BIOLOGICAL CONTROL OF QUARANTINE BACTERIAL PLANT DISEASES WITH LACTOBACILLUS PLANTARUM STRAINS. IMPROVEMENT OF FITNESS AND MONITORING

Núria Daranas Boadella

Universitat de Girona

DOCTORAL THESIS

Biological control of quarantine bacterial plant diseases with *Lactobacillus plantarum* strains. Improvement of fitness and monitoring

Núria Daranas Boadella

2018

Universitat de Girona

DOCTORAL THESIS

Biological control of quarantine bacterial plant diseases with *Lactobacillus plantarum* strains. Improvement of fitness and monitoring

Núria Daranas Boadella

2018

Doctoral Programme in Technology

Supervised by:

Dra. Anna Bonaterra Carreras

Dra. Esther Badosa Romañó

Dr. Emilio Montesinos Seguí

Tutor:

Dra. Anna Bonaterra Carreras

Thesis submitted to apply for the Doctoral degree by the University of Girona

Núria Daranas Boadella	Dra. Anna Bonaterra Carreras	Dra. Esther Badosa Romañó	Dr. Emilio Montesinos Seguí

Universitat de Girona

Dra. Anna Bonaterra Carreras, Professora titular de l'Àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona,

Dra. Esther Badosa Romañó, Investigadora de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona,

i

Dr. Emilio Montesinos Seguí, Catedràtic de l'Àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona,

DECLAREM:

Que el treball titulat "Biological control of quarantine bacterial plant diseases with *Lactobacillus plantarum* strains. Improvement of fitness and monitoring", que presenta Núria Daranas Boadella per a l'obtenció del títol de doctora per la Universitat de Girona, ha estat realitzat sota la nostra direcció.

I, perquè així consti i tingui els efectes oportuns, signem aquest document.

Dra. Anna Bonaterra | Dra. Esther Badosa | Dr. Emilio Montesinos

Girona, 13 de juliol de 2018

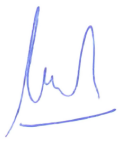
Universitat de Girona

Dra. Anna Bonaterra Carreras, Professora titular de l'Àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona, i directora dels projectes de recerca "Nuevas estrategias de control del fuego bacteriano. Bacterias del ácido láctico productoras de bacteriocinas (BALFUEBA)" (Ref. AGL2012-39880-C02-01) i "Metabolitos de fermentación para el control de bacterias fitopatógenas de cuarentena (METAPEP)" (Ref. AGL2015-69876-C2-1-R) del Ministerio de Economía y Competitividad, en els que es circumscriu la tesi doctoral titulada "Biological control of quarantine bacterial plant diseases with *Lactobacillus plantarum* strains. Improvement of fitness and monitoring" realitzada per **Núria Daranas Boadella**,

DECLARA QUE:

Aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal i de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona que participen en els esmentats projectes (Article 2. Apartat 2. RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

I, perquè així consti i tingui els efectes oportuns, signo aquest document.



Dra. Anna Bonaterra

Girona, 13 de juliol de 2018

Universitat de Girona

Dr. Emilio Montesinos Seguí, Catedràtic de l'Àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona, i director del projecte de recerca "Strategies to develop effective, innovative and practical approaches to protect major European fruit crops from pests and pathogens (DROPSA)" (Ref. FP7-KBBE.2013.1.2-04 613678) de European Union's Seventh Framework Programme, en el que es circumscriu la tesi doctoral titulada "Biological control of quarantine bacterial plant diseases with *Lactobacillus plantarum* strains. Improvement of fitness and monitoring" realitzada per **Núria Daranas Boadella**,

DECLARA QUE:

Aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal i de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona que participen en els esmentats projectes (Article 2. Apartat 2. RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

I, perquè així consti i tingui els efectes oportuns, signo aquest document.

Dr. Emilio Montesinos

Girona, 13 de juliol de 2018

A la meva família, a en Marc
i en especial a la memòria de l'Estel

AKNOWLEDGEMENTS

Mentre acabo d'enllestir la redacció de la tesi arriba el moment d'escriure unes paraules d'agraïment a totes les persones que d'una manera o d'una altra han contribuït a fer-ho possible. A tots, gràcies! De cada un n'he après alguna cosa, tant en l'àmbit professional com personal.

En primer lloc vull agrair la dedicació i el suport dels meus directors de tesi. Anna, m'has demostrat que la perseverança i la constància són clau per fer recerca. Gràcies per la teva predisposició a ajudar-me en la realització dels assajos sempre que ha fet falta. Esther, el teu optimisme i la teva convicció han sigut de gran ajuda per avançar dia rere dia. Gràcies per ajudar-me a buscar les solucions als entrebancs que ens anàvem trobant. Emili, gràcies per donar-me la oportunitat de realitzar la tesi en el grup de Patologia Vegetal i per haver aportant sempre la teva visió crítica. A tots tres, gràcies per la vostra implicació a nivell personal i per tots els consells que m'heu donat.

També vull fer un especial agraïment a tots els que m'han ajudat en el assajos d'hivernacle i de camp. Jesús, gràcies pels consells, ajuda en la preparació dels experiments i interès en el seu seguiment. Jordi, gràcies per facilitar-me l'espai a l'hivernacle del parc. Josep Pereda, gràcies pels consells i dedicació en la preparació del material vegetal. Pere Vilardell, gràcies per facilitar-nos camps de pomeres i pereres a on fer els assajos. No em vull deixar unes paraules d'agraïment per a la Daniela, en Narcís i la Malva, per la seva ajuda, rigorositat i bon ambient de treball.

A en Jordi també vull agrair-li el suport en temes de bioinformàtica. I a l'Anna Nadal i a la Maria Pla vull donar-los les gràcies pels seus consells en biologia molecular i per la seva voluntat per resoldre els dubtes que ens sorgien.

I would like to thank Dr. Francesco Spinelli the opportunity that he gave me to stay for two months in the Department of Agricultural and Food Sciences of University of Bologna and to carry out some experiments in these facilities. Antonio, Giampaolo, Irene, Francesco, thank you for your assistant during the stay.

A més, durant aquests anys de tesi he tingut la sort de compartir amb grans persones molts bons moments, tant a dins com a fora del laboratori. Gerard, Cristina, Bea, Anna Serra, Malva, Miquel, Laura Foix, Aina, Laura Montesinos, Isa, Mireia, Gemma, ha estat un plaer estar rodejada de tots vosaltres durant aquest temps. Gràcies per la vostra simpatia, bon humor, companyia i amistat. Segur que continuarem compartint més bons moments plegats. Gemma, mil gràcies per ser-hi sempre. Quina sort que vaig tenir de ser la teva "pupil·la" mentre tu feies la tesi.

També vull agrair a tots els que formeu o heu format part del grup de Patologia Vegetal durant aquests anys, Emili, Anna Bonaterra, Esther, Jesús, Cun, Isidre, Jordi, Lídia, Josep, Gemma, Mireia, Isa, Laura, Malva, Aina, Bea, Anna Serra, Alba, Yolanda, de qui he rebut ajuda i consells sempre que ho he necessitat i amb qui he compartit bones estones a congressos i celebracions.

Mónica, Dolors, Elena, Anna Maria, Maria Àngels, José Alberto, Núria Fiol i a tots als del menjador d'EQATA, gràcies pel vostre humor i bona companyia mentre esmorzàvem i dinàvem.

Per anar acabant vull també donar les gràcies al meus amics, els de sempre i els que he tingut la sort de trobar-me pel camí, per preocupar-se de com m'anava la tesi, animar-me a seguir endavant i distreure'm quan més ho necessitava.

Per últim, un agraïment molt especial a la meva família, tant als que hi són com als que sempre hi seran, per recolzar-me, cuidar-me i confiar sempre amb mi. Estel, el teu somriure el tinc ben present. No deixis mai de brillar! I a tu Marc, mil gràcies per ser-hi sempre, pel teu amor i per la teva paciència.

Núria

This work was supported by AGL2012-39880-C02-01 and AGL2015-69876-C2-1-R projects (Ministerio de Economía y Competitividad of Spain and FEDER of the European Union) and by FP7-KBBE.2013.1.2-04 613678 DROPSA project (European Union). Núria Daranas was the recipient of the predoctoral research grant 2015 FI_B00515 (Secretaria d'Universitats i Recerca, Departament d'Economia i Coneixement, Generalitat de Catalunya i Fons Social Europeu) and the mobility grant MOB2017 (Universitat de Girona).

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
BCA	Biological control agent
cDNA	Complementary DNA
CFU	Colony forming unit
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
Ea	*Erwinia amylovora*
EFSA	European Food Safety Authority
EMA	Ethidium monoazide
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolysaccharides
EU	European Union
FDA	Food and Drug Administration
gfp	Green fluorescent protein
GMO	Genetically modified organism
GRAS	Generally Regarded as Safe
IMC	Isothermal microcalorimetry
IPM	Integrated pest management
ITS	Internal transcribed spacer
kDa	Kilodalton
LAB	Lactic acid bacteria
*lux*AB	Luciferase enzyme
MLST	Multilocus sequence typing
MPN	Most probable number
mRNA	Messenger RNA
MRS	de Man, Rogosa and Sharpe
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Propidium monoazide
Psa	*Pseudomonas syringae* pv. *actinidiae*
QC-PCR	Quantitative-competitive PCR
qPCR	Real-time PCR
QPS	Qualified Presumption of Safety
RAPD	Random amplified polymorphic DNA

RH	Relative humidity
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription qPCR
SCAR	Sequence-characterized amplified regions
ST	Sequence type
USA	United States of America
VBNC	Viable-but-nonculturable
v-qPCR	Viability qPCR
Xap	*Xanthomonas arboricola* pv. *pruni*
Xf	*Xanthomonas fragariae*

LIST OF FIGURES AND TABLES

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This Ph.D. Thesis is presented as a compendium of three publications:

Daranas N., Roselló G., Cabrefiga J., Donati I., Francés J., Badosa E., Spinelli F., Montesinos E., Bonaterra A. (2018) Biological control of bacterial plant diseases with *Lactobacillus plantarum* strains selected by their broad-spectrum activity. Submitted to *Annals of Applied Biology* on 2 July 2018

Annals of Applied Biology has an impact factor of 2.046 and it is in the first quartile (Q1) in the agriculture, multidisciplinary category (ISI Journal Citation Reports Ranking 2017, Science Edition, published by Thompson Reuters).

Daranas N., Badosa E., Francés J., Montesinos E., Bonaterra A. (2018) Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. *PLoS ONE*, **13**, e0190931. DOI:10.1371/journal.pone.0190931

PLoS ONE has an impact factor of 2.766 and it is in the first quartile (Q1) in the multidisciplinary sciences category (ISI Journal Citation Reports Ranking 2017, Science Edition, published by Thompson Reuters).

Daranas N., Bonaterra A., Francés J., Cabrefiga J., Montesinos E., Badosa E. (2018) Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method. *Applied and Environmental Microbiology*, **84**, e00107-18. DOI:10.1128/AEM.00107-18

Applied and Environmental Microbiology has an impact factor of 3.633 and it is in the first quartile (Q1) in the biotechnology and applied microbiology category (ISI Journal Citation Reports Ranking 2017, Science Edition, published by Thompson Reuters).

SUMMARY

Fire blight of apple and pear, bacterial canker of kiwifruit, bacterial spot of stone fruits, and angular leaf spot of strawberry are plant diseases responsible for important crop losses worldwide, because the management options available are of limited efficacy. Their causal agents (*Erwinia amylovora*, Ea; *Pseudomonas syringae* pv. *actinidiae*, Psa; *Xanthomonas arboricola* pv. *pruni*, Xap; and *Xanthomonas fragariae*, Xf, respectively) are bacterial plant pathogens considered as quarantine organisms by the European and Mediterranean Plant Protection Organization (EPPO), as well as, by the European Union. The restrictions in the use of some effective chemical products, the increasing evolution of resistant pathogen populations to existing bactericides, as well as, the public concern about the negative impact of conventional pesticides on environment and human health have promoted the development of alternative and sustainable management tools. In particular, biological control is an alternative or complementary strategy, since the use of microbial biopesticides could be merged into an integrated management approach of plant diseases. Several microorganisms are commercially available as biological control agents (BCA) of fire blight, while little is known about antagonistic microorganisms for the biological control of bacterial canker of kiwifruit, bacterial spot of stone fruits, and angular leaf spot of strawberry.

This Ph.D. Thesis contributes to the development of a novel microbial biopesticide based on lactic acid bacteria (LAB) with broad-spectrum activity. LAB are well known as biopreservative agents in food and their potential use in crop protection has also been explored, but in a lesser extent. In addition, LAB are ubiquitous members of many plant microbiomes and are generally considered as safe by food safety agencies.

In the first part of this Ph.D. Thesis plant-associated LAB were screened for their *in vitro* antagonistic activity against Psa, Xap, and Xf. *Lactobacillus plantarum* CC100, PM411, and TC92 and *Leuconostoc mesenteroides* CM160 and CM209 were selected for showing broad spectrum of activity. The selected strains were studied in more detail by means of a multiple-pathosystem approach in potted plants of kiwifruit, *Prunus* sp., and strawberry under greenhouse conditions. *L. plantarum* PM411 and TC92 prevented infections of Psa, Xap, and Xf in their corresponding host plants. In addition, the biocontrol performance of both strains was comparable to reference products in the semi-field and field experiments performed. Therefore, the multifactorial screening based on *in vitro* and *in planta* assays against multiple pathogens was successful in selecting *L. plantarum* PM411 and TC92 as candidate BCA. The mechanism involved in the *in vitro* antibacterial activity against Psa, Xap, and Xf was based, at least in part, on a pH lowering effect and lactic acid production. Moreover, both strains were able to survive with similar rates on leaf surfaces. Interestingly, both strains were clearly distinguished due to their differential multilocus sequence typing (MLST) and random amplified polymorphic DNA (RAPD) profiles.

The establishment of suppressive populations of BCA, and also of LAB, on aerial plant surfaces, where many pathogens epiphytically grow, is suggested as a key factor for the biological

control. Aerial plant surfaces are generally considered as hostile environments for bacterial colonization due to ultraviolet radiation, nutrient limitation, and fluctuating temperature and water availability. In order to improve the epiphytic fitness of *L. plantarum* PM411 and TC92, in the second part of this Ph.D. Thesis, the water-stress tolerance was increased by means of a physiological adaptive strategy consisting of growing cells into a hyperosmotic medium until stationary phase, which also involves acid production. Adapted cells showed higher survival rates under *in vitro* desiccation than non-adapted cells. Interestingly, the response of PM411 and TC92 toward the adaptation treatment resulted in an increase in transcript levels of general stress-related genes, which generally remained unaltered during the subsequent desiccation challenge. However, differences between the transcription patterns of the two strains were observed, which were in agreement with a better performance of adapted cells of PM411 than TC92 on plant surfaces under low relative humidity environmental conditions. Indeed, the adaptation treatment increased the survival of PM411 cells in different host plants in the greenhouse (strawberry and kiwifruit leaves) and under field conditions (apple and pear blossoms). Moreover, the adaptation treatment provided more consistency in the PM411 biocontrol efficacy of Ea and Xf infections on apple and pear blossoms and strawberry leaves, respectively.

Monitoring methods that allow for the strain-specific detection and quantification of viable cells are required to distinguish the introduced BCA strain from autochthonous microbiota of the same species, and to evaluate its population dynamics on plant surfaces after field release. Therefore, in the last part of this Ph.D. Thesis, a viability quantitative PCR (v-qPCR) method was developed for the unambiguous detection and quantification of *L. plantarum* PM411 viable cells on aerial plant surfaces. The v-qPCR method relied on a sample pre-treatment with a nucleic acid-binding dye prior to qPCR. The nucleic acid-binding dye PEMAX was used to selectively detect and enumerate viable cells. Interestingly, a PM411 strain-specific molecular marker was identified within a region of a predicted prophage with mosaic architecture. Three TaqMan qPCR assays with different amplicon lengths (92, 188, and 317 bp) were designed, being the primer set amplifying a 188 bp DNA fragment the most suitable for v-qPCR. The reliability of the v-qPCR together with specific plate counting and qPCR in the monitoring of PM411 cell population on plant surfaces (pear, strawberry, and kiwifruit leaves and apple and pear blossoms) was simultaneously evaluated under greenhouse and field conditions. The three methods contributed to comprehend the behaviour of PM411 in different tissues, plant species and environmental conditions. The population estimation did not differ significantly between the three methods when conditions were conducive to bacterial survival. However, under stressful conditions, differences between methods were observed due to cell death or viable-but-nonculturable (VBNC) state induction. Therefore, qPCR overestimated and plate counting underestimated the viable population level of PM411 under growth limiting conditions.

RESUM

El foc bacterià de les pomeres i pereres, el xancre bacterià del kiwi, la taca bacteriana dels fruiters de pinyol i la taca angular de les fulles de maduixera són malalties que causen importants pèrdues en el cultiu de fruita a tot el món ja que les opcions disponibles pel seu control tenen una eficàcia limitada. Els agents causals (*Erwinia amylovora*, Ea; *Pseudomonas syringae* pv. *actinidiae*, Psa; *Xanthomonas arboricola* pv. *pruni*, Xap; i *Xanthomonas fragariae*, Xf, respectivament) són bacteris fitopatògens considerats organismes de quarantena per la Organització Europea i Mediterrània de Protecció Vegetal (EPPO) i la Unió Europea. El nombre limitat de productes químics eficaços, la creixent evolució de les poblacions de patògens resistents als bactericides existents, així com la preocupació social per l'impacte negatiu dels plaguicides convencionals sobre el medi ambient i la salut humana han fomentat el desenvolupament d'eines de control alternatives i sostenibles. Concretament, el control biològic mitjançant l'ús del bioplaguicides microbians és una estratègia alternativa que forma part del maneig integrat de malalties de plantes. Mentre que diversos microorganismes estan disponibles en el mercat com a agents de biocontrol (ABC) del foc bacterià, actualment hi ha poca informació sobre microorganismes antagonistes pel control biològic del xancre bacterià del kiwi, la taca bacteriana dels fruiters de pinyol i la taca angular de les fulles de maduixera.

Aquesta tesi doctoral contribueix en el desenvolupament d'un nou bioplaguicida microbià basat en bacteris de l'àcid làctic (BAL) amb activitat d'ampli espectre. Els BAL són àmpliament coneguts com a bioconservants en aliments i el seu potencial ús en protecció de cultius també ha estat explorat, tot i que en menor mesura. A més, els BAL formen part de la microbiota de les plantes i generalment estan qualificats com a segurs per les agències de seguretat alimentària.

En la primera part d'aquesta tesi doctoral es va avaluar l'antagonisme *in vitro* de BAL contra Psa, Xap i Xf. Les soques *Lactobacillus plantarum* CC100, PM411 i TC92 i *Leuconostoc mesenteroides* CM160 i CM209 es van seleccionar per la seva activitat d'ampli espectre. Les soques seleccionades es van estudiar amb més detall mitjançant assajos de biocontrol en condicions d'hivernacle en plantes de kiwi, *Prunus* sp. i maduixera. *L. plantarum* PM411 i TC92 van prevenir les infeccions de Psa, Xap i Xf en les corresponents plantes hostes. A més, en els experiments de semi-camp i camp realitzats, l'eficàcia d'ambdues soques va ser comprable a productes de referència. Per tant, l'aproximació multifactorial basada en assajos *in vitro* i *in planta* enfront múltiples patògens va permetre seleccionar *L. plantarum* PM411 i TC92 com a candidats ABC. El mecanisme implicat en l'activitat antibacteriana *in vitro* de les dues soques es basa, almenys en part, per l'efecte de la reducció de pH i la producció d'àcid làctic. A més, ambdues soques van presentar similars taxes de supervivència a la superfície de les fulles. Les soques *L. plantarum* PM411 i TC92 van ser clarament diferenciades pels perfils obtinguts mitjançant les tècniques "multilocus sequence typing" (MLST) i "random amplified polymorphic DNA" (RAPD).

L'establiment dels ABC, i també dels BAL, a les superfícies de la part aèria de les plantes, on molts patògens creixen de manera epifítica, és un factor clau en el control biològic. La part aèria de les plantes és generalment considerada un ambient hostil per a la colonització bacteriana degut a la radiació ultraviolada, la limitació de nutrients, i les fluctuacions de temperatura i disponibilitat d'aigua. Per tal de millorar l'aptitud epifítica de *L. plantarum* PM411 i TC92, en la segona part d'aquesta tesi doctoral es va incrementar la tolerància a l'estrès per manca d'aigua mitjançant una estratègia fisiològica d'adaptació que consisteix en fer créixer les cèl·lules en un medi hiperosmòtic fins a fase estacionària, que implica la producció d'àcid. Les cèl·lules adaptades van presentar nivells de supervivència enfront la dessecació *in vitro* més alts que les cèl·lules no adaptades. A més, la resposta de PM411 i TC92 al tractament d'adaptació va implicar un increment dels nivells de transcripció de gens relacionats amb l'estrès, els quals, en general, van romandre inalterats durant la posterior dessecació. Tot i així, es van observar diferències entre els patrons de transcripció de les dues soques coincidint amb un millor comportament de les cèl·lules adaptades de PM411 que les de TC92 a la superfície de les plantes en condicions de baixa humitat relativa. De fet, el tractament d'adaptació va incrementar la supervivència de les cèl·lules de PM411 en diferents plantes hostes en condicions d'hivernacle (fulles de maduixera i kiwi) i de camp (flors de pomera i perera). A més, el tractament d'adaptació va aportar més consistència en el biocontrol de les infeccions d'Ea i Xf en flors de pomera i perera i fulles de maduixera, respectivament, per part de la soca PM411.

Els mètodes de monitoratge que permeten la detecció específica a nivell de soca i la quantificació de cèl·lules viables són necessaris per distingir la soca de l'ABC introduïda de la microbiota autòctona de la mateixa espècie, i per avaluar les seves dinàmiques poblacionals a la superfície de les plantes després de l'aplicació a camp. En l'última part d'aquesta tesi doctoral es va desenvolupar un mètode basat en la PCR quantitativa de viables (v-qPCR) per la detecció i quantificació de cèl·lules viables de *L. plantarum* PM411 a la superfície de plantes. Aquest mètode es basava en un pretractament de la mostra amb un colorant que s'intercala amb el DNA previ a la tècnica de qPCR. L'agent intercalant PEMAX es va utilitzar per detectar i enumerar només cèl·lules viables mitjançant la qPCR. Es va identificar un marcador molecular específic de la soca PM411 en una regió d'un possible pròfag amb arquitectura de mosaic. Es van dissenyar tres assajos de qPCR amb amplicons de diferents llargades (92, 188 i 317 pb), essent l'assaig que amplificava un fragment de DNA de 188 pb el més adequat pel mètode v-qPCR. La idoneïtat de la tècnica v-qPCR juntament amb la de recompte en placa i qPCR en el monitoratge de la població de la soca PM411 a les superfícies de plantes (fulles de perera, maduixera i kiwi i flors de pomera i perera) es va avaluar de manera simultània en condicions d'hivernacle i camp. Els tres mètodes van contribuir a la comprensió del comportament de la soca PM411 en diferents òrgans i espècies de plantes i diferents condicions ambientals. La població estimada amb els tres mètodes no va diferir significativament quan les condicions eren favorables per la supervivència de PM411. En canvi, en condicions d'estrès, es van observar diferències entre els mètodes degut a la mort

cel·lular o a la inducció de l'estat de viables però no cultivables. En aquests casos la tècnica de qPCR va sobreestimar el nivell poblacional de viables de PM411 mentre que el recompte en placa el va subestimar.

RESUMEN

El fuego bacteriano del manzano y el peral, en chancro bacteriano del kiwi, la mancha bacteriana de los frutales de hueso y la mancha angular de las hojas de fresa son enfermedades que causan importantes pérdidas en los cultivos de fruta en todo el mundo debido a que las opciones disponibles para su control tienen una eficacia limitada. Los agentes causales (*Erwinia amylovora*, Ea; *Pseudomonas syringae* pv. *actinidiae*, Psa; *Xanthomonas arboricola* pv. *pruni*, Xap; y *Xanthomonas fragariae*, Xf, respectivamente) son bacterias fitopatógenas consideradas organismos de cuarentena por la Organización Europea y Mediterránea de Protección Vegetal (EPPO) y la Unión Europea. El número limitado de productos químicos eficaces, la creciente evolución de poblaciones de patógenos resistentes a los bactericidas existentes, así como la preocupación social por el impacto negativo de los plaguicidas convencionales sobre el medio ambiente y la salud humana han fomentado el desarrollo de herramientas de control alternativas y sostenibles. Concretamente, el control biológico mediante el uso de bioplaguicidas microbianos es una estrategia alternativa que forma parte del manejo integrado de enfermedades de plantas. Mientras que algunos microorganismos están disponibles en el mercado como agentes de biocontrol (ABC) del fuego bacteriano, actualmente hay poca información sobre microorganismos antagonistas para el control biológico del chancro bacteriano del kiwi, la mancha bacteriana de los frutales de hueso y la mancha angular de las hojas de fresa.

Esta tesis doctoral contribuye en el desarrollo de un nuevo bioplaguicida microbiano basado en bacterias del ácido láctico (BAL) con actividad de amplio espectro. Las BAL son ampliamente conocidas como bioconservantes en alimentos y su potencial uso en protección de cultivos también ha sido explorado, aunque en menor medida. Además, las BAL forman parte de la microbiota de las plantas y generalmente están calificadas como seguras para las agencias de seguridad alimentaria.

En la primera parte de esta tesis doctoral se evaluó el antagonismo *in vitro* de BAL contra Psa, Xap y Xf. Las cepas *Lactobacillus plantarum* CC100, PM411 y TC92 y *Leuconostoc mesenteroides* CM160 y CM209 se seleccionaron por su actividad de amplio espectro. Las cepas seleccionadas se estudiaron con mayor detalle mediante ensayos de biocontrol en condiciones de invernadero en plantas de kiwi, *Prunus* sp. y fresa. *L. plantarum* PM411 y TC92 previnieron las infecciones de Psa, Xap y Xf en las correspondientes plantas huéspedes. En los experimentos de semi-campo y campo realizados, la eficacia de ambas cepas fue comparable con productos de referencia. Por lo tanto, la aproximación multifactorial basada en ensayos *in vitro* e *in planta* frente a múltiples patógenos permitió seleccionar *L. plantarum* PM411 y TC92 como candidatos ABC. El mecanismo implicado en su actividad antibacteriana *in vitro* se basa, al menos en parte, por el efecto de la reducción de pH y la producción de ácido láctico. Además, ambas cepas presentaron similares tasas de supervivencia en la superficie de las hojas. Las cepas *L. plantarum* PM411 y

TC92 se diferenciaron claramente con los perfiles obtenidos mediante las técnicas “multilocus sequence typing” (MLST) y “random amplified polymorphic DNA” (RAPD).

El establecimiento de los ABC, y también de las BAL, en las superficies de la parte aérea de las plantas, donde muchos patógenos crecen de manera epífita, es un factor clave en el control biológico. La parte aérea de las plantas se considera generalmente un ambiente hostil para la colonización bacteriana debido a la radiación ultravioleta, la limitación de nutrientes y las fluctuaciones de temperatura y disponibilidad de agua. Con el fin de mejorar la aptitud epífita de *L. plantarum* PM411 y TC92, en la segunda parte de esta tesis doctoral se incrementó la tolerancia al estrés por déficit de agua mediante una estrategia de adaptación fisiológica que consiste en hacer crecer las células en un medio hiperosmótico hasta alcanzar la fase estacionaria, que conlleva la producción de ácido. Las células adaptadas presentaron mayores niveles de supervivencia frente a la desecación *in vitro* que las no adaptadas. La respuesta de PM411 y TC92 al tratamiento de adaptación implicó un incremento de los niveles de transcripción de genes relacionados con el estrés, los cuales, en general, permanecieron inalterados durante la posterior desecación. Aun así, se observaron diferencias entre los patrones de transcripción de las dos cepas coincidiendo con un mejor comportamiento de las células adaptadas de PM411 que las de TC92 en las plantas en condiciones de baja humedad relativa. El tratamiento de adaptación incrementó la supervivencia de PM411 en condiciones de invernadero (hojas de fresa y kiwi) y de campo (flores de manzano y peral). Además, el tratamiento de adaptación aportó a la cepa PM411 mayor consistencia en el biocontrol de las infecciones de Ea en flores de manzano y peral y de Xf en hojas de fresa.

Los métodos de monitorización que permiten la detección específica a nivel de cepa y la cuantificación de células viables son necesarios para distinguir la cepa del ABC introducida de la microbiota autóctona de la misma especie, y para evaluar sus dinámicas poblacionales en las superficies de las plantas después de la aplicación en campo. En la última parte de esta tesis se desarrolló un método basado en la PCR cuantitativa de viables (v-qPCR) para la detección y cuantificación de células viables de *L. plantarum* PM411. Este método se basaba en un pretratamiento de la muestra con un colorante que se intercala con el DNA previo a la técnica de qPCR. El agente intercalante PEMAX se utilizó para detectar y enumerar solamente células viables mediante qPCR. Se identificó un marcador molecular específico de la cepa PM411 en una región de un posible profago con arquitectura de mosaico. Se diseñaron tres ensayos de qPCR con amplicones de distintas longitudes (92, 188 y 317 pb), siendo el que amplificaba un fragmento de 188 pb el más apropiado para el método v-qPCR. La idoneidad de la técnica v-qPCR juntamente con la del recuento en placa y qPCR en la monitorización de la población de PM411 en la superficie de plantas (hojas de peral, fresa y kiwi y flores de manzano y peral) se evaluó de manera simultánea en condiciones de invernadero y campo. Los tres métodos contribuyeron en la comprensión del comportamiento de la cepa PM411 en distintos órganos, especies de plantas y condiciones ambientales. La población estimada con los tres métodos no difirió significativamente

cuando las condiciones eran favorables para la supervivencia de PM411. Por lo contrario, en condiciones de estrés, se observaron diferencias entre los métodos debido a la muerte celular o a la inducción del estado de viables pero no cultivables. En estos casos la técnica de qPCR sobreestimó el nivel poblacional de viables de PM411 mientras que el recuento en placa lo subestimó.

CHAPTER I

General Introduction

1 Bacterial diseases of fruit crops

Fruit and vegetable production is an important contributor to the European Union (EU) agricultural market (ca. 21%) (Freshfel Europe, 2017). In particular, the fruit sector stands for 6.8% of agricultural output in the EU with 36.4 million tonnes of fruit production in 2016, being Spain (10.6 million tonnes), Italy (8.7 million tonnes) and Poland (4.4 million tonnes) the most important producer countries (Eurostats, 2017). The main fruit categories produced in the EU are apples, pears, citrus, peaches and nectarines, kiwifruits, and melons/watermelons (Freshfel Europe, 2016; FAOSTAT, 2017). Spain is the leading supplier of fruits with almost 7.5 million tonnes shipped to other EU member states in 2015, having the largest European orchard area destined for fruit and citrus tree cultivation (33.5%) (Eurostats, 2014).

The production and quality of fruits are seriously threatened by bacterial, fungal and viral diseases that produce significant economic losses (ca. 16%) in agriculture worldwide (Oerke, 2006). The main bacterial diseases of fruit crops together with their causal agents are listed in Table 1.

Table 1. Bacterial diseases of fruit crops (EPPO, 2018a).

Bacterial pathogen	A1/A2 List [a]	Disease
Agrobacterium tumefaciens	-	Crown gall
'Candidatus Liberibacter africanus' *'Candidatus* Liberibacter asiaticus'	A1 A1	Citrus Huanglongbing
Erwinia amylovora	A2	Fire blight of apple and pear
Pseudomonas syringae pv. *actinidiae*	A2	Bacterial canker of kiwifruit
Pseudomonas syringae pv. *morsprunorum* *Pseudomonas syringae* pv. *syringae*	- -	Bacterial canker of stone fruits
Xanthomonas arboricola pv. *juglandis*	-	Bacterial blight of walnut
Xanthomonas arboricola pv. *pruni*	A2	Bacterial spot of stone fruits
Xanthomonas citri pv. *citri*	A1	Citrus canker
Xanthomonas fragariae	A2	Angular leaf spot of strawberry
Xylella fastidiosa subsp. *fastidiosa*	A2	Pierce's disease of grapevine
Xylella fastidiosa subsp. *multiplex* *Xylella fastidiosa* subsp. *sandyi*	A2 A2	Bacterial leaf scorch
Xylella fastidiosa subsp. *pauca*	A2	Citrus variegated chlorosis Olive quick decline syndrome

[a] EPPO A1 and A2 Lists of pests recommended for regulation as quarantine pests (updated at June 2018).

In particular, fire blight of apple and pear (*Erwinia amylovora*), bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*), bacterial spot of stone fruits (*Xanthomonas arboricola* pv. *pruni*), and angular leaf spot of strawberry (*Xanthomonas fragariae*) pose a serious challenge to European fruit production. In fact, these plant diseases are also a major concern in countries outside Europe. Actually, these diseases and pathogens were included in the EU-funded DROPSA project and have been the purpose of this Thesis. The causal agents of these diseases are bacterial pathogens catalogued as quarantine organisms by the European and Mediterranean Plant Protection Organization (EPPO), which includes these bacteria in the A2 list as locally present in the EPPO region (EPPO, 2017). In particular, *X. arboricola* pv. *pruni* is considered as a quarantine bacterium in the EU in plant propagation material (i.e. in nurseries) but not in the field, where it is very extended. In addition, *P. syringae* pathovars and *E. amylovora* belong to the "Top 10" plant pathogenic bacteria based on scientific and economic importance (Mansfield *et al*., 2012). The aforementioned bacterial plant diseases are described in more detail in the following paragraphs.

1.1 Fire blight of apple and pear

Fire blight is a substantial threat to global pome fruit production, causing high economic losses every year. The causal agent is *Erwinia amylovora* (hereafter, Ea), a Gram-negative bacterium that belongs to the family of *Enterobacteriaceae*. *E. amylovora* affects several plant species, mainly belonging to the *Rosaceae* family, such as fruit trees (*Pyrus* spp., *Malus* spp., *Cydonia* sp.) and ornamentals plants (*Cotoneaster* spp., *Crataegus* spp., *Pyracantha* spp.) (van der Zwet *et al*., 2012; EPPO, 2018a). Fire blight was first identified in 1780 in the United States of America (USA) and subsequently detected in New Zealand in 1920. Since then, *E. amylovora* has been reported worldwide where susceptible hosts are cultivated. Currently, fire blight is present in several countries of Europe, North America, the Mediterranean region, and occidental and central Asia (van der Zwet *et al*., 2012; Park *et al*., 2017). In Spain, the disease is established in some regions, while other zones are free of fire blight (MAPAMA, 2016). Typical symptoms are blackening of shoots, flowers, fruits and leaves, as if they had been swept by fire and often associated with droplets of ooze (exudates) on the surface of infected tissues (Figure 1). Necrotic lesions and cankers developed on woody tissues are also observed and are the sites where *E. amylovora* overwinters (Thomson, 2000). *E. amylovora* enters into the host plant via natural openings, such as the stigmas and nectarthodes of flowers, stomata, and hydathodes, or wounds. The pathogen begins to multiply in the intercellular spaces and is capable of rapid movement within plants and the establishment of systemic infections (Vanneste, 2000).

Figure 1. Fire blight symptoms on blossoms (A), vegetative shoot (B), and woody tissue (C) of pear tree. Images are courtesy of E. Montesinos (A and C) and J. Francés (B).

1.2 Bacterial canker of kiwifruit

Bacterial canker of kiwifruit is an emerging plant disease that has become one of the most serious limiting factors for kiwifruit production, causing substantial economic losses in worldwide cultivated crops (Vanneste *et al*., 2013; Donati *et al*., 2014). The causal agent is *Pseudomonas syringae* pv. *actinidiae* (hereafter, Psa), a Gram-negative bacterium that belongs to the family of *Pseudomonadaceae*. The two most economically important species of green-fleshed and yellow-fleshed kiwifruit in the world, *Actinidia deliciosa* and *Actinidia chinensis*, respectively, are both susceptible to Psa. Bacterial canker of kiwifruit was first reported in Japan in 1984 and was also found, subsequently, in Korea, Italy, and China. Since 2008, when an epidemic outbreak occurred in central Italy, Psa has been spread to a pandemic scale and, to date, Psa is present in several European countries. Moreover, in 2010 the pathogen was reported in New Zealand and Chile for the first time (Janse, 2012; EPPO, 2018a). Spain is considered as free of Psa, except for certain zones in Galicia and Asturias where Psa was first detected in 2011 and 2013, respectively, and outbreaks are under eradication (MAPAMA, 2018). The main symptoms include dark angular necrotic spots on leaves, often accompanied by a yellow chlorotic halo, and shoot wilting and dieback in addition to exudates from cankers on woody plant tissues (Froud *et al*., 2015) (Figure 2). Psa can enter into the host plant through stomata, flowers, leaf and fruit abscission scars, and wounds. Consequently, the pathogen can systemically invade the plant. When conditions are favourable for development of the disease, Psa can kill a kiwifruit vine within a few months (Vanneste *et al*., 2013; Donati *et al*., 2014).

Figure 2. Bacterial canker symptoms on leaf (A), shoots (B), and woody tissue (C) of kiwifruit. Images are courtesy of L. Montesinos (A) and I. Donati (B and C).

1.3 Bacterial spot of stone fruits

Bacterial spot produces a negative economic impact in the major producing areas of stone fruits and almond due to the decrease in quality and marketability of fruits, a reduction of orchard productivity, and an increase in costs of nursery productions (Palacio-Bielsa *et al*., 2010; Janse, 2012). The causal agent is *Xanthomonas arboricola* pv. *pruni* (hereafter, Xap), a Gram-negative bacterium that belongs to the family of *Xanthomonadaceae*. Xap affects a wide range of *Prunus* species including fruit crops, such as plum (*P. domestica*), nectarine, peach (*P. persica*), apricot (*P. armeniaca*), cherry (*P. avium*), almond (*P. dulcis*), and their hybrids, and ornamental species, such as cherry laurel (*P. laurocerasus*) (EPPO, 2018a). Bacterial spot of stone fruits was first described in the USA in 1903 on Japanese plum. Since then, the disease has been reported throughout the five continents and today it is distributed in the major stone-fruit-producing areas of the world. In Spain, Xap is present with a restricted distribution and outbreaks are under eradication (EPPO, 2018a). The main symptoms can be observed on leaves, fruits, twigs and branches, despite differences among the affected species. Symptoms include necrotic angular spots on leaves, twig and branch dieback, spots or sunken lesions on fruits, and stem cankers (Figure 3). Heavy infections lead to severe defoliation resulting in weakened trees (Scortichini, 2010). Infected dormant buds, leaf scars, and cankers are sites where the pathogen overwinters and are the sources of primary inoculum. Xap multiplies epiphytically, penetrates through natural openings (leaf stomata and fruit and twig lenticels) or wounds, and migrates systemically inside the plant (Lamichhane, 2014).

Figure 3. Bacterial spot symptoms on peach fruit (A and C) and leaves (B and C). Images are courtesy of G. Morales.

1.4 Angular leaf spot of strawberry

Angular leaf spot of strawberry is responsible for significant losses in strawberry fruit and nursery-plant production. The causal agent is *Xanthomonas fragariae* (hereafter, Xf), a Gram-negative bacterium that belongs to the family of *Xanthomonadaceae*. The predominant variety of cultivated strawberry, *Fragaria* x *ananassa*, is the primary host of *X. fragariae*. Angular leaf spot of strawberry was first reported in 1962 in the USA and, nowadays, it is widespread in most strawberry growing areas in Europe (Austria, Bulgaria, France, Spain, Germany, Portugal, Italy, Switzerland, Netherlands, Belgium, Finland), Asia (Taiwan, Iran), Africa (Ethiopia), South America (Argentina, Brazil, Paraguay, Uruguay, Venezuela) and North America (USA, Canada, Mexico) (Kim *et al*., 2016; EPPO, 2018a). *X. fragariae* is widespread in nurseries and is readily transmitted via asymptomatic planting stock with latent infection in fruit production fields. In Spain, that is the main European producer of fresh strawberries, *X. fragariae* is present with restricted distribution in producer regions (Andalucía) but it has the potential to establish there (EPPO, 2018a). Disease symptoms first appear on the abaxial side of the leaves as light green, angular, water-soaked spots and turn into reddish brown spots being visible on the adaxial side of the leaves (Figure 4). In addition, bacterial exudates and blight symptoms on pedicels, petioles and calyxes may appear (Kim *et al*., 2016). *X. fragariae* can easily invade leaves via the stomata and start to multiply through the intercellular spaces of parenchyma (Kastelein *et al*., 2014). The disease may become systemic due to pathogen colonization, causing collapse of the plant.

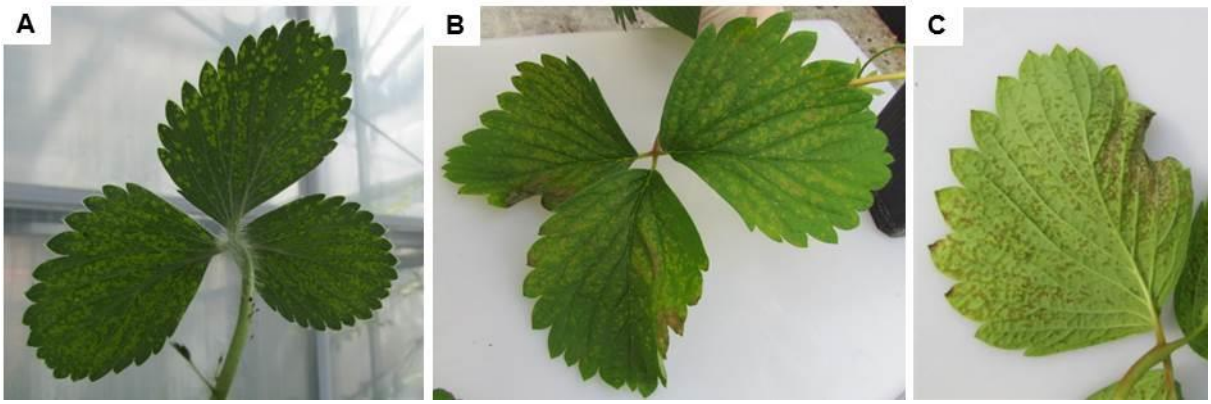


Figure 4. Angular leaf spot symptoms on abaxial (A and C) and adaxial (B) sides of strawberry leaves. Images obtained by N. Daranas.

2 Integrated management of bacterial plant diseases

The attainment of an effective management of the bacterial plant diseases described above is extremely difficult and a comprehensive understanding of the bacterial pathosystems is required. Since curative treatments are not available, the preventive crop protection strategy is preferable to avoid the arrival and the spread of the pathogen (Ea, Psa, Xap, and Xf) into the host plants (Norelli *et al*., 2003; Stefani, 2010; Donati *et al*., 2014; Luiz *et al*., 2017). An integrated management approach, including breeding of resistant or the least susceptible cultivars, cultural practices, and intervention with chemical and/or biological control with the support of plant disease forecasting models, typically represents the best strategy for effective and sustainable disease management (Sundin *et al*., 2016).

Firstly, adequate legislation is essential to ensure the use of healthy planting material and avoid the introduction and dissemination of the quarantine bacterial pathogens to new geographical locations. The phytosanitary passport is required to import to the EU plant material that can be host of Ea, Xap, and Xf, which are included as harmful organisms in the Council Directive 2000/29/EC. Besides, specific regions within the EU are designated as protected zones with the aim of maintaining them as free from Ea and Xap (Commission Implementing Regulation (EU) 2016/873).

Suitable agronomic practices are indispensable as protective measures to reduce the infection risk, the pathogen inoculum, and the incidence and severity of infections, as well as, to control the disease spreading. Some of the most important interventions are focused on the removal of infected plants, balanced fertilization, appropriate pruning and irrigation practices, and breeding of resistant or tolerant cultivars (Norelli *et al*., 2003; Pérez-Jiménez *et al*., 2012; Cameron & Sarojini, 2014; Lamichhane, 2014).

Control strategies based on preventive applications of chemical pesticides, such as copper formulations (copper hydroxide, copper oxychloride, copper sulphate) and antibiotics (streptomycin, oxytetracycline) have been traditionally applied to satisfactorily suppress epiphytic populations of Ea, Psa, Xap, and Xf in the field (Roberts *et al*., 1997; Norelli *et al*., 2003; Cameron & Sarojini, 2014; Lamichhane, 2014). However, the use of both copper and antibiotics faces problems of phytotoxicity, selection of resistant populations of plant pathogens, and environmental and food contamination (Roberts *et al*., 1997; Lalancette & Mcfarland, 2007; Cameron & Sarojini, 2014). In addition, copper induces the viable-but-nonculturable (VBNC) state in several plant-pathogenic bacteria, such as *E. amylovora*, which can maintain pathogenicity, as a survival strategy under adverse environmental conditions (Ordax *et al*., 2006). Besides, the use of antibiotics is not allowed for regulatory reasons in the EU because of the potential impact on the transfer of antibiotic resistance into clinical pathogens. With a greater concern on these negative effects, trends in crop protection have been oriented towards a reduction of reliance on conventional pesticides together with the compulsory implementation of integrated pest

management (IPM) principles in the EU (Directive 2009/128/EC). IPM is a decision-based process that involves coordinated use of multiple tactics for optimizing the management of all classes of pests in an ecologically responsible and an economically sound manner (Lamichhane *et al*., 2016). Consequently, the interest in effective and sustainable alternative strategies to conventional pesticides has increased to manage these quarantine fruit crop diseases.

An alternative approach to manage plant diseases is the use of novel compounds that have been described as elicitors of induced resistance in the host plant or as plant growth regulators, decreasing plant susceptibility to pathogen infections. Prohexadione-Ca, acibenzolar-S-methyl, and harpin have been reported as effective compounds to be included in the fire blight management strategy (Costa *et al*., 2006; Bastas & Maden, 2007; Balajoo *et al*., 2012), whilst chitosan and acibenzolar-S-methyl are potential tools for the management of bacterial canker of kiwifruit (Ferrante & Scortichini, 2010; Cellini *et al*., 2014). Synthetic antimicrobial peptides (AMP) have also been distinguished as prospective candidates of new pesticides since they may be active against a wide range of fungal and bacterial plant pathogens (Montesinos, 2007; Montesinos *et al*., 2012). Bactericidal activity of promising AMP against Ea and Psa has been reported (Badosa *et al*., 2007; Cameron *et al*., 2014; Cabrefiga & Montesinos, 2017). Recently, the application of plant endogenous peptides, such as plant elicitor peptides (Peps) has also been described to trigger plant defence response in *Prunus*-Xap pathosystem (Ruiz *et al*., 2018).

Biological control constitutes a worthy tactic though the use of biopesticides in the integrated management of bacterial plant diseases. In this context, biopesticides contain biological control agents (BCA), generally including beneficial microorganisms and bioactive compounds derived from their metabolism, as well as, natural products, such as byproducts/extracts from plants or animals, as active ingredients to supress bacterial pathogens (Chandler *et al*., 2011; Sundin *et al*., 2016; Montesinos & Bonaterra, 2017).

In addition, the use of reliable disease forecasting models should be taken into account to implement an effective control strategy (Llorente & Montesinos, 2013). They consist of a support system to guide the applications of pesticides or alternative plant protection products to ensure efficient disease management avoiding unnecessary sprays. These models are usually disease-specific and based on information about weather, crop, and pathogen. For example, Maryblyt (Lightner & Steiner, 1992) and Cougarblight (Smith, 1993) were developed for the fire blight risk assessment. Whereas forecasting systems for the bacterial canker of kiwifruit and the bacterial spot of stone fruits have been recently developed (Beresford *et al*., 2017; Morales *et al*., 2017, 2018).

3 Microbial biopesticides

Microbial biopesticides are made from BCA that include beneficial microorganisms or the compounds derived from their metabolism as active ingredients capable of suppressing population density or impact of a specific pest organism or pathogen (Glare *et al*., 2012). Specifically in protection of plant diseases, the biological control mechanism of microorganisms may be sustained by direct and indirect interactions with the pathogen. Direct interactions include (i) competition for nutrients or space, (ii) antagonism through production of antimicrobial compounds or lytic enzymes, (iii) parasitism, and (iv) interference with pathogen signals. Whereas indirect interaction is related to the induction of defence responses in plants towards the pathogen (Alabouvette *et al*., 2006; Bonaterra *et al*., 2012).

Microbial biopesticides have a range of attractive properties that favour their use in crop protection. They show multiple modes of action, which differ from those of conventional pesticides, since beneficial microorganisms may exhibit a combination of antagonistic mechanisms (Bonaterra *et al*., 2012). Therefore, there is less chance of resistance selection in a particular pathogen. In addition, microbial biopesticides usually have no pre-harvest interval and can be applied shortly before harvest (Kiewnick, 2007). Although microbial biopesticides generally have a narrow spectrum of activity that contributes to limit their impact on non-target organisms, this feature is commonly perceived as a disadvantage for placing the product on the market due to their low profit potential (Glare *et al*., 2012; Villaverde *et al*., 2014). Moreover, inconsistency in efficacy between trials or limited biocontrol achieved under field conditions due to the influences of biotic and abiotic factors is also a drawback (Alabouvette *et al*., 2006; Bonaterra *et al*., 2012). In this context, microbial biopesticides may be not appropriate for being used as stand-alone treatments and, therefore, their incorporation into an integrated programme can result in the achievement of the desired control level, whilst reducing the application of conventional pesticides (Spadaro & Gullino, 2005; Alabouvette *et al*., 2006).

During the last decades, many strains of microorganisms, including bacteria, yeasts and fungi, have been widely demonstrated as active in the control of different plant pathogens, such as bacteria and fungi causing aerial and root diseases (Bonaterra *et al*., 2012; Montesinos & Bonaterra, 2017), or effective against postharvest diseases (Nunes, 2012; Montesinos *et al*., 2015). Regarding fire blight management, several bacterial strains of *Bacillus* spp. (Aldwinckle *et al*., 2002; Broggini *et al*., 2005), *Pantoea* spp. (Ishimaru, 1988; Vanneste *et al*., 2002), and *Pseudomonas* spp. (Wilson & Lindow, 1993; Cabrefiga *et al*., 2007) have been selected and deeply characterized as BCA because of their ability to prevent or suppress the progress of the disease. While little is known about antagonistic microorganisms able to manage angular leaf spot of strawberry, bacterial canker of kiwifruit and bacterial spot of stone fruits since few studies have been reported so far (Biondi *et al*., 2009; Kawaguchi *et al*., 2014; Henry *et al*., 2016; Wicaksono *et al*., 2018).

The availability of microbial biopesticides for agricultural use in the EU market is limited in comparison with other countries (e.g. USA) due to the requirements of the legislation to register them (Montesinos, 2003). According to the EU Pesticides Database (European Commission, 2018), currently there are 44 strains, which belong to 21 species of microorganisms, approved as active substances as fungicide/bactericide for plant disease management, while five microorganisms are in pending status (Table 2).

Table 2. Biological control agents approved as active ingredients of microbial biopesticides for plant disease management in the European Union (updated at June 2018) (European Commission, 2018; EPPO, 2018b).

Active ingredient	Commercial name	Category [a]
Bacteria		
Bacillus amyloliquefaciens AH2 [b]	-	FU
B. amyloliquefaciens FZB24	Taegro	FU
B. amyloliquefaciens IT-45 [b]	-	FU
B. amyloliquefaciens MBI 600	Serifel, Subtilex	FU
B. amyloliquefaciens subsp. *plantarum* D747	Double Nickel, Triathlon, AmyloX	FU
Bacillus pumilus QST 2808	Sonata, Ballad	FU
Bacillus subtilis IAB/BS03 [b]	-	FU
B. subtilis QST 713	Serenade, Rhapsody, Cease	FU, BA
Pseudomonas chlororaphis MA342	Cedomon, Cerall	FU
Pseudomonas sp. DSMZ 13134	Proradix	FU
Streptomyces K61 (formerly *S. griseoviridis*)	Mycostop	FU
Streptomyces lydicus WYEC 108	Actinovate, Actino-Iron	FU, BA
Fungi		
Ampelomyces quisqualis AQ10	AQ-10	FU
Coniothyrium minitans CON/M/91-08 (DSM 9660)	Contans	FU
Fusarium sp. L13 [b]	-	FU
Gliocladium catenulatum J1446	Prestop	FU
Phlebiopsis gigantea (14 strains)	Rotstop	FU
Pythium oligandrum M1	Polyversum	FU
Trichoderma asperellum (formerly *T. harzianum*) ICC012, T25 and TV1	Bioten, Tusal, Tenet	FU
T. asperellum T34	Asperello	FU
Trichoderma atroviride (formerly *T. harzianum*) IMI 206040 and T11	Binab-T, Tusal	FU
T. atroviride I-1237	Tri-Soil, Esquive	FU
T. atroviride SC1	Vintec	FU
Trichoderma gamsii (formerly *T. viride*) ICC080	Tenet, Bioten	FU
Trichoderma harzianum T-22 and ITEM 908	Trianum-P, Rootshield, Plantshield	FU
Trichoderma polysporum IMI 206039	BinabT	FU
Verticillium albo-atrum (formerly *V. dahliae*) WCS850	Dutch Trig	FU
Yeast		
Aureobasidium pullulans DSM 14940 and DSM 14941	Blossom protect, Botector	FU, BA
Candida oleophila O	Nexy	FU
Metschnikowia fructicola [b]	-	FU
Saccharomyces cerevisiae LAS02	-	FU

[a] Category: FU, fungicide; BA, bactericide
[b] Pending approval

4 Development process of a microbial biopesticide

The discovery and development of a microbial biopesticide is a complex process that involves several steps and ends up with the registration procedure for its commercial use (Figure 5): (i) selection of candidate antagonistic microorganisms, (ii) identification and characterization of strains, (iii) assessment of mechanisms of action and the biocontrol efficacy in pilot tests, (iv) improvement of the competitiveness in the plant, (v) definition of mass production and formulation systems, (vi) development of specific monitoring methods, and (vii) biosafety and environmental impact studies.

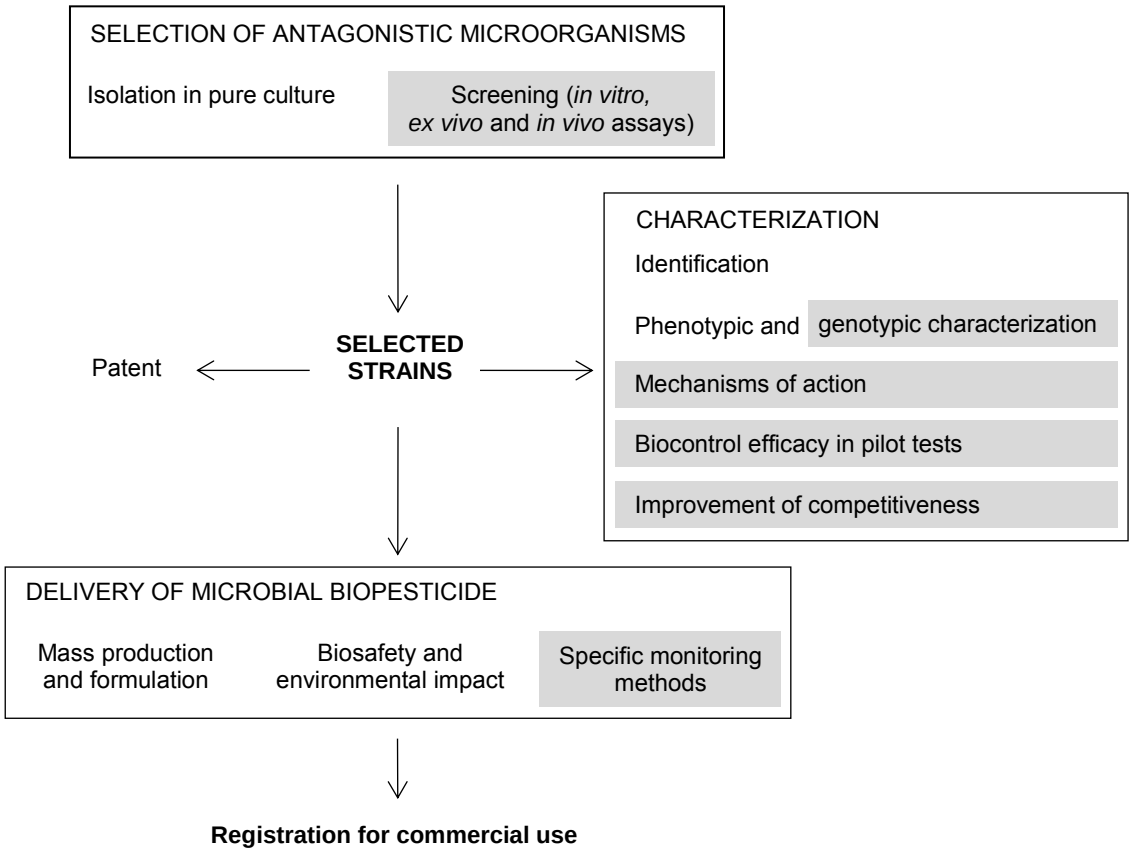


Figure 5. Scheme of the steps required for the development of a microbial biopesticide. Grey squares highlight the steps that have been tackled in this Ph.D. Thesis. Adapted from Montesinos & Bonaterra (2017).

The selection procedure consists of isolation and screening of microorganisms capable of supressing the targeted plant pathogens and reducing the disease levels.

Proper sampling at adequate niches can increase the probability to obtain candidate microorganisms for biological control (Montesinos & Bonaterra, 2017). For instance, samples may be taken from plant materials where there is evidence of the presence of beneficial microorganisms, such as healthy plants that survived the disease in epidemic areas (Montesinos,

2003), or near pathogen infection sites (Handelsman *et al*., 1990). In addition, antagonists may be isolated from samples obtained from the same natural environment where they will be introduced for the disease management to ensure their ecological adaptation. A careful choice of the isolation technique using enrichment approach and selective or differential media allows for the successful isolation of the specific microbial group of interest. Microorganisms with potential antagonistic properties and categorized as safe are highly appreciated for the development of microbial biopesticides. The isolation of hundreds of candidates is recommended since the presence of microorganisms with high antagonistic activity is relatively rare in the environment and it is restricted to the strain level (Montesinos, 2003).

The screening is a critical step because the type of microorganism selected depends on the method used (Montesinos, 2003). Screening procedures are usually specific to a given mechanism of action, such as antibiosis, competition or induction of plant defence (Montesinos & Bonaterra, 2017). Therefore, it is expected that only a portion of the antagonistic microbiota will be selected (Pliego *et al*., 2011). Efficacy bioassays are generally performed by means of *in vitro*, *ex vivo*, or *in planta* tests in a controlled laboratory environment to select isolates with biocontrol potential against targeted pathogens. Additionally, the use of molecular markers to prospect specifically antagonists by means of the specific detection of genes involved in the synthesis of secondary metabolites with antimicrobial properties seems to be a good strategy to increase the efficiency of screening procedures (Mora *et al*., 2011, 2015; Montesinos & Bonaterra, 2017). In a first screening round *in vitro* assays allow for a rapid throughput with clear discriminatory results. However, the selection criterion of *in vitro* screening method is only based on pathogen growth inhibition by means of the production of antimicrobial compounds as mode of action and may exclude the discovery of antagonists that control plant pathogens through other mechanisms. An appropriate screening procedure is based on *ex vivo* and *in planta* bioassays since the interaction between the host plant, the pathogen and the antagonist is taken into account, as well as, different mechanisms of action related to biological control (Köhl *et al*., 2011; Pliego *et al*., 2011). The multi-pathogen approach is recommended to select strains with broad spectrum of activity (Roberts *et al*., 2005; Suárez-Estrella *et al*., 2013; Haidar *et al*., 2016). Besides antagonistic efficacy, other relevant characteristics of the candidates related to suitability for commercial production and registration should be taken into account to guide the selection of attractive candidate antagonists (Köhl *et al*., 2011).

Once candidate strains are selected, it is necessary to proceed to their identification and characterization by phenotypic and genotypic analysis. The mechanisms involved in their biological control activity should also be determined. All this information is not only required for the registration and patenting of the potential antagonistic strains, but also for improving their efficacy. Understanding the mechanisms of action is also critical for the eventual improvement and effective use of the microbial biopesticide as a component of IPM strategies. In addition, since the relative dose of pathogen and BCA is an important factor determining the efficacy and consistency of

biological control, *in planta* bioassays under controlled environment make possible to evaluate disease management over a wide range of pathogen and BCA densities. Dose–response models have been developed to obtain quantitative parameters that describe the efficacy of the BCA, allowing for the comparison of different BCA and pathosystems (Montesinos & Bonaterra, 1996; Bonaterra *et al*., 2003; Francés *et al*., 2003; Trias *et al*., 2008a). Besides, these parameters may give information on the dose range of the BCA needed to provide reliable, economical biological control.

Before an antagonistic strain is seriously considered for the microbial biopesticide development, pilot trials (greenhouse and field bioassays) must be conducted in several pathosystems and under diverse environmental conditions to ensure a wide range of applicability, as well as, consistency in efficacy under real conditions (Montesinos, 2003).

One of the major limitations of microbial BCA is the inconsistent performance under field conditions due to biotic (host plant species, nutritional status, pathogen) and abiotic (temperature, relative humidity) factors. Fluctuations in water availability and temperature on aerial plant surfaces greatly affect microbial epiphytic life (Montesinos & Bonaterra, 2017). Therefore, improving the competitiveness of BCA to colonize and survive in the plant environment is a key step of the development process to improve stability and biocontrol efficacy of microbial biopesticides in the field (Bonaterra *et al*., 2012).

For the commercial launch of a microbial biopesticide, suitable and cost-effective mass production at the industrial scale system (solid or liquid fermentation) have to be carefully developed to obtain the greatest amount of efficacious cells in the shortest period of time and to optimize the production of bioactive metabolites (Nunes, 2012). Subsequently, developing an appropriate formulation (dry or liquid) is fundamental to increase shelf-life, improve delivery, enhance the persistence in the field, and maintain the biocontrol efficacy (Hynes & Boyetchko, 2006; Segarra *et al*., 2015). Thus, the use of protective additives compatible with the BCA is common and they can be incorporated at different points of the production-formulation process.

Biosafety studies have to be undertaken to guarantee the lack of adverse effects of the active ingredient and the formulated product in plants and animals, including humans. It is also required to perform risk assessment studies on traceability, residue analysis, and environmental impact (Montesinos, 2003). Thus, the development of reliable monitoring methods that accurately identify the released microorganism at strain level and track its population dynamics over time is a registration requirement (Bonaterra *et al*., 2012). These methods are useful for monitoring the fate and behaviour of a released strain in the environment and for the quality control during production and formulation of the microbial biopesticide.

Finally, for placing the microbial biopesticide on the EU market, the active substance (i.e. antagonistic strain) needs to be approved at EU level and the formulated product must be authorized at member state level (Regulation (EC) No 1107/2009).

5 Lactic acid bacteria as biological control agents

Lactic acid bacteria (LAB) are indigenous to food-related habitats, including plants (fruits, vegetables, and cereal grains), wine, milk, and meat (Di Cagno *et al*., 2008; Crowley *et al*., 2013). They are also part of the normal healthy flora of humans and animals readily found in the oral cavity and the intestinal tract. In addition, LAB are ubiquitous members of many plant microbiomes and are found in the phyllosphere, endosphere and rhizosphere of many plants (Fhoula *et al*., 2013; Shade *et al*., 2013; Minervini *et al*., 2015).

The term 'lactic acid bacteria' (LAB) describes a group of Gram-positive bacteria that share metabolic and physiological characteristics (Gänzle, 2015). LAB are non-spore forming, non-motile, catalase-negative, devoid of cytochromes, anaerobic aero-tolerant, acid-resistant, with cocci or rod shape. Typically, LAB are mesophilic bacteria, but they are also able to grow at temperatures ranging from 5 to 45 ºC. These bacteria produce lactic acid as the major end product of sugar fermentation (Stiles & Holzapfel, 1997; Holzapfel & Wood, 2014).

Two pathways for carbohydrate fermentation are employed by LAB species: the homofermentative pathway (glycolysis, Embden-Meyerhof-Parnas pathway) in which lactic acid is the primary product or the heterofermentative pathway (6-phosphogluconate/phosphoketolase pathway) in which many other metabolites, such as organic acids, carbon dioxide, acetate, ethanol and aromatic compounds are produced in addition to lactic acid (Stiles & Holzapfel, 1997; Gänzle, 2015).

This group of bacteria belongs to the phylum *Firmicutes* that encompasses 11 genera: *Lactobacillus* (by far the most investigated genus)*, Lactococcus, Streptococcus, Enterococcus*, *Pediococcus, Leuconostoc*, *Oenococcus, Tetragenococcus, Carnobacterium*, *Vagococcus and Weissella* (Stiles & Holzapfel, 1997; Papadimitriou *et al*., 2016).

The genus *Lactobacillus* is the largest group of LAB and comprises more than 150 different species (Siezen *et al*., 2010; da Silva Sabo *et al*., 2014). Among them, *Lactobacillus plantarum* (Figure 6) can be found in different ecological niches, both in fermented foods and plant material, as well as, in gastro-intestinal tract of human and animals (Siezen *et al*., 2010). *L. plantarum* has traditionally been used as starter cultures in food fermentations and some strains have also been highlighted as probiotic bacteria.

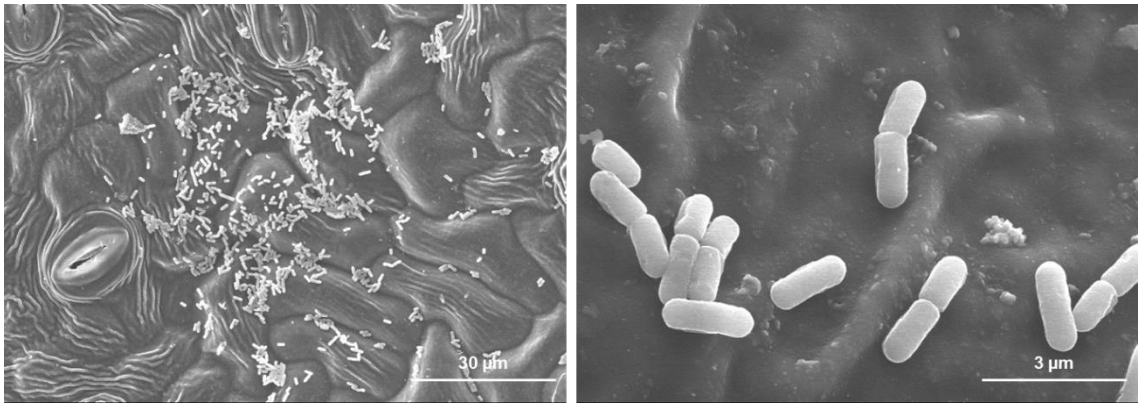


Figure 6. Scanning electron micrographs of *Lactobacillus plantarum* TC92 inoculated on *Prunus* sp. (rootstock GF-677) leaves. Images obtained by N. Daranas.

LAB are widely used as starter cultures in the manufacture of fermented foods for the dairy, meat, vegetable, beverage, and baking industries, as well as, in silage for animal feed. In the fermentation process, LAB not only contribute to food preservation, but also the development of organoleptic properties.

The interest in LAB as food biopreservatives to increase food safety is due to their antimicrobial activity. Moreover, LAB represent ideal candidates for commercial exploitation since some species/strains are designated with the Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) status by the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), respectively. Several studies have documented the efficacy of particular LAB strains as biopreservative agents to prevent spoilage and to extend the shelf life of a wide range of food (Table 3). These reported strains are able to inhibit the growth of foodborne bacterial pathogens, postharvest fungi and mycotoxigenic moulds in fruits, vegetables, cheese, bread, meat, and fish.

Table 3. Selected lactic acid bacteria strains as food biopreservative agents

Species	Strain	Isolated from	Antagonism *in vitro*	Food/Pathogen	Proposed mechanism	Reference
Lactobacillus plantarum	UG1	Dry sausage	*Listeria monocytogenes* *Bacillus cereus* *Clostridium perfringens* *Clostridium sporogenes*	N/A	Plantaricin UG1	Enan *et al*., 1996
Lactobacillus curvatus	ET30	Cold-smoked salmon (CSS)	N/A	CSS / *Listeria innocua*	Bacteriocin	Tomé *et al*., 2008
Lactobacillus delbrueckii	ET32					
Enterococcus faecium	ET05					
Leuconostoc mesenteroides	CM160 CM135 PM249	Fresh fruit and vegetables	*L. monocytogenes*	Apple and lettuce / *Salmonella typhimurium* *L. monocytogenes*	Organic acids, hydrogen peroxide, bacteriocins	Trias *et al*., 2008a, 2008b
Weissella cibaria	TM128					
W. cibaria	TM128	Fresh fruit and vegetables	N/A	Apple / *Penicillium expansum*	Organic acids	Trias *et al*., 2008c
Lactococcus lactis	FF441					
L. plantarum	TC97					
Leuconostoc citreum	TM319					
L. mesenteroides	AC318					
L. plantarum	S2, S18, S11	Fresh vegetables	*Staphylococcus aureus* *Salmonella typhimurium* *Escherichia coli*	N/A	None	Darsanaki *et al*., 2012
Lactobacillus casei	S7, S13					
Lactobacillus brevis	S22					
Pediococcus pentosaceus	54	Cheese	*P. expansum* *Penicillium digitatum* *Penicillium notatum* *Penicillium roqueforti* *Rhizopus stolonifer* *Fusarium culmorum* *Aspergillus fumigatus* *Rhodotorula mucilaginosa*	Pear / *P. expansum*	None	Crowley *et al*., 2013

Table 3. (continued)

Species	Strain	Isolated from	Antagonism *in vitro*	Food/Pathogen	Proposed mechanism	Reference
Pediococcus acidilactici *P. pentosaceus* *Lactobacillus sakei*	KTU05-7 KTU05-8 KTU05-10 KTU05-6	Sourdough	*Bacillus* spp. *Pseudomonas* spp. *E. coli* *S. typhimurium* *A. fumigatus* *Aspergillus niger* *Aspergillus versicolor* *F. culmorum* *Penicillium chrysogenum* *P. expansum* *Candida parapsilosis*	Bread / *Bacillus subtilis*	Organic acids, bacteriocins-like inhibitory substances (BLIS)	Cizeikiene *et al*., 2013
Weissella confusa *Enterococcus faecium* *Enterococcus* spp. *L. plantarum*	FS054, FS036 FS106 Several FS119	Rhizosphere of olive trees and desert truffle	*S. aureus* *L. monocytogenes* *P. expansum* *A. niger*	N/A	None	Fhoula *et al*., 2013
L. plantarum	Several (12)	Herbs, fruits and vegetables	*A. versicolor* *Cladosporium herbarum* *Penicillium solitum*	Cottage cheese / *Penicillium commune*	None	Cheong *et al*., 2014
Lactobacillus spp.	Several (13)		*Colletotrichum gloeosporioides* *Botrytis cinerea* *P. expansum* *Aspergillus flavus*		3-phenyllactic acid	Cortés-Zavaleta *et al*., 2014
L. plantarum	PCS20	N/A	*Clostridium* spp.	Pork ground meat / *Clostridium* spp.	None	Di Gioia *et al*., 2016
L. curvatus	BCS35	Fish product	N/A	Megrim fish / *Listeria* spp. (spoilage microbiota)	Bacteriocin	Gómez-Sala *et al*., 2016
Lactococcus piscium and Carnobacterium divergens (mixture of strains)	CNCM I-4031 V41	Fish	N/A	Cooked and peeled shrimp / *L. monocytogenes*	None	Saraoui *et al*., 2017

Some antagonistic LAB strains play also a role to control phytopathogens, such as fungi, oomycetes and bacteria (Table 4). The potential antagonism between plant-associated LAB and some foliar phytopathogenic bacteria was reported for the first time by Visser *et al*. (1986) with the ultimate aim to implement LAB for the biological control of bacterial plant diseases. Thenceforward, the application of LAB has been suggested as a promising alternative for the management of different plant diseases, such as bacterial spot in pepper plants (Shrestha *et al*., 2014), bacterial soft rot in cabbage (Tsuda *et al*., 2016), and fire blight in apple and pear trees (Roselló *et al*., 2013, 2017). Interestingly, some LAB strains are able to inhibit more than one phytopathogen and, therefore, they may allow for a wide range of plant protection (Trias *et al*., 2008c). The main advantage of using LAB as BCA is that they are not perceived as environmental and health hazards. Consequently, LAB would pose no risks for applications in edible crop production, exempting them from costly and time consuming regulatory approval processes. Beside acting as BCA, LAB have also been shown to be effective biofertilizers and biostimulants, improving nutrient availability, alleviating biotic and abiotic stresses, and directly stimulating plant growth (Hamed *et al*., 2011; Shrestha *et al*., 2014; Lamont *et al*., 2017).

Table 4. Selected lactic acid bacteria strains as potential biological control agents of crop plant diseases

Species	Strain	Isolated from	Antagonism *in vitro*	Crop/Pathogen	Proposed mechanism	Reference
Lactobacillus plantarum	L292	Plant surfaces and plant-associated products	*Xanthomonas campestris* *Erwinia carotovora* *Pseudomonas syringae*	Bean plant / *P. syringae*	None	Visser *et al*., 1986
Enterococcus mundtii *L. plantarum* *Leuconostoc mesenteroides*	MC6 TC110 XM360 PM456	Fresh fruit and vegetables	*X. campestris* *E. carotovora* *Monilinia laxa* *Botrytis cinerea*	N/A	Organic acids	Trias *et al*., 2008c
Lactobacillus spp.	KLF01	Rhizosphere of tomato	*Ralstonia solanacearum* *Xanthomonas axonopodis* pv. *citri*	Tomato / *R. solanacearum* Pepper / *X. campestris* pv. *vesicatoria*	None	Shrestha *et al*., 2009, 2014
Unidentified LAB Unidentified LAB	KLC02 KPD03	Unknown Unknown	*Xanthomonas campestris* pv. *vesicatoria* *Erwinia pyrifoliae* *E. carotovora*			
L. plantarum	IMAU10014	Fermented milk (koumiss)	*B. cinerea* *Alternaria solani* *Phytophthora drechsleri* Tucker *Fusarium oxysporum* *Glomerella cingulata*	N/A	Proteinaceous compounds, 3-phenyllactic acid, benzeneacetic acid, 2-propenyl ester	Wang *et al*., 2011, 2012
Weissella confusa *Lactococcus lactis* *L. plantarum* *L. mesenteroides* *Enterococcus durans* *Enterococcus faecium*	Several (11)	Rhizosphere of olive trees and desert truffle	*Pseudomonas savastanoi*	N/A	None	Fhoula *et al*., 2013
Enterococcus spp.	Several	Rhizosphere of olive trees and desert truffle	*B. cinerea* *Verticillium dahliae*	N/A	None	Fhoula *et al*., 2013
L. plantarum	TC92 PM411	Pear and tomato	*Erwinia amylovora* *P. syringae*	Pear and apple trees / *E. amylovora*	Pre-emptive colonization	Roselló *et al*., 2013, 2017
L. plantarum	SLG17	Silage	*Fusarium culmorum* *Fusarium graminearum*	N/A	None	Baffoni *et al*., 2015
L. plantarum	BY	Fermented food	N/A	Chinese cabbage / *Pectobacterium carotovorum* subsp. *carotovorum*	None	Tsuda *et al*., 2016

5.1 Antimicrobial activity of LAB

The success of LAB in preventing the growth and activity of pathogenic bacteria and undesirable spoilage microorganisms is mainly due to a wide diversity of mechanisms of action. They produce one or more antimicrobial metabolites, such as organic acids, carbon dioxide, diacetyl, hydroxide peroxide, and also other proteinaceous compounds like bacteriocins and antifungal peptides (Reis *et al*., 2012). In addition, LAB may exclude pathogens by pre-emptively colonizing plant tissues susceptible to infection (Visser *et al*., 1986; Tsuda *et al*., 2016), by competition for nutrient and space, or by inducing defence responses in plants towards the pathogen (Konappa *et al*., 2016).

The production of organic acids, mainly lactic and acetic, implies a decrease of the environmental pH, which generally restricts growth of both bacteria and fungi. Organic acids diffuse through the cytoplasmic membrane of the target organisms in their hydrophobic undissociated form and then reduce intracellular pH and stop metabolic activities (Dalié *et al*., 2010; Reis *et al*., 2012). Moreover, these acids functions as permeabilizers of the Gram-negative bacterial outer membrane promoting the effects of other antimicrobial substances (Alakomi *et al*., 2000).

Hydrogen peroxide is produced by most LAB in the presence of oxygen and its accumulation due to the inability to produce catalase oxidises the lipid membrane and cellular proteins of the target organisms (Dalié *et al*., 2010; Reis *et al*., 2012)

Bacteriocins constitute a heterogeneous group of ribosomally synthesized antimicrobial peptides released into the extracellular medium that are active against other bacteria, either in the same or related species (narrow spectrum) or across genera (broad spectrum) (Cotter *et al*., 2005). Bacteriocins are produced by both Gram-negative and Gram-positive bacteria, including many strains belonging to different genera of LAB. In Gram-positive bacteria, most bacteriocins are small in size (30-70 amino acids), have cationic properties and kill target cells by destabilizing the integrity of the inner membrane envelope (e.g. pore forming), causing disruption of the membrane-potential and/or leakage of cellular solutes that eventually leads to cell death (Nes *et al*., 2007). For many bacteriocins from Gram-positive bacteria, the inhibitory activity is not directed against only bacteria within the same species as the producer but also against other species and genera (Nes *et al*., 2007). Bacteriocin activity against Gram-negative bacteria has been shown, but, usually, when the integrity of the outer membrane has been previously compromised by specific agents or treatments, such as the use of chelating agents, by treatment with plant essential oils or by physical treatments, such as heating, freezing or high pressure processing (Cotter *et al*., 2005; Prudêncio *et al*., 2015).

Since the first classification of LAB bacteriocins proposed by Klaenhammer (1993), different schemes have been proposed taking it as a basis (Cotter *et al*., 2005; Drider *et al*., 2006; Savadogo *et al*., 2006; Todorov, 2009). Recently, it was proposed a slightly adjusted classification for LAB bacteriocins into three different classes that can accommodate the novel subclasses that

are appearing, based on the biosynthesis mechanism and biological activity (Alvarez-Sieiro *et al*., 2016). Class I includes small (less than 10 kDa) post-translationally modified peptides that are heat-stable and characterized by their content of several unusual amino acids, such as lanthionine and methyl-lanthionine. Among them, nisin, produced by *Lactococcus lactis,* is the best studied (Lubelski *et al*., 2008). Class II comprises small (less than 10 kDa), unmodified and heat-stable peptides classified as pediocin-like bacteriocins (Class IIa), two-peptide bacteriocins (class IIb), leaderless bacteriocins (class IIc), and non-pediocin-like, single-peptide bacteriocins (class IId). Pediocin PA-1 from *Pediococcus acidilactici* (Pucci *et al*., 1988), Lactococcin G from *L. lactis* (Nissen-Meyer *et al*., 1992), and Enterocin L50 from *Enterococcus faecium* (Cintas *et al*., 1998) are the most extensively studied representative pediocin-like bacteriocin, two-peptide bacteriocin, and leaderless bacteriocin, respectively. Class III encompasses unmodified large-molecular-weight (>10 kDa) and heat-labile antimicrobial bacteriocins with bacteriolytic or non-lytic mechanism of action. Zoocin A and helveticin J are one of the best-characterized LAB bacteriolysins and non-lytic bacteriocins, respectively (Joerger & Klaenhammer, 1990; Simmonds *et al*., 1996).

Since several species/strains of LAB possess the GRAS and QPS status for use in foods, the bacteriocins produced by them are generally considered as safe compounds with interesting properties (e.g. stability, antimicrobial activity, lack of toxicity, no flavour alteration). Semi-purified and purified LAB bacteriocins or bacteriocin-producing LAB cultures have been proposed as effective biopreservative agents to increase the shelf-life of fermented and nonfermented foods, whether of animal or vegetable origin, by eliminating or controlling spoilage microorganisms and foodborne pathogens (Cotter *et al*., 2005; Settanni & Corsetti, 2008). The production of bacteriocins by LAB is dependent on a number of factors which need to be considered for the optimization of fermentation process and enhance the bacteriocin production (Abbasiliasi *et al*., 2017). However, only nisin (Nisaplin™, Danisco, E324) and pediocin PA-1 (ALTA2431™, Quest) have been commercialized as food additives so far (Alvarez-Sieiro *et al*., 2016). Although the interest in bacteriocins or bacteriocin-producer strains to control plant pathogenic bacteria and fungi has been increased and several examples have been reported, the strains involved in these studies were not LAB (Pham *et al*., 2004; Parret *et al*., 2005; Hammami *et al*., 2012).

6 Improvement of microbial biopesticides

A challenge in the development of microbial biopesticides is to overcome the inconsistent performance of biological control. The establishment of a suppressive population of the BCA strain on aerial plant surfaces is critical in order to guarantee effective biological control of plant diseases. Colonization and survival of the BCA on the phyllosphere after spray application are greatly dependent on environmental conditions. Aerial plant surfaces show nutrient limitations and are exposed to ultraviolet radiation and changes in water availability and temperature. These conditions are generally considered inhospitable for bacterial growth and may cause significant declines in survival (Lindow & Brandl, 2003). Additionally, during the production process, including dehydration procedures and storage, BCA encounter various severe abiotic stresses, such as desiccation, high temperature and shear forces, which also reduce their viability and subsequent performance (Costa *et al*., 2000; Cabrefiga *et al*., 2014; Stephan *et al*., 2016). Consequently, tolerance to environmental stresses may contribute to the fitness traits of BCA, influencing the survival capacity on aerial plant surfaces and during the mass production and formulation steps (Hagen *et al*., 2009). Therefore, developing strategies to enhance the stress tolerance of BCA is essential for their efficacy and commercial application (Sui *et al*., 2015).

Approaches consisting of physiological adaptation of BCA to stress and improvement of formulation, as well as, genetic manipulation of microorganisms have been reported to improve the performance of microbial biopesticides (Figure 7). The ability of BCA to colonize and survive in the plant environment and, consequently, the efficacy of microbial biopesticides under a wide range of conditions are improved, expanding their spectrum of activity (Montesinos & Bonaterra, 2017).

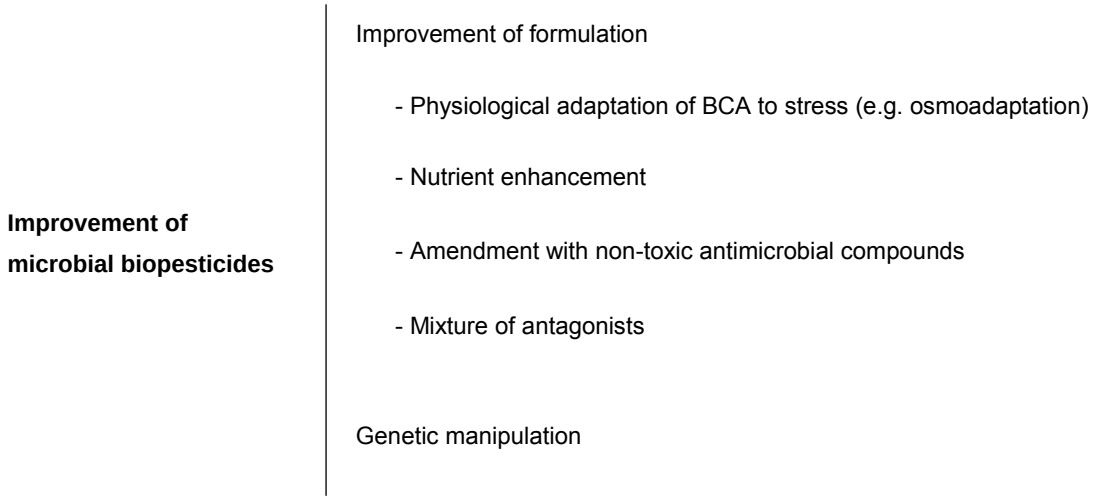


Figure 7. Approaches for the improvement of microbial biopesticides.

The physiological adaptation of BCA (bacteria and yeasts) by osmoadaptation has been reported as an effective approach to improve their behaviour in front of drying process and storage and to enhance epiphytic establishment on plant surfaces. Some examples are: the fire blight biological control agents *Pseudomonas fluorescens* EPS62e (Bonaterra *et al*., 2007; Cabrefiga *et al*., 2011, 2014) and *Pantoea agglomerans* E325 (Pusey & Wend, 2012), the postharvest biological control agents *P. agglomerans* EPS125 (Bonaterra *et al*., 2005), CPA-2 (Teixidó *et al*., 2006; Cañamás *et al*., 2008), and *Candida sake* CPA-1 (Teixidó *et al*., 1998), the *Fusarium* head blight biological control agents *Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 (Palazzini *et al*., 2009), and the *Aspergillus flavus* biological control agents *Kluyveromyces* spp. Y16 and Y25 (Montemarani *et al*., 2014). The osmoadaptation consists of inoculum preparation under osmotic stress conditions that provokes the intracellular accumulation of compatible solutes (osmolytes) to counterbalance the osmotic pressure of the environment and maintain cell turgor. These osmolytes include sugars, amino acids and their derivatives synthesized or taken from the growth medium. Therefore, environmental stress tolerance in microorganisms can be induced by cultivation under suboptimal conditions. Water-stress tolerance is an important component of bacterial fitness and is therefore relevant to understand, predict, and manipulate the ecological success of BCA (Hagen *et al*., 2009; Cabrefiga *et al*., 2011).

Another strategy to increase the fitness of a BCA upon delivery to the field is the nutritional enhancement. Nutrient availability is one of the factors that govern the efficacy of biological control. This strategy has been reported for improving biological control activity of several bacteria and yeasts against fungal postharvest pathogens (Janisiewicz *et al*., 1992; Nunes *et al*., 2001; Guetsky *et al*., 2002; Druvefors *et al*., 2005) and bacterial plant pathogens (Cabrefiga *et al*., 2011). This approach consists of the addition in the formulation specific nutrients that are preferably metabolized by the BCA but not by the pathogen, which results in a stimulation of growth and a better colonization of the antagonist. This method is especially useful for antagonists in which competition for limiting nutritional sources is the major mechanism of biocontrol (Janisiewicz, 1994). However, nutrients have to be carefully chosen for each particular BCA/pathogen system because nutritional enhancement may have unexpected effects, like potentiation of the pathogen activity.

The improvement of formulation of a microbial biopesticide can be achieved by mixing antagonists (bacteria, fungi and yeasts). This approach may increase efficacy and repeatability within experiments, as well as, provide a broad spectrum of protection against soilborne fungal pathogens (Roberts *et al*., 2005; Kim *et al*., 2008; Agustí *et al*., 2011; Yang *et al*., 2015), foliar bacterial pathogens (Roselló *et al*., 2017; Jetiyanon *et al.*, 2003; Jetiyanon & Kloepper, 2002) and postharvest pathogens (Janisiewicz, 1988; Guetsky *et al*., 2001; Calvo *et al*., 2003). An important requisite for designing effective strain mixtures is the use of compatible candidates that complement each other with different mechanisms of action and ecological attributes (Lutz *et al*., 2004; Stockwell *et al*., 2010; Nunes, 2012). The establishment of several BCA jointly may

collectively result in utilization of a greater range of nutrients required by the pathogen (Stockwell *et al*., 2010). Therefore, combination of antagonistic strains may permit more extensive colonization and, therefore, synergistic effect on biological control under a broad range of conditions (Spadaro & Gullino, 2005). However, despite using ecologically compatible antagonistic strains, the mixture may not provide additive or synergistic disease control due to mechanistic incompatibility, whereby the activity of one antagonist interferes with the mechanism of action employed by the other antagonist (Stockwell *et al*., 2010).

The amendment of BCA with non-toxic antimicrobial compounds, such as antimicrobial peptides, bioregulators, organic acids, essential oils, and food additives, is another strategy that has attracted much attention. The combination of two different and complementary modes of action may result in synergistic inhibitory effects. Remarkably improved biological control of plant and postharvest pathogens was reported by combining prohexadione-Ca and *P. agglomerans* P10c (Spinelli *et al*., 2012), salicylic or gibberellic acid and *Cryptococcus laurentii* strain (Yu *et al*., 2006, 2007), lactic acid and *L. plantarum* strains (Roselló *et al*., 2017), essential oils and *Bacillus amyloliquefaciens* or *L. plantarum* strains (Arrebola *et al*., 2010; Zamani-Zadeh *et al*., 2014), and sodium bicarbonate or ammonium molybdate and several antagonistic yeasts (Nunes *et al*., 2002b; Wan *et al*., 2003).

Another possibility to improve the antagonist effectiveness include the genetic manipulation of the BCA (Spadaro & Gullino, 2005). In this context, antagonists can be manipulated by molecular techniques to over-express mechanisms of biological control or foreign genes can be transferred to antagonists to increase tolerance to environmental stresses and to produce antimicrobial substances (Janisiewicz *et al*., 2008). Thus, this approach allows for the introduction of new traits to enhance biological control activity. However, regulation restrictions to release genetically modified organisms (GMO) into the environment have to be taken into account since genetic manipulation is an impediment for registration of a GM-biological control agent.

7 Monitoring of biological control agents

Monitoring population dynamics of microbial BCA once introduced in the environment is necessary. Specific analytical methods at strain level are required since autochthonous wild populations of the same species or genus may be present. Moreover, since the performance of BCA depends on their survival and colonization in the environment, monitoring methods should also be capable of enumerating the viable population fraction. A great number of monitoring methods have been described in the literature to track BCA (bacteria, fungi and yeasts) delivered in both rhizosphere and phyllosphere (Table 5). In all of these approaches, the detection method, which is based on the unambiguous identification of the BCA strain by a specific marker, and the quantification method, which allows for the assessment of its population level, are both defined. Monitoring methods can be divided into culture-based and culture-independent methods (Figure 8). However, a combination of them may be useful to get an in-depth study of the overall BCA population, being able to monitor both culturable, viable and dead cells (Gamalero *et al*., 2003). It is known that BCA may be undergone a large variety of processes following their introduction in the environment, including growth, death, physiological adaptation, and conversion to nonculturable cells (Van Elsas *et al*., 1998).

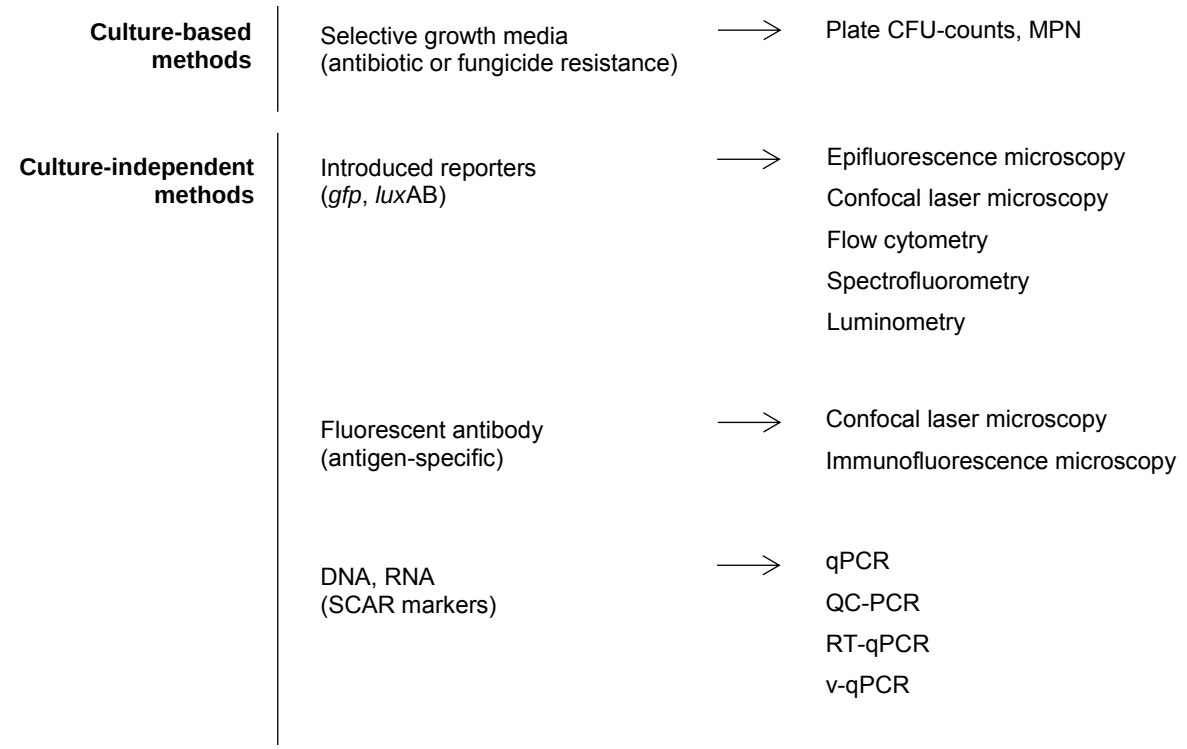


Figure 8. Quantitative analytical methods for monitoring biological control agents at strain level.

Table 5. Monitoring methods used to detect and quantify biological control agents

Quantification method	Detection marker	Biological control strain	Environment	Reference
CULTURE-BASED METHODS				
Plate CFU-counts	Ab-R	*Lactobacillus plantarum* PM411, TC92	apple and pear flowers	Roselló *et al*., 2013
		Pantoea agglomerans C9-1	apple and pear flowers	Johnson *et al*., 2000
		P. agglomerans EPS125	apple surface	Bonaterra *et al*., 2005
		Pseudomonas fluorescens EPS62e	apple and pear leaf, flower and fruit	Bonaterra *et al*., 2007
	Ab-R, Morph	*Pantoea vagans* C9-1 *P. fluorescens* A506	apple and pear flowers	Stockwell *et al*., 2010
	gfp gene	*Bacillus subtilis* ZJY-116 *Brevibacillus brevis* ZJY-1	spikes of barley	Zhang *et al*., 2005
		P. agglomerans C9-1	apple and pear flowers	Spinelli *et al*., 2005
	NYDA medium	*Candida sake* CPA-1	apple surface	Usall *et al*., 2001
	P. agglomerans specific-medium	*P. agglomerans* CPA-2	apple surface	Nunes *et al*., 2002a
CULTURE-INDEPENDENT METHODS				
Cell counting methods				
Immunofluorescence microscopy	fluorescent antigen-specific antibody	*Alcaligenes eutrophus* JMP134	rhizosphere	Kragelund & Nybroe, 1996
Confocal laser microscopy	fluorescent antigen-specific antibody	*P. fluorescens* DF57	rhizosphere	Hansen *et al*., 1997
Fluorescence microscopy, direct fluorescence scanning technique	*gfp* gene	*P. fluorescens* 1100-6	apple surface	Etebarian & Holberg, 2006
Flow cytometry, luminometry	*gfp* and *lux*AB gene	*P. fluorescens* SBW25	soil, wheat plants	Unge & Jansson, 2001
Confocal laser microscopy, epifluorescence microscopy	*gfp* and *gus* gene	*Clonostachys rosea* IK726	soil, rhizosphere	Lübeck *et al*., 2002
Confocal laser microscopy, flow cytometry, epifluorescence microscopy, spectrofluorometry	*gfp* gene	*P. fluorescens* A506	soil, rhizosphere	Tombolini *et al*., 1997; Lowder *et al*., 2000

Table 5. (continued)

Quantification method	Detection marker	Biological control strain	Environment	Reference
PCR-based methods				
QC-PCR	*plhA* gene	*P. fluorescens* CHA0	N/A	Rezzonico *et al*., 2003
qPCR (Scorpion probe)	SCAR marker	*Aureobasidium pullulans* L47	grape and cherry surface	Schena *et al*., 2002
qPCR (Sybr Green I dye)	ITS region	*Cystofilobasidium infirmominiatum* (Cim)	apple, pear and cherry surface	Spotts *et al*., 2009
	SCAR marker	*Epicoccum nigrum* 282	peach surface	Larena & Melgarejo, 2009b
		Pseudomonas brassicacearum MA250	rhizosphere	Holmberg *et al*., 2009
		P. fluorescens CHA0 *P. fluorescens* F113	rhizosphere	Von Felten *et al*., 2010
		T. atroviride T1	soil	Cordier *et al*., 2007
	tetB, *trpE(G)*, *yecA* gene	*B. amyloliquefaciens subsp. plantarum* UCMB5113, UCMB5033, UCMB5036	rhizosphere	Johansson *et al*., 2014
qPCR (TaqMan probe)	*aox1* gene	*T. harzianum* T22	rhizosphere	Horn *et al*., 2016
	dnaX gene	*Pseudomonas* sp. DSMZ 13134	rhizosphere	Mosimann *et al*., 2017
	genomic island	*P. agglomerans* E325	apple flowers	Braun-Kiewnick *et al*., 2012
	SCAR marker	*Candida oleophila* O	apple surface	Massart *et al*., 2005
	SCAR marker	*P. agglomerans* CPA-2	apple surface	Soto-Muñoz *et al*., 2014b
	SCAR marker	*P. fluorescens* EPS62e	apple flowers and leaves	Pujol *et al*., 2006
	SCAR marker	*T. harzianum* 2413	soil	Rubio *et al*., 2005
	yndJ gene	*B. amyloliquefaciens subsp. plantarum* D747, *B. subtilis* QST713	grape-berries surface	Rotolo *et al*., 2016

Table 5. (continued)

Quantification method	Detection marker	Biological control strain	Environment	Reference
qPCR (Sybr Green I dye or TaqMan probe	SCAR marker	*P. fluorescens* Pf153	rhizosphere	Von Felten *et al*., 2010; Mosimann *et al*., 2017
multiplex qPCR (TaqMan probe)	SCAR marker	*Trichoderma polysporum* IMI 206039 and *Trichoderma atroviride* IMI206040	soil	Feng *et al*., 2011
RT-qPCR (TaqMan probe)	ITS region	*T. harzianum* T-78	soil	Beaulieu *et al*., 2011
Viability qPCR (PMA dye)	SCAR marker	*P. agglomerans* CPA-2	orange surface	Soto-Muñoz *et al*., 2014a, 2015a
COMBINED METHODS				
CFU-counts combined with PCR	Ab-R, SCAR marker	*P. agglomerans* CPA-2	orange surface	Nunes *et al*., 2008
	Ab-R, Fu-R, SCAR marker	*Aureobasidium pullulans* Ach 1-1 and 1113-5	apple surface	El Hamouchi *et al*., 2008
	Fu-R, SCAR marker	*Epicoccum nigrum* 282	peach surface	Larena & Melgarejo, 2009b
	Fu-R, Morph, SCAR marker	*P. agglomerans* CPA-2	apple surface	Soto-Muñoz *et al*., 2014b
	Fu-R, ITS region	*Penicillium oxalicum* 212	soil	Larena & Melgarejo, 2009a
	SCAR marker	*B. subtilis* 101	rhizosphere	Felici *et al*., 2008
	Morph, SCAR marker, RBAM 007760 gene	*Bacillus amyloliquefaciens* CPA8	N/A	Gotor-Vila *et al*., 2016, Vilanova *et al*., 2018
	Ab-R, Fu-R, Morph, SCAR marker	*Pichia anomala* K	apple surface	De Clercq *et al*., 2003
CFU-counts or MPN combined with PCR	Ab-R, SCAR marker	*P. fluorescens* EPS62e	pear leaves	Pujol *et al*., 2005
QC-PCR combined with ELOSA	SCAR marker	*P. anomala* K	apple surface	Pujol *et al*., 2004

Ab-R, antibiotic resistance; CFU: colony-forming unit; ELOSA, enzyme-linked oligosorbent assay; Fu-R, fungicide resistance; *gfp,* green fluorescent protein; *gus*, β-glucuronidase gene; ITS, internal transcribed spacer; *lux*AB, luciferase gene; Morph, morphology; MPN, most probable number; PCR, polymerase chain reaction; QC-PCR, quantitative-competitive PCR; qPCR, real-time PCR; RT-qPCR, reverse transcription qPCR; SCAR, sequence characterized amplified region.

7.1 Culture-based methods

Culture-based methods consist of microbial growth on selective or semi-selective media (e.g. supplemented with antibiotics or fungicides) in which only the target strain is able to grow or can be differentiated from other strains (Bonaterra *et al*., 2012). The use of spontaneous mutant derivatives resistant to antibiotics for tracking BCA strains has been reported (Johnson *et al*., 2000; Bonaterra *et al*., 2005, 2007; Stockwell *et al*., 2010; Roselló *et al*., 2013). The population size is estimated by colony forming units (CFU) counting after dilution plating or using the most probable number (MPN) determination. Although these methods have been traditionally used mainly because of their easy performance and low cost, they show some limitations. Culture-based methods lack specificity because non-target microorganisms able to grow on the media with the same antibiotic resistance profile may be present in the environment (Badosa *et al*., 2004) and differentiation between strains regarding colony morphological characteristics is mostly difficult. Moreover, considering that part of the BCA population may enter into viable-but-nonculturable (VBNC) state as an adaptive response to cope with environmental stresses after field release (Oliver, 2005; Pinto *et al*., 2015), culture-based methods may underestimate the effective population size. Consequently, the development of strain-specific and culture-independent monitoring tools has received interest.

7.2 Culture-independent methods

7.2.1 Cell counting methods

Genetic engineering of BCA with exogenous reporter genes that encode enzymes or proteins responsible for bioluminescence or fluorescence has provided useful tools for monitoring several strains in environments (Tombolini *et al*., 1997; Unge *et al*., 1999; Lowder *et al*., 2000; Unge & Jansson, 2001; Lübeck *et al*., 2002; Spinelli *et al*., 2005; Zhang *et al*., 2005; Etebarian & Holberg, 2006; Bloemberg, 2007). The most promising described reporter genes are the *gfp* and *lux*AB genes. The *gfp* gene encodes the green fluorescent protein from jellyfish *Aequorea victoria* (fluorescence marker) and the *lux*AB gene encodes the luciferase enzyme from *Vibrio fischeri* (luminescent marker). The emission of fluorescence is detected from both viable and dead *gfp*-tagged cells, since it has no energy requirement. In contrast, only metabolically active cells tagged with *luxAB* gene are detected, since its gene expression and, consequently, the bioluminescence phenotype is dependent on cellular energy status. Simultaneous quantification of total population and metabolic activity based on the use of a dual *gfp/luxAB* marker system was reported to monitor *P. fluorescens* SBW25 (Unge *et al*., 1999). However, the main drawback is that the use of heterologous genes converts the BCA into a GMO, introducing difficulties for its further registration

and commercialisation in the EU. Therefore, this approach can be only used for monitoring studies under confined conditions.

Immunofluorescence techniques have also been used to track BCA (Kragelund & Nybroe, 1996; Hansen *et al*., 1997). They are based on the use of antigen-specific antibodies tagged with a fluorescent dye to detect the presence of the target strain (antigen). The use of different dyes provides an extremely powerful way to characterize the physiological state, activity or degree of viability of bacteria (Gamalero *et al*., 2003).

The quantification of tagged cells with fluorescent markers is achieved by fluorescence microscopy (epifluorescence and confocal laser microscopy), spectrofluorometry, or flow cytometry. In addition, information regarding the localization and distribution of the BCA *in planta* is obtained by visualization of microbial cells *in situ* (Spinelli *et al*., 2005).

7.2.2 PCR-based methods

Molecular methods based on the detection of nucleic acids by means of polymerase chain reaction (PCR) technique are increasingly used for monitoring BCA without regard to their cultivability. PCR-based methods are rapid, versatile, sensitive, and precise and allow for specific detection and quantification of microorganisms of interest (Martini *et al*., 2015). The possibility to differentiate the target strain from closely related strains of the same species is achieved by detecting a strain-specific region within its genome. Several DNA fingerprinting techniques provide the detection of natural polymorphisms within the target strain genome to obtain molecular markers. These techniques have been generally proved to be suitable since they do not require any prior knowledge of the target strain genome and because of their simplicity and high discrimination power. One of the most widely used techniques is the random amplified polymorphic DNA (RAPD), based on the use of a short single oligonucleotide of a random sequence as a primer for the amplification of genomic DNA under non-restrictive conditions (Williams *et al*., 1990). Other fingerprinting methods include the amplified fragment length polymorphism (AFLP), based on the detection of genomic restriction fragments (Vos *et al*., 1995), and the amplification of repetitive element sequences (rep-PCR), based on using oligonucleotide primers complementary to interspersed repetitive sequences (Versalovic *et al*., 1994). The comparison of fingerprinting patterns of related strains within the same species allows for the discrimination among them. Unique amplified fragments for the target strain are cloned and sequenced to generate sequence-characterized amplified regions (SCAR) in which specific primers can be designed for the specific and reproducible amplification of single DNA fragments. SCAR markers have been commonly used to develop monitoring methods for BCA (Schena *et al*., 2002; De Clercq *et al*., 2003; Massart *et al*., 2005; Pujol *et al*., 2005; Rubio *et al*., 2005; Cordier *et al*., 2007; Felici *et al*., 2008; Nunes *et al*., 2008; Holmberg *et al*., 2009; Larena & Melgarejo, 2009b; Von Felten *et al*., 2010; Feng *et al*., 2011; Gotor-Vila *et al*., 2016). When the target strain genome is available, exploiting sequence

polymorphisms in genes or sequences with known functions have also been reported for strain-specific marker detection in BCA (Johansson *et al*., 2014; Horn *et al*., 2016). Furthermore, genome comparative analysis also yielded genomic islands that probed to be strain-specific (Braun-Kiewnick *et al*., 2012). The use of phage-related sequences as genomic markers have been used to identify bacteria at strain level as well (Brandt & Alatossava, 2003; Coudeyras *et al*., 2008).

Once the strain-specific sequence has been identified, BCA detection in the environment consists of direct DNA extraction from samples followed by PCR with specific primers. However, conventional PCR does not allow accurate quantitative analysis. To overcome this limitation, the combination of plating on semi-selective media and CFU counts with PCR detection using specific primers was reported in order to detect and quantify viable and culturable cells of the target strain (De Clercq *et al*., 2003; Pujol *et al*., 2005; Felici *et al*., 2008; Nunes *et al*., 2008; Larena & Melgarejo, 2009b). Nevertheless, the population size may be underestimated because bacteria can enter in a VBNC state. The emergence of new techniques, such as the quantitative-competitive PCR (QC-PCR) and mainly the real-time PCR (qPCR), allows for the quantification of nucleic acids *in vitro* and the estimation of the population size of the BCA strain.

The QC-PCR is based on the co-amplification of the target DNA with an internal standard (IS) DNA, which competes for the primer binding during amplification. Quantification is achieved by comparing the relative yield of the PCR products amplified from the target and from the IS that should remain constant during the amplification (Rezzonico *et al*., 2003; Pujol *et al*., 2004).

The qPCR is nowadays the most widespread technique for specific detection and enumeration of BCA in different plant environments. The amplification reaction is monitored by detection of fluorescent signal using different chemistries that can be grouped into sequence non-specific (e.g. SYBR Green I dye) and sequence specific systems (e.g. hydrolysis probes, molecular beacons, Scorpion probes) (Schena *et al*., 2004). SYBR Green is an intercalating binding dye that binds to any double-stranded DNA and consequently emits fluorescent signal. Among the amplicon sequence-specific system, the hydrolysis probes, such as TaqMan probe, are the most utilized. TaqMan probe is an oligonucleotide probe fluorescently labelled that hybridises with the target DNA during amplification and its hydrolysis by the 5'-3' exonuclease activity of the DNA polymerase after each PCR cycle causes the emission of fluorescence. The advantage of fluorogenic probes over DNA binding dyes is that specific hybridisation between probe and target DNA sequence is required to generate a fluorescent signal (Schena *et al*., 2004). TaqMan probe is commonly employed for specific BCA strain detection (Massart *et al*., 2005; Rubio *et al*., 2005; Pujol *et al*., 2006; Beaulieu *et al*., 2011; Feng *et al*., 2011; Braun-Kiewnick *et al*., 2012; Soto-Muñoz *et al*., 2014b; Horn *et al*., 2016; Mosimann *et al*., 2017). The fluorescence emitted, which is proportional to the amount of PCR product, is captured during the amplification reaction and is plotted as a curve. The quantification of the population size of the BCA is achieved by interpolating cycle threshold (Ct) values of unknown samples with standard curves obtained after the amplification of serial dilutions of known quantities of the target DNA. The cycle threshold (Ct) is

the number of PCR cycles necessary to generate a fluorescent signal significantly higher than the background signal and is inversely related to the logarithm of the initial amount of target molecules. The biggest drawback of qPCR is the possible overestimation of the active BCA population since this method cannot differentiate between DNA from viable and dead cells, and non-degraded extracellular DNA (Josephson *et al*., 1993; Schena *et al*., 2004).

It is generally accepted that a cell must be intact, capable of reproduction, and metabolically active to be considered alive. A variety of techniques exist to assess the relatively proportions of viable and dead microorganisms in natural environment, which typically address one of the three aspects of microbial viability: (i) the existence of an intact, functional membrane, (ii) the presence of cellular metabolism or energy, or (iii) the possession of self-replicating DNA that can be transcribed into RNA, which if applicable, can subsequently be translated into protein (Emerson *et al*., 2017).

A possible strategy to exclude the detection of dead cells may be addressed by using RNA rather DNA as target molecule. mRNA is only produced by metabolically active cells and is degraded rapidly after cell death, making mRNA suitable to specifically detect viable microorganisms. In order to use RNA as target molecule, a reverse transcription step in which RNA is converted to cDNA is required before qPCR run (Schena *et al*., 2004, 2013). The reverse transcription (RT) coupled to qPCR has been reported for the determination and quantification of active cells of a *Trichoderma harzianum* strain in soil (Beaulieu *et al*., 2011). However, since RNA is less stable than DNA, RNA degradation may occur by inadequate sample manipulation and could lead to false negative or underestimation. In addition, since the expression level of many mRNA species greatly depends on the physiological status of cells (e.g. VBNC state) and environmental conditions, precise quantification may be not achieved.

An alternative approach for the preferential detection and quantification of viable cells is the viability qPCR (v-qPCR). This method relies on the use of nucleic acid intercalating dye as a sample pre-treatment prior to DNA isolation and the qPCR. The v-qPCR has been shown to be useful for quantification of viable foodborne pathogenic microorganisms in different food matrices (Rudi *et al*., 2005; Elizaquível *et al*., 2012; Martin *et al*., 2013; Elizaquível *et al*., 2014; Liu & Mustapha, 2014; Seinige *et al*., 2014), and probiotic and starter strains in milk and dairy products (Desfossés-Foucault *et al*., 2012; Villarreal *et al*., 2013). However, in the biocontrol field of plant pathogens v-qPCR has only been developed to monitor the strain *P. agglomerans* CPA-2 in citrus fruit in postharvest so far (Soto-Muñoz *et al*., 2015a, 2015b). The principle of viable-dead distinction is mainly based on membrane integrity (Fittipaldi *et al*., 2012). By applying a nucleic acid intercalating dye, such as propidium monoazide (PMA) or ethidium monoazide (EMA), to the sample prior to the nucleic acid extraction, the dye penetrate cells with damaged membranes and once inside the cell the dye binds to DNA. Light activation of these DNA-bound molecules results in a covalent linkage preventing PCR amplification of the modified DNA (Nocker *et al*., 2006; Fittipaldi *et al*., 2012; Elizaquível *et al*., 2014). Although EMA was the first reagent described, its

use has been limited since EMA can cross intact cell membranes of some specific species. PMA was later accepted as the best reagent due to high selectivity in penetrating only compromised membranes, possibly due to its higher charge relative to EMA (Nocker *et al*., 2006). However, PMA is unable to avoid PCR amplification of nonviable cells with unaltered membranes. Recently, a new approach, the PEMAX reagent, has been developed to improve the v-qPCR and extend the concept of viability PCR to cells with intact cell membrane structure but also with active metabolism (Codony, 2014; Codony *et al*., 2015; Agustí *et al*., 2017). PEMAX is a double dye technology that combines low levels of EMA (10 μM) with PMA (50 μM). These dyes have different size and charge. The smaller molecule (EMA) has a certain level of permeability in intact cellular membranes, but metabolically active cells are able to throw out this uptake by means of active efflux pumps. The second dye (PMA) is needed to complete dead cell or extracellular DNA neutralization when in the sample exists high amounts of dead cells with damaged cell membranes (Thanh *et al*., 2017). Viability assessment studies for monitoring bacterial pathogens have used the PEMAX reagent in the viability qPCR method (Lizana *et al*., 2017; Thanh *et al*., 2017). After the treatment with PEMAX, DNA from viable cells with intact membrane structure and active metabolism will be free of labelling and then detected and quantified by qPCR. The efficiency of the v-qPCR technique depends on a complex set of parameters that must be set up, such as the dye (type and concentration) and the length of the PCR amplicon (Fittipaldi *et al*., 2012).

8 Context and approach of this Ph.D. Thesis

Trends in crop protection are currently focused on the rational use of pesticides and encouraging the implementation of ecologically friendly alternative strategies. Among them, biological control based on microorganisms is an important component of integrated pest management. In this context, the interest in antagonist microorganisms as new active ingredients of biopesticides has increased in the last years. Currently, the EU has approved a significant number of microbial strains as biopesticides and they account for around a 12% of the total plant protection products.

The Plant Pathology group of the University of Girona started working on the development of innovative and sustainable technologies for the management of plant diseases (e.g. biological control) since 1993. Beneficial bacteria, such as *P. fluorescens* EPS62e and *P. agglomerans* EPS125 have been reported to control plant diseases of economic interest, such as fire blight of apple and pear trees and postharvest fungal rot diseases, respectively (Bonaterra *et al*., 2003; Cabrefiga, 2004). Different aspects of biocontrol have been studied for the development of these strains as BCA, such as the mechanisms of action (Cabrefiga *et al*., 2007), dose-response relationships in biological control processes (Montesinos & Bonaterra, 1996; Francés *et al*., 2006), colonization and traceability (Pujol *et al*., 2005, 2006, 2007) and physiological improvement of strains and optimization of formulations (Bonaterra *et al*., 2005, 2007; Cabrefiga *et al*., 2011, 2014). In the last decade, the research on BCA has been focused on *Bacillus* (Mora *et al*., 2011, 2015) and LAB. From a large collection of LAB strains isolated from plant sources, particular strains have been reported to control foodborne bacterial pathogens and postharvest fungi (Trias *et al.*, 2008a, 2008b, 2008c), and also fire blight of apple and pear (Roselló *et al.*, 2013, 2017). In addition, it has been developed an optimized mixed inoculum of *L. plantarum* strains with a co-formulant to increase the efficacy and consistency of biological control of fire blight.

According to the above mentioned background, the present Ph.D. Thesis is focused on (i) studying the applicability of LAB strains on crop protection to control emerging quarantine plant pathogens, (ii) improving the fitness of the most promising candidates in plant environment, and (iii) developing a method to monitor viable cells of the selected BCA strain in aerial plant surfaces. This Thesis has been presented as a compendium of three scientific papers that correspond to chapters III, IV, and V and are focused on the research performed to achieve the three mentioned goals.

For the screening of candidate BCA with broad-spectrum activity, a set of 55 LAB strains were used. These strains belong to a collection of 539 LAB strains isolated from fresh fruits and vegetables and natural and agricultural plant environments that were previously collected in two surveys (Trias, 2008; Roselló, 2016) by the research group of Plant Pathology of the University of Girona. The 55 strains were selected according to the *in vitro* antibacterial activity against some Gram-positive (*Staphylococcus aureus*, *Leuconostoc mesenteroides*, *B. subtilis*) and Gram-

negative (*Pseudomonas syringae*, *E. amylovora*, *Escherichia coli*) bacteria and the presence of bacteriocin-gene markers, such as *plnEF*, *mes* and *nis* genes encoding plantaricin, mesentericin and nisin, respectively (Roselló, 2016). The use of antimicrobial peptide gene markers has also assisted screening procedures in the selection of *Bacillus* strains as candidate BCA (Mora *et al*., 2011). Since the antimicrobial activity of bacteriocins has been widely reported (Savadogo *et al*., 2006; Alvarez-Sieiro *et al*., 2016), the presence of their biosynthetic genes was considered a valuable criterion of selection. In fact, genetic screening of LAB for bacteriocin-encoding genes has been reported in order to select candidate strains as food biopreservatives (Ben Omar *et al*., 2008; Knoll *et al*., 2008).

CHAPTER II

Objectives

The main objective of this Ph.D. Thesis was to develop a microbial biopesticide based on lactic acid bacteria (LAB) for the biological control of multiple quarantine bacterial plant diseases.

The specific objectives were:

1. Selection and characterization of LAB strains with broad-spectrum activity able to control bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*), bacterial spot of stone fruits (*Xanthomonas arboricola* pv. *pruni*) and angular leaf spot of strawberry (*Xanthomonas fragariae*).

2. Physiological improvement of the fitness of *Lactobacillus plantarum* strains to increase cell survival on aerial plant surfaces and the consistency between experiments of biological control of bacterial plant diseases.

3. Development of a strain-specific viability quantitative PCR method to monitor the viable population level of a *L. plantarum* strain on aerial plant surfaces.

This Thesis is presented as a compendium of three publications that correspond to chapters III, IV, and V and follows the same order as the objectives.

CHAPTER III. Biological control of bacterial plant diseases with *Lactobacillus plantarum* strains selected by their broad-spectrum activity. Submitted to *Annals of Applied Biology* on 2 July 2018.

CHAPTER IV. Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. *PLoS ONE*, **13**, e0190931.

CHAPTER V. Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method. *Applied and Environmental Microbiology*, **84**, e00107-18.

CHAPTER III

Biological control of bacterial plant diseases with *Lactobacillus plantarum* strains selected by their broad-spectrum activity

Daranas N, Roselló G, Cabrefiga J, Donati I, Francés J,

Badosa E, Spinelli F, Montesinos E, Bonaterra A

This manuscript has been submitted on 2 July 2018 for publication in Annals of Applied Biology

Figure 6 and the supporting information Figure S1 have been previously included in the Ph.D. Thesis of Gemma Roselló (Roselló 2016). These figures have not been previously published in any peer-reviewed journal. They have been included in the manuscript submitted to Annals of Applied Biology as part of the contribution of G. Roselló to this study.

ANNALS OF APPLIED BIOLOGY aab

Biological control of bacterial plant diseases with *Lactobacillus plantarum* strains selected by their broad-spectrum activity

Journal:	*Annals of Applied Biology*
Manuscript ID	AAB-2018-0206
Manuscript Type:	Original Article
Date Submitted by the Author:	02-Jul-2018
Complete List of Authors:	Daranas, Núria; Universitat de Girona, Institute of Food and Agricultural Technology Roselló, Gemma; Universitat de Girona, Institute of Food and Agricultural Technology Cabrefiga, Jordi; Universitat de Girona, Institute of Food and Agricultural Technology Donati, Irene; Alma Mater Studiorum -University of Bologna, Department of Agricultural Sciences Francés, Jesús; Universitat de Girona, Institute of Food and Agricultural Technology Badosa, Esther; Universitat de Girona, Institute of Food and Agricultural Technology Spinelli, Francesco; Alma Mater Studiorum -University of Bologna, Department of Agricultural Sciences Montesinos, Emilio; Universitat de Girona, Institute of Food and Agricultural Technology Bonaterra, Anna; Universitat de Girona, Institute of Food and Agricultural Technology
Key Words:	Bacterial Pathogens, Biological Control, Lactobacillus plantarum, Pseudomonas syringae pv. actinidiae, Xanthomonas arboricola pv. pruni, Xanthomonas fragariae

Biological control of bacterial plant diseases with *Lactobacillus plantarum* strains selected by their broad-spectrum activity

Running head: Biological control of bacterial plant diseases with *Lactobacillus plantarum*

N. Daranas[1], G. Roselló[1], J. Cabrefiga[1], I. Donati[2], J. Francés[1], E. Badosa[1], F. Spinelli[2], E. Montesinos[1], A. Bonaterra[1]

[1] Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Girona, Spain

[2] Department of Agricultural and Food Sciences (DISTAL), Alma Mater Studiorum, University of Bologna, Bologna, Italy

Correspondence

A. Bonaterra, Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Girona, Spain. Email: anna.bonaterra@udg.edu

ABSTRACT

Lactic acid bacteria were studied to control multiple pathogens that affect different crops, such as *Pseudomonas syringae* pv. *actinidiae* in kiwifruit, *Xanthomonas arboricola* pv. *pruni* in *Prunus*, and *Xanthomonas fragariae* in strawberry. A screening procedure based on *in vitro* and *in planta* assays against the three bacterial pathogens was successful in selecting potential strains as biological control agents. The antagonistic activity of 55 strains was first tested *in vitr*o and *Lactobacillus plantarum* CC100, PM411, and TC92, and *Leuconostoc mesenteroides* CM160 and CM209 were selected for showing broad-spectrum activity. The biocontrol efficacy of the selected strains was assessed using a multiple-pathosystem approach under greenhouse conditions and *L. plantarum* PM411 and TC92 prevented infections of all three pathogens in their corresponding host plants. In addition, the biocontrol performance of both strains was comparable to reference products in semi-field and field experiments. The *in vitro* inhibitory mechanism of PM411 and TC92 is based, at least in part, on a pH lowering effect and lactic acid production. Moreover, both strains showed similar survival rates on leaf surfaces. Both strains have differential multilocus sequence typing (MLST) and random amplified polymorphic DNA (RAPD) profiles.

KEYWORDS: *Lactobacillus plantarum*, bacterial plant diseases, *Xanthomonas arboricola* pv. *pruni*, *Pseudomonas syringae* pv. *actinidiae*, *Xanthomonas fragariae*

INTRODUCTION

Increased global trade together with climate change and limitations in plant protection products favour the emergence of new plant diseases and their establishment, causing significant crop losses (Yáñez-López *et al*., 2012; Lamichhane *et al*., 2015). Fruit production is threatened by several bacterial plant diseases, such as the bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), the bacterial spot of stone fruits caused by *Xanthomonas arboricola* pv. *pruni* (Xap), and the angular leaf spot of strawberry caused by *Xanthomonas fragariae* (Xf) (Donati *et al*., 2014; Lamichhane, 2014; Kim *et al*., 2016). Psa, Xap and Xf are considered quarantine organisms by the European and Mediterranean Plant Protection Organization (EPPO).

The management of these diseases relies mainly on preventive applications of bactericides containing copper compounds or antibiotics (Cameron & Sarojini, 2014; Lamichhane, 2014). However, the selection of resistant pathogen populations and phytotoxicity are the main drawbacks (McManus *et al*., 2002; Lalancette & Mcfarland, 2007). Overall, there is the need to reduce the reliance on conventional pesticides and to implement an integrated pest management (IPM) framework (Lamichhane *et al*., 2015). The plant defence elicitor acibenzolar-S-methyl (ASM) has been reported as a potential alternative compound for the management strategy of bacterial canker of kiwifruit (Cellini *et al*., 2014) and angular leaf spot of strawberry (Braun & Hildebrand, 2013).

Also, chitosan exhibits antimicrobial activity and acts as an elicitor of plant defence mechanisms, being a potential alternative agent to manage bacterial canker of kiwifruit (Cameron & Sarojini, 2014). Nevertheless, phytotoxicity and a high variability in the host plant response in the field have been reported, raising questions about their feasibility in crop protection (Reglinski *et al*., 2013). Therefore, the interest in selecting beneficial microorganisms for the development of biological control agents (BCA) has increased since microbial biopesticides are a key tool in IPM (Matyjaszczyk, 2015). Strains of bacteria, fungi and viruses are now commercially available to manage plant diseases and pests (Matyjaszczyk, 2015; Montesinos & Bonaterra, 2017). However, the efficacy of biological products may be variable between trials or low under field conditions, due to the influences of biotic and abiotic factors (Sundin *et al*., 2009). These limitations have stimulated the search for novel strains of microorganisms with a wide spectrum of antagonistic activity against plant pathogens. Lactic acid bacteria (LAB) constitute an interesting group often found in the plant-associated microbiome (Trias *et al*., 2008a; Zwielehner *et al*., 2008).

LAB are good candidates for the development of microbial biopesticides because they include some strains categorized as Generally Regarded as Safe (GRAS) by the U.S. Food and Drug Administration (FDA) and as having Qualified Presumption of Safety (QPS) by European Food Safety Authority (EFSA). Besides, many LAB strains show antimicrobial activity due to the production of active metabolites, such as organic acids, bacteriocins and several inhibitory bioactive compounds (Reis *et al*., 2012). LAB have been widely reported as biopreservatives of vegetables and fruits, inhibiting the growth of foodborne bacterial pathogens and spoilage fungi (Trias *et al*., 2008a, 2008b; Crowley *et al*., 2013). In addition, some LAB strains have also been reported as potential BCA against several bacterial plant pathogens (Visser *et al*., 1986; Roselló *et al*., 2013; Tsuda *et al*., 2016).

BCA must be carefully selected since not all species or strains confer plant protection against pathogens. Screening strategies enabling the selection of strains with pathogen suppressive activity include *in vitro* antagonism tests and the assessment of infection prevention in detached plant organs and whole plants (Köhl *et al*., 2011; Roselló *et al*., 2013; Haidar *et al*., 2016). Moreover, the strong commercial interest in LAB has stimulated studies to typify the most promising strains, being the identification and characterization at strain level a requirement for BCA registration. Typing techniques are based on DNA analysis and two of the most used are multilocus sequence typing (MLST) (de las Rivas *et al*., 2006; Tanganurat *et al*., 2009) and random amplified polymorphic DNA (RAPD-PCR) (López *et al*., 2008).

The aims of the present study were: (i) to screen plant-associated LAB by means of *in vitro* tests and select antagonistic strains with broad-spectrum activity against Psa, Xap, and Xf; (ii) to assess the biocontrol efficacy of the selected strains in preventing infections of the three pathogens in potted plants (kiwifruit, *Prunus*, and strawberry) in the greenhouse; (iii) to compare the biocontrol performance of the selected strains to reference products in semi-field and field

experiments; and (iv) to characterize the selected strains regarding the mechanisms involved in the *in vitro* antibacterial activity against Psa, Xap, and Xf and MLST and RAPD-PCR profiling.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 55 LAB isolates from the INTEA-CIDSAV culture collection were selected for this study (Table S1, Supporting information), which were isolated from several plant sources and identified at species level based on 16S rDNA sequences in previous works (Trias *et al*., 2008a, 2008b; Roselló *et al*., 2013). *L. plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* were discriminated using a PCR-based procedure (Chagnaud *et al*., 2001; Torriani *et al*., 2001) (details in Table S1). To monitor culturable populations of *L. plantarum* PM411 and TC92 in plant colonization studies, spontaneous mutants resistant to rifampicin (PM411R and TC92R) were used (Roselló *et al*., 2013).

Pseudomonas syringae pv. *actinidiae* (Psa) CFBP7286 (Collection Française de Bactéries Phytopathogènes, INRA, France), NCPPB3739 (National Collection of Plant Pathogenic Bacteria, Fera, UK), and IVIA 3700-1 (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain); *Xanthomonas arboricola* pv. *pruni* (Xap) CFBP3894 and CFBP5563; and *Xanthomonas fragariae* (Xf) IVIA XF349-9A and CECT549 (Colección Española de Cultivos Tipo, Valencia, Spain) were used as target bacteria in *in vitro* antagonism tests and efficacy assays in whole plants (Table S1). Psa CFBP7286-GFPuv, which is resistant to kanamycin, was used for colonization studies in kiwifruit plants (Spinelli *et al*., 2011).

Cultures were prepared for routine use from isolates preserved at -80ºC. All LAB isolates were grown on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) at 23ºC for 48 h. Psa and Xap were grown on Luria-Bertani (LB) agar at 23ºC for 24 h and Xf was grown on B medium (Hazel & Civerolo, 1980) at 23ºC for 24 h. Bacterial suspensions of LAB and pathogen strains were obtained by scraping colonies from agar surface and resuspending them in sterile distilled water. The suspensions were adjusted to 1-5 x 10^8 CFU mL^{-1} and confirmed by plate counting on the corresponding media.

Plant material and greenhouse conditions

Two-year-old plants and 15- to 30-day-old plantlets of kiwifruit (*Actinidia chinensis* var. *deliciosa* cv. Hayward) were obtained from two commercial nurseries (SolJardí, Jafre, Spain and Vitroplant, Cesena, Italy, respectively). *Prunus amygdalus* × *P. persica* (cv. GF-677) plantlets that were 15 to 30 days old and cold-stored strawberry plants (*Fragaria* × *ananassa* cv. Darselect) were obtained from two commercial nurseries (Agromillora Iberica, Barcelona, Spain and Planasa,

Valtierra, Spain, respectively). All plants were used when uniform growth stage was achieved and maintained in a greenhouse at 26 ± 2ºC, 60 ± 10% of relative humidity (RH) and a 16:8 h light:dark photoperiod. Standard NPK fertilization and irrigation, as well as, insecticide and miticide sprays were applied. In all efficacy assays, inoculated plants with Psa, Xap, or Xf were maintained in a class II greenhouse (quarantine biosafety greenhouse A2 level according to EPPO rules).

In vitro antagonistic activity

The antagonistic activity of 55 LAB isolates was assayed against Psa (NCPPB3739 and IVIA 3700-1), Xap (CFBP3894 and CFBP5563), and Xf (IVIA XF349-9A and CECT549) using *in vitro* tests. The experiment was performed twice by means of two different procedures. One assay was carried out by the agar spot test in lactose-bromocresol purple agar (LBP) as described previously (Trias *et al*., 2008a), with some modifications. Briefly, LAB isolates were spotted on LBP agar plates and incubated 24 h at 23°C. 0.5 mL of the target pathogen suspension at 5 x 10^7 CFU mL^{-1} were mixed in 4.5 mL of LBP (Psa and Xap) or B medium (Xf) soft agar (0.7% agar) and overlaid on the plate containing grown colonies of LAB. For the other assay, discs (5 mm diameter) cut from 24-hour-old cultures of LAB isolates on MRS agar were deposited on the surface of plates containing cultures of the target pathogen. 0.5 mL of the target pathogen suspension at 5 x 10^7 CFU mL^{-1} were mixed in 4.5 mL of LB (Psa and Xap) or B medium (Xf) soft agar (0.7% agar) and overlaid on the plate containing the same media. Controls of target pathogen growth in the corresponding media were performed in the absence of LAB. Plates were incubated at 23°C and the inhibition zone diameter was measured after 24 and 48 h.

In planta assays under greenhouse conditions

Efficacy assays were performed on potted plants of kiwifruit (two-year-old plants), *Prunus*, and strawberry under greenhouse conditions and five LAB isolates (*L. plantarum* CC100, PM411, TC92 and *Leuconostoc mesenteroides* CM160 and CM209) were evaluated. Plants were sprayed to runoff with a 10^8 CFU mL^{-1} suspension of LAB cells using a hand-sprayer (Herkules, Nuair, Robassomero, Italy). Then, plants were maintained in plastic bags to reach high RH conditions in the greenhouse. After 24 h, plants were inoculated with a suspension of the corresponding pathogen at 1-5 x 10^8 CFU mL^{-1} (IVIA 3700-1 of Psa, CFBP5563 of Xap, or CECT549 of Xf). The pathogen suspensions were mixed with diatomaceous earth (1 mg mL^{-1}) and applied using a hand-sprayer to runoff. Then, plant material was covered with plastic bags for 24 h and maintained in a class II greenhouse for 15-21 days. Streptomycin treated (Sigma, Missouri, USA) (100 mg L^{-1}) and water-treated plants were included as positive and negative controls, respectively. Disease incidence, calculated as the percentage of infected leaves, was determined in each replicate at 15-21 days post inoculation.

Monitoring inoculated LAB population on leaves

Colonization and survival studies of *L. plantarum* PM411 were performed on potted plants of kiwifruit (15- to 30-day-old plantlets) and strawberry, while the same studies for *L. plantarum* TC92 were performed on *Prunus*. Plants were sprayed to runoff with a 10^8 CFU mL^{-1} cell suspension of PM411R or TC92R using a hand-sprayer. After inoculation, plants were covered with plastic bags to reach high RH conditions and maintained in the greenhouse. The monitoring of PM411R or TC92R population levels was performed following the procedure described by Roselló *et al*. (2017). Three leaves were taken from each replicate at 0, 1, 2, 5, 8, and 10 days post inoculation. The population levels of PM411R or TC92R were expressed as Log_{10} CFU per leaf.

Effect of *Lactobacillus plantarum* PM411 on Psa survival in kiwifruit plants

Survival studies of Psa were performed on potted plants of kiwifruit (15- to 30-day-old plantlets). Plants were sprayed to runoff with a 10^8 CFU mL^{-1} cell suspension of PM411 using a hand-sprayer and covered with plastic bags to reach high RH conditions. After 24 h, plants were inoculated with a Psa suspension at 1-5 x 10^8 CFU mL^{-1} (CFBP7286-GFPuv) to runoff using a hand-sprayer. After inoculation, plants were covered with plastic bags and maintained in the greenhouse. Streptomycin (Sigma) (100 mg L^{-1}) and water-treated plants were included as controls. For monitoring epiphytic and endophytic Psa population, three leaves per replicate were sampled at 1 and 4 days post inoculation. Leaves were weighted and the epiphytic population was collected by vigorously homogenizing leaves in 20 mL of sterile PBS and 0.1% peptone for 5 min. The same leaves were also used to assess endophytic population according to the procedure described by Cellini *et al*. (2018). Both epiphytic and endophytic samples were serially diluted and appropriate dilutions were seeded onto LB agar plates amended with 100 µg mL^{-1} of kanamycin (Sigma) to select CFBP7286-GFPuv and 100 µg mL^{-1} of cycloheximide (Sigma) to avoid fungal growth. Plates were incubated at 23ºC for 48 h and green fluorescent colonies were counted under UV light. The epiphytic and endophytic population levels of Psa were expressed as Log_{10} CFU per leaf or g, respectively.

In planta assays under semi-field conditions

The efficacy of two selected LAB strains (*L. plantarum* PM411 and TC92) in controlling bacterial plant pathogens was studied in semi-field assays and compared to reference products.

Potted plants of kiwifruit (two-year-old plants) for Psa experiments were brought to an experimental orchard located at Zevio (Verona, Italy). Plants were treated with products and kept under field conditions for 7 days until being transported in a greenhouse for pathogen inoculation. The treatments performed were: (i) PM411 cell suspension at 10^8 CFU mL^{-1} prepared as described above, (ii) *Bacillus amyloliquefaciens* D747 (Amylo-X©, 25% w/w a.i., 5 x 10^{10} CFU g^{-1}, Biogard,

Monza Brianza, Italy) at 0.375 g a.i. L^{-1}, (iii) copper oxide (Nordox, 75% w/w a.i. Comercial Química Massó, Barcelona, Spain) at 0.45 g a.i. L^{-1}.

Potted plants of *Prunus* for Xap experiments and strawberry plants for Xf experiments were brought to the experimental orchard located at the Mas Badia Agricultural Experiment Station (Girona, Spain). Plants were treated with products and kept under field conditions for 7 days until being transported in a class II quarantine greenhouse for pathogen inoculation. The treatments performed were: (i) TC92 or PM411 cell suspension at 10^8 CFU mL^{-1} prepared as described above, (ii) *Bacillus subtilis* QST713 (Serenade Max®, 15.67% w/w a.i., Bayer Crop Science, Monheim am Rhein, Germany) at 0.55 g a.i. L^{-1}, (iii) chitosan (Biorend®, 2.5% v/v a.i., Bioagro, Santiago, Chile) at 7.5 g a.i. L^{-1}, (iv) acibenzolar-S-methyl (ASM, Bion®, 50% w/w a.i., Syngenta, Basel, Switzerland) at 0.075 g a.i. L^{-1}, (v) copper hydroxide (Kocide®, 35% w/w a.i Certis, Elche, Spain) at 1.05 g a.i. L^{-1} and (vi) kasugamycin (Kasumin®, 8% w/w a.i, Lainco, Barcelona, Spain) at 0.16 g L^{-1}. In all experiments, water-treated plants were included as control. All the treatments, except for kasugamycin, were applied twice, 7 and 1 days before inoculation, using a hand-sprayer to runoff.

Plants were spray-inoculated with the corresponding pathogen suspension at 10^8 CFU mL^{-1} (CFBP7286 of Psa, CFBP5563 of Xap, CECT549 of Xf). Spray application was performed as described in the greenhouse experiments. After inoculation, plants were maintained for 48 h in plastic bags to reach high RH and maintained in a class II greenhouse for the following days. The disease incidence was determined as described above per each replicate at 15-21 days post inoculation.

In planta Psa control assay in orchard conditions

The field experiment was performed in 2017 in a commercial orchard located in Sarna, close to Faenza (Emilia Romagna, Italy). The trial was carried out in *A. chinensis* var. *deliciosa* (cv. Hayward) orchard where the disease was present with a moderate pressure in the previous years. Standard cultural management (i.e. fertigation, green and winter pruning, thinning and assisted pollination) was adopted. The orchard was planted in 2006, with a pergola training system (4.7 × 2.4 m). For each plant (experimental design explained below) 4 shoots without Psa symptoms were selected and tagged at the beginning of the experiment. The treatments performed were: (i) PM411 cell suspension at 10^8 CFU mL^{-1} prepared as described above, (ii) *B. amyloliquefaciens* D747 (Amylo-X© 5 x 10^{10} CFU g^{-1}) at 0.375 g a.i. L^{-1}, and (iii) copper oxide (Nordox) at 0.45 g a.i L^{-1}. Water-treated plants were included as control. All the treatments were applied every 14 days or after each rainfall (≥4 mm of rain), in case the rain event occurred at 7 or more days after treatment. Copper application started at bud break (BBCH 03) and was repeated till fruit growth up to 30% the final size (BBCH 73). BCA application was performed at 10, 50 and 100% of blooming and was repeated till BBCH 73. Psa incidence was assessed twice during the season being the

second assessment after the stop of disease progression and was calculated as the percentage of symptomatic leaves on 20 shoots per repetition. Psa symptomatology was confirmed by molecular identification according to Gallelli *et al*. (2011).

Characterization of PM411 and TC92

Different methods were used to characterize the two selected strains *L. plantarum* PM411 and TC92. The role of different produced metabolites on the antibacterial activity against target pathogens was studied together with a genotypic characterization.

Antibacterial activity

Agar diffusion assays using cell-free supernatants (CFS) were performed. PM411 and TC92 strains were grown in 250 mL Erlenmeyer flasks with 100 mL of MRS broth for 24 h at 30°C with shaking (100 rpm). CFS were obtained by centrifugation (10,000 g for 10 min) (5810 R, Eppendorf, Hamburg, Germany) and were filtrated through 0.45 µm pore filter (Whatman FP30/0.45, Millipore, Bedford, USA). 20 µL of CFS was deposited on the surface of plates containing cultures of the target pathogens Psa (IVIA 3700-1), Xap (CFBP3894), and Xf (IVIA XF349-9A) prepared as described above (see *in vitro* antagonism tests, LB for Psa and Xap and B medium for Xf). Controls of target pathogen growth in the corresponding media were performed in the absence of CFS. Plates were incubated at 23°C and the diameter of the zone of inhibition was examined at 24 and 48 h. Fractions of CFS were exposed to different treatments (neutralized CFS, and neutralized CFS treated with proteinase K, trypsin, α-chimotrypsin or catalase) as described by Trias *et al*. (2008b) and the antimicrobial activity was assessed by agar diffusion assay as described above. Three independent replicates of each CFS fraction were performed. Lactic acid was quantified in CFS using Enzytec™ D-/L-Lactic Acid commercial kit (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) following the instructions of the manufacturer. 1:10 diluted CFS with redistilled water were used for the measurement. Two experiments were carried out with three independent replicates.

Molecular characterization of PM411 and TC92

Multilocus sequence typing analysis (MLST) and random amplified polymorphic DNA-polymerase chain reaction analysis (RAPD-PCR) were performed for strain genotypic characterization.

(i) DNA extraction. Genomic DNA from 45 *L. plantarum* isolates (INTEA-CIDSAV culture collection, Table S1) was extracted according to the method described by Llop *et al*. (1999). Previously, *L. plantarum* isolates were cultured in MRS medium at 23ºC for 48 h and cell suspensions were prepared at 10^8 CFU mL^{-1}. The concentration and purity of DNA was assessed

by spectrophotometric measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

(ii) PCR amplifications and conditions. Amplification mixtures and PCR conditions used in this study to perform MLST and RAPD-PCR analysis are described in Table 1. The amplification products were separated by electrophoresis on a 1.5% (w/v) agarose gel in 1X Tris-acetate EDTA (TAE) and stained with Sybr Safe (SYBR® Safe, Invitrogen, Life Technologies, Carlsbad, USA). Gel images were captured with an imaging system (FX-20M, Vilvert, Lourmat, France).

Table 1 Amplification mixtures and PCR conditions.

PCR approach	Amplification mixture [a]	PCR conditions [b]
MLST	1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 1 U *Taq* and 20 ng DNA (reaction vol, 25 µL)	95ºC for 10 min; 30 cycles of 95ºC for 30 s, 54ºC (*gdh*) or 48ºC (*pgm, ddl, gyrB, purK1, mutS*) for 60 s, 72ºC for 60 s; and elongation 72ºC for 10 min.
RAPD-PCR	1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 3.75 U *Taq*, and 50 ng DNA (reaction vol, 25 µL)	For P3 and P4: 94ºC for 3 min; 30 cycles of 94ºC for 1 min, 36ºC for 2 min, 72ºC for 2 min; and elongation at 72ºC for 2 min. For 512Fb, Inva1 and P7: 94ºC for 4 min; 45 cycles of 94ºC for 1 min, 35ºC for 1 min, 72ºC for 1 min; and elongation at 72ºC for 5 min.

[a] dNTP, deoxynucleotides (Invitrogen Life Technologies, Carlsbad, USA); primers (Sigma Aldrich, Barcelona, Spain); *Taq*, Taq DNA polymerase (Biotools, Madrid, Spain for MLST and Invitrogen for RAPD-PCR).

[b] PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems).

(iii) MLST analysis. PM411 and TC92 strains were analysed together with 43 *L. plantarum* isolates from INTEA-CIDSAV culture collection (Trias *et al*., 2008a, 2008b; Roselló *et al*., 2013), and 26 *L. plantarum* isolates from other studies (de las Rivas *et al*., 2006; Tanganurat *et al*., 2009). The housekeeping genes selected for MLST analysis were phosphoglucomutase (*pgm*), D-alanine-D-alanine ligase (*ddl*), B subunit of DNA gyrase (*gyrB*), ATPase subunit of the phosphoribosylaminoimidazole carboxylase (*purK1*), glutamate dehydrogenase (*gdh*) and DNA mismatch repair protein (*mutS*). The amplification and sequencing primers used were described previously by de las Rivas *et al*. (2006), except for *gdh* gene that the primers were designed in this work (gdhF 5'-GGTTACACCCATCCGTTAAT-3' and gdhR 5'-TTCTTCAAAAGTCCAGTCA-3', 901 bp fragment). Amplification products were purified with QIAEX desalting and concentrating DNA solutions kit (QIAGEN GmbH, Hilden, Germany) for direct sequencing using Automatic Sequencer 3730XL (Macrogen, Seoul, Korea). Sequence alignments and comparisons were performed using BioEdit Sequencing Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For

each gene, the sequences obtained were compared to the ones reported in the other studies, and allele numbers were assigned following the codes previously described (de las Rivas *et al*., 2006; Tanganurat *et al*., 2009). New allele sequences were deposited in the GenBank database under the accession numbers KT247498 (allele 4 *pgm*), KT247499 (allele 7 *ddl*), KT247496 (allele 9 *purK1*), KT247497 (allele 10 *purK1*), KT247501 (allele 11 *gdh*) and KT247500 (allele 9 *mutS*). Each strain was defined by an allele profile or sequence type (ST), derived from the combination of numbers corresponding to the alleles at the analysed loci. Sequences that differed, even at a single nucleotide site, were considered as distinct alleles (de las Rivas *et al*., 2006).

(iv) RAPD-PCR analysis. PCR amplification of each isolate was carried out with five primers, with arbitrarily chosen sequences, P3 (Tailliez *et al*., 1998), Inva1 (Rahn *et al*., 1992), 512Fb (Holt & Cote, 1998), P4 and P7 (Di Cagno *et al*., 2010) in separate reactions. Pattern analyses were performed with Image Lab™ version 4.1 software (Bio-Rad Laboratories, Hercules, USA).

Experimental designs and data analysis

In vitro antagonism tests consisted of three independent replicates of each LAB and were performed twice. The inhibition zone diameter of the 55 isolates against the six target pathogens were transformed to binary data (indicating the presence and absence of inhibition zones ("1" for present, "0" for absent)) for hierarchical cluster analysis. The simple matching coefficient of similarity using unweighted pair group method with the arithmetic average (UPGMA) as the cluster algorithm was used (Numerical Taxonomy System program package NTSYSpc, Exter Software, New York, USA).

Biocontrol efficacy assays in greenhouse and semi-field conditions consisted of three independent biological replicates per treatment with five plants per replicate. Two independent experiments were performed in all three pathosystems in greenhouse and semi-field experiments except for semi-field and monitoring assays in Psa that were performed once. The biocontrol efficacy assay against Psa in the field consisted of four randomized blocks of five plants per treatment. Each block was repeated in separated rows. Disease incidence and Psa population levels were subjected to analysis of variance (ANOVA) and mean values were compared using the least significant difference (LSD) test at $P < 0.05$. The analysis was performed with the GLM procedure of the PC-SAS (Version 8.2, SAS Institute Inc., Cary, USA).

The monitoring studies of LAB population consisted of three independent biological replicates with five plants per replicate and two independent experiments.

Allelic profiles of six housekeeping genes of each *L. plantarum* strain obtained by MLST analysis were used in order to investigate clonal complexes of strains by minimum spanning tree (MST) method (Bionumerics v7.5, Applied-Maths, Sint-Martens-Latem, Belgium). Polymorphic DNA bands obtained by RAPD analysis were transformed into a binomial matrix indicating the presence and absence of fragments ("1" for present, "0" for absent). A dendrogram with the five

series of RAPD-PCR profiles of the 45 *L. plantarum* isolates was generated using the Dice coefficient of similarity and the UPGMA method (NTSYSpc).

RESULTS

Antagonistic activity against bacterial plant pathogens

Several LAB isolates strongly inhibited growth (inhibition zone diameter > 10 mm) of the majority of the target bacteria. However, differences in sensitivity to inhibition by LAB were observed among the six target strains. The cluster analysis showed three main groups of antagonism spectrum at a similarity level of 0.55 (Fig. 1). Group 1 included 17 strains and showed none or low activity against Psa, moderate activity against Xap, and moderate to high activity against Xf. Group 2 included 33 strains that showed low to moderate activity against Psa, moderate to high activity against Xap and Xf. Interestingly, Group 3 included five strains, CC100, CM160, CM209, PM411, and TC92, that displayed a broad and generally high activity against all the target pathogens.

Control of Psa, Xap and Xf in plants under greenhouse conditions

L. mesenteroides CM160 and CM209 and *L. plantarum* CC100, PM411, and TC92 were selected for efficacy assays in greenhouse conditions due to their broad and high *in vitro* antagonism against Psa, Xap, and Xf. PM411 and TC92 strains consistently reduced Psa, Xap and Xf disease incidence in kiwifruit, *Prunus* and strawberry plants, respectively, in comparison with non-treated control in both experiments performed (Fig. 2). The efficacy of the *L. plantarum* strains in kiwifruit ranged from 84.5 to 96.3% for TC92 and from 70.0 to 75.4% for PM411; in *Prunus*, ranged from 59.1 to 69.3% for TC92 and from 45.5 to 65.5% for PM411; and in strawberry, ranged from 35.4 to 69.2% for TC92 and from 45.8 to 92.3% for PM411. PM411 and TC92 did not differ significantly from streptomycin, expect for PM411 in one experiment of Xf-strawberry pathosystem. In contrast, CC100, CM160, and CM209 did not reduce disease incidence in comparison to non-treated plants in some experiments.

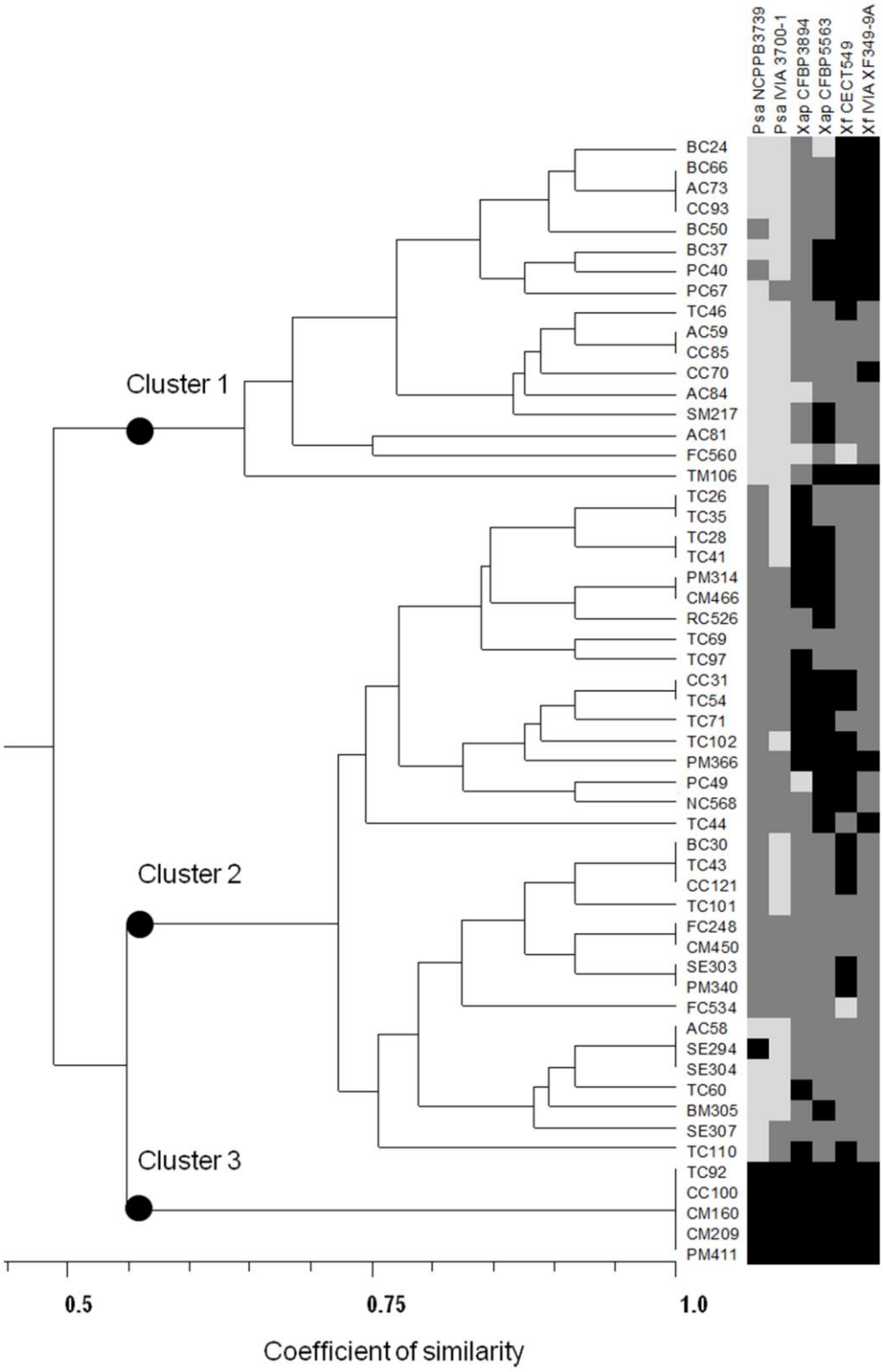


Fig. 1 Dendrogram of the *in vitro* antagonism spectrum of 55 lactic acid bacteria (LAB) strains. The target pathogens are listed at the top and correspond to strains NCPPB3739 and IVIA 3700-1 of *P. syringae* pv. *actinidiae* (Psa), strains CFBP3894 and CFBP5563 of *X. arboricola* pv. *pruni* (Xap) and strains CECT549 and IVIA XF349-9A of *X. fragariae* (Xf). Two independent experiments were performed. Clusters of strains are indicated by a circle dot. Colour legend indicates the inhibition activity: pale grey, negative in both experiments; dark grey, positive in one experiment; black, positive in both experiments. Cluster analysis was performed using unweighted pair group method with the arithmetic average (UPGMA) with the simple matching coefficient of similarity.

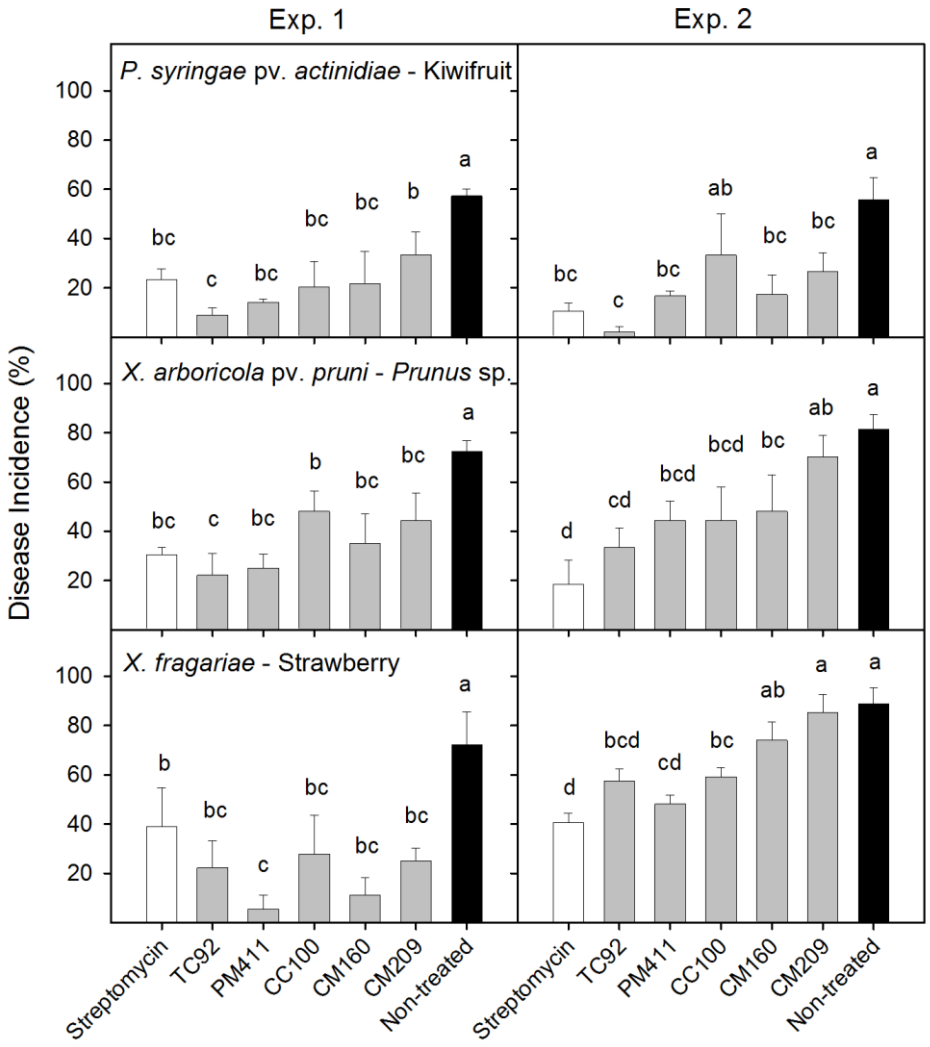


Fig. 2 Effect of the treatments with lactic acid bacteria (LAB) strains (grey bars) on *P. syringae* pv. *actinidiae* (Psa), *X. arboricola* pv. *pruni* (Xap), and *X. fragariae* (Xf) infections in kiwifruit, *Prunus* and strawberry plants, respectively, under greenhouse conditions. The effect of strains on disease incidence (%) was compared with streptomycin (white bars) and a non-treated control (black bars). Two independent experiments were performed (left and right panels). Values are the mean of three replicates and error bars represent the standard error of the mean. Bars with the same letter in the same panel do not differ significantly (P<0.05) according to the least significant difference (LSD) test. $LSD_{Psa\text{-}Exp.1}$=22.4; $LSD_{Psa\text{-}Exp.2}$=28.8; $LSD_{Xap\text{-}Exp.1}$=23.4; $LSD_{Xap\text{-}Exp.2}$=29.0; $LSD_{Xf\text{-}Exp.1}$=32.8; $LSD_{Xf\text{-}Exp.2}$=16.8.

Monitoring inoculated LAB population on leaves

The survival of *L. plantarum* PM411 and TC92 was monitored onto leaves of kiwifruit and strawberry plants (PM411) and leaves of *Prunus* plants (TC92) under greenhouse conditions (Fig. 3). After inoculation, the population level decreased approximately 2 log units between the 1st and the 10th day. In particular, the drop of population was observed during the first five days. Thereafter, the viable population remained stable throughout the following five days at a level of around 10^4 CFU per leaf.

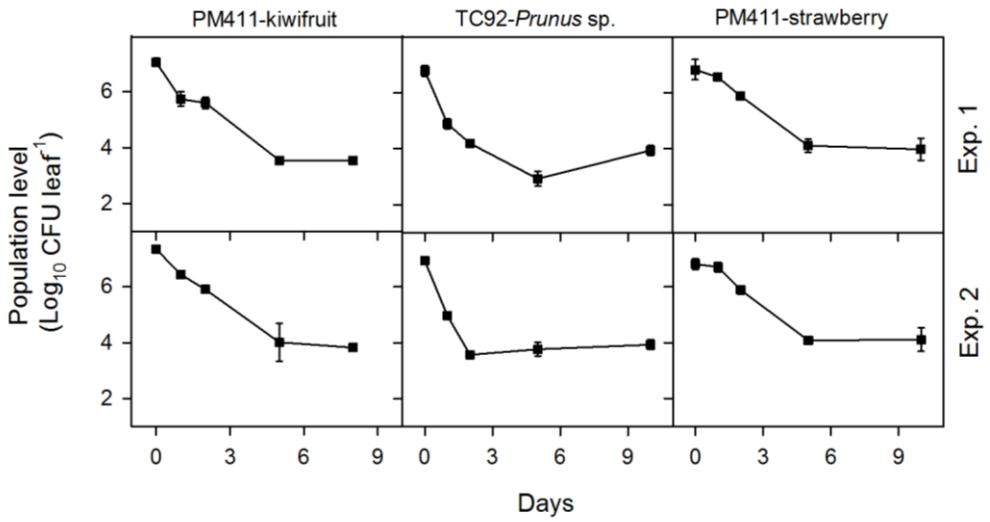


Fig. 3 Survival of *L. plantarum* PM411 in kiwifruit and strawberry plants and survival of *L. plantarum* TC92 in *Prunus* plants under controlled environmental conditions. Survival is shown as the population level (Log_{10} CFU $leaf^{-1}$). Values are the mean of the three replicates and error bars represent the standard error of the mean. Two independent experiments were performed (top and bottom panels).

Control of Psa, Xap and Xf in plants under semi-field and field conditions

Efficacy of TC92 and PM411 in controlling Xap and Xf, respectively, was compared to reference products in semi-field experiments (Fig. 4). The incidence of infection attained in *Prunus* plants treated with *L. plantarum* TC92 was significantly lower than in the non-treated control in both experiments performed. TC92 efficacy in controlling Xap on *Prunus* (41.5-55.0%) was not significantly different from *B. subtilis* QST713, chitosan, acibenzolar-S-methyl and kasugamycin in both experiments or from copper in only one. Strawberry plants treated with *L. plantarum* PM411 showed lower incidence of infections than the non-treated control in both experiments. PM411 efficacy (63.6 to 75.0%) in controlling Xf on strawberry did not differ significantly from *B. subtilis* QST713, acibenzolar-S-methyl, copper and kasugamycin in both experiments or from chitosan in only one.

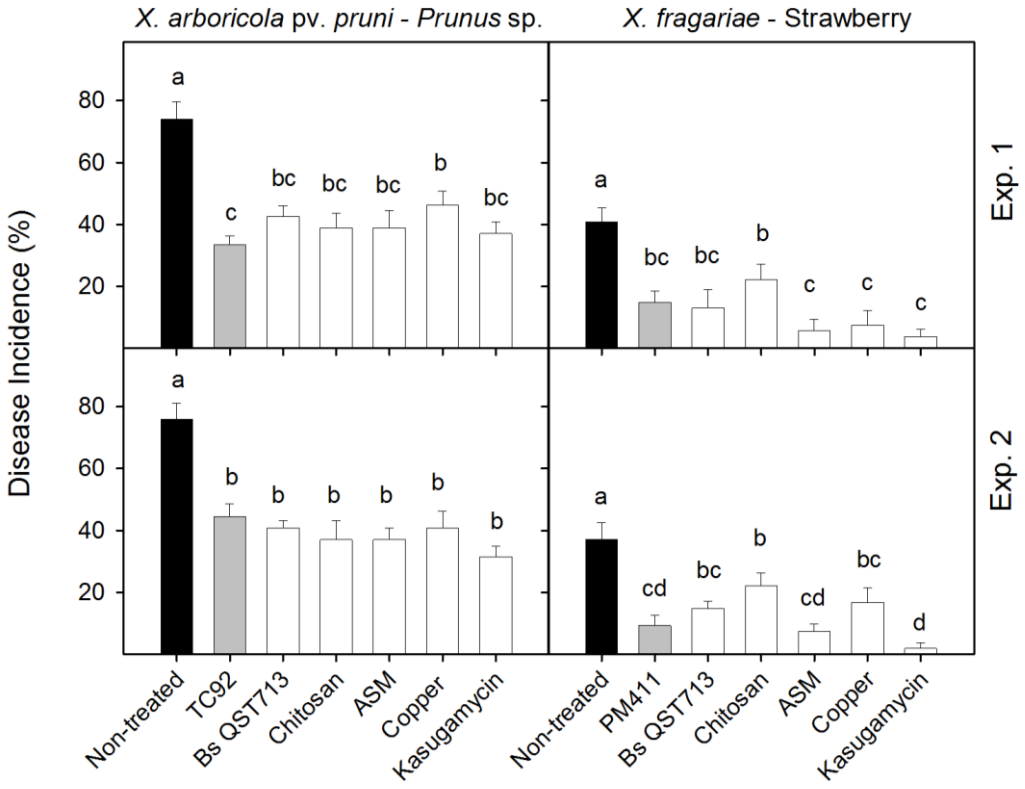


Fig. 4 Effect of the treatment with *L. plantarum* TC92 and PM411 (grey bars) on *X. arboricola* pv. *pruni* (Xap) infections in *Prunus* potted plants and on *X. fragariae* (Xf) infections in strawberry potted plants, respectively, in semi-field experiments. The effect of strains on disease incidence (%) was compared with different reference products, such as *B. subtilis* (Bs) QST713, chitosan, acibenzolar-S-methyl (ASM), copper, and kasugamycin (white bars) and a non-treated control (black bars). Two independent experiments were performed. Values are the mean of three replicates and error bars represent the standard error of the mean. Bars with the same letter in the same panel do not differ significantly (P<0.05) according to the least significant difference (LSD) test. $LSD_{Xap\text{-}Exp.1}$=12.7; $LSD_{Xap\text{-}Exp.2}$=13.0; $LSD_{Xf\text{-}Exp.1}$=12.8; $LSD_{Xf\text{-}Exp.2}$=10.6.

Efficacy of PM411 in controlling Psa was compared to reference products in a semi-field experiment (Fig. 5). PM411 was able to halve disease incidence in kiwifruit plants in comparison to non-treated control with significant differences. PM411 efficacy in controlling Psa on kiwifruit (54.2%) was similar to the ones of *B. amyloliquef*aciens D747 and copper without significant differences between them.

In the field experiment, performed in a commercial kiwifruit orchard, the natural Psa incidence in non-treated plants was 45.3%. PM411 was the most effective treatment in lowering disease incidence, attaining 50.3% of efficacy and no significant differences were observed in comparison with *B. amyloliquef*aciens D747 as a reference product, which showed 22.7% of efficacy (Fig. 5). Copper did not significantly reduce disease incidence in comparison to non-treated control, showing 37.1% of efficacy.

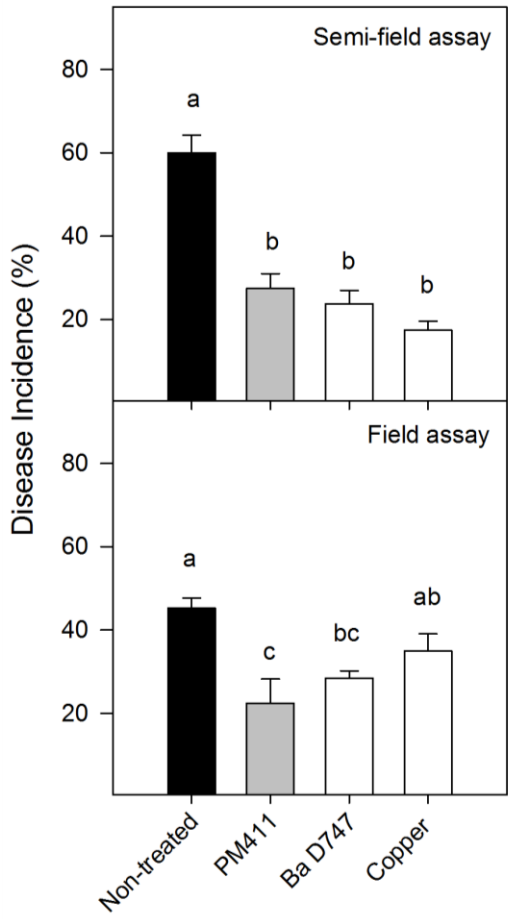


Fig. 5 Effect of the treatment with *L. plantarum* PM411 (grey bars) on *P. syringae* pv. *actinidiae* (Psa) infections in kiwifruit plants in semi-field and field experiments. The effect of PM411 on disease incidence (%) was compared with reference products, such as *B. amyloliquefaciens* (Ba) D747 and copper (white bars) and a non-treated control (black bars). Values are the mean of three replicates and error bars represent the standard error of the mean. Bars with the same letter in the same panel do not differ significantly (P<0.05) according to the least significant difference (LSD) test. $LSD_{Semi\text{-}field}$=13.5; LSD_{Field}=11.7.

Psa population suppression by PM411

The epiphytic and endophytic populations of Psa on leaves of potted kiwifruit plants treated with PM411 were monitored for 4 days post inoculation (dpi) of the pathogen at high RH conditions (Table 2). Population of Psa recovered from leaves of plants treated with PM411 was lower than that from untreated plants. Specifically, for the epiphytic population the reduction (1 log unit) was significant at 1 dpi, while for the endophytic population it was observed at 4 dpi. A reduction of 1.5 to 2 log units of Psa population was observed in streptomycin-treated plants.

Table 2 Effect of *L. plantarum* PM411 treatment on survival of *P. syringae* pv. *actinidiae* (Psa) in kiwifruit plants.

	Psa population levels [a]							
	epiphytic (Log_{10} CFU $leaf^{-1}$)				**endophytic (Log_{10} CFU g^{-1})**			
Treatment [b]	1 day		4 days		1 day		4 days	
PM411	6.1	b	5.5	ab	3.7	a	2.8	b
Streptomycin	5.4	c	5.0	b	2.7	b	< 2[c]	c
Non-treated	7.2	a	6.5	a	4.0	a	3.6	a
LSD	0.48		1.02		0.58		0.23	

[a] Values of Psa population levels are the mean of the three replicates one and four days post Psa inoculation on plants. Values with different letters in the same column are significantly different ($P < 0.05$) according to the least significant difference (LSD) test.

[b] Treatments were carried out one day before Psa inoculation.

[c] Log_{10} CFU g^{-1} value under detection limit.

PM411 and TC92 characterization

To understand the antibacterial activity of PM411 and TC92, cell-free supernatant (CFS) were analysed. CFS without pH adjustment (pH 3.8) showed antibacterial activity against the three target pathogens Psa, Xap, and Xf. However, the inhibitory activity was completely lost after CFS pH neutralization, while enzymatic treatments (tripsin, α-chimiotripsin, proteinase K and catalase) had no impact on the inhibitory effect (data not shown). A similar amount of a mixture of D- and L-lactic acid was quantified in CFS obtained from both strains (75.75 ± 0.63 mM for PM411 and 74.86 ± 1.02 mM for TC92), with D-lactic acid the predominant optical isomer in the mixture.

In relation to genotypic characterization, the minimum spanning tree analysis (MST) of different *L. plantarum* strains based on MLST data (Fig. S1, Supporting information) is shown in Fig. 6. Specifically, PM411 and TC92 were analysed together with other isolates, 43 from INTEA-CIDSAV culture collection and 26 from other studies. The six housekeeping genes yielded a total of 21 STs and the majority of the strains (66%) represented the ST16 (n=38) and ST1-15 (n=9). The 21 STs were grouped into three clonal complexes (CCs). CC1 consisted of 5 STs that accounted for 42 strains, including ST5 as the putative primary founder. CC2 consisted of ST1-15 represented by nine strains and ST17 that included one strain. CC3 was composed of ST20 and ST21 with four strains. The remaining STs were considered as singletons. Specifically, PM411 and TC92 were found in two different clonal complexes, TC92 in CC1 and PM411 in CC3. Strain PM411 shared ST21 with two strains from INTEA-CIDSAV collection. Strain TC92 shared ST16

with other 35 strains from INTEA-CIDSAV collection and with two strains studied by Tanganurat *et al*. (2009). The differences between TC92 and PM411 were found in *purK1* and *gdh*.

PM411 and TC92 together with 43 *L. plantarum* strains were subjected to RAPD-PCR analysis (Fig. 6). According to band profiles, strains were divided into three clusters at a coefficient of similarity higher than 0.6. TC92 shared cluster A with 22 strains and PM411 shared cluster B with other 2 strains.

Fig. 6 Minimum spanning tree of 71 *L. plantarum* isolates based on allelic profiles of genes *pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS* (A). Each circle corresponds to a sequence type (ST) and the size of the circle is proportional to the number of isolates within any given ST. Colour codes of isolates: green, INTEA-CIDSAV collection; red, de las Rivas *et al*. (2006); and blue, Tanganurat *et al*. (2009). The type of line between isolates indicates the strength of the genetic relationship between them (black, strong relationship; grey, intermediate relationship; and dotted line, weak relationship). The number of mutations between STs is also indicated for each relationship. STs that belong to the same clonal complex (CC) are shown as circles grouped in a grey area. Black arrows indicate that PM411 strain with ST21 belongs to CC3 and TC92 strain with ST16 belongs to CC1. Dendrogram of the randomly amplified polymorphic DNA-PCR (RAPD-PCR) patterns of 45 *L. plantarum* strains (INTEA-CIDSAV collection) using primers P3, P4, P7, 512Fb and Inva1 (B). Circle dots indicate the three main clusters as well as the singletons. Cluster analysis was performed by unweighted pair group method with the arithmetic average (UPGMA) with the Dice coefficient of similarity. Black arrows indicate TC92 and PM411 strains.

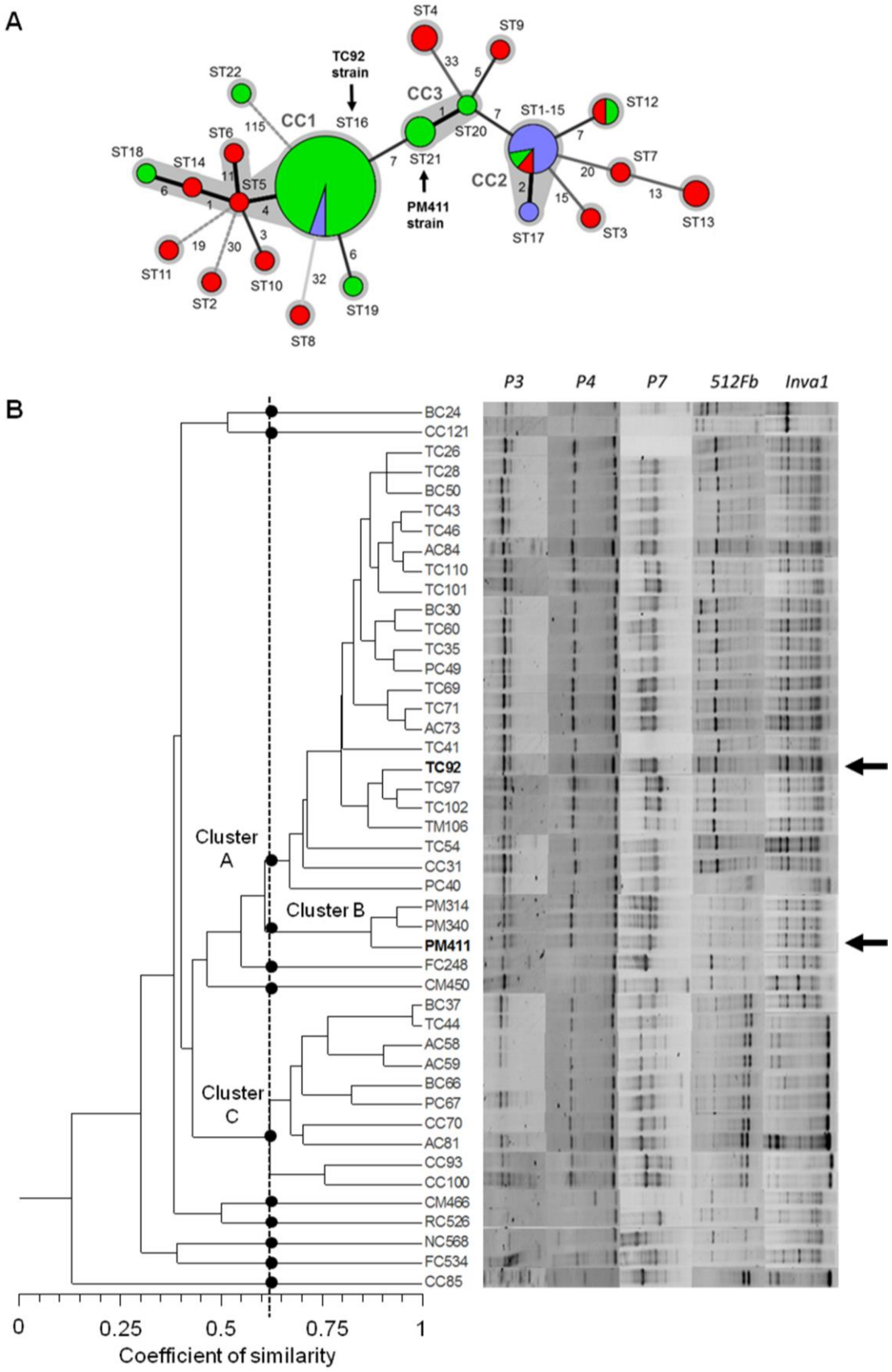

A
ST4
ST9
33
5
TC92 strain
ST22
CC3
1
7
ST1-15
ST12
CC1
ST16
115
ST6
ST20
7
ST18
ST14
11
ST5
ST21
CC2
20
ST7
6
1
4
PM411 strain
2
15
13
ST13
19
30
3
ST17
ST3
ST11
ST10
6
ST2
32
ST19
ST8
B
P3
P4
P7
512Fb
Inva1
BC24
CC121
TC26
TC28
BC50
TC43
TC46
AC84
TC110
TC101
BC30
TC60
TC35
PC49
TC69
TC71
AC73
TC41
TC92
TC97
TC102
TM106
TC54
CC31
PC40
PM314
PM340
PM411
FC248
CM450
BC37
TC44
AC58
AC59
BC66
PC67
CC70
AC81
CC93
CC100
CM466
RC526
NC568
FC534
CC85
Cluster A
Cluster B
Cluster C
0
0.25
0.5
0.75
1
Coefficient of similarity

Fig. 6

DISCUSSION

The growing interest in sustainable management of plant diseases caused by Psa, Xap, and Xf has stimulated the search for novel BCA. Several bacteria, such as *Pseudomonas* and *Xanthomonas*, were selected for their biocontrol activity against these pathogens, but these studies have been focused on a single pathogen (Biondi *et al*., 2009; Kawaguchi *et al*., 2014; Henry *et al*., 2016; Wicaksono *et al*., 2018). In the present work, a multi-disease approach was adopted to select potential antagonistic bacteria with a broad-spectrum activity. This strategy has been previously reported for the screening of BCA in other pathosystems (Haidar *et al*., 2016). Since some LAB strains have shown antagonistic activity against bacterial and fungal plant pathogens (Visser *et al*., 1986; Trias *et al*., 2008b; Wang *et al*., 2011; Fhoula *et al*., 2013) and denoted biocontrol efficacy on plants (Roselló *et al*., 2013; Shrestha *et al*., 2014; Tsuda *et al*., 2016; Roselló *et al*., 2017), plant-associated LAB have been considered as candidate for the development of BCA.

In the present study *in vitro* and *in planta* tests were used to select candidate BCA to control multiple bacterial plant pathogens in their corresponding host plants. 55 LAB isolates were screened for *in vitro* antibacterial activity against Psa, Xap, and Xf and CC100, CM160, CM209, PM411 and TC92 were selected for exhibiting a broad spectrum of antagonism. Other reports have also demonstrated a high antagonistic activity of certain strains of LAB against phytopathogenic bacteria, such as *Xanthomonas campestris, Pectobacterium carotovorum*, *Pseudomonas syringae*, *Pseudomonas savastanoi*, and *Ralstonia solanacearum* (Trias *et al*., 2008b; Fhoula *et al*., 2013; Shrestha *et al*., 2014). *In vitro* assays are commonly used for preliminary selection of antagonist strains from a huge number of candidates testing different phytopathogens as target (Kavroulakis *et al*., 2010; Mora *et al*., 2015). This strategy is mainly focused on the selection of antagonists whose mode of action is the secretion of antimicrobial compounds (Köhl *et al*., 2011), such as LAB (Ben Omar *et al*., 2008). Therefore, this method allowed for the preselection of BCA candidate strains.

In planta tests based on multiple-pathosystem approach were suitable to highlight strains with potential biocontrol ability and broad spectrum among the ones previously selected in *in vitro* tests. The biocontrol activity of PM411 and TC92 against Psa, Xap, and Xf was confirmed in plants under greenhouse conditions reducing pathogen infections similarly to streptomycin. In a previous study both strains were also selected in a screening procedure for exhibiting suppressive effect against *E. amylovora* in plant assays (Roselló *et al*., 2013).

The biocontrol efficacy of PM411 and TC92 against Psa, Xap, and Xf in the corresponding hosts was also observed in semi-field or field experiments, confirming that they could be useful in a wide range of conditions. Due to the regulatory restrictions of the quarantine pathogens in EU, the assessment of disease management strategies in orchards affected with these pathogens is practically unaffordable in designated protected zones, such as Spain. Therefore, semi-field

experiments, consisting of treatment applications under field conditions and pathogen inoculation in the quarantine greenhouse, have been performed in this study since they were previously reported as comparable to field experiments for biocontrol testing (Cabrefiga *et al*., 2011).

Biocontrol efficacy of the LAB selected strains was comparable to *B. amyloliquefaciens* D747, *B. subtilis* QST713, chitosan, ASM, copper, and kasugamycin. LAB strains could be considered as a promising alternative tool to be included in the disease management strategy due to the limitations that restrict the use of commercial products. In particular, phytotoxicity and pathogen resistance selection have been reported regarding copper compounds and antibiotics used for Psa, Xap, and Xf control (Roberts *et al*., 1997; Lalancette & Mcfarland, 2007; Colombi *et al*., 2017). While lack of consistency in efficacy has been demonstrated under some limited conditions concerning plant defence elicitors, such as ASM (Reglinski *et al*., 2013) and commercial microbial biopesticides based on *Bacillus* spp. against Psa (Monchiero *et al*. 2015).

Due to their interest as biological control agents, PM411 and TC92 were characterized regarding the mechanism involved in the antimicrobial activity against the pathogens, as well as, genetically typified for their development as BCA. Cell-free supernatants (CFS) obtained from cultures of both strains showed also inhibitory effect, indicating the presence of antimicrobial compounds. Indeed, lactic acid production was confirmed in cell cultures of PM411 and TC92. While plantaricin synthesis by both strains is expected due to the presence of biosynthetic genes (*plnEF, plnJK*) with similar levels of expression (Roselló *et al*., 2013; Daranas *et al*., 2018a). Since pH neutralization of CFS eliminated the antibacterial effect, acidic pH or the presence of organic acids could account for the main antimicrobial activity. Other studies reported organic acids as one of the main mechanisms through which antimicrobial activity of LAB is exerted against a broad spectrum of target bacteria (Arena *et al*., 2016). Although in this work plantaricins have not been proved to contribute in Psa, Xap, and Xf inhibition, their role should not be dismissed because acidification or acid-mediated cell membrane disruption may be required to exert an antagonistic effect (Alakomi *et al*., 2000). Even though hydrogen peroxide role in antimicrobial activity was not confirmed in CFS, its production is expected to be favoured on plant surfaces, where there are aerobic conditions, and to contribute to pathogen suppression as previously reported (Pridmore *et al*., 2008). Therefore, to deeply understand the multifactorial mode of action of PM411 and TC92 responsible for disease prevention in plants further studies are required.

Besides the production of antimicrobial metabolites, pre-emptive colonization of plant tissues susceptible to pathogen infection is an important mechanism underlying BCA effectiveness (Giddens *et al*., 2003). Since Psa, Xap, and Xf enter into the host plant through natural openings, such as leaf stomata, and wounds, the presence of PM411 and TC92 cells on the leaf surfaces prior to the arrival of pathogens might avoid infection. In particular, PM411 preventively sprayed on plants inhibited endophytic and epiphytic Psa population indicating a direct effect on pathogen and in accordance with the reduction of disease incidence in plant experiments. This inhibitory effect of PM411 and TC92 was also observed on *E. amylovora* on pear plant surfaces (Roselló *et al*.,

2017). The survival of PM411 and TC92 on leaves of kiwifruit, *Prunus*, and strawberry plants was confirmed in greenhouse experiments. Similar population levels were reached on the three different host plants and in agreement with their survival previously reported on pear plants (Roselló *et al*., 2017). A population decrease after spray was observed due to the harsh conditions in aerial plant surfaces. However, a constant level of 10^3-10^4 CFU leaf^{-1} of LAB strains was attained in the following ten days. This decline of population on leaves was also reported in other *L. plantarum* BCA, which, interestingly, attained higher population levels in wounded sites of leaves (Tsuda *et al*., 2016). Changes in water availability and temperature, nutrients limitation, or ultraviolet radiation on leaves can be transiently inadequate for BCA growth (Lindow & Brandl, 2003).

Although the performance of PM411 and TC92 regarding the suppression of pathogens is similar and both strains were identified as *L. plantarum*, they were clearly discriminated by RAPD-PCR analysis and belong to different clonal complexes according to MLST. Interestingly, these strains were analysed together with other plant-associated *L. plantarum* strains and both RAPD-PCR and MLST analysis displayed similar groups.

L. plantarum PM411 and TC92 are effective in preventing treatments to control bacterial pathogens representing different genera (*Pseudomonas, Xanthomonas*) and affecting different hosts (kiwifruit, *Prunus*, strawberry). Their exhibited broad-spectrum antagonism against bacterial pathogens is mainly based on antimicrobial metabolites. Moreover, they inhibited pathogen population on plant surfaces supressing infections. However, the population reduction of PM411 and TC92 under nonconductive conditions might compromise plant protection. To achieve the BCA population required for biocontrol, repeated spray applications may be necessary. Therefore, monitoring of viable cells could aid the design of a suitable delivery schedule of applications (Daranas *et al*., 2018b). Also, the improvement of BCA ecological fitness could be implemented (Daranas *et al*., 2018a). Further studies are needed to confirm the performance of PM411 and TC92 in the field under different agricultural and climatic conditions.

ACKNOWLEDGMENTS

Funding was provided by AGL2015-69876-C2-1-R (Spain Ministerio de Economía y Competitividad and FEDER of the European Union), by FP7-KBBE.2013.1.2-04 613678 DROPSA of the European Union, and by MPCUdG2016 grant (University of Girona). N. Daranas was recipient of a research grant 2015 FI_B00515 (Secretaria d'Universitats I Recerca, Departament d'Economia i Coneixement, Generalitat de Catalunya; ES, EU). The research group is accredited by SGR 2014-697 and TECNIO net from Departament d'Economia i Coneixement- ACCIÓ Catalonia.

REFERENCES

Alakomi H.-L., Skyttä E., Saarela M., Mattila-sandholm T., Latva-kala K., Helander I.M. (2000) Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*, **66**, 2001–2005.

Arena M.P., Silvain A., Normanno G., Grieco F., Drider D., Spano G., Fiocco D. (2016) Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers in Microbiology*, **7**, 464.

Ben Omar N., Abriouel H., Keleke S., Sánchez Valenzuela A., Martínez-Cañamero M., Lucas López R., Ortega E., Gálvez A. (2008) Bacteriocin-producing *Lactobacillus* strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. *International Journal of Food Microbiology*, **127**, 18–25.

Biondi E., Dallai D., Brunelli A., Bazzi C., Stefani E. (2009) Use of a bacterial antagonist for the biological control of bacterial leaf/fruit spot of stone fruits. *IOBC/WPRS Bulletin*, **43**, 277–281.

Braun P.G., Hildebrand P.D. (2013) Effect of sugar alcohols, antioxidants and activators of systemically acquired resistance on severity of bacterial angular leaf spot (*Xanthomonas fragariae)* of strawberry in controlled environment conditions. *Canadian Journal of Plant Pathology*, **35**, 20–26.

Cabrefiga J., Francés J., Montesinos E., Bonaterra A. (2011) Improvement of fitness and efficacy of a fire blight biocontrol agent via nutritional enhancement combined with osmoadaptation. *Applied and Environmental Microbiology*, **77**, 3174–3181.

Cameron A., Sarojini V. (2014) *Pseudomonas syringae* pv. *actinidiae*: chemical control, resistance mechanisms and possible alternatives. *Plant Pathology*, **63**, 1–11.

Cellini A., Buriani G., Rocchi L., Eondelli E., Savioli S., Rodriguez Estrada M.T., Cristescu S.M., Costa G., Spinelli F. (2018) Biological relevance of volatile organic compounds emitted during the pathogenic interactions between apple plants and *Erwinia amylovora*. *Molecular Plant Pathology*, **19**, 158–168.

Cellini A., Fiorentini L., Buriani G., Yu J., Donati I., Cornish D.A., Novak B., Costa G., Vanneste J.L., Spinelli F. (2014) Elicitors of the salicylic acid pathway reduce incidence of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidae*. *Annals of Applied Biology*, **165**, 441–453.

Chagnaud P., Machinis K., Coutte L.A., Marecat A., Mercenier A. (2001) Rapid PCR-based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. *Journal of Microbiological Methods*, **44**, 139–148.

Colombi E., Straub C., Künzel S., Templeton M.D., McCann H.C., Rainey P.B. (2017) Evolution of copper resistance in the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* through acquisition of integrative conjugative elements and plasmids. *Environmental Microbiology*, **19**, 819–832.

Crowley S., Mahony J., Van Sinderen D. (2013) Broad-spectrum antifungal-producing lactic acid bacteria and their application in fruit models. *Folia Microbiologica*, **58**, 291–299.

Daranas N., Badosa E., Francés J., Montesinos E., Bonaterra A. (2018a) Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. *PLoS ONE*, **13**, e0190931.

Daranas N., Bonaterra A., Francés J., Cabrefiga J., Montesinos E., Badosa E. (2018b) Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method. *Applied and Environmental Microbiology*, **84**, e00107-18.

de las Rivas B., Marcobal A., Muñoz R. (2006) Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiology*, **152**, 85–93.

Di Cagno R., Minervini G., Sgarbi E., Lazzi C., Bernini V., Neviani E., Gobbetti M. (2010) Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing *Lactobacillus plantarum* isolates from raw vegetables and fruits. *International Journal of Food Microbiology*, **143**, 246–253.

Donati I., Buriani G., Cellini A., Mauri S., Costa G., Spinelli F. (2014) New insights on the bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*). *Journal of Berry Research*, **4**, 53–67.

Fhoula I., Najjari A., Turki Y., Jaballah S., Boudabous A., Ouzari H. (2013) Diversity and antimicrobial properties of lactic acid bacteria isolated from rhizosphere of olive trees and desert truffles of Tunisia. *BioMed Research International*, **2013**, 1–14.

Gallelli A., Talocci S., L'aurora A., Loreti S. (2011) Detection of *Pseudomonas syringae* pv. *actinidiae*, causal agent of bacterial canker of kiwifruit, from symptomless fruits and twigs, and from pollen. *Phytopathologia Mediterranea*, **50**, 462–472.

Giddens S.R., Houliston G.J., Mahanty H.K. (2003) The influence of antibiotic production and pre-emptive colonization on the population dynamics of *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 and *Erwinia amylovora* in planta. *Environmental Microbiology*, **5**, 1016–1021.

Haidar R., Deschamps A., Roudet J., Calvo-Garrido C., Bruez E., Rey P., Fermaud M. (2016) Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control*, **92**, 55–65.

Hazel W., Civerolo E. (1980) Procedures for growth and inoculation of *Xanthomonas fragariae*, causal organism of angular leaf spot of strawberry. *Plant Disease*, **64**, 178–181.

Henry P.M., Gebben S.J., Tech J.J., Yip J.L., Leveau J.H.J. (2016) Inhibition of *Xanthomonas fragariae*, causative agent of angular leaf spot of strawberry, through iron deprivation. *Frontiers in Microbiology*, **7**, 1589.

Holt S.M., Cote G.L. (1998) Differentiation of dextran-producing *Leuconostoc* strains by a modified randomly amplified polymorphic DNA protocol. *Applied and Environmental Microbiology*, **64**, 3096–3098.

Kavroulakis N., Ntougias S., Besi M.I., Katsou P., Damaskinou A., Ehaliotis C., Zervakis G.I., Papadopoulou K.K. (2010) Antagonistic bacteria of composted agro-industrial residues exhibit antibiosis against soil-borne fungal plant pathogens and protection of tomato plants from *Fusarium oxysporum* f.sp. *radicis-lycopersici*. *Plant and Soil*, **333**, 233–247.

Kawaguchi A., Inoue K., Inoue Y. (2014) Biological control of bacterial spot on peach by nonpathogenic *Xanthomonas campestris* strains AZ98101 and AZ98106. *Journal of General Plant Pathology*, **80**, 158–163.

Kim D.-R., Gang G.-H., Jeon C.-W., Kang N.J., Lee S.-W., Kwak Y.-S. (2016) Epidemiology and control of strawberry bacterial angular leaf spot disease caused by *Xanthomonas fragariae*. *The Plant Pathology Journal*, **32**, 290–299.

Köhl J., Postma J., Nicot P., Ruocco M., Blum B. (2011) Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biological Control*, **57**, 1–12.

Lalancette N., Mcfarland K.A. (2007) Phytotoxicity of copper-based bactericides to peach and nectarine. *Plant Disease*, **91**, 1122–1130.

Lamichhane J.R. (2014) *Xanthomonas arboricola* diseases of stone fruit, almond, and walnut trees: progress toward understanding and management taxonomic relationships. *Plant Disease*, **98**, 1600–1610.

Lamichhane J.R., Arendse W., Dachbrodt-Saaydeh S., Kudsk P., Roman J.C., van Bijsterveldt-Gels J.E., Wick M., Messéan A. (2015) Challenges and opportunities for integrated pest management in Europe: A telling example of minor uses. *Crop Protection*, **74**, 42–47.

Lindow S.E., Brandl M.T. (2003) Microbiology of the phyllosphere. *Applied and Environmental Microbiology*, **69**, 1875–1883.

Llop P., Caruso P., Cubero J., Morente C., López M.M. (1999) A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods*, **37**, 23–31.

López I., Torres C., Ruiz-Larrea F. (2008) Genetic typification by pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) of wild *Lactobacillus plantarum* and *Oenococcus oeni* wine strains. *European Food Research and Technology*, **227**, 547–555.

Matyjaszczyk E. (2015) Products containing microorganisms as a tool in integrated pest management and the rules of their market placement in the European Union. *Pest Management Science*, **71**, 1201–1206.

McManus P.S., Stockwell V.O., Sundin G.W., Jones A.L. (2002) Antibiotic use in plant agriculture. *Annual Review of Phytopathology*, **40**, 443–465.

Monchiero M., Gullino M.L., Pugliese M., Spadaro D., Garibaldi A. (2015) Efficacy of different chemical and biological products in the control of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit. *Australasian Plant Pathology*, **44**, 13–23.

Montesinos E., Bonaterra A. (2017) Pesticides, Microbial, In *Reference Module in Life Sciences*. Amsterdam, Netherlands: Elsevier.

Mora I., Cabrefiga J., Montesinos E. (2015) Cyclic lipopeptide biosynthetic genes and products, and inhibitory activity of plant-associated *Bacillus* against phytopathogenic bacteria. *PLoS ONE*, **10**, e0127738.

Pridmore R.D., Pittet A.-C., Praplan F., Cavadini C. (2008) Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. *FEMS Microbiology Letters*, **283**, 210–215.

Rahn K., De Grandis S.A., Clarke R.C., McEwen S.A., Galán J.E., Ginocchio C., Curtiss R., Gyles C.L. (1992) Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes*, **6**, 271–279.

Reglinski T., Vanneste J.L., Wurms K., Gould E., Spinelli F., Rikkerink E. (2013) Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae*. *Frontiers in Plant Science*, **4**, 24.

Reis J.A., Paula A.T., Casarotti S.N., Penna A.L.B. (2012) Lactic acid bacteria antimicrobial compounds: Characteristics and applications. *Food Engineering Reviews*, **4**, 124–140.

Roberts P.D., Jones J.B., Chandler C.K., Stall R.E. (1997) Disease progress, yield loss, and control of *Xanthomonas fragariae* on strawberry plants. *Plant Disease*, **81**, 917–921.

Roselló G., Bonaterra A., Francés J., Montesinos L., Badosa E., Montesinos E. (2013) Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*. *European Journal of Plant Pathology*, **137**, 621–633.

Roselló G., Francés J., Daranas N., Montesinos E., Bonaterra A. (2017) Control of fire blight of pear trees with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid. *Journal of Plant Pathology*, **99** (Special issue), 111–120.

Shrestha A., Kim B.S., Park D.H. (2014) Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. *Biocontrol Science and Technology*, **24**, 763–779.

Spinelli F., Donati I., Vanneste J.L., Costa M., Costa G. (2011) Real time monitoring of the interactions between *Pseudomonas syringae* pv. *actinidiae* and *Actinidia* species. *Acta Horticulturae*, **913**, 461–465.

Sundin G.W., Werner N.A., Yoder K.S., Aldwinckle H.S. (2009) Field evaluation of biological control of fire blight in the eastern United States. *Plant Disease*, **93**, 386–394.

Tailliez P., Tremblay J., Dusko Ehrlich S., Chopin A. (1998) Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). *Systematic and Applied Microbiology*, **21**, 530–538.

Tanganurat W., Quinquis B., Leelawatcharamas V., Bolotin A. (2009) Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables. *Journal of Basic Microbiology*, **49**, 377–385.

Torriani S., Felis G.E., Dellaglio F. (2001) Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Applied and Environmental Microbiology*, **67**, 3450–3454.

Trias R., Bañeras L., Badosa E., Montesinos E. (2008a) Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International Journal of Food Microbiology*, **123**, 50–60.

Trias R., Bañeras L., Montesinos E., Badosa E. (2008b) Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. *International Microbiology*, **11**, 231–236.

Tsuda K., Tsuji G., Higashiyama M., Ogiyama H., Umemura K., Mitomi M., Kubo Y., Kosaka Y. (2016) Biological control of bacterial soft rot in Chinese cabbage by *Lactobacillus plantarum* strain BY under field conditions. *Biological Control*, **100**, 63–69.

Visser R., Holzapfel W.H., Bezuidenhout J.J., Kotze J.M. (1986) Antagonism of lactic acid bacteria against phytopathogenic bacteria. *Applied and Environmental Microbiology*, **52**, 552–555.

Wang H., Yan H., Shin J., Huang L., Zhang H., Qi W. (2011) Activity against plant pathogenic fungi of *Lactobacillus plantarum* IMAU10014 isolated from Xinjiang koumiss in China. *Annals of Microbiology*, **61**, 879–885.

Wicaksono W.A., Jones E.E., Casonato S., Monk J., Ridgway H.J. (2018) Biological control of *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, using endophytic bacteria recovered from a medicinal plant. *Biological Control*, **116**, 103–112.

Yáñez-López R., Torres-Pacheco I., Guevara-González R.G., Hernández-Zul M.I., Quijano-Carranza J.A., Rico-García E. (2012) The effect of climate change on plant diseases. *African Journal of Biotechnology*, **11**, 2417–2428.

Zwielehner J., Handschur M., Michaelsen A., Irez S., Demel M., Denner E.B.M., Haslberger A.G. (2008) DGGE and real-time PCR analysis of lactic acid bacteria in bacterial communities of the phyllosphere of lettuce. *Molecular Nutrition and Food Research*, **52**, 614–623.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Lactic acid bacteria and bacterial plant pathogen strains used in this study.

Figure S1 Dendrogram according to the MLST typing of 71 *L. plantarum* strains, including 45 strains of this study (Trias *et al*., 2008a, 2008b; Roselló *et al*., 2013) and 26 strains from other studies. The analysis included allelic profiles of genes *pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS*. Colour codes of strains: green, this study; red, de las Rivas *et al*. (2006); and blue, Tanganurat *et al*. (2009). Cluster analysis was performed by MEGA version 5.1 software (http://www.megasoftware.net) using the unweighted pair group method with arithmetic averages (UPGMA) and the Kimura two-parameter model (1000 Bootstrap method). Bootstrap confidence intervals, origin of the isolates, sequence type (ST) and allelic profiles (in brackets) are indicated.

Table S1. Lactic acid bacteria and bacterial plant pathogen strains used in this study

Species	Code strain [a]	Host	Geographical origin	Growth medium [b]
Lactic acid bacteria [c]				
Lactobacillus plantarum	AC58, AC59, AC73, AC81, AC84	Aubergine	Spain	MRS
	BC24, BC30, BC37, BC50, BC66	Chard	Spain	MRS
	CC31, CC100, CC121	Cucumber	Spain	MRS
	CC70, CC85, CC93	Cabbage	Spain	MRS
	CM450	Courgette	Spain	MRS
	CM466	Persimmon	Spain	MRS
	FC248, FC534	Fig	Spain	MRS
	NC568	Loquat	Spain	MRS
	PC40, PC49, PC67	Potato	Spain	MRS
	PM314, PM340, PM411, PM411R [d]	Pear	Spain	MRS
	RC526	Blackberry	Spain	MRS
	TC26, TC28, TC35, TC41, TC43, TC44, TC46, TC54, TC60, TC69, TC71, TC92, TC92R [d], TC97, TC101, TC102, TC110, TM106	Tomato	Spain	MRS
Lactobacillus pentosus	SE217, SE294, SE304, SE307	Soya beans	Spain	MRS
	BM305	Broccoli	Spain	MRS
Leuconostoc mesenteroides	CM160	Cherry	Spain	MRS
	CM209	Lettuce	Spain	MRS
	PM366	Peach	Spain	MRS
Lactococcus lactis	SE303	Soya beans	Spain	MRS
Non-identified	FC560	Fig	Spain	MRS
Bacterial plant pathogens				
Pseudomonas syringae pv. *actinidiae* (Psa)	CFBP7286, CFBP7286-GFPuv [e]	*Actinidia chinensis*	Italy	LB
	NCPPB3739	*Actinidia deliciosa*	Japan	LB
	IVIA 3700-1 [f]	*A. deliciosa*	Portugal	LB
Xanthomonas arboricola pv. *pruni* (Xap)	CFBP3894	*Prunus salicina*	New Zealand	LB
	CFBP5563	*Prunus persica*	France	LB
Xanthomonas fragariae (Xf)	IVIA XF349-9A [f]	*Fragaria vesca*	Spain	B medium
	CECT549	*Fragaria chiloensis* var. *ananassa*	USA	B medium

[a] CFBP, Collection Française de Bactéries Phytopathogènes (INRA, France); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); IVIA, Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain); NCPPB, National Collection of Plant Pathogenic Bacteria (Fera, UK).

[b] MRS (de Man, Rogosa and Sharpe), LB (Luria Bertani), B medium (Hazel & Civerolo 1980).

[c] INTEA-CIDSAV culture collection (Trias *et al*., 2008a, 2008b; Roselló *et al*., 2013). LAB strains were identified at species level based on 16S rDNA sequences. *L. plantarum* "group" was confirmed using species-specific primers (PLANT1/LOWLAC) (Chagnaud *et al*., 2001) by PCR amplification. Positive isolates for specie-specific PCR were then tested by multiplex PCR in a second step for the identification of *L. plantarum, L. paraplantarum* and *L. pentosus* with *recA* gene-based primers paraF, pentF, planF and pREV (Torriani *et al*., 2001).

[d] Spontaneous mutants of *L. plantarum* PM411 and TC92 strains resistant to rifampicin.

[e] Courtesy of Dr. F. Spinelli, Department of Agricultural and Food Sciences, University of Bologna, Italy (Spinelli *et al*., 2011).

[f] Courtesy of Dra. M. M. López, IVIA, Valencia, Spain.

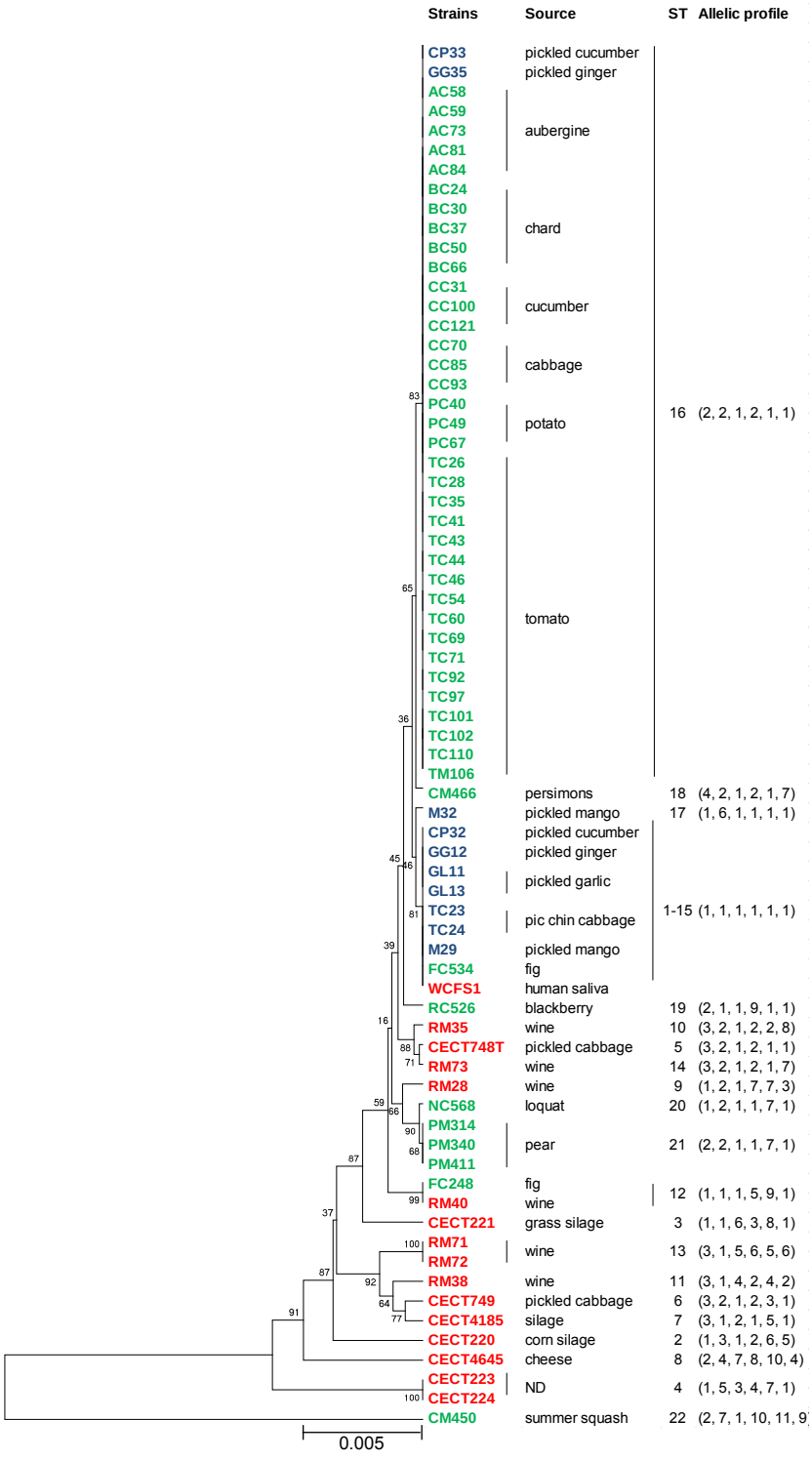


Figure S1 Dendrogram according to the MLST typing of 71 *L. plantarum* strains, including 45 strains of this study (Trias *et al*., 2008a, 2008b; Roselló *et al*., 2013) and 26 strains from other studies. The analysis included allelic profiles of genes *pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS*. Colour codes of strains: green, this study; red, de las Rivas *et al*. (2006); and blue, Tanganurat *et al*. (2009). Cluster analysis was performed by MEGA version 5.1 software (http://www.megasoftware.net) using the unweighted pair group method with arithmetic averages (UPGMA) and the Kimura two-parameter model (1000 Bootstrap method). Bootstrap confidence intervals, origin of the isolates, sequence type (ST) and allelic profiles (in brackets) are indicated.

CHAPTER IV

Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments

Daranas N, Badosa E, Francés J, Montesinos E, Bonaterra A

PLoS ONE

2018, 13(1):e0190931

DOI:10.1371/journal.pone.0190931

PLOS ONE

RESEARCH ARTICLE

Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments

Núria Daranas, Esther Badosa, Jesús Francés, Emilio Montesinos, Anna Bonaterra*

Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Girona, Spain

* anna.bonaterra@udg.edu

OPEN ACCESS

Citation: Daranas N, Badosa E, Francés J, Montesinos E, Bonaterra A (2018) Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. PLoS ONE 13(1): e0190931. https://doi.org/10.1371/journal.pone.0190931

Editor: Sudisha Jogaiah, Karnatak University, INDIA

Received: September 15, 2017

Accepted: December 24, 2017

Published: January 5, 2018

Data Availability Statement: All relevant data are within the paper.

Funding: Funding was provided by Spain MINECO (Ministerio de Economía y Competitividad) (AGL2012-39880-C02-01 and AGL2015-69876-C2-1-R) and FEDER of the European Union and in part by project FP7-KBBE.2013.1.2-04 613678 DROPSA of the European Union. N. Daranas was recipient of a research grant 2015 FI_B00515 (Secretaria d'Universitats i Recerca, Departament d'Economia i Coneixement, Generalitat de

Abstract

Lactobacillus plantarum strains PM411 and TC92 can efficiently control bacterial plant diseases, but their fitness on the plant surface is limited under unfavourable low relative humidity (RH) conditions. To increase tolerance of these strains to water stress, an adaptive strategy was used consisting of hyperosmotic and acidic conditions during growth. Adapted cells had higher survival rates under desiccation than non-adapted cells. Transcript levels and patterns of general stress-related genes increased immediately after the combined-stress adaptation treatment, and remained unaltered or repressed during the desiccation challenge. However, there were differences between strains in the transcription patterns that were in agreement with a better performance of adapted cells of PM411 than TC92 in plant surfaces under low RH environmental conditions. The combined-stress adaptation treatment increased the survival of PM411 cells consistently in different plant hosts in the greenhouse and under field conditions. Stress-adapted cells of PM411 had similar biocontrol potential against bacterial plant pathogens than non-adapted cells, but with less variability within experiments.

Introduction

The development of practical strategies for the sustainable management of plant diseases to minimize the use of environmentally aggressive pesticides pose a challenge to worldwide crop production [1–2]. The number of crops without efficient protection methods has increased in recent years and this fact has stimulated an increasing demand of beneficial plant-microbe interactions to withdraw biotic and abiotic stresses [3–5]. Thus, there is a need for finding new and more effective biological control agents (BCA) and biostimulants, and also for optimizing the methods by which these new products are made viable, durable, robust and economical [6–8]. *Lactobacillus plantarum* strains have been reported as novel BCA for bacterial diseases control such as fire blight of rosaceous plants [9–10].

The establishment of the BCA on plant organs, prior to the arrival of the pathogen, is a key factor to achieve an efficient biocontrol of aerial plant diseases. In many BCA, a population decline is often observed after application to plants, thus reducing biocontrol efficiency

Catalunya; ESF, EU). The research group is accredited by SGR 2014-697 and TECNIO net from Catalonia.

Competing interests: The authors have declared that no competing interests exist.

[11–12]. This decline of population is due to the shift from laboratory culture conditions to the growth-limiting plant surface [13]. In addition, microorganisms are exposed on the phyllosphere to ultraviolet radiation, nutrient limitation, and fluctuating water availability [14–15]. These reasons may explain the limited efficacy of lactic acid bacteria like *L. plantarum* strains observed under non-optimal environmental conditions.

However, lactic acid bacteria (LAB) are able to tolerate several stresses (pH, salt, heat, inhibitory compounds) [16], and as in other bacteria, *L. plantarum* have several mechanisms to enhance survival under stressful environments [17–19]. These mechanisms include the synthesis of chaperone proteins (GroES/GroEL and DnaK/DnaJ/GrpE), which assist the folding of misfolded proteins, and proteases (Clp family of proteins, such as ClpA, ClpB, ClpC, ClpE, ClpL and ClpX), which degrade damaged proteins; both regulated mainly by the transcriptional repressors CtsR and FtsH [20–21]. Also, small heat shock proteins (sHSP) and cold shock proteins (CSP) function as folding chaperones, and play an important role in maintaining membrane integrity under stress conditions. Cells exhibiting an increased synthesis of these proteins have shown a greater ability to survive under stress conditions [22–24]. In addition to stress related proteins, bacteriocins (e.g. plantaricins) may confer competitive advantages to *L. plantarum* [25–26] and their synthesis is affected by growth conditions and stress [27]. Also, the adhesion-like factor EF-Tu may function as an "envelope associated protein", which can be released from the cell when they experience osmotic shock [28–29]. As it has been suggested, transcriptional profiling of stress-related, bacteriocin-encoding and adhesion factor genes could be used as a tool to evaluate the suitability of adaptation strategies, and to compare strains adaptability to a stressful environment [30–33].

Different strategies have been used to increase BCA adaptation to stress and to improve its epiphytic fitness upon delivery to the field. For example, cells have been adapted during its cultivation to a sub-lethal dose of a specific physical or chemical stress [34–36] or to heat shock [37–38]. These adaptation treatments can significantly improve the subsequent performance of the microorganism under suboptimal conditions, not only to the specific stressing factor used during adaptation, but also to a range of different stresses, a phenomenon known as cross-protection [39–40]. In lactic acid bacteria it has been described that the exposure to a single stressor is commonly associated to the cross-tolerance to other stressors like acid [41], heat shock [42] or desiccation [43]. The development of cross-tolerance to stress has been described in *L. plantarum* strains from food processing and probiotics [24, 44]. However, there are no reports on tolerance to stress in *L. plantarum* biological control strains of plant diseases.

In the present work, we wanted to physiologically improve the fitness of *L. plantarum* strains PM411 and TC92, by means of a strategy of tolerance to desiccation stress and low relative humidity conditions that are common problems onto plant surfaces. We used a procedure consisting of the adaptation of cells to salt and/or acidic pH conditions during inoculum preparation. The response of both strains to stress was compared by means of: (1) the effect of different adaptation treatments on cell inactivation during desiccation stress, and on plant surfaces under controlled environment conditions at low relative humidity (RH), and (2) the transcriptional pattern of selected genes after the adaptation treatment and during the desiccation challenge. Finally, (3) the effect of the adaptation treatment on the survival of PM411 on different plant surfaces under greenhouse and field conditions and on the efficacy of biocontrol was studied.

Materials and methods

Bacterial strains and growth conditions

L. plantarum strains PM411 and TC92 were isolated from pear and tomato, respectively, as described previously [10, 45]. Strains were cultured in Man, Rogosa and Sharpe broth (MRS)

(Oxoid; Unipath Ltd., Basingstoke, Hampshire, England) at 30˚C in an orbital shaker at 80 rpm. Stock cultures were stored at -80˚C in MRS containing 20% (v/v) glycerol. To monitor culturable populations of PM411 and TC92 in plant colonization studies, spontaneous mutants resistant to rifampicin (PM411R and TC92R) were used [10]. *Erwinia amylovora* EPS101 isolated from an infected shoot of a Conference pear tree in Lleida (Spain) [35] and *Xanthomonas fragariae* CECT549 (Colección Española de Cultivos Tipo) were used in pathogen inoculation experiments. Bacterial suspensions of the pathogens were obtained from ultrafreeze-preserved cultures (-80˚C) grown overnight at 25˚C in Luria–Bertani (LB) agar for *E. amylovora* and in medium B agar [46] for *X. fragariae*. Colonies were scraped from the agar surface and suspended in sterile distilled water. The culture was adjusted to a cell density corresponding to 1 x 10^8 CFU mL^{-1} (OD_{600} of 0.12 and 0.40 for *E. amylovora* and *X. fragariae*, respectively) and diluted with sterile distilled water to obtain an appropriate concentration.

Adaptation treatments

PM411 and TC92 cells were cultured overnight in MRS broth until an OD_{600} of 0.8–1.0, harvested by centrifugation (10000 x g, 10 min at 10˚C), and washed in 50 mM sterile phosphate buffer (PBS, pH 7.0). Washed cells were resuspended in the corresponding adaptation medium at an initial OD_{600} of 0.2, or cultured accordingly. The adaptation treatments performed were acidic, hyperosmotic, stationary phase, combined-stress, and non-adapted (Fig 1). For the acidic treatment (A), cells were cultured until mid-log phase (final conditions OD_{600} = 0.6, pH 3.8 ± 0.2) in MRS broth at pH 4.0 (modified by the addition of HCl 1N). For the hyperosmotic treatment (O), cells were cultured until mid-log phase in MRS broth (pH 6.2) amended with NaCl 1 M (final conditions OD_{600} = 0.6, pH 5.3 ± 0.2). For the stationary phase treatment (S), cells were cultured in MRS broth at pH 6.2 until stationary phase (final conditions OD_{600} = 1.0, pH 3.8 ± 0.2) (no pH control). For the combined-stress treatment (C), cells were cultured in MRS broth (pH 6.2) amended with NaCl 1 M until stationary phase (final conditions OD_{600} = 1.0, pH of 3.8 ± 0.2). For non-adapted treatment (N), cells were cultured in MRS broth (pH 6.2), until mid-log phase (final conditions OD_{600} = 0.6, pH = 5.3 ± 0.2). All treatments were incubated at 30˚C with shaking at 80 rpm. Three independent biological replicates were prepared for each *L. plantarum* strain (PM411 and TC92) and treatment combination. Two independent experiments were performed.

Effect of adaptation treatments on cell survival during desiccation

Previously to the water stress challenge, cells were obtained from cultures grown under different adaptation conditions, as described above. In the first experiment, the adaptation treatments performed were A, O, S, C, and N, and subsequently cells were exposed to desiccation along a period of 6 days (long-term). In the second experiment, the adaptation treatments were A, C, and N, and the exposure to desiccation was along 5 h (short-term). In both experiments, cells from culture aliquots (25 ml) were harvested by centrifugation (10000 x g, 10 min at 10˚C), washed once in PBS and resuspended in 25 ml of sterile PBS (pH 7.0). In the case of O and C cells, the sterile PBS used to wash cells was amended with NaCl to a final concentration of 1 M. Then, aliquots of 1 ml were distributed into 1.5 ml Eppendorf tubes and harvested by centrifugation at 6000 x g for 5 min. The supernatant was discarded and the pellet containing cells was dried in a vacuum desiccator using silica gel as desiccant as described [34]. The tubes were maintained opened in the desiccator at 25˚C. In the first experiment, samples for cell survival analysis (an Eppendorf tube) were taken at 1, 2 and 6 days after the start of the desiccation challenge. In the second experiment, tubes were taken at 0.5, 1, 1.5, 2, 3, 4 and 5 h. The experimental design consisted of three independent biological replicates of each

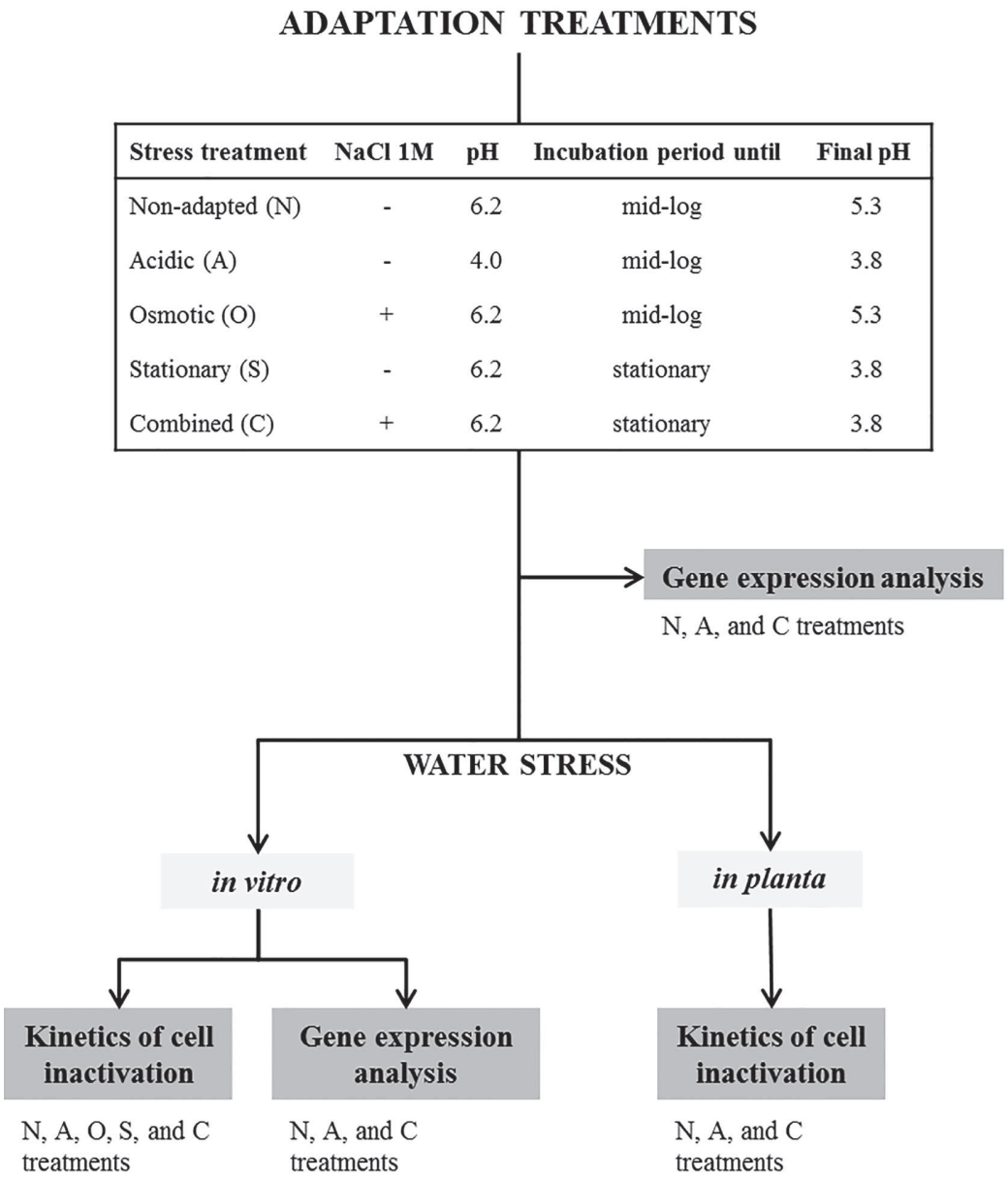


Fig 1. Schematic representation of the adaptation treatments and water stress challenge, and the analysis performed in *L. plantarum* PM411 and TC92 strains. Gene expression of cells was studied in non-adapted (N), acidic (A), and combined-stress (C) conditions after the adaptation treatments. After the treatments (N, A, and C) cells were exposed to water stress (i) *in vitro* for the study of cell inactivation kinetics and gene expression, and (ii) *in planta* for the study of cell inactivation kinetics.

https://doi.org/10.1371/journal.pone.0190931.g001

adaptation treatment and date of assessment, for each strain and experiment. For cell survival analysis, the pellets were resuspended in 1 ml of sterile PBS (pH 7.0) and appropriate dilutions of the suspensions were seeded onto MRS agar plates (Panreac, Barcelona, Spain) using a spiral plater system (Eddy Jet; IUL Instruments, Barcelona, Spain). Plates were incubated at 23˚C for 48 h. Colonies were counted using an automatic counter system (Countermat Flash; IUL Instruments) and counts were transformed to CFU ml^{-1} of culture. Non-desiccated cells of

each adaptation treatment (N, A, O, S, and C) served as controls. The inactivation of cells for each treatment was calculated as log N_0/N, where N_0 is the initial number of unstressed cells (control) and N the number of survivors.

Effect of adaptation treatments on survival of *L. plantarum* under low relative humidity conditions

Apple flowers. Open blossoms were collected from an experimental field of Golden Smoothie apple cultivar near Girona (Mas Badia Agricultural Experiment Station), taken to the laboratory under refrigeration, and used within 24 h. The procedure was essentially as described [47]. Briefly, detached flowers were maintained with the cut peduncle submerged in a sucrose solution in Eppendorf tubes and placed in transparent plastic boxes. Flowers were treated with a hand sprayer to the runoff point (0.4 ml per flower), with suspensions of PM411R and TC92R cells (10^8 CFU ml^{-1}) from A, C, or N adaptation treatments. Adapted and non-adapted cells were obtained as described above, and resuspended in quarter-strength Ringer's solution by setting the OD_{600} of cell suspensions to 0.25 (confirmed by colony counts, 10^8 CFU ml^{-1}). After the corresponding treatment, inoculated flowers were placed at 25±1˚C, with 16 h of fluorescent light/8 h dark and under low relative humidity (RH) conditions (50%). RH conditions were obtained in controlled environment chambers (SGC097.PFX.F, Fitotron, Sanyo Gallenkamp plc, UK) by placing $CaCl_2$ into the chamber as a humidity absorber. The experimental design consisted of three independent biological replicates of each adaptation treatment and date of assessment for each strain. Two independent experiments were performed. Samples of 5 flowers from each replicate were taken for monitoring population levels of PM411R or TC92R at 1, 2 and 6 days post inoculation. Flowers were homogenized with 20 ml of sterile PBS (pH 7.0) and 0.1% peptone using a stomacher (Masticator; IUL Instruments). Sample homogenates were diluted and appropriate dilutions were seeded onto MRS agar plates supplemented with 50 μg ml^{-1} of rifampicin (Sigma, Missouri, USA) to counter-select the corresponding strain inoculated. Plates contained 10 μg ml^{-1} of econazole nitrate salt (Sigma, Missouri, USA) to avoid fungal growth. Plates were incubated at 23˚C for 48 h and colonies were counted. The population levels of PM411R or TC92R were expressed as CFU per flower. A sample of recently inoculated flowers by each adapted (A and C) and non-adapted (N) strain served as a control. The inactivation index for each treatment was calculated as described above.

***Prunus* leaves.** *Prunus amygdalus* × *P. persica* plants of the rootstock GF-677 were obtained by micropropagation (Agromillora, Barcelona, Spain). Plants of 20 cm in length were grown in 10-cm-diameter plastic pots having 6 to 10 young leaves, and were maintained in a greenhouse at 26 ± 2˚C, 60 ± 10% of RH and a 16-h photoperiod, and were fertilized once a week with a 200 ppm N/P/K solution (20:10:20) and standard insecticide and miticide sprays were applied until use. Plants were inoculated with 10^8 CFU ml^{-1} cell suspensions of A, C, or N adapted PM411R and TC92R cells using a microsprayer until runoff (10 ml per plant). Cell suspensions were prepared as described above. Inoculated plants were placed into transparent plastic boxes at 25˚C, with 16 h of fluorescent light / 8 h dark and under low (60–70%)-RH conditions. Low RH conditions were obtained by placing $CaCl_2$ into the boxes as a humidity absorber. The experimental design consisted of three independent biological replicates (5 plants per replicate) of each adaptation treatment and date of assessment for each strain. Two independent experiments were performed. For monitoring population levels of PM411R or TC92R five leaves were taken from each replicate at 1, 2, 3, 4 and 7 days post-inoculation. Samples were processed and calculations were done as described above.

Transcriptional analysis during adaptation conditions and desiccation challenge

Expression patterns of PM411 and TC92 cells obtained immediately after growth under different adaptation treatments (A, C, and N) and after desiccation challenge were analysed. Total RNA was extracted from PM411 and TC92 cells grown under three adaptation conditions (A, C, and N), and exposed or non-exposed to desiccation challenge. A, C, and N cells were obtained following the procedure described above, immediately after growth (non-desiccated) or at different times during desiccation (0.5, 1, 1.5, 2, 3, 4 and 5 h) (Fig 1). At every sampling time dehydrated cells were suspended in 1 ml of sterile PBS (pH 7.0). RNA from 500 μl of cell suspensions was immediately stabilized in two volumes of RNA Protect Bacteria Reagent (Qiagen, Hilden, Germany). Total RNA was isolated according to the procedure recommended by Qiagen with minor modifications, involving enzymatic lysis together with proteinase K digestion, followed by mechanical disruption. The pellet was resuspended with 200 μl TE buffer containing 15 mg ml^{-1} lysozyme (Sigma). Besides, 20 μl of proteinase K and 6 μl of mutanolysin (Sigma) were added and samples were incubated at 37˚C for 45 min with shaking at 300 rpm. The mechanical disruption was performed adding 50 mg of acid-washed glass beads (Sigma, 150–600 μm) to the sample using the Tissulyser II instrument (Qiagen) at frequency of 30 s^{-1} for 5 min. Extracted total RNA was purified with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and RNA was resuspended in 50 μl RNase free water. The concentration and purity of RNA was assessed by spectrophotometric measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Residual DNA was removed using the Turbo DNA-free kit (Applied Biosystems, Foster City, USA) and cDNA was synthetized from RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The absence of chromosomal DNA contamination was confirmed by minus-reverse transcriptase control in quantitative real-time PCR (qPCR).

Quantitative real-time PCR was carried out in a 7500 Real-Time PCR System (Applied Biosystems) to assess the transcriptional level of *L. plantarum* genes associated to: (i) stress response, i.e. encoding molecular chaperones (*dnaK* and *groEL*), Clp proteins (*clpC* and *clpB*), small heat shock proteins (*hsp1* and *hsp3*), cold shock proteins (*cspP* and *cspL*), stress factors (*ctsR* and *ftsH*), and small redox protein (*trxB1*); (ii) adhesion factor protein (*efTU*), and (iii) plantaricin synthesis (*plnE* and *plnJ*). Optimized reactions included 10 μl SYBR® Green PCR Master Mix (Applied Biosystems), 7 μl RNase-free water, 1 μl of each forward and reverse primer (Table 1) at 2 μM, and 1 μl cDNA in a final volume of 20 μl. The thermal cycling conditions were as follows: 10 min at 95˚C for initial denaturation; 50 cycles of 15 s at 95˚C, and 1 min at 60˚C; and a final melting curve program of 60 to 95˚C with a heating rate of 0.5˚C/s. Each qPCR assay included duplicates of each cDNA sample, no-template and RNA controls to check for contamination. Measures were taken from each condition from three independent biological cultures. The lactate dehydrogenase D gene (*ldhD*) was used as internal control for data normalization [53].

Expression patterns of PM411 and TC92 cells obtained immediately after growth under different adaptation treatments (A, C, and N) were analysed and the normalized expression values of selected genes were quantified and $\log_2$ transformed. Standard curves for quantification of gene expression were created using decimal dilutions of recombinant plasmid DNA (target sequences were cloned into a vector pSpark® in *Escherichia coli* DH5α cells) corresponding to copy numbers ranging between 10^2 and 10^6. Ct values in each dilution were measured in triplicate using the optimized qPCR as described above and a negative non-template control was included in each run. Ct values were plotted against the logarithm

Table 1. Function of genes and primer sequences used in the transcriptional response study.

Function	Gene	Forward primer (5'—3')	Reverse primer (5'—3')	Reference
Chaperone proteins	*dnaK*	TCAACCGTGTCACCCAAGTA	TCCTTCAGTTGTGGCATTCA	[31]
	groEL	ACCGGATTGAAGATGCTTTG	AACCAGCATTTTCAGCGATT	[31]
Proteases	*clpB*	AGTTACCGGCGTCCATACTG	GACTCAAAGCCGTCTCAAG	[48]
	clpC	ATCCTTTCCTCGCGAATTTT	TGGCGTTCCTTCAGTCTTCT	[49]
Small heat shock proteins	*hsp1*	AGGTTGATGTCCCTGGTATTG	TTAAGACACCGTCAGCTTGG	[48]
	hsp2	TTACCTTCGCTATCCCGCAAC	CGGTGAAGTATGCTGACGAA	[50]
	hsp3	ATCCGCAGCTGCCTTCTTT	CGCGAGTGAACGTCAAACTG	[31]
Cold shock proteins	*cspP*	TACTGGTGAAGATGGCAACG	GAACAACGTTAGCAGCTTGTGG	[44]
	cspL	GTGAAGACGGTACCGATGTCTT	GTGGTTGAACGTTCGTTGCT	[44]
Stress factors	*ftsH*	GCAGCTACCTTCGAAGAATCCA	GGGAAACTTGGTTCAGCAACA	[51]
	ctsR	AATTTGGTCGATGATGCTGATG	TAAGTCCCGGTCCGTTAATCC	[51]
Small redox protein	*trxBI*	ATGGCAAAGAGTTACGACG	CCCACCATAGATTCCGCGAT	F: [52] R: this study
Adhesion protein	*efTU*	CCACGTAATAACGCACCAAC	TTCTGGTCGTATCGATCGTG	[29]
Plantaricin synthesis	*plnE*	GTTTTAATCGGGGCGGTTAT	ATACCACGAATGCCTGCAAC	[32]
	plnJ	TAAGTTGAACGGGGTTGTTG	TAACGACGGATTGCTCTGC	[32]
D-lactate dehydrogenase	*ldhD*	ACGCCCAAGCTGATGTTATATC	AGTGTCCCACGAGCAAAGTT	[53]

https://doi.org/10.1371/journal.pone.0190931.t001

of their initial template copy numbers and each standard curve was generated by a linear regression of the plotted points.

Besides, the effect of desiccation stress (1, 1.5, 2, 3 and 4h under desiccation) on the transcriptional response of selected genes of PM411 and TC92, depending on the adaptation treatments (A, C, and N), was analysed. In this case, the comparative critical threshold (ΔΔCt) method was used to assess the relative transcriptional level. Similar amplification efficiencies of all genes primer pairs were checked making the ΔΔCt method appropriate to calculate the relative expression (RE) level. The ΔCt of non-desiccated cells, at the corresponding cell-adaptation method (A, C, and N), was used as the calibrating condition to calculate RE level. Genes were considered to be up- or down-regulated if their RE levels were at least twofold (RE level = 2^1 or 2^{-1}) higher or less than the calibrator condition [54, 55].

Effect of combined stress adaptation treatment on the dynamics of population of PM411 on plant surfaces

Two experiments under greenhouse conditions and two field trials at the Mas Badia Agricultural Experiment Station (Girona, Spain) were performed.

Greenhouse experiments. Experiments were conducted in potted plants (10-cm-diameter plastic pots) of cv. Darselect strawberry and cv. Hayward kiwifruit. Plants were used when had 30 to 40 cm in length and 10 to 15 young leaves in the case of kiwifruit plants and 5 to 8 leaves per crown in strawberry plants. Plants were spray inoculated to runoff (6 and 20 mL per strawberry and kiwifruit plants, respectively) with a suspension of PM411R. The inoculum of the biological control agent was prepared as previously described, either adapted with the combined treatment (C) or non-adapted (N). Cells were harvested by centrifugation and resuspended in quarter-strength Ringer's solution to 5 x 10^7 CFU mL^{-1}. After the corresponding treatment, plants were maintained in a greenhouse at 26 ± 2˚C, 70 ± 10% of RH and a 16-h photoperiod. Three replicates of three plants per replicate were used for each adaptation treatment. Sample collection for monitoring population levels of PM411 was performed at 0, 1, 2, 3

and 6 days. Samples of three leaflets for strawberry plants and three leaves for kiwifruit plant were taken from each replicate of the corresponding treatment and sampling date.

Field experiments. Experiments were conducted in plots of cv. Golden Smoothie apple and cv. Comice pear trees. Treatments were distributed in a completely randomized block design with three replications of each treatment and 7 trees per replicate. In each tree two branches containing blossoms were tagged. Treatments corresponded to suspensions of PM411 non-adapted (N) or adapted (C). In both treatments cells were harvested by centrifugation and diluted in quarter-strength Ringer's solution to 5 x 10^7 CFU mL^{-1}. Two strategies were assayed, one doing a single application of bacteria to trees on 29 March 2017 (cv. Comice pear) and on 5 April 2017 (cv. Golden Smoothie apple), and in the second strategy two applications were performed (29 March and 4 April 2017 in the case of pear trees, and 5 April and 11 April 2017 in the case of apple trees). Open blossoms from tagged branches were spray inoculated until near runoff with the bacterial suspension using a hand-held 5 mL sprayer (3 mL per blossom). Weather conditions in the field plots were measured with a weather station located in the experimental field (Mas Badia, La Tallada d'Empordà) (Temperature, RH and rainfall). Sample collection for monitoring PM411 levels was performed at 0, 1, 2, 5, 6 and 7 days. Samples of two blossoms (4–6 flowers and accompanying leaves) were taken from each replicate of the corresponding treatment and sampling date. All the samples (leaves from the greenhouse experiment and blossoms from the field experiment) were homogenized in a sterile plastic bag with 30 mL of 0.05 M phosphate buffer (pH 7.0) and 0.1% peptone under shaking in an orbital shaker at 150 r.p.m. for 30 min at 4˚C. The extract was concentrated 10-fold by centrifugation at 10000 g for 10 min. The extract was serially diluted and appropriate dilutions were seeded onto MRS agar plates supplemented with 50 μg ml^{-1} of rifampicin and 10 μg ml-1 of econazole nitrate salt and population levels of PM411 were assessed as described.

Effect of combined stress adaptation treatment on efficacy of disease control

Two different assays were performed to control angular leaf spot of strawberry (*X. fragariae*) and fire blight of apple and pear (*E. amylovora*).

Angular leaf spot disease of strawberry. Plants were sprayed to runoff with 10 ml of a suspension of adapted or non-adapted cells of PM411. After 24 h, plants were inoculated with a suspension of Xf at 1x10^8 CFU ml^{-1}. Pathogen suspension was prepared in distilled water with diatomaceous earth (1 mg ml^{-1}) and applied using a hand-sprayer (at a pressure of 20 psi) to runoff with 6 ml of the suspension. After inoculation, plants were maintained for 48 h in plastic bags to allow a high relative humidity conditions. Then, plant material was maintained in the quarantine greenhouse at 26˚C (± 2˚C), 60% ± 10 of RH and a 16 h light-8 h dark photoperiod. The experimental design consisted of three replicates of two plants per replicate for each treatment. Kasugamycin treated (Kasumin, Lainco, Barcelona, Spain) (80 mg l^{-1}) and non-treated plants were included as controls.

Two experiments were performed. After two weeks from the pathogen inoculation the severity of infections was determined per each replicate. Severity of infections was determined as the level of infection of each leaf, according to the following scale: 0, no symptoms; 1, presence of small spots of necrosis (<25% of the leaf surface); 2, necrosis progression through the leaf (25–50%) and; 3, leaf mainly affected by necrosis (>50%). The mean severity level per leaf was calculated per each replicate of two plants.

Fire blight control. Open blossoms of pear and apple treated in the experimental orchard plot (see above) were collected at 1 day after treatment, to perform efficacy assays under controlled environmental conditions. The individual flowers used for inoculation with

E. amylovora were prepared as described [35], in single Eppendorf vials [47]. The hypanthium of flowers was then inoculated with 10 μL of a suspension of *E. amylovora* at 10^7 CFUmL^{-}1. The inoculated flowers were again placed in plastic boxes, and incubated at 25˚C and high RH for 5 days. The experimental design consisted of three replicates of five flowers per replicate for each treatment. Kasugamycin treated (80 mg l^{-1}), and non-treated controls inoculated with the pathogen alone were included. Two experiments were performed. Severity of infections on flowers was evaluated per each replicate after 5 days of pathogen inoculation. The severity of infections was determined as the level of infection of each inoculated flower according to a scale from 0 to 3 in function of the symptoms observed: 0, no symptoms; 1, partial hypanthium necrosis; 2, total hypanthium necrosis; 3, necrosis progression through peduncle.

Data analysis

To test the significance of the effect of adaptation treatments on inactivation of PM411 and TC92 to desiccation *in vitro*, and on flowers and leaves, and to test the effect of adaptation treatment of PM411 on the population level on plant surfaces and on biocontrol efficacy against *X. fragariae* and *E. amylovora*, a one-way analysis of variance (ANOVA) was performed. Means of the index of inactivation were separated according to the Tukey's test at $P \leq 0.05$. The statistical analyses were performed using GLM procedure of the PC-SAS (version 9.1; SAS Institute Inc., Cary, NC).

Results

Effect of adaptation treatments on survival of cells during desiccation

The behaviour of acidic (A), hyperosmotic (O), stationary phase (S), combined-stress (C) adapted and non-adapted (N) cells of PM411 and TC92 strains, during a long-term and a short-term period under desiccation stress was compared (Fig 2).

Over the long-term period of desiccation, the C adaptation treatment had the lowest inactivation values (less than 1 log after 6 days of desiccation), significantly different from the other adaptation treatments in both strains ($P < 0.001$). Inactivation values of A, S, and C cells were significantly lower than N cells in both strains ($P < 0.001$). S and A treated cells had inactivation values around 2 log in PM411, while in TC92 these values were around 4 log for A cells and 2 log for S cells. O treated cells had lower inactivation values than N cells in TC92 (specifically at 2 and 6 days under desiccation) but had similar inactivation in the case of PM411cells. Overall, PM411 N cells were less affected by desiccation stress than TC92 N cells.

The short-term experiment was performed once the best treatments were selected. Also in this case the inactivation values of A and C treated cells were significantly lower than in N cells in both strains ($P < 0.001$), being the C adaptation treatment which gave the lowest inactivation values. No significant differences ($P > 0.05$) were observed in inactivation among the treatments in both strains during the first 3 h. However, after this period, the inactivation values increased for A and N treatments. TC92 N cells reached higher inactivation values than PM411 N cells.

Effect of adaptation treatments on survival on flowers and leaves under low relative humidity conditions

The inactivation of PM411 and TC92 previously submitted to adaptation treatments was studied in apple flowers and *Prunus* leaves at low RH (Fig 3).

In both strains, A, C, and N treated cells reached lower inactivation values on flowers (1.1 to 1.5 log at 6 days post inoculation) than on leaves (3.5 to 4.3 log at 7 days post-inoculation).

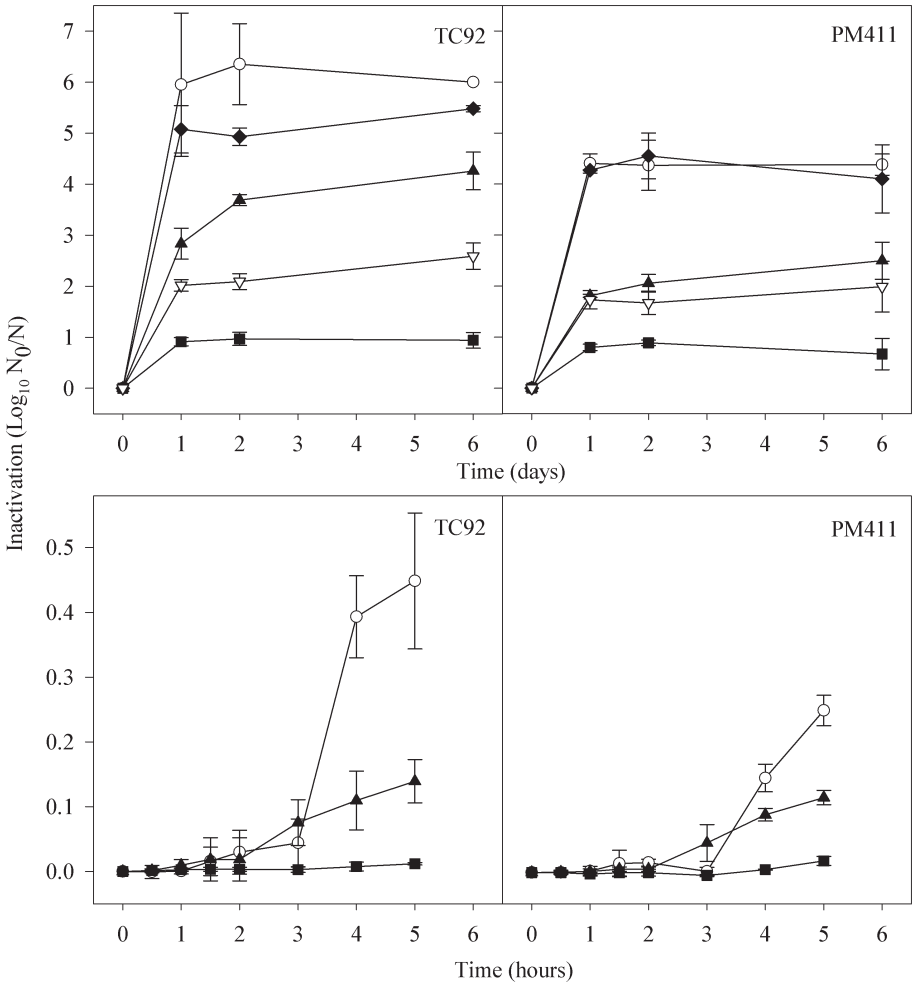


Fig 2. Effect of adaptation treatments on cell inactivation in *L. plantarum* TC92 and PM411 during desiccation. Long-term (top panels) and short-term (bottom panels) periods. Non-adapted, N (○), acid adapted, A (▲), hyperosmotic adapted, O (♦), stationary phase adapted, S (▽), and combined-stress adapted, C (■) cells. Inactivation is shown as log N_0/N, where N_0 is the initial number of cells and N is the number of survivors. Values are the mean of three independent biological replicates and error bars represent the standard deviation of the mean.

https://doi.org/10.1371/journal.pone.0190931.g002

In PM411, the inactivation of C treated cells was significantly lower than in A and N treated cells, in both plant materials ($P < 0.001$). Hence, the C treatment clearly improved PM411 cell survival, both on flowers and leaves. No significant differences in inactivation were observed between adapted and non-adapted TC92 cells, neither on flowers nor on leaves ($P > 0.05$) (except for C treated cells on leaves in day 2, which were significantly lower than A and N treated cells ($P < 0.05$)).

Globally, PM411 C cells showed lower inactivation than TC92 C cells. On leaf surfaces at low RH, inactivation values were 2 log in PM411 compared to 4 log in TC92. However, in blossoms, the inactivation in PM411 C adapted cells did not differ from TC92 cells.

Transcriptional response to adaptation treatments and desiccation stress

The effect of adaptation treatments and desiccation stress on the expression levels and pattern of selected genes in PM411 and TC92 cells was studied. Gene expression during adaptation in

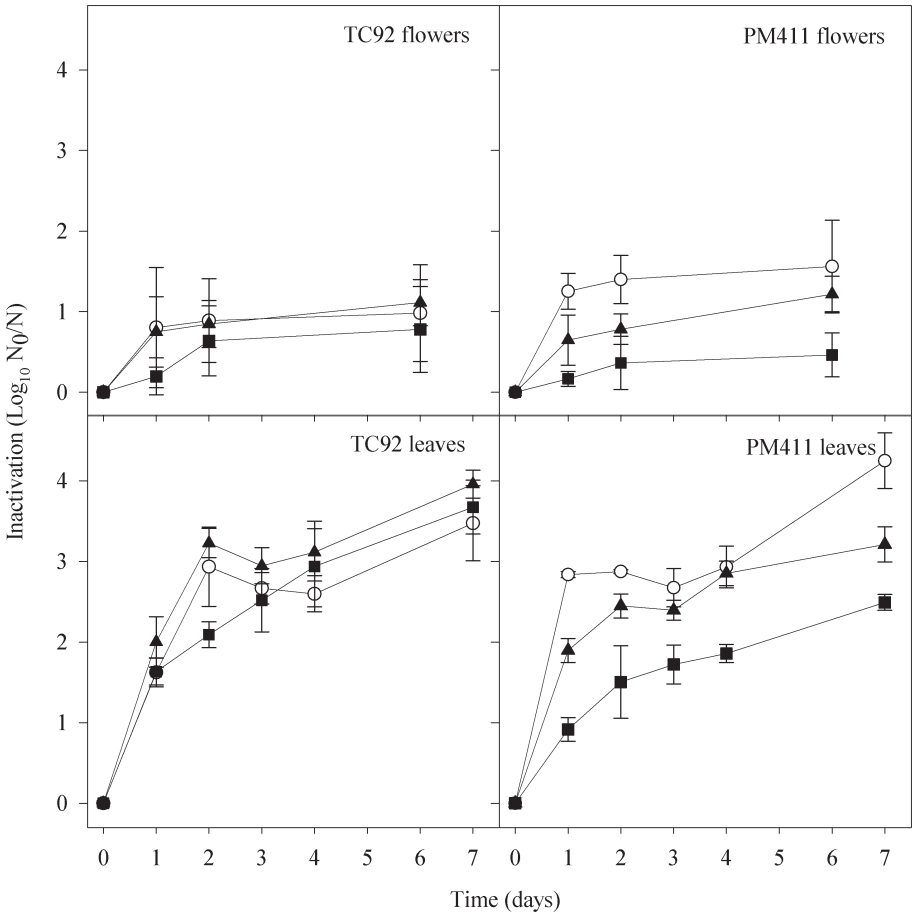


Fig 3. Effect of adaptation treatments on cell inactivation in *L. plantarum* TC92 and PM411 in apple flowers and *Prunus* leaves at low RH. Non-adapted, N (○), acid adapted, A (▲), and combined-stress adapted, C (■) cells. Inactivation is shown as log N_0/N, where N_0 is the initial number of cells and N is the number of survivors. Values are the mean of three independent biological replicates and error bars represent the standard deviation of the mean.

https://doi.org/10.1371/journal.pone.0190931.g003

acid (A), combined-stress (C), and non-adapted (N) conditions is shown in Fig 4. The expression pattern was quite similar in the two strains, but differed depending on treatments. A and N treatments showed similar expression pattern (in 10 out of 14 genes). However, the C treatment showed different expression levels in both strains (in 10 out of 14 genes) in comparison to A and N treatments. Expression values of stress related genes were higher in C than in N treated cells, for 10 genes in PM411 (*dnaK*, *groEL*, *clpB*, *clpC*, *hsp1*, *hsp3*, *cspL*, *ftsH*, *ctsR*, and *trxB1*), and for 7 genes in TC92 (*groEL*, *clpB*, *clpC*, *hsp1*, *hsp3*, *ftsH*, and *trxB1*). The adhesion factor *efTU* and the plantaricins *plnE* and *plnJ* had similar levels of expression in all treatments and in both strains. Interestingly, in C treated cells, in both strains, cold shock proteins (*cspP* and *cspL*) showed the lowest levels of expression of stress-related genes.

Gene expression during desiccation stress, depending on adaptation treatments, is shown in Fig 5. Non-adapted cells showed different desiccation stress response patterns in each strain. In PM411 N cells all genes were upregulated and, in general, their expression levels were kept upregulated over time. On the contrary, in TC92 N cells only *dnaK* and *groEL* genes were kept upregulated over time. So, TC92 N cells had a lower response capacity to desiccation stress than PM411. The transcriptional pattern of A treated cells did not differ significantly from N

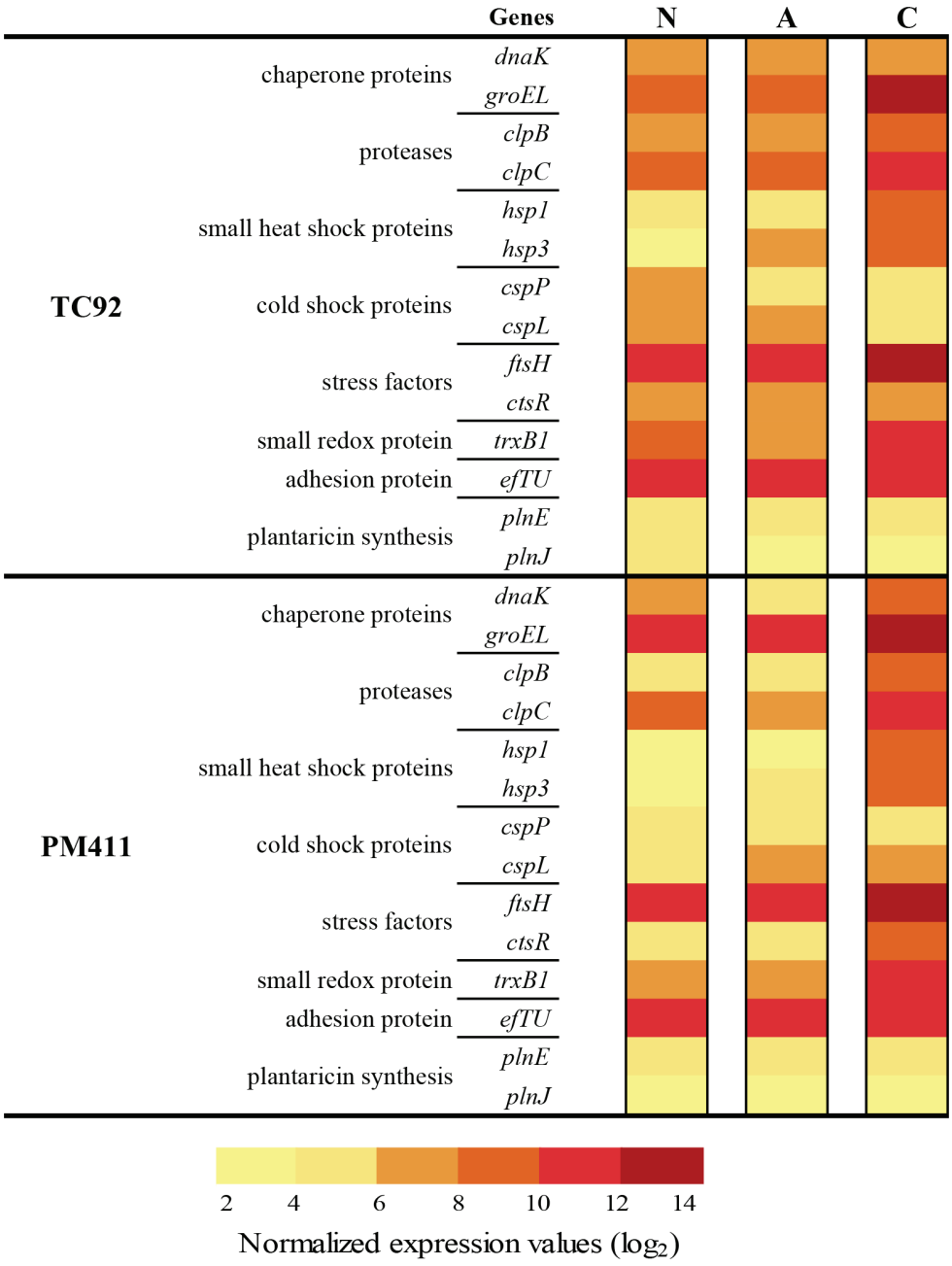


Fig 4. Transcriptional analysis during adaptation conditions in *L. plantarum* TC92 and PM411. Normalized expression values (log_2) of genes associated to stress response, adhesion factor protein, and plantaricin synthesis, in non-adapted (N), acid adapted (A), and combined-stress adapted (C). *ldhD* gene was used for data normalization. Three independent biological replicates were performed.

https://doi.org/10.1371/journal.pone.0190931.g004

cells in both strains. Interestingly, the expression pattern of C treated cells was different from the N and A cells in both strains. Most of the genes were downregulated at some singular point or were kept unchanged during the desiccation stress period. However, the expression levels of some particular genes were actually different between TC92 C and PM411 C cells. For example, *clpB*, *clpC*, *hsp1* and *hsp3* were upregulated only in TC92 under desiccation conditions for

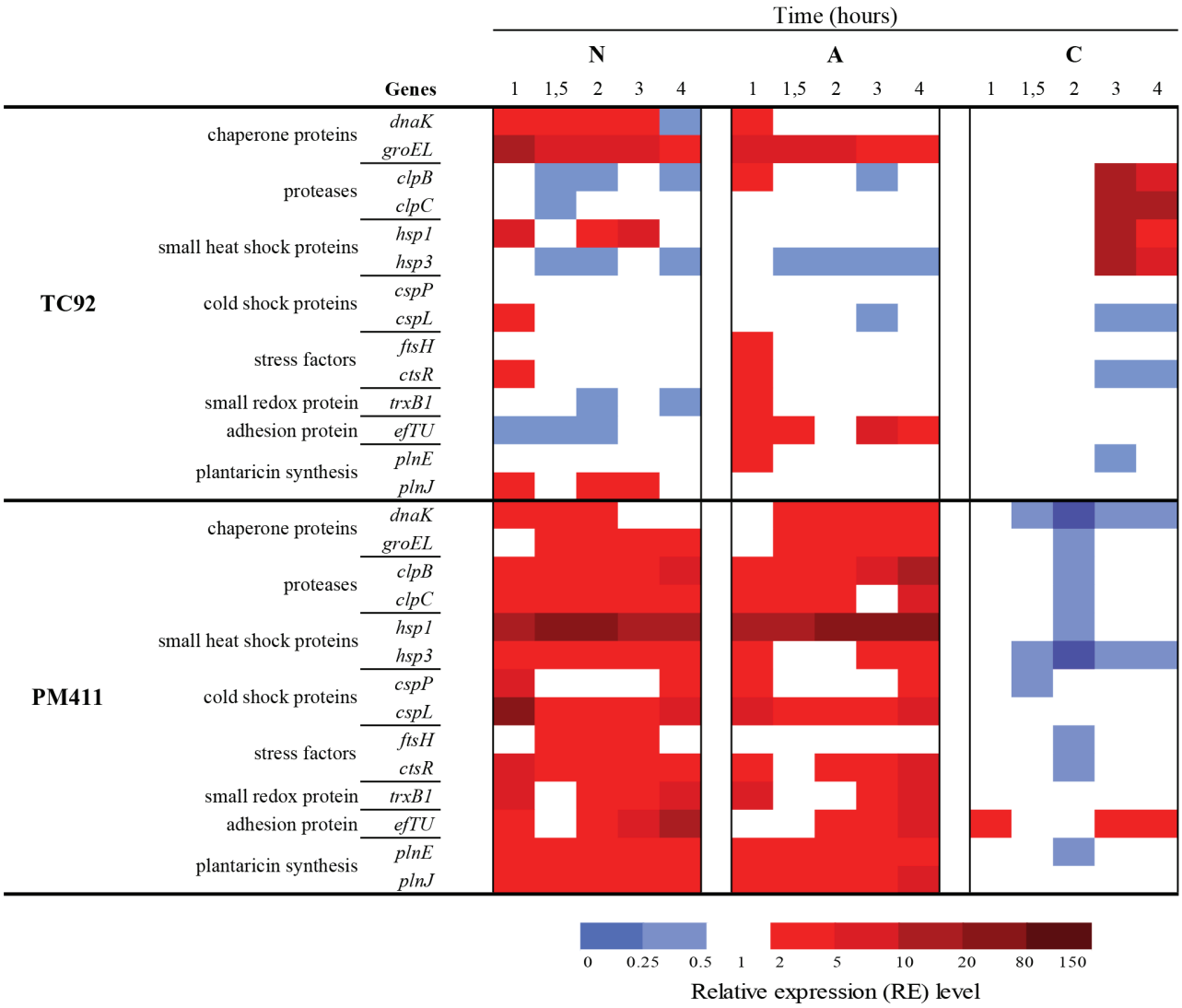


Fig 5. Transcriptional pattern of adapted cells of *L. plantarum* TC92 and PM411 during desiccation challenge. Non-adapted (N), acid adapted (A), and combined-stress adapted (C) cells. Samples during desiccation were taken at selected steps throughout a 4 h period. The relative expression (RE) level was assessed by the ΔΔCt method. The *ldhD* gene was used as the internal control. The ΔCt of non-desiccated cells for the corresponding adaptation method was defined as the calibrator. Three independent biological replicates were performed.

https://doi.org/10.1371/journal.pone.0190931.g005

3 hours, and their expression level was kept upregulated during the following hour. The *efTU* gene was upregulated in PM411 C cells while was unaltered in TC92 C cells.

Effect of adaptation on survival under greenhouse and field conditions and on efficacy of biological control

Levels of PM411 on plants under greenhouse conditions decreased slightly through time and were higher in kiwifruit than in strawberry plants (Fig 6). Differences in population levels were observed between adapted and non-adapted PM411 cells in the two experiments performed for each plant species.

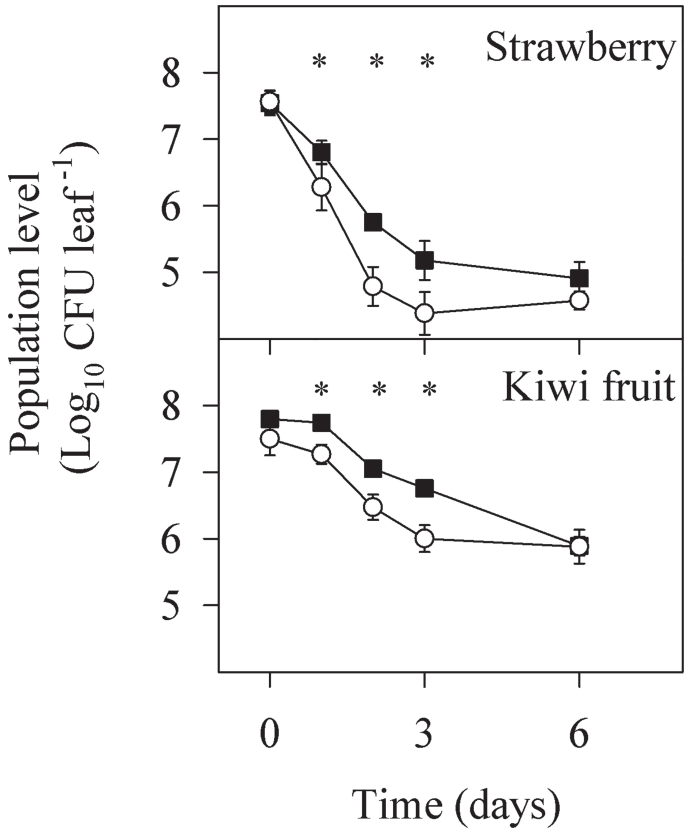


Fig 6. Effect of adaptation treatment on survival of PM411 in strawberry and kiwifruit leaves, under controlled environment greenhouse conditions. Plants were sprayed with non-adapted, N (○) or combined-stress adapted, C (■) cells. Values are the mean of three independent biological replicates and error bars represent the standard deviation of the mean. *, indicates significant differences between treatments according to the Tukey test.

https://doi.org/10.1371/journal.pone.0190931.g006

Population levels of PM411 decreased also through time on pear and apple blossoms under field conditions that were relatively dry (one single rainfall event in pear tree assay and moderate temperatures and low humidity in both pear and apple trees; Fig 7). Three days following field inoculation, population levels of PM411 decreased to steady-state values in non-adapted treatments (10^3–10^4 CFU per blossom), but were significantly higher in adapted treatments (10^4–10^5 CFU per blossom) (Fig 7). When a second spray of PM411 was applied under harsh conditions, also adapted cells survived better than non-adapted cells on both, pear and apple plants.

A decrease in disease severity was observed compared to the control in plants treated with both adapted (C) and non-adapted (N) cells of PM411, without significant differences between them (Fig 8). However, the C treatment had less variability compared to the N treatment and no significant differences between N and non-treated control could be detected in four out of six experiments performed. More in detail, the coefficients of variation in both experiments performed were for the C treatment 5.7 and 17.1% in strawberry, 8.3 and 15.7% in pear and 9.1 and 20% in apple, and those of the N treatment were 62.6 and 59% in strawberry, 55.1 and 52.7% in pear and 32.8 and 45.8% in apple. These results indicated that the C treatment gave more consistent efficacy.

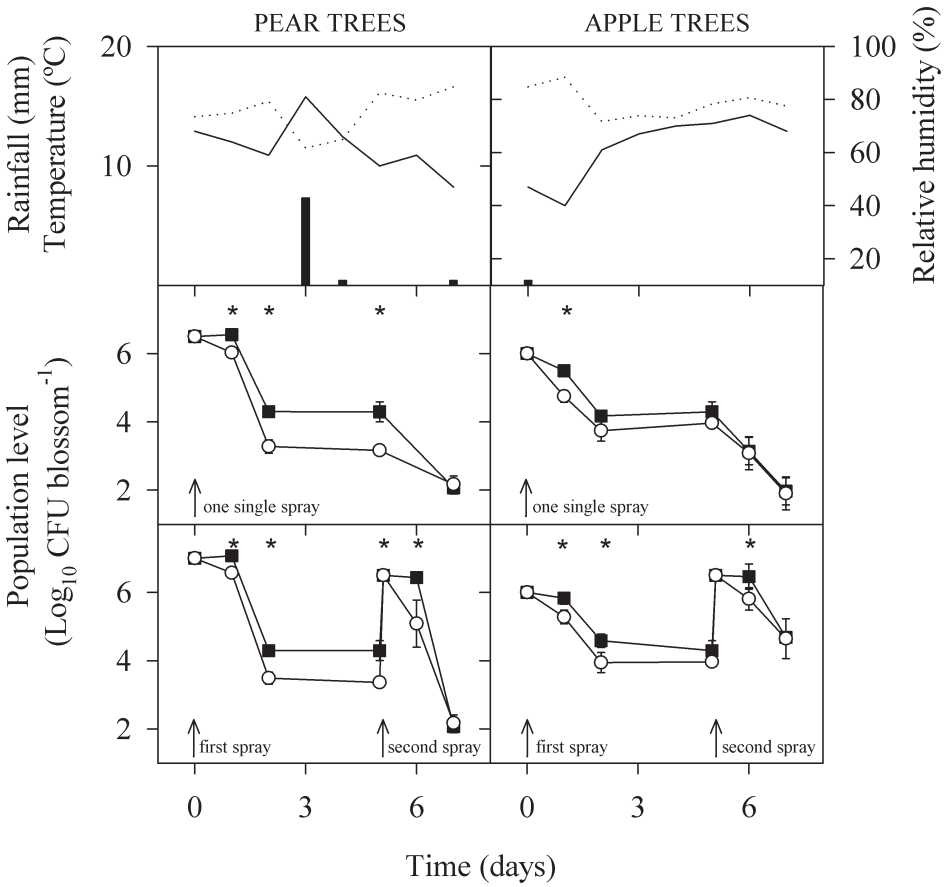


Fig 7. Effect of adaptation treatment on survival of PM411 in blossoms of 'Comice' pear and 'Golden Smoothie' apple under field conditions. Environmental conditions during the field experiments are shown in the upper panels. Temperature (dotted line), RH (bold line) and rainfall (vertical bars). Blossoms were treated in the field with either adapted cells (C) (■), or non-adapted cells (N) (○), using a single spray strategy or a repeated spray strategy. Values are the mean of three replicates. Error bars represent the 95% confidence interval of the mean. *, indicates significant differences between treatments according to the Tukey test.

https://doi.org/10.1371/journal.pone.0190931.g007

Discussion

In order to increase the tolerance to water stress of *L. plantarum* strains isolated from plant environments, and to improve its epiphytic survival under low relative humidity conditions, a strategy based on the adaptive growth under stress conditions has been developed. The combined-stress strategy consisted of adapting cells by growing them into a hyperosmotic medium until stationary-phase, which involves also acid production.

Our results with *L. plantarum* are in agreement with the findings in other *Lactobacillus* species from fermented foods, that reported a higher tolerance to stress of stationary-phase cells compared to exponential-phase cells [17, 19, 42, 56]. Also, the present results are in accordance with studies showing that cells obtained from non-controlled pH fermentations had better tolerance to stresses than cells from controlled pH experiments [57–59]. In addition, cells of *Lactobacillus* had enhanced tolerance to harsh conditions when grown under salt [60–62] or acid stress [20, 63].

The response of strains PM411 and TC92 toward the combined-stress treatment resulted in a strong protection against desiccation. The study of the gene expression responses

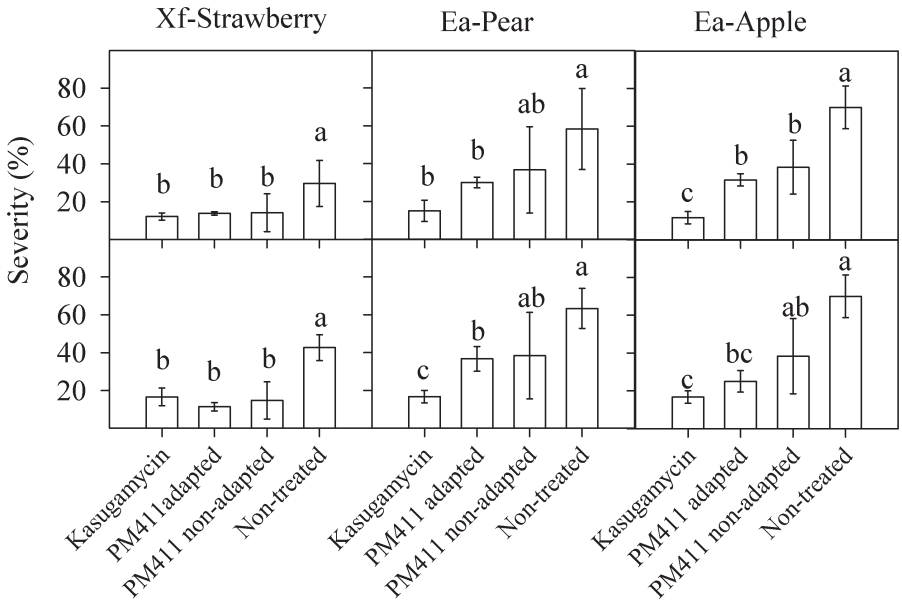


Fig 8. Effect of adaptation of PM411 on susceptibility of strawberry plants to infection by *X. fragariae* (Xf) and of pear and apple flowers to infection by *E. amylovora* (Ea). Plants and blossoms were treated under greenhouse and field conditions, respectively, with adapted PM411 cells, non-adapted PM411 cells, kasugamycin or non-treated. Strawberry plants were sprayed with Xf in the greenhouse. Once treated, pear and apple flowers were collected in the field and inoculated with *E. amylovora* under controlled environment conditions in the laboratory. Values of severity are the mean of three replicates. Error bars represent the 95% confidence interval of the mean. Bars for severity panels with the same letter in the same panel do not differ significantly ($P<0.05$) according to the Tukey test.

https://doi.org/10.1371/journal.pone.0190931.g008

immediately after the combined-stress treatment confirmed an increase in transcript levels of the stress-related genes (*DnaK*, *GroEL*, *ClpC*, *ClpB*, *Hsp1*, *Hsp3*, *CspP*, *CspL*, *CtsR*, *FtsH* and *TrxB1*), in comparison with non-adapted cells. Whereas, after desiccation challenge, transcript levels of the same genes remained unaltered or repressed in adapted cells while were overexpressed in non-adapted cells. This is in concordance with a better survival of adapted vs. non-adapted cells. These results suggest that cells after the combined-stress treatment have high levels of stress proteins that probably protect themselves from damage caused by a subsequent desiccation. Other studies performed in probiotic and dairy lactic acid bacteria have also shown that the adaptive response to stress involves the overproduction of several stress-related proteins, such as DnaK, GroEL, Clp, FtsH, that might give better survival under harsh environment [31, 43, 62, 64]. Interestingly, in our strains, the combined-stress adaptation treatment did not affect the expression level of the non-stress related genes *efTU*, *plnE* and *plnJ*. This is in agreement with the report that *plnE/F* and *efTU* gene expression in *L. plantarum* and *L. pentosus* was not affected by salt contents or pH variations in the growth medium [29, 32].

The acidic treatment, consisting of a single stress factor, showed less protection against desiccation than the combined treatment. In accordance, the gene expression pattern immediately after the acidic adaptation treatment and after desiccation challenge was quite similar to non-adapted cells in both strains. However, this can be a particular response of our strains, since other studies have shown differential expression of some genes in acid-adapted cells compared to non-adapted cells, specifically those related with malolactic fermentation and intracellular accumulation of histidine in *L. casei* [63] or stress-related genes (e.g. *clpL*) in *Oenococcus oeni* [22].

Differences in tolerance to desiccation were observed between PM411 and TC92 cells, in agreement with the gene expression patterns. PM411 non-adapted cells survived better than TC92 over the period of desiccation and showed upregulation of all the eleven stress-related genes analysed, whereas in TC92 only three genes were upregulated. Concerning the response to the combined adaptation treatment, both strains increased cell survival under desiccation but had slightly different stress-related gene expression patterns. After the adaptation treatment, PM411 showed overexpression in more genes than TC92. Moreover, throughout desiccation only in TC92 heat shock proteins (*hsp1* and *hsp3*) and proteases (*clpB* and *clpC*) were differentially upregulated after 3 h, probably due to cell damage and death, because it was at this time when inactivation in non-adapted cells increased.

In plant surfaces, under low RH conditions, the combined-stress adaptation treatment increased cell survival only in PM411. Therefore, we hypothesize that the differences in the stress-related gene expression patterns between the strains might be indicative of a better behaviour of PM411 adapted cells, with a high capacity to withstand stresses in plant surfaces. This is in agreement with the report on differences of tolerance to stress between strains of *L. plantarum* from fermented foods [56, 59]. In addition, differences in stress response between strains of *Pseudomonas fluorescens* antagonistic to *E. amylovora* have also been reported, which explained their performance in disease biocontrol [12, 55, 65]. Accordingly, in PM411 adapted with the combined treatment, the *efTU* gene was upregulated after desiccation challenge, while not changed in TC92. Interestingly, the *efTU* gene, which encodes an adhesion-like protein elongation factor, may play a role in adhesion. As reported, in *L. plantarum* the *efTU* gene is upregulated in the presence of mucus covering the intestinal surface suggesting its role in adhesion [29]. In addition, adhesion-like proteins were reported to be released from the cells of hiochi bacteria (*Lactobacillus* spp.) when they experience osmotic shock [28].

The combined-stress treatment improved survival of PM411 on plant surfaces under limiting environmental conditions (low RH) in different plant hosts. This beneficial effect was significant in *Prunus*, kiwifruit and strawberry leaves under greenhouse conditions, and in apple and pear flowers under controlled environment and also in the field. Under these unfavourable conditions, the strain did not multiply and only survived to steady-state populations, and adapted cells survive better than non-adapted cells. This effect was more important in leaves than in flowers. Probably this is because the water stress under low RH conditions is higher in leaves than in flowers. Leaves have waxy surfaces and are easily exposed to low RH conditions than flowers, and also provide limited nutrient resources to bacterial colonists. In contrast, flowers have internal structures (hypanthium and pistil) that can be colonized by *L. plantarum* where there are less prone to suffer sudden fluctuations in RH and have more nutrients available. Thus, bacteria may have more options of avoiding stresses in flowers than in leaves [14, 66]. The combined-stress treatment improved biocontrol efficacy since reduced variability within the trial, thus providing more consistency in disease suppression. A reduction of the within-experiment variability was also observed in other BCA and associated with an improvement of biocontrol [67]. This beneficial effect is related to the better survival performance of adapted PM411 on plant hosts.

Finally, strategies to adapt bacteria to stress conditions have been widely used in lactic acid bacteria to improve technological performance (fermented foods, probiotics), but not in the field of plant disease protection. Our results show that a combined adaptation treatment based on simultaneous hyperosmotic and acid stress, as well as cultivation until stationary phase, has beneficial effects on the survival of *L. plantarum* strains in response to desiccation and low relative humidity conditions on the plant surfaces. The effect was stronger in strain PM411 than in strain TC92, probably due to a better transcriptional response. This strategy increases

PM411 survival on plant surfaces and the performance of biocontrol of bacterial diseases, thus, helping to develop improved microbial pesticides.

Acknowledgments

We are grateful to Josep Pereda for help with greenhouse plants and to Pere Vilardell for help with field experiments.

Author Contributions

Conceptualization: Núria Daranas, Esther Badosa, Jesús Francés, Emilio Montesinos, Anna Bonaterra.

Formal analysis: Núria Daranas, Esther Badosa, Anna Bonaterra.

Funding acquisition: Núria Daranas, Emilio Montesinos, Anna Bonaterra.

Investigation: Núria Daranas, Esther Badosa, Jesús Francés.

Methodology: Núria Daranas, Esther Badosa, Jesús Francés, Emilio Montesinos, Anna Bonaterra.

Supervision: Esther Badosa, Jesús Francés, Emilio Montesinos, Anna Bonaterra.

Visualization: Núria Daranas.

Writing – original draft: Núria Daranas, Anna Bonaterra.

Writing – review & editing: Núria Daranas, Emilio Montesinos, Anna Bonaterra.

References

1. Lamichhane JR, Arendse W, Dachbrodt-Saaydeh S, Kudsk P, Roman JC, van Bijsterveldt-Gels JEM, et al. Challenges and opportunities for integrated pest management in Europe: A telling example of minor uses. Crop Prot. 2015; 74: 42–47.
2. Matyjaszczyk E. Products containing microorganisms as a tool in integrated pest management and the rules of their market placement in the European Union. Pest Manag Sci. 2015; 71: 1201–1206. https://doi.org/10.1002/ps.3986 PMID: 25652108
3. Timmusk S, Behers L, Muthoni J, Muraya A and Aronsson A-C. Perspectives and challenges of microbial application for crop improvement. Front. Plant Sci. 2017; 8:49. https://doi.org/10.3389/fpls.2017.00049 PMID: 28232839
4. Calvo P, Nelson LM, Kloepper J. Agricultural uses of plant biostimulants. Plant Soil 2014; 383: 3–41.
5. Jogaiah S, Abdelrahman M, Tran L-SP, Ito S-I. Different mechanisms of *Trichoderma virens*-mediated resistance in tomato against Fusarium wilt involve the jasmonic and the salicylic acid pathways. Mol. Plant Pathol. 2017; https://doi.org/10.1111/mpp.12571 PMID: 28605157
6. Pertot I, Alabouvette C, Hinarejos Esteve E. Franca S. Focus Group soil-borne diseases—The use of microbial biocontrol agents against soil—borne diseases. Agriculture & Innovation. 2016.
7. Govind SR, Jogaiah S, Abdelrahman M, Shetty HS, Tran L-SP. Exogenous trehalose treatment enhances the activities of defense-related enzymes and triggers resistance against downy mildew disease of pearl millet. Front. Plant Sci. 2016; 7:1593. https://doi.org/10.3389/fpls.2016.01593 PMID: 27895647
8. Jogaiah S, Mahantesh K, Sharathchnadra RG, Shetty HS, Vedamurthy AB, Tran L-SP. Isolation and evaluation of proteolytic actinomycete isolates as novel inducers of pearl millet downy mildew disease protection. Scientific Reports. 2016; 6: 30789. https://doi.org/10.1038/srep30789 PMID: 27499196
9. Roselló G, Bonaterra A, Francés J, Montesinos L, Badosa E, Montesinos E. Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*. Eur J Plant Pathol. 2013; 137: 621–633.
10. Roselló G, Francés J, Daranas N, Montesinos E, Bonaterra A. Control of fire blight of pear trees with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid. J. Plant Pathol. 2017; 99 (Special issue): 111–120.

11. Stockwell VO, Johnson KB, Loper JE. Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. Phytopathology. 1998; 88: 506–513. https://doi.org/10.1094/PHYTO.1998.88.6.506 PMID: 18944901

12. Hagen MJ, Stockwell VO, Whistler CA, Johnson KB, Loper JE. Stress tolerance and environmental fitness of *Pseudomonas fluorescens* A506, which has a mutation in *rpoS*. Phytopathology. 2009; 99: 679–688. https://doi.org/10.1094/PHYTO-99-6-0679 PMID: 19453226

13. Pujol M, Badosa E, Montesinos E. Epiphytic fitness of a biological control agent of fire blight in apple and pear orchards under Mediterranean weather conditions. FEMS Microbiol Ecol. 2007; 59: 186–193. https://doi.org/10.1111/j.1574-6941.2006.00227.x PMID: 17233751

14. Lindow SE, Brandl MT. Microbiology of the phyllosphere. Appl Environ Microbiol.2003; 69: 1875–1883. https://doi.org/10.1128/AEM.69.4.1875-1883.2003 PMID: 12676659

15. Vorholt JA. Microbial life in the phyllosphere. Nat Rev Microbiol. 2012; 10: 828–840. https://doi.org/10.1038/nrmicro2910 PMID: 23154261

16. Rubio R, Martin B, Aymerich T, Garriga M. The potential probiotic *Lactobacillus rhamnosus* CTC1679 survives the passage through the gastrointestinal tract and its use as starter culture results in safe nutritionally enhanced fermented sausages. Int J Food Microbiol. 2014; 186: 55–60. https://doi.org/10.1016/j.ijfoodmicro.2014.06.013 PMID: 24998181

17. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. Anton Leeuw Int J G. 2002; 82: 187–216.

18. Corcoran BM, Stanton C, Fitzgerald G, Ross RP. Life under stress: The probiotic stress response and how it may be manipulated. Curr Pharm Design. 2008; 14: 1382–1399.

19. Serrazanetti DI, Guerzoni ME, Corsetti A, Vogel R. Metabolic impact and potential exploitation of the stress reactions in lactobacilli. Food Microbiol. 2009; 26: 700–711. https://doi.org/10.1016/j.fm.2009.07.007 PMID: 19747603

20. Mills S, Stanton C, Fitzgerald GF, Ross RP. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microb Cell Fact. 2011; 10: S19. https://doi.org/10.1186/1475-2859-10-S1-S19 PMID: 21995734

21. Papadimitriou K, Alegría Á, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, et al. Stress physiology of lactic acid bacteria. Microbiol Mol Biol Rev. 2016; 80: 837–890. https://doi.org/10.1128/MMBR.00076-15 PMID: 27466284

22. Beltramo C, Desroche N, Tourdot-Marechal R, Grandvalet C, Guzzo J. Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium. Res Microbiol. 2006; 157: 267–274. https://doi.org/10.1016/j.resmic.2005.07.006 PMID: 16171980

23. Watson D, Sleator RD, Hill C, Gahan CG. Enhancing bile tolerance improves survival and persistence of *Bifidobacterium* and *Lactococcus* in the murine gastrointestinal tract. BMC Microbiol. 2008; 8:176. https://doi.org/10.1186/1471-2180-8-176 PMID: 18844989

24. van Bokhorst-van de Veen H, Abee T, Tempelaars M, Bron PA, Kleerebezem M, Marco ML. Short- and long-term adaptation to ethanol stress and its crossprotective consequences in *Lactobacillus plantarum*. Appl Environ Microbiol. 2011; 77: 5247–5256. https://doi.org/10.1128/AEM.00515-11 PMID: 21705551

25. Diep DB, Håvarstein LS, Nes IF. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol. 1996; 178: 4472–4483. PMID: 8755874

26. Saenz Y, Rojo-Bezares B, Navarro L, Diez L, Somalo S, Zarazaga M, et al. Genetic diversity of the *pln* locus among oenological *Lactobacillus plantarum* strains. Int J Food Microbiol. 2009; 134: 176–183. https://doi.org/10.1016/j.ijfoodmicro.2009.06.004 PMID: 19604592

27. da Silva Sabo S, Vitolo M, González JMD, de Souza Oliveira RP. Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. Food Res Int. 2014; 64: 527–536.

28. Nakamura J, Ito D, Nagai K, Umehara Y, Hamachi M, Kumagai C. Rapid and sensitive detection of hiochi bacteria by amplification of hiochi bacterial common antigen gene by PCR method and characterization of the antigen. J Ferment Bioeng. 1997; 185: 7019–7023.

29. Ramiah K, Van Reenen CA, Dicks LMT. Expression of the mucus adhesion genes *Mub*, *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR. Int J Food Microbiol. 2007; 116: 405–409. https://doi.org/10.1016/j.ijfoodmicro.2007.02.011 PMID: 17399831

30. Olguín N, Bordons A, Reguant C. Multigenic expression analysis as an approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions. Int J Food Microbiol. 2010; 144: 88–95. https://doi.org/10.1016/j.ijfoodmicro.2010.08.032 PMID: 20926151

31. Bové P, Russo P, Capozzi V, Gallone A, Spano G, Fiocco D. *Lactobacillus plantarum* passage through an oro-gastro-intestinal tract simulator: carrier matrix effect and transcriptional analysis of genes

associated to stress and probiosis. Microbiol Res. 2013; 168: 351–359. https://doi.org/10.1016/j.micres.2013.01.004 PMID: 23414698

32. Hurtado A, Reguant C, Bordons A, Rozès N. Expression of *Lactobacillus pentosus* B96 bacteriocin genes under saline stress. Food Microbiology. 2011; 28: 1339–1344. https://doi.org/10.1016/j.fm.2011.06.004 PMID: 21839383

33. Rizzello CG, Filannino P, Di Cagno R, Calasso M, Gobbetti M. Quorum-sensing regulation of constitutive plantaricin by *Lactobacillus plantarum* strains under a model system for vegetables and fruits. Appl Environ Microbiol. 2014; 80: 777–787. https://doi.org/10.1128/AEM.03224-13 PMID: 24242246

34. Bonaterra A, Camps J, Montesinos E. Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. FEMS Microbiol Lett. 2005; 250: 1–8. https://doi.org/10.1016/j.femsle.2005.06.028 PMID: 16002241

35. Cabrefiga J, Francés J, Montesinos E, Bonaterra A. Improvement of fitness and efficacy of a fire blight biocontrol agent via nutritional enhancement combined with osmoadaptation. Appl Environ Microbiol. 2011; 77: 3174–3181. https://doi.org/10.1128/AEM.02760-10 PMID: 21441337

36. Pusey PL, Wend C. Potential of osmoadaptation for improving *Pantoea agglomerans* E325 as biocontrol agent for fire blight of apple and pear. Biol Control. 2012; 62: 29–37.

37. Liu J, Wisniewski M, Droby S, Tian S, Hershkovitz V, Tworkoski T. Effect of heat shock treatment on stress tolerance and biocontrol efficacy of *Metschnikowia fructicola*. FEMS Microbiol Ecol. 2011; 80: 145–155.

38. Sui Y, Wisniewski M, Droby S, Liu J. Responses of yeast biocontrol agents to environmental stress. Appl Environ Microbiol. 2015; 81: 2968–2975. https://doi.org/10.1128/AEM.04203-14 PMID: 25710368

39. Boor KJ. Bacterial stress responses: what doesn't kill them can make them stronger. Plos Biol. 2006; 4: e23. https://doi.org/10.1371/journal.pbio.0040023 PMID: 16535775

40. Kultz D. Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol. 2005; 67: 225–257. https://doi.org/10.1146/annurev.physiol.67.040403.103635 PMID: 15709958

41. Pieterse B, Leer RJ, Schuren FHJ, van der Werf MJ. Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. Microbiol. 2005; 151: 3881–3894.

42. De Angelis M, Di Cagno R, Huet C, Crecchio C, Fox PF, Gobbetti M. Heat shock response in *Lactobacillus plantarum*. Appl Environ Microbiol. 2004; 70: 1336–1346. https://doi.org/10.1128/AEM.70.3.1336-1346.2004 PMID: 15006751

43. Desmond C, Fitzgerald GF, Stanton C, Ross RP. Improved stress tolerance of GroESL-overproducing *Lactococcus lactis* and probiotic *Lactobacillus paracasei* NFBC 338. Appl Environ Microbiol. 2004; 70: 5929–5936. https://doi.org/10.1128/AEM.70.10.5929-5936.2004 PMID: 15466535

44. Song S, Bae DW, Lim K, Griffiths MW, Oh S. Cold stress improves the ability of *Lactobacillus plantarum* L67 to survive freezing. Int J Food Microbiol. 2014; 18: 135–143.

45. Trias R, Bañeras L, Badosa E, Montesinos E. Bioprotection of Golden Delicious apple and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. Int J Food Microbiol. 2008; 123: 50–60. https://doi.org/10.1016/j.ijfoodmicro.2007.11.065 PMID: 18191266

46. Hazel WJ, Civerolo EL. Procedures for growth and inoculation of *Xanthomonas fragariae*, causal organism of angular leaf spot of strawberry. Plant Dis. 1980; 64: 178–181.

47. Pusey PL. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology. 1997; 87: 1096–1102. https://doi.org/10.1094/PHYTO.1997.87.11.1096 PMID: 18945005

48. Fiocco D, Capozzi V, Collins M, Gallone A, Hols P, Guzzo J, et al. Characterization of the CtsR stress response regulon in *Lactobacillus plantarum*. J Bacteriol. 2010; 192: 896–900. https://doi.org/10.1128/JB.01122-09 PMID: 19933364

49. Bron PA, Grangette C, Mercenier A, de Vos WM, Kleerebezem M. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J Bacteriol. 2004; 186: 5721–9. https://doi.org/10.1128/JB.186.17.5721-5729.2004 PMID: 15317777

50. Capozzi V, Weidmann S, Fiocco D, Rieu A, Hols P, Guzzo J, et al. Inactivation of a small heat shock protein affects cell morphology and membrane fluidity in *Lactobacillus plantarum* WCFS1. Res Microbiol. 2011; 162: 419–25. https://doi.org/10.1016/j.resmic.2011.02.010 PMID: 21349328

51. Fiocco D, Collins M, Muscariello L, Hols P, Kleerebezem M, Msadek T, et al. The *Lactobacillus plantarum ftsH* gene is a novel member of the CtsR stress response regulon. J Bacteriol. 2009; 191: 1688–94. https://doi.org/10.1128/JB.01551-08 PMID: 19074391

52. Serrano LM, Molenaar D, Wels M, Teusink B, Bron PA, de Vos WM, et al. Thioredoxin reductase is a key factor in the oxidative stress response of *Lactobacillus plantarum* WCFS1. Microb Cell Fact. 2007; 6: 29. https://doi.org/10.1186/1475-2859-6-29 PMID: 17725816

53. Fiocco D, Crisetti E, Capozzi V, Spano G. Validation of an internal control gene to apply reverse transcription quantitative PCR to study heat, cold and ethanol stresses in *Lactobacillus plantarum*. World J Microb Biot. 2008; 24: 899–902.

54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-delta delta CT) method. Methods. 2001; 25: 402–408. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

55. Desroche N, Beltramo C Guzzo J. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. J Microbiol Meth. 2005; 60: 325–333.

56. Parente E, Ciocia F, Ricciardi A, Zotta T, Felis GE, Torriani S. Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. Int J Food Microbiol. 2010; 144: 270–279. https://doi.org/10.1016/j.ijfoodmicro.2010.10.005 PMID: 21035223

57. Silva J, Carvalho AS, Ferreira R, Vitorino R, Amado F, Domingues P, et al. Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* to stress conditions during spray-drying. J Appl Microbiol. 2005; 98: 775–782. https://doi.org/10.1111/j.1365-2672.2004.02516.x PMID: 15715882

58. Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P. Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int Dairy J. 2004; 14: 835–847.

59. Ferrando V, Quiberoni A, Reinhemer J, Suárez V. Resistance of functional *Lactobacillus plantarum* strains against food stress conditions. Food Microbiol. 2015; 48: 63–71. https://doi.org/10.1016/j.fm.2014.12.005 PMID: 25790993

60. Kets E, Teunissen P, de Bont J. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. Appl Environ Microbiol. 1996; 62: 259–261. PMID: 16535214

61. Desmond C, Stanton C, Fitzgerald GF, Collins K, Ross RP. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int Dairy J. 2001; 11: 801–808.

62. Prasad J, McJarrow P, Gopal P. Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl Environ Microbiol. 2003; 69: 917–925. https://doi.org/10.1128/AEM.69.2.917-925.2003 PMID: 12571012

63. Broadbent JR, Larsen RL, Deibel V, Steele JL. Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. J Bacteriol. 2010; 192: 2445–2458. https://doi.org/10.1128/JB.01618-09 PMID: 20207759

64. van Bokhorst-van de Veen H, Bongers RS, Wels M, Bron PA, Kleerebezem M. Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation. Microb Cell Fact. 2013; 12: 112. https://doi.org/10.1186/1475-2859-12-112 PMID: 24238744

65. Stockwell VO, Hockett K, Loper JE. Role of RpoS in stress tolerance and environmental fitness of the phyllosphere bacterium *Pseudomonas fluorescens* strain 122. Phytopathology. 2009; 99: 689–695. https://doi.org/10.1094/PHYTO-99-6-0689 PMID: 19453227

66. Beattie GA, Lindow SE. Bacterial colonization of leaves: a spectrum of strategies. Phytopathology. 1999; 89: 353–359. https://doi.org/10.1094/PHYTO.1999.89.5.353 PMID: 18944746

67. Agustí L, Bonaterra A, Moragrega C, Camps J, Montesinos E. Biocontrol of root rot of strawberry caused by *Phytophthora cactorum* with a combination of two *Pseudomonas fluorescens* strains. J Plant Pathol. 2011; 93: 363–372.

CHAPTER V

Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method

Daranas N, Bonaterra A, Francés J, Cabrefiga J, Montesinos E, Badosa E

Applied and Environmental Microbiology

2018, 84:e00107-18

DOI:10.1128/AEM.00107-18

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PLANT MICROBIOLOGY

Monitoring Viable Cells of the Biological Control Agent *Lactobacillus plantarum* PM411 in Aerial Plant Surfaces by Means of a Strain-Specific Viability Quantitative PCR Method

Núria Daranas,[a] Anna Bonaterra,[a] Jesús Francés,[a] Jordi Cabrefiga,[a] Emilio Montesinos,[a] Esther Badosa[a]

[a]Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Girona, Spain

ABSTRACT A viability quantitative PCR (v-qPCR) assay was developed for the unambiguous detection and quantification of *Lactobacillus plantarum* PM411 viable cells in aerial plant surfaces. A 972-bp region of a PM411 predicted prophage with mosaic architecture enabled the identification of a PM411 strain-specific molecular marker. Three primer sets with different amplicon lengths (92, 188, and 317 bp) and one TaqMan probe were designed. All the qPCR assays showed good linearity over a 4-log range and good efficiencies but differed in sensitivity. The nucleic acid-binding dye PEMAX was used to selectively detect and enumerate viable bacteria by v-qPCR. The primer set amplifying a 188-bp DNA fragment was selected as the most suitable for v-qPCR. The performance of the method was assessed on apple blossoms, pear, strawberry, and kiwifruit leaves in potted plants under controlled environmental conditions, as well as pear and apple blossoms under field conditions, by comparing v-qPCR population estimations to those obtained by qPCR and specific plate counting on de Man-Rogosa-Sharpe (MRS)-rifampin. The population estimation did not differ significantly between methods when conditions were conducive to bacterial survival. However, under stressful conditions, differences between methods were observed due to cell death or viable-but-nonculturable state induction. While qPCR overestimated the population level, plate counting underestimated this value in comparison to v-qPCR. PM411 attained stable population levels of viable cells on the flower environment under high relative humidity. However, the unfavorable conditions on the leaf surface and the relatively dryness in the field caused an important decrease in the viable population.

IMPORTANCE The v-qPCR method in combination with plate counting and qPCR is a powerful tool for studies of colonization and survival under field conditions, to improve formulations and delivery strategies of PM411, and to optimize the dose and timing of spray schedules. It is expected that PEMAX v-qPCR could also be developed for monitoring other strains on plant surfaces not only as biological control agents but also beneficial bacteria useful in the sustainable management of crop production.

KEYWORDS biological control, viability-qPCR

The development of new biological control agents (BCA) to prevent crop diseases is receiving considerable attention. The use of BCA is in agreement with the principles and benefits of integrated pest management (IPM), reducing the application of conventional plant protection products. In this context, lactic acid bacteria (LAB), which have been extensively reported as food biopreservatives (1–4), show several features that make them candidate BCA of foliar bacterial plant diseases. Several LAB strains are antagonists of many plant-pathogenic bacteria and fungi (5–7) due to a wide diversity of mechanisms of action, such as the production of organic acids, bacteriocins, and

Received 12 January 2018 **Accepted** 28 February 2018

Accepted manuscript posted online 9 March 2018

Citation Daranas N, Bonaterra A, Francés J, Cabrefiga J, Montesinos E, Badosa E. 2018. Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method. Appl Environ Microbiol 84:e00107-18. https://doi.org/10.1128/AEM.00107-18.

Editor Eric V. Stabb, University of Georgia

Address correspondence to Anna Bonaterra, anna.bonaterra@udg.edu.

other inhibitory bioactive compounds, or to the competition for nutrients or colonization sites (8). Moreover, some LAB strains have been qualified as generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (FDA) and as having a qualified presumption of safety (QPS) by the European Food Safety Authority (EFSA). Concretely, the *Lactobacillus plantarum* PM411 strain has been selected in our laboratory as potential BCA due to its broad *in vitro* antagonistic activity against several plant-pathogenic bacteria. This strain synthesizes antimicrobial compounds, such as plantaricins EF and JK, and produces lactic acid, efficiently controlling fire blight disease of pear and apple plants (5, 9).

It is necessary to develop strain-specific quantitative methods for monitoring strain PM411 in the environment in order to study its ecological fitness and to optimize formulations and application strategies in the phyllosphere of plants (10–12). Metagenomic studies in apple trees have revealed the presence of *Lactobacillus* spp. as components of the phyllosphere (13, 14). Therefore, the monitoring method has to be able to discriminate strain PM411 from other inhabitants of the same species in plants. In addition, since the performance of a BCA requires its colonization and survival on plant surfaces, the development of methods capable of quantifying only viable cells is needed.

Several monitoring methods are commonly used to detect and quantify BCA at the strain level in environmental samples, but in most cases, they are unable to estimate only the viable or culturable population. Culture-based techniques combined with the use of an antibiotic-resistant mutant allow the quantification of a specific strain (11) but may underestimate (ca. 2 or 3 log units) the population size of the BCA under nonconducive conditions (15, 16). This is because some bacteria, including LAB species, such as *L. plantarum*, may enter in a viable-but-nonculturable state (VBNC) as a survival strategy to cope with environmental stress (17–19). VBNC cells retain some metabolic activity and intact membranes, despite minor changes in their composition (18, 20). In contrast, the population level may be overestimated if monitoring methods based on nucleic acid targets, such as real-time PCR (qPCR), are used, since DNA from viable and dead cells can be indifferently amplified (15, 21, 22). The viability quantitative PCR (v-qPCR) constitutes a method that allows the quantification of only viable cells. The method has been shown to be useful for quantification of viable foodborne pathogenic microorganisms, such as *Listeria monocytogenes* (23), *Escherichia coli* O157:H7 (23, 24), *Campylobacter* spp. (25, 26), and *Salmonella* spp. (23, 27) in different food matrices (e.g., fresh-cut vegetables, ground beef, chicken, and cooked ham). Viability assessment of LAB has also been studied to enumerate probiotic and starter strains in milk and dairy products (28, 29). Moreover, this methodology has been used to monitor a BCA strain of *Pantoea agglomerans* in citrus fruit (21).

For the development of a strain-specific v-qPCR assay, first, it is necessary to find a specific molecular marker in the strain that can be identified by comparative genomic analysis or even by fingerprinting techniques, such as randomly amplified polymorphic DNA-PCR (RADP-PCR) or amplified fragment length polymorphisms (AFLP) (30–32). In addition, for selectively detecting and enumerating viable bacteria, different nucleic acid-binding dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) are used in combination with qPCR (33–35). EMA and PMA can penetrate damaged cellular membranes and intercalate into DNA. Light activation of these DNA-bound molecules results in a covalent linkage preventing PCR amplification of the modified DNA. However, both dyes have some limitations. EMA can cross intact cell membranes of some bacterial species and cause, to some extent, inhibition of PCR amplification of viable cells (33). While PMA is highly selective in penetrating only compromised membranes, it is unable to avoid PCR amplification of nonviable cells with unaltered membranes. A new approach, the PEMAX reagent, has been developed recently to improve the v-qPCR and extend the concept of viability PCR to cells with intact cell membrane structure but also with active metabolism. This new approach consists of using an adequate level of EMA (<10 μM) mixed with PMA (≥ 20 μM) (36–38). Low levels of EMA can cross intact membranes and are accumulated in cells that lack the

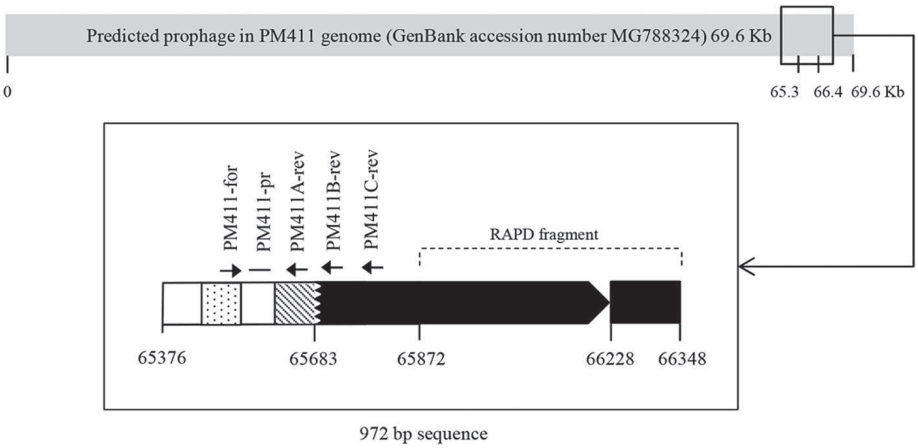


FIG 1 Description of the PM411 sequence (972 bp) in which strain-specific qPCR assays were designed. The sequence was located in the putative prophage (GenBank accession number MG788324) and revealed mosaic architecture. Black boxes show a 665-bp region with 79% identity with 32 *L. plantarum* strains, encoding a tail fiber and a hypothetical protein. The striped box shows a 90-bp region with ≤85% identity with 7 *L. plantarum* strains, encoding a tail fiber. The dotted box shows a 162-bp region with 80% identity with *L. plantarum* HFC8, encoding a hypothetical protein. White boxes show nonhomologous sequences. The primers and TaqMan probe and the region corresponding to RAPD fragment are indicated.

metabolic ability to offset its uptake. However, EMA is thrown out from metabolically active cells (36). The combination of EMA with PMA increases the strength of the DNA neutralization when samples contain high levels of dead cells with damaged membranes, but it also avoids amplification of DNA of cells with undamaged membranes and inactive metabolism. After the treatment of the bacterial suspension with PEMAX, DNA from viable cells with intact membrane structure and active metabolism (whether culturable or VBNC) will be free of labeling and then detected by qPCR (37).

To our knowledge, the application of the PEMAX reagent in the v-qPCR approach has been recently used in viability assessment studies for monitoring bacterial pathogens (e.g., *Legionella and Salmonella*) (39, 40) but not for the specific quantification of beneficial bacteria in plant environments.

The aim of the present work was to develop a strain-specific v-qPCR assay using the PEMAX system to detect and quantify viable cells of *L. plantarum* PM411 in aerial plant surfaces. The method has allowed monitoring of the survival of PM411 after artificial inoculation to plant material under different conditions and plant hosts, in comparison to qPCR and plate counting techniques.

RESULTS

Identification of an *L. plantarum* PM411-specific molecular marker. A discriminatory band (520 bp) was found in the PM411 strain by the RAPD-PCR method using the primer XD9 (described in Materials and Methods). Its sequence shared 86% identity with the *Lactobacillus* phage Sha1 (GenBank accession number HQ141411) and 81% identity with 32 *L. plantarum* strains available in the NCBI database. Detection for prophage DNA sequences within the PM411 genome using PHAST indicated three regions that were predicted to represent prophages. One of these regions with 69.6 kb (GenBank accession number MG788324) contained the RAPD sequence and was identified as the putative prophage Lactob_PLE3 (GenBank accession number NC_031125) with a score of 150 and with 80 coding DNA sequences (CDS) (see Table S1 in the supplemental material). In particular, the 520-bp sequence (found by RAPD-PCR) was located in the CDS 75 and 76 of the putative phage. Figure 1 shows a 972-bp fragment (from CDS positions 65376 to 66348), which includes the RAPD sequence, with mosaic architecture that contain homologous and nonhomologous sequences to strains available in the NCBI database. Its specificity was confirmed *in silico* since the whole 972-bp fragment was not found in any strain available in the database. The fragment contained homologous sequences, such as 665 bp that shared 79% identity with 32 *L. plantarum*

TABLE 1 Primers and TaqMan probes used for RAPD-PCR, rep-PCR, and qPCR analysis

Oligonucleotide, primer, or probe by analysis type[a]	Sequence (5′–3′)[b]	Amplicon length (bp)[b]	Reference or source
RAPD-PCR			
P3	CTGCTGGGAC	—	72
P4	CCGCAGCGTT	—	73
P7	AGCAGCGTGG	—	73
M13	GAGGGTGGCGGTTCT	—	73
Inva1	GTGAAATTATCGCCACGTTCGGCAA	—	74
512Fb	GATGCAGTCGACAATGTGGATGCT	—	75
XD9	GAAGTCGTCC	—	76
rep-PCR			
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	—	70
ERIC2	AAGTAAGTGACTGGGGTGAGCG		
REP-1R	IIIICGICGICATCIGGC	—	
REP-2	ICGICTTATCIGGCCTAC		
BOXA1R	CTACGGCAAGGCGACGCTGACG	—	
GTG_5	GTGGTGGTGGTGGTG	—	
qPCR			
PM411-for	AGATGCCAGCACTGGATTAAGC		This work
PM411-pr	FAM-TGCACGGCACAACTCAGGCGATT-TAMRA		
PM411A-rev	TTCATAGTAATCCCAGTGGTTTGG	92	
PM411B-rev	CCTTGTCGATACCAAAGTTAGCTATG	188	
PM411C-rev	CGGCGGCACCACCTT	317	

[a]ERIC, enterobacterial repetitive intergenic consensus sequence; REP, repetitive extragenic palindromic sequence; BOX, BOX sequence; GTG_5, polytrinucleotide $(GTG)_5$ sequence.
[b]—, variable size. Amplicon sizes listed are amplification products obtained by qPCR using PM411-for primer, PM411-pr TaqMan probe, and the corresponding reverse primer (PM411A-rev, PM411B-rev, or PM411C-rev).

strains, partially encoding a tail fiber and a hypothetical protein, 90 bp that shared ≤85% identity with 7 *L. plantarum* strains, partially encoding a tail fiber, and 162 bp that shared 80% identity with *L. plantarum* HFC8, partially encoding a hypothetical protein. However, the presence of the nonhomologous sequences enabled the identification of a strain-specific molecular marker. The strain specificity of the marker was confirmed by PCR using PM411-for and PM411C-rev primers (Table 1). With this approach, no amplification was obtained for any of the strains listed in Table 2, except for PM314 and PM340, which were confirmed to be clones of PM411 by repetitive element sequence-based PCR (rep-PCR) fingerprinting. *L. plantarum* PM411, PM314, and PM340 strains showed an identical banding pattern (data not shown). This common banding pattern was clearly different from other *L. plantarum* strains tested (CM450, FC248, TC92, and WCFS1).

Strain-specific qPCR designs. Departing from the PM411 strain-specific marker, TaqMan-based qPCR assays were developed. Three qPCR assays producing different amplicon lengths (92, 188, and 317 bp) were designed in the polymorphic region and checked (A, B, and C) in order to study their suitability in v-qPCR (Fig. 1). The shared forward primer (PM411-for) annealed with the sequence homologous to *L. plantarum* HFC8, the TaqMan probe (PM411-pr) with the sequence without homology, and the reverse primers (PM411A-rev, PM411B-rev, and PM411C-rev) with the region homologous to several *L. plantarum* strains (HFC8 nonincluded).

Specificity, sensitivity, and amplification efficiency of qPCR assays. At 4 ng of genomic DNA per qPCR (approximately 10^6 CFU or genomic equivalents), successful amplification of PM411 was achieved, with cycle threshold (C_T) values from 16.5 to 23 by the three strain-specific TaqMan-based qPCR assays developed. No amplification was observed with DNA from pure cultures of the large collection of strains of different species and genera (LAB and non-LAB bacteria) listed in Table 2. Only random fluorescence signals were observed at C_T values higher than 38 in 7 LAB strains and in some plant material washings without PM411 cells. Hence, the three qPCR assays were considered to be specific at the strain level.

TABLE 2 Bacterial strains used in this study

Species	Code strain[a]
LAB	
Lactobacillus brevis	CECT 4669
Lactobacillus buchneri	CECT 4111[b]
Lactobacillus collinoides	CECT 922[b]
Lactobacillus dextrinicus	CECT 4791[b]
Lactobacillus pentosus	10 strains isolated from plant sources[c]
Lactobacillus plantarum	PM411, PM314, PM340, TC54, TC92, FC248, CM450, CM466, RC526, FC534, 35 strains isolated from plant sources[c]
	CECT 221, CECT 223, CECT 748[b], CECT 749, CECT 4185, CECT 4308, CECT 4528, CECT 4645, CECT 5785, WCFS1 (syn. of LMG 9211), ATCC 8014
Lactobacillus sakei	CECT 980
Lactococcus lactis	3 strains isolated from plant sources,[c] CECT 539, CECT 984, CECT 4433
Leuconostoc citreum	1 strain isolated from plant sources[c]
Leuconostoc fallax	CECT 4701
Leuconostoc mesenteroides	12 strains isolated from plant sources,[c] CECT 219[b]
Pediococcus acidilactici	LMG 6411
Pediococcus parvulus	CECT 7350
Pediococcus pentosaceus	LMG 10740
Weissella cibaria	3 strains isolated from plant sources[c]
Non-LAB	
Bacillus subtilis	EPS201
Erwinia amylovora	PMV 6076
Escherichia coli	ATCC 5954
Pantoea agglomerans	EPS125
Pantoea vagans	7 EPS strains, C9-1[d]
Pseudomonas fluorescens	10 EPS strains, EPS62e
Pseudomonas syringae	7 EPS strains, EPS94
Staphylococcus aureus	ATCC 9144
Xanthomonas axonopodis pv. vesicatoria	2133-2

[a]CECT, Colección Española de Cultivos Tipo; BCCM/LMG, The Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology, Ghent University; ATCC, American Type Culture Collection; PMV, Laboratoire de Pathologie Moléculaire et Végétale, INRA/INA-PG, Paris, France; EPS, Escola Politècnica Superior-UdG, Spain; IVIA, Instituto Valenciano de Investigaciones Agrarias, Spain.
[b]Type strain.
[c]Trias et al. (4) and Roselló et al. (5).
[d]Strain provided by F. Rezzonico.

Standard curves of the three qPCR assays, which were prepared in flower washings, showed good linearity over a 4-log range (from 1×10^3 to 1×10^7 CFU · ml^{-1}, $R^2 = 0.99$), and the lowest limit of detection was 1×10^2 CFU · ml^{-1}. The equations of regression curves for the A, B, and C designs were $C_T = -3.3$ log CFU · ml^{-1} + 39.7 (for A), $C_T = -3.3$ log CFU · ml^{-1} + 42.8 (for B), $C_T = -3.4$ log CFU · ml^{-1} + 47.6 (for C). The corresponding amplification efficiencies (*E*) were 99.9% (A), 98.7% (B), and 98.9% (C). However, the three assays differed in sensitivity. The A design (92 bp) was the most sensitive, followed by the B design (188 bp), with the C design (317 bp) being the least sensitive. The comparison between the three standard curves led to the selection of the A and B assays for further experiments.

v-qPCR. The effect of different PEMAX concentrations and qPCR assay (different amplicon length) on the signal reduction (SR) (difference of C_T value [ΔC_T] between non-PEMAX-treated and PEMAX-treated samples) was determined on dead and viable cells (Fig. 2). On dead cells, significant differences of SR between concentrations of PEMAX were observed, and the highest SR in both qPCR assays (A and B) was obtained using 50 μM PEMAX. However, on viable cells, the different PEMAX concentrations did not show significant differences. Based on these results, the PEMAX concentration of 50 μM was chosen for further experiments.

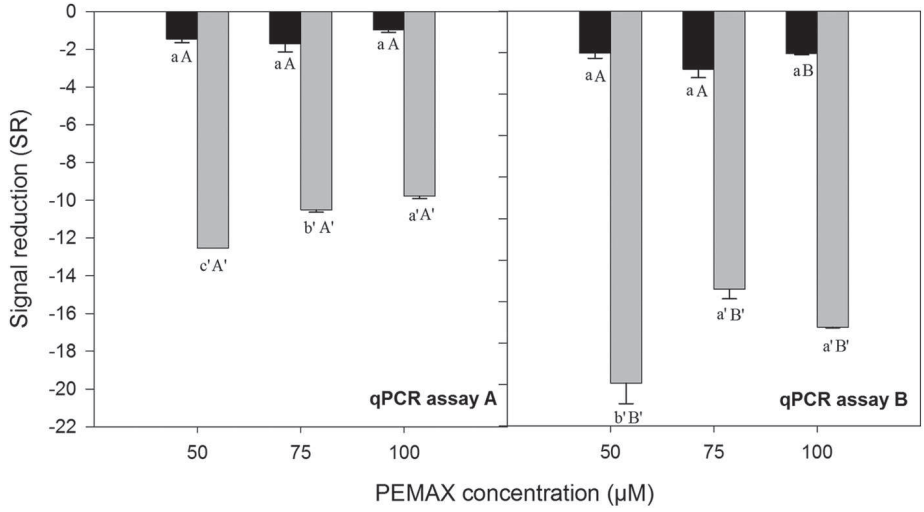


FIG 2 Signal reduction (SR) in viable (black) or dead (gray) cell suspensions with different concentrations of PEMAX reagent (50, 75, and 100 μM). SR is the difference between cycle threshold values (ΔC_T) of non-PEMAX and PEMAX-treated samples. TaqMan-based qPCR assays designated A (92 bp) and B (188 bp) were carried out. The results are shown as means from three independent replicates, and error bars represent standard deviations of the mean. Different capital letters (letters without apostrophe in viable cell suspensions and letters with apostrophe in dead cell suspensions) show significant differences between qPCR assays for each concentration of PEMAX ($P < 0.05$), according to the Tukey test. Different lowercase letters (letters without apostrophe in viable cell suspensions and letters with apostrophe in dead cell suspensions) in the same panel indicate significant differences between concentrations of PEMAX reagent ($P < 0.05$), according to the Tukey test.

When A and B qPCR assays were compared, on dead cells, a significantly higher SR was obtained by the B assay, with an amplicon length of 188 bp, than by the A assay, with an amplicon length of 92 bp, for all PEMAX concentrations (Fig. 2). On viable cells, only a significant higher SR was obtained with the B than the A assay using 100 μM PEMAX, whereas when using 50 and 75 μM PEMAX, no significant differences between assays were observed. The B assay was finally chosen.

Standard curves were developed in flower washings to check the v-qPCR method as a specific bacterial detection and quantification tool (Fig. 3). On viable cells, standard curves (each obtained in three independent experiments) were generated using the B assay, with or without PEMAX treatment. The correlation coefficient values ($R^2 = 0.99$) and the amplification efficiencies (83.5% with PEMAX and 86.2% without PEMAX) were comparable. The standard curves were linear over the range of 1×10^3 to 1×10^7 CFU · ml^{-1}, with and without PEMAX treatment. However, in the presence of PEMAX treatment, a shift of 2 cycles was observed regarding the nontreated samples. On dead cells treated with PEMAX, C_T values higher than 38 were obtained over the range from 1×10^3 to 1×10^7 CFU · ml^{-1}, meaning that the amplification was inhibited (Fig. 3). In mixtures of viable cells (from 1×10^3 to 1×10^7 CFU · ml^{-1}) and a fixed quantity of dead cells (1×10^6 CFU · ml^{-1}) treated with PEMAX, the standard curves (each obtained in two independent experiments) achieved a high correlation coefficient ($R^2 = 0.98$), with an amplification efficiency of 96.9% (Fig. 3). However, the C_T values of this standard curve obtained with the presence of dead cells in the sample were slightly smaller than those from only viable cells, especially when the concentrations of viable cells were low. Without PEMAX treatment, the qPCR assay was unable to differentiate between DNA from viable and dead cells.

Quantification of viable *L. plantarum* PM411 in aerial plant surfaces. PM411 was monitored on inoculated apple blossoms and leaves of pear, strawberry, and kiwifruit plants under controlled environment conditions (25°C, high or low relative humidity [rH]) by qPCR, v-qPCR, and plate counting (pc) (Fig. 4 and 5).

On apple blossoms, in both experiments performed, there were significant differences between qPCR (total cells) and the other two quantification methods, v-qPCR

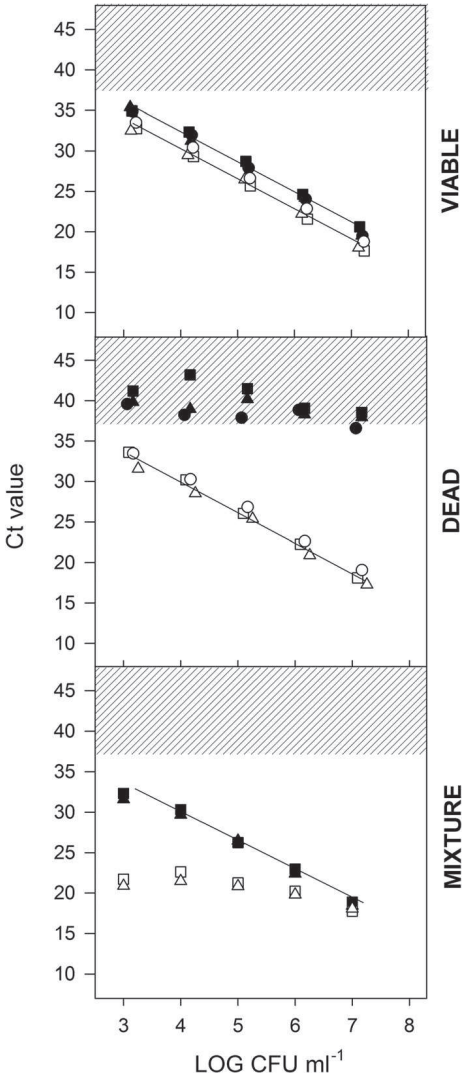


FIG 3 Cycle threshold (C_T) values obtained by TaqMan-based v-qPCR assay (B design) for a range of concentrations from 1×10^3 to 1×10^7 CFU · ml^{-1}. The experiment was performed with (i) only viable cells, (ii) only dead cells, and (iii) viable cells in the presence of 1×10^6 CFU · ml^{-1} of dead cells. Cells were treated with PEMAX reagent (black symbols) or not (white symbols) prior to DNA extraction. Three independent experiments represented by circle, triangle, and square symbols were carried out. The striped background represents the detection limit at C_T values of >38.

(viable cells) and pc (culturable cells), at some steps throughout the experiments (Fig. 4). After inoculation, the total population size decreased approximately 1 log unit between the 1st and 8th days, whereas the viable and culturable population decreased up to 3 log units. In particular, after a reduction of 1.5 log units during the first 24 h, the viable population remained stable throughout the following 2 days, at around 10^6 to 10^7 CFU per blossom, without significant differences compared to the total and cultivable populations. However, after this period, the amounts of viable and culturable cells were significantly lower than total cells. Under these conditions, there was a linear relationship between culturable (pc) and viable (v-qPCR) population levels ($y = 0.95x + 0.12$; $R^2 = 0.93$; $P < 0.001$).

On pear, strawberry, and kiwifruit leaves, significant differences between the three quantification methods (qPCR, v-qPCR, and pc) were observed at some steps throughout the experiments (Fig. 5). Total population level (qPCR) was significantly higher than viable (v-qPCR) and culturable (pc) population levels almost in all sampling days throughout the experiments. Interestingly, significant differences were also observed between the viable and culturable populations, with the quantification of viable cells

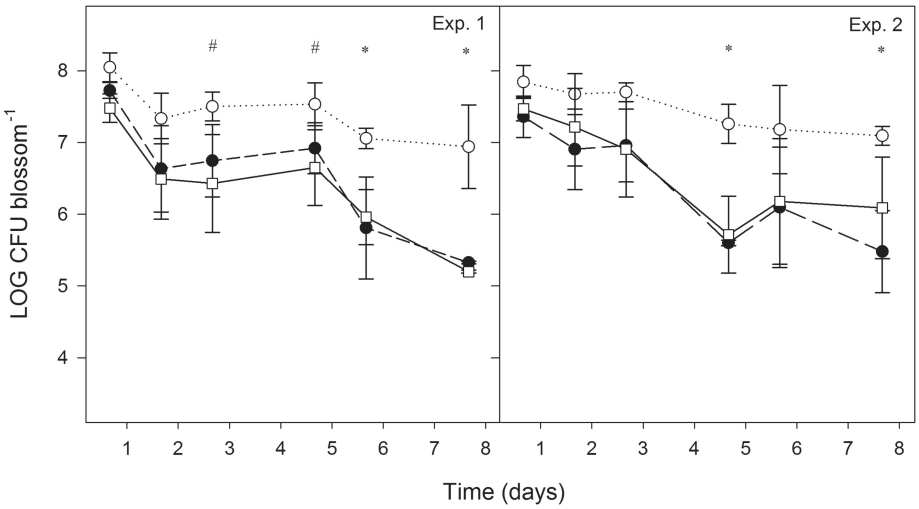


FIG 4 Population dynamics of *L. plantarum* PM411 estimated by qPCR (total) (○), v-qPCR (viable) (●), and plate counting (culturable) (□) on apple blossoms under controlled-environment conditions (25°C and high rH). Cells were sprayed onto the plant material at 10^8 CFU · ml^{-1}. The experiment was performed two times. Values are the means of three replicates, and error bars represent the standard deviation of the mean. *, significant differences between qPCR and v-qPCR/pc; #, significant differences between qPCR and pc, according to the Tukey test. Exp., experiment.

being significantly higher than the culturable cells, especially after 2 to 3 days under high rH and after only 1 day under low rH. In all the experiments, the total population decreased approximately 1 to 1.5 log units between the 1st and 6th days, whereas culturable and viable cells declined more, from 2 to 4 log units, depending on plant species and rH conditions. While on pear and strawberry leaves at high rH, the population reduction of viable and culturable cells was 3 to 4 log units, on kiwifruit leaves, it was 2 to 2.5 log units. Under low rH, the population decrease of viable and culturable cells on kiwifruit leaves during the 3 days postinoculation (2 to 2.5 log units for viable cells and 3 to 3.5 log units for culturable cells) was higher than under high rH (1 log unit for viable cells and 1.5 log units for culturable cells).

The population levels of PM411 were also monitored on apple and pear blossoms under field conditions, which were relatively dry (moderate temperatures and low humidity) with one single rainfall event in the pear tree assay (Fig. 6). Total population size differed significantly from viable and culturable population levels at some steps throughout the experiments, both on apple and pear blossoms. Two days following field inoculation (first or single spray), viable cells of PM411 decreased to steady-state values (10^3 to 10^5 CFU per blossom) both on apple and pear, without significant differences from culturable cells on apple flowers. However, on pear blossoms, the viable population was significantly higher than the culturable population after 1 to 2 days of PM411 inoculation. When a second spray of PM411 was applied, the three quantification methods (qPCR, v-qPCR, and pc) estimated the same population only immediately after the spray. After 1 day, PM411 population decreased, and significant differences in total population compared to viable and culturable populations were also observed both on pear and apple plants. However, only on pear blossoms was the viable population significantly higher than the culturable population after the second spray. From the different types of plant material and environmental conditions studied, it can be concluded that the populations of viable and culturable cells did not differ significantly under environmental conditions conducive for bacterial survival (on flowers under high rH), but they were different under harsh conditions, especially on leaves under low rH.

DISCUSSION

Monitoring the persistence and traceability of *L. plantarum* PM411 in plants is a key task for understanding its behavior in the crop environment and to improve formula-

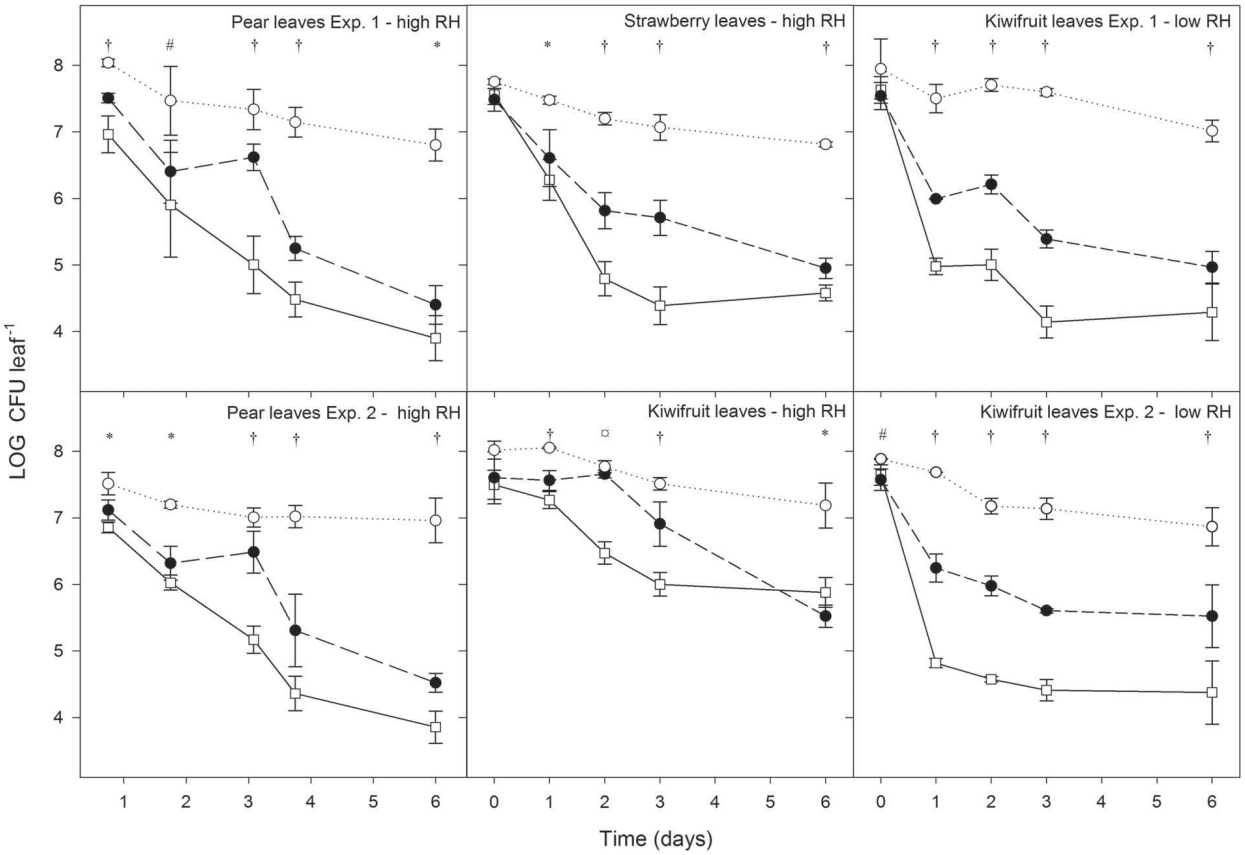


FIG 5 Population dynamics of *L. plantarum* PM411 estimated by qPCR (total) (○), v-qPCR (viable) (●), and plate counting (culturable) (□) on leaves of pear, strawberry, and kiwifruit plants under controlled-environment conditions (25°C with high or low rH). Cells were sprayed onto the plant material at 10^8 CFU · ml^{-1}. The experiments were performed two times, except for strawberry and kiwifruit plants under high rH. Values are the means of three replicates, and error bars represent the standard deviation of the mean. *, significant differences between qPCR and v-qPCR/pc; #, significant differences between qPCR and pc; †, significant differences between qPCR, v-qPCR, and pc; ¤, significant differences between qPCR/v-qPCR and pc, according to the Tukey test.

tions and delivery strategies for biological control of plant diseases. The fate and persistence of target microorganisms in the environment have been traditionally assessed with a variety of culture-dependent and independent methods (6–8, 10, 11). Among the various approaches used, PCR-based methods have been the most popular because they are very sensitive and specific to properly identify the inoculated strains, distinguishing them from the resident population. In the present study, we have developed a viability qPCR assay using PEMAX reagent for the unambiguous detection and quantification of *L. plantarum* PM411 viable cells in aerial plant surfaces. This method has two main advantages: to be specific at the strain level and to allow the quantification of only viable cells, whether culturable or VBNC.

In order to identify a strain-specific marker, a putative PM411-specific DNA region was identified using the RAPD-PCR technique that showed homology with part of the sequence of *Lactobacillus* phage Sha1 (41). However, this region was not sufficiently specific to PM411, since it was shared by 32 *L. plantarum* strains of the NCBI database. The RAPD sequence was located in one of the three prophage regions in the PM411 genome that was predicted to represent the Lactob_PLE3 phage (42). The occurrence of prophage DNA within bacterial genomes is common in LAB, such as *Lactobacillus* spp. (43–45). This putative prophage in the PM411 genome has mosaic architecture with homologous sequences to *L. plantarum* strains and also to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Enterococcus*, and *Listeria* phages (Table S1), which are alternated with nonhomologous sequences. This polymorphic structure allowed the identification of sequences to design a strain-specific marker. Prophages exhibit a high degree of

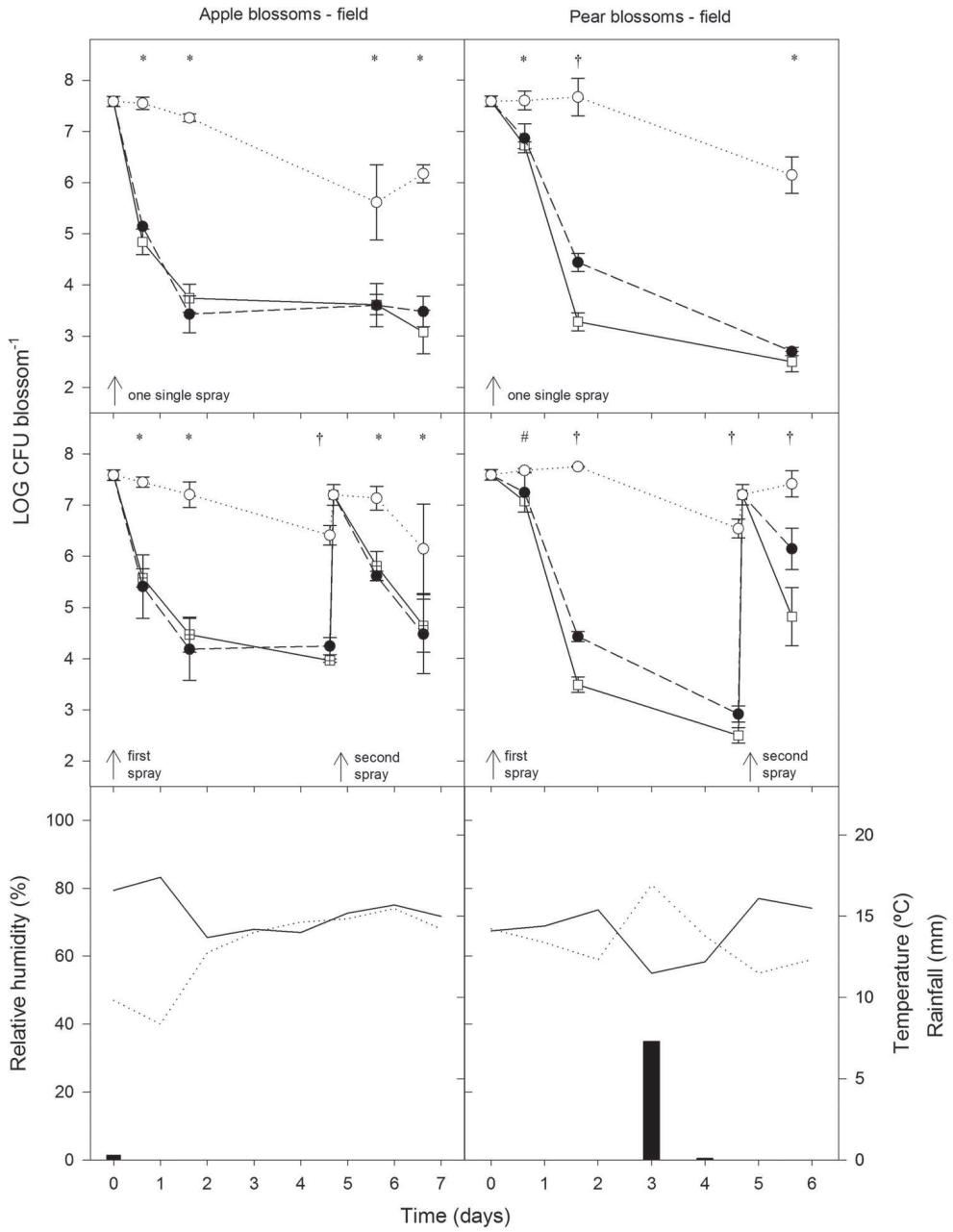


FIG 6 Population dynamics of *L. plantarum* PM411 estimated by qPCR (total) (○), v-qPCR (viable) (●), and plate counting (culturable) (□) on apple and pear blossoms under field conditions. Cells were sprayed onto the plant material at 10^8 CFU · ml^{-1}. One single spray or two sprays were performed both in pear and apple blossom experiments. Values are the mean of three replicates, and error bars represent the standard deviation of the mean. *, significant differences between qPCR and v-qPCR/pc; #, significant differences between qPCR and pc; †, significant differences between qPCR, v-qPCR and pc; ¤, significant differences between qPCR/v-qPCR and pc, according to the Tukey test. Mean daily temperature (black line), amount of rainfall (black bars), and relative humidity (dotted line) were monitored during the trials.

mosaicism (46) and have been found to contribute to interstrain genetic variability in bacteria (47, 48). Therefore, polymorphic sites within prophage sequences or prophage junction fragments in the genome can be used as indicators of genomic diversity. The presence of homologous phage genes spread in different bacterial strains might reflect phylogeny and suggests horizontal gene transfer between these related species (42). Several studies included the use of phage-related sequences as genomic markers. For example, regions of Lc-Nu and A2 phage sequences were used for strain-specific PCR primer design to identify *Lactobacillus rhamnosus* strains (49, 50). Moreover, the use of

prophage junction fragments as indicators of genomic diversity was already reported in other taxa, such as *Salmonella* and *Listeria* (47, 51).

The specificity of the PM411 marker was first confirmed *in silico* and by the absence of amplification signal by PCR in all the *L. plantarum* strains tested (except for PM314 and PM340, which were deemed to be clones of PM411), other plant-associated bacterial genera, and plant material washings from field samples. Since other strains of *L. plantarum* may be present in the crop environment (52), this specificity is a key factor in monitoring PM411. Although random amplifications with high C_T values (higher than 38) were observed for some nontarget strains and plant material washings, this phenomenon was previously described in the literature as a background no-template control (15), being irrelevant if remaining outside the range used to generate the standard curve, as occurred in the present study.

The sensitivity and reliability of the three qPCR assays with different amplicon lengths were evaluated mimicking conditions of field sampling by amending plant material washings with different concentrations of PM411, in order to ensure comparable qPCR efficiencies (53, 54). All the qPCR assays fulfill the requirements for satisfactory amplification. Moreover, the values obtained were similar to those reported in other qPCR assays designed to quantify several biological control agents (15, 32, 55).

The use of EMA and PMA coupled with qPCR is an efficient technique to distinguish between viable and dead cells in plant samples (25, 33). These systems have been used to detect foodborne pathogens in lettuce (56) and in fresh-cut vegetables (23), as well as to detect biological control agents in postharvest fruits (21). The new approach based on a double-dye reagent, PEMAX, improves the v-qPCR scope (36–38). In the present study, PEMAX was used to set up a viability qPCR method specific for PM411, and after PEMAX treatment of plant material washings, only DNA from PM411 cells with undamaged membrane and active metabolism was detected by qPCR.

In our work, the effect of PEMAX concentration was optimized to selectively detect viable PM411 cells, avoiding the amplification of heat-killed cells in plant material washings, in accordance with other reports that used PMA dye on different microorganisms (23, 27). According to our study, 50 μM PEMAX in a dead cell suspension in flower washings allowed the inhibition of DNA amplification, while viable cell suspensions were not affected.

In order to choose the best assay for v-qPCR, two designs of different amplicon lengths were compared, taking into account the inactivation of amplification in dead cells, while preserving the performance of qPCR (sensitivity, linearity, and efficiency). Our results showed that the best performance was obtained using the longer amplicon (188 bp). Although the optimal amplicon length for qPCR assays to guarantee method efficiency is less than 100 bp (57), in v-qPCR, longer DNA sequences are necessary (27). As reported, the probability of dye (EMA/PMA) binding in the target region of dead bacteria increases with the length of the DNA fragment (58). Since amplification efficiency and sensitivity of the reaction diminish when amplicon length increases, the reliability of the developed viability qPCR method (using the 188-bp amplicon and PEMAX) was evaluated on viable, dead, and a mixture of viable/dead cells of PM411. The quantification method developed was linear over the range of 1×10^3 to 1×10^7 CFU $\cdot$ ml^{-1}, and the obtained standard curves, using C_T values from three independent experiments, showed high correlation coefficient values and amplification efficiencies. Taking into account that the quantification limit was determined in the presence of a high level of dead cells, this sensitivity is similar to those reported in other methods developed to detect and quantify biological control agents (15, 21, 59). The slight increase in C_T values of PEMAX-treated samples compared to nontreated ones was previously reported in studies using PMA as a dye (24, 26). Considering that the PEMAX-qPCR method allowed the quantification of viable cells in the presence of 1×10^6 CFU $\cdot$ ml^{-1} of dead cells with high amplification efficiency, the methodology was suitable to monitor viable PM411 cells in plant samples.

The performance of the method was studied by comparing v-qPCR population estimation to those obtained by qPCR and specific plate counting on de Man-Rogosa-

Sharpe (MRS)-rifampin. Since *L. plantarum* PM411 is a biological control agent of fire blight of apple and pear (5, 9) and is capable of controlling other bacterial plant diseases, such as bacterial canker of kiwifruit and angular leaf spot of strawberry, plant species, like pear, apple, strawberry, and kiwifruit, were used for further experiments to evaluate the method. In addition, different environmental conditions for the BCA, such as blossoms or leaves, under controlled (high and low rH) or field conditions were analyzed. No significant differences were observed between the three methods when the conditions were conducive for bacterial survival. However, under harsh conditions, qPCR quantification overestimated the population level of PM411 (until 4 log units), indicating the presence of nondegraded DNA released from dead cells, and plate counting underestimated the population of the strain (until 2 log units), indicating induction of the VBNC state. Therefore, v-qPCR enabled the most accurate quantification of PM411 viable cells, whether culturable or VBNC, to monitor survival on aerial plant surfaces.

On flowers, under controlled-environment conditions of high rH, PM411 showed a transient drop in population level upon inoculation, probably due to the stressful conditions of the spray. However, after this initial decrease, PM411 attained stable population levels of viable cells for the following 2 days, reaching values from 10^6 to 10^7 CFU per blossom. In this period, viable population levels were not significantly different from those estimated by qPCR or plate counting. The usefulness of qPCR or plate counting as monitoring tools of BCA after delivery on plants was also confirmed in the BCA *Pseudomonas fluorescens* EPS62e that showed efficient colonization of blossoms (15, 60). This result is consistent with the fact that the flower environment is favorable for bacterial survival and colonization because of a high level of nutrients. Sugars, such as glucose and fructose, and amino acids, such as proline, asparagine, glutamic acid, and glutamine, are predominant in apple and pear flowers (61). It is expected that *L. plantarum* can reach stable populations in the flower environment, since it has the capacity to use a broad range of carbohydrates and amino acids (62). After 5 days on the flower surface, the population of PM411 clearly decreased, coinciding with the end of the life span of flowers. This survival reduction may be attributed to the nonconducive conditions as a result of the senescence of the tissues (13). At this period, population was overestimated by qPCR, indicating the presence of nondegraded DNA being released from dead cells. Studies conducted by other authors monitoring bacteria on plant surfaces confirmed the differences in population estimation between qPCR and plate counting (15, 21). Interestingly, all viable cells were culturable probably because conditions were not enough stressful to induce a VBNC state.

On the leaf surface, the PM411 population decreased more than on flowers after inoculation. The leaf environment is poor in carbon-containing nutrients and more exposed to fluctuations in temperature, UV radiation, and especially water availability (relative humidity and leaf wetness) (63, 64). Under these stress conditions, the induction of a VBNC state and cell death may explain significant differences between v-qPCR, qPCR, and plate counting. However, these differences were observed in leaf experiments both under high and low rH, probably meaning that the lack of nutrients is one of the most important limiting factors for PM411 survival. As reported in other bacteria, including LAB, stressful conditions (e.g., desiccation and starvation) can promote cells to enter in a VBNC state (17, 20). Consequently, VBNC cells were not quantified by plate counting, and the viable PM411 population was underestimated, as reported in other BCA monitoring studies under different stress conditions (15, 16, 65). As VBNC cells are still metabolically active and preserve membrane integrity, they should be considered the effective population of BCA since they can become culturable again when better conditions arrive (20). On flowers under field conditions, the viable population of PM411 dropped to around 10^3 to 10^5 CFU per blossom immediately after the spray (both in the single- or two-spray experiments). Although the nutritional conditions in flowers are expected to be optimal, the harsh environment, such as periods of low relative humidity ($<$70%) combined with UV radiation exposition, probably caused this

decrease in population. Interestingly, in a pear orchard, differences between v-qPCR and plate counting were observed, presumptively due to the induction of the VBNC state as an adaptive stress response of cells against suboptimal environmental conditions. It was reported in previous studies that *L. plantarum* PM411 increased transcript levels of stress-related genes under desiccation (66). However, in an apple orchard, similar populations of viable and culturable PM411 cells were observed. Therefore, differences in morphology and physicochemical environment between pear and apple flowers, as well as weather conditions registered in the field, may explain how a discrepancy between viable and culturable populations was only observed in apple flowers.

In our study, the unfavorable environmental conditions on the leaf surface and the relatively dry field conditions during the experiments seem to induce a VBNC state in PM411 cells.

Finally, we have developed a method for the specific detection and quantification of viable PM411 that has been evaluated and validated. The method is expected to be a reliable monitoring tool to estimate the viable population of the strain in aerial plant surfaces, and it will allow further studies of colonization and survival under field conditions, as well as improvements in formulations and delivery strategies. Data obtained from v-qPCR monitoring may indicate when PM411 should be released in the field to achieve the population required for biocontrol, since the decrease in the viable population can compromise the BCA efficacy. It is expected that PEMAX v-qPCR could also be developed for monitoring other bacterial strains on plant surfaces, not only biological control agents, but also other beneficial bacteria (e.g., biofertilizers and biostimulants) useful in the sustainable management of crop production.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA extraction. The bacterial strains used in this study are listed in Table 2. LAB strains were grown on de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, UK) at 23°C for 48 h. Non-LAB strains were grown on Luria-Bertani (LB) agar at 25°C for 24 h. *Escherichia coli* DH5α calcium competent cells were used for cloning procedures and were grown in LB medium at 37°C. A spontaneous mutant of wild-type *L. plantarum* PM411 resistant to rifampin, obtained as previously described (5), was used in this study. All strains were stored in 20% glycerol at −80°C. DNA was extracted according to the method described by Llop et al. (67) from pure bacterial suspensions (Table 2).

Strategy to identify a strain-specific molecular marker for *L. plantarum* PM411. All primers, PCR mixtures, and PCR conditions used in this study are described in Tables 1 and 3, and amplified fragments were analyzed using standard methods.

RAPD-PCR analysis was carried out to identify a PM411 strain-specific molecular marker. Seven primers were tested in strain PM411 and other selected *L. plantarum* strains (ATCC 8014, CECT 223, CECT 4528, CECT 5785, TC54, TC92, and WCFS1) by PCR. A potential PM411 strain-specific RAPD band was excised from the agarose gel, purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany), cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed into *E. coli* DH5α calcium competent cells. Cells were selected by antibiotic resistance in LB agar supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin (Sigma, MO, USA), and a PCR analysis with the universal primers T7 and Sp6 was done to confirm the insertion. The RAPD-PCR fragment was sequenced (Macrogen, Seoul, South Korea) and analyzed using the FinchTV 1.4.0 software (Geospiza, Seattle, WA, USA) and Multalin software (68). The specificity was ensured *in silico* using the BLAST program at the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

As the fragment identified by RAPD-PCR showed similarity with a prophage, raw data of sequenced *L. plantarum* PM411 genome were used for prophage region search and annotation using a phage finding tool (PHAge Search Tool [PHAST] [69]). The corresponding putative phage sequence was deposited in the GenBank database (accession number MG788324). The RAPD sequence was located in the putative prophage of *L. plantarum* PM411, and a 972-bp region (region from positions 65376 to 66348 of accession number MG788324) was checked *in silico* for specificity. This region was used in order to design a primer pair (PM411-for and PM411C-rev) using Primer-BLAST (Table 1). The primers were designed in the PM411-specific region. The specificity of the primers was tested using strains described in Table 2. The rep-PCR amplifications of PM411 and other *L. plantarum* strains listed in Table 2 (CM450, FC248, PM314, PM340, TC92, and WCFS1) were carried out for clone detection with the repetitive sequence-based oligonucleotides corresponding to ERIC, REP, BOXA1R, and GTG_5 (Table 1) (70).

Strain-specific qPCR designs and specificity, sensitivity, and amplification efficiency evaluation. Three TaqMan-based qPCR assays were designed (Table 1) within the strain-specific marker (region from positions 65376 to 66348 of accession number MG788324) to obtain three amplicons with different lengths. All of them shared the same forward primer (PM411-for) and probe (PM411-pr), but they had three different reverse primers (PM411A-ref, PM411B-rev, and PM411C-rev). Probes were labeled with the

TABLE 3 Amplification mixture and PCR conditions

PCR approach	Amplification mixture[a]	PCR conditions[b]
RAPD-PCR	1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM each primer, 3.75 U *Taq*, and 100 ng DNA (reaction vol, 50 μl)	For M13: 94°C for 3 min; 35 cycles of 94°C for 1 min, 40°C for 20 s, ramp to 72°C at 0.6°C/s for 20 min; elongation at 72°C for 2 min For P3 and P4: 94°C for 3 min; 30 cycles of 94°C for 1 min, 36°C for 2 min, 72°C for 2 min; and elongation at 72°C for 2 min For P7, Inva1, 512Fb, and XD9: 94°C for 4 min; 45 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min; and elongation at 72°C for 5 min
pGEM-T insert amplification	1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM primers T7 and Sp6, 1.875 U *Taq*, and 2 μl recombinant vector (reaction vol, 25 μl)	98°C for 2 min; 35 cycles of 98°C for 10 s, 45°C for 30 s, 72°C for 30 s; and elongation at 72°C for 12 min
PCR	1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM PM411-for and PM411C-rev, 1.75 U *Taq*, and 25 ng DNA (reaction vol, 25 μl)	95°C for 5 min; 30 cycles of 95°C for 45 s, 60°C for 40 s, for 72°C 40 s; and elongation at 72°C for 10 min
rep-PCR	1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.6 mg/ml BSA, 10% DMSO, 500 nM each forward and reverse primer for rep-PCR and ERIC-PCR or of the single primer for BOX-PCR and GTG_5-PCR, 2.5 U *Taq*, and 50 ng DNA (reaction vol, 25 μl)	95°C for 7 min; 30 cycles of 94°C for 1 min, 52°C for 1 min for ERIC- and BOX-PCR or 42°C 1 min for REP- and GTG_5-PCR, and 65°C for 8 min; and elongation at 65°C for 16 min
qPCR	1× TaqMan universal PCR master mix, 500 nM each forward and reverse primer, 200 nM GP probe or 250 nM RP1 or RP2 probe, and 20 ng DNA or 4 μl DNA sample (reaction vol, 20 μl)	50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 15 s and 60°C for 1 min

[a]dNTPs, dinucleoside triphosphates; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; *Taq*, *Taq* DNA polymerase (Invitrogen). TaqMan Universal PCR master mix is manufactured by Invitrogen.
[b]PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) and qPCR in a 7500 real-time PCR system (Applied Biosystems).

6-carboxyfluorescein (FAM) reporter dye at the 5′ end and with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye at the 3′ end. Primers and TaqMan probes were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA).

The specificity of the qPCR designs was tested after optimization of the concentrations of the primers and probe using bacteria listed in Table 2. A no-template control (NTC), using water instead of genomic DNA, and positive control with PM411 DNA were included in all PCR runs. All reactions were performed in triplicate and were carried out in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Standard curves were developed to check the sensitivity and efficiency of the qPCR assays by mixing several concentrations of PM411 cells with plant material washings. To obtain plant material washings, open blossoms of apple (cv. Golden Smoothee) and pear (cv. Comice) and leaves from potted plants of pear (cv. Conference), strawberry (cv. Darselect), and kiwifruit (cv. Hayward) were used. Two blossoms or three leaves were infused with 30 ml of 50 mM sterile phosphate buffer (PBS; pH 7.0) and 0.1% peptone in a 100-ml bottle and mixed in an orbital shaker (KS501 digital; IKA Labortechnik, Staufen, Germany) at 130 rpm for 30 min on ice (15, 60). Cell suspensions of PM411 were prepared in sterile distilled water at high concentration (10^9 CFU · ml^{-1}) and diluted to appropriate concentrations with plant material washings. The cell concentration of the first serial decimal dilution was checked by a measure of the optical density at 600 nm (OD_{600}), considering that 0.25 corresponds to 10^8 CFU · ml^{-1}, and this was confirmed by colony counts. The tested concentrations covered a 5-log range, from 1×10^3 to 1×10^8 CFU · ml^{-1}. An aliquot of plant material washings without PM411 cells was kept as a no-template control (NTC) sample.

DNA was isolated according to the method described by Schmidt et al. (71), with some modifications. Briefly, 1 ml of sample was centrifuged at 13,200 × *g* for 10 min, and the pellet was resuspended in 400 μl of TES buffer (50 mM Tris-HCl, 1 mM EDTA, 6.7% glucose). Cells were incubated with 100 μl of lysozyme at 20 mg · ml^{-1} (Sigma) and 6 μl of mutanolysin at 5,000 U · ml^{-1} (Sigma) for 1 h at 37°C with shaking (ThermoMixer F1.5; Eppendorf, Hamburg, Germany). After adding 15 μl of proteinase K at 20 mg · ml^{-1} (Qiagen) and 40 μl of 20% sodium dodecyl sulfate (SDS), samples were incubated at 60°C for 1 h. Then, mechanical disruption was performed transferring the sample into a 2-ml safe-seal microcentrifuge tube with 70 mg of acid-washed glass beads (Sigma) and using a TissueLyzer II instrument (Qiagen) at a frequency of 30 s^{-1} for 10 min. Glass beads and cell debris were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was purified adding 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) and mixed by vortexing for 5 s. Phases were separated by centrifugation at 16,000 × *g* for 5 min. The aqueous phase was mixed with 2 volumes of ice-cold ethanol and 0.1 volume of 4 M sodium acetate, and DNA was precipitated overnight at −20°C. DNA was collected by centrifugation at 16,000 × *g* for 30 min, and the pellet was washed in ice-cold 70% ethanol, dried, resuspended in 50 μl of water, and stored at −20°C until analyzed. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, USA).

qPCR was performed, and two no-template controls (NTC) were included in all PCR runs: (i) one using water instead of genomic DNA, and (ii) one using DNA isolated from plant material washings without PM411 cells. All reactions were performed in triplicate as described above. C_T values were plotted against the logarithm of the initial number of CFU · ml^{-1}, and standard curves were generated by a linear regression of the plotted points. Slopes were used to determine the amplification efficiency of each design using the equation E (%) = $(10^{-1/\text{slope}} - 1) \times 100$.

v-qPCR. (i) PEMAX concentration optimization. PEMAX reagent (GenIUL, Terrassa, Spain) was diluted in 500 μl of sterile bidistilled water to obtain a stock solution of 2,000 μM that was stored at −20°C in the dark until needed. To determine the optimal concentration of PEMAX (50, 75, and 100 μM), an appropriate volume of PEMAX stock solution (25, 37.5, or 50 μl) was added into 1 ml of viable or dead PM411 cell suspension, both adjusted to 1×10^6 CFU · ml^{-1} in apple flower washings. Dead cells were obtained by heating a cell suspension at 100°C for 15 min (10, 26) (ThermoMixer F1.5; Eppendorf). The loss of cell viability was checked by plating on MRS agar, followed by incubation for 48 h at 30°C. Then, samples were thoroughly mixed and incubated for 30 min in the dark at room temperature in an orbital shaker KS501 digital (IKA Labortechnik) at 130 rpm. Immediately, samples were photoactivated for 15 min with the PhAST Blue photoactivation system (GenIUL, Barcelona, Spain) set to 100% and transferred into DNA low-binding 1.5-ml tubes (Sarstedt, Nümbrecht, Germany). PEMAX-treated cells (viable and dead) were collected by centrifugation at 13,200 × *g* for 10 min and washed with 50 mM sterile PBS (pH 7.0) under the same centrifugation conditions. Samples of viable and dead cells, adjusted to 1×10^6 CFU · ml^{-1} in apple flower washings and without being treated with PEMAX were also used. DNA extraction of PEMAX-treated and non-PEMAX-treated samples was performed as described in the previous paragraph. Each experimental condition was assayed in two independent experiments.

(ii) Amplicon length effect. To study the influence of amplicon length in the effectiveness of the PEMAX treatment to suppress PCR amplification of dead cells, two independent qPCR assays (A and B) were performed. One unique forward primer (PM411-for) and probe (PM411-pr) and two reverse primers (PM411A-rev and PM411B-rev) were used to obtain two amplicons with different lengths (Table 1). qPCR was performed as described previously, and a no-template control (NTC) was included in each PCR run. All reactions were performed in triplicate.

The effect of PEMAX at different concentrations on DNA amplification suppression by qPCR assays A and B was tested in viable and dead cells and expressed as "signal reduction." Signal reduction was calculated by subtracting C_T values between non-PEMAX-treated and PEMAX-treated samples. Three independent experiments were performed.

(iii) Standard curve. To check the v-qPCR method as a specific bacterial detection and quantification tool, the sensitivity and amplification efficiency of the v-qPCR B design were evaluated by developing standard curves. Cell suspensions were prepared using viable, dead, or mixtures of PM411 cells in apple flower washings. Samples were prepared from covering a 5-log range (from 1×10^3 to 1×10^8 CFU · ml^{-1}) of viable or dead cells, obtained as described, and mixing the same concentration range of viable PM411 cells with a constant number of dead cells (1×10^6 CFU · ml^{-1}). From each sample, 1 ml was treated with PEMAX at 50 μM according to the procedure described previously. An aliquot of each sample without being treated with PEMAX was also used. DNA extraction was performed in PEMAX-treated and non-PEMAX-treated samples as described above. qPCR was performed using the B design (PM411-for, PM411-pr, and PM411B-rev), obtaining an amplicon size of 188 bp. qPCR was performed as described previously, including the two negative controls (NTC) mentioned above. All reactions were performed in triplicate. Standard curves were generated as described above. Three independent experiments were carried out.

Quantification of *L. plantarum* PM411 on plant material. Greenhouse experiments were performed in different plant materials, such as apple blossoms and pear, strawberry, and kiwifruit leaves. Two field trials on apple and pear blooming trees at the Mas Badia Agricultural Experimental Station (Girona, Spain) were also included.

Greenhouse experiments on leaves were conducted in potted plants (10-cm-diameter plastic pots) of pear (cv. Conference), strawberry (cv. Darselect), and kiwifruit (cv. Hayward). Plants were used when they were 30 to 40 cm in length, with 6 young leaves per shoot in pear plants, 10 to 15 young leaves in kiwifruit plants, and 5 to 8 leaves per crown in strawberry plants. Open blossoms of the Golden Smoothee apple cultivar were obtained from a commercial orchard near Girona (Spain). Flowery branches were collected and transported to the greenhouse under refrigeration, and the lower end of the branches was kept submerged in a 5% sucrose solution. To inoculate PM411, the plant material was sprayed to runoff (10 ml per pear plant, 6 ml per strawberry plant, 20 ml per kiwifruit plant, and 3 ml per open blossom) with the bacterial suspension at 10^8 CFU · ml^{-1}. All plant material was kept at 25°C, with a 16-h fluorescent light/8-h dark photoperiod. Treated flowery branches and potted plants were covered with plastic bags to reach high-rH conditions. A group of kiwifruit plants was maintained at low rH (70%) in controlled-environment chambers (SGC097.PFX.F; Fitotron, Sanyo Gallenkamp plc, UK). The experimental design consisted of three replicates of four pear potted plants, three strawberry and kiwifruit plants, or five flowery branches containing 15 blossoms in total. Experiments were carried out twice, except for strawberry and kiwifruit plants at high rH, which were performed once. Sampling for monitoring the PM411 population was performed immediately or at 12 h after inoculation and over six (plant assays) or eight (flower assays) days. Two blossoms, three leaflets of strawberry plants, and three leaves of pear or kiwifruit plants were taken from each replicate and sampling date.

Field experiments were conducted in plots of cv. Golden Smoothie apple and cv. Comice pear trees during the blooming period. Three replicates of 7 trees per replicate were used. In each tree, two branches containing blossoms were tagged. Two strategies were assayed in independent experiments:

one doing a single application of PM411 to trees (day 0), and a second strategy with two applications (days 0 and 5). Open blossoms from tagged branches were spray inoculated until near runoff with the bacterial suspension at 10^8 CFU · ml^{-1} using a handheld 5-ml sprayer (3 ml per blossom). Temperature, rH, and rainfall were measured with a weather station located in the experimental field (Mas Badia, La Tallada d'Empordà, Girona, Spain). Sample collection for monitoring PM411 levels was performed at 0, 1, 2, 5, 6, and 7 days. Samples of two blossoms (4 to 6 flowers and accompanying leaves) were taken from each replicate and sampling date.

Plant material washings were obtained as described above by homogenizing blossoms and leaves with 30 ml of 50 mM sterile PBS (pH 7.0) and 0.1% peptone at 130 rpm for 30 min on ice bath. Plant material washings from field experiments were 10-fold concentrated by centrifugation at 10,000 × *g* for 10 min of 20 ml of plant extract and resuspended in 2 ml of sterile PBS and 0.1% peptone. The population levels of PM411 on blossoms and leaves were determined using qPCR, v-qPCR, and plate counting.

For qPCR, DNA was isolated from 1 ml of each plant material washing, as explained above. DNA was evaluated in triplicate using the TaqMan-based qPCR assay B (PM411-for, PM411-pr, and PM411B-rev, 188 bp). The quantification was performed by means of a standard curve of the corresponding plant material washing (apple or pear blossoms or strawberry, kiwifruit, or pear leaves) specifically developed (linear range of 1×10^3 to 1×10^7 CFU · ml^{-1}, $R^2 = 0.99$, $E > 80\%$), and used in each plate run. The amount of total cells by qPCR was obtained by interpolating the C_T values from the unknown samples against the corresponding developed standard curve and expressed as $\log_{10}$ CFU per blossom or leaf.

For v-qPCR, previously to DNA isolation, 1 ml of sample was treated with PEMAX at 50 μM, according to the procedure described above. DNA extraction, qPCR assay, and quantification, using the corresponding standard curve with PEMAX treatment (linear range of 1×10^3 to 1×10^7 CFU · ml^{-1}, $R^2 = 0.99$, and $E > 80\%$), were carried out as described above.

For plate counting, plant material washings were serially diluted, and appropriate dilutions were seeded using a spiral plater (Eddy Jet; IUL Instruments, Barcelona, Spain) onto MRS agar plates supplemented with 50 μg · ml^{-1} rifampin (Sigma) to counterselect PM411 and 10 μg · ml^{-1} econazole nitrate salt (Sigma) to avoid fungal growth. Plates were incubated at 30°C for 48 h, and colonies were counted using an automatic counter system (Countermat Flash; IUL Instruments). The culturable population level of PM411 was expressed as the $\log_{10}$ CFU per blossom or leaf.

Statistical analysis. To test the significance of the effect of PEMAX concentration and qPCR design in the suppression of DNA amplification (signal reduction) on dead and viable cells of PM411, a two-way analysis of variance (ANOVA) was performed. To test the effect of the quantification method to estimate the PM411 population on plant surfaces for each sampling date, ANOVA was performed. Means of the ΔC_T (signal reduction) or CFU · $blossom^{-1}$ or CFU · $leaf^{-1}$ (population) were separated according to the Tukey's test at a P value of ≤0.05. The statistical analyses were performed using GLM procedure of the PC-SAS (version 9.1; SAS Institute, Inc., Cary, NC).

Accession number(s). The putative phage sequence of *L. plantarum* strain PM411 has been deposited in the GenBank database under the accession number MG788324.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00107-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

Funding was provided by AGL2015-69876-C2-1-R (Spain Ministerio de Economía y Competitividad and FEDER of the European Union), by FP7-KBBE.2013.1.2-04 613678 DROPSA of the European Union, and by grant MPCUdG2016 from the University of Girona. N. Daranas was the recipient of research grant 2015 FI_B00515 (Secretaria d'Universitats i Recerca, Departament d'Economia i Coneixement, Generalitat de Catalunya, Spain, European Union).

The research group is accredited by SGR 2014-697 and TECNIO net from Departament d'Economia i Coneixement–ACCIÓ Catalonia.

REFERENCES

1. Cheong EYL, Sandhu A, Jayabalan J, Le TTK, Nhiep NT, Ho THM, Zwielehner J, Bansal N, Turner MS. 2014. Isolation of lactic acid bacteria with antifungal activity against the common cheese spoilage mould *Penicillium commune* and their potential as biopreservatives in cheese. Food Control 46:91–97. https://doi.org/10.1016/j.foodcont.2014.05.011.
2. Di Gioia D, Mazzola G, Nikodinoska I, Aloisio I, Langerholc T, Rossi M, Raimondi S, Melero B, Rovira J. 2016. Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth. Int J Food Microbiol 235:53–59. https://doi.org/10.1016/j.ijfoodmicro.2016.06.019.
3. Gómez-Sala B, Herranz C, Díaz-Freitas B, Hernández PE, Sala A, Cintas LM. 2016. Strategies to increase the hygienic and economic value of fresh fish: biopreservation using lactic acid bacteria of marine origin. Int J Food Microbiol 223:41–49. https://doi.org/10.1016/j.ijfoodmicro.2016.02.005.
4. Trias R, Bañeras L, Badosa E, Montesinos E. 2008. Bioprotection of golden delicious apples and iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. Int J Food Microbiol 123:50–60. https://doi.org/10.1016/j.ijfoodmicro.2007.11.065.
5. Roselló G, Bonaterra A, Francés J, Montesinos L, Badosa E, Montesinos E. 2013. Biological control of fire blight of apple and pear with antagonistic

Lactobacillus plantarum. Eur J Plant Pathol 137:621–633. https://doi.org/10.1007/s10658-013-0275-7.
6. Trias R, Bañeras L, Montesinos E, Badosa E. 2008. Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. Int Microbiol 11:231–236.
7. Tsuda K, Tsuji G, Higashiyama M, Ogiyama H, Umemura K, Mitomi M, Kubo Y, Kosaka Y. 2016. Biological control of bacterial soft rot in Chinese cabbage by *Lactobacillus plantarum* strain BY under field conditions. Biol Control 100:63–69. https://doi.org/10.1016/j.biocontrol.2016.05.010.
8. Reis JA, Paula AT, Casarotti SN, Penna ALB. 2012. Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Eng Rev 4:124–140. https://doi.org/10.1007/s12393-012-9051-2.
9. Roselló G, Francés J, Daranas N, Montesinos E, Bonaterra A. 2017. Control of fire blight of pear trees with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid. J Plant Pathol 99:111–120.
10. Martini M, Moruzzi S, Ermacora P, Loi N, Firrao G. 2015. Quantitative real-time PCR and high-resolution melting (HRM) analysis for strain-specific monitoring of fluorescent pseudomonads used as biocontrol agents against soil-borne pathogens of food crops. Trends Food Sci Technol 46:277–285. https://doi.org/10.1016/j.tifs.2015.10.017.
11. Montesinos E. 2003. Development, registration and commercialization of microbial pesticides for plant protection. Int Microbiol 6:245–252. https://doi.org/10.1007/s10123-003-0144-x.
12. Bonaterra A, Badosa E, Cabrefiga J, Francés J, Montesinos E. 2012. Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. Trees 26:215–226. https://doi.org/10.1007/s00468-011-0626-y.
13. Shade A, McManus PS, Handelsman J. 2013. Unexpected diversity during community succession in the apple flower microbiome. mBio 4:e00602-12. https://doi.org/10.1128/mBio.00602-12.
14. Yashiro E, McManus PS. 2012. Effect of streptomycin treatment on bacterial community structure in the apple phyllosphere. PLoS One 7:e37131. https://doi.org/10.1371/journal.pone.0037131.
15. Pujol M, Badosa E, Manceau C, Montesinos E. 2006. Assessment of the environmental fate of the biological control agent of fire blight, Pseudomonas fluorescens EPS62e, on apple by culture and real-time PCR methods. Appl Environ Microbiol 72:2421–2427. https://doi.org/10.1128/AEM.72.4.2421-2427.2006.
16. Soto-Muñoz L, Torres R, Usall J, Viñas I, Solsona C, Teixidó N. 2015. DNA-based methodologies for the quantification of live and dead cells in formulated biocontrol products based on *Pantoea agglomerans* CPA-2. Int J Food Microbiol 210:79–83. https://doi.org/10.1016/j.ijfoodmicro.2015.06.013.
17. Papadimitriou K, Alegría Á Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, van Sinderen D, Varmanen P, Ventura M, Zúñiga M, Tsakalidou E, Kok J. 2016. Stress physiology of lactic acid bacteria. Microbiol Mol Biol Rev 80:837–890. https://doi.org/10.1128/MMBR.00076-15.
18. Oliver JD. 2005. The viable but nonculturable state in bacteria. J Microbiol 43:93–100.
19. Liu J, Deng Y, Peters BM, Li L, Li B, Chen L, Xu Z, Shirtliff ME. 2016. Transcriptomic analysis on the formation of the viable putative non-culturable state of beer-spoilage *Lactobacillus acetotolerans*. Sci Rep 6:36753. https://doi.org/10.1038/srep36753.
20. Pinto D, Santos MA, Chambel L. 2015. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. Crit Rev Microbiol 41:61–76. https://doi.org/10.3109/1040841X.2013.794127.
21. Soto-Muñoz L, Teixidó N, Usall J, Viñas I, Abadias M, Torres R. 2015. Molecular tools applied to identify and quantify the biocontrol agent *Pantoea agglomerans* CPA-2 in postharvest treatments on oranges. Postharvest Biol Technol 100:151–159. https://doi.org/10.1016/j.postharvbio.2014.10.004.
22. Josephson KL, Gerba CP, Pepper IL. 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl Environ Microbiol 59:3513–3515.
23. Elizaquível P, Sánchez G, Aznar R. 2012. Quantitative detection of viable foodborne *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. Food Control 25:704–708. https://doi.org/10.1016/j.foodcont.2011.12.003.
24. Liu Y, Mustapha A. 2014. Detection of viable *Escherichia coli* O157:H7 in ground beef by propidium monoazide real-time PCR. Int J Food Microbiol 170:48–54. https://doi.org/10.1016/j.ijfoodmicro.2013.10.026.
25. Rudi K, Moen B, Drømtorp SM, Holck AL. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71:1018–1024. https://doi.org/10.1128/AEM.71.2.1018-1024.2005.
26. Seinige D, Krischek C, Klein G, Kehrenberg C. 2014. Comparative analysis and limitations of ethidium monoazide and propidium monoazide treatments for the differentiation of viable and nonviable *Campylobacter* cells. Appl Environ Microbiol 80:2186–2192. https://doi.org/10.1128/AEM.03962-13.
27. Martin B, Raurich S, Garriga M, Aymerich T. 2013. Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham. Food Anal Methods 6:683–690. https://doi.org/10.1007/s12161-012-9460-0.
28. Villarreal MLM, Padilha M, Vieira ADS, Franco BD, Martinez RCR, Saad SMI. 2013. Advantageous direct quantification of viable closely related probiotics in Petit-Suisse cheeses under *in vitro* gastrointestinal conditions by propidium monoazide-qPCR. PLoS One 8:e82102. https://doi.org/10.1371/journal.pone.0082102.
29. Desfossés-Foucault E, Dussault-Lepage V, Le Boucher C, Savard P, Lapointe G, Roy D. 2012. Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification. Front Microbiol 3:350. https://doi.org/10.3389/fmicb.2012.00350.
30. Felici C, Vettori L, Toffanin A, Nuti M. 2008. Development of a strain-specific genomic marker for monitoring a *Bacillus subtilis* biocontrol strain in the rhizosphere of tomato. FEMS Microbiol Ecol 65:289–298. https://doi.org/10.1111/j.1574-6941.2008.00489.x.
31. Sisto A, De Bellis P, Visconti A, Morelli L, Lavermicocca P. 2009. Development of a PCR assay for the strain-specific identification of probiotic strain *Lactobacillus paracasei* IMPC2.1. Int J Food Microbiol 136:59–65. https://doi.org/10.1016/j.ijfoodmicro.2009.09.017.
32. Braun-Kiewnick A, Lehmann A, Rezzonico F, Wend C, Smits THM, Duffy B. 2012. Development of species-, strain- and antibiotic biosynthesis-specific quantitative PCR assays for *Pantoea agglomerans* as tools for biocontrol monitoring. J Microbiol Methods 90:315–320. https://doi.org/10.1016/j.mimet.2012.06.004.
33. Nocker A, Cheung C-Y, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67:310–320. https://doi.org/10.1016/j.mimet.2006.04.015.
34. Fittipaldi M, Nocker A, Codony F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Methods 91:276–289. https://doi.org/10.1016/j.mimet.2012.08.007.
35. Elizaquível P, Aznar R, Sánchez G. 2014. Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. J Appl Microbiol 116:1–13. https://doi.org/10.1111/jam.12365.
36. Codony F, Agustí G, Allué-Guardia A. 2015. Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR. Mol Cell Probes 29:190–192. https://doi.org/10.1016/j.mcp.2015.03.003.
37. Agustí G, Fittipaldi M, Codony F. 2017. False-positive viability PCR results: an association with microtubes. Curr Microbiol 74:377–380. https://doi.org/10.1007/s00284-016-1189-3.
38. Codony F. 2014. Procedimiento para la detección de células vivas, con las membranas celulares íntegras y funcionales, mediante técnicas de amplificación de ácidos nucleicos. Spanish patent ES2568527B1.
39. Lizana X, López A, Benito S, Agustí G, Ríos M, Piqué N, Marqués AM, Codony F. 2017. Viability qPCR, a new tool for *Legionella* risk management. Int J Hyg Environ Health 220:1318–1324. https://doi.org/10.1016/j.ijheh.2017.08.007.
40. Dinh Thanh M, Agustí G, Mader A, Appel B, Codony F. 2017. Improved sample treatment protocol for accurate detection of live *Salmonella* spp. in food samples by viability PCR. PLoS One 12:e0189302. https://doi.org/10.1371/journal.pone.0189302.
41. Yoon BH, Jang SH, Chang H-I. 2011. Sequence analysis of the *Lactobacillus* temperate phage Sha1. Arch Virol 156:1681–1684. https://doi.org/10.1007/s00705-011-1048-2.
42. Dieterle ME, Fina Martin J, Durán R, Nemirovsky SI, Sanchez Rivas C, Bowman C, Russell D, Hatfull GF, Cambillau C, Piuri M. 2016. Characterization of prophages containing "evolved" Dit/Tal modules in the genome of *Lactobacillus casei* BL23. Appl Microbiol Biotechnol 100:9201–9215. https://doi.org/10.1007/s00253-016-7727-x.
43. Villion M, Moineau S. 2009. Bacteriophages of *Lactobacillus*. Front Biosci 14:1661–1683. https://doi.org/10.2741/3332.

44. Abriouel H, Montoro BP, Del M, Casado Muñoz C, Knapp CW, Gálvez A, Benomar N. 2017. *In silico* genomic insights into aspects of food safety and defense mechanisms of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives. PLoS One 12:e0176801. https://doi.org/10.1371/journal.pone.0176801.
45. Liu C-J, Wang R, Gong F-M, Liu X-F, Zheng H-J, Luo Y-Y, Li X-R. 2015. Complete genome sequences and comparative genome analysis of *Lactobacillus plantarum* strain 5-2 isolated from fermented soybean. Genomics 106:404–411. https://doi.org/10.1016/j.ygeno.2015.07.007.
46. Morgan GJ, Pitts WB. 2008. Evolution without species: the case of mosaic bacteriophages. Br J Philos Sci 59:745–765. https://doi.org/10.1093/bjps/axn038.
47. Colavecchio A, D'Souza Y, Tompkins E, Jeukens J, Freschi L, Emond-Rheault J-G, Kukavica-Ibrulj I, Boyle B, Bekal S, Tamber S, Levesque RC, Goodridge LD. 2017. Prophage integrase typing is a useful indicator of genomic diversity in *Salmonella enterica*. Front Microbiol 8:1283 1–11. https://doi.org/10.3389/fmicb.2017.01283.
48. Brandt K, Tilsala-Timisjärvi A, Alatossava T. 2001. Phage-related DNA polymorphism in dairy and probiotic *Lactobacillus*. Micron 32:59–65. https://doi.org/10.1016/S0968-4328(00)00030-5.
49. Brandt K, Alatossava T. 2003. Specific identification of certain probiotic *Lactobacillus rhamnosus* strains with PCR primers based on phage-related sequences. Int J Food Microbiol 84:189–196. https://doi.org/10.1016/S0168-1605(02)00419-1.
50. Coudeyras S, Marchandin H, Fajon C, Forestier C. 2008. Taxonomic and strain-specific identification of the probiotic strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* group. Appl Environ Microbiol 74:2679–2689. https://doi.org/10.1128/AEM.02286-07.
51. Verghese B, Lok M, Wen J, Alessandria V, Chen Y, Kathariou S, Knabel S. 2011. *comK* prophage junction fragments as markers for *Listeria monocytogenes* genotypes unique to individual meat and poultry processing plants and a model for rapid niche-specific adaptation, biofilm formation, and persistence. Appl Environ Microbiol 77:3279–3292. https://doi.org/10.1128/AEM.00546-11.
52. Di Cagno R, Surico RF, Siragusa S, De Angelis M, Paradiso A, Minervini F, De Gara L, Gobbetti M. 2008. Selection and use of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. Int J Food Microbiol 127:220–228. https://doi.org/10.1016/j.ijfoodmicro.2008.07.010.
53. Sattler VA, Mohnl M, Klose V. 2014. Development of a strain-specific real-time PCR assay for enumeration of a probiotic *Lactobacillus reuteri* in chicken feed and intestine. PLoS One 9:e90208. https://doi.org/10.1371/journal.pone.0090208.
54. Fibi S, Klose V, Mohnl M, Weber B, Haslberger AG, Sattler VA. 2016. Suppression subtractive hybridisation and real-time PCR for strain-specific quantification of the probiotic *Bifidobacterium animalis* BAN in broiler feed. J Microbiol Methods 123:94–100. https://doi.org/10.1016/j.mimet.2016.02.011.
55. Rotolo C, De Miccolis Angelini RM, Pollastro S, Faretra F. 2016. A TaqMan-based qPCR assay for quantitative detection of the biocontrol agents *Bacillus subtilis* strain QST713 and *Bacillus amyloliquefaciens subsp. plantarum* strain D747 BioControl 61:91–101.
56. Liang N, Dong J, Luo L, Li Y. 2011. Detection of viable *Salmonella* in lettuce by propidium monoazide real-time PCR. J Food Sci 76: M234–M237. https://doi.org/10.1111/j.1750-3841.2011.02123.x.
57. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797.
58. Contreras PJ, Urrutia H, Sossa K, Nocker A. 2011. Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. J Microbiol Methods 87:89–95. https://doi.org/10.1016/j.mimet.2011.07.016.
59. Soto-Muñoz L, Teixidó N, Usall J, Viñas I, Torres R. 2014. Detection and quantification by PCR assay of the biocontrol agent *Pantoea agglomerans* CPA-2 on apples. Int J Food Microbiol 175:45–52. https://doi.org/10.1016/j.ijfoodmicro.2014.01.014.
60. Pujol M, Badosa E, Montesinos E. 2007. Epiphytic fitness of a biological control agent of fire blight in apple and pear orchards under Mediterranean weather conditions. FEMS Microbiol Ecol 59:186–193. https://doi.org/10.1111/j.1574-6941.2006.00227.x.
61. Pusey PL, Rudell DR, Curry EA, Mattheis JP. 2008. Characterization of stigma exudates in aqueous extracts from apple and pear flowers. HortScience 43:1471–1478.
62. Siezen RJ, Tzeneva VA, Castioni A, Wels M, Phan HTK, Rademaker JLW, Starrenburg MJC, Kleerebezem M, Molenaar D, van Hylckama Vlieg JET. 2010. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environ Microbiol 12:758–773. https://doi.org/10.1111/j.1462-2920.2009.02119.x.
63. Lindow SE, Brandl MT. 2003. Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883. https://doi.org/10.1128/AEM.69.4.1875-1883.2003.
64. Redford AJ, Bowers RM, Knight R, Linhart Y, Fierer N. 2010. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environ Microbiol 12:2885–2893. https://doi.org/10.1111/j.1462-2920.2010.02258.x.
65. Rezzonico F, Moënne-Loccoz Y, Défago G. 2003. Effect of stress on the ability of a *phlA*-based quantitative competitive PCR assay to monitor biocontrol strain *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol 69:686–690. https://doi.org/10.1128/AEM.69.1.686-690.2003.
66. Daranas N, Badosa E, Francés J, Montesinos E, Bonaterra A. 2018. Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. PLoS One 13:e0190931. https://doi.org/10.1371/journal.pone.0190931.
67. Llop P, Caruso P, Cubero J, Morente C, López MM. 1999. A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. J Microbiol Methods 37:23–31. https://doi.org/10.1016/S0167-7012(99)00033-0.
68. Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–10890. https://doi.org/10.1093/nar/16.22.10881.
69. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. 2011. PHAST: a fast phage search tool. Nucleic Acids Res 39:W347–W352. https://doi.org/10.1093/nar/gkr485.
70. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40.
71. Schmidt RJ, Emara MG, Kung L, Jr. 2008. The use of a quantitative real-time polymerase chain reaction assay for identification and enumeration of *Lactobacillus buchneri* in silage. J Appl Microbiol 105:920–929. https://doi.org/10.1111/j.1365-2672.2008.03834.x.
72. Tailliez P, Tremblay J, Dusko Ehrlich S, Chopin A. 1998. Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). Syst Appl Microbiol 21:530–538. https://doi.org/10.1016/S0723-2020(98)80065-9.
73. Di Cagno R, Minervini G, Sgarbi E, Lazzi C, Bernini V, Neviani E, Gobbetti M. 2010. Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing *Lactobacillus plantarum* isolates from raw vegetables and fruit. Int J Food Microbiol 143:246–253. https://doi.org/10.1016/j.ijfoodmicro.2010.08.018.
74. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, Curtiss R, III, Gyles CL. 1992. Amplification of an *invA* gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes 6:271–279. https://doi.org/10.1016/0890-8508(92)90002-F.
75. Holt SM, Cote GL. 1998. Differentiation of dextran-producing *Leuconostoc* strains by a modified randomly amplified polymorphic DNA protocol. Appl Environ Microbiol 64:3096–3098.
76. Moschetti G, Blaiotta G, Aponte M, Catzeddu P, Villani F, Deiana P, Coppola S. 1998. Random amplified polymorphic DNA and amplified ribosomal DNA spacer polymorphism: powerful methods to differentiate *Streptococcus thermophilus* strains. J Appl Microbiol 85:25–36. https://doi.org/10.1046/j.1365-2672.1998.00461.x.

Table S1. Characteristics of the putative prophage Lactob_PLE3 (69.6 Kb) in *Lactobacillus plantarum* PM411 genome according to PHAST bioinformatic tool.

#	CDS POSITION	BLAST HIT [1]	E-VALUE
1	1..12	attL ATAAAATAAAAA	0
2	complement(9359..10516)	PHAGE_Lactob_Lj965_NC_005355: putative integrase; PP_00012; phage(gi41179218)	2.00E-46
3	complement(10686..11354)	PHAGE_Sulfit_NYA_2014a_NC_027299: T5ord172-domain containing protein; PP_00013; phage(gi848469691)	4.00E-05
4	complement(11466..11897)	nucleotide-binding protein, UspA family [Lactobacillus plantarum JDM1]. gi|254555641|ref|YP_003062058.1|; PP_00014	2.00E-75
5	complement(11997..12254)	hypothetical; PP_00015	0
6	complement(12378..12554)	hypothetical protein LPST_C1709 [Lactobacillus plantarum subsp. plantarum ST-III]. gi|308180891|ref|YP_003925019.1|; PP_00016	3.00E-21
7	complement(12581..12670)	tRNA	0
8	complement(12724..13284)	hypothetical; PP_00017	0
9	complement(13376..13807)	PHAGE_Lactob_phig1e_NC_004305: hypothetical protein; PP_00018; phage(gi23455774)	2.00E-31
10	complement(13817..14191)	PHAGE_Lactob_phig1e_NC_004305: repressor; PP_00019; phage(gi23455773)	1.00E-22
11	14513..14722	PHAGE_Strept_phiARI0746_NC_031907: hypothetical protein; PP_00020; phage(gi100038)	1.00E-06
12	14726..14929	hypothetical protein JDM1_0477 [Lactobacillus plantarum JDM1]. gi|254555646|ref|YP_003062063.1|; PP_00021	1.00E-25
13	14929..15201	hypothetical protein JDM1_0478 [Lactobacillus plantarum JDM1]. gi|254555647|ref|YP_003062064.1|; PP_00022	1.00E-43
14	15339..15788	PHAGE_Lister_B025_NC_009812: gp52; PP_00023; phage(gi157325269)	3.00E-39
15	15775..15951	Hypothetical protein zj316_1378 [Lactobacillus plantarum ZJ316]. gi|448820901|ref|YP_007414063.1|; PP_00024	3.00E-21
16	15944..16129	hypothetical protein JDM1_0480 [Lactobacillus plantarum JDM1]. gi|254555649|ref|YP_003062066.1|; PP_00025	4.00E-28
17	16101..16271	Hypothetical protein zj316_1380 [Lactobacillus plantarum ZJ316]. gi|448820903|ref|YP_007414065.1|; PP_00026	4.00E-18
18	16274..16591	hypothetical; PP_00027	0
19	16579..16698	Hypothetical protein zj316_1381 [Lactobacillus plantarum ZJ316]. gi|448820904|ref|YP_007414066.1|; PP_00028	7.00E-09
20	16695..17174	PHAGE_Lactob_LfeSau_NC_029068: hypothetical protein; PP_00029; phage(gi985757759)	7.00E-33
21	17245..18585	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00030; phage(gi100040)	7.00E-153
22	18657..19298	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00031; phage(gi100041)	1.00E-63
23	19301..19924	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00032; phage(gi100042)	9.00E-30
24	19995..20789	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00033; phage(gi100043)	1.00E-74
25	20786..22060	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00034; phage(gi100044)	9.00E-123
26	22318..22659	phage protein [Lactobacillus plantarum JDM1]. gi|254555659|ref|YP_003062076.1|; PP_00035	3.00E-57
27	22640..22837	hypothetical; PP_00036	0
28	22830..23141	hypothetical protein Lp16_1925 [Lactobacillus plantarum 16]. gi|513841770|ref|YP_008121742.1|; PP_00037	8.00E-53
29	23145..23303	PHAGE_Lactob_Sha1_NC_019489: hypothetical protein; PP_00038; phage(gi418489791)	1.00E-09
30	23306..23431	hypothetical protein JDM1_0491 [Lactobacillus plantarum JDM1]. gi|254555660|ref|YP_003062077.1|; PP_00039	7.00E-14
31	23664..23954	PHAGE_Weisse_WCP30_NC_031101: XRE family transcriptional regulator; PP_00040; phage(gi100024)	3.00E-08
32	23951..24409	PHAGE_Lactob_Sha1_NC_019489: hypothetical protein; PP_00041; phage(gi418489796)	5.00E-22
33	24649..24960	hypothetical protein JDM1_0494 [Lactobacillus plantarum JDM1]. gi|254555663|ref|YP_003062080.1|; PP_00042	6.00E-52
34	24972..25385	Prophage protein [Lactobacillus plantarum ZJ316]. gi|448820916|ref|YP_007414078.1|; PP_00043	3.00E-71
35	25501..26490	hypothetical; PP_00044	0
36	26571..27293	hypothetical; PP_00045	0
37	27508..27918	PHAGE_Entero_phiEf11_NC_013696: SbcC domain protein; PP_00046; phage(gi282598758)	3.00E-12
38	27985..28200	PHAGE_Lactob_phig1e_NC_004305: hypothetical protein; PP_00047; phage(gi254854752)	8.00E-08
39	28184..28522	PHAGE_Lister_B025_NC_009812: gp65; PP_00048; phage(gi157325282)	7.00E-25
40	28522..28764	Prophage protein [Lactobacillus plantarum ZJ316]. gi|448820920|ref|YP_007414082.1|; PP_00049	3.00E-32
41	28782..29024	Prophage protein [Lactobacillus plantarum ZJ316]. gi|448820921|ref|YP_007414083.1|; PP_00050	6.00E-37
42	29132..29419	PHAGE_Bacill_vB_BhaS_171_NC_030904: putative Cro/Cl-type repressor; PP_00051; phage(gi100005)	1.00E-12
43	29416..31098	PHAGE_Bacill_vB_BhaS_171_NC_030904: XRE family transcriptional regulator; PP_00052; phage(gi100006)	3.00E-141
44	31117..32259	PHAGE_Staphy_P954_NC_013195: HK97 family phage portal protein; PP_00053; phage(gi257136404)	1.00E-93

45	32246..32998	PHAGE_Lactob_PLE2_NC_031036: hypothetical protein; PP_00054; phage(gi100004)	4.00E-45
46	33019..34197	PHAGE_Lister_LP_101_NC_024387: major capsid protein; PP_00055; phage(gi658607815)	5.00E-101
47	34336..34641	PHAGE_Bacill_vB_BhaS_171_NC_030904: endonuclease; PP_00056; phage(gi100011)	5.00E-10
48	34622..35011	PHAGE_Lister_B025_NC_009812: gp8; PP_00057; phage(gi157325226)	2.00E-20
49	35008..35415	PHAGE_Lister_LP_101_NC_024387: hypothetical protein; PP_00058; phage(gi658607819)	1.00E-14
50	35412..35834	PHAGE_Lister_LP_101_NC_024387: hypothetical protein; PP_00059; phage(gi658607820)	9.00E-15
51	35849..36454	PHAGE_Staphy_6ec_NC_024355: major tail protein; PP_00060; phage(gi658310345)	1.00E-21
52	36429..36701	PHAGE_Salmon_BP63_NC_031250: terminase small subunit; PP_00061; phage(gi100030)	6.00E-10
53	36773..37087	hypothetical protein JDM1_0510 [Lactobacillus plantarum JDM1]. gi|254555679|ref|YP_003062096.1|; PP_00062	1.00E-47
54	37111..37332	Prophage protein, tail tape measure protein [Lactobacillus plantarum ZJ316]. gi|448820933|ref|YP_007414095.1|; PP_00063	3.00E-32
55	37351..41895	PHAGE_Lactob_LF1_NC_019486: phage tail tape measure protein; PP_00064; phage(gi418489397)	0
56	41899..42720	PHAGE_Entero_phiFL1A_NC_013646: tail protein; PP_00065; phage(gi281416378)	3.00E-31
57	42740..46666	PHAGE_Lactob_ATCC8014_NC_019916: prophage tail super family protein; PP_00066; phage(gi431809813)	8.00E-75
58	46689..47174	PHAGE_Brocho_BL3_NC_015254: gp20; PP_00067; phage(gi327409412)	2.00E-09
59	47176..47610	hypothetical protein JDM1_0516 [Lactobacillus plantarum JDM1]. gi|254555685|ref|YP_003062102.1|; PP_00068	1.00E-74
60	47627..48010	PHAGE_Lactob_LfeSau_NC_029068: hypothetical protein; PP_00069; phage(gi985757745)	8.00E-32
61	48013..48207	PHAGE_Lactob_phijl1_NC_006936: hypothetical protein; PP_00070; phage(gi62327114)	3.00E-05
62	48207..48491	PHAGE_Lactob_phig1e_NC_004305: holin; PP_00071; phage(gi23455817)	1.00E-06
63	48491..49423	PHAGE_Entero_EF62phi_NC_017732: endolysin type Endo-N-acetylmuramidase; PP_00072; phage(gi384519788)	2.00E-40
64	49669..50103	PHAGE_Lactob_Lv_1_NC_011801: portal protein; PP_00073; phage(gi219563200)	2.00E-21
65	50066..51916	PHAGE_Lactob_Lv_1_NC_011801: protease-scaffold-major head protein; PP_00074; phage(gi219563201)	2.00E-65
66	52061..52378	hypothetical protein JDM1_0989 [Lactobacillus plantarum JDM1]. gi|254556156|ref|YP_003062573.1|; PP_00075	9.00E-33
67	52365..52712	PHAGE_Strept_Sfi19_NC_000871: putative head-tail joining protein; PP_00076; phage(gi9632902)	2.00E-11
68	52715..53119	PHAGE_Strept_DT1_NC_002072: putative tail component protein; PP_00077; phage(gi9632427)	3.00E-29
69	53119..53499	PHAGE_Strept_Sfi21_NC_000872: putative tail component protein; PP_00078; phage(gi9632947)	8.00E-16
70	53516..54169	phage major tail protein [Lactobacillus plantarum subsp. plantarum ST-III]. gi|308180857|ref|YP_003924985.1|; PP_00079	6.00E-113
71	54245..54619	hypothetical protein PEPE_0995 [Pediococcus pentosaceus ATCC 25745]. gi|116492758|ref|YP_804493.1|; PP_00080	8.00E-61
72	54892..59643	PHAGE_Strept_DT1_NC_002072: putative tail component protein; PP_00081; phage(gi29165636)	6.00E-176
73	59720..61495	PHAGE_Lactob_Ldl1_NC_026609: distal tail protein; PP_00082; phage(gi764162085)	1.00E-48
74	61559..63970	PHAGE_Lactob_Ldl1_NC_026609: baseplate protein tal-like protein; PP_00083; phage(gi764162086)	4.00E-129
75	63988..66228	PHAGE_Lactob_LP65_NC_006565: tail fiber; PP_00084; phage(gi56693145)	2.00E-139
76	66221..66463	PHAGE_Lactob_Sha1_NC_019489: hypothetical protein; PP_00085; phage(gi418489818)	3.00E-33
77	66467..66628	PHAGE_Lactob_Sha1_NC_019489: hypothetical protein; PP_00086; phage(gi418489819)	3.00E-22
78	66612..67118	PHAGE_Lactob_Sha1_NC_019489: prophage Lp2 protein 53-like protein; PP_00087; phage(gi418489820)	2.00E-68
79	67124..67594	PHAGE_Lactob_iLp1308_NC_028911: tail fiber protein; PP_00088; phage(gi971754939)	1.00E-13
80	67606..68634	PHAGE_Lactob_PLE3_NC_031125: hypothetical protein; PP_00089; phage(gi100023)	1.00E-79
81	68634..68903	PHAGE_Lactob_Sha1_NC_019489: phage-related holin; PP_00090; phage(gi418489822)	6.00E-32
82	68915..69277	PHAGE_Lactob_LP65_NC_006565: hypothetical protein; PP_00091; phage(gi56693137)	4.00E-23
83	69638..69649	attR ATAAAATAAAAA	0

[1]: Hits against Virus and prophage DB (marked in grey) and hits against Bacterial DB or GenBank file (not marked)

CHAPTER VI

General Discussion

Bacterial plant diseases, such as fire blight of apple and pear, bacterial canker of kiwifruit, bacterial spot of stone fruits, and angular leaf spot of strawberry, are responsible for important crop losses worldwide because few effective management options are available. Although chemical pesticides have been generally effective, the restrictions in the use of some products (e.g. antibiotics in the European Union), the increasing evolution of pathogen resistance to existing bactericides, as well as, the public concern about the negative impact of conventional pesticides on environment and human health, are promoting the development of alternative and sustainable management tools. In particular, biological control is an alternative or complementary strategy since the use of microbial biopesticides could be merged into an integrated management approach of crop protection (Sundin *et al*., 2016; Chandler *et al*., 2011).

Lactic acid bacteria (LAB) have many characteristics that make them attractive biological control agents (BCA) as active ingredients of microbial biopesticides. LAB are well known as biopreservative agents in food against pathogenic and spoilage microorganisms (Tomé *et al*., 2008; Trias *et al*., 2008a, 2008b; Lan *et al*., 2012; Cizeikiene *et al*., 2013; Crowley *et al*., 2013; Cheong *et al*., 2014; Di Gioia *et al*., 2016; Gómez-Sala *et al*., 2016; Saraoui *et al*., 2017). The potential use of LAB in crop protection has also been explored and some strains, mostly *Lactobacillus plantarum*, have been reported during the last decade as antagonists of plant pathogenic bacteria and fungi (Wang *et al*., 2011, 2012; Shrestha *et al*., 2014; Baffoni *et al*., 2015; Tsuda *et al*., 2016). However, there is no microbial biopesticides based on LAB available on a commercial scale so far. Bacterial BCA registered in the European Union (EU) belong to species of *Bacillus*, *Pseudomonas*, and *Streptomyces*. In addition, LAB are plant-associated microorganisms being ubiquitous members of flower, leaf and fruit microbiomes (Trias *et al*., 2008b; Zwielehner *et al*., 2008; Leff & Fierer, 2013; Shade *et al*., 2013; Williams *et al*., 2013). Interestingly, most LAB fulfil the requirements concerning biosafety since several species, including *L. plantarum*, are considered with the Qualified Presumption of Safety (QPS) status by the European Food Safety Agency (EFSA) (EFSA Panel on Biological Hazards, 2017) and certain strains are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA) (FDA, 2018). Consequently, it is expected that LAB strains would pose no risks for applications in edible crop protection and would not be limited to be approved as active ingredient of a microbial biopesticide regarding biosafety (Regulation (EC) No 1107/2009). Moreover, cost-effective strategies for industrial scale production of LAB biomass have been reported although these bacteria have numerous growth requirements (Schiraldi *et al*., 2003; Aguirre-Ezkauriatza *et al*., 2010; Krzywonos & Eberhard, 2011).

This Ph.D. Thesis contributes to the development of a novel microbial biopesticide based on LAB, specifically *L. plantarum*, as broad-spectrum BCA. Some of the phases involved in its development have been addressed, such as the screening of effective BCA together with their characterization, the improvement of their fitness onto plant surfaces, and the development of a strain-specific monitoring method.

Biopesticides with wide spectrum of application are really appreciated for the management of multiple diseases in different cropping systems or in a single crop since it remains an unfulfilled goal (Köhl *et al*., 2011; Droby *et al*., 2016). The first aim of this Thesis was the screening of LAB as candidate BCA with the main purpose to identify strains showing suppression toward a broad range of bacterial phytopathogens, such as *Pseudomonas syringae* pv. *actinidiae* (Psa), *Xanthomonas arboricola* pv. *pruni* (Xap), and *Xanthomonas fragariae* (Xf) (Chapter III of this Thesis). Similarly, a multi-pathogen approach for the screening of microbial antagonists was addressed in other studies as well (Roberts *et al*., 2005; Suárez-Estrella *et al*., 2013; Gava *et al*., 2018). In a preliminary phase, *L. plantarum* CC100, PM411, and TC92 and *Leuconostoc mesenteroides* CM160 and CM209 were preselected owing to high *in vitro* antagonistic activity against the three bacterial phytopathogens. The antibacterial activity of PM411, TC92, and CM209 was previously reported against *Erwinia amylovora,* as well as, against other target bacteria, such as *Pseudomonas syringae*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Roselló *et al*., 2013). These results are in agreement with previous reported antagonistic activity of certain *L. plantarum* and *L. mesenteroides* strains against Gram-positive and Gram-negative bacteria (Elegado *et al*., 2004; Ben Omar *et al*., 2008; Knoll *et al*., 2008; Darsanaki *et al*., 2012; Fhoula *et al*., 2013; Arena *et al*., 2016).

Since the correlation between *in vitro* and *in vivo* results is not always found as it has been reported by Parikh *et al*. (2018), the applicability of the preselected LAB strains as BCA was studied in more detail by means of *in planta* efficacy assays in greenhouse, in which the interaction between the host plant, the pathogen, and the antagonist is taken into consideration (Köhl *et al*., 2011). *L. plantarum* PM411 and TC92 consistently reduced infections in the three pathosystems (Psa-kiwifruit, Xap-*Prunus*, and Xf-strawberry). Indeed, both strains were also selected because in a previous report they showed biocontrol efficacy against *E. amylovora* in pear plants (Roselló *et al*., 2013, 2017).

Therefore, the multifactorial screening procedure based on *in vitro* and *in planta* assays against multiple pathogens was successful in selecting two very interesting BCA, *L. plantarum* PM411 and TC92, showing effective biological control of bacterial canker of kiwifruit, bacterial spot of stone fruits, and angular leaf spot of strawberry.

The characterization of BCA is required for their development as biopesticides and for the registration procedure. Therefore, once PM411 and TC92 strains were selected as candidate BCA, the mechanism of action involved in the pathogen suppression and the genotypic characterization were tackled (Chapter III). Organic acids, such as lactic acid, were the preferred mediators of *in vitro* inhibition of Psa, Xap, and Xf by both strains. Similarly, lactic and acetic acid, as the principal end products of the metabolism of probiotic LAB strains, have been attributed as the main antimicrobial agents against foodborne pathogens (Tejero-Sariñena *et al*., 2012). Although the widely reported antagonistic activity of bacteriocins against foodborne pathogens and spoilage bacteria (Allende *et al*., 2007) and the presence of the biosynthetic plantaricin genes *plnEF* and

plnJK in the two selected strains (Roselló *et al*., 2013) with similar levels of expression (this Thesis, Daranas *et al*., 2018a), the antagonistic role of plantaricins in PM411 and TC92 was not proved in this study. Gram-negative bacteria are naturally resistant to bacteriocins due to their outer membrane, which acts as an effective barrier. Nevertheless, lactic acid produced by LAB might promote the sensitization of Gram-negative bacterial pathogens towards bacteriocins destabilizing the outer membrane (Alakomi *et al*., 2000; Prudêncio *et al*., 2015). On leaf surfaces both strains were able to survive and, in particular, PM411 showed a suppression of Psa population. In order to better elucidate the multifactorial mechanism of PM411 and TC92 responsible for pathogen suppression, it would be interesting to determine the antimicrobial metabolites produced directly on plant surfaces. It is hypothesized that under aerobic conditions the production of lactic acid or other organic acids resulting from fermentation might be limited, whereas the formation of hydrogen peroxide could contribute to the antagonism. Accordingly to other authors, hydrogen peroxide accumulated by LAB in a cell suspension has shown inhibitory effect against foodborne pathogens (Ito *et al*., 2003).

Although the similarities explained above between PM411 and TC92 strains, multilocus sequence typing (MLST) together with random amplified polymorphic DNA-PCR (RAPD-PCR) analysis of several *L. plantarum* strains allowed a clear discrimination between both strains. This is in accordance with previous reports in which these genotypic methods provided different profiles within *L. plantarum* strains (de las Rivas *et al*., 2006; Tanganurat *et al*., 2009; Di Cagno *et al*., 2010 Xu *et al*., 2015). The concordance between clusters obtained using MLST and RAPD-PCR analysis provided robust results. De las Rivas *et al*. (2006) suggested that strains sharing the same sequence type (ST) might be clones. In particular, PM411 shared the same ST with two other *L. plantarum* strains from our collection (PM314 and PM340) which were isolated from the same sample (pear). The hypothesis that they could be clones was confirmed later by the amplification of repetitive element sequences (rep-PCR) (this Thesis, Daranas *et al*., 2018b). In contrast, TC92 shared the same ST with several strains but it is unlikely that they could be clones since they were isolated from different plant sources.

Acceptable and consistent performance under field conditions is critical for the success of any microbial biopesticide. Therefore, the efficacy of the two selected *L. plantarum* strains PM411 and TC92 in suppressing Psa, Xap, and Xf infections in plants was confirmed by means of semi-field and field assays and was comparable with reference products, including commercial microbial biopesticides, plant defence elicitors, copper and antibiotics. Nevertheless, variable biocontrol performance of PM411 and TC92 in the field is very likely due to fluctuating environmental conditions.

The establishment of suppressive populations of BCA on aerial plant surfaces, where many pathogens epiphytically grow, is suggested as a key factor for the development of protection against pathogens (Stockwell *et al*., 1998; Hagen *et al*., 2009; Stockwell *et al*., 2010). However,

aerial plant surfaces are generally considered as hostile environments for bacterial colonization since bacteria are exposed to ultraviolet radiation, nutrient limitation, and fluctuating temperature and water availability (Lindow & Brandl, 2003; Remus-Emsermann & Vorholt, 2014). Therefore, the capacity of *L. plantarum* to cope with stresses imposed in the field is likely to be a key factor contributing to epiphytic fitness. The second aim of this Thesis was the physiological improvement of fitness of the two selected *L. plantarum* strains PM411 and TC92 to increase cell survival on aerial plant surfaces and the consistency of biological control of bacterial plant diseases (Chapter IV).

Bacteria, including LAB, have evolved specific mechanisms to respond and to withstand harsh conditions and sudden environmental changes (stress-sensing system and defences) (van de Guchte *et al*., 2002; Bucka-Kolendo & Sokołowska, 2017). Stress defence mechanisms of LAB can be grouped according to their direct participation in preserving cell energy, protecting the cell envelope, and defending macromolecules (Papadimitriou *et al*., 2016). One of the most common consequences of a given stress condition in bacteria is the induction of protein misfolding. When this situation occurs, a set of molecular effectors, generally related to chaperones and proteases, are induced. While the task of chaperones is to protect functional proteins and to refold misfolded ones, proteases provide the last line of defence by removing irreversibly damaged proteins (Papadimitriou *et al*., 2016). In this Thesis, the transcriptional analysis focused on the expression patterns of stress-related genes together with kinetics of cell inactivation were proposed for the assessment of stress tolerance of PM411 and TC92 strains.

Water-stress tolerance is an important epiphytic fitness trait of BCA strains that influence their capacity to survive on aerial plant surfaces (Hagen *et al*., 2009). *L. plantarum* PM411 and TC92 differs in inherent desiccation tolerance and stress response. PM411 showed higher survival and a stronger stress response with a higher amount of upregulated stress-related genes during desiccation than TC92. These results are in agreement with differences observed in the survival of two strains of *P. syringae* in water-stressed environments that were also correlated with different gene expression profiles. In particular, the most tolerant strain exhibited a proactive response to osmotic stress based on upregulation of genes (Freeman *et al*., 2013). In this sense, diversity in intrinsic resistance to different stresses (Ferrando *et al*., 2015) and in stress response patterns (Parente *et al*., 2010) of *L. plantarum* strains has also been reported. Interestingly, the adhesion-like protein elongation factor *efTU*, whose role in intestinal surface adhesion has been reported in *L. plantarum* (Ramiah *et al*., 2007), was only upregulated in PM411. Therefore, it is likely that PM411 cells could show a better attachment on plant surfaces than TC92 cells. In addition, unlike TC92, PM411 cell colonies display a ropy phenotype which may be attributed to the production of exopolysaccharides (EPS) as previously reported for other strains (Dertli *et al*., 2015; Caggianiello *et al*., 2016). EPS are thought to contribute to the defence mechanism of bacterial cells against environmental stressors. For instance, the layer of extracellular polysaccharides surrounding microbial cell creates a microenvironment with increased water retention capacity and ensures

greater cell resistance against desiccation (Donot *et al*., 2012). In addition, EPS can also be involved in adhesion to surfaces and biofilm formation (Caggianiello *et al*., 2016). Therefore, it is hypothesized that the putative EPS could confer to PM411 increased adhesion ability to plant surfaces, as well as, protection against desiccation.

The improvement of microbial biopesticide competitiveness on plant surfaces under field conditions is a common step required for its development in order to overcome its variable performance of biocontrol due to fluctuating environmental conditions. In this Thesis, an enhancing water-stress tolerance strategy was developed to improve the epiphytic survival of *L. plantarum* PM411 and TC92 and, therefore, the consistency of their biocontrol efficacy. The water-stress adaptation treatment relies on growing cells into a hyperosmotic medium until stationary phase, which involves also acid production. In this sense, many other studies have reported the induction of adaptive response by applying a sublethal stress, mainly hyperosmotic stress, during inoculum preparation as an adaptation treatment to improve viability of BCA under water-stress conditions in the phyllosphere (Bonaterra *et al*., 2005, 2007; Palazzini *et al*., 2009; Cabrefiga *et al*., 2011) or during formulation and storage (freeze-drying) (Teixidó *et al*., 2005, 2006; Wang *et al*., 2010; Pusey & Wend, 2012; Cabrefiga *et al*., 2014). Preadaptation to one particular stress condition can also imply cells to resist to other stress conditions and this phenomenon is known as cross-protection. The ability to develop cross-protection responses have been confirmed by different biocontrol bacteria and yeasts (Teixidó *et al*., 2005, 2006; Liu *et al*., 2011). Interestingly, for the adaptation treatment, some studies combined saline osmotic stress and osmolyte amendment to the minimal growth media used (Bonaterra *et al*., 2005; Cabrefiga *et al*., 2011, 2014). Intracellular accumulation of compatible solutes, such as amino acids, is one of the most common strategies of bacteria to balance the osmotic pressure (Pichereau *et al*., 2000; Welsh, 2000). Consequently, this strategy contributes to improve survival of cells exposed later to drying conditions. Despite the limited capacities of *L. plantarum* to synthesize compatible solutes (Poolman & Glaasker, 1998), the adaptation treatment defined in this Thesis does not include the addition of osmolytes. The main reason is because the complex media de Man Rogosa and Sharpe (MRS) was used to reach high growth rates and it contains yeast and beef extract and amino acids, such as betaine and carnitine, are both present (Kets *et al*., 1996). The protective role of ionic solutes, such as betaine, on *L. plantarum* has been described as a response to osmotic stress (Kets *et al*., 1996; Zhao *et al*., 2014; Papadimitriou *et al*., 2016). Accordingly, the improvement of survival rates after drying have also been reported in probiotic *Lactobacillus* spp. by fermentation under suboptimal conditions (Prasad *et al*., 2003; Liu *et al*., 2014; Ferrando *et al*., 2015).

The adaptive response of PM411 and TC92 toward the water-stress adaptation treatment resulted in an increase in transcript levels of stress-related genes that encode chaperone proteins, proteases, small heat shock proteins, cold shock proteins, stress factors, and small redox proteins, which generally remained unaltered under the subsequent desiccation. Consequently, a strong protection from damage caused by the *in vitro* desiccation was achieved in adapted cells of both

strains. These results indicate that the induction of stress-related gene expression in PM411 and TC92 may be a key factor for the improvement of stress tolerance. Upregulation of general stress-related gene expression, such as chaperone proteins (*groEL*, *dnaK*) and stress factors (*ftsH*), has been previously involved in the stress response in *Lactobacillus* spp. when cells have been cultured under sublethal osmotic stress (Prasad *et al*., 2003; Bove *et al*., 2012). The preadaptation treatment provokes the modulation of cell physiology to adapt to the perceived stress and, thereby, indirectly enhances desiccation tolerance (Berninger *et al*., 2018). Similarly, changes in gene expression pattern during stress adaptation treatment and the improvement in subsequent stress tolerance have also been reported for BCA in the literature (Liu *et al*., 2011, 2012). As in other bacteria, adaptive response appears to be a usual mode of stress protection in LAB (van de Guchte *et al*., 2002). Although water-stress adapted cells of both strains showed similar kinetics of cell inactivation under desiccation *in vitro*, differences on plant surfaces under low relative humidity (RH) conditions were observed. The protective effect of adaptation treatment was stronger in PM411 that in TC92 and this fact is probably due to particular differences in the transcriptional patterns of these strains. In particular, in adapted cells of PM411 a higher number of stress-related genes showed increased expression values in comparison with non-adapted cells than in adapted cells of TC92. Also, while in PM411 adapted cells stress-related genes remained unaltered or downregulated during desiccation, in TC92 adapted cells genes that encode proteases and small heat shock proteins were upregulated. Therefore, PM411 strain was selected as a potential BCA showing higher water-stress tolerance and a better adaptive response than TC92.

According to these results, transcriptional analysis focused on particular stress-related genes may be highly-regarded as a tool to assess stress response of candidate strains as BCA against common environmental stresses during screening programs. Therefore, stress tolerance could be a relevant criterion for the selection of BCA. Additionally, this transcriptional analysis may also assist the development of a preadaptation treatment regarding to the adaptive response of cells.

An improvement of the establishment of adapted PM411 on the surface of strawberry and kiwifruit leaves in greenhouse and in apple and pear blossoms in the field was achieved in comparison with non-adapted PM411. Higher survival rates of adapted PM411 cells could be expected. These results indicate that other restrictive environmental variables different from desiccation, such as ultraviolet radiation, nutrient limitation, fluctuating temperature, rain, and wind might have directly influenced the capability of PM411 for the establishment on leaf surfaces and flowers. Although cross-protection is common among bacteria, including LAB (Bucka-Kolendo & Sokołowska, 2017), slight protection response against other stress factors was observed in water-stress adapted PM411 cells. Previous studies reported that osmoadaptation treatments in *P. agglomerans* BCA were not shown to be advantageous in the orchard (Cañamás *et al*., 2008; Pusey & Wend, 2012).

In this sense, it seems advisable to perform further studies to identify the detrimental effect of other environmental stress factors, such as ultraviolet radiation, temperature and wash off, on the

establishment of PM411 in the field. Several studies have been focused on finding out about the tolerance of bacterial and yeast BCA to abiotic stresses in order to improve them or as a decision support to select BCA (Cañamás *et al*., 2008; Lahlali *et al*., 2011; Segarra *et al*., 2015; Gava *et al*., 2018). In addition, the development of a formulation of PM411 containing adjuvants may offer a solution to protect cells against unfavourable environmental conditions and to improve their establishment and adherence on plant surfaces. For instance, humectants can reduce the effects of fluctuating wet and dry conditions, sunscreens might avoid adverse effect of sunlight by reflecting or absorbing ultraviolet radiation, and stickers may improve adherence to foliage and persistence in the event of wind and rain (Hynes & Boyetchko, 2006; Segarra *et al*., 2015). The assessment of different additives in formulations of BCA to promote their adherence and persistence on plant surfaces and, therefore, biocontrol efficacy in the orchard have been reported (Segarra *et al*., 2015; Cañamás *et al*., 2008). Moreover, freeze-dried formulations of adapted PM411 cells could also exhibit greater resistance to environmental conditions than fresh cells due to stress responses induced during the drying process. This occurrence has been reported in *Pseudomonas* and *Pantoea* spp. BCA (Stockwell *et al*., 1998; Cañamás *et al*., 2008; Cabrefiga *et al*., 2014).

Interestingly, the adaptation treatment developed in this Thesis provides more consistency in the PM411 biocontrol efficacy of Xf and Ea infections on strawberry leaves and apple and pear blossoms, respectively. Whereas it was reported that adaptation treatments of *Metschnikowia fructicola*, *Candida oleophila*, *Rhodosporidium paludigenum* (Wang *et al*., 2010; Liu *et al*., 2011, 2012), *B. subtilis* and *Brevibacillus* sp. (Palazzini *et al*., 2009), *P. agglomerans* (Bonaterra *et al*., 2005), and *Pseudomonas fluorescens* (Bonaterra *et al*., 2007; Cabrefiga *et al*., 2011) improved their biocontrol activity, other studies reported that adapted cells of *P. agglomerans* only maintained their efficacy (Teixidó *et al*., 2006; Cañamás *et al*., 2009).

Monitoring methods that allow for the specific detection and quantification of viable cells are required to evaluate the population dynamics of BCA on plant surfaces after field release. Since the efficacy of microbial biopesticides in controlling aerial plant diseases depends on their establishment on plant surfaces, knowing the population size of the BCA is really useful to predict its biocontrol efficacy. The third aim of this Thesis was the development of a viability quantitative PCR (v-qPCR) method to monitor the population dynamics of *L. plantarum* PM411 after artificial inoculation on aerial plant surfaces (Chapter V).

The quantification of BCA have been generally reported by strain-specific approaches, such as qPCR (Massart *et al*., 2005; Pujol *et al*., 2006; Cordier *et al*., 2007; Holmberg *et al*., 2009; Von Felten *et al*., 2010; Braun-Kiewnick *et al*., 2012; Mosimann *et al*., 2017) and plate CFU-counts in combination with PCR confirmation (De Clercq *et al*., 2003; Pujol *et al*., 2005; El Hamouchi *et al*., 2008; Felici *et al*., 2008; Nunes *et al*., 2008; Larena & Melgarejo, 2009b; Vilanova *et al*., 2018). However, these methodologies have some limitations. While qPCR is not capable of discriminating

between dead and viable cells, plate CFU-counts combined with PCR does not consider viable-but-nonculturable (VBNC) cells. An alternative molecular method could be the use of mRNA as a marker of viability since these levels rapidly decline after cell death and has an essential role in bacterial metabolism. Specific mRNA fragments from genes involved in bacterial survival and fitness have been reported as viability markers using reverse transcription real-time PCR (RT-qPCR) for evaluating survival of the phytopathogen *Xanthomonas citri* subsp. *citri* on plant material (Golmohammadi *et al*., 2012). The RT-qPCR method has also been reported for monitoring the population of the BCA strain *Trichoderma harzianum* T-78, although the detection marker (ITS region) is not specific at strain level (Beaulieu *et al*., 2011). The main drawback of using RT-qPCR as a strain-specific quantification approach is that finding a strain-specific mRNA sequence as an indicator of viability with steady expression levels may be unlikely. In addition, extensive studies, similar to those performed for the appropriate choice of reference genes for accurate normalization of gene expression data, should be required to ensure that the expression level of the marker does not change under different environmental conditions. Furthermore, handling RNA is tedious and cumbersome due to its sensitivity to degradation, limiting an accurate quantification.

Several culture-independent techniques different from molecular methods have been addressed for the viability assessment of probiotic bacteria in the food industry. Membrane integrity-based fluorescence staining coupled to flow cytometry has been reported as an efficient and time-saving detection method to monitor viability of probiotic bacteria in dried formulations. The LIVE/DEAD *Bac*Light viability kit, containing SYTO9 and propidium iodide (PI), allows for discriminating live and dead cells regarding cell membrane integrity. SYTO9 stains all cells fluorescent green, while PI penetrates only the cells whose cell membrane has been damaged, staining them red (Kramer *et al*., 2009). A rapid method based on isothermal microcalorimetry (IMC), which measures heat flow from biological processes produced by active bacterial cells, has been reported as a promising tool to be used in the quality control of dried probiotic formulations (Hernández-Garcia *et al*., 2017). The measurement of adenosine triphosphate (ATP) content, as the energy currency of all living cells, has been correlated with the number of metabolically active cells. The method involves the use of ATP-luciferase reaction kit based on the ATP-dependent luciferin-luciferase reaction and the measurement of luminescence (Fujimoto & Watanabe, 2013). However, all these viability approaches cannot be used to target specific bacterial strains and their use to monitor BCA in the plant environment is not acceptable since the BCA cannot be discriminated from other microorganisms that belong to the resident population.

Therefore, the viability quantitative PCR (v-qPCR) method, which relies on the sample pre-treatment with nucleic acid-binding dye prior to qPCR, was chosen in this Thesis since the quantification of viable cells, whether culturable or not, of a specific strain is feasible. The v-qPCR method has been widely reported for selective detection and quantification of foodborne pathogens (Rudi *et al*., 2005; Elizaquível *et al*., 2012; Martin *et al*., 2013; Liu & Mustapha, 2014; Seinige *et al*., 2014) and other relevant microorganisms in the food industry, such as probiotic (Desfossés-

Foucault *et al*., 2012; Villarreal *et al*., 2013). Nevertheless, in the biocontrol field few studies have tested v-qPCR for monitoring plant pathogens (Meng *et al*., 2016; Vilanova *et al*., 2017) and BCA (Soto-Muñoz *et al*., 2014a, 2015a) so far.

The PM411 strain-specific TaqMan qPCR was designed into a sequence of 972 bp with mosaic architecture located in a putative prophage in the PM411 genome. The first approach to detect polymorphisms within PM411 genome was based on RAPD-PCR technique. According to other authors, the RAPD-PCR technique has been successful to obtain specific molecular markers when working with other BCA and their genome sequences are not available (Pujol *et al*., 2005; Felici *et al*., 2008; Nunes *et al*., 2008; Holmberg *et al*., 2009; Von Felten *et al*., 2010; Gotor-Vila *et al*., 2016). However, in our study, the putative strain-specific band showed homology with part of a phage sequence together with other *L. plantarum* sequences in GenBank database and was not sufficiently specific to PM411. In contrast, RAPD-PCR has been useful to identify strain-specific molecular markers in *Lactobacillus* spp. (Galanis *et al*., 2015). Therefore, the second approach was the analysis of prophage DNA sequences in PM411 genome since they have been found to contribute to inter-strain genetic variability in bacteria, including *Lactobacillus* spp. (Brandt *et al*., 2001). The polymorphic structure of the putative prophage that contained the RAPD sequence allowed the identification of regions to design the strain-specific qPCR. These results are in agreement with previous reports in which phage-related sequences were used as a basis for designing strain-specific primers and probes in *Lactobacillus rhamnosus* using polymorphic sites (Brandt & Alatossava, 2003; Endo *et al*., 2012; Karjalainen *et al*., 2012). Therefore, it is hypothesized that the design of strain-specific qPCR assays for BCA based on polymorphisms of phage-related sequences may be a valuable strategy.

For the development of the v-qPCR method a number of considerations must be taken into account. In order to achieve the neutralization of DNA amplification from dead cells of PM411, the sample treatment with the nucleic acid-binding dye together with the amplicon length were optimized. PEMAX reagent was chosen as the nucleic acid-binding dye instead of ethidium monoazide (EMA) and propidium monoazide (PMA) that are widely known in v-qPCR. The main reason is that PEMAX extends the concept of viability to cells with intact cell membrane structure, but also with active metabolism, and may avoid detection of “ghost cells”, which have an intact membrane but are metabolically inactive (Codony *et al*., 2015), unlike EMA and PMA that cell membrane integrity is the only viability criterion (Nocker *et al*., 2006). Besides, targeting longer-than-average sequences in qPCR in combination with PEMAX treatment was found to be greatly beneficial for suppressing dead cell DNA amplification while preserving the performance of the method (sensitivity, linearity, and efficiency). These results are in agreement with previous reported studies focused on the effect of the amplicon length in v-qPCR to enumerate viable cells of *Salmonella* (Martin *et al*., 2013), *Vibrio anguillarum* and *Flavobacterium psychrophilum* (Contreras *et al*., 2011).

The reliability of the v-qPCR together with plate counting and qPCR in the monitoring of PM411 cell population on plant surfaces, both leaves and flowers, was simultaneously evaluated under greenhouse and field conditions. The three methods contributed to comprehend the behaviour of PM411 in different host plant tissues, species and environmental conditions. Under conducive conditions for bacterial survival (e.g. flowers at high RH), correlation within three methods was observed. Contrarily, under harsh conditions (e.g. leaf surfaces or field conditions) discrepancy between methods was observed due to the presence of nondegradated DNA from dead cells or VBNC cells. Therefore, qPCR overestimated the viable population level of PM411 under limited conditions, while plate counting underestimated this value.

For the placing of a microbial biopesticide on the EU market, the active substance (antagonist strain) needs to be approved at EU level and the formulated product must be authorized at member state level (Regulation (EC) No 1107/2009 and Directive 2009/128/EC). Besides toxicological studies to ensure safety, environmental risk assessment studies are required in order to accurately evaluate the persistence and dissemination of the microbial biopesticide upon delivery to the environment, as well as, its impact on biodiversity and the ecosystem (effects on non-target organisms) (Boivin & Poulsen, 2017). In the literature, environmental fate and behaviour of BCA under field conditions and during postharvest storage have been reported in order to provide valuable information for registering issues, as well as, to study the impact of formulation, application techniques, and weather conditions on the ecological fitness of BCA strains (Pujol *et al*., 2006, 2007; Soto-Muñoz *et al*., 2015a, 2015b; Gotor-Vila *et al*., 2017; Vilanova *et al*., 2018).

The v-qPCR method combined with plate counting and qPCR are expected to assist further studies for the development of PM411 as a BCA. These studies include the assessment of the persistence and dispersion of PM411 in the crop environment, ecological fitness studies under different weather conditions, the improvement of formulation to ensure reasonable shelf-life and maintain efficacy during long-term storage, and the establishment of a delivery schedule of PM411 based on its suppressive threshold level to guarantee crop protection. Indeed, it would be deeply interesting to study the minimum effective dose of PM411 for the biological control of plant diseases, considering the role of both culturable and VBNC cells since it is an unfilled topic.

Colonization studies of the BCA based on spatial distribution in plant tissues susceptible to pathogen infection may provide valuable information for a better knowledge of biocontrol performance. Therefore, the genetic engineering of PM411 with the *gfp* gene that encodes the green fluorescent protein could also be developed in the future as a monitoring tool with the aim to provide information about *in situ* spatial distribution of PM411 cells in the plant, as well as, the interaction between host plant, pathogen and PM411.

L. plantarum PM411 has been selected as a potential BCA of multiple quarantine bacterial pathogens in fruit crops. PM411 is capable of surviving on plant surfaces but it is not a good colonizer and, probably, the effect on biological control is due to its inundative application to the plant. Therefore, repetitive sprays and interlaced application of PM411 with conventional

agrochemical products, such as copper or other alternative biopesticides, should be taken into account as a disease management strategy to achieve an extended protective effect.

CHAPTER VII

Conclusions

1. The screening procedure based on a multi-pathogen approach by means of *in vitro* and *in planta* assays has been a successful strategy to select lactic acid bacteria (LAB) strains as candidate biological control agents (BCA) with broad-spectrum activity. *Lactobacillus plantarum* PM411 and TC92 have been selected for exhibiting *in vitro* antagonism against *Pseudomonas syringae* pv. *actinidiae* (Psa), *Xanthomonas arboricola* pv. *pruni* (Xap), and *Xanthomonas fragariae* (Xf), and consistently reducing infections of these pathogens in the corresponding host plants.

2. *L. plantarum* PM411 and TC92 have been confirmed as BCA since their efficacy in the biological control of bacterial canker of kiwifruit (Psa), bacterial spot of stone fruits (Xap), and angular leaf spot of strawberry (Xf) in semi-field and field assays was similar to commercial products, including microbial biopesticides, plant defence elicitors, and chemical pesticides.

3. The main mechanism of action of *L. plantarum* PM411 and TC92 involved in the *in vitro* suppression of Psa, Xap, and Xf has been attributed to organic acids, such as lactic acid.

4. Genetic characterization by multilocus sequence typing (MLST) and random amplified polymorphic DNA-PCR (RAPD-PCR) analysis allowed for the discrimination between PM411 and TC92 strains.

5. *L. plantarum* PM411 and TC92 differ in their tolerance and response to desiccation. Unlike TC92 strain, the water-stress response of PM411 included upregulation of the eleven stress-related genes considered in this study. The differences in the gene expression patterns have been related to higher survival rates in PM411 than in TC92 under desiccation.

6. The physiological adaptation strategy that consisted of growing cells into a hyperosmotic medium until stationary-phase, which involves also acid production, has enhanced the tolerance to water stress of *L. plantarum* PM411 and TC92. The response of both strains toward the adaptation treatment included an increase in transcript levels of stress-related genes and resulted in higher survival rates under desiccation.

7. Particular differences in the gene expression patterns between adapted cells of *L. plantarum* PM411 and TC92, both after the adaptation treatment and under desiccation, have been related to different water-stress tolerance of strains in plants. Unlike TC92 strain, the adaptation treatment led PM411 strain to withstand low relative humidity conditions on plant surfaces.

8. The adaptation treatment provided *L. plantarum* PM411 with a survival increase on plant surfaces and field conditions, and more consistency between experiments in suppressing fire blight in apple and pear blossoms and angular leaf spot in strawberry plants.

9. The viability quantitative PCR (v-qPCR) method using PEMAX reagent allowed the unambiguous detection and quantification of *L. plantarum* PM411 viable cells on aerial plant surfaces. A polymorphic region of a putative prophage in the PM411 genome was useful to design strain-specific primers and a TaqMan probe for the qPCR assay. The establishment of an adequate amplicon length allowed for sufficient suppression of dead cell DNA amplification by PEMAX treatment together with the preservation of the sensitivity of the qPCR method.

10. The combined use of v-qPCR, conventional qPCR, and specific plate counting methods has contributed to comprehend population dynamics of *L. plantarum* PM411 in different plant surfaces and environmental conditions. The unfavourable conditions on leaf surfaces and the relatively dry field conditions have induced the viable-but-nonculturable (VBNC) state and cell death in the PM411 population.

CHAPTER VIII

References

A

Abbasiliasi S., Tan J.S., Tengku Ibrahim T.A., Bashokouh F., Ramakrishnan N.R., Mustafa S., Ariff A.B. (2017) Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: a review. *RSC Advances*, **7**, 29395–29420.

Aguirre-Ezkauriatza E.J., Aguilar-Yáñez J.M., Ramírez-Medrano A., Alvarez M.M. (2010) Production of probiotic biomass (*Lactobacillus casei*) in goat milk whey: comparison of batch, continuous and fed-batch cultures. *Bioresource Technology*, **101**, 2837–2844.

Agustí G., Fittipaldi M., Codony F. (2017) False-positive viability PCR results: an association with microtubes. *Current Microbiology*, **74**, 377–380.

Agustí L., Bonaterra A., Moragrega C., Camps J., Montesinos E. (2011) Biocontrol of root rot of strawberry caused by *Phytophthora cactorum* with a combination of two *Pseudomonas fluorescens* strains. *Journal of Plant Pathology*, **93**, 363–372.

Alabouvette C., Olivain C., Steinberg C. (2006) Biological control of plant diseases: the European situation. *European Journal of Plant Pathology*, **114**, 329–341.

Alakomi H.-L., Skyttä E., Saarela M., Mattila-Sandholm T., Latva-Kala K., Helander I.M. (2000) Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*, **66**, 2001–2005.

Aldwinckle H.S., Bhaskara Reddy M.V., Norelli J.L. (2002) Evaluation of control of fire blight infection of apple blossoms and shoots with SAR inducers, biological agents, a growth regulator, copper compounds, and other materials. *Acta Horticulturae*, **590**, 325–331.

Allende A., Martínez B., Selma V., Gil M.I., Suáez J.E., Rodríguez A. (2007) Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. *Food Microbiology*, **24**, 759–766.

Alvarez-Sieiro P., Montalbán-López M., Mu D., Kuipers O.P. (2016) Bacteriocins of lactic acid bacteria: extending the family. *Applied Microbiology and Biotechnology*, **100**, 2939–2951.

Arena M.P., Silvain A., Normanno G., Grieco F., Drider D., Spano G., Fiocco D. (2016) Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers in Microbiology*, **7**, 464.

Arrebola E., Sivakumar D., Bacigalupo R., Korsten L. (2010) Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. *Crop Protection*, **29**, 369–377.

B

Badosa E., Ferre R., Planas M., Feliu L., Besalú E., Cabrefiga J., Bardají E., Montesinos E. (2007) A library of linear undecapeptides with bactericidal activity against phytopathogenic bacteria. *Peptides*, **28**, 2276–2285.

Badosa E., Moreno C., Montesinos E. (2004) Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions. *FEMS Microbiology Ecology*, **48**, 169–178.

Baffoni L., Gaggia F., Dalanaj N., Prodi A., Nipoti P., Pisi A., Biavati B., Di Gioia D. (2015) Microbial inoculants for the biocontrol of *Fusarium* spp. in durum wheat. *BMC Microbiology*, **15**, 242.

Balajoo O.M., Kesahavarzi M., Zahabi A., Danesh Y.R., Haghjuyan R. (2012) Protective effect of acibenzolar-s-methyl on fireblight severity in quince and characterization of the *Erwinia amylovora* strains involved. *Journal of Plant Pathology*, **94**, 211–214.

Bastas K.K., Maden S. (2007) Evaluation of host resistance inducers and conventional products for fire blight management in loquat and quince. *Phytoprotection*, **88**, 93–101.

Beaulieu R., López-Mondéjar R., Tittarelli F., Ros M., Pascual J.A. (2011) qRT-PCR quantification of the biological control agent *Trichoderma harzianum* in peat and compost-based growing media. *Bioresource Technology*, **102**, 2793–2798.

Ben Omar N., Abriouel H., Keleke S., Sánchez Valenzuela A., Martínez-Cañamero M., Lucas López R., Ortega E., Gálvez A. (2008) Bacteriocin-producing *Lactobacillus* strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. *International Journal of Food Microbiology*, **127**, 18–25.

Beresford R.M., Tyson J.L., Henshall W.R. (2017) Development and validation of an infection risk model for bacterial canker of kiwifruit, using a multiplication and dispersal concept for forecasting bacterial diseases. *Phytopathology*, **107**, 184–191.

Berninger T., González López Ó., Bejarano A., Preininger C., Sessitsch A. (2018) Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microbial Biotechnology*, **11**, 277–301.

Biondi E., Dallai D., Brunelli A., Bazzi C., Stefani E. (2009) Use of a bacterial antagonist for the biological control of bacterial leaf/fruit spot of stone fruits. *IOBC/WPRS Bulletin*, **43**, 277–281.

Bloemberg G. V (2007) Microscopic analysis of plant–bacterium interactions using auto fluorescent proteins. *European Journal of Plant Pathology*, **119**, 301–309.

Boivin A., Poulsen V. (2017) Environmental risk assessment of pesticides: state of the art and prospective improvement from science. *Environmental Science and Pollution Research*, **24**, 6889–6894.

Bonaterra A., Badosa E., Cabrefiga J., Francés J., Montesinos E. (2012) Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. *Trees*, **26**, 215–226.

Bonaterra A., Cabrefiga J., Camps J., Montesinos E. (2007) Increasing survival and efficacy of a bacterial biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. *FEMS Microbiology Ecology*, **61**, 185–195.

Bonaterra A., Camps J., Montesinos E. (2005) Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. *FEMS Microbiology Letters*, **250**, 1–8.

Bonaterra A., Mari M., Casalini L., Montesinos E. (2003) Biological control of *Monilinia laxa* and *Rhizopus stolonifer* in postharvest of stone fruit by *Pantoea agglomerans* EPS125 and putative mechanisms of antagonism. *International Journal of Food Microbiology*, **84**, 93–104.

Bove P., Capozzi V., Garofalo C., Rieu A., Spano G., Fiocco D. (2012) Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiological Research*, **167**, 187–93.

Brandt K., Alatossava T. (2003) Specific identification of certain probiotic *Lactobacillus rhamnosus* strains with PCR primers based on phage-related sequences. *International Journal of Food Microbiology*, **84**, 189–196.

Brandt K., Tilsala-Timisjärvi A., Alatossava T. (2001) Phage-related DNA polymorphism in dairy and probiotic *Lactobacillus*. *Micron*, **32**, 59–65.

Braun-Kiewnick A., Lehmann A., Rezzonico F., Wend C., Smits T.H.M., Duffy B. (2012) Development of species-, strain- and antibiotic biosynthesis-specific quantitative PCR assays for *Pantoea agglomerans* as tools for biocontrol monitoring. *Journal of Microbiological Methods*, **90**, 315–320.

Broggini G.A.L., Duffy B., Holliger E., Schärer H.-J., Gessler C., Patocchi A. (2005) Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro®) in a Swiss apple orchard. *European Journal of Plant Pathology*, **111**, 93–100.

Bucka-Kolendo J., Sokołowska B. (2017) Lactic acid bacteria stress response to preservation processes in the beverage and juice industry. *Acta Biochimica Polonica*, **64**, 459–464.

C

Cabrefiga J. (2004) Fire blight (*Erwinia amylovora*) of rosaceous plants. Pathogen virulence and selection and characterization of biological control agents. Ph.D. Thesis. University of Girona, Spain. Available at: https://www.tdx.cat/handle/10803/7924 [Accessed May 16, 2018].

Cabrefiga J., Bonaterra A., Montesinos E. (2007) Mechanisms of antagonism of *Pseudomonas fluorescens* EPS62e against *Erwinia amylovora*, the causal agent of fire blight. *International Microbiology*, **10**, 123–132.

Cabrefiga J., Francés J., Montesinos E., Bonaterra A. (2014) Improvement of a dry formulation of *Pseudomonas fluorescens* EPS62e for fire blight disease biocontrol by combination of culture osmoadaptation with a freeze-drying lyoprotectant. *Journal of Applied Microbiology*, **117**, 1122–1131.

Cabrefiga J., Francés J., Montesinos E., Bonaterra A. (2011) Improvement of fitness and efficacy of a fire blight biocontrol agent via nutritional enhancement combined with osmoadaptation. *Applied and Environmental Microbiology*, **77**, 3174–3181.

Cabrefiga J., Montesinos E. (2017) Lysozyme enhances the bactericidal effect of BP100 peptide against *Erwinia amylovora*, the causal agent of fire blight of rosaceous plants. *BMC Microbiology*, **17**, 39.

Caggianiello G., Kleerebezem M., Spano G. (2016) Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. *Applied Microbiology and Biotechnology*, **100**, 3877–3886.

Calvo J., Calvente V., de Orellano M.E., Benuzzi D., de Tosetti M.I.S. (2003) Improvement in the biocontrol of postharvest diseases of apples with the use of yeast mixtures. *BioControl*, **48**, 579–593.

Cameron A., Sarojini V. (2014) *Pseudomonas syringae* pv. *actinidiae*: chemical control, resistance mechanisms and possible alternatives. *Plant Pathology*, **63**, 1–11.

Cameron A., Zoysa G.H. De, Sarojini V. (2014) Antimicrobial peptides against *Pseudomonas syringae* pv. *actinidiae* and *Erwinia amylovora*: chemical synthesis, secondary structure, efficacy, and mechanistic investigations. *Biopolymers*, **102**, 88–96.

Cañamás T.P., Viñas I., Abadias M., Usall J., Torres R., Teixidó N. (2009) Acid tolerance response induced in the biocontrol agent *Pantoea agglomerans* CPA-2 and effect on its survival ability in acidic environments. *Microbiological Research*, **164**, 438–450.

Cañamás T.P., Viñas I., Usall J., Casals C., Solsona C., Teixidó N. (2008) Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2 Part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, **49**, 86–95.

Cellini A., Fiorentini L., Buriani G., Yu J., Donati I., Cornish D.A., Novak B., Costa G., Vanneste J.L., Spinelli F. (2014) Elicitors of the salicylic acid pathway reduce incidence of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidae*. *Annals of Applied Biology*, **165**, 441–453.

Chandler D., Bailey A.S., Tatchell G.M., Davidson G., Greaves J., Grant W.P. (2011) The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B*, **366**, 1987–1998.

Cheong E.Y.L., Sandhu A., Jayabalan J., Le T.T.K., Nhiep N.T., Ho T.H.M., Zwielehner J., Bansal N., Turner M.S. (2014) Isolation of lactic acid bacteria with antifungal activity against the common cheese spoilage mould *Penicillium commune* and their potential as biopreservatives in cheese. *Food Control*, **46**, 91–97.

Cintas L.M., Casaus P., Holo H., Hernandez P.E., Nes I.F., Håvarstein L.S. (1998) Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. *Journal of Bacteriology*, **180**, 1988–1994.

Cizeikiene D., Juodeikiene G., Paskevicius A., Bartkiene E. (2013) Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control*, **31**, 539–545.

Codony F. (2014) Procedimiento para la detección de células vivas, con las membranas celulares íntegras y funcionales, mediante técnicas de amplificación de ácidos nucleicos. Spanish patent, ES2568527B1.

Codony F., Agustí G., Allué-Guardia A. (2015) Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR. *Molecular and Cellular Probes*, **29**, 190–192.

Commission Implementing Regulation (EU) 2016/873 (2016) Commission Implementing Regulation (EU) 2016/873 of 1 June 2016 amending Regulation (EC) No 690/2008 recognising protected zones exposed to particular plant health risks in the Community. *Official Journal of the European Union*, **L 145**, 10-17.

Contreras P.J., Urrutia H., Sossa K., Nocker A. (2011) Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. *Journal of Microbiological Methods*, **87**, 89–95.

Cordier C., Edel-Hermann V., Martin-Laurent F., Blal B., Steinberg C., Alabouvette C. (2007) SCAR-based real time PCR to identify a biocontrol strain (T1) of *Trichoderma atroviride* and study its population dynamics in soils. *Journal of Microbiological Methods*, **68**, 60–68.

Cortés-Zavaleta O., López-Malo A., Hernández-Mendoza A., García H.S. (2014) Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, **173**, 30–35.

Costa E., Usall J., Teixidó N., Garcia N., Viñas I. (2000) Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology*, **89**, 793–800.

Costa G., Andreotti C., Spinelli F., Rademacher W. (2006) Prohexadione-Ca: more than a growth regulator for pome fruit trees. *Acta Horticulturae*, **727**, 107–116.

Cotter P.D., Hill C., Ross R.P. (2005) Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*, **3**, 777–789.

Coudeyras S., Marchandin H., Fajon C., Forestier C. (2008) Taxonomic and strain-specific identification of the probiotic strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* group. *Applied and Environmental Microbiology*, **74**, 2679–89.

Council Directive 2000/29/EC (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities*, **L 169**, 1–112.

Crowley S., Mahony J., Van Sinderen D. (2013) Broad-spectrum antifungal-producing lactic acid bacteria and their application in fruit models. *Folia Microbiologica*, **58**, 291–299.

D

Dalié D.K.D., Deschamps A.M., Richard-Forget F. (2010) Lactic acid bacteria – Potential for control of mould growth and mycotoxins: a review. *Food Control*, **21**, 370–380.

Daranas N., Badosa E., Francés J., Montesinos E., Bonaterra A. (2018a) Enhancing water stress tolerance improves fitness in biological control strains of *Lactobacillus plantarum* in plant environments. *PLoS ONE*, **13**, e0190931.

Daranas N., Bonaterra A., Francés J., Cabrefiga J., Montesinos E., Badosa E. (2018b) Monitoring viable cells of the biological control agent *Lactobacillus plantarum* PM411 in aerial plant surfaces by means of a strain-specific viability quantitative PCR method. *Applied and Environmental Microbiology*, 84, e00107-18.

Darsanaki R.K., Rokhi M.L., Aliabadi M.A., Issazadeh K. (2012) Antimicrobial activities of *Lactobacillus* strains isolated from fresh vegetables. *Middle-East Journal of Scientific Research*, **11**, 1216–1219.

da Silva Sabo S., Vitolo M., González J.M.D., de Souza Oliveira R.P. (2014) Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. *Food Research International*, **64**, 527–536.

De Clercq D., Cognet S., Pujol M., Lepoivre P., Jijakli M. (2003) Development of a SCAR marker and a semi-selective medium for specific quantification of *Pichia anomala* strain K on apple fruit surfaces. *Postharvest Biology and Technology*, **29**, 237–247.

de las Rivas B., Marcobal A., Muñoz R. (2006) Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiology*, **152**, 85–93.

Dertli E., Mayer M.J., Narbad A. (2015) Impact of the exopolysaccharide layer on biofilms, adhesion and resistance to stress in *Lactobacillus johnsonii* FI9785. *BMC Microbiology*, **15**, 8.

Desfossés-Foucault E., Dussault-Lepage V., Le Boucher C., Savard P., Lapointe G., Roy D. (2012) Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification. *Frontiers in Microbiology*, **3**, 350.

Di Cagno R., Minervini G., Sgarbi E., Lazzi C., Bernini V., Neviani E., Gobbetti M. (2010) Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing *Lactobacillus plantarum* isolates from raw vegetables and fruits. *International Journal of Food Microbiology*, **143**, 246–253.

Di Cagno R., Surico R.F., Siragusa S., De Angelis M., Paradiso A., Minervini F., De Gara L., Gobbetti M. (2008) Selection and use of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. *International Journal of Food Microbiology*, **127**, 220–228.

Di Gioia D., Mazzola G., Nikodinoska I., Aloisio I., Langerholc T., Rossi M., Raimondi S., Melero B., Rovira J. (2016) Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth. *International Journal of Food Microbiology*, **235**, 53–59.

Directive 2009/128/EC. 2009. Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. *Official Journal of the European Communities*, **L 309**, 71–86.

Donati I., Buriani G., Cellini A., Mauri S., Costa G., Spinelli F. (2014) New insights on the bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*). *Journal of Berry Research*, **4**, 53–67.

Donot F., Fontana A., Baccou J.C., Schorr-Galindo S. (2012) Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers*, **87**, 951–962.

Drider D., Fimland G., Héchard Y., McMullen L.M., Prévost H. (2006) The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, **70**, 564–582.

Droby S., Wisniewski M., Teixidó N., Spadaro D., Haissam Jijakli M. (2016) The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biology and Technology*, **122**, 22–29.

Druvefors U.A., Passoth V., Schnürer J. (2005) Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during airtight storage of wheat. *Applied and Environmental Microbiology*, **71**, 1865–1869.

E

EFSA Panel on Biological Hazards (2017) Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. *EFSA Journal*, **15**, 4664–4841.

Elegado F.B., Guerra M.A.R.V, Macayan R.A., Mendoza H.A., Lirazan M.B. (2004) Spectrum of bacteriocin activity of *Lactobacillus plantarum* BS and fingerprinting by RAPD-PCR. *International Journal of Food Microbiology*, **95**, 11–18.

El Hamouchi A., Bajji M., Friel D., Najimi B., Achbani E.H., El Jaafari S., Durieux A., Jijakli M.H. (2008) Development of SCAR markers and a semi-selective medium for the quantification of strains Ach 1-1 and 1113-5, two *Aureobasidium pullulans* potential biocontrol agents. *Postharvest Biology and Technology*, **50**, 216–223.

Elizaquível P., Aznar R., Sánchez G. (2014) Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. *Journal of Applied Microbiology*, **116**, 1–13.

Elizaquível P., Sánchez G., Aznar R. (2012) Quantitative detection of viable foodborne *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Food Control*, **25**, 704–708.

Emerson J.B., Adams R.I., Román C.M.B., Brooks B., Coil D.A., Dahlhausen K., Ganz H.H., Hartmann E.M., Hsu T., Justice N.B., Paulino-Lima I.G., Luongo J.C., Lymperopoulou D.S., Gomez-Silvan C., Rothschild-Mancinelli B., Balk M., Huttenhower C., Nocker A., Vaishampayan P., Rothschild L.J. (2017) Schrödinger's microbes: tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome*, **5**, 86.

Enan G., El-Essawy A.A., Uyttendaele M., Debevere J. (1996) Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. *International Journal of Food Microbiology*, **30**, 189–215.

Endo A., Aakko J., Salminen S. (2012) Evaluation of strain-specific primers for identification of *Lactobacillus rhamnosus* GG. *FEMS Microbiology Letters*, **337**, 120–125.

EPPO (2017) EPPO A2 List of pests recommended for regulation as quarantine pest. Available at: https://www.eppo.int/QUARANTINE/listA2.htm [Accessed March 14, 2018].

EPPO (2018a) EPPO Global Database. Available at: https://gd.eppo.int [Accessed January 25, 2018].

EPPO (2018b) Information on Plant Protection Products. Databases on registered plant protection products in Europe. Available at: https://www.eppo.int/PPPRODUCTS/information/ information _ppp.htm [Accessed February 12, 2018].

Etebarian R.H., Holberg P.L. (2006) Monitoring population levels of *Pseudomonas fluorescens* labelled with green fluorescent protein, using fluorescence microscopy and direct fluorescence scanning techniques. *Canadian Journal of Plant Pathology*, **28**, 125–130.

European Commission (2018) EU Pesticides database. Available at: http://ec.europa.eu/ food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN [Accessed February 12, 2018].

Eurostats (2017) Agricultural production - crops. Statistics explained. Available at: http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-_crops [Accessed January 18, 2018].

Eurostats (2014) Agricultural production - orchards. Statistical explained. Available at: http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production - orchards [Accessed January 18, 2018].

F

FAOSTAT (2017) Statistics Division of the Food and Agriculture Organization of the United Nations. Available at: http://faostat.fao.org [Accessed January 18, 2018].

FDA (2018) Generally Recognized as Safe (GRAS). Available at: https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm [Accessed June 17, 2018].

Felici C., Vettori L., Toffanin A., Nuti M. (2008) Development of a strain-specific genomic marker for monitoring a *Bacillus subtilis* biocontrol strain in the rhizosphere of tomato. *FEMS Microbiology Ecology*, **65**, 289–298.

Feng X.M., Holmberg A.-I.J., Sundh I., Ricard T., Melin P. (2011) Specific SCAR markers and multiplex real-time PCR for quantification of two *Trichoderma* biocontrol strains in environmental samples. *BioControl*, **56**, 903–913.

Ferrando V., Quiberoni A., Reinhemer J., Suárez V. (2015) Resistance of functional *Lactobacillus plantarum* strains against food stress conditions*. Food Microbiology*, **48**, 63–71.

Ferrante P., Scortichini M. (2010) Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathology*, **59**, 954–962.

Fhoula I., Najjari A., Turki Y., Jaballah S., Boudabous A., Ouzari H. (2013) Diversity and antimicrobial properties of lactic acid bacteria isolated from rhizosphere of olive trees and desert truffles of Tunisia. *BioMed Research International*, **2013**, 1–14.

Fittipaldi M., Nocker A., Codony F. (2012) Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods*, **91**, 276–289.

Francés J., Bonaterra A., Moreno M.C., Cabrefiga J., Badosa E., Montesinos E. (2006) Pathogen aggressiveness and postharvest biocontrol efficiency in *Pantoea agglomerans*. *Postharvest Biology and Technology*, **39**, 299–307.

Freeman B.C., Chen C., Yu X., Nielsen L., Peterson K., Beattie G.A. (2013) Physiological and transcriptional responses to osmotic stress of two *Pseudomonas syringae* strains that differ in epiphytic fitness and osmotolerance. *Journal of Bacteriology*, **195**, 4742–4752.

Freshfel Europe (2016) Annual activity report 2016. Available at: http://freshfel.org/about-freshfel/activity-reports/ [Accessed January 18, 2018].

Freshfel Europe (2017) Annual activity report 2017. Available at: http://freshfel.org/about-freshfel/activity-reports/ [Accessed January 18, 2018].

Froud K.J., Everett K.R., Tyson J.L., Beresford R.M., Cogger N. (2015) Review of the risk factors associated with kiwifruit bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae*. *New Zealand Plant Protection*, **68**, 313–327.

Fujimoto J., Watanabe K. (2013) Quantitative detection of viable *Bifidobacterium bifidum* BF-1 cells in human feces by using propidium monoazide and strain-specific primers. *Applied and Environmental Microbiology*, **79**, 2182–2188.

G

Galanis A., Kourkoutas Y., Tassou C.C., Chorianopoulos N. (2015) Detection and identification of probiotic *Lactobacillus plantarum* strains by multiplex PCR using RAPD-derived primers. *International Journal of Molecular Sciences*, **16**, 25141– 25153.

Gamalero E., Lingua G., Berta G., Lemanceau P. (2003) Methods for studying root colonization by introduced beneficial bacteria. *Agronomie*, **23**, 407–418.

Gänzle M.G. (2015) Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, **2**, 106–117.

Gava C.A.T., de Castro A.P.C., Pereira C.A., Fernandes-Júnior P.I. (2018) Isolation of fruit colonizer yeasts and screening against mango decay caused by multiple pathogens. *Biological Control*, **117**, 137–146.

Glare T., Caradus J., Gelernter W., Jackson T., Keyhani N., Rgen J., Hl K., Marrone P., Morin L., Stewart A. (2012) Have biopesticides come of age? *Trends in Biotechnology*, **30**, 250–258.

Golmohammadi M., Llop P., Scuderi G., Gell I., Graham J.H., Cubero J. (2012) mRNA from selected genes is useful for specific detection and quantification of viable *Xanthomonas citri* subsp. *citri*. *Plant Pathology*, **61**, 479–488.

Gómez-Sala B., Herranz C., Díaz-Freitas B., Hernández P.E., Sala A., Cintas L.M. (2016) Strategies to increase the hygienic and economic value of fresh fish: biopreservation using lactic acid bacteria of marine origin. *International Journal of Food Microbiology*, **223**, 41–49.

Gotor-Vila A., Teixidó N., Casals C., Torres R., De Cal A., Guijarro B., Usall J. (2017) Biological control of brown rot in stone fruit using *Bacillus amyloliquefaciens* CPA-8 under field conditions. *Crop Protection*, **102**, 72–80.

Gotor-Vila A., Teixidó N., Usall J., Dashevskaya S., Torres R. (2016) Development of a SCAR marker and a strain-specific genomic marker for the detection of the biocontrol agent strain CPA-8 *Bacillus amyloliquefaciens* (formerly *B. subtilis*). *Annals of Applied Biology*, **169**, 248–256.

Guetsky R., Elad Y., Shtienberg D., Dinoor A. (2002) Improved biocontrol of *Botrytis cinerea* on detached strawberry leaves by adding nutritional supplements to a mixture of *Pichia guilermondii* and *Bacillus mycoides*. *Biocontrol Science and Technology*, **12**, 625–630.

Guetsky R., Shtienberg D., Elad Y., Dinoor A. (2001) Combining biocontrol agents to reduce the variability of biological control. *Biological Control*, **91**, 621–627.

H

Hagen M.J., Stockwell V.O., Whistler C.A., Johnson K.B., Loper J.E. (2009) Stress tolerance and environmental fitness of *Pseudomonas fluorescens* A506, which has a mutation in *rpoS*. *Phytopathology*, **99**, 679–688.

Haidar R., Deschamps A., Roudet J., Calvo-Garrido C., Bruez E., Rey P., Fermaud M. (2016) Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control*, **92**, 55–65.

Hamed H.A., Moustafa Y.A., Abdel-Aziz S.M. (2011) *In vivo* efficacy of lactic acid bacteria in biological control against *Fusarium oxysporum* for protection of tomato plant. *Life Science Journal*, **8**, 462–468.

Hammami I., Jaouadi B., Bacha B., Rebai A., Bejar S., Nesme X., Rhouma A. (2012) *Bacillus subtilis* bacteriocin Bac 14B with a broad inhibitory spectrum: Purification, amino acid sequence analysis, and physicochemical characterization. *Biotechnology and Bioprocess Engineering*, **17**, 41–49.

Handelsman J., Raffel S., Mester E.H., Wunderlich L., Grau C.R. (1990) Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. *Applied and Environmental Microbiology*, **56**, 713–718.

Hansen M., Kragelund L., Nybroe O., Sørensen J. (1997) Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiology Ecology*, **23**, 353–360.

Henry P.M., Gebben S.J., Tech J.J., Yip J.L., Leveau J.H.J. (2016) Inhibition of *Xanthomonas fragariae*, causative agent of angular leaf spot of strawberry, through iron deprivation. *Frontiers in Microbiology*, **7**, 1589.

Hernández-Garcia A., Herrmann A.M., Håkansson S. (2017) Isothermal microcalorimetry for rapid viability assessment of freeze-dried *Lactobacillus reuteri*. *Process Biochemistry*, **55**, 49–54.

Holmberg A.-I.J., Melin P., Levenfors J.P., Sundh I. (2009) Development and evaluation of SCAR markers for a *Pseudomonas brassicacearum* strain used in biological control of snow mould. *Biological Control*, **48**, 181–187.

Holzapfel W.H., Wood B.J.B. (2014) *Lactic acid bacteria. Biodiversity and taxonomy*. Hoboken, NJ: Wiley-Blackwell.

Horn I.R., van Rijn M., Zwetsloot T.J.J., Basmagi S., Dirks-Mulder A., van Leeuwen W.B., Ravensberg W.J., Gravendeel B. (2016) Development of a multiplex Q-PCR to detect *Trichoderma harzianum* Rifai strain T22 in plant roots. *Journal of Microbiological Methods*, **121**, 44–49.

Hynes R.K., Boyetchko S.M. (2006) Research initiatives in the art and science of biopesticide formulations. *Soil Biology and Biochemistry*, **38**, 845–849.

I

Ishimaru C.A. (1988) Multiple antibiotic production by *Erwinia herbicola*. *Phytopathology*, **78**, 746–750.

Ito A., Sato Y., Kudo S., Sato S., Nakajima H., Toba T. (2003) The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Current Microbiology*, **47**, 231–236.

J

Janisiewicz W.J. (1988) Biocontrol of postharvest diseases of apple with antagonist mixtures. *Phytopathology*, **78**, 194–198.

Janisiewicz W.J. (1994) Enhancement of biocontrol of blue mold with the nutrient analog 2-deoxy-d-glucose on apples and pears. *Applied and Environmental Microbiology*, **60**, 2671–2676.

Janisiewicz W.J., Bastos Pereira I., Almeida M., Roberts D., Wisniewski M., Kurtenbach E. (2008) Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide. *Postharvest Biology and Technology*, **47**, 218–225.

Janisiewicz W.J., Usall J., Bors B. (1992) Nutricional enhancement of biocontrol of blue mold on apples. *Phytopathology*, **82**, 1364–1370.

Janse J.D. (2012) Bacterial diseases that may or do emerge, with (possible) economic damage for Europe and the Mediterranean basin: notes on epidemiology, risks, prevention and management on first occurrence. *Journal of Plant Pathology*, **94**, S4.5-S4.29.

Jetiyanon K., Fowler W.D., Kloepper J.W. (2003) Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Disease*, **87**, 1390–1394.

Jetiyanon K., Kloepper J.W. (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological Control*, **24**, 285–291.

Joerger M.C., Klaenhammer T.R. (1990) Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. *Journal of Bacteriology*, **172**, 6339–6347.

Johansson A.H., Bejai S., Niazi A., Manzoor S., Bongcam-Rudloff E., Meijer J. (2014) Studies of plant colonisation by closely related *Bacillus amyloliquefaciens* biocontrol agents using strain specific quantitative PCR assays. *Antonie van Leeuwenhoek*, **106**, 1247–1257.

Johnson K.B., Stockwell V.O., Sawyer T.L., Sugar D. (2000) Assessment of environmental factors influencing growth and spread of *Pantoea agglomerans* on and among blossoms of pear and apple. *Phytopathology*, **90**, 1285–1294.

Josephson K.L., Gerba C.P., Pepper I.L. (1993) Polymerase chain reaction detection of nonviable bacterial pathogens. *Applied and Environmental Microbiology*, **59**, 3513–3515.

K

Karjalainen H., Ahlroos T., Myllyluoma E., Tynkkynen S. (2012) Real-time PCR assays for strain-specific quantification of probiotic strains in human faecal samples. *International Dairy Journal*, **27**, 58–64.

Kastelein P., Krijger M., Czajkowski R., van der Zouwen P.S., van der Schoor R., Jalink H., van der Wolf J.M. (2014) Development of *Xanthomonas fragariae* populations and disease progression in strawberry plants after spray-inoculation of leaves. *Plant Pathology*, **63**, 255–263.

Kawaguchi A., Inoue K., Inoue Y. (2014) Biological control of bacterial spot on peach by nonpathogenic *Xanthomonas campestris* strains AZ98101 and AZ98106. *Journal of General Plant Pathology*, **80**, 158–163.

Kets E., Teunissen P., de Bont J. (1996) Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Applied and Environmental Microbiology*, **62**, 259–261.

Kiewnick S. (2007) Practicalities of developing and registering microbial biological control agents. *CAB Reviews*, **2**, 013.

Kim D.-R., Gang G.-H., Jeon C.-W., Kang N.J., Lee S.-W., Kwak Y.-S. (2016) Epidemiology and control of strawberry bacterial angular leaf spot disease caused by *Xanthomonas fragariae*. *The Plant Pathology Journal*, **32**, 290–299.

Kim Y.C., Jung H., Kim K.Y., Park S.K. (2008) An effective biocontrol bioformulation against Phytophthora blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions. *European Journal of Plant Pathology*, **120**, 373–382.

Klaenhammer T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, **12**, 39–85.

Knoll C., Divol B., du Toit M. (2008) Genetic screening of lactic acid bacteria of oenological origin for bacteriocin-encoding genes. *Food Microbiology*, **25**, 983–991.

Köhl J., Postma J., Nicot P., Ruocco M., Blum B. (2011) Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biological Control*, **57**, 1–12.

Konappa N.M., Maria M., Uzma F., Krishnamurthy S., Nayaka S.C., Niranjana S.R., Chowdappa S. (2016) Lactic acid bacteria mediated induction of defense enzymes to enhance the resistance in tomato against *Ralstonia solanacearum* causing bacterial wilt. *Scientia Horticulturae*, **207**, 183–192.

Kragelund L., Nybroe O. (1996) Competition between *Pseudomonas fluorescens* Ag1 and *Alcaligenes eutrophus* JMP134 (pJP4) during colonization of barley roots. *FEMS Microbiology Ecology*, **20**, 41–51.

Kramer M., Obermajer N., Bogovic Matijasić B., Rogelj I., Kmetec V. (2009) Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. *Applied Microbiology and Biotechnology*, **84**, 1137–1147.

Krzywonos M., Eberhard T. (2011) High density process to cultivate *Lactobacillus plantarum* biomass using wheat stillage and sugar beet molasses. *Electronic Journal of Biotechnology*, **14**. DOI: 10.2225/vol14-issue2-fulltext-10

L

Lahlali R., Brostaux Y., Jijakli M.H. (2011) Control of apple blue mold by the antagonistic yeast *Pichia anomala* strain K: screening of UV protectants for preharvest spplication. *Plant Disease*, **95**, 311–316.

Lalancette N., Mcfarland K.A. (2007) Phytotoxicity of copper-based bactericides to peach and nectarine. *Plant Disease*, **91**, 1122–1130.

Lamichhane J.R. (2014) *Xanthomonas arboricola* diseases of stone fruit, almond, and walnut trees: progress toward understanding and management taxonomic relationships. *Plant Disease*, **98**, 1600–1610.

Lamichhane J.R., Dachbrodt-Saaydeh S., Kudsk P., Messéan A. (2016) Toward a reduced reliance on conventional pesticides in European agriculture. *Plant Disease*, **100**, 10–24.

Lamont J.R., Wilkins O., Bywater-Ekegärd M., Smith D.L. (2017) From yogurt to yield: potential applications of lactic acid bacteria in plant production. *Soil Biology & Biochemistry*, **111**, 1–9.

Lan W., Chen Y., Wu H., Yanagida F. (2012) Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, **57**, 99–105.

Larena I., Melgarejo P. (2009a) Development of a method for detection of the biocontrol agent *Penicillium oxalicum* strain 212 by combining PCR and a selective medium. *Plant Disease*, **93**, 919–928.

Larena I., Melgarejo P. (2009b) Development of a new strategy for monitoring *Epicoccum nigrum* 282, a biological control agent used against brown rot caused by *Monilinia* spp. in peaches. *Postharvest Biology and Technology*, **54**, 63–71.

Leff J.W., Fierer N. (2013) Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PLoS ONE*, **8**, e59310.

Lightner G.W., Steiner P.W. (1992) Maryblyt™: A computer model for predicting of fire blight disease in apples and pears. *Computers and Electronics in Agriculture*, **7**, 249–260.

Lindow S.E., Brandl M.T. (2003) Microbiology of the phyllosphere. *Applied and Environmental Microbiology*, **69**, 1875–1883.

Liu J., Wisniewski M., Droby S., Norelli J., Hershkovitz V., Tian S., Farrell R. (2012) Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. *FEMS Microbiology Ecology*, **80**, 578–590.

Liu J., Wisniewski M., Droby S., Tian S., Hershkovitz V., Tworkoski T. (2011) Effect of heat shock treatment on stress tolerance and biocontrol efficacy of *Metschnikowia fructicola*. *FEMS Microbiology Ecology*, **76**, 145–155.

Liu X.T., Hou C.L., Zhang J., Zeng X.F., Qiao S.Y. (2014) Fermentation conditions influence the fatty acid composition of the membranes of *Lactobacillus reuteri* I5007 and its survival following freeze-drying. *Letters in Applied Microbiology*, **59**, 398–403.

Liu Y., Mustapha A. (2014) Detection of viable *Escherichia coli* O157:H7 in ground beef by propidium monoazide real-time PCR. *International Journal of Food Microbiology*, **170**, 48–54.

Lizana X., López A., Benito S., Agustí G., Ríos M., Piqué N., Marqués A.M., Codony F. (2017) Viability qPCR, a new tool for *Legionella* risk management. *International Journal of Hygiene and Environmental Health*, **220**, 1318–1324.

Llorente I., Montesinos E. (2013) Predicción de epidemias para el control de enfermedades de los cultivos. *Phytoma*, **252**.

Lowder M., Unge A., Maraha N., Jansson J.K., Swiggett J., Oliver J.D. (2000) Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. *Applied and Environmental Microbiology*, **66**, 3160–3165.

Lübeck M., Knudsen I.M.B., Jensen B., Thrane U., Janvier C., Jensen D.F. (2002) GUS and GFP transformation of the biocontrol strain *Clonostachys rosea* IK726 and the use of these marker genes in ecological studies. *Mycological Research*, **106**, 815–826.

Lubelski J., Rink R., Khusainov R., Moll G.N., Kuipers O.P. (2008) Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cellular and Molecular Life Sciences*, **65**, 455–476.

Luiz C., da Rocha Neto A.C., Franco P.O., Di Piero R.M. (2017) Emulsions of essential oils and aloe polysaccharides: antimicrobial activity and resistance inducer potential against *Xanthomonas fragariae*. *Tropical Plant Pathology*, **42**, 370–381.

Lutz M.P., Wenger S., Maurhofer M., Défago G., Duffy B. (2004) Signaling between bacterial and fungal biocontrol agents in a strain mixture. *FEMS Microbiology Ecology*, **48**, 447–455.

M

Mansfield J., Genin S., Magori S., Citovsky V., Sriariyanum M., Ronald P., Dow M., Verdier V., Beer S. V., Machado M.A., Toth I., Salmond G., Foster G.D. (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, **13**, 614–629.

MAPAMA (2018) Chancro bacteriano del kiwi. Available at: http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/chancro_bacteriano_kiwi/ [Accessed May 28, 2018].

MAPAMA (2016) Fuego Bacteriano (*Erwinia amylovora*). Available at: http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/fuego-bacteriano/ [Accessed May 28, 2018].

Martin B., Raurich S., Garriga M., Aymerich T. (2013) Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham. *Food Analytical Methods*, **6**, 683–690.

Martini M., Moruzzi S., Ermacora P., Loi N., Firrao G. (2015) Quantitative real-time PCR and high-resolution melting (HRM) analysis for strain-specific monitoring of fluorescent pseudomonads used as biocontrol agents against soil-borne pathogens of food crops. *Trends in Food Science & Technology*, **46**, 277–285.

Massart S., De Clercq D., Salmon M., Dickburt C., Jijakli M.H. (2005) Development of real-time PCR using Minor Groove Binding probe to monitor the biological control agent *Candida oleophila* (strain O). *Journal of Microbiological Methods*, **60**, 73–82.

Meng X., Chai A., Chen L., Shi Y., Xie X., Ma Z., Li B. (2016) Rapid detection and quantification of viable *Pseudomonas syringae* pv. *lachrymans* cells in contaminated cucumber seeds using propidium monoazide and a real-time PCR assay. *Canadian Journal of Plant Pathology*, **38**, 296–306.

Minervini F., Celano G., Lattanzi A., Tedone L., De Mastro G., Gobbetti M., De Angelis M. (2015) Lactic acid bacteria in durum wheat flour are endophytic components of the plant during its entire life cycle. *Applied and Environmental Microbiology*, **81**, 6736–6748.

Montemarani A., Nesci A., Etcheverry M. (2014) Production of *Kluyveromyces* spp. and environmental tolerance induction against *Aspergillus flavus*. *Annals of Microbiology*, **64**, 935–944.

Montesinos E. (2007) Antimicrobial peptides and plant disease control. *FEMS microbiology letters*, **270**, 1–11.

Montesinos E. (2003) Development, registration and commercialization of microbial pesticides for plant protection. *International Microbiology*, **6**, 245–252.

Montesinos E., Badosa E., Cabrefiga J., Planas M., Feliu L., Bardají E. (2012) Antimicrobial peptides for plant disease control. From discovery to application. In Rajasekaran K., Cary J.W., Jaynes J.M., and Montesinos E. (eds.) *Small Wonders: Peptides for Disease Control*. Washington, DC: American Chemical Society. pp. 235–261.

Montesinos E., Bonaterra A. (1996) Dose-response models in biological control of plant pathogens: an empirical verification. *Phytopathology*, **86**, 464–472.

Montesinos E., Bonaterra A. (2017) Pesticides, Microbial, In *Reference Module in Life Sciences*, Amsterdam, Netherlands: Elsevier.

Montesinos E., Francés J., Badosa E., Bonaterra A. (2015) Post harvest control. In Lugtenberg B. (ed.) *Principles of plant-microbe interactions*. *Microbes for sustainable agriculture*. Cham, Germany: Springer, pp. 193–202.

Mora I., Cabrefiga J., Montesinos E. (2011) Antimicrobial peptide genes in *Bacillus* strains from plant environments. *International Microbiology*, **14**, 213–223.

Mora I., Cabrefiga J., Montesinos E. (2015) Cyclic lipopeptide biosynthetic genes and products, and inhibitory activity of plant-associated *Bacillus* against phytopathogenic bacteria. *PLoS ONE*, **10**, e0127738.

Morales G., Llorente I., Montesinos E., Moragrega C. (2017) A model for predicting *Xanthomonas arboricola* pv. *pruni* growth as a function of temperature. *PloS ONE*, **12**, e0177583.

Morales G., Moragrega C., Montesinos E., Llorente I. (2018) Effects of leaf wetness duration and temperature on infection of *Prunus* by *Xanthomonas arboricola* pv*. pruni*. *PLOS ONE*, 13, e0193813.

Mosimann C., Oberhänsli T., Ziegler D., Nassal D., Kandeler E., Boller T., Mäder P., Thonar C. (2017) Tracing of two *Pseudomonas* strains in the root and rhizoplane of maize, as related to their plant growth-promoting effect in contrasting soils. *Frontiers in Microbiology*, **7**, 2150.

N

Nes I.F., Yoon S.-S., Diep D.B. (2007) Ribosomally synthesiszed antimicrobial peptides (bacteriocins) in lactic acid bacteria: a review. *Food Science and Biotechnology*, **16**, 675–690.

Nissen-Meyer J., Holo H., Håvarstein L.S., Sletten K., Nes I.F. (1992) A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *Journal of Bacteriology*, **174**, 5686–5692.

Nocker A., Cheung C.-Y., Camper A.K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*, **67**, 310–320.

Norelli J.L., Jones A.L., Aldwinckle H.S. (2003) Fire blight management in the twenty-first century using new technologies that enhance host resistance in apple. *Plant Disease*, **87**, 756–765.

Nunes C. (2012) Biological control of postharvest diseases of fruit. *European Journal of Plant Pathology*, **133**, 181–196.

Nunes C., Bajji M., Stepien V., Manso T., Torres R., Usall J., Jijakli M.H. (2008) Development and application of a SCAR marker to monitor and quantify populations of the postharvest biocontrol agent *Pantoea agglomerans* CPA-2. *Postharvest Biology and Technology*, **47**, 422–428.

Nunes C., Usall J., Teixidó N., Fons E., Viñas I. (2002a) Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on Golden Delicious apples. *Journal of Applied Microbiology*, **92**, 247–255.

Nunes C., Usall J., Teixidó N., Miró M., Viñas I. (2001) Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears. *European Journal of Plant Pathology*, **107**, 543–551.

Nunes C., Usall J., Teixidó N., Viñas I. (2002b) Improvement of *Candida sake* biocontrol activity against post-harvest decay by the addition of ammonium molybdate. *Journal of Applied Microbiology*, **92**, 927–935.

O

Oerke E.-C. (2006) Crop losses to pests. *Journal of Agricultural Science*, **144**, 31–43.

Oliver J.D. (2005) The viable but nonculturable state in bacteria. *The Journal of Microbiology*, **43**, 93–100.

Ordax M., Marco-Noales E., López M.M., Biosca E.G. (2006) Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. *Applied and Environmental Microbiology*, **72**, 3482–3488.

P

Palacio-Bielsa A., Roselló M., Cambra M.A., López M.M. (2010) First report on almond in Europe of bacterial spot disease of stone fruits caused by *Xanthomonas arboricola* pv. *pruni*. *Plant Disease*, **94**, 786–786.

Palazzini J.M., Ramirez M.L., Alberione E.J., Torres A.M., Chulze S.N. (2009) Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents on *Fusarium* head blight in wheat. *Biological Control*, **51**, 370–376.

Papadimitriou K., Alegría Á., Bron P.A., de Angelis M., Gobbetti M., Kleerebezem M., Lemos J.A., Linares D.M., Ross P., Stanton C., Turroni F., van Sinderen D., Varmanen P., Ventura M., Zúñiga M., Tsakalidou E., Kok J. (2016) Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, **80**, 837–890.

Parente E., Ciocia F., Ricciardi A., Zotta T., Felis G.E., Torriani S. (2010) Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. *International Journal of Food Microbiology*, **144**, 270–279.

Parikh L., Eskelson M.J., Adesemoye A.O. (2018) Relationship of *in vitro* and *in planta* screening: improving the selection process for biological control agents against *Fusarium* root rot in row crops. *Archives of Phytopathology and Plant Protection*, **51**, 156–169.

Park D.H., Lee Y.-G., Kim J.-S., Cha J.-S., Oh C.-S. (2017) Current status of fire blight caused by *Erwinia amylovora* and action for its management in Korea. *Journal of Plant Pathology*, **99**, 59–63.

Parret A.H.A., Temmerman K., De Mot R. (2005) Novel lectin-like bacteriocins of biocontrol strain *Pseudomonas fluorescens* Pf-5. *Applied and Environmental Microbiology*, **71**, 5197–5207.

Pérez-Jiménez R.M., De Cal A., Melgarejo P., Cubero J., Soria C., Zea-Bonilla T., Larena I. (2012) Resistance of several strawberry cultivars against three different pathogen. *Spanish Journal of Agricultural Research*, **10**, 502–512.

Pham H.T., Riu K.Z., Jang K.M., Cho S.K., Cho M. (2004) Bactericidal activity of Glycinecin A, a bacteriocin derived from *Xanthomonas campestris* pv. *glycines*, on phytopathogenic *Xanthomonas campestris* pv. *vesicatoria* cells. *Applied and Environmental Microbiology*, **70**, 4486–4490.

Pichereau V., Hartke A., Auffray Y. (2000) Starvation and osmotic stress induced multiresistances: influence of extracellular compounds. *International Journal of Food Microbiology*, **55**, 19–25.

Pinto D., Santos M.A., Chambel L. (2015) Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. *Critical Reviews in Microbiology*, **41**, 61–76.

Pliego C., Ramos C., de Vicente A., Cazorla F.M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant and Soil*, **340**, 505–520.

Poolman B., Glaasker E. (1998) Regulation of compatible solute accumulation in bacteria. Mol*ecular Microbiology*, **29**, 397–407.

Prasad J., McJarrow P., Gopal P. (2003) Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Applied and Environmental Microbiology*, **69**, 917–925.

Prudêncio C.V., Dos Santos M.T., Vanetti M.C.D. (2015) Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology. *Journal of Food Science and Technology*, **52**, 5408–5417.

Pucci M.J., Vedamuthu E.R., Kunka B.S., Vandenbergh P.A. (1988) Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Applied and Environmental Microbiology*, **54**, 2349–2353.

Pujol M., Badosa E., Cabrefiga J., Montesinos E. (2005) Development of a strain-specific quantitative method for monitoring *Pseudomonas fluorescens* EPS62e, a novel biocontrol agent of fire blight. *FEMS Microbiology Letters*, **249**, 343–352.

Pujol M., Badosa E., Manceau C., Montesinos E. (2006) Assessment of the environmental fate of the biological control agent of fire blight, *Pseudomonas fluorescens* EPS62e, on apple by culture and real-time PCR methods. *Applied and Environmental Microbiology*, **72**, 2421–2427.

Pujol M., Badosa E., Montesinos E. (2007) Epiphytic fitness of a biological control agent of fire blight in apple and pear orchards under Mediterranean weather conditions. *FEMS Microbiology Ecology*, **59**, 186–193.

Pujol M., De Clercq D., Cognet S., Lepoivre P., Jijakli M.H. (2004) Monitoring system for the biocontrol agent *Pichia anomala* strain K using quantitative competitive PCR-ELOSA. *Plant Pathology*, **53**, 103–109.

Pusey P.L., Wend C. (2012) Potential of osmoadaptation for improving *Pantoea agglomerans* E325 as biocontrol agent for fire blight of apple and pear. *Biological Control*, **62**, 29–37.

R

Ramiah K., van Reenen C.A., Dicks L.M.T. (2007) Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR. *International Journal of Food Microbiology*, **116**, 405–409.

Regulation (EC) No 1107/2009. 2009. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant products on the market and repealing Council Directive 79/117/EEC and 91/414/EEC. *Official Journal of the European Union*. **L309**, 1-50.

Reis J.A., Paula A.T., Casarotti S.N., Penna A.L.B. (2012) Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews*, **4**, 124–140.

Remus-Emsermann M.N.P., Vorholt J.A. (2014) Complexities of microbial life on leaf surfaces. *Microbe*, **9**, 448–452.

Rezzonico F., Moënne-Loccoz Y., Défago G. (2003) Effect of stress on the ability of a *phlA*-based quantitative competitive PCR assay to monitor biocontrol strain *Pseudomonas fluorescens* CHA0. *Applied and Environmental Microbiology*, **69**, 686–690.

Roberts D.P., Lohrke S.M., Meyer S.L.F., Buyer J.S., Bowers J.H., Baker C.J., Li W., de Souza J.T., Lewis J.A., Chung S. (2005) Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. *Crop Protection*, **24**, 141–155.

Roberts P.D., Jones J.B., Chandler C.K., Stall R.E. (1997) Disease progress, yield loss, and control of *Xanthomonas fragariae* on strawberry plants. *Plant Disease*, **81**, 917–921.

Roselló G. (2016) Characterization and improvement of plant-associated *Lactobacillus plantarum*. Novel biocontrol agents for fire blight disease. Ph.D. Thesis. University of Girona, Spain. Available at: https://www.tdx.cat/handle/10803/403404 [Accessed May 16, 2018].

Roselló G., Bonaterra A., Francés J., Montesinos L., Badosa E., Montesinos E. (2013) Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*. *European Journal of Plant Pathology*, **137**, 621–633.

Roselló G., Francés J., Daranas N., Montesinos E., Bonaterra A. (2017) Control of fire blight of pear trees with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid. *Journal of Plant Pathology*, **99**, 111–120.

Rotolo C., De Miccolis Angelini R.M., Pollastro S., Faretra F. (2016) A TaqMan-based qPCR assay for quantitative detection of the biocontrol agents *Bacillus subtilis* strain QST713 and *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747. *BioControl*, **61**, 91–101.

Rubio M.B., Hermosa M.R., Keck E., Monte E. (2005) Specific PCR assays for the detection and quantification of DNA from the biocontrol strain *Trichoderma harzianum* 2413 in soil. *Microbial Ecology*, **49**, 25–33.

Rudi K., Moen B., Drømtorp S.M., Holck A.L. (2005) Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Applied and Environmental Microbiology*, **71**, 1018–24.

Ruiz C., Nadal A., Montesinos E., Pla M. (2018) Novel Rosaceae plant elicitor peptides as sustainable tools to control *Xanthomonas arboricola* pv. *pruni* in *Prunus* spp. *Molecular Plant Pathology*, **19**, 418–431.

S

Saraoui T., Cornet J., Guillouet E., Pilet M.F., Chevalier F., Joffraud J.-J., Leroi F. (2017) Improving simultaneously the quality and safety of cooked and peeled shrimp using a cocktail of bioprotective lactic acid bacteria. *International Journal of Food Microbiology*, **241**, 69–77.

Savadogo A., Ouattara C.A.., Bassole I.H.., Traore S.A. (2006) Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, **5**, 678–683.

Schena L., Nicosia M.G.L.D., Sanzani S.M., Faedda R., Ippolito A., Cacciola S.O. (2013) Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. *Journal of Plant Pathology*, **95**, 7–24.

Schena L., Nigro F., Ippolito A., Gallitelli D. (2004) Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology*, **110**, 893–908.

Schena L., Sialer M.F., Gallitelli D. (2002) Molecular detection of strain L47 of *Aureobasidium pullulans* , a biocontrol agent of postharvest diseases. *Plant Disease*, **86**, 54–60.

Schiraldi C., Adduci V., Valli V., Maresca C., Giuliano M., Lamberti M., Cartenì M., De Rosa M. (2003) High cell density cultivation of probiotics and lactic acid production. *Biotechnology and Bioengineering*, **82**, 213–222.

Scortichini M. (2010) Epidemiology and predisposing factors of some major bacterial diseases of stone and nut fruit trees species. *Journal of Plant Pathology*, **92**, S1.73-S1.78.

Segarra G., Puopolo G., Giovannini O., Pertot I. (2015) Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens: the case of *Lysobacter capsici* AZ78. *Journal of Biotechnology*, **216**, 56–64.

Seinige D., Krischek C., Klein G., Kehrenberg C. (2014) Comparative analysis and limitations of ethidium monoazide and propidium monoazide treatments for the differentiation of viable and nonviable *Campylobacter* cells. *Applied and Environmental Microbiology*, **80**, 2186–2192.

Settanni L., Corsetti A. (2008) Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology*, **121**, 123–138.

Shade A., McManus P.S., Handelsman J. (2013) Unexpected diversity during community succession in the apple flower microbiome. *mBio*, **4**, e00602-12.

Shrestha A., Choi K., Lim C.K., Hur J.H., Cho S. (2009) Antagonistic effect of *Lactobacillus* sp: strain KLF01 against soil borne plant pathogen *Ralstonia solanacearum*. *The Korean Journal of Pesticide Science*, **13**, 45–53.

Shrestha A., Kim B.S., Park D.H. (2014) Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. *Biocontrol Science and Technology*, **24**, 763–779.

Siezen R.J., Tzeneva V.A., Castioni A., Wels M., Phan H.T.K., Rademaker J.L.W., Starrenburg M.J.C., Kleerebezem M., Molenaar D., van Hylckama Vlieg J.E.T. (2010) Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. *Environmental Microbiology*, **12**, 758–773.

Simmonds R.S., Pearson L., Kennedy R.C., Tagg J.R. (1996) Mode of action of a lysostaphin-like bacteriolytic agent produced by *Streptococcus zooepidemicus* 4881. *Applied and Environmental Microbiology*, **62**, 4536–4541.

Smith T.J. (1993) A predictive model for forecasting fire blight of pear and apple in Washington state. *Acta Horticulturae*, **338**, 153–160.

Soto-Muñoz L., Teixidó N., Usall J., Viñas I., Abadias M., Torres R. (2015a) Molecular tools applied to identify and quantify the biocontrol agent *Pantoea agglomerans* CPA-2 in postharvest treatments on oranges. *Postharvest Biology and Technology*, **100**, 151–159.

Soto-Muñoz L., Teixidó N., Usall J., Viñas I., Crespo-Sempere A., Torres R. (2014a) Development of PMA real-time PCR method to quantify viable cells of *Pantoea agglomerans* CPA-2, an antagonist to control the major postharvest diseases on oranges. *International Journal of Food Microbiology*, **180**, 49–55.

Soto-Muñoz L., Teixidó N., Usall J., Viñas I., Torres R. (2014b) Detection and quantification by PCR assay of the biocontrol agent *Pantoea agglomerans* CPA-2 on apples. *International Journal of Food Microbiology*, **175**, 45–52.

Soto-Muñoz L., Torres R., Usall J., Viñas I., Dashevskaya S., Teixidó N. (2015b) Environmental monitoring of the biocontrol agent *Pantoea agglomerans* CPA-2 applied to citrus fruit at preharvest. *Annals of Applied Biology*, **167**, 250–261.

Spadaro D., Gullino M.L. (2005) Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop Protection*, **24**, 601–613.

Spinelli F., Ciampolini F., Cresti M., Geider K., Costa G. (2005) Influence of stigmatic morphology on flower colonization by *Erwinia amylovora* and *Pantoea agglomerans*. *European Journal of Plant Pathology*, **113**, 395–405.

Spinelli F., Vanneste J., Costa G. (2012) Acylcyclohexanediones and biological control agents: combining complementary modes of action to control fire blight. *Trees*, **26**, 247–257.

Spotts R.A., Wallis K.M., Serdani M., O'Gorman D.T., Sholberg P.L. (2009) Real time polymerase chain reaction for rapid and quantitative determination of *Cystofilobasidium infirmominiatum* on the surfaces of apple, pear, and sweet cherry fruit. *Postharvest Biology and Technology*, **51**, 227–231.

Stefani E. (2010) Economic significance and control of bacterial spot/canker of stone fruits caused by *Xanthomonas arboricola* pv. *pruni*. *Journal of Plant Pathology*, **92**, S1.99-S1.103.

Stephan D., Da Silva A.-P.M., Bisutti I.L. (2016) Optimization of a freeze-drying process for the biocontrol agent *Pseudomonas* spp. and its influence on viability, storability and efficacy. *Biological Control*, **94**, 74–81.

Stiles M.E., Holzapfel W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, **36**, 1–29.

Stockwell V.O., Johnson K.B., Loper J.E. (1998) Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. *Phytopathology*, **88**, 506–513.

Stockwell V.O., Johnson K.B., Sugar D., Loper J.E. (2010) Control of fire blight by *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1 applied as single strains and mixed inocula. *Phytopathology*, **100**, 1330–1339.

Suárez-Estrella F., Arcos-Nievas M.A., López M.J., Vargas-García M.C., Moreno J. (2013) Biological control of plant pathogens by microorganisms isolated from agro-industrial composts. *Biological Control*, **67**, 509–515.

Sui Y., Wisniewski M., Droby S., Liu J. (2015) Responses of yeast biocontrol agents to environmental stress. *Applied and Environmental Microbiology*, **81**, 2968–2975.

Sundin G.W., Castiblanco L.F., Yuan X., Zeng Q., Yang C.-H. (2016) Bacterial disease management: challenges, experience, innovation and future prospects. *Molecular Plant Pathology*, **17**, 1506–1518.

T

Tanganurat W., Quinquis B., Leelawatcharamas V., Bolotin A. (2009) Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables. *Journal of Basic Microbiology*, **49**, 377–385.

Teixidó N., Cañamás T.P., Abadias M., Usall J., Solsona C., Casals C., Viñas I. (2006) Improving low water activity and desiccation tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments. *Journal of Applied Microbiology*, **101**, 927–937.

Teixidó N., Canamas T.P., Usall J., Torres R., Magan N., Viñas I. (2005) Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. *Letters in Applied Microbiology*, **41**, 248–252.

Teixidó N., Viñas I., Usall J., Sanchis V., Magan N. (1998) Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. *Journal of Applied Microbiology*, **84**, 192–200.

Tejero-Sariñena S., Barlow J., Costabile A., Gibson G.R., Rowland I. (2012) *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: evidence for the effects of organic acids. *Anaerobe*, **18**, 530–538.

Thanh M.D., Agustí G., Mader A., Appel B., Codony F. (2017) Improved sample treatment protocol for accurate detection of live *Salmonella* spp. in food samples by viability PCR. *PLoS ONE*, **12**, e0189302.

Thomson S. V (2000) Epidemiology of Fire Blight, In Vanneste J.L. (ed.) *Fire blight. The disease and its causative agent Erwinia amylovora*. Wallingford: CABI Publishing, pp. 9–36.

Todorov S.D. (2009) Bacteriocins from *Lactobacillus plantarum* - production, genetic organization and mode of action. *Brazilian Journal of Microbiology*, **40**, 209–221.

Tombolini R., Unge A., Davey M.E., de Bruijn F.J., Jansson J.K. (1997) Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. *FEMS Microbiology Ecology*, **22**, 17–28.

Tomé E., Gibbs P.A., Teixeira P.C. (2008) Growth control of *Listeria innocua* 2030c on vacuum-packaged cold-smoked salmon by lactic acid bacteria. *International Journal of Food Microbiology*, **121**, 285–294.

Trias R. (2008) Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables. Ph.D. Thesis. University of Girona, Spain. Available at: https://www.tesisenred.net/handle/10803/7932 [Accessed May 16, 2018].

Trias R., Badosa E., Montesinos E., Bañeras L. (2008a) Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. *International Journal of Food Microbiology*, **127**, 91–98.

Trias R., Bañeras L., Badosa E., Montesinos E. (2008b) Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International Journal of Food Microbiology*, **123**, 50–60.

Trias R., Bañeras L., Montesinos E., Badosa E. (2008c) Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. *International Microbiology*, **11**, 231–236.

Tsuda K., Tsuji G., Higashiyama M., Ogiyama H., Umemura K., Mitomi M., Kubo Y., Kosaka Y. (2016) Biological control of bacterial soft rot in Chinese cabbage by *Lactobacillus plantarum* strain BY under field conditions. *Biological Control*, **100**, 63–69.

U

Unge A., Jansson J. (2001) Monitoring population size, activity, and distribution of *gfp-luxAB*-tagged *Pseudomonas fluorescens* SBW25 during colonization of wheat. *Microbial Ecology*, **41**, 290–300.

Unge A., Tombolini R., Mølbak L., Jansson J.K. (1999) Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual *gfp-luxAB* marker system. *Applied and Environmental Microbiology*, **65**, 813–821.

Usall J., Teixidó N., Torres R., Ochoa de Eribe X., Viñas I. (2001) Pilot tests of *Candida sake* (CPA-1) applications to control postharvest blue mold on apple fruit. *Postharvest Biology and Technology*, **21**, 147–156.

V

van de Guchte M., Serror P., Chervaux C., Smokvina T., Ehrlich S.D., Maguin E. (2002) Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek*, **82**, 187–216.

van der Zwet T., Orolaza-Halbrendt N., Zeller W. (2012) *Fire blight: History, biology and management*. St. Paul, MN: APS Press.

Van Elsas J.D., Duarte G.F., Rosado A.S., Smalla K. (1998) Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. *Journal of Microbiological Methods*, **32**, 133–154.

Vanneste J.L. (2000) *Fire blight: The disease and its causative agent, Erwinia amylovora*. Wallingford, UK: CABI Publishing.

Vanneste J.L., Cornish D.A., Tanner D.J., Windner R., Chapman J.R., Taylor R.K., Mackay J.F., Dowlut S. (2013) Identification, virulence, and distribution of two biovars of *Pseudomonas syringae* pv. *actinidiae* in New Zealand. *Plant Disease*, **97**, 708–719.

Vanneste J.L., Cornish D.A., Yu J., Voyle M.D. (2002) P10C: A new biological control agent for control of fire blight which can be sprayed or distributed using honey bees. *Acta Horticulturae*, **590**, 231–235.

Versalovic J., Schneider M., Bruijn F. De (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, **5**, 25–40.

Vilanova L., Teixidó N., Usall J., Balsells-Llauradó M., Gotor-Vila A., Torres R. (2018) Environmental fate and behaviour of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 after preharvest application to stone fruit. *Pest Management Science*, **74**, 375–383.

Vilanova L., Usall J., Teixidó N., Torres R. (2017) Assessment of viable conidia of *Monilinia fructicola* in flower and stone fruit combining propidium monoazide (PMA) and qPCR. *Plant Pathology*, **66**, 1276–1287.

Villarreal M.L.M., Padilha M., Vieira A.D.S., Franco B.D.G. de M., Martinez R.C.R., Saad S.M.I. (2013) Advantageous direct quantification of viable closely related probiotics in Petit-Suisse cheeses under *in vitro* gastrointestinal conditions by propidium monoazide - qPCR. *PLoS ONE*, **8**, e82102.

Villaverde J.J., Sevilla-Morán B., Sandín-España P., López-Goti C., Alonso-Prados J.L. (2014) Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. *Pest Management Science*, **70**, 2–5.

Visser R., Holzapfel W.H., Bezuidenhout J.J., Kotze J.M. (1986) Antagonism of lactic acid bacteria against phytopathogenic bacteria. *Applied and Environmental Microbiology*, **52**, 552–555.

Von Felten A., Défago G., Maurhofer M. (2010) Quantification of *Pseudomonas fluorescens* strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-specific real-time PCR unaffected by the variability of DNA extraction efficiency. *Journal of Microbiological Methods*, **81**, 108–115.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

W

Wan Y.K., Tian S.P., Qin G.Z. (2003) Enhancement of biocontrol activity of yeasts by adding sodium bicarbonate or ammonium molybdate to control postharvest disease of jujube fruits. *Letters in Applied Microbiology*, **37**, 249–253.

Wang H., Yan H., Shin J., Huang L., Zhang H., Qi W. (2011) Activity against plant pathogenic fungi of *Lactobacillus plantarum* IMAU10014 isolated from Xinjiang koumiss in China. *Annals of Microbiology*, **61**, 879–885.

Wang H., Yan Y., Wang J., Zhang H., Qi W. (2012) Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. *PLoS ONE*, **7**, e29452.

Wang Y., Wang P., Xia J., Yu T., Lou B., Wang J., Zheng X.D. (2010) Effect of water activity on stress tolerance and biocontrol activity in antagonistic yeast *Rhodosporidium paludigenum*. *International Journal of Food Microbiology*, **143**, 103–108.

Welsh D.T. (2000) Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiology Reviews*, **24**, 263–290.

Wicaksono W.A., Jones E.E., Casonato S., Monk J., Ridgway H.J. (2018) Biological control of *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, using endophytic bacteria recovered from a medicinal plant. *Biological Control*, **116**, 103–112.

Williams J.G.K., Kubelik A.R., Livak K. enneth J., Rafalski J.A., Tingey S. V (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**, 6531–6535.

Williams T.R., Moyne A.-L., Harris L.J., Marco M.L. (2013) Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. *PLoS ONE*, **8**, e68642.

Wilson M., Lindow S.E. (1993) Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. *Phytopathology*, **83**, 117-123.

X

Xu H., Liu W., Zhang W., Yu J., Song Y., Menhe B., Zhang H., Sun Z. (2015) Use of multilocus sequence typing to infer genetic diversity and population structure of *Lactobacillus plantarum* isolates from different sources. *BMC Microbiology*, **15**, 241.

Y

Yang R., Fan X., Cai X., Hu F. (2015) The inhibitory mechanisms by mixtures of two endophytic bacterial strains isolated from *Ginkgo biloba* against pepper phytophthora blight. *Biological Control*, **85**, 59–67.

Yu T., Chen J., Chen R., Huang B., Liu D., Zheng X. (2007) Biocontrol of blue and gray mold diseases of pear fruit by integration of antagonistic yeast with salicylic acid. *International Journal of Food Microbiology*, **116**, 339–345.

Yu T., Wu G., Qi J.J., Zheng X.D., Jiang F., Zhan X. (2006) Improved control of postharvest blue mold rot in pear fruit by a combination of *Cryptococcus laurentii* and gibberellic acid. *Biological Control*, **39**, 128–134.

Z

Zamani-Zadeh M., Soleimanian-Zad S., Sheikh-Zeinoddin M., Amir S., Goli H. (2014) Integration of *Lactobacillus plantarum* A7 with thyme and cumin essential oils as a potential biocontrol tool for gray mold rot on strawberry fruit. *Postharvest Biology and Technology*, **92**, 149–156.

Zhang X., Zhang B., Zhang Z., Shen W., Yang C., Yu J., Zhao Y. (2005) Survival of the biocontrol agents *Brevibacillus brevis* ZJY-1 and *Bacillus subtilis* ZJY-116 on the spikes of barley in the field. *Journal of Zhejiang University Science*, **6B**, 770–777.

Zhao S., Zhang Q., Hao G., Liu X., Zhao J., Chen Y., Zhang H., Chen W. (2014) The protective role of glycine betaine in *Lactobacillus plantarum* ST-III against salt stress. *Food Control*, **44**, 208–213.

Zwielehner J., Handschur M., Michaelsen A., Irez S., Demel M., Denner E.B.M., Haslberger A.G. (2008) DGGE and real-time PCR analysis of lactic acid bacteria in bacterial communities of the phyllosphere of lettuce. *Molecular Nutrition & Food Research*, **52**, 614–623.