4 (Pieterse et al., 2000). The activation of the ethylene and JA- mediated mechanisms by Pseudomonads requires increased levels of these molecules which have shown to involve either the increased expression and activity of the enzymes involved in their biosynthesis or the enhanced production of their receptors (Pieterse *et al.*, 2000).

Downstream signaling lead to the activation of mitogen-activated protein kinase cascades (MAPKs) which results in the expression of effector molecules called pathogenesis-related (PR) proteins such as β-1,3-glucanases and chitinases with ultimately degrade the pathogen's cell wall or other structures causing their dead or hampering their multiplication and colonization (Chisholm *et al.*, 2006; Van Wees, Van der Ent and Pieterse, 2008). Among plant mechanisms to **limit pathogen's colonization** are the strengthening of cell walls through lignification, and the confinement of pathogens in an oxidative or dead environment through oxidative burst and hypersensitive response (HR). HR is a programmed cell death mechanism activated during SAR, which leads to the rapid death of infected cells and the formation of local necrotic lesions to restrict the growth of biotrophic pathogens (Czarny, Grichko and Glick, 2006). HR includes the production of ROS followed by the increase of levels of PR-proteins. To counteract the deleterious effects of oxidative stress on the plant tissue, ROS-scavenging enzymatic and non-enzymatic mechanisms are concomitantly activated. As occurs upon wounding and described in Section 1.1.3, those mechanisms include the productions of antioxidants (e.g. phenolic compounds, ascorbic acid, and glutathione) and ROS- scavenging enzymes (CAT, POX, PPO, APX, GSR, and CHS) (Lugtenberg, Dekkers and Bloemberg, 2001; Romanazzi *et al.*, 2016). Downstream signaling SA-mediated cascade leads to the binding of SA to CAT and APX with the formation of a phenolic radical that in turn is involved in lipid peroxidation resulting in the activation of defense-related gene expression (Kawano and Boutou, 2013). PR-proteins production has also been observed in papaya fruits treated with the BCA *P. putida* MGP1 (Shi *et al.*, 2013). In rice plants inoculated with *P. fluorescens* Pf1, increased lignification and activities of enzymes involved in lignin biosynthesis such as POX, PAL and 4-coumarate CoA ligase have been observed (Vidhyasekaran *et al.*, 2001). Those responses enhanced the plant resistance to the pathogen *Xhantomonas oxysporum* pv. *Orizae.* Moreover, it has been demonstrated that the enhancement of the defense response in wheat and tomato by biocontrol agents including *P.*

fluorescens CHAO involves the increased activity of POX, lipoxygenases, and glucanases (Silva *et al.*, 2004; Sari, Etebarian and Aminian, 2008; Daval *et al.*, 2011). In the same way, *Pseudomonas* spp. *EPB5* induced the activities of chitinases and β-1,3-glucanases, POX, PPO and PAL as well as increased phenolic compounds content in banana plants challenged with aphids and bunchy top virus (Harish *et al.*, 2009).

In addition to activate plant defense mechanisms, some Pseudomonads produce **ACC deaminase** (ACD). This is a cytoplasmic enzyme bound to the coenzyme pyridoxal phosphate which converts ACC, the precursor of ethylene, to ammonia and α-ketobutyrate, thereby limiting ethylene biosynthesis. The optimal conditions for this enzyme are a temperature of 30°C and pH 8.5 (Czarny, Grichko and Glick, 2006). ACD is normally present at basal levels in microorganisms and although it has low affinity for ACC, it can be activated by very low amounts of (100 nM) of this substrate (Gamalero and Glick, Bernard, 2015). The inhibition of ethylene biosynthesis results in a lowered level of 'stress' ethylene which can result in a decrease in the severity of infection by various phytopathogens and can contribute to delay ripening and senescence, extending the shelf-life of fresh-cut produce (Robison *et al.*, 2001).

Foodborne pathogens and the plant host

Foodborne pathogens such as *S. enterica* and *E. coli* H157:O7, despite not being specialized plant pathogens, have adapted to survive and colonize plant tissues (reviewed by Lim, Lee and Heu, 2014). They can attach to plant surfaces through external structures such as pili, fimbriae, curli and flagellum, or molecules from the capsule and cell wall (Schikora *et al.*, 2008, 2011). Afterwards, they can invade internal tissues through stomata, wounds or cut surfaces and activate a number of plant defense-related genes leading to the production of PR-proteins or evade plant defense mechanisms through the action of effectors injected by their Type 3 secretion systems (TTSS), similar to plant pathogens (Schikora *et al.*, 2008; Roy

et al., 2013). Therefore, enhancing plant immune response may be an efficient mechanism for BCA to fight both plant and human pathogens (Schikora *et al.*, 2011; Schikora, Garcia and Hirt, 2012).

Pseudomonas graminis CPA-7 as a biopreservative agent

Pseudomonas graminis CPA-7 is a bio-preservative strain that was isolated from whole 'Golden delicious' apple surface (Alegre, Viñas, Usall, Teixido, *et al.*, 2013) and it is deposited as CBS124167 in Centraalbureau voor Schimmelcultures (CBS) (Utrecht, The Netherlands). *P. graminis* species, first described by Behrendt *et al.* (1999), includes Gram-negative, aerobic, non-spore-forming, bacilli (0-5-1.0 x 35-50 pm) with polar flagella (15-20 °C). Like other strains described within the species, they form round, smooth and convex, with entire-margin colonies on TSA medium, whose color change from pale yellow to deep yellow with age and refrigerated temperatures. Moreover, they do not produce fluorescent pigments on BHI medium (Behrendt *et al.*, 1999; Mehnaz *et al.*, 2009; Crovadore *et al.*, 2016; Mikiciński, Sobiczewski, Puławska and Maciorowski, 2016).

CPA-7 can grow at temperatures from 6 to 30 ºC, but not above 33 ºC (Alegre, Viñas, Usall, Teixido, *et al.*, 2013). Metabolic characterization with the API 20NE system showed that CPA-7 is oxidase-negative and catalase- positive, do not reduce nitrate to nitrite, or ferment glucose to produce acid (Alegre, Viñas, Usall, Teixido, *et al.*, 2013).

CPA-7 showed inhibitory activity against several isolates of the foodborne pathogens *L. monocytogenes* and *S. enterica* subsp. *enterica* as well as on *E. coli* O157:H7 on various fresh-cut fruit (Alegre, Viñas, Usall, Anguera, *et al.*, 2013; Iglesias, 2017). Therefore, it has been patented as biocontrol agent in the EU (EP 2886665) (Viñas *et al.*, 2017) and the US (US8735136) (Viñas *et al.*, 2012). The effectiveness of the antagonistic activity of CPA-7 on foodborne pathogens varies depending on the level of inoculum and growth conditions (temperature,

packaging atmosphere, preservative compounds and the composition of the growth medium or food matrix) as well as on the target pathogenic microorganism. The highest effectiveness of CPA-7 in apple plugs has been observed at growth conditions close to optimal (20 ºC in air), with reductions by 2.8, 4.5 and 5.9 log_{10} of *S. enterica*, *E. coli* and *L. innocua*, respectively (Alegre, Viñas, Usall, Teixido, *et al.*, 2013). In the same conditions, *S. enterica*, *E. coli* and *L. innocua* were reduced by 2.8, 4.0, and 4.3 log_{10}, respectively, in peach plugs. Similarly, the best performances of CPA-7 against *L. monocytogenes* and *S. enterica* were observed in air storage at 20 °C on fresh-cut melon and pear, with reductions by 5.3 and 7.3 log_{10}, respectively, on melon (Abadias et al. 2014) and by 2.8 and 2.4 log_{10}, respectively, on pear (Iglesias *et al.*, 2018) after 2 d of storage. Based on those experiments, the best CPA-7'S antagonistic performance is obtained with inocula ranging from 10^7 to 10^8 CFU g^{-1}.

Extrinsic factors involved in **semi-commercial conditions** have shown differential effect on CPA-7's antagonistic activity against foodborne pathogens. For instance, treatment with the antioxidant NatureSeal® did not influence the inhibitory effect of CPA-7 (10^7 CFU mL^{-1}) against *S. enterica* on 'Golden delicious' apple wedges but reduced its effectiveness against *L. monocytogenes* after 7 d of MAP storage at abused refrigerated temperatures (10 °C), compared to air (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). In the same way, lowering temperature to 5 °C annulled CPA-7's inhibitory effect on *S. enterica* and reduced the efficacy for *L. monocytogenes* control by 2 log_{10}. Similarly, in fresh-cut 'Piel de sapo' melon, the effectiveness of CPA-7 (10^7 CFU mL^{-1}) against *L. monocytogenes* significantly decreased from 4.9 to < 1 log_{10} reduction, with the drop of temperature from 10 and 5 °C, in air storage for 9 d (Abadias et al. 2014). In the same experiment, MAP decreased the effectiveness of CPA-7 (10^7 CFU mL^{-1}) against *L. monocytogenes*, from 5.4 to < 1 log_{10} reduction, after 8 d at 10 °C while low temperature and MAP resulted in no effect on this pathogen (Abadias et al. 2014). Treatment of fresh-cut pear with an antioxidant solution containing

ascorbic acid, sodium citrate and calcium chloride (20 g L^{-1} each) resulted in no inhibitory effect of CPA-7 on *S. enterica* during 10 d in MAP at 5 °C, while the effectiveness at reducing *L. monocytogenes* decreased from 2.4 to 1.3 $log_{10,}$ compared to air (Iglesias *et al.*, 2018). In the same study, no inhibitory effect of CPA-7 on *S. enterica* or in *L. monocytogenes* was observed in MAP after 10 d at 10 °C.

The liquid production process of *P. graminis* CPA-7 has been optimized in laboratory bioreactors (2 and 5 L) and scaled up to 100 L-bioreactor in pilot plant facilities. A dry formulation is already available showing high viability after 21 months in refrigerated conditions (4°C) (Teixido et al., unpublished data).

Preliminary attempts to determine the **mode of action** of CPA-7 included testing the production of diffusible antimicrobials on solid medium using the pour-plate method. No antimicrobial activity of the cell free supernatants from CPA-7 overnight TSB-cultures was observed against *E. coli* O157:H7, *S. enterica, L. innocua, L. monocytogenes* CECT-4031, *Aeromonas hydrophila* ATCC 7966 or *P. marginalis* CECT-229 on PCA plates after 24 h of incubation at 30 °C (Alegre, Viñas, Usall, Teixido, *et al.*, 2013). The effect of CPA-7 on the plant host defense response has been assessed by studying the activity of the oxidative stress enzymes POX and PPO in fresh-cut 'Piel de sapo' melon treated with 5 g L^{-1} calcium chloride (Plaza *et al.*, 2016). Results showed an increase in POX activity after 8 d of storage at 5 °C in response to CPA-7 whereas no change was observed in PPO activity. Furthermore, the putative activation of the plant hypersensitive response was assessed in tobacco seedlings wound-inoculated in the leaves with a 10^9 CFU mL^{-1} CPA-7 suspension. Results showed no necrotic areas around inoculation sites, indicating that CPA-7 was unable to activate cell death mechanisms in the plant host (Alegre, 2012).

On the other hand, the lack of viable CPA-7 cells (below 50 CFU g^{-1}) after a static simulated gastric digestion of inoculated apple samples suggested that the

possibility for this antagonist to colonize the intestinal epithelium surface was unlikely (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). The experimental setup included a simulated mastication for 120 s in artificial saliva (6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$ and 1.2 g L^{-1} $NaHCO_3$) tempered at 37 °C followed by a shaking incubation with synthetic gastric fluid (2 M HCL, 0.3 g L^{-1} pepsin, pH 2.0) for 2 h at 37 °C, but excluded intestinal digestion.

1.2.3 Non-thermal physical methods

Physical methods applied for preserving fresh produce includes those aimed at reducing metabolic activity of both the plant product and its inhabiting microbiota, such as refrigeration and oxygen-restrictive packaging and storage, and those that show direct germicide activity and are used for decontamination. Emerging physical non-thermal antimicrobial technologies include ultrasounds, plasma, ionizing radiation, pulsed light, ultraviolet light, pulsed electric field, and high hydrostatic pressure (Mukhopadhyay and Ukuku, 2018). For **ultrasounds** treatment produce is immersed in a liquid, usually organic acids or chlorine dioxide solutions, and waves at frequencies from 20 to 100 kHz are applied allowing the reduction of both foodborne pathogens and residing spoilage microbiota (Sánchez *et al.*, 2015). **Ionizing radiation** of includes gamma rays from Cobalt-60 or Cesium-137 radioisotopes, electron beams, and X-rays (Yang *et al.*, 2013). Maximum doses of 1 to kGy are established for fruit and vegetables, respectively, in the EU (EFSA, 2011). Gamma rays, e-beams and X-rays interact with matter resulting in the formation of energetic electrons and molecular ions that disrupt microbial activity (Yang *et al.*, 2013), but in turn, they may cause fruit softening and reduce the ascorbic acid content and titratable acidity (Fan *et al.*, 2005). **Plasma** is composed of energetic reactive species, such as free radicals, electrons, ions, and ultraviolet photons, dissociated from gas molecules upon an energy input (Song *et al.*, 2015). Its antimicrobial activity is based on the combined action of generated ROS and UV photons and it has been effectively

used for decontamination of fresh produce (Song *et al.*, 2015; Bourke *et al.*, 2017). **Pulsed electric fields** technology is based on the application of short high power electrical pulses (μs to ms) resulting in both in the induction of the plant secondary metabolism and in increased microbial cell membrane permeability, leading to death (Gil, Selma, López-Gálvez, & Allende 2009). **High hydrostatic pressures** (400−600 MPa) inactivate pathogenic and spoilage microorganisms in fresh produce while preserving or enhancing nutritional and physical quality attributes (Westphal *et al.*, 2017). Ultraviolet light-methods are also effective for both decontamination and improvement of nutritional properties of fresh produce and will be more extensively described in the subsequent subsections.

Ultraviolet light

Ultraviolet (UV) light is an electromagnetic radiation which comprises long (315 to 400 nm, UV-A), medium (280 to 315 nm, UV-B) and short wavelength ranges (100 to 280 nm, UV-C) (Turtoi, 2013). UV-C is the non-ionizing portion of the UV spectrum which is used for surface decontamination of materials, water and food (Shama, 2007). UV-C is lethal to most microorganisms including viruses, bacteria, protozoa, molds and yeasts, mainly due to the formation of cyclobutane–pyrimidine dimers and 6–4 photoproducts in the DNA. The formation of such misstructured products prevents proper DNA transcription and replication which leads to mutagenesis and death (Bintsis, Litopoulou-Tzanetaki and Robinson, 2000). Other **deleterious effects** in microbial cells are abnormal ion flow, increased cell membrane permeability, and depolarization of the cell membrane (Wuytack *et al.*, 2003). However, at certain doses, UV-C light can also damage the plant cell membranes and organelles such as chloroplasts (i. e. disruption of structural integrity of thylakoids and destruction of plastoquinones) and mitochondria (Urban *et al.*, 2016) and promote photo-oxidative reactions leading to the production of ROS and peroxidation of membranes (Petit *et al.*, 2009). However, when applied at low doses, UV-C

irradiation can also induce beneficial biological reactions in fruit and vegetables, in a phenomenon known as hormesis (Shama, 2007).

The **advantages o**f UV-C technology include the activity against a broad range of spoilage and pathogenic microorganisms, the lack of soluble chemical toxic by-products, and the relatively low cost and energy consumption compared to other thermal and non-thermal pasteurization processes (Gayán, Condón and Álvarez, 2014). The major **limitations** of UV-C radiation are the low penetration (50–300 nm depth), which implies reduced effectiveness on rough solid surfaces and in liquids with high-turbidity or with organic matter in suspension, and on overpopulations of microorganisms forming layers and biofilms (Civello, Vicente and Martinez, 2006). Moreover, certain microorganisms can reverse the destructive effects of UV through light-dependent (photo reactivation) or independent (dark repair) DNA-repair mechanisms (reviewed by Sinha and Häder, 2002).

Direct antimicrobial effect

The antimicrobial effect of UV light **depends on** the dose, which in turn depends on the irradiance and the time of exposure (Turtoi, 2013). **Doses** ranging from 0.2 to 20 kJ m^{-2} were tested for the decontamination of fresh-cut produce with highly variable results depending on the strain and specie-specific **microbial sensitivity** to UV-, and to the type, shape, topography, and maturity stage of the commodity. In general vegetative microbial cells are more sensitive than spores. Yeasts and molds are more sensitive than bacteria, and within the last ones, the Gram-negative are more susceptible than the Gram-positive. In the same way, smooth **plant surfaces** allow better accessibility than rough ones (Gayán, Condón and Álvarez, 2014). In fresh-cut broccoli the susceptibility to UV-C decreased from *E. coli* to *S. enterica ser.* Enteritidis and *L. monocytogenes,* since effective doses for 1 log_{10} reduction were 0.02, 1.07 and 9.26 kJ m^{-2}, respectively (Martínez-Hernández, Navarro-Rico, *et al.*, 2015). Similar species sensitivity and

semi-logarithmic survival curves with upward concavities and a tail were observed by Schenk et al. (2008) in fresh-cut pear, with a reduction of the inactivation rate at irradiation times over 7 min (33 kJ m^{-2}) and increased effectiveness with the removal of peel. A dose of 0.9 kJ m^{-2} UV-C reduced *E. coli* O157:H7 by > 2 log_{10} in fresh-cut apple and pear while higher doses (7.2, and 10.5 kJ m^{-2}) achieved lower reductions (2.0 and 1.1 log_{10}) in strawberry and raspberry, respectively (Adhikari *et al.*, 2015). In the same experiment, the combination species resistance + food matrix characteristics resulted in considerably higher doses needed for reduction of *L. monocytogenes*: 3.75 kJ m^{-2} for a 1.6 log_{10} reduction on apple, 11.9 kJ m^{-2} for the same reduction on pear, while the latter dose only achieved 1.0 log_{10} reduction on cantaloupe and strawberry.

HORMETIC EFFECTS

Hormetic effects of UV-C include the delay of senescence and fruit ripening (Pombo et al., 2009; Severo et al., 2015), the increased production of health-promoting phytochemicals (Duarte-Sierra, 2015; Formica-Oliveira et al., 2017), and the induction of natural defense and elicitors against fungi and bacteria (Mintoff, Rookes and Cahill, 2015). The **delay of fruit softening** by UV-C was suggested to be related to changes in the amount and activities of enzymes and proteins involved in cell wall disassembly (Ribeiro, Canada and Alvarenga, 2012). For example, a decrease in the expression of genes encoding cell wall degrading enzymes and proteins (polygalacturonases, pectin-methylesterases and endoglucanase) has been observed in strawberry within the 24 h following irradiation with 4.1 kJ m^{-2} UV-C (Pombo *et al.*, 2009).

Hormetic UV-C treatments (3.7 kJ m^{-2}), also **delayed chlorophyll degradation** and carotenoid accumulation and increased polyamine content in tomato (Severo et al., 2015; Tiecher et al., 2013). Treatments with 7-10 kJ m^{-2} UV-C showed several hormetic effects in broccoli such as higher phenolic and ascorbic acid contents, higher antioxidant activity and delayed chlorophyll degradation. This latter

correlated with the reduced activities of enzymes involved in chlorophyll catabolism (chlorophyllase and magnesium-dechelatase) (Costa *et al.*, 2006; Lemoine *et al.*, 2007).

UV-C radiation also **activates the biosynthesis of metabolites** from the phenylpropanoid pathway and other bioactive compounds that promote human health as part of a complex sunscreen and antioxidant plant defense mechanism. Total phenolic compounds, flavonoids, terpenoids, carotenoids, phytoalexins (resveratrol), glucosinolates, and tanins have been reported to increase upon UV-C exposure in many fruits and vegetables in a dose dependent manner (reviewed by Alothman et al., 2009). Relatively high UV-C doses (10 kJ m^{-2}) alone or combined to UV-B increased glucobrassicin and glucoraphanin levels in fresh-cut broccoli (A.C. Formica-Oliveira *et al.*, 2017).

Similarly, treatment with 4.1 kJ m^{-2} UV increased the activities of defense-related enzymes such as PAL, β 1,3-glucanase, POX and PPO, as well as induced the expression of PR-genes in strawberry fruit, resulting in an **enhanced resistance** to a sequent infection by *B. cinerea* and less decay after 9 d of storage (Pombo *et al.*, 2011). Chitinase, β -1,3-glucanase and PAL gene-expression and activities were also induced in peach fruit after irradiation with 7.6 kJ m^{-2} (El Ghaouth et al., 2003). Similarly, UV-C exposure induced SAR in *A. thaliana* to subsequent infections by necrotrophic, hemi-biotrophic and biotrophic pathogens (*B. cinerea*, *P. syringae*, *Hyaloperonospora arabidopsidis*, respectively) (Shapiro and Zhang, 2001; Kunz *et al.*, 2008; Stefanato *et al.*, 2009). Recent studies also reported the increased production of plant defense response regulators such as JA and secondary messengers such as H_2O_2 in UV-C-treated tomato fruit and *A. thaliana* (Xu *et al.*, 2016; Scott *et al.*, 2018).

Pulsed light

Pulse Light (PL) technology is an emerging non-thermal technique used for the decontamination of surfaces of food or materials through the inactivation of microorganisms by short-duration and high-power pulses (Rowan, Valdramidis and Gómez-López, 2015). PL treatments with pulses up to 2 ms and at a maximum dose of 120 kJ m^{-2} are approved by the FDA for the decontamination of food (FDA-Food and Drugs Administration, 2015). Pulses are emitted in a broad-spectrum white light including wavelengths from UV to near infrared (200 - 1100 nm). They are generated by flash lamps filled with an inert-gas, generally xenon (Gómez-López et al. 2005). The major **advantages** of this technique are mainly the very short treatment times, the lower energy cost and expense of treatments compared to thermal technologies and the lack of residual compounds (Oms-Oliu, Martín-Belloso and Soliva-Fortuny, 2010).

The main **mechanism** of microbial inactivation by PL is attributed to the photochemical effect of UV-C on the DNA structure of bacteria, viruses, and other pathogens that prevent them from replicating and eventually leads to mutagenesis and death (Elmnasser *et al.*, 2007). Moreover, some microbicide effects are also attributed to the UV-A and UV-B fractions of the spectrum and to thermo-physical effects causing structural changes in microbial cell walls and membranes (Takeshita *et al.*, 2003; Gómez-López *et al.*, 2007; Cheigh *et al.*, 2012; B. Kramer, Wunderlich and Muranyi, 2017b). The **effectiveness** of PL treatment **depends** primarily **on** the food (surface topography, chemical composition) and type and amount of target microorganisms, as well as on the intensity and number of pulses (dose) (Gómez-López et al. 2005). Oils and proteins absorb UV while high roughness surfaces, overlapping of microorganisms at high population densities, cause shadows that prevent light from reaching everywhere, therefore reducing the dose and the microbicide effect (Gómez-López et al. 2005). On the other hand, low intensity may result in ineffectiveness

and too high intensity can result in negative photo-thermal effects, impairing color, texture and sensory characteristics in detriment to the quality of fresh-cut products (Oms-Oliu *et al.*, 2010; A. Ignat *et al.*, 2014). Factors such as distance from light source to target and propagation vehicle (air, water, fruit juice) also affect the level of energy than ultimately reaches the target (Gómez-López et al. 2007).

Wide ranges of PL doses and pulse frequencies have been assessed in **fresh-cut produce** showing differential **effectiveness** at reducing microbial populations and at maintaining or improving physical, nutritional and bioactive properties. PL treatments at 22.6 kJ m^{-2} increased β-carotene, poly-acetylene, and β-glucose content in fresh-cut carrot slices without affecting color (Aguiló-Aguayo *et al.*, 2017). High dose PL treatments (716 kJ m^{-2}) reduced initial counts of native microbiota (mesophilic and molds and yeasts) on fresh-cut apple by 0.8–0.9 log_{10} and kept them 1.2 log_{10} below the control after 7 d of refrigerated storage (Gómez *et al.*, 2012). Two-sided high frequency intense pulsed flashes for 45 or 180 s, equivalent to 675 and 2700 pulses of 70 kJ each, reduced mesophilic microorganisms in a range of 0.21 and 1.67 at 45 s/side, and between 0.56 and 2.04 at 180 s/side in minimally processed spinach, celeriac, green bell pepper, soybean sprouts, radicchio, carrot, iceberg lettuce and white cabbage (Gómez-López et al. 2005). PL (40, 60 or 80 kJ m^{-2}) showed no effect on initial psychrophilic bacteria in fresh-cut tomato slices, but the highest doses reduced yeasts and molds by 0.7 and 1.1 log_{10}, respectively. However, in that study firmness decreased due to the increased activity of pectinolytic enzymes throughout storage at 4 °C (Valdivia-Nájar, Martín-Belloso and Soliva-Fortuny, 2018). Low PL doses (10 kJ m^{-2}) reduced initial mesophilic native microbiota as well as inoculated *E. coli* and *L. innocua* by 2.5, 2.3 and 2.5 log_{10}, respectively in fresh-cut endive and by 1.7, 1.9 and 1.6, respectively in mung bean sprouts (B. Kramer, Wunderlich and Muranyi, 2017b). Similarly, PL (17.5 kJ m^{-2}) reduced the initial populations of *Lactobacillus brevis* and *L. innocua* in fresh-cut apple by 3.0

and 2.6 log_{10}, respectively, and kept native microbial populations below 1.70 log_{10} CFU cm^{-2} for 7 d at 4 °C, showing no improvement by increasing PL dose to up to 157.5 kJ m^{-2} (A. Ignat *et al.*, 2014).

Refrigeration

Refrigerated temperatures during processing, conservation, transport and retailing are crucial to preserve fresh-cut produce and minimize and/or inhibit the effects of wounding stress (Artés and Allende, 2014). Refrigerated reduce enzymatic activity as well as metabolic and respiration rates and ethylene production, delaying ripening and senescence (Rivera-López *et al.*, 2005; Zhan *et al.*, 2012; Fagundes, Carciofi and Monteiro, 2013). Storage temperatures just above freezing would be optimal for reducing metabolic rate. Nevertheless, temperature needs to be adequately adjusted in order to avoid damage such as chilling injury in sensitive commodities (Toivonen and Dell, 2002). Temperatures ranging from 0 to 5 °C are advisable to inhibit the respiration rise induced by processing and limit microbial growth in fresh-cut produce. However, many times these products are shipped and marketed at temperatures ranging from 5 to 10 °C (Morelli *et al.*, 2012; Kou *et al.*, 2014; Lundén *et al.*, 2014). To counteract this situation robust methods or combined strategies must be implemented to ensure safety and extend the shelf-life of fresh-cut products.

Modified atmosphere packaging

The modification of the normal composition of air (78% N_2; 21% O_2; 0.01% CO_2 and traces of noble gases) within packages headspace is a widely used conservation method for prolonging the shelf-life of fresh or minimally processed produce (Phillips, 1996). Two modalities of modified atmosphere are used: **controlled atmosphere storage**, where O_2 and CO_2 concentrations are continuously monitored and controlled throughout the storage period; and modified

atmosphere packaging, where the O_2 and CO_2 concentrations are modified initially, and change dynamically depending on the respiration rate of the commodity and the permeability of the film surrounding the produce (Erkan and Wang, 2006). **Modified Atmosphere Packaging (MAP)** can be carried by **active** modification through the displacement of gases in the package and their replacement with a desired mixture of gases; or by **passive or equilibrium** modification, established when the permeation rates for O_2 and CO_2 of the film matches the respiration rate of the packaged produce (Farber *et al.*, 2003). MAP reduces microbial growth as well as the metabolic process of the plant product, thereby reducing the rate of substrate depletion (Sandhya, 2010).

Both high (> 80 kPa) and low oxygen (< 8 kPa) active-MAP are alternatives that inhibit enzymatic activity and microbial growth, and prevent the moisture loss and the decay of fresh-cut fruits and vegetables (Lu and Toivonen, 2000; Chunyang *et al.*, 2010; Li *et al.*, 2012).

Super atmospheric O_2 can inhibit PPO activity via substrate or product inhibition (Lu and Toivonen, 2000). High O_2 may also inhibit anaerobic fermentation and activate the production of ROS which would damage vital cellular macromolecules thereby limiting the growth of both anaerobic and aerobic microorganisms (Day, 2003). High-oxygen atmosphere (> 90 kPa O_2) active-MAP reduced natural microbiota (mesophilic, yeasts and molds) and inhibited browning and respiration rate in fresh-cut egg-plant and mushrooms after 12 d at 4 °C (Li *et al.*, 2012). Similarly, 100 kPa O_2 reduced discoloration, browning degree, electrolyte leakage, and inhibited peroxidase activity in fresh-cut mushrooms after 8 d of refrigerated storage. However such conditions increased firmness and weight loss of the commodity as well (Liu *et al.*, 2010). High-O_2 (80 kPa O_2, 20 kPa CO_2) active-MAP using a high-barrier film also reduced the respiration rate and aerobic bacterial growth as well as weight-loss and total reducing-sugar decline, in fresh-cut onions for up to 5 d at room temperature (Chunyang *et al.*, 2010). High O_2 (50 or 90 kPa O_2) active-MAP showed anti-

browning and anti-microbial effect on fresh-cut lettuce stored for 6 d at 7 °C (López-gálvez *et al.*, 2015). Low-oxygen (4 kPa O_2, 5 kPa CO_2) and high-oxygen (100 kPa O_2) active-MAP delayed firmness-loss, prevented the decline in nutritional quality (soluble solid contents, reducing sugars, and ascorbic acid), and reduced microbial growth in pineapple slices stored for 9 d at 10 °C (Pan, Zhu and Li, 2015). However, low-O_2 was better at preserving chemical and microbiological quality when compared to high-O_2, therefore they were recommended as more suitable for that commodity. Similar low-oxygen active-MAP (4 kPa O_2, 10 kPa CO_2) preserved color, reduced translucency, respiration rate, and microbial populations in fresh-cut 'Cantaloupe' melon cubes for 9 d at 5 °C (Bai *et al.*, 2001). Alternatively, the pretreatment of whole apples with 1 or 100 kPa O_2 during 5, 12 or 19 d before processing resulted in the inhibition of cut-surface browning, flesh softening, and off-flavors production in 'Spartan' apple slices for over 14 d of storage at 1°C; being high-O_2 preferable than low-O_2 (Lu and Toivonen, 2000).

Low O_2 levels combined with relatively **high CO_2 levels** (10 kPa O_2 and 10 kPa CO_2) obtained using active-MAP showed good results at preserving the overall quality and organoleptic and physical attributes of fresh-cut 'Big Top' nectarines (Cozzolino *et al.*, 2018). Similar gases composition can be achieved by passive-MAP using semi-permeable films (O_2 transmission < 2mL in 24 h at 23 °C) allowing the preservation of quality parameters in fresh-cut apples (Gormley, Brunton and Butler, 2016). For fresh-cut fruit, due to their higher respiration rates, the time required to reach the equilibrium can represent a significant percentage of their short useful life (7–9 d). Therefore, the use of microperforated films (40 < 200 µm perforation diameter) with higher permeability quotient (~ 1) allowing simultaneously high-O_2 and CO_2 levels (e.g. 5-15 kPa O_2; 5-15 kPa CO_2) is appropriate (González-Buesa *et al.*, 2009). This technology is also suitable for produce with high respiration rates such as broccoli, cauliflower, carrot, mushrooms and spinach (Fernández-León *et al.*, 2013; Ayhan, 2017).

Microperforated films allowing 14 kPa O_2 and 7 kPa CO_2 levels have been used to extend the storability of fresh-cut apples, increasing their crispness and lowering ethylene production (Cliff *et al.*, 2010).

The effectiveness of MAP relays on the dynamic interaction between the product, the headspace atmosphere in the package, and the package itself (González-Buesa *et al.*, 2009). Several **variables** involved in that interaction are **intrinsic to the plant product**, some of them are conditioned by the processing process as previously discussed, but others by the respiration rate as a function of the temperature, type and physiological stage of the commodity, the optimum levels and the limits of O_2 and CO_2 that it tolerates depending on its internal air spaces (Sandhya, 2010). An additional number of variables are **related to the package** including the permeability of the material as a function of temperature, gas exchange area, free volume, weight of the product and relative humidity (Ayhan, 2017).

Metabolic processes such as respiration and ripening are accelerated by 2 to 3-fold for every 10 °C rise in temperature (González-Buesa *et al.*, 2009). Film permeability also increases with temperature rises, with CO_2 permeability responding more than O_2 permeability (Soltani, 2015). Low relative humidity can increase transpiration damage leading to desiccation and increased respiration (Fonseca, Oliveira and Brecht, 2002). Therefore, designing a proper MAP is a key step that should take into account the factors previously mentioned and should be adjusted to each commodity since incorrectly designed MAP may be ineffective or even shorten the shelf-life of the product. Mathematical modelling is an useful tool for selecting or designing an effective MAP and several models have been developed for fresh and fresh-cut produce (Ucherek, 2001; Fonseca, Oliveira and Brecht, 2002; Rodov *et al.*, 2007; González-Buesa *et al.*, 2009; Belay, Caleb and Opara, 2016).

1.2.4 INTEGRATED PRESERVATION STRATEGIES

A **hurdle technology** comprises a combination of preservation methods which can sequentially or synergistically result in better ensuring the safety of the food product than a single method strategy (Leistner, 2000). Combining MAP, refrigerated storage, and antioxidant treatments is one of the most extended hurdle technologies currently used in fresh-cut industry. The synergistic effect of antioxidant dipping (0.5 % ascorbic acid dip), high O_2 atmosphere (> 90%) MAP and chilling storage (4 °C) was effective at preserving the physical and biochemical quality as well as improving the microbiological quality of fresh-cut potato (Limbo and Piergiovanni, 2007) and egg-plant (Li *et al.*, 2012). The combination of active-MAP (100% N_2) with AA and $CaCl_2$-dipping inhibited most of the changes in color and texture-losses of fresh-cut apples regardless of the maturity stage for more than 30 d at refrigerated storage (Soliva-Fortuny, Oms-Oliu and Martìn-Belloso, 2002). The application of antioxidant mixtures such as 0.75% N-acetylcysteine + 0.75% glutathione combined to low-O_2 atmosphere MAP prevented browning, reduced ethylene production and maintained vitamin C, chlorogenic acid, and TAC in fresh-cut pear for 14 d at 4 °C (Llano *et al.*, 2016).

Combined decontamination strategies have also been assessed for fresh-cut processing showing in some cases **synergistic antimicrobial effects** without significantly affecting physicochemical properties. The combination of hot water treatments during 90 and 120 s followed by peroxyacetic dips, were effective to control microbial growth extending the shelf-life of fresh-cut 'Galia' melon up to 10 d at refrigerated storage (Silveira *et al.*, 2011). The combination of 1.5 % H_2O_2 at 50 °C and UV-C irradiation (3.78 kJ m^{-2}) had higher effect than the separate methods at reducing *Salmonella* spp. *P. fluorescens*, and *E. coli* on fresh-cut vegetables including iceberg lettuce, spinach leaves, onions, cauliflower, and broccoli, achieving reductions by 2 - 4 log_{10}, 3 - 4 log_{10} or 2.1 – 4.9 log_{10}, respectively, showing the highest effectiveness in leafy vegetables (Hadjok,

Mittal and Warriner, 2008). Combined PL + malic acid (20 pulses at 4 kJ m^{-2}/pulse + 2 % malic acid) treatments or PL + malic acid + 2 % alginate coating additively reduced *L. innocua* counts by 4.5 and 3.9 log_{10}, respectively, on fresh-cut mango and kept its populations below 6 log CFU g^{-1} over 14 days of storage, showing an increase in firmness in the case of the alginate-coated samples (Salinas-Roca *et al.*, 2016).

The antimicrobial effects of peroxyacetic acid and neutral electrolyzed water were improved when they were combined with UV-C (7.5 kJ m^{-2}), decreasing *E. coli* and *S. enteritidis* counts by about 3 log_{10} in fresh-cut Bimi® broccoli (Martínez-Hernández, Navarro-Rico, *et al.*, 2015). The combination of the antagonistic yeast (5 x 10^7 CFU $ml^{-1)}$ *Candida guilliermondii* and (5 kJ m^{-2}) UV-C in pear resulted in lower disease incidence and severity of infections by *P. expansum* and *B. cinerea* than each method applied alone (Xu and Du, 2012). Pre-treatment with UV-C elicited plant defense responses such as induced chitinase, β-1,3-glucanase, CAT, and POX activities without affecting the growth of *C. guilliermondii* on pear fruit wounds. However, sometimes physical and chemical or biological strategies when used combined interfere with each other instead of having a synergistic effect. For example, the combination of high PL 71.6 J cm^{-2} doses with antibrowning solutions (1% ascorbic acid, 0.1% calcium chloride) in fresh-cut apples showed similar initial reductions of native microbiota by 0.9 log_{10} but decreased the inhibitory effect throughout 7 d of storage, compared to PL treatments alone (Gómez *et al.*, 2012).

Overall, the need for ensuring safe minimally processed products while maintaining their physicochemical quality and nutritional properties has led to the development of novel preservation techniques like those investigated in the present work.

OBJECTIVES

GENERAL OBJECTIVE

To develop novel preservation strategies, alternative to chlorine, for improving the microbiological and physicochemical quality of minimally processed fruit and vegetables.

SPECIFIC OBJECTIVES

In order to ensure the safety of different food matrices meeting specific requirements according to their characteristics and ways of consumption, several preservation strategies were investigated: i) biopreservation, which was applied to fresh-cut fruit; ii) non-thermal physical methods, alone or combined with chemical methods, which were applied to minimally processed vegetables including those that are sold ready-to-use but are intended to be eaten cooked and those that are intended to be eaten raw; iii) preservation strategies integrating physical, chemical, and biological methods, which were evaluated for ready-to-eat vegetables which usually have a high microbial load. The following specific objectives were set:

1 To elucidate the mode of action of biopreservation using the bacterium *P. graminis* CPA-7 for the control of the foodborne pathogens *Salmonella enterica* subsp. *enterica, Escherichia coli* O157:H7, and *Listeria monocytogenes,* both *in vitro* and *in vivo*.

1.1 To evaluate the niche overlapping between CPA-7 and the foodborne pathogens *S. enterica* subsp. *enterica* ser. Enteritidis, *E. coli* O157:H7, and *L. monocytogenes,* regarding the *in vitro* utilization of individual sources of carbon and nitrogen.

1.2 To evaluate the *in vitro* production of siderophores, extracellular lytic enzymes, and biosurfactants by CPA-7.

1.3 To evaluate ability of CPA-7 to form biofilms on inert surfaces when grown in liquid medium.

1.4 To evaluate the antimicrobial effect of the non-volatile metabolites produced by CPA-7, *in vitro* and *in vivo*, on the growth both *in vitro* and in *vivo*, of foodborne pathogens.

1.5 To evaluate the effect of CPA-7 on the activation of enzymatic and non-enzymatic defense-response antioxidant mechanisms in fresh-cut apple upon air and MAP conditions.

1.6 To evaluate the effect of CPA-7 in interaction with the antioxidant and the storage system on the accumulation of ethylene and the respiration in fresh-cut apple at different maturities stages.

1.7 To evaluate the effect of the prior interaction on fresh-cut pear of CPA-7 and *L. monocytogenes* or *S. enterica* subsp. *enterica* ser. *Enteritidis* on their colonization capacities, using the human epithelium cellular model Caco-2.

2 To evaluate the effectiveness of a water-assisted ultraviolet light technology (WUV) for the decontamination of the native microbiota and inoculated *L. innocua* on fresh-cut broccoli.

2.1 To optimize the WUV conditions alone or in combination with peroxyacetic acid for the control of the native microorganisms in fresh-cut broccoli without significantly affecting its physicochemical quality.

2.2 To evaluate the effectiveness of WUV, alone or combined with peroxyacetic acid, for the control of *L. innocua* on fresh-cut broccoli.

2.3 To evaluate the effect of WUV alone or in combination with peroxyacetic on the physicochemical and bioactive properties of fresh-cut broccoli.

2.4 To determine the effect of the optimal WUV treatment for the control of *L. innocua* on fresh-cut broccoli throughout storage and after microwave processing.

3 To evaluate the effectiveness of pulsed light (PL) for the decontamination of native microbiota and inoculated *L. innocua* in fresh-cut broccoli.

   3.1 To optimize PL conditions for the control of native microbiota in fresh-cut broccoli throughout storage, without significantly affecting its physicochemical quality.

   3.2 To evaluate the effectiveness of the selected PL treatment for the control of yeasts and molds and *L. innocua* populations on fresh-cut broccoli throughout storage.

   3.3 To determine the effect of the selected PL treatment on the physicochemical quality and the bioactive properties of fresh-cut broccoli.

4 To evaluate the effectiveness of the combination of *P. graminis* CPA-7, WUV and peroxyacetic acid for the decontamination of inoculated *L. monocytogenes* and *S. enterica* subsp. *enterica* in fresh-cut ready-to-eat leafy greens.

   4.1 To optimize WUV conditions for the treatment of fresh-cut 'Iceberg' lettuce and baby spinach leaves without significantly affecting their physicochemical quality.

   4.2 To optimize WUV in combination with peroxyacetic acid for the decontamination of *L. monocytogenes* and *S. enterica* subsp. *enterica* on fresh-cut 'Iceberg' lettuce and baby spinach leaves.

   4.3 To evaluate the synergistic effect of the selected combination of WUV and peroxyacetic acid with *P. graminis* CPA-7 for the control of *L. monocytogenes* and *S. enterica* subsp. *enterica* on fresh-cut 'Iceberg' lettuce and spinach sprouts throughout storage.

OBJECTIUS

OBJECTIU GENERAL

Desenvolupar noves estratègies de higienització, alternatives al clor, per millorar la qualitat microbiològica i fisicoquímica de les fruites i hortalisses mínimament processades.

OBJECTIUS ESPECÍFICS

Per a garantir la seguretat de les diferents matrius alimentàries a la vegada que complir els requisits específics d'acord amb les seves característiques i formes de consum, s'han investigat diverses estratègies de higienització : i) la biopreservació, que es va aplicar a fruita mínimament processada; ii) els mètodes físics no tèrmics, sols o combinats amb mètodes químics, que s'apliquen a les hortalisses mínimament processades, incloses les que normalment es consumeixen cuites i les que es consumeixen crues ; iii) estratègies integrades físic-químic-biològiques , que es van avaluar per a vegetals llests per menjar que generalment tenen una elevada càrrega microbiana. Es van establir els següents objectius específics:

1 Dilucidar el mode d'acció de la biopreservació del bacteri *Pseudomonas graminis* CPA-7 per al control dels patògens transmesos pels aliments *Salmonella enterica* subsp. *enterica*, *Escherichia coli* O157: H7, i *Listeria monocytogenes*, tant *in vitro* com *in vivo*.

1.1 Avaluar la superposició de nínxol ecològic entre la soca de *P.graminis* CPA-7 i els patògens transmesos per les aliments *S. enterica* subsp. *enterica*, *E. coli* O157: H7 i *L. monocytogenes*, pel que fa a la utilització *in vitro* de fonts individuals de carboni i nitrogen.

1.2 Avaluar la producció *in vitro* de sideròfors, enzims lítics extracel·lulars i biosurfactants per la soca de *P. graminis* CPA-7.

1.3 Avaluar la capacitat de la soca de *P. graminis* CPA-7 per formar biofilms en superfícies inertes quan creix en medi líquid.

1.4 Avaluar l'efecte antimicrobià dels metabòlits no volàtils produïts per la soca *P. graminis* CPA-7, *in vitro* i *in vivo*, sobre el creixement tant *in vitro* com *in vivo* de patògens de transmissió alimentaria.

1.5 Avaluar l'efecte de la soca de *P. graminis* CPA-7 en l'activació de mecanismes antioxidants enzimàtics i no enzimàtics de resposta en poma mínimament processada en condicions d'aire o en atmosfera modificada passiva.

1.6 Avaluar l'efecte de la soca de *P. graminis* CPA-7 en la interacció amb l'antioxidant i el sistema d'emmagatzematge sobre l'acumulació d'etilè i la respiració en pomes tallades en diferents estats de maduració.

1.7 Avaluar l'efecte de la interacció prèvia en pera mínimament processada de la soca de *P. graminis* CPA-7 i *L. monocytogenes* o *S. enterica* subsp. *enterica* ser. Enteritidis en relació a les seves capacitats de colonització, utilitzant el model cel·lular de l'epiteli humà Caco-2.

2 Avaluar l'efectivitat de la llum ultraviolada en aigua per a la descontaminació de *L. innocua* i de la microbiota epifítica en bròquil mínimament processat.

2.0 Optimitzar les condicions de la llum ultraviolada aplicada en aigua o amb l'àcid peroxiacètic per al control dels microorganismes aerobis totals epifítics del bròquil mínimament processat sense afectar significativament la seva qualitat fisicoquímica.

2.1 Avaluar l'efectivitat de la llum ultraviolada en aigua o amb l'àcid peroxiacètic, per al control de *L. innocua* inoculada en el bròquil mínimament processat.

2.2 Avaluar l'efecte de la llum ultraviolada en aigua o amb l'àcid peroxoacètic en relació a les propietats físic-químiques i bioactives del bròquil mínimament processat.

2.3 Determinar l'efecte del tractament òptim de la llum ultraviolada per al control de *L. innocua* inoculada en bròquil mínimament processat durant l'emmagatzematge i després de la cocció al microones.

3 Avaluar l'efectivitat de la llum polsada per a la descontaminació de *L. innocua* i de la microbiota epifítica en bròquil mínimament processat.

3.1 Optimitzar les condicions de la llum polsada (PL) per al control de la microbiota epifítica en bròquil mínimament processat durant l'emmagatzematge, sense afectar significativament la seva qualitat fisicoquímica.

3.2 Avaluar l'efectivitat del tractament seleccionat de llum polsada per al control de llevats i floridures epifítiques i de les poblacions de *L. innocua* inoculada en bròquil mínimament processat durant l'emmagatzematge.

3.3 Determinar l'efecte del tractament de llum polsada (PL) seleccionat en relació a la qualitat fisicoquímica i les propietats bioactives del bròquil mínimament processat.

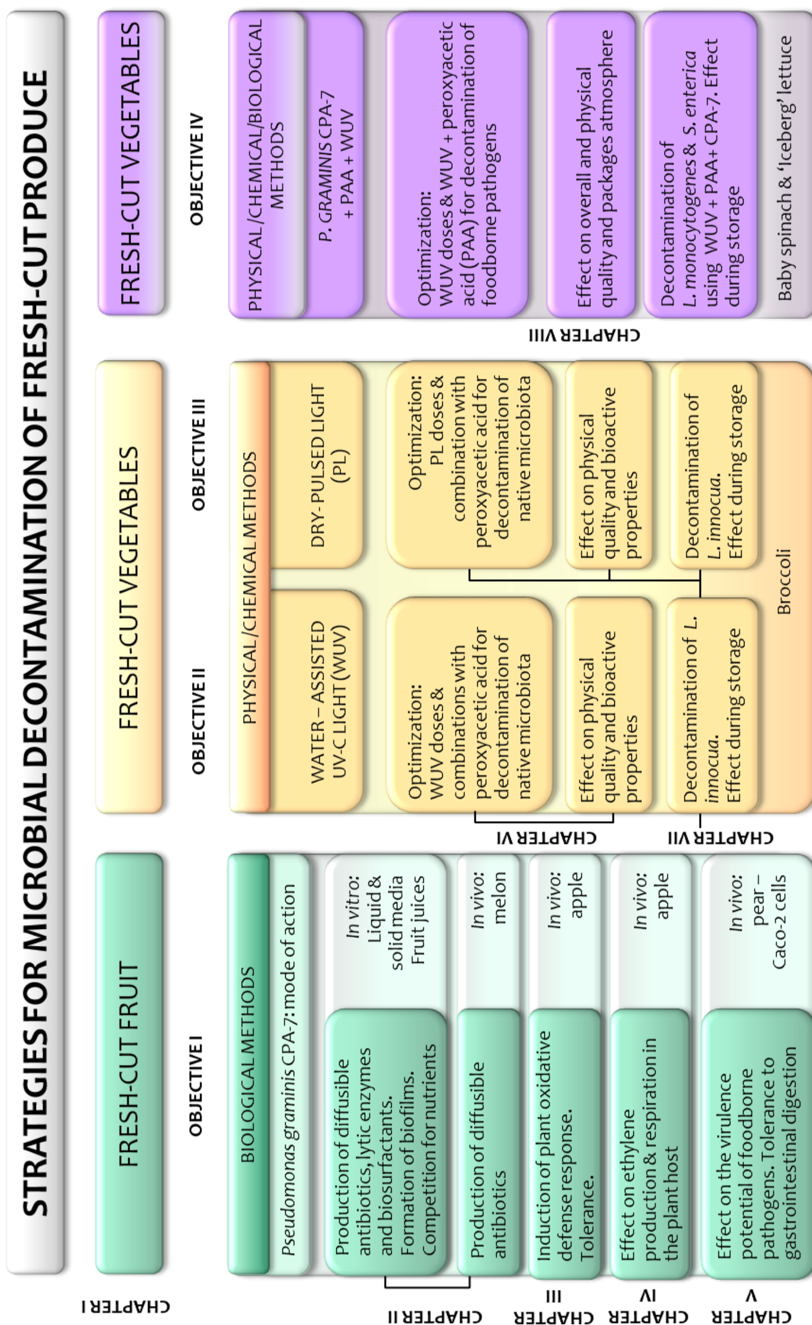
4 Avaluar l'efectivitat de la combinació de la llum ultraviolada, l'àcid peroxiacètic i la soca de *P. graminis* CPA-7, per a la descontaminació de *L. monocytogenes* i *S. enterica* subsp. *enterica* inoculades en hortalisses de fulla verda mínimament processades.

4.1 Optimitzar les condicions de la llum ultraviolada aplicada en aigua per al tractament de enciam "Iceberg" i brots d'espinacs mínimament processats sense afectar significativament la seva qualitat fisicoquímica.

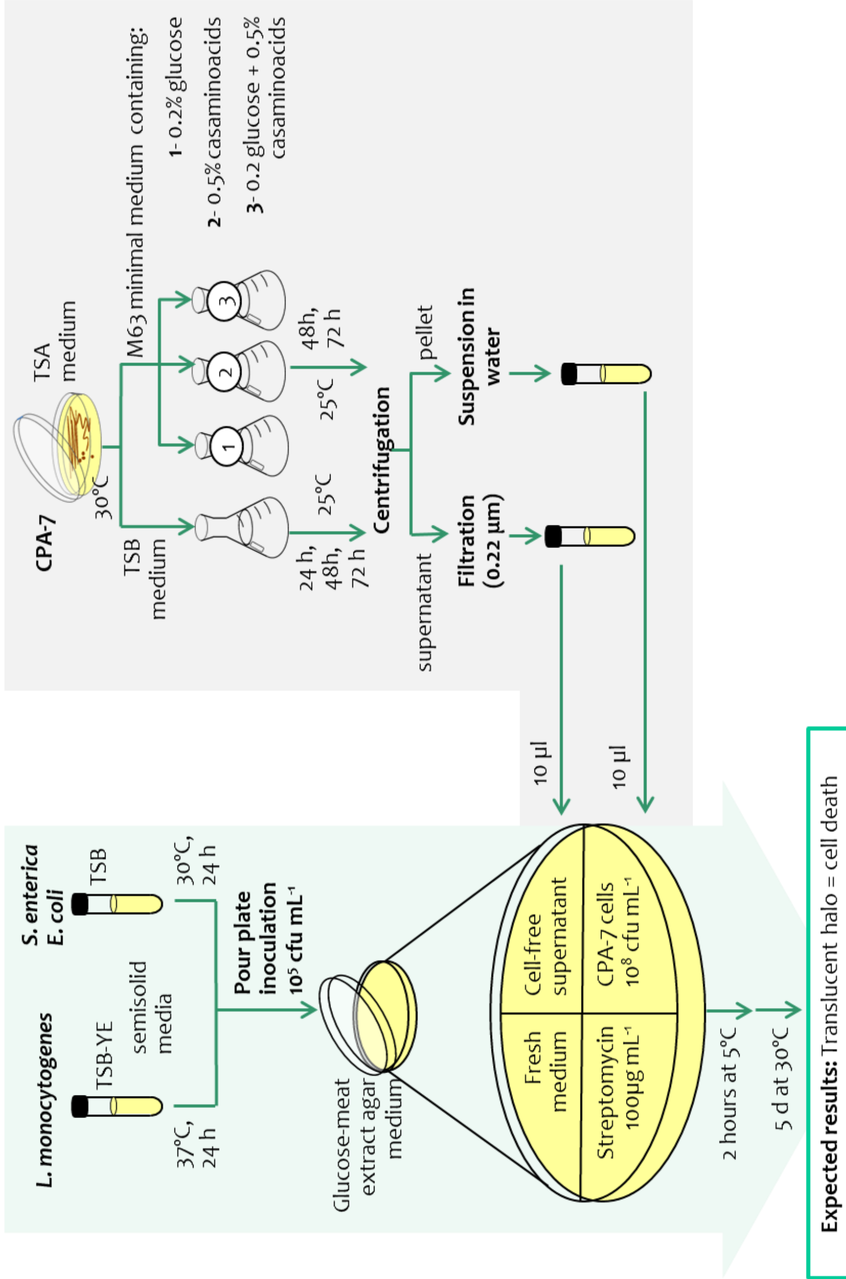
4.2 Avaluar la efectivitat del tractament de la llum ultraviolada en aigua o en l'àcid peroxiacètic per a la descontaminació de *L. monocytogenes* i *S. enterica* subsp. *enterica* inoculades en enciam "Iceberg" i en brots d'espinacs durant l'emmagatzematge

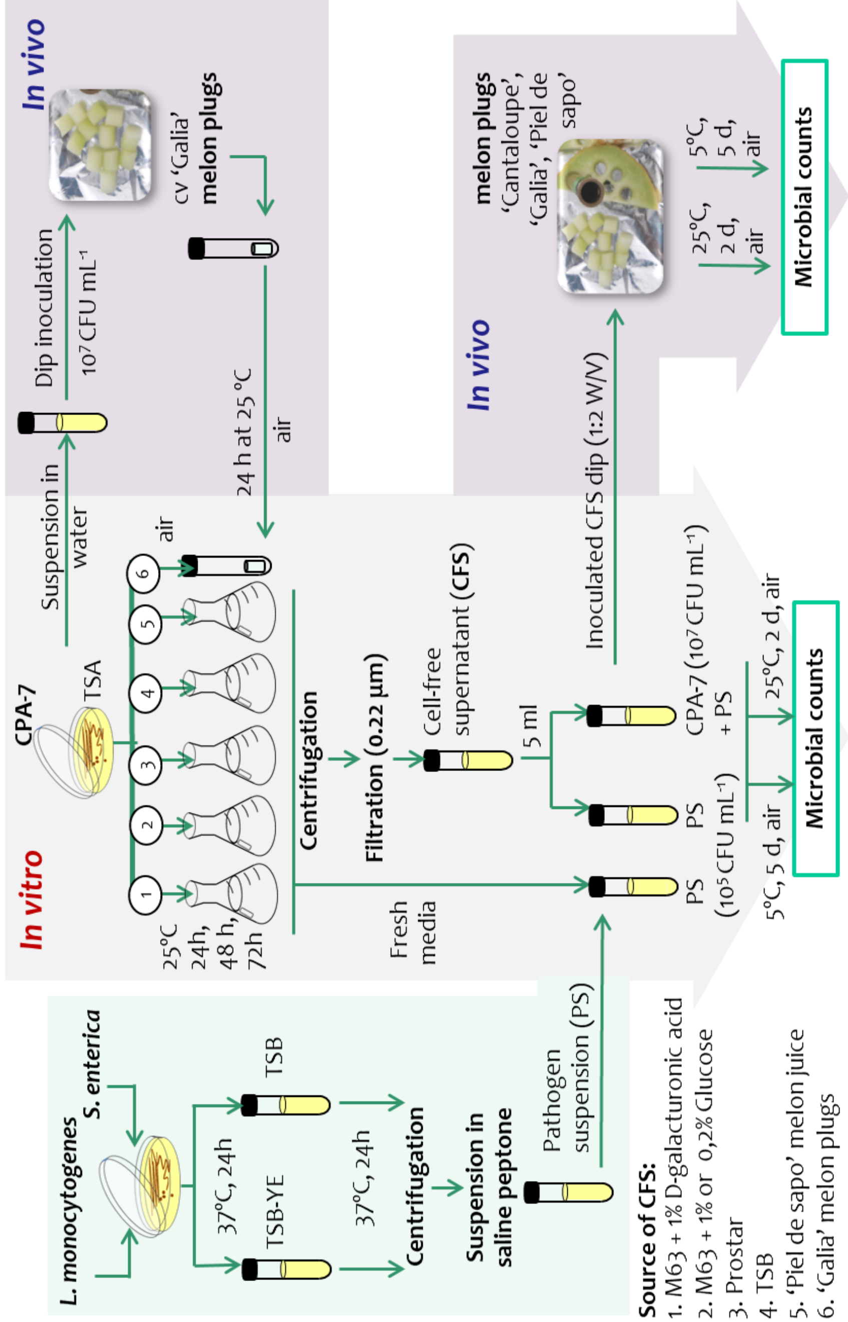
4.3 Avaluar l'efecte sinèrgic del pretractament amb una combinació de llum ultraviolada i àcid peroxiacètic i posterior inoculació amb la soca de *P. graminis* CPA-7 per al control de *L. monocytogenes* i *S. enterica* inoculats en enciam "Iceberg" i en brots d'espinacs durant l'emmagatzematge.

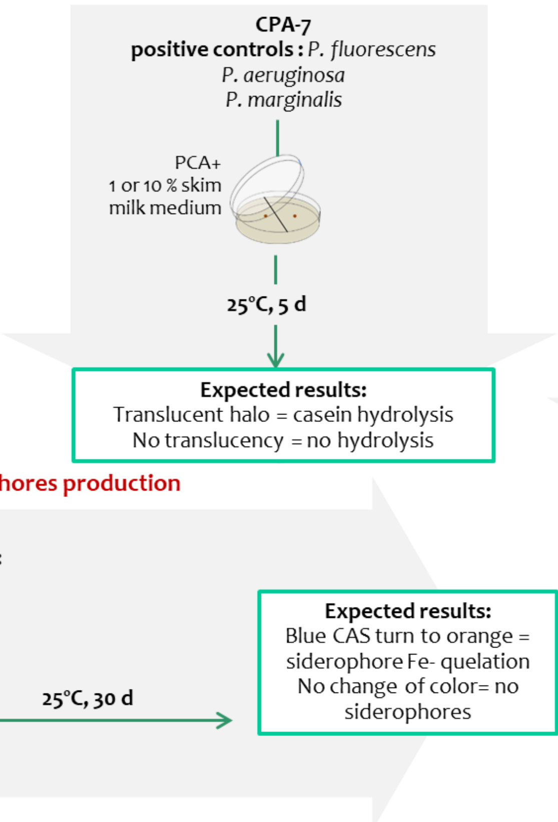
WORK SCHEDULE



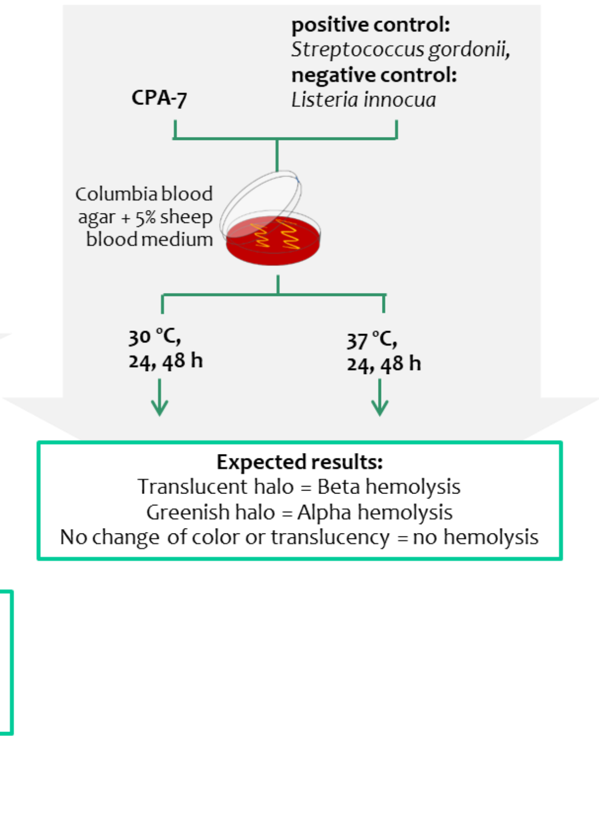
EXPERIMENTAL SETUP



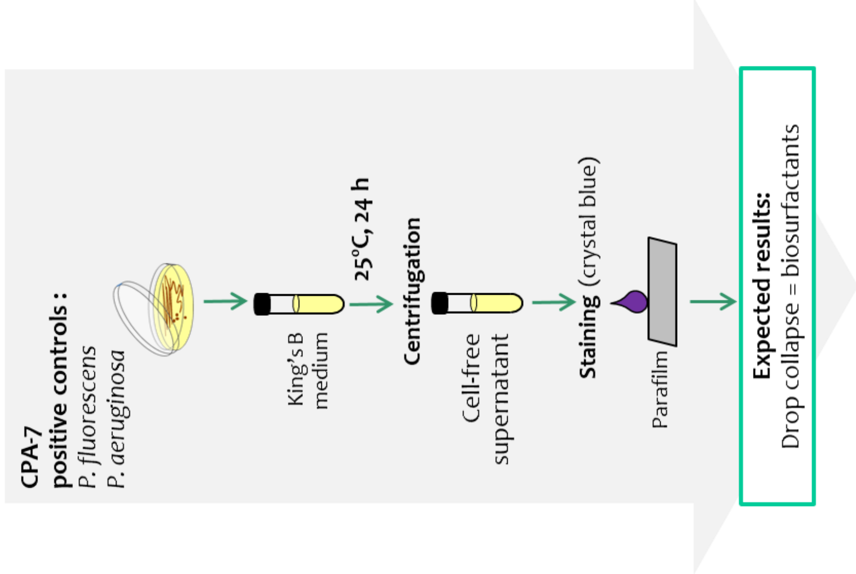

Production of diffusible antibiotics: effect in liquid media and melon juice and on melon plugs
In vitro
In vivo
L. monocytogenes
S. enterica
37ºC, 24h
TSB-YE
TSB
37ºC, 24h
Centrifugation
Suspension in saline peptone
Pathogen suspension (PS)
CPA-7
TSA
Suspension in water
Dip inoculation
10^7 CFU mL^-1
cv 'Galia' melon plugs
24 h at 25 ºC
air
25ºC
24h, 48 h, 72h
air
Centrifugation
Filtration (0.22 µm)
Cell-free supernatant (CFS)
Fresh media
5 ml
PS (10^5 CFU mL^-1)
PS
CPA-7 (10^7 CFU mL^-1) + PS
5ºC, 5 d, air
25ºC, 2 d, air
Microbial counts
Inoculated CFS dip (1:2 W/V)
melon plugs 'Cantaloupe', 'Galia', 'Piel de sapo'
25ºC, 2 d, air
5ºC, 5 d, air
Microbial counts
Source of CFS:
1. M63 + 1% D-galacturonic acid
2. M63 + 1% or 0,2% Glucose
3. Prostar
4. TSB
5. 'Piel de sapo' melon juice
6. 'Galia' melon plugs

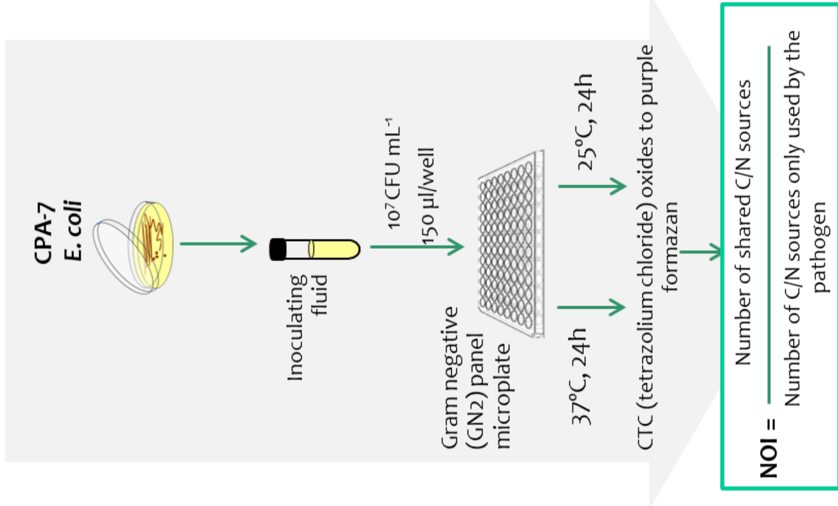


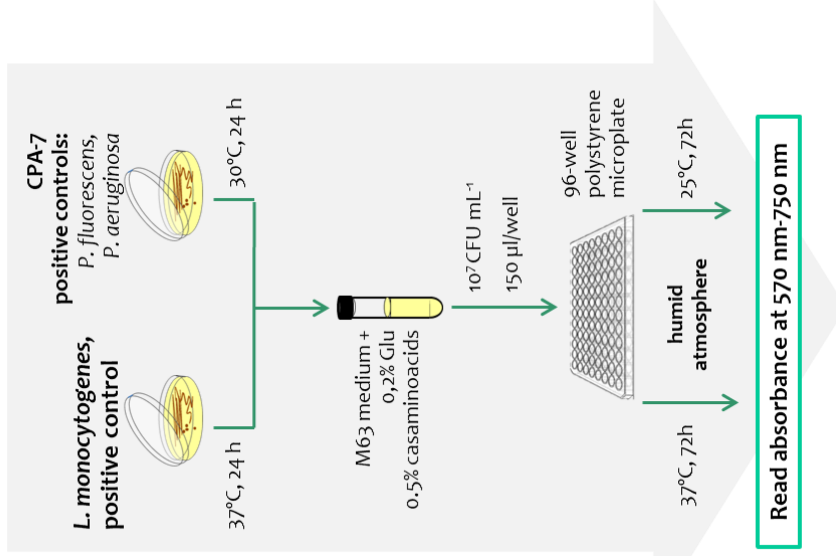
In vitro proteolytic activity
CPA-7
positive controls : P. fluorescens
P. aeruginosa
P. marginalis
PCA+
1 or 10 % skim
milk medium
25°C, 5 d
Expected results:
Translucent halo = casein hydrolysis
No translucency = no hydrolysis
In vitro siderophores production
CPA-7
positive controls :
P. fluorescens
P. aeruginosa
P. marginalis
CAS agar – TSA or
CAS-agar - BHI
25°C, 30 d
Expected results:
Blue CAS turn to orange =
siderophore Fe- quelation
No change of color= no
siderophores

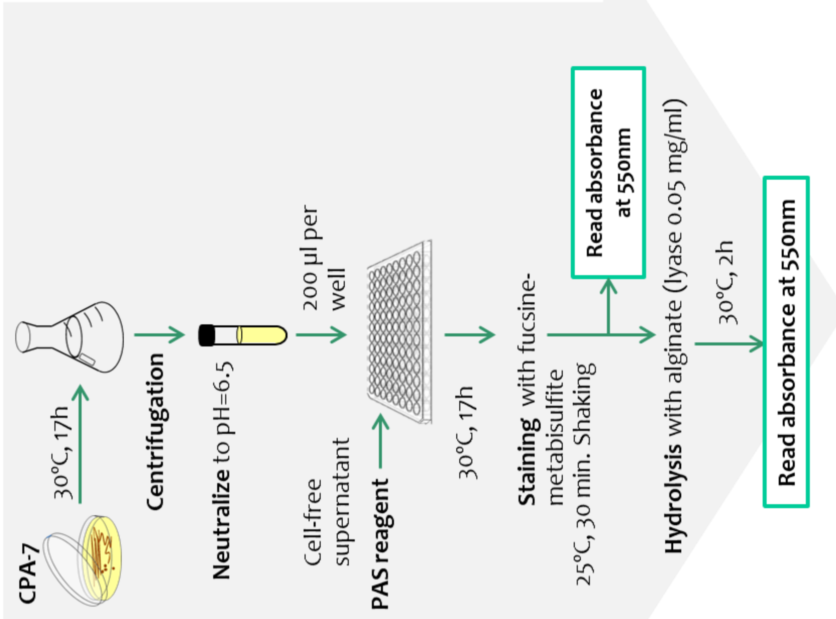


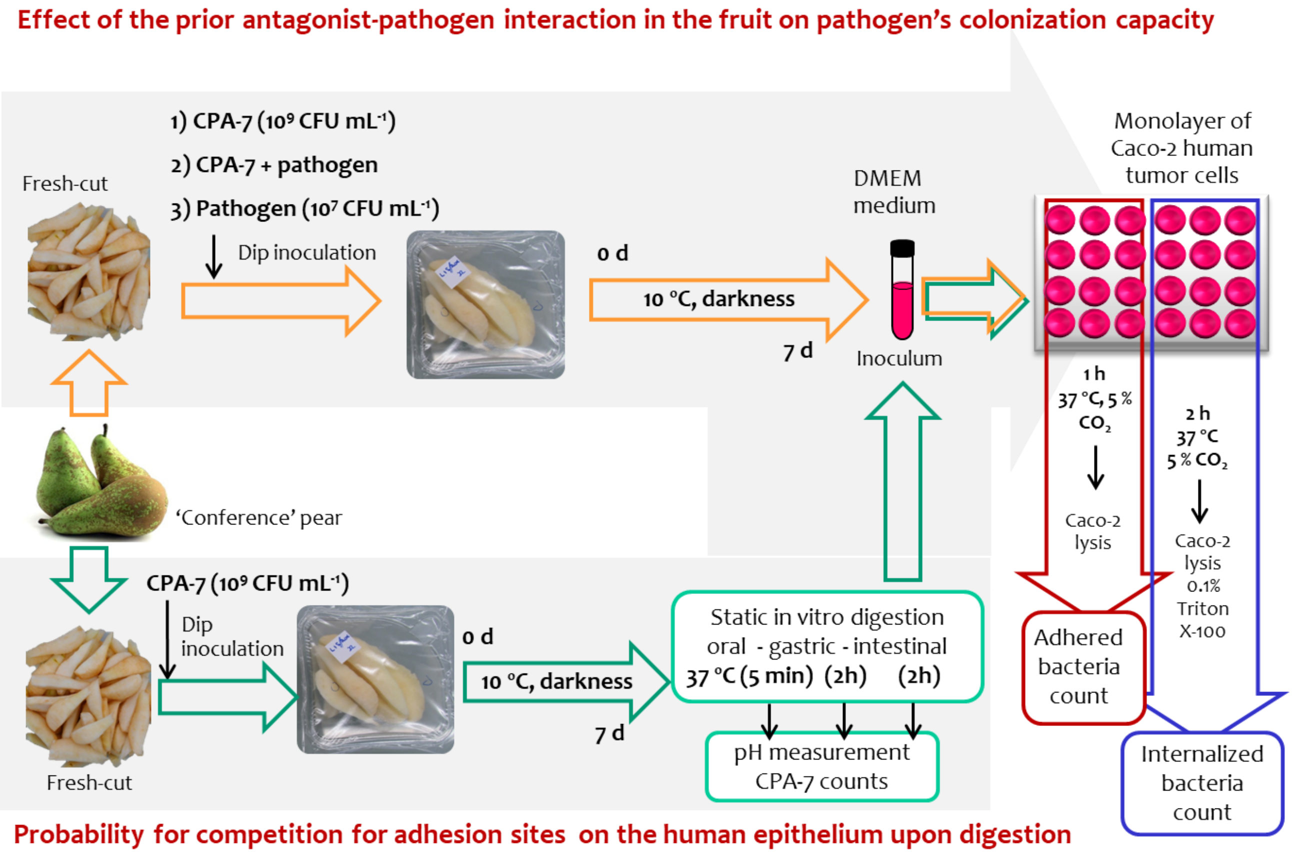
In vitro hemolytic activity
positive control:
Streptococcus gordonii,
negative control:
Listeria innocua
CPA-7
Columbia blood
agar + 5% sheep
blood medium
30 °C,
24, 48 h
37 °C,
24, 48 h
Expected results:
Translucent halo = Beta hemolysis
Greenish halo = Alpha hemolysis
No change of color or translucency = no hemolysis


In vitro biosurfactants production
CPA-7
positive controls :
P. fluorescens
P. aeruginosa
King's B medium
25°C, 24 h
Centrifugation
Cell-free supernatant
Staining (crystal blue)
Parafilm
Expected results:
Drop collapse = biosurfactants

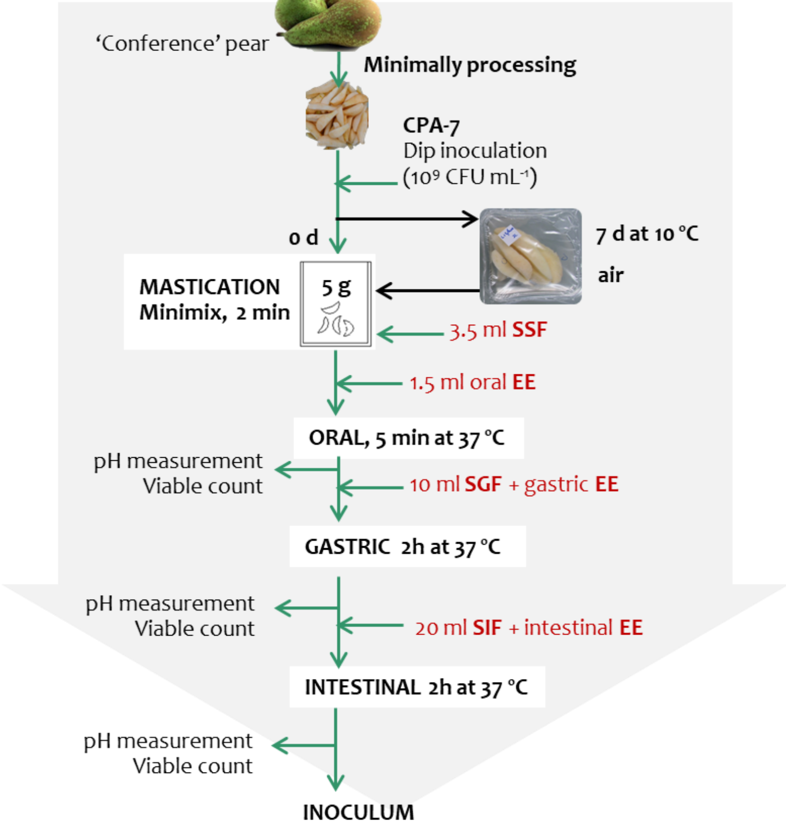

In vitro C/N single source use
CPA-7
E. coli
Inoculating fluid
10⁷ CFU mL⁻¹
150 µl/well
Gram negative (GN2) panel microplate
37°C, 24h
25°C, 24h
CTC (tetrazolium chloride) oxides to purple formazan
NOI =
Number of shared C/N sources
Number of C/N sources only used by the pathogen


In vitro biofilms formation
L. monocytogenes, positive control
CPA-7
positive controls:
P. fluorescens,
P. aeruginosa
37ºC, 24 h
30ºC, 24 h
M63 medium +
0,2% Glu
0.5% casaminoacids
10⁷ CFU mL⁻¹
150 µl/well
96-well polystyrene microplate
humid atmosphere
37ºC, 72h
25ºC, 72h
Read absorbance at 570 nm-750 nm


In vitro alginate production
CPA-7
30ºC, 17h
Centrifugation
Neutralize to pH=6.5
200 µl per well
Cell-free supernatant
PAS reagent
30ºC, 17h
Staining with fucsine-metabisulfite
25ºC, 30 min. Shaking
Read absorbance at 550nm
Hydrolysis with alginate (lyase 0.05 mg/ml)
30ºC, 2h
Read absorbance at 550nm


Effect of the prior antagonist-pathogen interaction in the fruit on pathogen's colonization capacity
Fresh-cut
1) CPA-7 (10⁹ CFU mL⁻¹)
2) CPA-7 + pathogen
3) Pathogen (10⁷ CFU mL⁻¹)
Dip inoculation
0 d
10 °C, darkness
7 d
DMEM medium
Inoculum
Monolayer of Caco-2 human tumor cells
1 h
37 °C, 5 % CO₂
Caco-2 lysis
Adhered bacteria count
2 h
37 °C
5 % CO₂
Caco-2 lysis
0.1% Triton X-100
Internalized bacteria count
'Conference' pear
CPA-7 (10⁹ CFU mL⁻¹)
Dip inoculation
Fresh-cut
0 d
10 °C, darkness
7 d
Static in vitro digestion
oral - gastric - intestinal
37 °C (5 min) (2h) (2h)
pH measurement
CPA-7 counts
Probability for competition for adhesion sites on the human epithelium upon digestion

In vitro static gastrointestinal digestion

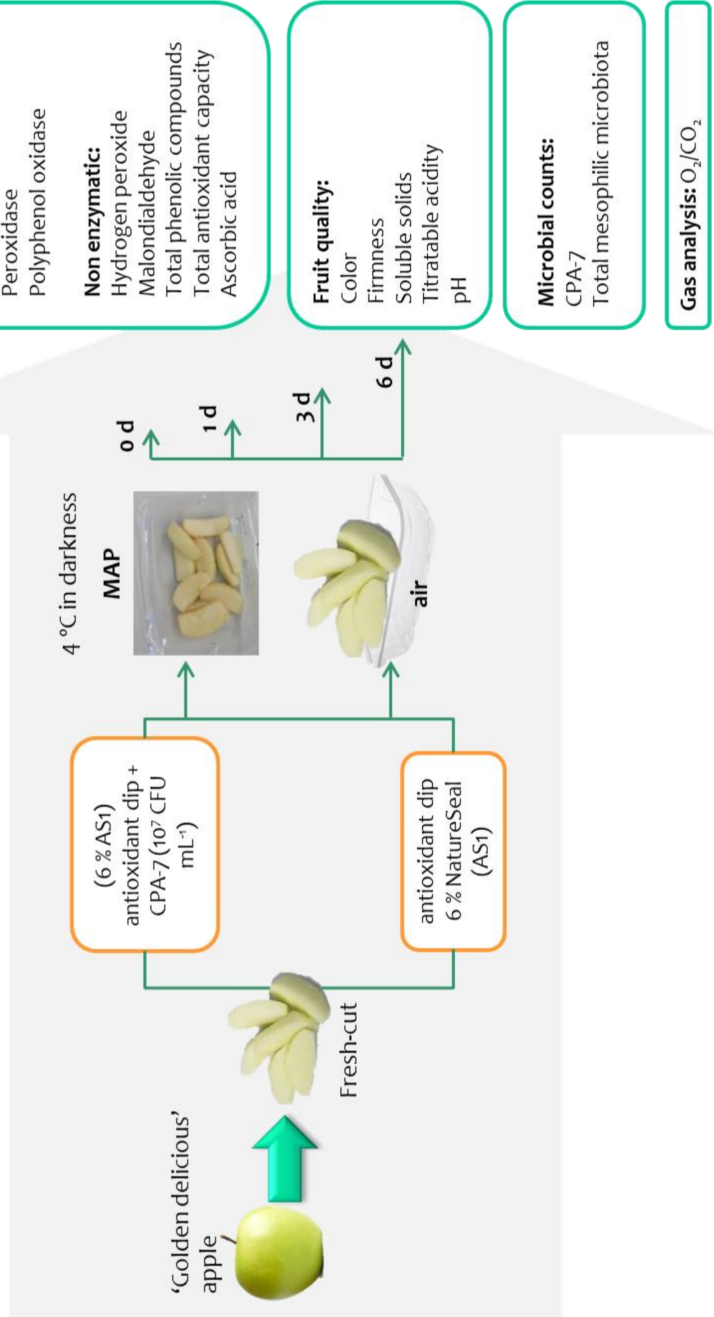


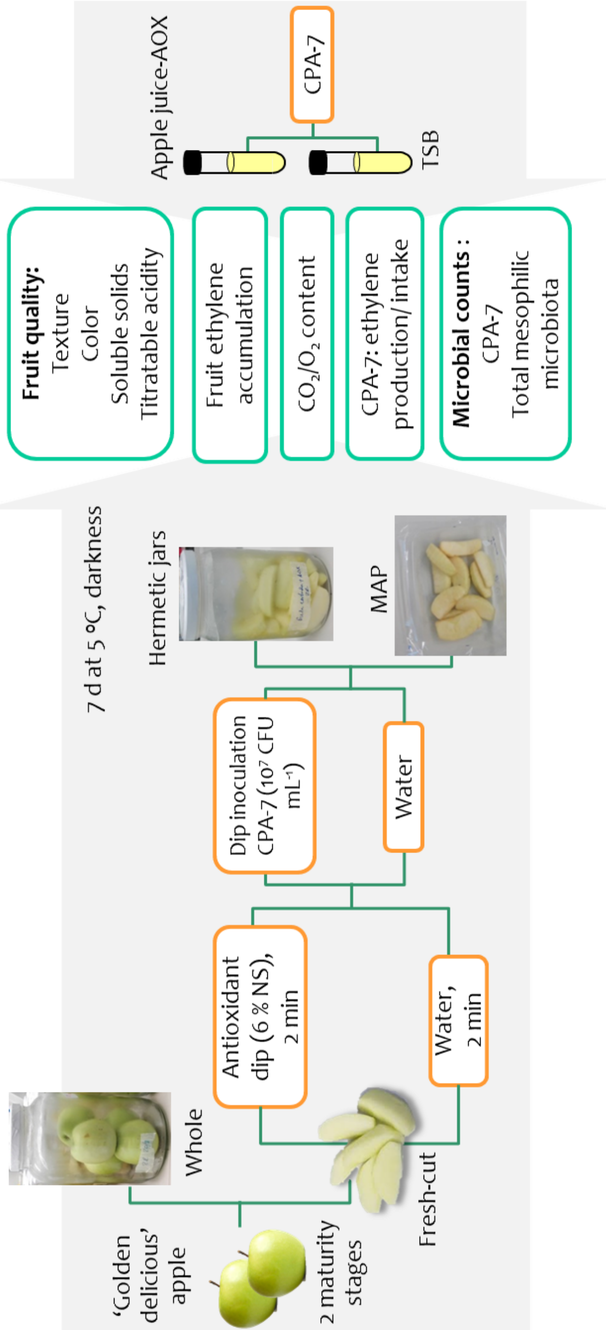
Electrolytes concentrations according to Minekus et al., 2014. Simulated salivary fluid (SSF), Simulated gastric fluid (SGF), Simulated intestinal fluid (SIF).

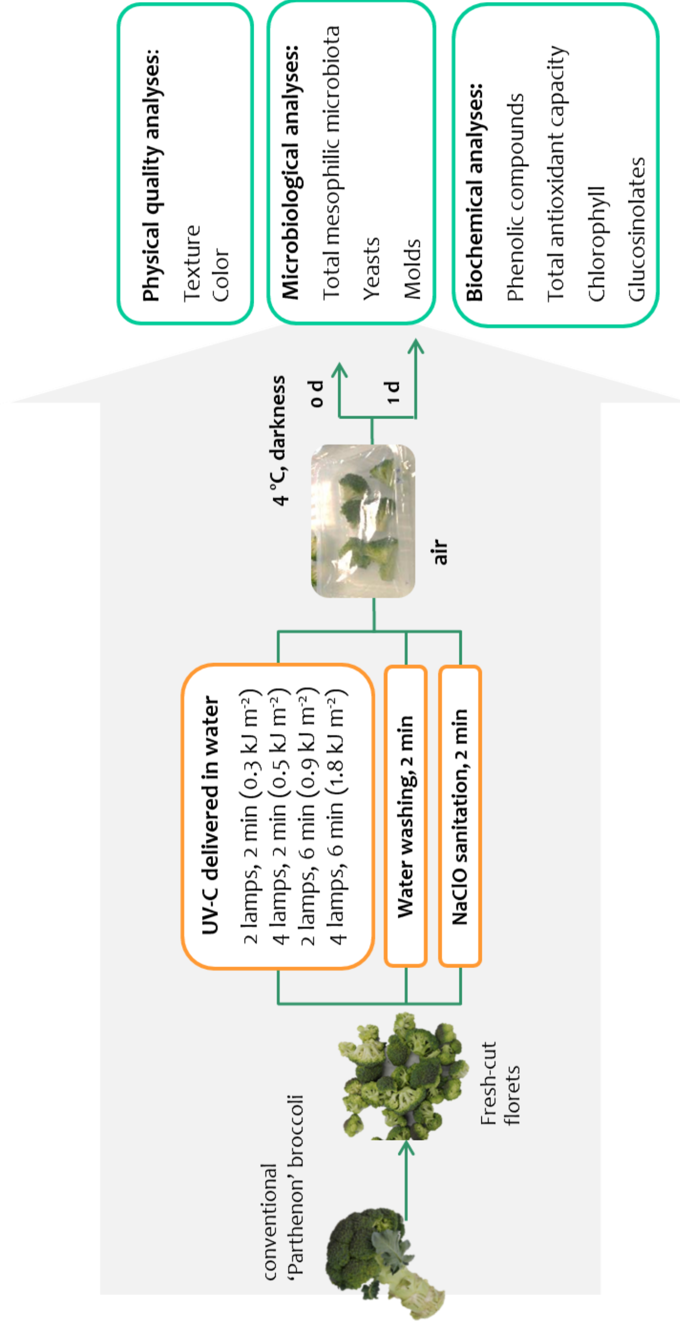
Electrolyte	SSF	SGF	SIF
	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}
K+	18.8	7.8	7.6
Na+	13.6	72.2	123.4
Cl-	19.5	70.2	55.5
H2PO4	3.7	0.9	0.8
HCO_3^-, CO_3^{2-}	13.7	25.5	85
Mg^{2+}	0.15	0.1	0.33
NH_4^+	0.12	1.0	-
Ca^{2+}	1.5	0.15	0.6

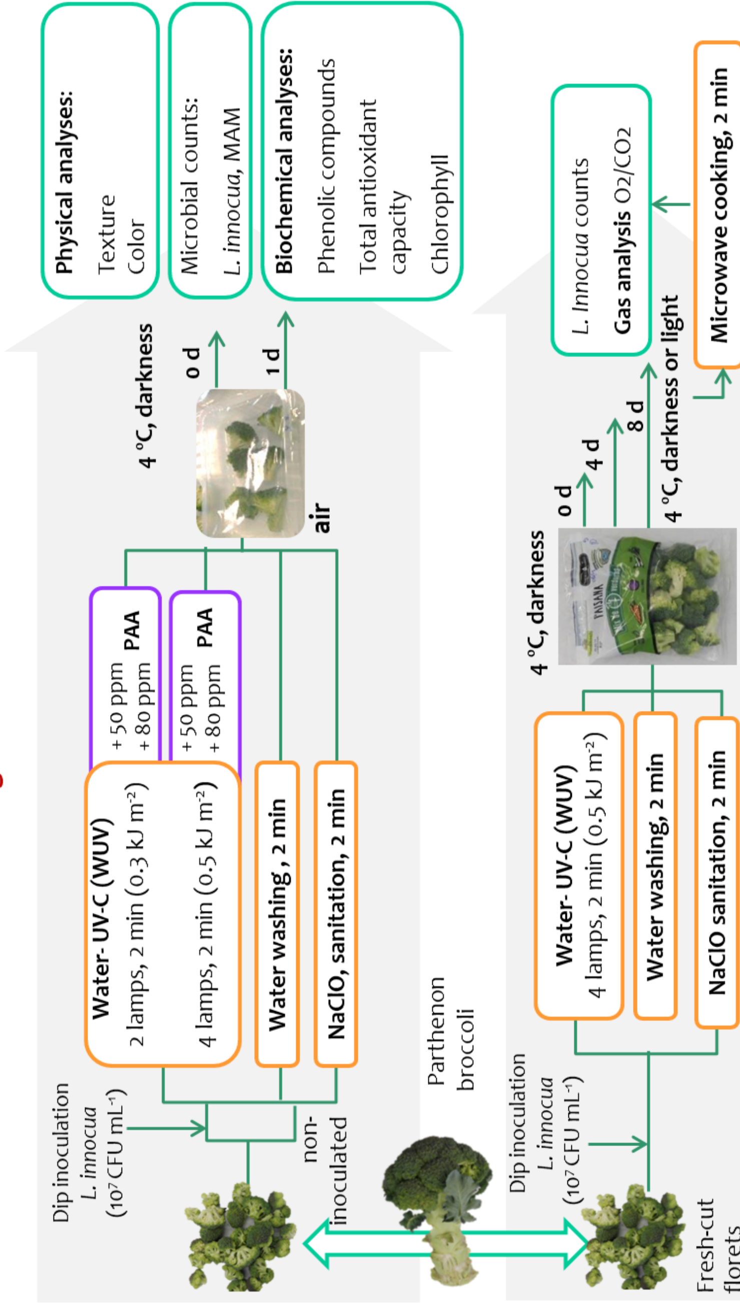
Enzymatic solutions (**EE**) according to Zudaire et al., 2017

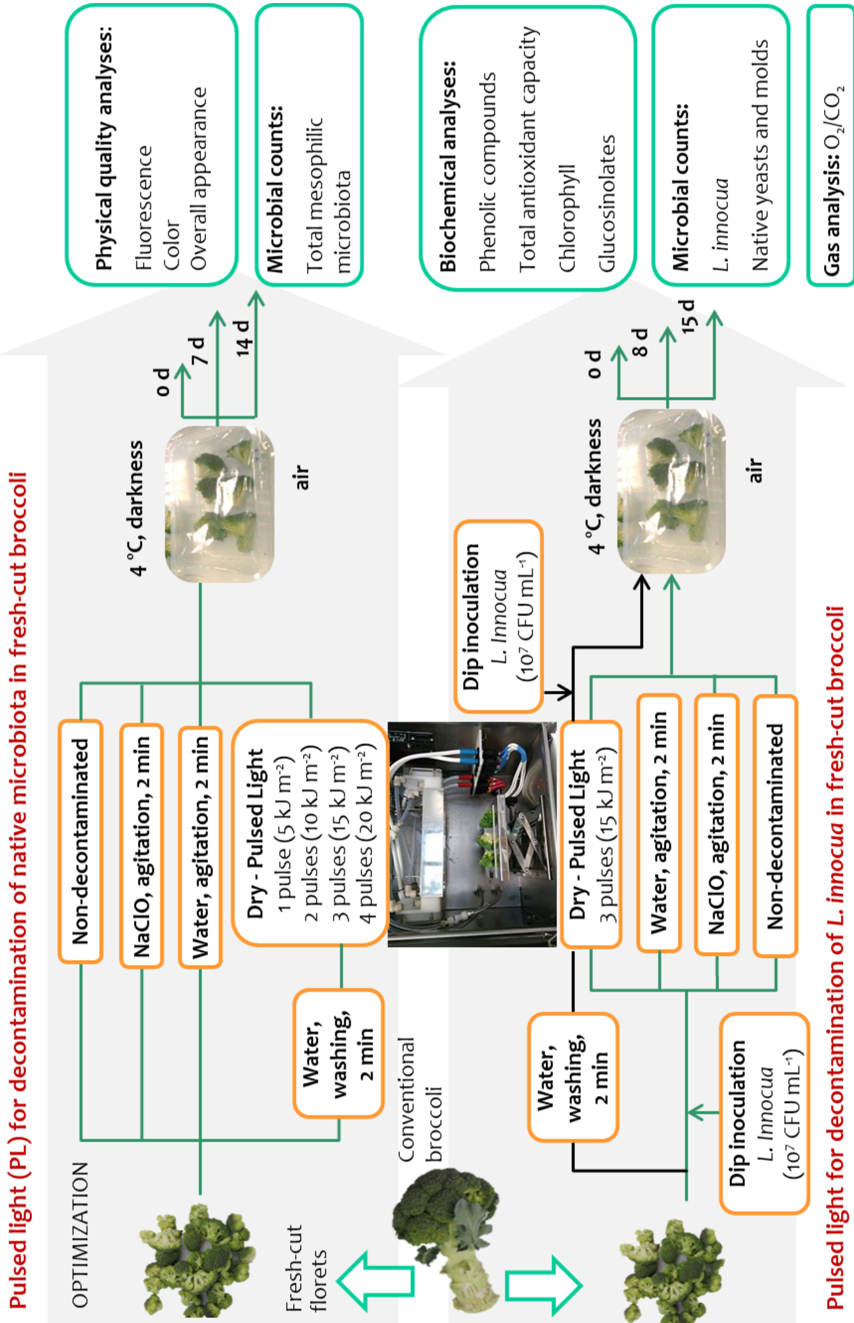
Enzyme	oral	gastric	intestinal
α-amylase	150 U mL^{-1}		
pepsin		4000 U mL^{-}	
pancreatin			100 U mL^{-1}
bile			10 mmol L^{-1}


Effect of CPA-7 on the fruit oxidative metabolism and tolerance to oxidative stress
'Golden delicious' apple
Fresh-cut
(6 % AS1) antioxidant dip + CPA-7 (10^7 CFU mL^-1)
antioxidant dip 6 % NatureSeal (AS1)
4 °C in darkness
MAP
air
0 d
1 d
3 d
6 d
Enzymatic activity:
Ascorbate peroxidase
Superoxide dismutase
Catalase,
Peroxidase
Polyphenol oxidase
Non enzymatic:
Hydrogen peroxide
Malondialdehyde
Total phenolic compounds
Total antioxidant capacity
Ascorbic acid
Fruit quality:
Color
Firmness
Soluble solids
Titratable acidity
pH
Microbial counts:
CPA-7
Total mesophilic microbiota
Gas analysis: O2/CO2

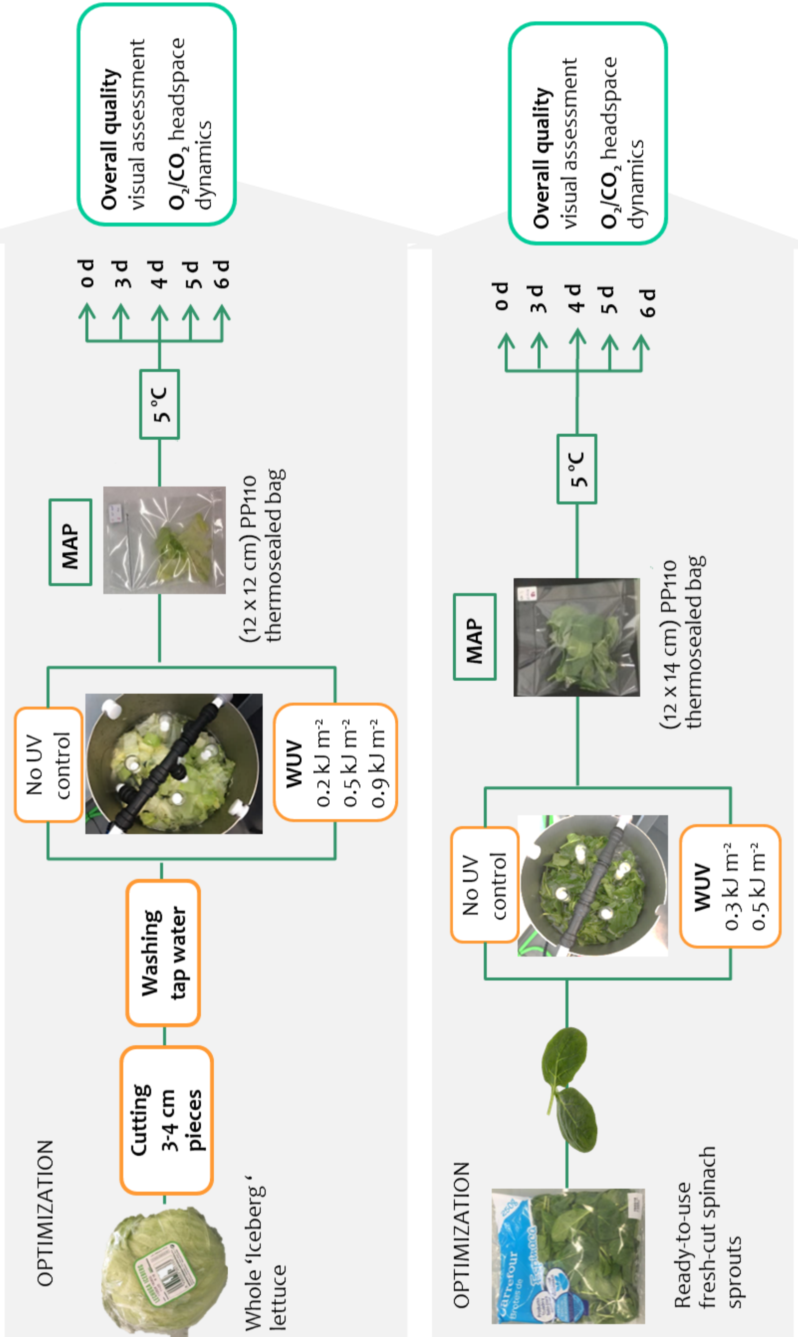

In vivo
Effect of CPA-7 on the fruit's respiration and ethylene accumulation
'Golden delicious' apple
2 maturity stages
Whole
Fresh-cut
Antioxidant dip (6 % NS), 2 min
Water, 2 min
Dip inoculation CPA-7 (10⁷ CFU mL⁻¹)
Water
7 d at 5 ºC, darkness
Hermetic jars
MAP
Fruit quality:
Texture
Color
Soluble solids
Titratable acidity
Fruit ethylene accumulation
CO₂/O₂ content
CPA-7: ethylene production/ intake
Microbial counts :
CPA-7
Total mesophilic microbiota
Apple juice-AOX
TSB
CPA-7
In vitro
Production or consumption of ethylene by CPA-7


Water-assisted UV-C (WUV) for decontamination of native microbiota in fresh-cut conventional broccoli
conventional
'Parthenon' broccoli
Fresh-cut
florets
UV-C delivered in water
2 lamps, 2 min (0.3 kJ m⁻²)
4 lamps, 2 min (0.5 kJ m⁻²)
2 lamps, 6 min (0.9 kJ m⁻²)
4 lamps, 6 min (1.8 kJ m⁻²)
Water washing, 2 min
NaClO sanitation, 2 min
4 °C, darkness
air
0 d
1 d
Physical quality analyses:
Texture
Color
Microbiological analyses:
Total mesophilic microbiota
Yeasts
Molds
Biochemical analyses:
Phenolic compounds
Total antioxidant capacity
Chlorophyll
Glucosinolates


Water-assisted UV-C (WUV) and peroxyacetic acid-assisted UV-C for the decontamination of native microbiota and L. innocua in fresh-cut organic broccoli
Dip inoculation L. innocua (10⁷ CFU mL⁻¹)
non-inoculated
Water- UV-C (WUV)
2 lamps, 2 min (0.3 kJ m⁻²)
4 lamps, 2 min (0.5 kJ m⁻²)
+ 50 ppm
+ 80 ppm
PAA
Water washing , 2 min
NaClO, sanitation, 2 min
air
4 °C, darkness
0 d
1 d
Physical analyses:
Texture
Color
Microbial counts:
L. innocua, MAM
Biochemical analyses:
Phenolic compounds
Total antioxidant capacity
Chlorophyll
Parthenon broccoli
Fresh-cut florets
Dip inoculation L. innocua (10⁷ CFU mL⁻¹)
Water- UV-C (WUV)
4 lamps, 2 min (0.5 kJ m⁻²)
Water washing, 2 min
NaClO sanitation, 2 min
4 °C, darkness
0 d
4 d
8 d
4 °C, darkness or light
L. Innocua counts
Gas analysis O2/CO2
Microwave cooking, 2 min
L. innocua DNA repair after during storage in dark or light. Survival to microwave cooking

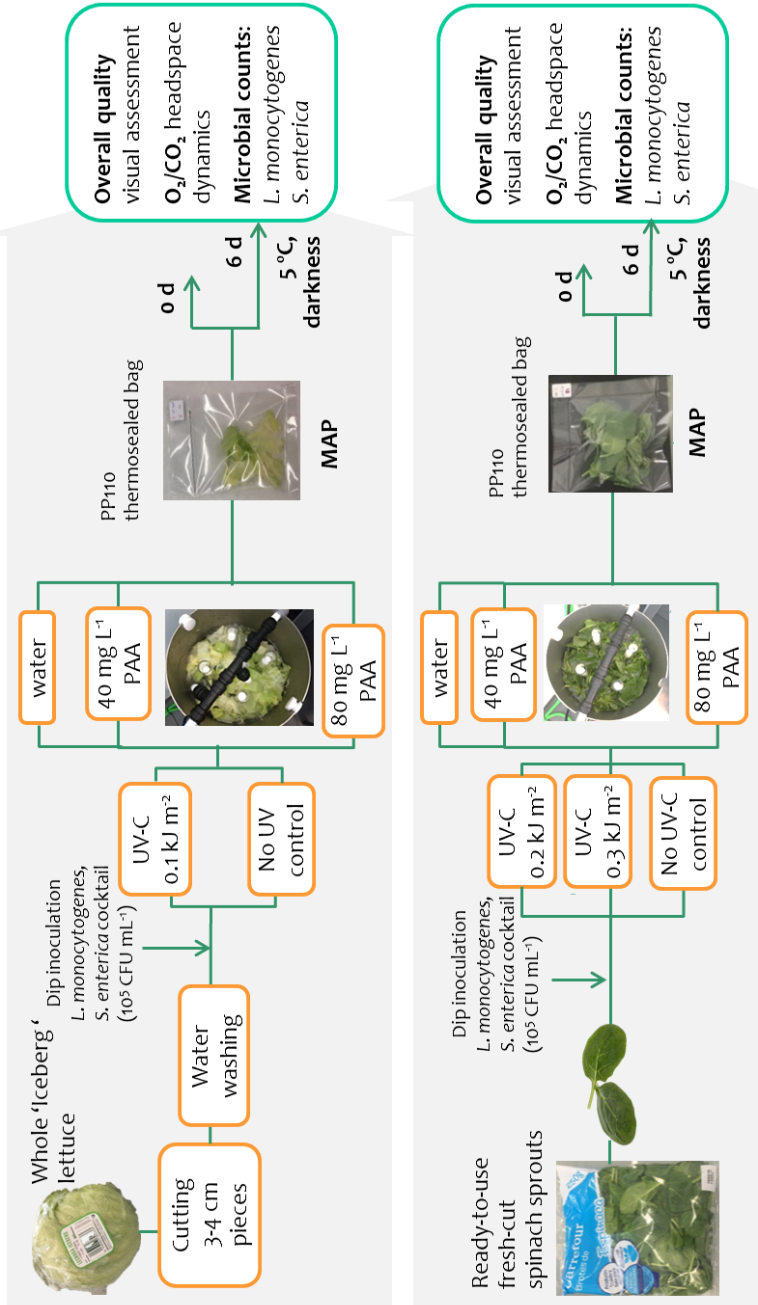


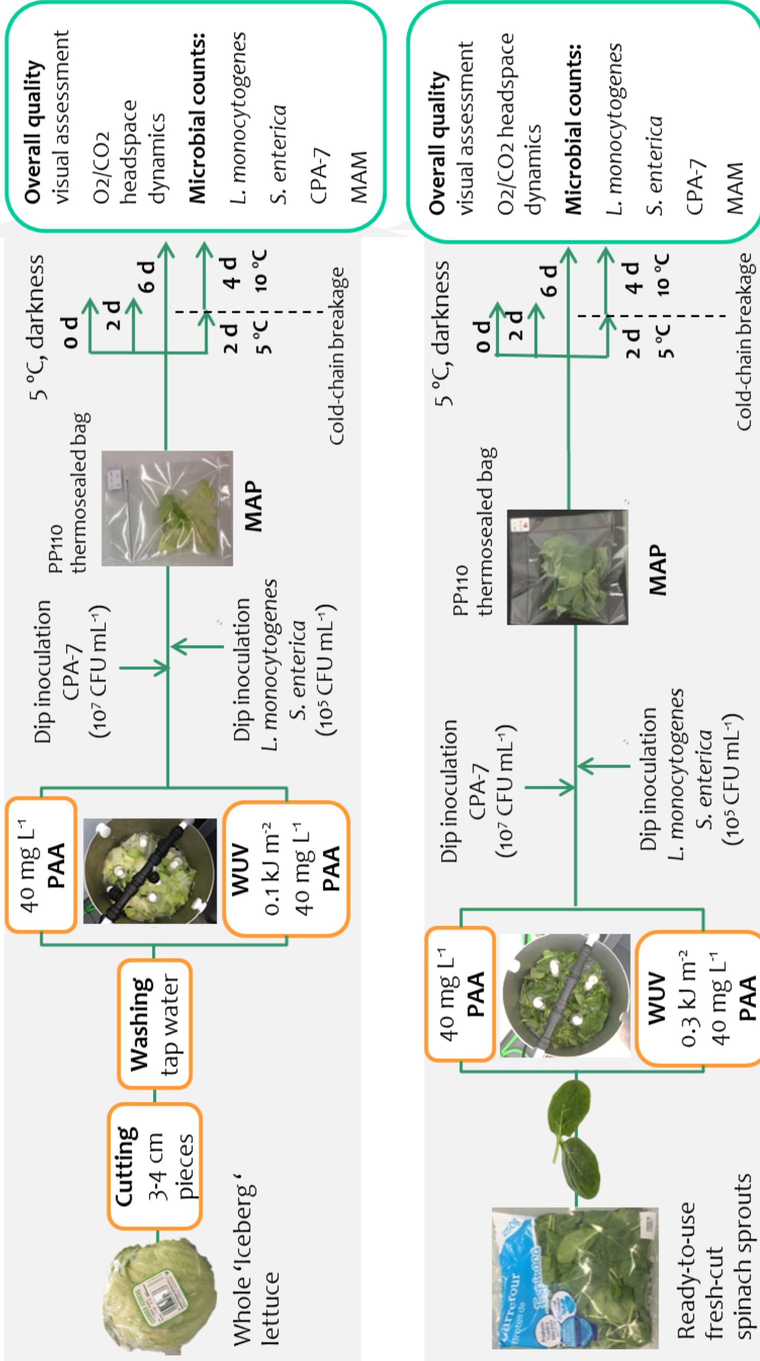
Pulsed light (PL) for decontamination of native microbiota in fresh-cut broccoli
OPTIMIZATION
Fresh-cut florets
Conventional broccoli
Non-decontaminated
NaClO, agitation, 2 min
Water, agitation, 2 min
Dry - Pulsed Light
1 pulse (5 kJ m⁻²)
2 pulses (10 kJ m⁻²)
3 pulses (15 kJ m⁻²)
4 pulses (20 kJ m⁻²)
Water, washing, 2 min
4 °C, darkness
air
0 d
7 d
14 d
Physical quality analyses:
Fluorescence
Color
Overall appearance
Microbial counts:
Total mesophilic microbiota
Pulsed light for decontamination of L. innocua in fresh-cut broccoli
Dip inoculation
L. Innocua
(10⁷ CFU mL⁻¹)
Water, washing, 2 min
Dry - Pulsed Light
3 pulses (15 kJ m⁻²)
Water, agitation, 2 min
NaClO, agitation, 2 min
Non-decontaminated
Dip inoculation
L. Innocua
(10⁷ CFU mL⁻¹)
4 °C, darkness
air
0 d
8 d
15 d
Biochemical analyses:
Phenolic compounds
Total antioxidant capacity
Chlorophyll
Glucosinolates
Microbial counts:
L. innocua
Native yeasts and molds
Gas analysis: O₂/CO₂



Water-assisted UV-C for 'Iceberg' lettuce and baby spinach leaves without affecting overall quality
OPTIMIZATION
Whole 'Iceberg' lettuce
Cutting 3-4 cm pieces
Washing tap water
No UV control
WUV
0.2 kJ m-2
0.5 kJ m-2
0.9 kJ m-2
MAP
(12 x 12 cm) PP110 thermosealed bag
5 °C
0 d
3 d
4 d
5 d
6 d
Overall quality
visual assessment
O2/CO2 headspace dynamics
OPTIMIZATION
Ready-to-use fresh-cut spinach sprouts
No UV control
WUV
0.3 kJ m-2
0.5 kJ m-2
MAP
(12 x 14 cm) PP110 thermosealed bag
5 °C
0 d
3 d
4 d
5 d
6 d
Overall quality
visual assessment
O2/CO2 headspace dynamics

UV-C delivered in water or in peroxyacetic acid for the control of *L. monocytogenes* and *S. enterica* in fresh-cut lettuce and baby spinach leaves





Integration of WUV with peroxyacetic acid and CPA-7 for the control of *L. monocytogenes* and *S. enterica* in fresh-cut lettuce and baby spinach leaves

CHAPTER II

Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7 against food-borne pathogens *in vitro* and on fresh-cut melon

Collazo, C., Abadias, M., Aguiló-Aguayo, I., Alegre, I., Chenoll, E., & Viñas, I. (**2017**). *LWT - Food Science and Technology*, 85, 301–308.
http://doi.org/https://doi.org/10.1016/j.lwt.2017.02.029

ABSTRACT

The present study was aimed at gaining insight into the mode of action of the antagonistic bacteria *Pseudomonas graminis* CPA-7, which has been previously identified as an effective biocontrol agent against *L. monocytogenes, S. enterica* and *E. coli* O157:H7 on fresh-cut fruit. *In vitro* experiments did not reveal any antimicrobial or proteolytic activity on solid media nor did they reveal any biosurfactant activity on hydrophobic surfaces. Metabolites produced by CPA-7 in two different culture media and on 'Galia' melon were unable to inhibit *L. monocytogenes* populations on 'Galia' melon plugs at 25 °C or 5 °C. In contrast, at 25 °C the population of this pathogen on 'Galia' plugs was reduced by 2.1 and 3.3 log-units when co-inoculated with the antagonist in water, after 24 and 48 h, respectively. CPA-7 did not form biofilms after 72 h at 25 °C (OD 0.03) or at 30 °C (OD 0.01) on polystyrene plates and the production of alginate was close to the negative control. Studies of nutritional profiles showed high overlap (NOI 0.92) between CPA-7 and *E. coli* O157:H7 regarding the use of carboxylic acids. This functional group could also contain putative targets for competition between CPA-7 and *S. enterica*, although overlapping was not restrictive enough (NOI 0.83).

2.1 INTRODUCTION

Biological control using native microbiota from fruits and vegetables surfaces is a method that matches the 'natural' and 'additive free' criteria, which can aid in reducing the risk of contracting food-borne diseases by pre-emptive exclusion of the pathogens involved. As these microorganisms have the advantage of being in their natural environment, they can easily colonize the food and inhibit pathogen growth when their populations are high enough (Belak and Maraz, 2015). In particular, diverse *Pseudomonas* spp. strains have the capacity to inhibit or suppress plant diseases and foodborne illnesses usually by a combined strategy that includes the production of antimicrobial compounds (Hernández-León *et al.*, 2015), siderophores (Solanki *et al.*, 2014) or hydrolytic enzymes, competition for nutrients (Saraoui *et al.*, 2016), rapid growth and formation of biofilms (Blumenstein *et al.*, 2015) and elicitation of resistance pathways in the host (Wang *et al.*, 2015).

P. graminis CPA-7 is a native strain from whole apple surfaces which inhibits the growth of *E. coli* O157:H7, *S. enterica* and *L. innocua* on fresh-cut fruits (Alegre, Viñas, Usall, Teixido, et al., 2013; Alegre, Viñas, Usall, Anguera, et al., 2013; Abadias et al., 2014). However, its effectiveness varies depending on the pathogen, preservation conditions and food matrix. The present study was aimed at gaining insight into the mode of action of *P. graminis* (CPA-7) for controlling the growth of *L. monocytogenes, E. coli* O157:H7 and *S. enterica* subsp. *enterica.*

2.2 MATERIALS AND METHODS

2.2.1 FRUIT PROCESSING

Different batches of melon (*Cucumis melo* L.) of three cultivars: 'Piel de sapo', 'Cantaloupe' and 'Galia' were purchased from local markets (Lleida, Spain). Fruits were washed with running tap water and surface-disinfected with 700 mL L^{-1} ethanol. Flesh plugs of approximately 1.2 cm of diameter and 1 cm thickness were cut with a cork borer. 'Piel de sapo' melon was also used to prepare fresh juice by crushing peeled fruit pieces in a blender. Juice was filtered using gauze pads, bottled and autoclaved at 90 °C for 10 min. Fruit quality parameters were determined before each experiment as described by Colas-Meda et al. (2017).

2.2.2 BACTERIAL STRAINS

Bacterial strains used in this study and their intended purpose in each section are listed in Table **II.**1. Culture conditions for each microorganism are described in each section.

2.2.3 BIOFILM FORMATION

Biofilm formation was assessed by a microtiter plate assay (O'Toole and Kolter, 1998). Briefly, microorganisms (Table 1) were cultured overnight in agitation as follows: CPA-7 and P. fluorescens, in tryptone soy broth (TSB, Biokar-Diagnostics, Beauvais, France) at 25 °C, *S. enterica* and *E. coli* in TSB at 37 °C and *L. monocytogenes*, in TSBYE (TSB supplemented with 6 g L-1 yeast extract) at 37 °C. One microliter of culture of each microorganism was added to 500 µL of M63 minimal medium (Pardee, Jacob and Monod, 1959) (supplemented with 2 g L-1 glucose; 5 g L-1 casaminoacids) in order to obtain inocula that were transferred (100 µL per well) to 96-well, polystyrene microplates (Grainier Bio-one, NC, USA).

Table II.1. Bacterial strains used in this study.

Microorganism	Serovar (identification code)	Purpose of use (section of this article)
Salmonella enterica subsp. *enterica*	Agona (ATCC BAA-707)	Pathogen cocktail (2.2.7) Nutrients profiling (2.2.9)
S. enterica subsp. *enterica*	Michigan (ATCC BAA-709)	Pathogen cocktail (2.2.7)
S. enterica subsp. *enterica*	Montevideo (ATCC BAA-710)	Pathogen cocktail (2.2.7)
S. enterica subsp. *enterica*	Gaminara (ATCC BAA-711)	Pathogen cocktail (2.2.7) Nutrients profiling (2.2.9)
S. enterica subsp. *enterica*	Enteritidis (CECT 4300)	Pathogen (2.2.3, 2.2.6)
Listeria monocytogenes	1a (CECT 4031)	Pathogen cocktail (2.2.7, 2.2.8)
L. monocytogenes	3a (CECT 933)	Pathogen cocktail (2.2.7, 2.2.8)
L. monocytogenes	4d (CECT 940)	Pathogen cocktail (2.2.7, 2.2.8) Nutrients profiling (2.2.9)
L. monocytogenes	1/2a (Lm 230) (UdL-IRTA, Lleida)	Pathogen cocktail (2.2.7, 2.2.8) Nutrients profiling (2.2.9)
Streptococcus gordonii	(CECT 804)	Hemolysis assay, positive control (2.2.5)
Listeria innocua	(CECT 910)	Negative control in hemolysis assay (2.2.5)
Pseudomonas graminis	(CPA-7), (UdL - IRTA, Lleida)	Antagonist (2.2.1-2.2.9)
Pseudomonas aeruginosa	(NCTC 12903)	Biosurfactants test, positive control (2.2.5)
Pseudomonas fluorescens	(1.62) Food Technology department collection, UdL, Lleida)	Positive control (2.2.3) Proteolysis test, positive control (2.2.5)
Escherichia coli	O157:H7, shigatoxin negative (NCTC 12900)	Pathogen (2.2.3, 2.2.6) Nutrients profiling (2.2.9)

Two plates were inoculated: one with CPA-7 and *P. fluorescens* which was incubated at 25 °C and the other with *L. monocytogenes, S. enterica* subsp. *enterica, E. coli* and CPA-7, which was incubated at 30 °C. Fresh M63 medium was included as negative control in both plates, which were stored statically during 72 h in a closed container with a moistened filter paper to maintain humid atmosphere. Optical density (OD) was measured at 570 and 750 nm (Epoch

Microplate Spectrophotometer, Biotek-Instruments, Winooski, USA). The experiment was performed twice and included six replicates per microorganism.

2.2.4 Alginate production

Production of alginate by CPA-7 was evaluated using supernatants from overnight cultures in TSB at 25 °C. Alginate detection was carried out using the Periodic acid Schiffs (PAS) procedure adapted to 96-well microplate format (Houghton *et al.*, 2014). Cell-free supernatants, cell pellets and fresh medium were subjected to hydrolysis with alginate-lyase (0.05 mg mL^{-1}) (Sigma-Aldrich, St Louis, USA) for 2 h at 30 °C. Quantification of the exopolysaccharides that were present in the samples before and after hydrolysis was carried out by interpolating OD_{550nm} values in a sodium alginate (PubChem CID: 5102882) (Sigma-Aldrich, St Louis, USA) standard curve. Three independent assays including three replicates of each sample were performed.

2.2.5 Proteolytic, hemolytic and biosurfactant activities

Casein proteolysis was evaluated on plate count agar (PCA, Biokar-Diagnostics, Beauvais, France) containing 0.1 g mL^{-1} skim milk powder (Vanderzant and Splittstoesser, 1992). After 72 h at 25 °C, the plates were flooded with 1 mol L^{-1} HCl. Hemolysis was tested on Columbia blood agar plates (Bioser, Barcelona, Spain) during 48 h. CPA-7 and the negative control (Table 1) were incubated at 30 °C and the positive control was incubated at 37 °C. Biosurfactants production was assessed by checking the collapse of a supernatant droplet previously stained with methylene blue (PubChem CID: 6099) on parafilm (Sigma-Aldrich, St Louis, USA) (Kuiper *et al.*, 2004). Supernatants were obtained after centrifugation (10,976 × *g* for 15 min at 10 °C) of 72 h cultures of each microorganism (Table 1) grown in King´s B medium. All experiments were performed three times and included three replicates of each microorganism.

2.2.6 Antimicrobial activity on solid media

The production of soluble antimicrobial substances by CPA-7 in two different culture media at different stages of growth was evaluated by the agar spot inoculation method (Alegre et al., 2013b) with some modifications. To obtain cell-free supernatants (CFS), CPA-7 was grown in TSB and in M63 minimal medium (pH=6.5) supplemented with 0.2% glucose, 0.5% casaminoacids or a combination of both at 25 °C. Supernatants were collected by centrifugation (10,976 × *g* for 15 min at 10 °C) at 24, 48 and 72 h and filtered through sterile, 0.2 µm-pore, nitrocellulose membranes (Millipore, County Cork, Ireland). Agar plates containing BSB medium (8g L^{-1} meat extract, 8g L^{-1} glucose, 14 g L^{-1} agar) were overlaid with soft BSB agar media (the same composition but with 7 g L^{-1} agar), previously inoculated with the correspondent pathogen strain (10^5 CFU mL^{-1}) (Table 1). Plates were marked into quadrants and 10 µL of **i**) CFS, **ii**) antagonist cell suspension (10^8 CFU mL^{-1}), **iii**) fresh culture medium and **iv**) streptomycin (100 µg mL^{-1}) were spotted onto the sections and allowed to soak into the agar for 2 h at 5 °C. Then, the plates were incubated for 72 h at 30 °C and checked for hyaline zones. Two independent assays to test the supernatants of each culture media at each sampling time were conducted, including three replicates per each pathogen.

2.2.7 Inhibitory effect on pathogens in melon juice

Cultivar (cv) 'Piel de sapo' melon juice was used to test the effect of any inhibitory soluble substance produced by CPA-7 in TSB medium until the stationary growth phase on *L. monocytogenes* (*Lm*) and *S. enterica* subsp. *enterica* (*Sal*) growth in a liquid matrix containing fruit components. Aliquots of a mixture containing 4 mL of melon juice and 1 mL of either CPA-7 CFS (prepared as described in section 2.6) or water were transferred to sterile glass tubes. Inocula of the antagonist and the pathogens were obtained as described by

Abadias et al. (2014) and added to the water-juice or the CFS-juice mixtures as follows: **i)** *Lm* + *Sal* (10^5 CFU mL^{-1} each), **ii)** *Lm* + *Sal* (10^5 CFU mL^{-1} each) + CPA-7 (10^7 CFU mL^{-1}), **iii)** CPA-7 (10^7 CFU mL^{-1}). Bacterial populations were estimated initially and after 5 d of incubation at 5 °C or 10 °C by plate count onto the appropriate media as described by Abadias et al. (2014) except that CPA-7 was grown in TSA. The experiment was carried out twice and included three replicate fruit plugs per treatment and sampling time.

2.2.8 Inhibitory effect on pathogens in melon plugs

The antagonistic effect of CPA-7 CFS from overnight TSB-cultures against a cocktail of *L. monocytogenes* (*Lm*) (Table 1) was tested on melon-flesh plugs of three different cultivars. Twelve melon plugs were dipped for 2 min at 185 rpm in 25 mL of water or CFS inoculated with *Lm* (10^5 CFU mL^{-1}) or with both *Lm* (10^5 CFU mL^{-1}) and CPA-7 (10^7 CFU mL^{-1}). A control of CPA-7 (10^7 CFU mL^{-1}) in water was also included. Samples were placed in 10 ml sterile glass tubes and analyzed just after inoculation and after incubation for 24 or 48 h at 25 °C or for five days at 5 °C. Microbial populations were estimated by plate count in three samples of each treatment at each sampling time as explained in the previous section.

Moreover, the effect of the metabolites produced by CPA-7 in ‘Galia’ melon during 24 and 48 h at 25 °C was also tested against *L monocytogenes* on ‘Galia’ melon plugs. For this, 25 g of the plugs was dip-inoculated in a suspension of CPA-7 (10^7 CFU/mL) in deionized water, drained, air-dried and homogenized with 75 mL of deionized water to obtain CFS as previously described. The resultant 24 and 48 h CPA-7-CFS, along with a non-inoculated Galia filtered supernatant were inoculated with *Lm* as described above and used to immerse ‘Galia’ plugs. Water treatments inoculated with **i)** *Lm,* **ii)** *Lm* + CPA-7 and **iii)** CPA-7 at the same concentrations of the previous experiment were included to corroborate the antagonistic activity of CPA-7. Samples were stored and analyzed by plate count

at the same sampling times and incubation temperatures of the previous experiment. All of the experiments were carried out twice and they included three replicates per treatment and sampling time.

2.2.9 NUTRITIONAL SIMILARITY

Individual nutritional profiles based upon single substrate utilization were defined for Gram-negative and Gram-positive bacteria (Table 1) using GN2 and GP2 MicroLog System, respectively, according to the manufacturer's instructions (Biolog, Hayward, USA). Briefly, colonies of each microorganism, grown in BUG-B (5% sheep blood)-AGAR (Biolog, Hayward, USA) at 30 °C, were re-suspended in GN/GP inoculation fluid (10^7 CFU mL^{-1}) and transferred to 96-well microplates (150 µL per well) which were incubated statically for 96 h at 30 °C. Transformation of tetrazolium chloride to purple-colored Formazan was visually recorded and analyzed as presence/absence data every 24 h for four days. Each strain was tested in duplicate in independent assays.

Nutritional similarity between CPA-7 and the pathogens was estimated by the niche overlap index (NOI) = number of compounds used by both the antagonist and the pathogen/number of compounds used by the pathogen (Ji and Wilson, 2002). NOIs were calculated for the 95 sources that were tested and for specific groups: carbohydrates, carboxylic acids, amino acids, polymers, amines/amides and miscellaneous (Dobranic and Zak, 1999). Niche size (NS) was calculated for each microorganism: NS = number of compounds used/95 and NS = number of compounds used/subtotals of each functional group that has been previously mentioned.

2.2.10 STATISTICAL ANALYSIS

Microbiological results measured as CFU mL^{-1} were transformed to log_{10} CFU g^{-1} fruit. Population increase of the pathogen was calculated for each treatment: log_{10} CFU g^{-1} fruit at a specific sampling time - log_{10} CFU g^{-1} fruit at initial time, and presented as mean ± standard error of the mean. In the biofilm formation experiment the mean values of OD_{570nm} - OD_{750nm} were calculated. OD results are expressed as mean ± standard error of the mean. Statistical analyses were performed using the Statistical software JMP (version 8.0.1 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity and accordingly, compared by using analysis of variance (ANOVA) or by Welch's test and separated by Tukey's test ($P < 0.05$).

2.3 RESULTS AND DISCUSSION

2.3.1 BIOFILM FORMATION

Production of biofilms by CPA-7 after 72 h at 25 °C (0.03 ± 0.01) or at 30 °C (0.012 ± 0.004) was close to the negative control (0.012 ± 0.003). These results differed significantly ($P < 0.05$) from the positive control *P. fluorescens* (0.48 ± 0.04) and *L. monocytogenes* (0.14 ± 0.03). Adhesiveness of *S. enterica* subsp. *enterica* ser. Enteritidis CECT 4300 was similar (0.03 ± 0.01) to the negative control (0.012 ± 0.004) while *E. coli* O157:H7 was significantly higher (0.048 ± 0.004). Minimal medium M63 supplemented with glucose and casaminoacids was selected for this test based on previous results, when it promoted the formation of biofilms by Pseudomonads such as *P. fluorescens* WCS365 on plastic surfaces (O'Toole and Kolter, 1998). However, it was not able to induce the production of significant amounts of biofilm by CPA-7 as it did for *L. monocytogenes*. The adaptive advantage of CPA-7, with respect to the pathogens tested, cannot be explained by its adherence capacity under the conditions examined although

under different conditions, biofilm formation could be possible (Ude *et al.*, 2006).

2.3.2 ALGINATE PRODUCTION

To assess the potential capacity of CPA-7 to adhere other surfaces, the production of alginate, reported as a matrix biofilm component of Pseudomonads such as *P. aeruginosa, P. fluorescens, P. putida, P. syringae P. mendocina* and *P. extremaustralis* (Govan, Fyfe, & Jarman, 1981; Raiger Iustman et al., 2014), was also quantified. However, the amounts of exopolysaccharides present in fresh culture medium (0.3 ± 0.2 mg mL^{-1}), cell-free supernatants (0.2 ± 0.2 mg mL^{-1}) and cell pellets (0.2 ± 0.1 mg mL^{-1}) were not significantly different. Likewise, they were similar to those obtained after hydrolysis with alginate lyase (0.4 ± 0.2 mg mL^{-1}, 0.2 ± 0.2 mg mL^{-1}, 0.3 ± 0.3 mg mL^{-1}, respectively) suggesting that any of the polysaccharides detected was alginate.

2.3.3 PROTEOLYTIC, HEMOLYTIC AND BIOSURFACTANT ACTIVITIES

Proteolytic activity was observed as translucent haloes of 5 mm width around the bacterial growth of *P. fluorescens* in PCA medium supplemented with skim milk, but no halo was obtained for CPA-7 (Fig. **II.**1A). Translucent haloes, that are characteristic of complete hemolysis, were not produced in Columbia blood agar plates by any of the microorganisms tested. A greenish-colored halo indicating incomplete hemolysis was observed surrounding the positive control growth and no halo was produced by CPA-7 or *L. innocua* (data not shown).

Additionally, production of biosurfactants was explored as they have shown microbial inhibitory activity and they can influence biofilm formation (Kuiper *et al.*, 2004). No reduction of the surface tension of King's B medium, suggesting

biosurfactant activity, was detected for CPA-7, whereas supernatant of *P. aeruginosa* resulted in a collapsed droplet (Fig. **II.**1B).

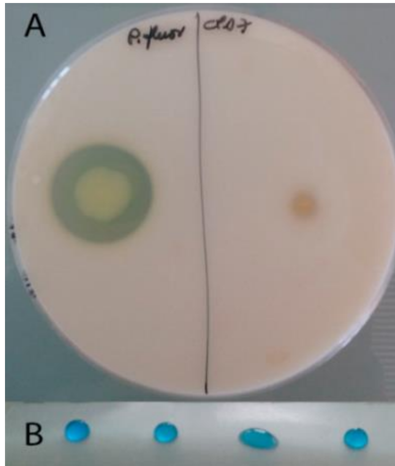


Figure II.1. **(A)** Proteolytic activity assay on PCA medium supplemented with 0.1 g mL^{-1} skim milk. Left section: positive control *Pseudomonas fluorescens,* right section: CPA-7. **(B)** Biosurfactant activity of 20 µl droplets of 72 h King's B medium - supernatants stained with methylene blue on a hydrophobic surface (parafilm). From left to right: water, fresh King's B medium, positive control (*P. aeruginosa*) and CPA-7. Each assay was repeated three times and included three replicates.

2.3.4 Antimicrobial activity on solid media

No inhibition zone indicating antimicrobial activity on any of the pathogens tested was observed for either the cell-free supernatants or CPA-7 cells, irrespective of the medium (TSB or M63) or the incubation time tested (24, 48, 72 h) (data not shown). This contrasted with the hyaline zone observed around the antibiotic spot (Fig. **II.**2). Overnight growth of CPA-7 in M63 medium was too low, thus it was not included in the study. In previous studies with overnight cultures of CPA-7 in TSB medium, no antimicrobial activity against *E. coli*, *S. enterica* subsp. *enterica or L. monocytogenes* was detected (Alegre, et al., 2013b).

In the present study, we extended the analysis to different stages of growth in the same rich medium and in a minimal medium because it has been reported that production of antibiotics by *Pseudomonas* spp. can be regulated by the presence or limitation of certain carbon and nitrogen sources and may differ

according to the growth stage (He, Xu, & Zhang, 2008; Kremmydas, Tampakaki, & Georgakopoulos, 2013).

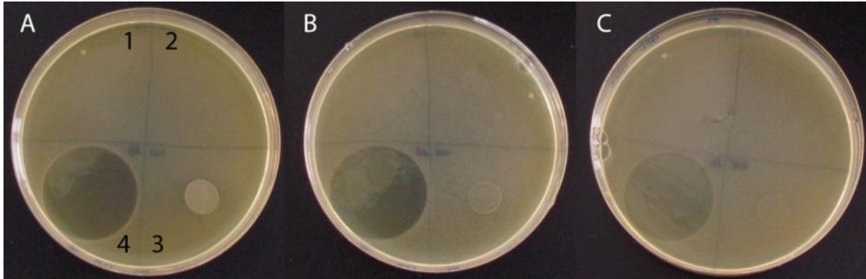


Figure II.2. Antibiosis diffusion assay on agar medium inoculated with: (A) *L. monocytogenes* CECT 4032, (B) *Salmonella enterica* var. *enterica* CECT 4300 or (C). *Escherichia coli* O157:H7. On each plate: 1. Fresh M63 medium, 2. CPA-7 cell-free supernatant obtained from 72 h culture in M63 medium, 3. CPA-7 cells (10^8 CFU/mL), 4. Streptomycin (100 µg mL^{-1}). The assay was repeated two independent times including three replicates per pathogen.

Growth conditions may influence the production by *Pseudomonas* spp. of certain groups of antibiotics, such as phenazines, pyoluteorin, pyrrolnitrin and 2,4 diacetylphloroglucinol, as well as cyclic lipopeptides and rhamnolipids (Hernández-León et al., 2015; Tupe et al., 2015; Ramette et al., 2011; Raaijmakers, de Bruijn, & de Kock, 2006). For instance, glucose repressed pyrrolnitrin synthesis and induced the production of phenazines in *P. chlororaphis* at higher levels during the stationary phase (Park *et al.*, 2011). As the CPA-7 CFS obtained from any of the assayed liquid media did not show lethal effects on the pathogens on solid media, a more sensitive method was used which entailed monitoring the effect in the progression of its populations in fruit matrices.

2.3.5 Inhibitory effect on pathogens in melon juice

The effect of the secondary metabolites produced by CPA-7 in TSB medium on the growth of *L. monocytogenes* or *S. enterica* subsp. *enterica* ser. Enteritidis in cv 'Piel de sapo' melon juice was compared with the direct pathogen-antagonist interaction in this matrix, at 5 °C and 10 °C. Initial populations of CPA-7 and *S.*

enterica were 8.0 ± 0.3 log_{10} CFU mL^{-1} and 5.8 ± 0.3 log_{10} $CFUmL^{-1}$, respectively. After five days of incubation at 5°C, the counts of *S. enterica* remained invariable in the four treatments tested. When incubated at 10 °C, *S. enterica* populations increased by an average of 2.4 ± 0.6 log_{10} in all treatments. No significant differences were observed between pathogen´s populations in the supernatant-treated and in the water control treatments.

Regarding *L. monocytogenes* populations, the initial counts were 5.4 ± 0.2 log_{10} CFU mL^{-1}, in all treatments. After 5 d of incubation, it increased by 1.2 and 2.3 log_{10} in the treatments stored at 5 °C and at 10 °C, respectively. *L. monocytogenes* populations were not reduced when inoculated in the supernatant-juice mix compared to the control juice. In the same way, no inhibition of *L. monocytogenes* growth was observed when co-inoculated with the antagonist cells in the supernatant-juice or in the water control at any of the incubation temperatures assayed.

Results showed that CPA-7 was not effective for controlling the populations of *S. enterica* or *L. monocytogenes* in melon juice, regardless of the temperature of incubation analyzed, even when inoculated two orders of magnitude above the pathogens, which has been previously observed as an effective proportion for biocontrol on apples slices at 5 °C and 20 °C and on melon plugs at 10 °C and 20 °C (Alegre et al., 2013; Abadias et al., 2014). Furthermore, metabolites produced by CPA-7 in cv 'Piel de sapo' melon juice did not show reduction of *L. monocytogenes* or *S. enterica* populations in any of the conditions that were tested, including the effect of the antagonist cells, which indicated that an alternative mechanism must have been responsible for the inhibitory effect observed in the previous experiments performed at 10 °C (Abadias et al., 2014). The fluidity of this matrix, although its composition is similar to that of fruit pieces, implies that local changes in the concentration of microbial growth and the accumulation of final metabolic products cannot occur, avoiding competition between bacterial species resulting from a near spatial distribution

(Wilson et al., 2002). The antagonistic activity of Pseudomonads such as *P. syringe* and *P. fluorescens* against *L. monocytogenes* and *S. enterica* has been previously reported to be hampered in fruit juices (Pacetto *et al.*, 2003).

2.3.6 Inhibitory effect on pathogens in melon plugs

The effect of CPA-7 CFS obtained from cultures in TSB to control *L. monocytogenes* populations was tested at the optimal growth temperature for CPA-7 (25 °C) and under refrigerated conditions (5 °C) in three different melon cultivars and the results were compared to the effectiveness of the antagonist cells. In the experiment carried out at 25 °C, the initial counts of CPA-7 on melon plugs of cultivars 'Cantaloupe', 'Galia' and 'Piel de sapo' were 5.8 ± 0.1, 5.8 ± 0.2 and 6.0 ± 0.3 $\log_{10}$ $CFUg^{-1}$ fruit, respectively (data not shown). After 24 h of incubation, control populations of CPA-7 increased by approximately 4 orders of magnitude in all cultivars. CPA-7 TSB-CFS had no inhibitory effect ($P < 0.05$) on *L. monocytogenes* populations in any of the melon cultivars that were tested, after 24 or 48 h of incubation (Table **II.**2).

In 'Galia' melon, co-inoculation of *L. monocytogenes* with CPA-7 in water resulted in a reduction of the pathogen's growth by 2.4 and 3.8 $\log_{10}$ with respect to the water control, after 24 and 48 h of incubation, respectively. In 'Piel de sapo' melon, co-inoculation with CPA-7 in water or in CFS, did not result in a significant reduction of *L. monocytogenes* populations, which differs from previous results obtained in similar experiments performed at 20 °C (Abadias et al., 2014). This could be related with the higher incubation temperature used in the present study which could also favor an increase of the growth rate of the pathogen and other mesophilic microorganisms present on the samples.

On 'Cantaloupe' melon, *L. monocytogenes* population growth declined by 1.8 $\log_{10}$ when co-inoculated with CPA-7 compared to the pathogen inoculated alone, in the water-treated samples at 48 h post-inoculation. However, no

significant reduction was observed at 24 h. Furthermore, when 'Cantaloupe' melon plugs were treated with TSB-CFS, in the presence of the antagonist cells, *L. monocytogenes* growth was reduced by 1.1 log_{10} at 24 and at 48 h, compared to the control.

Table **II**.2. *Listeria monocytogenes* population increase, calculated as log_{10}CFU g^{-1} fruit at a specific sampling time – log_{10}CFU g^{-1} fruit at initial time, on plugs of cv 'Galia', cv 'Piel de sapo' and cv 'Cantaloupe' melons, after incubation at 25 ºC in air, when inoculated alone or co-inoculated with *Pseudomonas graminis* (CPA-7) in water or in cell-free supernatants (CFS) obtained from 24 h cultures of CPA-7 in tryptone soy broth medium (TSB).

Treatment	cv 'Galia'		cv 'Piel de sapo'		cv 'Cantaloupe'	
	24 h	48 h	24 h	48 h	24 h	48 h
Lm (water)	5.0±0.1 a	5.1±0.1 a	4.9±0.2 a	4.9±0.1 a	4.5±0.3 ab	5.1±0.2 ab
Lm + CPA-7 (water)	2.6±0.6 b	1.3±0.3 b	5.0±0.4 a	4.5±0.3 a	3.9±0.1 b	3.3±0.4 c
Lm (TSB-CFS)	5.1±0.1 a	5.1±0.1 a	5.3±0.1 a	4.7±0.4 a	4.7±0.2 a	5.3±0.1 a
Lm + CPA-7 (TSB-CFS)	4.2±0.6 ab	4.2±0.7 a	4.4±0.4 a	4.2±0.3 a	3.7±0.3 b	4.2±0.2 bc

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments analyzed separately for each melon cultivar, according to Welch's test ($P < 0.05$) and Tukey's test for n=6.

At 5 °C, *L. monocytogenes* populations were not significantly reduced when inoculated in CPA-7 TSB-CFS compared to the control inoculated in water in any of the three melon cultivars (Table **II.**3). In 'Cantaloupe' melon, *L. monocytogenes* populations increased more on the plugs treated with the CFS than on the control treatment. A favorable combination of nutrients of the culture medium and the matrix could promote the pathogen's growth, but on the other hand it also enhanced the antagonistic activity of CPA-7. A general trend of reduction of *L. monocytogenes* populations was observed in the samples treated with CPA-7 cells in water compared with the treatments where the pathogen was inoculated alone in water or in TSB-CFS. However, no significant reduction could be determined on 'Piel de sapo' or 'Galia'. The loss of efficacy of the biocontrol exerted by the antagonist could be related to the variability of the composition and the structure of the matrix with the ripening stage. During this process, the

breakdown of the cell wall changes its texture, increasing the fluidization and modifying the compounds that accumulate in the microenvironment (Wilson et al., 2002). Furthermore, the proportion of sugars also changes (i.e. increase of sucrose and reduction of glucose and fructose) which could also diminish the possibility of competition, probably as a result of a lesser efficiency in using the available Carbon sources (Villanueva, Tenorio, Esteban & Mendoza, 2004).

As CPA-7 reduced *L. monocytogenes* growth on ‘Galia’ melon, the CFS obtained from cultures of the antagonist that was grown for 24 and 48 h in this matrix stored at 25 °C, were used to dip-inoculate ‘Galia’ melon plugs with this pathogen to determine whether the inhibitory effect was associated with the production of antimicrobial metabolites.

Table **II**.3. *Listeria monocytogenes* population increase, calculated as $\log_{10}$CFU/g fruit at 120 h post-inoculation – $\log_{10}$ CFUg^{-1} fruit at initial time, on plugs of cv ‘Galia’, cv ‘Piel de sapo’ and cv ‘Cantaloupe’ melons, after incubation at 5 ºC in air, when inoculated alone or co-inoculated with *P. graminis* (CPA-7) in water or in cell-free supernatants (CFS) obtained from 24 h cultures of CPA-7 in tryptone soy broth medium (TSB).

Treatment	cv 'Galia'	cv 'Piel de sapo'	cv 'Cantaloupe'
Lm (water)	1.4 ± 0.3 ab	1.1 ± 0.3 ab	1.5 ± 0.1 a
Lm + CPA7 (water)	0.4 ± 0.2 b	0.6 ± 0.2 b	1.7 ± 0.1 ab
Lm (TSB-CFS)	1.6 ± 0.2 a	1.7 ± 0.1 a	2.1 ± 0.2 b
Lm + CPA7 (TSB-CFS)	1.1 ± 0.4 ab	1.4 ± 0.1 a	1.6 ± 0.1 a

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments analyzed separately for each melon cultivar, according to Welch’s test ($P < 0.05$) and Tukey’s test for n=6.

The population increase of *L. monocytogenes* on ‘Galia’ plugs treated with the CPA-7-24 h or 48 h-CFS was similar ($P < 0.05$) to plugs treated with the supernatant obtained from non-inoculated melon, regardless of the temperature of incubation and the sampling time analyzed (Table **II.**4). These results indicate that the compounds produced as a result of the metabolic activity of CPA-7 on ‘Galia’ melon were not responsible for the reduction of growth of *L. monocytogenes* observed in this matrix in the conditions tested.

This suggests that it could be associated with the competition for nutrients and/or rapid replication of the antagonist during the logarithmic phase of growth.

Table II.4. *Listeria monocytogenes* population increase, calculated as log_{10}CFU per gram of fruit at a specific sampling time – log_{10}CFU per gram of fruit at initial time, on plugs of cv 'Galia' melon after incubation at 25 ºC or 5 ºC in air, when inoculated alone or co-inoculated with *Pseudomonas graminis* (CPA-7) in water or in cell-free supernatants (CFS) obtained from 24 or 48 h cultures of CPA-7 on 'Galia' melon plugs.

Treatment	**cv 'Galia'**		
	24 h at 25 °C	**48 h at 25 °C**	**120 h at 5°C**
Lm (water)	5.1 ± 0.1 a	4.7 ± 0.1 a	2.3 ± 0.1 a
Lm + CPA-7 (water)	2.4 ± 0.5 b	1.0 ± 0.2 c	1.4 ± 0.1 c
Lm (non-inoculated Galia supernatant)	4.8 ± 0.1 a	4.0 ± 0.1 b	3.0 ± 0.1 b
Lm (24h-Galia-CFS)	5.0 ± 0.2 a	4.1 ± 0.1 ab	2.5 ± 0.2 ab
Lm (48h-Galia-CFS)	5.3 ± 0.1 a	4.0 ± 0.2 b	2.5 ± 0.1 ab

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments, analyzed separately for each sampling time, according to a Welch's test ($P < 0.05$) and Tukey's test for n=6.

2.3.7 Nutritional similarity

GN2/GP2 microplates, initially developed for identification of Gram-negative bacteria, have shown to be useful for exploring interactions and functional diversity of microbial communities (Horemans, Smolders and Springael, 2013) and for approaching to niche overlapping in biological control interactions (Janisiewicz, 1996). In this study, nutritional niche size that was calculated including the 95 carbon/nitrogen sources, was similar for all of the Gram-negative microorganisms tested (Table **II.**5). However, the analysis of specific functional groups showed differences among them. For instance, *E. coli* used 31% more carbohydrates than CPA-7 of the 26 tested. Similar results were obtained when nutritional overlap indexes (NOI) were calculated for all sources, showing a high level of coexistence between CPA-7 and the pathogens tested.

However, NOIs calculated for specific functional groups allowed a better approach to competitive relations at an ecologically-significant resource dimension. Carbohydrates utilization (adonitol, L-arabinose, D-arabitol, L-fucose, D-galactose, α-D-glucose, m- inositol, D-mannitol, D-mannose, D-sorbitol) showed the lowest overlapping between the antagonist and the pathogens of all groups of compounds analyzed (Table 5). From the major soluble sugars (sucrose, glucose and fructose) present in melon, pear and apple, which are some of the fruits where CPA-7 has shown to be effective against the pathogens tested, this antagonist was able to use only glucose (Wu, Gao, Zhao, Liao, & Chen, 2007; Beaulieu, Lea, & Eggleston, 2003). This could be due to a high level of specialization and it could represent an advantage to effectively use this compound in the fruit matrix as *E. coli* and *S. enterica* also oxidized it and were not able to metabolize sucrose.

Carboxylic acids were the carbon sources that were most used by CPA-7 (64%) in relation to the total tested, whereas for both strains of *S. enterica* and for *E. coli* their use was close to 50%. Although the highest level of coincidence among the microorganisms analyzed was observed for the polymers (NOI 1.00), from the five tested only dextrin is present in food matrices and it is not naturally present in fruits. Thus, at a biologically significant level, carboxylic acids were the most probable targets for competiveness between *E. coli* O157:H7 and the antagonist, as NOI was above 0.9 (Blumenstein *et al.*, 2015). CPA-7 and both strains of *S. enterica* also matched in the use of this group of compounds although the level of overlapping was not restrictive enough (NOI 0.83). From the 25 organic acids tested, acetic acid, cis-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D,L-lactic acid, propionic acid, D-saccharic acid, succinic acid and bromosuccinic acid, could be used by the four microorganisms, while α-keto glutaric acid was only used by CPA-7 and *E. coli*. It is noteworthy that the shared use of citric and succinic acids, which are predominant organic acids in melons, suggests suitable targets for competition

between the antagonist and the three pathogens (Beaulieu, Lea and Eggleston, 2003).

Table II.5. Nutritional profile of *Pseudomonas graminis* (CPA-7), *E. coli* O157:H7 and *S. enterica* subsp. *enterica*, strains CECT 707 (Sal 707) and CECT 711 (Sal 711).

Functional group	Total	Niche size				NOI		
		E. coli	S.e 707	S.e 711	CPA7	CPA7 /*E. coli*	CPA7/ S.e 707	CPA7 /S.e 711
Carbohydrates	26	0.69	0.50	0.58	0.38	0.39	0.50	0.50
Aminoacids	20	0.50	0.55	0.60	0.55	0.70	0.73	0.75
Carboxylic acids	25	0.52	0.48	0.48	0.64	0.92	0.83	0.83
Amines/Amides	8	0.25	0.25	0.25	0.63	0.00	0.50	0.50
Polymers	5	0.20	0.60	0.60	0.60	1.00	1.00	1.00
Miscellaneous	11	0.73	0.73	0.73	0.73	0.88	0.88	0.88
Total	95	0.55	0.52	0.55	0.56	0.63	0.73	0.71

S.e (*S. enterica*), NOI (niche overlapping index= Total used by both antagonist and pathogen/Total used by pathogen), niche size = number of used/total tested

The use of amino acids by all the microorganisms analyzed was around 55% of the 20 that were tested. However, overlapping between the antagonist and the three pathogens evaluated was close to 0.7. L-proline, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid and L- glutamic acid were used by all of the microorganisms tested. L-pyroglutamic acid was metabolized by both CPA-7 and *S. enterica* CECT 711 while L-histidine was used by CPA-7 and both strains of *S. enterica*. All of these aminoacids except for L-pyroglutamic acid, have been identified in both cv 'Cantaloupe' and cv 'Galia', from which glutamic acid, aspartic acid and alanine are the most predominant in both of them, but they are almost to times more abundant in cv 'Cantaloupe' than in cv 'Galia' (Lamikanra *et al.*, 2000).

The GP2 MicroLog System was used to evaluate the use of 95 carbon and nitrogen sources by two strains of *L. monocytogenes* (Table 1). After 96 h of incubation, a change of colour was only detected for carbohydrates, 10 of which (arbutin, D-cellobiose, D-fructose, D-glucose, N-acetyl-D-glucosamine, D-

mannose, D-ribose, D-trehalose, turanose, D-xylose) were transformed by strain 230 and 8 of them (the above-mentioned, except for N-acetyl-D-glucosamine and D-fructose) by strain CECT940. From these compounds, only α-D-glucose and L-mannose were oxidized by both *L. monocytogenes* strains as well as by CPA-7 and could represent target for competiveness in fresh-cut fruit. This narrow use of carbon and nitrogen sources by both strains of *L. monocytogenes* could indicate that the technology used is not suitable for the analysis of these strains, although it has been used in comparative studies including several variants of other strains of *L. monocytogenes* (Kastbjerg, Hein-Kristensen and Gram, 2014). Similar technologies with a broader spectrum of carbon compounds (i. e. Phenotype microarrays, Biolog MicroArray™), has shown to be useful for selecting highly competitive potential biocontrol agents (Blumenstein et al., 2015; Fox, Leonard, & Jordan, 2011) and could be suitable to further analyze the *L. monocytogenes*/CPA-7 niche overlap.

2.4 FINAL REMARKS

Although the number of putative mechanisms involved in the antagonistic activity of CPA-7 that have been explored in this study, we were not be able to clarify its mode of action. We could not demonstrate inhibition of growth of the foodborne pathogens tested in agar plates, fruit juices or fruit pieces, using the supernatants from CPA-7 monocultures or the homogenates of 'Galia' melon plugs. These results suggest that the concentration of bactericidal compounds, if any, was insufficient to be detected using the methods that were applied in our assay, or that the inhibition of growth could be mediated by a contact- or proximity-dependent mechanism involving bactericidal compounds or quorum-sensing molecules which can achieve inhibition at very low concentrations. Furthermore, as the production of antimicrobial molecules can be medium dependent, the specific conditions tested do not completely exclude the possibility of this occurring in the presence of different Carbon/Nitrogen sources

or in specific restrictive or stressful conditions. On the other hand, niche differentiation that was estimated from *in vitro* Carbon/Nitrogen utilization profiles that were performed using GN2/GP2 plates did not provide enough information to establish a suitable number of targets for competiveness allowing explaining the antagonistic effect observed in fruit matrices. Therefore, further studies of niche overlapping including a wider range of Carbon/Nitrogen sources that could be targeted for competition between this antagonist and foodborne pathogens as well as the induction of the host defense mechanisms, need to be carried out in order to ascertain its mode of action. Nonetheless, the inability of CPA-7 to produce biosurfactants, to form biofilms or to secrete alginate, and the lack of proteolytic or hemolytic activities in the conditions tested, could represent additional advantages to evaluate this strain for the Qualified Presumption of Safety (QPS) list of microorganisms, which is required in the EU for market authorization of biological agents that are intentionally added to foods.

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CHAPTER III

Effect of *Pseudomonas graminis* strain CPA-7 on the ability of *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* to colonize Caco-2 cells after pre-incubation on fresh-cut pear

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ABSTRACT

To further gain insight into the mechanism by which the biopreservative bacterium *Pseudomonas graminis* CPA-7 develop its antimicrobial activity, we have examined the effect that the prior interaction established between this bacterium and two foodborne pathogens on fresh-cut pear, has on their capacity to colonize human epithelial cells (Caco- 2 cell line) which is crucial for establishing infection. CPA-7 inhibited the growth of *L. monocytogenes* and *S. enterica* subsp. *enterica* ser. Enteritidis by 5.5 and 3.1 log_{10}, respectively, after 7 d of interaction at 10 °C. Furthermore, CPA-7 attenuated the adherence of *S. enterica* to Caco-2 cells by 0.8 log_{10} regardless of the pre-adaptation on the fruit. Conversely, the adhesiveness of *L. monocytogenes* was not influenced by the interaction with the antagonist but it was reduced by 0.5 log_{10} after incubation on the food matrix. Pathogen-antagonist-food matrix interaction was associated to a significant reduction of the relative invasiveness of both pathogens, by 1.3 log_{10} in the case of *L. monocytogenes* and to an undetectable level (below 5 CFU g^{-1} fruit) for *S. enterica*. CPA-7 can adhere to and internalize into intestinal epithelium which enables it for competition. Its adherence positively correlates with the multiplicity of infection in respect to Caco-2 cells, increasing by 0.6 log_{10} in a range of 0.1:1 to 100:1. For the same levels of inoculum, internalized cells could only be detected after 7 d of pre-adaptation in the fruit (pH 4.5 - 5.0). However, the combination of simulated digestion and habituation on the fruit resulted in a significant reduction of CPA-7 populations (by 2 log_{10} more after 7 d of incubation than on inoculation day) as well as in the reduction of its adhesiveness (by 0.8 log_{10}) and invasiveness (to undetectable levels).

2.1 INTRODUCTION

Pseudomonas graminis strain CPA-7 is a Gram-negative bacteria with antagonistic effect on *L. monocytogenes*, *S. enterica* subsp. *enterica* and *E. coli* O157:H7 on fresh-cut apples, peaches, pears and melons (Abadias et al., 2014; Alegre et al., 2013a, b). Our work group has been working on elucidating the mechanisms through which this strain controls the populations of foodborne pathogens. Although a number of *in vitro* and *in vivo* experiments related to the production of antibiotics, biosurfactants and exo-proteases have been performed, its mode of action could not be elucidated (Collazo et al., 2017). Hence the impairment of pathogenesis-related characteristics of foodborne pathogens was hypothesized as a putative mechanism for antagonism.

To cause gastrointestinal infections, foodborne pathogenic bacteria must adhere to the epithelium, activating the release of enzymes and toxins that lead to the initiation of necrotic processes into the target cells facilitating their internalization into the tissue (Jankowska et al., 2008; Tamburro et al., 2015). Among foodborne pathogens, *Salmonella* spp. and *Listeria* spp. are typical intracellular pathogens, capable of surviving and actively replicating inside epithelial cells (Götz and Goebelt, 2010).

Reduction of the colonization capacities of foodborne pathogens by interfering with their attachment to the human intestinal epithelium or by altering their pathways to penetrate into the enterocytes is a mechanism to antagonize them which has been explored for probiotic bacteria (Feng et al., 2015; Tamburro et al., 2015). Anti-adherent abilities of probiotics can be mediated by the production of inhibitory substances and by exclusive competition because of their capacity to survive in low pH conditions, auto-aggregate or co-aggregate with pathogens and adhere to the human gastrointestinal tract (Pan et al., 2008; Vuotto et al., 2014). In such a way, *Lactobacillus acidophilus, L. casei and L. rhamnosus* were shown to actively reduce the adhesion of *S. enterica* and *L. monocytogenes* to

human adenocarcinoma cell lines to less than 50 % (Dutra et al., 2016; Tabasco et al., 2014). The ability of non-acidolactic probiotic bacteria such as *Clostridium butyricum* CB2 and *Enterococcus mundtii* ST4SA to impair foodborne pathogens adherence to intestinal epithelial cells have also been studied (Botes et al., 2008; Pan et al., 2008). However, to the best of knowledge, no information in the body of literature was found concerning the putative effect of non-probiotic antagonists, such as the one tested in the present study, on the behavior of the pathogens inhabiting contaminated food once they come into contact with the human gastrointestinal tract.

A great part of the studies that have focused on this matter have been carried out using Caco-2 cells, a human colon adenocarcinoma cell line, that reproduce morphological and functional properties of human intestinal epithelium. They differentiate in a similar manner to normal small intestinal cells, expressing characteristics of immature or mature enterocytes with functional brush border microvilli and apical hydrolases (Gaillard, Finlay and Line, 1996). After confluence (5 to 6 days of culture), the process of polarization of Caco-2 cells involves the whole monolayer while differentiation takes another ten days to complete (Gaillard, Finlay and Line, 1996). Polarization and differentiation of this cell line have shown to influence the invasion process of *Listeria* spp. and *Salmonella* spp. because surface molecules such as the receptors that mediate their internalization express themselves asymmetrically (Boumart et al., 2014; Jankowska et al., 2008).

The aim of this research was to study the effect of the interaction between the antagonist *P. graminis* CPA-7 and the enteropathogenic bacteria *L. monocytogenes* and *S. enterica* in a food matrix on the ability of these microorganisms to adhere to and to invade differentiated colorectal human adenocarcinoma cells Caco-2. For this, the antagonist and each pathogen were pre-incubated aerobically for seven days on fresh-cut pear upon abused refrigerated conditions (10 °C). Additionally, survival of CPA-7 during static

simulated gastrointestinal digestion and its subsequent adhesion to and invasion into Caco-2 cells was evaluated.

3.1 MATERIALS AND METHODS

3.1.1 FRUIT PROCESSING

Pears (*Pyrus communis* L cv. 'Conference') were obtained from local packing-houses (Lleida, Spain). Prior to the experimental studies, they were washed in running tap water, disinfected with 700 mL/L ethanol and air-dried at room temperature. Pears were peeled and cut into eight wedges using a slicer/corer or into plugs (cylinders of 1.2 cm diameter, 1 cm thickness) using a cork borer. Quality parameters: pH, soluble solids, firmness and titratable acidity were initially determined as described elsewhere (Colas-Meda et al., 2017).

3.1.2 BACTERIAL STRAINS AND CULTURE CONDITIONS

The strain *P. graminis* CPA-7 (Alegre et al., 2013b) was used as antagonist. Five strains of *S. enterica* subsp. *enterica*: ATCC BAA-707, ATCC BAA-709, ATCC BAA-710, ATCC BAA-711 and CECT 4300 belonging to serovars Agona, Michigan, Montevideo, Gaminara and Enteritidis, respectively, were used. Five strains of *L. monocytogenes*: CECT 4031, CECT 933, CECT 940, CECT 4032 and Lm 230/3 (Abadias *et al.*, 2008) belonging to serotypes 1a, 3a, 4d, 4b and 1/2a, respectively, were tested.

Strains of *S. enterica* and *L. monocytogenes* were grown individually as described by Abadias et al. (2014) in order to obtain concentrated suspensions. CPA-7 was grown in tryptone soy broth (TSB) and a concentrated suspension was obtained following the above mentioned procedure. Concentrations were checked by plating appropriate ten-fold dilutions in saline peptone (8.5 g L^{-1} NaCl and 1 g L^{-1}

peptone) onto Xylose Lysine Deoxycholate Agar (XLD-agar) for *S. enterica*, onto Palcam Agar Base with selective supplement, for *L. monocytogenes* and onto tryptone soy agar (TSA) for CPA-7. *S. enterica* and *L. monocytogenes* plates were incubated at 37 °C for 24 and 48 h, respectively, and CPA-7 was grown at 30 °C for 48 h. All synthetic culture media and supplements were purchased from Biokar Diagnostics, Beauveais, France.

3.1.3 Caco-2 cells culture conditions

Human intestinal Caco-2 cells (ECACC 86012202) were grown in 24-wells cell culture polystyrene plates (Falcon, USA) in Dulbecco's Modified Eagle Medium (DMEM 1X, Gibco, MA, USA) supplemented with 200 mL L^{-1} inactivated fetal bovine serum (Gibco, MA, USA) and 10 mL L^{-1} non-essential aminoacids solution as described by Jankowska et al. (2008). Plates were maintained at 37 °C in a humidified incubator at 5 % CO_2. For the experiments, cells were seeded at 5 x 10^4 cells per well and grown until differentiation (12-13 days), refreshing the culture medium every two days. Penicillin (20.000 U mL^{-1}) and streptomycin (20 mg mL^{-1}) were added to the culture medium except for 24 h prior to virulence assays. Antibiotics and aminoacids were purchased from Sigma-Aldrich, St Louis, USA.

3.1.4 Selection of pathogenic strains

As shown in Fig. **III.**1, preliminary trials were performed in order to select one strain of *S. enterica* subsp. *enterica* and one strain of *L. monocytogenes* out of five strains from of each species from our laboratory collection. Susceptibility of each strain to be effectively controlled by CPA-7 on pear plugs at 10 °C and high virulence, evaluated by their ability to adhere to and to invade Caco-2 cells were used as selective criteria. For the antagonist effectiveness test, bacterial suspensions containing 10^5 CFU mL^{-1} of each pathogenic strain inoculated alone or in combination with the antagonist, this latter inoculated at 10^7 CFU mL^{-1},

were prepared with deionized water. In the same way, a CPA-7 control treatment was included. Pear plugs were dip-inoculated at a ratio of 1:2 (pear weight: volume of bacterial suspension) for 2 min in agitation in an orbital tabletop shaker and subsequently drained and air-dried. Bacterial populations were analyzed in triplicated by plate count just after inoculation and after six days of incubation in sterile glass tubes at 10 °C, allowing air exchange. For this, 1 g of sample was homogenized in 9 mL of buffered peptone water (BPW, Oxoid LTD, UK) at 7 strokes/s during 2 min in a Bagmixer 100 (Minimix, Interscience, Saint Nom, France).

For virulence screening, each strain was inoculated individually in DMEM medium to achieve a final concentration of 10^5 CFU mL^{-1}. Afterwards, 12 wells of 24-well plates containing post-confluent differentiated Caco-2 cells were inoculated with 500 µL per replicate per treatment. Plates were incubated for 1 h in a humidified incubator at 37 °C and 5 % CO_2. Non-adhered bacterial cells were removed by two washes with phosphate buffered saline (PBS, 0.137 mol L^{-1} NaCl, 0.0027 mol L^{-1} KCl, 0.01 mol L^{-1} Na_2HPO_4, 0.0018 mol L^{-1} KH_2PO_4, pH 7.4) and Caco-2 cells from 6 wells/replicate were lysed with cold 1 mL L^{-1} Triton X-100 (Sigma, UK) and collected in sterile glass analysis tubes. The other 6 wells were incubated for 2 h in the same conditions with DMEM supplemented with Gentamicin (150 µg mL^{-1}) to kill extracellular bacteria. Viable adhered and internalized cells were estimated by plate count as previously described.

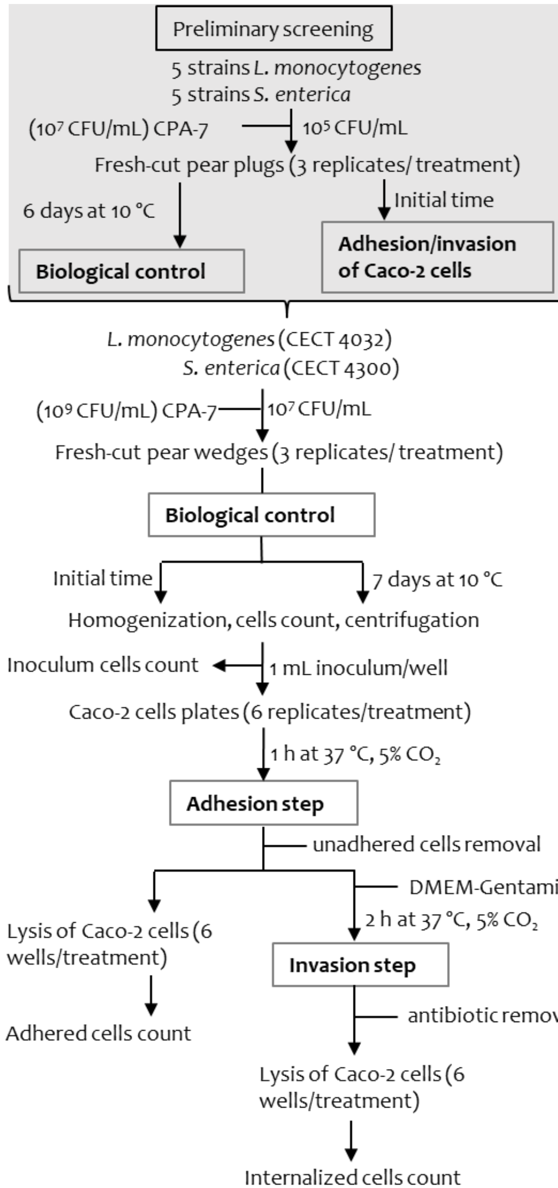

Fig. III.1. Diagram of the experimental design of biological control and adhesion/invasion assays.

3.1.5 ANTAGONIST-PATHOGEN INTERACTION ON THE FRUIT

Prior to *in vitro* virulence assays, the selected strains *L. monocytogenes* CECT 4032 and *S. enterica* subsp. *enterica* ser. Enteritidis (CECT 4300) were submitted to the interaction with CPA-7 in separate trials which included three treatments

that were prepared using deionized water: **i**) CECT 4032 or CECT 4300 control, **ii**) CPA-7 + CECT 4032 or CECT 4300 and **iii**) CPA-7 control. In all cases, concentrations of the pathogens (10^7 CFU mL^{-1}) and the antagonist (10^9 CFU mL^{-1}) were 2 log_{10} above the amount used in the selection phase, in order to detect bacterial cells at the end of the invasion process, considering the expected population reduction due to antagonism. Pear slices were dip-inoculated in each treatment at a ratio of 1:2 (pear weight: volume of bacterial suspension) as previously described. Samples were examined on the day of inoculation and after 7 d of incubation at 10 °C in 500 mL lidded-polyethylene trays not hermetically closed to allow air exchange. Populations of *S. enterica, L. monocytogenes* and CPA-7 were determined at each sampling time following the same procedure explained in section 2.2. For this, 10 g of pear of each sample was previously homogenized in 90 mL BPW within a 400 mL sterile full-page filter bag (Bagpage, Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set at 4.2 strokes/s for 90 s. The experiments were performed two independent times for each pathogen including three replicates per treatment and sampling time. Each replicate was analyzed in duplicate in the subsequent adhesion and invasion assays.

3.1.6 ADHESION TO AND INVASION INTO CACO-2 CELLS

Fifty milliliters of each pear homogenate were collected at each sampling time, individually centrifuged at 15777 × g for 20 min at 4 °C in a Sorvall Legen XTR centrifuge (Thermo Scientific, USA) and re-suspended in 13 mL of DMEM medium to obtain concentrated suspensions that were used as inocula for the adhesion and invasion assays. Afterwards, 1 mL of bacterial suspension per replicate per treatment was added to each of 12 wells containing 2 x 10^6 differentiated Caco-2 cells/well. Multiplicity of infection (MOI) was set at 0.1:1 (bacteria: Caco-2 cells) for both pathogens and at 1:1 for the antagonist control on the initial day of the experiments. The plates were incubated and analyzed

following the experimental scheme described in section 3.2.4 (Fig. **III**.1). Inoculated, adhered and internalized bacteria were estimated by plate count as previously described. Concomitantly, the viability of CPA-7 in the conditions assayed (37 °C in humid atmosphere with 5 % CO_2) was assessed. For this, 1 mL of the CPA-7 suspension (10^6 CFU/mL DMEM), was added to nine wells of a 24-wells plate and analyzed in triplicate by plate count after 1, 2 and 3 h of incubation, as described in section 3.2.2.

The impact of MOI in *S. enterica* and *L. monocytogenes* adhesion and invasion was evaluated using bacterial suspensions in a range of concentration from 10^2 to 10^7 CFU mL^{-1} DMEM. Then, 1 mL of each suspension was inoculated on Caco-2 plates and adhered and internalized cells were estimated following the procedure previously described. All experiments were performed twice.

3.1.7 SURVIVAL AND COLONIZATION ABILITIES OF CPA-7 IN A SIMULATED GASTROINTESTINAL TRACT

Additionally, to assess the possibility for competition between CPA-7 and the evaluated pathogens once in contact with the intestinal epithelium in a more realistic scenario, its survival under *in vitro* static gastrointestinal passage and the subsequent adhesion to and invasion into Caco-2 cells was tested. For this, pear wedges were inoculated as previously described and then submitted to simulated digestion at initial time or after seven days of storage at 10 °C in air. Gastrointestinal digestion protocol was adapted from that described by Zudaire et al. (2017) based on the standardized method of Minekus et al. (2014). Briefly, 5 g of inoculated pear wedges was placed into a sterile filter plastic bag (BagPage 80 mL, Interscience BagSystem, Saint Nom, France) with 3.5 mL of synthetic salival fluid tempered at 37 °C. The mixture was then homogenized 7 strokes/s during 2 min in a Bagmixer 100 (Minimix, Interscience, Saint Nom, France). Then, 1.5 mL of salivary enzymatic solution was added to the sample and incubated

statically at 37 °C for 5 min. Afterwards, the sample was mixed with 7.5 mL of synthetic gastric fluid and 2.4 mL of gastric enzymatic solution, adjusted to pH 3 with 1 mol L^{-1} NaOH and incubated statically for 2 h at 37 °C. Then, 11 mL of synthetic intestinal fluid and 9 mL of intestinal enzymatic solution was added to each sample and incubated statically at 37 °C for 2 h. After every phase, pH was measured and 1 mL of each sample was used to estimate CPA-7 populations by plate count as previously described. All enzymes were purchased from Sigma-Aldrich, St Louis, USA.

Subsequently, adhesiveness and invasiveness of CPA-7 before and after habituation on pear wedges followed by *in vitro* gastrointestinal simulation was tested in the same experimental conditions as previously described and following the same scheme. For this, resulting cells from the gastrointestinal passage were harvested by centrifugation at 15777 × *g* for 20 min at 4 °C, were diluted in 1 mL of DMEM medium at a concentration of 10^6 CFU g^{-1} fruit and used as the inoculum that was added to the Caco-2 plates. Adhered and internalized cells were estimated by plate count on TSA as previously described. These assays were performed in triplicate, two independent times.

3.1.8 Expression of results and statistical analysis

Microbiological data were calculated as colony forming units per milliliter (CFU mL^{-1}) and transformed to log_{10} CFU g^{-1} fruit. The adhesion and invasion efficiencies of each pathogen were expressed as logarithmic reductions calculated as follows: log_{10} (N_1/N_0); where N_1 refers to the bacterial count per gram of fruit at the end of the adhesion or invasion step and N_0 refers to the initial bacterial count per gram of fruit inoculated onto Caco-2 cells. Survival capacity of CPA-7 after each phase of gastrointestinal simulation was calculated as logarithmic reductions: log_{10} (N_1/N_0), where N_1 is the bacterial count per gram of fruit at the end of the analyzed digestion phase and N_0 is the initial count per

gram of fruit before digestion. All data were tested for agreement to normal distribution and homoscedasticity. The significance of the differences between factors were determined by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$). Statistical analyses were performed using Statistical software JMP (version 8.0.1 SAS Institute Inc., NC, USA).

3.2 RESULTS AND DISCUSSION

3.3.1 SELECTION OF PATHOGENIC STRAINS

Initial screening of five strains of each pathogen, *L. monocytogenes* and *S. enterica,* was carried out to select one strain of each species for the subsequent evaluation of the effect of the interaction with the antagonist on their virulence. Previous biological control assays had evidenced growth inhibition of cocktails of the analyzed strains by CPA-7 on fresh-cut fruit but individual evaluation of this effect had not been performed (Alegre et al., 2013a, 2013b; Abadias et al., 2014). In biological control assays, the initial populations of CPA-7 on the fruit were around 6.3 $\log_{10}$ CFUg^{-1} fruit which represents at least 2 $\log_{10}$ above each pathogen's population. Populations of *L. monocytogenes* strains were initially 3.9 $\log_{10}$ CFU g^{-1} fruit. After six days of incubation at 10 °C strains CECT 4032, CECT 940 and Lm 230/3 were reduced by more than 3 $\log_{10}$ when co-inoculated with the antagonist with respect to the control (Fig. **III.**2A).

Compared to the mentioned strains, CECT 4031 was significantly less inhibited (reduction by 2.6 $\log_{10}$) while no inhibitory effect was observed on strain CECT 933. On the other hand, initial populations of *S. enterica* isolates on fruit plugs were around 4.1 $\log_{10}$ CFU g^{-1} fruit. As observed on day six, strains BAA-711 and CECT 4300 were the most susceptible to biological control (reduced by more than 4 $\log_{10}$) (Fig. **III.**2A).

Regarding their virulence, *L. monocytogenes* CECT 4032, CECT 933, CECT 940 and Lm 230/3 and were the most adhesive to Caco-2 cells showing less reduction of adhered cells in respect of the initial inoculum (Fig. **III**.2B). In the same way, CECT 4032 was the most efficient in terms of invasion into epithelial cells.

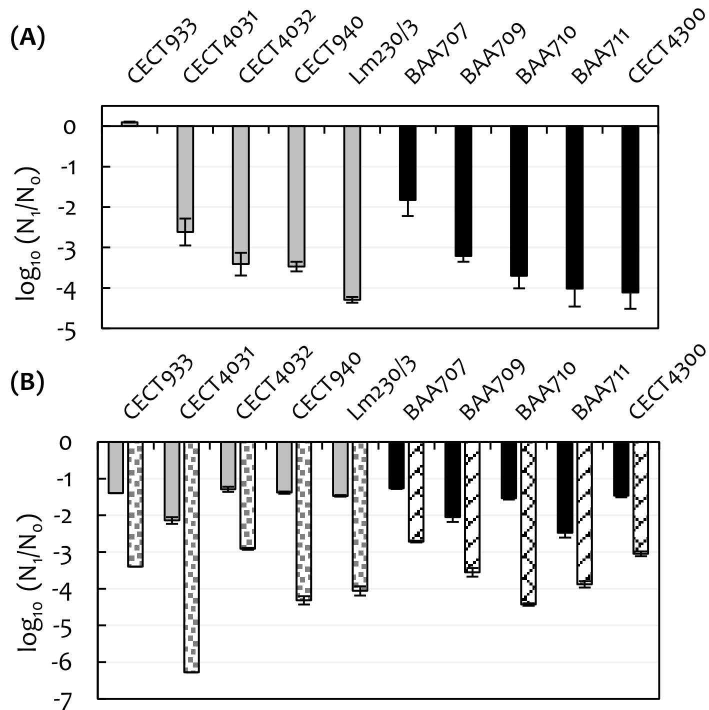

Fig. **III**.2. Preliminary screening including several strains of *L. monocytogenes* and *S. enterica* subsp. *enterica.* (**A**) Inhibition of growth of *L. monocytogenes* (▢) and *S. enterica* (■) when co-inoculated with *P. graminis* (CPA-7) on pear plugs after 6 d of aerobic incubation at 10 °C. (**B**) Non-adhered *L. monocytogenes* cells (■) and *S. enterica* cells (▢) and non-internalized *L. monocytogenes* cells (▨) and *S. enterica* cells (▨) when inoculated individually in DMEM onto Caco-2 cells. Columns represent the means of $\log_{10} (N_1/N_0)$, were (A) N_1 is the bacterial count of the pathogen-CPA-7 co-inoculated treatment at 6 d and N_0 is the bacterial count of the pathogen control at this sampling point, (B) N_1 is the bacterial count after the adhesion or the invasion step and N_0 is the bacterial count of the inoculum added to the Caco-2 monolayer. Each experiment was performed twice including three replicates per each strain. Error bars represent standard errors of the mean.

Among *S. enterica* strains, BAA-707, BAA-710 and CECT 4300 had similar attachment efficiencies that were higher than the observed for the strains BAA-

709 and BAA-711 (Fig. **III**.2B). BAA-707 and CECT 4300 were also the most invasive strains. According to the obtained results, *L. monocytogenes* CECT 4032 and *S. enterica* ser. Enteritidis (CECT 4300) isolates, which showed a combination of high virulence (adherence and invasion capacities) and marked susceptibility to CPA-7, were selected to investigate the antagonistic effect of the CPA-7 on their colonization properties.

3.3.2 Antagonist-pathogen interaction on the fruit

The interaction of *S. enterica* subsp. *enterica* ser. Enteritidis (CECT 4300) and *L. monocytogenes* CECT 4032 and CPA-7 was evaluated on pear wedges stored in plastic trays during 7 days at 10 °C in air. We selected 10 °C as it has shown to be a chilling temperature which better reproduces real conditions on refrigerated display cabinets where processed fruit and vegetable are usually stored in retail food stores (Morelli et al., 2012; Lundén et al., 2014). It is also a suitable refrigerated temperature for tracking changes in the populations of *S. enterica* and *L. monocytogenes* during biological control assays as it fits into the growth temperature range for both microorganisms (8 - 45 °C and 0 - 45 °C, respectively) (Khaleque and Bari, 2015).

At initial time, CPA-7 populations on the fruit were 7.5 log_{10} CFU/g fruit on average, which represent approximately 1.8 log_{10} above the populations of the pathogens (Fig. **III.**3). After seven days of incubation, CPA-7 showed significantly more effectiveness ($P < 0.0001$) at inhibiting the growth of *S. enterica* than of *L. monocytogenes* (reductions by 5.5 log_{10} and 3.1 log_{10}, respectively). Similar storage conditions has been previously used in experiments that have shown the effectiveness of CPA-7 at reducing the populations of cocktails of *S. enterica* and *L. monocytogenes,* by 4 and 3 log_{10} respectively, on fresh-cut melon and apple (Alegre et al., 2013a; Abadias et al., 2014).

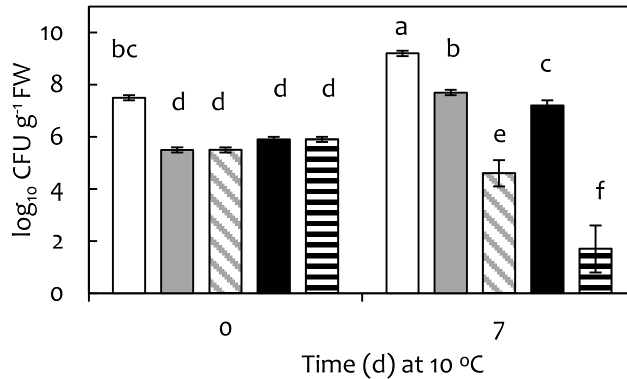

Figure III.3. Effect of *P. graminis* strain CPA-7 on the populations of *L. monocytogenes* CECT 4032 and *S. enterica* subsp. *enterica* ser. Enteritidis (CECT 4300) after 7 d of aerobic incubation on pear wedges at 10 °C. Columns represent the means of bacterial counts transformed to log_{10} CFU/g fruit of three replicates from two independent assays: ( ) CPA-7 control, ( ) CECT 4032 control, ( ) CECT 4032 co-inoculated with CPA-7, ( ) CECT 4300 control, ( ) CECT 4300 co-inoculated with CPA-7. Different letters above columns indicate significant differences among treatments according to analysis of variances (ANOVA) (n=6) and Tukey's test $P < 0.05$).

3.3.3 Adhesion to and invasion into Caco-2 cells

We evaluated the effect of the antagonist-foodborne pathogen interaction in a food matrix on the virulence of the pathogens, focusing on adhesion to and invasion of human gastrointestinal epithelial cells because these are preliminary steps in infection. Interference with the adherence and invasion of pathogens to epithelial cells has previously suggested as a mechanism for antagonism (Cells, Coconnier, Lie, Lorrot, & Servin, 2000; Burkholder & Bhunia, 2010).

Concentrated fresh-cut pear homogenates resulting from samples inoculated individually with CPA-7, *S. enterica* subsp. *enterica* ser. Enteritidis CECT 4300 and *L. monocytogenes* CECT 4032 or with a combination of each pathogen and the antagonist were obtained at initial day and after 7 d of storage at 10 °C and used as inocula for adhesion and invasion assays. Relative adhesion to Caco-2 cells of *L. monocytogenes* was not influenced by CPA-7 on inoculation day or after

storage while it was significantly reduced (by 0.5 $\log_{10}$) after habituation in the fruit matrix (Fig. **III.**4A). In similar experimental conditions the adhesion to Caco-2 cells of another strain of *L. monocytogenes* (Lm 230/3, serotype 1/2a) was reduced after habituation on fresh-cut pear (Colas-Meda, Viñas, *et al.*, 2017).

In contrast, the relative adherence of *S. enterica* was significantly reduced (by 0.8 $\log_{10}$) in the presence of the antagonist compared to the control treatment regardless of the habituation in the fruit (Fig. **III.**4A). Similarly, the relative adhesiveness of the CPA-7 control did not show to be influenced by the incubation period. Reduction of the adhesiveness of *S. enterica* in the presence of CPA-7 could be due to competition for sites on Caco-2 surfaces as the antagonist cells were two orders of magnitude more abundant than *S. enterica* cells. However, the same proportion was used for *L. monocytogenes* and no effect on adhesiveness was observed which could indicate that they do not compete for the same receptors. Previous works have shown that beneficial bacteria can express molecules that attach to glycolipids or glycoproteins which enable them to compete with enteropathogens for the same carbohydrate receptors in the gut (Neeser *et al.*, 2000). Mechanisms for adherence of antagonistic probiotic bacteria and have shown to be either nonspecific, which are mediated by electrostatic interactions and hydrophobic or steric forces; or specific, which involve the adhesin/receptor interaction and/or the secretion of extracellular proteinaceous adherence-promoting factors (Muñoz-Provencio et al., 2009; Wang et al., 2014; Sribuathong et al., 2014). However, CPA-7 is not a probiotic and so far it has not shown to be able of produce antimicrobial metabolites that could be linked to a putative ability to exclude or displace foodborne pathogens (Collazo, Abadias, *et al.*, 2017). Furthermore, it was reported that it fails to grow at temperatures higher than 33 °C on TSA plates (Alegre et al., 2013a). However, its viability in the conditions tested in the present study had not previously been assessed. We demonstrated that CPA-7 was able to survive and maintain its populations in DMEM medium at 37 °C in 5 %

CO_2 in humidified atmosphere for at least 3 h (data not shown). This would enable it for steric disruption or competition for adhesion sites in the conditions assayed in this study.

These results agree with previous experiments performed with isolates of *P. fluorescens* of different origins: MF37 (crude milk isolate) and MFN1032 (clinical isolate). These isolates had also been considered to be psychrotrophic and unable to grow at temperatures above 32 °C, but showed to be able to survive and even adapt to growth at 37 °C or above and subsequently adhere to and translocate across the cytoplasm to the basal membrane of differentiated intestinal epithelial cells (Caco-2/TC7) (Madi *et al.*, 2010a).

Regarding the capacity of invasion, no significant differences could be observed on inoculation day between the control and the CPA-7 co-inoculated treatment for any of the evaluated pathogens (Fig. **III.**4B). Nevertheless, after 7 d the number of non-internalized cells of *L. monocytogenes* when co-inoculated with CPA-7 increased by 1.3 $log_{10.}$ Thus, the invasion efficiency of this strain was significantly reduced compared to the control. In contrast with the effect observed on adhesiveness, habituation on the fruit did not influence the invasiveness of the *L. monocytogenes* when inoculated alone. Concerning *S. enterica*, the combined effect of the habituation on the fruit and the interaction with the antagonist resulted in the reduction of its efficiency of invasion. This is suggested by the fact that on day 7 no viable internalized cells of the co-inoculated treatment could be detected for inocula ranging from 10^3 to 10^5 CFU mL^{-1} while on inoculation day about 1.1 log_{10} CFU g^{-1} fruit of internalized cells was detected for an inoculum containing 10^5 CFU mL^{-1} (Fig. **III.**4B).

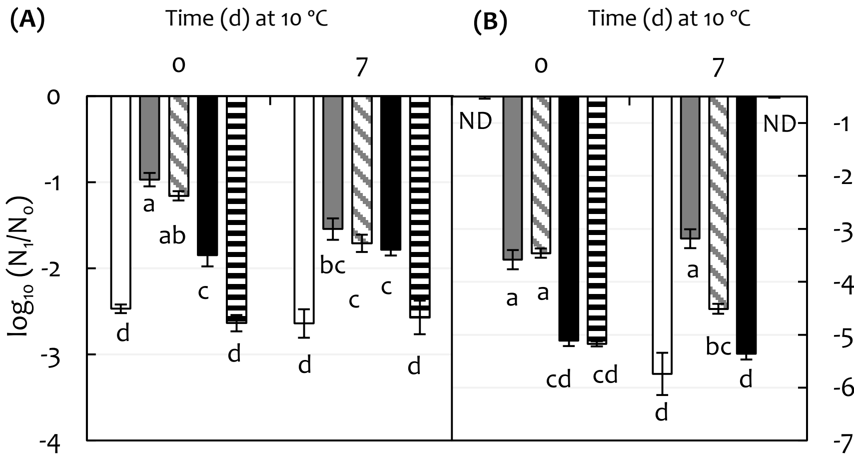

Figure III.4. Reduction of (A) adhesiveness and (B) invasiveness to Caco-2 cells of *L. monocytogenes* CECT 4032 and *S. enterica* subsp. *enterica* ser. Enteritidis CECT 4300 at initial time and after 7 d of aerobic incubation on pear wedges at 10 °C. Columns represent (☐) CPA-7 control, (■) CECT 4032 control (▧) CECT 4032 co-inoculated with CPA-7, (■) CECT 4300 control and (▤) CECT 4300 co-inoculated with CPA-7. Results are expressed as means of logarithmic reductions: $\log_{10}(N_1/N_0)$, where N_1 represent the cell counts after adhesion or invasion step and N_0 represent the cell counts of the inocula added to Caco-2 cells monolayers in each case. ND: not detected, internalized cells below detection limit of 5 CFU g^{-1} fruit. All assays were performed two independent times per each microorganism (n=12). Different letters represent significant differences among means of different treatments according to analysis of variance (ANOVA) and Tukey's test ($P < 0.05$).

On the other hand, preadaptation in the food matrix by itself did not influence the invasiveness of this pathogen since about 2.6 $\log_{10}$ CFU g^{-1} fruit of internalized cells were detected in the control treatment for both 10^5 and 10^6 CFU mL^{-1} inocula before and after incubation, respectively. Conversely, habituation on the fruit significantly affected the relative invasion of CPA-7 which increased from an undetectable level (below a detection limit of 5 CFU g^{-1} fruit) for a 10^6 CFU mL^{-1} inoculum on initial day, to about 2 $\log_{10}$ CFU g^{-1} for inocula that ranged in a similar concentration (10^5 to 10^9 CFU mL^{-1}) on the 7th day.

Variations in the concentrations of the inocula used for the adhesion/invasion assays were conditioned by pre-incubation in the food matrix which led to an increase of the control populations of *S. enterica*, *L. monocytogenes* and CPA-7 and a concomitant reduction of pathogen's populations due to the antagonistic

effect. Therefore, microbial population dynamics was related to a variation of the multiplicity of infection (MOI) in the subsequent adhesion and invasion assays. Analysis of the effect of different MOIs were performed using dilutions of each pathogen in DMEM medium showed that relative adhesion of *S. enterica* CECT 4300 remained stable for MOIs ranging from 0.0001:1 to 0.01 but it increased for an MOI of 1:1. For *L. monocytogenes* CECT 4032 the adhesion increased in a different MOI range (0.001:1 < 10:1 < 0.1:1) (data not shown). Increased relative adherence (by 0.6 $\log_{10}$) of CPA-7 to the monolayers positively correlated to the increase of multiplicity of infection of the antagonist with respect to Caco-2 cells (0.1:1 < 1:1) reaching to a saturation point at 100:1. Antagonistic strains of *Lactobacillus (L. plantarum* PD110 and *L. cellobiosus* RE 33) have also shown increasing adherence ability at inocula ranging from 10^6 to a saturation point of 2×10^8 CFUmL^{-1} (MOI 1:1 and 1:100, respectively) (Sribuathong, Saengprakai and Trevanich, 2014). Disrupted adhesion was attributed to steric impediment caused by bacterial clusters.

As for invasive capacities, we could not observe an effect of MOI on *S. enterica* internalization cells in a range of 0.0001:1 to 1:1 which corresponded to the levels obtained after interaction with the antagonist. The increase of MOI from 0.001:1 to 0.1:1 was associated to an increase of invasion of *L. monocytogenes.* However, invasiveness remained stable at higher MOI levels (10:1). These results agreed with previous reports regarding the adhesion and invasion capacities of *S. typhymurium* strain C52 to monolayers of intestine cells (Int-407), which varied between certain MOI ranges (0.1:1 = 1:1 < 100:1) (Kusters *et al.*, 1993). MOIs have previously shown to influence the efficiencies of infection of both *Salmonella* spp. and *Listeria* spp. at different levels according to the strain and to the experimental conditions tested (Kusters, Mulders-Kremers, Van Doornik, & Van der Zeijst, 1993; Kushwaha & Muriana, 2010).

The combined effect of cell-to-cell contact, food matrix microenvironment and storage conditions could have led to the modulation of the production or the

functionality of the molecules involved in adhesion, invasion and multiplication of the pathogens evaluated in the intestinal epithelium. Several reports have stated the role of environmental factors such as growth temperatures, osmotic stress, pH, anaerobiosis, and cell-to-cell signaling prior to infection, in the regulation of virulence genes of intracellular foodborne pathogens (Ivy, Chan, Bowen, Boor, & Wiedmann, 2010; Pricope-Ciolacu, Nicolau, Wagner, & Rychli, 2013; Zilelidou et al., 2015). For instance, cell-to-cell contact between several combinations of *L. monocytogenes* strains (e.g. Scott A serovar 4b and PL25 serovar 1/2b) co-cultured in a nutrient-rich broth resembling a food matrix, had an inhibitory effect in their growth as well as reduced their capacity to invade and multiply within Caco-2 cells (Zilelidou et al., 2015).

Molecular and physiological basis of virulence reduction have been associated to changes in expression of stress-response genes such as *sigB* as well as of virulence genes (*plcA, iap,* and *hly*) in *L. monocytogenes* of different serovars, during habituation in different food matrices at refrigerated temperatures (4 °C and 12 °C) (Rantsiou et al., 2012). Down-regulation of genes involved in the invasion process (including *actA*, *hly, inlA,* and *plcA*) leading to differential protein expression, has also resulted in reduced invasiveness in antagonist-pathogen interactions (*L. monocytogenes* CMCC54001 and *Bifidobacterium longum* NCC2705) (Tan et al., 2012).

3.3.4 Survival and colonization abilities of CPA-7 in a simulated gastrointestinal tract

In view of the positive results obtained in the adhesion and invasion assays, the abilities of CPA to survive simulated human gastrointestinal passage and to subsequently colonize the intestinal epithelium were evaluated. On the first day of the experiments, the populations of CPA-7 inoculated onto 'Conference' pear wedges remained unaltered after the oral phase but they were drastically

reduced after the gastric phase (by 6.5 log_{10}) (Fig. **III.**5). However, a significant population increase (by 2.7 log_{10}) was recorded after the intestinal phase. At this sampling point, CPA-7 was able to survive in acidic conditions, bile salts and corporal temperature of the host, but its population dynamics was positively correlated to changes of pH. In this way, bacterial number was drastically reduced after the gastric phase, as pH dropped from 6.4 to 2.0 and recovered along with the rise of pH to 7.5 during the intestinal phase.

During the 7 d of incubation on the fruit CPA-7 populations increased by 1.8 log_{10} but their ability to overcome simulated gastrointestinal conditions was compromised. Unlike the observed on inoculation day, initial populations were reduced by 0.7 log_{10} during the oral phase, then dropped significantly (by 6.0 log_{10}) during the gastric phase and remained stable during the intestinal phase. At this sampling time, the variations of pH were similar to the occurred on first day of analysis, being 6.0, 2.9 and 8.2 after the oral, gastric and intestinal phases, respectively.

However, after habituation in the food matrix surviving cells from the gastric phase were unable to grow during the subsequent phase, reaching similar numbers at the end of the gastrointestinal passage although initial population was 2 log_{10} higher. In spite of the low number of viable cells after gastrointestinal passage, the adhesion capacity of CPA-7 was the same regardless of the stress experienced during digestion, suggesting that cells that reach the intestinal epithelium could stablish competition for adhesion sites. In previous experiments no viable cells of this antagonist could be detected after simulated gastric digestion but assayed conditions (pH 2; 2 mmol L^{-1} HCl, 0.3 g L^{-1} pepsin) and food matrix (fresh-cut ‘Golden delicious’ apple) differed from those tested in the present work (Alegre, Viñas, Usall, Teixido, *et al.*, 2013).

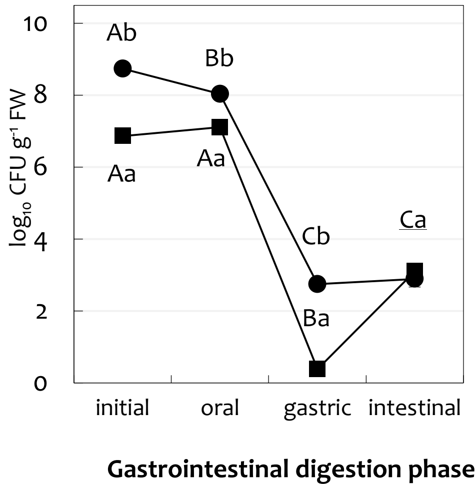

Figure III.5. *P. graminis* strain CPA-7 population dynamics during *in vitro* static gastrointestinal simulation at inoculation day (■) and after 7 d (●) of incubation on pear wedges at 10 °C. Symbols represent means of viable counts after each phase, from two independent assays (n=6). Different lowercase letters represent significant differences at each phase throughout time. Different capital letters indicate significant differences among phases at each sampling time according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent overlapped symbols.

We expected the population size of *L. monocytogenes* to remain invariable after the whole gastrointestinal passage and evaluated directly its ability to attach and to penetrate intestinal cells, based on the observations of a similar model of static gastrointestinal simulation, where bacterial counts of the strain CECT 4032 inoculated on fresh-cut 'Conference' pear remained stable at the end of gastrointestinal passage, after seven days of storage at 10 °C (Iglesias *et al.*, 2017). Similarly, populations of *L. monocytogenes* strain 230/3 inoculated on 'Conference' pear did not change along the passage through a simulated static gastrointestinal tract after 6 days of storage at 10 °C in air (Colas-Meda, Abadias, *et al.*, 2017).

3.4 CONCLUSIONS

The data presented herein showed that the interaction pathogen-antagonist-food matrix was associated to the reduction of the virulence features of the pathogens. Invasiveness of *S. enterica* subsp. *enterica* ser. Enteritidis CECT 4300 and *L. monocytogenes* CECT 4032 to Caco-2 cells were reduced after seven days

of co-incubation with *P. graminis* CPA-7. Adhesion to Caco-2 cells of the analyzed pathogens was differentially influenced by the antagonist and by habituation in the fruit. The obtained results could be explained by a combined effect of several processes, i) cell-contact-dependent competition established in the food matrix which could modulate the expression of genes involved in adhesion and invasion leading to the impairment by the antagonist of the pathogen's virulence traits associated with host colonization; ii) physical competition for adhesion to the epithelial membranes which is linked to the proportion of antagonist-pathogen cells and; to some extent, to the multiplicity of infection.

Further investigation should be carried out to determine the effect of the CPA-7 in the regulation of specific virulence-related genes of *L. monocytogenes* and *S. enterica* during co-incubation at different temperatures and storage conditions. To the best of our knowledge, this is the first study on the influence of the interaction of a non-probiotic antagonistic bacterium on fresh-cut fruit in the colonization abilities of human enteropathogenic bacteria.

ACKNOWLEDGEMENTS

This work has been supported by the CERCA Programme / Generalitat de Catalunya, the European Social Fund and the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya (grants FI-DGR-2015-0004 and 2015-FI-B100156). The authors are grateful to Dr. Sonia Marín and Dr. Jordi Voltas for their advice regarding the analysis of the results.

CHAPTER IV

Pseudomonas graminis strain CPA-7 differentially modulates the oxidative response in fresh-cut 'Golden delicious' apple depending on the storage conditions

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ABSTRACT

The oxidative response in fresh-cut antioxidant-treated 'Golden delicious' apples during chilling storage was differentially modified by the biopreservative bacterium *Pseudomonas graminis* CPA-7 depending on the storage conditions (passive modified atmosphere packaging (MAP) or air). Results showed that inoculation with CPA-7 had no influence on fruit quality parameters in any of the conditions tested. During the first 24 h both in air and in MAP, ascorbate peroxidase (APX) activity triplicated the initial level in response to CPA-7, reaching up to 4- fold the activity of non-inoculated fruit (control). From 24 h of storage in MAP, polyphenol oxidase (PPO) activity was sharply enhanced in response to CPA-7 attaining values up to 8-fold higher than that of the control at the end of the experiment, yet it was not paired to an increase in browning incidence. Concomitantly, at 24 h of storage in MAP, CPA-7 suppressed peroxidase (POX) and catalase (CAT) activities. Subsequently, after 3 d in such conditions, superoxide dismutase (SOD) and PPO activities were almost duplicated in the presence of CPA-7 compared to the control. On the other hand, when stored in air, POX showed a biphasic induction in response to CPA-7 after 1 d and 6 d of incubation. On day 6, this enzyme duplicated its activity in inoculated samples compared to the control regardless of storage conditions. Inoculation with CPA-7 led to the slowdown of the decline of antioxidant capacity in air, which contrasted with the response upon MAP conditions. These results suggest that CPA-7 may trigger the activation of the fruit defense-response thereby mitigating its oxidative damage. Such activation may play a role as a putative biocontrol mechanism against foodborne pathogen infections.

4.1 INTRODUCTION

Given the growing demand for ready-to-eat products and the concern of consumers about the use of chlorine for decontamination in the food industry, the use of biocontrol agents (BCA) is a bio-friendly method which has been envisaged as an alternative (Belak and Maraz, 2015; Leverentz et al., 2006). However, more research is needed to enlarge the information about the mode of action of BCA in order to obtain their approval and registration for industrial application.

The processing of fresh produce not only increases the risk for contamination with human and plant pathogens but also impacts on physicochemical quality due to the disruption of constitutive barriers and the leakage of cell components. During this process, the cutting of fruit flesh compromise compartmentalization in nearby cells allowing phenolic compounds located in vacuoles to get in contact with polyphenol oxidase (PPO), located in plastids, triggering the reaction known as enzymatic browning (Holderbaum *et al.*, 2010). Thus, ensuring the microbiological safety and physicochemical quality of fresh-cut products implies the need for combined strategies including biological (biocontrol agents), chemical (antimicrobial, anti-browning, texture-maintaining products) and physical methods (refrigerated storage and modified atmosphere packaging) which in turn, modulate fruit physiology and specifically, its oxidative metabolism (Reviewed by Parish et al., 2003; Spadaro and Droby, 2016).

Cold storage has shown to induce the accumulation of reactive oxygen species (ROS) in harvested fruit (Chiriboga *et al.*, 2013). However, differential response has been observed regarding the production of hydrogen peroxide (H_2O_2) and the induction of ROS - scavenging enzymes in processed apples under chilling conditions according to the type of preservative chemical compound used (Larrigaudiere *et al.*, 2008). Nevertheless, scarce information is available about

the oxidative changes in fresh-cut fruit at the enzymatic or metabolic level after treatment with biocontrol agents.

Besides PPO, other antioxidant enzymes such as peroxidase (POX) has also been linked to polyphenol-associated browning through a coordinated mechanism involving the accumulation of its substrate, hydrogen peroxide (H_2O_2), stimulated by the PPO-mediated generation of quinones (Jiang and Miles, 1993). Concomitantly, H_2O_2 is highly oxidant and may damage membrane and other cellular components through several mechanisms. Thus, in both fresh-cut and intact fruit, its removal is rapidly activated in order to protect plant cells from these damages. H_2O_2- scavenging mechanisms include enzymatic reactions which involve superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) found in the cytosol, chloroplasts and mitochondria of higher plants (Hung, Yu and Lin, 2005) as well as non-enzymatic antioxidants including phenolic compounds (vitamin E, flavonoids, phenolic acids among others); nitrogen compounds (alkaloids, amino acids and amines), carotenoids and chlorophyll derivatives (Panda, 2012).

The control of enzymatic browning in the fresh- cut produce industry is currently mainly based on the modulation of PPO activity through antioxidant formulations that commonly contain reducing compounds such as ascorbic acid and its derivatives, cysteine and glutathione (Rupasinghe *et al.*, 2005). These chemical compounds are hypothesized to control PPO activity either by reducing quinones to the native di-phenols or by reacting irreversibly with o- quinones to form stable colorless products (Nicolas et al., 1994; He and Luo, 2007).

In addition, oxidative metabolism is also activated in response to biotic stress as a part of induced plant defense mechanisms (Pieterse et al., 2000, 2014). Under the light of the increasing knowledge about plant-microorganism interactions, induced resistance has arisen as a method for controlling postharvest diseases (Reviewed by Da Rocha & Hammerschmidt, 2005; Walters and Fountaine, 2009).

Several studies have evidenced the protective effect of biotic agents applied in the roots (plant growth promoting rhizobacteria, PGPR) or in the green parts of the plant, through the activation of Induced Systemic Resistance (ISR) against plant pathogens invading distant organs (Attitalla, Johnson, Brishammar, & Quintanilla, 2001; Verhagen et al., 2010). Moreover, the application of strategies combining antagonists and chemical or physical methods have shown to reduce disease severity to fungal plant pathogens in cherry tomato fruit and peach (Yao and Tian, 2005; Zhao *et al.*, 2009). The investigation of these processes has revealed that biocontrol agents (BCA) activate defense-related enzymes such as phenylalanine ammonia lyase (PAL), POX, PPO and chalcone synthase among others. BCA have also shown to stimulate oxidative burst and lignin deposition in mechanically damaged fruit (Chen et al., 2000; Valentines et al., 2005). The activation of such mechanisms might play an important role in priming fruit active defense response allowing higher tolerance to a subsequent pathogen attack (Alkan and Fortes, 2015; Jain et al., 2012; Lim et al., 2014).

Pseudomonas graminis strain CPA-7 is an aerobic epiphyte bacillus which was isolated from whole apple (Alegre et al., 2013b). It has been used as BCA against foodborne pathogens such as *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* on fresh cut apples, peaches and melons (Abadias et al., 2014; Alegre et al., 2013a, 2013b). Although attempts to elucidate the mechanisms underlying its antagonistic activity have been carried out, they still remain poorly understood (Collazo et al., 2017). Thus, it was hypothesized that a putative mode of action for CPA-7 could be the activation of non-host pathogen oxidative-related defense mechanisms in fresh-cut fruit, leading to the control of pathogens populations, as it has been reported for several BCA (Reviewed by Spadaro and Droby, 2016). Accordingly, enzymatic and non-enzymatic oxidative metabolism of CPA-7-inoculated fresh-cut apples treated with an anti-browning compound were investigated in two different scenarios: i) aerobic conditions, more suitable

for the antagonist growth and ii) semi-commercial conditions (modified atmosphere packaging, MAP) more stressful for the antagonist growth.

4.2 MATERIALS AND METHODS

4.2.1 FRUIT PROCESSING

Apples (*Malus domestica* L. cv. Golden Delicious) were obtained from local packing-houses (Lleida, Catalonia, Spain). Prior to experimental assays, the fruit were washed with running tap water, surface disinfected with 700 mL L^{-1} ethanol, peeled with an electric fruit peeler and cut into eight wedges with a handheld corer/slicer. Wedges were kept in chilled (5°C) chlorinated tap water (pH 6) until subsequent treatment.

4.2.2 ANTAGONIST CULTURE CONDITIONS

For the antagonist inoculum preparation, a single colony of *Pseudomonas graminis* strain CPA-7, grown in tryptone soy agar plates (TSA, Biokar, Beauveais, France) during 48 h at 30 °C, was inoculated into 50 mL of tryptone soy broth (TSB, Biokar, Beauveais, France) and incubated in agitation (15.71 rad s^{-1}), in aerobic conditions at 25 °C for 24 h. Bacterial cells were harvested by centrifugation at 9800 × g for 10 min at 10 °C and re-suspended in sterile distilled water. The concentration of the suspension was checked by plate count of appropriate 10-fold dilutions in saline peptone (SP, 8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto TSA plates after incubation at 30 °C for 48 h.

4.2.3 INOCULATION, SAMPLING AND MICROBIOLOGICAL ANALYSIS

Suspensions of CPA-7 at a concentration of 10^7 CFU mL^{-1} were prepared in an antioxidant aqueous cold solution (4 °C) containing 60 g L^{-1} NatureSeal® AS1

(AS1, AgriCoat Ltd., Great Shefford, UK), a calcium ascorbate-based food grade antioxidant agent. Non-inoculated antioxidant solution was included as control. Fruit wedges were dipped-inoculated at a ratio of 1:2 (weight of fruit: volume of suspension) for 2 min in agitation (15.71 rad s^{-1}) in a tabletop orbital shaker (Unimax 1010, Heidolph, Germany).

After the drainage of the excess of water, samples were packaged (120 g per replicate) in 400 mL polyethylene terephthalate trays (ShelfMaster™ Pronto™, PlusPack, Denmark). Each tray was considered as a replicate and three replicates per treatment and sampling time were used. Each tray was thermosealed with 181.7 cm^2 of a 3-holed (60 - 80 μm diameter, 75 mm spacing) microperforated film (PDS Group, Murcia, Spain) composed of polyester anti-fog film (OALF, 14 μm of thickness) + oriented polypropylene film (OPP, 20 μm of thickness), to achieve passive modified atmosphere. In a parallel set of trays the film was macro perforated (nine extra holes per tray) in order to attain aerobic conditions.

Trays were stored at 5 °C in darkness and biochemical and microbiological analyses were performed at 0, 1, 3 and 6 d post-treatment. For biochemical tests, about 70 g of each sample was frozen with liquid nitrogen, grinded in a commercial grinder (Minimoka 6R-020, Coffeemotion, Lleida, Spain) and stored at -80 °C until analysis. For microbiological analysis, 10 g of apple from each tray was homogenized in 90 mL of buffered peptone water (BPW, Biokar, Beauvais, France) and tested by viable cells count at each sampling time. In inoculated samples, CPA-7 populations were analyzed on TSA plates incubated at 30 °C for 48 h. In non-inoculated samples, total mesophilic bacteria populations were determined on plate count agar plates (PCA, Biokar, Beauveais, France) after incubation at 25 °C for 72 h.

4.2.4 Physicochemical quality parameters of the fruit

Firmness, color and pH of fresh-cut wedges were determined initially and at each sampling time as described elsewhere (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). Low values of CIE coordinate L* and high values of a* were considered as indicators of surface browning intensity (Sapers and Douglas, 1987). Soluble solids and titratable acidity of each replicate were measured initially and at the end of storage. Soluble solids concentration at 20 °C was expressed as mass fraction of sugars relative to the fruit (%). Acidity was measured in 10 mL of pulp and was expressed as malic acid content (g L^{-1} juice).

4.2.5 Headspace gas composition

The headspace gas composition of each tray was measured at the end of storage using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) and expressed as percentages.

4.2.6 Biochemical analyses

Total antioxidant capacity and total phenolic content

Extracts for total phenolic content (TPC) and total antioxidant capacity (TAC) determination were prepared by mixing 3 g of frozen fresh pulp with 10 mL of a solution containing 19.7 mol L^{-1} methanol, 0.05 mol L-1 HCl. The mixture was held in agitation (20.94 rad s^{-1}) for 2 h and centrifuged at 24000 x g at 4 °C. TPC was quantified by measuring the optical density (OD) at 765 nm in a spectrophotometer (EONC, Biotek Instruments, Highland Park, VT, USA) after the reaction of 0.050 mL of each extract with 0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of 1.9 mol L^{-1} Na_2CO_3. TAC was determined by measuring OD at 593

nm of the above mentioned extracts following the Ferric Reducing Antioxidant Power (FRAP) protocol (Giné-Bordonaba and Terry, 2016).

H_2O_2 production and malondialdehyde content

Malondialdehyde (MDA) was quantified in 0.5 g of frozen fresh pulp as a marker of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS) assay described by (Martínez-Solano et al. (2005). The OD of the supernatants after reaction was measured at 532 nm and subtracted to the unspecific absorption read at 600 nm. Quantification of TBARS was calculated by its extinction coefficient of 155 mmol L^{-1} cm^{-1}. To determine hydrogen peroxide levels, 5 g of frozen fresh pulp was homogenized in 7.5 mL of 0.5 mol L^{-1} trichloroacetic acid, filtered through two layers of Miracloth (Textil Planas Oliverassa, Manresa, Spain), and centrifuged at 20 000 x g for 15 min at 4 ºC. H2O2 content was determined using the aqueous peroxide colorimetric assay, PeroxiDetect™ Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. This procedure is based on the measurement of the color change at 560 nm of the adduct formed by Fe3+ ion and xylenol orange (XO, PubChem CID 73041), during the oxidation by peroxide of Fe^{2+} to Fe^{3+} ions at acidic pH. The molar extinction coefficient of the XO-Fe^{3+} colored adduct in aqueous solution at 560 nm is 15 000.

Activities of antioxidant enzymes

Peroxidase and polyphenol oxidase

Total peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) were extracted from 10 g of frozen fresh pulp as described by Giné-Bordonaba et al. (2017). POX activity was measured as the optical density at 470 nm following the reaction of the extract with 10 mmol L^{-1} guaiacol and 10 mmol L^{-1} H_2O_2, according to the method described by Lurie et al. (1997). PPO activity was

determined at 400 nm in a reaction mixture containing 0.1 mol L^{-1} potassium phosphate buffer (pH 6) and 65 mmol L^{-1} pyrocatechol together with the correspondent enzyme extract as described by Vilaplana et al. (2006).

Superoxide dismutase and catalase

For the extraction of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), 5 g of frozen fresh pulp were homogenized in 15 mL 0.1 mol L^{-1} potassium phosphate buffer (pH 7.8), 2 mmol L^{-1} dithiothreitol (DTT), 50 g L^{-1} polyvinylpolypyrrolidone (PVPP), 0.1 mmol L^{-1} ethylene diamino tetracetic acid (EDTA) and 1.25 mmol L^{-1} polyethylene glycol. The homogenized was filtered, centrifuged and purified as described by Giné-Bordonaba et al. (2017). In turn, SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm following the method of Gianopolitis and Ries (1977).

Ascorbate peroxidase

For ascorbate peroxidase (APX; EC 1.11.1.11) extraction, 10 g of frozen fresh pulp was homogenized with 30 mL of 0.1 mol L^{-1} base phosphate buffer (pH 7.5) containing 0.8 mmol L^{-1} HCl, 1 mmol L^{-1} EDTA, filtered through two layers of Miracloth and centrifuged at 10 000 x g for 15 min at 4 °C. APX activity was determined at 290 nm during 10 min by monitoring the H_2O_2-dependent decomposition of ascorbate in a mixture containing 20 µL of the recovered supernatant and 280 µL of a reaction solution (0.22 mmol L^{-1} ascorbic acid, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} H_2O_2) (Nakano and Asada, 1981).

Protein content

Protein content of all extracts was determined by the Bradford method at 595 nm using a protein assay kit (Bio-Rad, München, Germany). Protein reagent was mixed with the correspondent phosphate buffer (pH 6, 7 or 7.8) used for each

enzyme extraction at a ratio of 1:3.6. Bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was used as standard.

Ascorbic/dehydroascorbic acid contents

Total vitamin C, ascorbic acid (AA) and dehydroascorbic acid (DHAA) content were measured at 0, 1 and 3 d post-treatment (corresponding to the sampling times where noticeable differences among treatments regarding APX activity were observed). Extracts were obtained from 0.5 g lyophilized apple pulp following the method described by Klimczak and Gliszczynska-Swiglo (2015). For AA determination, 5 μL of a dilution of each extract in 100 mL L^{-1} meta-phosphoric acid (at a ratio of 1:20.) was analyzed by the validated Ultra High Performance Liquid Chromatography - Photodiode Array (UHPLC-PDA) method (Spinola *et al.*, 2012). For total vitamin C determination, each extract was mixed with 50 mL L^{-1} dithiothreitol (DTT, Sigma-Aldrich, St Louis, MO, USA) at a ratio of 1:1. Subsequently, each mixture was diluted and analyzed as previously described. DHHA content was calculated by subtracting AA content from the total amount of vitamin C present in each sample.

4.2.7 Expression of results and statistical analysis

Enzyme activity was expressed in units of enzyme activity per milligram of protein (U mg^{-1} protein). For SOD, 1 U represents the amount of enzyme required to inhibit NBT reduction by 50 % of initial amount. For the rest of the analyzed enzymes, 1 U represents the quantity of enzyme responsible for a change in 1 absorbance unit per minute. Microbiological data was estimated as microbial counts per gram of fresh weight of fruit (CFU g^{-1} FW). Non-enzymatic antioxidant activities were expressed as g of the measured analyte (i.e. Gallic acid (GAE) or Fe^{3+}) per kilogram of fresh weight of fruit (g kg^{-1} FW). Oxidative stress markers (H_2O_2 and MDA) were expressed as μmol kg^{-1} fresh weight of fruit (μmol kg^{-1} FW).

Total vitamin C, AA and DHAA contents were expressed as g kg^{-1} FW. Data were analyzed using the general linear model procedure to determine differences among treatments and interaction effects using the statistical software JMP (version 11 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity of residues and accordingly, enzymatic activities and microbiological results were transformed to log_{10} of U mg^{-1} protein or CFU g^{-1} FW, respectively, Transformed results were schematically represented as back-transformed means ± standard error of the mean. Transformed and non-transformed means were compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).

4.3 RESULTS

4.3.1 MICROBIAL DYNAMICS

Initial populations of CPA-7 on the fruit were 5.53 ± 0.03 log_{10} CFU g^{-1} FW (Fig. **IV.**1A). Lower oxygen concentration associated to modified atmosphere packaging (MAP) slowed down CPA-7 growth compared to the samples stored in air, delaying the start of the exponential phase from 1 d to 3 d post-inoculation. CPA-7 final populations in the MAP-stored samples were 0.5 log_{10} below those in the samples stored in aerobic conditions. Populations of total mesophilic bacteria were initially the same (1.7 ± 0.2 log_{10} CFU g^{-1} FW) in all samples (Fig. **IV.**1B). However, differences in their growth by 1 log_{10} among air and MAP-stored samples became evident on day 6.

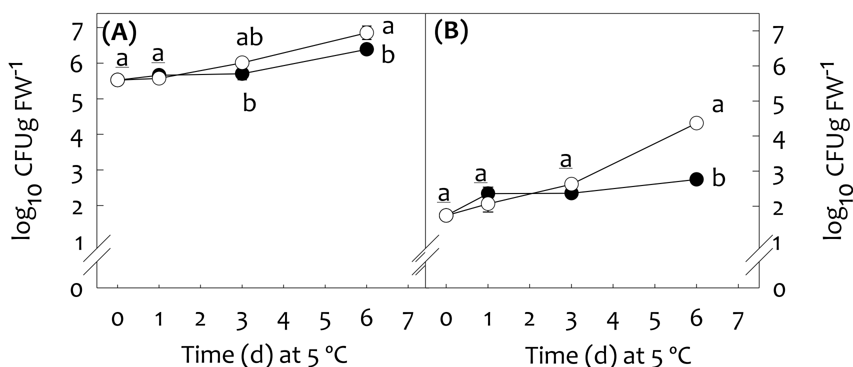

Figure IV.1. Population dynamics of (A) *P. graminis* CPA-7 and (B) total mesophilic bacteria on 'Golden delicious' fresh-cut apple wedges treated with antioxidant, during 6 d of storage at 5 °C in modified atmosphere packaging (MAP) (●) or in air (O). Each point represents the mean and error bars represent the standard error of the mean (n = 6). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means that correspond with overlapped symbols in the graph.

4.3.2 PHYSICOCHEMICAL QUALITY PARAMETERS OF THE FRUIT

No significant differences were observed among initial and final values of L* (lightness) and a* (redness) in the presence of CPA-7 (Table **IV**.1). Likewise, no differences were observed in the content of soluble solids or pH in response to the antagonist under any of the tested conditions throughout storage. Firmness was better maintained throughout the studied period in the presence of CPA-7 than in the non-inoculated control. In this latter it was reduced by 2.6 N at the end of storage in MAP compared to the initial value.

Table IV.1. Quality parameters of fresh-cut antioxidant-treated 'Golden delicious' apple wedges inoculated with *P. graminis* CPA-7 (CPA) or non-inoculated (NI) during storage in aerobic conditions (AIR) or in modified atmosphere packaging (MAP).

	Treatment	Time (days) at 5 °C			
		0	1	3	6
(L*)	**AIR NI**	78.1 ± 0.5 **aB**	80.9 ± 0.2 **aA**	79.7 ± 0.4 **a AB**	78.5 ± 0.6 **abcB**
	AIR CPA	78.5 ± 0.9 **aB**	77.5 ± 0.4 **bcB**	78.8 ± 0.6 **ab B**	78.5 ± 0.7 **abcB**
	MAP NI		78.2 ± 0.6 **bcA**	79.8 ± 0.5 **aA**	79.1 ± 0.5 **abA**
	MAP CPA		80.1 ± 0.3 **abA**	79.0 ± 0.5 **abA**	79.7 ± 0.4 **aA**
(a*)	**AIR NI**	-6.6 ± 0.4 **bB**	-6.4 ± 0.4 **aA**	-6.4 ± 0.3 **cdA**	-5.8 ± 0.5 **bcA**
	AIR CPA	-6.1 ± 0.6 **abB**	-6.0 ± 0.3 **bcB**	-6.5 ± 0.2 **abB**	-6.2 ±0.4 **bcB**
	MAP NI		-6.4 ±0.5 **bcAB**	-7.7 ± 0.1 **dB**	-6.4 ± 0.1 **cA**
	MAP CPA		-7.2 ± 0.5 **abA**	-6.0 ± .3 **cdA**	-6.5 ± 0.3 **cA**
Firmness (N)	**AIR NI**	13.1 ± 1.0 **aA**	12.1 ± 0.8 **aA**	12.0 ± 0.9 **aA**	11.5 ± 0.6 **aB**
	AIR CPA	13.0 ± 0.8 **aA**	12.8 ± 0.8 **aA**	12.5 ± 0.6 **aA**	13.0 ± 0 .6 **aA**
	MAP NI		14.3 ± 0.2 **aA**	12.2 ± 0.6 **aAB**	11.7 ± 0.3 **aB**
	MAP CPA		11.3 ± 1.5 **aA**	11.7 ± 0.5 **aA**	13.1 ± 0.9 **aA**
pH	**AIR NI**	3.8 ± 0.1 **aA**	4.0 ± 0.1 **aA**	3.9 ± 0.02 **aA**	3.7 ± 0.1 **aA**
	AIR CPA	4.1 ± 0.1 **aA**	3.9 ± 0.1 **aA**	4.1 ± 0.1**aA**	3.9 ± 0.2 **aA**
	MAP NI		3.85 ± 0.03**aA**	3.9± 0.1 **aA**	3.9 ± 0.1 **aA**
	MAP CPA		3.9 ± 0.1 **aA**	3.9 ± 0.1 **aA**	4.15 ± 0.04 **aA**
TA	**AIR NI**	3.3 ± 0.2 **aA**			3.8 ± 0.3 **aA**
	AIR CPA	3.22 ±0.02 **aA**			3.6 ± 0.5 **aA**
	MAP NI				3.5 ± 0.3 **aA**
	MAP CPA				3.6 ± 0.2 **aA**
SS	**AIR NI**	12.93 ± 0.04 **aA**			13.3 ± 0.9 **aA**
	AIR CPA	12.80 ± 0.06 **aA**			12.6 ± 0.1 **aA**
	MAP NI				13.3 ± 0.1 **aA**
	MAP CPA				12.6 ± 0.3 **aA**

Values are means of 3 replicates ± standard error of the mean. Different lowercase letters represent significant differences among treatments at each sampling time. Different uppercase letters represent significant t differences for each treatment throughout time according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$). TA: titratable acidity measured as (g L^{-1}) malic acid content. SS: soluble solids expressed as mass fraction to volume of juice (%).

4.3.3 HEADSPACE GAS COMPOSITION

Final gas measurements of trays headspace after storage at 5 °C showed a reduction of the O_2 content and an increase of the CO_2 content in MAP compared to air conditions (data not shown). However, gases composition was

similar for CPA-7-inoculated and non-inoculated samples both in MAP (16 ± 1 kPa O2; 5.2 ± 0.7 kPa O_2) and in air (20.6 ± 0.6 kPa O_2; 0.4 ± 0.1 kPa CO_2).

4.3.4 Biochemical analyses

Total antioxidant capacity and total phenolic content

Initial total antioxidant capacity (TAC), measured by the content of Fe^{3+}, was 13.6 ± 0.2 g kg^{-1} FW in all samples and it gradually decreased throughout storage (Fig. **IV.** 2A). However, the drop of TAC was significantly slower in presence of CPA-7 in aerobic conditions where it remained at 12.3 ± 0.4 g kg^{-1} FW from the initial day until day 3. No differences were observed among MAP-stored samples in the first three days of the experiment. However, on day 6 in MAP conditions, it was recorded a reduction by 0.8-fold in response to CPA-7 compared to the control.

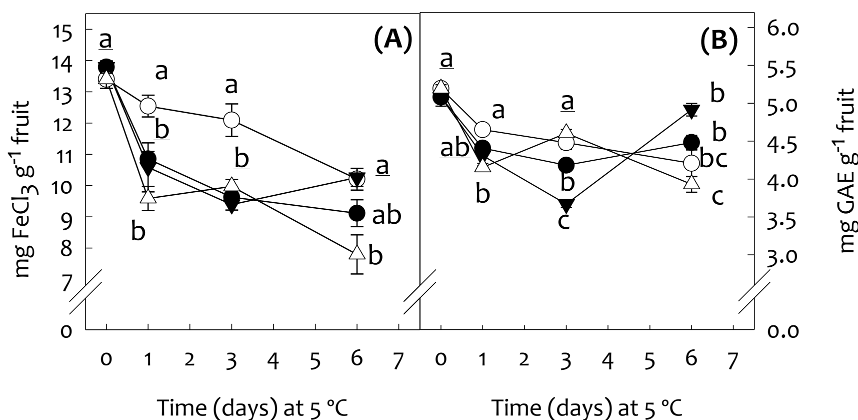

Figure IV.2. Changes in the levels of (A) Total Antioxidant Capacity, TAC, estimated from the amount of Fe^{3+} and (B) Total Phenolic Content, TPC, estimated from the amount of Gallic acid, in fresh-cut 'Golden delicious' apple wedges treated with antioxidant after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means that correspond with overlapped symbols in the graph.

Total phenolic content (TPC) was initially 5.1 ± 0.1 g kg^{-1} FW in all samples regardless of the presence of the antagonist (Fig**. IV.**2B). On day 3 and for both storage conditions, the inoculation with CPA-7 was associated to a significantly higher amount of TPC (4.5 ± 0.1 g kg^{-1} FW), than in the control samples (4.2 ± 0.04 g kg^{-1} FW in air and 3.7 ± 0.04 g kg^{-1} FW in MAP). In agreement to TAC, on day 6, TPC was reduced in MAP-stored samples in response to CPA-7 (4.1 ± 0.1 g kg^{-1} FW) compared to the control (4.9 ± 0.1 g kg^{-1} FW).

H_2O_2 PRODUCTION AND MALONDIALDEHYDE CONTENT

The low malondialdehyde (MDA) content (0.018 ± 0.001 µmol kg-1 FW) in all samples showed that no peroxidation of the membranes occurred under any of the conditions tested during the analyzed period (data not shown). In combination with the antioxidant, CPA-7 was able to erase the oxidative burst or at least that referred to H_2O_2 accumulation in the fruit stored in air throughout the analyzed period (Fig. IV.3). Contrastingly, in the MAP-stored samples H_2O_2 drastically increased ($P < 0.0001$) in the first 24 h reaching levels 1.4-fold higher in response to CPA-7 than in the non-inoculated control. In general, H_2O_2 content remained significantly higher ($P < 0.0001$) in the MAP-stored samples in the presence of CPA-7 than in the rest of the samples during the whole storage. In this sense, values remained 13-fold higher in inoculated samples than in the control at the end of storage.

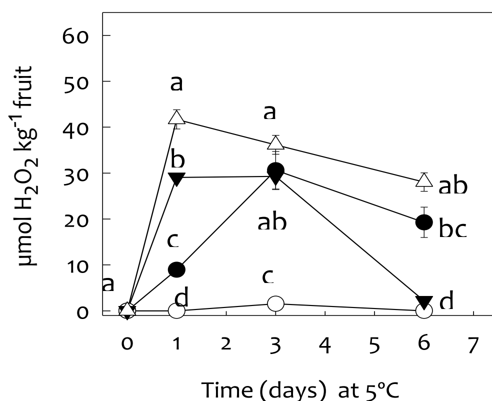

Figure IV.3. Changes in the levels of hydrogen peroxide (H_2O_2) in 'Golden delicious' fresh-cut apple wedges treated with antioxidant after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). Symbols represent means and error bars represent standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means that correspond with overlapped symbols in the graph.

Activities of antioxidant enzymes

Changes in the enzymatic antioxidant potential of 'Golden delicious' fresh-cut apple differed throughout storage according to the inoculation with the antagonist and/or to the storage conditions. SOD activity decreased in the first 24 h ($P < 0.0001$) from 5554 ± 42 U mg^{-1} protein to 4863 ± 111 U mg^{-1} protein in all samples, except for CPA-7-inoculated MAP-stored samples, in which it remained stable until day 3. In the presence of CPA-7, SOD activity reached values 1.5-fold higher than those observed for the control after 3 d in MAP conditions (Fig **IV**.4A). In contrast, on day 6, SOD activity was oppositely modulated by CPA-7 depending on the storage conditions; it was 0.7-fold lower than the control when stored in MAP and 0.6-fold higher than the control when stored in air.

Initially, PPO activity was 243 ± 7 U mg^{-1} protein in all samples and it was reduced by 0.5-fold in the first 24 h ($P < 0.001$) (Fig. **IV.**4C). Thereafter, it drastically increased in response to CPA-7 in MAP reaching almost 8-fold the activity

showed by the non-inoculated control stored in the same conditions. However, when stored in air, CPA-7 had no significant effect in PPO activity throughout the analyzed period. Activity of APX was initially around 348 ± 43 U mg^{-1} protein in all samples (Fig. **IV**.4D). During the first 24 h post-inoculation, APX activity was induced by 3-fold in response to the antagonist, compared to the initial value ($P < 0.0001$), when stored in air. At this sampling time, peaks of APX activity in response to CPA-7 triplicated the activities recorded for the controls, regardless of the storage conditions. An enhanced APX activity associated to the antagonist was maintained until day 3 (1.8 fold higher than the control) in air-stored samples. Immediately after inoculation, POX activity was inhibited in response to CPA-7 (by 0.6-fold compared to the control) (Fig. **IV.**4E). Thereafter, this enzyme showed biphasic activation in response to CPA-7 in aerobic conditions reaching values 1.5-fold and 1.7-fold higher than those observed for the control at 1 and 6 d post-inoculation, respectively. In contrast, POX activity was reduced in MAP conditions during the first 24 h post-inoculation ($P < 0.0001$), but it attained lower values in the presence of the antagonist (84 ± 5 U mg^{-1} protein) than in the control (98 ± 10 U mg^{-1} protein). However, longer storage in MAP was associated to an enhanced POX activity in the CPA-7-inoculated samples (2.6-fold higher) compared to the control. Immediately after processing, catalase activity was similar in all samples (237 ± 17 U mg^{-1} protein), regardless of any of the factors assayed (Fig. **IV.**4B).

However, it differentially changed in response to the antagonist in different storage conditions. It increased by 0.7-fold compared to the initial value when stored in air ($P < 0.001$), reaching the same level than the control after 24 h. In contrast, CAT activity in inoculated samples was inhibited in response to the antagonist when stored in MAP (by more than 0.6-fold compared to the control) on the 3rd d of storage.

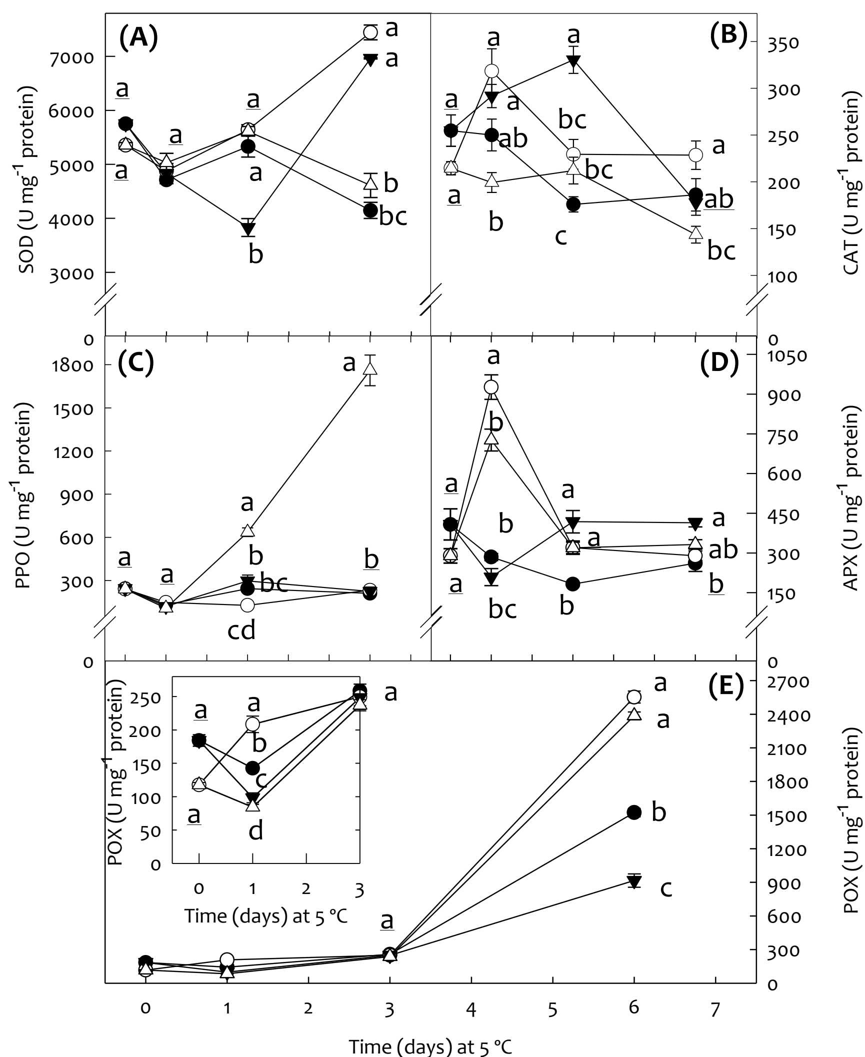

Figure IV.4. Changes in the activity of the enzymes A) superoxide dismutase (SOD), B) catalase (CAT), C) polyphenol oxidase (PPO), D) ascorbate peroxidase (APX) and E) peroxidase (POX) in 'Golden delicious' fresh-cut antioxidant-treated apple wedges, after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). The internal plot in graph E represents the first three sampling times at a smaller scale. Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means that correspond with overlapped symbols in the graph.

Ascorbic acid/dehydroascorbic acid contents

Because ascorbic acid (AA) can be easily converted into dehydroascorbic acid (DHAA) during fresh-cut fruit storage, both molecules were determined. Immediately after processing and antioxidant dipping, AA content in both inoculated and non-inoculated processed samples was similar (3.1 ± 0.2 g kg^{-1} FW) which triplicated the value observed for whole or intact fruit (0.21 ± 0.002 g kg^{-1} FW). AA content was slightly reduced (P = 0.005) in CPA-7-inoculated MAP-stored samples 24 h post-processing (2.1 ± 0.3 g kg^{-1} FW) compared to the initial value yet it was not significantly different than that observed for the control. DHAA (0.2 ± 0.1 g kg^{-1} FW) and vitamin C (2.8 ± 0.3 g kg^{-1} FW) contents remained invariable throughout storage and showed no differences between inoculated and non-inoculated apple wedges at any of the analyzed sampling points.

4.4 DISCUSSION

The processing of fresh produce implies the removal or damage of most of the constitutive barriers of plants and generally activates inducible defense mechanisms to protect themselves against pathogens (Reviewed by Hodges and Toivonen, 2008). Pseudomonads that have been used as biocontrol agents (BCA) have shown to enhance plant defense responses including cell-wall straightening, oxidative burst and the expression of genes that are usually activated by pathogens (Van der Ent, Van Wees and Pieterse, 2009). This phenomenon 'primes' plant metabolism allowing it to react faster and stronger upon a subsequent pathogen attack (Pieterse *et al.*, 2001). Among plant resistance responses, the activation of oxidative metabolism, including enzymatic and non-enzymatic pathways, has been previously established (Baxter et al., 2014; Hung et al., 2005; Sewelam et al., 2014). The increased activity of enzymes such as peroxidase (POX) which mediates the last steps of the biosynthesis of lignin and hydrogen peroxide (H_2O_2) as well as the oxidation

of phenolic compounds, entails the reinforcement of plant barriers, the activation of signaling pathways and the induction of defense genes in order to restore plant homeostasis (Hiraga, 2001). However, little is known about the oxidative response of fresh-cut fruit after inoculation with BCA. Thus, in the present study biochemical changes associated to the oxidative metabolism of fresh-cut apple in response to the BCA *P. graminis* CPA-7 at the enzymatic and non-enzymatic level were explored.

Results showed a differential oxidative response in the presence of CPA-7 depending on the storage conditions. As expected, H_2O_2 immediately accumulated in inoculated and non-inoculated MAP-stored samples after peeling, cutting and antioxidant dipping reaching a peak at 24 h. This increase attained a lower level but remained longer in the air-stored control. Similar H_2O_2 accumulation pattern was previously observed in non-inoculated fresh-cut 'Fuji' apples treated with ascorbic acid and stored in MAP conditions (Larrigaudiere *et al.*, 2008). However, in the presence of CPA-7, the oxidative burst was practically erased in air conditions keeping H_2O_2 levels close to zero during the whole experiment. This result is consistent with the absence of hypersensitive response in tobacco leaves and apple fruits after wound-inoculation with 10^9 CFU mL^{-1} of CPA-7 (Alegre, 2012).

The initial activation of ROS-scavenging mechanisms correlated with the wound-induced oxidative burst due to processing. Although the initial levels of all of the evaluated enzymes were similar regardless of inoculation with CPA-7, except for POX which was reduced in the presence of the antagonist, their subsequent dynamics differentially varied throughout storage. The quick and strong induction of SOD activity with the concomitant increase of H_2O_2 levels in the first hours after processing regardless of the antagonist presence, could be explained as a response to wounding that has been widely described (Reviewed by Toivonen and Brummell, 2008). Conversely, in the subsequent 24 h post-inoculation, while SOD activity dropped, CPA-7 induced POX, CAT and APX

activities in aerobic conditions which could contribute to quickly eliminate H_2O_2 as soon as it was produced. The sharp and transient induction of APX during the first 24 h post-processing was also evident in the MAP stored samples as a first line of defense against elevated H_2O_2 production.

The induction of APX is often related to the reduction of ascorbic acid which usually decreases during storage and processing of fruit and vegetables. Browning itself is among the processes associated to the degradation of ascorbic acid (Chisari, Barbagallo and Spagna, 2008). However, the obtained results showed that total vitamin C and ascorbic acid (AA) content did not correlated with the changes in APX activity induced by CPA-7 in any of the conditions tested. Initial levels of ascorbic acid were elevated compared to those in intact apples due to the addition of ascorbate as an antioxidant agent. In this context, exogenous ascorbic acid was probably not able to penetrate the cell membranes and therefore, could not be used as a substrate for APX to detoxify hydrogen peroxide. Endogenous ascorbate could be used as a substrate for APX in H_2O_2-scavenging reactions as suggested by the slight reduction of AA content observed in the CPA-7-inoculated MAP-stored samples 24 h post-inoculation, which corresponded to a higher APX activity. However, the lack of differences in AA content at this sampling time in respect of the control, do not support this hypothesis. Therefore, the addition of ascorbate as a preservative of fruit quality probably masked the endogenous ascorbate dynamics.

On the other hand, in the conditions assayed herein, as time passed, the stress associated to mechanical damage was added to that caused by changes in headspace gas composition as in MAP-stored samples; hence leading to the differential modulation of some scavenging enzymes aiming at maintaining redox homeostasis. In this way, from day 1 to 3, SOD activity peaked in the CPA-7-inoculated MAP-stored samples and subsequently, PPO and POX were sharply induced until the end of storage counteracting the inhibition of CAT. PPO and POX has shown to play important roles in the stress response in apple fruit but

the dynamics of their activities depends on both biotic and abiotic factors such as fruit cultivar, stage of development, temperature and preservative treatment, among others (Valentines *et al.*, 2005; Holderbaum *et al.*, 2010). The induction of POX activity in MAP conditions as previously observed in fresh cut 'Fuji' apple under chilling storage, has shown also to be dependent on the anti-browning treatment (Larrigaudiere *et al.*, 2008). Furthermore, the increase in POX activity without the concomitant increase of browning has previously been observed in 'Golden Delicious' apple after microbial inoculation (*Penicilium expansum)* and associated to lignin accumulation (Valentines *et al.*, 2005).

In contrast, in the air-stored samples the activation of SOD due to CPA-7 lasted until day 6 and it was paired to the induction of POX. Thus, stressful conditions were associated not only to increased ROS-scavenging enzymes activities but to changes in their balance. It has been previously observed that reduced CAT activity is sometimes compensated by the induction of APX and glutathione peroxidase (GPX) (Apel and Hirt, 2004). Similarly, Wang et al. (2014) observed that the treatment of loquat fruit with the antagonist *B. cereus* AR-156 induced SOD activity while reduced the activities of CAT and APX under aerobic conditions. This coordinated enzyme modulation enhanced the fruit defense response to the subsequent infection by the pathogen *Colletotricum acutatum*.

In the present experiment, differences in the oxidative metabolism upon MAP and air storage were not related to drastic changes in O_2 and CO_2 levels. Oxygen content in MAP conditions remained far from anoxia (16 %) and agreed with reported standardized levels expected for well-designed micro-perforated films, allowing the O_2 entry rate to be higher than the O_2 exit rate (Toivonen, 2009). Combinations of O_2 and CO_2 similar to those found in our study have been referred as suitable for avoiding secondary browning and off-odors formation as well as for enhancing the quality attributes of fresh-cut apple wedges (Cliff et al., 2010; Toivonen, 2006). In the same way, ethanol accumulation in fresh-cut apple has been observed to be avoided during the first 10 d of storage when O_2

availability in the package headspace is close to air (Rojas-Graü, Grasa-Guillem and Martín-Belloso, 2007).

The modulation of oxidative metabolism in fruit and vegetables in response to *Pseudomonas* spp. as observed in the present study, has been previously reported and correlated to the induction of plant resistance to several plant pathogens (Sangeetha *et al.*, 2010). For instance, combinations of a non-fluorescent *Pseudomonas* spp. strain NFP6, *P. fluorescens* Pf3a and *B. subtilis* induced up to 4-fold PAL, POX and PPO activities and increased by 3.6-fold the phenolic content in banana fruits challenged with crown rot pathogens when stored at 24 °C in plastic bags, reaching maximum values 5 d after the treatment (Sangeetha *et al.*, 2010). Likewise, *Pseudomonas corrugate* strain 13 and *Pseudomonas aerofasciens* strain 63-28 significantly induced PAL, POX and PPO in cucumber 2 to 5 d following treatment which elicited the defense response to subsequent infection with *Phytium aphanidermatum* (Chen *et al.*, 2000). Similarly, mixtures of fluorescent rhizosphere and endophytic Pseudomonads induced these enzymes when sprayed on banana plants which in turn, showed enhanced systemic resistance against bunchy top virus (Harish *et al.*, 2009). An analogous response regarding POX, PPO and SOD activities have been previously observed for other biocontrol agents such as *B. amyloliquefaciens* LJ02 after being sprayed on three leave-stage cucurbit seedlings (Li *et al.*, 2015).

Regarding, fresh-cut fruit and vegetables, scarce information is available regarding the effect of BCA on antioxidant or oxidative metabolism. An increase in PPO activity in fresh-cut 'Piel de sapo' melon in response to *P. graminis* CPA-7 was previously observed after 8 d of storage at 5 °C in aerobic conditions (Plaza *et al.*, 2016). However, in the same fruit but stored under MAP conditions, no effect was induced by CPA-7 compared to the control. Differences in sampling times between that study and ours could suggest a differential dynamics of PPO activity in response to CPA-7 depending on storage conditions and/or the fruit matrix. In this sense, the oxidative response of fresh produce upon stressful

conditions has been shown to be differential depending on the type of produce even to a cultivar level (Chisari et al., 2008; Reyes et al., 2006). During this process, the synergistic activation of PPO and POX has been reported to be affected by the nature of the oxidized phenols and thus influenced by the phenolic composition of each commodity which depends on several factors (Manach C, Scalbert A, Morand C, 2004; Tomás-Barberán and Espín, 2001).

The question still remains on whether the increase of ROS-scavenging enzymes activities such as SOD, CAT and POX, is only a fruit response or it is also a strategy deployed by the antagonist. This would confer this bacterium with the ability to tolerate highly oxidant conditions generated after the processing of fresh produce and could act as a pivotal mechanism of action to outcompete pathogens or spoilage microorganisms. Differences in the modulation pattern of the oxidative metabolism throughout storage observed in the present study did not correspond to great changes in CPA-7 populations as a function of storage conditions. The tracking of CPA-7 population dynamics showed that this bacterium was able to tolerate the changes produced in the fruit oxidative metabolism and to grow upon such conditions both in air and in MAP. Nevertheless, lower concentrations were observed in MAP stored apple wedges which may be related to lower oxygen availability. Previous semi-commercial trials performed with fresh-cut apples in conditions resembling those assayed herein showed similar CPA-7 population dynamics and revealed an inhibitory or bacteriostatic effect of this antagonist on *L. monocytogenes* depending on the storage temperature rather than on O_2 availability (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). From this perspective, the high resistance to ROS showed by the antagonistic yeasts *Cryptococcus laurentii* LS-28 and *Rhodotorula glutinis* LS-11 in apple and peach fruits has previously been associated with an enhanced antagonistic activity against the wound-inoculated pathogens *B. cinerea* and *P. expansum* (Castoria et al., 2003; Zhang et al., 2017).

On the other hand, concomitantly with changes at the enzymatic level, increased non-enzymatic antioxidant activity provides host tissue with additional defense tools to counteract ROS production induced by both biotic and abiotic stress (Hung, Yu and Lin, 2005). The initial accumulation of phenolic compounds, as observed in our trials regardless of the presence of the antagonist, has been extensively studied and reported for fresh-cut produce (Saxena, Bawa and Raju, 2009). Such accumulation may be related to the high production of H_2O_2 upon mechanical injury through an enhanced respiration or due to the activation of several and diverse metabolic pathways and/or the release of bound phenolic compounds by hydrolytic enzymes (Harish *et al.*, 2009).

Nevertheless, the subsequent reduction of total phenolic content was hindered at least until day 3, in the presence of CPA-7 when stored in aerobic conditions, thereby leading to a better maintenance of the fruit nutritional properties. Otherwise, in MAP conditions, CPA-7 was associated to a peak in the production of phenolic compounds on day 3 which contrasted with its reduction in the control samples. A positive correlation between the increase of phenolic compounds and PPO activity was observed only until day 3, since total phenolic compounds declined thereafter. However, changes in PPO did not correlate to an increase of browning incidence, which is likely related to reversal reactions mediated by the ascorbate added to the samples (Nicolas *et al.*, 1994) or to improved cell compartmentalization. In previous studies, changes in PPO activity did not either correlate with browning-indicative color parameters or total phenolic content in fresh-cut melon from several cultivars (Chisari, Barbagallo and Spagna, 2008).

Evidences showing the activation of fruit defense mechanisms after inoculation with CPA-7 could suggest that under these conditions, fruit response to fight subsequent microbial infection could be enhanced, as it has previously been discussed. However, further studies including the analysis of the differential gene expression and the modulation of the activity of antioxidant isoenzymes in

the plant and/or the antagonist upon challenge with foodborne pathogens are needed to prove this hypothesis and would improve the understanding of such mechanisms.

Regarding the effect of the antagonist on fruit quality, we observed a positive effect of CPA-7 on the maintenance of physical parameters such as firmness throughout storage in MAP conditions, unlike control samples. This result is supported by the maintenance of the integrity of cellular membranes which could be estimated from the measurement of MDA. No significant effect on pH or soluble solids was observed due to the antagonist during the studied period. Luminosity and redness were maintained throughout storage in inoculated and non-inoculated samples. Previous studies have shown either positive or negative effects in different types of commodities after inoculation with CPA-7, depending on the antioxidant treatment (Alegre et al., 2013a; Plaza et al., 2016). Therefore, the combination of CPA-7 and AS1 could be an alternative to be considered for maintaining both the microbiological and the physicochemical quality of fresh-cut apple.

4.5 CONCLUSIONS

The present findings indicated that CPA-7-inoculated antioxidant-treated fresh-cut apple stored in MAP, showed higher accumulation of phenolic compounds at certain moments of storage, compared to non-inoculated samples. On the other hand, during storage in air, inoculated apples maintained higher antioxidant capacity as well as higher phenolic content than the control. Based on these results, combined treatments of antioxidant compounds (i.e. AS1) and biological control agents such as CPA-7, could be used to modulate the antioxidant activity in fresh-cut apples attaining products with an improved bioactive capacity.

CPA-7 ability to tolerate or induce changes in the fruit oxidative metabolism and to grow upon such conditions could imply its high resistance to oxidative stress.

This ability would provide the evaluated antagonist with an adaptive advantage to out-compete plant or human foodborne pathogens in case of contamination or to prime the fruit response to pathogen attack. This would support the hypothesis that the modulation of oxidative metabolism could be a mode of action for antagonism.

The observed differential temporal enhancement of key antioxidant enzymes depending on the storage conditions suggests a synergistic activation of different pathways in response to the several factors presently evaluated (chilling temperature, MAP storage, antioxidant treatment and the use of CPA-7 as BCA). In turn, such changes at the biochemical level were not accompanied by a negative effect on the fruit quality. Thus, the combination of these preservative strategies may be an advisable hurdle technology to simultaneously maintain the microbiological and physicochemical quality of fresh-cut apple.

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CHAPTER V

Impact of *Pseudomonas graminis* CPA-7 on respiration and ethylene production in fresh-cut 'Golden delicious' apple as affected by the maturity stage and preservation strategy

Collazo, C., Giné-Bordonaba, J., Aguiló-Aguayo, I., Povedano, I., Bademunt, A., & Viñas, I.
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ABSTRACT

The effect of the biocontrol agent (BCA) *Pseudomonas graminis* CPA-7 on the accumulation of CO_2 and ethylene (C_2H_4) in fresh-cut apples at two maturity stages was evaluated in refrigerated conditions. The influence of factors involved in the preservation strategy applied upon commercial conditions such as the antioxidant (AOX) treatment and the storage system was included in the analysis. Regardless of the maturity stage, the BCA reduced C_2H_4 levels within the MAP atmosphere in AOX-untreated apples wedges, by 29 and 43% in immature and mature apples, respectively. Nevertheless, the addition of ascorbate as antioxidant counteracted this effect. *In vitro* tests suggested that the reduction of C_2H_4 levels was not associated to the uptake of this molecule by CPA-7. Interestingly, in non-inoculated samples AOX treatment showed contradictory effects on C_2H_4 production in MAP conditions by significantly reducing C_2H_4 levels in immature apples (by 23%) while increasing it in mature ones (by 40%). Similarly, CPA-7 had opposite effects on the CO_2 accumulation pattern depending on the storage system or the fruit maturity stage. In this sense, CPA-7 was associated to higher fruit respiratory activity at advanced maturity stages yet without inducing the fruit fermentative metabolism or altering fruit quality during a week of refrigerated storage. Overall, these results show that CPA-7 may contribute to the maintenance of the microbiological and physicochemical quality of fresh-cut apple by modulating the fruit ethylene production and/or respiration.

5.1 INTRODUCTION

The effect of the application of antagonists on fresh-cut produce in commercial conditions is influenced by internal factors like the type and maturity stage of the commodity and external factors such as temperature, preservative treatments as well as oxygen and carbon dioxide concentrations within packages. From the physiological stand, processed products essentially behave as wounded tissues where the disruption of cell compartmentalization lead to the mixture of cellular components with an increase of enzymatic and respiratory activities as well as an elevated production of ethylene (C_2H_4) (Hodges and Toivonen, 2008; Mahajan et al., 2014). C_2H_4 is, indeed, the main hormone controlling ripening in clicmateric fruit (Reid, Rhodes and Hulme, 1973) and its biosynthesis involves the transformation of S-adenosylmethionine into the precursor 1-aminocyclopropane (ACC) mediated by the enzyme ACC synthase (ACS). ACC is later converted to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). In apple, as a fruit with a climacteric behavior, the regulation of these two steps is auto-inhibitory during fruit development prior to ripening and auto-stimulatory at the onset of ripening (Tatsuki, Endo, & Ohkawa, 2007; Wang et al., 2009; Lelièvre, Latché, Jones, Bouzayen, & Pech, 1997). Processing implies the mechanical injury of fruit tissues which induces the activity and synthesis of ACS leading to the formation of "wound ethylene" whose accumulation may be enough to activate the clicmateric phase depending of the size and permeability of packages (Lamikanra, Imam, & Ukuku, 2005; Yu & Yang, 1980). However, the low availability of ACO in pre-climacteric apples is a limiting factor regulating ethylene production upon cutting due to the reduced capability for the conversion of ACC into ethylene (Lara and Vendrell, 2000). In the post-clicmateric stage the capacity for ethylene production is also reduced and response to wounding is more limited than in the clicmateric stage (Abeles, Morgan and Saltveit, 1993). Therefore, the physiological stage of the commodity

constitutes a key factor to take into account during the production of fresh-cut fruit products (Toivonen and Dell, 2002).

Respiration also shows a biphasic rise during the development of clicmateric commodities, the first one early in development and the second one during ripening or senescence. The second peak usually precedes the autocatalytic ethylene synthesis stage (Fonseca, Oliveira and Brecht, 2002). Moreover, respiration is also induced by cutting due to the loss of compartmentalization of the enzymes involved in the respiration pathways and its substrates, and the activation of key regulatory steps of glycolysis and the tricarboxylic acid cycle (Rolle and Chism, 1987).

The mechanical injury of cell membranes also activates the enzymatic degradation of its lipid components, with the formation of long-chain fatty acids whose α-oxidation also causes a rise in respiration (Rolle and Chism, 1987). It is also well stablished that 'wound ethylene' induces fruit respiration (Yu and Yang, 1980). Furthermore, an increase in CO_2 production also occurs in fresh-cut tissues due to the activation of cell repair processes, not only for obtaining energy but for the synthesis of replacement structural compounds (Gomez-Lopez, 2012). The accelerated oxidative breakage of organic substrates and the loss of structure of membranes, entailed by the above mentioned processes, are detrimental to the nutritional properties and the general quality of fresh-cut fruit (Soliva-Fortuny & Martín-Belloso, 2003).

To reduce both respiration and ethylene production several methods comprising chilling conditions and modified atmosphere packaging (MAP) are amongst the most currently used in the fresh-cut produce industry (Rupasinghe and Yu, 2013). The addition of biocontrol agents (BCA) such as *Pseudomonas* spp. is another method that could contribute to modulate ethylene levels thereby extending the shelf-life of fresh ready-to-eat products. Mechanisms for the modulation of plant ethylene metabolism by *Pseudomonas* spp. have been already documented

and may imply both its exacerbation or its reduction (Fatima & Anjum, 2017; Glick, 2014; Hase et al., 2003). To accomplish the first mentioned effect, pseudomonads enhance the plant capacity to transform the precursor ACC into ethylene, inducing the expression of C_2H_4-responsive genes (Hase et al., 2003). Consequently, systemic induced resistance (ISR) is triggered or primed allowing plants to respond better to a subsequent infection by a broad spectrum of pathogens (Van Wees et al., 1997). The C_2H_4 reducing effect has been observed in plants upon treatment with pseudomonads with ACC deaminase (ACD) activity (Hernández-León et al., 2015; Singh et al., 2015). ACD cleaves ACC into ammonia and α-ketobutyrate (Honma and Shimomura, 1978) lowering the amount of available ACC and therefore limiting ethylene synthesis (Glick, 2014). As a consequence of this process pseudomonads can delay ripening and senescence, promote growth, prime resistance mechanisms and alleviate deleterious ethylene-mediated plant stresses (Eckert et al., 2014; Glick, 2005; Wang, Knill, Glick, & Défago, 2000). Belonging to this bacterial group is *Pseudomonas graminis* CPA-7, a whole apple epiphyte biopreservative strain which controls foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica* on fresh cut fruit (Alegre et al., 2013a, 2013b; Abadias et al., 2014; Collazo et al., 2017) and modulates oxidative metabolism in fresh-cut apple (Collazo, Giné-Bordonaba, *et al.*, 2018). In an attempt to clarify its mode of action we also investigated the possibility for CPA-7 to modulate the ethylene metabolism in fresh-cut apple thereby influencing the defense response and/or the senescence of this fruit. With this in mind, we monitored the effect of the antagonist in fresh-cut apples as affected by several factors involved in production and commercial conditions (antioxidant treatment, packaging headspace gas composition and the maturity stage of the commodity). In addition, to assess the effect of CPA-7 metabolic activity in supplying exogenous ethylene or metabolizing the produced by the fruit, the ability of the antagonist to either produce or consume C_2H_4 was tested *in vitro*.

5.2 MATERIALS AND METHODS

5.2.1 ANTAGONIST INOCULUM PREPARATION

For inoculum preparation, *P. graminis* CPA-7 (Alegre, Viñas, Usall, Teixido, *et al.*, 2013) was grown overnight in 50 mL of tryptone soy broth (TSB, Biokar, Beauveais, France) at 25 °C in agitation. Cells were harvested by centrifugation at 9800 x g for 10 min and suspended in deionized sterile water. The concentration of the suspension was checked by viable plate count of appropriate ten-fold dilutions in saline peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto TSA plates after incubation at 30 °C for 48 h.

5.2.2 FRUIT PROCESSING

Apples (*Malus domestica* Borkh. cv. 'Golden delicious') used in this study were grown in local farms (Lleida, Catalonia, Spain) and collected in august, 2017 at two maturity stages (with a week of difference between harvests). Prior to experimental assays, apples were washed with running tap water, surface disinfected with 700 mL L^{-1} ethanol and either stored as such or processed (peeled with an electric fruit peeler and cut into eight wedges with a handheld corer/slicer). Wedges were kept in chilled (5 °C) chlorinated tap water (pH 6) until treatment and/or packaging.

5.2.3 *IN VITRO* ANALYSIS OF ETHYLENE PRODUCTION OR CONSUMPTION

PREPARATION AND INOCULATION OF LIQUID CULTURE MEDIA

In vitro assays were performed in order to evaluate the putative ethylene production or consumption by CPA-7 in a culture medium with a similar

composition to the fruit but discarding the changes due to the apple's native microbiota. For that, analysis glass tubes containing 10 mL of sterile apple juice were inoculated with CPA-7 to a concentration of 10^5 CFU mL^{-1}. Additionally, aliquots of TSB were prepared, inoculated and analyzed in the same way, to serve as a control treatment. For juices preparation, apple wedges were previously dipped in 6% NatureSeal® AS1 solution (AS1, AgriCoat Ltd., Great Shefford, UK), a calcium ascorbate-based product, or in cold deionized water, for 2 min in agitation (15.7 rad s^{-1}) in a tabletop orbital shaker (Unimax 1010, Heidolph, Germany). Then, juices were obtained in a commercial blender, subsequently filtered through cloth gauzes and either adjusted to pH 6.5 with 1 mmol L^{-1} NaOH or sterilized as such at 215 °C for 5 min and stored at 5 °C until use. Non-inoculated aliquots of each culture medium were also prepared and used as controls. Inoculated and non-inoculates cultures were stored in agitation in aerobic conditions for 7 d at 5 °C in the case of TSB and apple juice pH 4,5; and at 5 °C or at 25 °C in the case of apple juice pH 6.5. *In vitro* assays were repeated twice and included three replicates per treatment.

MICROBIAL DYNAMICS

CPA-7 population dynamics in each culture medium was tracked by viable plate count on TSA at 0, 1, 3, 6, and 7 d post-inoculation, as described in section 5.2.1.

CO_2 ACCUMULATION PATTERN

The headspace O_2 and CO_2 composition of each culture tube was measured at 0, 1, 3, 6, and 7 d post-inoculation using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark). Before each measurement tubes were hermetically closed for 12 h. CO_2 accumulation was expressed in mg mL^{-1} liquid culture medium.

C_2H_4 ACCUMULATION PATTERN

The ethylene accumulation patterns of cultures tubes previously sealed for 12 h were determined at 0, 1 and 3, 6 and 7 d post-inoculation. For that,1 mL of gas sample was withdrawn daily from each jar or tray with a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1,Tecknokroma, Barcelona, Spain). The injector and detector were kept at 180 °C and 280 °C, respectively. Quantification was carried out by comparing the gas chromatography signal of the samples to that of a 21 µL L^{-1} C_2H_4 standard (Carburos metálicos SL, Aragón, Spain). Ethylene accumulation within the storage atmosphere of the tubes was expressed as µL mL^{-1} culture medium. Putative ethylene degradation by CPA-7 was assessed by tracking the reduction of 1 mL of 21 µL L^{-1} C_2H_4 standard injected with a syringe in hermetically sealed tubes (containing 10 mL of either apple juice or TSB medium and 12 mL of headspace).

5.2.4 *IN VIVO* ANALYSIS OF C_2H_4 AND CO_2 ACCUMULATION PATTERNS

FRUIT TREATMENT AND PACKAGING

The experimental setup of *in vivo* tests is shown in Fig. **V.**1. For dip inoculation of apple wedges, CPA-7 suspensions at a concentration of 10^7 CFU mL^{-1} were prepared in cold deionized water (4 °C) or in cold 60 g L^{-1} AS1 antioxidant aqueous solution. Fruit wedges were dipped in the bacterial suspensions or in non-inoculated water or antioxidant solution as controls, at a ratio of 1:2 (weight of fruit: volume of solution) as described in section 2.3.1.

After the draining, treated apple wedges were packaged in two storage systems; hermetic jars (static system) and commercial trays (MAP). For the static system, 1 kg of intact apples was stored in 3.4 L jars and 500 g of apples wedges was stored in 1.7 L jars. Jars were equipped with a silicon septum for sampling the gas of the headspace. For MAP, 120 g of processed fruit were placed in 400 mL polyethylene terephthalate ShelfMaster™ Pronto™ trays (PlusPack, Denmark) and thermosealed with 181.7 cm^2 of a 3-holed (60 - 80 µm diameter, 75 mm spacing) multilayered microperforated film (polyester anti-fog film, OALF (14 µm of thickness) + oriented polypropylene, OPP (20 µm of thickness) (PDS Group, Murcia, Spain) to achieve passive modified atmosphere. Trays and jars were stored statically at 5 °C in darkness. Each tray/jar was considered as a replicate and three replicates per treatment and sampling time were included.

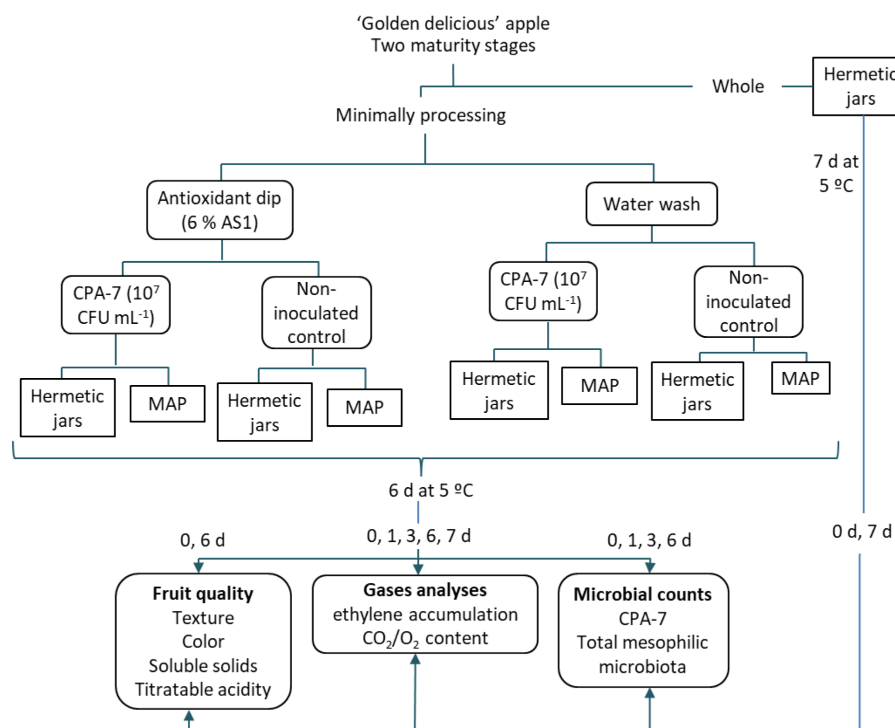

Figure V.1. Experimental workflow of *in vivo* trials

Microbial dynamics

CPA-7 as well as total mesophilic aerobic microorganisms' (MAM) population dynamics on apple wedges stored in trays were analyzed at 0, 1, 3 and 6 d post-inoculation. For that, 10 g of apple from each tray was homogenized in 90 mL buffered peptone water (BPW, Biokar, Beauvais, France) and analyzed by viable cells counts. CPA-7 was plated onto TSA plates and MAM were plated onto plate count agar plates (PCA, Biokar, Beauvais, France). Plates were incubated at 30 °C for 48 h or 3 d, respectively. In the same way, microbial populations in jar-stored apples were determined at 0 and 7 d post-inoculation. Microbiological data were expressed as colony forming units per gram of fresh weight of fruit (CFU g^{-1} FW) and transformed to log_{10} for subsequent statistical analyses.

CO_2 accumulation pattern

The headspace O_2 and CO_2 composition of each replicate stored in jars or in MAP (trays) was measured at 7 h post-inoculation and then at 1, 2, 3, 6, and 7 d as described in section 2.3.3. CO_2 accumulation was expressed relative to the fresh weight of fruit (mg kg^{-1} FW).

C_2H_4 accumulation pattern

To determine *in vivo* ethylene accumulation in trays and jars samples were taken 7 h post-treatment and then at 1, 3, 6, and 7 d. Ethylene accumulation within the storage atmosphere was expressed as μL kg^{-1} FW.

Fruit quality parameters

Texture of whole and processed apples as well as color, pH, soluble solids content (SSC) and titratable acidity (TA) of apple wedges were determined as

described elsewhere (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). In apples stored in jars (whole and processed) all quality parameters were measured initially and at the end of storage. In processed apples stored in MAP color was measured at 0, 1, 3 and 6 d post-treatment while pH, TA and SSC were measured initially and at the end of storage. Texture and pH were measured in five wedges per replicate per treatment at each sampling time. Two measures of color, one per side, were performed on five wedges per replicate per sampling time. Low values of CIE color parameter L* and high values of a* were considered as indicators of surface browning intensity (Sapers and Douglas, 1987). SSC at 20 °C was expressed as mass fraction (%). TA was measured in 10 mL of pulp and was expressed as malic acid content (g L^{-1} juice).

5.2.5 STATISTICAL ANALYSIS

Data were analyzed using the general linear model procedure to determine the treatment and interaction effects, with the statistical software JMP (version 11 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity of residues. Results were schematically represented as means ± standard deviation. Means were compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).

5.3 RESULTS AND DISCUSSION

5.3.1 *IN VITRO* ANALYSES: MICROBIAL DYNAMICS AND C_2H_4 PRODUCTION OR CONSUMPTION

To test the ability of CPA-7 to growth and produce or consume ethylene in sterile apple juice, microbial populations and gas headspace composition were measured throughout 7 days. As a control treatment, the same analyses were performed in TSB, a synthetic media usually used for CPA-7 culture in the

laboratory (Alegre, Viñas, Usall, Anguera, *et al.*, 2013; Abadias *et al.*, 2014). Results showed that CPA-7 initial populations (10^5 CFU mL^{-1}) increased by 1 log_{10} in TSB after 24 h at 5 °C and afterwards they remained stable up to day 6 (Fig. **V.** 2).

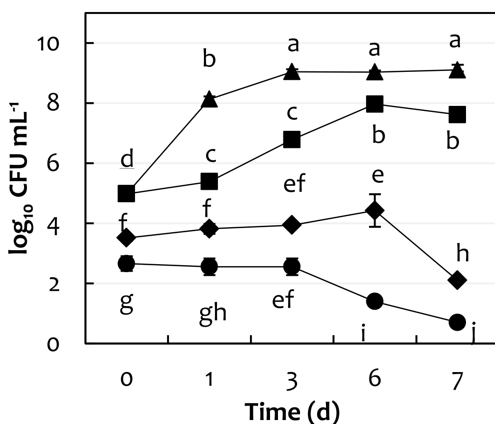

Figure V.2. CPA-7 *in vitro* population dynamics when grown in: apple juice pH 6.5 at 25 ºC (▲), apple juice pH 6.5 at 5 ºC (■), TSB medium at 5 ºC (◆), and apple juice pH 4.5 at 5 ºC (●). Symbols represent means and error bars represent standard deviation of the mean (n = 6). Different letters represent significant differences among treatments according to ANOVA and Tukey's test with a 95 % confidence ($p < 0.0001$).

No growth was observed in apple juice pH 4.5 but instead, the population gradually decreased to levels close to the detection limit (5 CFU mL^{-1}), thus the gas analysis of these samples was stopped after day 3. In accordance with this result, previous studies showed that CPA-7 failed to growth in synthetic liquid media at pH ranging from 4.5 to 5 (adjusted with inorganic and organic acids). Furthermore, CPA-7 grew better in media adjusted with malic acid than in those adjusted with citric acid (Iglesias, 2017). CPA-7 also showed limited growth in melon juices at 5 °C compared to pieces; being this fruit rich in citric acid despite of having a pH close to neutrality (Collazo et al., 2017). Therefore, the subsequent analyses were performed in apple juices with pH adjusted to 6.5.

Results showed that CPA-7 populations quickly increased (by 3 log_{10} in the first 24 h) in apple juice pH 6.5 incubated at 25 °C reaching the stationary phase (9 ± 0.1 log_{10} CFU mL^{-1}) before day 3 as expected for the optimal growth temperature (Alegre, Viñas, Usall, Teixido, *et al.*, 2013). When incubated at 5 °C CPA-7 populations in apple juice pH 6.5 increased slower than at 25 °C, reaching the

stationary phase on day 6 and attaining levels 2 log_{10} higher than when grown in TSB (pH 7.3). In general, the above presented results showed that apple juice pH 6.5 a suitable culture medium for CPA-7, showing similar population dynamics to that observed in fresh-cut melon (pH 6.4) incubated at a temperature within the optimal range (20 °C) (Abadias *et al.*, 2014).

CO_2 production was close to cero during the whole evaluated period in TSB cultures as well as apple juice pH 4.5 ones (data not shown), which was in accordance with the lower growth observed in those culture media compared to apple juice pH 6.5. Likewise, CO_2 accumulation patterns in apple juices (pH 6.5) correlated with the CPA-7 population's dynamics depending on the temperature of incubation. At 5 °C CO_2 accumulation remained stable throughout storage (0.04 ± 0.04 mg L^{-1} h^{-1}), while it increased from 1.5 ± 0.4 to 2.9 ± 0.9 mg L^{-1} h^{-1} at 25 °C.

Regardless of the temperature of storage no ethylene production was observed for CPA-7 whether it was grown in TSB or in apple juices (pH 6.5 or pH 4.5). In the same way, no differences in ethylene accumulation or in the antagonist population levels were detected in the inoculated samples after supplementation with exogenous C_2H_4 compared to the non-supplemented control at any of the analyzed sampling points in any of the culture media or incubation temperatures assayed (data not shown). Overall, the results obtained in *in vitro* assays showed that although CPA-7 is able to use the nutrients present in apple juice to grow, it is unable to produce or consume ethylene under the conditions tested.

5.3.2 *IN VIVO* ANALYSIS OF C_2H_4 AND CO_2 ACCUMULATION PATTERNS

MICROBIAL POPULATION DYNAMICS

In MAP-stored apples CPA-7 populations were initially 5.53 ± 0.04 and 5.90 ± 0.04 $\log_{10}$ CFU g^{-1} FW in samples from the first (H1) and the second harvest (H2), respectively. Afterwards, in samples upon the antioxidant effect, CPA-7 populations showed a slower growth and reached lower levels than in AOX-untreated samples (Fig. **V.**3A). Conversely, in apples from the second harvest no differences were observed between AOX-treated and untreated samples during the whole storage period (Fig**. V.**3B). Previous semi-commercial trials have shown contradictory results regarding the effect on antioxidant treatments on CPA-7 growth. For example, CPA-7 showed slower and less growth after 6 d at 5 °C on fresh-cut pear upon antioxidant treatments containing calcium and ascorbate and in similar MAP conditions (Iglesias *et al.*, 2018). On the contrary, Alegre et al. (2013a) selected AS1 as antioxidant treatment for commercial assays with CPA-7 in 'Golden Delicious' fresh-cut apple wedges as they observed no differences in growth between AS1-treated or untreated samples incubated at 10 °C during 2 d. Discrepancies in the effect of antioxidant treatments on CPA-7 growth among experiments might be related to not only to the commodity used but due to its maturity stage. Furthermore, CPA-7 grew more in H2 (by 1.2 ± 0.2 $\log_{10}$) than in H1 apples (by 0.8 ± 0.1 $\log_{10}$) regardless of the antioxidant application as previously observed for fresh-cut mangoes stored in containers with snap-fit lids at 5 °C for 8 d (Abeles, Morgan and Saltveit, 1993). On the other hand, when stored in jars, CPA-7 populations on apple wedges showed similar dynamics regardless of the harvest date or the antioxidant treatment (Fig**. V.**3C-D).

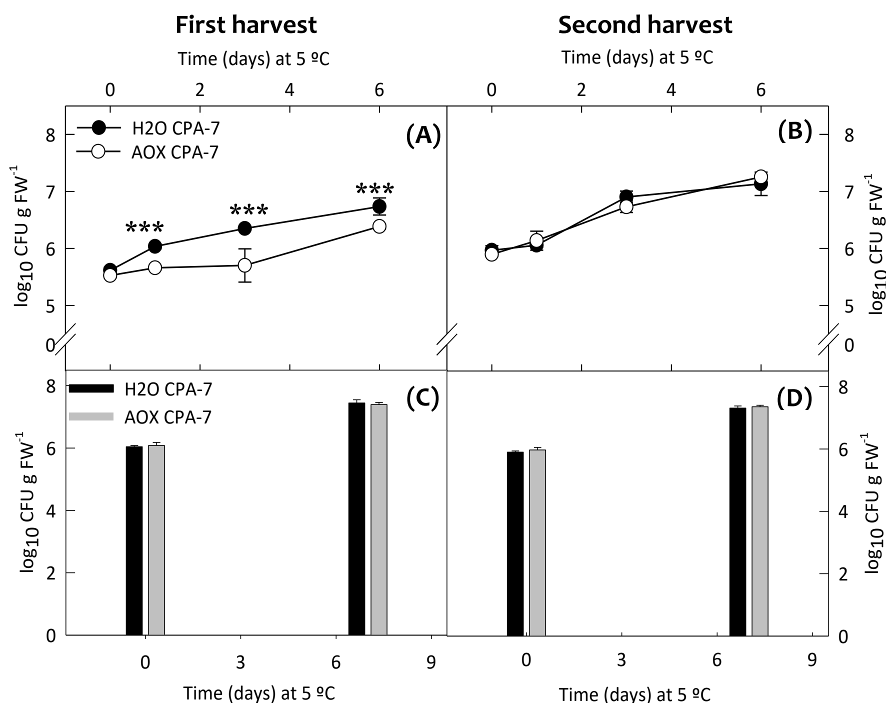

Figure **V**.3. CPA-7 population dynamics on antioxidant-treated (AOX) or untreated (H_2O) fresh-cut 'Golden delicious' apple wedges from the first (left column) and the second harvest (right column) dates during storage in trays (MAP conditions) (A-B) or in hermetically sealed jars (C-D) at 5 °C. Symbols represent means and error bars represent standard deviation of the mean (n = 6). Asterisks represent significant differences among treatments according to analysis of variances (ANOVA) and Tukey's test with a 95 % confidence (*** for $p < 0.0001$).

MAM populations were initially at the same levels in samples from both harvest dates (2.3 ± 0.2 log_{10} CFU g^{-1} FW). As observed for CPA-7, mesophilic bacteria grew more in the AOX-untreated H1 apples (by 1.9 ± 0.4 log_{10}) than in the rest of the samples (by 0.7 ± 0.1 log_{10}) (data not shown). Those results confirmed the inhibitory effect that ascorbate and calcium-containing antioxidant agents may have on microbial growth as previously observed in several studies performed with different fresh-cut fruit (Iglesias et al., 2018; Oms-Oliu et al., 2007).

CO_2 ACCUMULATION PATTERN

The headspace gas composition of packages was highly influenced by the permeability of the storage system and the physiological stage of the fruit (Supplementary Table 1). As expected, apples from the second harvest (H2) showed higher respiration rates than those from the first one (H1) thereby depleting faster O_2 levels. This led to anoxic conditions (0.4 ± 0.3 %) by day 7 of storage in the hermetic system in H2 inoculated samples while in H1 apples O_2 levels only decreased to 12 ± 1 % in the same period. In the permeable system a stable O_2 concentration ranging from 15 to 17 % was maintained throughout storage.

Like ethylene production, respiration is activated not only during ripening but also upon wounding-stress thus, during the processing of fresh-cut produce (Saltveit, 2016). Accordingly, our results showed an initial rise in CO_2 production by 40 % in processed apples compared to intact apples in both maturity stages (Fig. **V.**4G-H). The magnitude of the wound-response in the respiration rate was significantly higher in mature than in immature apples exceeding by 2.5-fold and 1.4-fold, respectively, the respiration rate of whole apples. The exacerbation of respiration rate in fresh-cut apple slices by 2 - 3 times compared to whole fruit had previously been reported (Lakakul, Beaudry, & Hernandez, 1999). The increased respiration in minimally processed fruit is mostly due to a physiological response to wounding which is tightly linked to the maturity stage, since the removal of the peel barely reduce the resistance to O_2 diffusion in apples (Fonseca et al., 2002). In general, we observed that the respiration rate reached the highest values immediately after processing and subsequently declined throughout storage regardless of the combination of factors analyzed.

In MAP storage, CO_2 accumulation in H1 AOX-untreated samples showed an increase by 16 % in the presence of CPA-7 compared to non-inoculated control, 24 h post-processing (Fig. **V.**4C). The addition of the antioxidant reversed this

behavior, being CO_2 production in inoculated samples up to 25 % lower than the control during the first two days in MAP conditions (**Fig. V.**4A). In H2 samples differences in CO_2 accumulation pattern were only observed on the 3rd day of MAP storage being remarkably higher in response to the antagonist than in the control whether they were treated (by 16 %) or not (by 22 %) with the antioxidant. This could be associated to the clicmateric peak of the product as it was correlated with a rise in ethylene production (Lelièvre *et al.*, 1997). According to this result, CPA-7 may enhance the clicmateric peak in processed apples at more advanced maturity stages in agreement with previous findings showing that once the autocatalytic phase of ripening has begun the effectiveness of preservative methods is considerably reduced and shelf-life is shortened (Rojas-Graü et al., 2007; Soliva-Fortuny, Oms-Oliu, & Martìn-Belloso, 2002). Previous experiments performed with fresh-cut 'Fuji' apple slices showed that according to storage conditions, reducing agents such as ascorbic acid influence other physiological processes in addition of preventing oxidation (Gil, Gorny and Kader, 1998). In that study, ascorbic acid dips reduced respiration rate as well as ethylene production in 'Fuji' apples stored in air (21% O_2, 0% CO_2) while increased respiration in MAP- stored (0% O_2, 0% CO_2, 100% N_2) apple slices (Gil, Gorny and Kader, 1998).

Contrastingly in the hermetic system (jars), no differences were observed between inoculated and non-inoculated apples during the first two days of storage regardless of the addition of the antioxidant or the harvest date. However, contradictory effects of CPA-7 on CO_2 accumulation according to the maturity stage and the treatment with the antioxidant were observed in the hermetic system from the 3rd day of storage even when population levels were not influenced by the mentioned factors. For instance, a decrease by 22% (Fig. **V.**4G) or an increase by 67% (Fig. **V.**4H) in CO_2 accumulation was observed in CPA-7-inoculated AOX-untreated samples from H1 and H2, respectively, compared to non-inoculated controls.

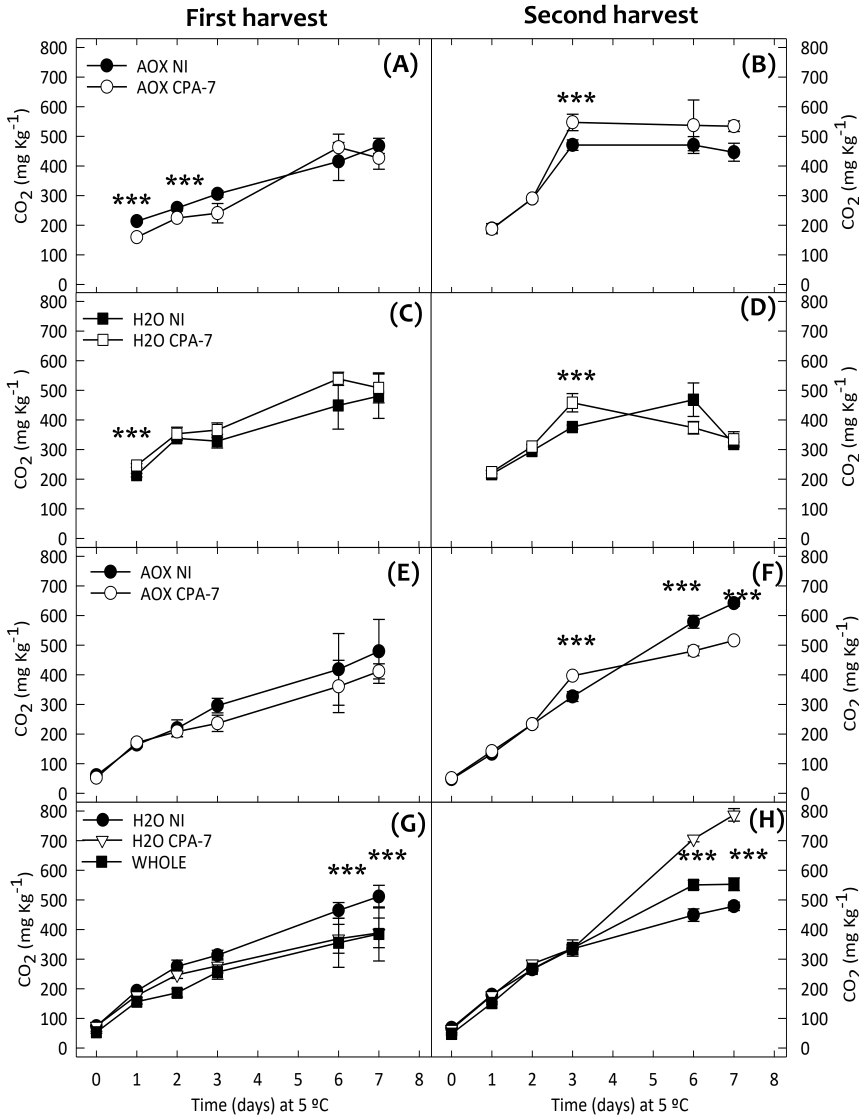

Figure V.4. CO_2 accumulation patterns in CPA-7-inoculated and non-inoculated (NI) fresh-cut 'Golden delicious' apple wedges from the first (left column) and the second harvest (right column) dates when stored in MAP (**A-D**) and in hermetic jars (**E-H**). Graphs A-B and E-F: apples treated with the antioxidant (AOX); graphs **C-D** and **G-H**: AOX-untreated samples. Symbols represent means of three biological replicates and error bars represent standard deviations. Asterisks represent significant differences according to an analysis of variances ANOVA and Tukey´s test with a 95 % confidence (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$). Asterisks below the lines represent differences only between whole and processed apples, regardless of the inoculation with CPA-7.

The impact of CPA-7 on CO_2 accumulation in the hermetic system was either eliminated (no differences in H1 samples) or inversed (higher by 22 % than the H2 control) upon the antioxidant treatment in both maturity stages (Fig. **V.**4E-F). The influence of external factors such as temperature and the gas headspace composition (O_2 and CO_2) of the packages on the respiration of fresh-cut commodities is generally recognized and thus it has been included in several models for the design of MAP technologies (Fagundes, Carciofi, & Monteiro, 2013; Lakakul et al., 1999). However the combination of these factors and biological or chemical preservatives has not been so well studied. Investigation on this matter is needed for developing models to predict the shelf-life of fresh-cut produce in commercial conditions.

C_2H_4 accumulation pattern

The initial ethylene production of intact apples from the second harvest date (H2) was 3.7-fold higher than those from the first one (H1) evidencing their more advanced physiological stage (Lelièvre *et al.*, 1997), thus they will be henceforth referred as mature and immature apples, respectively. In the same way, C_2H_4 production in intact H2 apples remained 3-fold higher than processed ones during the whole storage while it showed no differences in H1 fruit (Fig. **V.**5G-H) suggesting the pre-clicmateric stage of the fruit from the earliest harvest (Oetiker and Yang, 1995; Chaves and de Mello-Farias, 2006). The immediate effect of processing in C_2H_4 production was highly influenced by the maturity stage, showing a reduction in mature apples wedges compared to intact fruit and not differences in immature fruit. Contradictory effects of cutting on ethylene production, as previously observed in several clicmateric commodities, have been explained by differences in the physiological stage of the fruit and linked to differential expression patterns of the genes involved in the ethylene metabolism, before, during and after, the clicmateric phase (Bapat et al., 2010; Toivonen & Dell, 2002; Vilanova et al., 2017).

The impact of the application of CPA-7 on fresh-cut fruit ethylene metabolism cannot be separated from that of other factors such as the gas internal composition of packages, the maturity stage and the preservative chemical treatment which has previously shown to have significant influence on the physiology of this kind of products (Rojas-Graü, Grasa-Guillem and Martín-Belloso, 2007). In MAP storage, the ethylene accumulation pattern in processed AOX-untreated apples was different according to the maturity stage (Fig. **V.** 5, C-D) showing a peak on day 6 in H1 apples and on day 3 in H2 ones. Similarly, during the first days of storage, differential effects of the application of CPA-7 on the ethylene accumulation within the MAP atmosphere were observed in processed AOX-untreated apples, causing reduced C_2H_4 levels (by 30%) in immature apples on day 2 while it enhanced them by 11% in mature apples on day 3. However, as time passed, CPA-7 was associated to a reduction in C_2H_4 accumulation by about 29 % and 43 % in H1 and H2 apples, respectively. This may have in turn contributed to a reduction of the ethylene-mediated fruit senescence (Czarny, Grichko and Glick, 2006) and hence, partially explain the improved quality of CPA-7 inoculated apples.

The antioxidant treatment effectively reduced C_2H_4 levels in H1 apples upon MAP conditions during the whole storage while markedly enhanced it in H2 apples (Fig**. V.**5A-B). In general, CPA-7 effect on the C_2H_4 levels within MAP packages was suppressed by the antioxidant, except for the increase by 29% during the first 24 h post-processing in H1 apples. Likewise, no differences were observed regarding C_2H_4 accumulation within the MAP atmosphere in mature apples until day 6 when it dropped by 98 % in the presence of CPA-7.

In our trials, the reduction of C_2H_4 levels cannot be explained by the consumption of ethylene by the antagonist since the levels of this molecule remained invariable after its supplementation *in vitro,* both in TSB medium and in apples juices.

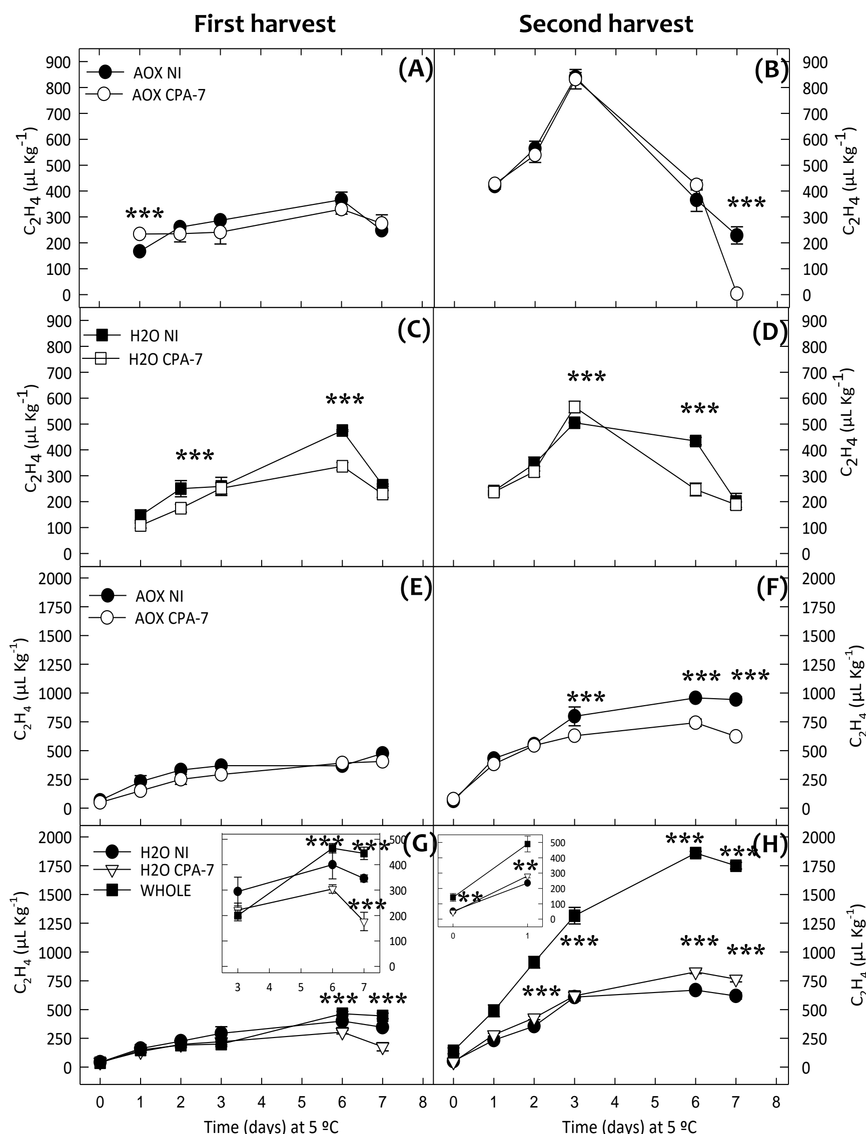

Figure V.5. Ethylene production in CPA-7-inoculated and non-inoculated (NI) fresh-cut 'Golden delicious' apples from the first (left column) and the second harvest (right column) dates when stored in MAP (A-D) or in hermetic jars (E-H). Graphs A-B and E-F: apples treated with the antioxidant (AOX); graphs C-D and G-H: AOX-untreated samples. Inserts in graphs G and H are plots of the same data in a smaller scale. Symbols represent means of three biological replicates and error bars represent standard deviations. Asterisks represent significant differences according to an analysis of variances ANOVA and a Tukey´s test with a 95 % confidence (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

Alternatively, the reducing effect of this bacterium on the fruit ethylene production could be associated to 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity. This hypothesis is suggested by the presence in the bacterial genome of a gene fragment encoding a protein (Collazo et al., unpublished results) that shares 98% homology with the ACC-deaminase (ACD) enzyme (GenBank Acc. No. WP_065986140.1) belonging to *P. graminis* strain P 294/08 (Behrendt *et al.*, 1999). However, further analysis should be performed in order to demonstrate the functionality of this gene in CPA-7. Following this thought, no remarkable reduction of C_2H_4 can be attributed to ACD activity soon after processing since the initial response to wounding has been suggested to deplete the existing pool of ACC available within plant cell through the rapid action of ACC oxidase (ACO), which has greater affinity for ACC than ACD (Glick, 2014). However, ACD activity could explain the reduction of the second ethylene peak, probably associated to *de novo* production of ACC that was observed in CPA-7-inoculated samples after 6 d of storage. The decrease in C_2H_4 levels at that sampling point was observed in both storage systems (MAP and hermetic) when O_2 concentration was similar (14 – 16%).

In the hermetic system, changes in the accumulation of ethylene in CPA-7-inoculated AOX-untreated apple wedges were only observed at the end of storage and they showed to be highly influenced by the maturity stage (Fig. **V.**5G-H). In H1 apples CPA-7 reduced the C_2H_4 accumulation pattern by 24 and 49% compared to the non-inoculated control after 6 and 7 days of storage, respectively (Fig 5G). In H2 apples, inoculation with CPA-7 triggered higher C_2H_4 levels (by up to 19%) after 6 d of storage (Fig. **V.**5H). The CPA-7 effect was either annulled (no differences in H1 apples) or reversed (decreased by 34 % at day 7 in H2 apples) upon antioxidant treatment (Fig. **V.**5E-F). Similarly, a reduction of C_2H_4 levels in wounded clicmateric melon inoculated with the biological control agent *Bacillus subtilis* EXWB1 was also observed by Wang et al. (2010) after 4 d of storage in hermetically sealed containers at 24 °C, which correlated with a

subsequent increase in the fruit defense response to fungal decay. In addition to the maturity stage, the differences in the C_2H_4 accumulation patterns observed at the end of storage in the hermetic system could also be influenced by O_2 availability, which varied from 12 – 13% for immature apples to 0.4 – 1.2 % for mature ones. However, reduced C_2H_4 accumulation in respect of the control was observed in mature apples stored in the hermetic system (2.9 – 1.7 O_2, 14% CO_2) when treated with the antioxidant. O_2 is a key factor in the regulation of *AcdS* gene, which encodes ACD (Singh *et al.*, 2015) but limited information is available regarding the influence of antioxidants in this process. Results showed that antioxidant (AOX) dipping of mature apples enhanced the accumulation of C_2H_4 both in the hermetic and the MAP storage system, regardless of the inoculation with the antagonist. Ascorbic acid-based antioxidant dipping has previously shown to increase ethylene production in fresh-cut Fuji apples upon refrigerated conditions at a different timing and extent according to oxygen availability and ripening stage, showing higher ethylene accumulation in MAP than air conditions and in ripe than in unripe fruit (Rojas-Graü, Grasa-Guillem and Martín-Belloso, 2007).

Fruit quality parameters

The initial firmness of whole apples was similar regardless of the moment of harvest (67 ± 3 N) yet the loss of firmness throughout storage was more evident in mature apples (H2) than in those from the first harvest (immature, H1): by 45 and 26 %, respectively. In general, no significant differences in firmness were observed among treatments or throughout storage in MAP or in jars, with values ranging from 13 ± 2 and 10 ± 1 N (data not shown). No significant reduction of texture was either observed upon inoculation with CPA-7 and MAP storage for 5 to 10 d at 5 or 10 °C in previous experiments performed in fresh-cut apples, melons and pears (Alegre, Viñas, Usall, Anguera, *et al.*, 2013; Abadias *et al.*, 2014;

Iglesias *et al.*, 2018). Those results suggest that CPA-7 does not show an enhanced pectinolytic activity leading to altered fruit firmness.

Soluble solids content (SSC) in unprocessed apples was initially higher in H2 apples than in H1 ones (Table **V.**1). It subsequently remained invariable throughout time in apples from both harvests. Interestingly, samples treated with the antioxidant showed an initial increase in the SSC in contrast with untreated samples regardless of the maturity stage. Higher contents in soluble solids were also observed for AOX- treated samples at the end of MAP storage in apples from the second harvest date. In general, inoculation with CPA-7 did not alter SSC in any of the evaluated conditions and hence agrees with previous studies conducted on a range of commodities (Alegre, Viñas, Usall, Anguera, *et al.*, 2013; Abadias *et al.*, 2014; Iglesias *et al.*, 2018). Similarly, apple pH was about the same (4.0 ± 0.2) and remained stable throughout the studied period for all the conditions tested.

Titratable acidity was initially lower in CPA-7 - inoculated H2 samples compared to the control while no differences were observed between inoculated and non-inoculated H1 apples (Table 1). The opposite effect was observed at the end of storage in the hermetic system. In MAP conditions, this parameter did not show differences in the presence of CPA-7 at the end of storage. For the AOX-treated samples no differences were observed between inoculated and non-inoculated samples regardless of the maturity stage. Previous works have shown that AOX treatments containing calcium and ascorbate contributed to maintain titratable acidity and soluble solids content in fresh cut 'Golden delicious' apples (Soliva-Fortuny, Ricart-Coll, & Martín-Belloso, 2005).

Table **V**.1. Physical quality parameters of intact (Whole) and fresh-cut 'golden delicious' apples from the 1st and the 2nd harvest dates, inoculated with CPA-7 (CPA) or non-inoculated (NI), upon antioxidant treatment (AOX) or not (H_2O), when stored in trays (MAP) or in hermetic jars.

		Jars and trays		**Trays**		**Jars**	
		day 0		**day 6**		**day 7**	
	Treatment	H1	H2	H1	H2	H1	H2
SS (%)	Whole	12.5 ± 0.1**aA**	13.3±0.01**aA**			14±1**aB**	13±0.9**aA**
	AOX NI	12.8 ±0.01**aA**	13.6±0.2**aA**	12.7±0.04 aA	13.3±0.03**aA**	12.3±0.4**aA**	12.5±0.2**aB**
	AOX CPA	12.9 ± 0.1**aA**	12.8±0.1**bA**	12.6 ± 0.1**aA**	12.6±0.01**aA**	12.8±0.8**aA**	13.1±0.2**aA**
	H2O NI	12.4 ± 0.1**bA**	12.4±0.04**cA**	12.5±0.03**aA**	11.6±0.04**bB**	12.1±0.2**aA**	12.4±0.4**aA**
	H2O CPA	12.4 ±0.01**bA**	12.3±0.02**cA**	12.3±0.2**aA**	11.7±0.01**bB**	11.2±0.4**aA**	12.6±0.1**aA**
TA ($g\ L^{-1}$)	Whole	3.2±0.1**aA**	4.4±0.2**aA**			2.9±0.4**cA**	4.6±0.4**aA**
	AOX NI	3.3±0.2**aA**	3.3±0.1**aA**	3.4±0.2**aA**	3.8±0.3**aA**	3.8±0.1**aB**	3.2±0.1**bA**
	AOX CPA	3.22±0.02**aA**	3.37±0.03**aA**	3.2±0.4**aA**	3.6±0.5**abA**	3.8±0.2**aB**	3.5±0.1**bA**
	H2O NI	3.2±0.1**aA**	2.8±0.5a**bA**	3.4±0.1**aA**	3.0±0.2**bcA**	4.1±0.1**aB**	3.2±0.1**bB**
	H2O CPA	3.4 ±0.1**aA**	3.1±0.04**bA**	3.4±0.3**aA**	3.1±0.3**bcA**	3.3±0.1**bA**	3.1±0.04**bA**

Results represent mean ± standard deviation. Different lowercase letters represent significant differences among treatments at each sampling time and uppercase letters represent significant differences throughout time for each treatment according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$). TA: titratable acidity calculated as malic acid content. SS: soluble solids expressed as mass fraction to juice volume.

Inoculation with CPA-7 had no influence on a* and L* color parameters compared to non-inoculated control in any of the conditions tested (harvest date, storage system, antioxidant treatment and/or storage period) (Fig. **V.**6). Similar results were obtained after 14 d of storage at 5 °C in a previous study testing the effect of the inoculation with CPA-7 on the quality of fresh-cut apples in semi-commercial conditions resembling the ones assayed in the present work (Alegre, Viñas, Usall, Anguera, *et al.*, 2013). In general, antioxidant treatment resulted in a reduction of a* after 1 d of MAP storage in inoculated and non-inoculated apples from both harvests (Fig. **V.**6C-D).

However, this effect was more marked in H1 apple wedges than H2 ones, which agreed with previous studies performed in apple wedges (Soliva-Fortuny et al., 2002). As observed for a*, antioxidant dipping significantly improved and

preserved lightness (L*) throughout storage in both storage systems and harvest dates regardless of the inoculation with the antagonist (Fig. **V.**6A-B, E-F). In general, CPA-7 showed no negative effect on fruit quality parameters when combined with the antioxidant in any of the storage systems tested.

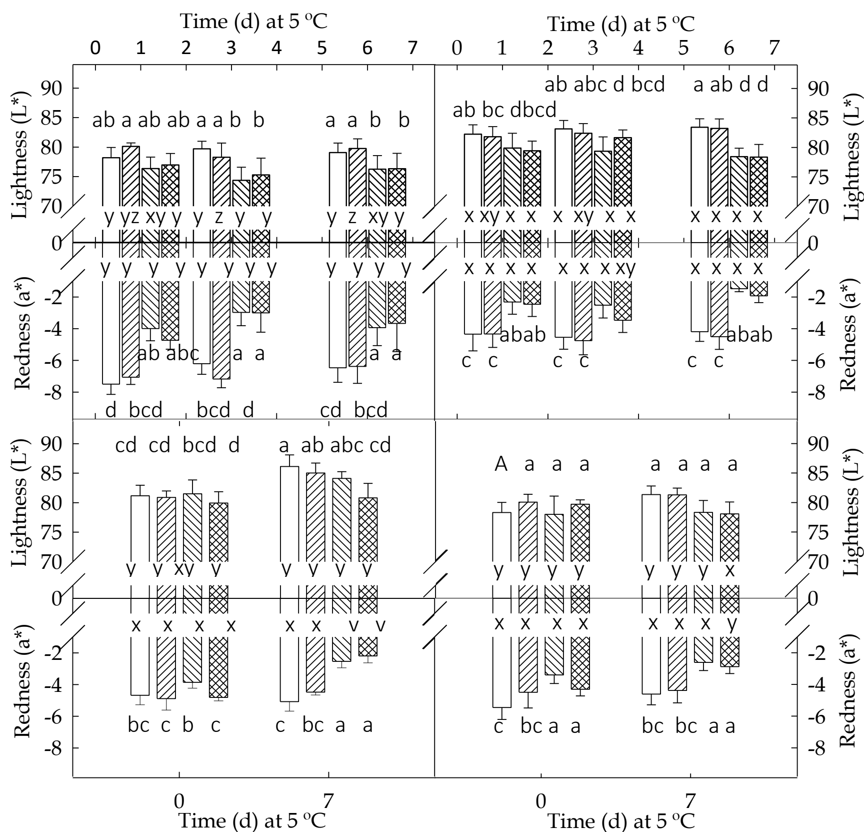

Figure **V**.6. CIE color parameters: L* (lightness) and a* (redness) of fresh-cut apple wedges from the 1st (left column) and the 2nd (right column) harvest dates when stored in trays (MAP): A-D, or in hermetic jars: E-F. Columns represent means of 10 measures per each of three replicate treatments of: non-inoculated antioxidant-treated apples (☐), CPA-7-inoculated antioxidant-treated apples (▨), non-inoculated water-washed apples (▧), CPA-7-inoculated water-washed (▩). Error bars represent standard deviations. Different capital letters represent significant differences among sampling times for each treatment. Different lowercase letters represent significant differences among treatments for each sampling time, according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$).

5.4 CONCLUSIONS

CPA-7 reduced the ethylene production in fresh-cut ‘golden delicious’ apples after 6 d in MAP which could potentially delay fruit senescence thereby increasing the shelf-life of these kind of products in commercial conditions. However, the inhibition of this effect by the ascorbate-based antioxidant treatment (AS1) limits the possibilities of the use of CPA-7-AS1 combination as a hurdle technology. Further studies on the effect of combined strategies including this biocontrol agent and different types of natural chemical preservatives and/or physical methods on the ethylene metabolism of fresh-cut apple, could lead to the advent of better ecofriendly alternatives to improve the feasibility of minimally processed products at a commercial level.

ACKNOWLEDGMENTS

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CHAPTER VI

Decontamination of fresh-cut broccoli with a water–assisted UV-C technology and its combination with peroxyacetic acid

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ABSTRACT

The effectiveness of a water-assisted UV-C (WUV) technology for the decontamination of fresh-cut broccoli from conventional and organic agricultural practices was evaluated as an alternative to chlorine sanitation. Several WUV doses (0.3 - 1.8 kJ m^{-2}) were tested alone or combined with peroxyacetic acid (PAA). Results showed that 0.5 kJ m^{-2} was sufficient to reduce natural total aerobic mesophilic microorganisms by 2 log_{10} in conventional broccoli without negative consequences on the physical quality. However, in order to achieve the same effect on organic broccoli, a combined application of at least 0.3 kJ m^{-2} and 50 mg L^{-1} PAA was required. Total antioxidant capacity (TAC) was enhanced by 42, 90 and 81% in conventional broccoli 24 h after treatment with 0.3, 0.5 and 1.8 kJ m^{-2}, respectively, compared to water-control. A similar trend was observed in organic broccoli, although the increase in TAC (by 22%) compared to the water-control was only significant when a dose of 0.5 kJ m^{-2} was used. Similarly, 0.5 kJ m^{-2} enhanced the sulforaphane content in conventional broccoli by 1.5 and 4-fold compared to water and chlorine-controls, respectively. WUV is a promising alternative technology to improve the microbiological and nutritional quality of fresh-cut broccoli.

6.1 INTRODUCTION

Broccoli is a vegetable belonging to the *Brassicaceae* family which contains high levels of phytochemicals including vitamins, minerals, flavonoids, and glucosinolates (Herr and Büchler, 2010). Major glucosinolates present in broccoli are glucoraphanin and glucobrassicin, which are precursors of the isothiocyanates sulforaphane and indole-3-carbinol, respectively (Song and Thornalley, 2007; Roy *et al.*, 2009). Isothiocyanates have been widely studied for their anticancer, anti-inflammatory, and antimicrobial properties (Conaway *et al.*, 2005; Munday *et al.*, 2008). Specifically, sulforaphane and indole-3-carbinol have shown chemoprotective activity against several cancer types (colon, bladder, breast, and lung among others) by stimulating cellular antioxidant systems, interfering with cytokine production and activity or by restricting tumor progression through the induction of cell cycle arrest and apoptosis (Cheung and Kong, 2010; Tortorella *et al.*, 2015; Radošević *et al.*, 2017). However, studies with animal models have shown that some glucosinolates and its degradation products might also have genotoxic effects (Latté, Appel and Lampen, 2011).

Public awareness about the healthy properties of food includes not only the nutritional properties but the agricultural practices due to the belief that organic products have higher nutritional content, less pesticides residues, and reduced environmental impact than conventional ones; therefore the preference for this kind of products is growing among vegetable consumers (Hoefkens *et al.*, 2010; Fess and Benedito, 2018). In this regard, several studies have shown that organic vegetables have higher and more varied resident microbiota as well as increased amounts of some nutrients (e.g. certain glucosinolates) than conventional ones, but depending on several factors including the cultivar, the physiological stage of the commodity at harvest, and the growing conditions (Hoefkens *et al.*, 2010; Maffei, Silveira and Catanozi, 2013; Maggio *et al.*, 2013; Pace *et al.*, 2013).

Commercialized as a fresh-cut product, the health-promoting properties of broccoli are added to its convenience, which is highly appreciated in current society. However, during processing the perishability of this vegetable increases due to the loss of integrity of the plant cell's physical barriers, allowing the leakage of nutrients and the mixing of cellular components, thereby improving the conditions for microbial activity and triggering physiological processes that are detrimental to the product quality (Artés, Gómez, Aguayo, Escalona, & Artés-Hernández, 2009; Toivonen & Dell, 2002). In order to counteract these effects, sanitation techniques and preservation methods are included in the processing workflow (Leistner, 2000). The microbial load of fresh produce is usually reduced through washes with chlorine, but growing concern about its side-products such as trihalomethanes, which are harmful to humans and environment, has urged researchers and producers to search for alternative sanitation methods (Parish et al., 2003).

Among non-chlorinated chemicals peroxyacetic acid (PAA), also known as peracetic acid, is one of the bio-friendly alternative sanitizers used in the fresh-cut industry since acetic acid, water and oxygen are the only formed side-products (Abadias, Alegre, Usall, Torres, & Viñas, 2011; Artés & Allende, 2014). Furthermore, it is effective in a broader range of temperatures (0 – 40 °C) and pH (3.0 - 7.5) than chlorine (Vandekinderen *et al.*, 2007). The action mechanism of PAA is mainly based on the oxidation of proteins and lipids of cell walls and membranes of bacterial cells, endospores, yeasts, and mold spores, thereby disrupting their permeability, inactivating key enzymes and inhibiting DNA-synthesis (Finnegan *et al.*, 2010). Furthermore, after its decomposition in acetic acid it can diffuse through the cell membrane of microorganisms reducing cytoplasmic pH, which in turn affects the functionality of enzymes, structural proteins, and DNA (Rossoni and Gaylarde, 2000; Rodgers *et al.*, 2004; Mani-López, García and López-Malo, 2012).

In addition to chemical methods, physical non-thermal technologies such as ultraviolet light (UV) have emerged in the food industry because of their many advantages. These include the effectiveness against a broad range of spoilage and pathogenic microorganisms, a non-toxic and 'residue free' status, minimal negative effect on organoleptic and nutritional properties, and relative low costs and energy consumption compared to thermal decontamination technologies (reviewed by Gayán, Condón, & Álvarez, 2014). UV light includes wavelengths in the range of 200 to 400 nm, from which short UV-C waves (200 - 280 nm) have the most effective germicidal effects (Bintsis, Litopoulou-Tzanetaki and Robinson, 2000). Antimicrobial effect of UV-C light is primarily based on the formation of pyrimidine dimers in the DNA which inhibit transcription and eventually lead to mutagenesis and cell death (Witkin, 1984). This technology has been mostly used for the decontamination of water and packages. Direct exposure of commodities to UV-C light in a dose range of 0.2 to 20 kJ m^{-2} has also been assessed for the sanitation of several fresh-cut fruit and vegetables with variable effectiveness depending on the dose applied and on factors intrinsic to the commodity (its constituents, physiological stage, surface topography, and number of cell layers) (Civello, Vicente and Martinez, 2006; Gayán, Condón and Álvarez, 2014). UV-C light has also shown several hormetic effects which improve the nutritional properties of broccoli, including the increase in glucosinolates, phenolic compounds and ascorbic acid contents and the delay of chlorophyll degradation (Costa *et al.*, 2006; Lemoine *et al.*, 2007; Martínez-Hernández, Artés-Hernández, Gómez, Formica, *et al.*, 2013; Gamage *et al.*, 2016; A.C. Formica-Oliveira *et al.*, 2017). However, the application and efficacy of UV light in air is limited by the shadowing effect and the potential overheating of the product which can affect its quality (Liu, Huang, & Chen, 2015). In an attempt to address those problems, the aim of the present study was to evaluate the effectiveness of a water–assisted UV-C light (WUV) technology, which allows the tridimensional application of UV-C light to the product, for the sanitation of fresh-cut broccoli and the improvement of its nutritional quality. This technology

also integrates the decontamination of the product by irradiation and by water washing, while simultaneously decontaminating the water bath. A combined strategy using WUV and PAA was assessed in organic broccoli, to further improve the effectiveness of WUV in a product potentially containing higher microbial load and more varied microbiome.

6.2 MATERIALS AND METHODS

6.2.1 PLANT MATERIAL PROCESSING

Broccoli (*Brassica oleracea* L var. *Italica* cv. Parthenon) heads from conventional and organic agricultural practices were purchased from a local distribution warehouse or farm, respectively, in Catalonia, Spain. Heads were stored in wrapped boxes at 4 ^{O}C for up to 2 d until they were cut into 2 - 3 cm diameter florets with a sharpened knife on the day of the experiment.

6.2.2 WATER–ASSISTED UV-C EQUIPMENT

A water-assisted laboratory scale equipment LAB-UVC-Gama (UV-Consulting Peschl España, Castellón, Spain) composed of a water tank equipped with a recirculating system and connected to a water pump (Fig. **VI.**1) was used in order to improve the accessibility of UV-C light to all sides of the product in respect of conventional UV-C chambers. Before WUV treatments, lamps were preheated for 15 min. Before and after each treatment, temperature was measured using an infrared thermometer DualTemp Pro (Labprocess distribuciones, Barcelona, Spain) and irradiance was measured through an orifice located in the lid of the equipment using a UV-sensor EasyH1 (Peschl Ultraviolet, Mainz, Germany).

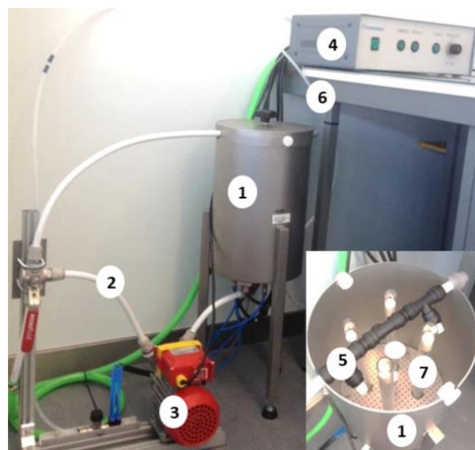

Figure VI.1. Water-assisted UV (WUV) light equipment setup: water tank (1) recirculating water circuit (2) water pump (maximum flow 1700 L h^{-1}) (3) power source (4). Pressurized water is introduced through a multiple-sprinkler adjustable device on the top (5), and pressured air, set at 1 bar, enters through the bottom of the tank for water bubbling (6). Four equidistant UV lamps (7) (17.2 W) emitting at 254 nm are located in water proofs quartz compartments inside the tank.

6.2.3 Sanitation of fresh-cut broccoli using Water-assisted UV-C; dose optimization

To optimize the sanitation procedure, approximately 300 g of conventional fresh-cut broccoli florets were immersed in 10.5 L of cold (5 ºC) tap water in agitation and submitted to four UV-C doses (0.3, 0.5, 0.9 and 1.8 kJ m^{-2}) by combining treatments with 2 or 4 lamps for 120 or 360 s of exposure. Doses were calculated as: the mean values of irradiance (W m^{-2}) of the several repetitions of the treatment x time of exposure (s). Washing broccoli florets for 120 s in agitated tap water or in 100 mg L^{-1} sodium hypochlorite solution with pH adjusted to 6.5 with ortho-phosphoric acid (Merck Millipore, Darmstadt, Germany), in the same proportion used for WUV treatments, were included as control treatments. After draining the excess of water and air-drying on the bench, some samples were immediately submitted to microbial analysis. For the analysis of the effect of treatments on biochemical parameters, processed florets were let at room temperature for a gap time of 6 h before freezing and storage. The rest of processed broccoli was stored at 5 ºC for 24 h in wrapped polystyrene trays before sampling and freezing for biochemical analysis.

6.2.4 Sanitation of fresh-cut broccoli using Water-assisted UV-C and UV-C in PAA

Considering the results obtained during the optimization phase, two UV-C doses (0.3 and 0.5 KJ m^{-2}) were selected based on their better suitability for industrial purposes (lower time of exposure and effectiveness regarding the control of microbial populations) and subsequently evaluated for the sanitation of organic broccoli following the procedure described in the previous section. Additionally, combined treatments including the selected UV-C doses and two doses of PAA (50 and 80 mg L^{-1}) were also tested. Sanitation with 50 or 80 mg L^{-1} PAA solutions, cold tap water or 100 mg L^{-1} hypochlorite solution without lighting the UV lamps, were included as control treatments.

6.2.5 Analysis of physical quality parameters

Physical quality parameters were evaluated in non-treated broccoli and in treated florets 6 h and 24 h after treatment. Superficial color of floret heads was determined by measuring CIE parameters L*, a* and b* with a chromameter (CR400, Minolta, Osaka, Japan) on two positions of 5 florets heads per treatment. Color results were interpreted according to the International Commission on Illumination (CIE) parameters: **L*** defines lightness (black to white) with values within 0 and 100; **a*** indicates redness when positive and greenness when negative; and **b*** represents yellowness to blueness corresponding to positive to negative values. Parameters a* and b* were expressed as hue angle (º) calculated as: 180 + arc tan(b*/a*) (McLellan, Lind and Kime, 1995). Firmness evaluation was performed using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., England, UK) by measuring the maximum force required for a compression platform (75 mm diameter) to cause a 10% deformation of a broccoli floret at 5 mm s^{-1}, for an activation threshold of 10 N. Overall visual assessment of quality in a 7 point hedonic scale (from **1**: dislike to **7**:

like very much) was evaluated two independent times by 23 untrained panelists. The evaluation panel was composed by 76% of women and 24% of men within the age ranges of 18-30 (69%) and 31-45 (31%).

6.2.6 Microbial analysis

Microbial populations were estimated before sanitation and immediately after. For this, three replicates of 25 g of florets were homogenized in 225 mL of buffered peptone water (BPW, Biokar, Beauvais, France) within a 400 mL sterile full-page filter bag (Bagpage, Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set at 4 strokes per s for 90 s. Total mesophilic aerobic microorganisms (MAM) were determined by plating the appropriate ten-fold dilutions in saline peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) on Plate Count Agar plates (PCA, Biokar, Beauvais, France) after incubation at 30 °C for 72 h. Native yeasts and molds were enumerated on Dichloran Rose Bengal Chloramphenicol agar plates (DRBC, Biokar, Beauvais, France) after incubation at 25 °C for 5 d. Viable counts of MAM, yeasts, and molds in water and chlorine baths were also performed. The analysis of chlorine baths was preceded by a neutralization step in Dey-Engley Neutralizing Broth (Sigma-Aldrich, Madrid, Spain). Bath samples were plated as previously described. Microbiological data were expressed as $\log_{10}$ of the colony forming units per gram of fresh weight of broccoli (CFU g^{-1} FW). Microbial reductions were calculated as: $\log_{10}(N_1/N_0)$, where N_1 is the microbial count of sanitized broccoli and N_0 is the microbial count of untreated broccoli.

6.2.7 Biochemical analysis

Approximately 70 g of florets per replicate, per treatment and per sampling time were frozen with liquid nitrogen, grinded using a commercial grinder (Minimoka

6R-020, Coffeemotion, Lleida, Spain), and stored at -80 °C until biochemical analysis.

Total antioxidant capacity (TAC) and total phenolic content (TPC) were measured in the supernatants resulting from the centrifugation at 24 000 x g for 20 min (at 4 °C) of the extracts obtained from a mix containing 3 g of frozen broccoli powder and 10 mL of extraction solution (19.7 mol L^{-1} methanol, 0.05 mol L^{-1} HCl), after agitation at 20.94 rad s^{-1} for 2 h. TAC was quantified using a spectrophotometer (EONC, Biotek Instruments, Highland Park, VT, USA) by the Ferric Reducing Antioxidant Power (FRAP) method and the DPPH (2,2 – diphenyl – 1 – picrylhydrazyl) free radical-scavenging activity method. The FRAP method was performed following the protocol of Benzie and Strain (1996) with some modifications (Giné-Bordonaba and Terry, 2016); OD was measured at 593 nm. The DPPH method, based on the described by Brand-Williams et al., (1995), was performed by measuring OD at 515 nm of a 1.5 mL reaction containing 1.4 mL of 1 mmol L^{-1} DPPH and 0.1 mL broccoli extract after 1 h incubation at room temperature in darkness. TPC was quantified by the Folin-Ciocalteu method (Singleton, Rossi Jr. and Rossi J A Jr., 1965), by measuring OD at 765 nm of a reaction containing 0.7 mL of each sample extract, 4.3 mL water and 0.5 mL Folin-Ciocalteu reagent, incubated for 5 min in darkness before adding 2 mL of 200 g L^{-1} Na_2CO_3 solution. Non-enzymatic antioxidant activities were expressed as g of the measured analyte (i.e. Gallic acid, GAE or ascorbic acid, AA) per kilogram of fresh weight of broccoli (g kg^{-1} FW).

Chlorophyll pigments were extracted from 2 g of frozen fresh broccoli using N,N dimethyl-formamide following the method of Moran (1982) and expressed as (mg kg^{-1} FW).

For glucosinolates extraction, 150 mg of processed broccoli, lyophilized after storage at 4 °C for 24 h, was mixed with 3 mL of a solution containing methanol: water (80:20, v:v) following the protocol described by Alarcón-Flores et al.

(2013). Glucosinolates content was determined by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) using an Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) coupled to an Agilent triple quadrupole mass spectrometer (6460A) with a Jet Stream ESI ion source (G1958-65138) (Alarcón-Flores *et al.*, 2013). Results were expressed as mg kg^{-1} of dry weight (DW). For the identification and quantification of glucosinolates, a multi-compound (5 mg L^{-1} of each standard) methanolic solution containing sulforaphane (Sigma-Aldrich, Steinheim, Germany), proigonitrin, gluconasturtin, glucoraphanin (PhytoLab GmbH & Co., Vestenbergsgreuth, Germany), glucotropaeolin, glucoerucin, and glucoiberin (Scharlab, Barcelona, Spain) was used.

6.2.8 Statistical analysis

All experiments were performed twice and included three biological replicates per treatment and sampling time. Physical, microbiological and biochemical data were analyzed using the statistical software JMP (version 11 SAS Institute Inc., NC, USA). All data were verified for normal distribution and homoscedasticity of residues and accordingly, means were compared by analysis of variances (ANOVA) and separated by Tukey's test ($P < 0.05$). Categorical data from overall quality assessment were analyzed by a logistic regression analysis ($P < 0.05$).

6.3 RESULTS AND DISCUSSION

6.3.1 Analysis of physical quality parameters

The evaluated WUV doses (0.3, 0.5, 0.9 and 1.8 kJ m^{-2}) did not affect the firmness of conventional broccoli when compared to the control treatments (Table **VI.**1). Similar results were obtained for organic broccoli when treated with 0.3 or 0.5 kJ m^{-2} (Table **VI.**2). Sanitation with PAA caused a reduction in the firmness of

organic broccoli 24 h post-treatment when compared to the water control ($p < 0.05$). Similarly, WUV had no effect on the color of conventional broccoli (Table **VI.**1).

Table **VI.**1. Physical quality parameters of fresh-cut conventional broccoli after sanitation with different UV-C doses using WUV, compared to water and chlorine washing.

				WUV (kJ m^{-2})			
Treatment		**Water**	**NaClO**	**0.3**	**0.5**	**0.9**	**1.8**
L*	0 h	42±2 a	40±3a	42±3 a	41±3 a	42±3 a	41±2 a
Hue (°)	0 h	129±5 a	127±6 a	128±5 a	127±5 a	128±5 a	127±4 a
Firmness (N)	0 h	13±5a	9±3a	9±3a	10±5 a	12±3 a	12±5 a
L*	24 h	43±2 a	42±3a	43 ± 2 a	42±2 a	42±2 a	42±2 a
Hue (°)	24 h	128±4 a	129±3a	129±5 a	124±8 ab	125±9 ab	128±4 a
Firmness (N)	24 h	12±3 a	20±7 a	16±4a	13±2a	16±4a	19±3 a

Values are means ± standard deviations (n=20). Different letters represent significant differences among treatment at each sampling time according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$)

Regarding organic broccoli, WUV or its combinations with PAA showed no effect on lightness (L*) (Table **VI.**2). However, 24 h after processing, the hue angle was slightly reduced ($p < 0.05$) in samples treated with 0.5 kJ m^{-2} and its combinations with PAA compared to the water control, although the highest reductions were observed for the PAA control treatments.

Nevertheless, color changes were not visually detected by the panelists during the analysis of the overall visual quality of samples collected at 6 or 24 h after treatment (data not shown). Hue angle has previously shown to better fit as a color parameter for measuring the progression of yellowing in broccoli florets during storage at 5 °C (Argüello *et al.*, 2017). UV-C doses ranging from 0.9 to 1.5 kJ m^{-2} have previously shown to contribute to color preservation in several broccoli varieties during a storage period of up to 23 days at 4 °C (Costa *et al.*, 2006; Martínez-Hernández *et al.*, 2011; Duarte-Sierra, 2015).

Table **VI.**2. Physical quality parameters of fresh-cut organic broccoli after sanitation with several UV-C doses using WUV or its combination with peroxyacetic acid, compared to water and chlorine (NaClO) washing.

		WUV ($kJ\ m^{-2}$), Peroxyacetic acid (PAA) ($mg\ L^{-1}$)									
		water	**Cl**	**50 PAA**	**80 PAA**	**0.3 $kJ\ m^{-2}$**	**0.3 + PAA 50**	**0.3 + PAA 80**	**0.5 $kJ\ m^{-2}$**	**0.5 + PAA 50**	**0.5 + PAA 80**
L*	6h	46 ± 4 bc	44 ± 3 c	46 ± 2 bc	47 ± 5 abc	45 ± 3 bc	45 ± 4 bc	45 ± 4 bc	48 ± 2 a	49 ± 2 a	51 ± 4 a
Hue (°)	6h	124±6 a	124±4 ab	117±7 b	119±5 b	124±5 a	125±4 a	122±7 ab	121±5 a	121±2 a	117±5 b
Firmness (N)	6h	19 ± 8 bc	22 ± 8 abc	14 ± 4 c	14 ± 7 c	20 ± 6 bc	26 ± 7 ab	22 ± 9 bc	33 ± 7 a	29±11 a	23±9 ab
L*	24h	45 ± 2 ab	43 ± 2 b	46 ± 3 ab	48 ± 3 a	43 ±2 b	45 ± 4 ab	45 ± 3 ab	45 ± 4 ab	47 ± 5 a	47 ± 3 a
Hue (°)	24h	127±3 a	126±3 a	117±6 c	120±5 bc	124±5 a	127±4 a	126±4 a	120±2 b	118±3 bc	119±3 bc
Firmness (N)	24h	23 ± 7 a	22 ± 9 a	16 ± 6 b	15 ± 8 b	23 ± 7 a	20 ± 6 ab	26 ± 7 a	29 ± 5 a	30 ± 4 a	25 ± 8 a

Numbers are means ± standard deviations (n = 20). Different letters represent significant differences among treatments at each sampling time according to an analysis of variances (ANOVA) and a Tukey's test ($P < 0.05$). H_2O: water control, NaClO: 100 $mg\ L^{-1}$ sodium hypochlorite

6.3.2 Microbial analysis

Sanitation using WUV; dose selection

Initial populations of MAM, molds and yeasts on fresh-cut conventional broccoli were 4.1 ± 0.1, 2.2 ± 0.1, and 2.3 ± 0.1 log_{10} CFU g FW^{-1}, respectively. After sanitation with 0.5 $kJ\ m^{-2}$ using WUV, a significant reduction of MAM by 2 ± 0.1 log_{10} compared to the untreated control was obtained ($p < 0.05$) (Fig. **VI.**2). Similar results were obtained after chlorine washing. No significant differences compared to the water control were observed after processing using higher doses (0.9 or 1.8 $kJ\ m^{-2}$). These results agreed with previous reports showing that the highest reduction of spoilage mesophilic microorganisms or pathogenic bacteria on fresh produce does not always correlate to higher UV doses. For example, Martínez-Hernández et al., (2015) after testing doses up to 15 $kJ\ m^{-2}$

using a dry UV technology, found that the maximum inactivation rate of *E. coli*, *S. enterica* and *L. monocytogenes* in inoculated Kaylan-hybrid broccoli was obtained while operating in the range from 0 to 2.5 kJ m^{-2}. Such lack of correlation between the dose and the extent of microbial reduction may has been due to putative structural changes occurred during treatments which may have influenced the response to UV or improved the conditions for microbial penetration into the plant tissue (Escalona et al., 2010; Graça et al., 2017).

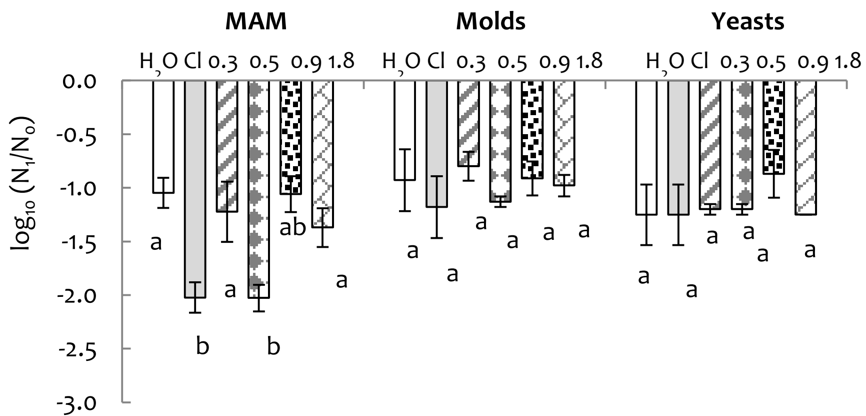

Figure VI.2. Logarithmic reductions of native total mesophilic aerobic microbiota (MAM), yeasts and molds populations on conventional fresh-cut broccoli sanitized (N_1) with: tap water (H_2O), a 100 mg L^{-1} chlorine solution (Cl) or with different WUV doses (0.3, 0.5, 0.9, 1.8 kJ m^{-2}), in respect of untreated broccoli (N_0). Columns represent means and error bars represent standard error of the mean (n=6). Different letters represent significant differences for each type of microorganism according to an analysis of variances (ANOVA) and a Tukey's test ($P < 0.05$).

The results obtained showed that water washing was enough to reduce the initial native molds and yeasts populations on conventional broccoli to values close to the detection limit (5 CFU mL^{-1}); thus the efficacy of WUV beyond water sanitation could not be established. The low initial levels of molds and yeasts populations compared to those previously reported for conventional broccoli from several varieties (4.9 to 6.5 $\log_{10}$ CFU g^{-1}) might be conditioned by the growing location, season and management system which influence the

composition and population levels of microbial communities (Argüello et al., 2017; Martínez-Hernández, Artés-Hernández, Gómez, & Artés, 2013; Wang et al., 2016).

MAM, yeasts and molds populations present in the water wash after the sanitation of conventional broccoli are shown in Table VI.3. No viable cells of yeasts and molds were detected after any of the assayed treatments. MAM populations in the water wash after treatment with 0.3 kJ m^{-2} WUV were 1.4 log_{10} lower that those present in the water wash after broccoli sanitation without switching on the UV lamps. Processing using 0.5 to 1.8 kJ m^{-2}, resulted in reductions ranging between 2.8 log_{10} (non-detected cells) and 1.3 ± 0.2 log_{10}, compared to the process water without UV.

After performing a UV treatment of the water bath for 2 additional minutes after broccoli sanitation, using 2 or 4 UV lamps, no viable cell was detected. This suggested that the viable cells detected in the water after broccoli sanitation could represent a combination of the cells that were circulating in the system during the UV treatment and failed to be exposed to the light and those that were protected within the product structure and passed to the water during the gap time from the light switching off until the withdrawing of the product from the equipment. A similar situation was described by Huang et al. (2015) when using a water-assisted pulsed light device for the decontamination of berries. Nevertheless, the use of 4 lamps instead of 2 would be advisable to better counteract the added effect of organic matter in suspension which is likely to occur at an industrial level when a higher amount of vegetal product is used.

Table VI.3. Populations (log_{10} CFU mL^{-1}) of total mesophilic aerobic microorganisms, yeasts and molds present in the wash after broccoli sanitation with several UV doses using WUV or its combination with peroxyacetic acid, compared to water and chlorine (NaClO) washing.

Broccoli	Treatment		MAM	Yeasts	Molds
conventional		H_2O	2.8 ± 0.2	0.4 ± 0.2	0.9 ± 0.3
		Cl	0.5 ± 0.2	nd	nd
	WUV (kJm^{-2})	0.3	0.4 ± 0.2	nd	nd
		0.5	1.0 ± 0.4	nd	nd
		0.9	0.9 ± 0.6	nd	nd
		1.8	0.8 ± 0.5	nd	nd
organic		H_2O	3.3 ± 0.5	2.5 ± 0.3	2.2 ± 0.4
		Cl	0.7 ± 0.5	nd	nd
	WUV (kJm^{-2})	0.5	1.1 ± 0.4	nd	nd
	PAA (mg L^{-1})	PAA50	0.7 ± 0.4	nd	nd
		PAA80	nd	nd	nd
	WUV (kJm^{-2})	0.3 + PAA50	nd	nd	nd
		0.3 + PAA80	0.8 ± 0.5	nd	nd
	WUV (kJm^{-2})	0.5	nd	nd	nd
	WUV (kJm^{-2}) + PAA (mg L^{-1})	0.5 + PAA50	nd	nd	nd
		0.5 + PAA80	nd	nd	nd

MAM: mesophilic aerobic microorganisms; PAA: peroxyacetic acid; WUV: water-assisted UV-C; nd: not detected, below detection limit (5 CFU mL^{-1}). Values are means ± standard deviations.

Sanitation using Water assisted UV-C and UV-C in PAA

In order to assess a worse-case scenario implying higher and more varied microbial load, fresh-cut broccoli from organic practices was used and the combination of WUV and PAA was tested to improve the efficacy of WUV (Renaud *et al.*, 2014; Wang *et al.*, 2016; Lupatini *et al.*, 2017). The initial populations of MAM, molds and yeasts on fresh-cut organic broccoli were 4.9 ± 0.1, 3.9 ± 0.3, and 3.4 ± 0.1 log_{10} CFU g FW^{-1}, respectively. The pH of the baths did not vary after treatments, being 5.2 ± 0.1 and 4.6 ± 0.1 for the 50 and 80 mg L^{-1} PAA solutions, respectively and 6.5 ± 0.1 for the chlorine solution. For the reduction of native MAM populations, the combined application of 0.3 kJ m^{-2} (2 lamps for 2 min) and 50 or 80 mg L^{-1} PAA were the most efficient treatments,

with reductions of 2 ± 0.2 log_{10}, compared to the untreated control (Fig. **VI.**3). However, the same efficacy was obtained with PAA and chlorine control treatments. Similarly, using a small scale laboratory version of a water-assisted UV-C technology, Liu et al. (2015) did not obtain a significant improvement of the UV treatment (7.9 mW cm^{-2} for 10 min) when combining it with 10 ppm chlorine, 100 ppm SDS, or 0.5% levulinic acid + 100 ppm SDS, for the reduction of *E. coli* and *Salmonella* spp. in dip-inoculated blueberries. Those researchers neither obtained differences among the dry, the wet UV technology and the chlorine control (100 mL L^{-1} NaClO, for 1 min). Other researchers obtained a reduction of MAM populations by 1.6 log_{10} in fresh-cut endives by washing them in cold water (4 ^{O}C) and then irradiating them with 1.2 kJ m^{-2} UV-C (Hägele et al., 2016). That reduction efficacy was improved to 2.1 log_{10} when warm water (45 ^{O}C) was used instead of cold water but, in both cases it was similar to that obtained with chlorine sanitation. The results obtained in the present study concerning the effectiveness of PAA compared to chlorine for microbial control contrasted to those obtained in previous experiments using a similar ratio of vegetal weight: volume of bath and time of exposure, when 100 mg L^{-1} PAA showed higher effectiveness than 100 mL L^{-1} chlorine for reducing *E. coli* and *Salmonella enteritidis* (reductions by 2–3 log) in fresh-cut kailan-hybrid broccoli (Martínez-Hernández, Navarro-Rico, et al., 2015). Such disagreement may be explained by the higher PAA concentration, different sensitivities to the sanitizers of the microorganisms tested or by a deeper colonization and establishment of the native microbiota compared to the inoculated one.

The results showed reductions of yeast populations ranging from 1.5 to 2.0 ± 0.1 log_{10} using 0.3 kJ m^{-2} + 80 mg L^{-1} PAA or the combination of 0.5 kJ m^{-2} + 50 or 80 mg L^{-1} PAA, which was significantly higher than the water control but similar to those obtained with the chemical control treatments. Molds populations were reduced by 1.0 to 1.6 ± 0.1 log_{10} when WUV treatments were combined with PAA, regardless of the dose applied. A similar reduction was obtained with 80 mg L^{-1}

PAA (1.4 ± 0.1 log_{10}) and chlorine (1.1 ± 0.2 log_{10}) which was significantly higher than that obtained with 50 mg L^{-1} PAA (0.4 ± 0.2 log_{10}).

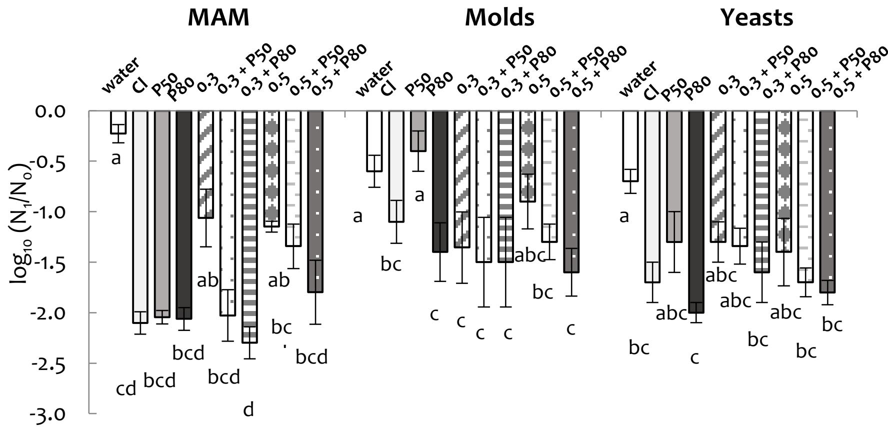

Figure VI.3. Logarithmic reductions of native microbial populations (mesophilic aerobic microorganisms (MAM), yeasts and molds on organic fresh-cut broccoli sanitized (N_1) with different WUV doses (0.3 and 0.5 kJ m^{-2}) in water or in 50 or 80 mg L^{-1} peroxyacetic acid (P50 and P80, respectively), in respect of untreated broccoli (N_0), as compared with tap water (H_2O) or 100 mg L^{-1} chlorine (Cl) washing. Columns represent means and error bars represent standard error of the mean (n=6). Different letters represent significant differences for each type of microorganism according to an analysis of variances (ANOVA) and a Tukey's test ($P < 0.05$).

Comparing the efficacy of WUV for the decontamination of conventional and organic broccoli, results showed that WUV, at doses of 0.3 and 0.5 kJ m^{-2}, was 50% less effective for the reduction of MAM in organic broccoli than in conventional broccoli compared to untreated controls. As expected for organic broccoli, reduced effectiveness of WUV for decontamination might be related to a higher and more heterogeneous initial microbial population, which could comprise various microbial species or strains with different sensitivity to UV (Lupatini *et al.*, 2017); to a different physiological stage of broccoli at harvest or to the stressful agricultural conditions which might influence the plant hormetic response (Hassenberg, Huyskens-Keil and Herppich, 2012).

Viable counts of the water baths after a single-use sanitation of organic broccoli showed that all of the studied WUV conditions reduced mesophilic microbial populations in a range of 2.3 ± 0.5 to 3.0 ± 0.5 log_{10} compared to water-washing, showing the same efficacy as chlorine-sanitation (Table 3). The populations of yeasts and molds present in the water wash after sanitation of organic broccoli were reduced to below the detection limit (5 CFU mL^{-1}) regardless of the assayed treatment. In agreement to our results, UV doses as low as 0.4 kJ m^{-2} achieved a 3.8 log_{10} reduction of total mesophilic bacteria in the water bath after sanitation of lamb's lettuce in semi-industrial conditions (Ignat *et al.*, 2015). In the present work the efficacy of chlorine for decontamination was evaluated only after a single use of the water bath. Since free chlorine concentration was reduced by 10% after sanitation, the efficacy of this treatment for the decontamination of a subsequent set of samples, as often occur at an industrial level, could also be diminished (Rodgers *et al.*, 2004). Furthermore, we observed that two additional minutes of WUV were enough to reduce the residual populations of MAM populations to below the detection limit, enabling water for potential reuse. However, a filtration step should be considered in order to reduce organic matter for up-scaled workflows (Fan, Huang and Chen, 2017). In this sense, despite the increasing turbidity and microbial load observed after several cycles of processing fresh-cut onions and endives at an industrial level, reductions from 0.6 to 1.3 log_{10} of bacterial populations in the water baths, have been recorded for each product, respectively, corroborating the usefulness of UV-C for reducing water consumption (Hägele et al., 2016; Selma et al., 2008). In addition to the UV dose, the water column thickness is an important factor to take into account in obtaining better results (Hägele *et al.*, 2016). Unlike previous experiments testing the combination of PAA and UV-C for wastewater disinfection at a pilot plant level, we observed no synergistic effect of UV-C and PAA (Caretti and Lubello, 2003).

6.3.3 Biochemical analysis

Total antioxidant capacity (TAC)

As measured by the DPPH method, the TAC of conventional broccoli 6 h after treatment with 0.3 kJ m^{-2} WUV was enhanced by 16% compared to the water-washed control (Fig. **VI.**4A). This difference increased to 42% at 24 h post-treatment. When compared to chlorine washing, irradiation with 0.3 kJ m^{-2} WUV increased TAC by 70 and 65%, after 6 h and 24 h, respectively. Increasing the UV-C dose to 1.8 kJ m^{-2} resulted in an increase in TAC by 22 and 80% in the WUV treated samples compared to the water-washed control, at 6 and 24 h post-treatment, respectively. Compared to the chlorine control, the difference was higher (by 80% at 6 h post-treatment), duplicating its value after 24 h. Treatment with 0.5 kJ m^{-2} showed no immediate effect on TAC but it duplicated the value observed for the water control, 24 h post-processing. In agreement with these results, Martínez-Hernández et al. (2011) obtained that in a certain range, higher UV-C doses (1.5 < 4.5 > 9; 4.5 > 15 kJ m^{-2}) correlated with higher TAC in Bimi® broccoli immediately after treatment. Higher antioxidant capacity was also detected in cv 'Cicco' broccoli florets 6 d after treatment with 10 kJ m^{-2} UV-C and storage at 20 ^{O}C, as measured by the DPPH method, although those differences were not significant at initial time (Costa *et al.*, 2006).

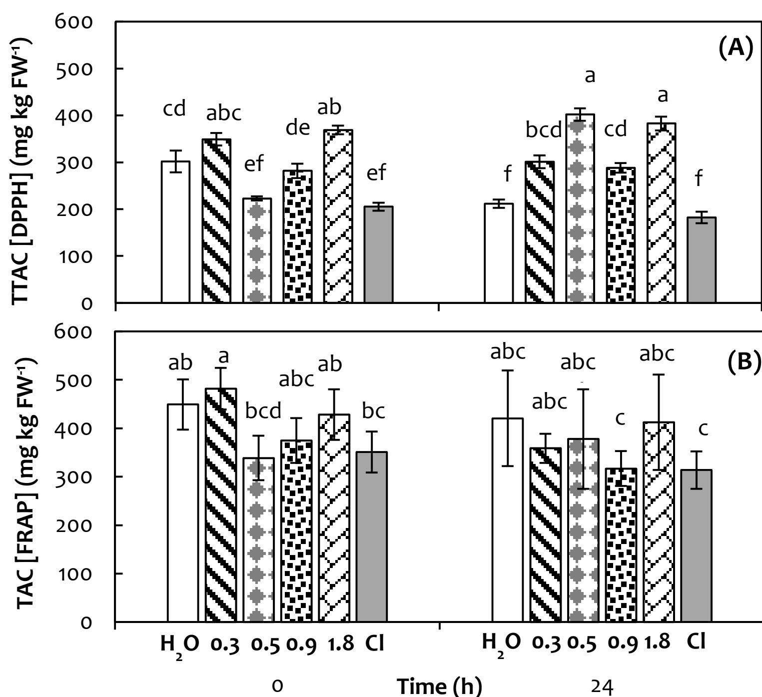

Figure VI.4. Total antioxidant capacity in fresh-cut conventional broccoli treated with different UV-C doses (0.3, 0.5, 0.9, and 1.8 kJ m^{-2}) using WUV as compared to sanitation with tap water (H_2O) or 100 mg L^{-1} chlorine (Cl). (A) Measured by the DPPH method, (B) measured by the FRAP method. Columns represent means and error bars represent standard deviations (n=6). Different letters represent significant differences among treatments at each sampling time according to analysis of variances (ANOVA) and Tukey test ($P < 0.05$).

Using the DPPH method, total antioxidant capacity in organic broccoli showed no variation (333 ± 11 mg kg^{-1}) after treatment with 0.3 or 0.5 kJ m^{-2} WUV compared to the water and chlorine controls, at 6 or 24 h post-treatment (Fig. **VI.**5A). No significant differences in TAC were observed between the samples treated with 0.3 kJ m^{-2} WUV alone and its combinations with 50 or 80 mg L^{-1} PAA. In contrast, the combined application of 0.5 kJ m^{-2} and 50 or 80 mg L^{-1} PAA resulted in poorer total antioxidant capacities than this WUV treatment alone, showing reductions by 27 and 35%, respectively, at 6 h post-treatment. However, such differences in TAC vanished after 24 h of incubation. Differential effect of UV-C in TAC, as measured by the DPPH method, have previously been observed

according to the broccoli variety and cultural practices (Martínez-Hernández, Artés-Hernández, Gómez, Formica, et al., 2013). The observed higher antioxidant capacity might has been due to an increase in the vitamin C and glutathione contents or to higher activities of antioxidant enzymes, as previously observed in UV—treated fresh-cut broccoli and red cabbage (Lemoine, Chaves, & Martínez, 2010; Zhang et al., 2017), but this hypothesis cannot be corroborated by our results.

When assessed using the FRAP method, differences in TAC were not so evident. An increase by 37% was observed in conventional broccoli 6 h after treatment wih 0.3 kJ m^{-2} WUV when compared to the chorine-washed control (Fig. **VI.**4B). However, differences between those WUV-treated samples and the water control were not significant at any of the analyzed times. In the same way, no differences were observed between the 1.8 kJ m^{-2}—treated samples and the water or the chlorine controls probably because of the higher variability obtained with this UV-C dose. In organic broccoli, treatments with WUV alone were the best of all of the assayed since maintained fresh-cut broccoli TAC (469 ± 11 mg kg^{-1}) similar to the water-washed control 6 h after treatment (Fig. **VI.**5B). Broccoli samples treated with 0.5 kJ m^{-2} showed an increased TAC (by 22%) compared to the water control, 24 h post-treatment.

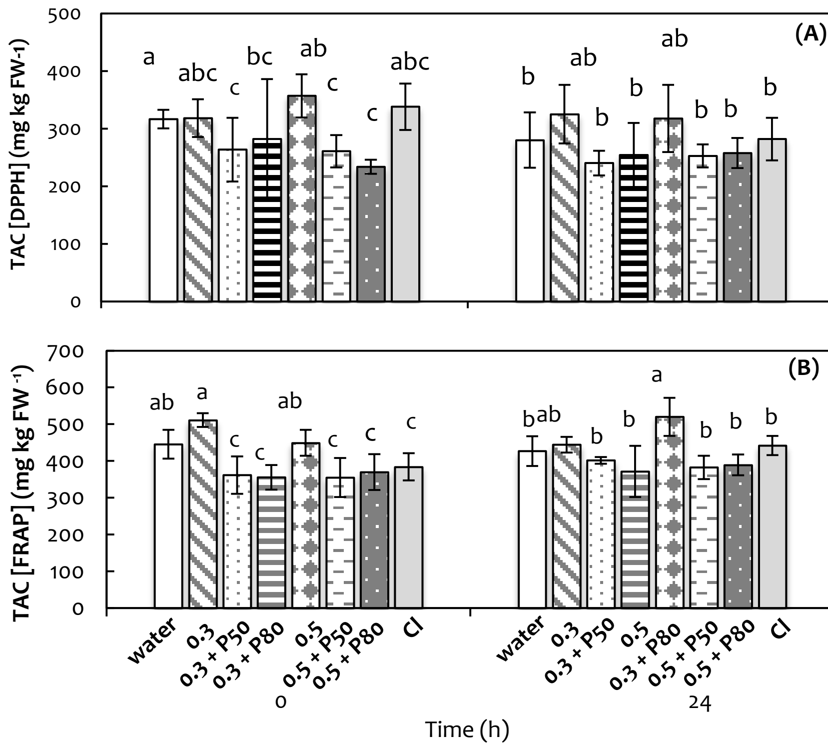

Figure VI.5. Total antioxidant capacity in fresh-cut organic broccoli treated with different UV-C doses (0.3 and 0.5 kJ m^{-2}) using WUV or its combination with 50 or 80 mg L^{-1} peroxyacetic acid (P50 or P80, respectively) as compared to sanitation with tap water (H_2O) or 100 mg L^{-1} chlorine (Cl) washes. (A) Measured by the DPPH method, (B) measured by the FRAP method. Columns represent means and error bars represent standard deviations (n=6). Different letters represent significant differences among treatments at each sampling time according to analysis of variances (ANOVA) and Tukey test ($P < 0.05$).

TOTAL PHENOLIC COMPOUNDS CONTENT (TPC)

No significant differences were observed in the phenolic compounds content of conventional broccoli among the evaluated treatments. Stable values around 77 ± 12 mg kg^{-1} remained during the analyzed period (data not shown). As observed for conventional broccoli, no variation in TPC could be detected in the samples treated with WUV or with the combined alternatives with PAA compared to the water-washed samples (48 ± 6 mg kg^{-1}) at 6 or 24 h posty-processing. The

putative induction of the phenylpropanoid metabolism could have taken longer than 24 h after UV-C radiation (Duarte-Sierra, 2015), as observed in Bimi® broccoli when using UV-C doses from 1.5 to 15 kJ m^{-2} (Martínez-Hernández et al., 2011). Lower TPC in organic broccoli compared to the conventional one, was unexpected since organic practices usually result in higher content in bioactive compounds (Valverde *et al.*, 2015). This could be due to a more advanced physiological stage at harvest of the conventional broccoli used in the present studies. Contradictory results regarding the TPC in broccoli florets after UV-C irradiation have been obtained by other authors, i.e. a reduction was observed using 10 kJ m^{-2} while an increase was obtained with 8 kJ m^{-2} (Costa et al., 2006; Lemoine et al., 2007).

CHLOROPHYLLS CONTENTS

Total chlorophyll content in conventional broccoli was 134 ± 21 μg kg^{-1} FW, with 67 ± 10 μg kg^{-1} FW of chlorophyll a, and 44 ± 8 μg kg^{-1} FW of chlorophyll b, regardless of the treatment applied. Thus, WUV did not affect the chlorophylls contents compared to the fresh-cut non-treated broccoli (data not shown) which is in line with the color retention discussed above and agree with previous results using similar UV-C doses (Duarte-Sierra, 2015; Martínez-Hernández et al., 2011). In the same way, no differences in the total chlorophyll content in organic broccoli (141 ± 19 μg kg^{-1} FW) nor in the chlorophyll a (69 ± 7 μg kg^{-1} FW) or b (47 ± 8 μg kg^{-1} FW) contents were observed among the analyzed treatments (data not shown).

GLUCOSINOLATES CONTENTS

Although more than 120 different glucosinolates have been identified in cruciferous vegetables, only some of these are present in high quantities. From the glucosinolates tested (glucoiberin, glucoraphanin, glucotropaeolin, proigonitrin, glucoerucin, and gluconasturtin), glucoraphanin was the only glucosinolate detected with contents ranging from 320 to 527 mg kg^{-1} DW (data

not shown). Glucoraphanin is the predominant glucosinolate in broccoli sprouts from several varieties (Verkerk, Tebbenhoff and Dekker, 2010; Westphal *et al.*, 2017), although in a previous work glucobrassicin and glucobrassicanapin were the most abundant found in ‘Parthenon’ variety (Fernández-León *et al.*, 2017). The glucosinolates composition and contents vary not only at the inter-variety level but also depending on the physiological stage, agricultural practices, pre-harvest treatments, and processing styles at the intra-variety level (Torres-Contreras et al., 2017; Valverde et al., 2015). For instance, Valverde et al. (2015) observed that the content in certain glucosinolates (i.e. glucobrassicin and neoglucobrassicin) was higher in organically than in conventionally produced broccoli, although those practices did not influence the content in glucoraphanin and its derived products. Factors intrinsic to the vegetal product such as those mentioned above, also influence the effect of UV-C irradiation on specific bioactive compounds, which is added to the effect of the dose applied (Reviewed by Civello, Vicente, & Martinez, 2006).

Treatment with 0.5 kJ m^{-2} WUV resulted in a 1.5-fold increase in sulforaphane content when compared to the water control and a 4-fold increase when compared to the chlorine-sanitized control. Higher WUV doses (2.3 kJ m^{-2}) enhanced the content in sulforaphane by 2-fold when compared to the water control, and by 5.5-fold when compared to the chlorine control, but at expenses of an increase in exposure time, from 2 to 10 min. Although high UV-C doses have shown to enhance the glucosinolates content in broccoli, they can entail a reduction of the product quality throughout storage (Duarte-Sierra, 2015).

In addition, a 0.6- and 0.7-fold decrease was observed in the glucoraphanin content in the 0.5 and 2.3 kJ m^{-2} WUV-treated samples when compared to the water-washed samples. No reduction was observed after chlorine sanitation. The conversion of glucoraphanin into sulforaphane can be enhanced by modulating physical factors such as temperature, pressure and pH during processing due to the activation of myrosinase, the enzyme that catalyzes this

reaction; in this way, positive results have been obtained with mild heat and high pressure treatments (Hanschen et al., 2017; Liu et al., 2017; Matusheski et al., 2004; Westphal et al., 2017). However, we found no reference regarding the effect of UV-C on this enzyme, which could explain the increase in the sulforaphane content in detriment of the glucoraphanin one. Thus, further studies on factors affecting the hydrolysis of glucosinolates would be helpful to clarify this issue. In others broccoli varieties, dry UV-C irradiation induced the content in several glucosinolates according to the dose, ranging from hormetic to high levels, and to the period of storage. For example, glucoraphanin levels increased in cv 'Everest' broccoli florets 24 h after treatment with 1.2 kJ m^{-2} UV; however no variation was obtained with a higher dose (3.6 kJ m^{-2}) (Nadeau *et al.*, 2012). In cv 'Diplomat' broccoli florets, the application of 1.2 and 3 kJ m^{-2} UV-C enhanced the titers of glucoraphanin and reduced those of glucobrassicin immediately after treatment, but afterwards the levels of the first one remained stable while those of the latter increased at 72 h (Duarte-Sierra, 2015). In Bimi® broccoli the increase of a glucoraphanin levels occurred 72 h after treatment with a higher UV-C dose (9 kJ m^{-2}) or with a UV-B + UV-C combination (9 + 15 kJ m^{-2}, respectively) (A.C. Formica-Oliveira *et al.*, 2017). However, in those experiments, the quantification of sulforaphane was not included.

6.4 CONCLUSIONS

The results obtained suggested that 0.5 kJ m^{-2} is a hormetic effective dose for the decontamination of natural microbiota and the enhancement of the nutritional quality of fresh-cut conventional broccoli, enabling water, energy and time savings, which are relevant to an upscale level. This UV-C dose significantly preserved nutritional quality associated with antioxidant and glucosinolates content in conventional broccoli compared to water and chlorine treatments. Therefore, this is a promising and economic technology to preserve the microbiological, physicochemical and nutritional quality of fresh-cut

conventional broccoli at an industrial level. Furthermore, sanitation using UV-C (0.5 kJ m^{-2}) delivered in 50 mg L^{-1} PAA was an effective strategy alternative to chlorine for reducing the potentially higher microbial load and the more varied native microbiota from organic broccoli, showing better efficacy than water-washing and WUV alone. However, current regulations do not allow the use of biocidal chemicals for organic produce. Therefore, the combination of WUV with mild heat treatments could be an alternative to be tested for this purpose.

ACKNOWLEDGEMENTS

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CHAPTER VII

Efficacy of water-assisted UV-C and dry-pulsed light for the decontamination of *Listeria innocua* in fresh-cut broccoli

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ABSTRACT

Two non-thermal technologies: water-assisted UV-C light (WUV) and pulsed light (PL) were applied for the decontamination of Listeria innocua in fresh-cut broccoli. Several UV-C doses were tested using both technologies and the combination of UV-C with peroxyacetic acid (PAA) was also evaluated. Moreover, the hormetic effect of PL at priming the plant response to a subsequent L. innocua infection, thereby improving the content in bioactive compounds was evaluated. Results showed higher reductions of *L. innocua* populations (from 1.7 to 2.4 log_{10}) with a WUV dose increase from 0.3 to 0.5 kJ m^{-2}. However, no improvement was obtained when delivering 0.3 kJ m^{-2} UV-C in PAA (50 or 80 mg L^{-1}). Applying 1 to 4 light pulses (0.5 to 2.0 kJ m^{-2} UV-C) did not control native aerobic mesophilic microbiota compared to water-washing during 7 d of refrigerated storage. Although a reduction of those microorganisms by 1.8 log_{10} compared to water-washing was observed 14 d after 4-pulse treatment, it was detrimental to the product quality. No direct or hormetic effects were observed on *L. innocua* populations in 3 pulses-treated samples (1.5 kJ m^{-2} UV-C). Nonetheless, that dose increased total phenolic content by 25 % compared to chlorine control, and enhanced total antioxidant capacity by 12% and 18%, compared to water and chlorine controls, respectively, 24 h post-processing. WUV is a promising technology for controlling foodborne pathogens populations thereby limiting the effects of cross-contamination during the processing of fresh-cut broccoli while dry-PL is not recommendable.

7.1 INTRODUCTION

Fresh-cut broccoli is a convenient source of health-promoting compounds including flavonoids such as kaempferol and quercetin, as well as of carotenoids, minerals and dietary fiber (Latté, Appel and Lampen, 2011; Ares, Nozal and Bernal, 2013). Beyond its nutritional properties, broccoli is highly valued due to the chemopreventive and anticancer effect of its sulfur-containing secondary metabolites glucosinolates, and its break-down products indoles and isothiocianates, which have shown to increase the activity of the phase II enzymes, and induce the cell cycle arrest and apoptosis of tumor cells in several cancer types such as lung, colorectal, breast and prostate (Higdon *et al.*, 2007; Clarke, Dashwood and Ho, 2008; Brown and Hampton, 2011). To counteract the negative issues related to the processing process such as the cross-contamination with foodborne pathogens, the increase of native microbial populations and the activation of metabolic process detrimental to the product quality, sanitation and preservative methods must be implemented during the production workflow. The reduction of microbial load is currently mainly carried in industry out by washes with chlorine. However, in order to reduce health and environmental risks entailed by the formation of halogenated side-products, alternative methods such as non-thermal physical technologies are being implemented.

UV-C irradiation is a non-ionizing technology used for microbial inactivation of surfaces (being food, liquids or packaging materials) through the application of wavelengths in the range 200 to 280 nm with the maximum DNA absorption between 260 and 265 nm (Gayán, Condón and Álvarez, 2014). UV- C induces the formation of DNA pyrimidine dimers, which inhibit transcription and replication and eventually lead to mutagenesis and cell death (Vishwakarma, Singh and Kewat, 2013). In addition, facing the potential generation of free radicals upon UV light irradiation, antioxidant mechanisms and production of shield molecules

are activated in the plant, leading to the increased accumulation of antioxidants such as glutathione and phenolic compounds, and other secondary metabolites such as glucosinolates (Lemoine, Chaves, & Martínez 2010). Therefore, as hormetic effects, UV-C indirectly act on microorganisms through the induction of the plant defense mechanisms against subsequent attacks and improve the bioactive properties of the plant products and stimulates the (Ribeiro, Canada, and Alvarenga, 2012; Shama, 2007). The use of two-sided dry UV- C radiation at doses from 0.4 to 15 kJ/m^2 is effective for reducing native microbial load and inoculated foodborne pathogens in fresh-cut broccoli from different varieties. Some of those doses also improved nutraceutical properties and preserved the product quality (Gamage, Heyes, Palmer, & Wargent 2016; Martínez-Hernández, Huertas, et al. 2015). However, at higher doses, UV- C can provoke overheating, changes in plant cell structure and permeability, increasing the leakage of nutrients, inducing softening and browning (Allende and Artes 2003). Thus, the application of low UV- C doses in water is an alternative contributing to maintain the product quality and improve the efficacy of this technology by increasing the surfaces exposed and limiting temperature rise. Previous attempts of combining low-dose UV and water baths have been carried out for the decontamination of natural mesophilic bacteria and foodborne pathogens (*Salmonella* spp. and *Escherichia coli*) on fresh produce and differential results have been obtained according to the methodology used for irradiation, the dose, the target microorganism and the food matrix (Hägele et al. 2016; Liu, Huang, and Chen 2015).

Pulsed light (PL) is another emerging non-thermal technology which involves the use of intense light pulses in a broad wavelength range (200– 1100 nm), comprising ultraviolet (200–400 nm), visible (400–700 nm) and near-infrared region (700–1100 nm), on the surface of either foods or packaging materials resulting in the inactivation of pathogenic and spoilage microorganisms (reviewed by Bhavya & Umesh Hebbar 2017). The main advantages of this

method are the very short treatment times, the relatively low energy expense and cost of treatments compared to thermal technologies and the lack of residual compounds (Ignat, Manzocco, Maifreni, Bartolomeoli, & Nicoli 2014). Microbial inactivation by PL is mainly due to the photochemical effect of UV light on microbial DNA, but photo-thermal and photo-physical effects damaging cell wall and cytoplasmic membrane have also being reported (Krishnamurthy *et al.*, 2010). PL efficacy is influenced by the pulse width and the peak of power, since both modulate the amount of UV-C radiation and the intensity of energy that are applied to the samples (Elmnasser *et al.*, 2007). PL has been used for the decontamination of several fresh-cut fruit (Charles, Vidal, Olive, Filgueiras, & Sallanon 2013; Ignat, Manzocco, Maifreni, Bartolomeoli, & Nicoli 2014) and vegetables (Gómez-López *et al.*, 2005; Ramos-Villarroel *et al.*, 2012; Hägele *et al.*, 2016), but no information was found in the body of literature about its use in fresh-cut broccoli.

In previous experiments our work group assessed the effect of a water-assisted UV-C technology (WUV) on the populations of natural mesophilic bacteria, yeasts and molds as well as on the antioxidants and glucosinolates content in fresh-cut broccoli. A significant reduction of native mesophilic bacteria and the improvement of bioactive properties were achieved without negatively affecting the physical quality (Collazo, Lafarga, *et al.*, 2018). In this context, the aims of this work were i) to further evaluate the effectiveness of WUV for the control of *Listeria innocua* inoculated on fresh-cut broccoli as a surrogate of the foodborne pathogen *L. monocytogenes,* immediately after treatment and throughout storage i) to test whether the increase of the UV-C dose or the substitution of water by peroxyacetic acid (PAA) improve the efficacy WUV and iii) to compare the efficacy of WUV for the decontamination of *L. innocua* with a pulse-light (PL) technology applied in air. Additionally, the effects of PL on the quality and the bioactive properties of fresh-cut broccoli were evaluated.

7.2 MATERIALS AND METHODS

7.2.1 PLANT MATERIAL

Broccoli (*Brassica oleracea* L var. *Italica*) heads were purchased from local farms from Lleida, Spain or Avignon, France and cut into 2 - 3 cm diameter florets with a sharpened knife.

7.2.2 BACTERIAL CULTURE AND INOCULATION

For inoculum preparation, *Listeria innocua* Seeliger strain CECT-910, a surrogate of *L. monocytogenes* (Francis and Beirne, 1997), was grown in tryptone soy broth (TSB, Biokar, Beauveais, France) supplemented with 6 g L^{-1} yeast extract (TSB-YE) at 37 °C for 24 h, harvested after centrifugation at 9800 x g for 10 min at 10 °C and diluted in saline solution (8.5 g L^{-1} NaCl). The concentration of the bacterial solution was determined by plate count in TSB-YE agar plates (15 g L^{-1} agar) after overnight incubation at 37 °C. Florets were dip-inoculated in a 10^7 CFU mL^{-1} *L. innocua* solution in deionized water at a ratio of 1:5 (g of broccoli: mL of bath) for 2 min in agitation. After draining the excess of water, samples were air- dried for 2 h before the decontamination treatments.

7.2.3 MULTISIDED DECONTAMINATION USING WUV AND ITS COMBINATION WITH PAA

WATER-ASSISTED UV-C EQUIPMENT

Water-assisted UV-C treatments (WUV) were performed in a laboratory scale equipment LAB-UVC-Gama (UV-Consulting Peschl España S.L., Spain) composed of a water tank containing four UV lamps, connected to a recirculating system activated by a water pump (see detailed description in Collazo et al. 2018).

Lamps were preheated for 15 min before each treatment. Before and after each treatment temperature was measured using an infrared thermometer DualTemp Pro (Labprocess distribuciones, Barcelona, Spain) and irradiance was measured through an orifice located in the lid of the equipment using a UV-sensor EasyH1 (Peschl Ultraviolet, Mainz, Germany).

Treatment and packaging

Inoculated fresh-cut broccoli florets were immersed for 120 s in agitated cold (5 ºC) tap water at a ratio of 1:35 (g of product: mL of solution) and concomitantly submitted to 0.3 kJ m^{-2} UV-C. In order to test the synergistic effect of the combination of WUV and a chemical compound alternative to chlorine, the same procedure was performed but substituting water by a 50 or 80 mg L^{-1} peroxyacetic acid solution. Washing broccoli florets for 120 s either in agitating tap water, peroxyacetic acid solutions (50 or 80 mg L^{-1}) or 100 mg L^{-1} sodium hypochlorite, the latter with pH adjusted to 6.5 with ortho-phosphoric acid (Merck Millipore, Darmstadt, Germany), were performed as control treatments. To further improve the efficacy of WUV a higher WUV dose was assayed (0.5 kJ m^{-2}) following the same procedure. Furthermore, to test the inhibitory effect of WUV on *L. innocua* growth throughout refrigerated storage, as well as the putative DNA repair in irradiated microbial cells upon exposure to daylight, and 120 g of 0.5 kJ m^{-2}- treated samples were microbiologically analyzed within the day or frozen with liquid nitrogen until subsequent biochemical analysis. The rest was stored in 20 x 10 cm thermosealed bags made of a 20 µm thick oriented polypropylene (OPP) microperforated film (PDS group, Murcia, Spain) for 8 d at 5 ºC, either exposed to daylight or in darkness. As control treatments, water and chlorine-washing were performed as previously described. UV doses were calculated as: irradiance (W m^{-2}) x seconds of exposure. All experiments were repeated twice and included three replicates per treatment and per sampling time.

MICROBIOLOGICAL ANALYSIS

After the drainage of the excess of water and air-drying at room temperature on the laboratory bench, 25 g of treated broccoli florets were homogenized with 225 mL of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, England) within a 400 mL sterile full-page filter bag (Bagpage, Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set at 4 strokes per second for 90 s. *L. innocua* viable counts were performed by plating the appropriate ten-fold dilutions of the homogenates in saline peptone (8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) onto selective Palcam agar (Biokar, Beauveais, France) and incubated at 37 °C for 48 h before counting. Populations in the samples stored at 5 °C, in light or darkness, were evaluated after 4 and 8 d of incubation, following the same procedure.

HEADSPACE GAS COMPOSITION

Headspace gas composition (O_2 and CO_2) of each replicate stored in the bags was measured at 4 and 8 d of refrigerated storage using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark).

7.2.4 TWO-SIDED DECONTAMINATION USING PULSED LIGHT (PL) IN AIR

PULSED LIGHT EQUIPMENT

PL treatments were performed in a laboratory scale system (CLARANOR S.A., Avignon, France) equipped with two automatic flash xenon lamps situated on the top of a closed chamber above a 47 x 95 mm platform adjusted to leave a 100 mm height from the top of the sample to the lamps (Fig. **VII**.1). Xenon flash lamps of the PL unit emitted short-time pulses (250 µs) of broad spectrum (200 – 1100 nm) light. Each pulse delivered 5 kJ m^{-2}, containing approximately 0.5 kJ m^{-2} of UV-C for an input of 3000 V at a distance of 100 mm from the lamps.

Temperature measurements were performed in all the samples using a noncontact infrared thermometer (TempTestr® IR, Oaklon, USA).

PHYSICAL QUALITY PARAMETERS

Superficial color of floret heads was determined by measuring CIE parameters L∗, a∗ and b∗ with a chromameter (CR400, Minolta, Osaka, Japan) on two positions of 5 florets per treatment. Color results were interpreted according to the CIE parameters: L*: lightness, ranging from black (0) to white (100); a*: greenness (negative) to redness (positive); b*: blueness (negative) to yellowness (positive). Parameters a* and b* were expressed as hue angle (°) calculated as: 180 + arc tan (b*/a*) (McLellan, Lind, & Kime, 1995). In order to characterize the impact of PL treatments on the plant photosystem, chlorophyll a fluorescence was measured each sampling time in the center of the heads of five florets after dark adaptation for 30 min using a handheld fluorimeter (Pocket-PEA, Hansatech Instrument, United Kingdom). During this period of darkness the electron acceptor center of photosystems II (PSII) are gradually re-oxidized enabling them to redo photochemistry (Stirbet and Govindjee, 2011). Measurements were performed after a 1 s of light induction with 3000 μmol m^{-2} s^{-1}. Results were expressed as the maximal quantum yield of photosystem II (Fv/Fm ratio), where Fv = Fm – Fo; Fm: maximum fluorescence yield in darkness, Fo: minimum fluorescence yield in darkness (Toivonen and DeEll, 2001).

MICROBIOLOGICAL ANALYSIS

For microbial counts of native MAM, samples of 25 g of PL-treated and untreated broccoli florets were homogenized in 225 mL of buffered peptone water (BPW) within a 400 mL sterile half-page filter bag (Bagpage, Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set at 4 strokes per s for 90 s. MAM populations were determined by plating the appropriate ten-fold dilutions in saline peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto Plate Count Agar plates (PCA) after incubation at 25 °C for 72 h. Yeasts and molds populations

were counted on Dichloran Rose Bengal Chloramphenicol agar plates (DRBC) after incubation at 25 ºC for 3 to 5 d. Culture media and its additives were purchased from Biokar (Beauvais, France) and the rest of chemical compounds were purchased from Sigma-Aldrich (Darmstadt, Germany). *L. innocua* counts were performed as described in Section 7.3.3.

SETTING UP OPTIMAL PULSED LIGHT DOSE

Selection of the optimal PL dose was based on the reduction of the native total aerobic mesophilic microbial (MAM) populations of broccoli (as described in Section 4.4.3) and the maintenance of its overall quality, color and fluorescence (Section 7.4.2) throughout refrigerated storage (at 0, 1, 7 and 14 d after treatment). Four doses were evaluated: 1, 2, 3 and 4 pulses, containing to 0.5, 1.0, 1.5 and 2 kJ m^{-2} UV-C. The first treatment was selected similar to the WUV dose. Before PL treatments broccoli florets were immersed in agitating tap water or in 100 mg mL^{-1} sodium hypochlorite in a proportion 1:5 (g of broccoli: mL of bath) for 2 min in a tabletop orbital shaker and let dry to air on the laboratory bench. Six broccoli florets were evenly distributed on the platform of the PL devise, submitted to light pulses on two opposite sides. Some samples were analyzed by microbial viable counts within the day or frozen in liquid nitrogen and stored at – 80 ºC until biochemical analysis. The rest of the samples were packaged in 250 mL polypropylene (PP) trays (Befor Technitrans, France), thermosealed with a six-hole macro-perforated polyethylene terephthalate - polyethylene (PETPE) film (Befor Technitrans, France) and stored for 14 d at 5 ºC in darkness. The headspace gas composition of trays was measured at 1, 7 and 14 d after treatment using a gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark).

DECONTAMINATION OF *L. INNOCUA*: TREATMENT AND PACKAGING

Based on the results of the optimization phase, a dose of 3 pulses of light, containing 1.5 kJ m^{-2} UV-C, was selected to further test the effectiveness of the

technology for the decontamination of native yeasts and molds or inoculated *L. innocua* populations (Fig. **VII.**1).

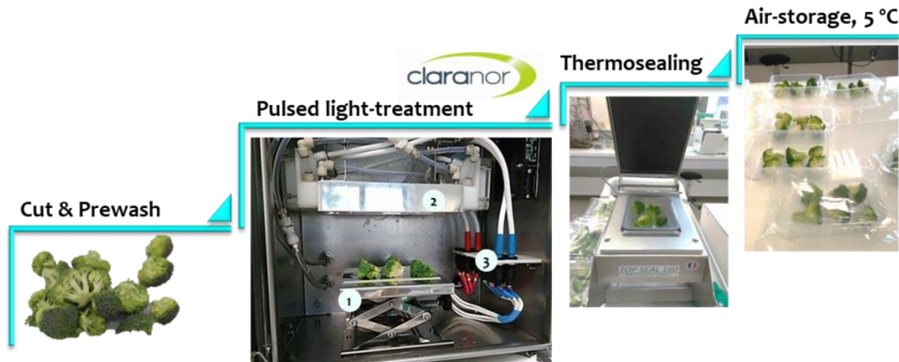

Figure **VII.**1. General workflow of pulsed light processing for the decontamination and control of *L. innocua* and native microbiota in fresh-cut broccoli. Pulsed-light chamber was equiped with an adjustable plattform (1), two lamps emitting in a wide wavelenght spectrum ranging from 200 – 1100 nm (2), and a power source (3000 V).

Prior to PL treatments, broccoli florets were washed in agitated tap water as described in the previous section or inoculated with *L. innocua* (see section 7.2.2). After inoculation or washing, six florets were evenly distributed on the platform of the PL unit and submitted to PL on two opposite sides. Non-treated *L. innocua*-inoculated samples and chlorine-decontaminated (100 mg L^{-1}) samples were included as controls. To test the antimicrobial hormetic of PL, via the activation of the plant defense response upon a subsequent infection with *L. innocua*, some pre-washed broccoli florets were PL-treated following the same procedure, and after 24 h of incubation at 5 ºC, were dip-inoculated with *L. innocua* as described in Section 7.2.2. To assess the effect of PL on *L. innocua* populations on broccoli, samples of 10 g of i) non-decontaminated control ii) PL-treated after inoculation and iii) PL treated before inoculation were homogenized in 90 mL of BPW and counted as described in section 7.3.3 in the same day of treatment or after 4 or 8 d of incubation at 5 ºC. In the processing

day, some samples were microbiologically analyzed or frozen with liquid nitrogen and stored at -80 °C for subsequent biochemical analyses. The rest of processed samples were packaged as described in the section 7.4.2 and stored for 15 d at 5 °C in darkness until sampling and further analysis. O_2 and CO_2 measurements of each tray were performed at 1, 8 and 15 d post-treatment as described in Section 7.4.2.

Biochemical analysis

Approximately 50 g of florets per replicate, per treatment and per sampling time was frozen with liquid nitrogen, grinded to powder (Grindomix GM 200, Retsch, Germany), and stored at -80 °C until biochemical analysis. Extracts for total antioxidant capacity (TAC) and total phenolic content (TPC) determinations were obtained by centrifugation at 24 000 x g for 20 min at 4 °C of a mixture containing 3 g of frozen broccoli powder and 10 mL of an aqueous solution (19.7 mol L^{-1} methanol and 0.05 mol L^{-1} HCl) previously agitated at 20.94 rad s^{-1} in an tabletop orbital shaker for 2 h. TAC was quantified by the Ferric Reducing Antioxidant Power (FRAP) method following the Benzie and Strain (1996) protocol with some modifications (Giné-Bordonaba and Terry, 2016) and by the DPPH (2,2 – diphenyl – 1 – picrylhydrazyl) free radical-scavenging activity method (Brand-Williams et al. 1995). For the DPPH determination, 1.5 mL of a reaction containing 1.4 mL of 1 mmol L^{-1} DPPH and 0.1 mL of broccoli extract was incubated for 1 h before colorimetric quantification. Optical density (OD) for TAC quantification was measured at 593 nm and 515 nm, following the FRAP and DPPH method, respectively. For the total phenolic content determination, a reaction containing 0.7 mL of each sample extract, 4.3 mL of water and 0.5 mL of Folin-Ciocalteu reagent, was incubated for 5 min in darkness, and then 2 mL of 200 g L^{-1} Na_2CO_3 solution was added before measuring OD at 765 nm, based on the method described by Singleton and Rossi (1965). Non-enzymatic antioxidant activities were expressed as g of the measured analyte (i.e. Gallic acid (GAE) or ascorbic acid) per kilogram of fresh weight of broccoli (g kg^{-1} FW). Standard

analytes and reagents were purchased from Sigma-Aldrich. Chlorophylls content was determined by measuring OD at 662, 645 and 670 nm of the supernatant resulting from the centrifugation at 15 000 x g for 5 min of a mixture containing 1 mL acetone and 100 mg of frozen treated or untreated broccoli powder, previously incubated for 15 min at 4 ^{O}C, following the protocol and math formulae described by Lichtenthaler and Buschmann (2001).

For glucosinolates extraction, triplicate samples of PL-treated broccoli as well as non-processed and water and chlorine- washed controls were stored for 24 h at 4 °C, frozen with liquid nitrogen and lyophilized. Then, 150 mg of each were mixed with 3 mL of a extraction solution (80:20, volume of methanol: volume of water) as described by Alarcón-Flores et al. (2013). Glucosinolates quantification was performed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) analysis using an Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) coupled to an Agilent triple quadrupole mass spectrometer (6460A) with a Jet Stream ESI ion source (G1958-65138 using a multi-compound standard methanoic solution containing 5 mg/L of sulforaphane (Sigma-Aldrich, Steinheim, Germany), gluconasturtin and glucoraphanin (PhytoLab GmbH & Co., Vestenbergsgreuth, Germany), glucotropaeolin, glucoerucin, glucoiberin and proigonitrin (Scharlab, Barcelona, Spain). Results were expressed as mg kg^{-1} of dry weight (DW).

7.2.5 Statistical analysis and expression of results

All experiments were repeated two times and included three biological replicates per treatment and sampling time. Resulting data were analyzed using the Statistical software JMP (version 8.0.1 SAS Institute Inc., NC, USA). All data were tested for agreement to normal distribution and homoscedasticity of residues. The significance of the differences and interactions among factors were determined by analysis of variance (ANOVA) and separated by Tukey's test

(P < 0.05). Microbiological data were calculated as colony forming units per milliliter (CFU mL^{-1}) and transformed to log_{10} CFU per gram of fresh weight of fruit (log_{10} CFU g FW^{-1}) before means comparison.

7.3 RESULTS

7.3.1 MULTISIDED DECONTAMINATION USING WATER-ASSISTED UV-C AND PAA-ASSISTED UV-C

MICROBIOLOGICAL ANALYSIS OF FRESH-CUT BROCCOLI & SANITATION SOLUTION

Initial *L. innocua* populations on broccoli florets ranged from 6.6 to 7.3 ± 0.4 log_{10} CFU g^{-1}. WUV treatments at a dose of 0.3 kJ m^{-2} reduced *L. innocua* populations by 1.7 ± 0.2 log_{10} in respect of untreated broccoli (Fig. **VII**.2). Substituting water by 50 or 80 mg L^{-1} peroxyacetic acid solutions (PAA) in the washing deposit did not improve those results.

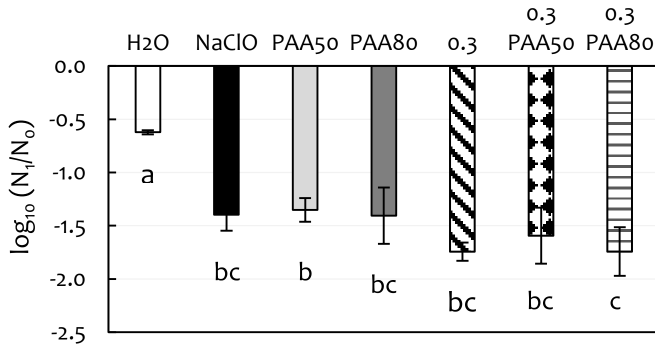

Figure **VII**.2 *L. innocua* population reductions after decontamination with WUV (0.3 kJ m^{-2}) or its combinations with 50 or 80 mg L^{-1} peroxyacetic acid (PAA) compared to water-washing (H2O) or chlorine-sanitizing (NaClO). N_0: initial population, N_1: final population. Columns represent means and error bars represent standard deviation (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test (P < 0.05).

Reductions obtained with WUV, regardless of the combination with peroxyacetic acid; were up to 2.8-fold higher than the reduction achieved by water-washing without UV, and similar to that obtained with the chlorine and the 50 or 80 mg L^{-1} peroxyacetic acid sanitation (1.4 ± 0.2 $\log_{10}$ reduction).

Increasing the UV dose to 0.5 kJ m^{-2} improved the effectiveness of WUV, with reductions by 40 % higher than that obtained with 0.3 kJ m^{-2} and by 75 % higher than that obtained by chlorine sanitation (Fig. **VII.**3). The survival of *L. innocua* after irradiation with 0.5 kJ m^{-2} WUV after 4 and 8 d of MAP storage at 5 ºC in darkness showed lower population increases by 1.5 ± 0.4 and 1 ± 0.2 $\log_{10}$ in each sampling day, respectively, than in the water-washed control. A similar result was obtained with chlorine sanitation. No DNA repair could be determined as measured by *L. innocua* population recovery in WUV-irradiated cells after 8 d of exposure to daylight in refrigerated storage compared to the populations stored in darkness or the water control.

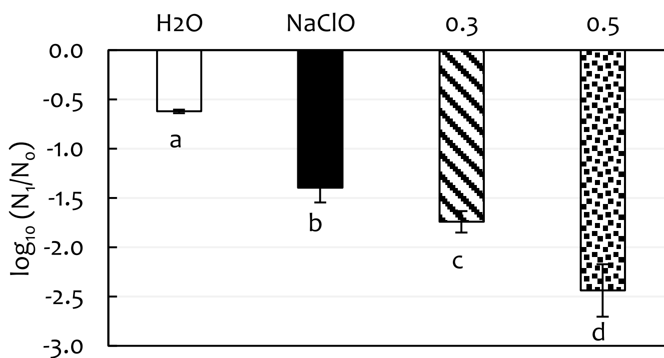

Figure **VII.**3 *L. innocua* population reductions after decontamination with several WUV doses (kJ m^{-2}) compared to water-washed (H_2O) or chlorine-sanitized (NaClO) controls. N_0: initial population, N_1: final population. Columns represent means and error bars represent standard deviations (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$).

No viable *L. innocua* cells were detected in the singe-used process water or PAA solutions after sanitation of inoculated broccoli either with 0.3 or 0.5 kJ m^{-2} WUV

or with any of the combinations of WUV and PAA (detection limit of 5 CFU mL^{-1}), which represented a 3 log_{10} reduction in respect of the single-used control process water.

GASES ANALYSIS

The gas headspace compositions of bags containing WUV-treated broccoli was similar to that of the water and chlorine controls after 4 d (16.1 kPa O_2; 4.1 kPa CO_2) and 8 d (17.9 kPa O_2; 2.8 kPa CO_2) of storage at 5 °C in darkness. No differences in the gas compositions of the bags were either observed between samples stored for 8 d in darkness compared to those exposed to daylight (data not shown).

7.3.2 TWO-SIDED DECONTAMINATION USING PULSED LIGHT

7.3.2.1 SETTING UP OPTIMAL PL DOSE

MICROBIOLOGICAL ANALYSIS

Initial total MAM populations on fresh broccoli were 5.5 ± 0.4 CFU g FW^{-1}. A reduction by 1 log_{10} was obtained after the application of PL regardless of the applied dose which was similar to that obtained by only water-washing the samples. In the same way, PL treatments showed no growth inhibition of MAM during the first 7 d of refrigerated storage, since populations reached 6.3 ± 0.2 CFU g FW^{-1} in all samples. However on day 14, samples treated with 4 pulses (2 kJ m^{-2} UV-C) showed a reduced growth (by 1.79 ± 0.04 CFU g FW^{-1}) compared to the rest of the samples (data not shown).

ANALYSIS OF PHYSICAL PARAMETERS

Color was maintained during the first 7 d of storage in all samples (Table **VII.**1). Lightness showed a trend to increase after 14 d of storage without significant differences among treatments. Hue angle was reduced after 14 d when treated

with the highest PL dose compared to initial time, which was similar to the observed for the water and chlorine controls, while no variation was detected in samples treated with 1 or 2 pulses. The visual analysis of overall quality showed some brownish nervure at the cut surface of irradiated sides and slight signs of dehydration in all PL-treated samples after 7 and 14 d of storage. However, the poorest appearance was observed in samples treated with 4 pulses (data not shown). Analysis of fluorescence showed at initial stress in samples treated with 3 and 4 pulses one day after treatment, as suggested by lower values of the maximum photochemical efficiency of photosystem II (Fv/Fm ratio) (0.79 ± 0.01) compared to the water control (0.82 ± 0.01). However, after 7 d of storage, the vegetable recovered the photosynthetic capacity showing Fv/Fm values that matched that of the water control.

GASES HEADSPACE COMPOSITION

Trays headspace composition remained close to ambient air (20.6 kPa O_2, 0 kPa CO_2) during the whole storage. During the first 24 h there was a decrease in O_2 content and an increase in CO_2 content in all samples. Afterwards the concentration of both gases stabilized during the rest of storage in all samples (Fig.**VII**.4). Samples treated with 4 pulses of light showed the most reduced O_2 and the highest CO_2 content after 1 d of storage, compared to the chlorine control, although no significant differences were observed in respect of the rest of the treatments.

In summary, results from the optimization phase suggested that 4 pulses of light was an excessive dose to maintain the physical quality parameters of broccoli. Thus, the immediate lower dose (3 light pulses corresponding to 1.5 kJ m^{-2} UV-C) was selected to subsequently test the effect of PL on artificially inoculated *L. innocua* and on naturally present yeasts and molds populations on fresh-cut broccoli.

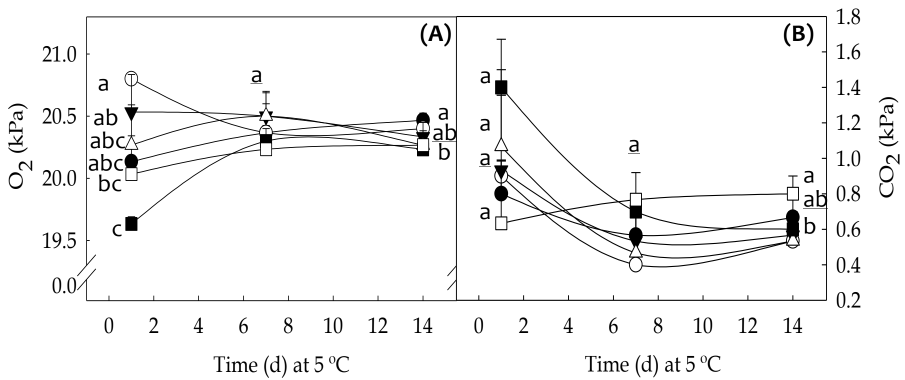

Figure **VII.**4 O_2 (A) and CO_2 (B) headspace composition of trays containing fresh-cut broccoli treated with different pulsed light doses: 1 pulse (○), 2 pulses (▼), 3 pulses (△) and 4 pulses (■) compared to water-washed (●) or chlorine-sanitized (□)controls, throughout refrigerated storage. Symbols represent means and error bars represent standard deviations (n=3). Different letters represent statistical differences according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$). Underlined letters represent equal means corresponding to overlapped symbols.

7.3.2.2 DECONTAMINATION WITH THE SELECTED PL DOSE

MICROBIOLOGICAL ANALYSIS

Initial populations of *L. innocua* after inoculation were 5.72 ± 0.33 CFU g FW^{-1} (Fig. 5A). No significant reduction compared to the water-washed control, was observed at any of the analyzed sampling points when using the pulsed-light technology, regardless of the dose and the moment of inoculation (before or after PL treatment). Significant reductions of *L. innocua* populations (by 0.5 ± 0.3, 0.8 ± 0.5, and 1.0 ± 0.3 log_{10}) were only observed for the chlorine-treated control immediately after treatment and after 1 and 8 d of storage, respectively. Native yeasts populations on broccoli were initially 3.3 ± 0.5 log10 CFU g FW^{-1}. Immediately after sanitation, a reduction by 0.7 ± 0.4 log_{10} of yeasts populations in respect of untreated broccoli was accomplished by water-washing the samples, whether applying or not light pulses afterwards (Fig. **VII.**5B). Similar

reduction compared to untreated broccoli was obtained at initial time for the chlorine control (1.1 ± 0.3 log_{10}).

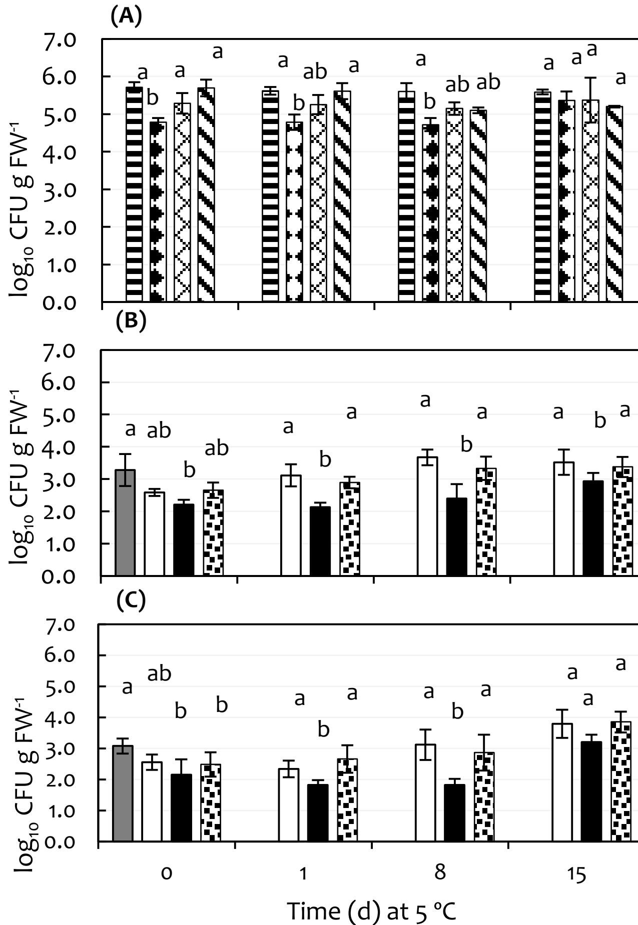

Figure VII. 5 Changes in microbial populations: (A) *L. innocua* and native (B) yeasts and (C) molds in fresh-cut broccoli treated with pulsed light (3 pulses, 1.5 kJ m^{-2} UV-C) compared to water or chlorine-washing, throughout refrigerated storage. (▩) unprocessed control, (☐) water control, (■) non-inoculated PL-treated, (▩) non-inoculated chlorine-sanitized control, (▤) *L. innocua* control, (▩)PL-treated after inoculation with *L. innocua*, (▩) Chlorine-sanitized after inoculation with *L. innocua*, (▧) PL-treated before inoculation with *L. innocua*. Columns represent means and error bars represent standard deviations (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test ($P < 0.05$).

Chlorine sanitation had an inhibitory effect on yeasts populations which was maintained during the whole storage period, showing reductions by 1 ± 0.2, 1.3 ± 0.4 and 0.6 ± 0.4 log_{10} compared to the water control after 1, 8, and 15 d, respectively. No effect of PL on yeasts populations was observed at any of the analyzed sampling points. Initial reductions of molds populations naturally present on broccoli (3.1 ± 0.2 log_{10} CFU g FW^{-1}) obtained by water-washing (by 0.5 ± 0.2 log_{10}) did not differ from the achieved with PL (by 0.6 ± 0.4 log_{10}) or chlorine sanitation (by 0.9 ± 0.5 log_{10}) (Fig. **VII.**5C). However, lower populations in respect of the water control (by 0.5 ± 0.2 and 1.1 ± 0.5 log_{10}) were observed at 1 and 8 d in chlorine-sanitized samples which contrasted with that observed for the PL treatment.

Analysis of physical parameters

Color (L* and hue angle) did not show significant differences among treatments for either inoculated or non-inoculated samples at any of the analyzed sampling points. In general, lightness increased throughout the incubation period (42 ± 1; 47 ± 1; 50 ± 1 at 1, 8, and 15 d, respectively). Hue angle decreased in all samples from 132 ± 2 to 127 ± 2 during the first 8 d of storage and then remained stable up to 15 d (125 ± 2) (data not shown). Off odors and poor appearance of samples were detected in all samples after 15 d of storage. Fv/Fm ratio was similar in all samples during the first 24 h post-processing (0.81 ± 0.02) and, in general, it subsequently showed a trend to decrease throughout storage, regardless of the sanitation method. However, significant differences in respect of the initial values were only observed for *L. innocua*-untreated control and *L. innocua*-chlorine disinfected control, both showing Fv/Fm values of 0.6 ± 0.2 at day 15 of storage.

Biochemical analysis

Twenty four hours after processing, total phenolic content (TPC) in PL-treated (3 pulses) non-inoculated samples increased by 15 % compared to unprocessed

broccoli (which initially had 65 ± 4 mg kg FW^{-1}), (Fig. **VII.**6A). Although this increase was not significant different from the water-washed control, it exceeded by 25 % the chlorine control. However, this effect did not remain throughout storage.

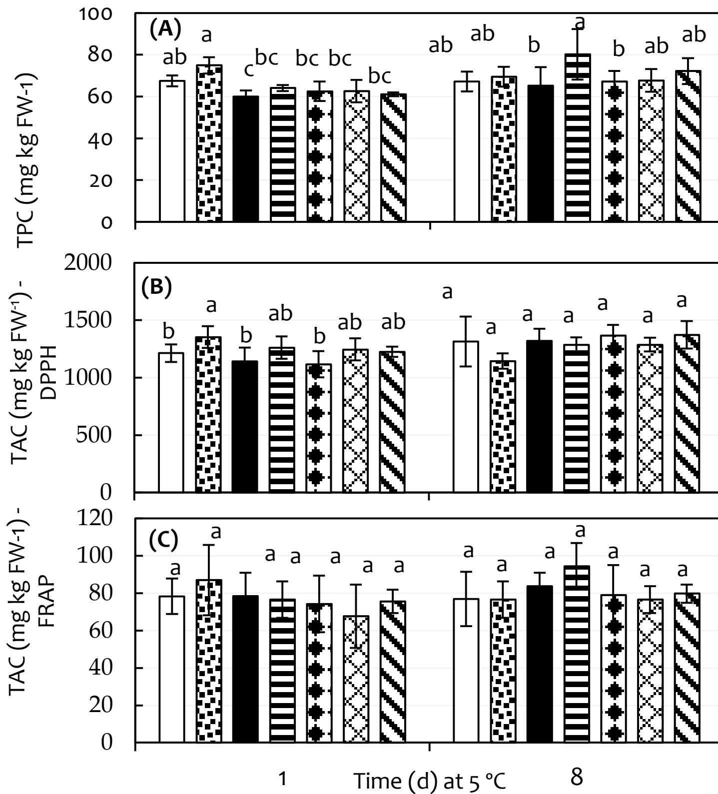

Figure **VII.**6 Changes in **(A)** total phenolic content (TPC) and total antioxidant capacity (TAC) measured by the DPPH **(B)** or FRAP **(C)** methods in fresh-cut non-inoculated or *L. innocua*-inoculated broccoli treated with pulsed light (3 pulses) compared to water or chlorine washing, throughout refrigerated storage.(☐) water-washed control, (▩) PL-treated, (■) non-inoculated chlorine-sanitized control, (▤) *L. innocua* control, (▩) PL-treated after inoculation with *L. innocua*, (▨) *L. innocua*- chlorine sanitized, (▧) PL-treated before inoculation with *L. innocua*. Columns represent means and error bars represent standard deviations (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test (P < 0.05).

In samples inoculated with *L. innocua* no differences were observed between PL-treated and the water or chlorine controls, 1 d after processing, but a decrease of the TPC by 13 % was observed for PL-treated inoculated samples compared to

the inoculated un-treated control, after 8 d of storage. As measured by the DPPH method, total antioxidant capacity (TAC) increased by 12 and 18 % in PL-treated samples compared to the water and chlorine-washed controls, respectively, 24 h post-processing (Fig. **VII.**6B). No variation was observed for inoculated samples upon PL treatment compared to the water or chlorine controls after 1 or 8 d of storage. When using the FRAP method no significant difference in TAC was observed for any of the treatments at 1 or 8 d of storage (Fig. **VII.**6C).

PL treatment did not alter the contents in chlorophylls a or b compared to the control samples, regardless of the inoculation with *L. innocua* or the storage time (Table **VII.**2).

Table **VII.**2 Chlorophylls and carotenoids contents in fresh-cut non-inoculated or *L. innocua*-inoculated broccoli treated with pulsed light (3 pulses, 1.5 kJ m^{-2} UV-C)

Treatment		Chlorophyll a ($mg\ kg\ FW^{-1}$)	Chlorophyll b ($mg\ kg\ FW^{-1}$)	Carotenoids ($mg\ kg\ FW^{-1}$)
1 d	H_2O	0.036 ± 0.007 a	0.011 ± 0.003 abc	3.9 ± 0.8 ab
	LP	0.034 ± 0.006 a	0.011 ± 0.002 abc	3.7 ± 0.7 ab
	Cl	0.018 ± 0.007 a	0.009 ± 0.005 abc	2.5 ± 0.1 b
	Li	0.033 ± 0.004 a	0.011 ± 0.002 ab	3.6 ± 0.4 b
	Li- LP	0.030 ± 0.004 a	0.009 ± 0.002 abc	3.5 ± 0.5 b
	Li- Cl	0.026 ± 0.003 a	0.008 ± 0.001 bc	2.8 ± 0.3 ab
	LP- Li	0.036 ± 0.008 a	0.010 ± 0.001 abc	4.1 ± 0.6 ab
8 d	H_2O	0.033 ± 0.005 a	0.011 ± 0.001 abc	4.1 ± 0.7 ab
	LP	0.028 ± 0.003 a	0.009 ± 0.002 abc	3.3 ± 0.4 ab
	Cl	0.035 ± 0.001 a	0.012 ± 0.002 abc	4.4 ± 0.4 a
	Li	0.028 ± 0.005 a	0.007 ± 0.004 c	3.4 ± 0.6 b
	Li- LP	0.028 ± 0.009 a	0.009 ± 0.003 abc	3.4 ± 0.7 b
	Li- Cl	0.030 ± 0.007 a	0.008 ± 0.002 bc	3.7 ± 0.4 ab
	LP- Li	0.028 ± 0.004 a	0.008 ± 0.002 bc	3.5 ± 0.4 ab

Treatments: H_2O (non-inoculated water-washed), Cl (non-inoculated chlorine-washed), PL (non-inoculated pulsed light-treated), Li (inoculated with *L. innocua*), Li- LP (inoculated with *L. innocua*-inoculated and PL-treated), Li Cl (inoculated with *L. innocua* and chlorine-washed), (LP-Li (treated with PL and then inoculated with *L. innocua*). Mean ± Standard deviation. Different letters represent different treatments according to analysis of variances (ANOVA) and Tukey's test (n=6)

From the detected glucosinolates, including two aromatic (gluconasturtiin and glucotropaeolin), one aliphatic (glucoraphanin), and one alkenyl (progoitrin) glucosinolates, glucoraphanin was the most abundant 24 h after processing (Table **VII.**3). PL preserved the glucoraphanin content obtained as a result of processing and washing with water, which exceeded by 32 % the initial content in unprocessed broccoli and chlorine-sanitized samples. In the same way, the content in the thiosulfate derived from glucoraphanin: sulforaphane was duplicated in PL-treated fresh-cut broccoli compared to non-processed one; although this increase was not significant different from the water and chlorine controls. No significant differences were observed for the rest of the analyzed glucosinolates as their content was very close to the detection limit (100 µg kg DW^{-1} for all glucosinolates except for gluconasturtin that was 25 µg kg DW^{-1}).

Table **VII.**3. Glucosinolates and sulforaphane contents in fresh-cut broccoli, 24 h after treatment with 3 light pulses (1.5 kJ m^{-2} UV-C) compared to water-washing or chlorine-sanitizing.

Treatment	Glucoraphanin (mg kg DW^{-1})	Gluconasturtin (mg kg DW^{-1})	Progoitrin (mg kg DW^{-1})	Sulforaphane (mg kg DW^{-1})
NP	635 ± 32 b	20 ± 8 a	5 ± 1 a	18 ± 4 b
H_2O	842 ± 113 a	27 ± 3 a	5 ± 1 a	31 ± 12 ab
PL	837 ± 63 a	11 ± 3 a	5 ± 1 a	40 ± 9 a
Cl	572 ± 34 b	15 ± 4 a	4 ± 1 a	18 ±10 ab

Treatments: NP: non-processed, H_2O (fresh-cut water-washed), Cl (fresh-cut chlorine-washed), PL (pulsed light-treated). Numbers are mean ± standard deviation. Different letters represent different treatments according to analysis of variances (ANOVA) and Tukey's test (n=6)

Gases headspace composition

Analysis of headspace composition of packages showed O_2 and CO_2 levels close to ambient air during the whole period of storage (data not shown). In non-inoculated samples, no differences in the O_2 or CO_2 contents among treatments were observed during the storage period except for 24 h after treatment, when the chlorine control showed a reduced CO_2 level (0.7 ± 0.2%) compared to both PL-treated and water-washed samples (1.2 ± 0.2%) while the O_2 content was

similar. In the samples inoculated with *L. innocua*, the O_2 or CO_2 contents were similar for all treatments.

7.4 DISCUSSION

As shown by the results the way of application of UV-C light significantly influences its effectiveness at controlling *L. innocua* on fresh-cut broccoli florets. While reductions of *L. innocua* by > 2 $\log_{10}$ were obtained at a dose of 0.5 kJ m^{-2} UV-C through a continuous multisided application using the water-assisted technology, the two-sided application of a three-fold higher UV-C dose (1.5 kJ m^{-2}) using the pulsed light technology applied in air, showed no effect neither immediately nor throughout storage. The higher efficacy of WUV may be explained by the two-method decontamination (by irradiation and by immersion) of the plant product and by the simultaneous decontamination of the process water. Besides, the rotation of the product increases the accessibility of UV light to a higher amount of microorganisms, as previously reported for water-assisted UV-C based technologies (Huang and Chen, 2014). In line with this thought, the observed lack of efficacy of PL (3 pulses, 1.5 kJ m^{-2} UV-C) at reducing *L. innocua* populations may have been partly due to the shadowing effect caused by the complicated topography of the plant material, which may have shielded much of the inoculated bacteria from the UV-C deleterious activity (Adhikari *et al.*, 2015).

We found that small increases of the WUV dose (from 0.3 to 0.5 kJ m^{-2}) resulted in a 40% improvement of the decontamination efficacy. Contrastingly, using PL, dose increases from 0.5 to 2.0 kJ m^{-2} UV-C showed no detectable effect on efficacy. In agreement with the WUV results, other researchers have reported a similar inactivation dose-response of foodborne pathogens on fresh-cut broccoli using UV-C light at dose levels below 2.0 kJ m^{-2}, which fits a Weibull model (Martínez-Hernández, Huertas, *et al.*, 2015). Similar behavior was observed for

inoculated populations of *E. coli, S. enterica* subsp. *enterica* ser. Enteritidis and *L. monocytogenes* on fresh-cut Kaylan broccoli treated with continuous UV-C light at doses ranging from 3.2 to 15 kJ m^{-2} (Martínez-Hernández *et al.*, 2011). Unlike our results, previous experiments testing the effectiveness of PL treatments at a range of fluences from 1.9 to 120 kJ m^{-2} have shown that the inactivation of naturally occurring microbiota also followed a Weibull model in fresh-cut shredded white cabbage, with significant effect on populations at very low fluence levels (no shoulder), but with small increases of efficacy with high increases of the dose (upward concavity and tail) (Izquier and Gómez-López, 2011; Levy *et al.*, 2012). It is possible that at higher doses we would observe similar dose-response behavior. However, since we noticed a significant diminishment of broccoli's quality at the presently assayed dose range, higher doses would not be suitable for a shelf-life of 7 d which is already shorter than that of currently commercialized products.

Increasing the PL dose should be tightly linked to the tracking of its effects on the physical and chemical quality of the target plant product. Several reports of the application of PL on fruits and vegetables have shown reductions of native or inoculated microorganisms with high PL doses, but either at the expense of high treatments times and high number of flashes, or of negatively affecting the product quality. For example, 2 log_{10} reductions of *L. innocua* on fresh-cut mushrooms were obtained using 120 kJ m^{-2} intense pulsed light (IPL) but also caused extensive damage of cytoplasm and cytoplasmic membrane of the product (Ramos-Villarroel et al. 2012). In the same food matrix, doses of 48 kJ m^{-2} were ineffective to reduce native MAM populations and increasing the dose to 120 and 480 kJ m^{-2} accomplished reductions by 1.4 log_{10} but caused browning and diminished texture (Oms-Oliu *et al.*, 2010). Within the range of doses used in the present study, 2 log_{10} reductions of MAM were obtained by applying 20 kJ m^{-2} PL to whole spinach leaves (Aguero *et al.*, 2016). The smooth surface topography of spinach and the limited physical damage applied during processing (only

detached) could account for the better results due to a lower surface for internalization of native microflora into the tissue and the less shadowing effect. In this sense, much lower effectiveness of PL (reductions of MAM by 0.34 log_{10}) was obtained in shredded spinach leaves treated with higher PL doses (675 light pulses of 7 kJ each at a distance of 12.3 cm from the platform), probably due to both more severe damage during processing and additional inflicted damage during PL treatment (Gómez-López *et al.*, 2005). In agreement with our trials, several authors have established low damage thresholds for UV-C treatment of fresh produce, therefore the dose must be adjusted for each commodity. For example, doses of 0.5 kJ m^{-2} have shown to be optimal for reducing decay in grapefruit but higher doses (1.5 kJ m^{-2}) caused rind browning and tissue necrosis (D'Hallewin *et al.*, 2000). Similarly, Baka et al. (1999) observed that 0.25 kJ m^{-2} UV-C slow down the ripening and senescence of strawberry fruits stored at 4 °C while 1.0 kJ m^{-2} damaged the fruit. However, taking previous results as a base for dose selection is un-accurate because the instrumental and experimental set-ups significantly influence the output of the technology, i.e. shorter distances from the light source, longer pulse widths as well as higher input voltages (which deliver higher amounts of UV-C in a single flash) have shown to have more microbicide effect than the additive dose of several flashes for the same final fluence (B. Kramer, Wunderlich and Muranyi, 2017a; Scott *et al.*, 2018). Furthermore, the effectiveness of PL for microbial reduction depends on many other factors such as the levels of the initial inoculated population and/or the levels and composition of the native microbiome and their sensitivity to UV-C (Ramos-Villarroel *et al.*, 2012; B Kramer, Wunderlich and Muranyi, 2017).

Regarding the permanence of UV-C antimicrobial effect, we observed no population recovery of *L. innocua* throughout storage in daylight –exposed cells compared to those stored in darkness, showing no DNA-repair of inactivated cells or at least, that they entered in a non-cultivable stage as it has been previously reported for *Listeria* spp. (Kramer and Muranyi, 2014; Lasagabaster

and Martínez- de Marañón, 2014). Moreover, reduced growth of *L. innocua* was maintained in WUV-treated samples until 8 d of storage suggesting that the activation of the defense mechanisms within the plant host may have prevented the population increase compared to the water-washed samples (Scott *et al.*, 2018). In PL-treated samples, only those exposed to 4 pulses showed a hormetic inhibition of MAM after 14 d of storage, but this effect was not observed for *L. innocua* in 3 pulse-treated samples. *Listeria* spp. have previously shown to be more resistance to both continuous UV-C light and PL treatments in fresh-cut produce (Ramos-Villarroel, Martín-Belloso and Soliva-Fortuny, 2011; Ramos-Villarroel *et al.*, 2012; Martínez-Hernández, Huertas, *et al.*, 2015).

In an attempt to further improve the efficacy of WUV (0.3 kJ m^{-2} WUV), it was combined with PAA (50 or 80 mg L^{-1}); however, we found no synergistic effect of both methods combined compared to the single treatments regardless of the PAA dose. In contrast, Martínez-Hernández and Navarro-Rico (2015) observed a synergistic effect (up to 2-fold more effectiveness than single treatments) when combining 100 mg L^{-1} PAA and 7.5 kJ m^{-2} UV-C for the control of *E. coli* and *S. enterica* on fresh-cut broccoli. The higher PAA and UV-C doses used in that experiment (by 10-fold) compared to those used in our trials as well as different target microorganisms could account for the discrepancies among the results. In previous experiments, we observed that the combination with PAA improved WUV efficacy for the decontamination of MAM in fresh-cut organic broccoli, although no improvement was obtained for the same purpose in conventional one (Collazo, Lafarga, *et al.*, 2018), suggesting that several factors influence the outcome of these decontamination strategies.

In the present study the selection of the PL doses was restricted to low-range fluences, comprising from 0.5 to 2.0 kJ m^{-2} UV-C, in order to achieve microbial reductions through both direct and hormetic effects while preserving the physical quality of fresh-cut florets throughout refrigerated storage. Regarding the effect on microbiological quality, we observed no reduction of native MAM

populations for any of the assayed PL doses, except for a reduced growth at the end of storage in samples treated with 4 pulses. However, a decline in the Fv/Fm ratio, which indicates stress at values below 0.83, was observed upon 3 and 4 pulses application, probably due to an increase in protective non-radiative energy dissipation or to photo-inhibitory damage to the PSII reaction center (Maxwell and Johnson, 2000). Although the photosynthetic capacity of the plant recovered after 7 d of storage in both cases, the increased respiration rate and the undesirable effects on overall physical quality observed in the 4 pulse-treated samples motivated the exclusion of this dose and the selection of 3 pulses for subsequent analysis. High O_2 consumption and CO_2 production are also indicators of the plant response to the physiological stress caused by mechanical wounding and pulsed light application, as it has previously been reported for other fresh-cut vegetables upon cutting and PL treatments at moderate doses (Mattos, Moretti and Yosino Da Silva, 2013).

The hormetic effect of continuous UV-C light regarding the activation of secondary metabolites biosynthesis and the delay of ripening and senescence has been extensively studied for several commodities (Alothman, Bhat and Karim, 2009; Artés-Hernández *et al.*, 2009; Martínez-Hernández *et al.*, 2011; Bravo *et al.*, 2012; Oms-Oliu *et al.*, 2012; Duarte-Sierra, 2015; A. C. Formica-Oliveira *et al.*, 2017; Darré *et al.*, 2017; Gogo *et al.*, 2017). In fresh-cut broccoli, continuous UV-C at a dose of 8 kJ m^{-2} increased total antioxidant activity as well as phenolic compounds and ascorbic acid contents. It also extended product's shelf-life by delaying senescence through the reduction of respiration rate and chlorophyll degradation during storage at 4 °C for up to 21 d (Costa, Vicente, Civello, Chaves, & Martínez 2006; Lemoine, Civello, Martínez, & Chaves 2007). In contrast, we observed no change in headspace composition or chlorophylls contents compared to the water control after 0.5 kJ m^{-2} WUV or 1.5 kJ m^{-2} PL treatments, during storage for 8 or 15 d, respectively. This contrasted with the increased respiration and negative changes in color parameters which were

eventually observed in chlorine-treated samples, implying that both WUV and PL technologies contributed better to preserve color and general quality than chlorine. In the same way, total phenolic content was maintained after PL treatment compared to water-washing while it was reduced after chlorine sanitation as it was observed in previous experiments performed by our work group in fresh-cut broccoli using WUV (Collazo, Lafarga, *et al.*, 2018). Furthermore, in agreement with previous results, in PL-treated samples TAC was enhanced during the first 24 h, as measured by DPPH method, showing certain hormetic effect (Martínez-Hernández *et al.*, 2011). TAC showed the same trend using the FRAP method but the variability of measures did not allow to detect significant differences, probably because of the interference of the elevated ascorbic acid content of broccoli with the activity of the standard used (Koh *et al.*, 2009). Higher measures and less interference were observed through DPPH method using gallic acid as standard (Stratil, Klejdus and Kubáň, 2006).

We observed that glucoraphanin (GP) was the most abundant glucosinolate in the broccoli samples, as it has been previously reported for several broccoli varieties (Gamage, 2015; Jones, Faragher, & Winkler, 2006; Torres-Contreras, Nair, Cisneros-Zevallos, & Jacobo-Velázquez, 2017). Both GP and sulforaphane content increased 24 h after processing. The activation of the glucosinolates synthesis pathways is one of the many responses to wounding mediated by the jasmonic acid or salicylic acid signaling pathways (Mikkelsen, 2003). Different cutting styles have shown to induce variations in specific glucosinolates as well as their transformation into thiocyanates due to the releasing of myrosinase enzyme from specialized cells after cutting (Chen and Andreasson, 2001; Torres-Contreras *et al.*, 2017). UV radiation (specifically wavelengths in the UV-B spectrum) at low doses (0.45 kJ m^{-2}) has also been demonstrated to elevate glucosinolates levels in broccoli sprouts which has been associated to the up-regulation of genes involved the biosynthetic pathway of aliphatic glucosinolates as well as of genes related to the response of pathogens and

herbivores (Mewis *et al.*, 2012). We observed that glucosinolates content did not increase any further 24 h after PL treatment that what it did after cutting and water washing. Continuous UV-C application (1.2 kJ m^{-2}) have previously shown to differentially activate the synthesis of certain glucosinolates (e.g. 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin and GP) while reducing others (glucobrassicin) in broccoli florets. The optimal moment to detect the significant accumulation of each glucosinolate compared to untreated control is also variable depending on the dose and the specific analyzed compound (from 3 d to 14 d after treatment) (Nadeau *et al.*, 2012; A.C. Formica-Oliveira *et al.*, 2017). The increase of the bioactive compounds such as lycopene in fresh-cut tomato plugs was only shown after PL treatments with very high doses (300 kJ m^{-2}) (Aguiló-Aguayo *et al.*, 2013) which would not be suitable for the treatment of minimally processed produce since significant reduction of weight and firmness and enzymatic activation due to membrane damage have been observed in this commodity for PL doses ranging from 40 to 80 kJ m^{-2} (Valdivia-Nájar, Martín-Belloso and Soliva-Fortuny, 2018). Alternatively, the application of PL in order to improve the nutritional properties could be adjusted in terms of not only to the type of commodity but to the production step. For instance, the application of low PL doses (2 pulses, 6 kJ m^{-2}) have shown to be more suitable than higher doses (up to 48 kJ m^{-2}) for improving quality of whole mangoes, by decreasing pectin-methylesterase activity, thereby maintaining firmness, which later resulted in an increased total phenolic content (TPC) in fresh-cut product (Lopes *et al.*, 2016). Contrastingly, no increase in TPC was observed in the same commodity when 80 kJ m^{-2} PL was applied during the processed stage (Charles *et al.*, 2013).

7.5 CONCLUSIONS

The present study showed that water-assisted UV (WUV) at a dose of 0.5 kJ m^{-2} effectively reduced *L. innocua* populations by more than 2 log_{10} on fresh-cut broccoli compared to water washing and that no viable cells were detected in single-used process water. Those results suggest that WUV is a promising residue-free technology alternative to chlorine useful for reducing pathogenic *Listeria* spp. in infected broccoli and for controlling cross contamination during processing. No further improvement of WUV effectiveness was obtained when combining it with peroxyacetic acid, thus maybe the combination with mild heat treatments should be assayed for this purpose. Low dose PL is not an efficient technology for the decontamination of fresh-cut broccoli since it showed no effectiveness at reducing inoculated *L. innocua* or native mesophilic populations. Nonetheless PL showed better results than chlorine washing regarding the nutritional value of fresh-cut broccoli, leading to an enhanced total antioxidant capacity and preserving total phenolic and glucosinolates contents.

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CHAPTER VIII

Integration of peroxyacetic acid-assisted UV-C and biopreservation using *Pseudomonas graminis* CPA-7 for controlling *Listeria monocytogenes* and *Salmonella enterica* in ready-to-eat 'Iceberg' lettuce and baby spinach leaves

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ABSTRACT

The direct antimicrobial effect of a UV-C delivered in water (WUV) or in peroxyacetic acid (PAA) for controlling *L. monocytogenes* and *S. enterica* populations in ready-to-eat 'Iceberg lettuce' and baby spinach leaves was evaluated during chilled storage in modified atmosphere packaging (MAP). The integration of the hormetic antimicrobial effect of pretreatments with UV-C in PAA and biocontrol using *Pseudomonas graminis* CPA-7 was assessed at 5 °C and upon a breakage of the cold-storage chain. In fresh-cut lettuce, 0.1 kJ m^{-2} UV-C treatments, in water or in 40 mg L^{-1} PAA, reduced the initial populations of both pathogens by up to 2.1 ± 0.7 log_{10}, improving the efficacy of water-washing by up to 1.9 log_{10}. The growth of both pathogens remained inhibited throughout storage. In baby spinach leaves, the combination of 0.3 kJ m^{-2} UV-C and 40 mg L^{-1} PAA reduced *S. enterica* and *L. monocytogenes* populations by 1.4 ± 0.2 and 2.2 ± 0.3 log_{10} respectively, which improved water-washing by 0.8 ± 0.2 log_{10}. Pretreating lettuce with UV-C in PAA reduced *L. monocytogenes* and *S. enterica* growth by up to 0.9 ± 0.1 log_{10}, in respect of the PAA-pretreated control after 6 d at 5 °C in MAP. Upon a cold-chain breakage, CPA-7 reduced *S. enterica* growth in PAA-pretreated lettuce samples by 0.2 ± 0.1 log_{10} in respect of non-inoculated control, whereas showed no effect on *L. monocytogenes* in any of both matrices. Low-dose UV-C in PAA is a suitable preservation strategy for improving the safety of ready-to-eat leafy greens via a dual direct-hormetic effect.

8.1 INTRODUCTION

Ready-to-eat green leafy salads are growingly demanded items because they combine convenience and a wide range of nutrients and bioactive phytochemicals which are recommended for a healthy diet (Artés and Allende, 2014). Nevertheless, as they are usually consumed row, they can become vehicles for human pathogens such as *Listeria monocytogenes* and *Salmonella enterica* (Franco & Destro, 2007; Sagoo, Little, Ward, Gillespie, & Mitchell, 2003; Sagoo, Little, & Mitchell, 2003). Outbreaks caused by several strains of the mentioned pathogenic species have been associated to contaminated lettuce in the European Union and the USA in the last years (Callejón *et al.*, 2015; EFSA, 2017). Cross-contamination with foodborne pathogens may occur during pre-harvest, through contaminated soil and irrigation water or due to organic fertilizers such as manures or sewage sludge (Brandl, 2006). During postharvest processing, inappropriate sanitation of tools, facilities surfaces, and process-water may also turn them into contamination sources (Artés and Allende, 2014).

To reduce the probability for process water to become a source of contamination, sanitation of fresh produce is carried out using antimicrobial solutions, since immersion of cut surfaces increases the probabilities for the infiltration of liquid into the tissues (Gorny *et al.*, 2006). The most used chemical sanitizer in food industry is chlorine due to its strong antibacterial activity and low costs (Hua and Reckhow, 2007). However, growing public concern about health and environmental negative effects of its by-products and the advent of restrictive regulations for its use in several countries (EC-European Comission for Health and Consummer Protection, 2005), have prompted the research and development of alternative decontamination strategies in food industry.

Short-wave Ultraviolet light (UV-C) has a direct deleterious effect on microbial DNA structure which leads to the inactivation and death of most types of

microorganisms without producing harmful byproducts (Gayán, Condón and Álvarez, 2014). Therefore, UV-based technologies have been used for the decontamination of food and food-contact surfaces, including equipment, tools, packages, liquids, powders and fresh produce (Bintsis, Litopoulou-Tzanetaki and Robinson, 2000; Fine and Gervais, 2004; Manzocco, Da Pieve and Maifreni, 2011; Charles *et al.*, 2013; Ignat *et al.*, 2015). When applied at high doses UV can damage plant tissues, being counterproductive for plant products shelf-life (Kovács and Keresztes, 2002). However, at low-doses, UV-C irradiation induces plant self-protective mechanisms against potential oxidative and mutagenic damages. This leads to increased synthesis and activities of antioxidant compounds and enzymes as well as pathogenesis-related proteins (PR proteins) thereby eliciting the defense response to a subsequent pathogenic infection (hormetic effect) (Allende *et al.*, 2006; Ou *et al.*, 2016; Scott *et al.*, 2017). Therefore, the perdurability of UV-C antimicrobial effects when applied to plant tissues can be attributed to both the reduction of the multiplication capacity of irradiated surviving microorganisms and to the increase of the negative pressure exerted through the elicitation of plant resistance mechanisms (Shama, 2007; Yun *et al.*, 2013). Thus, pre-treatment of commodities would potentially improve the safety of fresh-cut products by preventing the population increase and the establishment of foodborne pathogens throughout storage in case of cross-contamination during the production workflow.

Water-assisted alternatives of UV-C application (WUV) improve the accessibility of UV light to all sides of the product and reduce the probability of product overheating compared to conventional UV-C chambers (Huang and Chen, 2014; Huang *et al.*, 2015b; Collazo, Lafarga, *et al.*, 2018). They also integrate the effect of irradiation and immersion for reducing the microbial populations on the surface of fresh produce, while decontaminating the bath solution. Additionally, in order to exploit the synergistic effect of the simultaneous action of UV and other sanitation methods, several strategies combining UV with chemical

compounds or antagonistic agents have been used for improving the inactivation of microbial populations in several commodities (Koivunen and Heinonen-Tanski, 2005; Martínez-Hernández, Artés-Hernández, Gómez, Formica, *et al.*, 2013; Ou *et al.*, 2016; Park, Kang and Song, 2018). Among oxidizing chemical sanitizers, peroxyacetic acid (PAA) is a suitable alternative because of its wide microbial range of action, its robustness against pH and temperature switches and the non-toxicity of its by-products, water and acetic acid (Alvaro *et al.*, 2009). Our work group has previously evaluated the efficacy of a water-assisted technology, alone and combined with peroxyacetic acid, for the reduction of natural microbiota and *Listeria innocua* in fresh-cut broccoli (Collazo, Lafarga, et al., 2018; Collazo *et al*., unpublished results). The results showed similar or enhanced effectiveness in respect of chlorine sanitation, depending on the UV dose and the amount and type of target microorganism.

On the other hand, biopreservation has also been combined with physical sanitation methods for attempting to reduce the incidence of fungal diseases in fresh produce during postharvest (Xu & Du, 2012; Ou et al., 2016). Those experiments have shown promising results regarding the control of pathogens' populations through the activation of the plant defense mechanisms (Ou et al., 2016). Although foodborne human pathogens are not specifically pathogenic to plants, they have developed mechanisms allowing them to survive in intermediate plant hosts. Those mechanisms include the use of virulence factors to promote the adhesion to plant tissues (Xicohtencatl-Cortes, Sánchez Chacón, Saldaña, Freer, & Girón, 2009). Some of those molecules are also involved in the subsequent colonization of the human host, e. g. flagella-associated adhesins, Type 3 secretor system and surface-exposed aggregative fimbria/curli nucleator (Barak *et al.*, 2005; Torres *et al.*, 2005; Xicohtencatl-Cortes *et al.*, 2009). Therefore, competition for space, inhibition of pathogens' adhesiveness to plant surface, and induction of plant defense response, through biopreservation could be additional mechanisms to reduce pathogens' prevalence and establishment

in plant hosts as vehicles for transmission. We have previously assessed the inhibitory effect of the preservative strain *Pseudomonas graminis* CPA-7 for the control of *S. enterica* and *L. monocytogenes* in several fresh-cut commodities (Abadias et al., 2014; Alegre, Viñas, Usall, Anguera, et al., 2013; Alegre, Viñas, Usall, Teixido, et al., 2013; Iglesias, 2017; Iglesias, Abadias, Anguera, & Viñas, 2018). This effect showed to be associated to several mechanisms including the competition for ecological niche, the activation of the plant defense mechanisms and the reduction the colonization capacities of those pathogens (Collazo, Abadías, *et al.*, 2017; Collazo, Giné-Bordonaba, *et al.*, 2018).

With all this in mind, in the present work the direct antimicrobial effect of low-dose WUV treatment for the control of the foodborne pathogens *S. enterica* and *L. monocytogenes* was evaluated in fresh-cut lettuce and baby spinach leaves during chilled MAP storage. In an attempt of further improve the efficacy of WUV we tested the combination of WUV with different doses of peroxyacetic acid for the decontamination and control of those pathogens in the mentioned matrices as well as in process solutions. Additionally, pretreatments with WUV + PAA were integrated to subsequent inoculation with *P. graminis* CPA-7 to combine the hormetic antimicrobial effect of UV-C with the antagonistic activity of CPA-7 for the control of the mentioned foodborne pathogens during MAP refrigerated storage and upon a breakage of the cold chain of storage.

8.2 MATERIALS AND METHODS

8.2.1 PLANT MATERIAL, PROCESSING AND PACKAGING

Whole wrapped 'Iceberg' lettuce (*Lactuca sativa* var. capitata) and ready-to-use bagged baby spinach leaves (*Spinacia oleracea* L.) were purchased from local retail establishments in Lleida, Spain. The external leaves and core of 'Iceberg' lettuce were discarded and the rest was cut into 3-4 cm^2 pieces, washed in

chlorinated tap water, drained, spin-dried using a manual centrifuge and kept in trays overnight in air at 5 °C until use. Baby spinach leaves were un-bagged and stored in trays overnight in air at 5 °C without any additional discarding or processing until they were submitted to subsequent treatments. After treatment (described in sections 8.2.5, 8.2.6, and 8.2.7) vegetables were drained, spin-dried and 15 g samples were packaged in thermosealed 12 x 12 cm (lettuce) or 14 x 14 cm (spinach) polypropylene bags (PP110, ILPRA Systems Espanya SL, Mataró, Spain) to achieve passive MAP conditions. The film had a gas permeability of 110 and 500 cm^3/m^2 /day/bar for O_2 of and CO_2, respectively, at 23 °C and 0 % relative humidity.

8.2.2 Microbial culture conditions and inocula preparation

A cocktail containing five *L. monocytogenes* strains: CECT 4031, ser. 1a; CECT 4032, ser. 4b; CECT 933, ser. 3a; CECT 940, ser. 4a; and Lm203/3, ser. 1/2a (Abadias *et al*, 2008), and four *S. enterica* subsp. *enterica's* strains: BAA-707, ser. Agona; BAA-709, ser. Michigan; BAA-710, ser. Montevideo; and BAA-711 ser. Gaminara, was used as pathogenic inoculum. For its preparation, 5 mL of single strain overnight cultures in tryptone soy broth (TSB) or in TSB supplemented with 6 g L^{-1} yeast extract (TSB-YE) of *S. enterica* and *L. monocytogenes,* respectively, were incubated at 37 °C, mixed and centrifuged at 9800 x g for 10 min at 10 °C. The supernatant was discarded and bacterial cell pellets were suspended in 22.5 mL of aqueous saline solution (8.5 g L^{-1} NaCl). For antagonist inoculum preparation, *P. graminis* CPA-7 (Alegre, Viñas, Usall, Teixido, *et al.*, 2013) was seeded on TSA plates and incubated at 30 °C for 48 h. Single colonies were inoculated in TSB and incubated overnight at 25 °C. Bacterial cells were harvested as previously described and suspended in sterile deionized water. All synthetic culture media, buffers and supplements were purchased from Biokar Diagnostics, Beauveais, France.

8.2.3 MICROBIAL COUNTS

For microbial viable counts, 10 g samples were homogenized in 90 mL buffered peptone water within a 400 mL whole-filter bag (Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set at 4 strokes per s for 90 s. Appropriate 10-fold solutions in saline peptone (SP, 8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) of the homogenates were plated on plate count agar (PCA), Palcam agar or xylose-lysine-desoxycholate (XLD) for the determination of total mesophilic aerobic microorganisms (MAM), *L. monocytogenes,* or *S. enterica,* respectively. PCA plates were incubated at 25 °C for 3 d and XLD and Palcam plates were incubated at 37 °C for 24 or 48 h, respectively. Viable counts of each pathogen in the process solutions after single sanitation of each vegetable were performed as previously described. Presence/absence tests of neutralized process solutions in Dey-Engley medium were also performed to corroborate the inactivation of microorganisms. All synthetic culture media, buffers and supplements were purchased from Biokar Diagnostics, Beauveais, France.

8.2.4 OVERALL QUALITY AND HEADSPACE GAS COMPOSITION ASSESSMENT

Visual assessment of overall visual quality was performed by 6 untrained panelists using a 1 to 5 hedonic scale were 1 represented that only 25 % of the sample was profitable and the rest of the categories corresponded to 50, 75, 90 and 95 % profitability, respectively. The gas headspace composition of packages was measured using a handheld gas analyzer (CheckPoint O_2/CO_2, PBI Dansensor, Denmark).

8.2.5 WUV treatment preserving overall appearance: analyses and storage

Preliminary selection of the WUV dose was based on the visual assessment of the overall quality of treated samples from day 3 to 6 of storage in MAP at 5 °C in darkness at each time of analysis. Measurements of the O_2/CO_2 composition within packages were also performed as described in Section 8.2.4. For WUV treatments, batches of processed vegetables were immersed in agitated cold tap water (at a ratio of 0.03-0.04:1 weight of product: volume of water) using a water-assisted UV-C equipment composed of a 15 L deposit containing 4 UV lamps, connected to a water circuit, a water pump, and to a pressurized air entrance (for detailed description, see Collazo et al., 2018). Several WUV doses were assayed by combining 4 UV-C lamps and different times of exposure. For 'Iceberg' lettuce, 0.1, 0.3, and 0.5 kJ m^{-2}, corresponding to 1, 3 and 5 min of exposure, respectively, were tested. For spinach, 0.2 and 0.3 kJ m^{-2} treatments, corresponding to 2 and 3 min of exposure, respectively, were tested. Before each treatment, lamps were preheated until stabilization of the irradiance. Water-washing without turning on the UV lamps was performed as a control treatment. Before and after WUV treatments, temperature was measured using an infrared thermometer (DualTemp Pro, Labprocess distribuciones, Barcelona, Spain). Irradiance was measured using a UV-sensor EasyH1, Peschl Ultraviolet, Mainz, Germany).

8.2.6 UV-C in water or in PAA for decontamination: inoculation, treatment, analyses, and storage

Upon selection of the optimal WUV dose preserving quality, the effect of UV-C delivered in PAA was for the reduction of inoculated *L. monocytogenes* and *S. enterica* populations was assessed. Processed vegetables (see section 8.2.1) were dip-inoculated for 2 min in agitation in a solution containing pathogenic

inoculum (described in section 8.2.2) at a concentration of 10^5 CFU mL^{-1} of each strain. Inoculated samples were drained, spin-dried, and stored overnight in air at 5 °C. Afterwards, samples were immersed in agitated tap water or in 40 or 80 mg L^{-1} PAA solutions (average pH 5.7 and 4.7, respectively) (average of oxidation/reduction potential 478 and 526, respectively) and submitted to 0.1 kJ m^{-2} UV-C, in the case of lettuce and to 0.2 or 0.3 kJ m^{-2} in the case of spinach (based on the prior WUV-dose selection phase), using the WUV device. As controls, sanitation with water or the PAA solutions were performed using the same equipment without turning on the UV-C lamps. After sample-draining and spin-drying, initial microbial counts were performed as described in Section 8.2.3. Then, samples were packaged (Section 8.2.1) and stored at 5 °C for 6 d. Visual overall quality, headspace gas composition of packages (Section 8.2.4), and microbial counts (Section 8.2.3) were assessed at the end of storage.

8.2.7 Integration of UV-C, PAA and biopreservation: Treatment, inoculation, analyses, and storage

The experimental setup of this stage is showed in Fig. **VIII.**1. Prior inoculation, several batches of each processed vegetable were submitted to decontamination in the WUV equipment either in agitated cold 40 mg L^{-1} PAA solution at a ratio of 0.03-0.04:1 kg of product: L of solution without turning on the UV lamps, or with a combination of 40 mg L^{-1} PAA and WUV: 0.1 kJ m^{-2} for 'Iceberg' lettuce or 0.3 kJ m^{-2} for baby spinach. After treatment, samples were stored in trays at air overnight at 5 °C, and afterwards, they were dip-inoculated for 2 min in agitation in: pathogenic inoculum containing 10^5 CFU mL^{-1} of each bacterial strain, a mixture of pathogenic + antagonist inoculum containing 10^7 CFU mL^{-1} CPA-7 and 10^5 CFU mL^{-1} of each pathogenic strain, or in antagonist inoculum containing 10^7 CFU mL^{-1} CPA-7. Samples were drained, spin-dried and packaged as described in Section 8.4.1. Then, they were stored for up to 6 d at 5 °C or for 2 d at 5 °C followed by 4 d at 10 °C, to simulate a cold-chain breakage.

Before and after each treatment, concentration, pH and redox potential of PAA wash solutions were determined and temperature irradiance were measured as explained in Section 8.2.5.1. MAM counts were performed before initial washing, and before and after sanitation with WUV + PAA or PAA treatments, as described in Section 8.2.3. Pathogens' population dynamics throughout storage was tracked by viable counts at 0, 2 and 6 d of storage at 5 °C or at 6 d upon a cold-chain breakage (Fig. **VIII.**1). The assessment of the overall quality of processed vegetables as well as the O_2/CO_2 headspace composition of bags was performed at each sampling as described point during storage in Section 8.2.4.

8.2.8 Data processing and statistical analysis

All experiments were repeated twice and included three biological replicates for treatment and sampling time. Microbiological data were calculated as colony forming units per milliliter (CFU mL^{-1}) and transformed to $\log_{10}$ CFU g^{-1} before means comparison. Statistical analyses were performed using Statistical software JMP (version 8.0.1 SAS Institute Inc., NC, USA). Categorical data were analyzed through a contingency analysis using chi-square statistic ($n=12$, $P < 0.05$). Microbiological and physical data were verified for agreement to normal distribution and homoscedasticity of residues and accordingly, means were compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).

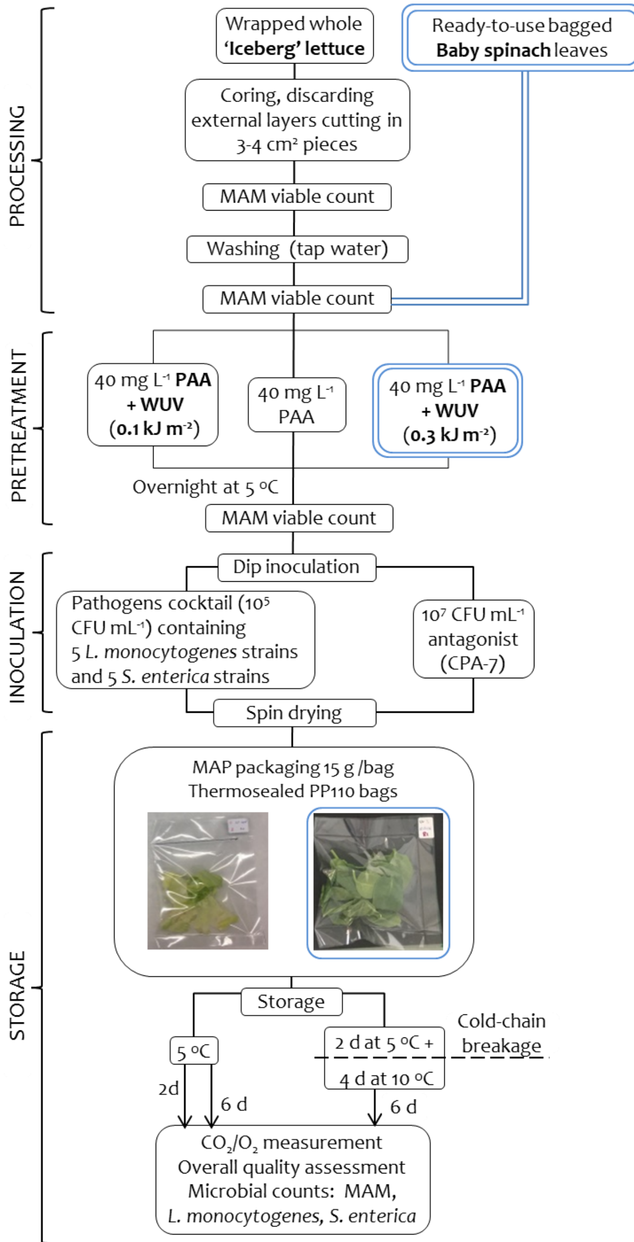

Figure VIII.1. Experimental setup of a combined preservation strategy comprising pretreatment with UV-C in peroxyacetic acid (PAA) before inoculation with the biopreservative bacterium *P. graminis* CPA-7, for controlling the populations of *S. enterica* and *L. monocytogenes*, in ready-to-eat 'Iceberg' lettuce and baby spinach leaves during refrigerated MAP storage and upon a breakage of a cold-chain of storage.

8.3 RESULTS AND DISCUSSION

8.3.1 WUV TREATMENTS PRESERVING OVERALL QUALITY

Trials testing the optimal WUV dose with less negative effects in the overall quality of fresh-cut 'Iceberg' lettuce showed that samples treated with WUV in a range of 0.1-0.3 kJ m^{-2} and stored in passive modified atmosphere, had a similar O_2/CO_2 composition (14 kPa / 6 kPa) that untreated controls until the end of storage (data not shown). However, at a higher dose (0.5 kJ m^{-2}) WUV provoked oxidative discoloration, a more marked reduction of O_2 levels (6.16 kPa) and an increase of CO_2 content (6.2 kPa) than the water-washed control (17.5 kPa O_2; 2.8 kPa CO_2) at the end of storage. In agreement with our results, increased respiration rates compared to the untreated control, positively correlated to the increase of the UV-C dose in a range of 0.4 to 8.1 kJ m^{-2} in 'Red Oak Leaf' and 'Lollo rosso' lettuces during MAP storage at 5 °C, which was attributed to physiological stress (Allende and Artes, 2003; Allende *et al.*, 2006). On the other hand, in our experiments the visual assessment of quality showed unacceptable quality of lettuce samples treated with 0.3 and 0.5 kJ m^{-2} after 6 d of MAP storage (data not shown); thus, the lowest dose (1 min of exposure, 0.1 kJ m^{-2}) was selected for the subsequent analysis. In Red Oak Leaf' lettuces, softening and browning of tissues were not detected in samples treated with UV-C at doses of 1.2 and 2.4, kJ m^{-2}, in respect of the control, whereas such negatives effects in addition to altered sensory quality were detected in samples treated with a higher dose (7.1 kJ m^{-2}) after 7 d at 5 °C (Allende *et al.*, 2006). This was associated to the production of free radicals and its deleterious effect on the cell wall(Allende <i>et al.</i>, 2006)(Allende <i>et al.</i>, 2006). The higher browning response of crisphead lettuce varieties such as 'Iceberg' to abiotic stress compared to Romaine and other varieties with less crispiness has been previously reported (Cantos, Espín and Tomás-Barberán, 2001).

As for baby spinach leaves, no differences in the overall appearance were observed among samples treated with WUV, regardless of the dose (data not shown). Gases analysis showed that treatment with WUV, regardless of the assayed dose (0.2 or 0.3 kJ m^{-2}), resulted in lower O_2 (17.6 kPa) content than the water-washed control (19.5 kPa), while no differences in the CO_2 contents (8.11 kPa) were observed among treatments. Therefore, both WUV doses were evaluated in the subsequent set of experiments. In accordance with these results, UV-C treatment of spinach leaves resulted in increased respiration rates compared to water control at doses ranging from 2.4 to 24 kJ m^{-2}, although no differences were observed among treatments (Escalona *et al.*, 2010). In that experiment, no reduction of quality was either detected for the lowest UV-C dose.

8.3.2 UV-C IN WATER OR IN PAA FOR MICROBIAL DECONTAMINATION

Upon the selection of 0.1 kJ m^{-2} as a WUV dose preserving the overall appearance of 'Iceberg' lettuce throughout storage, treatments with this dose applied in water or in PAA solutions were tested for microbial decontamination. Initial counts of *L. monocytogenes* and *S. enterica* in 'Iceberg' lettuce were 3.8 ± 0.1 and 4.0 ± 0.1 log_{10} CFU mL^{-1}, respectively. WUV treatment was effective for reducing *L. monocytogenes* initial levels by 2.1 ± 0.7 log_{10} in respect of inoculated populations, improving the efficacy of water washing by 1.9 log_{10}. The inhibition of *L. monocytogenes* growth was maintained throughout refrigerated MAP storage, showing no population increase in respect of initial levels (Fig. **VIII.**2A). Combining 0.1 kJ m^{-2} UV-C and 40 or 80 mg L^{-1} PAA did not enhance the initial reduction in respect of WUV or PAA alone but improved the effectiveness at inhibiting *L. monocytogenes'* growth during storage at 5 °C in respect of the PAA control treatment.

Similar initial reductions were obtained for *S. enterica* populations (by 2.0 ± 0.6 log_{10}) using 0.1 kJ m^{-2} WUV (Fig. **VIII.**2B), which improved water-washing efficacy by 1.7 log_{10}. Treatment with UV-C in PAA showed the same efficacy as WUV regardless of the PAA dose, maintaining *S. enterica* population 1.9 ± 0.7 log_{10} below the inoculated levels until the end of storage. Final populations reached 3 ± 0.7 log_{10} below those present in the water-washed samples. In agreement with our results, the sequential application of UV-C at fluencies > 1.5 kJ m^{-2} for the sanitation of *S. enterica* ser. Typhimurium in 'Iceberg' lettuce achieved > 2 log_{10} reduction of the internalized bacteria whereas no synergistic effect was observed when combining UV-C with PAA (80 mg L^{-1}) compared to UV-C alone (Ge, Bohrerova and Lee, 2013).

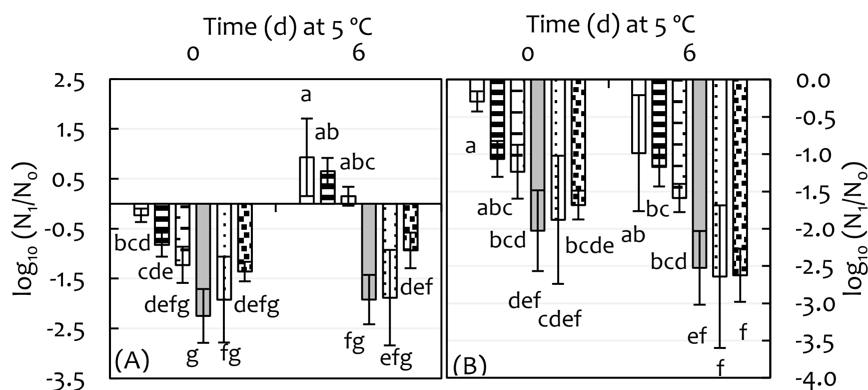

Figure **VIII**. 2. Selection of the optimal WUV + PAA combination for the decontamination of (A) *L. monocytogenes* and (B) *S. enterica* subsp. *enterica* in fresh-cut 'Iceberg' lettuce: Columns are means of the logarithmic reductions (log_{10} (N_1/N_0) of treated samples (N_1) in relation to initial inoculated non-treated samples (N_0): (☐) water control, (▤) 40 mg L^{-1} PAA: PAA40, (▤) 80 mg L^{-1} PAA: PAA80, (■) 0.1 kJ m^{-2} WUV, (▦) 0.1 kJ m^{-2} WUV + PAA40, (▩) UV-C + PAA80, and error bars are standard deviations (n=6). Different letters represent significant differences at each sampling time according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$).

In baby spinach leaves, processing with WUV delivered in water even at a higher dose (0.2 kJ m^{-2}) than that applied to lettuce, was not effective for reducing initial populations of any of the pathogens (4.3 ± 0.2 log_{10} CFU g^{-1}) compared to

water washing (Fig **VIII.**3 A and C). This limitation was overcome by substituting water by peroxyacetic acid solutions in the immersion deposit. The combination of 0.2 kJ m^{-2} UV-C and 40 mg L^{-1} PAA reduced *S. enterica* and *L. monocytogenes* initial populations by up to 0.9 ± 0.1 and 2 ± 0.1 log_{10}, respectively, which in the latter case was significantly better than the WUV treatment alone. Processing with 0.2 kJ m^{-2}, in water or in PAA, was ineffective for inhibiting *L. monocytogenes* throughout storage. Contrastingly, treatment with 0.2 KJ m^{-2} and 80 mg L^{-1} kept *S. enterica* populations 1.4 ± 0.2 log_{10} below the inoculated levels, which was significantly better than water-washing.

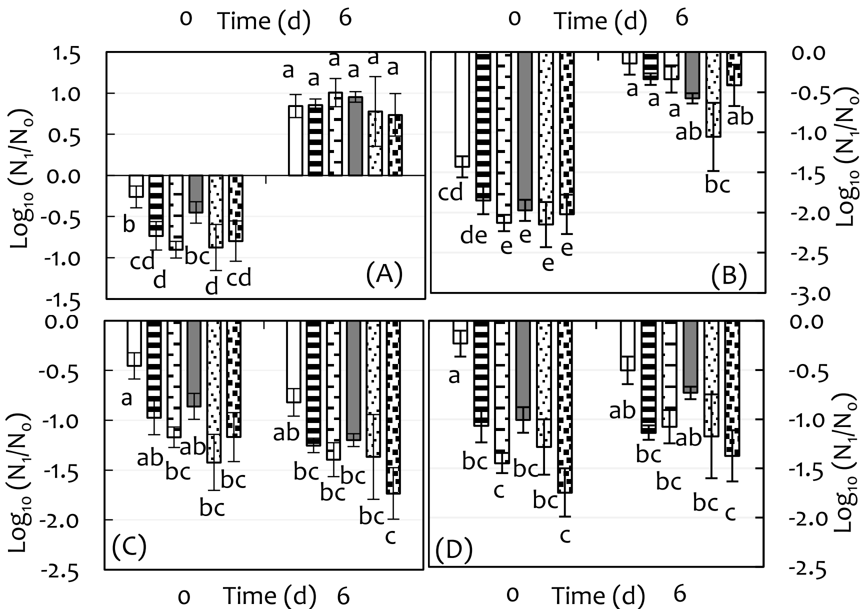

Figure **VIII**. 3. Selection of the optimal UV-C and PAA combination for the decontamination of (A and B) *L. monocytogenes* and (C and D) *S. enterica* in baby spinach leaves: Columns represent means of the logarithmic reductions in sanitized samples (N_1): ( ) water control, ( ) 40 mg L^{-1}PAA control, ( ) 80 mg L^{-1} PAA control, ( ) WUV-treated: 0.2 kJ m^{-2} in A and C or 0.3 kJ m^{-2} in B and D, ( ) 40 mg L^{-1}PAA + 0.2 kJ m^{-2} UV-C in A and C or 0.3 kJ m^{-2} UV-C in B and D, ( ) 80 mg L^{-1} PAA + 0.2 kJ m^{-2} UV-C in A and C) or 0.3 kJ m^{-2} UV-C in B and D, in relation to inoculated non-sanitized samples (N_0). Error bars represent standard deviations (n=6). Different letters represent significant differences among treatments at each sampling time according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$).

Increasing the dose from 0.2 to 0.3 KJ m^{-2} did not improve the efficacy of WUV at lowering initial *S. enterica* levels (0.9 ± 0.2 log_{10} reduction) but it enhanced the inactivation of *L. monocytogenes* to 2.0 ± 0.1 log_{10} (Fig. **VIII.**3 B). However, no further improvement was obtained by combining this dose of UV-C with PAA. The most effective treatment for the decontamination of baby spinach leaves was the combination of 0.3 kJ m^{-2} UV-C with 40 mg L^{-1} PAA which achieved initial reductions of *S. enterica* by 0.7 ± 0.2 log_{10} better than water washing and inhibited *L. monocytogenes* populations during storage at 5 °C keeping them 1.1 ± 0.4 log_{10} below the inoculated levels. Similarly, germicide effect was observed initially in spinach leaves treated with 2.4 kJ m^{-2} UV-C in a conventional chamber, and the inhibitory effect was maintained throughout 12 d of storage refrigerated storage (Escalona *et al.*, 2010).

The synergistic effect of integrated physic-chemical strategies involving UV-C irradiation for the decontamination of inoculated pathogens or native microbiota on fresh produce has also shown to be dose-dependent but also related to the way of treatment application, the food matrix, the target microorganism and the inoculation method (Fan, Huang and Chen, 2017). In white asparagus, treatment with 1 kJ m^{-2} UV-C showed no effect on indigenous MAM neither did it when combined with 3 or 4.5 mg L^{-1} of ozone (Hassenberg, Huyskens-Keil and Herppich, 2012). In contrast, UV-C irradiation (1.2 kJ m^{-2}) after washing in cold (4 °C) and warm (45 °C) water reduced native MAM in fresh-cut endive by 1.6 and 2.1 log_{10}, respectively, with minimal physiological stress (Hägele *et al.*, 2016). The germicidal effect of UV-C against *S. enterica* or *E. coli* superficial populations on blueberries was not improved after immersion in a water-assisted UV device containing hydrogen peroxide, sodium dodecyl sulfate, levulinic acid, or chlorine at room temperature compared to the water control (Liu, Huang and Chen, 2015). Similarly, pre-washing fresh-cut broccoli with 100 mg L^{-1} PAA before 7.5 kJ m^{-2} dry-UV-C irradiation showed no synergistic effect compared to the single treatments at reducing *S. enterica* or *E. coli* populations

throughout 7 d of MAP storage at 5 °C (Martínez-Hernández et al., 2015). However, spraying 1.5 % H_2O_2 at 50 °C on 'Iceberg' lettuce during the continuous application of dry-UV (0.38 kJ m^{-2}) enhanced *S. enterica* inactivation compared to the application of H_2O_2 or UV alone (Hadjok, Mittal and Warriner, 2008).

In the present work, although the combination of UV-C with PAA did not enhance the efficacy of the chemical treatments alone it inactivated both pathogens in the process solutions showing no viable cells (< 5 CFU mL^{-1}) after single-use sanitation of inoculated spinach or 'Iceberg' lettuce. This enabled solutions for reutilization, improving the efficacy of the decontamination step and lowering the production costs. In according with our results, the decontamination with 0.1 kJ m^{-2} UV-C of the process water resulting from lamb's lettuce washing, achieved > 5 log_{10} (below a detection limit of 10 CFU mL^{-1}) of both *L. monocytogenes* and *S. enterica,* when inoculated at 10^6-10^7 CFU mL^{-1} (Ignat *et al.*, 2015). In a previous work, a significant improvement of UV efficacy for the reduction of viable MAM cells in the process water of fresh-cut endive, was obtained with sequential sanitation with cold (4 °C) or warm (45 °C) water after pre-treatment with 1.2 kJ m^{-2} (Hägele *et al.*, 2016). The combination of O_3 with UV-C have also improved the efficacy of the UV-treatment alone at reducing the MAM levels in process water by up to 3 log_{10}, after sanitation of fresh-cut escarole and onion for 20 min (Selma *et al.*, 2008).

8.3.3 Integration of UV-C, PAA and biopreservation

Pretreatment with UV-C in PAA and subsequent inoculation with the biopreservative bacterium CPA-7 was hypothesized to synergistically act through direct antagonistic activity and indirect mechanisms involving the activation of the plant defense response by both UV-C and the biocontrol agent, thereby controlling pathogens and MAM populations throughout storage (reviewed by Urban, Charles, de Miranda, & Aarrouf, 2016). Results showed that initial

populations of MAM in lettuce and baby spinach leaves were 4.9 ± 0.2 and 6.9 ± 0.4 log_{10} CFU g^{-1}, respectively. Pretreating fresh-cut 'Iceberg' lettuce with UV-C in PAA resulted in a similar reduction of initial populations of MAM to that obtained after PAA sanitation (by 1.3 ± 0.2 log_{10}). As observed for lettuce, UV-C in PAA reduced initial MAM populations in baby spinach leaves with similar efficacy to that of PAA sanitation. However, reductions were less to half those observed for lettuce (by 0.5 ± 0.2 log_{10}), which showed that the efficacy of sanitation methods is conditioned by microbial population levels. No inhibition of MAM observed throughout storage for 6 d at 5 °C in lettuce or spinach samples pretreated with UV-C in PAA, reaching populations of 6.9 ± 0.4 and 7.7 ± 0.3 log_{10} CFU g^{-1}, respectively. Upon a breakage of the cold chain of storage, no differences in final MAM populations were either detected among samples of lettuce or spinach leaves (7.6 ± 0.3 and 8.8 ± 0.1 log_{10} CFU $g^{-1,}$ respectively) pretreated with any of the tested WUV + PAA combinations. Dose-dependent UV-C effect on native microbiota and its synergistic improvement by other chemical and physical factors seem to be largely linked to the food matrix. UV-C treatments at higher doses than those used in the present study have achieved significant reductions of MAM in other lettuce varieties, i.e. by > 1 log10 using 4.06 or 8.14 kJ m^{-2} in 'Lollo Rosso' lettuce and by 0.5-2 log_{10} using 0.8 to 8.14 kJ m^{-2} in 'Red Oak Leaf' lettuce (Allende & Artes, 2003; Allende & Artés, 2003). Similarly, UV-C irradiation of spinach leaves using doses ranging from 4.54 to 11.35 kJ m^{-2} has shown to reduce mesophilic microorganisms from 0.5 to 1 log_{10} (Artés-Hernández, Escalona, Robles, Martínez-Hernández, & Artés, 2009). However, in agreement with our results, no inhibitory effect throughout time was either observed in those experiments, compared to chlorine sanitation.

On the other hand, CPA-7 populations in both matrices were similar at the beginning of the experiment (5.9 log_{10} CFU g^{-1}) as well as at the end of storage at 5 °C (6.2 CFU g^{-1}) and upon a cold-chain breakage (6.8 log_{10} CFU g^{-1}), regardless of the pretreatment with UV-C in PAA. No differences among treatments were

detected regarding the O_2/CO_2 contents in the packages headspace at the end of refrigerated storage regardless of the inoculation with the antagonist, in any of the analyzed matrices. Final gases contents in baby spinach leaves were 19.8 kPa O_2/11.1 kPa CO_2 regardless of breakage of the cold-chain. In 'Iceberg' lettuce the CO_2 content was lower at the end of storage at 5 °C (11.0 kPa) than upon a breakage of the cold-chain (16.9 kPa), while the O_2 content was 19.5 kPa, regardless of the storage conditions. In both matrices the overall quality of samples inoculated with both pathogens and the antagonist was unacceptable at the end of storage and it was considered irrelevant because of the high initial microbial populations that were inoculated in order to obtain detectable levels using the viable count method.

Regarding the effect on inoculated pathogens, results showed no reduction in *L. monocytogenes* inoculated populations in fresh-cut lettuce during the first day of storage, regardless of the applied treatment (Fig. **VIII.**4 A). However, after 2 d of storage at 5 °C, *L. monocytogenes* populations were reduced by 0.2 ± 0.1 and 0.6 ± 0.2 $\log_{10}$ in samples treated with UV-C in PAA or with UV-C in PAA and then inoculated with CPA-7, respectively, compared to the 40 mg L^{-1} PAA-washed control. At day 6 of storage, *L. monocytogenes* populations were reduced by 0.8 ± 0.2 $\log_{10}$ in samples treated either with with UV-C in PAA or with UV-C in PAA and then inoculated with CPA-7 in respect of the PAA-sanitized ones. However, unlike previous reports in several commodities, we observed no synergistic improvement for the triple combination in respect of the double one. Previous reports have showed the enhancement of the UV-C effect regarding the activation of defense response to fungal diseases in several fruits. For instance, the incidence and severity of blue and gray mold, caused by *Penicillium expansum* and *Botrytis cinerea*, respectively, in pear fruits throughout storage at 20 °C for 15 d was reduced upon dip-inoculation with the antagonistic yeast *Candida guilliermondii* (5 x 10^7 CFU mL^{-1}) after 5 kJ m^{-2} UV-C pretreatment (Xu and Du, 2012). The integrated application of 4 kJ m^{-2} UV- C and *Candida tropicalis*

antagonistic yeast to pineapple maintained better the firmness and increased the resistance to the phytopathogenic fungus *Chalara paradoxa* (Ou *et al.*, 2016). Those effects correlated with lower activities of cell-wall degrading enzymes (pectin methylesterase, polygalacturonase, and cellulase) and with the enhancement of both non-enzymatic (total phenolic content via PAL activation) and enzymatic antioxidant mechanisms (catalase, superoxide dismutase and peroxidase) as well as the increase of PR protein activities (β-1,3-glucanase and CHT) in several fruits (El Ghaouth, Wilson and Callahan, 2003; Pombo *et al.*, 2011; Ou *et al.*, 2016). However, there is still a lack of information about green leaves response to plant or human pathogens.

On the other hand, with UV-C in PAA pretreatment did not show a significant reducing effect on *L. monocytogenes* in 'Iceberg' lettuce upon a cold-chain breakage. In such conditions, CPA-7 antagonistic activity against *L. monocytogenes* was highly variable among independent repetitions of the experiment and therefore, it was not statistically significant.

Regarding *S. enterica*, no differences were observed between the populations in samples treated with UV-C in PAA or with UV-C in PAA and then inoculated with CPA-7 during the first 2 d of storage. At day 6, a reduction by 0.5 ± 0.3 log_{10} was observed in WUV+PAA-treated samples in respect of the PAA (40 mg L^{-1})-sanitized control (Fig. **VIII.**4 B). In contrast to that observed for *L. monocytogenes*, at day 6 of storage upon a cold-chain breakage, CPA-7 was able to reduce *S. enterica* populations by 0.9 ± 0.1 log_{10} in respect of the non-inoculated control in 'Iceberg' lettuce samples pre-treated with 40 mg L^{-1} PAA, which would contribute to maintained the safety of the product in case of that event.

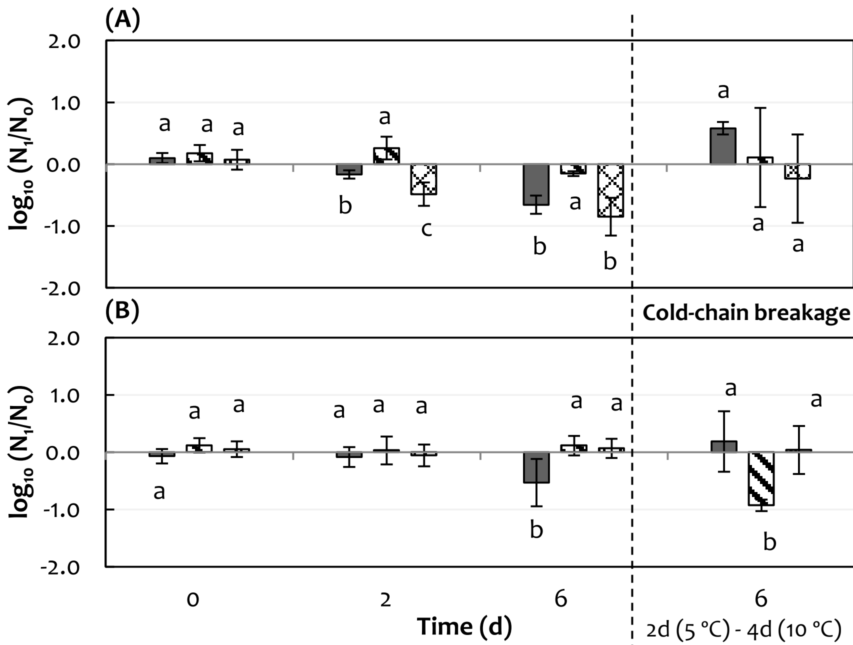

Figure **VIII.** 4. Logarithmic reductions of (A) *L. monocytogenes* and (B) *S. enterica* subsp. *enterica* populations in 'Iceberg' lettuce-treated samples (N_1) using the combination of 0.1 kJ m^{-2} WUV + 40 mg L^{-1} PAA (■), 40 mg L^{-1} PAA + CPA-7 (▧) or 0.1 kJ m^{-2} WUV + 40 mg L^{-1} PAA + CPA-7 (▩) in respect of the 40 mg L^{-1} PAA-washed control (N_0). Columns represent means and and error bars represent standard deviations (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$).

When testing pretreatment with UV-C (0.3 kJ m^{-2}) in PAA (40 mg L^{-1}) and sequential pretreatment with UV-C in PAA and then inoculation with CPA-7 in baby spinach leaves, no differences among treatments were observed until day 6 of refrigerated storage. At that day, samples pre-treated with UV-C in PAA showed a 0.2 ± 0.1 log_{10} reduction of *L. monocytogenes* populations in respect of PAA-pretreated control. At the same time of analysis, the sequential application of UV-C in PAA and then inoculation with CPA-7 inhibited *L. monocytogenes* growth by 0.4 ± 0.1 log_{10} in respect of the non-inoculated PAA-pretreated control (Fig. **VIII.**5 A).

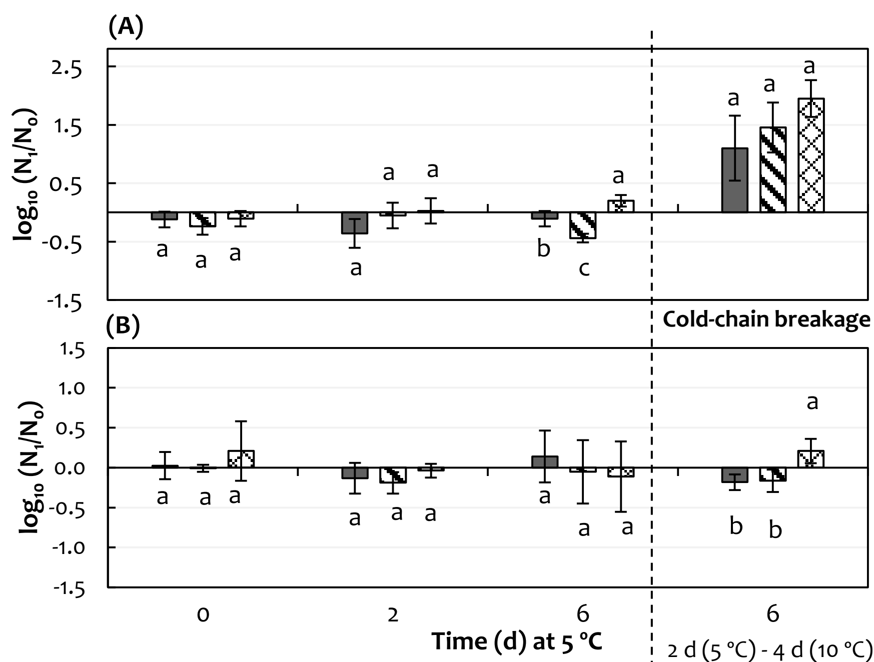

Figure **VIII**. 5. Logarithmic reductions of (**A**) *L. monocytogenes and* (**B**) *S. enterica* subsp. *enterica* in baby spinach leaves upon sanitation using the combination of 0.3 kJm^{-2} WUV + 40 mg L^{-1} PAA (▇), 40 mg L^{-1} PAA + CPA-7 (▧) or 0.3 kJ m^{-2} WUV + 40 mg L^{-1} PAA + CPA-7 (▩) (N_1) in respect of the 40 mg L^{-1} PAA-washed control (N_0). Columns represent means and and error bars represent standard deviations (n=6). Different letters represent significant differences according to analysis of variances (ANOVA) and Tukey's test ($p < 0.05$).

No significant differences in *L. monocytogenes* growth were observed upon a cold-chain breakage whether the biopreservative agent was present or not. Similarly, any of the evaluated treatments controlled *S. enterica* growth during the 6 d of storage at 5 °C (Fig. **VIII.**5 B). However, *S. enterica* growth was inhibited by 0.2 ± 0.1 $\log_{10}$ upon a breakage of the cold chain of storage in UV-C in PAA-pretreated samples compared to the PAA-pretreated control, regardless of the presence of the antagonist, which contrasted with the observed for *L. monocytogenes* in this food matrix. As observed for lettuce, no synergistic effect of the combination of pretreatment with UV-C in PAA and then inoculation with CPA-7 was observed in spinach leaves throughout storage at 5 °C or upon a

breakage of the cold-chain of storage compared to the CPA+PAA and the WUV+PAA double combinations.

8.4 CONCLUSIONS

WUV at doses ranging from 0.1 to 0.3 kJ m^{-2} allowed effective decontamination and growth inhibition of *S. enterica* and *L. monocytogenes* both in fresh-cut 'Iceberg' lettuce and in baby spinach leaves without significantly impacting respiration or overall quality. Delivering UV-C in PAA showed no synergistic reduction of pathogens' populations in respect of the individual control treatments, but it completely inactivated both pathogens in the solutions after a single-sanitation of the tested vegetables, improving the effectiveness of the chemical treatment alone. Pretreatment with UV-C in PAA and subsequent inoculation with CPA-7 synergistially inhibited *L. monocytogenes* growth after 2 d of refrigerated storage in 'Iceberg' lettuce but this effect was inconsistent in the rest of the conditions tested. In samples pretreated with PAA, the antagonistic activity of CPA-7 inhibited the growth of *L. monocytogenes* after 6 d of refrigerated MAP storage in baby spinach leaves and of *S. enterica* in 'Iceberg' lettuce upon a cold-chain breakage. This could contribute to the maintenance of safety in case of cross-contamination after sanitation and in a worse-case scenario including also a breakage of refrigerated storage. Low-dose WUV combined with PAA could be a suitable preservation strategy for improving the safety of ready-to-eat leafy greens.

ACKNOWLEDGEMENTS

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CHAPTER IX

OVERALL DISCUSSION

The lack of a single lethal step in the production workflow of fresh-cut industry entails the implementation of hurdle-technologies which comprises the disinfection of processed commodities as one of the most critical steps to guarantee the microbiological and physicochemical quality of final products. The advent of prohibitive and restrictive regulations to the use of chlorine as a disinfection method and the need for healthier and ecofriendly alternatives, motivated the present work.

9.1 BIOLOGICAL PRESERVATION STRATEGIES

UNRAVELING THE ACTIONS MECHANISMS OF BIOPRESERVATION USING THE BACTERIUM *P. GRAMINIS* CPA-7

The biopreservation of fresh-cut fruit using epiphytic biocontrol agents CPA-7 is an alternative that has been previously explored by our work group on several commodities (Alegre, 2012; Alegre, Viñas, Usall, Anguera, *et al.*, 2013; Alegre, Viñas, Usall, Teixido, *et al.*, 2013; Abadias *et al.*, 2014; Iglesias, 2017; Iglesias *et al.*, 2018). Those studies showed a differential effectiveness of CPA-7 antagonistic activity according to packaging and storage conditions, the addition of preservative agents, the target foodborne pathogen, and the commodity (Table **IX**.1).

Table IX.1. Effect of CPA-7 on *S. enterica* and *L. monocytogenes* growth on different fresh-cut fruit during storage at 10 °C in air, as obtained in previous experiments.

Fruit	Cultivar	$log_{10}(N_1/N_0)$		pH	SSC (%)	TA (g L-1)	Time (d)	Reference
		Lm	*Se*					
apple	Golden delicious	3.8	3.0	4.0	11.4	2.5 m	7	Alegre, *et al.*, 2013)
	Fuji	2.0	2.7	4.4	12.4	1.6 m	5	Iglesias, 2017
	Pink lady	2.0	2.7	3.6	15.4	4.4 m	5	Iglesias, 2017
pear	Conference	3.0	2.5	4.9	15.1	1.6 m	6	Iglesias *et al.*, 2018
mandarin	Ortanique	1.8	2.3	4.3	12.1	3.8 c	5	Iglesias, 2017
nectarine	Big Top	3.0	3.4	3.8	11.8	4.1 m	5	Iglesias, 2017
watermelon		2.0	2.0	5.5	11.5	1.2 c	5	Iglesias, 2017
melon	Piel de sapo	4.9	4.5	6.4	10.6	1.1 c	9	Abadias *et al.*, 2014
	Galia	1.3	0	6.4	10.4	1.0 c	5	Iglesias, 2017
	Cantaloupe	0	0	6.1	13.0	1.0 c	5	Iglesias, 2017
orange	Lane late	0	0	3.6	15.0	8.3 c	5	Iglesias, 2017
strawberry	Splendour	0	0	3.5	13.8	2.2 c	5	Iglesias, 2017
pineapple	Gold	0	0	3.5	13.8	6.0 c	5	Iglesias, 2017
kiwi	Haywar	0	0	4.4	12.5	9.4 c	5	Iglesias, 2017
mango	Kent	0	0	4.3	16.7	2.8 c	5	Iglesias, 2017

Titratable acidity TA: m: malic acid and c: citric acid. **SSC:** soluble solids content (%). **Pathogens:** *L. monocytogenes* (***Lm***), *S. enterica* (***Se***). **N_1:** final count of pathogen control treatment and **N_0:** final count of pathogen + CPA-7 co-inoculated treatment

As another step in the registration process of CPA-7 as a biopreservative strain, elucidating its mode of action to exert antagonistic activity against foodborne pathogens on fresh-cut commodities was our first goal. For that, we evaluated several action mechanisms including those that act directly on the pathogens affecting their growth and activity and those that act indirectly on them through the activation of the plant host defense response (Fig. **IX**.1).

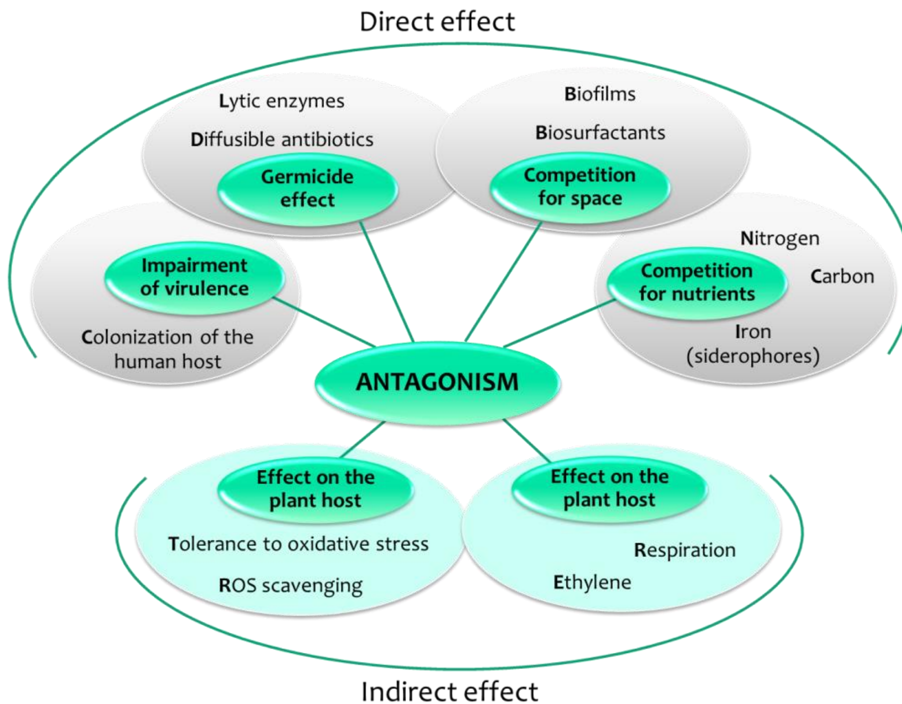

Figure IX.1. Putative action mechanisms evaluated in the present work.

Restriction of competitors' growth and activity: a multisided approach

To prevail in natural environments, where no microorganism is alone, each individual must deploy a series of mechanisms aimed at hampering the growth of the competing microbiota. Those mechanisms include depriving competitors from essential and limited resources such as nutrients and space -through rapid growth and enhanced adherence, efficient metabolic activity or secretion of specialized nutrient-sequestering molecules-, and altering the microenvironment -through the secretion of harmful metabolic wastes or toxins, or by misleading intercellular communication (Fig. **IX**.1) (Harrison *et al.*, 2008; Jordan *et al.*, 2014).

Previous experiments testing the antimicrobial effect of the secondary metabolites produced by CPA-7 during overnight growth in TSB, showed no lethal activity against *E. coli* O157:H7, *S. enterica*, *L. innocua*, *L. monocytogenes* CECT-4031, *A. hydrophila* ATCC 7966 or *P. marginalis* CECT-229 on nutrient agar (Alegre, Viñas, Usall, Teixido, *et al.*, 2013). However, as a part of our research we extended the analysis to the metabolites produced by CPA-7 in different growth conditions including: i) rich synthetic liquid medium (TSB, ph 7.2), ii) rich complex liquid medium (Prostar, pH 7; containing 1% molasses, 0.5% yeast extract, 1% Prostar protein (Brenntag Química, Granollers, Spain), iii) minimal salts liquid medium (M63, pH 6.5; supplemented with 1 % galacturonic acid or with glucose (0.2 % or 1 %)), iv) solid medium (meat extract-glucose-agar, MGA) 'Piel de sapo' melon juice and vi) melon pieces from varieties where this strain had shown differential effectiveness. The incubation period was extended to up to the stationary phase at optimal growth temperature (up to 72 h at 25 °C), based on reports showing that antibiotics production by Pseudomonads is enhanced at pH close to neutrality, temperatures in the optimal growth range (20-25 °C) and incubation periods of up to 5 d (Duffy and Défago, 1999; Park *et al.*, 2011). Glucose and soy peptone has previously been chosen as the optimal carbon and nitrogen sources, respectively, for the *in vitro* production of phenazine-1-carboxilic acid by *Pseudomonas* sp. M18G (He, Xu and Zhang, 2008). Furthermore, it has been demonstrated that the production of specific antibiotics by several *Pseudomonas* spp. is regulated in a strain-dependent manner by the presence and amount of certain carbon sources and minerals (Duffy and Défago, 1999). For example, DAPG production was induced by Fe^{3+} and sucrose in *P. fluorescens* F113, and by glucose in *P. fluorescens* Pf-5 and CHA0 (Duffy and Défago, 1999; Siddiqui and Shaukat, 2003). Glucose and glycerol as well as micronutrients such as Zn^{2+}, Mo^{2+}, and Co^{2+} also stimulated the production of DAPG, pyochelin, and its precursor SA, while repressed pyoluteorin in *P. fluorescens* CHA0 (Duffy and Défago, 1999). Similarly, glucose

enhanced phenazine production while suppressed pyrrolnitrin in *P. chlororaphis* O6 (Park *et al.*, 2011).

In contrast with those reports, the addition of the CFS obtained from cultures of CPA-7 in minimal or rich liquid media, to solid medium inoculated with *E. coli* H157:H7, *S. enterica* or *L. monocytogenes,* showed no detectable lethal activity against those pathogens. In the same experiment, no diffusible antimicrobial metabolites were produced by CPA-7 cells on that solid medium during 3 d of contact with the mentioned pathogens at 30 °C. This result contrasted with the inhibitory effect of the diffusible metabolites produced by the strain *P. graminis* 49M on the bacterial plant pathogen *Erwinia amylovora* in King's B plates after 48 h of contact at 26 °C (Mikiciński, Sobiczewski, Puławska and Malusa, 2016). In that study, the production of antimicrobials was related to the composition growth medium, since negligible antagonistic effects were observed on nutrient agar sucrose and on nutrient agar glycerol compared to king's B medium.

In view of the lack of lethal effect on solid medium, the inhibitory effect of the metabolites produced by CPA-7 during the growth in several liquid media and in melon juice until the stationary phase on *L. monocytogenes* and *S. enterica* growth was evaluated. For that, those pathogens where inoculated in the CFS from the mentioned media and their population dynamics was tracked during incubation for 48 h at 25 °C or for 5 d at 5 or 10 °C.

Prior to the evaluation of antagonism in melon juice, viability tests were performed in 'Cantaloupe', 'Galia' and 'Piel de sapo' melon juices in order to determine the optimal dilution for the antagonist growth as well as the moment of the onset of the stationary phase in each matrix. We observed that all the tested dilutions of each melon juice enabled similar CPA-7 growth and that the stationary phase was attained at 24 h of incubation at optimal growth temperature (Fig. **IX**.2).

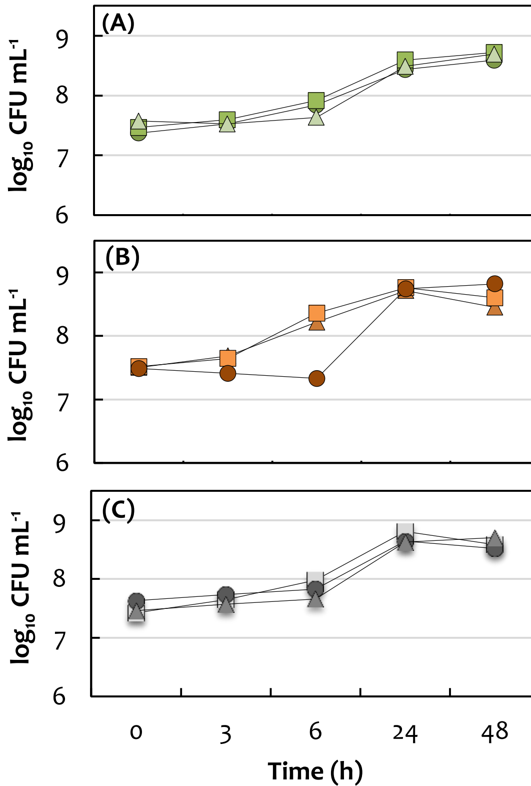

Figure IX.2. Viability of CPA-7 in several aqueous melon juice solutions (V of juice: V of water): (■) 1:2, (▲) 1:1, (●) 1:0 of (A) 'Galia', (B) 'Cantaloupe' and (C) 'Piel de sapo' cultivars, during storage at 25 °C in air.

Therefore, non-diluted juices were selected as culture media based on previous experiments showing that higher concentration of sugars in fruit juices promoted an increase in their consumption rate by certain bacterial antagonists (Guinebretiere, Morrison and Reich, 2000). 'Piel de sapo' melon juice was chosen due to its availability in the market, and 24 h CPA-7 incubated at 25 °C were used to obtain CFS.

In general, the metabolites produced by CPA-7 in the assayed liquid synthetic media or in 'Piel de sapo' melon juice did not inhibited the growth of any of the evaluated pathogens in the CFS neither after 48 h at 25 ° C nor after 5 d at 5 or at 10 °C, suggesting that CPA-7 was unable to produce antimicrobial compounds in such conditions. Moreover, the antagonistic activity of CPA-7 cells against *L.*

monocytogenes and *S. enterica* in melon juice was hindered, although both the antagonistic and the pathogenic bacteria were able to grow in that fruit matrix in the storage conditions tested. In contrast with these results, other researchers observed that certain bacterial antagonists were able to inhibit fungal germination and growth in fruit juices, which was not associated to the production of antimicrobial compounds but to an efficient use of sugars (up to 3-fold, compared to non-antagonistic strains) (Guinebretiere, Morrison and Reich, 2000).

Afterwards, the inhibitory effect of the CFS obtained from *in vitro* cultures of CPA-7 on the *in vivo* growth of *L. monocytogenes,* was tested on melon plugs from three varieties: 'Galia', 'Cantaloupe' and 'Piel de sapo', where CPA-7 had shown differential antagonistic activity (Table **IX**.1). The CFS obtained from 24 or 48 h cultures of CPA-7 in TSB did not inhibit *L. monocytogenes* growth on melon plugs of any the above mentioned varieties, in any of the storage conditions tested. In contrast, a similar recent work showed direct inhibitory activity of the CFS obtained from 3 d-cultures of *Pseudomonas* spp. QBA5 in a soy tryptone-containing medium on the conidia germination and mycelial growth of *B. cinerea* on tomato detached leaves and fruits (Gao *et al.*, 2018). In that work, the inhibitory effect was correlated to the concentration of the CFS and was therefore attributed to secreted antibiotics in the culture medium.

We found that when growth in co-culture on melon pieces from different varieties the antagonistic activity of CPA-7 was differential, in agreement with previous studies (Iglesias, 2017), cells against *L. monocytogenes*. After 48 h of co-incubation at 25 °C, CPA-7 reduced *L. monocytogenes* populations by 3.8 in 'Galia' and by 1.8 in 'Cantaloupe' melons, respectively, while no effect was observed in 'Piel de sapo' melon. At 5 °C, the inhibitory effect was highly variable among repetitions of the experiment, leading to not significant differences in respect of the control treatment for any the three melon cultivars tested. In a previous work testing CPA-7 antagonistic activity in the latter cultivar at 5 °C, the

ineffectiveness for controlling *L. monocytogenes* growth was also observed (Abadias *et al.*, 2014). Controversial results within repetitions of the experiment and with previous reports performed using the same varieties may be explained by differences in the maturity stage of the melon batches used which influence the chemical composition and the structure of the matrix and by the influence of growth temperature in microbial metabolic activity. For example, a study of sugars composition in 'Piel de sapo' melon during ripening showed significantly variations throughout this process: at early ripen stage (26 d after fruit set, 9.1 °Brix) glucose and fructose were in higher amounts (23 g kg^{-1}) than sucrose (15.9 g kg^{-1}) while at moderately ripen stage (35 d after fruit set, 11,9 °Brix), sucrose increased by 5-fold (82.3 g kg^{-1}) while glucose and fructose decrease almost to a half (15.1 and 14.2 g kg^{-1}, respectively) (Villanueva *et al.*, 2004). The inability of CPA-7 for using sucrose as a carbon source (as presented in chapter 2) could partially explain the reduced competitive effectiveness against foodborne pathogens in this matrix.

As results were most consistent in 'Galia' melon, CPA-7 CFS were obtained from fresh-cut pieces of this cultivar after 24 and 48 h of growth at optimal temperature and tested against both *L. monocytogenes* and *S. enterica* on fresh 'Galia' and 'Piel de sapo' melon plugs. In line with the results obtained in *in vitro* experiments, no inhibitory activity of the pathogens by any of the evaluated CFS was observed after incubation in air for 48 h at 25 °C or for 5 d at 5 °C. Control treatments co-inoculated with the pathogen and CPA-7 cells did reveal pathogen's growth inhibition in both storage conditions. CPA-7 genome analysis (Annex 1) showed a lack of genes encoding for several antibiotics present in fluorescent Pseudomonads including *phzA, C, D* (phenazines), *prnD* (pyrrolnitrin), *pltB, C, D* (pyoluteorin), and *phlA, C, B, D, E* (DPAG) (Collazo et al., unpublished results). These results together with those obtained in the many activity tests performed under several growth conditions, both *in vitro* and *in vivo* (Fig. **IX**.3)

at several incubation temperatures (5, 25, 30 °C), suggested that the production of antibiotics is highly unlikely as a mode of action for the antagonism of CPA-7.

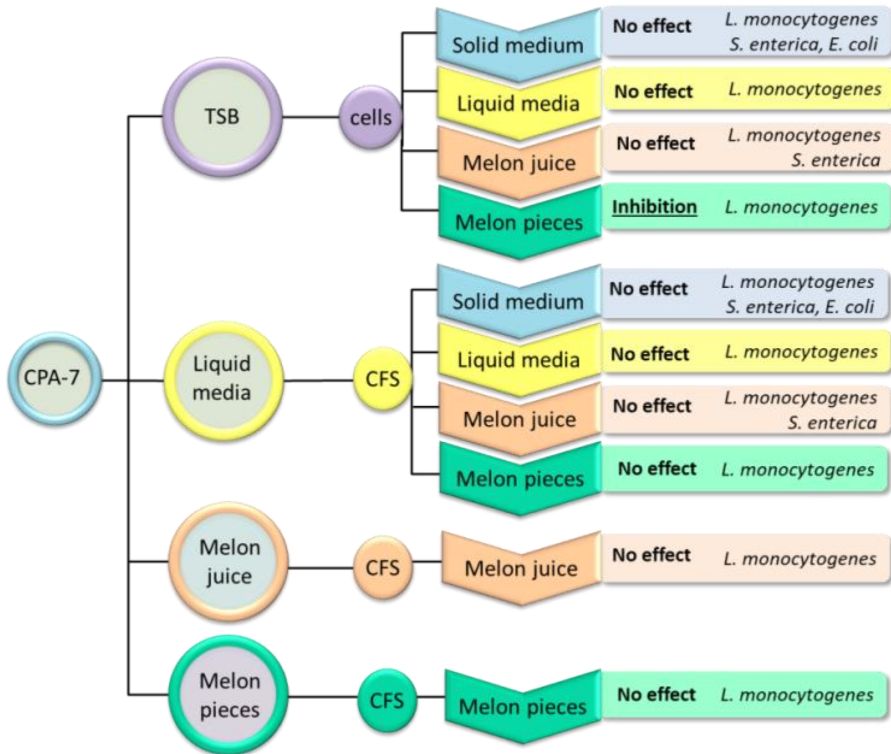

Figure IX.3. Summary of *in vitro* and *in vivo* analyses performed in the present work and results obtained when testing the antimicrobial activity of CPA-7 against foodborne pathogens.

The production of lytic enzymes associated to food spoilage (e.g. alkaline proteases, lipases, amylases, pectinases, β galactosidases) has been reported for many *Pseudomonas* spp. isolated from different food matrices (Nicodème *et al.*, 2005; Zambare, Nilegaonkar and Kanekar, 2011). As presented in chapter II, the alkaline protease activity by CPA-7 was evaluated on solid media (PCA containing 1 or 10 % of skim milk) after incubation at 30 °C for 48 h, since Pseudomonads production of extracellular proteases are usually detectable in the late log and the stationary phases (Rajmohan, Dodd and Waites, 2002). Results showed a very weak proteolytic activity deployed by CPA-7 in the medium containing 1% of

milk while no hyaline halo was observed around the antagonist colonies in the medium containing 10% of milk, which contrasted with the clear haloes observed around positive control's colonies (Fig. **IX**.4). In agreement with these results, an activation of alkaline protease activity by 48 h-cultures of several *P. chlororaphis* strains was observed when grown in minimal medium supplemented with 1% (v/v) skim milk, while this activity upon increasing the amount of milk to 5 % was only observed for some of those strains (Nicodème *et al.*, 2005). Extracellular heat-tolerant alkaline protease activity has been demonstrated for several strains of *P. fluorescens, P. chlororaphis,* and *P. fragi* among other milk isolates as well as for *P. aeruginosa* PAO1, showing a strain-dependent level of activity in function of protein concentration (1 to 10 % skim milk) and incubation temperature (7, 25 or 30 °C), and a high correlation with the presence of the *apr*X gene (Matéos *et al.*, 2015; Caldera *et al.*, 2016). This gene was not found in CPA-7 genome whereas two genes similar to those encoding for alkaline protease inhibitor (*inh*) were found (Annex 1). These results suggested that alkaline proteases, if produced by CPA-7, are secreted in low amounts and this production is not enhanced by higher amounts of protein in the medium.

On the other hand, in previous works indications of CPA-7 low extracellular lytic activities have been obtained, i.e. no gelatinase activity in API test and no detectable pectinolytic activity in peach and apple fruit wounds 3 or 6 d post-inoculation with 10^6, 10^7 or 10^8 CFU mL^{-1} CPA-7, when incubated at 10 or 20 °C (Alegre, 2012). In the same way, strain *P. graminis* 49M (10^8 CFU mL^{-1}) did not show pectinolytic activity on pear fruitlets, potato tubers, or in crystal violet pectate medium (CVP) after 48 h incubation at 27 °C (Mikiciński, Sobiczewski, Puławska and Maciorowski, 2016).

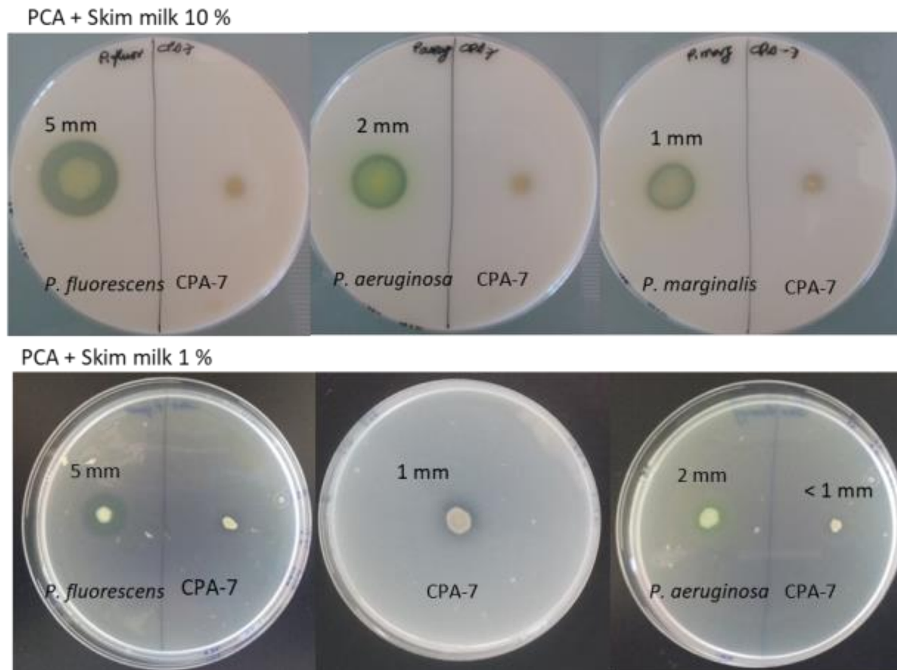

Figure **IX**.4. Alkaline protease activity test on Plate count agar medium + 1 or 10 % skim milk incubated at 25 ºC for 48 h: hyaline haloes around bacterial growth indicate protein degradation. Positive controls: *P. aeruginosa, P. fluorescens,* and *P. marginalis*.

Regarding the hemolytic activity, no change of color was observed around the colonies of CPA-7 or the negative control *L. innocua* on 5 % sheep blood-containing solid medium, after 48 h of incubation at 30 °C or 37 °C, respectively (Fig. **IX**.5). This contrasted with the greenish zone indicating incomplete hemolysis that surrounded the positive control *S. gordonii* colonies after 48 h at 37 °C. The lack of hemolytic activity showed the absence of a virulence trait that has been previously observed for pathogenic *Pseudomonas* spp. such as *P. aeruginosa* (Terada *et al.*, 1999), and represents a point in favor of the safeness of CPA-7 for human consumption.

Figure **IX**.5. *In vitro* hemolytic activity test: greenish halo indicates incomplete hemolysis; absence of halo indicates no hemolysis.

As for adherence, we could not demonstrate that this was an enhanced characteristic of CPA-7 that would explain its antagonistic activity. Unlike *P. fluorescens, E. coli* H157:O7 and *L. monocytogenes,* CPA-7 was unable to form detectable biofilms adhering to polystyrene micro-wells, when grown statically in minimal medium M63 supplemented with 2% glucose and 5% casaminoacids during 72 h and incubated either at 25 (Fig. **IX.**6) or 30 °C.

The selection of the culture medium was based on previous experiments showing that glucose stimulated biofilm formation, which was further increased by 2 or 3-fold upon the addition casaminoacids (O'Toole and Kolter, 1998). In that work, *P. fluorescens* WCS365, a rhizosphere-inhabiting biocontrol agent, was able to produce biofilms on several plastic hydrophobic surfaces, including polystyrene, after 10 h of incubation at room temperature. Moreover, two different growth temperatures were tested for CPA-7 in order to check for this ability in motile and sessile forms (Behrendt *et al.*, 1999). Motility has been well documented as an important factor related to biofilm formation by several bacteria (Bogino *et al.*, 2013; Yaron and Römling, 2014).

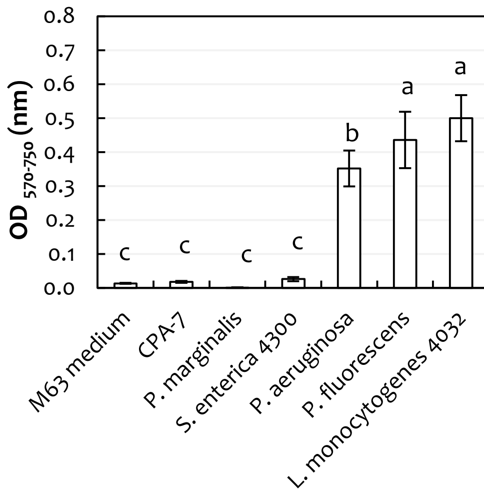

Figure IX.6. Biofilm formation test in polystyrene microplates when grown in M63 + 2% glucose + 5% CAA liquid medium during 72 h at 30 ºC. Columns are means and error bars are standard deviations (n=8). Different letters represent statistical differences according to analysis of variances (ANOVA) and Tukey's test ($p < 0.0001$).

As another approach to biofilm formation, we tested the production of alginate, a reported component of EPS in *P. aeruginosa* biofilms (Hentzer *et al.*, 2001), but no alginate production was detected in CPA-7 cell-free supernatants in the conditions tested: culture medium containing 17% casein peptone, 3% soy peptone, and 2,5% glucose (TSB) after 24 h of incubation at 25 °C, suggesting that a different polysaccharide might be the main component of *P. graminis* EPS in case of biofilms formation, or that the production of this compound could require different growth conditions. This is also suggested by the proven ability of another *P. graminis* strain (49M), epiphyte from the phyllosphere apple tree, which was able to form biofilms on polyvinyl microplates after 12 h of incubation at 27 °C in yeast peptone (1% peptone, 0.5% yeast extract) and in Luria Bertani, LB (1% casein enzymic hydrolysate, 0.5% yeast extract) liquid media; although EPS components were not studied (Mikiciński, Sobiczewski, Puławska and Malusa, 2016).

Deepening in the possibility for biofilms formation we also tested the ability of CPA-7 to produce biosurfactants during growth for 72 h in King's B liquid medium. Depending on their chemical structure, these molecules have previously proven multiple roles in Pseudomonads attachment, participating in

microbial interaction with surfaces, the initiation of biofilms and the maintenance of their structure as well as in their disruption (Kuiper *et al.*, 2004; Pamp and Tolker-Nielsen, 2007; Bogino *et al.*, 2013). Furthermore, a direct antimicrobial effect of biosurfactants has been observed in several BCA-pathogen interactions (reviewed by Raaijmakers *et al.*, 2010). However, we did not observe any biosurfactants production in the conditions tested.

In spite of the obtained results, reduced adhesion to inert surfaces cannot rule out the possibility for biofilm formation on plant tissues. Different carbon sources such as mannitol, malate, citrate, and glutamate, naturally present in fruits, can also stimulate biofilm formation and even rescue this ability in biofilm-defective mutants (O'Toole and Kolter, 1998), thereby indicating that several genetic pathways, involving or not motility structures and activated by varied compounds, may participate in their biosynthesis and regulation. Furthermore, the presence in CPA-7 genome of several genes related to the adhesion to surfaces and biosynthesis of alginate *algA, X, E, K* (Annex 1) suggest that the secretion of this compound as a component of EPS could be induced under certain conditions.

On the other hand, we explored competition for nutrients through the analysis of overlapping in the oxidation of specific nutrients between CPA-7 and several foodborne pathogens: *E. coli* O157:H7, two strains of *S. enterica* subsp. *enterica* and two strains of *L. monocytogenes* using GN2/GP2 microplates, which contain 95 single carbon/nitrogen sources (Table **IX**.2). This method provides data which point out to potential targets for competition among different microorganisms in natural environments, although interspecies interactions in microbial communities are much more complex and are influenced by numerous factors (Hibbing *et al.*, 2010; Stubbendieck and Straight, 2016). Nutrient overlapping indexes (NOIs) based on the single carbon/nitrogen sources utilization profiles showed that carboxylic acids were the most used compounds by CPA-7 (64 % in relation to the total tested) showing the highest overlapping levels in relation to

the tested Gram-negative pathogens: NOI (CPA7/*E. coli*) = 0.92 and NOI (CPA-7/*S. enterica*) = 0.83. Specifically, citric, malic, malonic, succinic, and gluconic acids, which are present in several fruits and vegetables, could be targets for competition (Flores, Hellín and Fenoll, 2012; Sandín-España *et al.*, 2016). In the same way, CPA-7 could stablish competition with both *S. enterica* and *E. coli* for glucose and sorbitol, among the majoritarian carbohydrates naturally present in apple, pear, nectarine, peach, and melon (fructose, glucose, sucrose and sorbitol) where it has shown antagonistic activity (Beaulieu, Lea and Eggleston, 2003; Wu *et al.*, 2007; Jovanovic-Malinovska, Kuzmanova and Winkelhausen, 2014; Kolniak-Ostek, 2016). Similarly, competition for glucose could be stablished in broccoli and lettuce (Jovanovic-Malinovska, Kuzmanova and Winkelhausen, 2014). Regarding aminoacids, asparagine, glutamine, proline, alanine, and leucine which are majoritarian in apples and melons, could be targets for competition between CPA-7 and *E. coli* or *S. enterica* in those commodities (Wu *et al.*, 2007). In the same way, histidine, also present although in lower amounts in those fruits, showed a shared use by CPA-7 and *S. enterica*. The exogenous application of aminoacids to apple wounds has previously shown that competition for these compounds was associated to the antagonism of the BCA *Aureobasidium pullulans* against *P. expansum* (Bencheqroun *et al.*, 2007).

However, antagonism based on competition for specific nutrients is highly dependent on the composition of the culture medium which, in the case of fruit and vegetable matrices, relays on the species, cultivar, pre-harvest treatments and conditions, storage conditions, and physiological stage of the commodity, as well as by the composition and abundance of the microbiome and the compounds they secrete into the extracellular environment (Moing *et al.*, 1998; Lamikanra *et al.*, 2000; Villanueva *et al.*, 2004; Chen *et al.*, 2007; Mahmood *et al.*, 2012; Jovanovic-Malinovska, Kuzmanova and Winkelhausen, 2014; Stubbendieck and Straight, 2016).

Table IX.2. Single carbon/nitrogen sources utilization profiles of CPA-7 and several foodborne pathogens when individually grown in GN2 microplates during 96 h at the optimal growth temperature for each microorganism. Shared use of nutrient sources in GP2 microplates are shown is shown when positive.

Group	carbon/nitrogen source	E.c	CPA-7	S.e 711	S.e 707	L.m 940	L.m 230
CH	N-acetyl -D-galactosamine	x					
CH	N-acetyl-D-glucosamine	x		x	x		X
CH	adonitol		**x**				
CH	L-arabinose	x	**x**	x	x		x
CH	D-arabitol		**x**				x
CH	γ-erythritol						
CH	D-cellobiose					X	X
CH	D-fructose	x			x		X
CH	L-fucose	x	**x**	x	x		
CH	D-galactose	x	**x**	x	x		
CH	gentibiose	x					
CH	α-D-glucose	x	**x**	x	x	X	X
CH	m- inositol		**x**	x	x		
CH	α -D-lactose						
CH	lactulose	x					
CH	maltose	x		x	x		
CH	D-mannitol	x	**x**	x	x		
CH	D-mannose	x	**x**	x	x	X	X
CH	D-melibiose	x		x	x		
CH	β-methylglucoside	x		x			x
CH	3-D-psicose	x		x			
CH	4-D-rafinose	x		x			
CH	L-rhamnose	x		x	x		
CH	D-sorbitol	x	**x**	x	x		
CH	sucrose						
CH	D-trehalose	x		x	x	X	X
CH	turanose	x				X	X
CH	xylitol						
CH	arbutin	NT	**NT**	NT	NT	X	X
CH	D-ribose	NT	**NT**	NT	NT	X	X
AA	L-alanine	x	**x**	x	x		
AA	L-alanyl-glycine	x	**x**	x	x		
AA	L-asparagine	x	**x**	x	x		
AA	L-aspartic acid	x	**x**	x	x		
AA	L-glutamic acid	x	**x**	x	x		
AA	glycyl-L-aspartic acid	x		x	x		
AA	glycyl-L-glutamic acid	x		x			
AA	L-histidine		**x**	x	x		
AA	hydroxy-L-proline						
AA	L-ornithine						

Group	carbon/nitrogen source	E.c	CPA-7	S.e 711	S.e 707	L.m 940	L.m 230
AA	L-Leucine		**x**				
AA	L-phenylalanine						
AA	L proline	x	**x**	x	x		
AA	L-pyroglutamic acid		**x**	x			
AA	D-serine				x		
AA	L-serine	x		x	x		
AA	L-threonine						
AA	D,L-carnitine						
AA	γ-amino butyric acid		**x**				
CA	acetic acid	x	**x**	x	x		
CA	cis-aconitic acid	x	**x**	x	x		
CA	citric acid	x	**x**	x	x		
CA	formic acid		**x**				
CA	D-galactonic acid lactone		**x**				
CA	D-galacturonic acid	x	**x**				
CA	D-gluconic acid	x	**x**	x	x		
CA	D-glucosaminic acid	x	**x**	x	x		
CA	D-glucuronic acid	x	**x**	x	x		
CA	α-hydroxy-butyric acid						
CA	β-hydroxy-butyric acid		**x**				
CA	γ-hydroxybutyric acid						
CA	hydroxypheyl acetic acid			x	x		
CA	itaconic acid		**x**				
CA	α –keto-butyric acid						
CA	α –keto-glutaric acid	x	**x**				
CA	α –keto-valeric acid						
CA	D,L-lactic acid	x	**x**	x	x		
CA	malonic acid						
CA	propionic acid	x		x	x		
CA	quinic acid						
CA	D-saccharic acid	x	**x**	x	x		
CA	sebacic acid						
CA	succinic acid	x	**x**	x	x		
CA	bromosuccinic acid	x	**x**	x	x		
AH	2,3-butanediol						
AH	glycerol	x	**x**	x	x		
AM	2-aminoethanol		**x**	x	x		
AM	succinamic acid		**x**				
AM	glucuronamide		**x**				
AM	L-alaninamide		**x**				
AM	phenylethylamine						
AM	putrescine		**x**				
PL	α-cyclodextrine					x	
PL	dextrine	x	**x**	x	x	x	
PL	tween 40		**x**	x	x		

Group	carbon/nitrogen source	E.c	CPA-7	S.e 711	S.e 707	L.m 940	L.m 230
PL	tween 80		**X**	x	x		
PL	glycogen						
PH	D,L-α-glycerol phosphate	x	**X**	x	x		
PH	glucose- 1-phosphate	x	**X**	x	x		
PH	glucose 6- phosphate	x	**X**	x	x		
NC	urocanic acid		**X**				
NC	inosine	x	**X**	x	x		x
NC	uridine	x	**X**	x	x		x
NC	thymidine	x		x	x		
ME	mono-methyl-succinate						
ME	methylpyruvate	x	**X**	x	x		

C/N source utilization: x: source used in GN2 microplates; X: source used in GP2 microplates; NT: not tested, source present in GP2 but not in GN2 plates. Microorganisms tested: E.c: *E.coli* O157:H7, S.e: *S. enterica* CECT 711 or CECT 707, L.m: *L. monocytogenes* 230/3 or CECT 940. Functional group: CH: carbohydrates; AA: aminoacids; CA: carboxylic acids; AH: alcohols; AM: amines/amides; PL: polymers; PH: phosphorylated compounds; NC: nitrogenous cyclic compounds; ME: methyl esters

Nevertheless, several copies of ABC transporter-like molecules related to the uptake and transmembrane transport of a variety of molecules (lipids, carbohydrates, aminoacids) were also found in CPA-7 genome (Annex 1), suggesting that this bacterium could have an adaptive advantage for outcompeting the surrounding microbiota in several conditions.

Although GN2/GP2 Biolog system has been used for assessing antagonist competitive potential and multiple microbial interactions, it has also been criticized by the lack of ecologically relevance and complexity of the C/N sources included (Preston-mafham, Lynne and Randerson, 2002; Horemans, Smolders and Springael, 2013; Halfeld-Vieira *et al.*, 2015). Furthermore, in the present study GN2/GP2 panel was not compatible with the growth and activity of *L. monocytogenes*, therefore the assessment of niche overlapping between CPA-7 and this species was very limited. In this way, after 96 h of incubation in GN2/GP2 plates CPA-7 and *L. monocytogenes* only showed putative competition for glucose, mannose, arabinose and arabitol, most of which are not important components of fruit or vegetables. Therefore, in order to clarify this interaction, further work using a more suitable technology should be tested.

Additionally, the secretion of siderophores as a specialized form of competition for nutrients was assessed on solid CAS-TSA or CAS-BHI half-plates, prepared as described by Milagres et al. (1999). Striate colonies of CPA-7 or of the positive controls *P. aeruginosa* and *P. fluorescens* were seeded along the edges of the rich medium to avoid the negative effect of CAS components on bacterial growth (Milagres, Machuca and Napoleão, 1999). After 1 d incubation a change of color from blue to orange-red (*P. aeruginosa*) or to orange-yellow (*P. fluorescens*) was observed in both in TSA-CAS and BHI-CAS plates. Similar halo orange-yellow could be observed on CPA-7 seeded BHI-CAS plates but not on TSA-CAS plates, from 7 d after incubation. The extent of this halo progressively increased over time concomitantly with the depletion of nutrients in the culture media (Fig. **IX**.7), suggesting the production of siderophores in this growth medium unlike in TSA. Differential production of siderophores according to the culture medium have been previously reported for other antagonistic Pseudomonads (Sasirekha and Srividya, 2016). Additionally, the presence in the CPA-7 genome of a gene similar to the *sbnA* gene, which encodes for the enzyme catalyzing the first step in the biosynthesis of the siderophore Staphyloferrin B in *Staphylococcus aureus* (Kobylarz *et al.*, 2016); as well as putative genes involved in reception and intake of ferric binding molecules produced by other microorganisms and in the regulation of those process (*pfeS, BfrD, fur*, TonB-dependent receptor) (Ochsner *et al.*, 2002; Armstrong, Brickman and Suhadolc, 2012), could account for the potential enhanced ability of this microorganism for the uptake of iron in starving conditions.

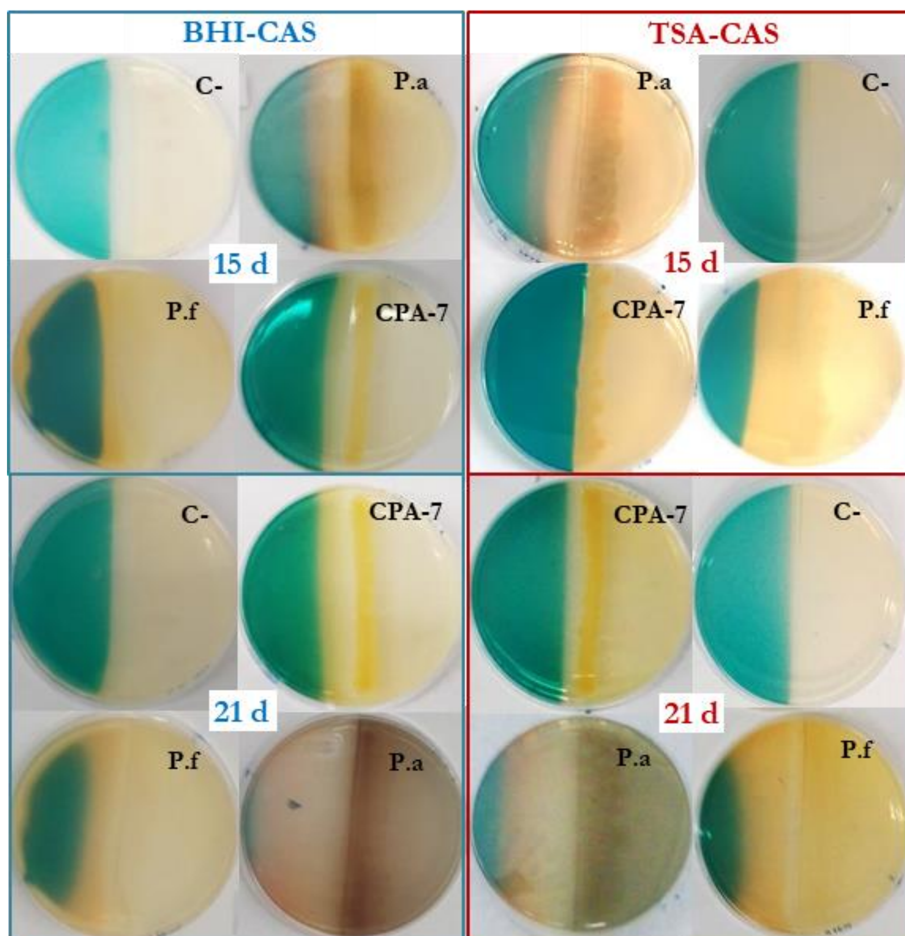

Figure IX.7. Siderophore-production CAS-agar test based on the method modified by Milagres et al., (1999), performed on TSA-CAS or BHI-CAS agar plates during incubation in humid atmosphere for up to 30 d at 30 ºC: C- (negative control), CPA-7, P.a (*P. aeruginosa*), P.f (*P. fluorescens*). Color turn from blue to orange or red indicate the removal of Fe^{3+} from the ferric-CAS complex by siderophores.

The ensemble of results presented and discussed in chapter 2 and highlighted in the present epigraph suggested that competition for nutrients, through an efficient utilization of specific carbon/nitrogen sources or minerals like iron, might be an effective mode of action for CPA-7 antagonism depending on the environmental conditions. This strategy is determined not only by the composition of the food matrix and the storage conditions, but by the spatial

distribution of the bacteria involved in the interaction. The lack of antagonistic effect of CPA-7 against foodborne pathogens when grown in co-cultures in melon juices or liquid media might be a result of differences in energy metabolism and metabolic end-products among sessile and planktonic bacteria (Skandamis and Nychas, 2012). Furthermore, solid matrices constraint bacterial growth and movement as well as diffusion of substances, allowing local accumulation of secondary metabolites, changes in pH, and depletion of nutrients, which contrast with the better distribution of solutes in liquid cultures (Popat *et al.*, 2012; LaSarre and Federle, 2013). Solid surfaces also allow cell-to-cell contact and communication through the secretion and proximal diffusion of quorum sensing signals (Wilson *et al.*, 2002; Hibbing *et al.*, 2010).

On the other hand, in the food environment, microorganisms surrounding the antagonistic agent may be foodborne pathogens. Thus, limiting their activity as a result of competition may entail the reduction of their virulence, thereby improving the safety of fresh-cut products. In this regard, we evaluated the effect of the interaction between CPA-7 and *L. monocytogenes* or *S. enterica* during up to 7 d on fresh-cut pear at 10 °C, on the subsequent ability of those pathogens to adhere and invade Caco-2 monolayers, a cellular model morphofunctionally resembling the human host intestinal epithelium (Sambuy *et al.*, 2005).

The results presented in chapter 3 showed that CPA-7 reduced the adhesion of *S. enterica* to Caco-2 cells by 0.8 $\log_{10}$, irrespective of the previous preadaptation on fresh-cut 'Conference' pear. In contrast to these results, *S. enterica* ser. Typhimurium adhesiveness and invasiveness to Caco-2 cells decreased after sequential passage through soil (1 d at 15 °C), lettuce leaves (1 d at 15 °C), and lettuce pieces (4 d at 5°C + 1 d at 20 °C), showing that the effect of the pre-adaptation period in a food matrix on the virulence traits of the pathogen depends on the strain, the commodity and the pre-incubation conditions (Oliveira *et al.*, 2011). Based on our findings showing that CPA-7 was able to

survive at 37 °C in DMEM medium, regardless of the pre-adaptation on the fruit matrix (pH 7.2) (Fig. **IX**.8), and that this bacterium adhered to the Caco-2 monolayer to the same extent that *S. enterica*, competition for adhesion sites on the intestinal epithelium could be one explanation for the reduced adhesiveness showed by the mentioned pathogen when co-inoculated with the antagonist.

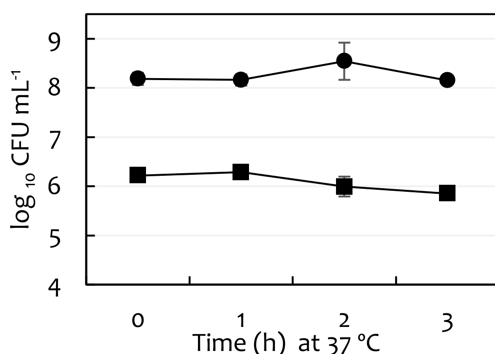

Figure **IX**.8. Viability of CPA-7 in DMEM medium at 37 °C in 5% CO_2 humid atmosphere, before (■) and after (●) pre-incubation on fresh-cut pear at 10 °C for 7 d. Results are presented as means ± standard deviations (n = 6). No significant differences ($p > 0.05$) in respect of the initial population were observed throughout time, according to analysis of variances (ANOVA).

Non-specific competition for adherence to intestinal epithelium has been explained by hydrophobic or steric impediment or by electrostatic interactions among microorganisms and is thereby influenced by population levels (Coconnier *et al.*, 2000; Servin, 2004). Other mechanisms such as co-aggregation with pathogens cells or saturation of the epithelium surface which involve structural or secreted adherence-promoting factors that specifically bind to carbohydrates, lipids or proteins on the pathogen or the host membranes, have been demonstrated for probiotics (Neeser *et al.*, 2000; Pan *et al.*, 2008; Lebeer, Vanderleyden and De Keersmaecker, 2010). For instance, molecules that specifically bind surface molecules of the human epithelial cells, such as carbohydrates (asialo-GM1 and mannose) and proteins (lectin) have been found in several lactobacilli and in *P. aeruginosa* (Laughlin *et al.*, 2000; Neeser *et al.*, 2000). They have shown similar structure to those identified in the motility structures of enterohemorragic *E. coli* and *Salmonella* spp. and could contribute to explain competition at the epithelium surface. In this case, CPA-7 inoculum was initially 1.1 log_{10} over that of *S. enterica* and the difference reached 3 log_{10} at

the end of the experiment, yet resulting in the same reduction of adhesion in both cases. This could be due to population numbers reached a saturation point leaving no available receptors for binding (Lee *et al.*, 2000).

In contrast, CPA-7 showed no effect on *L. monocytogenes'* adhesiveness in co-cultured samples compared to the control, regardless of habituation on the fruit, even when the antagonist outnumbered the pathogen by 1.5 and 1.8 $log_{10,}$ before and after the pre-incubation period, respectively. Contrastingly, habituation on pear wedges for 7 d reduced the adhesiveness of *L. monocytogenes* to Caco-2 cells by 0.5 log_{10}, both in control and in co-cultured samples. Similar effect of the food matrix on *L. monocytogenes* strain 230/3 (serovar 2a) adhesiveness was observed after pre-incubation on fresh-cut pear for 7 days at 10 °C, while invasion was unaffected (Colas-Meda *et al.*, 2017). Overall, the present findings suggested that specific competition targeting the same receptors for adhesion than *S. enterica* subsp. *enterica* or disrupting their functionality or structure could be a mechanism for CPA-7 to reduce *S. enterica* adhesiveness, which was not effective for preventing *L. monocytogenes's* adhesion.

In order to assess the possibility for competition in a more realistic scenario, CPA-7 was submitted to gastrointestinal digestion and subsequently, to adhesion and invasion tests with Caco-2 cells, to stablish the amount of viable bacteria that would reach the intestinal lumen before and after incubation for 7 d on the fruit matrix and the amount within those bacteria that would adhere to and invade into the epithelial cells. We found that CPA-7 populations were drastically reduced after gastric digestion (by > 6 log_{10}) irrespective of the pre-incubation on the pear. However, CPA-7 was able to grow during the intestinal phase before pre-habituation on the fruit, as previously observed for other Pseudomonads (Madi *et al.*, 2010b), and this capacity was compromised after 7 d-culture on pear pieces. Reduced capacities for overcoming gastrointestinal digestion have previously observed for *L. monocytogenes* after pre-incubation during 6 d at 5 or 10 °C in the same food matrix (Colas-Meda, Viñas, *et al.*, 2017).

On the other hand, the invasiveness of the evaluated pathogens was not affected by pre-incubation on pear pieces, while CPA-7 invasiveness, which was extremely limited before pre-incubation, became undetectable (< 5 CFU g^{-1}) after this period. Nevertheless, the invasiveness of both pathogens was reduced by > 4 $\log_{10}$ in co-cultures with CPA-7 compared to the single-inoculated controls, which suggests a putative modulation of their invasion mechanisms during the interaction in the fruit environment. Reduced invasion, cytotoxic and multiplication abilities of *L. monocytogenes, S. enterica*, and *E. coli* to human epithelial cells have previously been observed in co-cultures with other pathogenic or antagonist strains *in vitro* or in fruit matrices and have been associated to changes in virulence-genes expression (Medellin-Peña *et al.*, 2007; Medellin-Peña and Griffiths, 2009; Bayoumi and Griffiths, 2012; Rantsiou *et al.*, 2012; Tan *et al.*, 2012; Zilelidou *et al.*, 2015).

In summary, results exposed in chapter 3 showed that the interaction of CPA-7 with the foodborne pathogens *L. monocytogenes* and *S. enterica* in a food matrix decreased the invasiveness of both pathogens, which would result in a reduction of their virulence. CPA-7 was able to survive at 37 °C and 5% CO_2 and it was able of surviving to gastric digestion and of growing during the intestinal phase. Moreover, CPA-7 adhered to intestinal cells with a similar efficacy than *S. enterica,* all of which potentially enabled it for competition. However, CPA-7's populations were drastically reduced after gastric digestion and the capacity for their recovery was hindered by preadaptation in the fruit matrix which considerably limits the possibility for establishing competition at the intestinal level.

Indirect effect - Induction of the host's oxidative defense response

The induction of host defense mechanisms is a generally established mode of action of antagonistic rhizospheric Pseudomonads, which allows the plant to better respond to subsequent pathogenic infections (Chen *et al.*, 2000; Pieterse

et al., 2000, 2001, 2014; Verhagen *et al.*, 2010). However, very few has been studied about the effect of fruit-epiphytic Pseudomonads such as CPA-7 on the activation of defense mechanisms and specifically, on the oxidative response, which is also associated to wounding and therefore, is essential in fresh-cut produce.

As presented in chapter 4, we assessed the putative activation of defense responses in fresh-cut apple focusing on the oxidative metabolism as a mode of action of CPA-7. That effect was evaluated at refrigerated temperature and after antioxidant-dipping because these are established hurdles for the preservation of fresh-cut apples. In addition to these pre-requisites, the effect in aerobic conditions favoring the antagonist growth and activity was compared to that in MAP, to get a more practical approach. Results showed that the activity of some antioxidant enzymes was modulated in response to CPA-7 on fresh-cut apple wedges depending of the conditions. We observed that the initial response to wounding entailed the induction of SOD as a first line of defense against the rapid production of ROS. As a result of SOD dismutation reaction, a concomitant increase in H_2O_2 was observed, regardless of the antagonist application, as it has been reported in fresh-cut apples (Larrigaudiere *et al.*, 2008). Subsequently, to counteract the oxidative burst, enzymatic and non-enzymatic H_2O_2-scavenging mechanisms were activated in all samples but in a higher extent in the presence of the antagonist.

Singularities in the oxidative response in fresh-cut apple upon inoculation with CPA-7 compared to the control were observed 24 h after packaging. At that time, CPA-7 induced APX activity by 3-fold compared to the control in both storage conditions. However, CAT and POX activities were induced in air while suppressed in MAP. Similar POX and CAT modulation were observed in tomato plants within the 24 h after the foliar application of the combination BCA (*B. subtilis* AR12) and the pathogen (*Ralstonia solanacearum*) (Li *et al.*, 2008). Similarly, an initial peak in POX activity was observed in cabbage seeds during

the first 20 to 24 h post-inoculation with the BCA *P. fluorescens* UOMPfu-5; and a higher response was observed upon the combined treatment BCA + pathogen (*Xanthomonas campestris* pv. *Campestris)* (Umesha and Roohie, 2017). Concomitantly with the activation of enzymatic pathways, we observed that total antioxidant capacity total and phenolic content were higher in the presence of the antagonist than in the control when stored in air, probably due the induction of PAL. However, this effect was not observed in MAP. Such enhanced activity of enzymatic and non-enzymatic H_2O_2-scavenging mechanisms resulted in the maintenance of the levels of this molecule close to cero during the whole experiment in aerobic conditions, while in MAP a more progressive reduction was observed from 24 h post-treatment. Controlled oxidative burst was consistent with the lack of induction of hypersensitive response in tobacco leaves previously observed for CPA-7 as well as for the strain 49M of *P. graminis* (Alegre, 2012; Mikiciński, Sobiczewski, Puławska and Maciorowski, 2016).

Later in storage, a second activation peak of SOD activity (by 0.5-fold) in response to CPA-7 was observed at day 6 in air conditions which was counteracted by POX induction (by 0.7- fold), compared to non-inoculated control. Similarly, SOD and POX activation in wounded mangoes were observed from 3 to 5 d post-treatment with a chemical compound-BCA-pathogen combination (hexanal + *P. fluorescens* Pf1 + *Lasiodiplodia theobromae*) in air conditions (Parthasarathy *et al.*, 2016). In the same way, increased POX activity was associated to an enhanced response of wounded apples to *Botrytis mali* upon treatment with the BCA *P. fluorescens*, reaching the highest values on day 6 of incubation in air (Mikani, Etebarian and Aminian, 2011). In MAP, the second peak in SOD activation in response to the CPA-7 was observed at day 3 and it correlated with a drastic increase in PPO activity, reaching values 8-fold than non-inoculated control by the end of storage, which correlated with a significant reduction of its substrates (phenolic compounds). At the end of MAP storage, SOD activity dropped in inoculated samples while POX was strongly activated in

response to CPA-7 compared to the control, which resulted in the depletion of H_2O_2.

It remains still undetermined if the increased levels of antioxidant enzymes are only the result of an increased biosynthesis by the plant or also by the bacterium. CPA-7 genome harbors genes encoding for three different types of SODs (Mn-SOD, Fe-SOD and Cu-Zn-SOD), as well as for thiol-, glutathione-, and alkylhydro-peroxidases and several catalases (Annex 1), some of which have been described and predicted to be periplasmic or extracellular in other pathogenic or PGPR Pseudomonads (Paulsen *et al.*, 2005; Duan *et al.*, 2013; Ye *et al.*, 2014; Hernández-Salmerón, Moreno-Hagelsieb and Santoyo, 2017). Therefore, the increased production and secretion of these enzymes by this bacterium upon stressful conditions would enhance its tolerance to oxidative stress conferring it with an adaptive advantage for outcompete pathogens in the cut-edge oxidative environment or it could help to alleviate the oxidative burst in the plant.

In summary, the combined application of the antioxidant AS1 and CPA-7 in fresh-cut apple resulted in the activation of enzymes related to the defense mechanisms, the delay of the decay of both the antioxidant capacity and the phenolic compounds contents as well as the maintenance of the fruit quality parameters. Furthermore, CPA-7 was able to grow in apple wedges treated with AS1 antioxidant in both storage conditions attaining lower population levels in MAP than in air, but not to a great extent (by 0.4 log_{10}). Those facts suggests that the activation of defense pathways in the host and the tolerance of CPA-7 to oxidative stress conditions are some of the action mechanisms contributing to the antagonistic activity displayed by this strain against foodborne pathogens in similar conditions (Alegre, Viñas, Usall, Anguera, *et al.*, 2013).

The induction of plant ISR by Pseudomonads may involve the activation of ethylene and/or jasmonate signaling pathways (Iavicoli *et al.*, 2003; Ahn, Lee and

Suh, 2007). As an approach to this topic, we evaluated the induction of ethylene biosynthesis in fresh-cut apple at two maturity stages upon inoculation with CPA-7. Additionally, the effect of the interaction of the BCA with the antioxidant AS1 and the storage system (MAP or hermetic) were included in the analysis since they are essential hurdles within the preservation strategies of fresh-cut produce. Results, presented in chapter 5, showed that the maturity stage was a key factor determining the magnitude of the wound response, the effect of the antagonist on both the C_2H_4 and the CO_2 accumulation patterns in the fruit, as well as the effect of the antioxidant at controlling the last mentioned parameters. Wounding was associated to a reduced C_2H_4 accumulation in wedges compared to intact apples only at the more advanced maturity stage, while no variation was observed in immature ones. However, the initial C_2H_4 accumulation in immature and mature apples wedges was similar.

We observed that CPA-7 did not activate the production of C_2H_4 as a part of the defense response in fresh-cut apple but instead, it was associated to a reduced accumulation of this molecule in AOX-untreated MAP-stored samples from the 3rd d of storage. In the hermetic system, CPA-7 showed a C_2H_4-reducing effect only in immature apples while it acted oppositely in mature ones. The C_2H_4-reducing effect was not associated to the metabolization of this molecule by the antagonist since it was unable to reduce the levels of exogenous ethylene in apple juice or synthetic broth at refrigerated or optimal growth temperatures. Thus, this effect could be a result of the induction of ACD activity in CPA-7 as it has previously been reported for several BCA-PGPR Pseudomonads (Ramette *et al.*, 2011; Ali, Charles and Glick, 2012; Duan *et al.*, 2013). This hypothesis is suggested by the presence in the CPA-7's genome (Annex 1) of a gene sharing a 98 % homology with the gene encoding for the ACC-deaminase enzyme of the *P. graminis* type strain (294/08) (Behrendt *et al.*, 1999). The reduction of the ethylene levels after the 3rd d of MAP storage could contribute to the reduction of the C_2H_4-associated senescence of clicmateric fruits in semi-commercial

conditions. Nevertheless, the functionality of this gene and the conditions inducing its expression as well as the ACD activity remain to be established.

However, the C_2H_4-reducing effect of CPA-7 was hindered in the presence of the antioxidant, which in turn resulted in an enhanced production of this molecule in more mature apples, reaching values up to 40% higher than those measured in AOX-untreated samples on the 3rd day of MAP storage. In general, AOX effect was indeed highly influenced by the fruit maturity stage: it effectively reduced C_2H_4 production and respiration in immature samples but it sharply increased the peak in ethylene production and failed to inhibit respiration in mature non-inoculated apples, regardless of the storage system. These results corroborate those from previous experiments showing a reduced effectiveness of ascorbic acid-based AOX treatments for the preservation of fresh-cut in 'Fuji' apples in MAP storage (Rojas-Graü, Grasa-Guillem and Martín-Belloso, 2007). Furthermore, these results together with the fact that higher maturity stages also diminish the effectiveness of CPA-7 antagonistic activity against foodborne pathogens in fresh-cut melon, as obtained in the present work, alert about the importance of controlling the ripeness stage during the selection of the commodities used for fresh-cut produce production.

9.2 Non-Thermal Physical Preservation Strategies

9.2.1 Water-assisted UV-C and PAA-assisted UV-C

In addition to biopreservation we assayed a novel physical non-thermal technology integrating the decontamination of the plant product by UV irradiation and by immersion while simultaneously decontaminating the washing solution. This technology allows a better accessibility of UV-C light to all sides of the product and reduce the risk of overheating it, both of which are limitations of conventional UV-C chambers (Liu, Huang and Chen, 2015). Along with the

direct antimicrobial effect we evaluated the hormetic effect that has been reported for low-dose UV-C irradiation which results in the activation of the plant defense response to potential UV damage due to its mutagenic activity and the production of free radicals, thereby inducing the production of antioxidant and protective compounds such as phytoalexins, polyphenols, ascorbic acid, and glutathione, that scavenge the produced ROS and absorb damaging UV light wavelengths (Lemoine, Chaves and Martínez, 2010; Scott *et al.*, 2017; Zhang *et al.*, 2017).

Fresh-cut broccoli

Firstly, we evaluated the effectiveness of WUV for the decontamination of the native microbiota of fresh-cut broccoli florets from conventional and organic agricultural practices. In order to determine a hormetic dose allowing the improvement of the microbiological and the biochemical quality of conventional broccoli without compromising its overall visual quality, texture or color, we tested four UV-C doses (0.3, 0.5, 0.9, and 1.8 kJ m^{-2}) by combining two levels of irradiance (through the use of 2 or 4 UV lamps) and two exposure times (120 or 360 s). Results showed that 0.5 kJ m^{-2} was the best assayed treatment, reducing MAM populations by 2 log_{10} compared to the untreated control, which was similar to chlorine washing efficacy. The application of lower (0.3 kJ m^{-2}) or higher doses (0.9 and 1.8 kJ m^{-2}) failed to reduce MAM populations compared to the water control. This was in line with previous results showing that the reduction of native microbiota is not always proportionally correlated to the increase of UV-C dose, probably due to the differential sensitivity or tolerance of the microorganisms and the plant response threshold (Martínez-Hernández, Huertas, *et al.*, 2015; Graça *et al.*, 2017). Yeasts and molds populations on broccoli florets were reduced to levels close to the detection limit just with water washing, thus the usefulness of WUV beyond this effect could not be established. On the other hand, after broccoli sanitation with any of the assayed WUV doses, yeasts and molds suspended in the process water were reduced by

0.4 and 0.9 log_{10} in respect of water-washing, respectively, reaching undetectable levels and MAM were reduced by values ranging from 1.7 to 2.8 log_{10}. Furthermore, any of the assayed doses affected the firmness or the color parameters immediately after treatment or after 24 h of refrigerated storage. Based on those results and in the better fitness of shorter treatment times for industrial applications, we discarded 0.9 and 1.8 kJ m^{-2} and continued the analyses using the two lower doses.

For further improving the WUV effectiveness in a worst case scenario including higher microbial load and more heterogeneous microbiome (Lupatini *et al.*, 2017), we tested the combination of WUV and peroxyacetic acid for the decontamination of the native microbiota of fresh-cut broccoli from organic practices. We observed that in such case, WUV treatments with 0.3 or 0.5 kJ m^{-2} alone were not sufficient to reduce initial MAM populations beyond water-washing. Using the combination of 0.3 kJ m^{-2} and 50 or 80 mg L^{-1} PAA resulted in 2 log_{10} reduction, which was 10-fold more effective than water-washing and was similar result to the obtained in conventional broccoli using 0.5 kJ m^{-2} WUV alone. Due to higher initial yeast and molds titers compared to conventional broccoli, a reduction by 1.5 log_{10} of molds was obtained with any of the WUV and PAA combinations, whereas a reduction by 1.8 log_{10} of yeasts was achieved with 0.3 + kJ m^{-2} and 80 mg L^{-1} PAA or with 0.5 kJ m^{-2} and either of both PAA doses. In both cases, the results improved by 1 log_{10} the efficacy of water-washing. The reductions of MAM and yeasts obtained with the 0.3 kJ m^{-2} + PAA (50 or 80 mg L^{-1}) treatments were similar to that obtained with the PAA controls. However, the combined treatments were better at preserving broccoli quality since the use of PAA alone was associated to a reduction of firmness and the hue angle, 24 after treatment. Therefore, 0.5 m^{-2} + 50 mg L^{-1} PAA was the best alternative to reduce native microbiota in organic broccoli.

On the other hand, when used for the decontamination of inoculated microbiota in organic broccoli, WUV treatments at a dose of 0.3 kJ m^{-2} were effective for

reducing *L. innocua* populations by 1.7 $\log_{10}$ in respect of untreated control whereas combining this dose with 50 or 80 mg L^{-1} PAA did not improve the results. Increasing WUV dose to 0.5 kJ m^{-2} improved the reducing effect on *L. innocua* populations to 2.4 $\log_{10}$ reduction. In the same way, this inhibitory effect was maintained during 4 and 8 d of MAP storage at 5 ^{O}C in darkness, showing population increases below those observed for the water-washed control (by 1.5 and 1 $\log_{10}$, respectively). A similar result was obtained with chlorine sanitation. On the other hand, *L. innocua* was reduced to undetectable levels (< 5 CFU mL^{-1}) in the process bath after broccoli sanitation when using WUV alone or combined with PAA, regardless of the dose, which was at least 3 $\log_{10}$ more effective than a single-use water wash.

We evaluated the effect of WUV (0.5 kJ m^{-2}) on the resistance mechanisms of *L. innocua* to stress, including the putative recovery of its populations due to its ability to repair DNA damages caused by UV-C irradiation, during storage exposed to white light. We also evaluated the survival of this species to 2 min microwave cooking (800 W), before and after 8 d of refrigerated storage in MAP.

No differences in *L. monocytogenes'* growth between samples exposed or not to daylight were observed (Fig. **IX**.9). This suggested that the applied UV-C dose did not activate the pathogen's DNA repair systems or at least that repaired irradiated cells remained in an uncultivable stage (Kramer and Muranyi, 2014; Lasagabaster and Martínez- de Marañón, 2014). In the same way, no changes in survival after microwave cooking were observed for WUV treated and untreated samples, regardless of exposure or not to daylight, showing levels close to detection limit (5 CFU g^{-1}) in all cases.

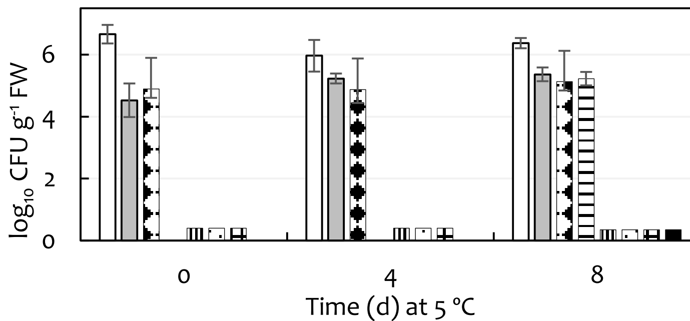

Figure IX.9. Survival of *L. innocua* after WUV (0.5 kJ m^{-2}) treatment during storage in MAP: (☐) water-washed control, (▒) chlorine-washed control, (▦) WUV-treated stored in darkness, (▤) WUV-treated stored in light, and after microwave cooking: (▥) water-washed control, (⬚) chlorine-washed control, (▦) WUV-treated stored in darkness, (■) WUV-treated stored in light. Bars represent means from dual independent experiments and error bars represent standard deviations (n=6).

Regarding the hormetic effect of WUV on the biochemical quality all of the assayed UV-C doses increased TAC in conventional broccoli 24 h after treatment, as previously reported for several UV-C doses and broccoli varieties (Lemoine, Chaves and Martínez, 2010; Martínez-Hernández *et al.*, 2011; A.C. Formica-Oliveira *et al.*, 2017), unlike chlorine treatment. In contrast with those previous works we did not observed an increase in phenolic content, probably because the activation of the phenylpropanoid pathway was not significant during the first 24 h, as has been reported in other studies (Martínez-Hernández *et al.*, 2011; Duarte-Sierra, 2015). Therefore, the increased TAC might be explained by increased amounts of other antioxidant compounds (AA, GSH) or the induction of enzymatic pathways (Zhang *et al.*, 2017). We could not observe any effect on the chlorophylls or carotenoid content upon any of the applied treatments, unlike previous reports in UV-C-treated broccoli (Martínez-Hernández *et al.*, 2011; Costa *et al.*, 2006).

In summary, the application of 0.5 kJ m^{-2} WUV allowed the reduction of MAM populations in conventional broccoli with the same efficacy as chlorine, increasing total antioxidant capacity and sulforaphane content without affecting

the product's physical quality. Combining UV-C and PAA allowed reductions of native MAM, yeasts and molds populations in organic broccoli with the same efficacy as the obtained with WUV alone in conventional broccoli, without significantly affecting its physical quality. Doses of 0.3 and 0.5 kJ m^{-2} WUV did not reduce native microbiota populations of organic broccoli compared to the water-washed control but allowed the reduction of inoculated microbiota (*L. innocua*) by more than 2 log_{10}.

Ready-to-eat leafy greens

WUV was also assayed for the sanitation of fresh-cut green leafy vegetables. Preliminary dose selection (0.1, 0.2, 0.3 and 0.5 kJ m^{-2}) was based on the effect of WUV on the general appearance of the processed vegetables and on the dynamics of the gas headspace composition of packages during storage in darkness for 6 d at 5 °C in MAP. Results showed that WUV at doses between 0.1 and 0.3 kJ m^{-2} preserved the general appearance of fresh-cut 'Iceberg' lettuce throughout storage. Furthermore, 0.5 kJ m^{-2} WUV correlated with an accelerated O_2 reduction compared to the water control. This effect has previously observed in fresh-cut 'Read Oak Leaf' lettuce' and was associated to physiological stress (Allende and Artes, 2003). Therefore, this dose was discarded, and 0.1 kJ m^{-2} was selected for further experiments. Two WUV doses were tested for the sanitation of baby spinach leaves, 0.2 and 0.3 kJ m^{-2}, both of which preserved overall quality and showed no detectable effect on respiration compared to the water control. Therefore, both doses were assayed for the next stage of experiments. Is this stage the effectiveness of WUV for the reduction of bacterial populations resulting from dip-inoculation of each vegetable in a multi-pathogenic cocktail containing 5 strains of *S. enterica* and 4 strains of *L. monocytogenes* (5 log_{10} CFU mL^{-1} each), was evaluated immediately after treatment and throughout 6 d of MAP refrigerated storage in darkness. Additionally, in order to further improve WUV efficacy, the effect of the combination of the selected WUV dose (s) with

either 40 or 80 mg L^{-1} PAA for the sanitation and growth inhibition of both pathogen's populations during MAP refrigerated storage, was also assessed.

Results revealed that 0.1 kJ m^{-2} alone reduced initial populations of *S. enterica* and *L. monocytogenes* in 'Iceberg' lettuce by > 2 log_{10} and the inhibition remained until the end of storage. Combining WUV (0.1 kJ m^{-2}) with PAA did not resulted in synergistic effect for sanitizing this matrix compared to the individual control treatments, but allowed to inactivate pathogens populations in the sanitation solutions improving the results obtaining by using PAA alone. In baby spinach leaves, increasing the WUV dose from 0.2 to 0.3 kJ m^{-2} WUV, improved the effectiveness for the sanitation of *L. monocytogenes* (from 0.5 to 2.2 log_{10} reduction) immediately after treatment, regardless of the PAA dose, while showed no improvement for the control of *S. enterica* (0.9 log_{10} reduction). Nevertheless, the combination of 0.3 kJ m^{-2} WUV + 40 mg L^{-1} PAA was the best alternative to control both pathogens in this matrix as well as in the sanitizing solution, compared to PAA sanitation alone. The best selected doses allowed an improvement by about 1 log_{10} for the reduction of both pathogens in lettuce and about 2 log_{10} in spinach, compared to water-washing.

9.2.2 PULSED LIGHT

FRESH-CUT BROCCOLI

Pulsed light was assessed as another non-thermal technology alternative to chlorine for the decontamination of fresh-cut conventional broccoli since it has been effective for reducing the populations of a wide range of microorganisms in various fruit and vegetables with different irregular surfaces in very short treatment times, but it had never been assayed in this commodity (Luksiene *et al.*, 2012; Xu and Wu, 2016). As PL spectrum includes UV light, its hormetic effect on the enhanced ability of the plant to respond to a subsequent pathogenic attack, an on the nutritional quality, including the increase of antioxidants and

glucosinolates contents, the delay of chlorophylls degradation, were also evaluated. We started the analyses with an optimization stage aimed at reducing native MAM populations while preserving physical quality parameters (overall appearance and color) during 14 d of refrigerated storage in air at darkness.

Results showed that PL treatments with 5, 10, and 15 kJ m^{-2} were ineffective to reduce MAM populations compared to water-washing during 14 d of refrigerated storage. Treatment with 4 pulses (20 kJ m^{-2}) reduced mesophilic microorganisms by 1.8 log_{10} in respect of water-washing on day 14, but it was detrimental to the overall quality of the product. PL doses up to 4-fold higher (60 and 80 kJ cm^{-2}) than the highest dose presently assayed, have been used to treat fresh-cut tomato slices achieving reductions of molds and yeasts by 0.7 and 1.1 log_{10}, respectively, whereas were ineffective against psychrophilic bacteria (Valdivia-Nájar, Martín-Belloso and Soliva-Fortuny, 2018). However, at such levels, PL exposure entailed higher weight and texture losses throughout storage than the untreated control, in a dose dependent manner. Therefore, the immediate lower dose, 3 pulses of light (15 kJ m^{-2}) was selected for subsequent analyses testing the immediate reduction of inoculated *L. innocua* and native microbiota (molds, yeasts and MAM) in fresh-cut broccoli and the persistence of the antimicrobial and nutritional-enhancing effects throughout storage for up to 15 d at 5 °C. Results showed that the assayed PL dose was ineffective for decontamination compared to water-washing, regardless of whether *L. innocua* was inoculated before or after PL treatments. Hence, no direct or indirect germicidal effects were observed. Regarding the nutritional attributes of the commodity upon PL application, TAC was increased by 12 and 18% in PL-treated samples compared to the water and chlorine-washed controls, respectively, 24 h post-processing. However, we found no significant change in phenolic compounds, carotenoids, chlorophylls, or glucosinolates compared to the water control, unlike previous reports showing that exposure of broccoli to the UV-C or UV-B portions of white light induced genes related to the secondary

metabolism and increased the accumulation of those compounds (Mewis *et al.*, 2012; Formica-Oliveira *et al.*, 2017; Darré *et al.*, 2017). In general, PL treatments, at the assayed doses and applied in air, were ineffective for improving the microbiological quality of fresh-cut broccoli.

9.2.3 PHYSICAL-CHEMICAL-BIOLOGICAL INTEGRATED STRATEGIES

INTEGRATION OF PAA-ASSISTED UV-C AND CPA-7 FOR THE SANITATION OF READY-TO-EAT LEAFY GREENS

Pretreatment with WUV + PAA synergistically enhanced (by 2-fold) the inhibitory effect of CPA-7 on *L. monocytogenes* growth in 'Iceberg' lettuce after 2 d of refrigerated storage in MAP. Other authors have reported that the dip-inoculation with antagonistic yeasts after pretreatment with UV-C further improved the UV-C hormetic effects resulting in enhanced resistance to subsequent infection with fungal plant pathogens and better maintenance of quality throughout storage in several fruits (Xu and Du, 2012; Ou *et al.*, 2016). Those effects have been correlated with lower activities of cell-wall degrading enzymes and with the enhancement of both non-enzymatic and enzymatic antioxidant mechanisms as well as the increase in activities of PR-protein (Ou *et al.*, 2016). Thus, based on the results presented in chapter 4 showing the induction of antioxidant enzymatic mechanisms in fresh-cut apple by CPA-7, it would be logical to think that this bacterium could potentially contribute to induce these processes in baby spinach. Therefore, further gene-expression and enzymatic activity studies of such mechanisms in ready-to-eat vegetables would complement the present results.

On the other hand, the antagonistic activity of CPA-7 prevented *L. monocytogenes* growth after 6 d of chilled storage in samples pretreated with PAA, but that effect was not observed in lettuce. Contrastingly, upon a cold-chain breakage, CPA-7 prevented the growth of *S. enterica* both in lettuce and in

spinach (by 0.9 ± 0.1 and 0.2 ± 0.1 log_{10}, respectively, in respect of the PAA-control). In such conditions, CPA-7 showed an inconsistent effect on *L. monocytogenes* populations in 'Iceberg' in lettuce, while showed no effect at all on this pathogen in baby spinach leaves. Even when the effectiveness of CPA-7's antagonistic activity vary upon the interaction with the several factors involved in hurdle preservation strategies, including this bacterium as a another hurdle may improve the outcome of presently used strategies regarding the microbial safety of fresh-cut produce in certain conditions, and it is matter worthy to be further studied.

GENERAL CONCLUSIONS

BIOPRESERVATION USING THE BACTERIUM *P. GRAMINIS* CPA-7: UNRAVELING THE ACTIONS MECHANISMS AGAINST FOODBORNE PATHOGENS

CPA-7 biopreservative activity is carried out through a combination of several action mechanisms including competition for ecological niche, reduction of pathogen's colonization abilities, and modulation of enzymatic and non-enzymatic antioxidant mechanisms involved in the plant host defense response.

This general conclusion is supported by the following findings:

COMPETITION FOR NUTRIENTS

CPA-7 showed a restrictive overlapping index (NOI 0.92) in respect of *E. coli* O157:H7 for the *in vitro* use of organic acids, and showed a shared use of a several carbon and nitrogen sources naturally present in fruits and vegetables, with *E. coli* O157:H7, and *S. enterica* subsp. *enterica,* which suggested putative targets for *in vivo* competition.

CPA-7 was able to produce siderophores when grown at 30 °C in Brain heart infusion-Chrome azurol S (BHI-CAS) solid medium but not in Trytone soy agar-Chrome azurol S (TSA-CAS), which suggested that competition for iron could be an action mechanism for biocontrol under certain growth conditions.

COMPETITION FOR SPACE

CPA-7 did not form biofilms when grown statically in liquid M63 minimal medium supplemented with 0.2% glucose and 0.5% casaminoacids or in tryptone soy broth medium during 72 h either at 25 or at 30 °C.

CPA-7 did not show biosurfactant activity when grown in liquid King's B medium for 24 h at 25 °C.

DIRECT ANTIMICROBIAL EFFECT

CPA-7 was unable to produce detectable non-volatile compounds with antimicrobial activity against E. coli O157:H7, *S. enterica* or *L. monocytogenes*, during its growth until the stationary phase neither in liquid media (M63 minimal medium supplemented with 0.2% glucose and/or 0.5 % casaminoacids or tryptone soy broth medium) or on solid medium (glucose meat extract agar), nor in 'Piel de melon' juice or on 'Galia' melon pieces.

CPA-7 showed no proteolytic activity on plate count agar supplemented with 10% skim milk, nor hemolytic activity on Columbia blood agar.

IMPAIRMENT OF FOODBORNE PATHOGENS' PATHOGENIC ACTIVITY AND COMPETITION FOR SITES AT THE INTESTINAL EPITHELIUM SURFACE

CPA-7 reduced by 0.8 $\log_{10}$ the adhesiveness of *S. enterica* subsp. *enterica* ser. *Enteritidis* regardless of the habituation on fresh-cut 'Conference' pear, whereas showed no effect on *L. monocytogenes* adhesiveness, irrespective of the habituation on the fruit.

After pre-incubation on fresh-cut pear for 7 d at 10 °C, CPA-7 reduced the invasiveness of both *L. monocytogenes* and *S. enterica* subsp. *enterica* ser. *Enteritidis* to Caco-2 human tumor cell line, which reduced their pathogenic potential.

A putative competition with pathogens for adhesion sites at the human intestinal epithelium surface was enabled by CPA-7's ability to adhere to Caco-2 cells regardless of pre-incubation on fresh-cut 'Conference' pear.

The probabilities for competition at the intestinal epithelium surface were narrowed by the drastic reduction (by ≥ 6 log_{10}) of CPA-7 populations after gastrointestinal digestion.

The probabilities for competition between CPA-7 and *S. enterica* or *L. monocytogenes* at the intestinal epithelium surface were even more limited after pre-incubation for 7 d at 10 °C on fresh-cut 'Conference' pear, because it reduced its adhesiveness to Caco-2 cells (by 0.8 log_{10}) in respect of inoculation day.

INDUCTION OF PLANT HOST RESISTANCE MECHANISMS

CPA-7 induced a 4-fold increase in ascorbate peroxidase activity in fresh-cut 'Golden delicious' apple 24 h post-processing both in air and in MAP compared to non-inoculated fruit, while suppressed peroxidase and catalase activities in MAP.

CPA-7 induced a biphasic activation of polyphenol oxidase at 24 h and at 6 d of storage in MAP, reaching levels 2-fold higher than those in non-inoculated control at the end of the experiment, yet without significant increase in browning.

CPA-7 induced by 2-fold the activities of superoxide dismutase and polyphenol oxidase after 3 d in MAP conditions, compared to non-inoculated control.

After the initial increase in total phenolic content in fresh-cut 'Golden delicious' apple as a response to wounding, CPA-7 delayed the decay in the content of those compounds until day 3 of air storage, thereby leading to a better maintenance of fruit nutritional properties.

Inoculation with CPA-7 slowed down the decline of antioxidant capacity in air, while showed no effect upon MAP conditions.

CPA-7 reduced the accumulation of ethylene in fresh-cut 'Golden delicious' apple wedges during refrigerated (5 °C) MAP storage, which could contribute to a delay of fruit senescence, but this effect was inhibited by the ascorbate-based antioxidant treatment.

PRESERVATION OF FRESH-CUT VEGETABLES USING WATER-ASSISTED UV-C AND PAA-ASSISTED UV-C

Water-assisted UV light delivered in water or in peroxyacetic acid, was effective for controlling both native microbiota and *L. innocua* on fresh-cut broccoli, as well as for increasing its contents in antioxidants and sulforaphane.

Low-dose UV-C delivered in PAA controlled *S. enterica* and *L. monocytogenes* in fresh-cut 'Iceberg' lettuce and baby spinach leaves as well as in the sanitation solution, improving the safety of the vegetable products and the efficiency of the process.

Those general conclusions are supported by the following findings:

UV-C IN WATER OR PEROXYACETIC ACID FOR THE DECONTAMINATION OF FRESH-CUT BROCCOLI

Exposure of fresh-cut conventional broccoli to 0.5 kJ m^{-2} WUV achieved a 2 log_{10} reduction of total mesophilic microorganisms which was the same efficacy showed by chlorine sanitation.

Treatments with 0.5 kJ m^{-2} WUV increased total antioxidant capacity by 90% and sulforaphane content by 1.5-fold in fresh-cut conventional broccoli in respect of the water control, without significantly affecting the product's physical quality.

Treatments with UV-C delivered in water or in PAA, preserved the phenolic and chlorophylls contents in fresh-cut conventional and organic broccoli.

WUV at doses ranging from 0.5 to 1.8 kJ m^{-2} reduced MAM populations from 1.3 to 2.8 log_{10} and yeasts and molds by > 2.5 log_{10} in the water used for a single sanitation of either organic or conventional broccoli.

Treatments with 0.3 kJ m^{-2} WUV reduced by 1.7 log_{10} *L. innocua* populations in fresh-cut organic broccoli in respect of inoculated levels. The efficacy was increased to a 2.4 log_{10} reduction with the increase of the dose (0.5 kJ m^{-2}).

The inhibitory effect of the treatment with 0.5 kJ m^{-2} WUV on *L. innocua* populations in fresh-cut organic broccoli remained during 8 d of refrigerated MAP storage.

No reversibility of the UV-C (0.5 kJ m^{-2}) deleterious effect, as measured by *L. innocua* population's recovery, was detected after 8 d in MAP at 5 ºC, regardless of exposure daylight.

WUV treatments alone, at doses of 0.3 or 0.5 kJ m^{-2}, did not reduce the native mesophilic aerobic microbiota of fresh-cut organic broccoli compared to the water-washed control.

UV-C (0.5 kJ m^{-2}) delivered in 50 mg L^{-1} PAA improved the effect of UV-C delivered in water for the sanitation of fresh-cut organic broccoli, achieving a 2 log_{10} reduction of native total mesophilic aerobic microorganisms and 1 log_{10} reductions of native yeasts and molds populations, without significantly affecting the product's physical quality.

UV-C IN WATER OR PEROXYACETIC ACID FOR THE DECONTAMINATION OF READY-TO-EAT LEAFY GREENS

Treatments with WUV reduced *S. enterica* and *L. monocytogenes* populations at doses of 0.2 or 0.3 kJ m^{-2} in fresh-cut 'Iceberg' lettuce and baby spinach leaves, respectively, without negative effects on respiration or overall quality.

Increasing the WUV dose from 0.3 to 0.5 kJ m^{-2} improved the effectiveness for reducing *L. monocytogenes* populations in baby spinach leaves immediately after treatment but did not improve the reduction of *S. enterica.*

UV-C delivered in 40 mg L^{-1} PAA, at doses of 0.1 kJ m^{-2} for fresh-cut 'Iceberg' lettuce or 0.3 kJ m^{-2} for baby spinach leaves, improved the control of *L. monocytogenes* in spinach in respect of UV-C delivered in water but showed no improvement in lettuce.

Native total mesophilic microbiota was not reduced after treatment with 0.2 kJ m^{-2} WUV + 40 mg L^{-1} PAA in 'Iceberg' lettuce or with 0.5 kJ m^{-2} UV-C delivered in 40 mg L^{-1} PAA in baby spinach leaves, either immediately after treatment or after 6 d of refrigerated MAP storage.

UV-C (0.2 or 0.5 kJ m^{-2}) delivered in 40 mg L^{-1} PAA completely inactivated *L. monocytogenes* and *S. enterica* in the solutions resulting from a single sanitation of 'Iceberg' lettuce or baby spinach leaves.

PRESERVATION OF FRESH-CUT BROCCOLI USING PULSED LIGHT

Pulsed light technology was not effective at reducing the populations of either native microbiota or *L. innocua* in fresh-cut broccoli without reducing the overall quality, but increased the antioxidant activity and preserved the glucosinolates contents.

This general conclusion is supported by the following findings:

Pulsed light at doses of 5, 10, and 15 kJ m^{-2} did not reduce the native microbiota of fresh-cut conventional broccoli either immediately after treatment nor during storage for 14 d at 5 °C in air at darkness.

Four pulses of light (20 kJ m^{-2}) reduced the native total mesophilic aerobic microbiota on fresh-cut conventional broccoli by 1.8 log_{10} in respect of the water-washed control after 14 d of refrigerated storage in air at darkness, but it was detrimental to the overall quality of the product.

Three pulses of light (15 kJ m^{-2}) showed no direct or hormetic antimicrobial effect on inoculated *L. innocua* on fresh-cut conventional broccoli during 14 d of refrigerated storage in air at darkness.

Three pulses of light increased total antioxidant capacity by 12 and 18% in fresh-cut conventional broccoli compared to the water and chlorine-washed controls, respectively, 24 h post-processing.

Three pulses of light preserved the chlorophylls, glucosinolates and phenolic contents in fresh-cut broccoli during aerobic refrigerated storage, compared to the water-washed control.

PRESERVATION OF FRESH-CUT VEGETABLES USING PHYSICAL-CHEMICAL-BIOLOGICAL INTEGRATED STRATEGIES

The integration of a pretreatment consisting of UV-C delivered in PAA, followed by inoculation with CPA-7 could be a suitable chlorine-alternative sanitation strategy for controlling foodborne pathogens in leafy greens in case of cross-contamination before or after the decontamination step.

This general conclusion is supported by the following findings:

Pretreatment with UV-C in PAA followed by inoculation with CPA-7 improved *S. enterica* growth inhibition in baby spinach leaves after 6d in MAP at 5 °C compared to the controls inoculated with CPA following PAA pretreatment or pretreated with UV-C in PAA; whereas showed no synergistic effect on this pathogen in 'Iceberg' lettuce.

Inoculation with CPA-7 following the pretreatment with UV-C in PAA showed synergistic effect for the reduction of *L. monocytogenes* populations after 2d in modified atmosphere packaging at 5 °C in lettuce but was inconsistent for the rest of the evaluated conditions.

CPA-7 controlled *S. enterica* populations upon a cold-chain breakage in 'Iceberg' lettuce but this ability was hampered by the WUV in PAA pre-treatment.

CONCLUSIONS GENERALS

BIOPRESERVACIÓ MITJANÇANT LA SOCA BACTERIANA *P. GRAMINIS* CPA-7: ELUCIDANT ELS MECANISMES D'ACCIÓ CONTRA ELS PATÒGENS DE TRANSMISSIÓ ALIMENTÀRIA

L'activitat biopreservant de la soca *Pseudomonas graminis* CPA-7 es duu a terme mitjançant una combinació de diversos mecanismes d'acció, incloent-hi la competència per al nínxol ecològic, la reducció de les habilitats de colonització dels patògens i l'activació de mecanismes antioxidants enzimàtics i no enzimàtics que intervenen en la resposta de defensa de la planta.

Aquesta conclusió general està basada en els següents resultats:

COMPETÈNCIA PER A NUTRIENTS

La soca *P. graminis* CPA-7 va mostrar un índex de solapament restrictiu (NOI 0.92) respecte a E. coli O157: H7 en quant a l'ús in vitro d'àcids orgànics així com va mostrar un ús compartit amb E. coli O157: H7 i *S. enterica* subsp. *enterica*, de algunes fonts de carboni i nitrogen presents naturalment en fruites i hortalisses, el que va suggerir objectius probables per a la competència *in vivo*.

La soca *P. graminis* CPA-7 va poder produir sideròfors a 30 ° C en el medi sòlid Brain heart infusion-Chrome azurol S (BHI-CAS), però no en Tryptone soy agar- Chrome azurol S (TSA-CAS), el que va suggerir que la competència pel ferro podria ser un mecanisme d'acció en determinades condicions.

COMPETÈNCIA PER L'ESPAI

La soca *P. graminis* CPA-7 no va formar biofilms quan va créixer estàticament en medi de cultiu líquid mínim M63 suplementat amb 0,2% de glucosa i 0,5% de

casaminoàcids o en el medi de cultiu Tryptone soy broth durant 72 hores a 25 o a 30 ° C.

La soca *P. graminis* CPA-7 no va mostrar activitat biosurfactant quan es cultivà en medi líquid King's B durant 24 hores a 25 °C.

EFECTE ANTIMICROBIÀ DIRECTE

No es va detectar activitat antimicrobiana dels compostos no volàtils produïts per la soca *P. graminis* CPA-7 durant el seu creixement fins a la fase estacionària ni en medis líquids: medi mínim M63 suplementat amb 0,2% de glucosa i / o 0,5% de casaminoàcids o en medi Tryptone soy broth, ni en el medi sòlid glucosa extracte de carn agar, ni en suc de meló "Pell de gripau" o en peces de meló "Galia".

La soca *P. graminis* CPA-7 no va mostrar cap activitat proteolítica en medi sòlid suplementat amb 10% de llet desnatada, ni activitat hemolítica en medi Columbia agar.

REDUCCIÓ DEL POTENCIAL PATOGÈNIC I COMPETÈNCIA PER A LLOCS D'ADHESIÓ A LA SUPERFÍCIE DEL EPITELI INTESTINAL HUMÀ

La soca *P. graminis* CPA-7 redueix la adhesivitat de *S. enterica* subsp. *enterica* ser. Enteritidis en 0.8 $\log_{10}$ respecte al control, independentment de la preadaptació en pera "Conference" mínimament processada, mentre que no va mostrar cap efecte sobre l'adhesió de L. monocytogenes, independentment de la preadaptació en la fruita.

Després de la pre-incubació en pera mínimament processada durant 7 d a 10 °C, la soca *P. graminis* CPA-7 va reduir la invasió de *L. monocytogenes* i *S. enterica* subsp. *enterica* ser. Enteritidis a la línia cel·lular tumoral humana Caco-2, reduint el seu potencial patogènic.

La capacitat de la soca *P. graminis* CPA-7 per adherir-se a les cèl·lules Caco-2 va mostrar la possibilitat per el establiment de competència amb els patògens per a llocs d'adhesió a la superfície epitelial de l'intestí humà, independentment de la pre-incubació en la pera "Conference" mínimament processada.

Les probabilitats de competència en la superfície de l'epiteli intestinal es van reduir després de la digestió gastrointestinal per la reducció dràstica (≥ 6 log10) de les poblacions de la soca *P. graminis* CPA-7.

Les probabilitats de competència entre la soca *P. graminis* CPA-7 *i S. enterica* subsp. *enterica* ser. Enteritidis o *L. monocytogenes* a la superfície de l'epiteli intestinal varen ser encara més limitades després de la pre-incubació durant 7 d a 10 °C en pera "Conference" mínimament processada, ja que es va reduir la seva capacitat de adherència a les cèl·lules Caco-2 en 0,8 log10 respecte al dia d'inoculació.

INDUCCIÓ DELS MECANISMES DE DEFENSA DE LA PLANTA

La soca *P. graminis* CPA-7 va induir un augment de 4 vegades de l'activitat de l'ascorbat peroxidasa en pomes "Golden delicious" en comparació amb fruites no inoculades, 24 hores després del processament, tant a l'aire com a atmosfera modificada passiva (MAP), mentre que va suprimir les activitats de peroxidasa i catalasa en MAP.

La soca *P. graminis* CPA-7 va induir una activació bifàsica de la polifenol oxidasa a les 24 hores i a els 6 d d'emmagatzematge en atmosfera modificada passiva, assolint nivells 2 vegades més alts que el control al final de l'experiment, encara que sense un augment significatiu del enfosquiment.

La soca *P. graminis* CPA-7 va induir les activitats de la superóxido dismutasa i la polifenol oxidasa per 2 vegades respecte al control no inoculat després de 3 d en atmosfera modificada passiva.

Després de l'augment inicial del contingut fenòlic total en pomes "Golden delicious" com a resposta al tall, la soca *P. graminis* CPA-7 va alentir la reducció del contingut d'aquests compostos fins al dia 3 d'emmagatzematge a l'aire, el que va conduir a un millor manteniment de les propietats nutritives de la fruita.

La inoculació amb la soca *P. graminis* CPA-7 va frenar la disminució de la capacitat antioxidant de la poma a l'aire, mentre que no va mostrar efecte en la atmosfera modificada passiva.

La soca *P. graminis* CPA-7 va reduir l'acumulació d'etilè en la poma 'Golden delicious' mínimament processada durant emmagatzematge en atmosfera modificada passiva a 5 °C, el que podria contribuir al retard de la senescència de la fruita, però aquest efecte va ser inhibit pel tractament antioxidant amb ascorbat càlcic.

HIGIENITZACIÓ DE VEGETALS DE QUARTA GAMMA AMB LLUM ULTRAVIOLADA-C EN MEDI LÍQUID

La llum ultraviolada C en aigua o en àcid peroxiacètic, va ser efectiva tant per a controlar la microbiota epifítica y els patògens de transmissió alimentaria del bròquil de quarta gamma com per augmentar el seu contingut en compostos bioactius.

El tractament amb la llum ultraviolada a dosis baixes amb àcid peroxiacètic va ser efectiu per a controlar *S. enterica* i *L. monocytogenes* en enciam "Iceberg"

mínimament processat i en brots de espinacs així como en la solució d'higienització, millorant la seguretat del producte i l'eficiència del procés.

Aquestes conclusions generales estan basades en els següents resultats:

LA LLUM ULTRAVIOLADA EN AIGUA O EN ÀCID PEROXOACÈTIC PER A LA HIGIENITZACIÓ DE BRÒQUIL DE IV GAMMA

L'exposició de bròquil de producció convencional a la llum ultraviolada en aigua a 0.5 kJ m-2 va reduir les poblacions dels microorganismes mesòfils totals amb la mateixa eficàcia que va mostrar la higienització amb clor (2 log10).

El tractament amb 0.5 kJ m-2 de llum ultraviolada en aigua van augmentar la capacitat antioxidant total del bròquil de producció tradicional en un 90% i el contingut de sulforafà per 1,5 vegades respecte al control rentat amb aigua, sense afectar significativament la qualitat física del producte.

Els tractaments amb llum ultraviolada en aigua o en àcid peroxoacètic , van preservar els continguts fenòlics i en clorofil·les en el bròquil mínimament processat, tant els de producció orgànica com els convencional.

La llum ultraviolada en aigua a dosis que van de 0,5 a 1,8 kJ m-2 van reduir les poblacions de microorganismes aerobis mesòfils d'1,3 a 2,8 log_{10} així com de llevats i floridures en mes de 2,5 log_{10}, en el aigua després de la higienització en el bròquil de quarta gamma, orgànic o convencional.

L'efecte germicida de la llum ultraviolada en L.innocua en bròquil orgànic mínimament processat es va incrementar al augment la dosis, aconseguint reduccions de 1,7 log10 i 2.4 log_{10} al utilitzar 0,3 kJ m^{-2} i 0,5 kJ m^{-2}, respectivament.

L'efecte inhibitori del tractament amb 0,5 kJ m^{-2} de llum ultraviolada en aigua en la població de *L. innocua* en bròquil orgànic de quarta gamma es va

mantenir durant 8 d d'emmagatzematge refrigerat en atmosfera modificada passiva, a diferencia del control rentat amb aigua.

La població de *L. innocua* no es va recuperar durant els 8 d d'exposició a la llum blanca o la foscor després del tractament amb 0.5 kJ m^{-2} de llum ultraviolada en aigua , suggerint l'absència de reversibilitat de l'efecte perjudicial de la llum ultraviolada- C durant l'emmagatzematge en atmosfera modificada passiva .

Els tractaments de llum ultraviolada en aigua a dosis de 0,3 o 0,5 kJ m^{-2}, no van reduir la microbiota epifítica del bròquil orgànic recentment tallat en comparació amb el control rentat amb aigua.

El tractament de bròquil orgànic de quarta gamma amb llum ultraviolada (0.5 kJ m^{-2}) en àcid peroxoacètic (50 mg L^{-1}) va millorar la eficàcia respecto a la aplicació amb aigua aconseguint una reducció des microorganismes mesòfils totals en 2 log_{10} i dels llevats i floridures en 1 log_{10}, sense afectar significativament la qualitat física del producte.

LA LLUM ULTRAVIOLADA EN AIGUA O EN ÁCID PEROXIACÉTIC PER A LA HIGIENITZACIÓ DE VERDURES DE QUARTA GAMMA

Els tractaments amb llum ultraviolada en aigua entre 0.1 y 0.3 kJ m^{-2} van permetre la descontaminació i la inhibició del creixement de *S. enterica* i de *L. monocytogenes* durant l' emmagatzematge, tant en enciam "Iceberg" mínimament processat com en brots de espinacs, sense afectar significativament a la respiració o la qualitat general.

L'augment de la dosi de la llum ultraviolada en aigua de 0,3 a 0,5 kJ m^{-2} per el tractament de brots d'espinacs va millorar l'efectivitat en quant a la reducció inicial de les poblacions de *L. monocytogenes* però no de *S. enterica.*

L'aplicació de la llum ultraviolada en àcid peroxiacètic (40 mg L^{-1}) a les dosis de 0.1 kJ m^{-2} y 0.3 kJ m^{-2} no va donar com a resultat una reducció sinèrgica de les poblacions dels patògens en enciam "Iceberg" mínimament processat o en brots de espinacs respecte als controls físic o químic, però va permetre la inactivació dels dos patògens en les solucions resultants del procés de higienització després del sanejament únic de les dos matrius, millorant la efectivitat del tractament químic individual.

La substitució del aigua per el àcid peroxiacètic (40 mg L^{-1}) en la aplicació de la llum ultraviolada acoblada a immersió per a enciam "Iceberg" mínimament processat (0.1 kJ m^{-2}) i brots d'espinacs (0.3 kJ m^{-2}), va millorar l'eficàcia en el control de *L. monocytogenes* en espinacs, però no en l'enciam.

L'aplicació de la llum ultraviolada en àcid peroxiacètic (40 mg L^{-1}) en enciam "Iceberg" mínimament processat i en brots d'espinacs, a dosis de 0,1 kJ m^{-2} i 0,3 kJ m^{-2} respectivament, no van reduir la microbiota aeròbia mesòfila total en aquestes verdures.

Preservació de bròquil de IV gamma amb llum polsada

La tecnologia de llum polsada no va ser eficaç a l'hora de reduir les poblacions de microbiota autòctona ni de *L. innocua* en bròquil de IV gamma sense reduir la qualitat general, però va augmentar l'activitat antioxidant i va conservar els continguts de glucosinolats a diferencia de la higienització amb clor.

Aquesta conclusió general esta basada en els següents resultats:

La llum polsada a dosis de 5, 10 i 15 kJ m^{-2} no va reduir la microbiota nativa del bròquil de producció convencional de IV gamma ni immediatament després del tractament ni durant l'emmagatzematge durant 14 d a 5 °C en l'aire i a la foscor.

El tractament de bròquil de producció convencional mínimament processat amb 4 polsos de llum (20 kJ m^{-2}) va reduir la microbiota mesòfila aeròbia total en 1.8 log10 respecte al control rentat amb aigua després de 14 d d'emmagatzematge refrigerat a l'aire i a la foscor, però va ser perjudicial per a la qualitat global del producte.

El tractament de bròquil de producció convencional mínimament processat amb 3 polsos de llum (15 kJ m^{-2}) no va mostrar efectes antimicrobians directes o hormètics sobre L. innocua durant 14 d d'emmagatzematge refrigerat a l'aire i a la foscor.

El tractament de bròquil de producció convencional mínimament processat amb 3 polsos de llum va augmentar l'activitat antioxidant total en un 12 i un 18% en comparació amb els controls rentats amb aigua o clor, respectivament, 24 h després del processat.

El tractament de bròquil de producció convencional mínimament processat amb 3 polsos de llum va preservar els continguts en clorofil·les, glucosinolats i compostos fenòlics en comparació amb el control rentat amb aigua durant l'emmagatzematge refrigerat en aire.

PRESERVACIÓ DE VEGETALS DE MÍNIMAMENT PROCESSATS EMPRANT ESTRATÈGIES INTEGRADES FÍSIC–QUÍMIC–BIOLÒGIQUES

El pretractament de vegetals de fulla verda llestos pel consum amb llum ultraviolada C en àcid peroxoacètic a dosis baixes podria implementar-se com estratègia de higienització alternativa al clor per millorar la seguretat d'aquests productes, ja que permet el control de patògens de transmissió alimentaria en el cas de contaminació creuada tant abans com després del procés.

Aquesta conclusió general està recolzada pels següents resultats:

L'efecte sinèrgic del pre-tractament amb llum ultraviolada C en àcid peroxoacètic i la biopreservació utilitzant la soca *P. graminis* CPA-7 va permetre una major inhibició de *L. monocytogenes* en enciam 'Iceberg' de quarta gamma als 2 d en atmosfera modificada passiva a 5 °C respecte als tractaments controls per separat, però va ser inconsistent en la resta de les condicions avaluades.

El pretractament de la llum ultraviolada en àcid peroxiacètic va inhibir el creixement de *L. monocytogenes* i de *S. enterica* en enciam 'Iceberg' de quarta gamma i *L. monocytogenes* en brots de espinacs respecte al control pretractat sol amb àcid peroxoacètic, durant 6 dies en atmosfera modificada passiva a 5 °C.

L'activitat antagònica de la soca *P. graminis* CPA-7 va controlar les poblacions de *S. enterica* en enciam 'Iceberg' de quarta gamma davant d'un possible trencament de la cadena de fred respecte al control tractat amb àcid peroxoacètic, però no va mostrar efecte en aquest patogen en brots de espinacs o en *L. monocytogenes* en ninguna de les dos matrius alimentaries avaluades.

FURTHER WORK

The results obtained in the present work provide novel information regarding the characterization of the action mechanisms of biopreservation and about the effectiveness of sanitation strategies combining physical, chemical and biological methods for the preservation of minimally processed vegetables. At the same time, they open the doors to a wide range of possibilities in which it would be interesting to deepen.

WIDENING THE KNOWLEDGE ABOUT COMPETITION FOR ECOLOGICAL NICHE

Since single carbon and nitrogen sources included in the GP2/GN2 system were not very representative of the composition of fruit and vegetables and therefore, their usefulness for determining restrictive overlapping in the use of different groups of compounds between CPA-7 and *S. enterica* or *L. monocytogenes* were limited. Furthermore, these systems failed to identify most of the compounds that can be used by the last mentioned microorganism. Therefore, a more suitable technology should be tested to elucidate putative targets for competition in a more realistic approach. One possibility could be the use of Phenotype Microarrays Biolog system, which includes 855 different carbon/nitrogen sources and has been used for the selection of biocontrol agents with highly competitive potential (Blumenstein *et al.*, 2015). GeoChip gene array is another proposed technology which has also been useful to study the functional structure and the metabolic diversity of microbial communities on inert and biological environments (Carter *et al.*, 2012). Additionally, experiments using diffusion chambers would complement the results by establishing a relation between growth inhibition and cell-to-cell contact and the subsequent reversal of this effect through the addition of the putative target for competition (Nilsson *et al.*, 2005; Saraoui *et al.*, 2016).

Additionally, based on the indications obtained in the present work about siderophores production by CPA-7 in certain culture media which suggested that this bacterium might have a competitive advantage against other microorganisms under ferric starvation, further expression analysis of the genes involved in the biosynthesis and regulation of siderophores in such conditions could be assayed. Moreover, the isolation of those molecules, their identification and quantification should be performed to characterize this mode of action in the fresh-cut produce environment.

Furthermore, studies of CPA-7 attachment and biofilms formation on fresh produce surfaces would complement the knowledge about its ability for competing for space.

EXPLOITING THE IMPAIRMENT OF FOODBORNE PATHOGENS' VIRULENCE

Our finding that the prior interaction between CPA-7 and the foodborne pathogens *S. enterica* and *L. innocua* on fresh-cut fruit reduced their capacity for internalizing the human host intestinal epithelium and thereby, their abilities to produce infection, opens the doors to the possibility of targeting storage as a moment for preventing disease development. Identifying the mechanisms and molecules involved in this effect would clarify the optimal conditions for this effect to occur and enable the possibility of enhancing it. Putative regulatory molecules could include quorum sensing signals as well as disrupters of pathogens intraspecific communication or regulators of the expression of virulence genes (Rasmussen and Givskov, 2006).

EXPLOITING THE EFFECT OF CPA-7 ON THE PLANT HOST RESPONSE

DELAY OF SENESCENCE: A POSSIBILITY FOR EXTENDING THE SHELF-LIFE OF FRESH PRODUCE

Indications about the modulation by CPA-7 of the accumulation of ethylene in fresh-cut apple wedges throughout refrigerated storage, and the presence of a gene encoding for ACC-deaminase in the genome of this bacterium, point out to its potential use for extending fresh-cut product shelf life through the reduction of stress-associated ethylene levels, thereby delaying senescence processes. In order to exploit that possibility, analyses of the expression of genes involved in the biosynthesis of this enzyme as well as enzymatic activity tests should be carried out to corroborate the functionality of ACD in CPA-7 and its activation upon stressful conditions.

ENHANCING THE PLANT DEFENSE RESPONSE FOR CONTROLLING FOODBORNE PATHOGENS

Although CPA-7 did not induce the increase of ethylene levels in fresh-cut apples, further pathogen-BCA co-inoculated trials testing the induction of the expression of JA and C_2H_4-responsive genes (such as *jar1, coi1 etr1, ein2*) should be conducted since it has been observed that the role of these molecules in ISR downstream signaling may be a result of an enhanced capacity to react to them, rather than of an increase in their production (Pieterse, 2001; van Loon, Geraats and Linthorst, 2006).

Furthermore, gene-expression analyses of the enzymes involved in ROS-scavenging mechanisms identified in the genome of CPA-7 would contribute to elucidate its resistance mechanisms to the oxidative stress associated to fresh-cut products which in turn, would provide adaptive advantage to this bacterium for outcompeting plant and foodborne pathogens in this environment (Castoria *et al.*, 2003; Parafati *et al.*, 2015; Spadaro and Droby, 2016).

DEVELOPMENT OF NOVEL COMBINED STRATEGIES FOR THE DECONTAMINATION OF FRESH-CUT VEGETABLES

We found that the substitution of water by a peroxyacetic acid aqueous solution was an effective alternative for improving the effect of WUV on the endemic microbiota of fresh-cut organic broccoli. However, biocidal chemicals are not currently allowed for this use in organic produce. Therefore, other integrated technologies such as combined WUV + mild heat treatments could be a currently approved alternative to be tested for this purpose.

Another approach for the decontamination of organic produce would be the development of a water-assisted alternative of the pulsed light technology similar to WUV, evaluated in the present work. A similar approach has already shown to be effective for the decontamination of native spoilage and inoculated pathogenic microbiota in other commodities (Huang and Chen, 2014; Huang *et al.*, 2015a) and its very short treatment times are highly advantageous for industrial applications.

REFERENCES

Abadias, M., Alegre, I., Usall, J., Torres, R. and Viñas, I. (2011) 'Evaluation of alternative sanitizers to chlorine disinfection for reducing foodborne pathogens in fresh-cut apple', *Postharvest Biology and Technology*, 59(3), pp. 289–297. doi: https://doi.org/10.1016/j.postharvbio.2010.09.014.

Abadias, M., Altisent, R., Usall, J., Torres, R., Oliveira, M. and Viñas, I. (2014) 'Biopreservation of fresh-cut melon using the strain *Pseudomonas graminis CPA-7.*', *Postharvest Biology and Technology*, 96, pp. 69–77. doi: https://doi.org/10.1016/j.postharvbio.2014.05.010.

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

Abdel-Daim, A., Hassouna, N., Hafez, M., Ashor, M. S. A. and Aboulwafa, M. M. (2013) 'Antagonistic activity of *Lactobacillus* isolates against *Salmonella typhi in vitro.*', *BioMed research international*, Article ID, p. 12 pp. doi: 10.1155/2013/680605.

Abdelhamid, A. G., Esaam, A. and Hazaa, M. M. (2018) 'Cell free preparations of probiotics exerted antibacterial and antibiofilm activities against multidrug resistant E. coli', *Saudi Pharmaceutical Journal*. King Saud University. doi: 10.1016/j.jsps.2018.03.004.

Abeles, F. B., Morgan, P. W. and Saltveit, M. E. J. (1993) 'Ethylene in Plant Biology.', *The Quarterly Review of Biology*. The University of Chicago Press, 68(1), pp. 116–117. doi: 10.1086/417964.

Adhikari, A., Syamaladevi, R. M., Killinger, K. and Sablani, S. S. (2015) 'Ultraviolet-C light inactivation of Escherichia coli O157: H7 and Listeria monocytogenes on organic fruit surfaces', *International Journal of Food Microbiology*. Elsevier B.V., 210, pp. 136–142. doi: 10.1016/j.ijfoodmicro.2015.06.018.

Agbor, T. A. and Mccormick, B. A. (2011) 'Salmonella effectors: Important players modulating host cell function during infection', *Cellular Microbiology*, 13(12), pp. 1858–1869. doi: 10.1111/j.1462-5822.2011.01701.x.

Aguayo, E., Escalona, V. H. and Artes, F. (2004) 'Metabolic behavior and quality changes of whole and fresh processed melon', *Journal of Food Science*, 69(4), pp. SNQ148-SNQ155. doi: 10.1111/j.1365-2621.2004.tb06356.x.

Aguero, M. V., Jagus, R. J., Martin-Belloso, O. and Soliva-Fortuny, R. (2016) 'Surface decontamination of spinach by intense pulsed light treatments: Impact on quality attributes', *Postharvest Biology and Technology*, 121, pp. 118–125. doi: 10.1016/j.postharvbio.2016.07.018.

Aguiló-Aguayo, I., Charles, F., Renard, C. M. G. C., Page, D. and Carlin, F. (2013) 'Pulsed light effects on surface decontamination, physical qualities and nutritional composition of tomato fruit', *Postharvest Biology and Technology*. Elsevier B.V., 86, pp. 29–36. doi: 10.1016/j.postharvbio.2013.06.011.

Aguiló-Aguayo, I., Gangopadhyay, N., Lyng, J. G., Brunton, N. and Rai, D. K. (2017) 'Impact of pulsed light on colour, carotenoid, polyacetylene and sugar content of carrot slices', *Innovative Food Science and Emerging Technologies*. Elsevier, 42(March), pp. 49–55. doi: 10.1016/j.ifset.2017.05.006.

Ahn, I.-P., Lee, S.-W. and Suh, S.-C. (2007) 'Rhizobacteria-induced priming in Arabidopsis is dependent on ethylene, jasmonic acid, and NPR1.', *Molecular plant-microbe interactions : MPMI*, 20(7), pp. 759–768. doi: 10.1094/MPMI-20-7-0759.

Akram, N. A., Shafiq, F. and Ashraf, M. (2017) 'Ascorbic Acid-A Potential Oxidant Scavenger and Its Role in Plant Development and Abiotic Stress Tolerance', *Frontiers in Plant Science*, 8(April). doi: 10.3389/fpls.2017.00613.

Alarcón-Flores, M. I., Romero-González, R., Martínez-Vidal, J. L. and Frenich, A. G. (2013) 'Multiclass determination of phytochemicals in vegetables and fruits by ultra high performance liquid chromatography coupled to tandem mass spectrometry', *Food Chemistry*, 141(2), pp. 1120–1129. doi: 10.1016/j.foodchem.2013.03.100.

Albrich, J. M., McCarthy, C. A. and Hurst, J. K. (1981) 'Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase', *Proceedings of the National Academy of Sciences of the United States of America*, 78(1), pp. 210–214. doi: 10.1073/pnas.78.1.210.

Alegre, I. (2012) *Capacidad de crecimiento de patógenos de transmisión alimentaria en manzana y melocotón mínimamente procesados y nuevas estrategias de intervención para mejorar su seguridad microbiológica*, *TDX (Tesis Doctorals en Xarxa)*. Available at: http://www.tdx.cat/handle/10803/84172 (Accessed: 20 November 2017).

Alegre, I., Viñas, I., Usall, J., Anguera, M., Altisent, R. and Abadias, M. (2013) 'Antagonistic effect of *Pseudomonas graminis* CPA-7 against foodborne pathogens in fresh-cut apples under simulated commercial conditions.', *Food microbiology*, 33, pp. 139–148. doi: https://doi.org/10.1016/j.fm.2012.09.007.

Alegre, I., Viñas, I., Usall, J., Teixido, N., Figge, M. J. and Abadias, M. (2013) 'Control of foodborne pathogens on fresh-cut fruit by a novel strain of *Pseudomonas graminis*.', *Food microbiology*, 34, pp. 390–399. doi: https://doi.org/10.1016/j.fm.2013.01.013.

Alexander, L. (2002) 'Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening', *Journal of Experimental Botany*, 53(377), pp. 2039–2055. doi: 10.1093/jxb/erf072.

Ali, H. M., El-Gizawy, A. M., El-Bassiouny, R. E. I. and Saleh, M. A. (2015) 'Browning inhibition mechanisms by cysteine, ascorbic acid and citric acid, and identifying PPO-catechol-cysteine reaction products', *Journal of Food Science and Technology*, 52(6), pp. 3651–3659. doi: 10.1007/s13197-014-1437-0.

Ali, S., Charles, T. C. and Glick, B. R. (2012) 'Delay of flower senescence by bacterial endophytes expressing 1-aminocyclopropane-1-carboxylate deaminase', *Journal of Applied Microbiology*, 113(5), pp. 1139–1144. doi: 10.1111/j.1365-2672.2012.05409.x.

Alkan, N. and Fortes, A. M. (2015) 'Insights into molecular and metabolic events associated with fruit response to post-harvest fungal pathogens', *Frontiers in Plant Science*, 6(October), pp. 1–14. doi: https://doi.org/10.3389/fpls.2015.00889.

Allende, A. and Artes, F. (2003) 'UV-C radiation as a novel technique for keeping quality of fresh processed " Lollo Rosso " lettuce', *Food Research International*, 36, pp. 739–746. doi: 10.1016/S0963-9969(03)00054-1.

Allende, A., McEvoy, J. L., Luo, Y., Artes, F. and Wang, C. Y. (2006) 'Effectiveness of two-sided UV-C treatments in inhibiting natural microflora and extending the shelf-life of minimally processed "Red Oak Leaf" lettuce', *Food Microbiology*, 23(3), pp. 241–249. doi: 10.1016/j.fm.2005.04.009.

Alothman, M., Bhat, R. and Karim, A. A. (2009) 'Effects of radiation processing on phytochemicals and antioxidants in plant produce', *Trends in Food Science and Technology*. Elsevier Ltd, 20(5), pp. 201–212. doi: 10.1016/j.tifs.2009.02.003.

Altenhoefer, A., Oswald, S., Sonnenborn, U., Enders, C., Schulze, J., Hacker, J. and Oelschlaeger, T. A. (2004) 'The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens', *FEMS Immunology and Medical Microbiology*, 40(3), pp. 223–229. doi: 10.1016/S0928-8244(03)00368-7.

Alvaro, J. E., Moreno, S., Dianez, F., Santos, M., Carrasco, G. and Urrestarazu, M. (2009) 'Effects of peracetic acid disinfectant on the postharvest of some fresh vegetables', *Journal of Food Engineering*. Elsevier Ltd, 95(1), pp. 11–15. doi: 10.1016/j.jfoodeng.2009.05.003.

Anand, T., Chandrasekaran, A., Kuttalam, S., Senthilraja, G. and Samiyappan, R. (2010) 'Integrated

control of fruit rot and powdery mildew of chilli using the biocontrol agent *Pseudomonas fluorescens* and a chemical fungicide', *Biological Control*, 52(1), pp. 1–7. doi: 10.1016/j.biocontrol.2009.09.010.

Andino, A. and Hanning, I. (2015) '*Salmonella enterica*: Survival, colonization, and virulence differences among serovars', *Scientific World Journal*. Hindawi Publishing Corporation, 2015(Table 3). doi: 10.1155/2015/520179.

Apel, K. and Hirt, H. (2004) 'Reactive Oxygen Species: metabolism, oxidative stress, and signal transduction', *Annual Review of Plant Biology*, 55(1), pp. 373–399. doi: https://doi.org/10.1146/annurev.arplant.55.031903.141701.

Ares, A. M., Nozal, M. J. and Bernal, J. (2013) 'Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds', *Journal of Chromatography A*. Elsevier B.V., 1313, pp. 78–95. doi: 10.1016/j.chroma.2013.07.051.

Argüello, P., Ayuso, M. C., Gonzalez-Gomez, D., Bohoyo, D., Delgado-Adamez, J. and Bernalte, M. J. (2017) 'Behaviour of fresh cut broccoli under different modified atmosphere conditions', *Emirates Journal of Food and Agriculture*, 29(3), pp. 188–197. doi: 10.9755/ejfa.2016-07-907.

Armstrong, S. K., Brickman, T. J. and Suhadolc, R. J. (2012) 'Involvement of multiple distinct Bordetella receptor proteins in the utilization of iron liberated from transferrin by host catecholamine stress hormones', *Molecular Microbiology*, 84(3), pp. 446–462. doi: 10.1111/j.1365-2958.2012.08032.x.

Arpita, S., Subroto, D., Pinaki, B. and Bidyut, B. (2010) 'Inhibition of Polyphenol Oxidase in Banana , Apple and Mushroom By Using Different Anti- Browning Agents Under Different Conditions', *International Journal of Chem*, 8(5), pp. 550–558.

Artés-Hernández, F., Escalona, V. H., Robles, P. A., Martínez-Hernández, G. B. and Artés, F. (2009) 'Effect of UV-C radiation on quality of minimally processed spinach leaves', *Journal of the Science of Food and Agriculture*, 89(3), pp. 414–421. doi: 10.1002/jsfa.3460.

Artés, F. and Allende, A. (2005) 'Minimal processing of vegetables, fruits and juices', in *Emerging Technologies for Food Processing: An Overview*. Elsevier Ltd, pp. 678–716. doi: 10.1016/B978-0-12-676757-5.50028-1.

Artés, F. and Allende, A. (2014) 'Minimal Processing of Fresh Fruit, Vegetables, and Juices', in *Emerging Technologies for Food Processing*. 2nd edn. CRC Press, Boca Raton, FL, pp. 583–597. doi: 10.1016/B978-0-12-411479-1.00031-0.

Artés, F., Gómez, P., Aguayo, E., Escalona, V. and Artés-Hernández, F. (2009) 'Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities', *Postharvest Biology and Technology*, 51(3), pp. 287–296. doi: 10.1016/j.postharvbio.2008.10.003.

Attitalla, I. H., Johnson, P., Brishammar, S. and Quintanilla, P. (2001) 'Systemic resistance to *Fusarium* wilt in tomato induced by *Phytopthora cryptogea*', *Journal of Phytopathology*, 149(7–8), pp. 373–380. doi: DOI: 10.1111/j.1439-0434.2001.tb03865.x.

Audenaert, K., Pattery, T., Cornelis, P. and Höfte, M. (2002) 'Induction of Systemic Resistance to *Botrytis cinerea* in Tomato by *Pseudomonas aeruginosa* 7NSK2: Role of Salicylic Acid, Pyochelin, and Pyocyanin', *Molecular Plant-Microbe Interactions*, 15(11), pp. 1147–1156. doi: 10.1094/MPMI.2002.15.11.1147.

Autret, N., Raynaud, C., Dubail, I., Charbit, A. and Berche, P. (2003) 'Identification of the Agr Locus of Listeria monocytogenes : Role in Bacterial Virulence Identification of the agr Locus of Listeria monocytogenes : Role in Bacterial Virulence', *Infection and Immunity*, 71(8), pp. 4463–4471. doi: 10.1128/IAI.71.8.4463.

Ayhan, Z. (2017) 'Effect of Packaging on the Quality and Shelf-life of Minimally Processed / Ready to Eat Foods', *Akademil Gida Food Journal*, 9(4), pp. 36–41.

REFERENCES

Bai, J. H., Saftner, R. A., Watada, A. E. and Lee, Y. S. (2001) 'Modified Atmosphere Maintains Quality of Fresh-cut Cantaloupe (*Cucumis melo* L .)', *JFS: Sensory and Nutritive Qualities of Food Modified*, 66(8), pp. 1207–1211.

Baka, M., Mercier, J., Corcuff, R., Castaigne, F. and Arul, J. (1999) 'Photochemical treatment to improve storability of fresh strawberries', *Journal of Food Science*, 64(6), pp. 1068–1072. doi: 10.1111/j.1365-2621.1999.tb12284.x.

Banach, J. L., Sampers, I., Haute, S. Van and van der Fels-Klerx, H. J. (2015) 'Effect of disinfectants on preventing the cross-contamination of pathogens in fresh produce washing water', *International Journal of Environmental Research and Public Health*, 12(8), pp. 8658–8677. doi: 10.3390/ijerph120808658.

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., Smyth, T. J. and Marchant, R. (2010) 'Microbial biosurfactants production, applications and future potential', *Applied Microbiology and Biotechnology*, 87(2), pp. 427–444. doi: 10.1007/s00253-010-2589-0.

Bansal, V. and Siddiqui, M. W. (2015) 'Minimally Processed Foods', (October). doi: 10.1007/978-3-319-10677-9.

Bapat, V. A., Trivedi, P. K., Ghosh, A., Sane, V. A., Ganapathi, T. R. and Nath, P. (2010) 'Ripening of fleshy fruit: Molecular insight and the role of ethylene', *Biotechnology Advances*. Elsevier Inc., 28(1), pp. 94–107. doi: doi: 10.1016/j.biotechadv.2009.10.002.

Barak, J. D., Gorski, L., Naraghi-arani, P. and Charkowski, A. O. (2005) 'Salmonella enterica Virulence Genes Are Required for Bacterial Attachment to Plant Tissue', *Appl. Environ. Microbiol.*, 71(10), pp. 5685–5691. doi: 10.1128/AEM.71.10.5685.

Barrett, D. M., Beaulieu, J. C. and Shewfelt, R. (2010) 'Color, flavor, texture, and nutritional quality of fresh-cut fruits and vegetables: Desirable levels, instrumental and sensory measurement, and the effects of processing', *Critical Reviews in Food Science and Nutrition*, 50(5), pp. 369–389. doi: 10.1080/10408391003626322.

Barth, M., Hankinson, T. R., Zhuang, H. and Breidt, F. (2009) *Microbiological Spoilage of Foods and Beverages*. doi: 10.1007/978-1-4419-0826-1.

Baselice, A., Colantuoni, F., Lass, D. a., Gianluca, N. and Antonio Stasi (2014) 'EU Consumers Perceptions of Fresh-Cut Fruit and Vegetables Attributes: a Choice Experiment Model', in *Agricultural & Applied Economics Association's (AAEA) anual meeting*, pp. 1–23. Available at: http://ageconsearch.umn.edu/bitstream/170527/2/paper_AAEA_2014_Baselice.pdf.

Baxter, A., Mittler, R. and Suzuki, N. (2014) 'ROS as key players in plant stress signalling', *Journal of Experimental Botany*, 65(5), pp. 1229–1240. doi: https://doi.org/10.1093/jxb/ert375.

Bayer-Crop Science-España (2018) *Serenade® Max. General information*. Available at: https://www.cropscience.bayer.es/Productos/Fungicidas/Serenade-Max.aspx (Accessed: 2 May 2018).

Bayoumi, M. A. and Griffiths, M. W. (2012) 'In vitro inhibition of expression of virulence genes responsible for colonization and systemic spread of enteric pathogens using Bifidobacterium bifidum secreted molecules', *International Journal of Food Microbiology*, 156(3), pp. 255–263. doi: 10.1016/j.ijfoodmicro.2012.03.034.

Beaulieu, J. C., Lea, J. M. and Eggleston, G. (2003) 'Sugar and organic acid variations in commercial Cantaloupes and their inbred parents.', *Journal of the American Society for Horticultural Science*, 128, pp. 531–536.

Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. and Seyfarth, W. (1999) 'A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov.', *International journal of systematic bacteriology*, 49, pp. 297–308. doi: 10.1099/00207713-49-1-297.

Belak, A. and Maraz, A. (2015) 'Antagonistic effect of *Pseudomonas* sp. CMI-1 on foodborne

pathogenic *Listeria monocytogenes*', *Food Technology and Biotechnology*, 53, pp. 223–230. doi: https://dx.doi.org/10.17113/2Fftb.53.02.15.3731.

Belay, Z. A., Caleb, O. J. and Opara, U. L. (2016) 'Modelling approaches for designing and evaluating the performance of modified atmosphere packaging (MAP) systems for fresh produce: A review', *Food Packaging and Shelf Life*. Elsevier Ltd, 10, pp. 1–15. doi: 10.1016/j.fpsl.2016.08.001.

Belletti, N., Lanciotti, R., Patrignani, F. and Gardini, F. (2008) 'Antimicrobial efficacy of citron essential oil on spoilage and pathogenic microorganisms in fruit-based salads', *Journal of Food Science*, 73(7). doi: 10.1111/j.1750-3841.2008.00866.x.

Bencheqroun, S. K., Bajji, M., Massart, S., Labhilili, M., Jaafari, S. El and Jijakli, M. H. (2007) 'In vitro and in situ study of postharvest apple blue mold biocontrol by Aureobasidium pullulans: Evidence for the involvement of competition for nutrients', *Postharvest Biology and Technology*, 46(2), pp. 128–135. doi: 10.1016/j.postharvbio.2007.05.005.

Beneduzi, A., Ambrosini, A. and Passaglia, L. M. P. (2012) 'Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents', 4, pp. 1044–1051.

Benzie, I. F. F. and Strain, J. J. (1996) 'The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay', *Analytical Biochemistry*, 239(1), pp. 70–76. doi: 10.1006/abio.1996.0292.

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P. and Frankel, G. (2010) 'Fresh fruit and vegetables as vehicles for the transmission of human pathogens', *Environmental Microbiology*, 12(9), pp. 2385–2397. doi: 10.1111/j.1462-2920.2010.02297.x.

Bhavya, M. L. and Umesh Hebbar, H. (2017) 'Pulsed light processing of foods for microbial safety', *Food Quality and Safety*, 1(August), pp. 187–201. doi: 10.1093/fqsafe/fyx017.

Bintsis, T., Litopoulou-Tzanetaki, E. and Robinson, R. K. (2000) 'Existing and potential applications of ultraviolet light in the food industry - A critical review', *Journal of the Science of Food and Agriculture*, 80(6), pp. 637–645. doi: 10.1002/(SICI)1097-0010(20000501)80:6<637::AID-JSFA603>3.0.CO;2-1.

Blumenstein, K., Albrectsen, B. R., Hultberg, M., Sieber, T. N., Helander, M. and Witzell, J. (2015) 'Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease', *Biocontrol*, 60, pp. 655–667.

Boeing, H., Bechthold, A., Bub, A., Ellinger, S., Haller, D., Kroke, A., Leschik-Bonnet, E., Müller, M. J., Oberritter, H., Schulze, M., Stehle, P. and Watzl, B. (2012) 'Critical review: Vegetables and fruit in the prevention of chronic diseases', *European Journal of Nutrition*, 51(6), pp. 637–663. doi: 10.1007/s00394-012-0380-y.

Bogino, P. C., Oliva, M. de las M., Sorroche, F. G. and Giordano, W. (2013) 'The role of bacterial biofilms and surface components in plant-bacterial associations', *International Journal of Molecular Sciences*, 14(8), pp. 15838–15859. doi: 10.3390/ijms140815838.

Bonifield, H. R. and Hughes, K. T. (2003) 'Flagellar Phase Variation in Salmonella enterica Is Mediated by a Posttranscriptional Control Mechanism Flagellar Phase Variation in Salmonella enterica Is Mediated by a Posttranscriptional Control Mechanism', 185(12), pp. 3567–3574. doi: 10.1128/JB.185.12.3567.

Botes, M., Loos, B., Van Reenen, C. A. and Dicks, L. M. T. (2008) 'Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments', *Archives of Microbiology*, 190, pp. 573–584.

Boumart, Z., Velge, P. and Wiedemann, A. (2014) 'Multiple invasion mechanisms and different intracellular behaviors: A new vision of *Salmonella*-host cell interaction', *FEMS Microbiology Letters*, 361(1), pp. 1–7.

Bourke, P., Ziuzina, D., Han, L., Cullen, P. J. and Gilmore, B. F. (2017) 'Microbiological interactions with cold plasma', *Journal of Applied Microbiology*, 123(2), pp. 308–324. doi: 10.1111/jam.13429.

Boyd, A. and Chakrabarty, A. (1995) '<i>Pseudomonas aeruginosa<i/> biofilm: role of the alginate exopolysaccharide', *Journal of Industrial Microbiology*, 15, p. 162–168.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995) 'Use of a free radical method to evaluate antioxidant activity', *LWT - Food Science and Technology*, 28(1), pp. 25–30. doi: 10.1016/S0023-6438(95)80008-5.

Brandl, M. T. (2006) 'Fitness of Human Enteric Pathogens on Plants and Implications for Food', *Annual Review of Phytopathology*, 44, pp. 367–392. doi: 10.1146/annurev.phyto.44.070505.143359.

Braun, P. G., Hildebrand, P. D., Ells, T. C., Kobayashi, D. C. and van den Berg, H. (2001) 'Evidence and characterization of a gene cluster required for the production of viscosin, a lipopeptide biosurfactant, by a strain of Pseudomonas fluorescens', *Canadian Journal of Microbiology*, 47(4), pp. 294–301. doi: tps://doi.org/10.1139/w01-017oi.org/10.1139/w01-017.

Bravo, S., García-Alonso, J., Martín-Pozuelo, G., Gómez, V., Santaella, M., Navarro-González, I. and Periago, M. J. (2012) 'The influence of post-harvest UV-C hormesis on lycopene, β-carotene, and phenolic content and antioxidant activity of breaker tomatoes', *Food Research International*. Elsevier Ltd, 49(1), pp. 296–302. doi: 10.1016/j.foodres.2012.07.018.

Brown, K. K. and Hampton, M. B. (2011) 'Biological targets of isothiocyanates', *Biochimica et Biophysica Acta - General Subjects*. Elsevier B.V., 1810(9), pp. 888–894. doi: 10.1016/j.bbagen.2011.06.004.

Buchanan, R. L., Gorris, L. G. M., Hayman, M. M., Jackson, T. C. and Whiting, R. C. (2017) 'A review of Listeria monocytogenes: An update on outbreaks, virulence, dose-response, ecology, and risk assessments', *Food Control*. Elsevier Ltd, 75, pp. 1–13. doi: 10.1016/j.foodcont.2016.12.016.

Buchanan, R. L., Stahl, H. G. and Whiting, R. C. (1989) 'Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of Listeria monocytogenes', *Journal of food protection*, 52(12), pp. 844–851.

Buchrieser, C. (2007) 'Biodiversity of the species Listeria monocytogenes and the genus Listeria', *Microbes and Infection*, 9(10), pp. 1147–1155. doi: 10.1016/j.micinf.2007.05.002.

Bultreys, A., Gheysen, I., Maraite, H. and De Hoffmann, E. (2001) 'Characterization of Fluorescent and Nonfluorescent Peptide Siderophores Produced by Pseudomonas syringae Strains and Their Potential Use in Strain Identification', *Applied and Environmental Microbiology*, 67(4), pp. 1718–1727. doi: 10.1128/AEM.67.4.1718-1727.2001.

Burkholder, K. M. and Bhunia, A. K. (2010) '*Listeria monocytogenes* uses *Listeria* adhesion protein (LAP) to promote bacterial transepithelial translocation and induces expression of LAP receptor Hsp60', *Infection and Immunity*, 78, pp. 5062–5073.

Cabrefiga, J., Bonaterra, A. and Montesinos, E. (2007) 'Mechanisms of antagonism of Pseudomonas fluorescens EPS62e against Erwinia amylovora, the causal agent of fire blight', *International Microbiology*, 10(2), pp. 123–132. doi: 10.2436/20.1501.01.18.

Caldera, L., Franzetti, L., Van Coillie, E., De Vos, P., Stragier, P., De Block, J. and Heyndrickx, M. (2016) 'Identification, enzymatic spoilage characterization and proteolytic activity quantification of Pseudomonas spp. isolated from different foods', *Food Microbiology*. Elsevier Ltd, 54, pp. 142–153. doi: 10.1016/j.fm.2015.10.004.

Callejón, R., Rodríguez-Naranjo, M., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. and Troncoso, A. (2015) 'Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes', *Foodborne Pathogens and Disease*, 12(1), pp. 32–38. doi: https://doi.org/10.1089/fpd.2014.1821.

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S. and Cabanes, D. (2011) 'The arsenal of virulence

factors deployed by *Listeria monocytogenes* to promote its cell infection cycle', *Virulence*, 2(5), pp. 379–394. doi: 10.4161/viru.2.5.17703.

Cantos, E., Espín, J. C. and Tomás-Barberán, F. A. (2001) 'Effect of wounding on phenolic enzymes in six minimally processed lettuce cultivars upon storage', *Journal of Agricultural and Food Chemistry*, 49(1), pp. 322–330. doi: 10.1021/jf000644q.

Caretti, C. and Lubello, C. (2003) 'Wastewater disinfection with PAA and UV combined treatment: A pilot plant study', *Water Research*, 37(10), pp. 2365–2371. doi: 10.1016/S0043-1354(03)00025-3.

Carter, M. Q., Xue, K., Brandl, M. T., Liu, F., Wu, L., Louie, J. W., Mandrell, R. E. and Zhou, J. (2012) 'Functional metagenomics of <i>Escherichia coli<i/> O157:H7 interactions with spinach indigenous microorganisms during biofilm formation', *PLoS ONE*, 7(9). doi: 10.1371/journal.pone.0044186.

Castoria, R., Caputo, L., De Curtis, F. and De Cicco, V. (2003) 'Resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action.', *Phytopathology*, 93, pp. 564–572. doi: https://doi.org/10.1094/PHYTO.2003.93.5.564.

CDC- Center for Disease Control and Prevention (2018) *Reports of Selected Salmonella Outbreak Investigations*, *Morbidity and Mortality Weekly Report*. Available at: https://www.cdc.gov/salmonella/outbreaks.html (Accessed: 18 January 2018).

CDC-Center for Disease Control and Prevention (2006) *Multistate Outbreak of Escherichia coli serotype O157:H7 Infections Associated with Consumption of Fresh Spinach*, *Morbidity and Mortality Weekly Report*.

CDC - Center for Disease Control and Prevention (2016) *Multistate Outbreak of Listeriosis Linked to Packaged Salads Produced at Springfield, Ohio Dole Processing Facility (Final Update)*, *Morbidity and Mortality Weekly Report*. Available at: https://www.cdc.gov/listeria/outbreaks/bagged-salads-01-16/index.html (Accessed: 16 January 2018).

Center for Food Safety and Applied Nutrition (2017) 'Standards for the Growing , Harvesting , Packing , and Holding of Produce for Human Consumption : What You Need to Know About the FDA Regulation : Guidance for Industry Small Entity Compliance Guide', (September), pp. 1–30.

Charles, F., Vidal, V., Olive, F., Filgueiras, H. and Sallanon, H. (2013) 'Pulsed light treatment as new method to maintain physical and nutritional quality of fresh-cut mangoes', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 18, pp. 190–195. doi: 10.1016/j.ifset.2013.02.004.

Chaves, A. L. S. and de Mello-Farias, P. C. (2006) 'Ethylene and fruit ripening: From illumination gas to the control of gene expression, more than a century of discoveries', *Genetics and Molecular Biology*, 29(3), pp. 508–515. doi: 10.1590/S1415-47572006000300020.

Cheigh, C. I., Park, M. H., Chung, M. S., Shin, J. K. and Park, Y. S. (2012) 'Comparison of intense pulsed light- and ultraviolet (UVC)-induced cell damage in Listeria monocytogenes and Escherichia coli O157:H7', *Food Control*. Elsevier Ltd, 25(2), pp. 654–659. doi: 10.1016/j.foodcont.2011.11.032.

Chen, C., Belanger, R. R., Benhamou, N. and Paulitz, T. C. (2000) 'Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*', *Physiological and Molecular Plant Pathology*, 56, pp. 13–23. doi: https://doi.org/10.1006/pmpp.1999.0243.

Chen, J., Wang, Z., Wu, J., Wang, Q. and Hu, X. (2007) 'Chemical compositional characterization of eight pear cultivars grown in China.', *Food Chemistry*, 104, pp. 268–275. doi: 10.1016/j.foodchem.2006.11.038.

Chen, S. and Andreasson, E. (2001) 'Update on glucosinolate metabolism and transport', *Plant Physiology and Biochemistry*, 39(9), pp. 743–758. doi: 10.1016/S0981-9428(01)01301-8.

Cheong, Y. H., Chang, H.-S., Gupta, R., Wang, X., Zhu, T. and Luan, S. (2002) 'Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses

in Arabidopsis.', *Plant physiology*, 129(2), pp. 661–677. doi: 10.1104/pp.002857.1.

Cheung, K. L. and Kong, A.-N. (2010) 'Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention', *The AAPS Journal*, 12(1), pp. 87–97. doi: 10.1208/s12248-009-9162-8.

Chilled Food Association (2010) *Produce wash protocol*. Available at: https://www.chilledfood.org/ (Accessed: 26 April 2018).

Chiriboga, M. A., Saladié, M., Giné Bordonaba, J., Recasens, I., Garcia-Mas, J. and Larrigaudière, C. (2013) 'Effect of cold storage and 1-MCP treatment on ethylene perception, signalling and synthesis: Influence on the development of the evergreen behaviour in "Conference" pears', *Postharvest Biology and Technology*. Elsevier B.V., 86, pp. 212–220. doi: https://doi.org/10.1016/j.postharvbio.2013.07.003.

Chisari, M., Barbagallo, R. N. and Spagna, G. (2008) 'Characterization and role of polyphenol oxidase and peroxidase in browning of fresh-cut melon', *Journal of Agricultural and Food Chemistry*, 56(1), pp. 132–138. doi: https://doi.org/10.1021/jf0721491.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006) 'Host-microbe interactions: Shaping the evolution of the plant immune response', *Cell*, 124(4), pp. 803–814. doi: 10.1016/j.cell.2006.02.008.

Choudhary, D. K., Prakash, A. and Johri, B. N. (2007) 'Induced systemic resistance (ISR) in plants: Mechanism of action', *Indian Journal of Microbiology*, 47(4), pp. 289–297.

Chunyang, H., Xiqing, Y., Fei, L. and Binxin, S. (2010) 'Effect of High Oxygen Modified Atmosphere Packaging on Fresh-Cut Onion Quality', pp. 124–128.

Cisneros-Zevallos, L. (2003) 'The use of controlled postharvest abiotic stresses as a tool for enhancing the nutraceutical content and adding-value', *Journal of Food Science*, 68(5), pp. 1560–1565. doi: 10.1111/j.1365-2621.2003.tb12291.x.

Civello, P. M., Vicente, A. R. and Martinez, G. A. (2006) 'UV-C technology to control postharvest diseases of fruits and vegetables', in Troncoso-Rojas;, R., Tiznado-Hernández, M., and González-León, A. (eds) *Recent Advances in alternative postharvest technologies to control fungal diseases in fruits & vegetables*. Kerala, India: Transworld Research Network.

Claessen, D., Rozen, D. E., Kuipers, O. P., Søgaard-Andersen, L. and van Wezel, G. P. (2014) 'Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies.', *Nature reviews. Microbiology*. Nature Publishing Group, 12(2), pp. 115–24. doi: 10.1038/nrmicro3178.

Clarke, J. D., Dashwood, R. H. and Ho, E. (2008) 'Multi-targeted prevention of cancer by sulforaphane', *Cancer Letters*, 269(2), pp. 291–304. doi: 10.1016/j.canlet.2008.04.018.

Clements, A., Young, J. C., Constantinou, N. and Frankel, G. (2012) 'Infection strategies of enteric pathogenic Escherichia coli', *Gut Microbes*, 3(2), pp. 71–87. doi: 10.4161/gmic.19182.

Cleveland, J., Montville, T. J., Nes, I. F. and Chikindas, M. L. (2001) 'Bacteriocins: Safe, natural antimicrobials for food preservation', *International Journal of Food Microbiology*, 71(1), pp. 1–20. doi: 10.1016/S0168-1605(01)00560-8.

Cliff, M. A., Toivonen, P. M. A., Forney, C. F., Liu, P. and Lu, C. (2010) 'Quality of fresh-cut apple slices stored in solid and micro-perforated film packages having contrasting O2 headspace atmospheres', *Postharvest Biology and Technology*. Elsevier B.V., 58(3), pp. 254–261. doi: 10.1016/j.postharvbio.2010.07.015.

Coconnier, M. H., Lievin, V., Lorrot, M. and Servin, A. L. (2000) 'Antagonistic activity of *Lactobacillus acidophilus* LB against intracellular *Salmonella enterica* serovar Typhimurium infecting human enterocyte-like Caco-2/TC7 cells', *Applied and Environmental Microbiology*, 66, pp. 1152–1157.

Colas-Meda, P., Abadias, M., Oliveira, M., Usall, J. and Viñas, I. (2017) 'Influence of fruit matrix and

storage temperature on the survival of *Listeria monocytogenes* in gastrointestinal simulation', *Food control*, 73, pp. 1045–1052.

Colas-Meda, P., Viñas, I., Oliveira, M., Anguera, M., Serrano, J. C. E. and Abadias, M. (2017) 'Exposure to minimally processed pear and melon during shelf life could modify the pathogenic potential of *Listeria monocytogenes*', *Food Microbiology*, 62, pp. 275–281.

Collazo, C., Abadias, M., Aguiló-Aguayo, I., Alegre, I., Chenoll, E. and Viñas, I. (2017) 'Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7 against food-borne pathogens *in vitro* and on fresh-cut melon', *LWT - Food Science and Technology*, 85, pp. 301–308. doi: https://doi.org/10.1016/j.lwt.2017.02.029.

Collazo, C., Abadías, M., Colás-Medà, P., Iglesias, M. B., Granado-Serrano, A. B., Serrano, J. and Viñas, I. (2017) 'Effect of Pseudomonas graminis strain CPA-7 on the ability of Listeria monocytogenes and Salmonella enterica subsp. enterica to colonize Caco-2 cells after pre-incubation on fresh-cut pear', *International Journal of Food Microbiology*. Elsevier, 262(September), pp. 55–62. doi: 10.1016/j.ijfoodmicro.2017.09.003.

Collazo, C., Giné-Bordonaba, J., Aguiló-Aguayo, I., Povedano, I., Bademunt, A. and Viñas, I. (2018) '*Pseudomonas graminis* strain CPA-7 differentially modulates the oxidative response in fresh-cut "Golden delicious" apple depending on the storage conditions', *Postharvest Biology and Technology*. Elsevier, 138, pp. 46–55. doi: 10.1016/j.postharvbio.2017.12.013.

Collazo, C., Lafarga, T., Aguiló-aguayo, I., Marín-sáez, J., Abadias, M. and Viñas, I. (2018) 'Decontamination of fresh-cut broccoli with a water – assisted UV-C technology and its combination with peroxyacetic acid', *Food Control*. Elsevier, 93(April), pp. 92–100. doi: 10.1016/j.foodcont.2018.05.046.

Conaway, C. C., Wang, C. X., Pittman, B., Yang, Y. M., Schwartz, J. E., Tian, D., McIntee, E. J., Hecht, S. S. and Chung, F. L. (2005) 'Phenethyl isothiocyanate and sulforaphane and their N-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice', *Cancer Research*, 65(18), pp. 8548–8557. doi: 10.1158/0008-5472.CAN-05-0237.

Cornelis, P. and Matthijs, S. (2002) 'Minireview Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads : not only pyoverdines', *Environmental Microbiologyvironmental*, 4, pp. 787–798.

Cossart, P. (2011) 'Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes.', *Proceedings of the National Academy of Sciences of the United States of America*, 108(49), pp. 19484–91. doi: 10.1073/pnas.1112371108.

Cossart, P. and Sansonetti, P. J. (2004) 'Bacterial invasion: the paradigm of enteroinvasive pathogens', *Science*, 304(April), pp. 242–248.

Costa, A., Lourenco, A., Civera, T. and Brito, L. (2018) 'Listeria innocua and Listeria monocytogenes strains from dairy plants behave similarly in biofilm sanitizer testing', *LWT - Food Science and Technology*. Elsevier, 92(February), pp. 477–483. doi: 10.1016/j.lwt.2018.02.073.

Costa, L., Vicente, A. R., Civello, P. M., Chaves, A. R. and Martínez, G. A. (2006) 'UV-C treatment delays postharvest senescence in broccoli florets', *Postharvest Biology and Technology*, 39(2), pp. 204–210. doi: 10.1016/j.postharvbio.2005.10.012.

Cotter, P. D., Hill, C. and Ross, R. P. (2005) 'Bacteriocins : developing innate immunity for food', *Nature Reviews Microbiology*, 3, pp. 777–788. doi: 10.1038/nrmicro1240.

Cozzolino, R., Cefola, M., Pace, B., Malorni, L. and Martignetti, A. (2018) 'Quality , sensory and volatile profiles of fresh-cut big top nectarines cold stored in air or modified atmosphere packaging', *International Journal Food Science and Technology*, pp. 1–8. doi: 10.1111/ijfs.13758.

Crovadore, J., Calmin, G., Chablais, R., Cochard, B., Schulz, T., Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. and Seyfarth, W. (2016) 'Whole-Genome Sequence of Pseudomonas graminis Strain UASWS1507 , a Potential Biological Control Agent and Biofertilizer Isolated in

Switzerland', 4(5), pp. 4–5. doi: 10.1128/genomeA.01096-16.Copyright.

Czarny, J. C., Grichko, V. P. and Glick, B. R. (2006) 'Genetic modulation of ethylene biosynthesis and signaling in plants', *Biotechnology Advances*, 24(4), pp. 410–419. doi: 10.1016/j.biotechadv.2006.01.003.

D'Hallewin, G., Schirra, M., Pala, M. and Ben-Yehoshua, S. (2000) 'Ultraviolet C irradiation at 0.5 kJ·m-2 reduces decay without causing damage or affecting postharvest quality of Star Ruby grapefruit (C. paradisi Macf.)', *Journal of Agricultural and Food Chemistry*, 48(10), pp. 4571–4575. doi: 10.1021/jf000559i.

Darré, M., Valerga, L., Ortiz Araque, L. C., Lemoine, M. L., Demkura, P. V., Vicente, A. R. and Concellón, A. (2017) 'Role of UV-B irradiation dose and intensity on color retention and antioxidant elicitation in broccoli florets (Brassica oleracea var. Italica)', *Postharvest Biology and Technology*. Elsevier B.V., 128, pp. 76–82. doi: 10.1016/j.postharvbio.2017.02.003.

Daval, S., Lebreton, L., Gazengel, K., Boutin, M., Guillerm-Erckelboudt, A.-Y. and Sarniget, A. (2011) 'The biocontrol bacterium <i-*Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus *Gaeumannomyces graminis* var . *tritici* on wheat roots', *Molecular Plant Pathology*, 12(9), pp. 839–854. doi: 10.1111/J.1364-3703.2011.00715.X.

Day, B. P. F. (2003) 'Industry Guidelines for High Oxygen MAP of Fresh Prepared Produce', *Acta Horticulturae*, 599, pp. 81–90.

Dell'Erba, A., Falsanisi, D., Liberti, L., Notarnicola, M. and Santoro, D. (2007) 'Disinfection by-products formation during wastewater disinfection with peracetic acid', *Desalination*, 215(1–3), pp. 177–186. doi: 10.1016/j.desal.2006.08.021.

Demidchik, V. (2015) 'Mechanisms of oxidative stress in plants: From classical chemistry to cell biology', *Environmental and Experimental Botany*. Elsevier B.V., 109, pp. 212–228. doi: 10.1016/j.envexpbot.2014.06.021.

Demoz, B. T. and Korsten, L. (2006) 'Bacillus subtilis attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens', *Biological Control*, 37(1), pp. 68–74. doi: 10.1016/j.biocontrol.2005.11.010.

Dianese, A. C., Ji, P. and Wilson, M. (2003) 'Nutritional similarity between leaf-associated nonpathogenic bacteria and the pathogen is not predictive of efficacy in biological control of bacterial spot of tomato', *Applied and Environmental Microbiology*, 69(6), pp. 3484–3491. doi: 10.1128/AEM.69.6.3484-3491.2003.

Djibaoui, D. and Bensoltane, A. (2005) 'Effect of iron and growth inhibitors on siderophores production by Pseudomonas fluorescens', *African Journal of Biotechnology*, 4(7), pp. 697–702. doi: 10.5897/AJB2005.000-3129.

Dobranic, J. K. and Zak, J. C. (1999) 'A microtiter plate procedure for evaluating fungal functional diversity.', *Mycologia*. Mycological Society of America, 91, pp. 756–765.

Dong, Y.-H., Wang, L.-H. and Zhang, L.-H. (2007) 'Quorum-quenching microbial infections: mechanisms and implications', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362(1483), pp. 1201–1211. doi: 10.1098/rstb.2007.2045.

Dong, Y. H., Xu, J. L., Li, X. Z. and Zhang, L. H. (2000) 'AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora.', *Proceedings of the National Academy of Sciences of the United States of America*, 97(7), pp. 3526–31. doi: 10.1073/pnas.060023897.

Dong, Y. H., Zhang, X. F., Xu, J. L. and Zhang, L. H. (2004) 'Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference', *Applied and Environmental Microbiology*, 70(2), pp. 954–960. doi: 10.1128/AEM.70.2.954-960.2004.

Draget, K. I. and Taylor, C. (2011) 'Chemical, physical and biological properties of alginates and their

biomedical implications', *Food Hydrocolloids*. Elsevier Ltd, 25(2), pp. 251–256. doi: 10.1016/j.foodhyd.2009.10.007.

Duan, J., Jiang, W., Cheng, Z., Heikkila, J. J. and Glick, B. R. (2013) 'The Complete Genome Sequence of the Plant Growth-Promoting Bacterium Pseudomonas sp. UW4', *PLoS ONE*, 8(3). doi: 10.1371/journal.pone.0058640.

Duarte-Sierra, A. (2015) *Abiotic stress hormesis: Hormetic stresses to maintain quality and enhance glucosinolates and phenolic compounds in broccoli (Brassica oleracea var. italica) during storage*. University of Laval, Quebec, Canada. Available at: www.theses.ulaval.ca/2015/32086/32086.pdf.

Dubern, J.-F., Lagendijk, E. L., Lugtenberg, B. J. J. and Bloemberg, G. V (2005) 'The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445.', *Journal of bacteriology*, 187(17), pp. 5967–5976. doi: 10.1128/JB.187.17.5967-5976.2005.

Dubern, J.-F., Lugtenberg, B. J. J. and Bloemberg, G. V (2006) 'The ppuI-rsaL-ppuR quorum-sensing system regulates biofilm formation of <i>Pseudomonas putida<i/> PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II.', *Journal of bacteriology*, 188(8), pp. 2898–906. doi: 10.1128/JB.188.8.2898-2906.2006.

Duffy, B. K. and Défago, G. (1999) 'Environmental factors modulating antibiotic and siderophore biosynthesis by <i>Pseudomonas fluorescens<i/> biocontrol strains.', *Applied and environmental microbiology*, 65(6), pp. 2429–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=91358&tool=pmcentrez&rendertype=abstract (Accessed: 13 March 2015).

Duffy, B. K. and Défago, G. (1999) 'Environmental factors modulating antibiotic and siderophore biosynthesis by Pseudomonas fluorescens biocontrol strains', *Applied and Environmental Microbiology*, 65(6), pp. 2429–2438.

Dutra, V., Silva, A., Cabrita, P., Peres, C., Malcata, X. and Brito, L. (2016) 'Lactobacillus plantarum LB95 impairs the virulence potential of Gram-negative and Gram-positive food-borne pathogens in HT-29 and Vero cell cultures.', *Journal Medical Microbiology*, 65, pp. 28–36.

Dwivedi, D. and Johri, B. N. (2003) 'Antifungals from fluorescent pseudomonads : Biosynthesis and regulation', *Current Science*, 85(12), pp. 53–58.

EC- European Comission for Health and Consummer Protection (2016) 'Commission Implementing Regulation (EU) 2017/656 Of 19 December 2016', *Official Journal of the European Union*, L304(72), pp. 20–30. doi: http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf.

EC-European Comission for Health and Consumer Protection (2007) 'Commission regulation (EC) No 1441/2007', *Official Journal of the European Union*, 322(1441), pp. 2–29.

EC-European Comission for Health and Consummer Protection (2002) 'Risk Profile on the Microbiological Contamination of Fruits and Vegetables Eaten Raw', *Report of the Scientific Committee on Food of European Comission for Health and Consummer Protection*, (April), p. 45. Available at: http://europa.eu.int/comm/food/fs/sc/scf (Accessed: 16 January 2018).

EC-European Comission for Health and Consummer Protection (2005) 'Regulation (EU) No 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin, and amending Council Directive 91/414/EEC', *Official Journal of the European Union*, L70, pp. 28–39. Available at: https://www.fsai.ie/uploadedFiles/Legislation/Food_Legisation_Links/Pesticides_Residues_in_food/Regulation_EC_No_396_2005.pdf.

Eckert, C., Xu, W., Xiong, W., Lynch, S., Ungerer, J., Tao, L., Gill, R., Maness, P.-C. and Yu, J. (2014) 'Ethylene-forming enzyme and bioethylene production.', *Biotechnology for biofuels*, 7(1), p. 33. doi: 10.1186/1754-6834-7-33.

EFSA (2017) 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016', *EFSA Journal*, 15(12). doi: 10.2903/j.efsa.2017.5077.

EFSA, E. F. S. A. (2011) 'Scientific Opinion on the efficacy and microbiological safety of irradiation', *EFSA Journal*, 9(4), pp. 1–88. doi: 10.2903/j.efsa.2011.2103.

El-zawahry, A. M., Khalil, A. E. M., Allam, A. D. A. and Mostafa, R. G. (2015) 'Effect of the Bio-agents (Bacillus megaterium and Trichoderma album) on Citrus Nematode (Tylenchulus semipenetrans) Infecting Baladi orange and Lime Seedlings', *Journal of Phytopathology and Pest Management*, 2(2), pp. 1–8.

Elmnasser, N., Guillou, S., Leroi, F., Orange, N., Bakhrouf, A. and Federighi, M. (2007) 'Pulsed-light system as a novel food decontamination technology: a review', *Canadian Journal of Microbiology*, 53(7), pp. 813–821. doi: 10.1139/W07-042.

Van der Ent, S., Van Wees, S. C. M. and Pieterse, C. M. J. (2009) 'Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes', *Phytochemistry*. Elsevier Ltd, 70(13–14), pp. 1581–1588. doi: https://doi.org/10.1016/j.phytochem.2009.06.009.

Erkan, M. and Wang, C. Y. (2006) 'Modified and controlled atmosphere storage of subtropical crops', *Stewart Postharvest Review*, 5. doi: 10.2212/spr.2006.5.4.

Escalona, V. H., Aguayo, E., Martínez-Hernández, G. B. and Artés, F. (2010) 'UV-C doses to reduce pathogen and spoilage bacterial growth in vitro and in baby spinach', *Postharvest Biology and Technology*, 56(3), pp. 223–231. doi: 10.1016/j.postharvbio.2010.01.008.

Espin, J. C., Veltman, R. and Wichers, H. (2000) 'The oxidation of L -ascorbic acid catalysed by pear tyrosinase', *Physiologia Plantarum*, 109, pp. 1–6. doi: 10.1034/j.1399-3054.2000.100101.x.

European Food Safety Authority (EFSA) (2016) 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015', *EFSA Journal*, 14(12), p. 231 pp. doi: 10.2903/j.efsa.2016.4634.

Fagundes, C., Carciofi, B. A. M. and Monteiro, A. R. (2013) 'Estimate of respiration rate and physicochemical changes of fresh-cut apples stored under different temperatures', *Food Science and Technology*, 33(1), pp. 60–67. doi: 10.1590/S0101-20612013005000023.

Fan, X., Huang, R. and Chen, H. (2017) 'Application of ultraviolet C technology for surface decontamination of fresh produce', *Trends in Food Science and Technology*. Elsevier, 70(October), pp. 9–19. doi: 10.1016/j.tifs.2017.10.004.

Fan, X., Niemera, B., Mattheis, J., Zhuang, H. and Olson, D. (2005) 'Quality of fresh-cut apple slices as affected by low-dose ionizing radiation and calcium ascorbate treatment', *JFS: Sensory and Nutritive Qualities of Food*, 70(2).

Farber, J. N., Harris, L. J., Parish, M. E., Beuchat, L. R., Suslow, T. V., Gorney, J. R., Garrett, E. H. and Busta, F. F. (2003) 'Microbiological Safety of Controlled and Modified Atmosphere Packaging of Fresh and Fresh-Cut Produce', *Comprehensive Reviews in Food Science and Food Safety*, 2(s1), pp. 142–160. doi: 10.1111/j.1541-4337.2003.tb00032.x.

Fatima, S. and Anjum, T. (2017) 'Identification of a potential ISR determinant from *Pseudomonas aeruginosa* PM12 against *Fusarium* wilt in tomato', *Frontiers in Plant Science*, 8, pp. 1–14. doi: 10.3389/fpls.2017.00848.

FDA-Food and Drugs Administration (2015) *Irradiation in the production, processing and handling of food*, *CFR - Code of Federal Regulations Title 21*. Available at: http://www.accessdata.fda.gov/ (Accessed: 20 April 2018).

FDA - Food and Drugs Administration (2018) *Foods and drugs for human comsumption. Secondary direct food additives permitted in food for human consumption*, *Electronic Code of Federal Regulations*. Available at: https://www.ecfr.gov/cgi-bin/ (Accessed: 19 January 2018).

Feng, J., Liu, P., Yang, X. and Zhao, X. (2015) 'Screening of immunomodulatory and adhesive *Lactobacillus* with antagonistic activities against *Salmonella* from fermented vegetables.', *World Journal of Microbiology and Biotechnology*. Springer Netherlands, 31, pp. 1947–1954.

Fernández-León, A. M., Fernández-León, M. F., González-Gómez, D., Ayuso, M. C. and Bernalte, M. J. (2017) 'Quantification and bioaccessibility of intact glucosinolates in broccoli "Parthenon" and Savoy cabbage "Dama"', *Journal of Food Composition and Analysis*. Elsevier Inc., 61, pp. 40–46. doi: 10.1016/j.jfca.2016.11.010.

Fernández-León, M. F., Fernández-León, A. M., Lozano, M., Ayuso, M. C., Amodio, M. L., Colelli, G. and González-Gómez, D. (2013) 'Retention of quality and functional values of broccoli "Parthenon" stored in modified atmosphere packaging', *Food Control*. Elsevier Ltd, 31(2), pp. 302–313. doi: 10.1016/j.foodcont.2012.10.012.

Fernando, W. G. D., Nakkeeran, S. and Zhang, Y. (2006) 'Biosynthesis of antibiotics by PGPR and its relation in biocontrol of plant diseases', in (ed.), Z. A. S. (ed.) *PGPR: Biocontrol and Biofertilization*. Springer Dordrecht, The Netherlands, pp. 67–109. doi: 10.1007/1-4020-4152-7_3.

Fess, T. L. and Benedito, V. A. (2018) 'Organic versus conventional cropping sustainability: A comparative system analysis', *Sustainability (Switzerland)*, 10(1). doi: 10.3390/su10010272.

Fine, F. and Gervais, P. (2004) 'Efficiency of Pulsed UV Light for Microbial Decontamination of Food Powders', *Journal of Food Protection*, 67(4), pp. 787–792. doi: 10.4315/0362-028X-67.4.787.

Finnegan, M., Linley, E., Denyer, S. P., McDonnell, G., Simons, C. and Maillard, J. Y. (2010) 'Mode of action of hydrogen peroxide and other oxidizing agents: Differences between liquid and gas forms', *Journal of Antimicrobial Chemotherapy*, 65(10), pp. 2108–2115. doi: 10.1093/jac/dkq308.

Fleckenstein, J. M., Hardwidge, P. R., Munson, G. P., Rasko, D. A., Sommerfelt, H. and Steinsland, H. (2010) 'Molecular mechanisms of enterotoxigenic Escherichia coli infection', *Microbes and Infection*. Elsevier Masson SAS, 12(2), pp. 89–98. doi: 10.1016/j.micinf.2009.10.002.

Flores, P., Hellín, P. and Fenoll, J. (2012) 'Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry', *Food Chemistry*. Elsevier Ltd, 132(2), pp. 1049–1054. doi: 10.1016/j.foodchem.2011.10.064.

Fonseca, S. C., Oliveira, F. A. R. and Brecht, J. K. (2002) 'Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: A review', *Journal of Food Engineering*, 52(2), pp. 99–119. doi: 10.1016/S0260-8774(01)00106-6.

Formica-Oliveira, A. C., Martínez-Hernández, G. B., Díaz-López, V., Artés, F. and Artés-Hernández, F. (2017) 'Effects of UV-B and UV-C combination on phenolic compounds biosynthesis in fresh-cut carrots', *Postharvest Biology and Technology*. Elsevier B.V., 127, pp. 99–104. doi: 10.1016/j.postharvbio.2016.12.010.

Formica-Oliveira, A. C., Martínez-Hernández, G. B., Díaz-López, V., Artés, F. and Artés-Hernández, F. (2017) 'Use of postharvest UV-B and UV-C radiation treatments to revalorize broccoli byproducts and edible florets', *Innovative Food Science & Emerging Technologies*. Elsevier, 43(May), pp. 77–83. doi: 10.1016/j.ifset.2017.07.036.

Fox, E. M., Leonard, N. and Jordan, K. (2011) 'Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates', *Applied and Environmental Microbiology*, 77, pp. 6559–6569.

Francis, G. A. and Beirne, D. O. (1997) 'Effects of gas atmosphere, antimicrobial dip and temperature on the fate of Listeria innocua and Listeria monocytogenes on minimally processed lettuce', *International Journal of Food Science and Technology*, 32(2), pp. 141–151. doi: 10.1046/j.1365-2621.1997.00390.x.

Franco, B. D. G. M. and Destro, M. T. (2007) 'Minimally Processed Vegetable Salads : Microbial Quality Evaluation', *Journal of Food Protection*, 70(5), pp. 1277–1280.

Friedly, E. C., Crandall, P. G., Ricke, S., O'Bryan, C. A., Martin, E. M. and Boyd, L. M. (2008) 'Identification of Listeria innocua surrogates for Listeria monocytogenes in hamburger patties', *Journal of Food Science*, 73(4). doi: 10.1111/j.1750-3841.2008.00719.x.

Fukuzaki, S. (2006) ‘Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes.’, *Biocontrol science*, 11(4), pp. 147–57. doi: 10.4265/bio.11.147.

Gaillard, J. L., Finlay, B. B. and Line, C.-C. (1996) ‘Effect of cell polarization and differentiation on entry of *Listeria monocytogenes* into the enterocyte-like Caco-2 cell line.’, *Infection and Immunity*, 64, pp. 1299–1308.

Gálvez, A., Abriouel, H., Benomar, N. and Lucas, R. (2010) ‘Microbial antagonists to food-borne pathogens and biocontrol’, *Current Opinion in Biotechnology*. Elsevier Ltd, 21(2), pp. 142–148. doi: 10.1016/j.copbio.2010.01.005.

Gamage, G. (2015) *Effectiveness of UV-C irradiation on controlling growth of* L . monocytogenes *on fresh cut broccoli*. Massey University of New Zealand.

Gamage, G. R., Heyes, J. A., Palmer, J. S. and Wargent, J. (2016) ‘Antimicrobial effect of UV-C treated fresh-cut broccoli against *Listeria monocytogenes*’, *Acta Horticulturae*, 1120, pp. 187–192. doi: 10.17660/ActaHortic.2016.1120.28.

Gamalero, E. and Glick, Bernard, R. (2015) ‘Bacterial Modulation of Plant Ethylene Levels’, *Plant Physiology*, 169(January), p. pp.00284.2015. doi: 10.1104/pp.15.00284.

Gandhi, M. and Chikindas, M. L. (2007) ‘Listeria: A foodborne pathogen that knows how to survive’, *International Journal of Food Microbiology*, 113(1), pp. 1–15. doi: 10.1016/j.ijfoodmicro.2006.07.008.

Gao, P., Qin, J., Li, D. and Zhou, S. (2018) ‘Inhibitory effect and possible mechanism of a Pseudomonas strain QBA5 against gray mold on tomato leaves and fruits caused by Botrytis cinerea’, *PLoS ONE*, 13(1), pp. 1–15. doi: 10.1371/journal.pone.0190932.

Garcia, E. and Barrett, D. M. (2002) ‘Preservative treatments for fresh-cut fruits and vegetables’, *CRC Press*, p. 32. doi: 10.1201/9781420031874.ch9.

Garland, J. and Mills, a L. (1991) ‘Classification and Characterization of Heterotrophic Microbial Communities Based on Patterns of Community-level Sole Carbon-source Utilization’, *Appl. Environ. Microbiol.*, 57(8), pp. 2351–2359.

Gayán, E., Condón, S. and Álvarez, I. (2014) ‘Biological aspects in food preservation by ultraviolet light: a review’, *Food and Bioprocess Technology*, 7(1), pp. 1–20. doi: 10.1007/s11947-013-1168-7.

Ge, C., Bohrerova, Z. and Lee, J. (2013) ‘Inactivation of internalized Salmonella Typhimurium in lettuce and green onion using ultraviolet C irradiation and chemical sanitizers’, *Journal of Applied Microbiology*, 114(5), pp. 1415–1424. doi: 10.1111/jam.12154.

El Ghaouth, A., Wilson, C. L. and Callahan, A. M. (2003) ‘Induction of Chitinase, β-1,3-Glucanase, and Phenylalanine Ammonia Lyase in Peach Fruit by UV-C Treatment’, *Phytopathology*, 93(3), pp. 349–355. doi: 10.1094/PHYTO.2003.93.3.349.

Gianopolitis, G. and Ries, S. (1977) ‘Superoxide dismutases. I. Ocurrence in higher plants.’, *Plant Physiology*, 59, pp. 309–314. doi: https://doi.org/10.1104/pp.59.2.309.

Gil, M., Gorny, J. and Kader, A. (1998) ‘Reponses of’Fuji’ apples to ascorbic acid and low oxygen atmosphere’, *HortScience*, 33(2), pp. 305–309.

Gil, M. I., Selma, M. V., López-Gálvez, F. and Allende, A. (2009) ‘Fresh-cut product sanitation and wash water disinfection: Problems and solutions’, *International Journal of Food Microbiology*. Elsevier B.V., 134(1–2), pp. 37–45.

Gill, S. S. and Tuteja, N. (2010) ‘Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants’, *Plant Physiology and Biochemistry*. Elsevier Masson SAS, 48(12), pp. 909–930. doi: 10.1016/j.plaphy.2010.08.016.

Giné-Bordonaba, J., Echeverria, G., Ubach, D., Aguiló-Aguayo, I., López, M. L. and Larrigaudière, C. (2017) ‘Biochemical and physiological changes during fruit development and ripening of two sweet cherry varieties with different levels of cracking tolerance’, *Plant Physiology and*

Biochemistry, 111, pp. 216–225. doi: https://doi.org/10.1016/j.plaphy.2016.12.002.

Giné-Bordonaba, J. and Terry, L. A. (2016) 'Effect of deficit irrigation and methyl jasmonate application on the composition of strawberry (Fragaria x ananassa) fruit and leaves', *Scientia Horticulturae*. Elsevier B.V., 199, pp. 63–70. doi: https://doi.org/10.1016/j.scienta.2015.12.026.

Glick, B. R. (2005) 'Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase', *FEMS Microbiology Letters*, 251(1), pp. 1–7. doi: 10.1016/j.femsle.2005.07.030.

Glick, B. R. (2014) 'Bacteria with ACC deaminase can promote plant growth and help to feed the world', *Microbiological Research*. Elsevier GmbH., 169(1), pp. 30–39. doi: 10.1016/j.micres.2013.09.009.

Gogo, E. O., Opiyo, A. M., Hassenberg, K., Ulrichs, C. and Huyskens-Keil, S. (2017) 'Postharvest UV-C treatment for extending shelf life and improving nutritional quality of African indigenous leafy vegetables', *Postharvest Biology and Technology*. Elsevier, 129(March), pp. 107–117. doi: 10.1016/j.postharvbio.2017.03.019.

Gomez-Lopez, V. M. (ed.) (2012) *Decontamination of Fresh and Minimally Processed Produce*. Edition il. John Wiley & Sons.

Gómez-López, V. M., Devlieghere, F., Bonduelle, V. and Debevere, J. (2005) 'Intense light pulses decontamination of minimally processed vegetables and their shelf-life', *International Journal of Food Microbiology*, 103(1), pp. 79–89. doi: 10.1016/j.ijfoodmicro.2004.11.028.

Gómez-López, V. M., Lannoo, A. S., Gil, M. I. and Allende, A. (2014) 'Minimum free chlorine residual level required for the inactivation of Escherichia coli O157: H7 and trihalomethane generation during dynamic washing of fresh-cut spinach', *Food Control*. Elsevier Ltd, 42, pp. 132–138. doi: 10.1016/j.foodcont.2014.01.034.

Gómez-López, V., Ragaert, P., Debevere, J. and Devlieghere, F. (2007) 'Pulsed light for food decontamination : a review', *Trends in Food Science & Technology*, 18, pp. 464–473. doi: 10.1016/j.tifs.2007.03.010.

Gómez, D., Azón, E., Marco, N., Carramiñana, J. J., Rota, C., Ariño, A. and Yangüela, J. (2014) 'Antimicrobial resistance of Listeria monocytogenes and Listeria innocua from meat products and meat-processing environment', *Food Microbiology*. Elsevier Ltd, 42, pp. 61–65. doi: 10.1016/j.fm.2014.02.017.

Gómez, P. L., García-loredo, A., Nieto, A., Salvatori, D. M., Guerrero, S. and Alzamora, S. M. (2012) 'Effect of pulsed light combined with an antibrowning pretreatment on quality of fresh cut apple', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 16, pp. 102–112. doi: 10.1016/j.ifset.2012.05.011.

González-Buesa, J., Ferrer-Mairal, A., Oria, R. and Salvador, M. L. (2009) 'A mathematical model for packaging with microperforated films of fresh-cut fruits and vegetables', *Journal of Food Engineering*. Elsevier Ltd, 95(1), pp. 158–165. doi: 10.1016/j.jfoodeng.2009.04.025.

Gormley, T. R., Brunton, N. and Butler, F. (2016) 'Quality and Antioxidant Properties of Fresh-cut Apple Wedges from 10 Cultivars During Modified Atmosphere Packaging Storage', *Food Science and Technology*, 17(3), pp. 267–276. doi: 10.1177/1082013210382448.

Gorny, J. R., Giclas, H., Gombas, D. and Means, K. (2006) *Commodity Specific Food Safety Guidelines for the Lettuce and Leafy Greens Supply Chain*. Available at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM169008.pdf (Accessed: 6 January 2018).

Gorny, J. R., Hess-Pierce, B., Cifuentes, R. A. and Kader, A. A. (2002) 'Quality changes in fresh-cut pear slices as affected by controlled atmospheres and chemical preservatives', *Postharvest Biology and Technology*, 24(3), pp. 271–278. doi: 10.1016/S0925-5214(01)00139-9.

Götz, A. and Goebelt, W. (2010) 'Glucose and glucose 6-phosphate as carbon sources in extra- and

intracellular growth of enteroinvasive *Escherichia coli* and *Salmonella enterica*.', *Microbiology*, 156, pp. 1176–1187.

Govan, J. R. W., Fyfe, J. A. M. and Jarman, T. R. (1981) 'Isolation of alginate-producing mutants of *Pseudomonasfluorescens* , *Pseudomonas putida* and *Pseudomonas mendocina*', *Journal of General Microbiology*, 125, pp. 217–220.

Graça, A., Santo, D., Quintas, C. and Nunes, C. (2017) 'Growth of *Escherichia coli*, *Salmonella enterica* and *Listeria* spp., and their inactivation using ultraviolet energy and electrolyzed water, on "Rocha" fresh-cut pears', *Food Control*, 77, pp. 41–49. doi: 10.1016/j.foodcont.2017.01.017.

Grimont, P. and Weill, F.-X. (2007) 'Antigenic Formulae of the Salmonella serovars, (9th ed.) Paris: WHO Collaborating Centre for Reference and Research on Salmonella', *Institute Pasteur.*, (January), pp. 1–166.

Guilbaud, M., Piveteau, P., Desvaux, M., Brisse, S. and Briandet, R. (2015) 'Exploring the diversity of Listeria monocytogenes biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype', *Applied and Environmental Microbiology*, 81(5), pp. 1813–1819. doi: 10.1128/AEM.03173-14.

Guinebretiere, M. H., Morrison, N. and Reich, M. (2000) 'Isolation and Characterization of Antagonists for the Biocontrol of the Postharvest Wound Pathogen Botrytis cinerea on Strawberry Fruits', *Journal of Food Protection*, 63(3), pp. 386–394.

Haas, D., Blumer, C. and Keel, C. (2000) 'Biocontrol ability of fluorescent pseudomonads genetically dissected : importance of positive feedback regulation', *Current Opinion in Biotechnology*, 11, pp. 290–297.

Hadjok, C., Mittal, G. S. and Warriner, K. (2008) 'Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide', *Journal of Applied Microbiology*, 104(4), pp. 1014–1024. doi: 10.1111/j.1365-2672.2007.03624.x.

Hägele, F., Nübling, S., Schweiggert, R. M., Baur, S., Weiss, A., Schmidt, H., Menegat, A., Gerhards, R. and Carle, R. (2016) 'Quality improvement of fresh-Cut endive (Cichorium endivia L.) and recycling of washing water by low-dose UV-C irradiation', *Food and Bioprocess Technology*. Food and Bioprocess Technology, 9(12), pp. 1979–1990. doi: 10.1007/s11947-016-1782-2.

Halfeld-Vieira, B. D. A., da Silva, W. L. M., Schurt, D. A., Ishida, A. K. N., de Souza, G. R. and Nechet, K. D. L. (2015) 'Understanding the mechanism of biological control of passionfruit bacterial blight promoted by autochthonous phylloplane bacteria', *Biological Control*. Elsevier Inc., 80, pp. 40–49. doi: 10.1016/j.biocontrol.2014.09.011.

Han, C., Ji, Y., Li, M., Li, X., Jin, P. and Zheng, Y. (2016) 'Influence of wounding intensity and storage temperature on quality and antioxidant activity of fresh-cut Welsh onions', *Scientia Horticulturae*. Elsevier B.V., 212, pp. 203–209. doi: 10.1016/j.scienta.2016.10.004.

Hanschen, F. S., Klopsch, R., Oliviero, T., Schreiner, M., Verkerk, R. and Dekker, M. (2017) 'Optimizing isothiocyanate formation during enzymatic glucosinolate breakdown by adjusting pH value, temperature & dilution in Brassica vegetables & Arabidopsis thaliana', *Scientific Reports*. Nature Publishing Group, 7(January), pp. 1–15. doi: 10.1038/srep40807.

Harish, S., Kavino, M., Kumar, N., Balasubramanian, P. and Samiyappan, R. (2009) 'Induction of defense-related proteins by mixtures of plant growth promoting endophytic bacteria against Banana bunchy top virus', *Biological Control*. Elsevier Inc., 51(1), pp. 16–25. doi: https://doi.org/10.1016/j.biocontrol.2009.06.002.

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H. and Busta, F. F. (2003) 'Outbreaks associated with fresh produce: incidence, growth and survival of pathogens in fresh and fresh-cut produce', *Comprehensive Reviews in Food Science and Food Safety*, 2(s1), pp. 78–141. doi: 10.1111/j.1541-4337.2003.tb00031.x.

Harrison, F., Paul, J., Massey, R. C. and Buckling, A. (2008) ‘Interspecific competition and siderophore-mediated cooperation in <i>Pseudomonas aeruginosa<i/>.’, *The ISME journal*, 2, pp. 49–55. doi: 10.1038/ismej.2007.96.

Hase, S., Van Pelt, J. A., Van Loon, L. C. and Pieterse, C. M. J. (2003) ‘Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection’, *Physiological and Molecular Plant Pathology*, 62(4), pp. 219–226. doi: 10.1016/S0885-5765(03)00059-6.

Hass, D. and Keel, C. (2003) ‘Regulation of antibiotic production in root-colonizing Pseudomonas ...’, *Annual Review of Phytopathology*, 41, pp. 117–153.

Hassenberg, K., Huyskens-Keil, S. and Herppich, W. B. (2012) ‘Impact of postharvest UV-C and ozone treatments on microbiological properties of white asparagus (*Asparagus officinalis* L.)’, *Journal of Applied Botany and Food Quality*, 85(2), pp. 174–181.

He, L., Xu, Y.-Q. and Zhang, X.-H. (2008) ‘Medium factor optimization and fermentation kinetics for phenazine-1-carboxylic acid production by *Pseudomonas* sp. M18G.’, *Biotechnology and bioengineering*, 100, pp. 250–9.

He, Q. and Luo, Y. (2007) ‘Enzymatic browning and its control in fresh-cut produce’, *Stewart Postharvest Review*, 3(6), pp. 1–7. doi: https://doi.org/10.1016/j.biocontrol.2009.06.002.

Hentzer, M., Teitzel, G. M., Balzer, G. J., Molin, S., Givskov, M., Matthew, R., Heydorn, A. and Parsek, M. R. (2001) ‘Alginate overproduction affects <i>Pseudomonas aeruginosa<i/> biofilm structure and function’, *Journal of bacteriology*, 183(18), pp. 5395–5401. doi: 10.1128/JB.183.18.5395.

Hernández-León, R., Rojas-Solís, D., Contreras-Pérez, M., Orozco-Mosqueda, M. del C., Macías-Rodríguez, L. I., Reyes-de la Cruz, H., Valencia-Cantero, E. and Santoyo, G. (2015) ‘Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains.’, *Biological Control*, 81, pp. 83–92.

Hernández-Salmerón, J. E., Moreno-Hagelsieb, G. and Santoyo, G. (2017) ‘Genome Comparison of *Pseudomonas fluorescens* UM270 with Related Fluorescent Strains Unveils Genes Involved in Rhizosphere Competence and Colonization’, *Journal of Genomics*, 5(7), pp. 91–98. doi: 10.7150/jgen.21588.

Herr, I. and Büchler, M. W. (2010) ‘Dietary constituents of broccoli and other cruciferous vegetables: Implications for prevention and therapy of cancer’, *Cancer Treatment Reviews*. Elsevier Ltd, 36(5), pp. 377–383. doi: 10.1016/j.ctrv.2010.01.002.

Hibbing, M. E., Fuqua, C., Parsek, M. R. and Peterson, S. B. (2010) ‘Bacterial competition: surviving and thriving in the microbial jungle.’, *Nat Rev Microbiol.*, 8(1), pp. 15–25. doi: 10.1038/nrmicro2259.Bacterial.

Higdon, J. V., Delage, B., Williams, D. E. and Dashwood, R. H. (2007) ‘Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis’, *Pharmacological Research*, 55(3), pp. 224–236. doi: 10.1016/j.phrs.2007.01.009.

Hiraga, S. (2001) ‘A Large Family of Class III Plant Peroxidases’, *Plant and Cell Physiology*, 42(5), pp. 462–468. doi: https://doi.org/10.1093/pcp/pce061.

Ho, N. K., Aleah C, H., Kathene, J. and Sherman, P. (2012) ‘Pathogenicity, host responses and implications for management of enterohemorrhagic Escherichia coli O157:H7 infection.’, *Canadian journal of gastroenterology.*, 27(5), pp. 281–285.

Hodges, D. M. and Toivonen, P. M. A. (2008) ‘Quality of fresh-cut fruits and vegetables as affected by exposure to abiotic stress’, *Postharvest Biology and Technology*, 48(2), pp. 155–162. doi: https://doi.org/10.1016/j.postharvbio.2007.10.016.

Hoefkens, C., Sioen, I., Baert, K., De Meulenaer, B., De Henauw, S., Vandekinderen, I., Devlieghere, F.,

Opsomer, A., Verbeke, W. and Camp, J. Van (2010) 'Consuming organic versus conventional vegetables: The effect on nutrient and contaminant intakes', *Food and Chemical Toxicology*. Elsevier Ltd, 48(11), pp. 3058–3066. doi: 10.1016/j.fct.2010.07.044.

Holderbaum, D. F., Kon, T., Kudo, T. and Guerra, M. P. (2010) 'Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: Dynamics during fruit development', *HortScience*, 45(8), pp. 1150–1154.

Honma, M. and Shimomura, T. (1978) 'Metabolism of 1-aminocyclopropane-1-carboxylic acid', *Agricultural and Biological Chemistry*, 42(10), pp. 1825–1831. doi: 10.1080/00021369.1978.10863261.

Horemans, B., Smolders, E. and Springael, D. (2013) 'Carbon source utilization profiles suggest additional metabolic interactions in a synergistic linuron-degrading bacterial consortium', *FEMS Microbiology Ecology*, 84, pp. 24–34.

Houghton, D., Wilcox, M. D., Brownlee, I. A., Chater, P., Seal, C. J. and Pearson, J. P. (2014) 'Method for quantifying alginate and determining release from a food vehicle in gastrointestinal digesta', *Food Chemistry*. Elsevier Ltd, 151, pp. 352–357.

Hua, G. and Reckhow, D. A. (2007) 'Comparison of disinfection byproduct formation from chlorine and alternative disinfectants', *Water Research*, 41(8), pp. 1667–1678. doi: 10.1016/j.watres.2007.01.032.

Huang, Y. and Chen, H. (2014) 'A novel water-assisted pulsed light processing for decontamination of blueberries', *Food Microbiology*. Elsevier Ltd, 40, pp. 1–8. doi: 10.1016/j.fm.2013.11.017.

Huang, Y., Hung, Y., Hsu, S., Huang, Y. and Hwang, D. (2008) 'Application of electrolyzed water in the food industry', *Food Control*, 19(4), pp. 329–345. doi: http://dx.doi.org/10.1016/j.foodcont.2007.08.012.

Huang, Y., Sido, R., Huang, R. and Chen, H. (2015a) 'Application of water-assisted pulsed light treatment to decontaminate raspberries and blueberries from Salmonella', *International Journal of Food Microbiology*. Elsevier B.V., 208, pp. 43–50. doi: 10.1016/j.ijfoodmicro.2015.05.016.

Huang, Y., Sido, R., Huang, R. and Chen, H. (2015b) 'Application of water-assisted pulsed light treatment to decontaminate raspberries and blueberries from *Salmonella*', *International Journal of Food Microbiology*. Elsevier B.V., 208, pp. 43–50. doi: 10.1016/j.ijfoodmicro.2015.05.016.

Hudson, J. A. and Mott, S. J. (1993) 'Growth of Listeria monocytogenes, Aeromonas hydrophila and Yersinia enterocolitica in pate and a comparison with predictive models', *Int J Food Microbiol*, 20(1), pp. 1–11. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8251302.

Hung, S. H., Yu, C. W. and Lin, C. H. (2005) 'Hydrogen peroxide functions as a stress signal in plants', *Botanical Bulletin of Academia Sinica*, 46, pp. 1–10. doi: https://doi.org/10.1083/jcb.200409170.

Iavicoli, A., Boutet, E., Buchala, A. and Métraux, J.-P. (2003) 'Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0.', *Molecular plant-microbe interactions : MPMI*, 16(10), pp. 851–8.

Ibusquiza, P. S., Herrera, J. J. R. and Cabo, M. L. (2011) 'Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by Listeria monocytogenes', *Food Microbiology*. Elsevier Ltd, 28(3), pp. 418–425. doi: 10.1016/j.fm.2010.09.014.

Iglesias, M. (2017) *Bioconservación de pera mínimamente procesada mediante el uso de Pseudomonas graminis CPA-7 y Lactobacillus rhamnosus GG y su efecto en la calidad de la fruta y en la modificación del potencial patogénico de Listeria monocytogenes María Belén Iglesias Val*.

Iglesias, M. B., Abadias, M., Anguera, M. and Viñas, I. (2018) 'Efficacy of *Pseudomonas graminis* CPA-7 against *Salmonella* spp. and *Listeria monocytogenes* on fresh-cut pear and setting up of the conditions for its commercial application', *Food Microbiology*, 70, pp. 103–112. doi:

10.1016/j.fm.2017.09.010.

Iglesias, M. B. B., Viñas, I., Colás-Medà, P., Collazo, C., Serrano, J. C. E. C. E. and Abadias, M. (2017) 'Adhesion and invasion of Listeria monocytogenes and interaction with Lactobacillus rhamnosus GG after habituation on fresh-cut pear', *Journal of Functional Foods*, 34, pp. 453–460. doi: 10.1016/j.jff.2017.05.011.

Ignat, A., Manzocco, L., Bartolomeoli, I., Maifreni, M. and Nicoli, M. C. (2015) 'Minimization of water consumption in fresh-cut salad washing by UV-C light', *Food Control*. Elsevier Ltd, 50, pp. 491–496. doi: 10.1016/j.foodcont.2014.09.036.

Ignat, A., Manzocco, L., Maifreni, M., Bartolomeoli, I. and Nicoli, M. C. (2014) 'Surface decontamination of fresh-cut apple by pulsed light: Effects on structure, colour and sensory properties', *Postharvest Biology and Technology*. Elsevier B.V., 91, pp. 122–127. doi: 10.1016/j.postharvbio.2014.01.005.

Ignat, A., Manzocco, L., Maifreni, M., Bartolomeoli, I. and Nicoli, M. C. (2014) 'Surface decontamination of fresh-cut apple by pulsed light: Effects on structure, colour and sensory properties', *Postharvest Biology and Technology*. Elsevier B.V., 91, pp. 122–127. doi: 10.1016/j.postharvbio.2014.01.005.

Ilic, S., Odomeru, J. and LeJeune, J. T. (2008) 'Coliforms and prevalence of Escherichia coli and foodborne pathogens on minimally processed spinach in two packing plants.', *Journal of food protection*, 71(12), pp. 2398–2403.

Ireton, K. (2007) 'Entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells', *Cellular Microbiology*, 9(6), pp. 1365–1375. doi: 10.1111/j.1462-5822.2007.00933.x.

Ivy, R. A., Chan, Y. C., Bowen, B. M., Boor, K. J. and Wiedmann, M. (2010) 'Growth temperature-dependent contributions of response regulators, σ B , PrfA, and motility factors to Listeria monocytogenes invasion of Caco-2 cells.', *Foodborne Pathogens and Disease*, 7(11), pp. 1337–1349. doi: DOI: 10.1089/fpd.2010.0563.

Izquier, A. and Gómez-López, V. M. (2011) 'Modeling the pulsed light inactivation of microorganisms naturally occurring on vegetable substrates', *Food Microbiology*. Elsevier Ltd, 28(6), pp. 1170–1174. doi: 10.1016/j.fm.2011.03.010.

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Pærregaard, A., Sandström, B., Tvede, M. and Jakobsen, M. (1999) 'Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans', *Applied and Environmental Microbiology*, 65(11), pp. 4949–4956.

Jahid, I. K. and Ha, S. (2012) 'A review of microbial biofilms of produce : future challenge to food safety', *Food Science and Biotechnology*, 21(2), pp. 299–316. doi: 10.1007/s10068-012-0041-1.

Jamalizadeh, M., Etebarian, H. R., Aminian, H. and Alizadeh, A. (2011) 'A review of mechanisms of action of biological control organisms against post-harvest fruit spoilage', *Bulletin OEPP/EPPO*, 41, pp. 65–71.

Janisiewicz, W. (1996) 'Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples.', *Phytopathology*, 86, pp. 473–479.

Janisiewicz, W. J. and Jeffers, S. N. (1997) 'Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage', *Crop Protection*, 16(7), pp. 629–633. doi: 10.1016/S0261-2194(97)00048-3.

Janisiewicz, W. and Korsten, L. (2002) 'Biological control of postharvest diseases of fruits', *Annual Review of Phytopathology*, 40(1), pp. 411–441. doi: 10.1146/annurev.phyto.40.120401.130158.

Jankowska, A., Laubitz, D., Antushevich, H., Zabielski, R. and Grzesiuk, E. (2008) 'Competition of

Lactobacillus paracasei with *Salmonella enterica* for adhesion to Caco-2 cells', *Journal of Biomedicine and Biotechnology*, p. 357964.

Ji, P. and Wilson, M. (2002) 'Assessment of the importance of similarity in carbon source utilization profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato', *Applied and Environmental Microbiology*, 68, pp. 4383–4389.

Jiang, Y. and Miles, P. W. (1993) 'Generation of H2O2 during enzymic oxidation of catechin', *Phytochemistry*, 33(1), pp. 29–34. doi: https://doi.org/10.1016/0031-9422(93)85391-4.

Jones, R. B., Faragher, J. D. and Winkler, S. (2006) 'A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (Brassica oleracea var. italica) heads', *Postharvest Biology and Technology*, 41(1), pp. 1–8. doi: 10.1016/j.postharvbio.2006.03.003.

Jordan, K., Dalmasso, M., Zentek, J., Mader, A., Bruggeman, G., Wallace, J., De Medici, D., Fiore, A., Prukner-Radovcic, E., Lukac, M., Axelsson, L., Holck, A., Ingmer, H. and Malakauskas, M. (2014) 'Microbes versus microbes: control of pathogens in the food chain.', *Journal of the science of food and agriculture*, 94(15), pp. 3079–89. doi: 10.1002/jsfa.6735.

Joshi, K., Mahendran, R., Alagusundaram, K., Norton, T. and Tiwari, B. K. (2013) 'Novel disinfectants for fresh produce', *Trends in Food Science and Technology*. Elsevier Ltd, 34(1), pp. 54–61. doi: 10.1016/j.tifs.2013.08.008.

Jovanovic-Malinovska, R., Kuzmanova, S. and Winkelhausen, E. (2014) 'Oligosaccharide profile in fruits and vegetables as sources of prebiotics and functional foods.', *International Journal of Food Properties*, 17(January 2014), pp. 949–965. doi: 10.1080/10942912.2012.680221.

Kader, A. and Barret, D. M. (no date) 'Classification, composition of fruits and postharvest maitenance of quality', in *Postharvest Maintenance of Fruit*.

Kadurugamuwa, J. L. and Beveridge, T. J. (1996) 'Bacteriolytic Effect of Membrane Vesicles from Pseudomonas aeruginosa on Other Bacteria Including Pathogens : Conceptually New Antibiotics', *Journal of Bacteriology*, 178(10), pp. 2767–2774.

Kalia, V. C. (2013) 'Quorum sensing inhibitors: An overview', *Biotechnology Advances*. Elsevier Inc., 31(2), pp. 224–245. doi: 10.1016/j.biotechadv.2012.10.004.

van Kan, J. A. L. (2006) 'Licensed to kill: the lifestyle of a necrotrophic plant pathogen', *Trends in Plant Science*, 11(5), pp. 247–253. doi: 10.1016/j.tplants.2006.03.005.

Karakurt, Y. and Huber, D. J. (2004) 'Ethylene-induced gene expression, enzyme activities, and water soaking in immature and ripe watermelon (Citrullus lanatus) fruit', *Journal of Plant Physiology*, 161(4), pp. 381–388. doi: 10.1078/0176-1617-01221.

Kastbjerg, V. G., Hein-Kristensen, L. and Gram, L. (2014) 'Triclosan-induced aminoglycoside-tolerant *Listeria monocytogenes* isolates can appear as small-colony variants', *Antimicrobial Agents and Chemotherapy*, 58, pp. 3124–3132.

Kathariou, S. (2002) 'Listeria monocytogenes virulence and pathogenicity, a food safety perspective.', *Journal of food protection*, 65(11), pp. 1811–1829. doi: 10.4315/0362-028X-65.11.1811.

Kawano, T. and Boutou, F. (2013) 'Salicilic acid-induced local and long distance signaling models in plants', in Baluska, F. (ed.) *Long-Distance Systemic Signaling and Communication in Plants*. Springer-Verlag Berlin Heidelberg, pp. 23–52. doi: 10.1007/978-3-642-36470-9.

Khaleque, L. and Bari, A. (2015) 'Facts on Foodborne Pathogens', in Latiful Bari and Dike O. (ed.) *Foodborne Pathogens and Food Safety*. FL: CRC Press, pp. 35–81.

Klimczak, I. and Gliszczynska-Swiglo, A. (2015) 'Comparison of UPLC and HPLC methods for determination of vitamin C', *Food Chemistry*, 175, pp. 100–105. doi: 10.1016/j.foodchem.2014.11.104.

Kobylarz, M. J., Grigg, J. C., Liu, Y., Lee, M. S. F., Heinrichs, D. E. and Murphy, M. E. P. (2016)

‘Deciphering the Substrate Specificity of SbnA, the Enzyme Catalyzing the First Step in Staphyloferrin B Biosynthesis’, *Biochemistry*, 55(6), pp. 927–939. doi: 10.1021/acs.biochem.5b01045.

Koh, E., Wimalasiri, K. M. S., Chassy, A. W. and Mitchell, A. E. (2009) ‘Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli’, *Journal of Food Composition and Analysis*, 22(7–8), pp. 637–643. doi: 10.1016/j.jfca.2009.01.019.

Koivunen, J. and Heinonen-Tanski, H. (2005) ‘Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments’, *Water Research*, 39(8), pp. 1519–1526. doi: 10.1016/j.watres.2005.01.021.

Kolniak-Ostek, J. (2016) ‘Chemical composition and antioxidant capacity of different anatomical parts of pear (Pyrus communis L.)’, *Food Chemistry*. Elsevier Ltd, 203, pp. 491–497. doi: 10.1016/j.foodchem.2016.02.103.

Korkea-aho, T. L., Papadopoulou, A., Heikkinen, J., von Wright, A., Adams, A., Austin, B. and Thompson, K. D. (2012) ‘<i>Pseudomonas<i/> M162 confers protection against rainbow trout fry syndrome by stimulating immunity’, *Journal of Applied Microbiology*, 113, pp. 24–35. doi: 10.1111/j.1365-2672.2012.05325.x.

Kou, L., Luo, Y., Ingram, D. T., Yan, S. and Jurick, W. M. (2014) ‘Open-refrigerated retail display case temperature profile and its impact on product quality and microbiota of stored baby spinach’, *Food Control*. Elsevier Ltd, 47, pp. 686–692.

Koutsoumanis, K. P., Kendall, P. A. and Sofos, J. N. (2004) ‘A comparative study on growth limits of Listeria monocytogenes as affected by temperature, pH and aw when grown in suspension or on a solid surface’, *Food Microbiology*, 21(4), pp. 415–422. doi: 10.1016/j.fm.2003.11.003.

Koutsoumanis, K. P., Kendall, P. a and Sofos, J. N. (2003) ‘Effect of Food Processing-Related Stresses on Acid Tolerance of Listeria monocytogenes’, *Society*, 69(12), pp. 1–4. doi: 10.1128/AEM.69.12.7514.

Kovács, E. and Keresztes (2002) ‘Effect of gamma and UV-B/C radiation on plant cells’, *Micron*, 33(2), pp. 199–210. doi: 10.1016/S0968-4328(01)00012-9.

Kramer, B. and Muranyi, P. (2014) ‘Effect of pulsed light on structural and physiological properties of Listeria innocua and Escherichia coli’, *Journal of Applied Microbiology*, 116(3), pp. 596–611. doi: 10.1111/jam.12394.

Kramer, B., Wunderlich, J. and Muranyi, P. (2017a) ‘Impact of treatment parameters on pulsed light inactivation of microorganisms on a food simulant surface’, *Innovative Food Science and Emerging Technologies*. Elsevier, 42(April), pp. 83–90. doi: 10.1016/j.ifset.2017.05.011.

Kramer, B., Wunderlich, J. and Muranyi, P. (2017b) ‘Pulsed light decontamination of endive salad and mung bean sprouts in water’, *Food Control*, 73(2), pp. 367–371. doi: 10.1016/j.foodcont.2016.08.023.

Kramer, B., Wunderlich, J. and Muranyi, P. (2017) ‘Recent findings in pulsed light disinfection’, *Journal of Applied Microbiology*, 122(4), pp. 830–856. doi: 10.1111/jam.13389.

Kremmydas, G. F., Tampakaki, A. P. and Georgakopoulos, D. G. (2013) ‘Characterization of the biocontrol activity of Pseudomonas fluorescens strain X reveals novel genes regulated by glucose’, *PloS one*, 8, pp. 1–11.

Krishnamurthy, K., Tewari, J. C., Irudayaraj, J. and Demirci, A. (2010) ‘Microscopic and spectroscopic evaluation of inactivation of staphylococcus aureus by pulsed UV light and infrared heating’, *Food and Bioprocess Technology*, 3(1), pp. 93–104. doi: 10.1007/s11947-008-0084-8.

Kuiper, I., Lagendijk, E. L., Pickford, R., Derrick, J. P., Lamers, G. E. M., Thomas-Oates, J. E., Lugtenberg, B. J. J. and Bloemberg, G. V. (2004) ‘Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down

existing biofilms', *Molecular Microbiology*, 51, pp. 97–113.

Kunz, B. A., Dando, P. K., Grice, D. M., Mohr, P. G., Schenk, P. M. and Cahill, D. M. (2008) 'UV-Induced DNA Damage Promotes Resistance to the Biotrophic Pathogen Hyaloperonospora parasitica in Arabidopsis', *Plant Physiology*, 148(2), pp. 1021–1031. doi: 10.1104/pp.108.125435.

Kushwaha, K. and Muriana, P. M. (2010) 'Comparison of invasiveness among surface-adherent variants of *Listeria monocytogenes* in Caco-2 cell culture assays', *International Journal Food Microbiology*. Elsevier B.V., 138, pp. 166–171. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2009.11.024.

Kusters, J. G., Mulders-Kremers, G. A. W. M., Van Doornik, C. E. M. and Van der Zeijst, B. A. M. (1993) 'Effects of multiplicity of infection, bacterial protein synthesis, and growth phase on adhesion to and invasion of human cell lines by *Salmonella typhimurium*', *Infection and Immunity*, 61, pp. 5013–5020.

Lakakul, R., Beaudry, R. M. and Hernandez, R. J. (1999) 'Modeling respiration of apple slices in modified-atmosphere packages', *Journal of Food Science*, 64(1), pp. 105–110. doi: 10.1111/j.1365-2621.1999.tb09870.x.

Lamia, B., Messaoud, B., Ahmed, C. and Lakhdar, K. (2017) 'Activity of plant growth promoting rhizobacteria (PGPRs) in the biocontrol of tomato Fusarium wilt', *Plant Protection Science*, 53(No. 2), pp. 78–84. doi: 10.17221/178/2015-PPS.

Lamikanra, O. (2005) 'Mechanical injury of fresh produce', in Lamikanra, O., Imam, S. H., and Ukuku, D. O. (eds) *Produce deterioration: pathways and prevention*. Boca Ratón, FL: CRC Press, pp. 79–176.

Lamikanra, O., Chen, J. C., Banks, D. and Hunter, P. A. (2000) 'Biochemical and microbial changes during the storage of minimally processed Cantaloupe', *Journal of Agricultural and Food Chemistry*, 48, pp. 5955–5961.

Lara, I. and Vendrell, M. (2000) 'Development of ethylene-synthesizing capacity in preclimacteric apples: interaction between abscisic acid and ethylene', *J. Amer. Soc. Hort. Sci.*, 125(4), pp. 505–512.

Larrigaudiere, C., Ubach, D., Soria, Y., Rojas-Grau, M. A. and Martin-Belloso, O. (2008) 'Oxidative behaviour of fresh-cut "Fuji" apples treated with stabilising substances', *J Sci Food Agric*, 88, pp. 1770–1776. doi: https://doi.org/10.1002/jsfa.3279.

Lasagabaster, A. and Martínez- de Marañón, I. (2014) 'Survival and growth of Listeria innocua treated by pulsed light technology: Impact of post-treatment temperature and illumination conditions', *Food Microbiology*. Elsevier Ltd, 41, pp. 76–81. doi: 10.1016/j.fm.2014.02.001.

LaSarre, B. and Federle, M. J. (2013) 'Exploiting Quorum Sensing To Confuse Bacterial Pathogens', *Microbiology and Molecular Biology Reviews*, 77(1), pp. 73–111. doi: 10.1128/MMBR.00046-12.

Latté, K. P., Appel, K. E. and Lampen, A. (2011) 'Health benefits and possible risks of broccoli - An overview', *Food and Chemical Toxicology*, 49(12), pp. 3287–3309. doi: 10.1016/j.fct.2011.08.019.

Laughlin, R. S., Musch, M. W., Hollbrook, C. J., Rocha, F. M., Chang, E. B. and Alverdy, J. C. (2000) 'The key role of *Pseudomonas aeruginosa* PA-I lectin on experimental gut-derived sepsis', *Annals of Surgery*, 232, pp. 133–142. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1421122/.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S. C. J. (2010) 'Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens.', *Nature reviews. Microbiology*. Nature Publishing Group, 8, pp. 171–184. Available at: http://dx.doi.org/10.1038/nrmicro2297.

Lee, Y. K., Lim, C. Y., Teng, W. L., Ouwehand, A. C., Tuomola, E. M. and Salminen, S. (2000) 'Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria.', *Applied and Environmental Microbiology*, 66(9), pp. 3692–

3697. doi: 10.1128/AEM.66.9.3692-3697.2000.Updated.

Leistner, L. (2000) 'Basic aspects of food preservation by hurdle technology.', *International Journal of Food Microbiology*, 55(1-3), pp. 181–186.

Lelièvre, J. M. ., Latché, A. ., Jones, B., Bouzayen, M. . and Pech, J. C. (1997) 'Ethylene and Fruit Ripening', *Physiologia Plantarum*, 101, pp. 727–739. doi: 10.1007/s00344-007-9002-y.

Lemoine, M., Civello, P., Martínez, G. and Chaves, P. (2007) 'Influence of postharvest UV-C treatment on refrigerated storage of minimally processed broccoli (*Brassica oleracea* var. Italica)', *J Sci Food Agric*, 87, pp. 1132–1139. doi: 10.1002/jsfa.2826.

Lemoine, M. L., Chaves, A. R. and Martínez, G. A. (2010) 'Influence of combined hot air and UV-C treatment on the antioxidant system of minimally processed broccoli (Brassica oleracea L. var. Italica)', *LWT - Food Science and Technology*. Elsevier Ltd, 43(9), pp. 1313–1319. doi: 10.1016/j.lwt.2010.05.011.

Leverentz, B., Conway, W. S., Janisiewicz, W., Abadias, M., Kurtzman, C. P. and Camp, M. J. (2006) 'Biocontrol of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* serovar poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists', *Applied and Environmental Microbiology*, 72(2), pp. 1135–1140. doi: https://dx.doi.org/10.1128/AEM.72.2.1135-1140.2006.

Leverentz, B., Janisiewicz, W. and Conway, W. (2002) 'Biological Control of Minimally Processed Fruits and Vegetables', *Microbial Safety of Minimally Processed Foods*, (December 2002). doi: doi:10.1201/9781420031850.ch15.

Levy, C., Aubert, X., Lacour, B. and Carlin, F. (2012) 'Relevant factors affecting microbial surface decontamination by pulsed light', *International Journal of Food Microbiology*. Elsevier B.V., 152(3), pp. 168–174. doi: 10.1016/j.ijfoodmicro.2011.08.022.

Li, S. M., Hua, G. G., Liu, H. X. and Guo, J. H. (2008) 'Analysis of defence enzymes induced by antagonistic bacterium Bacillus subtilis strain AR12 towards Ralstonia solanacearum in tomato', *Annals of Microbiology*, 58(4), pp. 573–578. doi: 10.1007/BF03175560.

Li, X., Jiang, Y., Li, W., Tang, Y. and Yun, J. (2012) 'Effects of ascorbic acid and high oxygen modified atmosphere packaging during storage of fresh-cut eggplants', *Food Science and Technology International*, 20(2), pp. 99–108. doi: 10.1177/1082013212472351.

Li, X., Long, Q., Gao, F., Han, C., Jin, P. and Zheng, Y. (2017) 'Effect of cutting styles on quality and antioxidant activity in fresh-cut pitaya fruit', *Postharvest Biology and Technology*. Elsevier B.V., 124, pp. 1–7. doi: 10.1016/j.postharvbio.2016.09.009.

Li, Y., Gu, Y., Li, J., Xu, M., Wei, Q. and Wang, Y. (2015) 'Biocontrol agent *Bacillus amyloliquefaciens* LJ02 induces systemic resistance against cucurbits powdery mildew', *Frontiers in Microbiology*, 6(AUG), pp. 1–15. doi: https://dx.doi.org/10.3389/fmicb.2015.00883.

Licciardello, G., Strano, C. P., Bertani, I., Bella, P., Fiore, A., Fogliano, V., Venturi, V. and Catara, V. (2012) 'N-acyl-homoserine-lactone quorum sensing in tomato phytopathogenic Pseudomonas spp. is involved in the regulation of lipodepsipeptide production', *Journal of Biotechnology*. Elsevier B.V., 159(4), pp. 274–282. doi: 10.1016/j.jbiotec.2011.07.036.

Lichtenthaler, H.K. and Buschmann, C. (2001) 'Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy.', in Wrolstad, R.E., Acree, T.E., An, H., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F. and Sporns, P. (ed.) *Current Protocols in Food Analytical Chemistry (CPFA),*. New York,: John Wiley and Sons, p. F4.3.1-F4.3.8.

Lim, H. S., Kim, Y. S. and Kim, S. D. (1991) 'Pseudomonas-Stutzeri Ypl-1 Genetic-Transformation and Antifungal Mechanism against Fusarium-Solani, an Agent of Plant-Root Rot', *Applied and Environmental Microbiology*, pp. 510–516.

Lim, J., Lee, D. H. and Heu, S. (2014) 'The interaction of human enteric pathogens with plants', *The*

Plant Pathology Journal, 30(2), pp. 109–116. doi: 10.5423/PPJ.RW.04.2014.0036.

Lim, J. Y., Yoon, J. and Hovde, C. J. (2010) 'A brief overview of Escherichia coli O157:H7 and its plasmid O157.', *Journal of microbiology and biotechnology*, 20(1), pp. 5–14. doi: 10.4014/jmb.0908.08007.

Limbo, S. and Piergiovanni, L. (2007) 'Minimally processed potatoes Part 2 . Effects of high oxygen partial pressures in combination with ascorbic and citric acid on loss of some quality traits', *Postharvest Biology and Technology*, 43, pp. 221–229. doi: 10.1016/j.postharvbio.2006.08.015.

Lin, Y., Lin, Y., Lin, H., Ritenour, M. A., Shi, J., Zhang, S., Chen, Y. and Wang, H. (2017) 'Hydrogen peroxide-induced pericarp browning of harvested longan fruit in association with energy metabolism', *Food Chemistry*. Elsevier Ltd, 225, pp. 31–36. doi: 10.1016/j.foodchem.2016.12.088.

Liu, C., Huang, Y. and Chen, H. (2015) 'Inactivation of Escherichia coli O157: H7 and Salmonella enterica on blueberries in water using ultraviolet light', *Journal of Food Science*, 80(7), pp. M1532–M1537. doi: 10.1111/1750-3841.12910.

Liu, M., Pirrello, J., CHERVIN, C., Roustan, J.-P. and Bouzayen, M. (2015) 'Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation', *Plant Physiology*, 169(December), p. pp.01361.2015. doi: 10.1104/pp.15.01361.

Liu, Q., Cedric Tan, C. S., Yang, H. and Wang, S. (2017) 'Treatment with low-concentration acidic electrolysed water combined with mild heat to sanitise fresh organic broccoli (*Brassica oleracea*)', *LWT - Food Science and Technology*. Elsevier Ltd, 79, pp. 594–600. doi: 10.1016/j.lwt.2016.11.012.

Liu, Z., Wang, X., Zhu, J. and Wang, J. (2010) 'Effect of high oxygen modified atmosphere on post-harvest physiology and sensorial qualities of mushroom', *International Journal Food Science and Technology*, 45, pp. 1097–1103. doi: 10.1111/j.1365-2621.2010.02199.x.

Llano, K. R. A., Marsellés-Fontanet, A. R., Olgamartín, B. and Soliva-Fortuny, R. (2016) 'Impact of pulsed light treatments on antioxidant characteristics and quality attributes of fresh-cut apples', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 33, pp. 206–215. doi: 10.1016/j.ifset.2015.10.021.

Lomonaco, S., Nucera, D. and Filipello, V. (2015) 'The evolution and epidemiology of Listeria monocytogenes in Europe and the United States', *Infection, Genetics and Evolution*. Elsevier B.V., 35, pp. 172–183. doi: 10.1016/j.meegid.2015.08.008.

van Loon, L. C., Geraats, B. P. J. and Linthorst, H. J. M. (2006) 'Ethylene as a modulator of disease resistance in plants', *Trends in Plant Science*, 11(4), pp. 184–191. doi: 10.1016/j.tplants.2006.02.005.

Lopes, M. M. A., Silva, E. O., Canuto, K. M., Silva, L. M. A., Gallão, M. I., Urban, L., Ayala-Zavala, J. F. and Miranda, M. R. A. (2016) 'Low fluence pulsed light enhanced phytochemical content and antioxidant potential of "Tommy Atkins" mango peel and pulp', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 33, pp. 216–224. doi: 10.1016/j.ifset.2015.12.019.

López-gálvez, F., Ragaert, P., Haque, A., Eriksson, M., Christine, M., Labeke, V. and Devlieghere, F. (2015) 'High oxygen atmospheres can induce russet spotting development in minimally processed iceberg lettuce', *Postharvest Biology and Technology*. Elsevier India Pvt Ltd, 100, pp. 168–175. doi: 10.1016/j.postharvbio.2014.10.001.

Lu, C. and Toivonen, P. M. A. (2000) 'Effect of 1 and 100 kPa O 2 atmospheric pretreatments of whole " Spartan " apples on subsequent quality and shelf life of slices stored in modified atmosphere packages', *Postharvest Biology and Technology*, 18, pp. 99–107.

Lugtenberg, B. J. J., Dekkers, L. and Bloemberg, G. V. (2001) 'Molecular determinants of rhizosphere colonization by *Pseudomonas*', *Annual Review of Phytopathology*, 39(1), pp. 461–490. doi: 10.1146/annurev.phyto.39.1.461.

Luksiene, Z., Buchovec, I., Kairyte, K., Paskeviciute, E. and Viskelis, P. (2012) 'High-power pulsed light for microbial decontamination of some fruits and vegetables with different surfaces', *Journal of*

Food, Agriculture and Environment, 10(3–4), pp. 162–167.

Lundén, J., Vanhanen, V., Myllymäki, T., Laamanen, E., Kotilainen, K. and Hemminki, K. (2014) 'Temperature control efficacy of retail refrigeration equipment', *Food Control*, 45, pp. 109–114.

Lupatini, M., Korthals, G. W., de Hollander, M., Janssens, T. K. S. and Kuramae, E. E. (2017) 'Soil microbiome is more heterogeneous in organic than in conventional farming system', *Frontiers in Microbiology*, 7(JAN), pp. 1–13. doi: 10.3389/fmicb.2016.02064.

Lurie, S., Fallik, E., Handros, A. and Shapira, R. (1997) 'The possible involvement of peroxidase in resistance to *Botrytis cinerea* in heat treated tomato fruit', *Physiological and Molecular Plant Pathology*, 50, pp. 141–149. doi: https://doi.org/10.1006/pmpp.1996.0074.

Luukkonen, T., Heyninck, T., Rämö, J. and Lassi, U. (2015) 'Comparison of organic peracids in wastewater treatment: Disinfection, oxidation and corrosion', *Water Research*, 85, pp. 275–285. doi: 10.1016/j.watres.2015.08.037.

Maddula, V. S. R. K. (2008) *Quorum sensing and phenazines are involved in biofilm formation in <i>Pseudomonas chlororaphis<i/> strain 30-84.* UNIVERSITY OF ARIZONA.

Madi, A., Svinareff, P., Orange, N., Feuilloley, M. G. and Connil, N. (2010a) '*Pseudomonas fluorescens* alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells.', *Gut pathogens*. BioMed Central Ltd, 2, p. 16.

Madi, A., Svinareff, P., Orange, N., Feuilloley, M. G. and Connil, N. (2010b) '*Pseudomonas fluorescens* alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells.', *Gut pathogens*, 2(1), p. 16. doi: 10.1186/1757-4749-2-16.

Maffei, D. F., Silveira, N. F. de A. and Catanozi, M. da P. L. M. (2013) 'Microbiological quality of organic and conventional vegetables sold in Brazil', *Food Control*. Elsevier Ltd, 29(1), pp. 226–230. doi: 10.1016/j.foodcont.2012.06.013.

Maggio, A., De Pascale, S., Paradiso, R. and Barbieri, G. (2013) 'Quality and nutritional value of vegetables from organic and conventional farming', *Scientia Horticulturae*. Elsevier B.V., 164, pp. 532–539. doi: 10.1016/j.scienta.2013.10.005.

Mahajan, P. V., Luca, A. and Edelenbos, M. (2014) 'Impact of mixtures of different fresh-cut fruits on respiration and ethylene production rates', *Journal of Food Science*, 79(7), pp. 1366–1371. doi: 10.1111/1750-3841.12512.

Mahmood, T., Anwar, F., Abbas, M., Boyce, M. C. and Saari, N. (2012) 'Compositional variation in sugars and organic acids at different maturity stages in selected small fruits from Pakistan', *International Journal of Molecular Sciences*, 13(2), pp. 1380–1392. doi: 10.3390/ijms13021380.

Mahn, A. and Reyes, A. (2012) 'An overview of health-promoting compounds of broccoli (Brassica oleracea var. italica) and the effect of processing', *Food Science and Technology International*, 18(6), pp. 503–514. doi: 10.1177/1082013211433073.

Malik-Kale, P., Winfree, S. and Steele-Mortimer, O. (2012) 'The bimodal lifestyle of intracellular Salmonella in epithelial cells: Replication in the cytosol obscures defects in vacuolar replication', *PLoS ONE*, 7(6), pp. 1–10. doi: 10.1371/journal.pone.0038732.

Manach C, Scalbert A, Morand C, et al. (2004) 'Bioavailability, Polyphenols: Food sources and', *The American Journal of Clinical Nutrition*, 79, pp. 727–747. doi: 10.1038/nature05488.

Mani-López, E., García, H. S. and López-Malo, A. (2012) 'Organic acids as antimicrobials to control Salmonella in meat and poultry products', *Food Research International*. Elsevier Ltd, 45(2), pp. 713–721. doi: 10.1016/j.foodres.2011.04.043.

Manzocco, L., Da Pieve, S. and Maifreni, M. (2011) 'Impact of UV-C light on safety and quality of fresh-cut melon', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 12(1), pp. 13–17. doi: 10.1016/j.ifset.2010.11.006.

Martín-Diana, A. B., Rico, D., Frías, J., Henehan, G. T. M., Mulcahy, J., Barat, J. M. and Barry-Ryan, C. (2006) 'Effect of calcium lactate and heat-shock on texture in fresh-cut lettuce during storage', *Journal of Food Engineering*, 77(4), pp. 1069–1077. doi: 10.1016/j.jfoodeng.2005.08.037.

Martín-Espada, M. C., D'ors, A., Bartolomé, M. C., Pereira, M. and Sánchez-Fortún, S. (2014) 'Peracetic acid disinfectant efficacy against Pseudomonas aeruginosa biofilms on polystyrene surfaces and comparison between methods to measure it', *LWT - Food Science and Technology*. Elsevier Ltd, 56(1), pp. 58–61. doi: 10.1016/j.lwt.2013.11.013.

Martínez-Hernández, G. B., Artés-Hernández, F., Gómez, P. A. and Artés, F. (2013) 'Comparative behaviour between kailan-hybrid and conventional fresh-cut broccoli throughout shelf-life', *LWT - Food Science and Technology*. Elsevier Ltd, 50(1), pp. 298–305. doi: 10.1016/j.lwt.2012.05.010.

Martínez-Hernández, G. B., Artés-Hernández, F., Gómez, P. A., Formica, A. C. and Artés, F. (2013) 'Combination of electrolysed water, UV-C and superatmospheric O_2 packaging for improving fresh-cut broccoli quality', *Postharvest Biology and Technology*. Elsevier B.V., 76, pp. 125–134. doi: 10.1016/j.postharvbio.2012.09.013.

Martínez-Hernández, G. B., Gómez, P. A., Pradas, I., Artés, F. and Artés-Hernández, F. (2011) 'Moderate UV-C pretreatment as a quality enhancement tool in fresh-cut Bimi® broccoli', *Postharvest Biology and Technology*, 62(3), pp. 327–337. doi: 10.1016/j.postharvbio.2011.06.015.

Martínez-Hernández, G. B., Huertas, J. P., Navarro-Rico, J., Gómez, P. A., Artés, F., Palop, A. and Artés-Hernández, F. (2015) 'Inactivation kinetics of foodborne pathogens by UV-C radiation and its subsequent growth in fresh-cut kailan-hybrid broccoli', *Food Microbiology*, 46, pp. 263–271. doi: 10.1016/j.fm.2014.08.008.

Martínez-Hernández, G. B., Navarro-Rico, J., Gómez, P. A., Otón, M., Artés, F. and Artés-Hernández, F. (2015) 'Combined sustainable sanitising treatments to reduce *Escherichia coli* and *Salmonella enteritidis* growth on fresh-cut kailan-hybrid broccoli', *Food Control*, 47, pp. 312–317. doi: 10.1016/j.foodcont.2014.07.029.

Martínez-Solano, J. R., Sánchez-Bel, P., Egea, I., Olmos, E., Hellin, E. and Romojaro, F. (2005) 'Electron beam ionization induced oxidative enzymatic activities in pepper (*Capsicum annuum* L.), associated with ultrastructure cellular damages', *Journal of Agricultural and Food Chemistry*, 53(22), pp. 8593–8599. doi: 10.1021/jf050994i.

Matéos, A., Guyard-Nicodème, M., Baglinière, F., Jardin, J., Gaucheron, F., Dary, A., Humbert, G. and Gaillard, J. L. (2015) 'Proteolysis of milk proteins by AprX, an extracellular protease identified in Pseudomonas LBSA1 isolated from bulk raw milk, and implications for the stability of UHT milk', *International Dairy Journal*, 49, pp. 78–88. doi: 10.1016/j.idairyj.2015.04.008.

Matthijs, S., Baysse, C., Koedam, N., Tehrani, K. A., Verheyden, L., Budzikiewicz, H., Schäfer, M., Hoorelbeke, B., Meyer, J., Greve, H. De and Cornelis, P. (2004) 'The Pseudomonas siderophore quinolobactin is synthesized from xanthurenic acid , an intermediate of the kynurenine pathway', *Molecular microbiology*, 52(2), pp. 371–384. doi: 10.1111/j.1365-2958.2004.03999.x.

Mattos, L. M., Moretti, C. L. and Yosino Da Silva, E. Y. (2013) 'Effects of modified atmosphere packaging on quality attributes and physiological responses of fresh-cut crisphead lettuce', *CYTA - Journal of Food*, 11(4), pp. 392–397. doi: 10.1080/19476337.2013.777124.

Matusheski, N. V., Juvik, J. A. and Jeffery, E. H. (2004) 'Heating decreases epithiospecifier protein activity and increases sulforaphane formation in broccoli', *Phytochemistry*, 65(9), pp. 1273–1281. doi: 10.1016/j.phytochem.2004.04.013.

May, T. B., Shinabarger, D., Maharaj, R., Kato, J., Chu, L., Devault, J. D., Roychoudhury, S., Zielinski, N. a., Berry, A., Rothmel, R. K., Misra, T. K. and Chakrabarty, a. M. (1991) 'Alginate synthesis by <i>Pseudomonas aeruginosa<i/>: A key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients', *Clinical Microbiology Reviews*, 4(2), pp. 191–206. doi: 10.1128/CMR.4.2.191.

McFadden, M., Loconsole, J., Schockling, A. J., Nerenberg, R. and Pavissich, J. P. (2017) 'Comparing

peracetic acid and hypochlorite for disinfection of combined sewer overflows: Effects of suspended-solids and pH', *Science of the Total Environment*. Elsevier B.V., 599–600, pp. 533–539. doi: 10.1016/j.scitotenv.2017.04.179.

McLellan, M. R., Lind, L. R. and Kime, R. W. (1995) 'Hue Angle determinations and statistical analysis for multiquadrant Hunter L, a, b data', *Journal of Food Quality*, 18(3), pp. 235–240. doi: 10.1111/j.1745-4557.1995.tb00377.x.

Medellin-Peña, M. J. and Griffiths, M. W. (2009) 'Effect of molecules secreted by Lactobacillus acidophilus strain La-5 on Escherichia coli O157:H7 colonization', *Applied and Environmental Microbiology*, 75(4), pp. 1165–1172. doi: 10.1128/AEM.01651-08.

Medellin-Peña, M. J., Wang, H., Johnson, R., Anand, S. and Griffiths, M. W. (2007) 'Probiotics affect virulence-related gene expression in Escherichia coli O157:H7', *Applied and Environmental Microbiology*, 73(13), pp. 4259–4267. doi: 10.1128/AEM.00159-07.

Mehnaz, S., Weselowski, B., Aftab, F., Zahid, S., Lazarovits, G. and Iqbal, J. (2009) 'Isolation, characterization, and effect of fluorescent pseudomonads on micropropagated sugarcane', *Canadian Journal of Microbiology*, 55(8), pp. 1007–1011. doi: 10.1139/W09-050.

Mewis, I., Schreiner, M., Nguyen, C. N., Krumbein, A., Ulrichs, C., Lohse, M. and Zrenner, R. (2012) 'UV-B irradiation changes specifically the secondary metabolite profile in broccoli sprouts: Induced signaling overlaps with defense response to biotic stressors', *Plant and Cell Physiology*, 53(9), pp. 1546–1560. doi: 10.1093/pcp/pcs096.

Michel-Briand, Y. and Baysse, C. (2002) 'The pyocins of Pseudomonas aeruginosa', *Biochimie*, 84, pp. 499–510.

Mikani, A., Etebarian, H. R. and Aminian, H. (2011) 'Changes in Peroxidase and phenols activity in apple fruit inoculated with antagonistic *Pseudomonas fluorescens* isolates and *Botritis mali*', *Pakistan Journal of Biological Sciences*, 14(18), pp. 854–861.

Mikiciński, A., Sobiczewski, P., Puławska, J. and Maciorowski, R. (2016) 'Control of fire blight (Erwinia amylovora) by a novel strain 49M of Pseudomonas graminis from the phyllosphere of apple (Malus spp.)', *European Journal of Plant Pathology*, 145(2), pp. 265–276. doi: 10.1007/s10658-015-0837-y.

Mikiciński, A., Sobiczewski, P., Puławska, J. and Malusa, E. (2016) 'Antagonistic potential of *Pseudomonas graminis* 49M against *Erwinia amylovora*, the causal agent of fire blight.', *Archives of Microbiology*, 198(6), pp. 531–539. doi: 10.1007/s00203-016-1207-7.

Mikkelsen, M. D. (2003) 'Modulation of CYP79 Genes and Glucosinolate Profiles in Arabidopsis by Defense Signaling Pathways', *Plant Physiology*, 131(1), pp. 298–308. doi: 10.1104/pp.011015.

Milagres, A. M. F., Machuca, A. and Napoleão, D. (1999) 'Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay', *Journal of Microbiological Methods*, 37(1), pp. 1–6. doi: 10.1016/S0167-7012(99)00028-7.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrì, F., Boutrou, R., Corredig, F. M., Dupont, D., Dufour, F. C., Egger, L., Golding, M., Karakaya, L. S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., Mcclements, D. J., Enard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W. and Brodkorb, A. (2014) 'A standardised static *in vitro* digestion method suitable for food – an international consensus', *Food Funct. Food Funct*, 5, pp. 1113–1124.

Minibayeva, F., Beckett, R. P. and Kranner, I. (2015) 'Roles of apoplastic peroxidases in plant response to wounding', *Phytochemistry*. Elsevier Ltd, 112(1), pp. 122–129. doi: 10.1016/j.phytochem.2014.06.008.

Mintoff, S. J. L., Rookes, J. E. and Cahill, D. M. (2015) 'Sub-lethal UV-C radiation induces callose, hydrogen peroxide and defence-related gene expression in Arabidopsis thaliana', *Plant Biology*, 17(3), pp. 703–711. doi: 10.1111/plb.12286.

Moing, A., Svanella, L., Rolin, D., Gaudillère, M., Gaudillère, J.-P., Monet, R., Gaudillere, M. and Gaudillere, J. P. (1998) 'Compositional Changes during the Fruit Development of Two Peach Cultivars Differing in Juice Acidity', *Journal of the American Society for Horticultural Science*, pp. 770–775. Available at: http://journal.ashspublications.org/content/123/5/770.abstract.

Moran, R. (1982) 'Formulae for determination of chlorophyllous pigments extracted with n,n-dimethylformamide.', *Plant physiology*, 69(6), pp. 1376–1381. doi: 10.1104/pp.69.6.1376.

Morelli, E., Noel, V., Rosset, P. and Poumeyrol, G. (2012) 'Performance and conditions of use of refrigerated display cabinets among producer/vendors of foodstuffs', *Food Control*. Elsevier Ltd, 26(2), pp. 363–368.

Morello, J. E., Pierson, E. A. and Pierson, L. S. (2004) 'Negative cross-communication among wheat rhizosphere bacteria: Effect on antibiotic production by the biological control bacterium Pseudomonas aureofaciens 30-84', *Applied and Environmental Microbiology*, 70(5), pp. 3103–3109. doi: 10.1128/AEM.70.5.3103-3109.2004.

Moroni, O., Kheadr, E., Boutin, Y., Lacroix, C. and Fliss, I. (2006) 'Inactivation of adhesion and invasion of food-borne *Listeria monocytogenes* by bacteriocin-producing *Bifidobacterium* strains of human origin', *Applied and Environmental Microbiology*, 72(11), pp. 6894–6901. doi: 10.1128/AEM.00928-06.

Mortureux, M. (2012) 'Avis de l' Agence nationale de sécurité sanitaire de l' alimentation, de l' environnement et du travail', *Maisons-Alfort*, 33(0), pp. 1–17. doi: 2011-SA-0133.

Mukhopadhyay, S. and Ukuku, D. O. (2018) 'The role of emerging technologies to ensure the microbial safety of fresh produce, milk and eggs', *Current Opinion in Food Science*. Elsevier Ltd, (December 2017), pp. 1–10. doi: 10.1016/j.cofs.2018.01.013.

Munday, R., Mhawech-Fauceglia, P., Munday, C. M., Paonessa, J. D., Tang, L., Munday, J. S., Lister, C., Wilson, P., Fahey, J. W., Davis, W. and Zhang, Y. (2008) 'Inhibition of urinary bladder carcinogenesis by broccoli sprouts', *Cancer Research*, 68(5), pp. 1593–1600. doi: 10.1158/0008-5472.CAN-07-5009.

Mundi, A., Delcenserie, V., Amiri-Jami, M., Moorhead, S. and Griffiths, M. W. (2013) 'Cell-free preparations of Lactobacillus acidophilus strain La-5 and Bifidobacterium longum strain NCC2705 affect virulence gene expression in Campylobacter jejuni.', *Journal of food protection*. United States, 76(10), pp. 1740–1746. doi: 10.4315/0362-028X.JFP-13-084.

Muñoz-Provencio, D., Llopis, M., Antolín, M., De Torres, I., Guarner, F., Pérez-Martínez, G. and Monedero, V. (2009) 'Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix', *Archives of Microbiology*, 191, pp. 153–161.

N.Teixidó, Torres, R., Viñas, I., M.Abadias and Usall, J. (2011) 'Biological control of postharvest diseases in fruit and vegetables', in *Protective Cultures, Antimicrobial Metabolites and Bacteriophages for Food and Beverage Biopreservation*. Elsevier, pp. 364–402. doi: 10.1533/9780857090522.3.364.

Nadeau, F., Gaudreau, A., Angers, P. and Arul, J. (2012) 'Changes in the level of glucosinolates in broccoli florets (*Brassica oleracea* var. italica) during storage following postharvest treatment with UV-C', *Acta Horticulturae*, 945, pp. 145–148.

Nagarajkumar, M., Bhaskaran, R. and Velazhahan, R. (2004) 'Involvement of secondary metabolites and extracellular lytic enzymes produced by Pseudomonas fluorescens in inhibition of Rhizoctonia solani , the rice sheath blight pathogen', *Microbiological Research*, 159, pp. 73–81. doi: 10.1016/j.micres.2004.01.005.

Nakano, Y. and Asada, K. (1981) 'Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts', *Plant Cell Physiol*, 22, pp. 867–880. doi: https://doi.org/10.1093/oxfordjournals.pcp.a076232.

Neeser, J. R., Granato, D., Rouvet, M., Servin, A., Teneberg, S. and Karlsson, K. a (2000) '*Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria.', *Glycobiology*, 10, pp. 1193–1199.

Nicodème, M., Grill, J. P., Humbert, G. and Gaillard, J. L. (2005) 'Extracellular protease activity of different Pseudomonas strains: Dependence of proteolytic activity on culture conditions', *Journal of Applied Microbiology*, 99(3), pp. 641–648. doi: 10.1111/j.1365-2672.2005.02634.x.

Nicolas, J. J., Richard-Forget, F. C., Goupy, P. M., Amiot, M. and Aubert, S. Y. (1994) 'Enzymatic browning reactions in apple and apple products', *Critical Reviews in Food Science and Nutrition*. Taylor & Francis, 34(2), pp. 109–157. doi: https://doi.org/10.1080/10408399409527653.

Nielsen, M. N. and Sørensen, J. (1999) 'Chitinolytic activity of Pseudomonas fluorescens isolates from barley and sugar beet rhizosphere', *FEMS Microbiology Ecology*, 30(3), pp. 217–227. doi: 10.1016/S0168-6496(99)00058-6.

Nilsson, L., Hansen, T. B., Garrido, P., Buchrieser, C., Glaser, P., Knøchel, S., Gram, L. and Gravesen, A. (2005) 'Growth inhibition of Listeria monocytogenes by a nonbacteriocinogenic Carnobacterium piscicola', *Journal of Applied Microbiology*, 98(1), pp. 172–183. doi: 10.1111/j.1365-2672.2004.02438.x.

Nunes, C., Usall, J., Teixidó, N., Fons, E. and Viñas, I. (2002) 'Post-harvest biological control by Pantoea agglomerans (CPA-2) on Golden Delicious apples', *Journal of Applied Microbiology*, 92(2), pp. 247–255. doi: 10.1046/j.1365-2672.2002.01524.x.

O'Toole, G. a. and Kolter, R. (1998) 'Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis', *Molecular Microbiology*, 28, pp. 449–461.

Ochsner, U. A., Wilderman, P. J., Vasil, A. I. and Vasil, M. L. (2002) 'GeneChip® expression analysis of the iron starvation response in Pseudomonas aeruginosa: Identification of novel pyoverdine biosynthesis genes', *Molecular Microbiology*, 45(5), pp. 1277–1287. doi: 10.1046/j.1365-2958.2002.03084.x.

Oetiker, J. H. . and Yang, S. F. (1995) 'The role of ethylene in fruit ripening', *Acta Horticulturae*, 398, pp. 167–178. doi: DOI: 10.17660/ActaHortic.1995.398.17.

Oh, S. A., Kim, J. S., Park, J. Y., Han, S. H., Dimkpa, C., Anderson, A. J. and Kim, Y. C. (2013) 'The RpoS sigma factor negatively regulates production of IAA and siderophore in a biocontrol <i>Rhizobacterium<i/>, <i>Pseudomonas chlororaphis<i/> O6.', *The plant pathology journal*, 29(3), pp. 323–9. doi: 10.5423/PPJ.NT.01.2013.0013.

Oliveira, M., Viñas, I., Colás, P., Anguera, M., Usall, J. and Abadias, M. (2014) 'Effectiveness of a bacteriophage in reducing Listeria monocytogenes on fresh-cut fruits and fruit juices', *Food Microbiology*. Elsevier Ltd, 38, pp. 137–142. doi: 10.1016/j.fm.2013.08.018.

Oliveira, M., Wijnands, L., Abadias, M., Aarts, H. and Franz, E. (2011) 'Pathogenic potential of Salmonella Typhimurium DT104 following sequential passage through soil, packaged fresh-cut lettuce and a model gastrointestinal tract', *International Journal of Food Microbiology*. Elsevier B.V., 148(3), pp. 149–155. doi: 10.1016/j.ijfoodmicro.2011.05.013.

Ölmez, H. and Kretzschmar, U. (2009) 'Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact', *LWT - Food Science and Technology*. Swiss Society of Food Science and Technology, 42(3), pp. 686–693. doi: 10.1016/j.lwt.2008.08.001.

Oms-Oliu, G., Aguiló-Aguayo, I., Martín-Belloso, O. and Soliva-Fortuny, R. (2010) 'Effects of pulsed light treatments on quality and antioxidant properties of fresh-cut mushrooms (Agaricus bisporus)', *Postharvest Biology and Technology*, 56(3), pp. 216–222. doi: 10.1016/j.postharvbio.2009.12.011.

Oms-Oliu, G., Martín-Belloso, O. and Soliva-Fortuny, R. (2010) 'Pulsed light treatments for food

preservation. A review', *Food and Bioprocess Technology*, 3(1), pp. 13–23. doi: 10.1007/s11947-008-0147-x.

Oms-Oliu, G., Odriozola-Serrano, I., Soliva-Fortuny, R., Elez-Martínez, P. and Martín-Belloso, O. (2012) 'Stability of health-related compounds in plant foods through the application of non thermal processes', *Trends in Food Science and Technology*, 23(2), pp. 111–123. doi: 10.1016/j.tifs.2011.10.004.

Oms-Oliu, G., Soliva-Fortuny, R. and Mart??n-Belloso, O. (2007) 'Effect of ripeness on the shelf-life of fresh-cut melon preserved by modified atmosphere packaging', *European Food Research and Technology*, 225(3–4), pp. 301–311. doi: 10.1007/s00217-006-0415-9.

Ou, C., Liu, Y., Wang, W. and Dong, D. (2016) 'Integration of UV-C with antagonistic yeast treatment for controlling post-harvest disease and maintaining fruit quality of Ananas comosus', *BioControl*. Springer Netherlands, 61(5), pp. 591–603. doi: 10.1007/s10526-016-9740-5.

Pace, G., Lima, P., Machado, T. M., Furtado, N. R., Fleuri, F., Cavalcante, M., Vieira, D. S., Oliveira, L. M. and Vianello, F. (2013) 'Influence of sanitation on the physico-chemical and microbiological quality of organic and conventional broccoli', *African Journal of Biotechnology*, 12(18), pp. 2456–2463. doi: 10.5897/AJB2012.2997.

Pacetto, A., Bella, P., Catara, V., La Rosa, R. and Cirvilleri, G. (2003) 'Survival of biocontrol *Pseudomonas* strains and human pathogens in fruit juices.', in Iacobellis, N. S., Collmer, A., Hutcheson, S. W., Mansfield, J. W., Morris, C. E., Murillo, J., Schaad, N. W., Stead, D. E., Surico, G., and Ullrich, M. S. (eds) Pseudomonas syringae *and related pathogens: Biology and Genetic*. Dordrecht: Springer Netherlands, pp. 107–115.

Pamp, S. J. and Tolker-Nielsen, T. (2007) 'Multiple roles of biosurfactants in structural biofilm development by Pseudomonas aeruginosa', *Journal of Bacteriology*, 189(6), pp. 2531–2539. doi: 10.1128/JB.01515-06.

Pan, X., Wu, T., Zhang, L., Song, Z., Tang, H. and Zhao, Z. (2008) '*In vitro* evaluation on adherence and antimicrobial properties of a candidate probiotic *Clostridium butyricum* CB2 for farmed fish', *Journal of Applied Microbiology*, 105, pp. 1623–1629.

Pan, Y., Zhu, J. and Li, S. (2015) 'Effects of pure oxygen and reduced oxygen modified atmosphere packaging on the quality and microbial characteristics of fresh-cut pineapple', *Fruits*, 70(2), pp. 101–108.

Panda, S. K. (2012) 'Assay guided comparison for enzymatic and non-enzymatic antioxidant activities with special reference to medicinal plants', *Intech*, Chapter 15, pp. 381–400. doi: 10.5772/50782.

Pandey, S., Fartyal, D., Agarwal, A., Shukla, T., James, D., Kaul, T., Negi, Y. K., Arora, S. and Reddy, M. K. (2017) 'Abiotic Stress Tolerance in Plants: Myriad Roles of Ascorbate Peroxidase', *Frontiers in Plant Science*, 8(April), pp. 1–13. doi: 10.3389/fpls.2017.00581.

Parafati, L., Vitale, A., Restuccia, C. and Cirvilleri, G. (2015) 'Biocontrol ability and action mechanism of food-isolated yeast strains against <i>Botrytis cinerea<i/> causing post-harvest bunch rot of table grape', *Food Microbiology*. Elsevier Ltd, 47, pp. 85–92. doi: 10.1016/j.fm.2014.11.013.

Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garrett, E. H., Farber, J. N. and Busta, F. F. (2003) 'Methods to reduce/eliminate pathogens from fresh and fresh-cut produce', *Comprehensive Reviews in Food Science and Food Safety*, 2(s1), pp. 161–173. doi: 10.1111/j.1541-4337.2003.tb00033.x.

Park, J. B., Kang, J. H. and Song, K. Bin (2018) 'Combined treatment of cinnamon bark oil emulsion washing and ultraviolet-C irradiation improves microbial safety of fresh-cut red chard', *Lwt*. Elsevier, 93(March), pp. 109–115. doi: 10.1016/j.lwt.2018.03.035.

Park, J. Y., Oh, S. A., Anderson, A. J., Neiswender, J., Kim, J.-C. and Kim, Y. C. (2011) 'Production of the antifungal compounds phenazine and pyrrolnitrin from Pseudomonas chlororaphis O6 is differentially regulated by glucose.', *Letters in applied microbiology*, 52, pp. 532–7.

Parret, A. H. A. and Mot, R. De (2002) 'Bacteria killing their own kind : novel bacteriocins of Pseudomonas and other γ -proteobacteria', *Trends in Microbiology*, 10(3), pp. 107–112. doi: https://doi.org/10.1016/S0966-842X(02)02307-7.

Parret, A. and De Mot, R. (2000) 'Novel bacteriocins with predicted tRNase and pore-forming activities in Pseudomonas aeruginosa PAO1 [1]', *Molecular Microbiology*, 35(2), pp. 472–473. doi: 10.1046/j.1365-2958.2000.01716.x.

Parthasarathy, S., Thiribhuvanamamala, G., Subramanian, K. S. and Prabakar, K. (2016) 'Bacterial antagonists and hexanal-induced systemic resistance of mango fruits against Lasiodiplodia theobromae causing stem-end rot', *Journal of Plant Interactions*, 11(1), pp. 158–166. doi: 10.1080/17429145.2016.1252068.

Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S. A., Mavrodi, D. V., DeBoy, R. T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R. J., Durkin, A. S., Brinkac, L. M., Daugherty, S. C., Sullivan, S. A., Rosovitz, M. J., Gwinn, M. L., Zhou, L., Schneider, D. J., Cartinhour, S. W., Nelson, W. C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E. A., Pierson, L. S., Thomashow, L. S. and Loper, J. E. (2005) 'Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5', *Nature Biotechnology*, 23(7), pp. 873–878. doi: 10.1038/nbt1110.

Petit, A. N., Baillieul, F., Vaillant-Gaveau, N., Jacquens, L., Conreux, A., Jeandet, P., Clément, C. and Fontaine, F. (2009) 'Low responsiveness of grapevine flowers and berries at fruit set to UV-C irradiation', *Journal of Experimental Botany*, 60(4), pp. 1155–1162. doi: 10.1093/jxb/ern361.

Phillips, C. A. (1996) 'Review: modified atmosphere packaging and its effects on the microbiological quality and safety of produce', *International Journal of Food Science and Technology*, 31, pp. 463–479. doi: 10.1046/j.1365-2621.1996.00369.x.

Pierson, L. S. and Pierson, E. a. (2010) 'Metabolism and function of phenazines in bacteria: Impacts on the behavior of bacteria in the environment and biotechnological processes', *Applied Microbiology and Biotechnology*, 86, pp. 1659–1670. doi: 10.1007/s00253-010-2509-3.

Pieterse, C. M. J. (2001) 'Rhizobacteria-mediated induced systemic resistance: triggering, signaling and expression', *European Journal of Plant Pathology*, 107, pp. 51–61. doi: 10.1023/A:1008747926678.

Pieterse, C. M. J., Van Pelt, J. A., Van Wees, S. C. M., Ton, J., Léon-Kloosterziel, K. M., Keurentjes, J. J. B., Verhagen, B. W. M., Knoester, M., der Sluis, I., Bakker, P. A. H. M. and Van Loon, L. C. (2001) 'Rhizobacteria-mediated Induced Systemic Resistance: Triggering, Signalling and Expression', *European Journal of Plant Pathology*, 107(1), pp. 51–61. doi: https://doi.org/10.1023/A:100874792.

Pieterse, C. M. J., Van Pelt, J. a, Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Metraux, J.-P. and Van Loon, L. C. (2000) 'Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production', *Physiological and Molecular Plant Pathology*, 57(3), pp. 123–134. doi: https://doi.org/10.1006/pmpp.2000.0291.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M. and Bakker, P. A. H. M. (2014) 'Induced systemic resistance by beneficial microbes.', *Annual Review of Phytopathology*, 52(1), pp. 347–375. doi: https://doi.org/10.1023/A:1008747926678.

Plaza, L., Altisent, R., Alegre, I., Viñas, I. and Abadias, M. (2016) 'Changes in the quality and antioxidant properties of fresh-cut melon treated with the biopreservative culture *Pseudomonas graminis* CPA-7 during refrigerated storage', *Postharvest Biology and Technology*, 111, pp. 25–30. doi: https://doi.org/10.1016/j.postharvbio.2015.07.023.

Pombo, M. A., Dotto, M. C., Martínez, G. A. and Civello, P. M. (2009) 'UV-C irradiation delays strawberry fruit softening and modifies the expression of genes involved in cell wall degradation', *Postharvest Biology and Technology*, 51(2), pp. 141–148. doi: 10.1016/j.postharvbio.2008.07.007.

Pombo, M. A., Rosli, H. G., Martínez, G. A. and Civello, P. M. (2011) 'UV-C treatment affects the expression and activity of defense genes in strawberry fruit (Fragaria×ananassa, Duch.)', *Postharvest Biology and Technology*. Elsevier B.V., 59(1), pp. 94–102. doi: 10.1016/j.postharvbio.2010.08.003.

Popat, R., Crusz, S. a., Messina, M., Williams, P., West, S. a. and Diggle, S. P. (2012) 'Quorum-sensing and cheating in bacterial biofilms', *Proceedings of the Royal Society B: Biological Sciences*, 279, pp. 4765–4771. doi: 10.1098/rspb.2012.1976.

Prakash, B., Veeregowda, B. M. and Krishnappa, G. (2003) 'Biofilms : A survival strategy of bacteria', *Current Science*, 85(9).

Prasad, A., Sedlářová, M., Kale, R. S. and Pospíšil, P. (2017) 'Lipoxygenase in singlet oxygen generation as a response to wounding: In vivo imaging in Arabidopsis thaliana', *Scientific Reports*, 7(1), pp. 1–10. doi: 10.1038/s41598-017-09758-1.

Preston-mafham, J., Lynne, B. and Randerson, P. F. (2002) 'Analysis of microbial community functional diversity using sole-carbon-source utilisation protoles - a critique', *FEMS microbiology ecology*, 42, pp. 1–14. doi: 10.1111/j.1574-6941.2002.tb00990.x.

Pricope-Ciolacu, L., Nicolau, A. I., Wagner, M. and Rychli, K. (2013) 'The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes*', *International Journal of Food Microbiology*. Elsevier B.V., 166, pp. 59–64.

Raaijmakers, J. M., de Bruijn, I. and de Kock, M. J. D. (2006) 'Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation.', *Molecular plant-microbe interactions : MPMI*, 19, pp. 699–710.

Raaijmakers, J. M., de Bruijn, I., Nybroe, O. and Ongena, M. (2010) 'Natural functions of lipopeptides from Bacillus and Pseudomonas: More than surfactants and antibiotics', *FEMS Microbiology Reviews*, 34(6), pp. 1037–1062. doi: 10.1111/j.1574-6976.2010.00221.x.

Radošević, K., Srček, V. G., Bubalo, M. C., Rimac Brnčić, S., Takács, K. and Redovniković, I. R. (2017) 'Assessment of glucosinolates, antioxidative and antiproliferative activity of broccoli and collard extracts', *Journal of Food Composition and Analysis*, 61, pp. 59–66. doi: 10.1016/j.jfca.2017.02.001.

Ragaert, P., Devlieghere, F. and Debevere, J. (2007) 'Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables', *Postharvest Biology and Technology*, 44(3), pp. 185–194. doi: 10.1016/j.postharvbio.2007.01.001.

Raiger Iustman, L. J., Tribelli, P. M., Ibarra, J. G., Catone, M. V., Solar Venero, E. C. and López, N. I. (2014) 'Genome sequence analysis of *Pseudomonas extremaustralis* provides new insights into environmental adaptability and extreme conditions resistance.', *Extremophiles*, 19, pp. 207–220.

Rajmohan, S., Dodd, C. E. R. and Waites, W. M. (2002) 'Enzymes from isolates of Pseudomonas fluorescens involved in food spoilage', *Journal of Applied Microbiology*, 93(2), pp. 205–213. doi: 10.1046/j.1365-2672.2002.01674.x.

Ramette, A., Frapolli, M., Saux, M. F., Gruffaz, C., Meyer, J., Défago, G., Sutra, L. and Moënne-loccoz, Y. (2011) '*Pseudomonas protegens* sp . nov ., widespread plant-protecting bacteria producing the biocontrol compounds 2 , 4-diacetylphloroglucinol and pyoluteorin.', *Systematic and Applied Microbiology*. Elsevier GmbH., 34, pp. 180–188.

Ramos-Villarroel, A. Y., Aron-Maftei, N., Martín-Belloso, O. and Soliva-Fortuny, R. (2012) 'The role of pulsed light spectral distribution in the inactivation of Escherichia coli and Listeria innocua on fresh-cut mushrooms', *Food Control*. Elsevier Ltd, 24(1–2), pp. 206–213. doi: 10.1016/j.foodcont.2011.09.029.

Ramos-Villarroel, A. Y., Martín-Belloso, O. and Soliva-Fortuny, R. (2011) 'Bacterial inactivation and quality changes in fresh-cut avocado treated with intense light pulses', *European Food Research and Technology*, 233(3), pp. 395–402. doi: 10.1007/s00217-011-1533-6.

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P. and Silva, C. L. M. (2013) 'Fresh fruits and vegetables - An overview on applied methodologies to improve its quality and safety', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 20, pp. 1–15. doi: 10.1016/j.ifset.2013.07.002.

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. and Swerdlow, D. L. (2005) 'Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002', *Emerging Infectious Diseases*, 11(4), pp. 603–609. doi: 10.3201/eid1104.040739.

Rantsiou, K., Mataragas, M., Alessandria, V. and Cocolin, L. (2012) 'Expression of virulence genes of *Listeria monocytogenes* in food.', *Journal of Food Safety*, 32, pp. 161–168.

Rasmussen, T. B. and Givskov, M. (2006) 'Quorum-sensing inhibitors as anti-pathogenic drugs', *International Journal of Medical Microbiology*, 296, pp. 149–156. doi: doi:10.1016/j.ijmm.2006.02.005.

Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., Soliva-Fortuny, R. and Martín-Belloso, O. (2009) 'Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials', *Comprehensive Reviews in Food Science and Food Safety*, 8(3), pp. 157–180.

Redondo-Nieto, M., Barret, M., Morrissey, J., Germaine, K., Martínez-Granero, F., Barahona, E., Navazo, A., Sánchez-Contreras, M., Moynihan, J. A., Muriel, C., Dowling, D., O'Gara, F., Martín, M. and Rivilla, R. (2013) 'Genome sequence reveals that Pseudomonas fluorescens F113 possesses a large and diverse array of systems for rhizosphere function and host interaction', *BMC Genomics*, 14(1), pp. 1–17. doi: 10.1186/1471-2164-14-54.

Reid, M., Rhodes, J. and Hulme, A. (1973) 'Changes in ethylene and CO2 during the ripening of apples', *Journal Science Food Agriculture*, 24, pp. 971–979. doi: 10.1002/jsfa.2740240815.

Remorini, D., Landi, M., Tardelli, F., Lugani, A., Massai, R., Graziani, G., Fogliano, V. and Guidi, L. (2015) 'Effect of Chlorine Dioxide and Ascorbic Acid on Enzymatic Browning and Shelf Life of Fresh-Cut Red Delicious and Granny Smith Apples', *Journal of Food Processing and Preservation*, 39(6), pp. 2925–2934. doi: 10.1111/jfpp.12544.

Renaud, E. N. C., Lammerts van Bueren, E. T., Paulo, M. J., van Eeuwijk, F. A., Juvik, J. A., Hutton, M. G. and Myers, J. R. (2014) 'Broccoli Cultivar Performance under Organic and Conventional Management Systems and Implications for Crop Improvement', *Crop Science*, 54(4), p. 1539. doi: 10.2135/cropsci2013.09.0596.

Renumarn, P., Srilaong, V., Uthairatanakij, A., Kanlayanarat, S. and Jitareerat, P. (2014) 'The effects of immersion methods and concentration of ozonated water on the microbial counts and the quality and sensory attributes of fresh-cut broccoli', *International Food Research Journal*, 21(2), pp. 533–539.

Reyes, L. F., Villarreal, J. E. and Cisneros-Zevallos, L. (2006) 'The increase in antioxidant capacity after wounding depends on the type of fruit or vegetable tissue', *Food Chemistry*, 101(3), pp. 1254–1262. doi: 10.1016/j.foodchem.2006.03.032.

Ribeiro, C., Canada, J. and Alvarenga, B. (2012) 'Prospects of UV radiation for application in postharvest technology', *Emirates Journal of Food and Agriculture*, 24(6), pp. 586–597. doi: 10.9755/ejfa.v24i6.14677.

Ribet, D. and Cossart, P. (2015) 'How bacterial pathogens colonize their hosts and invade deeper tissues', *Microbes and Infection*. Elsevier Masson SAS, 17(3), pp. 173–183. doi: 10.1016/j.micinf.2015.01.004.

Richard-Forget, F. C. and Gauillard, F. A. (1997) 'Oxidation of Chlorogenic Acid, Catechins, and 4-Methylcatechol in Model Solutions by Combinations of Pear (*Pyrus communis* Cv. Williams) Polyphenol Oxidase and Peroxidase: A Possible Involvement of Peroxidase in Enzymatic Browning [†]', *Journal of Agricultural and Food Chemistry*, 45(7), pp. 2472–2476. doi: 10.1021/jf970042f.

Rico, D., Martín-Diana, A. B., Frías, J. M., Barat, J. M., Henehan, G. T. M. and Barry-Ryan, C. (2007) 'Improvement in texture using calcium lactate and heat-shock treatments for stored ready-to-eat carrots', *Journal of Food Engineering*, 79(4), pp. 1196–1206. doi: 10.1016/j.jfoodeng.2006.04.032.

Riemann, H. and Cliver, O. (1998) 'Escherichia Coli O157:H7', *Veterinary Clinics of North America: Food Animal Practice*, 14(1), pp. 41–48. doi: https://doi.org/10.1016/S0749-0720(15)30278-4.

Rivera-López, J., Vázquez-Ortiz, F. A., Ayala-Zavala, J. F., Sotelo-Mundo, R. R. and González-Aguilar, G. A. (2005) 'Cutting Shape and Storage Temperature Affect Overall Quality of Fresh-cut Papaya cv. "Maradol"', *Journal of Food Science*, 70(7), pp. s482–s489. doi: 10.1111/j.1365-2621.2005.tb11496.x.

Roach, T., Colville, L., Beckett, R. P., Minibayeva, F. V., Havaux, M. and Kranner, I. (2015) 'A proposed interplay between peroxidase, amine oxidase and lipoxygenase in the wounding-induced oxidative burst in Pisum sativum seedlings', *Phytochemistry*. Elsevier Ltd, 112(1), pp. 130–138. doi: 10.1016/j.phytochem.2014.06.003.

Robison, M. M., Griffith, M., Pauls, K. P. and Glick, B. R. (2001) 'Dual role for ethylene in susceptibility of tomato to Verticillium wilt', *Journal of Phytopathology*, 149(7–8), pp. 385–388. doi: 10.1046/j.1439-0434.2001.00639.x.

Roble, C., Gormley, T. and Butler, F. (2009) 'Efficacy of Natureseal ® AS1 browning inhibitor in fresh-cut fruit salads applications, with emphasis on apple wedges', *Journal of Horticultural Science & Biotechnology*, (62), pp. 62–67. doi: https://doi.org/10.1080/14620316.2009.11512597.

Da Rocha, A. B. and Hammerschmidt, R. (2005) 'History and perspectives on the use of disease resistance inducers in horticultural crops', *HortTechnology*, 15(3), pp. 518–529.

Rodgers, S. L., Cash, J. N., Siddiq, M. and Ryser, E. T. (2004) 'A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe.', *Journal of food protection*, 67(4), pp. 721–31.

Rodov, V., Horev, B., Goldman, G., Vinokur, Y. and Fishman, S. (2007) 'Model-driven Development of Microperforated Active Modified- Atmosphere Packaging for Fresh-cut Produce', *Acta Horticulturae*, 746, pp. 83–88.

Rojas-Graü, M. A., Grasa-Guillem, R. and Martín-Belloso, O. (2007) 'Quality changes in fresh-cut Fuji apple as affected by ripeness stage, antibrowning agents, and storage atmosphere.', *Journal of food science*, 72(1), pp. 36–43. doi: 10.1111/j.1750-3841.2006.00232.x.

Rolle, R. S. and Chism, G. W. (1987) 'Physiological consequences of minimaly processed fruits and vegetables', *Journal of Food Quality*, 10(10), pp. 157–177. doi: 10.1111/j.1745-4557.1987.tb00856.x.

Romanazzi, G., Sanzani, S. M., Bi, Y., Tian, S., Gutiérrez Martínez, P. and Alkan, N. (2016) 'Induced resistance to control postharvest decay of fruit and vegetables', *Postharvest Biology and Technology*, 122, pp. 82–94. doi: 10.1016/j.postharvbio.2016.08.003.

Roongsawang, N., Hase, K., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S. (2003) 'Cloning and Characterization of the Gene Cluster Encoding Arthrofactin Synthetase from Pseudomonas sp. MIS38 Niran', *Chemestry and Biology*, 10(2), pp. 869–880. doi: DOI 10.1016/j.chembiol .2003.09.004.

Rossoni, E. M. M. and Gaylarde, C. C. (2000) 'Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy', *International Journal of Food Microbiology*, 61(1), pp. 81–85. doi: 10.1016/S0168-1605(00)00369-X.

Rowan, N. J., Valdramidis, V. P. and Gómez-López, V. M. (2015) 'A review of quantitative methods to describe efficacy of pulsed light generated inactivation data that embraces the occurrence of viable but non culturable state microorganisms', *Trends in Food Science and Technology*, 44(1), pp. 79–92. doi: 10.1016/j.tifs.2015.03.006.

Roy, D., Panchal, S., Rosa, B. a and Melotto, M. (2013) 'Escherichia coli O157:H7 induces stronger plant immunity than Salmonella enterica Typhimurium SL1344.', *Phytopathology*, 103, pp. 326–32. doi: 10.1094/PHYTO-09-12-0230-FI.

Roy, M. K., Juneja, L. R., Isobe, S. and Tsushida, T. (2009) 'Steam processed broccoli (*Brassica oleracea*) has higher antioxidant activity in chemical and cellular assay systems', *Food Chemistry*, 114(1), pp. 263–269. doi: 10.1016/j.foodchem.2008.09.050.

Ruiz-Cruz, S., Acedo-Félix, E., Díaz-Cinco, M., Islas-Osuna, M. A. and González-Aguilar, G. A. (2007) 'Efficacy of sanitizers in reducing Escherichia coli O157:H7, Salmonella spp. and Listeria monocytogenes populations on fresh-cut carrots', *Food Control*, 18(11), pp. 1383–1390. doi: 10.1016/j.foodcont.2006.09.008.

Rupasinghe, H. P. V., Murr, D. P., Deell, J. R. and Odumeru, J. (2005) 'Influence of 1-methylcyclopropene and natureseal on the quality of fresh-cut "Empire" and "Crispin" apples', *Journal of Food Quality*, 28(3), pp. 289–307. doi: https://doi.org/10.1111/j.1745-4557.2005.00035.x.

Rupasinghe, H. P. V. and Yu, L. J. (2013) 'Value-Added Fruit Processing for Human Health', *InTech*, (Vi). Available at: http://dx.doi.org/10.5772/53161.

Sagoo, S. ., Little, C. L., WArd, L., Gillespie, I. A. and Mitchell, R. T. (2003) 'Microbiological Study of Ready-to-Eat Salad Vegetables from Retail Establishments Uncovers a National Outbreak of Salmonellosis', *Journal of Food Protection*, 66(3), pp. 403–409.

Sagoo, S. K., Little, C. L. and Mitchell, R. T. (2003) 'Microbiological Quality of Open Ready-to-Eat Salad Vegetables: Effectiveness of Food Hygiene Training of Management', *Journal of Food Protection*, 66(9), pp. 1581–1586.

Salinas-Roca, B., Soliva-Fortuny, R., Welti-Chanes, J. and Martín-Belloso, O. (2016) 'Combined effect of pulsed light, edible coating and malic acid dipping to improve fresh-cut mango safety and quality', *Food Control*. Elsevier Ltd, 66, pp. 190–197. doi: 10.1016/j.foodcont.2016.02.005.

Saltveit, M. E. (2016) 'The three responses of plant tissue to wounding', *Acta Horticulturae*, 1141, pp. 13–20. doi: 10.17660/ActaHortic.2016.1141.2.

Sambuy, Y., Angelis, I. De, Ranaldi, G., Scarino, M. L., Stammati, A. and Zucco, F. (2005) 'The Caco-2 cell line as a model of the intestinal barrier: in£uence of cell and culture-related factors on Caco-2 cell functional characteristics 1', *Cell*, 21, pp. 1–26.

Sánchez, G., Elizaquível, P., Aznar, R. and Selma, M. V. (2015) 'Virucidal effect of high power ultrasound combined with a chemical sanitizer containing peroxyacetic acid for water reconditioning in the fresh-cut industry', *Food Control*, 52, pp. 126–131. doi: 10.1016/j.foodcont.2014.12.021.

Sandhya (2010) 'Modified atmosphere packaging of fresh produce: Current status and future needs', *LWT - Food Science and Technology*. Elsevier Ltd, 43(3), pp. 381–392. doi: 10.1016/j.lwt.2009.05.018.

Sandín-España, P., Mateo-Miranda, M., López-Goti, C., De Cal, A. and Alonso-Prados, J. L. (2016) 'Development of a rapid and direct method for the determination of organic acids in peach fruit using LC-ESI-MS', *Food Chemistry*, 192, pp. 268–273. doi: 10.1016/j.foodchem.2015.07.012.

Sangeetha, G., Thangavelu, R., Usha Rani, S., Muthukumar, A. and Udayakumar, R. (2010) 'Induction of systemic resistance by mixtures of antagonist bacteria for the management of crown rot complex on banana', *Acta Physiologiae Plantarum*, 32(6), pp. 1177–1187. doi: https://doi.org/10.1007/s11738-010-0513-y.

Sapers, G. M. and Douglas, F. W. (1987) 'Measurement of enzymatic browning at cut surfaces and in juice of raw apple and pear fruits.', *Journal Food Science*, 52, pp. 1285–1261. doi: https://doi.org/10.1111/j.1365-2621.1987.tb14057.x.

Sapers, G. M., Miller, R. L., Pilizota, V. and Mattrazzo, A. M. (2001) 'Antimicrobial treatments for

minimally processed cantaloupe melon', *Journal of Food Science*, 66(2), pp. 345–349. doi: 10.1111/j.1365-2621.2001.tb11344.x.

Saraoui, T., Fall, P. A., Leroi, F., Antignac, J.-P., Chéreau, S. and Pilet, M. F. (2016) 'Inhibition mechanism of Listeria monocytogenes by a bioprotective bacteria Lactococcus piscium CNCM I-4031', *Food Microbiology*, 53, pp. 70–78.

Sari, E., Etebarian, H. R. and Aminian, H. (2008) 'Effects of Pseudomonas fluorescens CHA0 on the resistance of wheat seedling roots to the take-all fungus Gaeumannomyces graminis var.*tritici*', *Plant Production Science*, 11(December 2007), pp. 298–306. doi: 10.1626/pps.11.298.

Sarniguet, A., Kraust, J., Henkelst, M. D., Muehlchent, A. M. and Lopertt, J. E. (1995) 'The sigma factor e affects antibiotic production and biological control activity of Pseudomonasfluorescens Pf-5 (pyrrolnitrin/pyoluteorin/2,4-diacetylphloroglucinol/stationary phase/biocontrol/rpoS gene)', *Microbiology*, 92(December), pp. 12255–12259. doi: 10.1073/pnas.92.26.12255.

Sasirekha, B. and Srividya, S. (2016) 'Siderophore production by Pseudomonas aeruginosa FP6, a biocontrol strain for Rhizoctonia solani and Colletotrichum gloeosporioides causing diseases in chilli', *Agriculture and Natural Resources*. Elsevier Ltd, 50(4), pp. 250–256. doi: 10.1016/j.anres.2016.02.003.

Saxena, A., Bawa, A. S. and Raju, P. S. (2009) 'Phytochemical changes in fresh-cut jackfruit (*Artocarpus heterophyllus* L.) bulbs during modified atmosphere storage', *Food Chemistry*. Elsevier Ltd, 115(4), pp. 1443–1449. doi: https://doi.org/10.1016/j.foodchem.2009.01.080.

Sayyed, R. Z., Badgujar, M. D., Sonawane, H. M., Mhaske, M. M. and Chincholkar, S. B. (2005) 'Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads', *Indian Journal of Biotechnology*, 4(4), pp. 484–490.

Schenk, M., Guerrero, S. and Alzamora, S. M. (2008) 'Response of some microorganisms to ultraviolet treatment on fresh-cut pear', *Food and Bioprocess Technology*, 1(4), pp. 384–392. doi: 10.1007/s11947-007-0029-7.

Schikora, A., Carreri, A., Charpentier, E. and Hirt, H. (2008) 'The dark side of the salad: Salmonella typhimurium overcomes the innate immune response of arabidopsis thaliana and shows an endopathogenic lifestyle', *PLoS ONE*, 3(5). doi: 10.1371/journal.pone.0002279.

Schikora, A., Garcia, A. V. and Hirt, H. (2012) 'Plants as alternative hosts for Salmonella', *Trends in Plant Science*. Elsevier Ltd, 17(5), pp. 245–249. doi: 10.1016/j.tplants.2012.03.007.

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P. and Hirt, H. (2011) 'Conservation of Salmonella infection mechanisms in plants and animals', *PLoS ONE*, 6(9). doi: 10.1371/journal.pone.0024112.

Scott, G., Dickinson, M., Shama, G. and Rupar, M. (2018) 'A comparison of the molecular mechanisms underpinning high-intensity, pulsed polychromatic light and low-intensity UV-C hormesis in tomato fruit', *Postharvest Biology and Technology*. Elsevier, 137(April 2017), pp. 46–55. doi: 10.1016/j.postharvbio.2017.10.017.

Scott, G., Rupar, M., Fletcher, A. G. D., Dickinson, M. and Shama, G. (2017) 'A comparison of low intensity UV-C and high intensity pulsed polychromatic sources as elicitors of hormesis in tomato fruit', *Postharvest Biology and Technology*. Elsevier B.V., 125, pp. 52–58. doi: 10.1016/j.postharvbio.2016.10.012.

Selma, M. V., Allende, A., López-Gálvez, F., Conesa, M. A. and Gil, M. I. (2008) 'Disinfection potential of ozone, ultraviolet-C and their combination in wash water for the fresh-cut vegetable industry', *Food Microbiology*, 25(6), pp. 809–814. doi: 10.1016/j.fm.2008.04.005.

Servin, A. L. (2004) 'Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens', *FEMS Microbiology Reviews*, 28(4), pp. 405–440. doi: 10.1016/j.femsre.2004.01.003.

Severo, J., Tiecher, A., Pirrello, J., Regad, F., Latché, A., Pech, J. C., Bouzayen, M. and Rombaldi, C. V.

(2015) 'UV-C radiation modifies the ripening and accumulation of ethylene response factor (ERF) transcripts in tomato fruit', *Postharvest Biology and Technology*. Elsevier India Pvt Ltd, 102, pp. 9–16. doi: 10.1016/j.postharvbio.2015.02.001.

Sewelam, N., Jaspert, N., Van Der Kelen, K., Tognetti, V. B., Schmitz, J., Frerigmann, H., Stahl, E., Zeier, J., Van Breusegem, F. and Maurino, V. G. (2014) 'Spatial H2O2 signaling specificity: H2O2 from chloroplasts and peroxisomes modulates the plant transcriptome differentially', *Molecular Plant*, 7(7), pp. 1191–1210. doi: https://doi.org/10.1093/mp/ssu070.

Shama, G. (2007) 'Process challenges in applying low doses of ultraviolet light to fresh produce for eliciting beneficial hormetic responses', *Postharvest Biology and Technology*, 44(1), pp. 1–8. doi: 10.1016/j.postharvbio.2006.11.004.

Shapiro, A. D. and Zhang, C. (2001) 'The Role of NDR1 in Avirulence Gene-Directed Signaling and Control of Programmed Cell Death in Arabidopsis', *Plant Physiol.*, 127(November), pp. 1089–1101. doi: 10.1104/pp.010096.1.

Sharma, P., Jha, A. B., Dubey, R. S. and Pessarakli, M. (2012) 'Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions', *Journal of Botany*, 2012, pp. 1–26. doi: http://dx.doi.org/10.1155/2012/217037.

Sharma, R. R., Singh, D. and Singh, R. (2009) 'Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review', *Biological Control*, 50(3), pp. 205–221.

Shastry, S. and Prasad, M. S. (2002) 'Extracellular protease from Pseudomonas sp. (CL 1457) active against Xanthomonas campestris', *Process Biochemistry*, 37(6), pp. 611–621. doi: 10.1016/S0032-9592(01)00249-7.

Shen, C., Norris, P., Williams, O., Hagan, S. and Li, K. W. (2016) 'Generation of chlorine by-products in simulated wash water', *Food Chemistry*. Elsevier Ltd, 190, pp. 97–102. doi: 10.1016/j.foodchem.2015.04.146.

Shi, J., Liu, A., Li, X. and Chen, W. (2013) 'Control of Phytophthora nicotianae disease, induction of defense responses and genes expression of papaya fruits treated with Pseudomonas putida MGP1', *Journal of the Science of Food and Agriculture*, 93(3), pp. 568–574. doi: 10.1002/jsfa.5831.

Siddiqui, I. A. and Shaukat, S. S. (2003) 'Suppression of root-knot disease by Pseudomonas fluorescens CHA0 in tomato : importance of bacterial secondary metabolite ', *Soil Biology and Biochemistry*, 35, pp. 1615–1623. doi: 10.1016/j.soilbio.2003.08.006.

Silva, H. S. A., Da Silva Romeiro, R., Macagnan, D., De Almeida Halfeld-Vieira, B., Pereira, M. C. B. and Mounteer, A. (2004) 'Rhizobacterial induction of systemic resistance in tomato plants: Non-specific protection and increase in enzyme activities', *Biological Control*, 29(2), pp. 288–295. doi: 10.1016/S1049-9644(03)00163-4.

Silveira, A. C., Aguayo, E., Escalona, V. H. and Artés, F. (2011) 'Hot water treatment and peracetic acid to maintain fresh-cut Galia melon quality', *Innovative Food Science and Emerging Technologies*. Elsevier Ltd, 12(4), pp. 569–576. doi: 10.1016/j.ifset.2011.02.010.

Singh, R. P., Shelke, G. M., Kumar, A. and Jha, P. N. (2015) 'Biochemistry and genetics of ACC deaminase: A weapon to "stress ethylene" produced in plants', *Frontiers in Microbiology*, 6(SEP), pp. 1–14. doi: 10.3389/fmicb.2015.00937.

Singleton, V. L., Rossi Jr., J. A. and Rossi J A Jr. (1965) 'Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents', *American Journal of Enology and Viticulture*, 16(3), pp. 144–158. doi: 10.12691/ijebb-2-1-5.

Sinha, R. P. and Häder, D.-P. (2002) 'UV-induced DNA damage and repair: a review', *Photochemical & Photobiological Sciences*, 1(4), pp. 225–236. doi: 10.1039/b201230h.

Siroli, L., Patrignani, F., Serrazanetti, D. I., Gardini, F. and Lanciotti, R. (2015) 'Innovative strategies based on the use of bio-control agents to improve the safety, shelf-life and quality of minimally

processed fruits and vegetables', *Trends in Food Science and Technology*. Elsevier Ltd, 46(2), pp. 302–310. doi: 10.1016/j.tifs.2015.04.014.

Siroli, L., Patrignani, F., Serrazanetti, D. I., Tabanelli, G., Montanari, C., Gardini, F. and Lanciotti, R. (2015) 'Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce', *Food Microbiology*. Elsevier Ltd, 47, pp. 74–84. doi: 10.1016/j.fm.2014.11.008.

Skandamis, P. N. and Nychas, G. J. E. (2012) 'Quorum sensing in the context of food microbiology', *Applied and Environmental Microbiology*, 78(16), pp. 5473–5482. doi: 10.1128/AEM.00468-12.

Söderqvist, K., Ahmed Osman, O., Wolff, C., Bertilsson, S., Vågsholm, I. and Boqvist, S. (2017) 'Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad', *Infection Ecology & Epidemiology*. Taylor & Francis, 7(1), p. 1328963. doi: 10.1080/20008686.2017.1328963.

Solanki, M. K., Singh, R. K., Srivastava, S., Kumar, S., Kashyap, P. L., Srivastava, A. K. and Arora, D. K. (2014) 'Isolation and characterization of siderophore producing antagonistic rhizobacteria against *Rhizoctonia solani*', *Journal of Basic Microbiology*, 54, pp. 585–596.

Soliva-Fortuny, R. C. and Martín-Belloso, O. (2003) 'New advances in extending the shelf-life of fresh-cut fruits: A review', *Trends in Food Science and Technology*, 14(9), pp. 341–353. doi: 10.1016/S0924-2244(03)00054-2.

Soliva-Fortuny, R. C., Ricart-Coll, M. and Martín-Belloso, O. (2005) 'Sensory quality and internal atmosphere of fresh-cut Golden Delicious apples', *International Journal of Food Science and Technology*, 40(4), pp. 369–375. doi: 10.1111/j.1365-2621.2004.00934.x.

Soliva-Fortuny, R., Oms-Oliu, G. and Martìn-Belloso, O. (2002) 'Effects of ripeness stages on the storage atmosphere , color, and textural properties of minimally processed apple slices', *Journal of food science*, 67, pp. 1958–1963. doi: 10.1111/j.1365-2621.2002.tb08752.x.

Soltani, M. (2015) 'Modified Atmosphere Packaging : A Progressive Technology for Shelf-Life Extension of Fruits and Vegetables Modified Atmosphere Packaging ; A Progressive Technology for Shelf-Life Extension of Fruits and Vegetables', *Journal of Applied Packaging Research*, 7(3), pp. 33–59.

Song, A. Y., Oh, Y. J., Kim, J. E., Song, K. Bin, Oh, D. H. and Min, S. C. (2015) 'Cold plasma treatment for microbial safety and preservation of fresh lettuce', *Food Science and Biotechnology*, 24(5), pp. 1717–1724. doi: 10.1007/s10068-015-0223-8.

Song, L. and Thornalley, P. J. (2007) 'Effect of storage, processing and cooking on glucosinolate content of Brassica vegetables', *Food and Chemical Toxicology*, 45(2), pp. 216–224. doi: 10.1016/j.fct.2006.07.021.

Spadaro, D. and Droby, S. (2016) 'Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists', *Trends in Food Science and Technology*. Elsevier Ltd, 47, pp. 39–49. doi: 10.1016/j.tifs.2015.11.003.

Spadaro, D. and Gullino, M. L. (2004) 'State of the art and future prospects of the biological control of postharvest fruit diseases', *International Journal of Food Microbiology*, 91, pp. 185–194. doi: 10.1016/S0168-1605(03)00380-5.

Spinola, V., Mendes, B., Camara, J. S. and Castilho, P. C. (2012) 'An improved and fast UHPLC-PDA methodology for determination of L-ascorbic and dehydroascorbic acids in fruits and vegetables. Evaluation of degradation rate during storage', *Analytical and Bioanalytical Chemistry*, 403(4), pp. 1049–1058. doi: 10.1007/s00216-011-5668-x.

Sribuathong, S., Saengprakai, J. and Trevanich, S. (2014) '*In vitro* anti-adherent assessment of selected lactic acid bacteria isolates against *Salmonella typhimurium* and *Listeria monocytogenes* to Caco-2 cells.', *Journal of Food Safety*, 34, pp. 270–282.

Stefanato, F. L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C. G., Métraux, J. P. and Schoonbeek, H. J. (2009) 'The ABC transporter BcatrB from Botrytis cinerea exports camalexin and is a virulence factor on Arabidopsis thaliana', *Plant Journal*, 58(3), pp. 499–510. doi: 10.1111/j.1365-313X.2009.03794.x.

Stirbet, A. and Govindjee (2011) 'On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient', *Journal of Photochemistry and Photobiology B: Biology*. Elsevier B.V., 104(1–2), pp. 236–257. doi: 10.1016/j.jphotobiol.2010.12.010.

Stratil, P., Klejdus, B. and Kubáň, V. (2006) 'Determination of total content of phenolic compounds and their antioxidant activity in vegetables - Evaluation of spectrophotometric methods', *Journal of Agricultural and Food Chemistry*, 54(3), pp. 607–616. doi: 10.1021/jf052334j.

Stubbendieck, R. M. and Straight, P. D. (2016) 'Multifaceted interfaces of bacterial competition', *Journal of Bacteriology*, 198(16), pp. 2145–2155. doi: 10.1128/JB.00275-16.

Surjadinata, B. B. and Cisneros-Zevallos, L. (2003) 'Modeling Wound-induced Respiration of Fresh-cut Carrots (Daucus carota L.)', *Journal of Food Science*, 68(9), pp. 2735–2740. doi: 10.1111/j.1365-2621.2003.tb05797.x.

Suttirak, W. and Manurakchinakorn, S. (2010) 'Potential Application of Ascorbic Acid, Citric Acid and Oxalic Acid for Browning Inhibition in Fresh-Cut Fruits and Vegetables', *Walailak Journal of Science and Technology*, 7(1), pp. 5–14. Available at: http://masterorg.wu.ac.th/file/ird-20100706-X09s3.pdf.

Tabasco, R., Fernández de Palencia, P., Fontecha, J., Peláez, C. and Requena, T. (2014) 'Competition mechanisms of lactic acid bacteria and bifidobacteria: Fermentative metabolism and colonization.', *LWT - Food Science and Technology*. Elsevier Ltd, 55, pp. 680–684. Available at: http://dx.doi.org/10.1016/j.lwt.2013.10.004.

Taguchi, Y., Hyakumachi, M., Horinouchi, H. and Kawane, F. (2003) *Biological control of rice blast disease by Bacillus subtilis IK-1080*, *Japanese Journal of Phytopathology*. doi: 10.3186/jjphytopath.69.85.

Takeshita, K., Shibato, J., Sameshima, T. and Fukunaga, S. (2003) 'Damage of yeast cells induced by pulsed light irradiation', *International Journal of Food Microbiology*, 85, pp. 151–158. doi: 10.1016/S0168-1605(02)00509-3.

Tamburro, M., Sammarco, M. L., Ammendolia, M. G., Fanelli, I., Minelli, F. and Ripabelli, G. (2015) 'Evaluation of transcription levels of inlA, inlB, hly, bsh and prfA genes in *Listeria monocytogenes* strains using quantitative reverse-transcription PCR and ability of invasion into human CaCo-2 cells', *FEMS microbiology letters*, 362, pp. 1–7.

Tan, Q., Xu, H., Chen, T., Li, P., Aguilar, Z. P., Xu, D., Ming, X., Xu, F. and Wei, H. (2012) 'Differential expression of virulence and stress fitness genes during interaction between *Listeria monocytogenes* and *Bifidobacterium longum*', *Bioscience, Biotechnology, and Biochemistry*, 76, pp. 699–704.

Tarr, P. and Neill, M. (2001) 'Escherichia coli O157:H7', *Gastroenterology Clinics*, 30(3), pp. 735–751. doi: http://dx.doi.org/10.1016/S0889-8553(05)70208-9.

Tatsuki, M., Endo, A. and Ohkawa, H. (2007) 'Influence of time from harvest to 1-MCP treatment on apple fruit quality and expression of genes for ethylene biosynthesis enzymes and ethylene receptors', *Postharvest Biology and Technology*, 43(1), pp. 28–35. doi: 10.1016/j.postharvbio.2006.08.010.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C. and Viñas, I. (2001) 'Improving control of green and blue molds of oranges by combining Pantoea agglomerans (CPA-2) and sodium bicarbonate', *European Journal of Plant Pathology*, 107(7), pp. 685–694. doi: 10.1023/A:1011962121067.

Terada, L. S., Johansen, K. a, Nowbar, S., Vasil, A. I. and Vasil, M. L. (1999) 'Pseudomonas aeruginosa Hemolytic Phospholipase C Suppresses Neutrophil Respiratory Burst Activity Pseudomonas aeruginosa Hemolytic Phospholipase C Suppresses Neutrophil Respiratory Burst Activity', 67(5), pp. 2371–2376.

Tiecher, A., de Paula, L. A., Chaves, F. C. and Rombaldi, C. V. (2013) 'UV-C effect on ethylene, polyamines and the regulation of tomato fruit ripening', *Postharvest Biology and Technology*. Elsevier B.V., 86, pp. 230–239. doi: 10.1016/j.postharvbio.2013.07.016.

Toivonen, P. and Dell, J. (2002) 'Physiology of fresh-cut fruits and vegetables', in Lamikanra, _O (ed.) *Fresh-cut fruit and vegetables. Science, technology and market*. CRC Press, Boca Raton, FL, p. 452. doi: 10.1201/9781420031874.

Toivonen, P. M. A. (2006) 'Fresh-cut apples : Challenges and opportunities for multi-disciplinary research', *Canadian Journal of Plant Science*, 86, pp. 1361–1368. doi: 10.13140/2.1.4789.3123.

Toivonen, P. M. A. (2009) 'Benefits of combined treatment approaches to maintaining fruit and vegetable quality', in Sivakumar, D. (ed.) *Fresh Produce*. Global Science Books, pp. 58–64.

Toivonen, P. M. A. and Brummell, D. A. (2008) 'Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables', *Postharvest Biology and Technology*, 48(1), pp. 1–14. doi: https://doi.org/10.1016/j.postharvbio.2007.09.004.

Toivonen, P. M. A. and DeEll, J. R. (2001) 'Chlorophyll fluorescence, fermentation product accumulation, and quality of stored broccoli in modified atmosphere packages and subsequent air storage', *Postharvest Biology and Technology*, 23(1), pp. 61–69. doi: 10.1016/S0925-5214(01)00086-2.

Tomás-Barberán, F. A. and Espín, J. C. (2001) 'Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables', *Journal of the Science of Food and Agriculture*, 81(9), pp. 853–876. doi: 10.1002/jsfa.885.

Torres-Contreras, A. M., Nair, V., Cisneros-Zevallos, L. and Jacobo-Velázquez, D. A. (2017) 'Stability of bioactive compounds in broccoli as affected by cutting styles and storage time', *Molecules*, 22(4), pp. 1–15. doi: 10.3390/molecules22040636.

Torres, A. G., Jeter, C., Langley, W. and Matthysse, A. G. (2005) 'Differential Binding of Escherichia coli O157 : H7 to Alfalfa , Human Epithelial Cells , and Plastic Is Mediated by a Variety of Surface Structures', *Applied and Environmental Microbiology*, 71(12), pp. 8008–8015. doi: 10.1128/AEM.71.12.8008.

Tortorella, S. M., Royce, S. G., Licciardi, P. V. and Karagiannis, T. C. (2015) 'Dietary sulforaphane in cancer chemoprevention: the role of epigenetic regulation and HDAC inhibition', *Antioxidants & Redox Signaling*, 22(16), pp. 1382–1424. doi: 10.1089/ars.2014.6097.

Tremonte, P., Succi, M., Coppola, R., Sorrentino, E., Tipaldi, L., Picariello, G., Pannella, G. and Fraternali, F. (2016) 'Homology-based modeling of universal stress protein from listeria innocua up-regulated under acid stress conditions', *Frontiers in Microbiology*, 7(DEC). doi: 10.3389/fmicb.2016.01998.

Tupe, S. G., Kulkarni, R. R., Shirazi, F., Sant, D. G., Joshi, S. P. and Deshpande, M. V. (2015) 'Possible mechanism of antifungal phenazine-1-carboxamide from *Pseudomonas* sp. against dimorphic fungi *Benjaminiella poitrasii* and human pathogen *Candida albicans*', *Journal of Applied Microbiology*, 118, pp. 39–48.

Turtoi, M. (2013) 'Ultraviolet light treatment of fresh fruits and vegetables surface : A review', *Journal of Agroalimentary Processes and Technologies*, 19(3), pp. 325–337.

Ucherek, M. (2001) 'How to design perforated polymeric films for modified atmosphere packs (MAP)', *Packaging Technology and Science*, 14(6), pp. 253–266. doi: 10.1002/pts.557.

Ude, S., Arnold, D. L., Moon, C. D., Timms-Wilson, T. and Spiers, A. J. (2006) 'Biofilm formation and

cellulose expression among diverse environmental *Pseudomonas* isolates', *Environmental Microbiology*, 8, pp. 1997–2011.

Ukuku, D. O. and Fett, W. F. (2002) 'Relationship of Cell Surface Charge and Hydrophobicity to Strength of Attachment of Bacteria to Cantaloupe Rind †', *Journal of Food Protection*, 65(7), pp. 1093–1099.

Umesha, S. and Roohie, R. K. (2017) 'Role of Pseudomonas fluorescens and INA against Black Rot of Cabbage', *Journal of Phytopathology*, 165(4), pp. 265–275. doi: 10.1111/jph.12558.

Upadhyay, A. and Srivastava, S. (2008) 'Characterization of a new isolate of Pseudomonas fluorescens strain Psd as a potential biocontrol agent', *Letters in Applied Microbiology*, 47(2), pp. 98–105. doi: 10.1111/j.1472-765X.2008.02390.x.

Urban, L., Charles, F., de Miranda, M. R. A. and Aarrouf, J. (2016) 'Understanding the physiological effects of UV-C light and exploiting its agronomic potential before and after harvest', *Plant Physiology and Biochemistry*, 105, pp. 1–11. doi: 10.1016/j.plaphy.2016.04.004.

Uroz, S., D'Angelo-Picard, C., Carlier, A., Elasri, M., Sicot, C., Petit, A., Oger, P., Faure, D. and Dessaux, Y. (2003) 'Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria', *Microbiology*, 149(8), pp. 1981–1989. doi: 10.1099/mic.0.26375-0.

US Food and Drug Administration (2008) *Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables*, *OMB Control No. 0910-0609*. Available at: https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ProducePlantProducts/ucm064458.htm (Accessed: 16 January 2018).

Valdivia-Nájar, C. G., Martín-Belloso, O. and Soliva-Fortuny, R. (2018) 'Impact of pulsed light treatments and storage time on the texture quality of fresh-cut tomatoes', *Innovative Food Science and Emerging Technologies*. Elsevier, 45(August 2017), pp. 29–35. doi: 10.1016/j.ifset.2017.08.007.

Valencia Fruits (2017) *La IV gama de frutas y hortalizas espera crecer un 10% hasta 2020*, *Valencia Fruits*. Available at: http://valenciafruits.com/la-iv-gama-de-frutas-y-hortalizas-espera-crecer-un-10-hasta-2020/ (Accessed: 26 January 2018).

Valentines, M. C., Vilaplana, R., Torres, R., Usall, J. and Larrigaudière, C. (2005) 'Specific roles of enzymatic browning and lignification in apple disease resistance', *Postharvest Biology and Technology*, 36(3), pp. 227–234. doi: 10.1016/j.postharvbio.2005.01.002.

Valverde, J., Reilly, K., Villacreces, S., Gaffney, M., Grant, J. and Brunton, N. (2015) 'Variation in bioactive content in broccoli (*Brassica oleracea* var. italica) grown under conventional and organic production systems', *Journal of the Science of Food and Agriculture*, 95(6), pp. 1163–1171. doi: 10.1002/jsfa.6804.

Vandekinderen, I., Van Camp, J., De Meulenaer, B., Veramme, K., Denon, Q., Ragaert, P. and Devlieghere, F. (2007) 'The effect of the decontamination process on the microbial and nutritional quality of fresh-cut vegetables', *Acta Horticulturae*, 746, pp. 173–179.

Vanderzant, C. and Splittstoesser, D. (eds) (1992) *Compendium of methods for the microbiological examination of foods.* Washington DC: American Public Health Association.

Vatsa, P., Sanchez, L., Clement, C., Baillieul, F. and Dorey, S. (2010) 'Rhamnolipid biosurfactants as new players in animal and plant defense against microbes', *International Journal of Molecular Sciences*, 11(12), pp. 5095–5108. doi: 10.3390/ijms11125095.

Van de Velde, F., Fenoglio, C., Piagentini, A. M. and Pirovani, M. E. (2018) 'Modeling the Impact of the Type of Cutting and Storage Temperature on the Bioactive Compound Content, Phenylpropanoid Metabolism Enzymes and Quality Attributes of Fresh-Cut Strawberries', *Food and Bioprocess Technology*. Food and Bioprocess Technology, 11(1), pp. 96–109. doi: 10.1007/s11947-017-1996-y.

Verhagen, B. W. M., Trotel-Aziz, P., Couderchet, M., Höfte, M. and Aziz, A. (2010) '*Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence responses in grapevine', *Journal of Experimental Botany*, 61(1), pp. 249–260. doi: 10.1093/jxb/erp295.

Verkerk, R., Tebbenhoff, S. and Dekker, M. (2010) 'Variation and distribution of glucosinolates in 42 cultivars of Brassica oleracea vegetable crops', *Acta Horticulturae*, 856, pp. 63–69. doi: 10.17660/ActaHortic.2010.856.7.

Vidhyasekaran, R., Kamala, N., Ramanathan, A., Rajappan, K., Paranidharan, V. and Velazhahan, R. (2001) 'Induction of Systemic Resistance by Pseudomonas fluorescens Pfl against Xanthomonas oryzae pv . oryzae in Rice Leaves', *Phytoparasitica*, 29, pp. 155–166.

Vilanova, L., Vall-llaura, N., Torres, R., Usall, J., Teixidó, N., Larrigaudière, C. and Giné-Bordonaba, J. (2017) '*Penicillium expansum* (compatible) and *Penicillium digitatum* (non-host) pathogen infection differentially alter ethylene biosynthesis in apple fruit', *Plant Physiology and Biochemistry*, 120, pp. 132–143. doi: 10.1016/j.plaphy.2017.09.024.

Vilaplana, R., Valentines, M. C., Toivonen, P. and Larrigaudiere, C. (2006) 'Antioxidant potential and peroxidative state of `Golden smoothee' apples treated with 1-methylcyclopropene', *J. Amer. Soc. Hort. Sci.*, 131(1), pp. 104–109.

Villanueva, M. J., Tenorio, M. D., Esteban, M. A. and Mendoza, M. C. (2004) 'Compositional changes during ripening of two cultivars of muskmelon fruits', *Food Chemistry*, 87, pp. 179–185.

Viñas, I., Abadias, M., Usall, J., Teixido, N. and Torres, R. (2017) 'Biological culture of a strain of the species Pseudomonas graminis and use of said culture as antagonist for the biocontrol of pathogenic bacteria (EU patent)', *Bulletin 2017/05*.

Viñas, I., Abadias, M., Usall, J., Teixido, N. and Torrres, R. (2012) 'Biological culture of a strain of the Pseudomonas graminis species, use of said culture as antagonist for biological control of pathogenic bacteria and method for treating fruit that comprises the step of applying to the fruit, a preparation that c'. Institut de Recerca I Tecnologia Agroalimentaries (ES); Universitat de Lleida (ES). doi: 9168268.

Vishwakarma, S., Singh, R. P. and Kewat, R. N. (2013) 'Increasing Shelf Life of Fruits and Vegetables Through UV Irradiation Process', *Trends in Bioscience*, 6(4), pp. 329–333.

Vuotto, C., Longo, F. and Donelli, G. (2014) 'Probiotics to counteract biofilm-associated infections: promising and conflicting data.', *International journal of oral science*, 6, pp. 189–194. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25257882.

Wallace, R. L., Hirkala, D. L. and Nelson, L. M. (2017) 'Postharvest biological control of blue mold of apple by Pseudomonas fluorescens during commercial storage and potential modes of action', *Postharvest Biology and Technology*. Elsevier, 133(March), pp. 1–11. doi: 10.1016/j.postharvbio.2017.07.003.

Walters, D. R. and Fountaine, J. M. (2009) 'Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions', *The Journal of Agricultural Science*, 147(05), p. 523. doi: 10.1017/S0021859609008806.

Wang, A., Tan, D., Tatsuki, M., Kasai, A., Li, T., Saito, H. and Harada, T. (2009) 'Molecular mechanism of distinct ripening profiles in "Fuji" apple fruit and its early maturing sports', *Postharvest Biology and Technology*, 52(1), pp. 38–43. doi: 10.1016/j.postharvbio.2008.09.001.

Wang, C., Knill, E., Glick, B. and Défago, G. (2000) 'Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into Pseudomonas fluorescens strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities.', *Can J Microbiol.*, 46(10), pp. 898–907. doi: 10.1139/w00-071.

Wang, D., Yu, J. M., Pierson, L. S. and Pierson, E. A. (2012) 'Differential regulation of phenazine biosynthesis by RpeA and RpeB in Pseudomonas chlororaphis 30-84', *Microbiology (United*

Kingdom), 158(7), pp. 1745–1757. doi: 10.1099/mic.0.059352-0.

Wang, G., Ning, J., Zhao, J., Hang, F., Tian, F., Zhao, J., Chen, Y., Zhang, H. and Chen, W. (2014) 'Partial characterisation of an anti-Listeria substance produced by *Pediococcus acidilactici* P9.', *International Dairy Journal*, 34(2), pp. 275–279. doi: 10.1016/j.idairyj.2013.08.005.

Wang, G., Zhao, Y., Tian, F., Jin, X., Chen, H., Liu, X., Zhang, Q., Zhao, J., Chen, Y., Zhang, H. and Chen, W. (2014) 'Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*.', *Food Control*. Elsevier Ltd, 44, pp. 49–57.

Wang, L., Jin, P., Wang, J., Jiang, L., Zhang, S., Gong, H., Liu, H. and Zheng, Y. (2015) 'In vitro inhibition and in vivo induction of defense response against *Penicillium expansum* in sweet cherry fruit by postharvest applications of *Bacillus cereus* AR156', *Postharvest Biology and Technology*. Elsevier India Pvt Ltd, 101, pp. 15–17.

Wang, W., Wang, H., Feng, Y., Wang, L., Xiao, X., Xi, Y., Luo, X., Sun, R., Ye, X., Huang, Y., Zhang, Z. and Cui, Z. (2016) 'Consistent responses of the microbial community structure to organic farming along the middle and lower reaches of the Yangtze River', *Scientific Reports*. Nature Publishing Group, 6(June), pp. 1–11. doi: 10.1038/srep35046.

Wang, X., Wang, L., Wang, J., Jin, P., Liu, H. and Zheng, Y. (2014) '*Bacillus cereus* AR156-induced resistance to *Colletotrichum acutatum* is associated with priming of defense responses in loquat fruit.', *PloS one*, 9(11), p. e112494. doi: https://doi.org/10.1371/journal.pone.0112494.

Wang, Y., Xu, Z. ., Zhu, P. ., Liu, Y. ., Zhang, Z. ., Mastuda, Y. ., Toyoda, H. . and Xu, L. (2010) 'Postharvest biological control of melon pathogens using Bacillus subtilis EXWB1', *Journal of Plant Pathology*, 92(3), pp. 645–652. doi: http://dx.doi.org/10.4454/jpp.v92i3.309.

Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Van 'T Westende, Y. A. M., Hartog, F. and Van Loon, L. C. (1997) 'Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria', *Molecular Plant-Microbe Interactions*, 10(6), pp. 716–724. doi: 10.1094/MPMI.1997.10.6.716.

Van Wees, S. C., Van der Ent, S. and Pieterse, C. M. (2008) 'Plant immune responses triggered by beneficial microbes', *Current Opinion in Plant Biology*, 11(4), pp. 443–448. doi: 10.1016/j.pbi.2008.05.005.

Weller, D. M., Landa, B. B., Mavrodi, O. V, Schroeder, K. L., De La Fuente, L., Blouin Bankhead, S., Allende Molar, R., Bonsall, R. F., Mavrodi, D. V and Thomashow, L. S. (2007) 'Role of 2,4-diacetylphloroglucinol-producing fluorescent <i>Pseudomonas<i/> spp. in the defense of plant roots.', *Plant biology (Stuttgart, Germany)*, 9(1), pp. 4–20. doi: 10.1055/s-2006-924473.

Westphal, A., Riedl, K. M., Cooperstone, J. L., Kamat, S., Balasubramaniam, V. M., Schwartz, S. J. and Böhm, V. (2017) 'High-pressure processing of broccoli sprouts: Influence on bioactivation of glucosinolates to isothiocyanates', *Journal of Agricultural and Food Chemistry*, 65(39), pp. 8578–8585. doi: 10.1021/acs.jafc.7b01380.

Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W. and Loper, J. E. (1998) 'The two-component regulators GacS and GacA influence accumulation of the stationary phase sigma factor RpoS and the stress response in Pseudomonas fluorescens Pf-5', *J. Bacteriol.*, 180(24), pp. 6635–6641.

Wilson, C. and Wisniewski, M. (1989) 'Biological Control of Postharvest Diseases of Fruits and Vegetables: An Emerging Technology', *Annual Review of Phytopathology*. Annual Reviews, 27(1), pp. 425–441. doi: 10.1146/annurev.py.27.090189.002233.

Wilson, M. and Lindow, S. E. (1994) 'Ecological similarity and coexistence of epiphytic Ice-nucleating (Ice+) <i>Pseudomonas syringae<i/> strains and a non-Ice-nucleating (Ice-) biological control agent', *Applied and Environmental Microbiology*, 60(9), pp. 3128–3137.

Wilson, P. D. G., Brocklehurst, T. F., Arino, S., Thuault, D., Jakobsen, M., Lange, M., Farkas, J., Wimpenny, J. W. T. and Van Impe, J. F. (2002) 'Modelling microbial growth in structured foods: Towards a unified approach', *International Journal of Food Microbiology*, 73, pp. 275–289.

Wisniewsky, M. a, Glatz, B. a, Gleason, M. L. and Reitmeier, C. a (2000) 'Reduction of Escherichia coli O157:H7 counts on whole fresh apples by treatment with sanitizers.', *Journal of food protection*, 63(6), pp. 703–708.

Witkin (1984) 'Ultraviolet mutagenesis and inducible DNA repair in *Caulobacter crescentus*.', *Molecular & general genetics : MGG*, 197(3), pp. 399–402. doi: 10.1007/BF00329935.

Wu, J., Gao, H., Zhao, L., Liao, X. and Chen, F. (2007) 'Chemical compositional characterization of some apple cultivars.', *Food Chemistry*, 103, pp. 88–93.

Wuytack, E. Y., Phuong, L. D. T., Aertsen, a, Reyns, K. M. F., Marquenie, D., De Ketelaere, B., Masschalck, B., Van Opstal, I., Diels, a M. J. and Michiels, C. W. (2003) 'Comparison of sublethal injury induced in Salmonella enterica serovar Typhimurium by heat and by different nonthermal treatments.', *Journal of food protection*, 66(1), pp. 31–7. doi: 10.4315/0362-028X-66.1.31.

Xavier, J. and Foster, K. R. (2007) 'Cooperation and conflict in microbial biofilms', *Proc. Natl. Adac. Sci. USA*, 104, pp. 876–881.

Xicohtencatl-Cortes, J., Sánchez Chacón, E., Saldaña, Z., Freer, E. and Girón, J. a (2009) 'Interaction of Escherichia coli O157:H7 with leafy green produce.', *Journal of food protection*, 72(00), pp. 1531–1537.

Xu, L. and Du, Y. (2012) 'Effects of yeast antagonist in combination with UV-C treatment on postharvest diseases of pear fruit', *BioControl*, 57(3), pp. 451–461. doi: 10.1007/s10526-011-9400-8.

Xu, W., Wang, T., Xu, S., Li, F., Deng, C., Wu, L., Wu, Y. and Bian, P. (2016) 'UV-C-Induced alleviation of transcriptional gene silencing through plant–plant communication: Key roles of jasmonic acid and salicylic acid pathways', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. Elsevier B.V., 790, pp. 56–67. doi: 10.1016/j.mrfmmm.2016.04.003.

Xu, W. and Wu, C. (2016) 'The impact of pulsed light on decontamination, quality, and bacterial attachment of fresh raspberries', *Food Microbiology*. Elsevier Ltd, 57, pp. 135–143. doi: 10.1016/j.fm.2016.02.009.

Yang, S. F. and Hoffman, N. E. (1984) 'Ethylene biosynthesis and its regulation in higher plants', *Annual Review of Plant Physiology*, 35(1), pp. 155–189.

Yang, Y., Meier, F., Ann Lo, J., Yuan, W., Lee Pei Sze, V., Chung, H. J. and Yuk, H. G. (2013) 'Overview of recent events in the microbiological safety of sprouts and new intervention technologies', *Comprehensive Reviews in Food Science and Food Safety*, 12(3), pp. 265–280. doi: 10.1111/1541-4337.12010.

Yang, Y., Mikš-Krajnik, M., Zheng, Q., Lee, S. B., Lee, S. C. and Yuk, H. G. (2016) 'Biofilm formation of Salmonella Enteritidis under food-related environmental stress conditions and its subsequent resistance to chlorine treatment', *Food Microbiology*, 54, pp. 98–105. doi: 10.1016/j.fm.2015.10.010.

Yao, H. J. and Tian, S. P. (2005) 'Effects of a biocontrol agent and methyl jasmonate on postharvest diseases of peach fruit and the possible mechanisms involved', *Journal of Applied Microbiology*, 98(4), pp. 941–950. doi: 10.1111/j.1365-2672.2004.02531.x.

Yaron, S. and Römling, U. (2014) 'Biofilm formation by enteric pathogens and its role in plant colonization and persistence', *Microbial Biotechnology*, 7(6), pp. 496–516. doi: 10.1111/1751-7915.12186.

Ye, L., Hildebrand, F., Dingemans, J., Ballet, S., Laus, G., Matthijs, S., Berendsen, R. and Cornelis, P. (2014) 'Draft Genome Sequence Analysis of a Pseudomonas putida W15Oct28 Strain with Antagonistic Activity to Gram-Positive and Pseudomonas sp. Pathogens', *PLoS ONE*, 9(11). doi: 10.1371/journal.pone.0110038.

Yoruk, R. and Marshall, M. M. R. (2003) 'Physicochemical properties and function of plant polyphenol oxidase: a review', *Journal of Food Biochemistry*, 27(352), pp. 361–422.

Yu, Y. B. and Yang, S. F. (1980) ‘Biosynthesis of wound ethylene.’, *Plant physiology*, 66(2), pp. 281–285. doi: 10.1104/pp.66.2.281.

Yun, J., Yan, R., Fan, X., Gurtler, J. and Phillips, J. (2013) ‘Fate of E. coli O157:H7, Salmonella spp. and potential surrogate bacteria on apricot fruit, following exposure to UV-C light’, *International Journal of Food Microbiology*. Elsevier B.V., 166(3), pp. 356–363. doi: 10.1016/j.ijfoodmicro.2013.07.021.

Zambare, V., Nilegaonkar, S. and Kanekar, P. (2011) ‘A novel extracellular protease from Pseudomonas aeruginosa MCM B-327: Enzyme production and its partial characterization’, *New Biotechnology*. Elsevier B.V., 28(2), pp. 173–181. doi: 10.1016/j.nbt.2010.10.002.

Zetzmann, M., Sánchez-Kopper, A., Waidmann, M. S., Blombach, B. and Riedel, C. U. (2016) ‘Identification of the agr peptide of Listeria monocytogenes’, *Frontiers in Microbiology*, 7(JUN), pp. 1–11. doi: 10.3389/fmicb.2016.00989.

Zhan, L., Hu, J., Li, Y. and Pang, L. (2012) ‘Combination of light exposure and low temperature in preserving quality and extending shelf-life of fresh-cut broccoli (Brassica oleracea L.)’, *Postharvest Biology and Technology*, 72, pp. 76–81. doi: 10.1016/j.postharvbio.2012.05.001.

Zhang, J., Yuan, L., Liu, W., Lin, Q., Wang, Z. and Guan, W. (2017) ‘Effects of UV-C on antioxidant capacity, antioxidant enzyme activity and colour of fresh-cut red cabbage during storage’, *International Journal of Food Science and Technology*, 52(3), pp. 626–634. doi: 10.1111/ijfs.13315.

Zhang, L. H. and Dong, Y. H. (2004) ‘Quorum sensing and signal interference: Diverse implications’, *Molecular Microbiology*, 53(6), pp. 1563–1571. doi: 10.1111/j.1365-2958.2004.04234.x.

Zhang, Z., Chen, J., Li, B., He, C., Chen, Y. and Tian, S. (2017) ‘Influence of oxidative stress on biocontrol activity of *Cryptococcus laurentii* against blue mold on peach fruit.’, *Frontiers in Microbiology*, 8(February), pp. 1–10. doi: https://doi.org/10.3389/fmicb.2017.00151.

Zhao, X. (2017) ‘Biofilm formation and control strategies of foodborne pathogens: food safety perspectives’, *Royal Society of Chemestry Advances*. Royal Society of Chemistry, 7(iii), pp. 36670–36683. doi: 10.1039/C7RA02497E.

Zhao, Y., Tu, K., Su, J., Tu, S., Hou, Y., Liu, F. and Zou, X. (2009) ‘Heat treatment in combination with antagonistic yeast reduces diseases and elicits the active defense responses in harvested cherry tomato fruit’, *Journal of Agricultural and Food Chemistry*, 57(16), pp. 7565–7570. doi: 10.1021/jf901437q.

Zhu, Q., Gooneratne, R. and Hussain, M. (2017) ‘Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels’, *Foods*, 6(3), p. 21. doi: 10.3390/foods6030021.

Zilelidou, E. A., Rychli, K., Manthou, E., Ciolacu, L., Wagner, M. and Skandamis, P. N. (2015) ‘Highly invasive *Listeria monocytogenes* strains have growth and invasion advantages in strain competition.’, *PLoS ONE*, 10, pp. 1–17.

Zudaire, L., Viñas, I., Abadias, M., Simó, J., Echeverria, G., Plaza, L. and Aguiló-Aguayo, I. (2017) ‘Quality and bioaccessibility of total phenols and antioxidant activity of calçots (*Allium cepa* L.) stored under controlled atmosphere conditions’, *Postharvest Biology and Technology*. Elsevier, 129, pp. 118–128.

ANNEX

Annex 1. Functional assignment to some open reading frames from the genome of *Pseudomonas graminis* CPA-7 related to the action mechanisms for biocontrol.

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
ACC deaminase activity	461519576	1-aminocyclopropane-1-carboxylate deaminase	gi|1272443179| PHX45591.1	acdS
	461483936	Lrp/AsnC family transcriptional regulator	WP_065988473.1	lrp
Adhesion/ motility/ biofilms	461552293	cystine ABC transporter substrate-binding protein	PF00497.12	fliY
	461571079	flagellin homolog domain protein	UniRef100_Q2PHB2	FliC
	461570849	flagellar hook-associated -terminus family protein	PF02465.10	fliD
	461571564	flagellar protein FliS	TIGR00208	fliS
	461570434	flagellar hook-basal body complex protein FliE	TIGR00205	fliE
	461571269	flagellar M-ring protein FliF	TIGR00206	fliF
	461570909	flagellar motor switch protein FliG	TIGR00207	fliG
	461570704	flagellar assembly FliH family protein	PF02108.8	fliH
	461571157	flagellar protein export ATPase FliI	TIGR03496	fliI
	461570758	flagellar basal body-associated FliL family protein	PF03748.6	fliL
	461570858	flagellar motor switch protein FliM	TIGR01397	fliM
	461570592	flagellar motor switch protein FliN	TIGR02480	fliN
	461570538	flagellar biosynthetic protein FliP	TIGR01103	fliP
	461570724	flagellar biosynthetic protein FliQ	TIGR01402	fliQ
	461571231	flagellar biosynthetic protein FliR	TIGR01400	fliR
	461570564	RNA polymerase sigma factor%2C FliA/WhiG family protein	TIGR02479	fliA
	461482533	twitching mobility protein	UniRef100_P24559	pilT
	461478449	type IV-A pilus assembly ATPase PilB	TIGR02538	pilB
	461547432	type IV pilus secretin PilQ family protein	TIGR02515	pilQ
	461571171	flagellar basal-body rod protein FlgB	TIGR01396	flgB
	461571108	flagellar basal-body rod protein FlgC	TIGR01395	flgC

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Adhesion/ motility/ biofilms	461570938	flagellar hook capping family protein	PF03963.6	flgD
	461570761	flagellar basal-body rod protein FlgG	TIGR02488	flgG
	461570917	flagellar L-ring family protein	PF02107.8	flgH
	461571054	flagellar P-ring family protein	PF02119.8	flgI
	461570688	flagellar hook-associated protein	TIGR02492	flgK
	461571328	flagellar hook-associated protein 3	TIGR02550	flgL
	461571002	flagellar biosynthetic protein	TIGR00328	flhB
	461570488	flagellar biosynthesis protein	TIGR01398	flhA
	461570738	flagellar biosynthetic protein	TIGR03499	flhF
	461571139	flagellar biosynthetic protein	UniRef100_E2XNY5	flhB
	461482304	hybrid sensor histidine kinase/response regulator	WP_065991176.1	
	461570643	chemotaxis protein CheA	WP_065987313.1	CheA
	461482268	YqgE/AlgH family protein	WP_065991165.1	algH
	461491738	mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase	TIGR01479	algA
	461491938	alginate biosynthesis protein AlgF	UniRef100_Q887Q8	algF
	461492057	alginate biosynthesis protein AlgJ	UniRef100_Q887Q7	algJ
	461491741	MBOAT family protein	PF03062.11	algI
	461491423	alginate lyase	UniRef100_Q9L7P2	algL
	461491651	alginate biosynthesis protein AlgX	UniRef100_Q887Q4	algX
	461492041	poly(beta-D-mannuronate) C5 epimerase	UniRef100_P59828	algG
	461491626	alginate production protein AlgE	UniRef100_P18895	algE
	461491633	sel1 repeat family protein	PF08238.4	algK
	461547807	response regulator	PF00072.16	algR
	461506851	alginate biosynthesis transcriptional regulatory protein AlgB	UniRef100_P23747	algB
Bacteriocins/ antibiotics	461519434	CvpA family protein	WP_065986402.1	CvpA
	461478293	Protein CreA	KPW47840.1	CreA
	461552763	PhzF family phenazine biosynthesis protein	WP_024916075.1	Phz

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Lytic activity	461556751	cell wall hydrolase	WP_065989196.1	
	461520913	alkaline proteinase inhibitor	WP_108122885.1	inh
Oxidative stress	461491732	glutathione peroxidase family protein	PF00255.11	btuE
	461503076	glutathione peroxidase family protein	PF00255.11	gpwA
	461545952	glutathione peroxidase family protein	PF00255.11	gpx1
	461563883	glutathione peroxidase family protein	PF00255.11	btuE
	461503781	superoxide dismutase [Fe]	UniRef100_P09223	sodB
	461496760	superoxide dismutase [Cu-Zn]	UniRef100_B2K562	sodC
	461518927	peroxiredoxin Ohr subfamily protein	TIGR03561	ohrB
	461528659	peroxiredoxin osmC	UniRef100_P0C0L2	osmC
	461494496	peroxiredoxin	TIGR03137	ahpC
	461571279	peroxidase ycdB	UniRef100_P31545	ycdB
	461474904	catalase/peroxidase HPI	TIGR00198	katG
	461514762	thiol peroxidase	UniRef100_P0A864	tpx
	461497557	alkyl hydroperoxide reductase F subunit	TIGR03140	ahpF
	461520866	alkyl hydroperoxidase AhpD family core domain protein	TIGR00778	ahpD
	461536098	superoxide dismutase [Mn/Fe]	UniRef100_P23744	sodB
	461502717	catalase activity - F;	UniRef100_P46206	katB
	461474904	catalase/peroxidase I activity - F; response to oxidative stress	TIGR00198	katG
	461496489	response to oxidative stress - P; catalase activity	PF00199.11	srpA
	461518041	catalase activity - F; transition Mn ion binding - F;	PF05067.4	katN
	461536716	response to oxidative stress - P; catalase activity - F;	UniRef100_D7I3B6	cat
	461506782	catalase B activity - F; cytosol - C; heme binding - F;	UniRef100_Q55DH8	catB
Quorum sensing	461482179	homoserine O-acetyltransferase	TIGR01392	metX
	461496158	acyl-homoserine lactone acylase quiP	UniRef100_Q9I4U2	quiP

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Quorum sensing	461536622	homoserine dehydrogenase	UniRef100_Q3SKI5	-
	461507444	homoserine kinase	TIGR00938	thrB
	461488886	beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabA	TIGR01749	fabA
	461536146	beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ	TIGR01750	fabZ
	461482436	methionine biosynthesis protein MetW	TIGR02081	metW
	461508497	adenosylmethionine-8-amino-7-oxononanoate transaminase	TIGR00508	bioA
	461508961	methionine adenosyltransferase	TIGR01034	metK
	461517311	S-adenosylmethionine decarboxylase proenzyme	TIGR03331	speD
	461545913	peptide methionine sulfoxide reductase MsrB	UniRef100_Q48FR2	msrB
	461529001	peptide-methionine (S)-S-oxide reductase	TIGR00401	msrA
	461560610	methionine aminopeptidase type I	TIGR00500	map
	461471069	methionine synthase	TIGR02082	metH
	461537156	methionine aminopeptidase type I	TIGR00500	map
	461536387	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	TIGR00113	queA
	461498244	peptide-methionine (S)-S-oxide reductase	TIGR00401	msrA
	461538366	swarming and quorum sensing, carbon storage regulator	TIGR00202	csrA
	461506072	swarming and quorum sensing, carbon storage regulator	TIGR00202	csrA
	461506418	swarming and quorum sensing, carbon storage regulator	TIGR00202	csrA
	461508606	DEAD/DEAH box helicase family protein	PF00270.21	rhlE1
	461483389	DEAD/DEAH box helicase family protein	PF00270.21	rhlE2
	461503559	ATP-dependent RNA elastase; EAD/DEAH box helicase family protein	PF00270.21	rhlB
Regulatory factors	461474366	RNA polymerase sigma factor RpoE	TIGR02939	rpoE
	461536874	RNA polymerase sigma factor RpoS	TIGR02394	rpoS
	461508086	RNA polymerase sigma factor RpoD	TIGR02393	rpoD
	461573578	DNA-directed RNA polymerase beta subunit	TIGR02013	rpoB

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Regulatory factors	461573419	RNA polymerase Rpb1%2C domain 1 family protein	PF04997.4	rpoC
	461573692	DNA-directed RNA polymerase beta' subunit	TIGR02386	rpoC
	461573643	DNA-directed RNA polymerase alpha subunit	TIGR02027	rpoA
	461503848	RNA polymerase sigma factor sigma-70 family protein	TIGR02937	rpoE
	461503627	RNA polymerase sigma-54 factor	TIGR02395	rpoN
	461570743	RNA polymerase sigma factor sigma-70 family protein	TIGR02937	rpoT
	461566482	DNA-directed RNA polymerase omega subunit	TIGR00690	rpoZ
Secretion systems/ effectors	461483323	type I secretion system permease/ATPase	WP_07489260 9	
	461508016	type IV secretion/conjugal transfer ATPase VirB4 family protein	TIGR00929	trbE
	461483323	type I secretion system ATPase family protein	TIGR01842	prtD
	461483616	type I secretion membrane fusion HlyD family protein	TIGR01843	eexE
	461483463	type I secretion outer membrane TolC family protein	TIGR01844	eexF
	461483543	type I secretion outer membrane TolC family protein	TIGR01844	lapE
	461483501	type I secretion membrane fusion HlyD family protein	TIGR01843	hlyD
	461491807	hlyD secretion family protein	PF00529.12	yhcQ
	461549094	type I secretion membrane fusion %2C HlyD family protein	TIGR01843	hlyD
	461549291	type I secretion outer membrane %2C TolC family protein	TIGR01844	tolC
	461509362	type IV / VI secretion system %2C DotU family domain protein	TIGR03349	vasF
	461529286	type I secretion outer membrane TolC family protein	TIGR01844	siiCB
	461529311	type I secretion system ATPase family protein	TIGR03375	siiBA
	461528993	type I secretion membrane fusion HlyD family protein	TIGR01843	cyaD
	461528988	type VI secretion system protein VasD	UniRef100_D5 C7Z4	vasD
	461529346	type VI secretion ATPase ClpV1 family	TIGR03345	clpV
	461529177	type VI secretion system effector Hcp1 family protein	TIGR03344	hcp

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Secretion systems/ effectors	461529047	type IV / VI secretion system DotU family domain protein	TIGR03349	dotU
	461574586	hlyD secretion family protein	PF00529.12	farA
	461521269	type I secretion system ATPase family protein	TIGR01842	aprD
	461521014	type I secretion membrane fusion HlyD family protein	TIGR01843	tliE
	461521273	type I secretion outer membrane TolC family protein	TIGR01844	tliF
	461520961	type III secretion regulator YopN/LcrE/InvE/MxiC	TIGR02568	hrpJ
	461521114	type III secretion HrcV family protein	TIGR01399	hrcV
	461521563	type III secretion protein HrpQ	UniRef100_Q2LJ02	hrpQ
	461521142	type III secretion apparatus H+-transporting two-sector ATPase	TIGR02546	hrcN
	461521323	type III secretion protein HrpO	UniRef100_E2MBV5	hrpO
	461521499	type III secretion protein HrpP	UniRef100_E2MBV6	hrpP
	461521551	type III secretion protein hrcQa	UniRef100_O85093	hrcQa
	461521017	type III secretion protein HrcQb	UniRef100_O85094	hrcQb
	461521046	type III secretion HrpO family protein	TIGR01403	hrcS
	461521022	type III secretion apparatus protein SpaR/YscT/HrcT	TIGR01401	epaR
	461556731	type III secretion protein HrpT	UniRef100_Q2LJ11	hrpT
	461556694	type III secretion protein HrpG	UniRef100_E2MBW8	hrpG
	461556681	type III secretion apparatus protein YscI/HrpB C-terminal domain	TIGR02497	hrpB
	461499669	hlyD secretion family protein	PF00529.12	farA
	461499579	hlyD secretion family protein	PF00529.12	hlyD
	461532512	type VI secretion system effector Hcp1 family protein	TIGR03344	ap002
	461478207	bacterial type II secretion system F domain protein	PF00482.15	pilC
	461473741	type I secretion outer membrane TolC family protein	TIGR01844	tolC
	461547432	type IV pilus secretin PilQ family protein	TIGR02515	pilQ
	461528153	haemolysin secretion/activation ShlB/FhaC/HecB family protein	PF03865.5	-

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Secretion systems/ effectors	461491648	hopJ type III effector family protein	PF08888.3	ptO
	461529177	type VI secretion system effectorHcp1 family protein	TIGR03344	hcp
	461499638	putative type III effector protein	UniRef100_C8 BNW6	-
	461532512	type VI secretion system effector Hcp1 family protein	TIGR03344	ap002
	461483213	type I bacteriocin secretion ATP-binding protein; ABC-type bacteriocin/antibiotic exporters		
Siderophores production/ secretion/ reception	461532396	tonB-dependent siderophore receptor family protein	TIGR01783	fiu
	461564436	tonB-dependent siderophore receptor family protein	TIGR01783	bfrF2
	461556699	ferripyoverdine receptor	UniRef100_ P48632	fpvA
	461534863	ferritin-like domain protein	PF00210.16	dps
	461536566	ferredoxin-NADP reductase	UniRef100_ Q44532	fpr
	461536353	ferredoxin 1	UniRef100_ P0A122	fdxA
	461536282	ferredoxin 2Fe-2 type ISC system	TIGR02007	fdx
	461478719	ferric uptake regulation protein	UniRef100_ Q03456	fur
	461539716	ferritin-like domain protein	PF00210.16	bfr
	461533353	fecCD transport family protein; ferric iron binding - F; cellular iron ion homeostasis - P	PF01032.10	eitB
	461564462	fecR family protein; Fe2+-dicitrate sensor; membrane component;	PF04773.5	fecR
	461574339	ABC transporter ATP-binding protein YojI; ABC-type siderophore export system; fused ATPase and permease components;	UniRef100_ P33941	yojI
Transmembrane transporters	461552534	inner membrane amino-acid ABC transporter permease protein yecS	UniRef100_ P0AFT3	yecS
	461482248	ABC transporter family protein	PF00005.19	ftsE
	461482224	amino ABC transporter permease 3-TM region His/Glu/Gln/Arg/opine family domain protein	TIGR01726	glnP
	461491772	ABC transporter family protein	PF00005.19	troB
	461503536	amino ABC transporter permease 3-TM region His/Glu/Gln/Arg/opine family domain protein	TIGR01726	yhdX
	461503999	inner membrane amino-acid ABC transporter permease protein yhdY	UniRef100_ P45768	yhdY

Functional group	Query ID	Protein description	Similar to protein	Similar to gene
Transmembrane transporters	461503396	ABC transporter family protein	PF00005.19	ttg2A
	461504327	putative phospholipid ABC transporter-binding protein mlaD	UniRef100_ P64605	mlaD
	461558952	electron transport complex RnfABCDGE type B subunit	TIGR01944	rnfB
	461558906	amino ABC transporter permease 3-TM region His/Glu/Gln/Arg/opine family domain protein	TIGR01726	hisM
	461558913	amino ABC transporter permease 3-TM region His/Glu/Gln/Arg/opine family domain protein	TIGR01726	hisQ

For fully sequencing, strain *Pseudomonas graminis* CPA-7 was grown in tryptone soy broth (TSB, Oxoid) for 24 h at 30 °C and centrifuged at 9800 x g during 10 min for cells harvesting. Total DNA was extracted using the "High Pure PCR" kit (Roche Diagnostics GmbH, Penzberg, Germany) and 15 µg were used to construct a Paired End (PE) library following the standard protocol 454 LifeScience/Roche. The library was run in a GS FLX Titanium Series 454 LifeScience/Roche sequencer using the Titanium chemistry. *De novo* assembly was run using the parameters by default in the Newbler assembler v2.8. All scaffolds and contigs were included in genome annotation using Interprot and Blast. In the present annex only some open reading frames showing functions related to the action mechanisms for biocontrol were included.