# Tissue age, orchard location and disease management influence the composition of fungal and bacterial communities present on the bark of apple trees

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Originality significance statement: This is the first study to examine the effects of tissue age, orchard location, sampling time and disease management on the taxonomic composition of the bark-associated fungal and bacterial communities of apple bark. here we found that the introduction of scab-resistant apple cultivars, and the consequent reduction in the fungicide applications, partially changes the taxonomic composition of bark-associated microbiota .

## Summary

Plants host microbial communities that can be affected by environmental conditions and agronomic practices. Despite the role of bark as a reservoir of plant pathogens and beneficial microorganisms, no information is available on the effects of disease management on the taxonomic composition of the bark-associated communities of apple trees. We assessed the impact of disease management strategies on fungal and bacterial communities on the bark of a scab-resistant apple cultivar in two orchard locations and for two consecutive seasons. The amplicon sequencing revealed that bark age and orchard location strongly affected fungal and bacterial diversity. Microbiota dissimilarity between orchards evolved during the growing season and showed specific temporal series for fungal and bacterial populations in old and young bark. Disease management did not induce global changes in the microbial populations across locations and seasons, but specifically affected the abundance of some taxa according to bark age, orchard location and sampling time. Therefore, the disease management applied to scab-resistant cultivars, which is based on a limited use of fungicides, partially changed the taxonomic composition of bark-associated fungal and bacterial communities, suggesting the need for a more accurate risk assessment regarding possible pathogen outbreaks.

## Keywords

*Malus domestica*, bark microbiota, metabarcoding, integrated disease management, low-input disease management, scab-resistant apple cultivar

## Introduction

Plants are colonised by a wide range of microorganisms that can establish beneficial (mutualistic), detrimental (pathogenic) or neutral (commensal) interactions with their host (Hassani *et al.*, 2018). Some plant-associated microorganisms exert beneficial effects on plant growth and health by producing phytohormones, enhancing nutrient uptake and providing protection against stress (Berendsen *et al.*, 2012; Vorholt, 2012; Bulgarelli *et al.*, 2013). As a consequence of its multiple properties, the plant microbiota impacts plant fitness, crop production and fruit quality (Gilbert *et al.*, 2014; Zarraonaindia *et al.*, 2015; Hassani *et al.*, 2018). Microbial ecology is increasingly focussing attention on factors that can influence the composition of plant-associated microbial communities (Busby *et al.*, 2017) and their possible negative or positive effects on plant hosts (Mendes *et al.*, 2013). In particular, environmental conditions (Bokulich *et al.*, 2014; Shen *et al.*, 2018), tissue age (Vorholt, 2012; Arrigoni *et al.*, 2018) and plant genotype (Correa *et al.*, 2007; Whipps *et al.*, 2008; Arrigoni *et al.*, 2018) are key determinants of the microbiota taxonomic structure. For example, geographical location influences the structure and the composition of fungal and bacterial communities associated with grapevine (Bokulich *et al.*, 2014; Perazzolli *et al.*, 2014; Mezzasalma *et al.*, 2017; Vitulo *et al.*, 2019) and lettuce (Rastogi *et al.*, 2012). Environmental factors (e.g. temperature, relative humidity and precipitation) can shape the structure of plant-associated microbial communities and explain the taxonomic dissimilarity across locations (Bokulich *et al.*, 2014; Perazzolli *et al.*, 2014; Agler *et al.*, 2016; Campisano *et al.*, 2017). Moreover, agronomic practices can influence the microbial community structure and disease management affects the plant-associated microbiota of apple trees (Ottesen *et al.*, 2009, 2016; Yashiro and McManus, 2012; Leff and Fierer, 2013; Glenn *et al.*, 2015; Abdelfattah *et al.*, 2016; Wassermann *et al.*, 2019; Karlsson *et al.*, 2017). In particular, the microbial community composition of apple fruits (Leff and Fierer, 2013; Abdelfattah *et al.*, 2016; Wassermann *et*

*al.*, 2019) and leaves (Ottesen *et al.*, 2009, 2016; Yashiro and McManus, 2012; Glenn *et al.*, 2015) differed under organic and conventional disease management.

The apple (*Malus domestica*) is one the most important fruit crops worldwide and it represents the dominant fruit sector in Europe in terms of economic relevance (Forti and Henrard, 2016). The apple tree is therefore a valid host for investigating the dynamics of the associated microbiota and for analysing potential environmental and agronomic impacts on the taxonomic structure of the microbial communities. The majority of commercially relevant cultivars are susceptible to several destructive diseases, such as apple scab, powdery mildew and fire blight (Jones and Aldwinckle, 1990). Apple scab, caused by *Venturia inaequalis*, is the most important disease in temperate climates (Bowen *et al.*, 2011) and frequent fungicide treatments have to be applied for its control in integrated disease management (Soriano *et al.*, 2009; Belete and Boyraz, 2017). Anti-scab fungicides represent the largest fraction of apple disease management treatments (Didelot *et al.*, 2016) and the introduction of scab-resistant apple cultivars can considerably reduce fungicide applications (Ellis *et al.*, 1998; Simon *et al.*, 2011; Didelot *et al.*, 2016). However, the fungicides applied to control apple scab can have side effects on secondary pathogens, such as *Diplodia seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015; Arrigoni *et al.*, 2019) or the sooty blotch and flyspeck disease complex (Weber *et al.*, 2016). Therefore, the introduction of scab-resistant apple cultivars, with the consequent reduction in the use of fungicide sprays under low-input disease management, may cause the outbreak of secondary diseases (Warner, 1991; Ellis *et al.*, 1998). However, no information is available on the effects of the reduction in fungicide treatments on the taxonomic structure of apple bark-associated microbial communities.

Most of the studies on the impacts of disease management have focused on soil (Shade *et al.*, 2013a; Hartmann *et al.*, 2015), leaf (Leff and Fierer, 2013; Perazzolli *et al.*, 2014; Glenn *et al.*, 2015; Karlsson *et al.*, 2017) and fruit microbiota (Ottesen *et al.*, 2009, 2016; Yashiro and McManus, 2012; Jensen *et al.*, 2013; Leff and Fierer, 2013; McGarvey *et al.*, 2015;

Abdelfattah *et al.*, 2016; Wassermann *et al.*, 2019). Although bark is a reservoir of plant pathogens and beneficial microorganisms (Buck *et al.*, 1998; Martins *et al.*, 2013; Arrigoni *et al.*, 2018), the effect of disease management strategies on bark-associated microbial communities has not been investigated in apple trees. The aim of this study is to understand the effect of bark age, orchard location and disease management on the taxonomic structure of fungal and bacterial communities associated with the bark of a scab-resistant apple cultivar (namely Fujion) in two different locations and for two consecutive seasons.

## Results

### *Composition of bark-associated fungal and bacterial communities*

Fungal and bacterial communities of old (three/four-year-old shoots) and young bark (one-year-old shoots) were analysed in the two orchards (orchard 1 and orchard 2) at six time points over two consecutive seasons (from T0 to T5) under low-input and integrated disease management (Figs. S1 and S2; Tables S1, S2 and S3) and a total of 2,423 fungal (9,843,605 filtered sequences) and 7,856 bacterial (8,746,945 filtered sequences) sequence variants (SVs) were obtained (Fig. S3; Tables S4, S5 and S6). Of the fungal and bacterial SVs, 46.3 and 70.0% were assigned to taxa at family level, whereas 36.6 and 44.8% were assigned to taxa at genus level, respectively. Bark fungal communities were dominated by SVs belonging to *Aureobasidium*, *Leptosphaeria*, *Tumularia*, *Davidiella*, *Alternaria*, *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* in terms of both relative (Fig. S4A) and absolute abundance, as assessed by quantitative real-time PCR (qPCR; Fig. S4B). Dominant bacterial SVs belonged to *Frondihabitans*, *Deinococcus*, *Amnibacterium*, *Hymenobacter*, *Sphingomonas*, *Kineococcus* and *Curtobacterium*, in terms of relative (Fig. S4C) and absolute abundance as assessed by qPCR (Fig. S4D).

Principal coordinate analysis (PCoA) and principal component analyses (PCA) classified fungal (Figs. 1A, 1B, S5A and S5B) and bacterial (Figs. 1E, 1F, S5E and S5F)

samples according to bark age and orchard location on the first and second axis, respectively. Conversely, fungal and bacterial communities did not cluster according to disease management and sampling time in the PCoA (Figs. 1C, 1D, 1G and 1H) and PCA (Figs. S5C, S5D, S5G and S5H) analysis, suggesting that these two factors have limited effects on bark-associated fungal and bacterial communities. The permutational multivariate analyses of variance (PERMANOVA) on Bray-Curtis dissimilarities and weighted UniFrac distances showed significant differences in fungal and bacterial communities according to bark age ($p < 1.0 \times 10^{-4}$), orchard location ($p < 1.0 \times 10^{-4}$) and sampling time ($p < 1.0 \times 10^{-4}$), but not according to disease management ($p > 0.05$; Table S7). Likewise, culturable fungal and bacterial data clustered according to bark age and orchard location, but not according to disease management and sampling time (Figs. S5I, S5J and S6), with significant effects observed for bark age ($p = 9.7 \times 10^{-4}$ and $p = 3.9 \times 10^{-4}$, respectively) and orchard location for old bark ($p = 8.1 \times 10^{-4}$ and $p = 5.2 \times 10^{-6}$, respectively) on generalised linear models (GLMs; Table S7). Having identified bark age and orchard location as major drivers of fungal and bacterial communities, changes on temporal dynamics and differential abundance of fungal and bacterial taxa were investigated specifically for bark age, orchard location, disease management and sampling time, as reported in the following paragraphs.

*Bark age affected the taxonomic structure and temporal dynamics of bark-associated fungal and bacterial communities*

Differences between old and young bark were found in temporal series of alpha diversity (estimated with the Shannon index) and richness (expressed as the number of observed SVs) at the first four and at the last three time points for fungal and bacterial communities, respectively (Fig. 2). In particular, fungal alpha diversity and richness were higher in old bark than in young bark (Figs. 2A and 2B), according to generalised additive models (GAMs; $p < 2 \times 10^{-16}$ and $p < 2 \times 10^{-16}$, respectively). More specifically, deviation curves (Figs. 2A and 2B)

and GLMs (Table S8) indicated higher fungal alpha diversity and richness in old bark than in young bark at all time points, except richness at T5. Bacterial alpha diversity and richness were higher in old bark than in young bark according to GAMs ($p = 4.78 \times 10^{-16}$ and $p = 5.13 \times 10^{-16}$, respectively), with major differences at late time points, according to deviation curves (Figs. 2C and 2D) and GLMs (Table S8).

Fungal and bacterial communities were dominated by the Ascomycota and Actinobacteria phyla, *Dothioraceae*, *Microbacteriaceae* and *Cytophagaceae* families (Figs. S7A and S7B; Tables S9 and S10). SVs assigned to *Capronia*, *Devriesia*, *Exophiala*, *Leptosphaeria, Rhodotorula* and *Tumularia* showed higher relative abundance in old bark than in young bark at T2 and T5, whereas SVs assigned to *Aureobasidium, Bulleromyces, Cryptococcus* and *Filobasidium* showed lower relative abundance in old bark than in young bark (Fig. S7C; Table S11). *Davidiella* and *Sporobolomyces* included some SVs with increased and other SVs with decreased relative abundance in old bark compared to young bark, as a possible differential adaptation of species belonging to the same genus. Bacterial taxa included SVs with higher relative abundance in old bark than in young bark, such as *Amnibacterium*, *Deinococcus*, *Friedmanniella, Modestobacter*, *Mucilaginibacter*, *Nocardioides*, *Novosphingobium, Sphingomonas* and *Spirosoma* (Fig. S7D; Table S12). As found for fungal SVs, some bacterial genera (e.g. *Hymenobacter, Massilia* and *Methylobacterium*) included some SVs with increased and others with decreased relative abundance in old bark compared to young bark.

*Orchard location affected the taxonomic structure and temporal dynamics of bark-associated fungal and bacterial communities*

Orchard location affected microbial populations of apple bark and the dissimilarity of fungal populations between the two orchards was higher in old bark than in young bark (GAMs: $p < 2 \times 10^{-16}$, Fig. 3A). Temporal series of fungal communities showed a stable profile in old bark

and two peaks in young bark, as a possible adaptation of old bark-associated communities to the environmental conditions. For bacterial communities, the temporal series of the dissimilarity between the two orchards showed a peak at T2 and it was lower in old bark than in young bark (GAMs: $p < 2 \times 10^{-16}$; Fig. 3B). Moreover, the dissimilarity between old and young bark was higher in orchard 2 than in orchard 1 for the majority of the time points for both fungal (Fig. 3C) and bacterial (Fig. 3D) communities.

Clusters of orchard-specific genera were found in the heatmap of fungal relative abundance (Fig. S8A) and taxa known to be affected by geographical location, such as Eurotiomycetes, Pleosporales and *Davidiella*; (Bokulich *et al.*, 2014; Shen *et al.*, 2018), differed in relative abundance between orchard 1 and orchard 2 (Table S13A). Clusters of orchard-specific genera cannot be easily found in the bacterial heatmap (Fig. S8B), but the relative abundance of seven and 17 taxa was higher and lower in orchard 2 than in orchard 1, respectively (Table S13B). The relative abundance of Proteobacteria and Bacteroidetes, which were affected by the environmental temperature in grapevine (Campisano *et al.*, 2017), differed in orchard 1 and orchard 2 (Table S13B). Likewise, the relative abundance of possible drought-affected *Curtobacterium* and Rhizobiales taxa (Naylor *et al.*, 2017; Santos-Medellín *et al.*, 2017) varied according to orchard location.

*Disease management affects the taxonomic structure of bark-associated fungal and bacterial communities*

Significant changes in relative abundance between low-input and integrated disease management were observed for some taxa according to bark age and orchard location, at specific time points as compared to T0 ($p \leq 0.05$). In particular, for fungal communities (Fig. 4; Table S14), an increase in the relative abundance of SVs belonging to Ascomycota (SV_24), *Diaporthe* (SV_156) and Pleosporales (SV_34) was found under low-input disease management compared to integrated disease management at more than three time points. The

relative abundance of *Bullera* (SV_91) and *Filobasidium* (SV_36) decreased under low-input disease management compared to integrated disease management at two time points. The relative abundance of *Alternaria* (SV_184), *Devriesia* (SV_53) and *Diaporthe* (SV_156) was higher under low-input disease management than under integrated disease management, whereas that of *Aureobasidium* (SV_309), *Filobasidium* (SV_36) and *Sporobolomyces* (SV_31 and SV_151) was lower.

Disease management affected the bacterial community structure according to bark age and orchard location, and changes occurred mainly in orchard 2 (Fig. 5; Table S15). In particular, the relative abundance of 19 SVs of *Sphingomonas* and of 14 SVs of *Hymenobacter* decreased under low-input disease management compared to integrated disease management in young bark. Contrasting relative abundance profiles were observed for some SVs belonging to the same genus (*Amnibacterium*, *Curtobacterium*, *Hymenobacter*, *Kineococcus*, *Massilia*, *Pseudomonas* and *Roseomonas*), as a consequence of possible differential adaptation of bacterial species to disease management. Although potential functions can be only partially hypothesised by taxonomic identification at genus level, the relative abundance of potential apple pathogens (*Alternaria* and *Diaporthe*) and potential plant pathogens (*Devriesia*) increased under low-input disease management compared to integrated disease management, whereas that of some SVs belonging to potential biocontrol genera decreased (*Aureobasidium*, *Filobasidium*, *Methylobacterium*, *Sphingomonas* and *Sporobolomyces*; Figs. 5 and 6).

Absolute abundance data assessed by qPCR, confirmed the disease management-related changes of fungal and bacterial communities, highlighting decreases in the abundance of SVs belonging to *Rhodotorula* (SV_893) and *Sphingomonas* (SV_79, SV_89, SV_132 and SV_214) and increases in the abundance of SVs belonging to *Cryptococcus* (SV_514) and *Curtobacterium* (SV_799 and SV_2547) under low-input disease management compared to integrated disease management (Table S16).

## Discussion

The bark of perennial crops can act as a possible reservoir of pathogenic and beneficial microorganisms (Buck *et al.*, 1998; Martins *et al.*, 2013; Arrigoni *et al.*, 2018) and plant-associated microbial communities are shaped by environmental factors (Bokulich *et al.*, 2014; Glenn *et al.*, 2015; Abdelfattah *et al.*, 2016; Shen *et al.*, 2018). Since the effects of disease management strategies on plant-associated microorganisms are highly variable (Ottesen *et al.*, 2009; Leff and Fierer, 2013; Perazzolli *et al.*, 2014; Glenn *et al.*, 2015; Abdelfattah *et al.*, 2016; Vitulo *et al.*, 2019; Wassermann *et al.*, 2019), the impact of low-input disease management on apple trees cannot be easily inferred from results obtained on other crops. We therefore assessed the effect of tissue age, orchard location, seasonality and disease management on the composition of bark-associated fungal and bacterial communities of a scab-resistant apple cultivar. As previously shown for apple (He *et al.*, 2012; Glenn *et al.*, 2015; Arrigoni *et al.*, 2018; Shen *et al.*, 2018), blackcurrant (Vepštaitė-Monstavičė *et al.*, 2018), European beech (Cordier *et al.*, 2012) and grapevine (Bokulich *et al.*, 2014) tissues, we found that apple bark was dominated by *Alternaria, Aureobasidium, Cryptococcus, Davidiella, Leptosphaeria, Rhodotorula* and *Sporobolomyces*. Bacterial communities were dominated by the *Amnibacterium, Curtobacterium, Deinococcus, Frondihabitans, Hymenobacter* and *Sphingomonas* genera and have been previously found on apple bark (Arrigoni *et al.*, 2018), flower (Shade *et al.*, 2013b) and leaf (Glenn *et al.*, 2015), confirming that bark is a reservoir of complex microbial communities (Buck *et al.*, 1998; Martins *et al.*, 2013; Arrigoni *et al.*, 2018; Vitulo *et al.*, 2019). In particular, fungal and bacterial SVs assigned to genera with beneficial and detrimental properties were found, such as potential apple pathogens (e.g. *Alternaria, Diaporthe, Diplodia* and *Phoma*), potential pathogens of other plants (e.g. fungi: *Devriesia, Entyloma, Leptosphaeria*; bacteria: *Ralstonia* and *Rathayibacter*) and potential biocontrol agents (e.g. fungi: *Aureobasidium, Cryptococcus,*

*Filobasidium*, *Rhodotorula*, *Sporobolomyces*; bacteria: *Methylobacterium*, *Nocardioides* and *Sphingomonas*). The potential properties of bark-associated microorganisms were assigned according to the possible interactions of the identified genera with plant hosts, but the same taxon can also include potential pathogenic and/or beneficial microorganisms of the same or other species (e.g. *Curtobacterium*, *Pantoea* and *Pseudomonas*). Unfortunately, the amplicon sequencing approach allowed poor taxonomic resolution and only taxonomic identifications at the genus level were used. Therefore, it is not possible to infer the precise properties of plant-associated microorganisms and only potential functions can be hypothesised by this study, indicating that further functional and molecular analyses are required to identify plant-associated microorganisms at deep taxonomic resolution.

Bark age was one of the major drivers of the richness, diversity and taxonomic composition of bark-associated communities. Alpha diversity and richness of fungal and bacterial communities were generally higher in old bark than in young bark and these differences have been associated with the rough bark surface, which may allow water retention and protection from adverse conditions (Arrigoni *et al.*, 2018). Bark hosts a greater bacterial diversity and richness than fruits and leaves (Martins *et al.*, 2013; Morrison-Whittle *et al.*, 2017; Vitulo *et al.*, 2019), indicating that it is a stable microbial habitat in perennial plants (Vitulo *et al.*, 2019). Therefore, the long permanence over time of the old bark possibly leads to the establishment of a diversified microbial community through dispersal, drift and selection processes, as described for annual plants (Dini-Andreote and Raaijmakers, 2018).

In addition to bark age, the taxonomic structure of bark-associated fungal and bacterial communities differed according to the geographic location, as previously found on apple fruits (Shen *et al.*, 2018; Vepštaitė-Monstavičė *et al.*, 2018), grapevine bark (Vitulo *et al.*, 2019) and grapevine fruits (Mezzasalma *et al.*, 2018; Morrison-Whittle and Goddard, 2018). In particular, the orchard location influenced the relative abundance of those fungal and bacterial genera that were previously shown to be affected by i) geographical location, such as

Eurotiomycetes and *Davidiella* (Bokulich *et al.*, 2014), Pleosporales (Shen *et al.*, 2018), Rhodospirillales and Enterobacteriales (Mezzasalma *et al.*, 2017); ii) temperature, such as Proteobacteria and Bacteroidetes (Campisano *et al.*, 2017), and iii) drought, such as *Curtobacterium* and Rhizobiales (Naylor *et al.*, 2017; Santos-Medellín *et al.*, 2017). Although potential microbial functions can be only partially hypothesised by taxonomic identification at the genus level, the relative abundance of fungal genera comprising potential apple pathogens (*Alternaria*, *Diaporte*, *Diplodia*, *Erwinia* and *Phoma*) and potential biocontrol agents (*Filobasidium*, *Paraconiothyrium*, *Rhodotorula* and *Sporobolomyces*) differed in the two orchards. However, the effects of orchard location were dependent on bark age and seasonal fluctuations. In particular, the dissimilarity between the two orchards was higher for fungal and lower for bacterial communities in old bark than in young bark, suggesting a differential adaptation of eukaryotes and prokaryotes.

In agreement with previous observations on organic- and conventionally-managed apple leaf (Glenn *et al.*, 2015) and fruit (Ottesen *et al.*, 2009, 2016; Abdelfattah *et al.*, 2016), the effect of disease management was associated with changes in the relative abundance of just some specific taxa, rather than with global changes in the microbial populations. In particular, the relative abundance of *Alternaria*, *Davidiella* and *Cryptococcus* increased under low-input disease management, as previously observed under biodynamic management of grapevine (Morrison-Whittle *et al.*, 2017). Likewise, the *Methylobacteriaceae* and *Nocardioidaceae* families were affected by disease management, in agreement with previous observations on apple fruits (Leff and Fierer, 2013). More specifically, relative abundance profiles suggested an increase in potential apple pathogens (*Alternaria* and *Diaporthe*) and a decrease in some potential biocontrol agents (*Aureobasidium*, *Filobasidium*, *Methylobacterium*, *Sphingomonas* and *Sporobolomyces*) under low-input disease management. Moreover, low-input disease management indicated an increase in the relative abundance of SVs belonging to other potential biocontrol genera (*Cryptococcus*, *Nocardioides* and *Pedobacter*), suggesting

possible effects on the equilibrium between potential pathogenic and potential beneficial microorganisms that will possibly require further investigation in the future.

To conclude, we found that fungal and bacterial communities residing on apple tree bark were influenced primarily by tissue age, orchard location and sampling time. Low-input disease management did not affect the structure of bark fungal and bacterial communities globally, but it did affect the abundance of some taxa according to bark age, orchard location and sampling time. The introduction of scab-resistant apple cultivars, and the consequent reduction in the use of fungicide treatments, may partially change the taxonomic composition of bark-associated fungal and bacterial communities. These results also suggest the need for a more accurate risk assessment and monitoring in orchards under low-input disease management, in order to predict the risk of pathogen outbreaks and to implement suitable surveillance procedures.

## Experimental Procedures

### *Experimental design*

Apple plants of the scab-resistant cultivar 'Fujion' were analysed in two experimental orchards in northern Italy, namely orchard 1 and orchard 2 (Fig. S1; Table S1). The two locations were chosen because they are characterised by an average two week-delay in the phenological phases of orchard 2 as compared to orchard 1 (Tables S2 and S3). The integrated and a low-input disease management strategies for apple scab control were applied to each orchard in two separated field plots located 100 m apart from each other, for two consecutive seasons (2016 and 2017) starting from T0 (orchard 1, 24 March 2016; orchard 2, 30 March 2016; Fig. S1, Tables S2 and S3). The integrated and low-input disease management practices adopted were those typically applied to scab-susceptible (Belete and Boyraz, 2017) and scab-resistant cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016) in commercial orchards, respectively. The number of treatments per season ranged from 12 (orchard 2, 2017) to 24

(orchard 1, 2016) and from five (orchard 2, 2017) to seven (orchard 1 and orchard 2, 2016) for integrated and low-input disease management respectively, according to orchard location and season. Daily maximum and minimum temperature and rainfall values were recorded in both orchards by meteorological stations located nearby (Fig. S2; Tables S2 and S3).

*Sample collection, apple bark microbiota isolation and sequencing*

Samples were collected at six time points (form T0 to T5) over two consecutive seasons (2016 and 2017) from each location (orchard 1 and orchard 2) and for each disease management strategy (low-input and integrated disease management), corresponding to the phenological phases (Chapman and Catlin, 1976) of i) pink cluster (T0 and T3), ii) 30 mm of fruit diameter (T1 and T4) and iii) two weeks before harvest (T2 and T5) in 2016 and 2017, respectively. From each orchard location, disease management strategy and time point, bark samples were collected in triplicate (named from 1 to 3) and each replicate consisted of a pool of five randomly selected plants (plant pool) collected according to a split-plot sampling design, as previously reported (Arrigoni *et al.*, 2018). From each plant, samples were collected from randomly chosen barks of three/four-year-old shoots (old bark) or one-year-old shoots (young bark). Each sample consisted of 30 bark curls (0.5 g) that were ground into sterile stainless steel jars with 2.5 ml of cold sterile 0.85% NaCl solution as previously described (Arrigoni *et al.*, 2018). The viability of culturable fungi and bacteria was assessed using the classical plating method and the number of bacterial and fungal colony-forming units (CFUs) per gram of bark fresh weight (CFUs/g) was determined on selective media (Arrigoni *et al.*, 2018).

DNA extraction, amplification of the fungal internal transcribed spacer 2 (ITS2; primer ITS3 forward 5′-CATCGATGAAGAACGCAG-3′ and ITS4 reverse 5′-TCCTSSSCTTATTGATATGC-3′) and bacterial V5-V7 region of 16S rDNA (primer 799 forward 5′-AACMGGATTAGATACCCKG-3′ and 1175 reverse 5′-ACGTCRTCCCCDCCTTCCT-3′), DNA purification, indexing, quantification and library

preparation for the Illumina MiSeq sequencing (PE300) were carried out as previously described (Arrigoni *et al.*, 2018). Fungal and bacterial sequences the of the 144 samples [two tissue ages (old and young bark), two orchards (orchard 1 and orchard 2), two disease managements (low-input and integrated) six time points (from T0 to T5) and three replicates] were deposited at the Sequence Read Archive of NCBI (https://www.ncbi.nlm.nih.gov/bioproject) under the BioProject number PRJNA495750.

*Absolute bacterial and fungal quantification using quantitative real-time PCR*

The total amount of fungal and bacterial DNA in bark samples was determined by qPCR as previously reported (Stefanini *et al.*, 2017). The same primer pairs used for ITS2 amplification and sequencing were used for fungal quantification, while the V7-V8 region of 16S rDNA was amplified using the primer 1175 forward (5'-AGGAAGGHGGGGAYGACGT-3') and 1392 reverse (5'-ACGGGCGGTGTGTRC-3') (Hanshew *et al.*, 2013) for bacterial quantification. For the latter, the forward qPCR primer was in common with the reverse primer of sequencing and the reverse qPCR primer differed from that used for sequencing in order to ensure the homogeneity of the amplified DNA length (Stefanini *et al.*, 2017). Standard curves were constructed using serial dilutions of PCR products ($2.0 \times 10^{1}$; $2.0 \times 10^{0}$, $2.0 \times 10^{-1}$; $2.0 \times 10^{-2}$ and $2.0 \times 10^{-3}$ ng/µl) of the ITS and 16S rRNA region, purified by NucleoSpin Gel Cleanup purification kit (Macherey-Nagel, Germany) and quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) with a Qubit 2.0 fluorometer (Thermo Fisher Scientific). The qPCR assay was carried out using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Roche, Germany) with 0.30 µM each primer and 2 µl DNA template in a total volume of 20 µl. Amplification was performed using a Roche Light Cycler 480 (Roche) with the following program: 95°C for 3 min; 35 cycles of 95°C for 3 sec, 59°C for 30 sec and a melting analysis from 60°C to 95°C to determine the amplification specificity. Absolute fungal and bacterial abundances were

then calculated as the product of SV relative abundance and the total fungal and bacterial DNA amount (ng/mg) detected in the sample, respectively (Stefanini *et al.*, 2017).

*Bioinformatic analysis*

Sequences were processed using the open-source MICCA (v1.7.0) software (Albanese *et al.*, 2015). Briefly, raw forward and reverse ITS sequences were truncated at 250 bp and were merged (Edgar and Flyvbjerg, 2015). Overlapping paired-end reads with an overlap length smaller than 60 bp and with more than 15 mismatches were discarded. Merged reads shorter than 275 bp and with an error rate higher than 0.50% were removed. Filtered fungal sequences were denoised using the UNOISE algorithm (Edgar, 2016). Denoising methods were applied in order to correct sequencing errors (Callahan *et al.*, 2016; Amir *et al.*, 2017; Nearing *et al.*, 2018) and determine real biological sequences at single nucleotide resolution by generating amplicon SVs (Amato *et al.*, 2018). Fungal SVs were taxonomically classified using the Ribosomal Database Project (RDP) Classifier v2.11 and the UNITE database (Kõljalg *et al.*, 2005) using the default confidence of 80%. SVs were aligned against the UNITE database (clustered at 85%, release 2017/12/01) using VSEARCH v2.3.4 (Rognes *et al.*, 2016) and SVs with no hit exceeding a 75% similarity threshold with the UNITE sequences (i.e. all taxonomic ranks) were discarded. For bacterial data, raw forward and reverse 16S reads were truncated at 250 bp and merged (Edgar and Flyvbjerg, 2015). After forward and reverse primer trimming, merged reads shorter than 360 bp and with an error rate higher than 0.75% were removed. Filtered bacterial sequences were denoised with UNOISE (Edgar, 2016) and the resulting SVs were taxonomically classified using the RDP Classifier v2.11 (Wang *et al.*, 2007) using the default confidence of 80%. After taxonomic classification, SVs corresponding to chloroplasts, green algae and plants were manually discarded. Multiple sequence alignment was performed on fungal and bacterial SVs using MUSCLE v3.8.31 (Edgar, 2004) and phylogenetic tree was inferred using FastTree v2.1.8 (DeSantis *et al.*, 2006; Price *et al.*, 2010). Each fungal and

bacterial genus was classified as potential apple pathogen, potential pathogen of other plants, potential biocontrol agent, potential plant growth promoter or as genus with potential neutral effects on plants according to literature search on functional properties of the comprised species, as previously reported (Arrigoni *et al.*, 2018). Genera comprising both potential pathogenic and potential beneficial properties were assigned to more than one class. Potential classes, references and a brief description of the comprised species are reported for each bacterial and fungal genus (Tables S11-S16).

*Statistical analysis*

Statistical analyses were performed using R v3.4.2, 'phyloseq' v1.25.3 (McMurdie and Holmes, 2013) and 'vegan' v2.5-2 packages (Dixon, 2003). Samples were rarefied without replacement at 15000 (ITS) and 30000 (16S) reads per sample (rarefied data). PCoA and PCA were carried out using the R package 'vegan', in order to identify the major drivers of the fungal and bacterial taxonomic structure on the first and second axis. Likewise, heatmaps were plotted using the R package 'pheatmaps' and hierarchical clustering was computed on the base 10 logarithm of relative abundance and CFUs/g values, using euclidean distance and complete linkage, in order to visualise the major drivers on culturable microbial populations.

PERMANOVA was performed with the 'adonis' function available in the R package 'vegan' (9,999 permutations) on Bray-Curtis dissimilarities and weighted UniFrac distances to verify the significant differences on fungal and bacterial taxonomic structure found in the PCA and PCoA analysis (design formula: 'distance ~ bark age × orchard location × disease management' and 'distance ~ bark age × orchard location × time point'). Culturable fungal and bacterial data was analysed using GLMs available in the R package 'stats' (quasi-Poisson family), testing the effect of location within each bark age on CFUs/g values (design formula 'CFUs/g ~ bark age + bark age : location'), in order to verify the significant differences on the major drivers found in the heatmap analysis.

After identification of the major drivers of the fungal and bacterial taxonomic structure, GAMs fitted on temporal series was used to assess bark age and orchard location effects according to the sampling time. More specifically, GAMs was used on rarefied data to assess the effect of bark age on alpha diversity (Shannon index) and richness (number of observed SVs) and to assess dissimilarities (distance) between the two orchard locations on temporal series. GAMs were trained using the R package 'mgcv' with the restricted maximum likelihood (REML) estimator (Pinheiro and Bates, 2009; Saw *et al.*, 2017). Deviation curves represent the deviations of the young barks from the overall time-richness relationships. Given a time point, the deviation is significant if the 95% confidence interval does not include the zero value (Saw et al., 2017). The negative binomial family was used for the number of observed SVs, whereas the gaussian family was applied for the Shannon alpha diversity and for the dissimilarity between the two orchard locations. GLMs restricted to the time points T2 and T5 were used on rarefied data to test the effect of bark age on the observed SVs and Shannon alpha diversity subtracting the baseline for each plant pool (replicate; design formula: 'alpha index ~ plant pool + bark age').

The differential abundance test was carried out by the R package DESeq2 (Love *et al.*, 2014) using the non-rarefied data (original counts), as suggested in (McMurdie and Holmes, 2014). *P*-values were false discovery rate (FDR)-corrected using the Benjamini–Hochberg procedure implemented in DESeq2, in order to assess significant effects ($p \leq 0.05$) of bark age (bark age test), orchard location (orchard location test) and disease management (disease management test) on SV relative and absolute abundance. More precisely, in the 'bark age test' (at time points T2 and T5), only SVs with at least five read counts (Love *et al.*, 2014) in more than six samples were considered. The effect of bark age was tested subtracting the baseline for each plant pool (paired test, design formula: 'relative abundance ~ plant pool + bark age'). The 'orchard location test' was performed at genus level and only genera with at least ten read counts in more than 18 samples were considered. The effect of location was

tested subtracting the baseline for each bark age (paired test, design formula: 'relative abundance ~ bark age + location'). The 'disease management test' was performed at SV level to identify taxa whose change of relative or absolute abundances with time (with respect to T0) was dependent on disease management (design formula: 'relative abundance ~ disease management + time point + disease management : time point') on SVs with at least ten normalised counts in more than 18 samples. Analysis design formulas reported above are given using the R syntax for the 'formula' function.


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## Conflict of interest

The authors declare that they have no conflict of interest

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693 **Figure legends**

694

695 **Fig. 1.** Principal coordinate analysis (PCoA) of bark-associated fungal and bacterial
696 communities. PCoA is reported for A-D, fungal and E-H, bacterial communities of bark
697 collected from three/four-year-old shoots (old) and one-year-old shoots (young) in orchard 1
698 and orchard 2 at six time points over two consecutive seasons (from T0 to T5) under low-
699 input and integrated disease management. The fungal and bacterial PCoA is reported with
700 different colours to highlight effects of: A and E, bark age; B and F, orchard location; C and
701 G, disease management and D and H, sampling time. Significant effects of bark age (old *vs.*
702 young bark), orchard location (orchard 1 *vs.* orchard 2) and sampling time were found ($p < 1$
703 $\times 10^{-4}$; Table S7).

704

705 **Fig. 2.** Temporal series of alpha diversity and richness of bark-associated fungal and bacterial
706 communities. Temporal series of A and B, fungal and C and D, bacterial alpha diversity
707 (estimated with Shannon index) and richness (expressed as number of observed sequence
708 variants, SVs) are reported for bark samples collected from three/four-year-old shoots (old)
709 and one-year-old shoots (young) in two orchards at six time points over two consecutive
710 seasons (from T0 to T5). Data of low-input and integrated disease management were
711 considered and generalised additive models (GAMs) were fitted on temporal series for old
712 and young bark. The plot below each chart indicates the GAM deviation curve (black line)
713 with the 95% confidence interval (grey area). Given a time point, the deviation is significant
714 if the 95% confidence interval does not include the zero value (dotted line). GAMs revealed
715 significant differences between old and young bark in the alpha diversity (fungi: $p < 2 \times 10^{-16}$,
716 bacteria: $4.78 \times 10^{-16}$) and richness (fungi: $p < 2 \times 10^{-16}$, bacteria: $p = 5.13 \times 10^{-12}$).
717 Significant differences between old and young bark were assessed at T2 and T5 using

718 generalised linear models (GLMs, Table S8). Fungal and bacterial taxa with significant
719 changes in relative abundance between old and young bark are listed in Tables S11 and S12.
720
721 **Fig. 3.** Dissimilarity of bark-associated fungal and bacterial communities between the two
722 orchard locations. Temporal series of A, fungal and B, bacterial dissimilarity between the two
723 orchard locations (orchard 1 *vs.* orchard 2) are reported for bark collected from three/four-
724 year-old shoots (old) and one-year-old shoots (young) at six time points (from T0 to T5) in
725 two consecutive seasons. Data of low-input and integrated disease management were
726 considered and generalised additive models (GAMs) of dissimilarity between orchard 1 and
727 orchard 2 were fitted on temporal series. GAMs revealed significant differences between the
728 temporal series of old and young bark in the fungal ($p < 2 \times 10^{-16}$) and bacterial communities
729 ($p < 2 \times 10^{-16}$). The plot below each chart indicates the GAM deviation curve (black line) with
730 the 95% confidence interval (grey area). Given a time point, the deviation is significant if the
731 95% confidence interval does not include the zero value (dotted line). C, fungal and D,
732 bacterial dissimilarity between the old and young bark was assessed for each orchard location
733 and time point and significant differences between orchard 1 and orchard 2 are reported
734 (asterisks) for each time point according to generalised linear models (GLMs; $p \leq 0.05$). Taxa
735 with significant changes in relative abundance between orchard 1 and orchard 2 are listed in
736 Table S13.
737
738 **Fig. 4.** Fungal sequence variants affected by disease management. Phylogenetic trees of
739 fungal sequence variants (SVs) with significant changes in relative abundance between low-
740 input and integrated disease management in bark collected from A and C, three/four-year-old
741 shoots (old) and B and D, one-year-old shoots (young) in orchard 1 and orchard 2. Changes in
742 relative abundance were assessed for each time point (circle from T1 to T5) as compared to
743 T0 and significantly stronger increase (blue scale) or decrease (red scale) under low-input

744 disease management compared to integrated disease management are indicated for each time
745 point and SV (differential abundance test; $p \leq 0.05$). Coloured dots indicate SVs with
746 taxonomic classification. Fungal genera comprising potential apple pathogens (AP), plant
747 pathogens (PP) and biocontrol agents (BC) are specified in the legend. Only SVs with
748 significant changes in at least one time point, orchard location and tissue age are shown
749 (Table S14).
750
751 **Fig. 5.** Bacterial sequence variants affected by disease management. Phylogenetic trees of
752 bacterial sequence variants (SVs) with significant changes in relative abundance between low-
753 input and integrated disease management in bark collected from three/four-year-old shoots
754 (old) and one-year-old shoots (young) in orchard 1 and orchard 2. Changes in relative
755 abundance were assessed for each time point (circle from T1 to T5) as compared to T0 and
756 significantly stronger increase (blue scale) or decrease (red scale) under low-input disease
757 management compared to integrated disease management are indicated for each time point
758 and SV (differential abundance test; $p \leq 0.05$). Coloured dots indicate SVs with taxonomic
759 classification. Bacterial genera comprising potential apple pathogens (AP), plant pathogens
760 (PP), biocontrol agents (BC) and plant growth promoters (PG) are specified in the legend.
761 Only SVs with significant changes in at least one time point, orchard location and tissue age
762 are shown (Table S15).
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765 **Supporting Information**

766 Additional Supporting Information may be found in the online version of this article at the
767 publisher's web-site:

768

769 **Fig. S1.** Orchard locations studied in this work.

770 **Fig. S2.** Climatic conditions and disease management strategies.

771 **Fig. S3.** Fungal and bacterial DNA amount.

772 **Fig. S4.** Relative and absolute abundances of dominant fungal and bacterial sequence
773 variants.

774 **Fig. S5.** Principal component analysis (PCA) of bark-associated communities and heatmap of
775 culturable fungi and bacteria.

776 **Fig. S6.** Culturable bark-associated fungi and bacteria.

777 **Fig. S7.** Overview of bark-associated fungal and bacterial phyla, families and genera.

778 **Fig. S8.** Heatmap of bark-associated fungal and bacterial communities.

779

780 **Table S1.** Geographical location and characteristics of the orchard 1 and orchard 2.

781 **Table S2.** Disease management and climatic data of the 2016 season.

782 **Table S3.** Disease management and climatic data of the 2017 season.

783 **Table S4.** Fungal sequence variants (SVs) of apple bark.

784 **Table S5.** Bacterial sequence variants (SVs) of apple bark.

785 **Table S6.** Summary of total filtered sequences and observed sequence variants obtained for
786 fungi and bacteria of apple bark.

787 **Table S7.** Effect of bark age, orchard location, disease management and sampling time on the
788 diversity of fungal and bacterial communities and on culturable fungi and bacteria.

789 **Table S8.** Effect of bark age on alpha diversity and richness

790 **Table S9.** Taxonomic overview of bark-associated fungal communities.

**Table S10.** Taxonomic overview of bark-associated bacterial communities.

**Table S11.** Fungal taxa comprising sequence variants with significant changes in relative abundance between bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark).

**Table S12.** Bacterial taxa comprising sequence variants with significant changes in relative abundance between bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark).

**Table S13.** A, fungal and B, bacterial taxa with significant changes in relative abundance between orchard 1 and orchard 2.

**Table S14.** Fungal sequence variants with significant changes in relative abundance between low-input and integrated disease management.

**Table S15.** Bacterial sequence variants with significant changes in relative abundance between low-input and integrated disease management.

**Table S16. A**, fungal and **B**, bacterial sequence variants with significant changes in absolute abundance between low-input and integrated disease management.