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The drivers of vine-plant root microbiota endosphere composition include both abiotic and plant-specific factors

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ABSTRACT

Microorganisms associated with plants are determinant for their fitness, but also in the case of vine grapes, for the quality and quantity of the wine. Plant microbiota is, however highly variable in space despite deterministic recruitment from the soil reservoir. Therefore, understanding the drivers that shape this microbiota is a key issue. Most studies that have analysed microorganisms associated with vines have been conducted at large scales (e.g., over 100 km) and have analysed the bulk soil and the rhizosphere. In this study, we focused on the root-microbiota endosphere, the most intimate fraction of microorganisms associated with plants. We sampled vine roots in 37 fields distributed throughout a vineyard to investigate drivers shaping the grapevine microbiota at the α- (i.e., within-field) and γ- (i.e., between-field) diversity scales. We demonstrated that vine endospheric microbiota differed according to both the edaphic and plant-specific parameters including cultivar type and age. This work supports the idea of an existing *microbial terroir* occurring within a domain and offers a new perspective for winemakers to include the *microbial terroir* in their management practices.

KEYWORDS

root microbiota, endosphere, vineyard, environmental factors, plant-specific factors, small scales

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4616

INTRODUCTION

Microorganism-plant interactions are the basis of fertility ecosystem services (e.g., van der Heijden *et al.*, 2008). Indeed, microorganisms associated with their host plant (i.e., plant microbiota) affect not only plant mineral and hydric nutrition but also plant health, by improving plant resistance to drought and pathogenic attacks (e.g., Rolli *et al.*, 2015; Vandenkoornhuyse *et al.*, 2015; Compant *et al.*, 2019). Microbiota can then deeply affect the fitness of plants. In the case of grapevines, microorganism composition has been shown to not only influence plant growth and development but also the quality and the quantity of the resulting wine, for instance by shaping the volatile compound profiles in grapes (Knight *et al.*, 2015; Belda *et al.*, 2017). Microorganisms are recruited from the soil microbial reservoir by the plant to form the rhizosphere from which a fraction of the microorganisms can colonise the inner part of the plant (i.e., the endosphere) (Vandenkoornhuyse *et al.*, 2015). This process has been suggested to be mainly determinist (Morrison-Whittle and Goddard, 2015). Microorganism distribution is still highly variable from one place to the other and understanding the drivers of microorganism variability is, therefore, a key issue for wine production. So far, drivers shaping microbial distribution in vineyards have been analysed using the biogeography framework (*sensu* MacArthur & Wilson, 1967). Analyses have thus focused on large geographical scales of more than 100 km, in continental and inter-regional studies. These studies have analysed different plant compartments (root, leaf, grape) and soil types (Bokulich *et al.*, 2014; Knight *et al.*, 2015; Oliveira *et al.*, 2018), and only a very small number examined vine microbiota at smaller geographic scales. For instance, Miura *et al.*, (2017) demonstrated differences in the grape and leaf microbiota among six Chilean vineyards located 35 km apart. Differences in fungal grape microbiota were shown to depend on the geographical distance between them following a distance-decay relationship, validating the biogeography hypothesis (Miura *et al.*, 2017). Knight *et al.*, (2020) investigated soil microbiota at a smaller scale, in four different vineyards situated within a 2-km radius. They observed geographic differences among fungal communities driven by local environmental heterogeneity, whereas they found no specific pattern when they analysed fermentative yeasts (Knight *et al.*, 2020). A recent study provided the first evidence of local

soil properties producing variations of fungal and bacterial communities within a single vineyard (Liang *et al.*, 2019). Despite the use of similar practices and of a very limited number of vine genotypes, soil microbial communities near the vine plants were strongly heterogeneous, and was only partly explained by soil characteristics (Liang *et al.*, 2019). Because endophytes are recruited in the soil microbiota, this local-scale patchiness of the soil microbiota likely shapes the microbial composition of the individual vines.

However, despite its tremendous importance, the root-endospheric microbiota has been widely overlooked to date in vineyards, and most agricultural crops. At the vineyard scale, non-random variations in the endophytic bacterial and fungal communities are likely explained by the heterogeneous patterns of soil components. Indeed, microbial recruitment to form the root-endospheric microbiota is mainly shaped by the soil reservoir, which can differ depending on changes in the physical-chemical properties of the soil (Plassart *et al.*, 2019). Soil organic carbon, pH, the C/N ratio and phosphorus contents are the main factors that have a significant influence on bacterial and fungal consortia in soil microbial reservoirs in vineyards described so far (Zarraonaindia *et al.*, 2015; Liang *et al.*, 2019), along with grape-surface microbiota (Bokulich *et al.*, 2014). In addition, because the recruitment of endophytic bacterial and fungal communities is plant-dependent, these assemblages are likely shaped by plant-specific factors such as grape type (cultivar type), growing seasons, the age of the vine and the rootstock genotype (Zarraonaindia *et al.*, 2015; Novello *et al.*, 2017; Marasco *et al.*, 2018; Berlanas *et al.*, 2019). In addition to the strong economic interest linked to vine plant culture, vine plants offer the ideal plant model to study the rules of microbiota assembly because vines are highly genetically homogeneous perennial plants. Differences in the cultivar microbiota can be assumed to be linked to a genotype effect and the preferential associations of microorganisms with a particular host genotype due to both passive barriers to colonization and active recruitment by the plants, especially through root exudates (Dennis *et al.*, 2010). Recruitment may also vary over time depending on microbial succession processes and plant requirements during their different developmental stages or to face and buffer different kinds of stress (Bulgarelli *et al.*, 2013; Vives-Peris *et al.*, 2020). First, microbial communities are hypothesised to depend on the composition of the first

species to colonise (priority effect) (Werner and Kiers, 2015) while preferential selection by the plants can actively occur later (host preference) (Vandenkoornhuyse *et al.,* 2002). Changes in the plant microbiota over time are expected to depend on the seasonal dynamics (Davison *et al.,* 2011) but also throughout plant development (i.e., interannual changes related to ageing). Roots are therefore organs of prime importance for the study of microorganisms associated with the vine as they form an ecological filter and act as a reservoir of specialised microorganisms for the other plant compartments (Vandenkoornhuyse *et al.,* 2015; Martínez-Diz *et al.,* 2019).

To advance our understanding of the factors driving microorganisms assembly, we analysed the effect of abiotic variables and plant-specific factors on the endophytic microbiota associated with vine roots. To this end, we worked in a vineyard domain located in Margaux (France) where the bedrock and soils are assumed to be homogeneous, and practices are similar across fields. We hypothesised that the composition of both the bacterial- and fungal-root-endosphere is determined by abiotic and plant-specific factors (i.e., the cultivar and the age of the plants). To address these hypotheses, we analysed variations in the bacterial and fungal composition of vine plants (i) at the scale of the vineyard (γ-diversity scale) and (ii) within a single field (α-diversity scale). We analysed root endosphere derived SSU rRNA gene amplicon fragments (bacteria and fungi) from 296 samples recovered from 37 fields within a single vineyard (Experimental design, Figure 1).

MATERIALS AND METHODS

1. Study site and field selection

We analysed the root microbiota in individual vine plants collected in a single 66-ha vineyard located in Margaux (*protected designation of origin;* "AOP" in French "*Appellation d'Origine Protégée*")
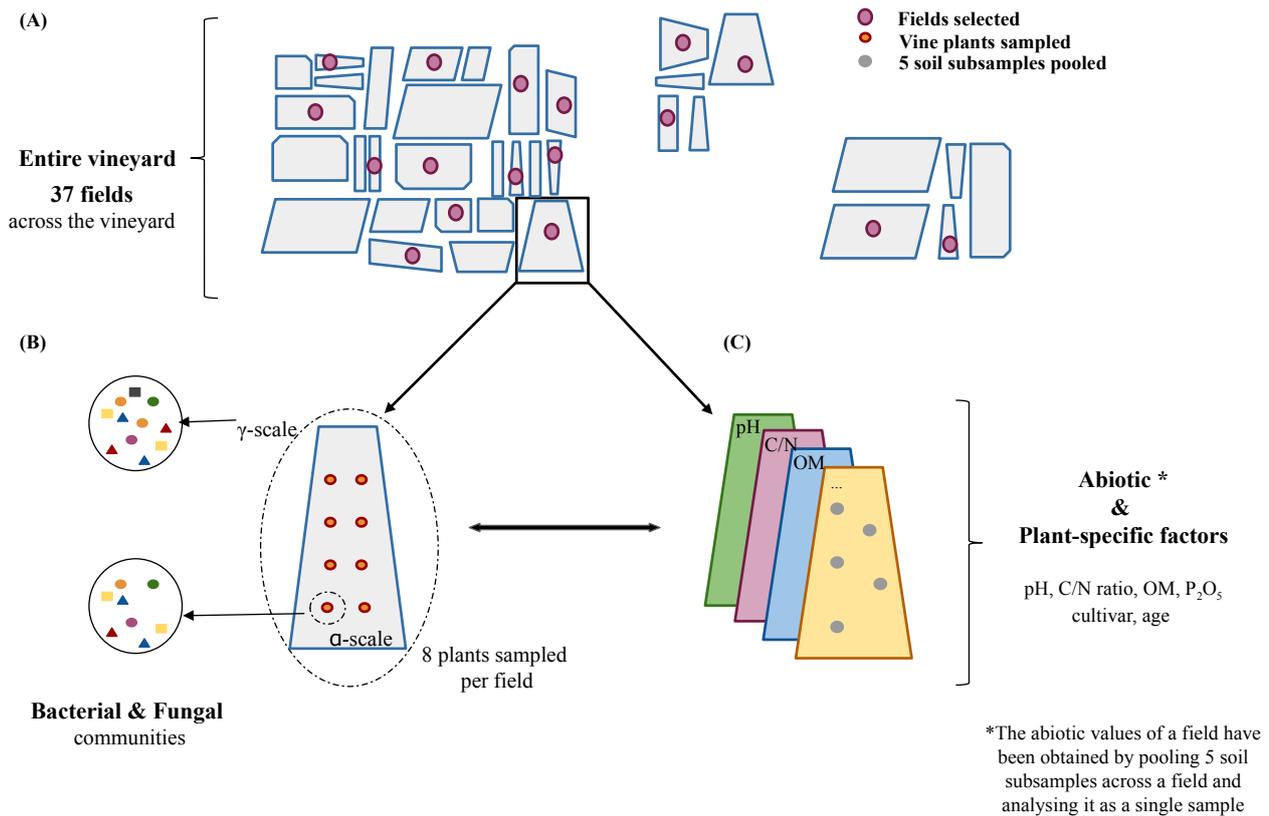
FIGURE 1. Presentation of the experimental design.

(A) Scheme of the vineyard and distribution of the 37 sampled fields.

(B) Plan showing the location of the 8 plants sampled in each of the 37 fields. The α-scale corresponds to the individual scale (one vine plant); the γ-scale represents the group of individuals at the field scale. The bacterial and fungal microbiota in each sample were identified using mass sequencing of amplified SSU rRNA gene fragments.

(C) Environmental factor data obtained from the vine-makers dataset to test their effect on the bacterial and fungal communities at both α- and γ-scales. The abiotic values have been obtained by analysing a pool of 5 soil subsamples of a field.

in France (45°2'12.73 "N; 0°40'9.84 "O) in June 2018. The region is characterised by a temperate climate with an average temperature of 14.2 °C (min: 1.6 °C; max: 36.3 °C) and cumulative annual precipitation of 934mm. This vineyard has been managed with biodynamic practices for several years and is actively engaged in practising sustainable viticulture. We sampled 37 fields with a balanced number of fields with either Cabernet-Sauvignon or Merlot cultivars, and that included all the different ages of the vines in the vineyard. The range of the ages of vine plants were between 3 and 80 years old (Table S1) and the two studied cultivars included the same number of fields per range of ages. In each field, eight equidistant vine plants were selected to cover the field variability (mean field area 0.79 +/- 0.36 ha) all of the same age and genotype (Figure1).

2. Characteristics of the environmental variables

Each field was characterised by abiotic factors linked to soil conditions, biotic factors linked to the type of cultivar (Cabernet-Sauvignon and Merlot), and the age of the vine. In all 37 fields, abiotic factors were measured including the C/N ratio, organic matter content (OM), pH and P_2O_5 (g/kg) (Figure 1, Table S1). These edaphic parameters were selected because they are known to potentially impact the soil microorganisms and thus possibly the plant recruitable microorganism reservoir (e.g., Santoyo *et al.,* 2017). The analyses were conducted on 37 soils samples collected at a depth of 25 cm. These soil samples were composed of a mix of five soil sub-samples per field representing different areas of a field. All the measurements were performed by Auréa AgroSciences (Ardon, France) under the request of the wine-makers for other vineyard management purposes. We compared these measures through time (i.e., through years, with measures not performed at the same period of the year) and found very similar values (e.g., highest range of variation in pH within a given field of 0.2). It can be suggested homogeneity. Thus, we used these edaphic values per parcel as an environmental proxy for the 8 sampled plants within a parcel. Within a given field, we thus concluded the homogeneity of edaphic parameters. To satisfy the assumption of independence among variables, we checked that these variables were not correlated (i.e., Pearson's tests showed that none of the correlation estimates were above 0.7) (Dormann *et al.,* 2013).

3. Root sampling and DNA extraction

We sampled 296 vine roots in June 2018 at the vine flowering stage, a period of very active vegetative growth. Roots were collected with a spade near the stem, to obtain a homogeneous root stage at a depth of from 5 to 20 cm. The roots were removed manually using gloves, placed in individual hermetic bags and stored in cooled boxes. The roots were then thoroughly washed first with tap water and then with a 5 ‰ Triton™ X100 solution for 10 minutes. They were rinsed several times using sterile ultra-pure water, placed in sterile micro-tubes and stored at -80 °C until DNA extraction. The total root DNA was extracted at the GENTYANE platform (Clermont-Ferrand, France) using magnetic bead technology (sbeadex™) on an oKtopure™ LGC Genomics automat. After the sample's mechanical grinding, nucleic acids were captured with the sbeadex™ magnetics particles. After washing steps to remove impurities the nucleic acid was eluted in the Beadex elution buffer. DNA concentration was measured using Hoechst 33258 reagent and a TECAN Infinite® 1000 instrument. DNA extracts were then stored at -20 °C until amplicon processing.

4. Molecular work

All the 296 extracted DNAs were normalised at 10 ng/µl using TE 0.1X to ensure the use of similar DNA concentrations for all the PCRs. The fungal community in vine roots was analysed by amplifying an 18S rRNA gene fragment with the specific fungal primers NS22b (5′-AATTAAGCAGACAAATCACT-3′) and SSU817 (5′-TTAGCATGGAATAATRRAATAGGA-3′). The bacterial community was analysed from PCR amplification of a 16S rRNA gene fragment (V5-V7 16S regions) using the bacterial primers 799F (5′-AACMGGATTAGATACCCKG-3′) and 1223R (5′-CCATTGTAGTACGTGTGTA-3′). These primers were chosen because they do not amplify plant DNA and allow, *in silico*, the best bacterial sequence recruitment from both Sylva and GreenGenes databases compared to the other commonly used primer sets (unpublished in silico benchmark analysis). Both specific primers were customised to include sample tags and Illumina® adaptors. To ensure a normalised PCR mix and avoid contamination, Illustra™PuReTaq Ready-to-go beads (GE Healhcare®) were used for all the amplifications. The two primer sets and the PCR conditions associated with the specific target are detailed in Vannier *et al.,* (2018). PCR products were cleaned with AMpureXP magnetic

beads (Agencourt®) using an Agilent Bravo Automated liquid handling platform (Agilent®) and quantified with a Quant-iT PicoGreen™ dsDNA Assay Kit to allow normalization at the same concentration. Library preparation and sequencing were performed at the Human and Environmental Genomics Platform (HEG platform, Rennes, France). Briefly, a second PCR using the Smartchip-Real-Time PCR machine (Takara) performed multiplex tagging (up to 384 amplicons in one-step PCR), the resulting tagged-amplicon pool was purified (AMpureXP, Agencourt®) and quantified using Kapa Library Quantification Kit-Illumina® on an LC480 LightCycler qPCR machine (Roche®). The library was sequenced on an Illumina MiSeq instrument (PE-2x250 cycles).

5. Sequence trimming and clustering

The base-calling step was performed on the MiSeq instrument with CASAVA v1.8 software (Illumina). Sequence data trimming consisted of removing primers and deleting reads containing unidentified bases using Cutadapt software. As we mixed fungal and bacterial amplicons, we processed the fastq files to separate the two types of reads based on their specific DNA sequence (homemade script–EcogenO platform) and then analysed them separately. These good-quality sequences were then processed with the FROGS pipeline (Escudié *et al.,* 2018) using the standard protocol for bacterial reads and after a particular pre-processing step for fungi (Kozich *et al.,* 2013). The FROGS pipeline notably includes SWARM as a clustering strategy, which enables analysis of reads without using the standard identity threshold of classical OTUs (97 %), and rather to produce what we call herein 'sequence-clusters'. In comparison to the ASV method (Callahan *et al.,* 2017), this clustering strategy and the subsequent filtering (see below) have the advantage to limit the over-estimation of sample richness (Escudié *et al.,* 2018) induced for instance by a multicopies artefact. The following steps were performed as recommended by Escudié *et al.,* 2018. Briefly, these steps consisted of using a specific algorithm for de-noising and a chimera removal process followed by a stringent filter to avoid artificial sequence-clusters. To ensure high quality and stringent analysis, only sequences present in three different samples (i.e., at least in 1 % of the samples) were retained and included in the sequence-clusters. Reads were filtered using the quality of the affiliations with a threshold of at least 95 % coverage and 95 % identity (BLAST).

Finally, the taxonomic affiliations were determined using Silva132 16S rRNA for bacteria (Quast *et al.,* 2012) and Phymyco-DB databases for fungi (Mahé *et al.,* 2012).

The stringent parameters applied in the FROGS pipeline resulted in the production of data matrices containing 2,778,222 reads for bacteria and 1,549,109 reads for fungi. Rarefaction curves were generated using R (version 3.6.1) with the function 'rrarefy' in the vegan 2.5-6 package. To perform subsequent statistical analysis, we normalised the number of reads for each bacterial and fungal dataset. Based on graphical observations (rarefaction curves), we normalised to the same number of reads for bacteria and fungi to 4264 and 1447 reads per sample respectively (Figure S1). Samples with fewer reads were removed from the dataset resulting in 34 fields for bacterial datasets and 36 fields for fungal datasets (i.e., a field being removed when more than 50 % of the samples did not satisfy the sequencing depth). The final matrices containing 1051 bacterial and 158 fungal sequence-clusters were finally used for the statistical analyses. We calculated sequence-cluster richness and the diversity index (Simpson's evenness) using the R 'vegan' package (Table S2) at two spatial scales: the vineyard level (γ-diversity scale) and the within-field level (α-diversity scale). Measures at the γ-scale were calculated using the total number of sequence-clusters and the mean number of reads. For all the statistical analyses, we decided to work at the phylum level because a large part of the sequence-clusters observed were unknown at the species level and a large proportion of them are unknown at higher taxonomic levels. We checked for the redundancy of diversity indices (Richness and Simpson indices) by making sure their correlation (Pearson's test) was below 0.70 (Dormann *et al.,* 2013). None of the estimates were higher than 0.70, except the correlation estimate (0.75) between the sequence-cluster richness of δ-Proteobacteria and the Simpson index of γ-Proteobacteria.

6. Statistical analyses

We analysed the effect of environmental factors on microbiota structure with a combination of multivariate analysis (Canonical correspondence analysis–CCA) and univariate models.

Canonical correspondence analysis (CCA) was used to explore whether the microbial sequence-cluster composition (i.e., bacterial and fungal microbiota composition) depended on plant-specific factors or abiotic variables.

Separate analyses were performed for "all bacteria/fungi" communities, i.e., the entire pools of both microbial communities, and the subsequent phyla separately. We coupled the matrix of the sequence-cluster composition under the constraint of the plant-specific/abiotic variables matrix (age, cultivar, pH, C/N ratio, organic matter (OM), P_2O_5). Plant genotypes (i.e., rootstock genotype and scion genotype) sampled within a field were identical, but rootstock genotype varied among fields. However, the rootstock genotype was unknown for some of the parcels independently of the age of the parcel. Therefore, we did not include rootstock genotype in the analyses. Furthermore, we tested the rootstock effect on the partial data set and found no detectable effect on the microbiota (i.e., *p > 0.05*). Rootstock variations were thus considered herein as a background noise expected of the same range across age categories.

Bacterial and fungal sequence-cluster composition of the fields was assessed using the normalised number of reads for sequence-clusters. The significance of the independent variables in the CCA structure was tested using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. These analyses were performed using the 'vegan' package.

We used respectively linear (γ-diversity scale) and linear mixed models (α-diversity scale) to analyse the effect of abiotic factors (pH, C/N ratio, OM and P_2O_5) and plant-specific factors (age and cultivar) on sequence-cluster richness and evenness for both fungi and bacteria. For the α-scale analysis, we considered 'field' as a random effect. All the models (α- and γ-scale) were performed using a model-averaging method (Burnham and Anderson, 2002).
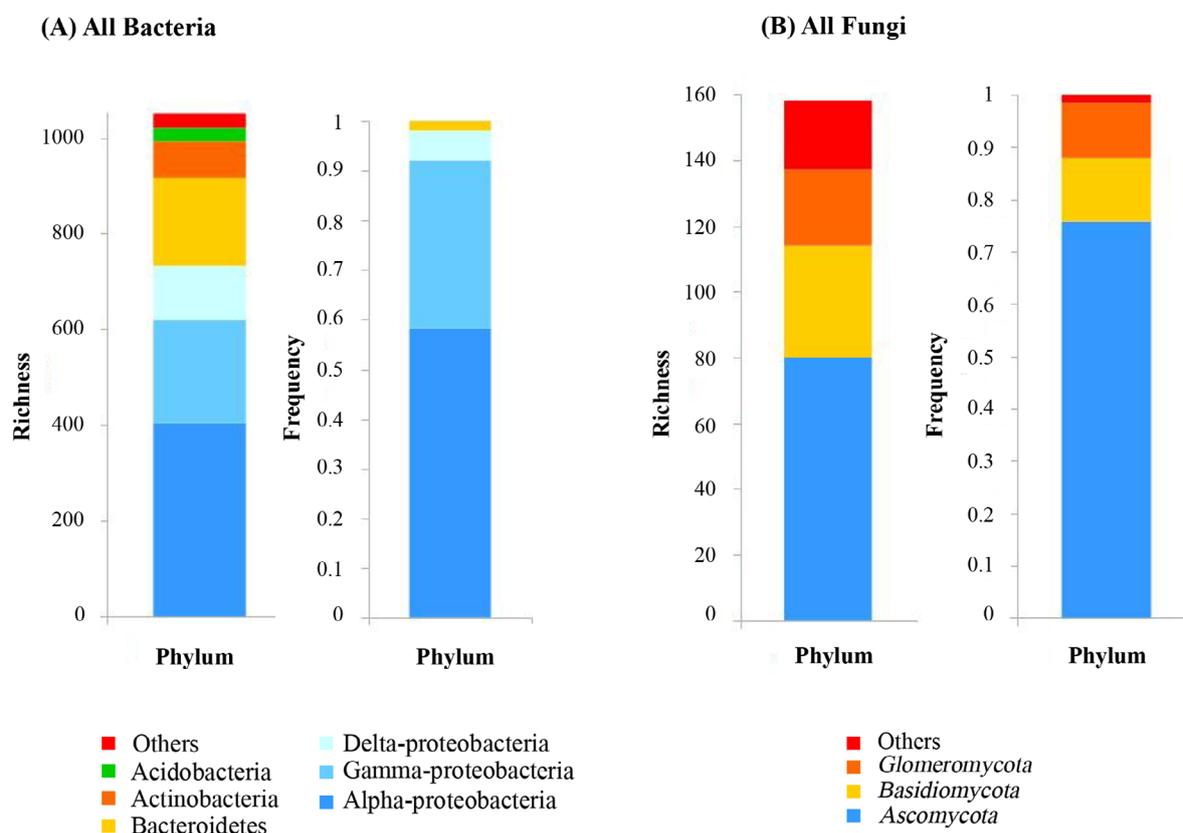
FIGURE 2. Description of the microbial communities at vineyard scale.

Sequence-cluster richness and relative abundance of the root endospheric bacterial (A) and fungal (B) microbiota of the vine plant at the phylum level.

(A) N= 34 fields. The «others» group contains phyla with limited number of sequence-clusters (10 or fewer sequence-clusters): Chlamydiae, Chloroflexi, Dependentiae, Fibrobacteres, Firmicutes, Gemmatimonadetes, Nitrospirae, Spirochaetes, Verrucomicrobia. Three representative sub-phyla of Proteobacteria, Alpha-proteobacteria, Gamma-proteobacteria and Delta-proteobacteria are represented to provide the required level of information.

(B) N=36 fields. The «others» group contains phyla with a limited number of sequence-clusters (less than 10 sequence-clusters): *Chytridiomycota*, *Zygomycota*, Multi-affiliation.

The model-averaging method enables the calculation of better estimates (i.e., higher mathematical accuracy) as well as more reliable confidence intervals than standard methods like model selection (Fletcher, 2018). All possible models (combination of explanatory variables) were generated and ranked using the Akaike information criterion corrected for small sample sizes (AICc). We produced full-model averaged estimates of each independent variable across the most parsimonious models (ΔAICc < 2) and visually checked the normality of the residuals. We considered independent variables to be significant if their 95 % confidence intervals did not overlap with zero (Burnham and Anderson., 2002). Finally, we calculated the proportion of variance (adjusted R^2 for the γ-scale and marginal R^2, conditional R^2 for the α-scale) of the most parsimonious model (ΔAICc = 0) as the values did not significantly differ from the values of the averaged models (Nakagawa and Schielzeth, 2013).

All statistical analyses were performed using R.3.6.2 (R Core Team). Linear mixed models, model averaging and associated R^2 were calculated using the following packages: 'lmerTest', 'lme4', 'nmle', 'car', 'MuMIn' and 'AICcmodavg'.
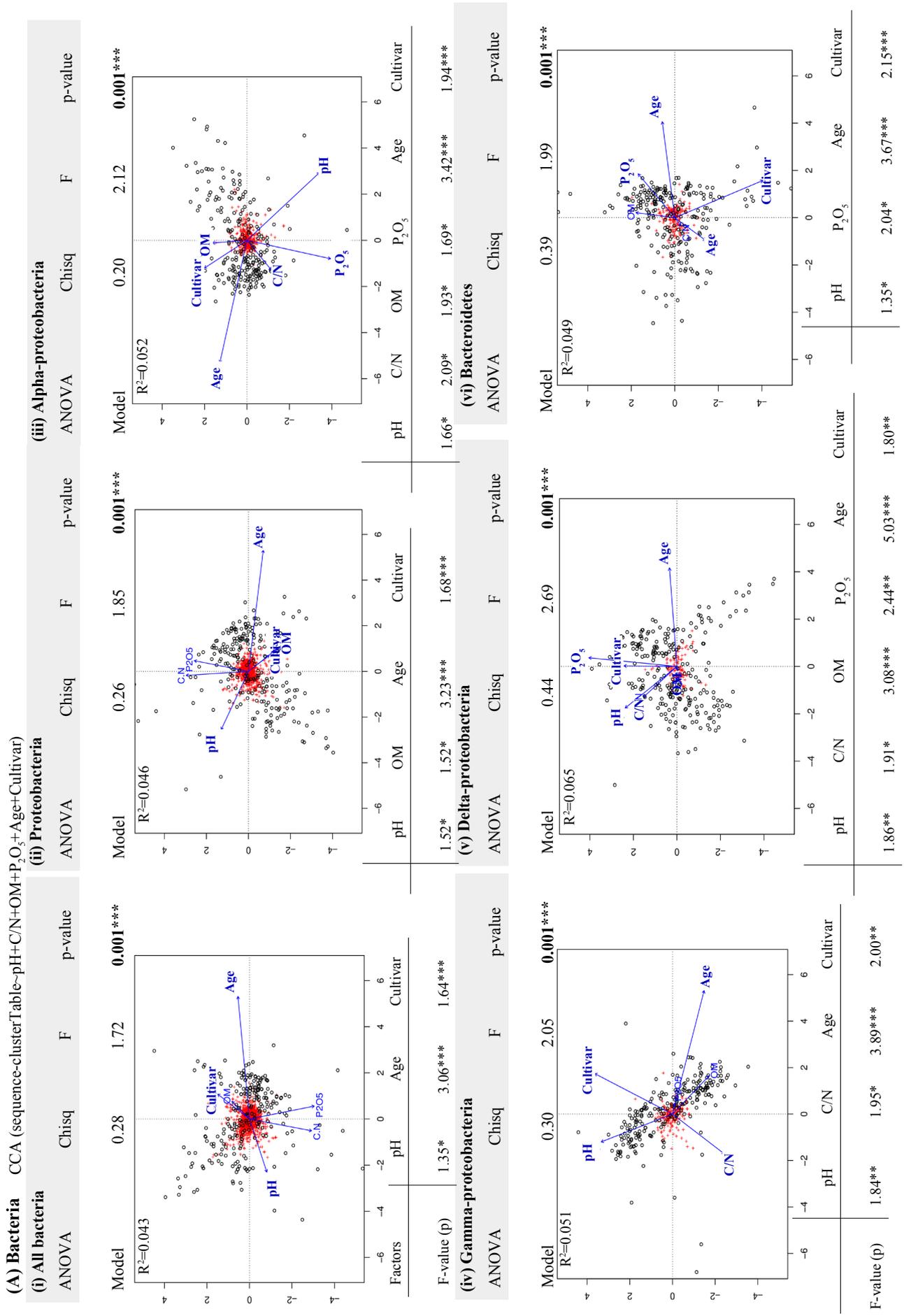
RESULTS

1. Composition of the vine root endospheric microbiota

Sequencing depths of both fungal and bacterial microbiota colonizing vine roots were sufficient to limit sequence-cluster subsampling (Figure S1). At the vineyard scale (i.e., the total microbial pool), the bacterial community in the root endosphere was dominated by the Proteobacteria phylum (733 sequence- clusters representing 72 % of the sequences in terms of relative abundance) (Figure 2). Within Proteobacteria, α-, γ- and δ-Proteobacteria classes accounted for 404 (43 % of the sequences), 215 (25 % of the sequences) and 114 (4 % of the sequences) sequence-clusters respectively. The Bacteroidetes phylum represented 184 (12 % of the sequences) of the sequence-clusters. The vine-root microbiota contained 158 fungal sequence-clusters mainly composed of *Ascomycota* (80 sequence-clusters, 76 % of the sequences in term of relative abundance), *Basidiomycota* (34 sequence-clusters, 12 % of the sequences), and *Glomeromycota* (23 sequence-clusters, 10 % of the sequences) (Figure 2).
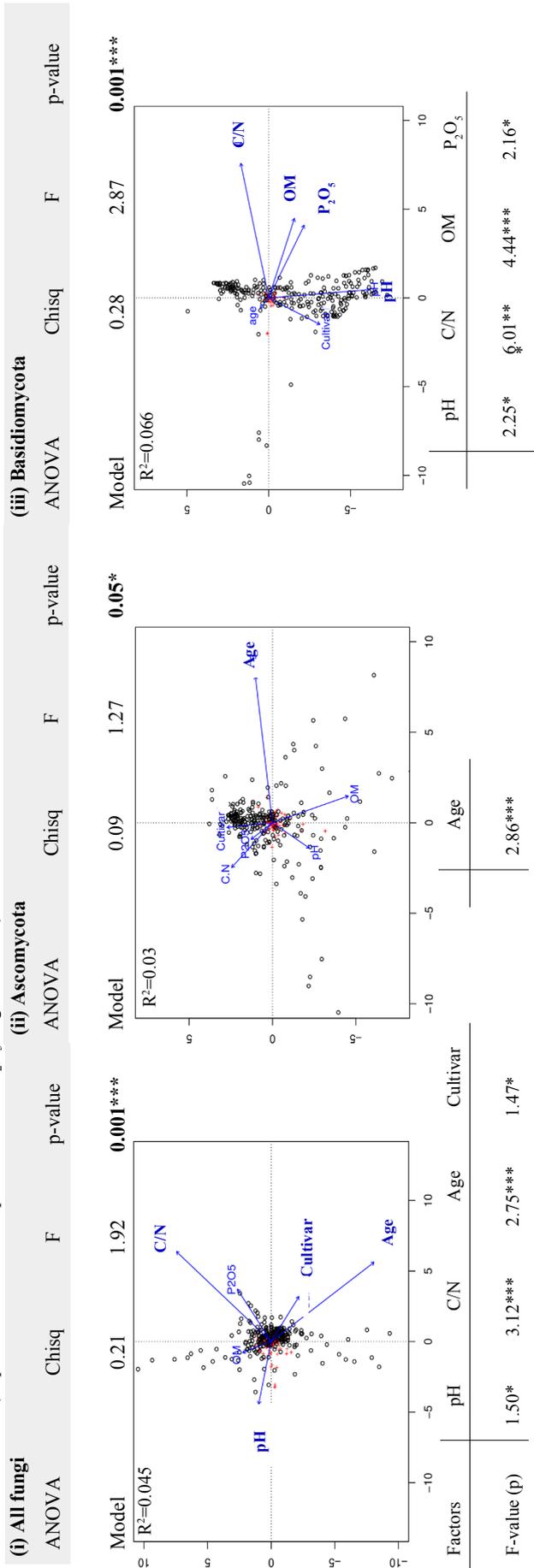
2. Drivers of the bacterial community in vine roots at both α- and γ-diversity scales

We used canonical correspondence analysis (CCAs) to test for potential shifts in bacterial community compositions depending on the factors tested. At the α-scale, we recorded significant effects of vine age, cultivar, and pH on the bacterial microbiota composition for the "all bacteria" community and all subsequent phyla (Figures 3A and S2A). The impact of OM, C/N ratio, and P_2O_5 on bacterial composition depended on the phylum considered (Figures 3A and S2A). The composition of α-Proteobacteria, δ-Proteobacteria, Actinobacteria and Acidobacteria communities was significantly influenced by all the factors tested (i.e., vine age, cultivar, pH, C/N, OM and P_2O_5) (Figures 3A and S2A). The composition of Proteobacteria, γ-Proteobacteria and Bacteroidetes was significantly affected by the OM, the C/N ratio and the P_2O_5 concentration respectively as well as the other factors shared by all the phylum (vine age, cultivar and the pH) (Figure 3A). At the γ-diversity scale, we found no significant relationship between the tested factors and bacterial composition, except for Actinobacteria that depended on the age of the vine (Figure S2B).
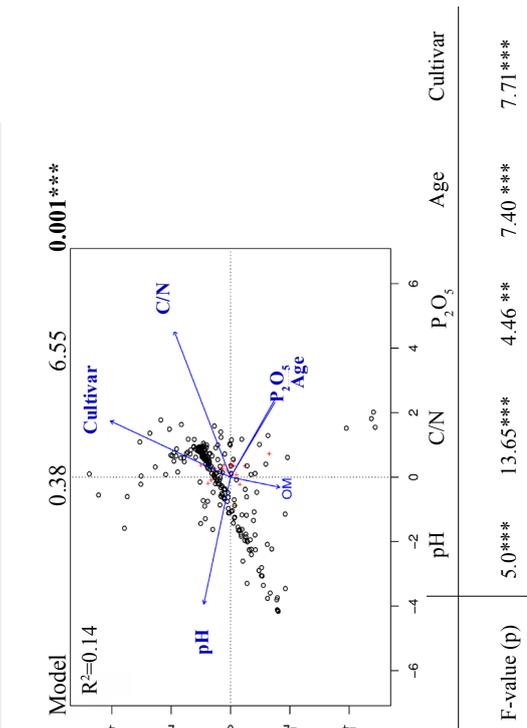
We used linear mixed models and calculated the full-model averaged estimates of each independent variable to test whether abiotic and plant-specific factors impacted bacterial richness and evenness. Unexpectedly, the effects of abiotic and plant-specific factors on bacterial-microbiota colonizing vine-roots varied depending on the taxa. The sequence-cluster richness of the bacteria microbiota ("all bacteria") and Proteobacteria were positively related with soil pH and OM at the α-diversity scale (Table 1A; Figure S3A). The sequence-cluster richness of the γ-proteobacteria increased with higher soil OM content (Table 1A; Figure S3A). Conversely, the sequence-cluster richness of the Actinobacteria and Acidobacteria were only impacted by plant-specific factors (Table 1A; Figure S3A). Indeed, the sequence-cluster richness of the Actinobacteria was higher in the Cabernet-Sauvignon cultivar than in Merlot and the sequence-cluster richness of the Acidobacteria increased with the age of the vine plants (Table 1A; Figure S3A). Interestingly, at the γ-scale, the sequence-cluster richness of almost all the bacterial taxa found to be significant ("all bacteria", Proteobacteria, α-Proteobacteria and Acidobacteria) was positively related with soil P_2O_5 content and independent of all other factors (Table 1C).

(A) Bacteria CCA (sequence-clusterTable~pH+C/N+OM+P₂O₅+Age+Cultivar)

(i) All bacteria

ANOVA	Chisq	F	p-value
Model	0.28	1.72	**0.001***

R²=0.043

Factors	pH	Age	Cultivar
F-value (p)	1.35*	3.06***	1.64***

(ii) Proteobacteria

ANOVA	Chisq	F	p-value
Model	0.26	1.85	**0.001***

R²=0.046

	pH	OM	Age	Cultivar
	1.52*	1.52*	3.23***	1.68***

(iii) Alpha-proteobacteria

ANOVA	Chisq	F	p-value
Model	0.20	2.12	**0.001***

R²=0.052

pH	OM	Age	Cultivar
1.66*	2.09*	1.93*	1.69*	3.42***	1.94***

(iv) Gamma-proteobacteria

ANOVA	Chisq	F	p-value
Model	0.30	2.05	**0.001***

R²=0.051

pH	C/N	Age	Cultivar
1.84**	1.95*	3.89***	2.00**

(v) Delta-proteobacteria

ANOVA	Chisq	F	p-value
Model	0.44	2.69	**0.001***

R²=0.065

pH	C/N	OM	P₂O₅	Age	Cultivar
1.86**	1.91*	3.08***	2.44**	5.03***	1.80**

(vi) Bacteroidetes

ANOVA	Chisq	F	p-value
Model	0.39	1.99	**0.001***

R²=0.049

pH	P₂O₅	Age	Cultivar
1.35*	2.04*	3.67***	2.15***

(B) Fungi   CCA (sequence-clusterTable~pH+C/N+OM+P$_2$O$_5$+Age+Cultivar)

(i) All fungi

ANOVA	Chisq	F	p-value
Model	R²=0.045	0.21	1.92	0.001***

Factors	pH	C/N	Age	Cultivar
F-value (p)	1.50*	3.12***	2.75***	1.47*

(ii) Ascomycota

ANOVA	Chisq	F	p-value
Model	R²=0.03	0.09	1.27	0.05*

	Age
	2.86***

(iii) Basidiomycota

ANOVA	Chisq	F	p-value
Model	R²=0.066	0.28	2.87	0.001***

	pH	C/N	OM	P$_2$O$_5$
	2.25*	6.01**	4.44***	2.16*

(iv) Glomeromycota

ANOVA	Chisq	F	p-value
Model	R²=0.14	0.38	6.55	0.001***

	pH	C/N	P$_2$O$_5$	Age	Cultivar
F-value (p)	5.0***	13.65***	4.46**	7.40***	7.71***

FIGURE 3. Effect of abiotic and plant-specific factors on the composition of the endospheric (A) bacterial and (B) fungal communities inhabiting the vine plant roots at the α-scale.

Percentages of explained deviance and significance were calculated using ANOVA-like permutation tests with canonical correspondence analysis (CCA). Only major bacterial phyla are represented (A) on the figure. All fungal phyla are represented (B). ANOVA tables show the significance for the CCA model.

Factors tested: age of the vine plant, cultivar (Merlot or Cabernet-Sauvignon), OM (Organic Matter), C/N ration (carbon/nitrogen ratio), pH and P$_2$O$_5$. Factors which significantly influence the microbial composition are reported on tables.

Values are given (F-value) for the «all bacteria» / «all fungi» communities and the associated phyla when the explained deviance is significant ($*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$). Black dots represent the samples and red crosses represent sequence-clusters.

TABLE 1. Summary of the models calculated by model averaging that explain bacterial and fungal sequence-cluster richness and evenness at two scales of diversity analysis.

Alpha scale		pH			C/N ratio			OM		
(A) Richness	Inter	E	Z	CI	E	Z	CI	E	Z	CI
All Bacteria	194.95	15.56	**2.34***	**2.5/28.6**				17.87	**3.36 *****	**7.4/28.2**
Proteobacteria	134.86	12.32	**2.2***	**1.3/23.3**				14.17	**3.12 ****	**5.4/22.9**
Gamma-proteobacteria	47.01							4.92	**3.52 *****	**2.2/7.7**
Actinobacteria	17.03									
Acidobacteria	4.89									
All Fungi	37.00									
Ascomycota	18.90									
(B) Evenness										
Actinobacteria	0.75									
Gamma scale		pH			C/N ratio			OM		
(C) Richness	Inter	E	Z	CI	E	Z	CI	E	Z	CI
All Bacteria	657.46									
Proteobacteria	465.37									
Alpha-proteobacteria	271.42									
Acidobacteria	13.44									
Ascomycota	47.05									
Glomeromycota	13.18									
(D) Evenness										
Alpha-proteobacteria	0.97				-0.0026	**2.3***	**-0.005/-0.0005**	0.007	**2.07***	**0.0004/0.014**
Actinobacteria	0.83									
Acidobacteria	0.74									

Models are based on variables of abiotic factors (pH, C/N ratio, OM and P_2O_5) and plant-specific factors (age and cultivar)

(A) Richness of the bacteria and fungi at the α-scale - (B) Evenness of the bacteria and fungi at the α-scale

(C) Richness of the bacteria and fungi at the γ-scale - (D) Evenness of the bacteria and fungi at the γ-scale

The table summarizes the model-averaged estimates (E), value «z-score» (Z) when significant *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and 95 % confidence interval (CI) of the independent variable(s) according to the AICc framework. Confidence intervals that did not encompass zero are in bold.

R^2m and R^2c describe the α-scale and adjusted-R^2 the γ-scale and were calculated for the best model.

(Table 1 continues on next page).

(Table 1 continued from previous page).

Alpha scale	P₂O₅			Age			Cultivar			Model
(A) Richness	E	Z	CI	E	Z	CI	E	Z	CI	**R²m (R²c)**
All Bacteria										0.082 (0.13)
Proteobacteria										0.088 (0.17)
Gamma-proteobacteria										0.085 (0.15)
Actinobacteria							-3.07	**2.48***	**-5.5/-0.6**	0.13 (0.24)
Acidobacteria				0.057	**3.11 ** **	**0.02/0.09**				0.10 (0.33)
All Fungi							4.52	**3.14 ** **	**1.7/7.3**	0.057 (0.15)
Ascomycota							3.59	**4.47 *** **	**2.0/5.2**	0.13 (0.19)
(B) Evenness										
Actinobacteria				-0.003	**3.69 *** **	**-0.005/-0.001**				0.17 (0.34)

Gamma scale	P₂O₅			Age			Cultivar			**Model**
(C) Richness	E	Z	CI	E	Z	CI	E	Z	CI	**Adjusted R²**
All Bacteria	304.13	**2.31***	**46.4/561.8**							0.136
Proteobacteria	224.56	**2.42***	**42.4/406.7**							0.157
Alpha-proteobacteria	124.04	**2.30***	**18.2/229.9**							0.134
Acidobacteria				0.09	**2.04***	**0.003/0.18**				0.102
Ascomycota							4.72	**2.21***	**0.54/8.89**	0.107
Glomeromycota				-0.061	**2.32***	**-0.11/-0.009**				0.118
(D) Evenness										
Alpha-proteobacteria										0.153
Actinobacteria				-0.003	**2.46***	**-0.0045/-0.0005**				0.219
Acidobacteria				0.0021	**2.35***	**0.0004/0.004**				0.142

Considering evenness at both α- and γ-scales, we only observed few significant effects of environmental factors on the bacterial communities and age was the only plant-specific factor altering the evenness of the bacterial communities (Table 1B, Table 1D). Indeed, α-Proteobacteria communities were more equitable with a high soil OM content but were less diverse with a higher C/N ratio at the γ-scale (Table 1D). Actinobacteria communities were more equitable in old vine plants but were dominated by certain sequence-clusters in young individuals at both α- and γ-scales (Table 1B, Table 1D; Figure S3A). Acidobacteria displayed higher equitability with increasing ages of the vine plants at both the γ-scales (Table 1D).

To summarise the bacterial results, we found that soil pH caused shifts within the "all bacteria" community as well as enrichment of the microbial pool whereas the age of the vine plants and the cultivar influenced the composition but not the richness of the "all bacteria" community (Figure 3A and Table 1A). At the phylum level, significant effects of the soil pH and the soil OM, as well as the cultivar and the age of vine plants resulted in shifts of communities linked with either enrichment (Proteobacteria) or impoverishment (Actinobacteria, Acidobacteria) of the bacterial communities (Figures 3A; 2SA and Table 1A). Considering the other phyla, the impacts of the tested factors on microbial community structure caused shifts within the communities but no change in the bacterial richness (Figure 3A and Table 1A).

3. Drivers of the fungal community in vine roots at both α- and γ-diversity scales

At the α-diversity scale, we found significant effects of the pH, C/N ratio, vine age, and cultivar on the "all fungi" community composition (Figure 3B). *Basidiomycota* composition was also influenced by the concentration of OM and P_2O_5 in the soil (Figure 3B). Changes in the composition of the *Glomeromycota* community were caused by the following factors: pH, C/N ratio, P_2O_5, age of the vine and cultivar (Figure 3B). At the γ-diversity scale, both the C/N ratio and the age of the vine significantly explained the fungal composition in the roots of the "all fungi" community (Figure S2B). We also demonstrated important effects of the pH, the C/N ratio, the age of the vine and the cultivar on the *Glomeromycota* community composition in vine roots (38.46 % significantly explained deviance; Figure S2B).

The effects of abiotic and plant-specific factors on fungal richness and evenness varied depending on the phylum considered. At the α-diversity scale, the sequence-cluster richness of the fungal microbiota ("all fungi") community was related to the cultivar alone and was higher in the Merlot cultivar (Table 1A; Figure S3B). This was mostly due to changes in the *Ascomycota* community, which was the only fungal phylum influenced by the factors we tested (Table 1A; Figure S3B). At the γ-scale, the sequence-cluster richness of the *Ascomycota* also responded to the cultivar and was higher in Merlot than in Cabernet-Sauvignon (Table 1C; Figure S3B). Interestingly, at the γ-scale, the *Glomeromycota* richness decreased with the age of the vine plants (Table 1C). Considering fungal evenness at both scales, no significant effects of either soil or plant-specific factors were found.

To summarise the fungal results, we found that the fungal community was richer in Merlot and the composition of fungi differed in Merlot and Cabernet-Sauvignon, whereas the C/N ratio and the vine age affected the composition of the entire fungal community ("all fungi") but did not change sequence-cluster richness (Figure 3B; S2B and Table 1A). At the phylum level, either the abiotic or the plant-specific factors were involved in changes in the fungal communities but did not alter their richness (Figure 3B; S2B and Table 1A).

DISCUSSION

1. Endospheric root microbial communities

In this study, we analysed the vine root-endosphere composition by characterizing both bacterial and fungal communities. The root endosphere mostly originates from the soil reservoir after passive or active recruitment (Bulgarelli *et al.,* 2012; Vandenkoornhuyse *et al.,* 2015) and because of the significant impact of the root endosphere on plant health and growth, the endospheric microbiota may be key to better define the *microbial terroir* of vineyards.

This analysis of the vine root endosphere microbiota was performed at the level of a single domain at Margaux (France) to ensure homogeneous viticulture across the sampled plots. Elsewhere the gravelly sandy soils are known to be similar among parcels with no elevation and limited physico-chemical variations. From the sampled roots, a total of 1051 bacterial and 158 fungal sequence-clusters composed the vine-root endospheric microbiota in this study. The composition of the root endospheric microbiota in

the vine plants (i.e., Proteobacteria and *Ascomycota* as the major phyla) was comparable in size to other studies analysing roots endospheres of different types of plants as maize (Gomes *et al.,* 2018), agave (Coleman-Derr *et al.,* 2016), herbaceous plants (*Glechoma hederacea*) (Vannier *et al.,* 2018) and vine plants (e.g., for review Liu *et al.,* 2019). In vine plants, a recent study compared the bacterial and fungal microbiota of different bio-compartments and similarly as herein, demonstrated that the composition of the roots microbiota was dominated by the same two main phyla, Proteobacteria and *Ascomycota* (Deyett and Rolshausen, 2020; Liu and Howell, 2020). This observation of selective filtering leading to a high proportion of Proteobacteria and *Ascomycota* in the plant microbiota endosphere has been recently explained by both plant-microbiome co-evolution and niche adaptation (Trivedi *et al.,* 2020).

2. Multi-scale spatial heterogeneity of plant microbiota

Our multi-scale sampling design enabled us to demonstrate marked spatial heterogeneity in plant microbiota at the plant individual scale (α-scale) and the scale of a group of individuals in the field (γ-scale). As hypothesised, this variability was explained by changes in environmental factors including abiotic factors, but also in plant-specific factors.

Because the global γ-diversity scale corresponds to the sum of α-diversity + β-diversity (i.e., β-diversity measuring the difference between individuals), low β-diversity (i.e., γ-diversity ~ α-diversity) could lead to similar responses to environmental factors at both α- and γ- scales, while strong β-diversity could lead to divergent responses between the α- and γ-diversity scales. It should be noted that differences in the detected statistical signal could also result from differences in the statistical power of the analysis between the α- and γ-diversity scales, i.e., sampling units are 8-fold higher at the α-scale than at the γ-scale. Most of the relationships we detected differed at both diversity scales, for instance, the effects of the pH and OM on "all bacteria" community richness and the cultivar on the "all fungi" community richness were significant at α-scale but not at γ-scale. Reciprocally, a few relationships were detected at the γ-scale but not at the α-scale, for example, an increase in bacterial richness with an increase in soil P_2O_5. P_2O_5 concentration may thus be heterogeneous particularly at a very fine scale and hence affect local microbial richness. Furthermore, we cannot exclude the existence of spatial variation

and possible patchiness in the P_2O_5 bioavailability. Another example of such a relationship detected at the γ-scale but not at the α-scale was a reduction in *Glomeromycota* richness in older individual vines compared to that in younger ones. This may be due to higher convergence in species composition among mature individuals in a given field (i.e., lower beta-diversity), leading to lower total species richness at the field scale. This convergence among individuals might be the result of a stronger influence of a host-preference effect in more mature individuals than in younger ones. Bulgarelli *et al.,* (2013) defined the root microbiota as a succession of dynamic processes combining primary colonization of microbes from the soil/ rhizosphere to the roots and subsequent host recruitment of proper microorganisms depending on host requirements (Bulgarelli *et al.,* 2013). Our results suggest that both mechanisms – priority effect and host-preference – are likely to be inversely related and to depend on the stage of succession of the microbial assemblage in relation to the development of the host plant.

3. Bacterial- and fungal-root endosphere composition shaped by environmental factors

Confirming our hypotheses, environmental factors were seen to shape bacterial and fungi communities through changes in the sequence-cluster identity and to a lesser extent, by modifying their richness and/or relative evenness.

A pattern shared by the two taxonomic groups was the strong effect of pH. pH is widely recognised as an important driver of microbial communities, notably because it can act on microbial enzyme activity or can regulate the ionization balance in the soil, thereby affecting the accessibility of nutrients for microorganisms (Burns *et al.,* 2015; Liang *et al.,* 2019; Pacifico *et al.,* 2019). Most relationships between P_2O_5, C/N and OM and root endosphere microbial communities differed between bacteria and fungi but also within each group, suggesting fine adjustments of microbiota composition depending on these factors. For instance, the "all fungi" community was particularly affected by the C/N ratio whereas the "all bacteria" community appeared unaffected by this factor. As the C/N ratio is usually considered as a key proxy for nutrient accessibility (Zhang and Elser, 2017), changes in soil C/N ratio likely impact fungal biosynthesis processes as well as food-seeking strategies (Cleveland and Liptzin, 2007). At a finer scale of taxonomy, we showed a stronger effect of the C/N ratio on the *Glomeromycota* compared to the effect on the *Basidiomycota* community.

All the members of *Glomeromycota* are involved in arbuscular mycorrhization and are thus very important microorganisms known to supply their host plants with mineral nutrients and water and to improve host-plant resistance to different kinds of stress (e.g., Smith and Read, 2009), as already demonstrated in vine plants (Nicolás *et al.,* 2015; Trouvelot *et al.,* 2015). In exchange for these services, *Glomeromycota* receive carbon synthesised by their host (e.g., Smith and Read, 2009; Johnson, 2010). If the C/N ratio decreases (the N content is higher) in some cases, the vine plants would have easier and more direct access to nitrogen and this would directly impact the *Glomeromycota* community. Therefore, as suggested by our results, the *Glomeromycota* community is more likely to be affected by changes in the C/N ratio than other components of the fungal microbiota communities.

4. Vine plants shaped their own bacterial and fungal root endosphere microbiota

We also demonstrated a general influence of cultivar and age on microbial composition but not necessarily an effect on richness. This suggests that these parameters induce a shift in sequence-cluster composition rather than affecting the number of species recruited. This shift may result from ecological and biological mechanisms. Like *Glomeromycota*, which show no host-plant specificity, it can be assumed that most members of the vine microbiota are not specific to their host. They are associated with a given host due to host-plant filtration of colonisers.

In agreement with this interpretation, different authors have reported filtering effects of the roots (e.g., Bulgarelli *et al.,* 2012) depending on the type of plant and/or plant genotype (Zarraonaindia *et al.,* 2015; Marasco *et al.,* 2018; Martínez-Diz *et al.,* 2019) involved in the recruitment of microorganisms. The analysis of grape and soil samples from three different cultivars showed that some bacterial sequence-clusters were specific to a particular cultivar (Mezzasalma *et al.,* 2018). Berlanas *et al.,* (2019) analysed microbiota in mature and young vineyards by focusing on the rhizosphere compartment and reported that, instead of a clear distinction between microbial communities in young and mature vineyards, differences were rather due to many different factors that included rootstock genotype. To our knowledge, our results also emphasised for the first time, a strong age effect of the vine on its root endosphere microbiota. This may be linked to a succession of processes involved in the colonization of roots by microorganisms over the vine's lifetime (Chang and Turner, 2019). However, we cannot exclude an indirect effect of the history of management of the individual vine plants on these natural processes. Rootstock genotype plays a role in shaping and recruiting the endophytic microbial communities of the vine plants (D'Amico *et al.,* 2018; Marasco *et al.,* 2018). However, this age-related effect on the root endospheric microbiota could not be statistically linked to a rootstock genotype effect (see methods) and a plant-maturity related niche differentiation is more likely.

5. Toward a *microbial terroir* at the vineyard scale

The idea of an existing *microbial terroir* in vineyards is gradually growing (Gilbert *et al.,* 2014; van Leeuwen *et al.,* 2018). Our results reveal new dimensions of the spatial profile of microbial communities in vineyards at the level of field in a highly homogeneous winegrower's domain in terms of soil bedrock and soil type (smaller geographic scale). This work is pivotal in that it demonstrates in vine plants that the endospheric microbiota differs according to both the environment and plant-specific parameters including cultivar and the age of the vine. If these new small-scale dimensions are confirmed by subsequent analyses (i.e., seasonal variability) and if these observed microbial variations are consistent with impacts on grapevine phenotypes, our work could offer wine-makers the opportunity to include this dimension in their production with the use of spatial maps of this *microbial terroir*. Because variations in microbial communities exist at the level of a single vineyard and in nearby locations, it is reasonable to suggest that understanding the complex interactions between bacteria, fungi and the vine plants could help vine-growers adapt their management practices and finally intensify the specificity of a vine and enhance the final product.

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