

### Intra- and inter-annual changes in root endospheric microbial communities of grapevine are mainly deterministic

Marine Biget, Cendrine Mony, Tingting Wang, Ning Ling, Adèle Miteul, Olivier Jambon, Romain Causse-Védrines, Sophie Michon-Coudouel, Maxime Hervé, Véronique Chable, et al.

#### ▶ To cite this version:

Marine Biget, Cendrine Mony, Tingting Wang, Ning Ling, Adèle Miteul, et al.. Intra- and inter-annual changes in root endospheric microbial communities of grapevine are mainly deterministic. Plant and Soil, 2023, 10.1007/s11104-023-06262-6. hal-04251671

#### HAL Id: hal-04251671 https://univ-rennes.hal.science/hal-04251671

Submitted on 11 Dec 2023

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- 1 Intra- and inter-annual changes in root endospheric microbial communities of
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#### grapevine are mainly deterministic

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#### 30 Abstract

Background and aims Considering the plant microbiota, temporal changes are
expected depending on plant development stages and environmental pressures because
of modifications in plant requirements and available soil microbial reservoir.

Methods Herein, we analyzed the composition of root endosphere microbiota of grafted vine plants using two grapevine cultivars (Merlot and Cabernet-Sauvignon as scion grafted on rootstocks of different clones) as models both sampled in a single vineyard at three dates over a period of two growing years.

38 Results Highly conserved temporal patterns were found in the two cultivars. Intra-39 annual changes in microbial community composition were recorded whereas 40 convergent microbial communities were observed on the two September dates. In 41 particular, the increase in Actinobacteria and decrease in Glomeromycota in September 42 were interpreted as shifts in the microbiota community patterns related to plant 43 physiological requirements (e.g. water supply). A high proportion of non-random 44 assembly of the root endospheric bacterial community confirmed the deterministic 45 influence of the plant or/and the environment in microbial recruitment over time. The 46 modified normalized stochasticity ratio (MST) showed that deterministic processes of 47 assembly (MST < 50%) were commonplace despite the changes in the root microbiota 48 composition observed among sampling dates.

49 Conclusion Our study suggests an intra-annual rhythm of microbiota shifts, marginally 50 random, with a succession within the root-microbiota endosphere likely governed by 51 active plant filtering. A better knowledge of microbial-recruitment at work, seems 52 important for both fundamental and applied perspectives.

2

- 53 Keywords: root microbiota, endosphere, temporal dynamics, deterministic assembly,
- 54 microbiota shifts, grapevine

#### 55 Introduction

56 Plant development and survival are largely dependent on a remarkable diversity of 57 microorganisms (Compant et al., 2019; Lemanceau et al., 2017; Vandenkoornhuyse et 58 al., 2015). These microorganisms, either epiphytes or endophytes, can live in all plant 59 organs and tissues. All plants worldwide are colonized by many different types of 60 microorganisms, the most frequently studied being bacteria and fungi (Müller et al., 61 2016). These plant-associated microorganisms that form plant microbiota are deeply 62 involved in supplying the plant with water and nutrients (Trivedi et al., 2020; 63 Vandenkoornhuyse et al., 2015). They help their host face biotic and abiotic stresses 64 such as drought and solar radiation (Trivedi et al., 2020; Vannier et al., 2015; Yang et 65 al., 2013). It is worth noting that microbial communities display dynamic fluctuations. 66 In continental ecosystems, dynamic fluctuations through seasons have been evidenced 67 and were found correlated with environmental constraints including temperature and 68 precipitation (Liu & Howell, 2021). Deterministic processes of microbiota assembly 69 driving changes through time can thus be hypothesized. In grafted plants, as far as we 70 know, determinism in changes through time in the microbiota composition within their 71 host-plant (i.e. the microbiota endosphere) has never been formally analyzed.

Endospheric microorganisms associated with plants originate from the environment, mostly from the soil, which thus serves as a microbial reservoir (Vandenkoornhuyse et al., 2015; Xiong et al., 2021; Zarraonaindia et al., 2015). The processes behind plant colonization shaping microbial assemblage composition are thought to be mainly deterministic, i.e. shaped by host selection (Morrison-Whittle et al., 2015; Xiong et al., 2021; and in grafted plants, Biget et al., 2023; Dries et al., 2021; Marasco et al., 2022; Vink et al., 2021). Deterministic processes are assumed to be primarily linked to plant-

4

79 microorganism interactions. However, randomness and neutral processes can occur80 (Werner & Kiers, 2015).

81 Two main mechanisms may be related to temporal fluctuations in the plant microbiota 82 community composition. Firstly, the development stage of the plant influences plant 83 microbiota over time (Chaparro et al., 2014; Copeland et al., 2015; Jumpponen & Jones, 84 2010; Mougel et al., 2006). This can be explained by changes in plant needs, which 85 thus modify the microbial assemblages recruited by the plants at the rhizosphere stage 86 and inside the roots (Berg & Smalla, 2009; Chaparro et al., 2014). Gradual changes 87 may also occur ranging from random colonization by species living close to the roots 88 to preferential selection of particular species by plants (i.e. host plant preference) 89 (Vandenkoornhuyse et al., 2002) and filtration by preferential carbon allocation to the 90 best cooperators (i.e. deterministic changes) (Kiers et al., 2011). In addition, microbiota 91 modifications can be linked to the development of new roots to colonize and to temporal 92 modifications in root characteristics, shape, and functions that may modify the space 93 available for microbial colonization (Iannucci et al., 2021; Pérez-Jaramillo et al., 2017; 94 Saleem et al., 2018) coming together with grape phenology with, for instance, drastic 95 changes in grape metabolism during grape ripening stage, leading to pectin and 96 cellulose degradation, acidity decreasing, and sugars accumulation in berries (Liu & 97 Howell, 2021; Renouf et al., 2005).

98 Secondly, environmental pressures influence plant microbiota over time. It has been 99 empirically noticed that microbial communities vary over time, both between seasons 100 and years (Liu & Howell, 2021; Zarraonaindia et al., 2015). In a year, climate 101 conditions may vary strongly depending on seasons, resulting in changes in soil 102 parameters (e.g. water availability, C/N ratio). These environmental changes over the

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103 year can lead to dynamic changes in the reservoir of recruitable microorganisms, and 104 hence in the composition of microorganisms recruited by the plant. In addition, plants 105 respond to changes in environmental conditions and buffer abiotic stresses by recruiting 106 particular microorganisms from the soil reservoir (Vannier et al., 2015; Vives-Peris et 107 al., 2020). However, so far, very few studies have investigated temporal variations in 108 plant microbiota endosphere, the most intimate fraction of the plant microbiota. We 109 used herein grapevine as a model to assess the endospheric microbiota dynamics 110 because grapevine has arguably a number of advantages, notably they are perennial, 111 genetically homogeneous (i.e. herein, when made of particular scion clone grafted on a 112 particular rootstock clone) and are usually managed very similarly at a given location 113 and in a given vineyard domain.

114 We formulated different hypotheses. (i) The composition of both the bacteria and fungi 115 on the grapevine varies over time. Assuming the existence of a major impact of the 116 plant development stage as well as of modifications in the soil parameters in the 117 different seasons, (ii) microbial differences between growing periods would be more 118 pronounced than annual variations (same sampling date in two different years). (iii) If 119 these temporal changes occur as a response to environmental constraints (changes in 120 plant selection and abiotic conditions), these changes in the plant microbiota result from 121 a combination of deterministic and stochastic processes, with the main factor driving 122 recruitment being the soil reservoir, followed by plant-related factors (genotype/stage). 123 To test these hypotheses, we sampled roots in a single vineyard domain located in 124 Margaux (France). At this location, the bedrock is considered homogeneous and 125 vineyard management is similar among fields. The root endosphere of grafted 126 grapevine plant only have been analysed. Amplified fragment (amplicons) of fungal 127 and bacterial small sub-unit rRNA gene obtained from root samples collected from the

6

same individual plants through time (three sampling campaigns) were sequenced toaddress the hypotheses.

#### 130 Materials and Methods

#### 131 Experimental design and study site

132 We selected individual vine plants in a single 66-ha vineyard located in Margaux, 133 France, (45°2'12.73" N; 0°40'9.84" W). Organic and biodynamic practices have been 134 used in the vineyard for 12 years. We sampled 25 fields (8 plant individuals per field), 135 the number of fields containing Cabernet Sauvignon CS (12 fields) and Merlot M (13 136 fields) clones (Fig. 1) all of similar age ranges. These two cultivars representing the 137 upper part (scion) were grafted on rootstock 101-14 mainly. However, it can be 138 highlighted that overall, three rootstocks types were used according to different 139 combinations (Table S1) and M and CS rootstocks are not strictly identical. Within one 140 plot, the plants were of the same genotype (i.e. same scion and rootstock clones). We 141 analyzed the composition of the microbial community associated with roots of the two 142 grapevine cultivars in September 2018 (S18), June 2019 (J19), and September 2019 143 (S19) (June: flowering stage and September: mature grapes before harvest). The same 144 individual grapevines were sampled in the three sampling campaigns (Fig. 1). The 145 region is characterized by a temperate climate with average temperatures of 14.2 °C 146 (2018) and 14 °C (2019); and cumulative annual precipitation of 934mm (2018) and 147 1,112mm (2019). Fig. S1 shows the average daily temperature and cumulative 148 precipitation per day recorded in the three sampling campaigns, two in 2018 and one in 149 2019 by the DEMETER weather station located in Margaux, http://www.meteo-150 agriculture.eu.

7

#### 151 Sampling and DNA extraction

152 On each date, 200 similar non-lignified vine root fragments (i.e. roots produced during 153 the current year) were collected to analyze variations in the grapevine root microbiota 154 over time (25 fields x 8 plant individuals). For each individual plant,  $\sim$  1g of healthy 155 and non-lignified roots, of similar shape and diameter were picked close to the stem at 156 a depth of 5-20 cm and placed in individual hermetic bags and stored in cooled packs. 157 At the laboratory, the roots were thoroughly washed in tap water, then immersed in a 158 5‰ Triton X100 solution for 10 minutes and finally thoroughly rinsed several times 159 using sterile ultra-pure water (Lê Van et al., 2017). The washed roots were placed in 160 sterile micro-tubes and stored at -80 °C until DNA processing. A representative healthy 161 rootlet sub-sample (100 mg) of the cleaned roots was used for the DNA extraction. 162 Total root DNA was extracted and quantified at the GENTYANE platform (Clermont-163 Ferrand, France) using magnetic bead technology (Sbeadex) on an Oktopure LGC 164 Genomics automat with Hoechst 33258 reagent. The DNA samples were then stored at 165 -20 °C until amplicon production.

#### 166 Molecular biology and sequencing

167 All 600 extracted DNA were normalized at  $10 \text{ ng/}\mu\text{L}$  to ensure PCR amplifications were 168 the same for all the samples. Endospheric fungi were targeted by amplifying an 18S 169 rRNA gene fragment with fungal primers NS22b (5'-170 AATTAAGCAGACAAATCACT-3') SSU817 (5'and 171 TTAGCATGGAATAATRRAATAGGA-3'). Endophytic bacteria were analyzed by 172 PCR amplification of a 16S rRNA gene fragment (V5-V7 region of the 16S gene) using 173 the bacterial primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1223R (5'-174 CCATTGTAGTACGTGTGTA-3'). The sets of primers were customized to

<sup>8</sup> 

175 incorporate sample tags and Illumina® adaptors. To provide normalized PCR mix and 176 avoid contamination, Illustra<sup>TM</sup>PuReTaq Ready-to-go beads (GE Healhcare®) were 177 used for both bacterial and fungal amplifications. The PCR conditions associated with 178 the specific targets have been detailed previously (Vannier et al., 2018). The following 179 molecular steps were performed on the EcogenO platform (Rennes, France): briefly, 180 PCR products were purified with AMpureXP magnetic beads (Agencourt®) and 181 quantified with a Quant-iT PicoGreen<sup>™</sup> dsDNA Assay Kit to insure normalization of 182 the PCR products at the same concentration. A second PCR using the Smartchip-Real 183 Time PCR machine (Takara) performed multiplex tagging, the resulting tagged-184 amplicon pool was purified (AMpureXP, Agencourt<sup>®</sup>) and quantified using the Kapa 185 Library Quantification Kit-Illumina®. The bacterial and fungal libraries were finally 186 sequenced on an Illumina MiSeq (PE-2x250 and PE-2x300 cycles for bacterial- and 187 fungal-amplifications respectively, with 3 sequencing runs each). As routine in 188 amplicon sequencing at EcogenO platform, a PCR negative control passing all the steps 189 of the library construction was included in the sequencing procedure.

#### 190 Raw data processing and clustering

Base calling 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. The sequences were then processed using the FROGS pipeline following the recommendations (Escudié et al., 2018). The FROGS pipeline uses SWARM as a sequence agglomeration strategy to produce "sequence clusters".

Briefly, the filtering steps recommended (Escudié et al., 2018) comprised a specializedalgorithm for de-noising and a chimera removal step to exclude artificial sequence

<sup>9</sup> 

clusters. Further filtering enabled removal of all sequence clusters identified in fewer
than three samples and with a threshold of 0.005% of reads. After taxonomic affiliations
using Silva138 16S rRNA for bacteria (Quast et al., 2012) and Phymyco-DB databases
for fungi (Mahé et al., 2012), a final filtering step was applied to affiliated sequence
clusters (95% coverage and 95% identity (BLAST)). The amplicon sequences did not
contain any plastidial or plant mitochondrial DNA sequence. Bacterial or fungal DNA
only was amplified and sequenced.

206 The resulting contingency tables contained 14 765 700 reads for bacteria and 12 483 207 626 reads for fungi. The sequenced negative control did not contain any detectable 208 contaminating DNA. Rarefaction curves were produced using R ("vegan" package). 209 Sequencing depths of both bacterial and fungal microbiota were sufficient to limit 210 sequence cluster subsampling (Fig. S2). To perform statistical analysis, because the 211 slopes of the curves tended toward a plateau (Fig. S2), we normalized the number of 212 reads at 8 500 and 3 700 reads for the bacterial- and fungal- dataset respectively. For 213 the bacteria, we discarded only 13 samples (13/960 samples representing 1.4%) to 214 rarefy at 8500 reads per sample. For the fungi, we discarded 37 samples (37/960 215 samples representing 3.85%) to rarefy at 3700 reads per sample. None of the fields were 216 composed of less than four out of the eight samples.

The final matrices containing 947 bacterial and 529 fungal sequence clusters were then used for statistical analyses. We calculated sequence cluster richness using the R "vegan" package. Index calculations and statistical analyses were performed for the dominant phyla (i.e., phyla representing the most sequence clusters with a threshold set at 5% of the total sequence clusters per date at the vineyard scale for the two microbial communities).

10

#### 223 Statistical analyses

224 Statistical analyses were performed using R software version 4.0.4. We analyzed the 225 effect of the sampling date (S18, J19 and S19) on root endospheric microbiota structure 226 with a combination of multivariate analysis, univariate models, and neutral models. We 227 checked if the microbial communities inhabiting the two cultivars differed using RDA 228 (Redundancy analyses; Fig. S3). RDA were performed on Hellinger distances 229 computed from sequence cluster matrices. The cultivar effect was assessed by a 230 permutation test (999 repetitions) using the 'RVAideMemoire' package (Hervé, 2020). 231 Because there was a significant effect of the cultivar in the two taxonomic groups (Fig. 232 S3), all the subsequent statistical analyses were conducted separately on each cultivar 233 to test the effect of the sampling date.

#### 234 Effect of the sampling date on microbial assemblage structure

235 Partial principal coordinate analyses (PCoAs) were performed to assess the distribution 236 pattern of root endospheric microbiota. PCoAs were based on Hellinger distances 237 computed from sequence cluster matrices (Legendre & Gallagher, 2001). The 238 individual grapevine was considered as a conditional effect to account for the biological 239 dependency of the samples. The effect of the sampling date was assessed by a 240 permutation test applied on the first PCoA factorial axes (function envfit () in the R 241 'vegan' package, 999 permutations). Pairwise comparisons were performed in the same 242 way, with p-values adjusted using false discovery rate correction (Benjamini & 243 Hochberg, 1995). PCoAs were also used to assess the effect of rootstocks on the 244 microbial composition within each cultivar. Variation partitioning analysis (VPA) was 245 used to test the effect of rootstock and sampling time on the community composition 246 within each cultivar. We compared the difference in the Bray-Curtis distance to

<sup>11</sup> 

compare the microbial variation between intra- and inter-annual time point underdifferent cultivars and under the combination of scion and rootstock.

We used Wald tests applied to linear mixed models to test whether the sampling date impacted microbial richness. We constructed all the models using the richness index as the explanatory variable, the "individual grapevine" as random effect, and the "sampling date" as fixed effect. The linear mixed models and subsequent tests were performed using the R package 'lme4' (Bates et al., 2014), 'car' (Fox & Weisberg, 2018) and 'emmeans' (Searle et al., 1980).

#### 255 Sampling dates differences on stochastic and deterministic assemblage processes

256 The Sloan neutral community model (NCM) was used to determine the influence of 257 neutral processes on the bacterial and fungal community assembly inhabiting the root 258 (Sloan et al., 2006). This neutral model predicts the relationship between sequence 259 cluster detection frequency and their relative abundance (Burns et al., 2016; 260 Venkataraman et al., 2015). The model predicts that species that are more abundant in 261 the metacommunity (total source pool) are more expected to disperse and be randomly 262 present in different sampling conditions, whereas species with low abundance are more 263 likely to get lost due to ecological drift (Burns et al., 2016). In this study, we conducted 264 neutral model analyses assuming the three-date metacommunity (i.e., the total dataset 265 of a given cultivar) was the source of microbial communities. One neutral model was 266 generated per sampling date and per taxonomic group (fungi and bacteria). The adequacy of model fitting was assessed through  $R^2$  values (0< $R^2$ <1) (Sloan et al., 2006). 267 268 The sequence clusters from each matrix were then separated into three classes 269 depending on their occurrence frequency based on a 95% confidence interval of the 270 neutral community model predictions. The 95% confidence intervals around all fitting

12

271 statistics were obtained by bootstrapping with 1 000 replicates. The three categories 272 corresponded to the sequence clusters that were more frequent than expected (above 273 class), less frequent than expected (below class), and within the confidence interval 274 (neutral class). The model also calculated the estimate rate "m" (read as a measure of 275 dispersal limitation) using a non-linear least squares fitting (NLS- 'minpack.lm' 276 package) (Elzhov et al., 2010). The m value gives the probability of dispersal 277 substitution from the metacommunity due to random loss of species in the local 278 community (Burns et al., 2016). Therefore, higher m values likely show less dispersal 279 limitation. The R code used for this analysis originated from (Burns et al., 2016). We 280 further analyzed the sequence clusters specific to each class (above, below, and neutral 281 classes) and extracted their taxonomic affiliations to identify the main specific 282 taxonomic patterns that define each class associated with each sampling date.

283 The modified normalized stochasticity ratio (MST) was further used to quantify the 284 relative importance of deterministic and stochastic process in community assembly 285 with 50% as the boundary point between more deterministic (MST<50%) and more stochastic (MST>50%) assembly (Ning et al., 2019). The MST value was calculated 286 287 based on the Jaccard distance estimated by using "tNST" functions in 'NST' package 288 (Ning et al., 2019). We divided all the samples into three time points to test the influence 289 of time series on the balance between determinism and stochasticity, and also divided 290 them into combinations of scion and rootstock.

13

#### 291 **Results**

#### 292 Description of the microbial composition of root endosphere

The Cabernet Sauvignon (CS) matrices contained 945 (12 fields, 288 samples) and 523
(12 fields, 278 samples) bacterial and fungal sequence clusters respectively while the
Merlot (M) matrices contained 943 (13 fields, 307 samples) bacterial sequence clusters

and 519 (13 fields, 519 samples) fungal sequence clusters.

#### 297 Effect of the sampling date on microbial community composition and richness

The effect of sampling date on microbial community composition was analyzed through PCoA. PCoA analyses demonstrated clear differences in both bacterial and fungal communities among sampling dates, whatever the cultivar (p < 0.001; Fig. 2). The effect of the sampling date explained 44.2% and 41.6% ( $R^2$  in Fig. 2A, B) of the variance of the bacterial community and 25.2% and 26.9% of the variance of the fungal community ( $R^2$  in Fig. 2C, D) in Cabernet Sauvignon and Merlot respectively.

304 Regarding communities composition, J19 and S19 displayed the highest dissimilarity 305 (Bray-Curtis distance) difference, while S18 and S19 presented the lowest difference, 306 with similar ranges for all bacterial and fungal communities of both cultivars (p < 0.05, 307 Fig. S4; Fig. S5). The richness of the fungal microbiota ("all fungi") was mainly 308 explained by Ascomycota (the most numerous in terms of richness), and richness was 309 lowest in J19 for both cultivars compared to S18 and S19. Reciprocally, 310 Glomeromycota richness was significantly higher in J19 for both cultivars (Fig. 3C, D) 311 while Basidiomycota richness had the same pattern for Merlot cultivar only. In contrast 312 to fungi, bacteria showed no clear pattern of variation among sampling dates, except 313 for Actinobacteria (lowest richness in J19 whatever the cultivar; Fig. 3A, B).

14

and S19 but only in the Merlot cultivar (Fig. 3B).

### 316 Effect of sampling date on the stochastic and deterministic assemblages of the

317 microbial communities

To decipher the stochastic and deterministic processes in shaping microbial 318 319 communities depending on the sampling date, we conducted neutral community model 320 analyses (NCM) (Fig. 4A, B). Overall, the occurrence frequency of bacteria analyses was partly defined by the neutral model ( $R^2=35.5\sim43.9$  for Cabernet Sauvignon and 321  $R^2 = 29.5 \sim 44.1$  for Merlot; Fig. 4A, B). However, for the fungal taxa, we did not record 322 323 a proper fit to the model (negative  $R^2$  values Fig. S6A, B) for three out of the six models. Proper fits were found only for the J19 and S19 analyses ( $R^2 = 14.8$  and  $R^2 = 6.14$ ; Fig. 324 S6A) and the J19 community analyses ( $R^2=15.5$ ; Fig. S6B) for Cabernet Sauvignon 325 326 and Merlot, respectively.

327 In the two grapevine cultivars and on all three sampling dates, some sequence clusters 328 of bacterial taxa occurred more frequently (10.7 to 53.9%, yellow dots) or less 329 frequently (30.1 to 42%, green dots) than predicted by the neutral models (Fig. 4A, B, 330 single bar charts). The yellow sequence clusters suggest active selection and 331 maintenance by the host plant while the green taxa suggest active selection against the 332 host (Fig. 4A, B). The stochastic fraction accounted for from 13.6 to 56.9% of 333 cumulative microbial occurrence, thereby rejecting the null hypothesis of random 334 assembly of the plant microbiota (hypothesis 4) (Fig. 4A, B, single bar charts).

Community composition defining the three classes (above, below and neutral partitions)
was not evenly distributed among phyla (Fig. 4A, B, combined bar charts). Clear
differences were observed for Alphaproteobacteria, Gammaproteobacteria, Bacteroidia

15

as well as Actinobacteria between the two deterministic partitions (Fig. 4A, B, "above"
and "below" in the combined bar charts) indicating that within a taxonomic group some
sequence clusters were deterministically selected and maintained by the host plant
while others were negatively influenced by the host plant or by the local environment
(Fig. 4A, B, "above" and "below" in bar charts).

343 To further quantify the relative importance of deterministic processes in shaping plant 344 associated microbiota, and to complement the NCM analyses, the modified normalized 345 stochastic ratio showed that MST value of bacterial and fungal communities was below 346 the 50% boundary point, i.e. deterministic processes of assembly played a preponderant 347 role in both bacterial and fungal communities composition in the vine root endosphere 348 (Fig. 5; Fig. S7). The estimated minimum proportion of determinism in the community 349 composition of endospheric microbial communities was 53% in S19 bacterial 350 community of Merlot. MST values depended on sampling date with higher values for 351 J19 compared to S18 and S19 for fungi; and increasing values across sampling dates 352 for bacteria.

353 Discussion

#### 354 Composition of the grapevine root endosphere

In this study, we analyzed the bacterial and fungal communities inhabiting the root endosphere and deciphered the temporal dynamics that structure microbial communities at the level of a vineyard. Proteobacteria (Alphaproteobacteria and Gammaproteobacteria) and Ascomycota were the main phyla forming the root endospheric microbiota in the grapevines in line with the results of most studies that analyzed grapevine microbial communities in different compartments, areas and climate (Bettenfeld et al., 2022). Although the microbial composition of the main phyla

<sup>16</sup> 

remained stable and was similar to that described in most microbial studies of grapevine
(Deyett et al., 2020; Swift et al., 2021), we observed specific changes in phyla
abundances depending on the sampling date.

# 365 Intra- versus inter-annual variations affect both microbial composition and 366 richness

367 In this present study, as expected, we observed consistent changes in the microbial 368 communities of the grapevine roots depending on the sampling date, with changes in 369 the composition, richness and selection of specific taxa (hypothesis 1). In both cultivars, 370 we showed that intra-annual variations in the composition of microbial communities in 371 the same year (June 2019 and September 2019) were stronger than those observed in a 372 given growing period in two different years (September 2018 and September 2019) 373 (hypothesis 2). Similarly, but in a different compartment, the rhizosphere, a recent study 374 showed changes in the bacterial and fungal richness of grapevine related to the sampling 375 date during the year (Berlanas et al., 2019). Elsewhere, in grafted grapevine, it has been 376 shown that the combination of both scion and rootstock determine, at least in part, the 377 root microbiota composition (Marasco et al., 2022) with a more pronounced effect of 378 rootstock as driver of the deterministic process of assembly of the grapevine root 379 microbiota (Biget et al., 2023). Considering the observed changes in the grapevine root 380 microbiota composition through time, we noticed that (i) almost all scion and rootstock 381 combinations showed less variation within than between years (Fig. S5) (ii) the 382 assembly process considering the different time points under almost all combinations 383 of rootstock and scion is deterministic (Fig S7) which is not coherent with the 384 observation (i.e. less variation between years) despite a confirmed effect of the 385 rootstock type on the microbial composition (Fig S8 and S9). Taken altogether, and

17

despite the limits of these analyses of the rootstock effect (i.e. redundancy between factors field and rootstock-scion combination), the results (Figs S5-S9) suggest that the changes observed among sampling dates in the grapevine root endosphere microbiota composition are not a consequence of divergences related to the rootstock type but rather seemingly convergent response patterns related to external effector.

391 Regarding the composition of the bacterial community, we particularly observed that 392 Actinobacteria were significantly richer in September than in June, whereas 393 Bacteroidota and Myxococcota were richer in June. Actinobacteria have been described 394 to improve plant mineral and hydric nutrition (Govindasamy et al., 2014) and may thus 395 have been recruited by the plant in greater numbers in September to buffer abiotic 396 stresses at the end of the summer. Monthly rainfall and mean temperature were 36.8 mm and 20.6°C, 134.0 mm and 20.9°C, 71.0 mm and 20.0°C for Sept 18, June 19 and 397 398 Sept 19 respectively. It can thus be suggested that the microbiota composition changes 399 are related to water availability. In support to this, analyses of synchronic effect of 400 drought on the microbiota composition have previously demonstrated changes in the 401 plant endosphere microbiota composition (Andreo-Jimenez et al., 2019).

402 Some genera among Actinobacteria, particularly Streptomyces, are known to control 403 pathogens by producing antibiotic molecules (Doumbou et al., 2001; Schrey & Tarkka, 404 2008). Thus, these *Streptomyces* can help to suppress phytopathogens and plant 405 diseases. We detected specific Streptomyces sequence clusters at the two September 406 sampling dates (the same sequence-clusters were identified in September 2018 and 407 September 2019, Table S3). Because the critical development stage of grapevines is at 408 the end of grape ripening, it can be suggested that the plants may secrete specific 409 exudate compounds to recruit Actinobacteria to face both biotic and abiotic stresses.

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The increase in Bacteroidota in June can be explained by their role in plant nutrition. Indeed, Bacteroidota have been reported to be involved in organic phosphorus mineralisation (Lidbury et al., 2021). Recruiting Bacteroidota in June, when the plants reach their highest vegetative growth phase, may help them to face increasing demand for mineral nutrition. Changes in bacterial communities between the flowering and the harvest periods (mature grapes) appeared to be in line with developmental grapevine stages, both involving differential plant needs.

417 The composition of the fungal community was also strongly influenced by the sampling 418 date, with higher Basidiomycota (only for Merlot) and Glomeromycota richness in June 419 and higher Ascomycota richness in September. A recent study investigated intra-annual 420 changes in fungal communities of different bio-compartments, which supports our 421 results on the root endosphere (Liu & Howell, 2021). They correlated weather 422 conditions with changes in fungal composition and observed dramatic changes in 423 microbiota composition and diversity especially at the veraison stage, just after the fruit 424 set period (Liu & Howell, 2021). Glomeromycota forming Arbuscular Mycorrhiza (AM) 425 are known to be involved in supplying minerals and water to plants and in improving 426 resistance to plant diseases (Smith & Read, 2010). Thus, changes in the patterns of AM 427 fungal community composition over time that have already been evidenced (Bennett et al., 2013; Vandenkoornhuyse et al., 2002) may be correlated with the plant 428 429 development stage and plant requirements over the seasons. Beyond this aspect, the 430 shifts in AM symbiosis composition may be partly attributable to a demonstrated 431 priority effect (i.e. the effect of order of arrival on subsequent community development) 432 (Werner & Kiers, 2015).

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19

Many endospheric Basidiomycota and Ascomycota that form positive associations with

434 plants (i.e. endophytic fungi) are known as plant "protective agents" (Porras-Alfaro & 435 Bayman, 2011) that promote plant resistance to environmental stresses including 436 drought, salinity, heat, insects, and pathogens (Hardoim et al., 2015; Porras-Alfaro & 437 Bayman, 2011). For instance, under drought conditions, rice has an enriched fungal 438 endosphere that has been interpreted as positive recruitment to buffer drought 439 conditions (Andreo-Jimenez et al., 2019). Fungal endophytes are known to induce a 440 plant systemic resistance (ISR) while these fungal symbionts escape this immune 441 response (Hardoim et al., 2015). In addition, it has been demonstrated *in vitro* that some 442 of these recruited fungi are able to produce antifungal metabolites (Hardoim et al., 2015) 443 which might modify the diversity of the root-fungal endosphere. Further analysis including an absolute quantification of each fungus composing the grapevine 444 445 mycobiota might provide complementary information to reinforce conclusions related 446 to the changes observed. More widely, the temporal changes in root-endosphere 447 community composition over time raise the question of the extent of the determinism 448 in the process of assembly.

#### 449 Stochastic versus deterministic process of assembly of the grapevine root 450 endosphere over time

Recent advances in statistical analyses have allowed to partition deterministic and ecologically neutral processes of assembly using a null model framework (Burns et al., 2016) (see Materials and Methods for the rationale). For fungi, the absence of a fit of the neutral community model (NCM) analysis did not allow partitioning with statistical support for stochastic and deterministic processes of assembly. However, considering bacteria, we were able to estimate that the deterministic part accounted for 43 to 84% for the two grapevine cultivars (Fig. 4) (hypothesis 3). This appeared consistent with

20

458 non-random assembly of bacterial communities inhabiting the root endosphere of 459 grafted grapevine plants, although the grafted plants were compared to the ungrafted 460 plants under the holobiont concept (Biget et al., 2023). In our study, we defined a new 461 non-random assemblage process of the grafted plant along a seasonal variation. This 462 new process seems independent from the rootstock genotype, a factor supposedly 463 inducing variance (i.e. a random-like part of the partitioning). The endosphere 464 composition dominated by deterministic assembly processes can be explained by active 465 rejection of bacteria by the host (bar charts showing the cumulative occurrence 466 frequencies of sequence clusters in Fig. 4), and to a lesser extent to sequence clusters 467 actively maintained and selected by the host plant. Previous studies have showed that 468 the endophytic communities in *Pinus* were structured by host age and environmental 469 factors (Carper et al., 2018). It has been shown in plant microhabitats (roots, shoots) 470 that deterministic processes of assembly were primarily responsible for community 471 assembly likely related to plant filtration processes (Guo et al., 2021). The determinism 472 governing the community assembly also suggested thinly tuned filtering processes in 473 grapevine root-endosphere composition over time in this study. The specific selection 474 of key taxa by the host plant can be interpreted as a process to face particular 475 environmental conditions or to meet specific needs. Increasing evidence has explained 476 selection and regulation of rhizospheric microbial communities through root exudates 477 (Broeckling et al., 2008; Chaparro et al., 2013; Chaparro et al., 2014; Voges et al. 2019; 478 Zhalnina et al., 2018). Depending on their development stage, plants can secrete 479 different types of compounds, including specific sugars and amino acids as well as more 480 complex secondary metabolites (Cotton et al., 2019; Jacoby et al., 2020; Voges et al., 481 2019), that act as signals for microbial assemblages of the rhizosphere habitat (Chaparro 482 et al., 2014). It has been recently shown that coumarins solubilized iron under particular

21

483 oxygen and pH conditions thus stimulating rhizospheric microbial growth in these 484 specific conditions (McRose et al., 2023). It is thus not only the control of carbon 485 released by plants but also the physicochemical status which modulates the rhizosphere 486 composition. Modifications in rhizosphere microbiota and in plant microbiota 487 evidenced herein over time may thus be a consequence, at least partially, of both 488 changes in the root secondary metabolites excreted (Dang et al., 2021; Kaur & Suseela, 489 2020; Voges et al., 2019) and physicochemical modifications related to water 490 availability. The rhizosphere being the antechamber of the endosphere, this process 491 should act together with the plant immune system and the mechanism of preferential C 492 allocation to the best cooperators (Kiers et al., 2011). This process would explain the 493 determinism observed in microbiota assembly in root-endosphere samples.

#### 494 Convergence of temporal shifts in the microorganisms root-endosphere 495 composition between the two cultivars

496 A key result of this work is the convergence of the temporal dynamics of 497 microorganisms between the two cultivars, Cabernet Sauvignon and Merlot. The 498 rootstock genotypes used in Cabernet Sauvignon (CS) are not strictly comparable to 499 Merlot (M) (Table S1). At least two plots contained a specific rootstock genotype 500 associated with Merlot scion. From this and because we have recently demonstrated 501 that rootstock is the key driver of endospheric microbiota composition (Biget et al., 502 2023) we expected to observe differences among grafted Carbernet Sauvignon and 503 Merlot. Conversely to the expectation over drivers than plant material can be the 504 observed convergence in the microbiota composition. It can be argued that the 505 environmental conditions (lower water availability) in September might cause a 506 convergent change in the endospheric microbiota composition. It is possible that in

22

addition to the environmental stress, the plant growth developmental stage is also important to consider. If this is true, the high convergence observed in the endospheric microbiota composition can be interpreted as empirical evidence of deterministic selection during plant growth and development. This interpretation is in agreement with recent findings in the maize microbiome, i.e. the plant developmental stage as driver of differentiation in ecological functions of the plant-microbiota and consequently of the recruited microorganisms forming this microbiota (Xiong et al., 2021).

514 The root microbiota has been characterized as a combination of dynamic processes 515 (primary colonization of microbes from the surrounding soil followed by host 516 recruitment) (Bulgarelli et al., 2013). Our results suggest that these mechanisms – 517 priority effect and host preference – are likely to be similar regarding the type of the 518 cultivar analysed and greatly expand on the development of the host plant. Although 519 variations in microbial composition depending on cultivar type and on the genotype 520 have been proposed, most of the work done so far acknowledged that the geographical 521 location and local environmental conditions (climate/soil parameters) had a bigger impact on microbial assembly (Bokulich et al., 2014; Marasco et al., 2018; 522 523 Zarraonaindia et al., 2015; Zhang et al., 2019). We have worked herein at the level of a 524 vineyard domain to limit the effect of bedrock and related soils characteristics, 525 viticulture practices and environmental conditions and confirmed differences in the 526 microorganism endosphere community composition in between Cabernet Sauvignon 527 and Merlot (Fig. S3) and more importantly demonstrated that the two cultivars 528 analyzed responded temporally in the same way, i.e. same key intra- and inter-cultivar 529 variations in the microbial-endosphere structure through time. To confirm our results, 530 the temporal dynamics of the microbiota should now be tested on a larger number of 531 cultivars at a given location under similar viticultural practices.

<sup>23</sup> 

#### 532 Conclusion

533 The study of microbial temporal dynamics advanced our understanding of the 534 mechanisms governing their assemblages and demonstrated tight relationships between 535 microbial assemblages and plant phenology and adaptation to environmental conditions. 536 Here, we showed similar patterns between the two cultivars, both cultivars displayed a 537 deterministic recruitment of specific microorganisms and harbour similar trend in 538 microbial communities variation. Beyond this framework, our understanding of the 539 mechanisms behind the deterministic changes, including the relative effect of the host 540 plant versus abiotic conditions, remains incomplete. Our results stress the complex 541 interactions that take place between a host and its microbiota over time. Understanding 542 microbial assembly rules would help preserve grapevine health, and possibly will allow 543 to understand the particularity of vintages and intensify the spirit of the wine (*microbial* 544 *terroir*). However, fundamental research is still needed to understand the mechanisms 545 of microorganisms recruitment and dynamics in the endosphere. One promising 546 prospect would be to analyse in gnotobiotic conditions (i.e. with the use of a culture 547 collection) how plants attract specific microorganisms (e.g. dynamic analyses of 548 exudates composition emitted) and also to better decipher the importance of source-549 sink transitions for carbon in relation to the developmental stage of the plants.

24

#### 550 Acknowledgements

551 This work was supported by a grant from the CNRS-EC2CO program (HOLOBIONT 552 project), CNRS-IRP program (M-AGRI project) and by the "Agence Nationale de la 553 Recherche et de la Technologie" (ANRT) through an industrial CIFRE agreement 554 between Château Palmer, Château Latour, Sovivins, Pépinière de Salettes and the 555 University of Rennes 1 (Agreement N° 2017/1579). We are grateful to the 556 GENTYANE and, within the ANAEE-France research infrastructure, to the EcogenO 557 platforms for help with DNA extraction and amplicon sequencing, respectively. We are 558 also grateful to the Genotoul platform Toulouse Midi-Pyrenees and GenOuest for 559 access to their bioinformatics pipeline and storage facilities and the Ecolex platform for 560 help during sampling. We deeply acknowledge all the trainees and people at Château 561 Palmer for their help during the sampling campaigns, especially Sylvain Fries, Oriane 562 Heulliet, Hélène Sarkis for their help performing this work. We are also very grateful 563 to July Hémon (EcogenO, molecular analysis) and Achim Quaiser (sampling). We 564 finally acknowledge Daphne Goodfellow for English editing.

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#### 808 Statements and Declarations

#### 809 Funding

- 810 This work was supported by a grant from the CNRS-EC2CO program (HOLOBIONT
- 811 project), CNRS-IRP program (M-AGRI project) and by the "Agence Nationale de la
- 812 Recherche et de la Technologie" (ANRT) through an industrial CIFRE agreement
- 813 between Château Palmer, Château Latour, Sovivins, Pépinière de Salettes and the
- 814 University of Rennes 1 (Agreement N $^{\circ}$  2017/1579).

#### 815 **Competing interests**

816 The authors declared no conflicts of interest.

#### 817 Author contribution

MB, CM, SP, VC and PV conceived the project. PV, CM and MB set up the methodology of the study. MB, CM, OJ, NL and PV conducted the sampling. MB conducted lab experiments with the help of AM. RCV and SMC performed the sequencing and MB and TW analyzed the sequence data. MB and TW performed the statistical analyses with advice from CM, MH and NL. MB wrote the first draft of the paper. All the authors gave their final approval for publication.

#### 824 Data Accessibility

- Data are available on Figshare https://figshare.com/s/6c6ca1fee254f88e0598.
- 826 Sequence data are available at European Nucleotide Archive (ENA) under accession
- 827 number PRJEB47615.

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#### 828 Figures



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#### 830 Fig.1 Experimental design and sampling scheme.

- 831 (A) Map of the study site located near Bordeaux (France) in the Medoc wine region.
- 832 (B) Scheme representing the nearby location of the 25 fields across the vineyard and
- 833 the 8 equidistant grapevines sampled in each field.
- 834 (C) Sampling dates of root collection associated with plant developmental stage:
- 835 September 2018 and 2019 (S18/S19 mature grapes) and June 2019 (J19 flowering
- 836 period).

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Analyses were done on the sequence-cluster matrix of the bacterial (A, B) and fungal (C, D) communities and for the two cultivars considered: the Cabernet-Sauvignon (A, C) and the Merlot (B, D). Results from the permutation tests testing the effect of the sampling dates (\*\*\*: p<0.001) and  $R^2$ , percentage of variance explained, are included on the plots. Statistical results from the pairwise comparisons are also described on the plots (\*\*: p<0.01; \*\*\*: p<0.001; ns: p>0.05). S18-yellow: September 2018; J19-black: June 2019 and S19-blue: September 2019.

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Fig. 3 Effect of the sampling date on bacterial and fungal sequence-cluster richness
at the α-diversity scale.

Linear mixed models for each sampling campaign were used to test whether the sampling date influenced microbial richness (grapevine individual considered as random effect). (A), (B) Results of bacterial communities for Cabernet-Sauvignon and Merlot cultivars respectively. (C), (D) Results of fungal communities for the Cabernet-Sauvignon and the Merlot cultivars respectively. S18-yellow: September 2018; J19black: June 2019 and S19-blue: September 2019.



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#### Fig. 4 Partition of deterministic and ecologically neutral processes of bacterial community assembly using neutral community model (NCM).

(A) S18/J19/S19-Cabernet-Sauvignon and (B) S18/J19/S19-Merlot, served as the
community source to create neutral models. The first graphs showed the occurrence
frequencies predicted through the NCM. The sequence-clusters occurring more
frequently than expected were dotted in yellow while the ones occurring less frequently

were colored in green. The solid blue line indicates the best fit of the model and the dashed ones indicate 95% confidence intervals. The dashed lines indicate the envelope of neutrally. "m" (estimate rate) and  $R^2$  ( $0 < R^2 \le 1$ , adequacy of the model fitting) are indicated on the graphs. The single bar charts indicated cumulative sequence-clusters occurrence frequencies of Above, Neutral and Below partitions; the combined bar charts represent ranked cumulative relative abundance of taxonomic groups of the three sequence-cluster partitions



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#### 871 Fig. 5 Modified normalized stochasticity ratios (MST) estimated to measure the

#### 872 relative importance of deterministic processes of assembly.

- 873 Bar plots showed the bacterial and fungal MST values under different treatments. S18-
- 874 yellow: September 2018; J19-black: June 2019 and S19-blue: September 2019.