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The epiphytic grapevine microbiome is shaped by a variety of variables at various geographical scales, including biogeography, cultivar, and vintage.

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Research Article

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Abstract

Microbial terroir is established by a variety of variables that influence the makeup of the grapevine microbiome. Less is known about the assembly processes of the epiphytic grapevine microbiome, while the majority of research has concentrated on the grape must microbiome. Our goal was to separate the effects of cultivar, vintage, and geographic location on the epiphytic fungal and bacterial populations of grapevine, both within (regional scale) and across (national scale) Greek viticultural zones. We postulated that: (i) these three variables have varying effects at various geographical scales; and (ii) bacteria and fungus react differentially to the factors influencing the grapevine microbiome. We examined leaves and berries from Greek cultivars (i) Vidiano and Agiorgitiko, which are found in three geographically separated areas (ranging from 100 to >600 km), and (ii) Roditis and Sideritis, which are found in several terroir units within the Aigialeia viticultural zone. Findings: While cultivar becomes a significant driver at the viticultural zone scale, amplicon sequencing study revealed substantial regional characteristics on the epiphytic microbiota across viticultural zones. Compared to bacterial communities, fungal colonies responded better to every factor examined. Differential abundance (DA) analysis revealed dominant amplicon sequence variants (ASVs) of bacteria (Sphingomonas, Masillia, Streptococcus, Staphylococcus, Enterobacterales) and fungi (Alternaria, Cladosporium, Nigrospora, Aureobasidium, Vishniacozyma) that showed patterns specific to cultivar and geography. In conclusion, our findings indicate that the epiphytic grapevine microbiome is shaped by several causes at various geographical scales, with fungal and bacterial populations exhibiting distinct reactions to the structural features under investigation.

KEYWORDS

terroir, vintage, grapevine microbiota, cultivar, and biogeography

1 | INTRODUCTION

A varied microbial population that supports the grapevine (*Vitis vinifera*) influences its development, health, and production (Rolli et al., 2017). Due to its significance in establishing the distinct metabolic profile of wine, the end result of grape fermentation, the makeup of the grapevine microbiome has garnered significant interest in recent years (Liu et al., 2019). The significance of the native grapevine microbiota in the development of the distinctive organoleptic character of wine was confirmed by recent high-throughput sequencing investigations, which also identified certain microbes as makers of desirable aromatic compounds (Bokulich et al., 2016; Liu et al., 2020). According to Deyett and Rolshausen (2020) and Zarraonaindia et al. (2015),

the microbiome of grapevines varies between aboveground and belowground plant parts, with rhizosphere soil and roots sustaining more varied and different microbial communities than leaves and grapes. However, the grape microbiome is mostly found in soil (Liu et al., 2020; Morrison-Whittle & Goddard, 2018). Biogeography, cultivar, and vintage are the most significant drivers of the grapevine microbiome, which follows nonrandom assembly pathways from soil to endophytic and grape must (Bokulich et al., 2014; Miura et al., 2017; Portillo et al., 2016). Terroir (Leeuwen & Gerard, 2006) refers to the combination of these elements, soil physicochemical characteristics, climate (Liu et al., 2020), and viticultural management techniques (Morrison-Whittle et al., 2017). In this context, Gilbert et al. (2014) proposed that the grapevine microbiota is largely responsible for the regional uniqueness of a vineyard and its grapes, and they came up with the term "microbial terroir" to describe the specific makeup of the grapevine microbiome. While the epiphytic grapevine microbiome (the surface of leaves and berries) has received less attention (Portillo et al., 2016), the majority of prior research has examined the effects of bio-geography, genotype, and vintage on the composition of the pre-fermentation must (Bokulich et al., 2014; Kamilari et al., 2021; Li et al., 2022; Morrison-Whittle & Goddard, 2018) and soil microbiome (Gobbi et al., 2022; Mezzasalma et al., 2015). In a groundbreaking research, Bokulich et al. (2014) shown that whereas cultivar and vintage were less significant confounding variables impacting microbial composition, biogeography was the greater determinant determining must microbiome. The majority of research has attempted to separate the effects of one or two of these factors (e.g., biogeography for a single cultivar or cultivar in different regions) (Liu et al., 2020; Zhang et al., 2020) (Mezzasalma et al., 2018; Vitulo et al., 2019), but the combined effects of all three factors in the formation of the grapevine microbiome have not been thoroughly investigated. Numerous scales have been used to study the impact of biogeography: vineyard size (Bokulich et al., 2016), viticultural zone scale (Knapp et al., 2021), national scale (Liu et al., 2020; Vitulo et al., 2019), and most recently, world scale for vineyard soils (Gobbi et al., 2022). The degree of similarity of microbial community composition decreases with increasing geographic distance, according to all of these studies, suggesting that geographical considerations have an impact on microbial community assemblages. The majority of these studies have been conducted at a single geographic scale: (i) at vineyard scale (<5 km), without providing information about the placement of the studied vineyards in distinct terroir units (an area within a viticultural zone where vineyards share similar edaphoclimatic and cultivation conditions) (Aguilar et al., 2020; Zarraonaindia et al., 2015); (ii) at viticultural zone scale (5–100 or 2020). Therefore, we still don't know which variables influence the grapevine microbiome at various geographical scales. This is especially true for the epiphytic grapevine microbiota, which is thought to be most impacted by environmental factors. The influence of cultivars on the grape microbiota has been emphasised in other research. The unique physical and physiological characteristics of the berries of the various grapevine varieties were primarily responsible for these effects (Bokulich et al., 2014; Portillo et al., 2016). Zhang et al. (2019) shown that grapevine genotype has a significant impact on the makeup of the bacterial communities of leaf samples when geographical variables are not present. Vintage will have a significant impact on the formation of the grapevine microbiome because of the significant yearly fluctuations in climate, which are predicted to increase in the future years due to climate change. The impact of vintage on the grapevine microbiome has been noted in earlier research, albeit the degree of this impact may differ for bacteria and fungi (Bokulich et al., 2014; Liu et al., 2020). Our goal was to separate the effects of biogeography, cultivar, and vintage on the epiphytic grapevine microbiome composition at two different experimental scales: (a) nationally across various viticultural zones and (b) viticultural zone-level across various terroir units. The primary scientific questions posed were: (i) how different geographical scales affect the influence and relative importance of bio-geography, cultivar, and vintage on the epiphytic grapevine microbiome; and (ii) whether fungal and bacterial epiphytic communities follow similar assembly mechanisms and react differently to the factors influencing the epiphytic grapevine microbiome. In order to confirm our theories, we used amplicon sequencing to identify the

bacterial and fungal communities on the leaves and grapes of four significant Greek grapevine cultivars: (i) Vidiano and Agiorgitiko, which were collected from vineyards in three different viticultural zones in Greece; and (ii) Roditis and Sideritis, which were collected from vineyards in different terroir units within the same viticultural zone. Lastly, we identified the core microbiome of the two cultivars under study in the Aigialeia viticultural zone with the goal of identifying microorganisms that may possess particular advantageous characteristics for the indigenous cultivars Roditis and Sideritis.

.MATERIALS AND METHODS

| Grapevine samples

In September and October of 2019 and 2020, leaves and grapes were harvested from specific vineyards arranged at two different geographic scales: (a) National geographical scale, which comprised four different viticultural zones in Greece: north (Thessaloniki), central (Athens), south (Crete), or southwest (Nemea, Peloponnese); and (b) Viticultural zone scale, which involved the viticultural zone of Aigialeia, located in north Peloponnese, Western Greece (Supporting Information: Figure S1). Depending on their national and regional distribution, various cultivars were examined at different scales. We tested the cultivars Agiorgitiko and Vidiano at the national level, which are distinguished by both their availability in germplasm collections and their wider spread. The cultivars Roditis and Sideritis, which are particularly significant for the viticultural zone of Aigialeia, the highest altitude viticultural zone in Greece (862 m), were sampled at the viticultural zone scale. The national germplasm collection of the Hellenic Agricultural Organization—Demeter in Thessaloniki, Thermi, and Athens, Lykovrisi, provided samples of grapes and leaves from the cultivars Agiorgitiko and Vidiano. Ten grapevines of each cultivar are grown in two distinct rows in the same vineyard, separated by 50–100 meters, in those collections. Additional samples for Vidiano and Agiorgitiko were gathered from commercial vineyards in Nemea, Peloponnese, and Herakleio, Crete, respectively. Every field that was tested was grown organically, and no pesticides were used. Samples for Sideritis and Roditis were taken from two commercial vineyards per cultivar, each situated in a distinct terroir unit. We tasted a vineyard in the Koutsoura region, which represents terroir A, for Roditis. The sea climate vineyard in the Petsakoi area, which represents terroir B, has an impact on the 800-meter-high vineyard. At 530 meters above sea level, the vineyard is influenced by the continental climate. We gathered samples for Sideritis from two commercial vineyards: (i) in south Achaia (terroir A) and (ii) in the Diakofto area (terroir B). Supporting Information contains information on the location of the vineyards tested as well as the physicochemical characteristics of the various vineyards' soils: Table S1. Supporting Information: Table S2 lists the climatic features of the several viticultural zones taken into consideration in the research. In both years, six geographically dispersed grapevines were tested in each vineyard. On the day of harvest, two healthy grape bunches and ten fully grown, healthy leaves were taken from each grapevine using a sterile blade. Prior to downstream analysis, all samples were promptly kept at -20°C in sterile plastic bags.

1.1 | DNA extraction

As previously mentioned, the DNA from the epiphytic bacteria that colonise grapevine leaves and berries was extracted (Katsoula et al., 2021). In short, 3–4 g of leaves and berries were immersed in an isotonic phosphate buffered saline 1× solution, and the epiphytic microorganisms' cells were separated from the plant tissue surface by intermittent sonication in an ultrasonic bath for 8 minutes and vortexing every 2 minutes for 10 seconds. After removing the plant tissues, the fluid was centrifuged for 15 minutes at 4°C at 9000 rpm. After removing the supernatant, the microbial pellet was resuspended in the extraction buffer of the DNeasy Power soil DNA isolation kit (QIAGEN), which was then used to extract DNA. Agarose gel (1%) electrophoresis was used to evaluate the extracted DNA's integrity, and a Qubit Fluorometric device (Invitrogen) was used to quantify it using the Quant-iT kit.

1.2 | Amplicon sequencing analysis

Using a HiSeq Illumina platform in Rapid Mode to obtain 2×250 bp paired-end reads of the amplicons, the bacterial and fungal communities of leaves and berries were identified through amplicon sequencing of the V4 region of the 16S rRNA gene and the ITS2 region, respectively, at Admera Health. Our own procedure, which is detailed in Vasileiadis et al. (2015), served as the basis for amplification, which enabled sample-wise multiplexing during sequencing. In short, isolated DNA was diluted to a final concentration of $0.2 \text{ ng } \mu\text{l}^{-1}$ for bacteria and $1 \text{ ng } \mu\text{l}^{-1}$ for fungus. The sample-wise amplicon indexing was then made possible by a two-step amplification procedure. The first stage was amplifying the bacterial 16S rRNA gene using the revised primers 515f-806r (Lorenzini et al., 2019) in accordance with the Earth Microbiome Project methodology (Lozupone et al., 2007). Primers ITS7F-ITS4R were used for the first amplification of the ITS2 region in relation to fungi (Martins et al., 2013). A second polymerase chain reaction (PCR) of seven cycles utilising the identical primers with sample-specific 5' overhangs came after the initial amplification phase, which included 28 cycles using the previously described primers. Q5® High-Fidelity DNA Polymerase (NEB) was used for all PCR. Tables S3 and S4 of the Supporting Information provide primers, PCR conditions, and reagents, respectively. Following the manufacturer's recommendations, Amplicon libraries were cleaned using the NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel) before being submitted for sequencing.

1.3 | Bioinformatic analysis

Flexbar v3.0.3 was used to demultiplex amplicon sequences to their original samples, preventing barcode mismatches. The dada2 v1.14.1 (Callahan et al., 2016) package of the R program v.4.0.2 (R Core Team, n.d.) was then used to quality-trim and filter the sequences and look for chimerical sequences. The Silva v.138 (Yilmaz et al., 2014) and UNITE ITSv.8.2 (04.02.2020) (Morrison-Whittle et al., 2017) referenced databases for bacteria and fungus, respectively, were used to build and taxonomically assign amplicon sequence variations (ASVs). ASVs with an annotation probability score of at least 80% were categorised to the lowest feasible taxonomical rank, while those not classified at the Kingdom and Phylum level were not included in downstream analysis. The sequencing data was uploaded with a BioProject accession number (ID: PRJNA834626) to the NCBI's Sequence Read Archive.

1.4 | Statistical analysis

Using the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software, α -diversity indices such as observed or predicted (abundance based coverage estimators [ACE]) richness (zero order diversity, representative of the identified different ASV numbers), Shannon (first order diversity, representative of the less dominant ASVs), inverse Simpson (second order diversity, representative of the more dominant ASVs; Jost, 2006), and Fisher's α (representative of the highly dominant ASVs; Fisher et al., 1943). The differences in α -diversity indices were evaluated using either the nonparametric Kruskal-Wallis followed by the Wilcoxon rank-sum post hoc test (if the ANOVA requirements were not satisfied) or the parametric analysis of variance (ANOVA) followed by Tukey's post hoc analysis. The Student's t-test or its non-parametric equivalent, the Wilcoxon rank sum test, were used for pairwise comparisons. The impacts of cultivar, vintage, and biogeography (or terroir units at viticultural zone size) on the β -diversity of the epiphytic grapevine microbiome were also assessed using the ASV matrices of bacteria and fungus. Nonmetric multidimensional scaling (NMDS) and permutational multivariate ANOVA (PERMANOVA) were used to assess the effects of various conditions on the β -diversity of bacteria and fungus. The Bray-Curtis algorithm was used to perform NMDS with dissimilarity matrices, and PERMANOVA using 999 permutations, as implemented in the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software, was used to determine the association of microbiome composition with the covariates of interest, the percentage of variance explained by those factors, and the variation partitioning between factors. To ascertain the effect of geographic distance between sample locations on the makeup of the bacterial and fungal communities, we used distance-decay relationship analysis. Mantel tests (9999 permutations) were used to compare the physical distances and the Bray-Curtis

dissimilarities of the microbial community using the Pearson correlation technique. Additionally, we built random forest models to represent each factor's contribution to the grapevine epiphytic microbiome's composition as reported by Bekris et al. (2021). In a nutshell, equivalent read numbers were obtained by subsampling the data matrix with replacement. Next, using the randomForest v4.6.14 R-software package (Liaw & Wiener, 2002), the pime v0.1.0 R-software package (Roesch et al., 2020) carried out random forest supervised classification models. 33% of the data were used as out-of-bag (OOB) samples for the model error evaluation, and 66% of the samples were used to generate each random forest model with 1000 trees. To identify bacterial and fungal taxa that were significantly linked to particular viticultural zones, cultivars, or terroir units, differential abundance (DA) tests utilising the Kruskal-Wallis and Wilcoxon rank-sum post hoc tests were used. The false discovery rate (FDR) algorithm was used to adjust the p value for multiple hypothesis testing, with an adjusted p value cut-off of 0.05. Based on the DA tests, heatmaps were created to show ASVs whose relative abundance was positively correlated with viticultural zones, cultivars, or terroir units. Additionally, we identified the core microbiome for the Sideritis and Roditis cultivars in the Aigialeia viticultural zone. To minimise noise, we filtered microbial taxa (for each community) and kept those that appeared in at least 60% of the samples in each cultivar at a constant detection threshold of 0.1% relative abundance.

The R package ggplot2 v3.3.3 was used to create all diagrams, and the microbiome package was used to create core heatmaps (Shetty & Lahti, 2019). The Github repository at https://github.com/ELEPAP/Grapevine_epiphytic_microbiome contains the complete analysis code.

2 | RESULTS

2.1 | The grapevine fungal community and factors shaping its structure at different geographical scales

2.1.1 | The composition of the fungal community

A total of 6699 ASVs were assigned to 2,541,923 nonchimeric quality sequences for fungus (3188 - 52,356). All samples' rarefaction curves plateaued, indicating that the fungal diversity was sufficiently covered by our sequencing effort (Supporting Information: Figure S2a). Ascomycota and Basidiomycota made up the majority of the fungal phyllosphere and carposphere groups. Dothideomycetes dominated the epiphytic fungal communities regardless of cultivars and geographical areas, however other fungal groups became significant in various viticultural zones, cultivars, or vintages (Supporting Information: Figure S3). In contrast to the samples of Sideritis, where Leotiomycetes and Microbotryomycetes shown an increase in their relative abundance in both years, we observed a significantly greater relative abundance of Agaricomycetes in the carposphere and phyllosphere of Roditis.

2.1.2 | Factors affecting the α -diversity of the epiphytic fungal community of grapevine

The α -diversity of fungus was significantly impacted by biogeography and vintage when examining different viticultural zones. In particular, samples from Athens compared to Thessaloniki and all samples from 2019 compared to 2020 showed substantially higher values ($p < 0.05$) of richness (ACE and Observed) (Supporting Information: Figure S4). In the Aigialeia viticultural zone, we found that the cultivar Roditis had significantly higher values ($p < 0.05$) of all diversity indices than Sideritis, with terroir A of the former and terroir B of the latter exhibiting significantly higher values ($p < 0.05$) between the two terroir units per cultivar (Supporting Information: Figure S5).

2.1.3 | Factors affecting the β -diversity of the epiphytic fungal community of grapevine at different geographical scales

Regardless of the geographical scale under investigation, preliminary examination of the amplicon sequencing data indicated a minimal impact of grapevine tissue (leaves or berries) on the makeup

of the fungal population ($p > 0.05$). Therefore, while analysing the β -diversity, samples of leaves and berries were taken into account jointly (as a single sample type collectively defined as the epiphytic grapevine microbiome). First, we examined the impact of several elements in various viticultural zones. Samples were categorised by viticultural zone and vintage using NMDS analysis (Figure 1), although cultivar had a minor clustering impact (Supporting Information: Figure S6). The larger effects of the viticultural zone and vintage on the fungal community were confirmed by PERMANOVA-based variation partitioning analysis, which contributed 21.1% and 11.7% of the total variance, respectively ($p < 0.001$), while cultivar only explained 2% of the variance ($p < 0.002$) (Table 1). Additionally, random forest analysis revealed that, in contrast to cultivar, which had an OOB error value of 20.64%, biogeography (viticultural zone) and vintage were the best explanatory factors with low OOB error values (1.25% and 0%, respectively) (Supporting Information: Figure S7a). We observed a distinct grouping of samples based on cultivar when the data were evaluated at the viticultural zone scale (Figure 2a), while vintage seemed to have a lesser clustering impact (Supporting Information: Figure S8c). PERMANOVA confirmed that cultivar had a greater impact (21.3% of the variation) than vintage (4.9% of the variance, $p < 0.001$) (Table 1). The fungal data set's variation was shown to be most significantly influenced by the terroir unit (26.9%, $p < 0.001$). Vintage seemed to have a role in sample grouping within each cultivar (Supporting Information: Figure S8a,b), however further examination of the β -diversity for each cultivar independently revealed a strong clustering of the samples of the various terroir units examined per cultivar (Figure 2b,c). Sideritis (54.5%, $p < 0.001$) had a greater impact than Roditis (20.9%, $p < 0.001$), according to PERMANOVA, which confirmed the considerable influence of terroir unit in structuring the fungal population per crop. Additionally, random forest analysis revealed that the examined explanatory factors (terroir unit, cultivar, and vintage) demonstrated very low OOB error values (1.04%, 0%, and 0%, respectively) (Supporting Information: Figure S7b). Significant correlations were found between the dissimilarity index values and the geographical distances (a) within the Aigialeia viticultural zone (0–60 km, Mantel test $r = 0.43$, $p < 0.0001$) with linear regression analysis $r^2 = 18.7\%$ and $p < 2.2e-16$, and (b) across viticultural zones (100–600 km, Mantel test 0.30, $p < 0.001$) with linear regression analysis $r^2 = 8.6\%$ and $p < 2.2e-16$ (Supporting Information: Figure S9).

|Fungal ASVs as signatures of viticultural zones at national geographical scale

Members of the fungal community whose relative abundance was linked to a specific area were also identified. Multiple viticultural zones were linked to very numerous *Alternaria* (e.g., 00001, 00032, 00018, 00019) and *Nigrospora* (00027) ASVs (Figure 3a). The viticultural zone of Nemea was linked to ASVs from *Aureobasidium*, *Pyronema*, and *Ustilago*, whereas *Cladosporium* and *Devriesia* were linked to Crete. Thessaloniki was favourably connected with ASVs from *Didymellaceae*, *Periconia*, *Ustilaginaceae*, *Pseudopithomyces*, *Curvularia*, and *Xylariales*, while Athens was positively correlated with an ASV from *Ganoderma*.

|Fungal ASVs as signatures of cultivars Roditis and Sideritis at viticultural zone scale

The relative abundance of ASVs from *Helotiales*, *Aureobasidium*, *Ganoderma*, and *Peniophora* was shown to be strongly positively correlated with the cultivar *Roditis* and *Nigrospora*, *Sporobolomyces*, *Sporidiobolaceae*, and *Vischniacozya* with the cultivar *Sideritis* (Figure 3b). Additionally, we identified the core microbiome of the cultivars *Roditis* and *Sideritis*, which was made up of (i) several less abundant ASVs that were specific to each cultivar, as shown in Supporting Information: Figure S10 and Table S5, and (ii) dominant ASVs (*Alternaria*, *Aureobasidium*, *Cladosporium*, *Capnodiales*, *Pleosporaceae*, *Vishniacozya*) that were shared between the two cultivars and represented cumulatively >60% of the total fungal community.

2.2 | The grapevine bacterial community and factors shaping its structure at different geographical scales

2.2.1 | The composition of the bacterial community

36,187 ASVs were assigned to a total of 3,641,387 quality bacterial sequences (902 – 90,893). All samples' rarefaction curves plateaued, indicating that the bacterial diversity was sufficiently covered by our sequencing effort (Supporting Information: Figure S2b). Proteobacteria (53.8% average relative abundance), Actinobacteriota (18.46%), Firmicutes (12.7%), Bacteroidota (5.54%), Myxococcota (2.49%), and Cyanobacteria (1.97%) dominated the grapevine bacterial community (Supporting Information: Figure S11).

(a)

(b)

FIGURE 1; NMDS analysis of the fungal microbiome in grapevine samples taken from the cultivars Vidiano and Agiorgitiko in vineyards from various Greek viticultural zones (Thessaloniki, Athens, Crete/Nemea). Samples are arranged by vintage (b) and viticultural zone (a). Each graph provides variance partitioning and the p value for the factor under study. Nonmetric multidimensional scaling, or NMDS.

2.2.2 Factors affecting the α -diversity of the epiphytic bacteria community of grapevine

While cultivar and vintage had no discernible influence, we found that the epiphytic bacterial population in grapevines in the areas of Thessaloniki and Athens had a considerably greater α -diversity ($p < 0.05$) throughout the viticultural zone when compared to Crete (Supporting Information: Figure S12). The α -diversity of the epiphytic bacterial population in the Aigialeia viticultural zone was not significantly impacted by any of the conditions examined (Supporting Information: Figure S13). Factors influencing the epiphytic bacterial community's β -diversity at various geographic scales comparable to the fungal microbiome, samples from leaves and berries were taken into consideration in our research since the carposphere and phyllosphere harboured comparable bacterial populations. The bacterial assemblages in the various viticultural zones were the first thing we examined. The samples were weakly clustered by viticultural zone and vintage, according to NMDS analysis (Figure 4), but no

TABLE 1 PERMANOVA-based

variation partitioning analysis of the fungal and bacterial grapevine microbial communities					
Factor	Degrees of freedom	Sum of squares	F. model	R^2 (%)	p Value (>F)
Fungi across viticultural zones (Agiorgitiko, Vidiano)					
Vintage	1	1.7781	18.961	11.7	0.001***
Cultivar	1	0.3058	3.2609	2.0	0.002**
Viticultural zone	3	3.2153	11.428	21.1	0.001***
Residuals	106	9.9403	65.2		
Fungi within viticultural zone (Roditis, Sideritis)					
Vintage	1	0.6619	9.602	4.9	0.001***
Cultivar	1	2.8393	41.186	21.3	0.001***
Terroir unit	3	3.6007	26.116	26.9	0.001***
Residuals	91	6.2733	0.40562	46.9	
Fungi Roditis					
Vintage	1	0.9555	10.532	15.0	0.001***
Terroir unit	1	1.3286	14.646	20.9	0.001***
Residuals	45	4.0823	64.1		
Fungi Sideritis					
Vintage	1	0.2414	6.561	5.8	0.002**
Terroir unit	1	2.2720	61.747	54.5	0.001***

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Residuals 45 1.6558 39.7

Bacteria across viticultural zones (Agiorgitiko, Vidiano)

Vintage	1	1.701	4.2222	3.5	0.001***
Cultivar	1	0.495	1.2287	1.0	0.044*
Viticultural zone	3	3.232	2.6744	6.7	0.001***
Residuals	106	42.707		88.7	

Bacteria within viticultural zone (Roditis, Sideritis)

Vintage	1	1.902	5.0096	4.7	0.001***
Cultivar	1	2.510	6.6138	6.1	0.001***
Terroir unit	3	1.965	2.5883	4.8	0.001***
Residuals	91	34.542		84.4	

Bacteria Roditis

Vintage	1	1.7822	4.3769	8.6	0.001***
Terroir unit	1	0.6639	1.6304	3.2	0.009**
Residuals	45	18.3236		88.2	

Bacteria Sideritis

Vintage	1	1.2176	3.6237	6.9	0.001***
Terroir unit	1	1.3010	3.8721	7.4	0.001***
Residuals	45	15.1201		85.7	

Note: Levels of statistical significance: ***p < 0.001; **p < 0.01; *p < 0.05. PERMANOVA stands for permutational multivariate analysis of variance. It was clear that the samples were lustring by cultivar (Supporting Information: Figure S14). The poor but statistically significant contributions of biogeography (6.7%, p < 0.001) and vintage (3.5%, p < 0.001) to the data set variance were confirmed by PERMANOVA-based variation partitioning analysis, while cultivar had an even less explanatory power (1.0%, p < 0.044) (Table 1). The poor explanatory power of all variables was confirmed by random forest analysis, with OOB error rates ranging from 6.03% for vintage

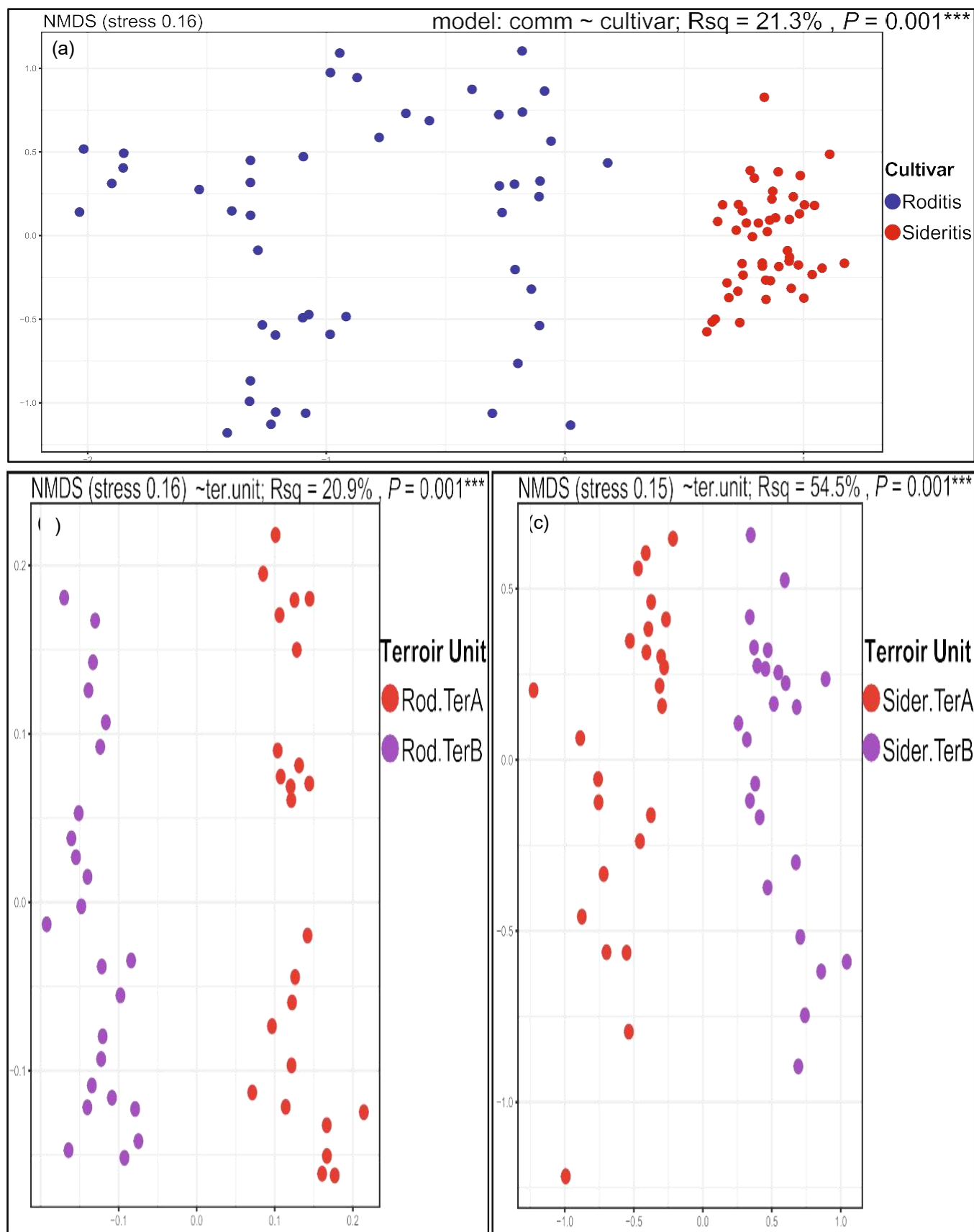


FIGURE 2; NMDS analysis of the fungal microbiome in grapevine samples taken from the cultivars Sideritis and Roditis in vineyards from various terroir units in the Aigialeia viticultural zone. Samples are arranged by cultivar (a). Additionally, samples of the cultivars Sideritis (c) and

Roditis (b) were examined independently and categorised using terroir units. Each graph provides variance partitioning and the p value for the factor under study. Nonmetric multidimensional scaling, or NMDS, to 33.96% and 41.15% for cultivar and viticultural zone, respectively (Supporting Information: Figure S7c). In Aigialeia, NMDS study of the parameters influencing the epiphytic bacterial population in a particular viticultural zone showed a substantial clustering of samples based on cultivar and vintage (Figure 5). With cultivar accounting for 6.1% ($p < 0.001$) and vintage for 4.7% ($p < 0.001$) of the overall variation, PERMANOVA confirmed the greater impact of these two variables on the β -diversity of the epiphytic bacterial population. Beyond these, we observed that the composition of the epiphytic bacterial population was highly influenced by the terroir unit, which explained 4.8% ($p < 0.001$) of the variation (Table 1). When examining the impact of terroir unit (and vintage) within each cultivar, we found that, in the case of Roditis, the samples were better grouped according to the vintage rather than along the terroir unit, whereas the Sideritis samples were clearly less grouped according to the terroir unit and vintage (Supporting Information: Figure S15). PERMANOVA revealed that the bacterial community's vintage and terroir unit explanatory strengths differed.

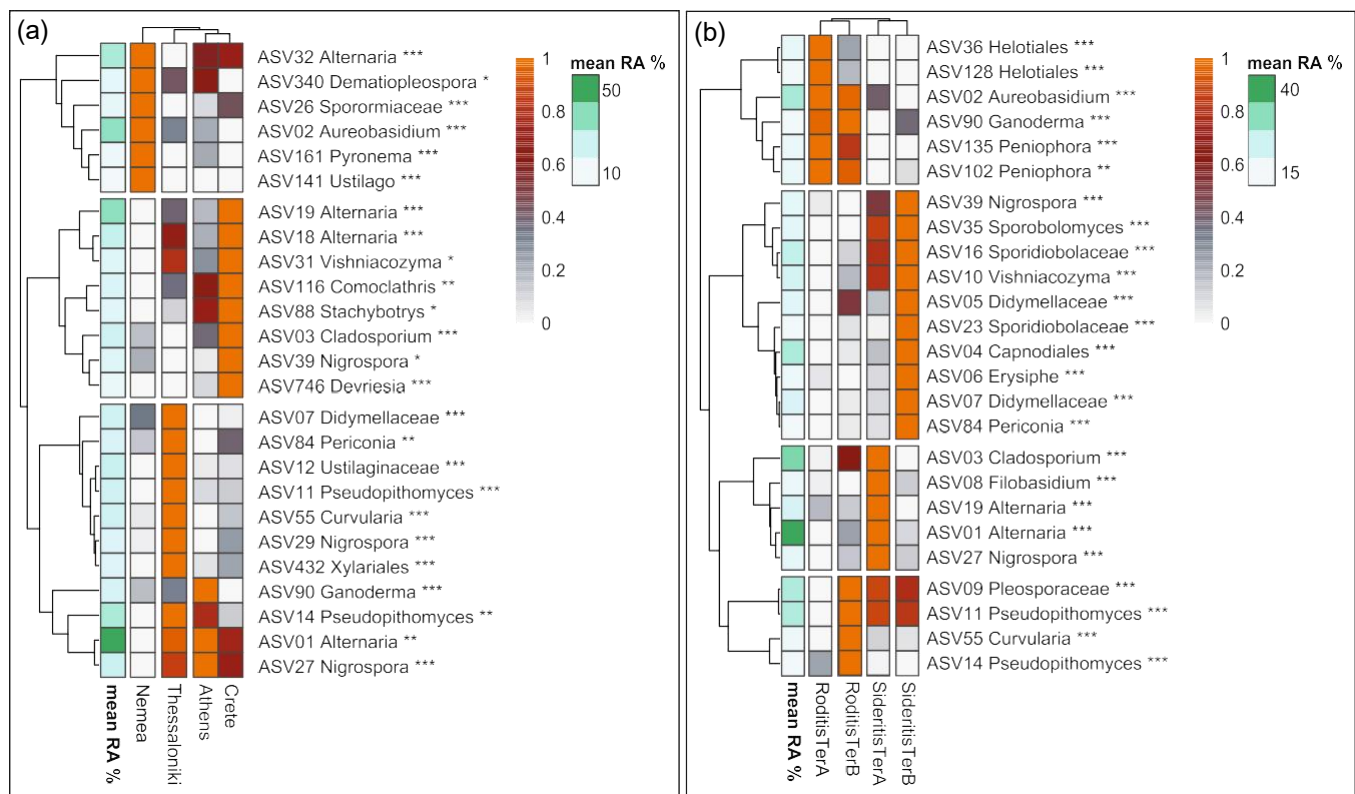


FIGURE 3 ; DA heatmaps displaying fungal ASVs whose relative abundance demonstrated a significant positive connection ($p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$) with certain cultivars (Roditis/Sideritis) in the Aigialeia viticultural zone (b) or with particular viticultural zones (Athens, Thessaloniki, Nemea/Crete) (a). DA stands for differential abundance, and ASVs for amplicon sequence variations. In contrast to Sideritis, where both vintage and terroir units had comparable explanatory strengths (6.9% and 7.4%, respectively, $p < 0.001$), Roditis exhibited a greater explanatory strength (8.6%, $p < 0.001$) compared to terroir units

(3.2%, $p < 0.009$) (Table 1). With OOB error values ranging from 11.46% for vintage and 12.5% for cultivar to 34.38% for terroir unit, random forest analysis further demonstrated the inadequate explanatory power of all variables (Supporting Information: Figure S7d). The epiphytic bacterial communities' distance-decay association study revealed a minor influence of geographic distances at the viticultural zone scale (Mantel test $r = 0.06$, $p < 0.05$), with linear regression analysis r^2 of 0.3% and $p < 9.2e-5$ (Supporting Information: Figure S16). On the other hand, we observed a larger association between the community dissimilarities and rising geographical distances when looking across viticultural zones (Mantel test $r = 0.30$, $p < 0.0001$, linear regression $r^2 = 8\%$ and $p < 2.2e-16$).

2.2.3 Bacterial ASVs as signatures of viticultural zones at national geographical scale

ASVs from *Skermanella* and *Massilia* were positively correlated with many regions (Figure 6a). *Sphingomonadaceae* ASVs were positively correlated with Nemea. ASVs from *Enterobacterales* were linked to Athens and Thessaloniki, while ASVs from *Staphylococcus* and *Streptococcus* were linked to Crete (Figure 6a).

2.2.4 | Bacterial ASVs as signatures of cultivars Roditis and Sideritis at viticultural zone scale

Roditis was strongly correlated with ASVs from *Streptococcus* (2) and *Staphylococcus* (1), while *Sideritis* was favourably correlated with many ASVs from *Sphingomonas* (7) and *Methylobacterium* (7) (Figure 6b). Low abundance ASVs specific to each cultivar made up the epiphytic core bacterial microbiome of the two cultivars. *Sphingomonas*, *Staphylococcus*, *Masillia*, *Methylobacterium*, *Acinetobacter*, *Streptococcus*, *Corynebacterium*, *Hymenobacter*, *Skermanella*, and *Acetobacteraceae* made up the core bacterial microbiome of Roditis (Supporting Information: Figure S17 and Table S6), while ASVs attributed to *Blastococcus*, *Skermanella*, *Staphylococcus*,

***Methylobacterium*, *Masillia*, *Sphingomonadaceae*, *Microbacteriaceae* and *Hymenobacter*.**

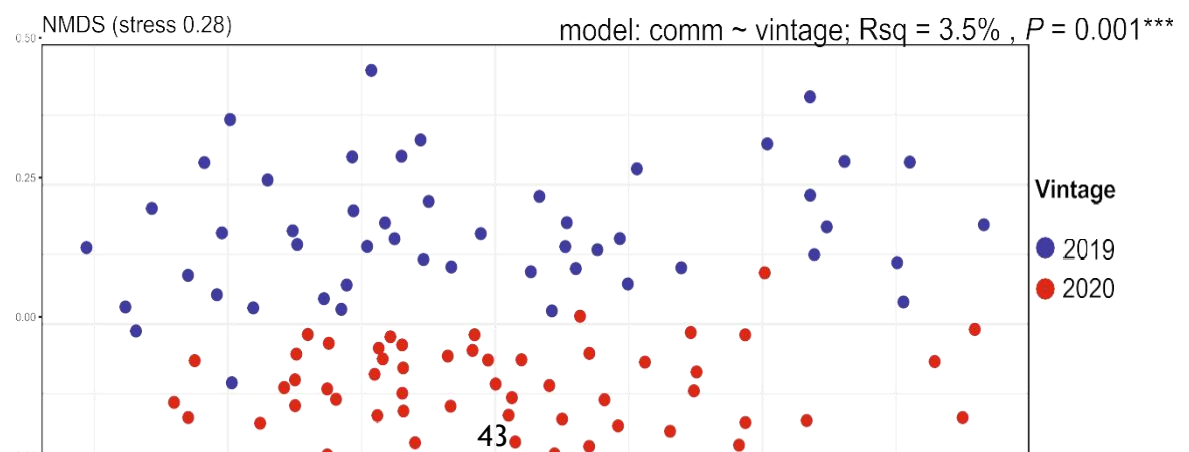
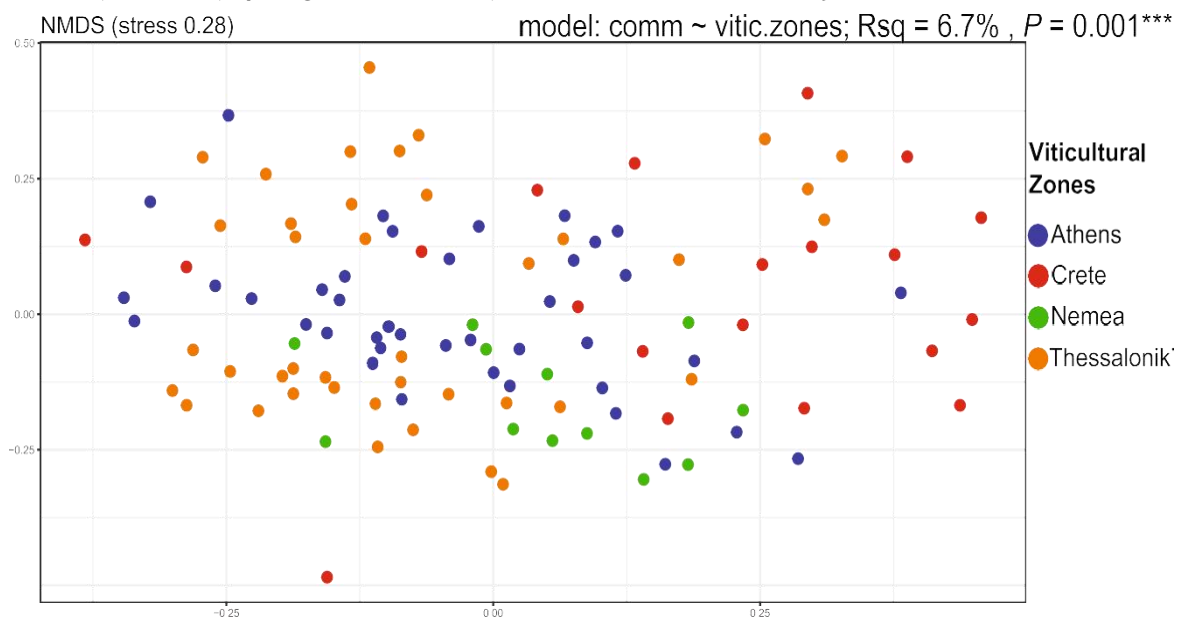


FIGURE 4; NMDS analysis of the bacterial microbiome in grapevine samples taken from the cultivars Vidiano and Agiorgitiko in vineyards from various Greek viticultural zones (Thessaloniki, Athens, Crete/Nemea). Samples are arranged by vintage (b) and viticultural zone (a). Each graph provides variance partitioning and the p value for the factor under study. Nonmetric multidimensional scaling, or NMDS.

3 DISCUSSION

The primary source of microorganisms influencing wine quality and chemical profile is the grapevine microbiome. In particular Greek grapevine varieties at various geographic scales, we focused on the epiphytic phyllospheric and carpospheric fungal and bacterial populations colonising the interface between the plant surface and the atmosphere.

Factors shaping the epiphytic fungal community in grapevine

The first issue we posed was whether the epiphytic grapevine microbiota is shaped by distinct processes at various geographic scales. Geographical location was shown to be the most significant element influencing the epiphytic fungus community when examining the grapevine microbiome in various viticultural locations (distances 100 - >600 km).

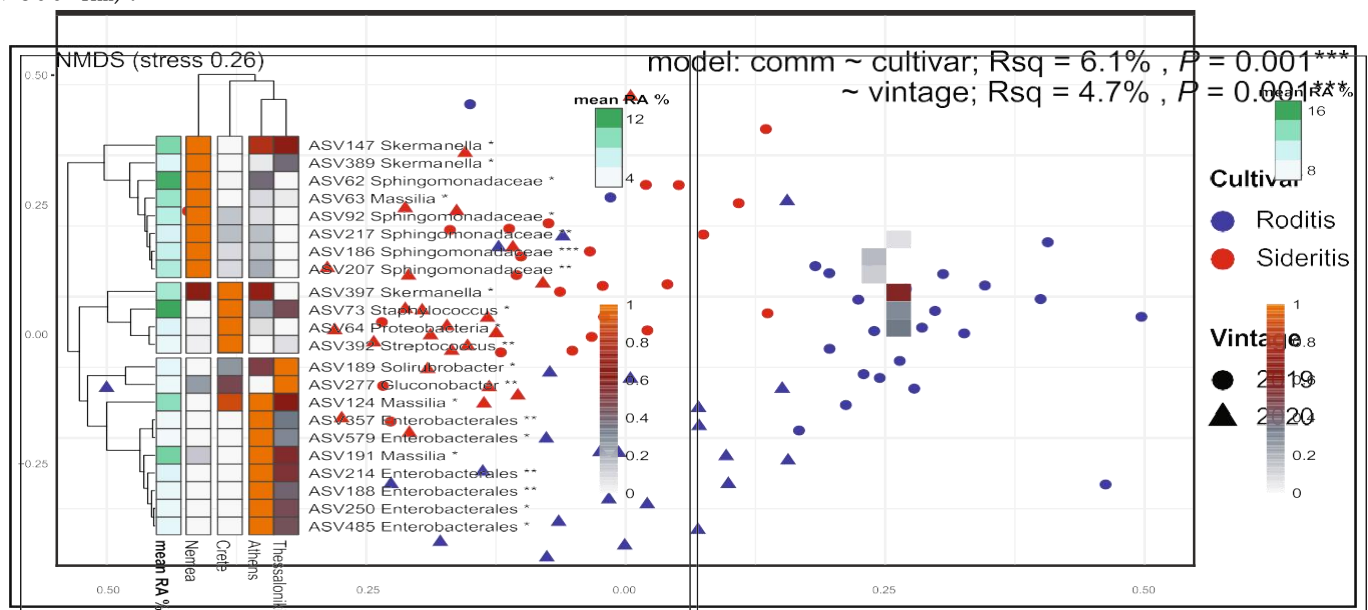


FIGURE 5 NMDS analysis of the bacterial microbiome in grapevine samples from the cultivars Sideritis and Roditis found in vineyards from various terroir units in the Greek viticultural zone of Aigialeia. Samples are arranged by vintage (caption code) or cultivar (colour code). Each graph provides variance partitioning and the p value for the factor under study. Nonmetric multidimensional scaling, or NMDS.

little impact, as shown by variance partitioning and confirmed by random forest analysis. Few studies have examined the variables influencing the grapevine's epiphytic fungus ecology at broad geographic ranges. Strong regional characteristics in the carposphere fungal population of Shiraz and Cabernet Sauvignon cultivars found in two geographically separated Australian viticultural zones were described by Kioroglou et al. (2019), while cultivars and vintage had a less significant impact. Strong regional patterns in the composition of the fungal community were also found in China (Li et al., 2022), California (Bokulich et al., 2014), Greece (Chalvantzi et al., 2021), Cyprus (Kamilari et al., 2021), and New Zealand (Morrison-Whittle & Goddard, 2018), according to earlier research on the must fungal microbiome at comparable geographical scales. Only vintage seemed to have a significant impact when other confounding variables, such as cultivar and vintage, were examined at the national geographic level. According to Bokulich et al. (2014), there was a slight vintage influence on the fungal must community's composition, which also differed across the cultivars under study. Since the epiphytic fungus community is more vulnerable to environmental and climatic factors than the must microbiome, it is anticipated that vintage will have a greater impact on the latter. We also examined the epiphytic fungus population at a viticultural zone size, where environmental condition differences and distances between vineyards were less than 100 km, in order to offer a thorough response to our original idea. We examined the epiphytic microbiome of the two cultivars in vineyards allocated to different terroir units of the viticultural zone of Aigialeia, in contrast to the majority of research that have investigated grapevine microbiome assemblage processes at the regional level in randomly chosen vineyards. We observed that, as shown by variance partitioning analysis and confirmed by random forest analysis, the cultivar becomes the most significant driver of the makeup of the epiphytic fungus population at the regional scale. Additionally, Singh et al. (2018) shown that cultivar seems to be the primary predictor of the grape fungal community within the same viticultural zone. The carposphere microbiome has been shown to be significantly influenced by cultivar-specific characteristics such as the colour and thickness of berry skin and bunch characteristics (Bokulich et al., 2014; Mezzasalma et al., 2018). Both cultivars in our research provide berries that range in hue from white to pink, but they vary in other ways: In contrast to Roditis, a small-berried cultivar with semithick skin that is distinguished by not very large and dense bunches, Sideritis's berries are enormous and thick-skinned, growing in notably large but not very dense bunches. These variations might account for the significant cultivar filtering impact on the epiphytic

3.1 | Factors shaping the epiphytic bacterial communities in grapevine

To address our second scientific issue, if the fungal and bacterial epiphytic grapevine communities are susceptible to distinct assembly processes, we expanded our investigation to the bacterial epiphytic microbiome. The bacterial population exhibited similar structural patterns at the national geographic scale as fungi, with biogeography being the most significant factor, followed by cultivar and vintage, which had negligible impacts. At the viticultural zone scale, where cultivar and secondly vintage become significant influencers of the epiphytic bacterial ecology, similar structural patterns were also seen. Terroir units seem to have an impact on the bacterial population for each cultivar, although Sideritis appears to be more affected. This is due, as previously mentioned, to the cultivar's larger distance between the two terroir units under study. Variance partitioning analysis and random forest analysis showed that the impact of the factors (biogeography, cultivar, terroir unit, and vintage) on the bacterial communities was weaker, even though the structural patterns of the bacterial and fungal grapevine communities were largely similar. The limited impact of geographic distance on epiphytic bacterial populations at the viticultural zone scale, which only becomes important across viticultural zones, was confirmed by distance-decay relationship analysis. Additionally, prior research has shown that, in contrast to fungi, epiphytic and must bacterial populations are less impacted by distance (Bokulich et al., 2014; Liu et al., 2020; Miura et al., 2017). Due to their larger propagules (1–30 μm diameter) as opposed to bacteria's (0.25–8 μm diameter), fungus are

limited in their ability to spread across long distances in the atmosphere (Smets et al., 2016; Wilkinson et al., 2012). Our results imply that the epiphytic bacterial grapevine population's composition is influenced by the same parameters as the fungal community, but with considerably lesser impacts, indicating the involvement of other variables not taken into account in our investigation.

3.2 | Members of the epiphytic grapevine microbiome showing regional signatures

We also searched the epiphytic grapevine microbiome for individuals linked to certain cultivars (within viticultural zone) or regions (across viticultural zones). The epiphytic fungal population was dominated by *Alternaria*, *Aureobasidium*, *Cladosporium*, and *Nigrospora*, which were antagonistic to grapevine plant pathogenic fungi regardless of geographic scale (Pantelides et al., 2015). According to Del Frari et al. (2019) and Lorenzini & Zapparoli (2014), *Alternaria* and *Cladosporium* are worldwide fungi that have been discovered as saprobes, plant diseases, and plant helpful microbes. Grapevine metabolism may be regulated by *nigrospora* (Yang et al., 2016). An intriguing finding was the obvious correlation between the Nemea area and *Pyronema*, which are recognised for their ability to break down pyrolysed organic matter (Fischer et al., 2021). This is most likely due to the recent fires that occurred in the Nemea viticultural zone in 2017. The widespread occurrence of *Skermanella* and *Massilia* in several geographic locations was a characteristic of the epiphytic grapevine bacterial community. Although their possible role is uncertain, individuals of these genera have been identified as part of the epiphytic grapevine microbiome (Leveau & Tech, 2011; Mezzasalma et al., 2018).

3.3 | The core epiphytic microbiome of the cultivars Roditis and Sideritis

Strong filtering effects on the epiphytic grapevine microbiome were seen when we concentrated on the Aigialeia area and identified the core microbiota of the cultivars Roditis and Sideritis. A few very prevalent ASVs from *Alternaria*, *Aureobasidium*, *Cladosporium*, and *Capnodiales* that were shared by the two cultivars dominated the core fungal microbiome. Conversely, each cultivar's distinct and non-numerically dominating members made up the bacterial core microbiome, with *Sphingomonas*, *Masillia*, and *Methylobacterium* having the highest abundance. These genera of bacteria dominate the grapevine's phyllosphere and carposphere (Martins et al., 2013). *Methylobacterium* and *Sphingomonas* are referred to be ant growth boosters (Kutschera, 2007) and biological control agents (Innerebner et al., 2011). Although their oenological impact is yet unclear, Bokulich et al. (2012) demonstrated that *Sphingomonas* and *Methylobacterium* could survive the vinification process. The goal of ongoing core microbiome-driven culturomics is to separate epiphytic bacteria from the grapevine microbiome and evaluate their potential as biological control agents or boosters of plant development.

4 | CONCLUSIONS

In order to determine the parameters influencing the epiphytic grapevine microbiome at the national and regional sizes, we examined the microbiome of four emblematic Greek cultivars at various geographic scales. We demonstrated that several determinants operate at various geographical scales: cultivar became the most significant driver at the viticultural zone scale, whereas biogeography was the primary predictor of the epiphytic grapevine microbiome across viticultural zones. In contrast to the bacterial community, whose composition demonstrated a weaker response to the confounding factors that were most likely driven, the epiphytic fungal grapevine community was more responsive to the structural factors under study and showed distinct distance-decay patterns regarding community similarity at both geographic scales.

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