Inoculation of stigma colonizing microbes to apple stigmas alters microbiome structure and reduces the occurrence of fire blight disease

Running title: Flower microbiome manipulation on disease control

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Abstract:

Flowers secrete nutrient rich exudates that support the growth of an assemblage of microorganisms, including both beneficial and pathogenic members, most of which belong to the phylum Proteobacteria. Given the potential role of the microbiome in plant health, manipulating the microbiome to promote growth of beneficial members holds promise in controlling plant diseases. In this study we inoculated four different bacterial strains that were originally isolated from apple stigmas, alone or in mixtures of increasing complexity, onto apple flowers during bloom. We tested if such treatments would influence fire blight occurrence, a disease caused by *Erwinia amylovora,* and if we could detect a shift in the structure of the microbiome due to the treatments. We show that various inoculations did influence the occurrence of fire blight, although the level of disease suppression was dependent upon specific bacterial strains. Furthermore, treatments using different strains or strain mixtures predominantly resulted in increased representation of the inoculated strains, suggesting that disease suppression was due to an alteration of the stigma microbiome structure. Compared to treatments with single strains, a *Pantoea-Pseudomonas* strain mixture produced a homogeneous microbiome structure with less inter-flower variability. Findings from this study suggest the microbiome on the flower stigma can be manipulated through microbial inoculation. Due to flowers' short life span yet important role in plant disease infection, even a shot-term influence on microbiome composition may result in significant decreases in disease susceptibility.

Keywords: microbiome manipulation, flower, fire blight, stigma

Introduction:

Flowers play a critical role in lifecycles of Angiosperm plants by providing a platform for diverse pollination possibilities resulting in the union of sperm with ovules. During pollination, flowers produce nutrient rich exudates, including stigma exudates, pollen exudates, and nectar to promote germination of pollen grains and / or to attract pollinators (Crawford and Yanofsky, 2008). Such exudates also support the growth of many microorganisms, including both those that are beneficial or pathogenic to the host (Spinelli et al., 2005; Pusey et al., 2009; Aleklett et al., 2014).

Our recent studies characterized the composition and assembly of the microbiome on apple flowers (Steven et al., 2018), with a focus on the stigma (Cui et al., 2020). In general, the apple flower microbiome is dominated by bacteria in the phylum *Proteobacteria* (>90%), with small contributions by *Cyanobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Steven et al., 2018). Temporal patterns in the stigma microbiome development revealed a diverse bacterial community at the early stage of bloom evolving into a community dominated by two families within the phylum Proteobacteria, the *Pseudomonadaceae* and *Enterobacteriaceae* (Cui et al., 2020). This suggests members within the *Pseudomonadaceae* and *Enterobacteriaceae* are best-adapted for the stigma environment, and form the core members of the apple stigma microbiome (Pusey et al., 2009; Stockwell et al., 2010; Cui et al., 2020).

Fire blight, caused by a bacterial pathogen *Erwinia amylovora*, is an important disease of Rosaceous plants such as apples and pears (van der Zwet et al., 2012). One of the major entry points of *E. amylovora* to host plants is flowers, and successful infection of flowers is highly dependent on establishing an epiphytic pathogen population on

stigma portion of flowers during bloom (Thomson, 1986). Stigmatic colonization by *E. amylovora* is influenced by various biotic and abiotic factors, such as temperature, humidity, flower age and morphology (Steiner, 1990; Pusey, 2000b). To date, no sustainable and completely effective control methods have been developed for this disease, and control mainly relies on the use of copper formulates and the antibiotic streptomycin (Vanneste et al., 1995; Sundin et al., 2016). However, such practices are becoming increasingly limited due to concerns about the phytotoxicity caused by copper and the recent development and spread of the streptomycin resistance in *E. amylovora* populations (McGhee et al., 2011; Sundin et al., 2016). An alternative tool for plant disease management is biological control (biocontrol), the use of a beneficial microorganism to suppress plant disease (Wilson et al., 1992; Johnson and Stockwell, 1998; Bonaterra et al., 2007; Pusey et al., 2009; Sundin et al., 2009; Ait Bahadou et al., 2018). In order for these treatments to be effective, the inoculated microbe needs to colonize the host, in essence becoming a member of the microbiome. Yet, there exists a significant knowledge gap concerning how plant microbiomes are acquired, develop, and how ecological processes such as invasion occur.

There is evidence that microbiome composition can influence plant disease development (Berendsen et al., 2018; Purahong et al., 2018; Morella et al., 2019). A large number of plant pathogens, such as *Pseudomonas syringae*, *Erwinia amylovora*, and *Xanthomonas citri*, grow epiphytically on plant surface prior to causing infection in the plants (Pusey, 2000a; Brunings and Gabriel, 2003; Xin and He, 2013). By secreting carbon to the plant surface, plants recruit an assemblage of microbes to compete with the pathogens for nutrients and space, thereby acting as a first line of defense (Leben, 1965;

Weller et al., 2002). With regards to fire blight control, previous studies have investigated inoculating beneficial bacterial strains to open flowers as biological control (Sundin et al., 2009; Stockwell et al., 2010; Stockwell et al., 2011). Selection of such strains often initiated from an in vitro test for antibiosis, or on detached flowers, followed by field testing (Pusey, 1997; Johnson and Stockwell, 2000). Strains tested for disease control efficacy include *Pseudomonas fluorescens* A506, *Pantoea vagans* C9-1, and *Bacillus amyloliquefaciens* QST 713 (Chen et al., 2009; Stockwell et al., 2010; Pusey et al., 2011; Ait Bahadou et al., 2018; Mikiciński et al., 2020). Although these treatments have shown promising results in suppressing fire blight disease occurrence and reduced pathogen populations, the downstream effects on the composition of the microbiome have not been assessed.

In this study, we used the apple flower as model to test the hypothesis that manipulating the structure of the microbiome would be associated with changes in disease incidence. Furthermore, we predicted that more complex inoculations consisting of multiple bacterial strains would potentially outperform single species inoculations by forming a more diverse microbiome structure. Our results demonstrate that inoculating natural stigma-habitating bacteria during bloom altered the structure of the stigma microbiome and reduced fire blight disease incidence. However contrary to our hypothesis, more complex inoculations were not associated with better performance as a biocontrol. Instead, the presence of a specific bacterium, with no observed antagonistic activity, was responsible for decreasing fire blight. Taken together these data indicate the utility of microbiome manipulation as a tool to control fire blight while also shedding light on factors that control the assembly and composition of the flower microbiome.

106

107 **Results**

108 *Selection of strains for microbiome manipulation.*

109 A culture collection of 88 bacterial strains was obtained from the stigmas of apple
110 flowers cultivar 'Early Macoun' collected from Lockwood Farm, CT (41.406 N, 72.906
111 W). 16S rRNA gene sequencing placed the isolates in 3 phyla and 5 families (Fig. S1).
112 We chose four strains for testing their efficacy in suppressing fire blight disease (Table 1,
113 Fig. S1B). The rationale for choosing these four strains was as follows. *Pseudomonas* and
114 *Pantoea* as there is a history of using related strains in fire blight control (Stockwell et al.,
115 2010; Stockwell et al., 2011). *Enterobacter* was chosen because it was closely related to a
116 highly abundant OTU found in our previous work (Cui et al., 2020). We anticipated that
117 it might inhibit pathogen colonization by niche exclusion, by effectively monopolizing
118 the stigma environment. Finally, *Curtobacterium* was selected as an out-group. Our
119 previous data showed that bacteria within the phylum Proteobacteria generally accounted
120 for >90% of the microbial diversity of the stigma (Cui et al., 2020). We wanted to test if a
121 bacterium outside this phylum could colonize the stigma and observe the interaction with
122 disease development.

123 We also wanted to design more complex strain mixtures in order to test if
124 increasing the complexity of the inoculated strains would affect disease suppression. A
125 co-inoculation assay demonstrated that the strains tested did not antagonize each other
126 when cultured under an *in vitro* condition (Fig. S2). We employed *Pseudomonas* and
127 *Pantoea* as the base strains for the mixtures based upon the previous use of related strains
128 in fire blight biocontrol (Stockwell et al., 2010). The strain mixtures increased in

129 complexity from 2 to 4 strains with equal representation of each component strain (mix 1,
130 *Pseudomonas* and *Pantoea*; mix 2, *Pseudomonas*, *Pantoea,* and *Enterobacter*; mix 3,
131 *Pseudomonas*, *Pantoea, Enterobacter,* and *Curtobacteria*).
132
133 *Impact of microbiome manipulation on fire blight disease occurrence.*
134 The microbial inoculum consisted of overnight cultures diluted to a final density
135 of 4 x 10^7 colony forming units (CFU) ml^{-1}. Water was used as a negative control. The
136 seven inoculations, including four single strain treatments and three strain mixtures, were
137 spray-inoculated onto open flowers of apple trees twice during the period of bloom at the
138 point at which *ca.* 50% and 70% of flowers were observed to be open. To evaluate
139 whether the microbial treatments had any impact on the occurrence of fire blight disease,
140 we inoculated the pathogen *E. amylovora* to flowers four days after the initial microbial
141 treatments. The antibiotic streptomycin was used as a positive control treatment, and was
142 sprayed to flowers twice: 2 hours and 24 hours post pathogen inoculation. The percentage
143 of flowers that developed disease symptoms was assessed three weeks later. A total of
144 33.2% of water-treated and 11.7% of streptomycin-treated flowers developed fire blight
145 symptoms (Fig. 1). Compared to the water treatment, flowers that received a treatment of
146 any single or multiple strains showed a trend towards lower disease incidence, although
147 the difference was only significant for the *Pantoea* alone and the various strain mixtures
148 (Fig. 1). None of the treatments achieved the same disease reduction as the streptomycin
149 treatment, yet the *Pseudomonas*-*Pantoea*, and the four-strain mixture produced
150 statistically similar results (Fig. 1). Among the single microbe treatments, flowers treated
151 with *Pseudomonas* or *Pantoea* showed lower disease incidence than that of *Enterobacter*

and *Curtobacterium*, though the *Pseudomonas* treatment was not significantly different than the water control (Fig. 1). Compared to the single strain treatments, the strain mixtures did reduce disease incidence, however they were not significantly different than the *Pantoea* alone (Fig. 1). These data indicate that *Pantoea* may be the core contributor to the control of fire blight on apple flowers, although its activity may be partially enhanced with the addition of other flower microbiome members.

Microbiome composition after manipulation.

To determine whether the inoculated microbes influenced the stigma microbiome, we performed 16S rRNA gene sequencing to evaluate the microbiome composition of each individual flower sample three days after the second microbial treatment (right before pathogen inoculation). A total of 11,610,049 high-quality filtered sequences were obtained from 78 samples with the number of sequences ranging from 68,767 to 190,579 (Table S1). These sequences were clustered into 147,102 operational taxonomic units (OTUs, mean 2,823 per sample) at 100% sequence similarity.

The Bray-Curtis distances among samples of each microbial treatment were visualized using NMDS, which showed that samples of the same microbial treatment clustered together and separated from samples from other treatments (PERMANOVA, $P = 0.001$) (Fig. 2A). This result was confirmed by β-disper test ($P = 0.002$) and suggests that the inoculated microbes did indeed affect the microbiome structure. Samples of the control dataset (water-treated) showed a large dispersion, suggesting a high variation of microbiome structure among individual flowers under natural conditions (Fig. 2A). Among different single-strain treatments, higher level of variation in the microbiome

175 structure among individual flowers was observed in *Pseudomonas* (red) and
176 *Curtobacterium* (yellow) treatments than the *Pantoea* (green) and *Enterobacter*
177 treatments (light blue, Fig. 2A), suggesting the impact of microbial manipulation to the
178 microbiome structure is largely strain dependent. The structures of microbiome
179 manipulated by mixtures of three (dark blue) and four (purple) microbes largely
180 overlapped (Fig. 2A), suggesting addition of multiple strains drove the structure of the
181 microbiome to a similar composition. Notably, a consistent observation was that the
182 treatments that included the *Pantoea* strain showed tighter clustering between samples,
183 suggesting a smaller variability in microbiome structure between individual stigmas (Fig.
184 2A).

185 Diversity of the stigma communities was assessed by calculating the Shannon's
186 Diversity index. Samples of different single-strain treatments displayed various levels of
187 diversity, with the diversity in *Enterobacter* treated sample being the lowest in
188 comparison to others. In samples treated with double-, triple-, and quadruple-strain
189 mixtures, we observed an increase in microbial diversity as the number of strains
190 included in the treatment increased (Fig. 2B). Differences in diversity were further
191 supported by the similar patterns in community richness and evenness (Fig. 2B, lower
192 panels). These findings suggest that inoculation of different strains and strain mixtures to
193 flowers affected the flower microbiome resulting in different community structures and
194 diversity.

195

196 *Phylum level taxonomic bins and the Enterobacteriaceae and Pseudomonadaceae ratio*
197 *(*$R_{E/P}$*)*.

198 To investigate how the microbial inoculations affected the composition of the
199 stigma microbiome, 16S rRNA gene sequences were classified to the phylum level.
200 Twenty-four phyla were detected across the different treatments. Excluding the
201 *Curtobacterium* treatment, samples were composed predominantly of *Proteobacteria*
202 (94.1-99.9%) with a small percentage of *Actinobacteria* (0.1-5.9%). This included the
203 water control, with no bacterial treatment (Fig. 3A). In the samples manipulated with
204 *Curtobacterium*, a member of the *Actinobacteria*, the percentage of *Proteobacteria* was
205 reduced to a mean of 53.8% (Fig 3A). Looking at the ratio of *Actinobacteria* sequences in
206 each of the flowers treated with *Curtobacterium,* a wide variation between flowers was
207 observed, with *Proteobacteria* sequences still being dominant in several of the individual
208 samples (Fig. 3B). Moreover, samples manipulated with the four-microbe mixture also
209 exhibited a slight decrease in percentage of *Proteobacteria*, which could be explained by
210 the colonization of *Curtobacterium* (Fig. 3A). These findings indicated that although
211 *Curtobacterium* is not typically an abundant core member of the stigma microbiome, it is
212 still able to predominate on stigmas when given a competitive advantage by artificial
213 inoculation.
214 We have previously shown that two families of bacteria tend to predominate on
215 the stigma, the *Enteorbacteriaceae* (*En*) and the *Pseudomonadaceae* (*Ps*) (Steven et al.,
216 2018; Cui et al., 2020). We determined the ratio of *En* to *Ps* ($R_{E/P}$) of individual flowers
217 across the treatments. The $R_{E/P}$ was reshaped by the various microbiome manipulations.
218 The $R_{E/P}$ of water treated dataset varied significantly (243%) among the samples (Fig.
219 3C). Some microbial treatments still displayed high variations in the $R_{E/P}$ (such as the
220 single strain treatments of *Pseudomonas* and *Curtobacterium*), yet many of other

treatments tended to stabilize the $R_{E/P}$ between individuals as observed in single strain treatments of *Pantoea* and *Enterobacter*, and all strain mixture treatments (Fig. 3C). The effect was most pronounced in the single strain treatments of *Pantoea* and *Enterobacter*, which became almost exclusively dominated by the *En* to the exclusion of the *Ps* (Fig. 3C). Interestingly, the *Pseudomonas* treatment did not result in a similar exclusion of the *En,* suggesting that *Pseudomonas* is not as adept at monopolizing the stigma environment. Similarly, the multiple strain mixtures that included *Pseudomonas* consistently showed a higher ratio of *En*, suggesting that *Enterobacteriaceae* consistently outcompetes the *Pseudomonadaceae,* and achieves higher relative abundances.

Identification of the inoculated strains in the microbiome

The sequences in each dataset were classified to the OTU level (100% sequence identity) and representative sequences for each OTU were interrogated against the isolate sequences. Four OTUs were identified that shared 100% sequence identity to the isolates, OTU1-*Enterobacter*, OTU2-*Pseudomonas,* OTU3-*Pantoea*, and OTU4-*Curtobacterium.* The relative abundance of each OTU was employed as a metric to determine the isolate's abundance in the stigma microbiome. OTU1 was the most abundant and conserved in the dataset (relative abundance ranging from 0.6% to 94.2% of recovered sequences), followed by OTU2 (0-94.9%), OTU3 (0-72.2%) and OTU4 (0-92.1%). In the water-treated control samples, the majority of OTUs were accounted for by OTU1 (mean 62.2%) and OTU2 (mean 6.7%) with a large abundance of other OTUs (rare, 31.1%; Fig. 4). OTU abundance was generally predictable from the treatments, in that the inoculated OTUs were enriched in the treatment datasets (Fig. 4). There were two notable

exceptions. OTU1-*Enterobacter* was highly abundant whether or not it was included in the inoculation. OTU1 showed the lowest abundance when it was co-inoculated with *Pseudomonas*. However, including other bacteria in the inoculation once again allowed OTU1 to dominate the stigma microbial community. The second exception to an inoculated strain gaining an advantage in the microbiome was OTU3-*Pantoea*. When inoculated alone it achieved a mean relative abundance of 42.0%. Yet, when co-inoculated with *Pseudomonas* only accounted for 0.04% of sequences. Yet, the mean relative abundance of *Pseudomonas* in the *Pseudomonas*-*Pantoea* mixture (9.6%) was significantly lower than when *Pseudomonas* was sprayed alone (75.4%). Moreover, the co-inoculation of *Pseudomonas* and *Pantoea* together resulted in a relative abundance of OTU1-*Enterobacter* similar to when *Enterobacter* was inoculated alone. This apparent paradox of co-inoculating *Pseudomonas* and *Pantoea* benefitting the non-inoculated *Enterobacter* may point to a competition between two organisms opening a niche for a third. Taken together, these observations point to complex interactions in the plant microbiome that underpin our need to characterize the functional ecology of this system, and documenting the cooperative and antagonistic relationships between community members.

In vitro antagonistic test of selected strains against E. amylovora

The selection criteria of the strains tested in this study were not the biocontrol activities but rather their representation of different members of the stigma microbiome. Our final goal was to determine the potential mechanisms of disease suppression by the inoculated strains in an *in vitro assay.* Of all four strains tested, only *Pseudomonas*

showed inhibition against the growth of *E. amylovora* on the Lysogen Broth (LB) agar medium (Fig. 5). Interestingly, despite the antagonistic activity towards *E. amylovora,* the *Pseudomonas* strain did not show the highest disease inhibition (Fig 5). Instead, the *Pantoea* strain, alone or in a strain mixture, showed the greatest disease suppression (Fig. 5). One potential explanation for the reduced disease incidence could be attributable to niche exclusion of *E. amylovora* on apple stigmas, which is consistent with previous observations (Giddens et al., 2003). Yet, when the *Pantoea* was mixed with *Pseudomonas*, disease suppression was similar to *Pantoea* alone, or in the other strain mixtures (Fig. 5), but the relative abundance of *Pantoea* dropped to >1% of recovered 16S rRNA sequences (Fig. 4). This suggests that the presence of *Pantoea* is still potentially inhibitory to fire blight development even when present in low relative abundance. Thus, these data suggest that *Pantoea* antagonistic activities were not apparent on LB media and would not have been predicted from the relative abundance of *Pantoea*-related sequences (particularly in the *Pseudomonas*-*Pantoea* mixture). These data highlight the importance of monitoring the microbiome *in situ* to assess the complex interactions that occur between members and how they may interact with outcomes such as disease suppression.

Discussion

In this study, we demonstrated that the microbiome structure can be altered by artificial inoculation, which resulted in attenuated disease occurrence. Many trials in manipulation of the microbiome with the intention to influence host-microbe interactions, suppress diseases, or to promote host-beneficial traits have been attempted (Brugman et

al., 2018). The outcomes of such efforts vary significantly, and are highly influenced by the host environment, nutrient stage and the manipulation approaches (Berg and Koskella, 2018; Brugman et al., 2018). Unlike many host environments, flowers are nutrient rich but also directly exposed to the natural environment, providing an easy access for manipulation. Compared to some environments that require a long term, steadfast microbiome intervention to produce beneficial effects such as root microbiome (Bakker et al., 2013), flowers have a shorter window of bloom, thus the microbiome manipulation could be short-term, yet the influence to disease control and plant health could still be significant.

Taxonomic diversity is an important measure of microbiome structure and function (Trivedi et al., 2012; Hu et al., 2016). Our data suggests that the diversity of the microbial community on the apple stigma is not directly correlated with the occurrence of fire blight. For example, similar taxonomic diversity was observed on flowers treated with *Enterobacter* and a mixture of *Pseudomonas* and *Pantoea,* although the disease occurrence of such treatments differed (Fig. 1 & 2B). Furthermore, among treatments of double-, triple-, and quadruple-strain mixtures, although the diversity gradually increased in accordance with the increasing number of strains, no significant difference in their disease protection was observed (Fig. 1 & 2B, Fig. S3). In other plant hosts, such as tomato, the level of disease protection ability of a constructed bacterial community was also found to be independent of overall bacterial diversity (Berg and Koskella, 2018). Thus, the hypothesis that a more complex microbial inoculation would result in better disease suppression was not supported. Here, our data point to a trend that treatments with *Pantoea* generally lead to better disease suppression than treatments that didn't.

The natural flowers harbored varied microbiome communities resulting in unique structures. The largest effect of the microbial treatments on the microbiome was a reduction in the inter-individual variability, with the exception of treatments with *Pseudomonas* and *Curtobacterium*. Particularly, microbial treatments using *Pseudomonas-Pantoea* based double-, triple-, and quadruple-strain mixtures resulted in the smaller distances between samples in the NMDS clustering (Fig 2A) and the similarity in the $R_{En/Ps}$ between replicate flowers (Fig. 3C). This is important as it suggests that there should also be more uniformity in any phenotypic effects induced by the microbiome. A common observation is that only a proportion of flowers sprayed with *E. amylovora* in the field will later go on to develop fire blight (Sundin et al., 2009; Cui et al., 2020). Similarly, many biocontrol strains have been described with excellent antagonistic potential under *in vitro* or *in planta* conditions, but present inconsistent effectiveness and year-to-year variation under field conditions (Johnson and Stockwell, 1998; Sundin et al., 2009). One potential reason for the variability observed at the individual flower level and for inter-annual irregularity could be caused by the large inter-individual disparity in microbiome structure between flowers. Thus, by shifting the microbiome to a state that is predictable, repeatable, stable, and that also induces disease resistance, we may be able to manage such variations. While none of the manipulations employed here attained the same disease reduction as the streptomycin treatment, they did produce a highly conserved microbiome structure (Fig. 4) and up to a ~45% decrease fire blight (Fig. 1). This suggests that a microbiome informed treatment strategy may be a viable path to controlling plant diseases.

335 Recent work trying to predict community assembly from first principals found
336 that complex communities seeded on a glucose minimal medium predictably adopted a
337 state dominated by members of the *Enterobacteriaceae* and *Pseudomonadaceae* in a
338 phenomenon termed "emergent simplicity" (Goldford et al., 2018). The predictability of
339 this assembly points to certain metabolic rules, based on cross-feeding, determining the
340 final state of the microbiome (Turner et al., 1996; Treves et al., 1998; Estrela et al., 2020).
341 Here we observe a similar phenomenon in a natural environment. The ratio of families
342 and the particular members of the microbiome can be seeded early in the development of
343 the stigma microbiome, leading to a predictable community structure. Untangling the
344 metabolic interactions between community members will better inform mechanisms by
345 which the microbiome can be shifted to potentially beneficial states.

346 One limitation of this study is some LB broth ingredients were sprayed to flowers
347 during the microbial treatments, as the overnight culture of the bacterial inoculum was
348 directly diluted in water prior to application on flowers. This limitation is caused by the
349 need of large volume of inoculum for field application and the unavailability of a large
350 centrifuge that can process litters of bacterial culture to remove the media ingredients.
351 The dilution factor for the bacterial cultures was approximately 25-fold and the media
352 content was largely depleted after bacterial growth, thus the amount of nutrients
353 remaining in the bacterial inoculum was likely minimal. However, the leftover nutrients
354 and potential secondary metabolites were not present in the water controls so could
355 account for some of the observed differences. Thus, during the analysis of the
356 microbiome data, we only directly compared the different microbial treatments. The

357 microbiome structure, composition, and diversity in water controls were provided as a
358 reference for the development of the microbiome in the absence of manipulation.

359 In summary, our data points to a common state of the stigma microbiome being
360 dominated by bacteria in two families, the *Enterobacteriaceae* and *Pseudomonadaceae*.
361 However, a probiotic spray in the field can determine the particular members that make
362 up this state. Treating flowers with certain microbial members, particularly a strain of
363 *Pantoea* was associated with disease suppression. Multi-species inoculations showed that
364 there are complex interactions between the treatment strains, but there is some evidence
365 that a more complex inoculation can enhance disease resistance. These data will inform
366 both treatments for fire blight as well as well as informing the ecological principals and
367 interactions that determine the structure and function of the flower microbiome.

368 .

369 **Materials and Methods**

370 ***Sampling site***

371 To exclude the effects of host and environmental conditions, studies were
372 conducted with flowers from 27 trees of the same apple cultivar 'Golden smoothie'
373 (*Malus domestica*) on MM104 rootstock planted in two adjacent blocks at Lockwood
374 Farm, Hamden, Connecticut, 41.406 N 72.906 W) in a 16' X 21' spacing. All trees were
375 the same age (planted in 1970) and were maintained under the same management
376 program (https://ag.umass.edu/fruit/publications/2020-new-england-tree-fruit-
377 management-guide). The trees were used in fire blight research in previous years but
378 were subject to extensive pruning to remove the overwinter inoculum prior to the
379 flowering season. During bloom, approximately 300 flowers were produced on each tree.

Weather data (temperature and humidity) prior to and during bloom (from May 1st to May 30th 2019) is summarized in Table S2.

Isolation of stigma colonizing bacteria

Stigma colonizing bacteria were isolated from stigma portion of 'Macoun' apple flowers at full bloom on May 8th 2018. The stigma portion of an single apple flower was dissected and placed in a sterile 1.5 ml microcentrifuge tube containing 200 µl of 0.5 x phosphate-buffered saline (PBS). A total of 100 single flowers from 6 trees were sampled. The microcentrifuge tubes containing stigma were placed in a water bath sonicator for 5 minutes followed by 30 seconds of vortexing. Five microliters of the PBS from each tube was spread on LB agar plates containing cycloheximide (50 µg ml^{-1}) to inhibit fungal growth. Single colonies of different morphologies from each plate were re-streaked onto a fresh LB agar plate to obtain pure cultures. More than 1000 bacterial isolates were obtained. The 16S rRNA of the isolates was PCR amplified using 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) and sequenced by Sanger sequencing. The 88 strains described in this study is a representative subset based on strains' colony morphology.

Bacterial treatments of open flowers

Bacterial strains of *Pseudomonas* spp. strain CT-1059, *Pantoea* spp. strain CT-1039, *Enterobacter* spp. strain CT-1341 and *Curtobacterium* spp. strain CT-1342 were stored at -80°C in 20% glycerol. All strains were cultured in LB medium at 28°C overnight and adjusted OD600 = 1.0 (approximately 10^9 CFU/ml). The 27 apple trees were randomly labeled into nine treatment groups, with three replicate trees in each treatment group. Each treatment group received with one of the nine treatments below: 1.

Pseudomonas, 2. *Pantoea*, 3. *Enterobacter*, 4. *Curtobacterium*, 5. *Pseudomonas-Pantoea*, 6. *Pseudomonas-Pantoea-Enterobacter*, 7. *Pseudomonas-Pantoea-Enterobacter-Curtobacterium,* 8. Water and 9. Streptomycin. Microbial treatments, regardless of single strain or strain mixtures, contains equal amount of the total bacteria (4×10^7 CFU/ml). Six hundred milliliters of overnight bacterial culture (adjusting OD600 =1.0) directly diluted into 14.2 L of water and used for spray. Equal amounts of ingredient bacteria were mixed together at the site of application. Water was used as negative control.

Microbial and water treatments were applied twice, once at 50% bloom (May 8th, 2019) and again at 70% bloom (May 9th). Approximately 3.8 L of bacterial suspensions at the final concentration of 4×10^7 CFU/ml were applied to each tree. *E. amylovora* was inoculated onto the flowers by spraying a 1×10^6 CFU/ml bacterial suspension on May 12th (100% bloom), immediately after sampling of stigma microbiome. Antibiotics streptomycin (100 ppm) was applied on the same day (May 12th), 2 hours later after *E. amylovora* inoculation, and again at 24 hours after the first inoculation.

Sampling for the stigma microbiome and quantification of disease incidence

Sampling of stigma microbiomes was performed on May 12th 2019 at 100% bloom, three days after the second microbial treatments. Ten flowers were collected from a labeled branch of each treated tree, from which the unopened flowers were removed prior to microbial treatment to ensure all flowers received the inoculum. The stigma portion of each individual flower was harvested with sterile scissors and placed in a sterile 1.5 ml microcentrifuge tube. Collected samples were kept on ice during transportation and were stored at -80°C until DNA isolation.

Three weeks after pathogen inoculation (May 30th), inoculated flowers were rated for the development of blossom blight symptoms (black withering, dying of the apple flowers, and emergence of ooze droplets). Percentage of diseased flower clusters in the total number of flower clusters was determined for each tree (Johnson and Temple, 2013). For each individual tree, disease symptoms were rated on more than 200 flower clusters by three individual people.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from the surface of stigmas collected from each individual flower. Stigmas were submerged in 200 µl of 0.5x PBS buffer in a microcentrifuge tube. Epiphytic microbes were removed from stigma surface by water bath sonication for 5 mins and followed by vortex for 30 seconds. Stigmas were removed from the tube and the DNA was extracted from the solution using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The isolated DNA in 100 µl of water was further concentrated by precipitating in 100% ethanol. Isolated DNA was used as template in a PCR reaction to amplify the V4 region of the bacterial 16S rRNA gene using the 515f/806r primer set with both primers containing a 6-bp barcode unique to each sample (Sinclair et al., 2015). The amount of template DNA added in the PCR reaction (25 µl) ranged from 10.0 ng to 20.0 ng as determined by Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA). Peptide nucleic acid (PNA) clamps to block the PCR amplification of apple plastid and mitochondrial sequences were included in the reaction as previously described in Steven et al. (2018) (Steven et al., 2018). Successful PCR amplifications at the correct amplicon size were confirmed by gel electrophoresis.

449 The PCR products were purified and normalized using the SequalPrep normalization
450 plate kit (Invitrogen, CA, USA). Pyrosequencing was conducted on an Illumina MiSeq
451 platform through services provided by the Yale Center for Genome Analysis (YCGA).
452
453 ***Bioinformatics and statistical analysis***
454 Illumina sequencing reads were assembled into contigs and quality screened using
455 mothur v1.39.5 (Schloss et al., 2009). Sequences that were at least 253 bp in length,
456 contained no ambiguous bases, and no homopolymers of more than 8 bp were used in the
457 analysis. Chimeric sequences were identified by using the VSEARCH (Rognes et al.,
458 2016) as implemented in mothur, and all potentially chimeric sequences were removed.
459 To maintain a similar sampling effort between samples, samples with less than 10,000
460 sequences per sample were also removed. The resulting sequence counts per sample are
461 presented in Table S1. Negative control (PCR using sterile H_2O as a template) was also
462 included in sequence datasets. The sequences data are deposited at the Sequence Read
463 Archive under accession number PRJNA628946.
464 Sampling effort was normalized to the depth of the smallest sample and
465 operational taxonomic units (OTUs) were defined at 100% sequence identity, employing
466 the OptiClust algorithm in mothur (Westcott and Schloss, 2017). Taxonomic
467 classification of sequences was performed with the Ribosomal Database Project (RDP)
468 classifier against the SILVA v132 reference alignment in mothur (Wang et al., 2007;
469 Quast et al., 2012). Non-metric multidimensional scaling (NMDS) was used to visualize
470 the pairwise distances among samples with Bray-Curtis distances in the Vegan package
471 in R (Dixon, 2003). Descriptive diversity statistics were calculated in mothur. The non-

parametric Shannon's Diversity Index and disease incidence in each treatment were generated with the ggplot2.0 package for R (Wickham, 2016). Statistically significant differences in diversity statistics were identified with a one-way ANOVA and Tukey-Kramer post hoc test in the agricolae package in R.

In vitro co-inoculation assay and antagonistic assay

Overnight LB cultures of *Pseudomonas* spp. strain CT-1059, *Pantoea* spp. strain CT-1039, *Enterobacter* spp. strain CT-1341 and *Curtobacterium* spp. strain CT-1342 were adjusted OD600 = 1.0 (approximately 10^8 CFU/ml). A narrow line of each strain inoculum was streak both on horizontal and perpendicular orientation on LB agar plate using an inoculum loop. For antagonistic assay, LB agar plate so that a tested strain, inoculated in the center of the plate. After 48 hours post-incubation, *E. amylovora* 110 were stamped onto the plates using a prong dipped in bacterial suspension (approximately 10^2 CFU/ml). Each treatment had 3 biological replicates and the experiment was repeated twice.

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgments

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Reference:

Ait Bahadou, S., Ouijja, A., Karfach, A., Tahiri, A., and Lahlali, R. 2018. New potential bacterial antagonists for the biocontrol of fire blight disease (Erwinia amylovora) in Morocco. Microb Pathog 117:7-15.

Aleklett, K., Hart, M., and Shade, A. 2014. The microbial ecology of flowers: an emerging frontier in phyllosphere research. Botany 92:253-266.

Bakker, P.A., Berendsen, R.L., Doornbos, R.F., Wintermans, P.C., and Pieterse, C.M. 2013. The rhizosphere revisited: root microbiomics. Front Plant Sci 4:165.

Berendsen, R.L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W.P., Burmølle, M., Herschend, J., Bakker, P.A., and Pieterse, C.M. 2018. Disease-induced assemblage of a plant-beneficial bacterial consortium. The ISME journal 12:1496-1507.

Berg, M., and Koskella, B. 2018. Nutrient- and Dose-Dependent Microbiome-Mediated Protection against a Plant Pathogen. Curr Biol 28:2487-2492 e2483.

Bonaterra, A., Cabrefiga, J., Camps, J., and Montesinos, E. 2007. Increasing survival and efficacy of a bacterial biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. FEMS Microbiol Ecol 61:185-195.

Brugman, S., Ikeda-Ohtsubo, W., Braber, S., Folkerts, G., Pieterse, C.M.J., and Bakker, P. 2018. A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research. Front Nutr 5:80.

Brunings, A.M., and Gabriel, D.W. 2003. Xanthomonas citri: breaking the surface. Molecular plant pathology 4:141-157.

Chen, X.-H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., and Borriss, R. 2009. Difficidin and bacilysin produced by plant-associated Bacillus amyloliquefaciens are efficient in controlling fire blight disease. Journal of biotechnology 140:38-44.

Crawford, B.C., and Yanofsky, M.F. 2008. The formation and function of the female reproductive tract in flowering plants. Curr Biol 18:R972-978.

Cui, Z., Huntley, R., Zeng, Q., and Steven, B. 2020. Temporal and spatial dynamics in the apple flower microbiome in the presence of the phytopathogen Erwinia amylovora. ISME J https://doi.org/10.1038/s41396-020-00784-y.

Dixon, P. 2003. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14:927-930.

Estrela, S., Vila, J.C.C., Lu, N., Bajic, D., Rebolleda-Gomez, M., Chang, C.-Y., and Sanchez, A. 2020. Metabolic rules of microbial community assembly. Biorxiv https://doi.org/10.1101/2020.03.09.984278.

Giddens, S.R., Houliston, G.J., and 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. Environ Microbiol 5:1016-1021.

Goldford, J.E., Lu, N., Bajic, D., Estrela, S., Tikhonov, M., Sanchez-Gorostiaga, A., Segre, D., Mehta, P., and Sanchez, A. 2018. Emergent simplicity in microbial community assembly. Science 361:469-474.

Hu, J., Wei, Z., Friman, V.P., Gu, S.H., Wang, X.F., Eisenhauer, N., Yang, T.J., Ma, J., Shen, Q.R., Xu, Y.C., and Jousset, A. 2016. Probiotic Diversity Enhances Rhizosphere Microbiome Function and Plant Disease Suppression. mBio 7.

Johnson, K.B., and Stockwell, V.O. 1998. Management of fire blight: a case study in microbial ecology. Annu Rev Phytopathol 36:227-248.

Johnson, K.B., and Stockwell, V.O. 2000. Biological control of fire blight. in: Fire Blight, the Disease and its Causal Agent, Erwinia amylovora (ed. J.L. Vanneste), CAB International.

Johnson, K.B., and Temple, T.N. 2013. Evaluation of strategies for fire blight control in organic pome fruit without antibiotics. Plant Disease 97:402-409.

Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annual Review of Phytopathology 3:209-230.

McGhee, G.C., Guasco, J., Bellomo, L.M., Blumer-Schuette, S.E., Shane, W.W., Irish-Brown, A., and Sundin, G.W. 2011. Genetic analysis of streptomycin-resistant (Sm(R)) strains of Erwinia amylovora suggests that dissemination of two genotypes is responsible for the current distribution of Sm(R) E. amylovora in Michigan. Phytopathology 101:182-191.

Mikiciński, A., Puławska, J., Molzhigitova, A., and Sobiczewski, P. 2020. Bacterial species recognized for the first time for its biocontrol activity against fire blight (Erwinia amylovora). European Journal of Plant Pathology 156:257–272.

Morella, N.M., Zhang, X., and Koskella, B. 2019. Tomato seed-associated bacteria confer protection of seedlings against foliar speck caused by Pseudomonas syringae. The Phytobiomes Journal 3:177-190.

Purahong, W., Orrù, L., Donati, I., Perpetuini, G., Cellini, A., Lamontanara, A., Michelotti, V., Tacconi, G., and Spinelli, F. 2018. Plant microbiome and its link to plant health: Host species, organs and Pseudomonas syringae pv. actinidiae infection shaping bacterial phyllosphere communities of kiwifruit plants. Frontiers in Plant Science 9:1563.

Pusey, P. 2000a. The role of water in epiphytic colonization and infection of pomaceous flowers by Erwinia amylovora. Phytopathology 90:1352-1357.

Pusey, P., Stockwell, V., Reardon, C., Smits, T., and Duffy, B. 2011. Antibiosis activity of Pantoea agglomerans biocontrol strain E325 against Erwinia amylovora on apple flower stigmas. Phytopathology 101:1234-1241.

Pusey, P.L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology 87:1096-1102.

Pusey, P.L. 2000b. The role of water in epiphytic colonization and infection of pomaceous flowers by Erwinia amylovora. Phytopathology 90:1352-1357.

Pusey, P.L., Stockwell, V.O., and Mazzola, M. 2009. Epiphytic bacteria and yeasts on apple blossoms and their potential as antagonists of Erwinia amylovora. Phytopathology 99:571-581.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F.O. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research 41:D590-D596.

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584.

582 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A.,
583 Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn,
584 D.J., and Weber, C.F. 2009. Introducing mothur: open-source, platform-independent,
585 community-supported software for describing and comparing microbial communities.
586 Appl Environ Microbiol 75:7537-7541.
587 Sinclair, L., Osman, O.A., Bertilsson, S., and Eiler, A. 2015. Microbial community composition and
588 diversity via 16S rRNA gene amplicons: evaluating the illumina platform. PloS one
589 10:e0116955.
590 Spinelli, F., Ciampolini, F., Cresti, M., Geider, K., and Costa, G. 2005. Influence of stigmatic
591 morphology on flower colonization by Erwinia amylovora and Pantoea agglomerans. Eu.
592 J. Plant Pathol. 113:395-405.
593 Steiner, P.W. 1990. Predicting apple blossom infections by Erwinia amylovora using the
594 MARYBLYT model. Acta Hortic. 273:139-148.
595 Steven, B., Huntley, R., and Zeng, Q. 2018. The influence of flower anatomy and apple cultivar
596 on the apple flower phytobiome. The Phytobiomes Journal 2:171-179.
597 Stockwell, V., Johnson, K., Sugar, D., and Loper, J. 2010. Control of fire blight by Pseudomonas
598 fluorescens A506 and Pantoea vagans C9-1 applied as single strains and mixed inocula.
599 Phytopathology 100:1330-1339.
600 Stockwell, V.O., Johnson, K.B., Sugar, D., and Loper, J.E. 2011. Mechanistically compatible
601 mixtures of bacterial antagonists improve biological control of fire blight of pear.
602 Phytopathology 101:113-123.
603 Sundin, G.W., Werner, N.A., Yoder, K.S., and Aldwinckle, H.S. 2009. Field evaluation of biological
604 control of fire blight in the eastern united states. Plant Disease 93:386-394.
605 Sundin, G.W., Castiblanco, L.F., Yuan, X., Zeng, Q., and Yang, C.H. 2016. Bacterial disease
606 management: challenges, experience, innovation and future prospects: Challenges in
607 Bacterial Molecular Plant Pathology. Mol Plant Pathol 17:1506-1518.
608 Thomson, S. 1986. The role of the stigma in fire blight infections. Phytopathology 76:476-482.
609 Treves, D.S., Manning, S., and Adams, J. 1998. Repeated evolution of an acetate-crossfeeding
610 polymorphism in long-term populations of Escherichia coli. Mol Biol Evol 15:789-797.
611 Trivedi, P., He, Z., Van Nostrand, J.D., Albrigo, G., Zhou, J., and Wang, N. 2012. Huanglongbing
612 alters the structure and functional diversity of microbial communities associated with
613 citrus rhizosphere. ISME J 6:363-383.
614 Turner, P.E., Souza, V., and Lenski, R.E. 1996. Tests of ecological mechanisms promoting the
615 stable coexistence of two bacterial genotypes. Ecology 77:2119-2129.
616 van der Zwet, T., Orolaza-Halbrendt, N., and Zeller, W. 2012. Fire blight history, biology, and
617 management. . American Phytopathological Society Press.
618 Vanneste, J., Ede, F., and Yu, J. 1995. Influence of delayed inoculation on efficacy of Erwinia
619 herbicola and streptomycin to control fire blight. Biological and Cultural Tests for
620 Control of Plant Diseases 10:46.
621 Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. 2007. Naive Bayesian classifier for rapid
622 assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ.
623 Microbiol. 73:5261-5267.
624 Weller, D.M., Raaijmakers, J.M., Gardener, B.B., and Thomashow, L.S. 2002. Microbial
625 populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev
626 Phytopathol 40:309-348.
627 Westcott, S.L., and Schloss, P.D. 2017. OptiClust, an improved method for assigning amplicon-
628 based sequence data to operational taxonomic units. MSphere 2:e00073-00017.
629 Wickham, H. 2016. ggplot2: elegant graphics for data analysis. Springer.

630 Wilson, M., Epton, H.A.S., and Sigee, D.C. 1992. Biological control of fire blight of hawthorn
631 (Crataegus monogyna) with fluorescent Pseudomonas spp. under protected conditions.
632 Journal of Phytopathology 136:16-26.
633 Xin, X.-F., and He, S.Y. 2013. Pseudomonas syringae pv. tomato DC3000: a model pathogen for
634 probing disease susceptibility and hormone signaling in plants. Annual review of
635 phytopathology 51:473-498.

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638 **Table 1.** Taxonomic assignments of stigma isolated bacteria used for this study.

isolates	Blastn by NCBI		Relationship to representative OTU sequences		Accession number
	Closest BLAST match	Sequence identity (%)	Closest match	Sequence identity (%)	
Pseudomonas spp. CT-1059	*Pseudomonas graminis*	100	OTU2*	100	MT396635
Pantoea spp. CT-1039	*Pantoea agglomerans*	100	OTU3	100	MT396714
Enterobacter spp. CT-1341	*Enterobacter spp.*	100	OTU1	100	MT396725
Curtobacterium spp. CT-1342	*Curtobacterium flaccumfaciens*	100	OTU4	100	MT396712

639 * The OTU numbers correspond to the OTU numbers listed in Figure 4.

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641

642 **FIGURE LEGENDS**

643 **Fig. 1.** Percentage of apple flower clusters that developed fire blight symptoms. Bacterial
644 treatments were applied at 50% and 70% bloom, respectively, with three individual apple
645 trees included in each treatment. *E. amylovora* strain 110 was applied to all trees at 100%
646 bloom three days after bacterial inoculations. Change in disease incidence compared to
647 the water treated control are indicated in brackets. Different letters denote statistical
648 significance ($P < 0.05$, identified by ANOVA comparisons of means, employing a post-
649 hoc Tukey-Cramer test for multiple comparisons). Control: flowers sprayed with H_2O;
650 Streptomycin: flowers sprayed with antibiotics streptomycin (100 ppm); *Pseu*:
651 *Pseudomonas* spp.; *Panto*: *Pantoea* spp.; *Entero*: *Enterobacter* spp.*; Curto*:
652 *Curtobacterium* spp.

653 **Fig. 2.** (**A**) Non-metric Multidimensional Scaling (NMDS) plot displaying relationships
654 of stigma microbial community composition in samples from water treated control (black
655 X) and different bacterial treatments. Distances were calculated using the Bray-Curtis
656 metric and the stress value of the ordination is indicated. Statistically significant
657 differences in clustering were evaluated via both PERMANOVA and the β-disper test
658 and P-values (95% confidence) are indicated. (**B**) Comparative analysis of community
659 diversity (Shannon index), richness (number of recovered OTUs) and evenness
660 (Shannon's evenness) among stigma samples. Different letters denote statistically
661 significant differences (P value < 0.05) identified by ANOVA comparisons of means,
662 employing a post-hoc Tukey-Cramer test for multiple comparisons. The control datasets
663 are included for reference only and were not included in the statistical comparisons.

Control: flowers sprayed with sterile H_2O; *Pseu: Pseudomonas* spp.*; Panto: Pantoea* spp.; *Entero*: *Enterobacter* spp.; *Curto: Curtobacterium* spp.

Fig. 3. (**A**) Mean relative abundance of the predominant bacterial phylum *Proteobacteria*. (**B**) Relative abundance of the *Actinobacteria* and *Proteobacteria* on individual flowers manipulated with *Curtobacterium*. The category "rare" represents the sum of the remaining taxa. (**C**) Relative abundance of the two predominant bacterial families upon water and microbial treatments. Each dot represents a single flower sample. The ratio of *Enterobacteriaceae* to *Pseudomonadaceae* is presented on the top of each treatment with standard division. Statistical significance was determined by ANOVA comparisons of means, employing a post-hoc Tukey-Cramer test for multiple comparisons. Control: flowers sprayed with sterile H_2O; *Pseu: Pseudomonas* spp.*; Panto: Pantoea* spp.; *Entero*: *Enterobacter* spp.; *Curto: Curtobacterium* spp.

Fig. 4 Identification of inoculated strains in the microbiome. Each column represents the microbial composition of a single flower. The four OTUs with 100% sequence identity to the inoculated strains are displayed, and the category "other" represents the sum of the remaining taxa. OTU1: *Enterobacter* spp. strain CT-1341, OTU2: *Pseudomonas* spp. strain CT-1059, OTU3: *Pantoea* spp. strain CT-1039 and OTU4: *Curtobacterium* spp. strain CT-1342.

Fig. 5 *In vitro* antagonism of strain (**A**) *Pseudomonas* spp.*,* (**B**) *Pantoea* spp., (**C**) *Enterobacter* spp. and (**D**) *Curtobacterium* spp. against *E. amylovora* strain 110. The tested strains were pre-cultured on LB agar plates for 48 hours. *E. amylovora* 110 was inoculated onto the plates using a prong dipped in bacterial suspension.

686 **Fig. S1** Genus level identification **(A)** and Phylogenetic relationship **(B)** of the 88
687 bacterial isolates from apple stigma, with strains used in this study for microbiome
688 manipulation highlighted. The phylogeny was built based on the full-length 16S rRNA
689 sequence alignment, using the maximum-likelihood method with the GTR model.

690 **Fig. S2** Interactions between the four selected bacterial strains of *Enterobacter* spp. strain
691 CT-1341*, Pseudomonas* spp. strain CT-1059*, Pantoea* spp. strain CT-1039 and
692 *Curtobacterium* spp. strain CT-1342 in cross-streak test.

693 **Fig. S3** Correlation analysis between microbiome diversity and fire blight disease
694 incidence (R2 = 0.280, P = 0.00) in AVONA test of linear effects model.

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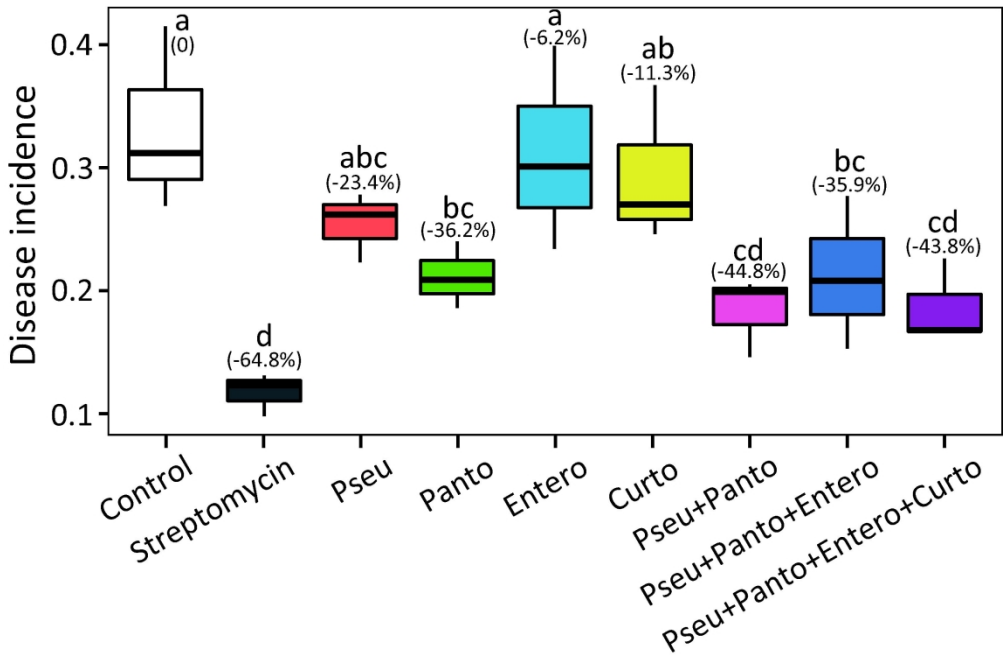

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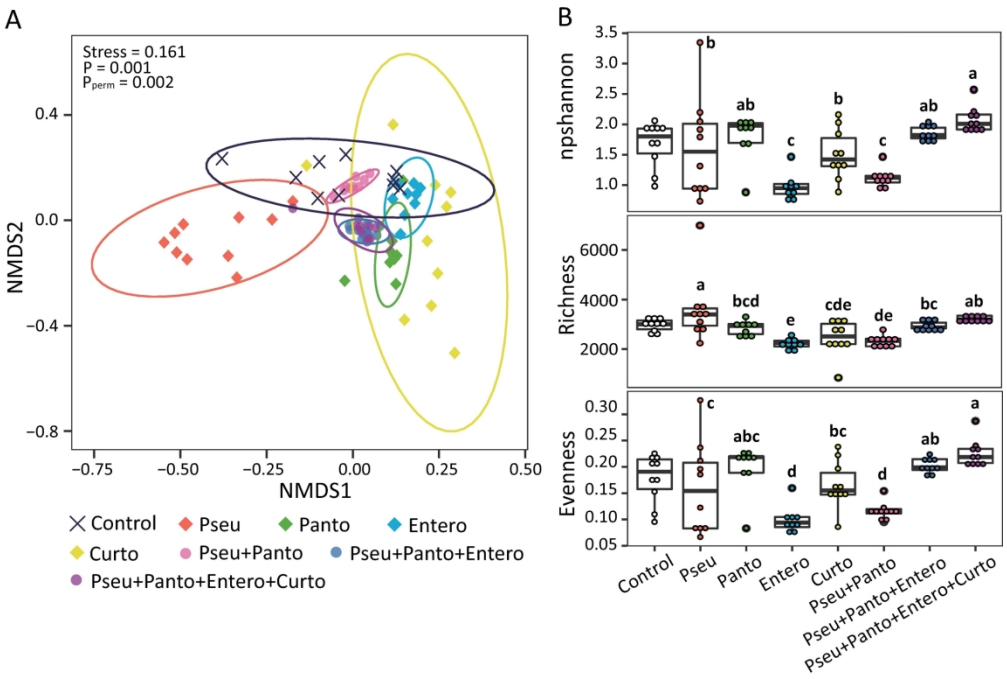

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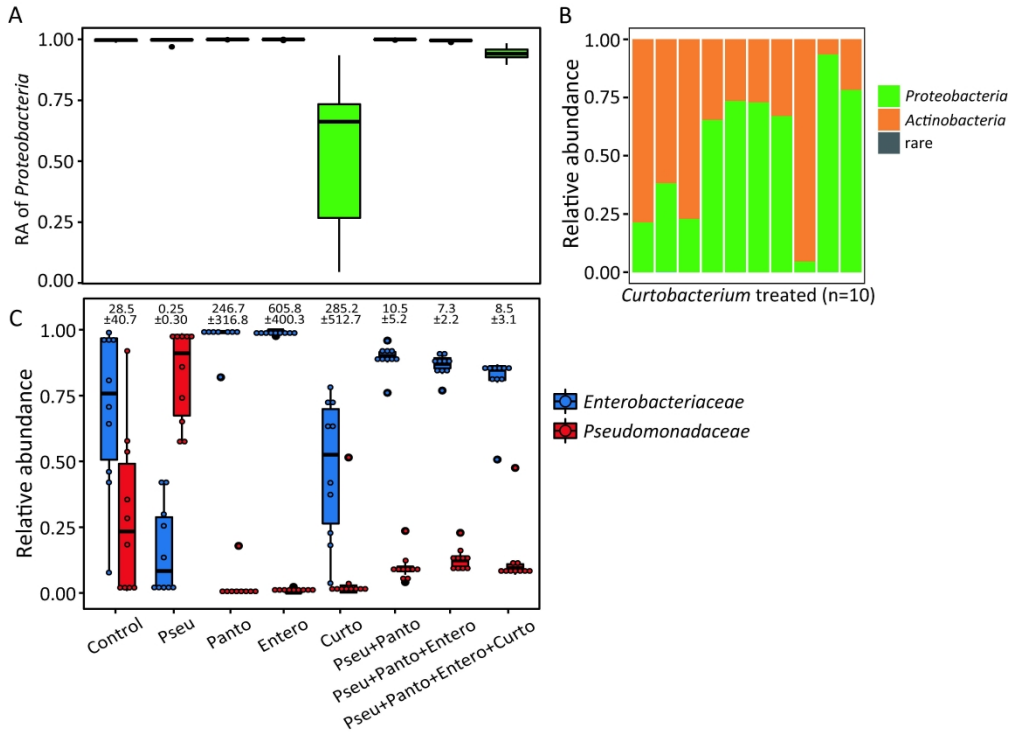

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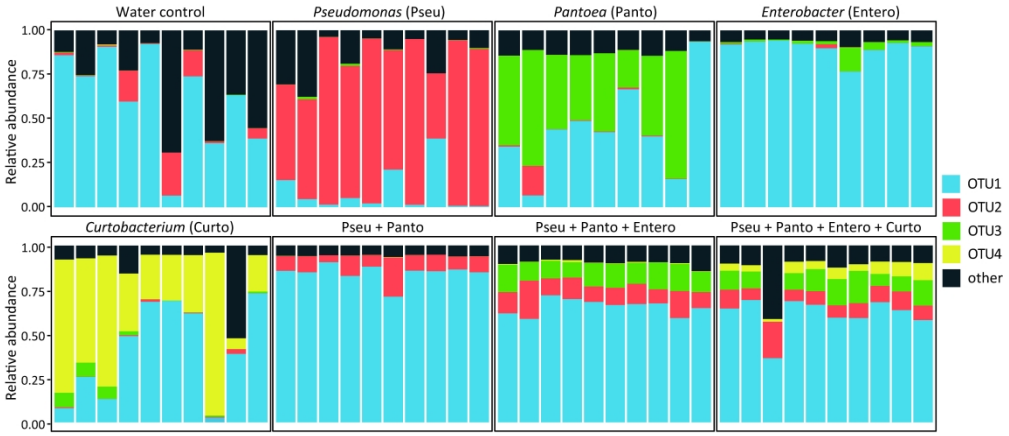

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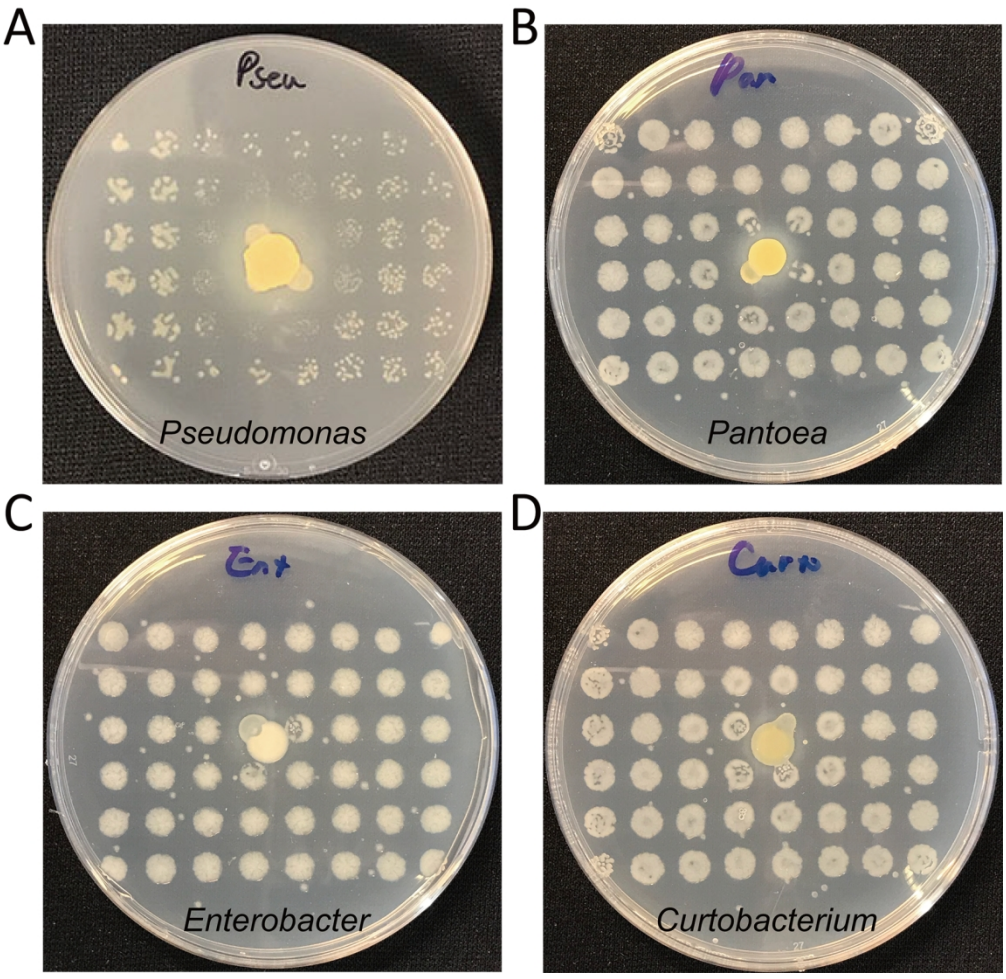

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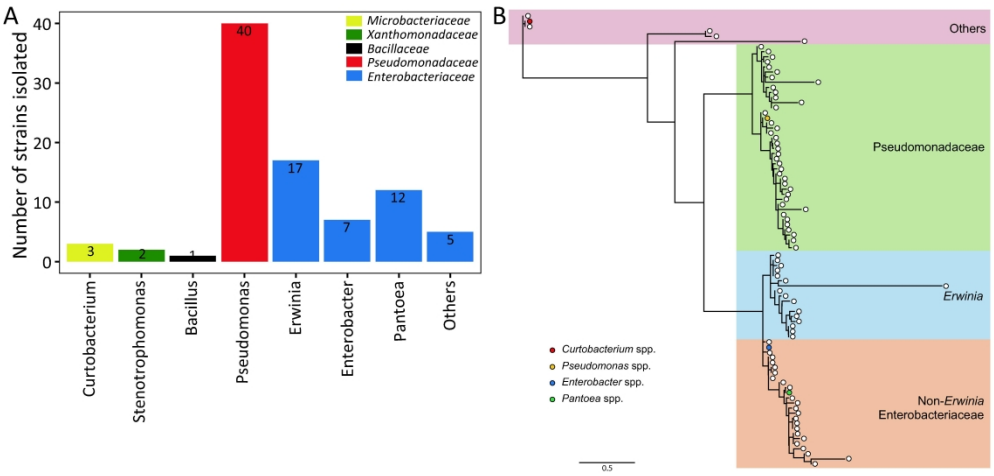

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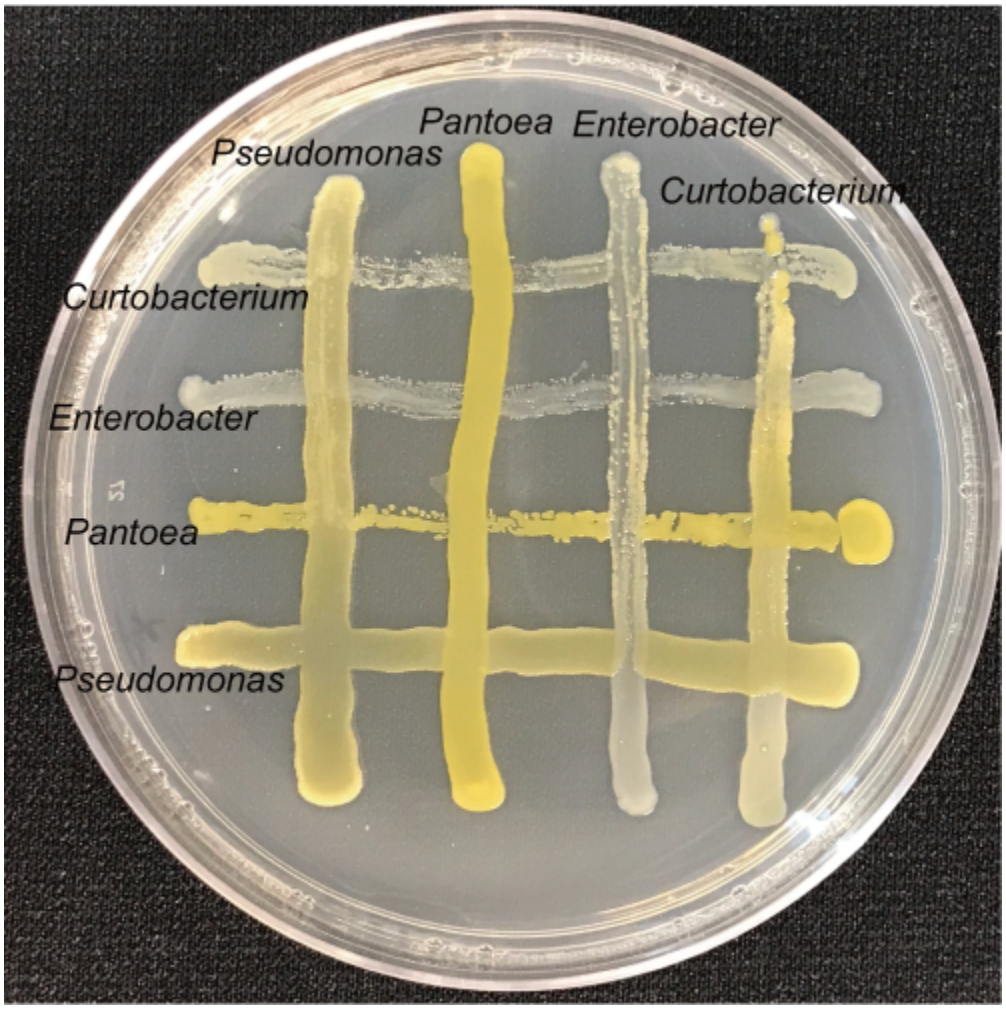

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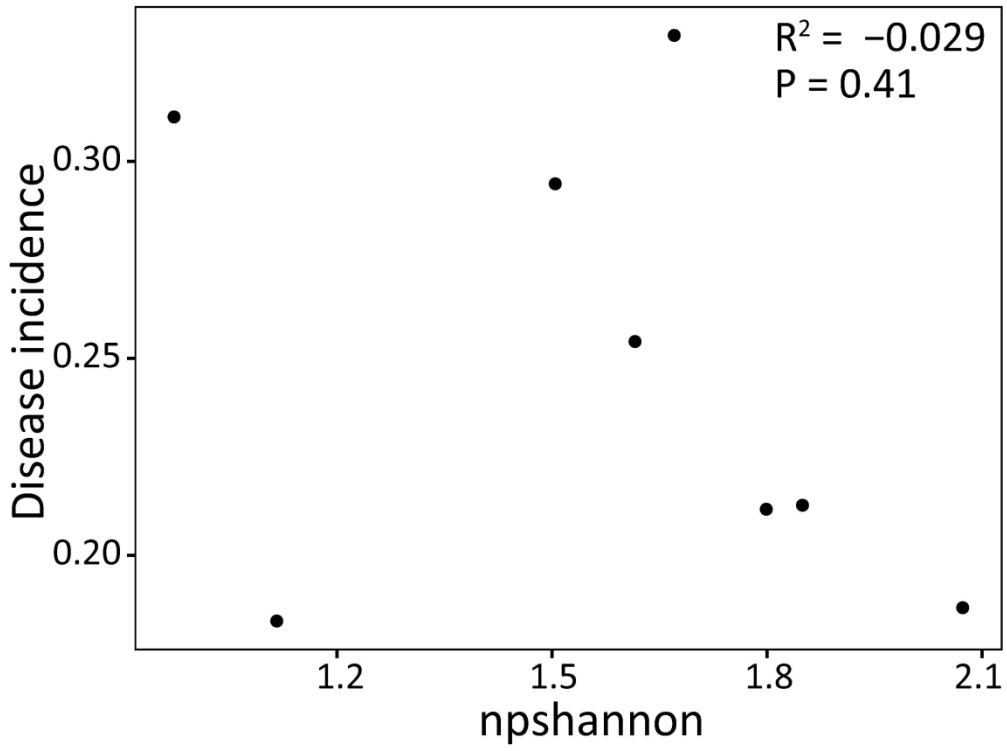

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