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

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Running Head: Strain-specific viability qPCR of *L. plantarum*

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## ABSTRACT

A viability qPCR (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 for selectively detecting and enumerating 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, and pear and apple blossoms under field conditions, by comparing

v-qPCR population estimation to those obtained by qPCR and specific plate counting on MRS-rifampicin. 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 non-culturable 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 flower environment under high relative humidity. However, the unfavourable conditions onto the leaf surface and the relatively dryness in the field caused an important decrease of viable population.

**IMPORTANCE**

The v-qPCR method in combination with plate counting and qPCR is a powerful tool for studies of colonization and survival in field conditions, to improve formulations and delivery strategies of PM411, or 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 biological control agents but also beneficial bacteria useful in the sustainable management of crop production.

## INTRODUCTION

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 other inhibitory bioactive compounds, or to the competition for nutrients or colonization sites (8). Moreover, some LAB strains have been qualified as GRAS (Generally Regarded As Safe) by the US Food and Drug Administration (FDA) and as QPS (Qualified Presumption of Safety) 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 are needed.

Several monitoring methods are commonly used to detect and quantify BCA at 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. two or three log units) the population size of the BCA under non-conducive conditions (15, 16). This is because some bacteria, including LAB species, such as *L. plantarum*, may enter in a viable but non-culturable state (VBNC) as a survival strategy to cope with environmental

stress (17–19). VBNC cells retain some metabolic activity and remain intact membranes, despite minor changes in their composition (18, 20). By 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 qPCR (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* (25, 26), and *Salmonella* (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) or amplified fragment length polymorphisms (AFLP) (30–32). In addition, for selectively detecting and enumerating viable bacteria, different nucleic acid-binding dyes 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 causes 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 non-viable 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 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 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 labelling 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 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 a *L. plantarum* PM411-specific molecular marker

A discriminatory band (520 bp) was found in PM411 strain by RAPD-PCR method using the primer XD9 (described in material and methods section). Its sequence shared 86% identity

126 with the *Lactobacillus* phage Sha1 (GenBank accession number HQ141411), and 81%
127 identity with 32 *L. plantarum* strains available in the NCBI database. Detection for prophage
128 DNA sequences within the PM411 genome using PHAST indicated three regions that were
129 predicted to represent prophages. One of these regions with 69.6 Kb (GenBank accession
130 number MG788324) contained the RAPD sequence and was identified as the putative
131 prophage Lactob_PLE3 (GenBank accession number NC_031152) with a score of 150 and
132 with 80 coding DNA sequences (CDS) (Table S1). In particular, the 520 bp sequence (found
133 by RAPD-PCR) was located in the CDS 75 and 76 of the putative phage. Figure 1 shows a
134 972 bp fragment (from 65376 to 66348 CDS position), which includes the RAPD sequence,
135 with mosaic architecture that contain homologous and non-homologous sequences to strains
136 available in the NCBI database. Its specificity was confirmed *in silico* since the whole 972 bp
137 fragment was not found in any strain available in the database. The fragment contained
138 homologous sequences, such as 665 bp that shared 79% identity with 32 *L. plantarum*,
139 partially coding a tail fiber and a hypothetical protein, 90 bp that shared 85% or less identity
140 with 7 *L. plantarum*, partially coding a tail fiber, and 162 bp that shared 80% identity with *L.*
141 *plantarum* HFC8, partially coding a hypothetical protein. However, the presence of the non-
142 homologous sequences enabled the identification of a strain-specific molecular marker. The
143 strain specificity of the marker was confirmed by PCR using PM411-for and PM411C-rev
144 primers (Table 2). With this approach, no amplification was obtained for any of the strains
145 listed in Table 1, except for PM314 and PM340 which were confirmed to be clones of
146 PM411 by repetitive element sequences-based PCR (rep-PCR) fingerprinting. *L. plantarum*
147 PM411, PM314, and PM340 strains showed identical banding pattern (data not shown). This
148 common banding pattern was clearly different from other *L. plantarum* strains tested
149 (CM450, FC248, TC92, and WCFS1).
150

**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 non-included).

**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 (Ct) 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 1. Only random fluorescence signals were observed at Ct 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 strain level.

Standard curves of the three qPCR assays, which were prepared in flower washings, showed good linearity over a 4-log range (from 1 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$, $R^2$ = 0.99) and the lowest limit of detection was 1 x $10^2$ CFU $ml^{-1}$. The equations of regression curves for the A, B and C designs were: Ct = -3.3 log CFU $ml^{-1}$ + 39.7 (A); Ct = -3.3 log CFU $ml^{-1}$ + 42.8 (B); Ct = -3.4 log CFU $ml^{-1}$ + 47.6 (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), and the C design (317 bp) being the less sensitive. The comparison between the three standard curves led the selection of the A and B assays for further experiments.

**Viability quantitative PCR (v-qPCR)**

The effect of different PEMAX concentrations and qPCR assay (different amplicon length) on the signal reduction (SR) (difference of Ct value, ΔCt, between non-PEMAX 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 of 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.

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 B than by A assay using 100 µM of PEMAX, whereas using 50 and 75 µM of PEMAX no significant differences between assays were observed. Then, 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 by 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 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$, with and without PEMAX treatment. However, in the presence of PEMAX treatment a shift of 2 cycles was

observed regarding the non-treated samples. On dead cells treated with PEMAX Ct values higher than 38 were obtained over the range from 1 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$, meaning that the amplification was inhibited (Fig. 3). In mixtures of viable cells (from 1 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$) and a fixed quantity of dead cells (1 x $10^6$ 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 Ct 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 (25ºC, high or low relative humidity (RH)) by qPCR, v-qPCR, and plate counting (pc) (Figs. 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 (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 the 8th day, 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 two days, around $10^6$-$10^7$ CFU per blossom, without significant differences compared to the total and cultivable population. However, after this period viable and culturable cells were significantly lower than total cells. Under these conditions, there

225 was a linear relationship between culturable (pc) and viable (v-qPCR) population levels (y =
226 0.95 x + 0.12; $R^2$ = 0.93; $P < 0.001$).
227 On pear, strawberry, and kiwifruit leaves, significant differences between the three
228 quantification methods (qPCR, v-qPCR, and pc) were observed at some steps throughout the
229 experiments (Fig. 5). Total population level (qPCR) was significantly higher than viable (v-
230 qPCR) and culturable (pc) population level almost in all sampling days throughout the
231 experiments. Interestingly, significant differences were also observed between the viable and
232 culturable population, being the quantification of viable cells significantly higher than the
233 culturable cells, especially after 2-3 days under high RH and after only 1 day under low RH.
234 In all the experiments, the total population decreased approximately 1-1.5 log units between
235 the 1st and the 6th day, whereas culturable and viable cells declined more, from 2 to 4 log
236 units, depending on plant species and RH conditions. While on pear and strawberry leaves at
237 high RH the population reduction of viable and culturable cells was 3-4 log units, on kiwifruit
238 leaves was 2-2.5 log units. Under low RH the population decrease of viable and culturable
239 cells on kiwifruit leaves during the three days post inoculation (2-2.5 log units for viable and
240 3-3.5 log units for culturable) was higher than under high RH (1 log unit for viable and 1.5
241 log units for culturable).
242 Population levels of PM411 were also monitored on apple and pear blossoms under field
243 conditions, which were relatively dry (moderate temperatures and low humidity) with one
244 single rainfall event in the pear tree assay (Fig 6). Total population size differed significantly
245 from viable and culturable population levels at some steps throughout the experiments, both
246 on apple and pear blossoms. Two days following field inoculation (first or single spray),
247 viable cells of PM411 decreased to steady-state values ($10^3$–$10^5$ CFU per blossom) both on
248 apple and pear, without significant differences to culturable cells in apple flowers. However,
249 on pear blossoms the viable population was significantly higher than the culturable

population after 1-2 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 one day, PM411 population decreased and significant differences in total population compared to viable and culturable population were also observed both on pear and apple plants. However, only on pear blossoms the viable population was 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 population of viable and culturable cells did not differ significantly when the environmental conditions were conducive for bacterial survival (on flowers under high RH), but 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 behaviour in the crop environment and to improve formulations 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 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 phage Lactob_PLE3 (42). The occurrence of prophage DNA within bacterial genomes is common in LAB, such as *Lactobacillus* (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 non-homologous sequences. This polymorphic structure allowed the identification of sequences to design a strain-specific marker. Prophages exhibit a high degree of mosaicism (46) and have been found to contribute to inter-strain 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 could 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 that were deemed to be clones of PM411), other plant-associated bacteria 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 to monitor PM411. Although random amplifications with high CT values (higher than 38) were observed for

some non-target strains and plant-material washings, this phenomenon was previously described in the literature as a background no-template control (15), being not relevant if remain 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 length 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 fulfil 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), and also 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 of PEMAX in 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 length 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 diminishes 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 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$ and the obtained standard curves, using Ct 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 than those reported in other methods developed to detect and quantify biological control agents (15, 21, 59). The slight increase of Ct values of PEMAX treated samples compared to non-treated 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 x $10^6$ CFU $ml^{-1}$ 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 MRS-rifampicin. Since *L. plantarum* PM411 is a biological control agent of fire blight of apple and pear (5, 9) and is also 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 of evaluation of 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 analysed. 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 non-degraded DNA released from dead cells, and plate counting underestimated the population of the strain (until 2 log units), indicating the induction of VBNC state. Therefore, v-qPCR let the most accurately quantification of PM411 viable cells, whether culturable or VBNC, to monitor it survival on aerial plant surfaces.

On flowers, under environmental controlled conditions of high RH, PM411 showed a transient drop of 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 two 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 favourable for bacteria survival and colonization because of 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 flower environment since it has the capacity to use a broad range of carbohydrates and amino acids (62). After five days onto the flower surface, the population of PM411 clearly decreased, coinciding with the end of the lifespan of flowers. This survival reduction may be attributed to the non-conducive conditions as a result of the senescence of the tissues (13). At this period, population was overestimated by qPCR indicating the presence of non-degraded DNA 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 VBNC state.

Onto 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 of temperature, UV radiation, and especially water availability (relative humidity and leaf wetness) (63, 64). Under these stress conditions the induction of 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 (i.e. desiccation, 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 as 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 $10^3$-$10^5$ CFU per blossom immediately after the spray (both in the single or two sprays experiments). Although the nutritional conditions in flowers are expected to be optimal, probably the harsh environment, such as periods of low relative humidity (<70%) combined with UV radiation exposition, caused this decrease of population. Interestingly, in pear orchard, differences between v-qPCR and plate counting were observed, presumptively, due to the induction of 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 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 that discrepancy between viable and culturable populations was only observed in apple flowers.

In our study the unfavourable environmental conditions onto the leaf surface and the relatively dry field conditions during the experiments seems 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 will allow further studies of colonization and survival in field conditions, and to improve 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 of 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, biostimulants) useful in the sustainable management of crop production.

## MATERIALS AND METHODS

### Bacterial strains, growth conditions and DNA extraction

Bacterial strains used in this study are listed in Table 1. LAB strains were grown on de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, UK) at 23ºC for 48 h. Non-LAB strains were grown on Luria-Bertani (LB) agar at 25ºC for 24 h. *Escherichia coli* DH5α calcium competent cells were used for cloning procedures and were grown in LB

425 medium at 37ºC. A spontaneous mutant of the wild-type *L. plantarum* PM411 resistant to
426 rifampicin, obtained as previously described (5), was used in this study. All strains were
427 stored in 20% glycerol at −80°C. DNA was extracted according to the method described by
428 Llop et. al. (67) from pure bacterial suspensions (Table 1).
429
430 **Strategy to identify a strain-specific molecular marker for *L. plantarum* PM411**
431 All primers, PCR mixtures and PCR conditions used in this study are described in Table 2
432 and 3, and amplified fragments were analysed by standard methods.
433 RAPD-PCR analysis was carried out to identify a PM411 strain-specific molecular marker.
434 Seven primers were tested in the strain PM411 and other selected *L. plantarum* strains
435 (ATCC 8014, CECT 223, CECT 4528, CECT 5785, TC54, TC92, and WCFS1) by PCR. A
436 potential PM411 strain-specific RAPD band was excised from the agarose gel, purified with a
437 QIAquick gel extraction kit (Qiagen, Hilden, Germany), cloned into the pGEM®-T Easy
438 Vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α calcium competent
439 cells. Cells were selected by antibiotic resistance in LB agar supplemented with 100 µg $ml^{-1}$
440 of ampicillin (Sigma, Missouri, USA) and a PCR analysis with the universal primers T7 and
441 Sp6 was done to confirm the insertion. The RAPD-PCR fragment was sequenced (Macrogen,
442 Seoul, Korea), and analysed using FinchTV 1.4.0 software (Geospiza, Seattle, WA, USA)
443 and Multalin software (74). The specificity was ensured *in silico* using the BLAST program
444 at NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/ accessed 4 December 2017).
445 As the fragment identified by RAPD-PCR showed similarity with a prophage, raw data of
446 sequenced *L. plantarum* PM411 genome was used for prophage region search and annotation
447 using a phage finding tool (PHAST, PHAge Search Tool (75)). The corresponding putative
448 phage sequence was deposited in the Genbank database (accession number MG788324). The
449 RAPD sequence was located in the putative prophage of *L. plantarum* PM411 and a 972 bp

region (region from 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 2). Primers were designed in the PM411 specific region. The specificity of the primers was tested using strains described in Table 1.

rep-PCR amplifications of PM411 and other *L. plantarum* strains listed in Table 1 (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 2) (73).

**Strain-specific qPCR designs and specificity, sensitivity, and amplification efficiency evaluation**

Three TaqMan-based qPCR assays were designed (Table 2) within the strain-specific marker (region from 65376 to 66348 of accession number MG788324) to obtain three amplicons with different length. All of them shared the same forward primer (PM411-for) and probe (PM411-pr), but they had three different reverse primers (PM411A-, PM411B- and PM411C-rev). Probes were labelled with the 6-carboxyfluoresceine (FAM) reporter dye at 5' end and with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye at 3' end. Primers and TaqMan probes designs were carried out using the Primer Express 3.0 software (Applied Biosystem, Foster City, USA).

Specificity of the qPCR designs was tested after optimization of the concentration of the primers and probe using bacteria listed in Table 1. Non-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 Biosystem, Foster City, USA).

474 Standard curves were developed to check the sensitivity and efficiency of the qPCR assays by
475 mixing several concentrations of PM411 cells with plant-material washings. To obtain plant-
476 material washings open blossoms of apple (cv. Golden Smoothee) and pear (cv. Comice) and
477 leaves from potted plants of pear (cv. Conference), strawberry (cv. Darselect) and kiwifruit
478 (cv. Hayward) were used. Two blossoms or three leaves were infused with 30 ml of 50 mM
479 sterile phosphate buffer (PBS, pH 7.0) and 0.1% peptone in a 100 ml bottle and mixed in an
480 orbital shaker (KS501 digital; IKA Labortechnik, Staufen, Germany) at 130 rpm for 30 min
481 on ice (15, 60). Cell suspensions of PM411 were prepared in sterile distilled water at high
482 concentration ($10^9$ CFU $ml^{-1}$) and diluted to appropriate concentrations with plant-material
483 washings. The cell concentration of the first serial decimal dilution was checked by $OD_{600}$
484 measure, considering that 0.25 corresponds to $10^8$ CFU $ml^{-1}$, and was confirmed by colony
485 counts. The tested concentrations covered a 5-log range, from 1 x $10^3$ to 1 x $10^8$ CFU $ml^{-1}$.
486 An aliquot of plant-material washings without PM411 cells was kept as non-template control
487 (NTC) sample.
488 DNA was isolated according to the method described by Schmidt et al. (76) with some
489 modifications. Briefly, 1 ml of sample was centrifuged at 13200xg for 10 min and the pellet
490 was resuspended in 400 µl of TES buffer (50 mM Tris-HCl, 1 mM EDTA, 6.7% glucose).
491 Cells were incubated with 100 µl of lysozyme at 20 mg $ml^{-1}$ (Sigma) and 6 µl of mutanolysin
492 at 5000 U $ml^{-1}$ (Sigma) for 1 h at 37ºC with shaking (ThermoMixer F1.5; Eppendorf,
493 Hamburg, Germany). After adding 15 µl of proteinase K at 20 mg $ml^{-1}$ (Qiagen) and 40 µl of
494 20% sodium dodecyl sulfate (SDS), samples were incubated at 60ºC for 1 h. Then,
495 mechanical disruption was performed transferring the sample into a 2 ml safe-seal
496 microcentrifuge tube with 70 mg of acid-washed glass beads (Sigma) and using Tissulyser II
497 instrument (Qiagen) at frequency of 30 $s^{-1}$ for 10 min. Glass beads and cell debris were
498 removed by centrifugation at 12000xg 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 16000xg 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 -20ºC. DNA was collected by centrifugation at 16000xg for 30 min and the pellet was washed in ice-cold 70% ethanol, dried, resuspended in 50 µl of water, and stored at -20ºC until analysed. The amount and purity of DNA samples were determined spectrophotometrically (Nanodrop ND-1000 Spectrophotometer; Thermo Fisher Scientific, USA).

qPCR was performed, and two non-template controls (NTC) were included in all PCR runs: (i) using water instead of genomic DNA, and (ii) using DNA isolated from plant-material washings without PM411 cells. All reactions were performed in triplicate as described above. Ct 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 from the equation E (%) = ($10^{-1/slope}$ – 1) x 100.

**Viable quantitative PCR (v-qPCR)**

*PEMAX concentration optimization.* PEMAX reagent (GenIUL, Terrassa, Spain) was diluted in 500 µl of sterile bidistilled water to obtain a stock solution of 2000 µM that was stored at -20ºC 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 at 1 x $10^6$ CFU $ml^{-1}$ in apple flower washings. Dead cells were obtained by heating a cell suspension at 100ºC 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 30ºC. Then, samples were thoroughly mixed

524 and incubated for 30 min in the dark at room temperature in an orbital shaker KS501 digital
525 (IKA Labortechnik) at 130 rpm. Immediately, samples were photo-activated for 15 min with
526 the PhAST Blue photoactivation system (GenIUL, Barcelona) set to 100% and transferred
527 into DNA low binding 1.5 ml tubes (Sarstedt, Nümbrecht, Germany). PEMAX treated cells
528 (viable and dead) were collected by centrifugation at 13200xg for 10 min and washed with
529 sterile 50 mM sterile PBS (pH 7.0) under the same centrifugation conditions. Samples of
530 viable and dead cells, adjusted at 1 x $10^6$ CFU $ml^{-1}$ in apple flower washings, without being
531 treated with PEMAX were also used. DNA extraction of PEMAX and non-PEMAX treated
532 samples was performed as described. Each experimental condition was assayed in two
533 independent experiments.
534 *Amplicon length effect.* To study the influence of amplicon length in the effectiveness of the
535 PEMAX treatment to suppress PCR amplification of dead cells, two independent qPCR
536 assays (A and B) were performed. One unique forward primer (PM411-for) and probe
537 (PM411-pr), and two reverse primers (PM411A-, PM411B-rev) were used to obtain two
538 amplicons with different lengths (Table 2). qPCR was performed as described previously and
539 non-template control (NTC) was included in each PCR run. All reactions were performed in
540 triplicate.
541 The effect of PEMAX, at different concentrations, on DNA amplification suppression, by
542 qPCR assays A and B, was tested in viable and dead cells and expressed as "signal
543 reduction". Signal reduction was calculated by subtracting Ct values between non-PEMAX
544 and PEMAX-treated samples. Three independent experiments were performed.
545 *Standard curve.* To check the v-qPCR method as a specific bacterial detection and
546 quantification tool, the sensitivity and amplification efficiency of the v-qPCR B design was
547 evaluated by developing standard curves. Cell suspensions were prepared using viable, dead,
548 or mixtures of PM411 cells in apple flower washings. Samples were prepared from covering

549 a 5-log range (from 1 x $10^3$ to 1 x $10^8$ CFU $ml^{-1}$) of viable or dead cells obtained as described
550 and mixing the same concentration range of viable PM411 cells with a constant number of
551 dead cells (1 x $10^6$ CFU $ml^{-1}$). From each sample, 1 ml was treated with PEMAX at 50 µM
552 following the procedure described previously. An aliquot of each sample without being
553 treated with PEMAX were also used. DNA extraction was performed in PEMAX and non-
554 PEMAX treated samples as described. qPCR was performed using the B design (PM411-for,
555 PM411-pr, PM411B-rev), obtaining an amplicon size of 188 bp. qPCR was performed as
556 described previously, including the two negative controls (NTC) mentioned above. All
557 reactions were performed in triplicate. Standard curves were generated as described above.
558 Three independent experiments were carried out.
559
560 **Quantification of *L. plantarum* PM411 on plant material**
561 Greenhouse experiments were performed in different plant materials, such as apple blossoms
562 and pear, strawberry and kiwifruit leaves. Two field trials on apple and pear blooming trees at
563 the Mas Badia Agricultural Experimental Station (Girona, Spain) were also included.
564 Greenhouse experiments on leaves were conducted in potted plants (10-cm-diameter plastic
565 pots) of pear (cv. Conference), strawberry (cv. Darselect), and kiwifruit (cv. Hayward). Plants
566 were used when they had 30 to 40 cm in length, with 6 young leaves per shoot in pear plants,
567 10 to 15 young leaves in kiwifruit plants, and 5 to 8 leaves per crown in strawberry plants.
568 Open blossoms of Golden Smoothee apple cultivar were obtained from a commercial orchard
569 near Girona (Spain). Flowery branches were collected, transported to the greenhouse under
570 refrigeration, and the lower end of the branches maintained submerged in a 5% sucrose
571 solution. To inoculate PM411 the plant material was sprayed to runoff (10 ml per pear plant,
572 6 ml per strawberry plant, 20 ml per kiwifruit plant, 3 ml per open blossom) with the bacterial
573 suspension at $10^8$ CFU $ml^{-1}$. All plant material was kept at 25ºC, with 16 h of 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 that 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 (flowers assays) days. Two blossoms, three leaflets of strawberry plants, 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 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 (day 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 hand-held 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 (4–6 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 tenfold concentrated by centrifugation at 10000xg for 10 min of 20 ml of plant extract and resuspended in two ml of

sterile PBS and 0.1% peptone. The population level of PM411 on blossoms and leaves was 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, 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 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$, $R^2$ = 0.99 and E over 80%), and used in each plate run. The amount of total cells by qPCR was obtained by interpolating the Ct 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 following the procedure described above. DNA extraction, qPCR assay and quantification, using the corresponding standard curve with PEMAX treatment (linear range of 1 x $10^3$ to 1 x $10^7$ CFU $ml^{-1}$, $R^2$ = 0.99 and E over 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 Instrument, Barcelona, Spain) onto MRS agar plates supplemented with 50 µg $ml^{-1}$ of rifampicin (Sigma) to counter-select PM411 and 10 µg $ml^{-1}$ of econazole nitrate salt (Sigma) to avoid fungal growth. Plates were incubated at 30ºC 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 $log_{10}$ CFU per blossom or leaf.

**Statistical analysis**

623 To test the significance of the effect of PEMAX concentration and qPCR design in the
624 suppression of DNA amplification (signal reduction) on dead and viable cells of PM411 a
625 two-way analysis of variance (ANOVA) was performed. To test the effect of the
626 quantification method to estimate the PM411 population on plant surfaces for each sampling
627 date a one-way analysis of variance (ANOVA) was performed. Means of the ΔCt (signal
628 reduction) or CFU blossom$^{-1}$ or leaf$^{-1}$ (population) were separated according to the Tukey's
629 test at $P \leq 0.05$. The statistical analyses were performed using GLM procedure of the PC-
630 SAS (version 9.1; SAS Institute Inc., Cary, NC).
631
632 **Accession number**
633 The putative phage sequence of *L. plantarum* PM411 strain has been deposited in GenBank
634 database with the accession number MG788324.
635
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644
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## FIGURE CAPTIONS

**Figure 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*, coding a tail fiber and a hypothetical protein. Striped box shows a 90 bp region with 85% or less identity with 7 *L. plantarum*, coding a tail fiber. Dotted box shows a 162 bp region with 80% identity with *L. plantarum* HFC8, coding a hypothetical protein. White boxes show non-homologous sequences. The primers and TaqMan probe and the region corresponding to RAPD fragment are indicated.

**Figure 2.** Signal reduction (SR) in viable (black) or dead (grey) cell suspensions with different concentrations of PEMAX reagent (50, 75 and 100 µM). SR is the difference between cycle threshold value (ΔCt) of non-PEMAX and PEMAX-treated sample. TaqMan-based qPCR assays designated as A (92 bp) and B (188 bp) were carried out. Results are shown as means from three independent replicates and error bars represent standard deviation 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.

**Figure 3.** Cycle threshold (Ct) values obtained by TaqMan-based v-qPCR assay (B design) for a range of concentrations from 1 x $10^3$ to 1 x $10^7$ 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 x $10^6$ 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 >38 Ct values.

**Figure 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 (25ºC, high RH). Cells were sprayed onto the plant material at $10^8$ CFU $ml^{-1}$. The experiment was performed two times. Values are the mean of three replicates and error bars represent the standard deviation of the mean. *, indicates significant differences between qPCR and v-qPCR/pc; and #, between qPCR and pc, according to the Tukey test.

**Figure 5.** Population dynamics of *L. plantarum* PM411 estimated by qPCR (total) (○), v-qPCR (viable) (●), and plate counting (culturable) (□) on leaves of pear, strawberry, kiwifruit plants under controlled environment conditions (25ºC, 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 mean of three replicates and error bars represent the standard deviation of the mean. *, indicates significant differences between qPCR and v-qPCR/pc; #, between qPCR and pc; †, between qPCR, v-qPCR and pc; ¤ between qPCR/v-qPCR and pc according to the Tukey test.

**Figure 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. *, indicates significant differences between qPCR and v-qPCR/pc; #, between qPCR and pc; †, between qPCR, v-qPCR and pc; ¤ 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 trials.

929 **Table 1.** Bacterial strains used in this study

| Species | Code strain |
|---|---|
| **Lactic acid bacteria (LAB)** | |
| *Lactobacillus brevis* | CECT [a] 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 and 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 [d] 9211), ATCC [e] 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 [f] 6076 |
| *Escherichia coli* | ATCC 5954 |
| *Pantoea agglomerans* | EPS125 |
| *Pantoea vagans* | 7 EPS strains, C9-1 [g] |
| *Pseudomonas fluorescens* | 10 EPS [h] strains, EPS62e |
| *Pseudomonas syringae* | 7 EPS strains, EPS94 |
| *Staphylococcus aureus* | ATCC 9144 |
| *Xanthomonas axonopodis* pv. *vesicatoria* | 2133-2 [i] |

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

940 **Table 2.** Primers and TaqMan probes used for RAPD-PCR, rep-PCR and qPCR analysis

| Oligonucleotides | | Sequence (5'-3') | Amplicon length (bp) | Reference |
|---|---|---|---|---|
| **RAPD-PCR** | | | | |
| | P3 | CTGCTGGGAC | - | (68) |
| | P4 | CCGCAGCGTT | - | (69) |
| | P7 | AGCAGCGTGG | - | (69) |
| | M13 | GAGGGTGGCGGTTCT | - | (69) |
| | Inva1 | GTGAAATTATCGCCACGTTCGGCAA | - | (70) |
| | 512Fb | GATGCAGTCGACAATGTGGATGCT | - | (71) |
| | XD9 | GAAGTCGTCC | - | (72) |
| **rep-PCR** | | | | |
| ERIC [a] | ERIC1R | ATGTAAGCTCCTGGGGATTCAC | - | |
| | ERIC2 | AAGTAAGTGACTGGGGTGAGCG | | |
| REP [b] | REP-1R | IIIICGICGICATCIGGC | - | (73) |
| | REP-2 | ICGICTTATCIGGCCTAC | | |
| BOX [c] | BOXA1R | CTACGGCAAGGCGACGCTGACG | - | |
| $GTG_5$ [d] | $GTG_5$ | GTGGTGGTGGTGGTG | - | |
| **qPCR** | | | | |
| | PM411-for | AGATGCCAGCACTGGATTAAGC | | |
| | PM411-pr | FAM-TGCACGGCACAACTCAGGCGATT-TAMRA | | |
| | PM411A-rev | TTCATAGTAATCCCAGTGGTTTGG | 92 [e] | This work |
| | PM411B-rev | CCTTGTCGATACCAAAGTTAGCTATG | 188 [e] | |
| | PM411C-rev | CGGCGGCACCACCTT | 317 [e] | |

941 -, variable size

942 [a] Enterobacterial repetitive intergenic consensus sequence

943 [b] Repetitive Extragenic Palindromic sequence

944 [c] BOX sequence

945 [d] Polytrinucleotide $(GTG)_5$ sequence

946 [e] Amplification product obtained by qPCR using PM411-for primer, PM411-pr TaqMan
947 probe and the corresponding reverse primer (PM411A, B, or C).

948

949 **Table 3.** Amplification mixture and PCR conditions

950

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

951

952 [a] PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) and qPCR in a
953 7500 real-time PCR system (Applied Biosystem)
954 [b] Taq DNA Polymerase (Invitrogen)
955 [c] TaqMan™ Universal PCR Master Mix (Invitrogen)
956

Figure 1

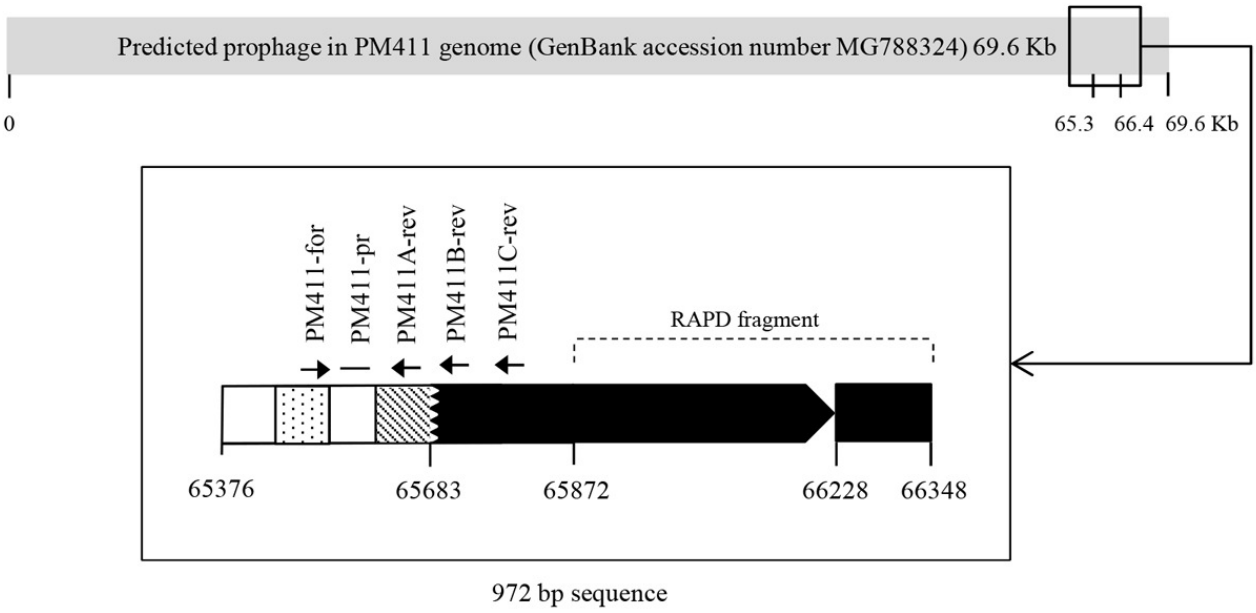

Figure 2

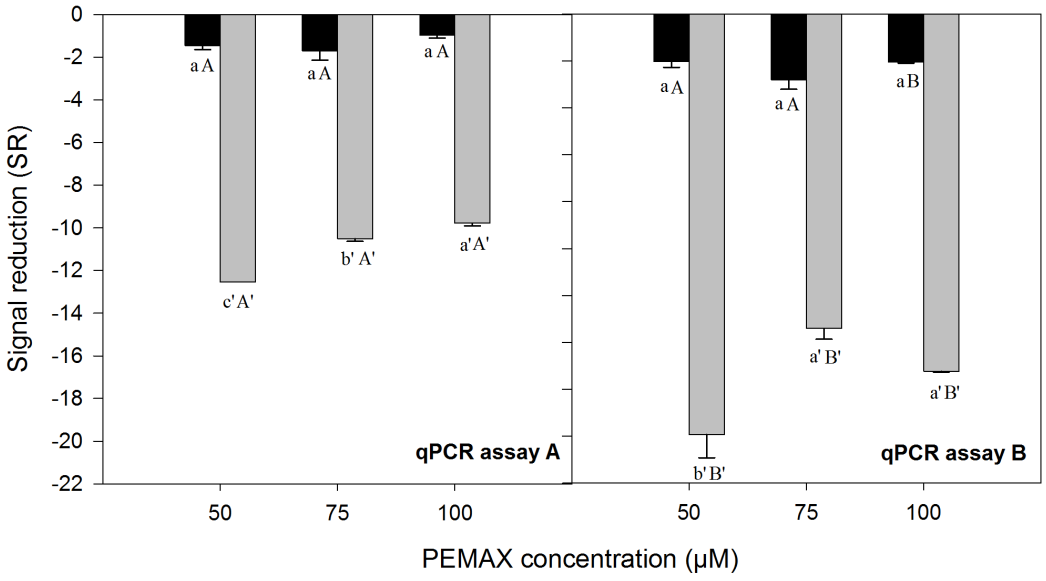

Figure 3

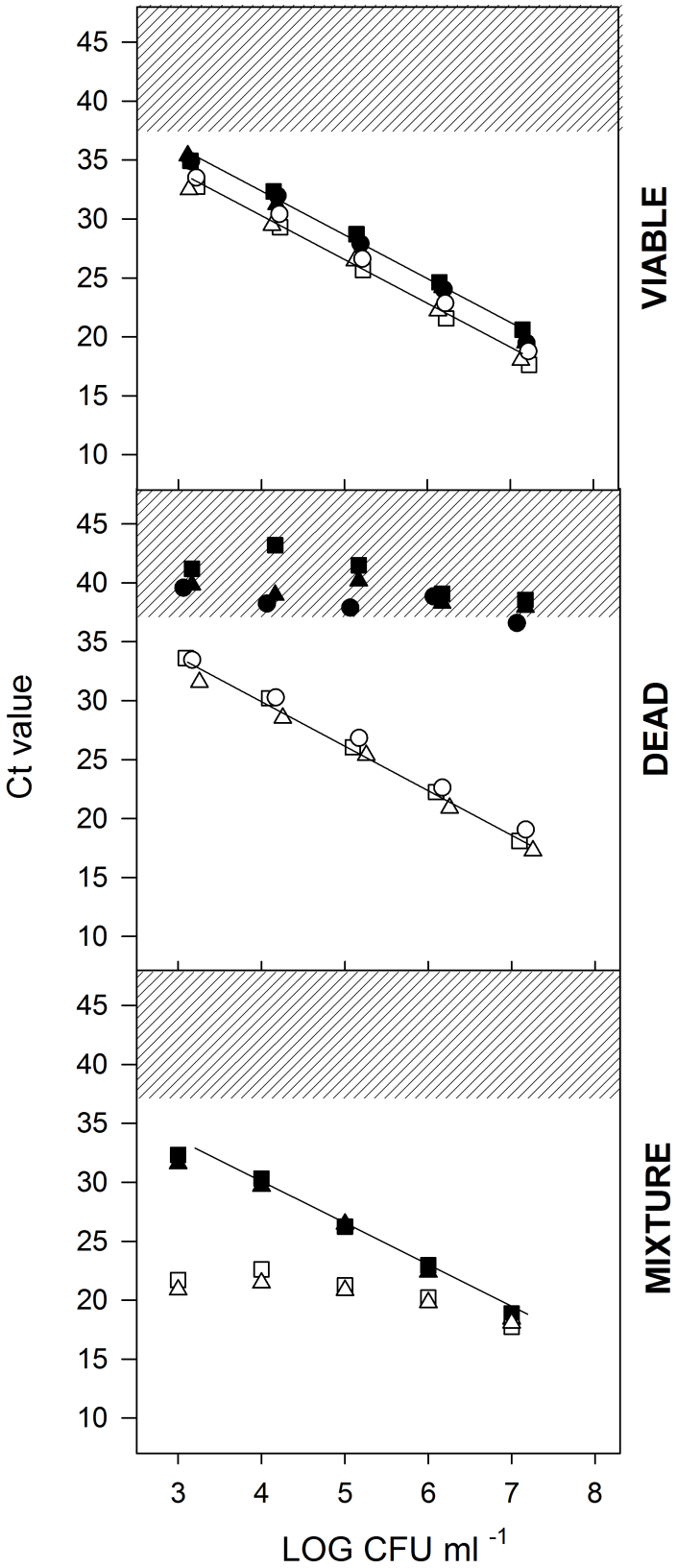

Figure 4

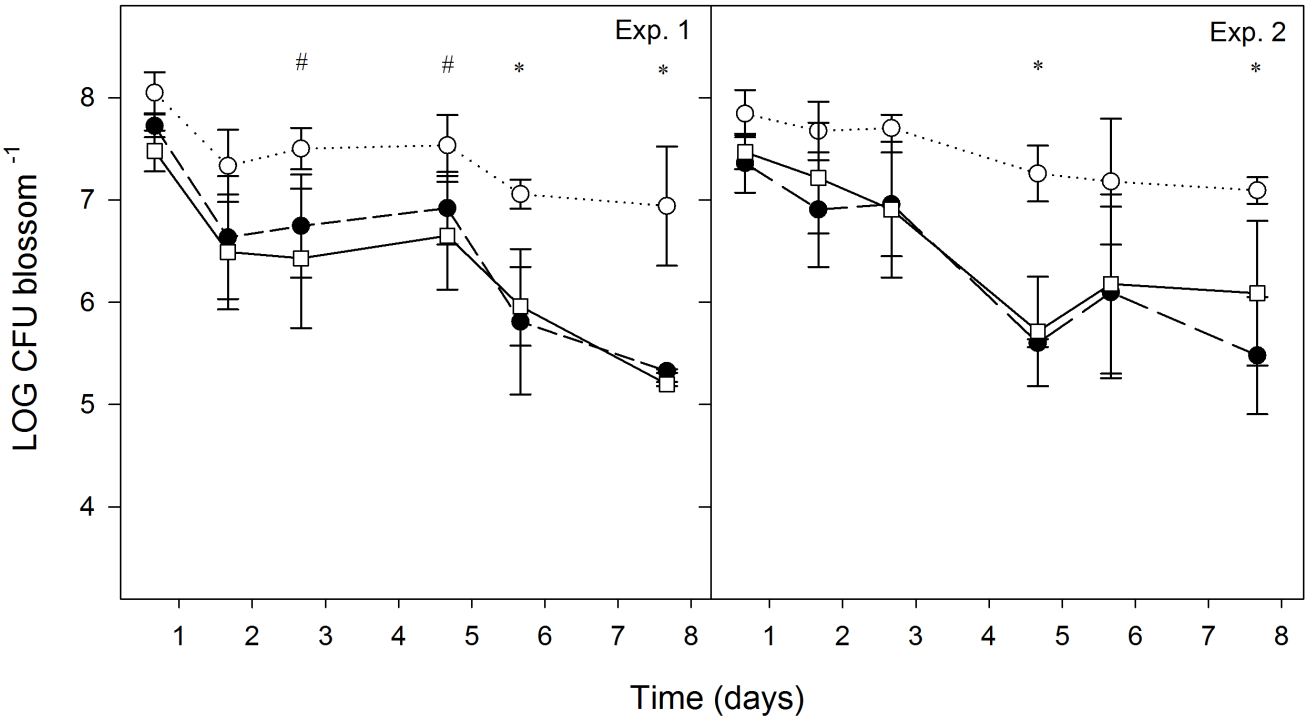

Figure 5

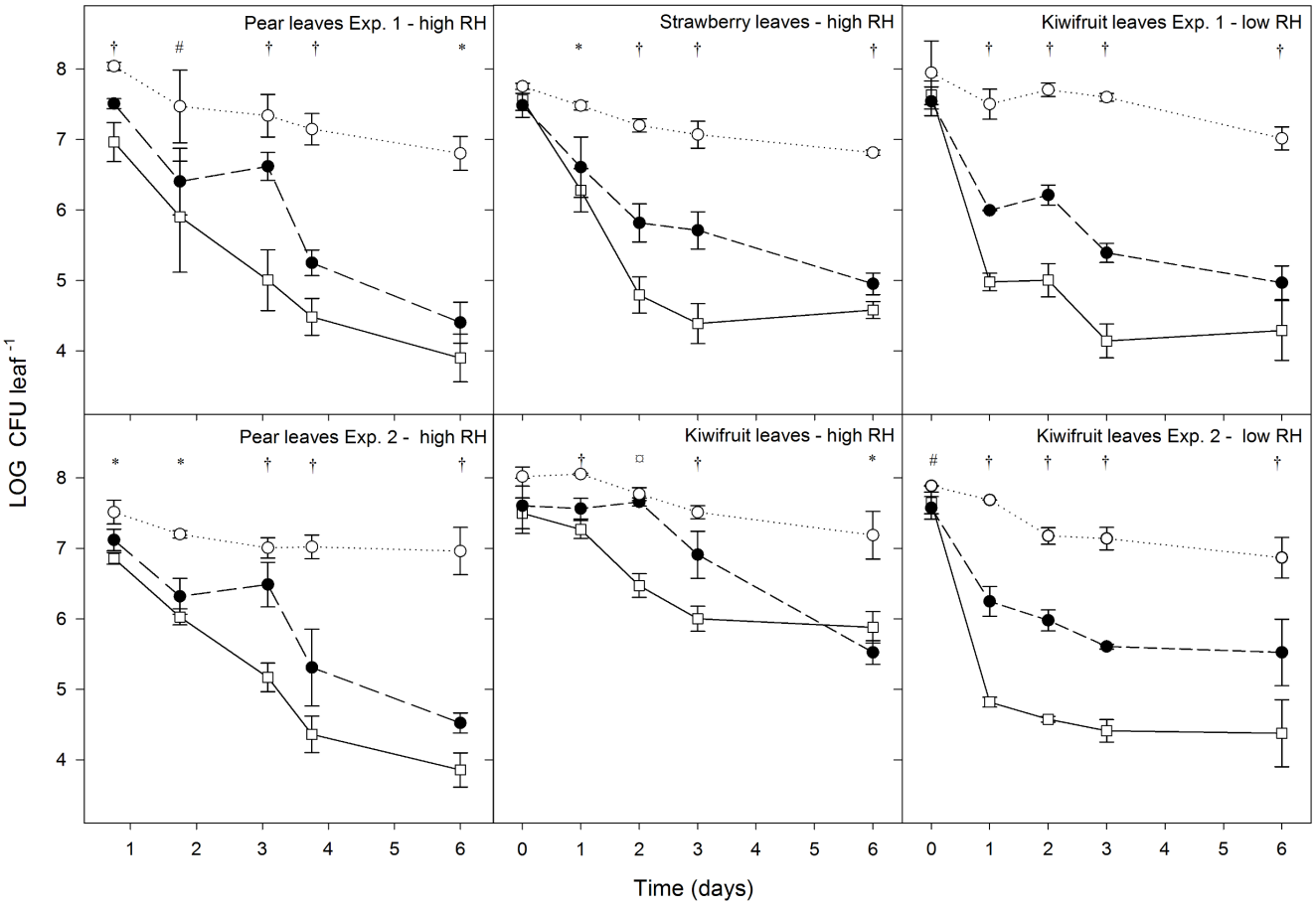

Figure 6