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Shifts in the structure of rhizosphere bacterial communities of avocado after Fusarium dieback

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ABSTRACT

The rhizosphere microbiome is critical for plant growth and protection against plant pathogens. However, rhizosphere microbial communities are likely to be restructured upon plant infection by fungal pathogens. Our objective was to determine the shifts in rhizosphere bacterial communities of avocado trees (*Persea americana* Mill.) after Fusarium dieback (FD), a disease triggered by the symbiotic fungi of invasive ambrosia beetles (*Euwallacea kuroshio* and *Euwallacea* sp. nr. *fornicatus*), using 16S rDNA gene amplicon sequencing and a culture-dependent approach. Rhizosphere soil samples were collected from five asymptomatic and five FD-symptomatic avocado trees in a Californian orchard. Sequence analysis showed that diversity metrics of the rhizosphere bacterial communities associated with asymptomatic avocado trees were larger than those of communities from FD-symptomatic trees. Moreover, FD produced significant shifts in rhizobacterial community structure, which were mainly caused by rare OTUs. Bacterial taxa such as Armatimonadetes, *Sporocytophaga* or *Cellvibrio* were exclusively associated with the rhizosphere of asymptomatic trees and may act as an insurance mechanism against fungal invasions. Conversely, genera such as *Myxococcus* or *Lysobacter*, which have been described as effective biocontrol agents against *Fusarium oxysporum*, *Colletotrichum gloeosporioides* or *Rhizoctonia* spp., among other phytopathogens, were only found in the rhizosphere of FD-symptomatic trees. The culturable bacterial communities in the rhizosphere of both FD-symptomatic and asymptomatic trees were dominated by isolates from the *Bacillus* and *Pseudomonas* genera, indicating that potential biocontrol agents against FD may be isolated from healthy and diseased avocado trees. Altogether, our results showed that FD elicited shifts in the avocado rhizosphere microbiome, which could potentially affect soil microbial processes, and provide a basis for the selection of biocontrol agents that could be used for FD prevention.

1. Introduction

The rhizosphere microbiome plays a crucial role for plant growth and health, as bacteria and fungi associated with the rhizosphere may enhance plant nutrient acquisition, confer tolerance to stressful abiotic conditions, produce plant growth promoting phytohormones or emit antimicrobial compounds that protect their host against pathogen infections (Philippot et al., 2013). Any change in the structure and composition of the rhizosphere microbiome is thus likely to influence plant growth and productivity (Berendsen et al., 2012).

Rhizosphere microbial communities are shaped by several biotic and abiotic factors, such as soil physico-chemical parameters, local climatic conditions, or by the host plant species, genotype and physiological stage (Bakker et al., 2013; Compant et al., 2019; Trivedi et al., 2020). An increasing amount of evidence also points to the plant health status as a fundamental driver of rhizosphere microbial assemblages (Berendsen

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et al., 2012, 2018). Soil-borne pathogens, for instance, can directly affect rhizosphere microbial communities through competition for space and nutrients or through the production of antimicrobial metabolites, or indirectly through quantitative and qualitative changes in the root exudates emitted by the host plant (Yang et al., 2001). Aboveground pests and pathogens can also influence rhizosphere microorganisms through the induction of systemic defense responses and the subsequent modification of root exudation patterns (Bais et al., 2006; Kim et al., 2019).

The effect of plant diseases on the structure and composition of rhizosphere microbial communities has been investigated in several economically important crops, such as cotton, citrus, ginseng or tomato (Zhang et al., 2011; Trivedi et al., 2012; Wu et al., 2016; Wei et al., 2018), with different outcomes. These contrasting results indicate that the nature of the pathogenic agent greatly influences the subsequent changes in the rhizosphere microbiome. Wei et al. (2018) reported that plant infection by *Ralstonia solanacearum* reduced the abundance and diversity of various bacterial taxa within the tomato rhizosphere microbiome and simplified bacterial interaction networks, which is consistent with results from Trivedi et al. (2010) in Huanglongbing-infected citrus trees. Contrastingly, Wu et al. (2018) reported a higher microbial abundance in the rhizosphere of root-rot affected ginseng than in that of healthy plants and Yang et al. (2001) found a higher rhizobacterial diversity in avocado trees infected by *Phytophthora cinnamomi* than in non-infected trees. Nevertheless, most studies confirmed a shift in rhizosphere microbial assemblages after plant infection and a subsequent modification of the soil microbial functions.

The United States of America are among the world's ten most producers of avocado (*Persea americana* Mill.), California being the principal producing state with approximately 90% of the national production (FAO, 2018). However, several fast-spreading diseases are hampering avocado production, such as Phytophthora root rot caused by *P. cinnamomi* or the recently discovered Fusarium dieback (FD), caused by *Fusarium euwallaceae* and *F. kuroshium*, among other fungi, and vectored by two invasive shot hole borers (*Euwallacea* sp. nr. *fornicatus* and *E. kuroshio*, also known as Polyphagous shot hole borer (PSHB) and Kuroshio shot hole borer (KSHB) respectively) (Eskalen et al., 2012; Guevara-Avendaño et al., 2018; Na et al., 2018). Our objective was therefore to investigate the rhizosphere bacterial communities of asymptomatic and FD-symptomatic avocado trees, as a first step to understand the ecological implications of FD-induced shifts in the avocado rhizosphere core microbiome and to unravel possible bacterial taxa associated with the disease. We focused on the core microbiome of each tree condition (FD-symptomatic vs. asymptomatic avocado trees), defining core microorganisms as those that are sufficiently dependent on the host to be consistently found across different plant health status. Core microbial taxa are thought to be successful rhizosphere colonizers due to their co-adaptation with their hosts and thus to be critical for plant health (Schlatter et al., 2020). Moreover, core microbiomes have been determined as crucial to identify key microbial taxa that could help enhance plant performance under stress and could therefore provide useful information for the design of biocontrol microbial consortia (Busby et al., 2017). Consequently, we used culture-independent and culture-dependent approaches to 1) determine the shifts in the structure of rhizosphere bacterial communities of avocado after FD and 2) isolate potential biocontrol agents that could be used for mitigating the impact of FD in avocado orchards. The obtained bacterial isolates could be screened for antifungal and plant growth promoting activities, both *in vitro* and *in planta*, to assess their ability to induce plant systemic defense responses and to reduce the disease incidence and severity. Moreover, identifying the changes in the avocado rhizosphere microbiome following FD could provide useful disease diagnostic tools and assist in the identification of plant growth-promoting bacteria (PGPR).

2. Materials and methods

2.1. Sample collection

Rhizosphere soil samples were collected in an avocado orchard located at Escondido (33°07′29″N 117°04′51″W) in San Diego County, California, in December 2015, as described in Guevara-Avendaño et al. (2018). Briefly, five asymptomatic and five FD-symptomatic avocado trees were randomly selected. FD symptoms included entry points of KSHB in the bark of trunk and branches, observation of galleries and wood discoloration after bark removal and dieback of the branches (Eskalen et al., 2013). Four samples of rhizosphere soil were collected per tree, considering the four cardinal points as sampling points, approximately 50 cm away from the trunk and at a depth of 5–10 cm. A composite sample was then prepared for each tree. Samples were transported in a cooler and immediately processed in the laboratory (Eskalen Lab., UC Riverside) for DNA extraction and isolation of culturable bacteria. Roots were shaken to remove loose soil and the remaining soil, which was strongly adhered to the roots, was recovered as rhizosphere soil.

2.2. Soil DNA extraction and sequencing

The MoBio PowerSoil® DNA Isolation Kit (QIAGEN, The Netherlands) was used to extract total DNA from rhizosphere soil samples (n = 10), following manufacturer's instructions. DNA extraction products were sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing of the V1–V3 16S rRNA regions, using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 518R (5′-GTATTACCGCGGCTGCTGG-3′), in a 2 × 250 bp paired-end run on a Illumina MiSeq platform. Data were deposited in the Sequence Read Archive of NCBI under accession number PRJNA689051.

2.3. Isolation of culturable bacteria from avocado rhizosphere soil

Isolation of culturable bacteria was carried out from eight of the ten rhizosphere soil samples (four from asymptomatic and four from FD-symptomatic trees, respectively), as described in Guevara-Avendaño et al. (2018, 2019). Suspensions were prepared from rhizosphere soil samples by resuspending 1 g of soil in 99 ml of sterile distilled water. Dilutions (1:10 and 1:100) were subsequently prepared with sterile distilled water. Culturable bacteria were isolated by inoculating 100 μl of both dilutions onto Luria Bertani medium (LB, Difco). Each dilution was inoculated in triplicate. Bacterial isolates were re-streaked until pure cultures were obtained and clustered into morphotypes based on colonial and cellular morphological criteria (shape, edge, elevation, surface, consistency, color, transmitted light, reflected light and Gram staining of pure cultures). All isolates were preserved in LB medium with 20% of glycerol at −20 °C.

Up to three bacterial isolates per morphotype were processed for molecular analysis and sequencing of the 16S rDNA region. Bacterial DNA was extracted with the DNeasy® Blood & Tissue kit (QIAGEN, The Netherlands), following manufacturer's instructions for Gram-negative and Gram-positive bacteria. The extracted DNA concentration was quantified with a BioSpec-nano spectrophotometer (Shimadzu Biotech, U.S.A.) and the quality of extraction products was verified by gel electrophoresis. The 16S rDNA region was then amplified by PCR using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR reactions (50 μl) contained 0.4 mM of each primer, 0.2 mM of each dNTP, 1.25 mM $MgCl_2$, 5 μl Taq buffer 10X, 1 U Taq DNA polymerase (QIAGEN, The Netherlands) and 25–150 ng template DNA. PCR amplification was performed in a SureCycler GA8800A thermal cycler (Agilent Technologies, U.S.A.) with an initial denaturation step at 95 °C for 4 min, followed by 30 cycles of amplification at 95 °C for 45 s, 53 °C for 45 s, and 72 °C for 2 min, and a final extension step at 72 °C for 5 min. The resulting PCR products were

purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, U.S.A.) or Purelink®Quick Gel Extraction Kit (QIAGEN, The Netherlands). The concentration of purified PCR products was quantified using a BioSpec-nano spectrophotometer (Shimadzu-Biotech, U.S.A.). The quality of purified PCR products was verified by agarose gel electrophoresis. Samples were sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing. Sequences were deposited in GenBank (accession numbers MW424648 to MW424760).

2.4. Sequence analyses

Sequences obtained in the culture-independent approach were analyzed as follows: quality analysis of paired-end reads was performed on FastQC v.0.11.5 (Andrews, 2010). Sequences of low quality (shorter than 50 bp or with a quality score ≤ 20) were removed from the analysis with PRINSEQ v.0.20.4 (Schmieder and Edwards, 2011). Reads were joined using QIIME v.1.8.0 (Caporaso et al., 2010). Removal of chimeras was conducted with MOTHUR v.1.25.0 (Schloss et al., 2009), using the Greengenes database (v13_8_99) as the template for the 16S marker. The remaining sequences were clustered into Operational Taxonomic Units (OTUs), picked by open-reference command using a 97% similarity threshold and the most frequent sequence per OTU was selected as the representative sequence. Taxonomy was assigned following the RDP method and using the Greengenes 13_8 reference database (DeSantis et al., 2006). OTUs corresponding to chloroplastic, archaeal and mitochondrial DNA were removed, as well as OTUs with less than 0.01% of relative abundance per sample.

Diversity analyses were carried out with data normalized to 69,400 reads per sample. Rarefaction curves were drawn for the number of observed OTUs, and for Shannon and Simpson diversity indices. Differences in alpha diversity between asymptomatic and FD-symptomatic trees were evaluated with Wilcoxon Rank-Sum tests with a FDR correction in R v.3.2.3 (R Core Team, 2015) and were considered statistically significant when $P \leq 0.05$. A cluster analysis based on Bray-Curtis dissimilarity was implemented to observe OTUs grouping depending on avocado tree condition (asymptomatic vs. FD-symptomatic trees). A Principal Coordinates Analysis (PCoA) was performed to observe differences in the structure of rhizosphere bacterial communities associated with asymptomatic and FD-symptomatic trees, based on weighted and unweighted Unifrac distances (Lozupone and Knight, 2005). An analysis of similarity (ANOSIM) was applied to detect significant differences in beta diversity between both tree conditions.

Sequences obtained from culturable bacteria were manually checked and edited using BioEdit v.7.2.5 (Hall, 1999). A reference dataset was constructed with the obtained sequences and their three closest matches as retrieved from the GenBank nucleotide database (www.ncbi.nlm.nih.gov). All sequences were aligned using the multiple sequence alignment T-coffee method (Notredame et al., 2000), and the alignment was manually edited with Gblocks v.0.91b (Castresana, 2000). A Maximum Likelihood phylogenetic tree was constructed in MEGA 7 (Kumar et al., 2016) using a General Time Reversible model with discrete Gamma distribution (GTR + G + I) and a bootstrap method using 100 replicates.

3. Results

3.1. Rhizosphere bacterial communities associated with FD-symptomatic and asymptomatic avocado trees

An average of 453,301 ± 15,937 raw sequences of 265 bp-length was obtained per sample. After quality filtering and removal of non-bacterial and rare OTUs, the remaining high-quality reads (113,528 ± 7510 sequences on average per sample) were clustered into 6705 OTUs at a 97% sequence similarity. As the objective of this work was to focus on the core microbiome of each tree condition (FD-symptomatic vs. asymptomatic avocado trees), sequence filtering was performed in order to consider only OTUs that were present in at least four of five samples per condition. After applying this filter, an average of 82,029 ± 8497 reads was obtained per sample, which were clustered into 1406 OTUs (Table S1).

Rarefaction curves based on the number of observed OTUs and on Shannon and Simpson indices reached a plateau (Fig. S1), indicating that an adequate sampling depth was achieved. Alpha-diversity analyses showed that richness (measured as the number of observed OTUs) and diversity (estimated by Shannon and Simpson indices) of the avocado rhizosphere bacterial community were significantly higher in asymptomatic than in FD-symptomatic trees (Fig. 1; $n = 10$; $P < 0.05$).

Cluster analysis (Fig. 2) showed that sequences from the avocado rhizosphere core microbiome were clustered into two groups, which mostly corresponded to FD-symptomatic and asymptomatic trees. However, one sample collected from an asymptomatic tree (Asympt 2) was grouped with the cluster of samples from FD-symptomatic trees, while one sample from a FD-symptomatic tree (FD-sympt 5) was found within the cluster of samples from the rhizosphere of asymptomatic trees.

Beta-diversity analyses (ANOSIM) showed that rhizosphere bacterial communities associated with FD-symptomatic avocado trees were significantly different from those associated with asymptomatic trees, especially when only considering the presence or absence of bacterial OTUs (unweighted Unifrac metric, $R = 1$, $P < 0.05$, Fig. 3a). When taking into account the relative abundance of OTUs within the community, the low similarity between the rhizosphere bacterial communities remained, although the differences between both conditions were less marked (weighted Unifrac metric, $R = 0.268$, $P < 0.05$, Fig. 3b).

As shown by the Venn diagram, most OTUs (57.3%) were shared by both conditions (Fig. 4a). A Wilcoxon test showed that the relative abundances of 15 of the 805 shared OTUs were significantly different between FD-symptomatic and asymptomatic trees (Table S2; $P < 0.05$). Of the total 1406 OTUs, 14.4% were exclusively detected in the rhizosphere of FD-symptomatic trees while 28.4% were only found in that of asymptomatic trees. Exclusive OTUs with unique taxonomic assignment at the class, order, family or genus level are presented in Table 1. Interestingly, sequences from *phylum* Armatimonadetes were exclusively found in the rhizosphere of asymptomatic avocado trees. Other taxa that were exclusively associated with asymptomatic trees included Frankiaceae and *Kribella* (Actinobacteria), or *Cellvibrio* (Gammaproteobacteria). Conversely, bacterial genera such as *Myxococcus* or *Lysobacter* were only found in the rhizosphere of FD-symptomatic trees (Table 1).

In general, no differences were detected in the relative abundance of bacterial taxa when studied at taxonomic ranks higher than the genus level, which suggests that differences between both conditions reside mostly at the OTU level. At the *phylum* level, avocado rhizosphere bacterial communities from both conditions were dominated by Proteobacteria, followed by Bacteroidetes, Actinobacteria and Acidobacteria (Fig. 4b). The most abundant bacterial taxa found in the avocado rhizosphere core microbiome included bacterial families Cytophagaceae and Chitinophagaceae (Bacteroidetes), Sinobacteraceae and Rhodospirillaceae (Proteobacteria), and genera such as *Rhodoplanes* (Proteobacteria), *Flavobacterium* (Bacteroidetes), or *Nitrospira* (Nitrospirae). To determine if differences could be found in the relative abundance of dominant OTUs within the avocado rhizosphere core microbiome, a heatmap was generated considering the 50 most abundant OTUs (Fig. 4c). The least abundant OTUs (<0.01%) were grouped in the category "Others". A Wilcoxon signed-rank test was subsequently carried out to assess significant differences in the relative abundance of dominant bacterial OTUs in the rhizosphere of both tree conditions. Only one of the dominant OTUs (OTU NR.396, corresponding to the Gammaproteobacteria class) presented a significant difference in its relative abundance in the rhizosphere of asymptomatic and FD-symptomatic trees, and was significantly more abundant in asymptomatic trees (Wilcoxon test, $P < 0.05$; Table S2). These results indicate that differences in the taxonomic composition of rhizosphere bacterial

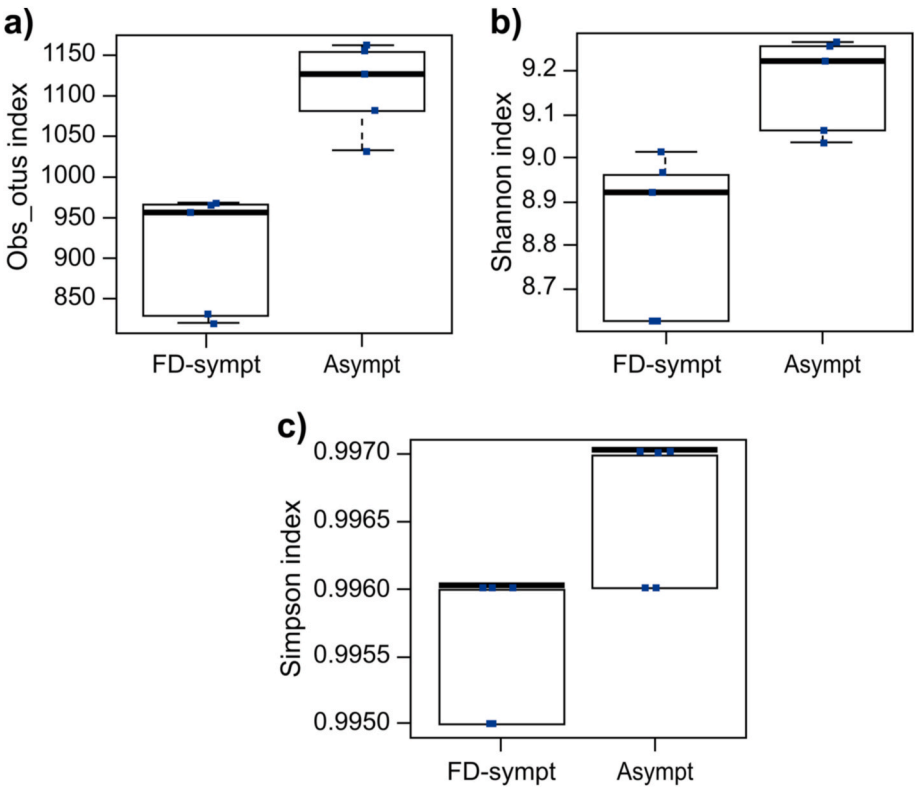

Fig. 1. Box plots of rhizosphere bacterial communities associated with FD-symptomatic and asymptomatic avocado trees. a. Number of observed OTUs; b. Shannon diversity index; c. Simpson index (Wilcoxon test, n = 10, $P < 0.05$).

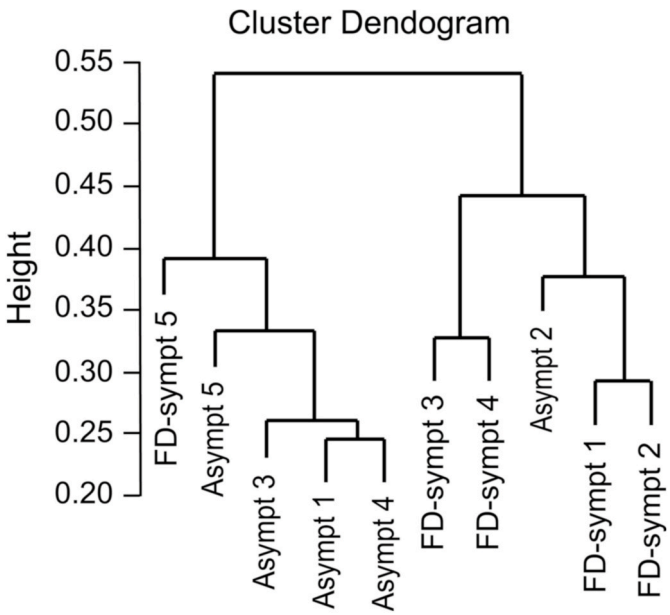

Fig. 2. Cluster analysis of rhizosphere bacteria associated with asymptomatic and FD-symptomatic avocado trees. The analysis was carried out from 1406 OTUs based on Bray-Curtis dissimilarity.

communities from FD-symptomatic and asymptomatic trees are given by rarer OTUs.

3.2. Culturable bacteria associated with the rhizosphere of FD-symptomatic and asymptomatic avocado trees

A total of 150 bacterial isolates were obtained from eight processed rhizosphere soil samples. Eighty-six bacterial isolates were retrieved from four rhizosphere samples collected from FD-symptomatic trees and 64 from four rhizosphere samples collected from asymptomatic trees. The obtained bacterial isolates were clustered into 83 morphotypes, based on colonial and cellular criteria. In total, 113 bacterial isolates were processed for sequencing (60 and 53 isolates from the rhizosphere of FD-symptomatic and asymptomatic trees, respectively) (Table S3).

The phylogenetic analysis (Fig. 5) clustered the obtained bacterial sequences into two phyla: Firmicutes and Proteobacteria. The bacterial isolates retrieved from the rhizosphere of FD-symptomatic and asymptomatic trees principally belonged to the Firmicutes *phylum* (81.7% and 81.1% of total bacterial isolates, respectively), followed by the Gammaproteobacteria class (16.7% and 18.9% of bacterial isolates from FD-symptomatic and asymptomatic avocado trees, respectively). One bacterial isolate (M35), obtained from the rhizosphere of a FD-symptomatic tree, belonged to the Alphaproteobacteria class and was identified based on the phylogenetic analysis as *Brevundimonas bullata*. Within the Firmicutes *phylum*, sequences obtained from samples collected from both tree conditions mostly belonged to the *Bacillus* genus; interestingly, sequences from the *Staphylococcus* genus were only retrieved in rhizosphere samples from FD-symptomatic avocado trees.

4. Discussion

4.1. Changes in the avocado rhizosphere bacterial communities associated with FD

We investigated the FD-induced differences in the structure and composition of the avocado rhizosphere core microbiome. Our findings show that diversity metrics of bacterial communities in the rhizosphere of FD-symptomatic trees were lower than those of communities associated with asymptomatic avocado trees, and that FD produced significant shifts in rhizobacterial community structure, as evidenced by the ANOSIM and cluster analyses. Differences in the taxonomic composition

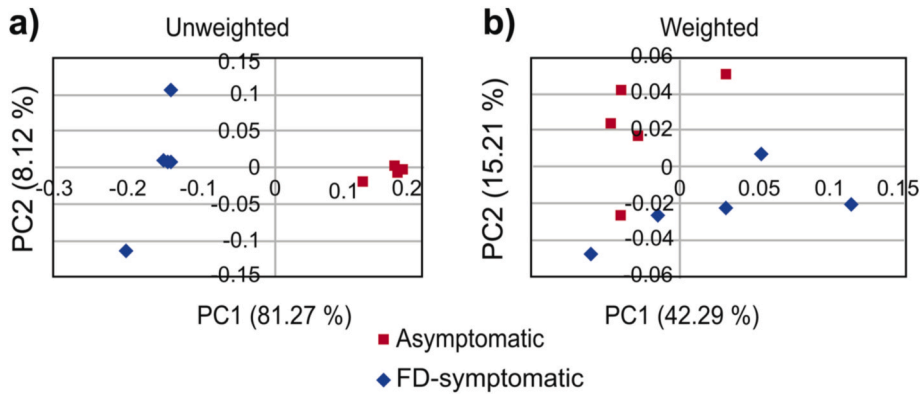

Fig. 3. PCoA biplot for a. unweighted; b. weighted Unifrac metrics of rhizosphere bacterial communities associated with asymptomatic (red) and FD-symptomatic (blue) avocado trees.

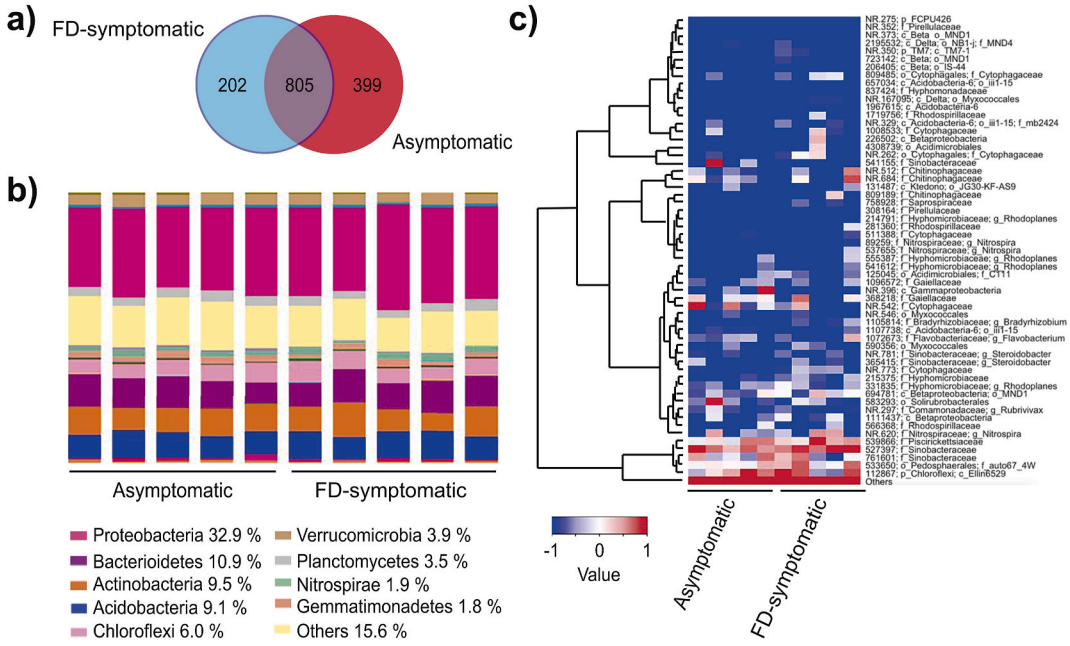

Fig. 4. a. Venn diagram representing the number of OTUs in the rhizosphere of asymptomatic and FD-symptomatic avocado trees. b. Relative abundance of bacterial phyla in the rhizosphere of asymptomatic and FD-symptomatic avocado trees. Bacterial phyla with less than 0.01% of relative abundance were collapsed in the category "Others". c. Heat-map of log2-transformed relative abundances of the 50 most abundant OTUs in the rhizosphere of asymptomatic and FD-symptomatic avocado trees. Less abundant OTUs (<0.01%) were collapsed in the category "Others". The ID number of each OTU and its taxonomic assignment based on the Greengenes database are presented.

of bacterial communities from both conditions were also detected, although these differences were found at the OTU level and seemed to be restricted to rare taxa.

The observed lower rhizosphere bacterial diversity in FD-symptomatic trees is consistent with findings from other studies, which report a similar decrease in diversity when plants are infected by microbial pathogens. This was shown for example by Filion et al. (2004) for black spruce affected by root rot, or by Trivedi et al. (2010) for citrus trees infected with Huanglongbing disease. Conversely, positive or neutral effects of microbial diseases on the rhizosphere microbial diversity have been reported in wilted *Lilium davidii* (Shang et al., 2016) and in avocado trees infected with Phytophthora root rot (Yang et al., 2001; Solís-García et al., 2021). These contrasting results suggest that alterations in the diversity of rhizosphere microbial communities may largely depend on the phytopathogenic agent, its infection mechanism, and on the host plant defense response. Several reports have investigated the restructuring of rhizosphere microbiomes by *Fusarium* spp., with different results. Fusarium wilt in cucumber, caused by *F. oxysporum* f. sp. *cucumerinum,* decreased the abundance of endophytic actinobacteria at the root level (Cao et al., 2020), whilst Fusarium wilt in tomato, caused by *F. oxysporum* f. sp. *lycopersici* (*Fol*), reduced that of Proteobacteria (Wan et al., 2017). These findings contrast with results from the present study where no effect of Fusarium wilt was detected at the phylum level. However, consistently with our results, *Fol* reduced the rhizosphere bacterial diversity in tomato (Zhou et al., 2020). These authors also reported the enrichment in potential biocontrol agents in healthy tomato roots, such as *Bacillus, Pseudomonas* and *Streptomyces*, which may help tomato to gain resistance against *Fol* (Zhou et al., 2020).

Our results also showed a shift in the structure of the avocado rhizosphere core microbiome associated with FD-symptomatic trees. Cluster analysis evidenced two separate sample groups, mostly corresponding to FD-symptomatic and asymptomatic trees. The presence of one sample from the opposite tree condition within each cluster indicates that variables other than FD may influence the avocado rhizosphere core microbiome. Although sampling was performed within the same orchard to minimize the influence of microclimate, soil type, tree

Table 1
Taxonomic assignment of bacterial OTUs exclusively found in the rhizosphere of asymptomatic or FD-symptomatic avocado trees.

Phylum	Class	Order	Family	Genus	No. of OTUs	Relative abundance (%)
Asymptomatic trees						
Acidobacteria	Chloracidobacteria	11–24			1	0.02
	EC1113				1	0.01
	iii1-8	DS-18			1	0.02
Actinobacteria	Actinobacteria	Actinomycetales	Frankiaceae		1	0.01
			Nocardioidaceae	*Kribbella*	1	0.01
Armatimonadetes	Fimbriimonadia	Fimbriimonadales			1	0.03
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Sporocytophaga*	1	0.01
			Flammeovirgaceae	*Marinoscillum*	1	0.01
	Flavobacteriia	Flavobacteriales	Cryomorphaceae	*Crocinitomix*	1	0.02
Chlorobi	BSV26	VC38			2	0.05
Chloroflexi	Ktedonobacteria	Thermogemmatisporales	Thermogemmatisporaceae		1	0.03
	SAR202				1	0.03
Cyanobacteria	4C0d-2	SM1D11			1	0.01
Gemmatimonadetes	Gemmatimonadetes	C114			2	0.03
OD1	ABY1				2	0.03
	Mb-NB09				1	0.04
OP11	OP11-4				2	0.02
Planctomycetes	Phycisphaerae	mle1-8			1	0.02
		Pla1			1	0.02
	Planctomycetia	Gemmatales	Isosphaeraceae		1	0.01
Proteobacteria	Alphaproteobacteria	BD7-3			3	0.06
	Deltaproteobacteria	MIZ46			1	0.01
		Myxococcales	Polyangiaceae		2	0.02
	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Cellvibrio*	1	0.01
		Legionellales	Coxiellaceae	*Aquicella*	1	0.01
		Xanthomonadales	Xanthomonadaceae	*Arenimonas*	2	0.02
TM7	TM7-3				2	0.03
Verrucomicrobia	Pedosphaerae	Pedosphaerales	Ellin515		1	0.03
FD-symptomatic trees						
Acidobacteria	Sva0725	Sva0725			2	0.03
Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Demequina*	1	0.01
Chlorobi	BSV26	C20			1	0.02
	SJA-28				1	0.01
Chloroflexi	Chloroflexi	AKIW781			1	0.02
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	1	0.01
Proteobacteria	Deltaproteobacteria	Entotheonellales	Entotheonellaceae	*Candidatus Entotheonella*	1	0.01
		Myxococcales	Myxococcaceae	*Myxococcus*	1	0.01
		NB1-j	JTB38		2	0.02
	Gammaproteobacteria	Alteromonadales	OM60		1	0.01
		Marinicellales	Marinicellaceae		1	0.01
		Xanthomonadales	Xanthomonadaceae	*Lysobacter*	1	0.01

Taxonomic assignment based on the Greengenes database.

age and cultivar on the rhizosphere microbiome, other factors such as time since infection or disease severity could not be controlled, as often in field studies. It is thus possible that an asymptomatic tree was in fact at the early stage of the infection, which could explain its clustering within the FD-symptomatic condition. Regardless, the modification of the rhizosphere bacterial community structure was confirmed by the beta-diversity analysis, which clearly separated samples from both tree conditions. This segregation was especially visible when considering the incidence of OTUs rather than their relative abundance, most likely because the observed differences were given by rare OTUs. Rare soil microbes have been shown to play crucial roles in plant health, by preventing the establishment of pathogens within the microbial community or promoting plant defense mechanisms (Hol et al., 2010; Jousset et al., 2017), and our results support the recent idea that the "rare biosphere" is an important driver of diversity (Lynch and Neufeld, 2015).

An entire *phylum,* Armatimonadetes, was exclusively detected in the rhizosphere of asymptomatic avocado trees. This *phylum* was discovered to be relatively frequent in samples obtained from oligotrophic environments (freshwater lakes, forest soils, hot springs) (Tamaki et al., 2011), although it has also been found in the rhizosphere of several plants (Sarria-Guzmán et al., 2016; Ferreira de Araujo et al., 2019). The relative abundance of Armatimonadetes has been positively correlated to plant growth (Ma et al., 2020). Nevertheless, as members of Armatimonadetes are difficult to isolate and study in pure cultures (Hu et al., 2014), the ecological importance of this *phylum* remains poorly understood. Further research aiming at exploring culture conditions and metabolic functions of *phylum* Armatimonadetes are therefore needed. Other taxa that were exclusively found in the rhizosphere of asymptomatic trees included *Kribbella*, *Sporocytophaga*, *Marinoscillum*, *Crocinotomix*, *Cellvibrio*, *Aquicella* and *Arenimonas*. Whilst most species of the *Kribbella* genus have been isolated from the soil (Trujillo et al., 2006), it is noteworthy that sequences from *Marinoscillum* and *Crocinotomix* were retrieved from the avocado rhizosphere. These bacterial genera have mainly been found in marine ecosystems (Bowman, 2003; Seo et al., 2009), although the presence of *Marinoscillum* was recently detected in the rhizosphere of different halophyte plants (Alzubaidy et al., 2016; Yuan et al., 2016; Yamamoto et al., 2018). *Sporocytophaga* and *Cellvibrio* are known for their cellulose- and chitin-degrading capacities and for their ability to use different carbon and nitrogen sources (Liu et al., 2014; Ciancio et al., 2016). In particular, *Cellvibrio* has been reported to enhance plant growth and productivity, for example through auxin production (Lévesque et al., 2020; Zhang et al., 2020) and elicit plant defense responses through the digestion of fungal cell walls by chitinases and other chitin-specific enzymes (Tuveng et al., 2016; Jaiswal et al., 2017). This suggests that its presence in asymptomatic trees may enhance plant fitness and help reduce disease incidence. Several studies have described a decrease in the abundance of PGPR following a phytopathogen infection. For example, Huanglongbing reduced the presence of *Burkholderia, Lysobacter, Pseudomonas, Bacillus* and

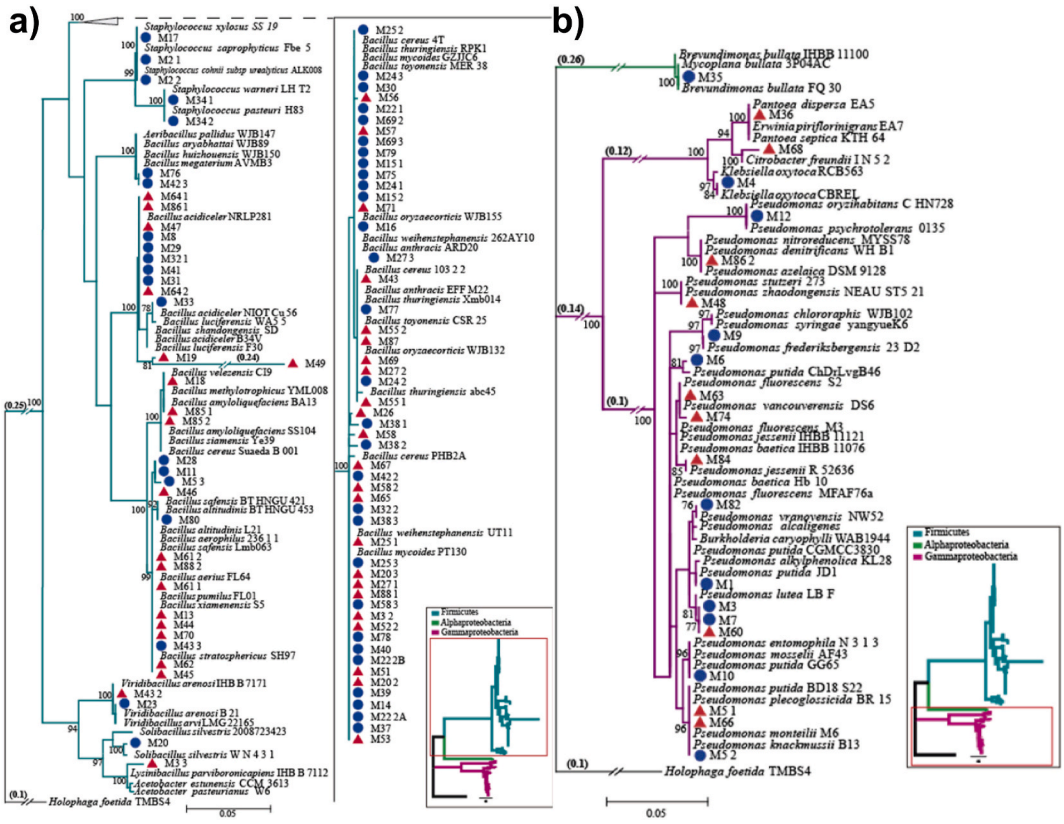

Fig. 5. Maximum-likelihood tree of 16S rRNA sequences from bacteria isolated from the rhizosphere of asymptomatic (red triangles) and FD-symptomatic (blue circles) avocado trees. The phylogenetic analysis was based on the General Time Reversible model with Gamma distribution. Numbers between brackets indicate the branch length. The numbers at the nodes are bootstrap values based on 100 replications (>75%). a. Firmicutes *phylum*. b. Proteobacteria *phylum* with Alphaproteobacteria and Gammaproteobacteria classes.

Paenibacillus, among other PGPR, in the citrus rhizosphere (Trivedi et al., 2010). Phytophthora root rot caused by *Phytophthora cinnamomi* decreased the relative abundance of Actinobacteria, Rhizobiales and *Bacillus* spp. in the rhizosphere of avocado trees (Solís-García et al., 2020), which is consistent with findings by Wei et al. (2018) in tomato plants infected by *Ralstonia solanacearum*. Interestingly, our results also show that *Aquicella*, a pathogenic bacterium, was exclusively found in the rhizosphere of asymptomatic trees, while it was associated with the rhizosphere of konjac plants infected by bacterial soft rot (*Pectobacterium* spp.) (Wu et al., 2017) or with that of sweet peppers infected with Phytophthora blight (Zhang et al., 2019).

Conversely, bacterial genera such as *Demequina*, *Gemmatimonas*, *Myxococcus* and *Lysobacter* were only detected in the rhizosphere of FD-symptomatic trees. *Demequina* is a bacterial genus that has been mainly associated with marine environments (Park et al., 2016), although it was recently reported in bark samples from pear trees (Arrigoni et al., 2018) and in soil of apple orchards, where its abundance was strongly correlated to plant growth (Peruzzi et al., 2017). *Gemmatimonas* was recently associated with the suppression of Panama disease in banana, which is caused by *F. oxysporum* f. sp. *cubense* (Shen et al., 2019). Plants are able to attract beneficial microorganisms to counteract a pathogen infection and induce the expression of defense genes through shifts in root exudation patterns (Berendsen et al., 2012; Liu et al., 2021), which could also explain the exclusive presence of *Myxococcus* and *Lysobacter* in the rhizosphere of FD-symptomatic trees. These taxa have been described as biocontrol agents against several pathogenic fungi and oomycetes, such as *F. oxysporum*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Rhizoctonia* spp., *Sclerotinia* spp. or *Verticillium* spp., among others (Bull et al., 2002; Liu et al., 2019).

Future efforts should be directed at elucidating how the avocado rhizosphere microbiome impacts the plant metabolome, as it could affect plant attraction to KSHB. As previously shown by Badri et al. (2013), the effect of soil microorganisms on aboveground pests is likely to be mediated by changes in the host plant metabolome, which calls for more studies investigating how the avocado rhizosphere microbiome affects plant emission of attractive volatile compounds or its production of defensive compounds aboveground. Moreover, isolating PGPR is crucial to be able to assess their antifungal activity, their ability to induce plant systemic resistance, and thus their contribution to plant fitness and disease protection.

4.2. Culturable bacteria associated with the rhizosphere of FD-symptomatic and asymptomatic avocado trees

The culturable bacterial communities in the rhizosphere of both FD-symptomatic and asymptomatic avocado trees were dominated by Firmicutes, principally from the *Bacillus* genus. Isolates from the *Pseudomonas* genus (Gammaproteobacteria) were also commonly retrieved from the rhizosphere of trees in both conditions. Although the composition of the culturable bacterial community is largely influenced by culture conditions such as growth medium, time and temperature of incubation, the predominance of *Bacillus* spp. and *Pseudomonas* spp. is not surprising as these genera are relatively simple to culture (Hugenholtz, 2002). However, it is noteworthy that the Firmicutes *phylum* was poorly represented in the bacterial community when assessed through 16S rRNA amplicon sequencing. This is consistent with other reports regarding the avocado rhizosphere microbiome, where the Firmicutes *phylum* was not found among the dominant taxa (Shu et al., 2019; Solís-García et al., 2021).

The *Bacillus* and *Pseudomonas* genera comprise PGPR with well-described antifungal properties, such as the production of antimicrobial lipopeptides (Cazorla et al., 2007; Cawoy et al., 2015) and 2,

4-diacetylphloroglucinol (DAPG) (Haas and Defago, 2005) or the emission of antifungal volatile compounds (Yuan et al., 2012; Ossowicki et al., 2017). Bacterial isolates from both genera have been previously found in the rhizosphere of Mexican avocado trees and have successfully inhibited the mycelial growth of *F. euwallaceae* associated with PSHB, *F. kuroshium* associated with KSHB and *F. solani,* through the emission of cyclo-lipopeptides and volatile organic compounds such as ketones and pyrazines (Guevara-Avendaño et al., 2020). Some of the isolates obtained in this study have also been investigated for their antifungal properties against *F. euwallaceae* and *F. kuroshium* with promising results in *in vitro* assays, and should be further assessed *in planta* as they represent potential biocontrol agents against FD (Guevara-Avendaño et al., 2018, 2019).

Interestingly, isolates from the *Staphylococcus* genus were only obtained from the rhizosphere of FD-symptomatic trees. Although *Staphylococcus* spp. have usually been studied for their role as opportunistic human pathogens, their presence in the soil and rhizosphere of several plant species has been previously recorded (Berg et al., 2005). Furthermore, *Staphylococcus* species may act as PGPR by enhancing plant mineral nutrition (Ipek et al., 2011), improving plant tolerance to salinity stress (Orhan, 2016), or by displaying antifungal activity (Sadfi-Zouaoui et al., 2008; Reverchon et al., 2019). Opportunistic pathogens are able to produce a wide range of antimicrobial compounds and *Staphylococcus* spp. may thus have been recruited by the plant upon infection by *F. kuroshium* (Berg et al., 2005). Altogether, these results indicate that potential biocontrol agents against FD may also be isolated from diseased trees, confirming the hypothesis stating that biocontrol agents could be selected from effective colonizers of the diseased rhizosphere and successful competitors of the disease-inducing pathogen (Ellis, 2017).

5. Conclusion

Our findings indicated that FD decreased the diversity and affected the structure of bacterial communities associated with the avocado rhizosphere. The observed shifts in community structure seemed to be caused by rare and exclusive OTUs, which may play a crucial role in plant health. Our results also showed that the culturable bacterial communities associated with the rhizosphere of both asymptomatic and symptomatic tree conditions were dominated by *Bacillus* and *Pseudomonas* spp., which are promising candidates for the biological control of FD. Future studies should aim at investigating the FD-induced changes in microbial functions at the rhizosphere level, in order to understand the implications of this emerging disease for soil ecological processes and orchard productivity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2021.100333.

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