DEPARTMENT OF
Biochemistry &
Biotechnology
UNIVERSITY OF THESSALY

University of Thessaly

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Study of the role of vine and vinification microbiome from local grapevine cultivars on winemaking and the final wine quality

A thesis submitted by

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For the degree of

Doctor of Philosophy

November 2023

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«Μελέτη του ρόλου του μικροβιώματος
τοπικών ποικιλιών αμπέλου και της οινοποίησης
στην ποιότητα των τελικών οινικών προϊόντων»

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Acknowledgments

This thesis was performed in the Laboratory of Plant and Environmental Biotechnology of the Department of Biochemistry and Biotechnology of the University of Thessaly, during 2019-2023. Firstly, I would like to thank my supervisor, Professor D. G. Karpouzas for the great honor to make me a member of his laboratory team, to work with him and for the unique chance to gain all the knowledge and experience I got. He put trust on me, being at the same time supporting, guiding, inspirative, very patient and kind, preparing me for my next steps in the best way he could, by making it possible to complete and in the end to be ready to present my PhD thesis.

Next, I would like to thank my advisory committee members K. Papadopoulou, Professor in the Department of Biochemistry and Biotechnology of University of Thessaly and D. Kouretas, Professor in the Department of Biochemistry and Biotechnology of University of Thessaly, for their guidance and their support all these years.

I would also like to thank the rest of the committee members S. Vasileidis, Associate Department of Biochemistry and Biotechnology of University of Thessaly, K. Liadaki, Associate Department of Biochemistry and Biotechnology of University of Thessaly, A. Veskoukis, Assistant Professor of Department of Nutrition and Dietetics, University of Thessaly, and last but not least Professor J. Klironomos, University of Sharjah, UAE, for helping with their advice and constructive comments on the finalization of this thesis.

Finally, I would also like to thank the PostDoc researcher Fotios Bekris, for the collaboration during all this thesis, for valuable support in all the processes and his guidance.

Summary

Grapevine phyllosphere and carposphere harbors microbial communities whose assemblage mechanisms are under the direct influence of the inner and outer plant environment. The epiphytic grapevine microbiome has a notable influence on plant performance and physiology and further on the vinification process and final wine quality. Its structure and composition can vary significantly depending on different vineyard mediated factors with clear reflections both on plant health and on the winemaking process. In this thesis we aimed to disentangle the factors and conditions that determine the composition of the epiphytic (phyllospheric and carpospheric) grapevine microbiome and the wood grapevine microbiome, with the latter focusing on its role in the establishment of grapevine trunk diseases (GTDs). Further, we determined, under near full scale spontaneous and commercial vinification conditions, the influence of vineyard-mediated and vinification-associated factors on microbial succession and on the antioxidant, antimutagenic and anticancer profile of the produced wines facilitating the establishment of correlations between vinification microbiome and wine properties. Finally we explored in spontaneous and inoculated vinifications microbial succession (via amplicon sequencing) and the metabolic pathways and functions employed by the vinification microbiota to cope with the fermentation conditions and lead to the production of wine (via metatranscriptomic analysis).

Firstly, we assessed the wood microbiome of grapevines asymptomatic and symptomatic to GTDs in three major Greek cultivars, each cultivated at geographically different zones. We noticed that biogeography/cultivar (lumped factor) was the main determinant of both fungal and bacterial communities, while the GTD status, differentiated asymptomatic from symptomatic vines only in the cultivar Xinomavro, demonstrating its higher resistance towards GTDs and thus complex interactions of cultivar-biogeography. Moreover, we observed a cultivar-dependent association of *P. chlamydospora, K. variispora, Fomitiporia spp* and *Diaporthe,* all previously proposed as GTDs causal agents with symptomatic grapevines suggesting their direct involvement in the establishment of GTDs. Random forest analysis identified *P. chlamydospora, K. variispora,* along with *Cladosporium spp.* and *A. alternata* as early and accurate predictors of GTDs, offering a valuable microbiome-mediated analytical tools for early identification of GTDs in vineyards. As for the

bacterial microbiome, the notable negative co-occurrence of *Bacillus* and *Streptomyces* with *Phaemoniella*, *Phaeoacremonium* and *Seimatosporium*, members of the GTD pathobiome, in asymptomatic grapevines suggested a potential suppressive role of these bacterial towards GTDs that needs to be further verified through directed isolation and further characterization of their suppressive potential *in vitro* and *in planta*.

Secondly, we analyzed, via amplicon sequencing, the epiphytic (carposphere and phyllosphere) grapevine microbiome in four emblematic Greek grapevine cultivars, at different geographical scales aiming to unravel the effects of different structural factors on the composition of the grapevine microbiome. When looking at larger geographical scales, across different viticultural zones, biogeography was the main determinant of the epiphytic fungal and bacterial communities. However, at regional scales, within the same viticultural zone of Aigialeia, cultivar becomes the most important determinant factor. When the analysis was performed separately for each of the two studied cultivars in the viticultural zone of Aigialeia (cv. Roditis and Sideritis) strong effects of the *terroir* units were evident. In addition, the epiphytic fungal grapevine microbiota exhibited stronger responses to the studied factors and clear distance–decay patterns at both studied scales, unlike the bacterial community which showed weaker responses to the confounding factors and weak distance-decay patterns.

Thirdly, we extended our study and we investigated the effects of vineyard-mediated factors on vinification microbiome under different vinification strategies, directly relevant to current winemaking practices. We showed that under realistic scale-wise and process-wise wine making practices, vineyard-mediated factors, such as cultivar and vintage, remain operative along the vinification process, unlike *terroir* whose effect, although significant at start (in vineyard and early fermentation stages), becomes less important as fermentation progresses. The strategy of vinification employed also strongly influenced the composition of the vinification microbiota, with the effect varying between fungi and bacteria. The fungal community showed a universal pattern of NS yeasts and filamentous fungi dominating at the early stages of fermentation being displaced, though by indigenous or inoculated *Saccharomyces* strains as the fermentation progresses. On the other hand, bacterial community succession varied, with the most common pattern mirrored in an initially rich in AAB bacterial community replaced by *Oenococcus* at the end of fermentation. Vintage and

vinification type were also the stronger determinants of the antioxidant, anticancer and antimutagenic profile of the produced wines. Further analysis identified significant, and universal across cultivars, positive correlations between members of the vinification microbiota like the NS yeasts *Torulaspora debrueckii* and *Lachancea quebecensis* with the anticancer and the antioxidant properties of wines. These findings could be exploited towards a microbiota-modulated vinification process that will lead to high-quality wines with desirable properties and enhanced regional identity.

Finally, we evaluated, in spontaneous and commercial vinifications (both being amended with preservatives) with grapes of the Greek cultivar Vidiano, the composition and succession of the fungal and bacterial microbiome and explored, via metatranscriptomic analysis, the metabolic pathways and functions employed by the vinification microbiome along the fermentation. The stage of vinification was identified as the most important structural factor of the vinification microbiome while the type of vinification process employed also contributed but to a lower extent, being most probably a result of the universal use of preservatives in both vinifications. The fungal community showed a wide phylogenetic diversity of NS yeasts and *Saccharomyces* at early fermentation stages which was replaced by the complete dominance of inoculated or indigenous *Saccharomyces* strains as the fermentation progressed. The bacterial community was composed of LAB and AAB, with *Oenococcus* showing a progressive increase along the fermentation process. Metatranscriptomic analysis reinforced the strong influence of vinification stage on the metabolic activity of the microbiome. When data from the different fermentation stages were analyzed separately, we noted strong signatures of the different vinification strategies employed (spontaneous and inoculated) at the first and middle stages of fermentation. Overall, we noted upregulation of genes mostly originated from *S. cerevisiae* and other yeasts that were associated with stress response mechanisms, transportation, glycolysis and ethanol production in line with the biochemical environment of fermentations.

The results of this thesis have serious practical implications for grapevine plant health (Chapter 2 and 3) and the wine making sector (Chapter 3, 4 and 5), while it offered benchmarking knowledge and diagnostic tools that could be further optimized and used in the vineyard management and the wine making process.

Γενική Περίληψη

Η φυλλόσφαιρα και η καρπόσφαιρα του αμπελιού φιλοξενούν μικροβιακές κοινότητες των οποίων οι μηχανισμοί δόμησης επηρρεάζονται τόσο από το το εξωτερικό περιβάλλον όσο και από το περιβάλλον του ίδιου του φυτού. Το επιφυτικό μικροβίωμα επηρρεάζει σημαντικά την απόδοση και την υγεία του αμπελιού και επιπλέον τη διαδικασία οινοποίησης και την ποιότητα του τελικού οίνου. Η δομή και η σύνθεσή του μπορεί να ποικίλει ανάλογα με την επίδραση που ασκείται από τους παράγοντες που επικρατούν στον αμπελώνα, ανακλώντας αυτές τις επηρροές τόσο στην υγεία όσο και στη διαδικασία της οινοποίησης, Στη συγκεκριμένη διατριβή, στοχεύσαμε στο να διασαφινίσουμε τους παράγοντες και τις συνθήκες που καθορίζουν τη σύσταση και του επιφυτικού (φυλλοσφαιρικού και καρποσφαιρικού) μικροβιώματος του αμπελιού και του μικροβιώματος του ξύλου στο αμπέλι, με το το τελευταίο να επικεντρώνεται περισσότερο στο ρόλο του μικορβιώματος όσον αφορά τις ασθένειες που προσβάλλουν τον κορμό του αμπελιού. Περαιτέρω, προσδιορίσαμε την επίδραση των παραγόντων που σχετίζονται με τον αμπελώνα και την οινοποίηση στη μικρβιακή διαδοχή κατά την οινοποίηση, υπό συνθήκες αυθόρμητης και ελεγζόμενης οινοποίησης, καθώς και την αντίστοιχη επίδρασή τους στο αντιοξειδωτικό, αντιμεταλλαξιγόνο και αντικαρκινικό προφίλ των παραγόμενων οίνων, διευκολύνοντας έτσι την εδραίωση συσχετίσεων μεταξύ του μικροβιώματος της οινοποίησης και των τελικών ιδιοτήτων των οίνων. Τέλος, μέσω αυθόρμητης και ελεγχόμενης οινοποίησης (μέσω amplicon sequencing) διερευνήσαμε τη μικροβιακή διαδοχή και τις μεταβολικές οδούς που χρησιμοποιεί η μικροβιακή κοινότητα της οινοποίησης για να ανταποκριθεί στις συνθήκες της ζύμησης και να καταλήξει στην παραγωγή ου οίνου (μέσω μετα-μεταγραφωματικής ανάλυσης).

Αρχικά, εξετάσαμε το μικροβίωμα του ξύλου σε αμπέλια συμπτωματικά και μη συμπτωματικά σε ασθένειες κορμού τοου αμπελιού, σε τρεις κύριες ελληνικές ποικιλίες αμπέλου, καλλιεργημένες σε γεωγραφικά διαφορετικές ζώνες. Παρατηρήσαμε ότι ο παράγοντας της ποικιλίας/βιογεωγραφίας (ως συνδυασμένος παράγοντας) ήταν ο κύριος καθοριστικός και για τις μυκητιακές και για τις βακτηριακές μικροβιακές κοινότητες, ενώ ο παράγοντας της ασθένειας του κορμού διαφοροποίησε τα υγιή από τα ασθενή πρέμνα μόνο στην ποικιλία Ξινόμαυρο, δείχνοτας τη μεγαλύτερη ανθεκτικότητα της ποικιλίας αυτής ως προς τις συγκεκριμένες ασθένειες και κατ'επεκταση τις πολύπλοκες αλληλεπιδράσεις μεταξύ ποικιλίας και βιογεωγραφίας. Επιπροσθέτως, παρατηρήσαμε μια συχέτιση βασιζόμενη στην ποικιλία των μικροοργανισμών P. chlamydospora, K. variispora, Fomitiporia spp και Diaporthe, που έχουν προταθεί ως αιτιολογικοί παράγοντες για τις ασθένειες κορμού του αμπελιού σε ασθενή πρέμνα, υποδηλώνοντας την άμεση εμπλοκή τους στην εδραίωση αυτων των ασθενειών. Μέσω της ανάλυσης με τον αλγόριθμό Random Forest, εντοπίσαμε τους μικροοργανισμούς P. chlamydospora, K. Variispora, μαζί με τους Cladosporium spp. και A. alternata ως πρώιμους προγνωστικούς παράγοντες των ασθενειών κορμού του πρέμνου, διαθέτοντάς τους ως ένα χρήσιμο μικροβιακό εργαλείο ανάλυσης για την πρώιμη ταυτοποίηση αυτών των ασθενειων κορμού του αμπελιού. Όσον αφορά το βακτηριακό μικροβίωμα, η αξιοσημείωτη αρνητική συνεμφάνιση, σε υγιή πρέμνα, των Bacillus και Streptomyces με μικροβιακά στελέχη των Phaemoniella, Phaeoacremonium και Seimatosporium, με

τα τρία τελευταία να αποτελούν μέλη του παθοβιώματος των ασθενειών κορμού του αμπελιού, υποδηλώνει την πιθανή κατασταλτική τους δράση ως προς τις ασθένειες κορμού του αμπελιού, που ωστόσο πρέπει να διερευνυθεί περεταίρω, μέσω στοχευμένης απομόνωσης και χαρακτηρισμού αυτού του κατασταλτικού δυναμικού τους, in vitro αλλά και σε φυτά.

Κατά δεύτερον, αναλύσαμε μέσω amplicon sequencing το επιφυτικό μικροβίωμα του αμπελιού (σε καρπόσφαιρα και φυλλόσφαιρα), από τέσσερις ελληνικές εμβληματικές ποικιλίες αμπελιού, προερχόμενες από διαφορετικές γεωγραφικές κλίμακες, στοχεύοντας στο να προσδιορίσουμε τις επιδράσεις των διαφορετικών δομικών παραγόντων στη σύνθεση του μικροβιώματος του αμπελιού. Εξετάζοντας σε ευρεία γεωγραφική κλίμακα, κατά μήκος διαφορετικών βιογεωγραφικών περοχών, η βιογεωγραφία αποτέλεσε τον κύριο καθοριστικό παράγοντα στον σχηματισμού των επιφυτικών μυκητιακών και βακτηριακών μικροβιακών κοινοτήτων. Ωστόσο, σε επίπεδο τοπικής κλίμακας, εντός της αμπελουργικής περιοχής της Αιγιάλειας, κύριος καθοριστικός παράγοντας γίνεται η ποικιλία. Πραγματοποιώντας την ανάλυση ξεχωριστά για καθεμια από τις δυο ποικιλίες στην περιοχή της Αιγιάλειας (Ροδίτη και Σιδερίτη), οι επιδράσεις του terroir γίνονται εμφανείς. Επιπροσθέτως, η επιφυτική μυκητιακή μικροβιακή κοινότητα του αμπελιού ανταποκρίθηκε ισχυρότερα ως προς τους δομικούς παράγοντες που μελετήθηκαν, με πιο ξεκάθαρα μοτίβα και στις δύο κλίμακες που εξετάστηκαν , σε αντίθεση με τη βακτηριακή επιφυτική κοινότητα που έδειξε ασθενέστερη ανταπόκριση στους προς μελέτη παράγοντες, με ασθενέστερα μοτίβα σύνθεσης.

Τρίτον, επεκτείναμε τη μελέτη μας στο να διερευνήσουμε τις επιδράσεις των παραγόντων που σχετίζονται με τον αμπελώνα στο μικροβίωμα της οινοποίησης, υπό διαφορετικούς τύπους οινοποίησης κατά τις εφαρμοζόμενες οινοποιητικές πρακτικές. Δείξαμε ότι, υπό τις τρέχουσες ρεαλιστικές πρακτικές της οινοποιητικής διαδικασίας, παράγοντες που σχετίζονται με τον αμπελώνα, όπως η ποικιλία και το έτος συγκομιδής, παραμένουν ενεργοί κατά τη διαδικασία της οινοποίησης, ενώ η επίδραση του terroir, παρ’ότι σημαντική αρχικά (στο στάδιο του αμπελώνα και στα αρχικά στάδια της οινοποίησης), γίνεται όλο και λιγότερο αισθητή κατά τη διεξαγωγή και εξέλιξη της οινοποίησης. Η στρατηγική οινοποίησης που ακολουθήθηκε επηρρέασε εξίσου σημαντικά το μικροβίωμα της οινοποίησης, με την επίδραση αυτή να ποικίλει μεταξύ μυκήτων και βακτηρίων. Η μυκητιακή μικροβιακή κοινότητα παρουσίασε ένα κοινό γενικά μοτίβο από ζύμες που δεν αποτελούν στελέχη σακχαρομύκητα και νηματώδεις μύκητες να κυριαρχούν στα αρχικά στάδια της οινοποίησης, που αντικαταστάθηκε όμως από αυτόχθονα ή εμβολιασμένα στελέχη αακχαρομύκητα κατά την εξέλιξη της ζύμωσης. Από την άλλη πλευρά, η μικροβιακή διαδοχή της βακτηριακής κοινότητας διέφερε, με το πιο κοινό μοτίβο που ακολουθήθηκε να απαρτίζεται από μια αρχικά βακτηριακή κοινότητα πλούσια σε βακτήρια οξικού οξέως, που αντικάταστάθηκε όμως από στελέχη του Oenococcus στο τέλος της ζύμωσης. Το έτος συγκομιδής και ο τύπος οινοποίησης αποτέλεσαν επίσης σημαντικούς παράγοντες καθορισμού του αντιοξειδωτικού, αντιμεταλλαξιγόνου και αντικαρκινικού προφίλ των παραγόμενων οίνων. Η περεταίρω ανάλυση, εντόπισε σημαντικές και κοινές μεταξύ των ποικιλιών θετικές συσχετίσεις μελών της μικροβιακής κοινότητας, όπως οι ζύμες-μη στελέχη

σακχαρομύκητα Torulaspora delbrueckii και Lachancea quebecensis με τις αντικαρκινικές και αντιοξειδωτικές ιδιότητες των οίνων. Τα ευρήματα αυτά θα μπορούσαν να αξιοποιηθούν περετάιρω μέσω μιας μικροβιακά μεσολαβούμενης οινοποιητικής διαδικασίας, που θα οδηγούσε στην παραγωγή οίνων υψηλής ποιότητας, με επιθυμητές ιδιότητες και ενισχυμένη τοπική ταυτότητα.

Τέλος, με σταφύλια της ελληνικής ποικιλίας Βιδιανό, αξιολογήσαμε σε αυθόρμητη και εμπορική ζύμωση (και οι δύο με την παρέμβαση συντηρητικών), τη σύνθεση και τη μικροβιακή διαδοχή του μυκητιακού και βακτηριακού μικροβιώματος και διερευνήσαμε, μέσω μέτα-μεταγραφωματικής ανάλυσης, τις μεταβολικές οδούς και τις λειτουργίες που ακολουθεί το μικροβίωμα της οινοποίησης, κατά τη διεξαγωγή της ζύμωσης. Το στάδιο οινοποίησης αναγνωρίστηκε ως ο σημαντικότερος δομικός παράγοντας του μικροβιώματος της οινοποίησης, ενώ και ο τύπος οινοποίησης που ακολουθήθηκε συνέβαλε, αλλά σε μικρότερο βαθμό, πιθανότατα ως αποτέλεσμα της κοινής χρήσης συντηρητικών και στις δύο οινοποιήσεις. Η κοινότητα των μυκήτων παρουσίασε ευρεία φυλογενετική ποικιλομορφία από ζύμες-μη σακχαρομύκητες στα αρχικά στάδια ζύμωσης, που αντικαταστάθηκε από την πλήρη κυραρχία στελεχών σακχαρομήκητα, εμβολιασμένων ή γηγενών, καθώς η ζύμωση εξελισσόταν. Η βακτηριακή κοινότητα αποτελούνταν από βακτήρια γαλακτικού και οξικού οξέος, με στελέχη του Oenococcus να παρουσιάζουν προοδευτική αύξηση κατά τη διάρκεια της ζύμωσης. Η μετα-μεταγραφωματική ανάλυση ενίσχυσε την ισχυρή επίδραση του σταδίου οινοποίησης στη μεταβολική δραστηριότητα του μικτοβιώματος της οινοποίησης. Όταν αναλύθηκαν ξεχωριστά τα δεδομένα για το κάθε στάδιο οινοποίησης, παρατηρήσαμε ισχυρές επιδράσεις του τύπου οινοποίησης που ακολουθήθηκε (αυθόρμητη και ελεγχόμενη) στην αρχή και τη μέση της ζύμωσης. Συνολικά, παρατηρήσαμε μια γονιδιακή έκφραση επαγόμενη από στελέχη του σακχαρομύκητα και άλλες ζύμες, που συσχετίστηκαν με μηχανισμούς απόκρισης σε συνθήκες στρες, στη μεταφορά, τη γλυκόλυση και την παραγωγή αιθανόλης, σε συμφωνία με το βιοχημικό περιβάλλον των ζυμώσεων.

Τα αποτελέσματα της συγκεκριμένης διατριβής εμπλέκονται σημαντικά στην υγεία των φυτών του αμπελιού (Κεφάλαια 2 και 3) και στον τομέα της παραγωγής οίνου (Κεφάλαια 3, 4 και 5), ενώ προσφέρουν σημαντικές γνώσεις και διαγνωστικά εργαλεία που μπορούν να βελτιστοποιηθούν περαιτέρω και να χρησιμοποιηθούν στη διαχείριση του αμπελώνα και τη διαδικασία παραγωγής οίνου.

List of abbreviations

GTDs: Grapevine trunk diseases

BCAs: Biocontrol agents

ASVs: Amplicon sequence variants

OTUs: Operational taxonomic units

CTAB: Cetyltetramethyl ammonium bromide

NMDS: Non-metric multidimensional scaling

PERMANOVA: Permutational multivariate analysis of variance

DA: Differential abundance

OOB: Out of bag.

NS: Non-Saccharomyces

AAB: Acetic Acid Bacteria

LAB: Lactic Acid Bacteria

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ABTS: 2,2′-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid)

RPA: Reducing Power Assay

PRA: Plasmid Relaxation Assay

MDA-MB-231: Monroe Dunaway Anderson -Metastatic Breast-231 cancer cells

HepG2: Human Hepatocarcinoma cell lines

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Chapter 1

General introduction

1. General introduction

Plants, as all higher organisms, are entities living in association with a large variety of microbes that surround or colonize them. The complex and dynamic relationship of plant-associated microbes with their host-plant and the microbial contribution in plant survival and evolution introduce the concept of the "holobiont" (Zilber-Rosenberg & Rosenberg, 2008). Within holobiont, the genome of the host-plant along with all genomes derived from its associated microbiota have in total established the term of "hologenome" (Sharifi and Ryu, 2017; Zilber-Rosenberg & Rosenberg, 2008).

The distinct microbial patterns observed across different plant compartments, indicate plant-part-based microbial populations, on both the internal of plants tissues (Hardoim et al., 2015) and the outside of their tissues, from the above ground episphreric parts (Vorholt, 2012) to the rhizosphere and the bulk soil (Lopes and Schachtman, 2023; Reinhold-Hurek et al., 2015; Philippot et al., 2013)

The plant associated microbiota, namely *phytomicrobiome*, consists of a multitude of microbial members that have developed either symbiotic or dysbiotic relationships with their host plants. Symbiosis relies on associates with microbial members characterized by neutral or beneficial effects on host plants, like promotion and stimulation of plant growth, fitness and productivity, that enable the plant holobiont to survive towards a wide range of stress, abiotic factors and diverse environments (Backer et al., 2018; Birhane et al., 2012; Berendsen et al., 2012). On the opposite, the imbalance of this relationship and the loss of host's capacity to regulate its microbiota (Choi et al., 2020; Wei et al., 2019), is usually associated with perturbations in plant health and induction of stress or disease symptoms on the host plant (Levy et al., 2017; Paasch et al., 2021; Petersen et al., 2014), leading to the condition of dysbiosis.

1.1 The plant holobiont

1.1.1. Plant -microbe associations

Microorganisms, having developed an interconnected relationship with plants, constitute a cornerstone for their life, health and survival. Various microbial groups are met on a plant habitat from aboveground (epiphytes) to belowground plant compartments (rhizosphere microbes), including bacteria, archaea, fungi, oomycetes, viruses, protozoa and algae (Bordenstein et al., 2015; Roughgarden et al., 2018; Baedke et al., 2020). These microbial populations have exhibited strong associations

with different plant tissues, favored by morphological, physiological, compositional and functional properties (Vandenkoornhuyse et al., 2015). Subsequently, microbial functioning constitutes one of the key controllers of the processes, the dynamics and the adaptations of microbial community (Fanin et al., 2018; Liang et al., 2019).

The symbiotic interactions established as an outcome of all those stable plant-microbe associations, can be neutral, harmful or can have beneficial effects on the host organism, involving commensalism, parasitism, pathogenicity and mutualism. Commensalism and mutualism, based on host's ability to provide substances to the symbiont (captured, ingested or generated), enable them to survive together, either independently or depending on each other, respectively, with no negative effects (Bogitsh, Carter & Oeltmann, 2019; Fukui, 2014). Parasitism, though, relies on symbiont's physical presence and colonization by taking resources from the host, causing possible negative effects on host's fitness (Newton et al., 2010; Fukui 2014). One step further, pathogenicity is exhibited when parasites, acting as pathogens, are able to actively harm the host plant for their own trophic benefit, while even causing host-necrosis. However, in some cases, whether a microbe is going to act as mutualist, commensal, or pathogen depends on the hosts and the microbes associated in this interaction, on genetic and molecular mechanisms, the external environment (Song et al., 2020; Baltrus 2017) and the between microbes antagonism (e.g. competition for the same resource or the exchange of beneficial metabolites) (Xu et al., 2020). Consequently, symbiotic roles are not so restricted, but found to be context-dependent in a way that they cannot always be assigned to a specific category.

1.1.2. Plant compartments acting as microbial habitats

The colonization of plants by microorganisms does not follow a random pattern, it is a not stochastic process (Cardinale et al., 2015) where each distinct compartment of the plant (rhizosphere, endosphere, phyllosphere) presents a distinct and specific microbial niche with relative specific functions and requirements (Berg et al., 2016).

1.1.2.1 Soil and rhizosphere

Soil, constitutes one of the most dense and heterogeneous habitats characterized by a complex composition of minerals, organic matter and a penetrating network of water- and air-filled pore spaces (aeration), being influenced by a multitude of biotic and abiotic factors (Orgiazzi et al., 2016). Decomposition, nutrient cycling and other vital

ecosystem functions are performed within this knotted and enriched habitat, while symbiotic relationships between plants and the native microbiota are also formed. The below ground bulk soil sets the foundation for physicochemical and biological properties by supporting plant growth (Philippot et al., 2013). Plant roots, the inside soil plant part, are surrounded by a narrow zone which is the interface between the absorbing roots and the bulk soil, known as the rhizosphere, which is the area where the recycling and materials - energy transfer take place (Mendes et al., 2013; Hinsinger et al., 2009). Within this intermediate area, an exchange of substances is performed, where plants absorb their required nutrients that flow from the bulk soil to the rhizosphere, under the direct secretion of organic matter by the plant root-system to the bulk soil (Pronk et al., 2022).

Soil and root microbiota, involved in ecological interactions and functions like carbon and nitrogen biogeochemical cycles (Mosley et al., 2022), are largely made up of bacteria and fungi that have crucial roles in keeping the soil healthy and fertile, (Berendsen et al., 2012; Mauchline and Malone, 2017) and plant exudates. Microbial assemblages in the soil and rhizosphere ecosystem are supported by microorganisms (Zarraonaindia et al., 2015; Bai et al., 2015), whose establishment is the result of either commensal, pathogenic, or beneficial relationships with their hosts as well as to each other. However, this can undergo modifications under the influence of different plant species (Liu et al., 2020a; Schmitz et al., 2022), land practices or fluctuations in environmental conditions, such as soil temperature, nutrient availability, acidity (pH) or humidity (Rahman et al., 2021).

Soil pathogens are part of soil microbial communities, acting as plant antagonists (Bever et al., 2015) or as permanent parts of the community or as frequent transient inhabitants, with a consistent presence for specific parts of their life cycle (Oliver et al., 2015). Many of them, though, may harm plants by leading to limited plant growth, crop loss and reduced agricultural production. (Yin et al., 2021). On the opposite, several fungal and bacterial beneficial species, useful for plant growth and development (Huang et al., 2014), set a barrier to plant pathogen, by enhancing plants capacity to obtain soil nutrients, promoting plants tolerance to abiotic stressors (Song et al., 2020) such as drought or salinity (de Vries et al., 2020; Schmitz et al., 2022) and competing with pathogens for space and resources (Hacquard et al., 2016; Kwak et al., 2018).

1.1.2.2 Upper plant parts

The aerial region of plant biosphere, including leaves, stems, flowers, fruits and pollens, has a central role in plant life, with unique morphological and physicochemical properties (Lindow and Brandl, 2003; Knief et al., 2010). Being at the first interface of interaction between plants and their environment (Chen et al., 2020; Mandal et al., 2023), this region is characterized as an heterogenous and harsh habitat, exposed to rapid and/or extreme fluctuations of temperature, humidity and ultraviolet radiation along with desiccation and limited availability in nutrients. Additional structural properties, like the external coverage by hydrophobic-waxy cuticle layers around the upper and lower foliate epidermis and its direct influence by plant metabolism, in combination with all the previous characteristics, render this environment a particularly inhospitable area for microbes to colonize and thrive (Bashir et al., 2022; Vorholt, 2012; Bringel & Couée, 2015; Hirsch, 2008). These habitat features, although universal, can undergo spatial alterations, counted on host genotype, plant developmental stage, biogeography, seasonality, agricultural practices or atmosphere pollution levels (Lindow and Brandl, 2003; Bao et al., 2020; Qian et al., 2020; Xiong et al., 2021).

Despite the poor growth conditions of this oligotrophic environment and the relatively lower microbial diversity compared to soil, numerous microorganisms eventually colonize and exploit this surface, comprising algae, bacteriophages, but mainly numerous bacteria, filamentous fungi, yeasts and protists (Vorholt, 2012). Within this microbial community, microbes dwelling on plant surface make the epiphytic microbiome, while those colonizing the internal tissues represent the endophytic microbiome (Leveau, 2019; Schlechter et al., 2019). The microbial components of the epiphytic microbiome originate from plant and field genetic heritage (vertically) or from environmental and neighboring microbial reservoirs (Bright and Bulgheresi, 2010).

Microbial associations on plant surface range from epiphytic commensals and mutualistic symbionts to pathogens (Kowalski et al., 2015), with the last being able to infect a wide spectrum of host plants being the main cause of devastation in natural ecosystems and crop yields. Such species, like *Pseudomonas syringae*, may colonize constantly the aboveground external and internal tissues, suppress host immunity and cause diseases in multiple economically important plant species (Mansfield et al., 2012; Morris et al., 2013; Xin et al., 2018; Agbavor et al., 2022). Some others, like

Fusarium species, known to be prevalent on leaf surfaces or on stems and roots, can lead to serious plant diseases of pitch canker (Herron et al., 2015), stem wilt and root rot (Zhang et al., 2021) or crown disease (Hafizi, Salleh and Latiffah, 2013).

Besides pathogens, most epiphytic microorganisms live as commensals on their host plants, affecting plant wellbeing by supporting stress tolerance, promoting plant growth, degrading environmental pollutants and suppressing diseases (Liu et al., 2020b; Zhu et al., 2023; Xu et al., 2021; Agha et al., 2022). Using mechanisms of regulation, they can contribute on the improvement of plant adaptability. Some members, by producing DNA protection proteins and stress alleviating enzymes, can manipulate reactive oxygen species (ROS) generated upon exposure to solar radiation, providing protection on plant surface (Delmotte et al., 2009). Biofilm formation is also a way of helping both the host and microbes to thrive under environmental stress, a mechanism used by bacteria, to facilitate water, oxygen and nutrients diffusion and avoid dessication (Costerton et al., 1994). Bacterial and fungal members of the epiphytic microbial communities contribute on preventing and controlling plant diseases. The ability of epiphytic bacteria to support the production of plant growth-promoting metabolites and improve grain yield, by consuming plant-secreted substances (e.g. methanol), is well documented (Abadi et al., 2020; Sanjenbam et al., 2022; Ajijah et al., 2023). Also, in the same frame, epiphytic fungi contribute to mineral absorption facilitating in forest ecosystems carbon, nitrogen, and phosphorus recycling (Sivakumar et al., 2020).

Agricultural crops are of great economic importance for the global market stressing the need to shed light on the factors controlling the structure and function of their associated microbiome under field conditions. This will enable us to effectively manipulate plant microbiome towards higher agricultural production and crop health.

1.2. Grapevine microbiome

Grapevine, deriving from species *Vitis vinifera L.*, is a crop plant of global interest, exhibiting high adaptability in diverse environments, allowing the expansion of viticulture globally (Palliotti et al., 2014). Grapevine harvests of fresh fruits can be consumed as they are or dried to form raisins, but their largest proportion yearly is, mostly, destined to be used as starting material for winemaking (Garcia-Lomillo and Gonzalez-SanJose, 2017). This way, wine production and viticulture, having been recognized globally as two of the most well-established agricultural production

sectors, are the focus of scientific research over the last years (Cimini and Moresi, 2022). Winemaking output is undoubtably linked with the initial grapes quality. The generated grape must, containing also solid parts of grape berries, release numerous compounds from berries inner and outer skin, during vinification (Ribéreau-Gayon et al., 2006). In this regard, the microbiota harboured in grapes has a key role in the production of metabolites and eventually in the whole outcome of the vinification process (Bokulich et al., 2016; Portillo et al., 2016; Oliveira et al., 2018; Anagnostopoulos et al., 2019; Liu et al., 2019; Griggs et al., 2021; Camilo et al., 2022). However, the interest on grapes microbiota is not limited to the ripened fruits at harvest, known to affect fermentation and wine quality (Belda et al., 2017; Mezzasalma et al., 2018), but it expands to the whole grapevine microbiome. Vineyard conditions together with the grapevine microbiome reflect on the sanitary state of the grapevine plant and eventually on grapes quality (Barata et al., 2012). Grapevines suffer from a number of plant diseases that can affect their condition and production ability, with underlined effects on the final products, concerning grapes as fruits, the wine production process and resulted wine's chemical composition and sensory traits (Steel et al., 2013; Lisov et al., 2023). In order to protect grapevine derived products and secure plant health sustainable and effective crop protection strategies should be implemented. In this frame, the reduction in the use of synthetic pesticides has been a main goal at EU level while at the same we are seeking of novel biobased crop protection solutions with microbial pesticides being a promising and safe alternative (Silva-Valderrama et al., 2021; Esteves et al., 2023; Vidal et al., 2023).

1.2.1. The epiphytic grapevine microbiome

1.2.1.1. Factors influencing the epiphytic grapevine microbiome

Grapevine carposphere, the habitat associated with grape surface, is a niche which is getting influenced both by the vineyard environment and the grapevine tissues and organs, receiving inputs and being affect by external abiotic and biotic factors and the grapevine metabolism. Epiphytic grapevine microbiota, under the influence of this unstable habitat, adapts its composition (Rastogi et al., 2013; Perazzolli et al., 2014), but to which extend and under which circumstances is not totally clear. Although each grapevine compartment (above, inner, below-ground) comprises distinct microbial communities (Martins et al., 2013; Swift et al., 2021; Bettenfeld et al., 2022), overlaps and transfers of microorganisms between above ground and below ground grapevine

compartments have been. Soil, roots and trunk bark microbial diversity are pools for the grapevine epiphytic microbiota (Martins et al., 2013; Ramirez et al., 2020 ; Darriaut et al., 2022 ; Liu et al., 2019), whilst microorganisms of the endophytic grapevine microbiota seem to migrate and colonize the plant surface (Pacifico et al., 2019; Compant et al., 2011; West et al., 2010). Carposphere microbial communities of grapevine, were also shown to be evidently regulated along the vegetative cycle (Pinto et al., 2014; Kecskeméti et al., 2016; Guzzon et al., 2022; Liu and Howell, 2021). Although noticeable, these interplays describe relationships of grapevine epiphytic microbiota with the close surrounding environment only. In fact, more complex interactions implicating geographical location, topography, climatic conditions and annual influences or the type of grape cultivar seem to be operative in the assemblage of the grapevine microbiota. But lets see which are the most important factors shaping the grapevine microbiota.

Biogeography

Epiphytic grapevine microorganisms are exposed to the environmental conditions of a specific region where grapevines are located like geological and topographical characteristics, the location, the altitude and also the microclimate (precipitation, UV radiation, wind, temperature) and seasonal fluctuations. When exposed to these regional conditions epiphytic grapevine associated microbial communities are expected to exhibit structurally distinct regional patterns. Indeed, obvious biogeographical patterns have been identified in grapevine epiphytic fungal and bacterial populations on grapes and leaves of distinct viticultural zones (Papadopoulou et al., 2022) and on fresh grape-musts (Bokulich et al., 2014). Li et al (2021) in a study characterizing the fungal communities in spontaneous fermentations from grapes of Chinese regions with distict climatological differences, described evident biogeographical patterns on fungal communities. Also, Mezzasalma et al (2018), when investigating the bacterial community structure on grape surface of different cultivars from three locations with distinct climatic features underlined the role of geographical origin on the shape of the bacterial microbiome. In line with all the above, geographical origin impact was identified by Kioroglou et al. (2019) as the major factor shaping the fungal community of grape carposphere in distinct wine-grape growing regions in Australia.

Cultivar

Grapevine cultivar seems to have a major role in shaping the epiphytic microbial communities. A plenty of different grapevine cultivars have been described (This et al., 2006), reflecting the genetic variability and plasticity of the grapevine genome and expressing this diversity through structural and functional differences (Costa et al., 2012; Catacchio et al., 2019) with relative impacts on the associated epiphytic microbiome. Mezzasalma et al. (2018), studying the soil and grapes microbiota from the international and diffused Cabernet Sauvignon, Syrah, and Sauvignon Blanc cultivars, indicated bacterial members (OTUs) strongly shared between the these two niches that could act as cultivar fingerprints. The selection pressure exerted by different grapevine genotypes leading to distinct combinations of phyllosphere-carposphere microbial communities among cultivars was also described by Singh et al. (2018), in a study regarding a wide range of different grapevine cultivars in France. Similarly, Awad et al. (2023), when studied the epiphytic microbiome from bark, buds and grape berries among 36 grapevine cultivars grown in the same environment, reported a clear divergence of microbial patterns and microbial species characteristic of the host genotype, while in a previous study (Awad et al., 2020), using metagenomics, they had also suggested the role of cultivar's genotype on shaping the associated microbial community with unique cultivar microbial fingerprints. A cultivar background of bacterial assemblages was also revealed by Zhang et al. (2019), in a study focused on grape berry surface microbiota from six different grapevine cultivars in China. Moreover, Bokulich et al (2014) described clear cultivar-patterns in the fungal community of must from cultivars Cabernet Sauvignon, Chardonnay and Zinfandel, while, under a different approach, evidence by Steiner et al. (2021) demonstrated the valuable differences between the response of Pinot noir and Chasselas cultivars to N- fixing microbes.

Vintage

Vintage influences, relied on anual climatic fluctuations and environmental variations exhibit evident impacts on the epiphytic grapevine microbial communities. Kecskemeti et al. (2016), in a study conducted over the same vineyard plots across consecutive years, reported considerable difference on microbial communities along the years even in cases where then plots studies were under the same management practices. Abiotic factors such as fluctuations on climatic conditions from year to

year, might have direct impacts on microbial epiphytic and carposphere communities (Ren et al., 2015; Balint et al., 2015; Huang et al., 2023a). On the other hand, Bokulich et al. (2014), did not detect significant changes across the different vintages in fresh grape musts. Other studies, performed across and within viticultural zones with distinct spatial or altitude features, identified clear vintage signatures in the composition of the carposphere microbiome (Papadopoulou et al., 2022; Steenwerth et al., 2021; Cureau et al., 2021). Similar vintage effects were reported on the assemblage of native yeast populations monitored on fresh grapes (Castrillo and Blanco, 2022)

Terroir

The complex interplay of all parameters predominating in a given region meaning climatic conditions, soil, location (geography, altitude), flora and fauna and the implicated agricultural practices, are collectively called "terroir". The regional distinctiveness of grapevine associated microbial communities, being under the influence of all the above factors is known as microbial *terroir*. Liu D. et al (2020a), in a comprehensive study assessing longitudinal vineyards in Australia, identified significant correlations between vineyard conditions and the fungal grapevine microbiome with wine metabolic profile, suggesting regional wine traits under the effect of terroir. In a study of Miura et al (2017), terroir signatures were evident in the fungal community of grapes but they were less clear in the bacterial community. Similar observations of terroir-dependent patters within viticultural zones were obtained by Kazou et al. (2023) for both bacterial and fungal grape microbiome in the region of Nemea (Greece). Additional evidence for the important effect of *terroir* in shaping the grape microbiome was presented by Jara et al. (2016) who showed clear terroir signatures on the grapevine yeast community in a wider viticultural region and in vineyards located in different Chilean valleys.

1.2.2. Epiphytic grapevine bacterial communities

Proteobacteria are the predominant phyla detected on grapevine surface tissues, as also reported for other plants, followed by *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* (Perazzolli et al., 2014; Pinto et al., 2014, 2015; Portillo and Mas, 2016; Portillo et al., 2016; Zhang et al., 2019; Ding et al., 2021; Papadopoulou et al., 2022; Cobos et al., 2022; Morgan et al., 2017). When looking at the genus level, the

epiphytic bacterial community of grapevine is mostly composed of *Pseudomonas*, *Sphingomonas*, *Methylobacterium* and *Acinetobacter*, found to be strongly associated with both grapes and leaves, while members of *Massilia*, *Bacillus* and *Kocuria* are known dwellers of the grapevine phyllosphere (Martins et al., 2013; Leveau and Tech, 2011 ; Ding et al., 2021 ; Wei et al., 2022a; Deyett and Rolshausen, 2019; Wassermann et al., 2021). In addition *Lactobacillus*, *Pediococcus*, *Leuconostoc* and frequently *Oenococcus*, known as lactic acid bacteria (LAB), have been detected as main members of the epiphytic grapevine microbiome in several grape cultivars on vineyards in different countries (Sieiro et al. 1990; Bae et al., 2006; Wei et al., 2022a; Gobbi et al., 2020). Some of the members of the epiphytic grapevine microbiome (e.g. *Bacillus*, *Pseudomonas*, *Acinetobacter*) have been shown to produce plant hormones like gibberellins, which play important role in various physiological plant processes (Mohamed et al., 2012; Kang et al., 2014; Kang et al., 2009; Radhakrishnan et al., 2016). Several of these bacterial members associated with leaves and grapes are known to be plant growth-promoters carrying activit against grapevine pathogens (Leveau and Tech, 2011).

1.2.3. Epiphytic grapevine fungal communities

The epiphytic grapevine fungal community is characterized by the predominance of *Ascomycetes* followed by *Basiodiomycetes* (Wassermann et al., 2021; Agiular et al., 2020; Barata et al., 2012; Awad et al., 2023; Lorenzini et al., 2019). The most common and abundant fungal members of the epiphytic grapevine microbiome are represented in the genera *Aureobasidium*, *Cladosporium*, *Alternaria*, *Aspergillus* and *Filobasidium*, most of them shared as common dwellers of plant surfaces for several other types of plants and crops (Pinto et al., 2018; Dimakopoulou et al., 2008; Tao et al., 2014; Awad et al., 2023; Zhang et al., 2019; Lorenzini et al., 2016; Singh et al., 2019; Regecova et al., 2022; Nemcova et al., 2015; Gobbi et al., 2020; Chen et al., 2022). Besides filamentous fungi, the epiphytic grapevine microbiota is composed of yeasts like *Metschnikowia* and *Hanseniaspora* (Zhu et al., 2021; Regecova et al., 2022; Nemcova et al., 2014). Some of these fungi are beneficial to grapevines, like *Aureobasidium* which competes and has antibiotic activity against the grapevine pathogen *Botrytis cinerea* (Carmichael et al., 2019; Rathnayake et al., 2018). Other fungi can act as plant growth promoters, like *Aspergillus*, that besides being pathogenic and producing toxins, they are capable of phytohormone production (Xu et

al., 2018; Asaf et al., 2018; Galeano et al., 2021). However there are also members of the epiphytic grapevine microbiome that are pathogenic to the host plant, like *Cladosporium* that has been associated with grape rot or *Alternaria* associated with mycotoxins production (Briceno et al., 2008; Prendes et al., 2021).

1.2.4. Grapevine trunk microbiome

1.2.4.1. Grapevine Trunk Disease (GTDs) – associated pathobiome

Despite the grapevine microbiota of leaves and grapes, the microbiota of woody-grapevine-parts like the grapevine trunk has been studied due to its implication in the development of grapevine trunk diseases (GTDs). GTDs are a complex of fungal diseases that has been identified as one of the most important threats for viticulture worldwide, widely influencing vineyards, declining vineyard longevity and ending to devastating grapevine productivity loss and vine death (Bertsch et al., 2013; Gramaje et al., 2018; Mundy et al., 2018). Members of the fungal microbiome that are associated with GTDs constitute the fungal pathobiome of GTDs (Hrycan et al., 2020; Diaz et al., 2023). They are either latent inhabitants of the trunk and they express their pathogenicity only under certain conditions or they infect the grapevine (e.g. via pruning wounds) and then colonize the trunk and other woody and lignified parts of the grapevine (Mondello et al., 2018; Mugnai et al., 1999; Bertsch et al., 2013; Kenfaoui et al., 2022). The main reported forms of the GTD complex, mostly encountered in mature grapevines (10-15 years and over) is composed of *Eutypa* dieback, *Esca* complex disease, *Phomopsis* dieback and *Botryosphaeria* dieback, the last one affecting also young grapevines along with *Black-foot* disease and *Petri* disease (Hofstetter et al., 2012; Gramaje et al., 2018; Cobos et al., 2022; Kenfaoui et al., 2022; Azevedo-Nogueira et al., 2022). Ascomycetes and Basidiomycetes are considered the most common members of the GTD pathobiome encompassing *Phaeomoniella chlamydospora, Phaeoacremonium spp* and *Fomitiporia Mediterannea* correlated with *Esca, Botryosphaeriaceae* family members (e.g. species of *Neofusicoccum, Botryosphaeria, Diplodia*) linked to *Botryosphaeria* dieback, *Eutypa lata* often associated with *Eutypa* dieback and *Phomopsis* dieback being often associated with *Phomopsis* and *Diaporthe* species (Martín et al., 2022; Úrbez-Torres, 2011; Fourie et al., 2004; Aroca et al., 2006; Edwards and Pascoe, 2001; van Niekerk et al., 2005; Lolas et al., 2020; Úrbez-Torres et al., 2013; Kaliterna et al., 2012; Spetik et al., 2023; Diaz et al., 2023). Symptoms of GTDs include visible malformations,

physiological disorders and functional declines mostly on wood grapevine tissues, appearing as necrotic wedges within the wood, but also on leaf tissues with relevant foliar dark spots, chlorosis or necrotic margins (Gramaje and Armengol, 2011; Serra et al., 2018; Lecomte et al., 2012). GTDs dispersion is favored by agricultural practices, fungal spores transmission through waterflow conditions (rain splash, wind) or via soil transfer (Cloete et al., 2011; Trouillas et al., 2011; Nerva et al., 2019). There are not main chemical means to combat GTDs after the ban of sodium arsenite. The withdrawal of this chemical for safety reasons has favored the outburst of GTDs in vineyards globally (Songy et al., 2019; Bruez et al., 2021; Trouvelot et al., 2023).

1.2.4.2. GTDs microbial biocontrol agents (BCAs)

To ensure high product quality and high crop yields emphasis has been put in the last few years on the development and use of eco-friendly crop protection strategies instead of application of synthetic pesticides whose use is to be reduced by 50% in 2030, as dictated by the European Green Deal (Dy et al., 2018; Prajapati et al., 2020; Singh and Negi, 2022). In this frame, the development of novel biocontrol agents (BCAs) has been explored (Altieri et al., 2023; Ons et al., 2020; Mesguida et al., 2023; He et al., 2021). The biological control of GTDs involves the application of beneficial (micro)organisms, frequently isolated from vineyards, aiming to suppress the population of fungi participating the GTDs pathobiome. In this frame, *Trichoderma* species have been found to reduce *Esca* appearance (Pertot et al., 2016; Bigot et al., 2020). In addition, *Clonostachys rosea* and *Cladosporium* strains have exhibited positive antagonistic activity to GTDs pathogens (Silva-Valderamma et al., 2021). Haidar et al. (2016), revealed significant antagonistic activities of *Pantoea* and *Enterobacter* strains on *Neofusicoccum parvum*. Similarly, indigenous to grapevine strains of *Aureobasidium* has showed potential inhibitory effects on *Botryosphaeria* dieback-related fungi (Pinto et al., 2018). Furthermore, a strain of *Pythium oligandrum*, dwelling on grapevine rhizophere, was also identified as an efficient BCA against major aggressive fungal-GTDs pathogens (Daraignes et al., 2018; Benhamou et al., 2012; Gerbore et sl., 2014).

1.3. Vinification microbiome

1.3.1. Vinification: the wine-making fermentation process

Grapevine epiphytic microbial communities have received large attention not only because of their interactions and effects on grapevine state but mostly due to their strong influence on the wine making process. The microbiome of grapes reflects features and characteristics of their vineyard of origin which are expected to influence the vinification process and eventually the attributes of the produced wine (Bokulich et al., 2016; Belda et al., 2017; Liu et al., 2019; Kamilari et al., 2021; Griggs et al., 2021; Welsh et al., 2023). Wine production involves grapes harvesting and crushing leading to the generation of grape must which is then subjected to alcoholic fermentation (AF) (Mills et al., 2007). The alcoholic fermentation (AF) involves a number of biological and chemical reactions for the transformation of carbohydrates contained in grape-must to ethanol. The AF is carried out by a complex microbial community originated from grape (inner part and carposphere) that is characterized by certain successional patterns. The vinification microbiota consists of yeasts (Saccharomyces and NS), filamentous fungi, prokaryotes with lactic and acetic acid bacteria showing the higher prevalence (Wang et al., 2016; Barata et al., 2012; Camilo et al., 2022). This vinification microbiota evolves, adapts and shapes along AF in response to the changes in the levels of alcohol, acidity, temperature, sugars concentration, oxygen presence, nutrients availability, produced metabolic compounds and commercial additives (Lin et al., 2014; Wang et al., 2014; Hao et al., 2021; Morales et al., 2015; Morgan et al., 2019).

Vinification types

Grape fermentation relying solely on the indigenous grape microbiota, called spontaneous fermentation, has attracted attention by the wine making industry as a strategy to enrich the regional character of the produced wines. However, spontaneous fermentation is considered a risky approach due to increasing incident of wine spoilage or reduced quality of the produced wine. Hence, inoculation of grape juice with commercial selected strains of *Saccharomyces cerevisiae* and *Oenococcus oeni*, enables a better management of the AF and is the globally preferred winemaking practice to ensure the control of wine flavor (Parapouli et al., 2020; Guindal et al., 2023; Maitre et al., 2014; Walker and Stewart, 2016; Liu et al., 2021). However, recent studies suggest that commercial (inoculated) vinifications result in the

production of wines with a reduced aromatic complexity and reduced regional identity, since the diversity of native strains is suppressed (Philipp et al., 2021; Piao et al., 2015; Beltran et al., 2002; Comitini et al., 2011; Scacco et al., 2012). Hence, particular and spatial fingerprints of the indigenous microbiota that have been highlighted to enhance wine regionality in the concept of "wine terroir", attracted the interest of research and winemaking sector to focus again on the use of native strains in wine fermentations (Bokulich et al., 2016; Herve, 2020; Jara et al., 2016; Lock et al., 2019; Zhou et al., 2021; Capitello et al., 2021; Bagheri et al., 2017)

Vinification stages

At the beginning of alcoholic fermentation, regardless of inoculation practices, the microbiota is composed of yeasts (weakly or strongly fermenting yeasts), filamentous fungi and bacteria, mostly originated by the grapes, sugar levels are high and alcohol levels are low (Jolly et al., 2003; Wang et al., 2015; Bagheri et al., 2020; Ghosh et al., 2015). As fermentation progresses and the amount of produced ethanol increases, most of the members of the early stages microbiota are outgrown by strongly fermentative and ethanol tolerant fungi and bacteria which dominate at the middle and the end of fermentation (Bagheri et al., 2015; Portillo and Mas, 2016; Tristezza et al., 2016; Setati et al., 2015; Jolly et al., 2003; Wei et al., 2022; Wang et al., 2015; Huang et al., 2023b). The metabolism and growth along the process is linked with nutrients and oxygen availability, ethanol accumulation and physicochemical conditions (Mendoza et al., 2009).

1.3.2 Vinification fungal microbiome

Yeasts in winemaking alcoholic fermentation, are the key mediators for must transformation to wine, based on their ability to compete successfully for nutrients in habitats with freely available saccharides, like vinification, by metabolizing sugars to ethanol and carbon dioxide (Piskur et al., 2006, Tofalo an Suzzi, 2016). Among these yeasts, *Saccharomyces cerevisiae* is the preferred by wine industry, due to its ability of completing alcoholic fermentation, by growing and proliferating in sugar-rich habitats, under both aerobic and anaerobic conditions, exhibiting resistance in alcohol and in the presence of SO_2, while at the same time promote the desirable sensory profile of wine (Merico et al., 2007; Beltran et al., 2002; Ciani et al., 2004; Swiegers and Pretorius, 2005). Besides *Saccharomyces*-yeasts, *non-Saccharomyces* (NS) yeasts

have also attracted the attention of the wine making industry due to their well documented positive effects on wine fermentation, and particularly for their contribution in the sensory features of wines (Jolly et al., 2014; Lappa et al., 2020). Despite the undoubtable effectiveness of *S. cerevisiae* in fermentation, the regional character provided in wine by the indigenous NS yeasts has risen the interest for their use, in order to emphasize the regional character of the produced wine (Ciani and Comitini, 2011; Benito et al., 2019; Jolly et al., 2014). The presence and performance of NS yasts is substantial at the early stages of fermentation, due to their presence at high numbers on the fermented grapes compared to *S. cerevisiae* that is found in low abundance in grapes (Goddard et al., 2010). Although, NS yeasts represent a potential sources of wine spoilage (Padilla et al., 2016; Borren and Tian, 2021), many of them have been isolated and they are currently commercially available as starter cultures like *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans*, that they are used as single inocula or in combination with *S. cerevisiae* (Roudil et al., 2019; Varela et al., 2017; Windholtz et al., 2021; Vejarano and Gil-Calderon, 2021; Tufariello et al., 2021).

1.3.3 Vinification bacterial microbiome

Major bacterial components of the bacterial microbiome are the lactic acid bacteria (LAB), responsible for the malolactic fermentation (MLF), a secondary fermentation after the initial alcoholic fermentation, through which the L-malic acid is transformed into the less acidic L-lactic acid and carbon dioxide. This process results in a decline of wine acidity and a microbiological stabilization and modification of wine aroma (Liu et al., 2002). The most common LAB encountered in vinification belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Weissella*, among which, *Oenococcus oeni* and *Lactobacillus plantarum* are the most frequently used to control the MLF (Lerm et al., 2010; Liu et al., 2014, Virdis et al., 2021; Reguant et al., 2021, Andorra et al., 2019). *O. oeni*, the most known, established and effective LAB, exhibits the higher tolerance and adaptability of all LAB to high ethanol levels, acidity, and SO_2 (Onetto et al., 2021; Balmaseda et al., 2022). Moreover, it contributes positively to wine sensory characters, unlike *Lactobacillus* and *Pediococcus*, that tend to produce undesirable sensory characteristics (Bartowsky et al., 2009). Noteworthily, LAB are also involved in the production of several enzymes like glucosidases, esterases, decarboxylases with essential role on wine sensory

properties (Matthews et al., 2004; Kieliszek et al., 2021; Capello et al., 2017; Rivas et al., 2022). Acetic acid bacteria (AAB) constitute the second major group of bacteria implicated in wine making. AAB are well adapted to various sugar and ethanol rich environments, capable to efficiently oxidize ethanol to acetic acid and thus commonly used in vinegar production. This characteristic makes them considered as spoilage microbial components for wine industry (Bartowsky and Henschke, 2008; Raspor and Goranivic, 2008). Main representatives of AAB encountered in vinification belong to *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter*. They are characterized by their high capacity to oxidize ethanol to acetic acid (Gomes et al., 2018; Andres-Barrao et al., 2013; Nakano and Fukaya, 2008). AAB in vinification are derived from grape surface where they thrive, but they could also get dominant in grape must during incomplete fermentation and in cases where wines are unintentionally exposed to air, being favored by the exposure to oxygen (Valera et al., 2011; Guillamon and Mas, 2011). However, they have been reported to multiply under anaerobic or semi-anaerobic conditions during all stages of winemaking (Joyeux et al., 1984).

1.3.4. Grapevine and vinification microbiome influence on wine properties

Grapes harbor a complex microbiome reflected on must and having an essential role in vinification and eventually in the establishment of wine sensory traits, quality and properties. It is known that the organoleptic characteristics, encompassing taste, aroma and visual attributes, of wines are of great significance for the overall flavor perception and the consumers preferences (Polaskova et al., 2008; Tempere et al., 2019). In many studies, desirable organoleptic characteristics of wines have been associated, through correlation testing, with specific members of the vinification microbiota and especially to native grapes yeasts. Known fermentative yeasts, like *Lachancea* sp., commonly detected on grapes, have been associated with the production of higher alcohols, esters and terpenes, contributing to wine aroma (Morata et al., 2018; Gobbi et al., 2013; Jolly et al., 2014; Sumby et al., 2010). Similarly, *Hanseniaspora* sp., has been correlated with unique fruity sensory attributes, while its most characteristic produced compounds that confer derirable aroma to wines are acetate esters and aldehydes (Medina et al., 2012; Martin et al., 2018). Additionally, *Metschnikowia* and *Torulaspora* yeasts have been associated with more flowery and smokey notes (Medina et al., 2012; Gonzalez-Royo et al., 2015).

Weak fermentative yeasts, like *Pichia and Rhodotorula*, contribute enzymes that lead to the formation of volatiles with increasing fruity aromas (Calabretti et al., 2012). Besides the aesthetic value of wines, their chemical composition in polyphenols (tannins, anthocyanins), organic acids, minerals and volatiles seems to confer further desirable properties like activities against cancers, cardiotoxicity, oxidative stress, cell proliferation cell apoptosis and metabolic regulations. (Duan et al., 2021; Nemzer et al., 2022; Lin et al., 2021). All these findings highlight the key role of the grapevine and vinification microbiome on the establishment of wine chemosensory characteristics that could be exploited to enhance the quality and the regional identity of the produced wines.

1.4. Main Objectives of the Thesis

In the frame of this thesis we determined the composition of the grapevine bacterial and fungal communities in Greek cultivars using amplicon sequencing of the 16S rRNA gene and the ITS genomic region, respectively. Firstly we explored the factors shaping the fungal and bacterial microbiome of GTDs in diseased and healthy grapevines across different viticultural zones, considering biogeography/cultivar (lumped factor) and the GTDs status as potential shaping factors. Based on the data obtained we identified via random forest and network analysis early indicators and potential suppressors of the GTDs. We further focused on the above ground plant part and explored the main determinants (biogeography, cultivar, vintage and terroir units) of the epiphytic (leaves and grapes) grapevine microbiome across different geographic scales: across and within viticultural zones. Our hypothesis was that different factors would be operative in shaping the epiphytic grapevine microbiome at different geographical scales. Finally, since the epiphytic grapevine microbiome is directly associated with the vinification process, we extended this study to the vinification process and monitored microbial succession under different vinification strategies (spontaneous with or without SO_2 addition and commercial). In this frame we explored if the same vineyard-related shaping factors remain operative during vinification and how they affect the antioxidant, antimutagenic and anticancer activities of the produced wines. Last, we identified consistent correlations between members of the vinification microbiome and the antioxidant, anticancer and antimutagenic activities of wine. In the final part of this thesis we employed metagenomic and metatranscriptomic analysis to disentangle the metabolic potential

and activity of the vinification microbiome under spontaneous and commercial fermentations. So the main objectives of this thesis were:

- To identify (i) the factors (cultivar/biogeography and GTD condition) that shape the grapevine wood microbiome and the fungal wood pathobiome, and (ii) microbial indicators and interactors that could be used as predictors of GTD establishment and as potential pole of BCAs for GTDs respectively.
- To determine the composition of the epiphytic microbial communities on emblematic Greek cultivars and explore the contribution of different factors like biogeography, cultivar, vintage and terroir on the composition of the epiphytic grapevine microbiome at different geographic scales: across and within viticultural zones.
- To determine microbial succession under different vinification strategies and identify how different vineyard- and vinification-mediated factors are shaping the vinification microbiome and affect the antioxidant, antimutagenic and anticancer activities of the produced wines.
- To disentangle the metabolic potential and metabolic activity of members of the vinification microbiome under different fermentation strategies using meta-omic approaches.

1.5. References

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Chapter 2

Grapevine wood microbiome analysis identifies key fungal pathogens and potential interactions with the bacterial community implicated in grapevine trunk disease appearance

The work presented in Chapter 2 is included in the following article:

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Environ Microbiome. 2021 Dec 4;16(1):23. doi: 10.1186/s40793-021-00390-1.

2.1 Background

Grapevine trunk diseases (GTDs) are considered a major threat for viticulture (Mungai et al., 1999). However, the ban of sodium arsenate, the main chemical used for their control, along with changes in cultivation practices and the use of infected planting material resulted in a strong upsurge in the incidence of GTDs globally (Bertsch et al., 2009; Elena et al., 2018; Gramaje et al., 2018) with estimated annual economic losses summing to 1.132 million euros (Hofstetter et al., 2012). GTDs are considered a complex of diseases that could be distinguished into three major groups: *Eutypa*, *Esca* and *Botryosphaeria* diebacks (Bertsch et al., 2013). Several fungi have been linked with GTDs like: (1) *Eutypa lata* and other *Eutypa* species associated with *Eutypa* dieback (Kuntzmann et al., 2010), (2) *Phaeomoniella chlamydospora*, *Phaeoacremonium spp*., and *Fomitiporia mediterranea* associated with esca decline (Crous et al., 1996) and (3) different genera of *Botryosphaeriaceae* (i.e. *Neofusicoccum, Lasiodiplodia, Diplodia, Botryosphaeria*) associated with the *Botryosphaeria* dieback (Punithalingam, 1976; Larignon et al., 2001; Crous et al., 2006). GTDs appear mostly in mature vines (> 8 years old) causing central, black necrosis and/or white rot that eventually lead to vine stunting and in acute cases to apoplexy (Surico et al., 2006). Foliar symptoms often appear years after infestation (Bertsch et al., 2013) and show transitory patterns of appearance (Lecomte et al., 2012). Fungal pathogens have not been recovered yet from symptomatic leaves suggesting that leaf symptoms are associated with the production and translocation to leaves of (1) secondary metabolites produced by the wood pathogens (Evidente et al., 2010; Andolfi et al., 2013; Reveglia et al., 2019; Cimmino et al., 2020) (2) phytotoxic by-products of wood decay or phytotoxic metabolites produced by the plant–fungi interaction (Del Frari et al., 2021).

Several studies have used culture-dependent approaches to isolate the causal agents of GTDs from symptomatic and comparatively from asymptomatic vines and/or from necrotic or soft rot tissues of vines (Elena et al., 2018; Hofstetter et al., 2012; Bruez et al., 2014). These studies suggested that all fungi previously identified as causal agents of GTDs were found at equivalent abundance levels in symptomatic and asymptomatic vines, implying that they are either latent pathogens or members of the endophytic vine microbiome causing symptoms when edaphoclimatic factors and vine genotype are conducive (Hofstetter et al., 2012). Culture-dependent approaches and low-resolution molecular tools (i.e. Single-Strand Conformation Polymorphism)

failed to unravel the full diversity of the endophytic fungal microbiome in vines (Elena et al., 2018; Bruez et al., 2014; Abed-Ashtiani et al., 2019). This is due to the limited cultivability of most environmental microorganisms (Hofer, 2018) and the bias of culture- dependent methods towards fast growing strains (Bridge et al., 2001). A few recent studies using amplicon sequencing provided deeper insights into the composition of wood fungal microbiome in GTD symptomatic and asymptomatic vines. For example, Bruez et al. (2020) did not identify compositional differences in the communities colonizing non-necrotic tissues of symptomatic and asymptomatic vines. Differences became evident when necrotic tissues were studied comparatively. In those tissues *F. mediterranea* dominated the fungal community. Similar studies in Portugal and Australia did not identify major differences in the fungal microbiome in asymptomatic vs symptomatic vines (Del Frari et al., 2019; Niem et al., 2020). A recent study by Bruez et al. (2021) showed that sodium arsenate application in esca diseased grapevines resulted in significant changes in the wood fungal microbiome with the most striking result being the strong inhibition of *F. mediterranea*. All these studies focused on a single cultivar in a single vineyard and have not considered differences in the resistance of vine cultivars to GTDs (Bertsch et al., 2013; Markakis et al., 2018) or the role of biogeography in the manifestation of symptoms in vineyards.

Besides fungi, the endophytic bacterial microbiome of grapevine and its role in GTDs is largely unexplored. Grapevines support a diverse bacterial community from roots to berries. The latter have been the focus of most vine microbiome studies (Bokulich et al., 2014; Zarraonaindia et al., 2015; Deyett et al., 2020). Wood-associated bacteria might have a suppressive effect on GTDs. They could act as forerunners of fungal wood pathogens facilitating the infection of wood tissues or as late comers colonizing necrotic or white rot tissues. To date only a few studies have investigated comparatively the composition of the bacterial community in the trunk of asymptomatic and symptomatic vines. Bruez et al. (2015) identified *Bacillales*, *Enterobacteriales* and *Xanthomonadales* as the most abundant bacterial orders colonizing the trunk of grapevines. However, they failed to identify consistent differences in the composition of the bacterial communities in asymptomatic and symptomatic vines. Similar results were obtained in recent studies using amplicon sequencing (Bruez et al., 2020; Niem et al., 2020).

We tested the hypotheses that (1) the composition of the fungal wood microbiome differs between asymptomatic and symptomatic vines with GTD-associated fungi being the key drivers of this differentiation, (2) the composition of the bacterial community differs between asymptomatic and symptomatic vines and specific bacterial taxa would be potential interactors with GTD-associated fungi and (3) vine genotype, biogeography and vine GTD condition interactively determine the composition of the vine wood microbiome and the fungal wood pathobiome. These hypotheses were evaluated in asymptomatic and GTD-symptomatic vines from three Greek cultivars, each cultivated in geographically distinct viticultural zones, using amplicon sequencing of the 16S rRNA gene for bacteria and the ITS2 region for fungi.

Multivariate analysis and machine learning approaches identified members of the wood fungal microbiome that could act as GTD predictors, and network analysis revealed microbial interactions relevant for the appearance of the disease.

2.2 Materials and Methods

2.2.1. Collection of samples

Wood samples were collected during July–August 2019 from vineyards located in three important and geographically distinct viticultural zones in north-west (Amyntaio), central-south (Nemea, Peloponnese) and southern Greece (Crete) (Supplementary Fig. S1). These regions are mainly cultivated with the Greek cultivars Xinomavro, Agiorgitiko and Vidiano, respectively. The three viticultural zones are characterized by different climatic conditions: (1) The Amyntaio region is humid (mean annual precipitation 611 mm) and cool (mean annual temperature 12.2 °C, max 18.4 °C and min 6.7 °C) (2) The Nemea region is equally humid (mean annual precipitation 611.3 mm) but warmer than Amyntaio (mean annual temperature 15.8 °C, max 22.5 °C and min 8.7 °C) (3) The Heraklion region in Crete is the driest (mean annual precipitation 464 mm) and warmest (mean annual temperature 17.8 °C, max 21.9 °C and min 14.0 °C) of the viticultural zones studied. In each zone, three vineyards, with the age of grapevines varying from 10 to 15 years, were selected for collecting samples. Details on the management practices, age, pruning system and rootstocks used in the nine vineyards of the current study are given in Table 1. In each

vineyard, three vines without any visible GTD symptom on leaves and wood, and three vines exhibiting typical foliar GTD symptoms (including leaf discoloration, tiger-stripe chlorosis or necrosis and plant wilting) in the last two years were selected and nominated as asymptomatic and symptomatic vines, respectively. The only exception was recorded for the cultivar Agiorgitiko where for technical reasons asymptomatic plants were sampled only from one of the three vineyards. Each one of the vines selected as symptomatic or asymptomatic were destructively sampled. Specifically, wood samples from the cordons and the trunk of the selected vines were removed using a sterile saw and transferred in the laboratory. Then, cordon and trunk samples were cut longitudinally to identify the extent of necrosis in these wood tissues. Wood samples from asymptomatic plants showing extensive necrotic areas were excluded from further sampling. Overall, samples from symptomatic plants were characterized by extensive necrotic areas, whereas samples from asymptomatic plants were largely free of necrotic areas. Based on this visible inspection, wood chips from necrotic tissues of symptomatic vines and wood chips from non-necrotic tissues of asymptomatic vines were collected using a sterile drill and stored at − 20 °C until further use. White rots were occasionally found adjoining necrotic areas and they were sampled along with necrotic tissues but they were not considered separately. A detailed list of the samples used in the study is given in Supplementary Table S1.

Table 1. Management practices, pruning practices, age and rootstocks used in the studied vineyards.

Vineyard	Cultivar	Management	Training/Pruning	Age (years)	Rootstocs
Amyntaio 1	Xinomavro	Conventional[a]	Double cordon trained, spur pruned	10	1103P
Amyntaio 2	Xinomavro	Conventional	Double cordon trained, spur pruned	10	1103P
Amyntaio 3	Xinomavro	Conventional	Double cordon trained, spur pruned	12	SO_4
Nemea 1	Agiorgitiko	Conventional	Double cordon trained, spur pruned	15	110 Richter
Nemea 2	Agiorgitiko	Conventional	Double cordon trained, spur pruned	15	110 Richter
Nemea 3	Agiorgitiko	Conventional	Double cordon trained, spur pruned	12	110 Richter
Heraklelon 1	Vidiano	Conventional	Double cordon trained, spur pruned	10	110 Richter
Heraklelon 2	Vidiano	Conventional	Double cordon trained, spur pruned	15	110 Richter
Heraklelon 3	Vidiano	Conventional	Double cordon trained, spur pruned	13	110 Richter

[a] Conventional type of management involves application of fungicides and insecticides with the active ingredients used varying largely between viticultural zones and even vineyards

2.2.2. DNA extraction

DNA from the wood samples was extracted using the Cetyltetramethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980) with slight modifications. Briefly, 250 mg of the collected wood tissue were homogenized in a chilled pestle and mortar using liquid nitrogen. 1200 µl (600 µl in two doses) of 2X CTAB extraction

buffer (100mMTris- HCl, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 0,5% v/v β-mecraproethanol) were added and homogenized. The samples were incubated at 65 °C for 30–60 min with occasional vortexing. Samples were centrifuged at 10,000 rpm for 10 min and the supernatant (~ 400 μl) was transferred to new tubes. 600 μl of chloroform:isoamyl alcohol (24:1) were added and mixed by inverting the tube several times. Samples were centrifuged at 13,000 rpm for 15 min. The aqueous phase was transferred into a new tube and 600 μl of chloroform were added. The tubes were centrifuged at 13,000 rpm for 10 min. Then, 1 volume of chilled isopropanol was added, followed by quick and gentle inversion and incubated at − 20 °C for 1 h. The DNA pellet was precipitated at 13,000 rpm for 20 min, washed twice with 70% ethanol and precipitated at 13,000 rpm for 3 min. The DNA pellet was then suspended in 50 μl of pre-heated TE-buffer. DNA integrity was initially assessed via agarose gel (0.8%) electrophoresis and DNA concentrations were determined using the Quant-iT kit with a Qubit Fluorometric device (Invitrogen, USA).

2.2.3. Amplicon sequencing analysis

The bacterial and fungal wood microbiome of vines was determined via amplicon sequencing of the V4 region of the 16S rRNA gene and the ITS2 region respectively, using a HiSeq, Illumina platform in Rapid Mode 2 × 250 paired-end in Admera Health company (New Jersey, USA). Amplification, facilitating sample-wise multiplexing during sequencing, was based on our in-house protocol described in detail in Vasileiadis et al. (2015). Briefly, extracted DNA was diluted to a final concentration of 1 ng μl−1 for fungi and 0.2 ng μl−1 for bacteria. We followed a two-step amplification process, which allowed indexing of our samples. In the first step, the bacterial 16S rRNA gene was amplified with the updated version of the primers 515f-806r (Parada et al., 2016) following the protocol of the earth microbiome project (Caporaso et al., 2018). Regarding fungi, a first amplification step of the ITS2 region was performed with primers ITS7F-ITS4R (White et al., 1990; Ihrmark et al., 2012). The first amplification step included 28 cycles using the primers mentioned above, followed by a second PCR of 7 cycles using the same primers with sample-specific 5’ overhangs. All PCR reactions were conducted with the use of Q5® High- Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA). Primers, PCR conditions and the reagents used are given in Supplementary Tables S2 and S3 respectively. Amplicon libraries were cleaned up with the use of NucleoMag® NGS Clean-up and

Size Select Kit (Macherey- Nagel, Duren, Germany) according to manufacturer instructions and they were sent for sequencing. Amplification of non-template controls gave no amplicons and their sequencing provided particularly low number of reads, hence they were not considered in the analysis.

2.2.4. Bioinformatic analysis

Sequence demultiplexing to their samples of origin was performed with flexbar v3.0.3 (Dodt et al., 2012) not allowing any barcode mismatches. Sequences were then quality-trimmed/ filtered and checked for chimerical sequences with the dada2 v1.14.1 (Callahan et al., 2016) package of the R software v.4.0.2 (R Core Team, 2020). Amplicon sequence variants (ASVs) were assembled and taxonomically assigned using the Silva v.138 (Yilmaz et al., 2014) and UNITE ITS v.8.2 (04.02.2020) (Abarenkov et al., 2020) reference databases for bacteria and fungi, respectively. ASVs not classified at Kingdom and Phylum level were excluded from downstream analysis. Whereas we included in the analysis ASVs with 80% or higher taxonomy annotation bootstrap confidence in the domain/kingdom taxa as previously suggested (Kozich et al., 2013). ASV classifications are provided at the lowest taxonomical rank passing this threshold.

2.2.5. Statistical analysis

The ASV matrices of bacteria and fungi were used to assess the effects of vine GTD condition and the lumped factor biogeography/cultivar on the β-diversity of the wood microbiome. Non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) were used to assess β-diversity differences for bacterial and fungal communities between symptomatic and asymptomatic vines either collectively for all cultivars/viticultural zones or separately for each viticultural zone/cultivar. NMDS was performed with dissimilarity matrices using the Bray–Curtis algorithm, while the association of microbiome composition with the covariates of interest and the determination of the % of variance explained by those factors were accessed with PERMANOVA (Lozupone et al., 2007) using 999 permutations as implemented in the Vegan v2.5–7 (Oksanen et al., 2019) package of the R software. Differential abundance (DA) tests, using the Kruskal–Wallis and the Wilcoxon rank-sum post-hoc tests, were employed to identify bacterial and fungal taxa that were significantly associated with symptomatic and asymptomatic vines. P-

value adjustment was conducted with false discovery rate (FDR) algorithm with implemented adjusted P value cut-off values of 0.05. Heatmaps were produced to depict ASVs whose relative abundance was positively correlated with symptomatic or asymptomatic vines according to the DA tests.

The GTD-associated fungal genera and the fungal and bacterial genera that participate in 10% of the samples with at least 1% relative abundance were used in network analysis aiming to identify positive or negative co-occurrence networks. Spearman correlation tests were performed among microbial genera and the data obtained were used as adjacency matrix for the weighted network analysis performed with the igraph v1.2.6 (Csardi et al., 2006) package of the R software. The minimum spanning tree algorithm was used for filtering the adjacency matrix choosing the shortest possible combination of connections among tree-nodes on the generated undirected networks (Drago et al., 2017). Afterwards, the sub-community clusters were identified according to local densities. The force-directed Fruchterman-Reingold layout (Fruchterman and Reingold, 1991) was used for plotting the final network.

We further selected fungal ASVs to define potential predictors of the GTD status of grapevines via Random Forest analysis. The data matrix was initially subsampled with replacement to equal read numbers (rarefied) as suggested by Weiss et al. (2017). Then, a series of Random Forest supervised classification models were performed by the pime v0.1.0 R-software package (Roesch et al., 2020), which relies on the RandomForest v4.6.14 R-software package (Liaw et al., 2002). Briefly, low prevalence taxa were filtered out for a range of minimum prevalence values (from 5 to 65% at 5% increments) for each sample. Each Random Forest model was run with 1000 trees, using 66% of the samples for building the model and 33% of the samples as out of bag (OOB) samples for the model error assessment. The OBB rate for each prevalence level was calculated for the dataset and the best compromise between model error rate and the remaining dataset reads was selected. The Random Forest analysis was implemented on a dataset of 63 samples (18 from asymptomatic and 45 from symptomatic vines) and 1182 taxa. Overall, the 40% of minimum prevalence cutoff was selected as the best compromise between model error and ASV/sequence numbers for the model, corresponding to 118,161 sequences and 22 ASVs, with an internal OOB error rate 6.25%. Following the Random Forest-based pime approach, the Kruskal–Wallis non-parametric ANOVA equivalent test coupled with a post hoc Wilcoxon rank sum analysis was performed for further filtering out ASVs showing no

significant differences between GTD sample groups. This reduced the dataset size to 10 ASVs and 50,649 sequences, which was re-tested for its sample classification accuracy with Random Forests according to the aforementioned parameters.

All data figures for the analysis were produced with the R package ggplot2 v3.3.3 (Wickham et al., 2009). The sequencing data were submitted to Sequence Read Archive of NCBI with bioproject accession number PRJNA738455. The full analysis code can be found in the Github repository in the link: https:// github. com/ Fotis bs/ Grape vine_ wood_ micro biome-. git

2.3 Results

2.3.1. Fungal microbiome

2.3.1.1. Composition of the wood fungal microbiome

In total 1,043,027 non chimeric quality fungal sequences (sequence numbers in the analyzed samples ranged from 1413 to 33,223) were obtained and they were assigned to 1378 ASVs. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the diversity of endophytic fungi in vines (Supplementary Fig. S2a). Overall, the fungal wood microbiome was composed of *Ascomycota* (75.2–97.6%, mean 88.7%) and *Basidiomycota* (2.4–23.3%, mean 10.8%). In total 31 fungal Classes were identified with the most abundant being *Dothideomycetes* (33.9–77.2%, mean 46.2%), *Sordariomycetes* (15.9% – 32.8%, mean 21.8%), *Eurotiomycetes* (1.3–21.2%, mean 13%), *Agaricomycetes* (0.2–20.9%, mean 8.1%), *Leotiomycetes* (0.1–18%, mean 4.6%) and *Lecanoromycetes* (0.3–4.1%, mean 2%) summing to a total relative abundance of 93.5–97.6% (Supplementary Fig. S3).

2.3.1.2. Factors structuring the wood fungal microbiome of vines

Preliminary analysis of the amplicon sequencing data suggested a non-significant effect of the type of plant tissue studied (cordon or trunk) on the fungal community and hence, it was not considered as an explanatory variable in our analysis. When samples from all viticultural zones/cultivars were considered, NMDS analysis showed a clear grouping ($p < 0.01$) of the samples according to viticultural zone/cultivar (Fig. 1a), but no clustering according to their GTD condition (symptomatic vs asymptomatic plants) (Fig. 1b). Still, PERMANOVA showed that GTD condition and viticultural zone/cultivar had a statistically significant effect and explained 3.5% ($p <$

0.001) and 22.7% ($p < 0.001$) of the dataset variance, respectively (Supplementary Table S4).

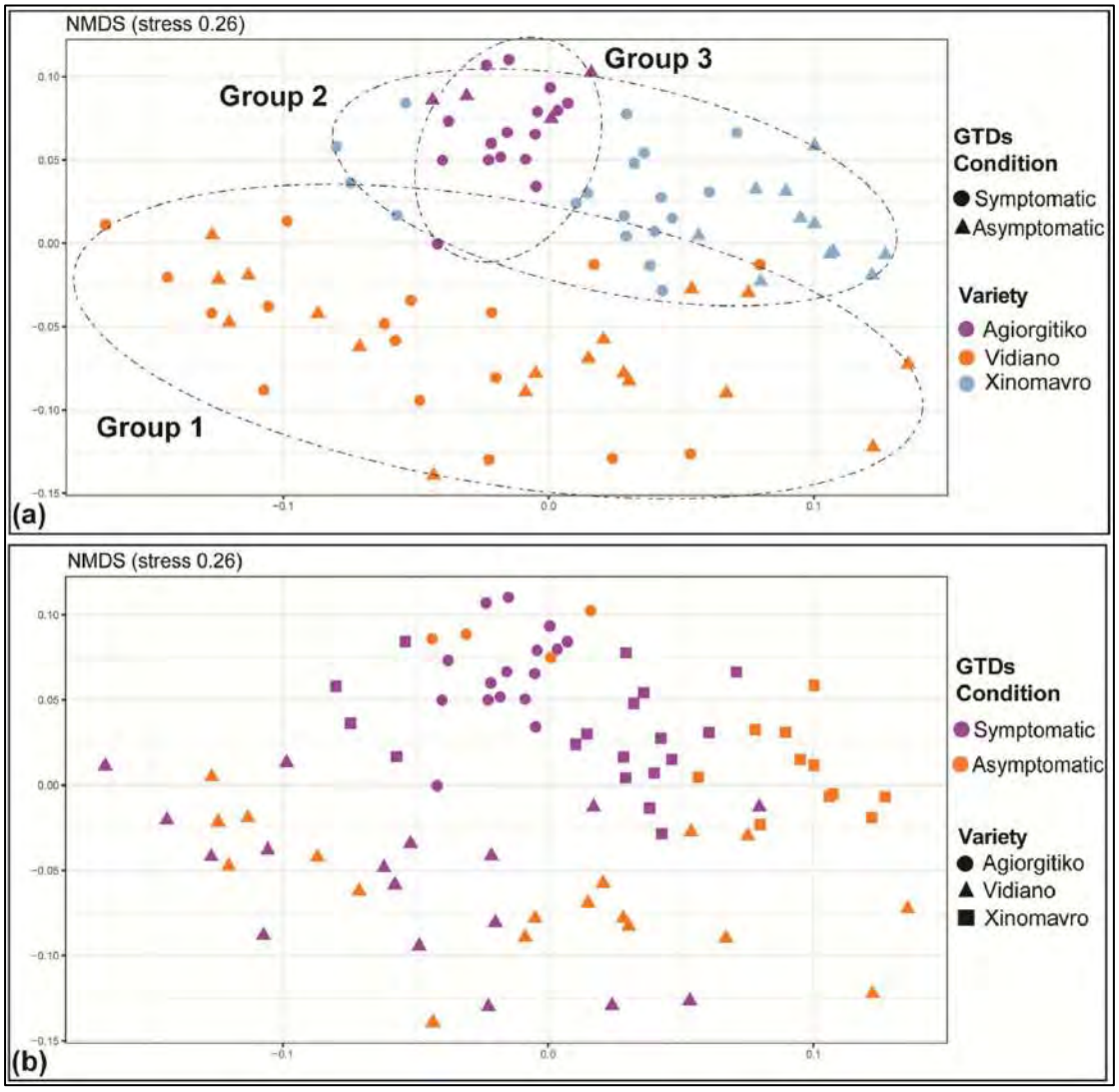

Fig. 1 NMDS ordination of wood samples, analyzed for their fungal microbiome, according to their location/cultivar (Agiorgitiko, Xinomavro, Vidiano) (a) and their GTD status (asymptomatic vs symptomatic) (b)

We further analyzed the differences in the β-diversity of the fungal microbiome imposed by GTDs separately for each genotype/viticultural zone. Regarding Xinomavro, NMDS analysis showed a clear separation of samples collected from GTD asymptomatic and -symptomatic plants (Fig. 2b). Further PERMANOVA showed that GTD condition was the most significant factor ($p < 0.001$) explaining 13.9% of the variance, while vineyard topography (in the same viticultural zone) contributed 8.6% ($p < 0.05$) (Supplementary Table S4). In contrast, NMDS analysis for the cultivars Agiorgitiko and Vidiano showed a marginal ($p < 0.05$) or not clear

separation ($p > 0.05$) respectively, of the symptomatic and asymptomatic samples (Fig. 2a, c).

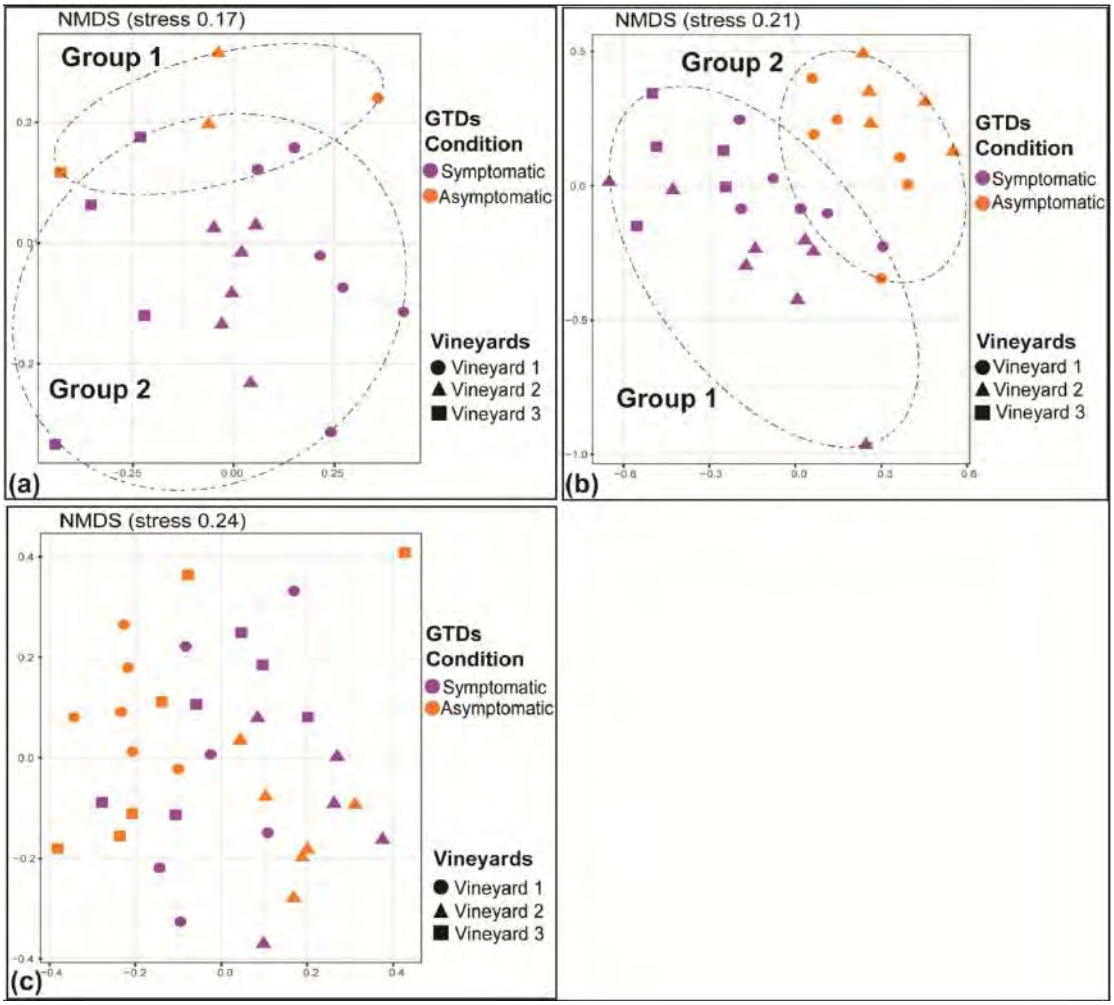

Fig.2. NMDS ordination of the fungal microbiome in wood samples collected from different locations/cultivars like: a Agiorgitiko b Xinomavro c Vidiano and analyzed according to their GTD status (asymptomatic vs symptomatic)

PERMANOVA further indicated that the GTD condition of plants explained 8.4% and 4.2% of the variance for Agiorgitiko and Xinomavro respectively and had a marginally significant ($p < 0.05$) or non-significant ($p > 0.05$) effect on the fungal β-diversity. Still for these two cultivars, the vineyard location (within the same viticultural zone) was the most significant factor ($p < 0.001$) explaining 20.5% and 15% of the variance in each case (Supplementary Table S4).

2.3.1.3. Fungi of the wood microbiome associated with symptomatic and asymptomatic vines

We further attempted to define ASVs whose relative abundance significantly differs between asymptomatic and symptomatic plants. DA heatmaps were constructed collectively for all cultivars/viticultural zones and separately for each cultivar/viticultural zone. When all cultivars were considered together only three ASVs showed significant association with either asymptomatic or symptomatic vines (Fig. 3a). ASV102 assigned to the genus *Alternaria*, showed a consistently higher relative abundance in asymptomatic compared to symptomatic plants. Conversely, ASV027 and ASV068, assigned to *Acremonium alternatum* and *Kalmusia variispora* respectively, showed significantly higher relative abundance in the symptomatic vines. We expanded our analysis and looked separately in each cultivar/viticultural zone for fungal ASVs with differential abundance patterns in symptomatic vs asymptomatic plants. Regarding the cultivar Agiorgitiko, we identified 11 ASV ($p < 0.05$) that showed differential abundance between asymptomatic and symptomatic vines. Amongst them, ASVs belonging to *Eutypa spp*., *Lophiostoma cynaroidis*, *Sporidiobolaceae*, *Malassezia restricta*, *Didymellaceae*, *Amphisphaeriaceae*, *Teloschistaceae*, *Seimatosporium vitis* and *Muriphaeosphaeria viburni* were associated with asymptomatic plants, unlike AS0V52, assigned to the genus *Fomitiporia*, which was amongst the most abundant ASVs of the wood microbiome, and it was systematically associated with symptomatic plants (Fig. 3b). Regarding the cultivar Xinomavro, we identified a single ASV, assigned to genus *Neosetophoma*, that was associated with symptomatic vines (Fig. 3c). In contrast, ASVs belonging to the genera *Alternaria*, *Curvularia* and the family *Nectriaceae* were associated with asymptomatic vines. In the cultivar Vidiano, we identified ASVs belonging to *P. chlamydospora*, one of the most dominant ASVs in our dataset (ASV014), *Acremonium sordidulum*, *Diaporthe spp*., *K. variispora* and *A. alternatum*, all associated ($p < 0.05$) with symptomatic vines (Fig. 3d), compared to ASVs belonging to *M. restricta* and *Catenulostroma spp.*, which showed a consistent association with asymptomatic vines.

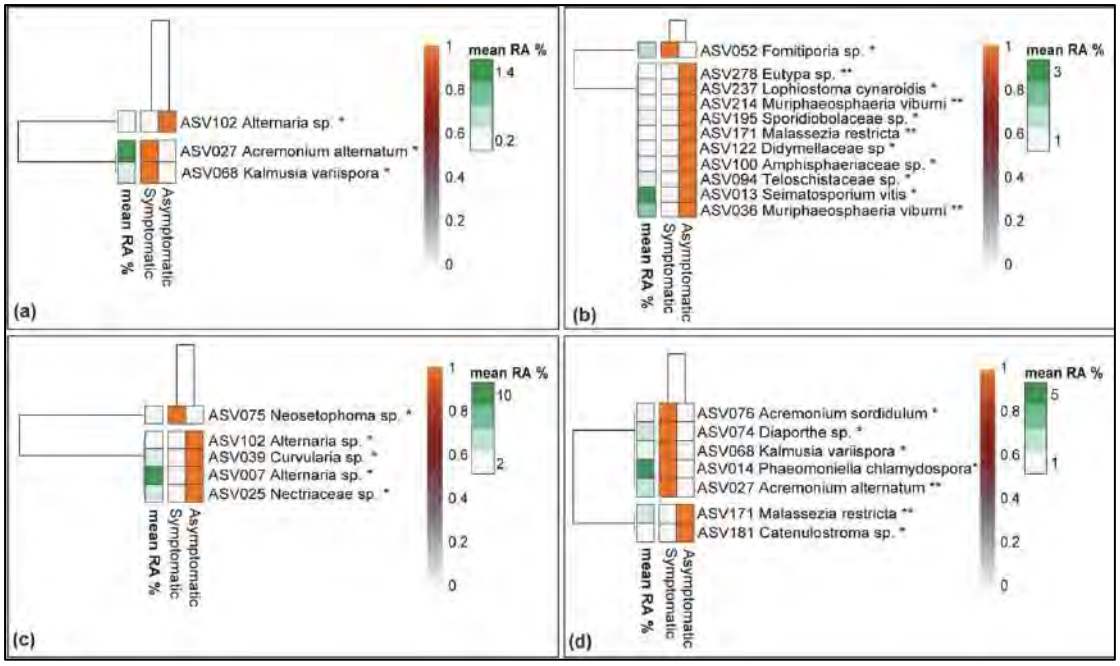

Fig.3. Differential abundance (DA) heatmaps presenting fungal ASVs whose relative abundance showed a significant positive correlation ($p < 0.05$*, $p < 0.01$**) with the wood samples from vines with (symptomatic) or without (asymptomatic) GTD symptoms. Results are presented collectively for all cultivars/locations (a) and separately for each variety/location (b) Agiorgitiko (c) Xinomavro and (d) Vidiano.

2.3.1.4. Differential abundance of fungal ASVs belonging to genera associated with GTDs

We then focused our analysis on fungal ASVs with relative abundance > 0.01% that are taxonomically assigned to genera/species previously identified as causal agents of GTDs like *P. chlamydospora*, *Phaeoacremonium spp*., *Fomitiporia spp*., *Diplodia spp*., *Phellinus rhamni*, *Seimatosporium vitis*, *K. variispora*, *Dothiorella rosulata*, *Neofusicoccum spp*., *Eutypa spp*., *Botryosphaeria spp*., *Lasiodiplodia spp*., *Neofabrea spp*., *Didymosphaeria spp*., *Diaporthe spp*. In total 61 ASVs were identified and used in the analysis with their relative abundance ranging from 0.03 to 12.9% (Supplementary Table S5). The differential abundance of these fungal ASVs in symptomatic and asymptomatic vines was statistically compared collectively for all geographical locations/cultivars together and separately (Supplementary Fig. S4). When data for all cultivars/viticultural zones were considered together, *P. chlamydospora*, (ASV014, $p < 0.01$) and *K. variispora* (ASV068, $p < 0.001$) showed significantly higher relative abundance in symptomatic vs asymptomatic vines (Supplementary Fig. S4a). When data for each cultivar/ viticultural zone were

analyzed separately, in the cultivar Agiorgitiko we identified two ASVs belonging to *S. vitis* (ASV013 and ASV338, $p < 0.01$) that showed significantly higher relative abundance in asymptomatic vines, in contrast to a *Fomitiporia* ASV ($p < 0.01$), which prevailed in the symptomatic vines (Supplementary Fig. S4b). For the cultivar Xinomavro (Supplementary Fig. S4c), we identified six ASVs all showing increased relative abundance in symptomatic vines including *P. chlamydospore* ($p < 0.001$), *Phaeoacremonium iranianum* ($p < 0.001$), *K. variispora* ($p < 0.01$), *Neosetophoma spp*., (ASV075 and ASV159, $p < 0.01$). Finally, for Vidiano, five ASVs showed significantly higher relative abundance in symptomatic vs asymptomatic vines. They were assigned to *P. chlamydospore* ($p < 0.01$), *K. variispora* ($p < 0.01$) and *Diaporthe spp*., ($p < 0.01$) (Supplementary Fig. S4d).

2.3.2. Wood bacterial microbiome

2.3.2.1. Composition of the wood bacterial microbiome of vines

In total 1,632,972 quality sequences for bacteria (sequence numbers in the analyzed samples ranged from 3318 to 51,768) were obtained and they were assigned to 7908 ASVs. Rarefaction curves reached a plateau in al samples suggesting that our sequencing effort adequately covered the diversity of endophytic bacteria in vines (Supplementary Fig. S2b). Overall, the wood bacterial microbiome was composed of 30 phyla. *Proteobacteria* (24.8–77.4%, mean relative abundance 49.5%), *Actinobacteriota* (11.4–22.8%, mean 17.6%), *Bacteroidota* (5.5– 20.8%, mean 13.4%), *Firmicutes* (0.4–49.4%, mean 9.3%), *Planctomycetota* (0.1–5.4%, mean 1.9%), *Abditibacteriota* (0.7–2.4%, mean 1.4%), *Verrucomicrobiota* (0.2–3.2%, mean 1.4%), *Acidobacteriota* (0.2–2.8%, mean 1.1%) and *Chloroflexi* (0.02–1.84%, mean 0.8%) were the most dominant phyla, all summing to a relative abundance of 94.4–99.3% (Supplementary Fig. S5a). Regarding *Proteobacteria*, α- (14.4–69.8%, mean relative abundance 39.9%) and *γ-Proteobacteria* (5.3–15%, mean 9.5%) were the most dominant classes. An interesting observation was the dominance of *Firmicutes* in the asymptomatic vines of the cultivar Xinomavro.

2.3.2.2. Factors structuring the wood bacterial microbiome

.In line with the fungal wood microbiome, we observed that the two plant parts sampled, cordons and trunk, supported similar bacterial communities and hence this factor was not considered further in our analysis. When samples from all viticultural

zones/cultivars were considered, NMDS analysis showed a clear grouping ($p < 0.01$) of the samples according to viticultural zone/cultivar (Fig. 4a), whereas no clustering according to the GTD conditions of samples (symptomatic vs asymptomatic) was observed (Fig. 4b). Still PERMANOVA showed that GTD condition and cultivar/viticultural zone exerted a statistically significant effect ($p < 0.001$) on bacterial community structure explaining 5.2 and 25.5% of the dataset variance respectively (Supplementary Table S4).

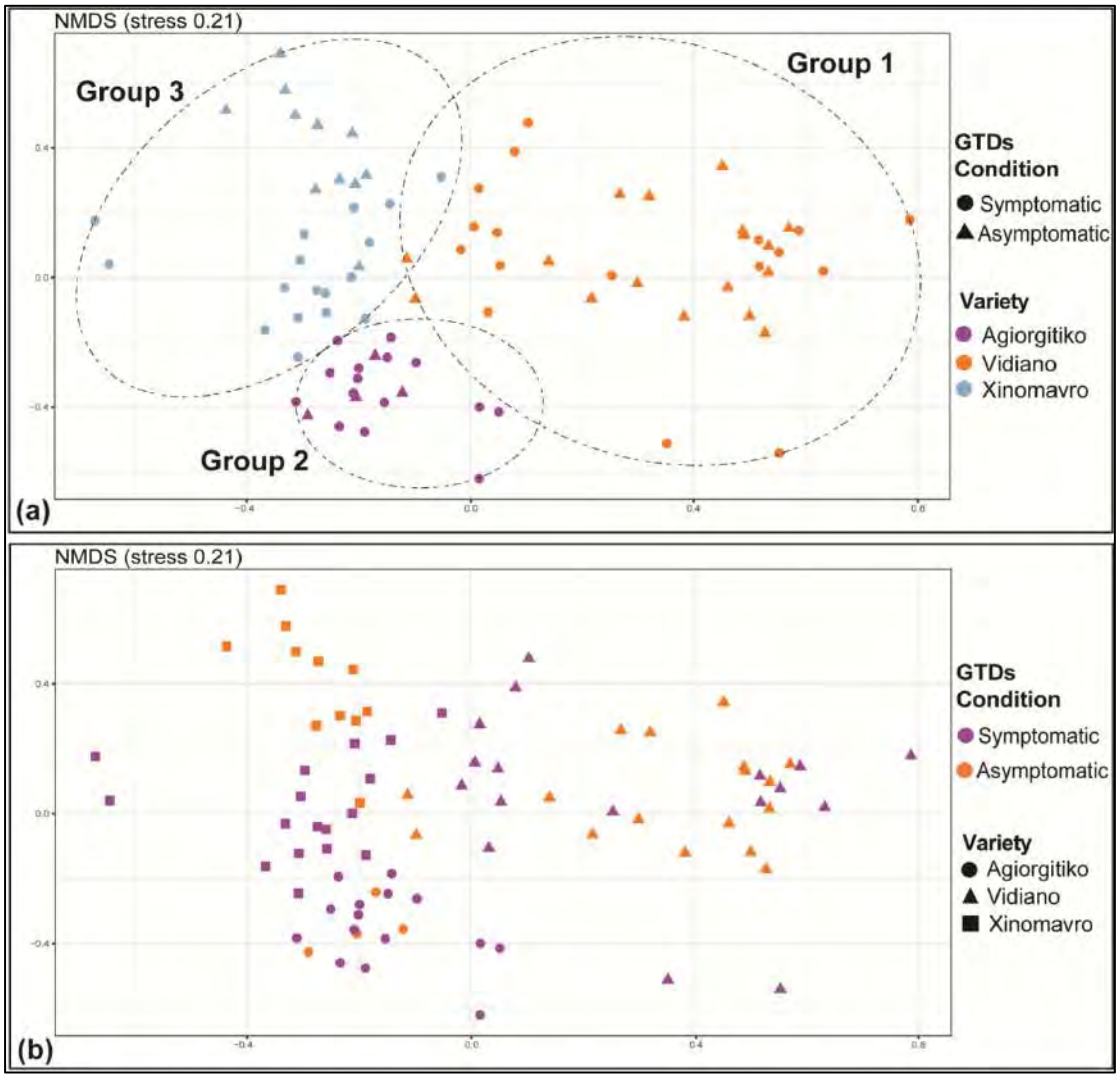

Fig.4. NMDS ordination of wood samples, analyzed for their bacterial microbiome, according to their location/cultivar (Agiorgitiko, Xinomavro, Vidiano) (a) and their GTD status (asymptomatic vs symptomatic) (b)

We further analyzed our data separately for each viticultural zone/cultivar aiming to explore further the effect of GTD occurrence on the composition of the bacterial microbiome. Regarding the cultivar Xinomavro, NMDS analysis showed a clear

separation of the samples from symptomatic and asymptomatic vines ($p < 0.01$) (Fig. 5b). PERMANOVA verified the significant effect of the GTD plant condition on the composition of the wood bacterial community ($p < 0.001$), which explained 19.7% of the variance, whereas vineyard topography (within the viticultural zone of Amyntaio) did not have a significant effect ($p > 0.05$) (Supplementary Table S4). Regarding the other two cultivars, we noted a weak clustering of the samples according to their GTD status only in the case of Vidiano ($p < 0.05$) (Fig. 5c). PERMANOVA analysis revealed that GTD condition and vineyard topography (at the viticulture zone scale) explained 5.3% ($p > 0.05$) and 13.6% ($p < 0.05$) of the variance respectively for Agiorgitiko, and 6% ($p < 0.01$) and 12.1% ($p < 0.01$) for Vidiano (Supplementary Table S4).

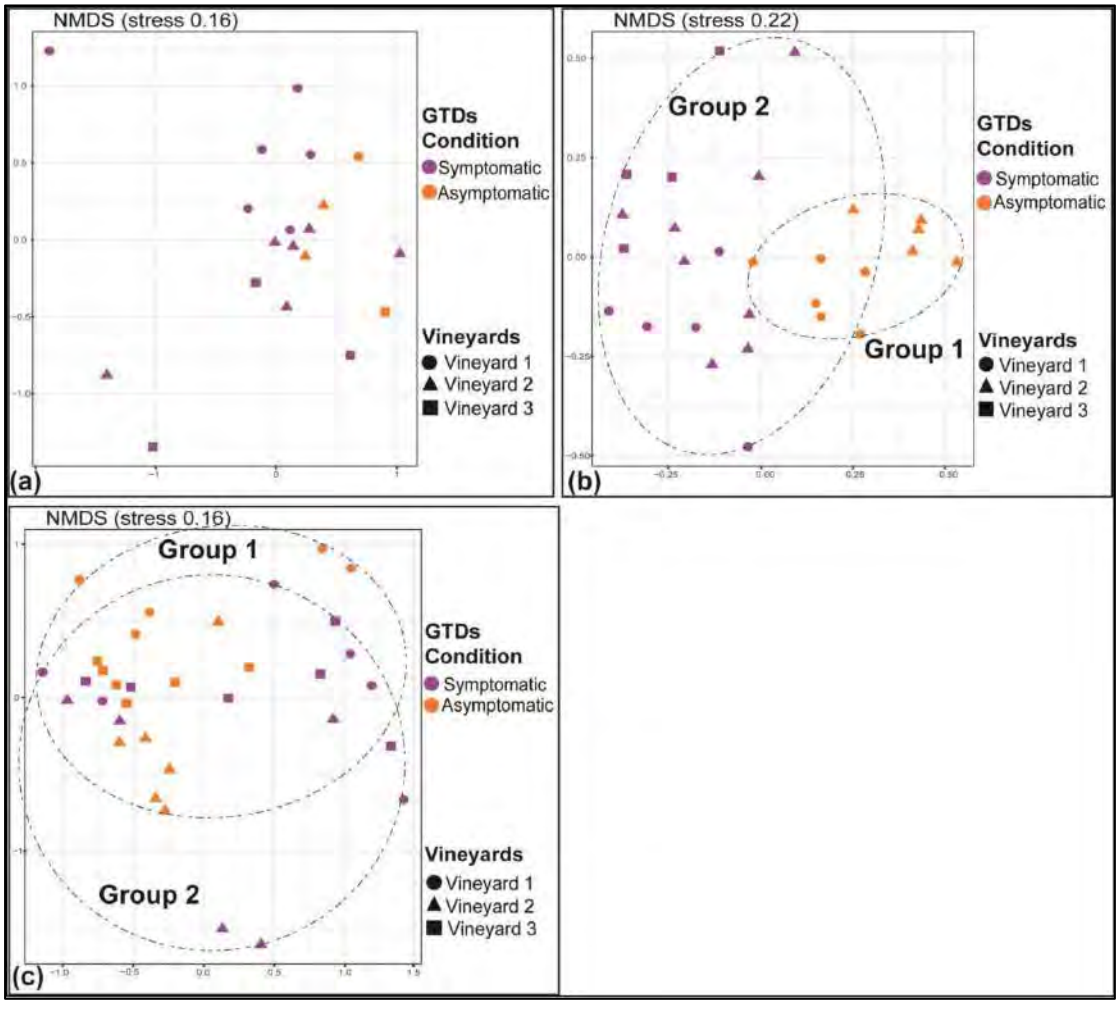

Fig.5. NMDS ordination of the bacterial microbiome in wood samples collected from different locations/cultivars like: (a) Agiorgitiko (b) Xinomavro (c) Vidiano and analyzed according to their GTD status (asymptomatic vs symptomatic)

2.3.2.3. Wood microbiome bacteria associated with symptomatic and asymptomatic vines

DA heatmaps pointed to ASVs that were consistently associated with symptomatic or asymptomatic vines. When data from all viticultural zone/cultivars were considered, we identified several ASVs, mostly belonging to *Rhizobiaceae*, that showed consistently higher relative abundance in symptomatic vines (Fig. 6a). On the other hand, five *Streptomyces*, ten *Bacillus*, five *Bacillaceae*, and four *Corynebacterium* ASVs were consistently present in the asymptomatic vines. When the same analysis was conducted separately for each viticultural zone/cultivar, we identified four ASVs, all belonging to *Bacillaceae*, that were associated ($p < 0.05$) with asymptomatic vines of the cultivar Agiorgitiko (Fig. 6b). Similarly, we noted five *Bacillaceae*, eleven *Bacillus* and six *Streptomyces* ASVs that were systematically associated ($p < 0.05$) with asymptomatic vines of the cultivar Xinomavro (Fig. 6c). Regarding, Vidiano six ASVs belonging to *Rhizobiaceae* were systematically associated with symptomatic vines (Fig. 6d), whereas ASVs belonging to *Sphingomonadaceae* (2), *Corynebacterium* (4), *Bacillaceae* (2) and Acinetobacter (2) were associated with asymptomatic vines.

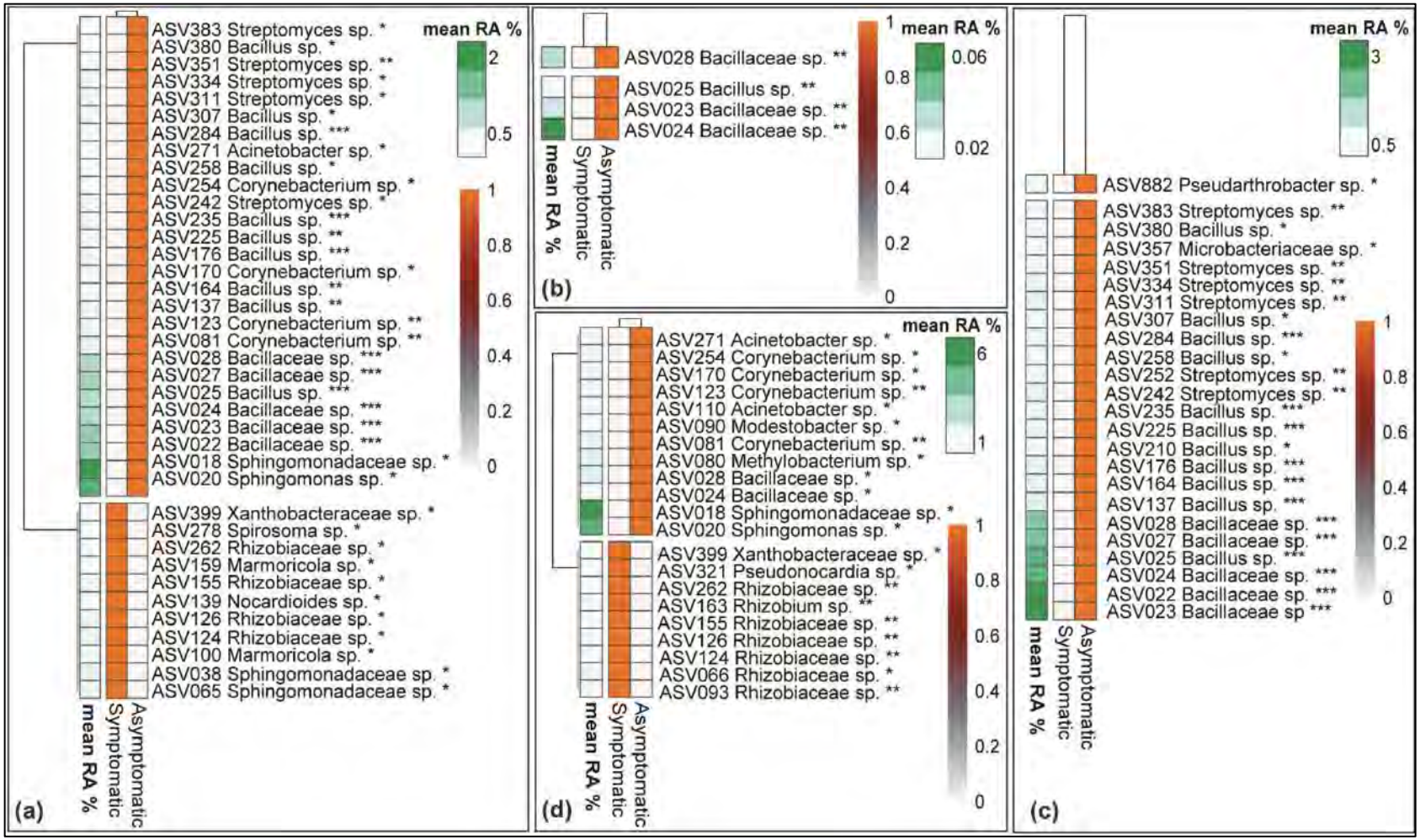

Fig. 6 Differential abundance (DA) heatmaps showing bacterial ASVs whose relative abundance showed a significant positive correlation ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***) with the wood samples from vines with (symptomatic) or without (asymptomatic) GTD symptoms. Results are presented collectively for all cultivar/locations (a) and separately for each cultivar/location (b) Agiorgitiko (c) Xinomavro and (d) Vidiano

2.3.3. Network analysis of the wood microbiome

Network analysis within the fungal microbiome across all cultivars revealed a significant negative co-occurrence pattern between *Alternaria* and the two GTD-linked fungal genera *Phaeomoniella* and *Phaeoacremonium* in the asymptomatic but not in the symptomatic vines (Supplementary Fig. S6). Network analysis between the GTD-associated fungi and the bacterial microbiome in all cultivars revealed a significant negative co-occurrence pattern between the GTD-relevant fungal genera *Phaeomoniella* and *Seimatosporium* with *Bacillus*, and of *Phaeoacremonium* with *Streptomyces*, only in the asymptomatic grapevines (Supplementary Fig. S6).

2.3.4. Random forest analysis of the fungal wood microbiome

To further explore our fungal dataset for possible marker ASVs for asymptomatic and symptomatic samples, a Random Forest supervised learning approach was used. This analysis showed that the fungal microbiome had strong discriminative power and pointed to 10 ASVs that could be considered as predictors of the GTD status of the vines. Hence ASVs belonging to *Cladosporium spp*., (ASV004, $p < 0.05$, most dominant), *P. chlamydospore* (ASV014, $p < 0.05$, dominant), *K. variispora* (ASV068, $p < 0.001$), *A. alternatum* (ASV027, $p < 0.01$) and *Lentitheciaceae* (ASV154, $p < 0.01$) were all predictors of symptomatic plants (Fig. 7). Whereas ASVs belonging to *Fusarium solani* (ASV132, $p < 0.038$), *Alternaria spp*., (ASV102 $p < 0.0$ and ASV007 $p < 0.001$), *Curvularia spp.,* (ASV039, $p < 0.02$) and *Nectriaceae* (ASV025, $p < 0.001$) were highlighted from the model as identifiers of asymptomatic vines. These ASVs had an overall (for both symptomatic and asymptomatic samples) OOB error rate of 7.94% (Additional file 1: Fig. S7). When using the five ASV identifiers for symptomatic samples we noted a high classification accuracy with OOB error rates of 0% suggesting that all 45 symptomatic samples were classified correctly. In contrast, we observed a low classification accuracy when using the ASV indicators of asymptomatic plants with an OOB error rate of 27%.

Fig. 7 Random Forest heatmap showing rarefied ASVs whose max relative abundance showed a significant positive correlation ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***) with the wood samples from vines with (symptomatic) and without (asymptomatic) GTD symptoms

2.4. Discussion

We determined the composition of the wood fungal microbiome in symptomatic and asymptomatic vines from three different Greek cultivars, each located in a geographically distinct viticultural zone, aiming to identify consistent drivers of the disease. In this quest we used amplicon sequencing approaches and relevant statistical and machine learning tools to identify potential biotic drivers of GTDs. We further expanded our study to the bacterial wood microbiome expecting to identify interactors with the fungal microbiome.

2.4.1. Fungal microbiome

The wood fungal microbiome of all studied cultivars was dominated by *Ascomycota*, while *Basidiomycota* constituted a substantially smaller fraction with *Agaricomycetes* being the most prevalent amongst them. Similar compositional patterns, denoting a clear dominance of *Ascomycota* in the wood microbiome of vines, have been reported in most previous studies (Bruez et al., 2020; Del Frari et al., 2019; Deyett and Rolshausen, 2020). An interesting pattern was the consistent and significant ($p < 0.05$) increase in the contribution of *Agaricomycetes* in symptomatic vines in all studied cultivars. This observation might be associated with the capacity of members of this fungal taxon to induce white rots in wood tissues, including *Fomitiporia spp*., a key colonizer of white rot tissues in vine exhibiting GTD symptoms (Mungai et al., 1999). Recently, Paolinelli et al. (2020) suggested that the increase in the

Basidiomycota/Ascomycota ratio observed in symptomatic over asymptomatic vines could be used as an early warning for the establishment of GTDs in vines.

We first explored the factors shaping the wood fungal microbiome and identified viticultural zone and/or cultivar, considered as a lump factor based on our experimental setup, as the main determinant of the fungal community. In contrast, we noted a weak differentiation of the wood fungal microbiome between symptomatic and asymptomatic samples. To date several studies have explored the effect of biogeography, cultivar and vintage on the grapevine microbiome focusing on above-ground (berries, leaves) (Bokulich et al., 2014; Mezzasalma et al., 2018; Morisson-Whittle and Goddard, 2018) and/or below-ground plant associated compartments (soil, rhizosphere, roots) (Zarraonaindia et al., 2015; Martinez-Diz et al., 2019), while the trunk microbiome has attracted little attention. These studies have identified biogeography as a main determinant of the non-random assemblage of the fungal vine microbiome, especially when microbiomes are looked at the macro-geographical scale, whereas at regional scale (micro-geographical scale) other confounding factors like cultivar and vintage become important (Bokulich et al., 2014). The wood fungal microbiome, as determined in our study, seems to share assemblage mechanisms with the other grapevine compartments. Although we could not distinguish between cultivar and biogeography, we speculate that the latter has possibly a stronger effect in shaping the fungal wood microbiome considering the wide geographical scale of our study. The limited dissimilarity of the fungal microbiome between asymptomatic and symptomatic vines, when the viticultural zone/cultivar factor was not considered, is in line with the previous studies, which, following similar sampling strategies, did not identify significant differences between symptomatic and asymptomatic vines (Elena et al., 2018; Briez et al., 2014; Del Frari et al., 2019; Niem et al., 2020).

We further determined the differences in the fungal wood microbiome separately for each cultivar/viticultural one, taking under consideration the variable climatic conditions that prevail in the studied viticultural zones and the different sensitivity of the studied cultivars to GTDs. Indeed, Agiorgitiko and Vidiano are more susceptible to GTDs compared to the more resistant Xinomavro (Markakis et al., 2017). We noted that the three cultivars exhibited different responses in their assembly of the fungal microbiome in symptomatic and asymptomatic vines. Hence, Xinomavro was the only cultivar that assembled clearly distinct endophytic fungal communities in symptomatic vs asymptomatic plants. This result may be due to the

clear visual distinction between potentially healthy and diseased plants, which is facilitated by the higher resistance of the cultivar Xinomavro to GTDs. Still, we could not exclude the contribution of the local climatic conditions, that varied in the three viticultural zones studied (see Methods), on the differential response of the studied cultivars to the assemblage of the wood microbiome in asymptomatic and symptomatic vines.

DA analysis identified members of the wood fungal microbiome that were specifically associated with asymptomatic or symptomatic vines. Considering the whole dataset, *Acremonium alternatum*, one of the most dominant ASV in the dataset overall, and *K. variispora*, were identified as indicators of symptomatic plants. The former is a common saprotrophic endophyte, previously isolated as grapevine endophyte (Hofstetter et al., 2012; Del Frari et al., 2019) not exhibiting plant pathogenic traits. *K. variispora* has been characterized as a common wood fungus of vine, that causes irregular discoloration of vascular tissues, necrotic spots, carcinomas, and grapevine decline (Bruez et al., 2014). In Greece, this fungus has been described as a causal agent of wood necrosis and fruit rot on apples (Ntasiou et al., 2021). The fungus produces two polyketide massarilactones that were phytotoxic, reproducing grapevine decline symptoms on plant leaves (Cimmino et al., 2020). Conversely, we identified an *Alternaria* ASV that was consistently associated with asymptomatic plants regardless of cultivar/viticultural zones. Fungi of this genus are common members of the cultivable grapevine wood microbiome (Hofstetter et al., 2012; Del Frari et al., 2019). Their consistent association with asymptomatic vines suggests a role in suppression of GTDs, further reinforced by the negative co-occurrence network pattern of *Alternaria* with hallmark GTD fungal genera like *Phaemomoniella* and *Phaeoacromonium*. In line with this Del Frari et al. (2019) showed that a consortium composed of four endophytic fungi, including *A. alternata*, isolated from asymptomatic vines was able to decrease *P. chlamydospora* infection in planta. When looking at each cultivar separately, we noted different GTD-associated pathogens to be consistently present in symptomatic plants like *Fomitiporia spp*., in Agiorgitiko, *P. chlamydospora*, *K. variispora* and *Diaporthe spp*., in Vidiano. Bruez et al. (2020) identified *Fomitiporia* as the single member of the wood microbiome that was consistently more abundant in white rot wood tissues of visibly healthy and diseased plants. They suggested that it is the key for the onset of the white rot syndrome collaborating with primary vascular tissue colonizers like *P.*

chlamydospora, and *Phaeoacremonium spp*.; both of these fungi were prevalent in our study, and the latter dominatedin the symptomatic Xinomavro vines. The key role of *Fomitiporia*, along with the two trachemycotic fungi (*P. chlamydospore* and *Phaeoacremonium sp*.) and other confounding factors like climatic conditions and vineyardage, in leaf symptoms manifestation was recently highlighted in two comprehensive reviews by Morreti et al. (2021) and Del Frari et al. (2021).

A range of wood endophytic fungi previously reported as members of the wood grapevine microbiome like *L. cynaroidis* (Bruez et al., 2020) and *M. restricta* (Del Frari et al., 2019) but also fungi that have been reported as wood pathogens involved in GTDs like *Eutypa* spp., and *S. vitis* in Xinomavro (Reisenzein et al., 2020; Lawrence et al., 2018), were associated with asymptomatic vines. The consistent presence of these pathogens in asymptomatic plants is not surprising, and previous studies have also reported the common occurrence of GTD-related pathogens in asymptomatic plants (Elena et al., 2018; Hofstetter et al., 2012)

A Random Forest supervised learning model was usedto identify members of the fungal microbiome that could be used as predictors of GTD symptomatic samples. This approach pointed to *P. chlamydospora* and *K. variispora*, both being members of the GTD-related pathobiome, along with *A. alternatum* and *Cladosporium* spp., not previously reported as wood pathogens in grapevine, as highly accurate predictors of symptomatic plants. Wood microbiome analysis of grapevines based on these predictors could be used as an early warning for the appearance of GTDs. Further testing with a wider dataset will verify the utility of such an approach in controlling the spread of GTDs in global vineyards.

2.4.2. Bacterial microbiome

The bacterial wood microbiome was overly dominated by *Proteobacteria*, and mostly *α*- and *γ-proteobacteria*, followed by *Actinobacteria* and *Bacteroidetes*, in line with previous studies (Bruez et al., 2020; Deyett et al., 2020; Bruez et al., 2015). Amongst those phyla, members of the families *Sphingomonadaceae* and *Bacillaceae* were the most prevalent. Previous culture-dependent and culture-independent efforts to record the grapevine wood bacteriome have identified *Bacillaceae* and *Sphingomonadaceae* amongst the most abundant members of this community (Bruez et al., 2020; West et al., 2010). The bacterial community shared similar assembly mechanisms with the fungal community, and it was strongly driven by biogeography/ cultivar as a lumped

factor, unlike GTD status which did not show a high discriminatory power. Previous studies looking at the vine microbiome suggested that biogeography was the most important factor shaping bacterial communities in berries (Bokulich et al., 2014) vineyard soil (Coller et al., 2019; Gobbi et al., 2020) and the bark of grapevines (Vitulo et al., 2019) The few studies that have explored the composition of the bacterial wood microbiome identified limited differences between symptomatic and asymptomatic vines (Bruez et al., 2020; Bruez et al., 2015), in accordance with our findings. However, in line with the fungal microbiome, we noted cultivar- / viticultural zone-specific differentiation between symptomatic and asymptomatic vines in the cultivar Xinomavro. It is not clear what may be the driving factors for the distinct assemblages of both fungal and bacterial communities in the Xinomavro cultivar compared with the other two, more susceptible to GTD cultivars. On-going analysis of the wood microbiome of this cultivar in symptomatic and asymptomatic vines across viticultural zones will shed further light into this hypothesis.

When looking for bacterial drivers of the distinction between asymptomatic and symptomatic plants, we identified a strong enrichment of *Bacillus* (and widely of members of the family *Bacillaceae*) and *Streptomyces* in the former. This enrichment was particularly prominent in the cultivars Xinomavro and Agiorgitiko, while in Vidiano we noted, in addition to *Bacillus*, an enrichment of *Corynebacterium*. Bruez et al. (2015) identified *Bacillus spp.* as major components of the wood bacterial community in vines; however, they did not observe a significant differentiation in their abundance between symptomatic and asymptomatic plants. This was further confirmed in a follow up study on the same vineyard using high throughput sequencing (Bruez et al., 2020). In the same study *Streptomyces* showed a significantly higher abundance in asymptomatic plants (7% vs < 0.1%), in line with our findings. The consistent prevalence of *Bacillus* and *Streptomyces* in the asymptomatic plants and their negative co-occurrence patterns with the GTD pathogens *Phaeoacremonium, Phaeomoniella* and *Seimatosporium* in asymptomatic vines could be associated with the capacity of members of these bacterial genera to act as biocontrol agents and/or plant growth promoters in several plants including grapevines (Alfonzo et al., 2009; Viane et al., 2016). Indeed Rezgui et al. (2016) and Haidar et al. (2016) reported a high suppressive activity of endophytic *Bacillus* strains isolated from vines against GTD-related pathogens like *P. chlamydospora, Lasidiodiplodia pseudotheobromae, Neofusicoccum parvum* and *Schizophyllum*

commune, acting through the production of VOCs, antibiotics or by inducing systematic resistance in the plant host. Alvarez-Perez et al. (2017) isolated an endophytic *Streptomyces* from asymptomatic grapevines that suppressed *P. chlamydospore* and *Phaeoacremonium minimum* in planta and in vitro. Similarly, Trotel-Aziz et al. (2021) showed that a *Bacillus subtilis* strain, isolated from grapevine rhizosphere, was able to attenuate *Botryosphaeria* dieback by triggering host immune responses and detoxifying phytotoxins produced by *N. parvum*, mechanisms further verified by genomic analysis of the studied strain (Leal et al., 2021). On-going high-throughput isolation effort will explore this rich endophytic bacterial community of asymptomatic plants for novel potential bacterial control agents against GTDs.

2.5. Conclusions

We studied the wood microbiome of grapevines from three Greek cultivars located in three geographically distinct viticultural zones following a systematic sampling approach distinguishing between GTD-symptomatic and asymptomatic plants. Geographical location and/ or cultivar were the main determinants of both fungal and bacterial microbiome, unlike the GTD status of grapevines that differentiated between asymptomatic and symptomatic plants only for the most resistant to GTDs cultivar Xinomavro, suggesting complex interactions between cultivar/biogeography and the wood fungal pathobiome. *P. chlamydospora*, *K. variispora*, *Fomitiporia* spp., *Diaporthe* spp., all previously proposed as causal agents of GTDs, along with *Acremonium* sp. (non GTD-linked fungus) were consistently associated with symptomatic vines in a cultivar-dependent manner. In addition, the first two of these pathogens, along with *Cladosporium* spp. and *A. alternatum* were identified, via Random Forest analysis, as highly accurate predictors of symptomatic vines. The bacterial microbiome in asymptomatic plants was dominated by *Bacillus* and *Streptomyces,* which showed a negative co-occurrence pattern with *Phaeomoniella*, *Phaeoacremonium* and *Seimatosporium.* This suggested a role of in the suppression of GTDs, a hypothesis which warrants verification through isolation and phenotypic characterization of these bacteria. Overall, we provide evidence that GTD symptomatic plants support a wood fungal microbiome showing cultivar and/or biogeography-dependent patterns. Specific members of this fungal wood microbiome could be used as a proxy to distinguish between healthy and diseased vines. Further

tests with similar datasets from different viticultural regions or at large geographical scale would verify the universal utility of this approach. Potential interactions between the bacterial and fungal wood microbiome in GTD-free vines should be further pursued in the quest for discovery of novel grapevine-accustomed biocontrol agents.

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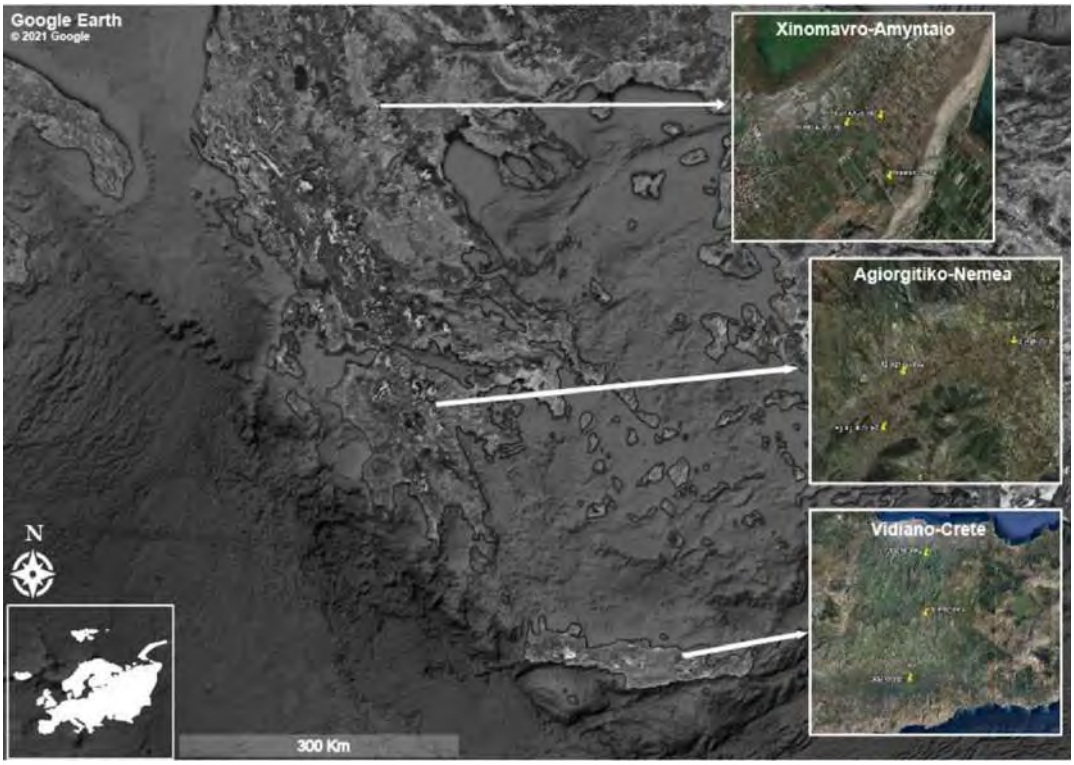
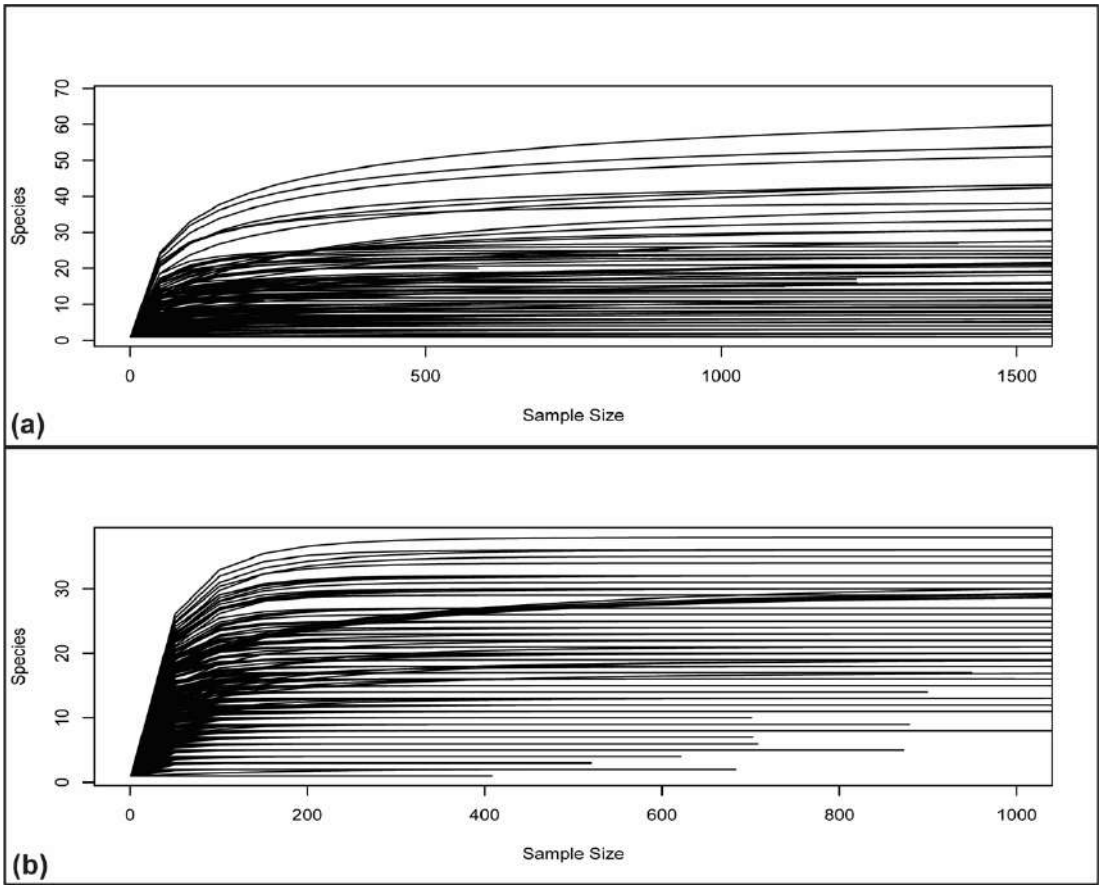
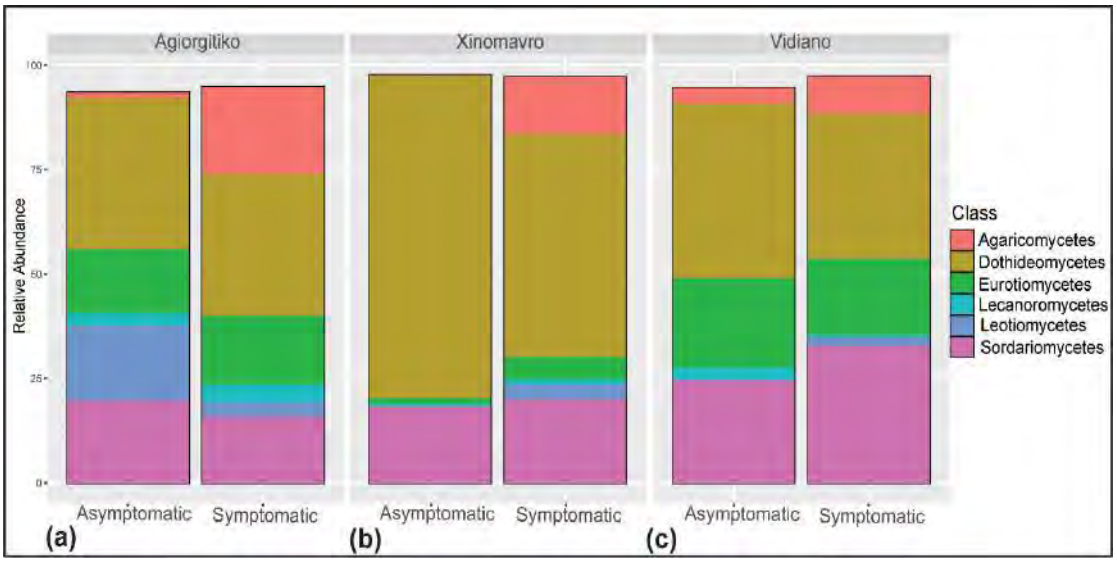
2.7 Annex I – Supplementary data



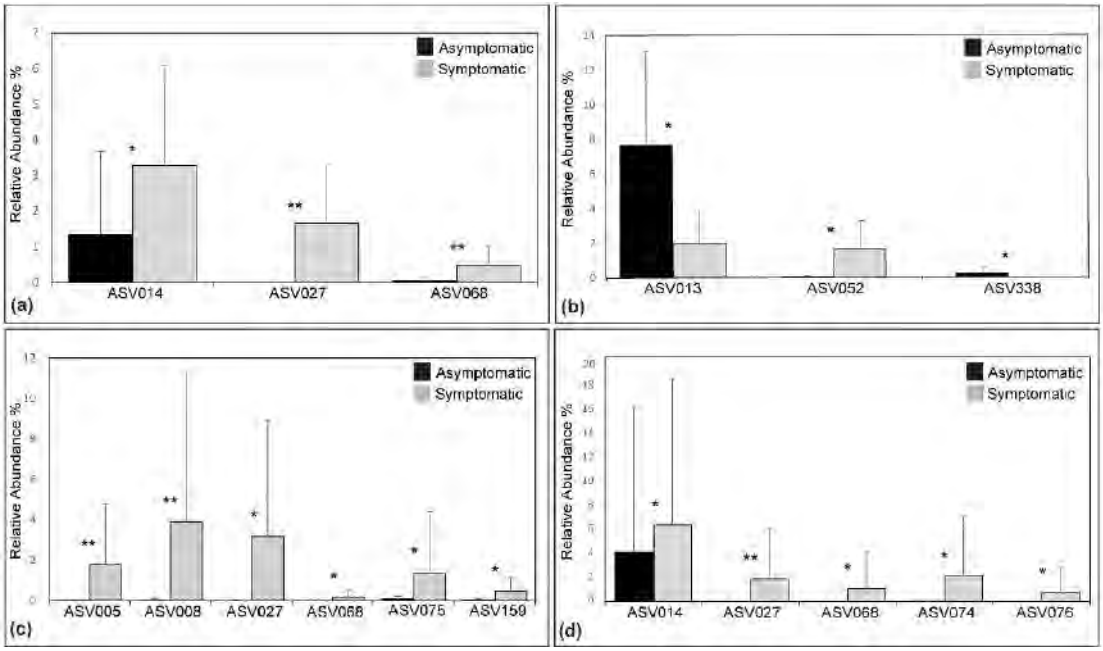
Supplementary Figure S1. A map of Greece showing the sampling sites (Amyntaio, Nemea and Crete). Vineyards of Xinomavro, Agiorgitiko and Vidiano cultivars were sampled in Amyntaio, Nemea and Crete regions, respectively.



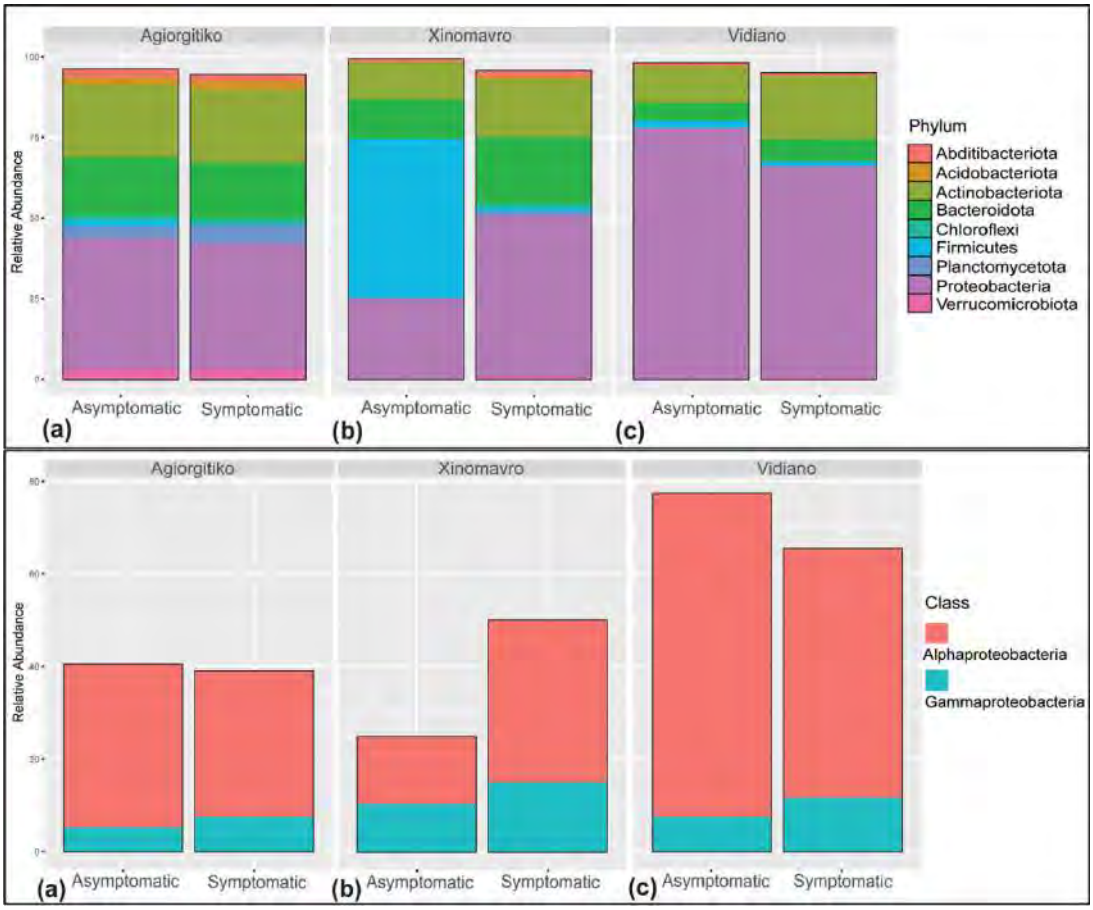
Supplementary Figure S2. Rarefaction curves for the wood samples analyzed for their fungal (a) and bacterial microbiome (b).



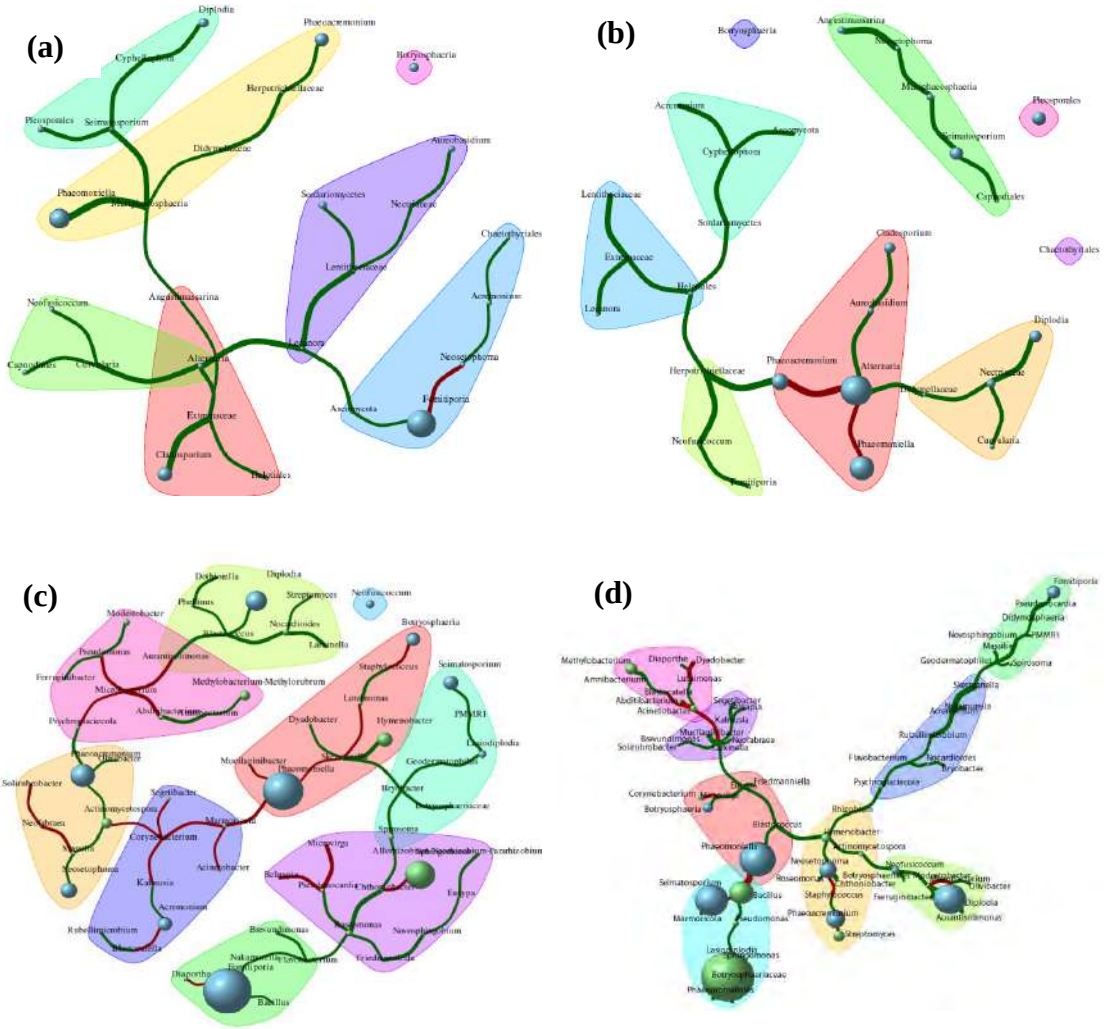
Supplementary Figure S3. Stacked bar plots showing the composition of the fungal community (at the class taxonomic level) in wood samples collected from GTD asymptomatic and symptomatic vines of the cultivars Agiorgitiko (a), Xinomavro (b), Vidiano (c) each located in a distinct viticultural zone in Greece.



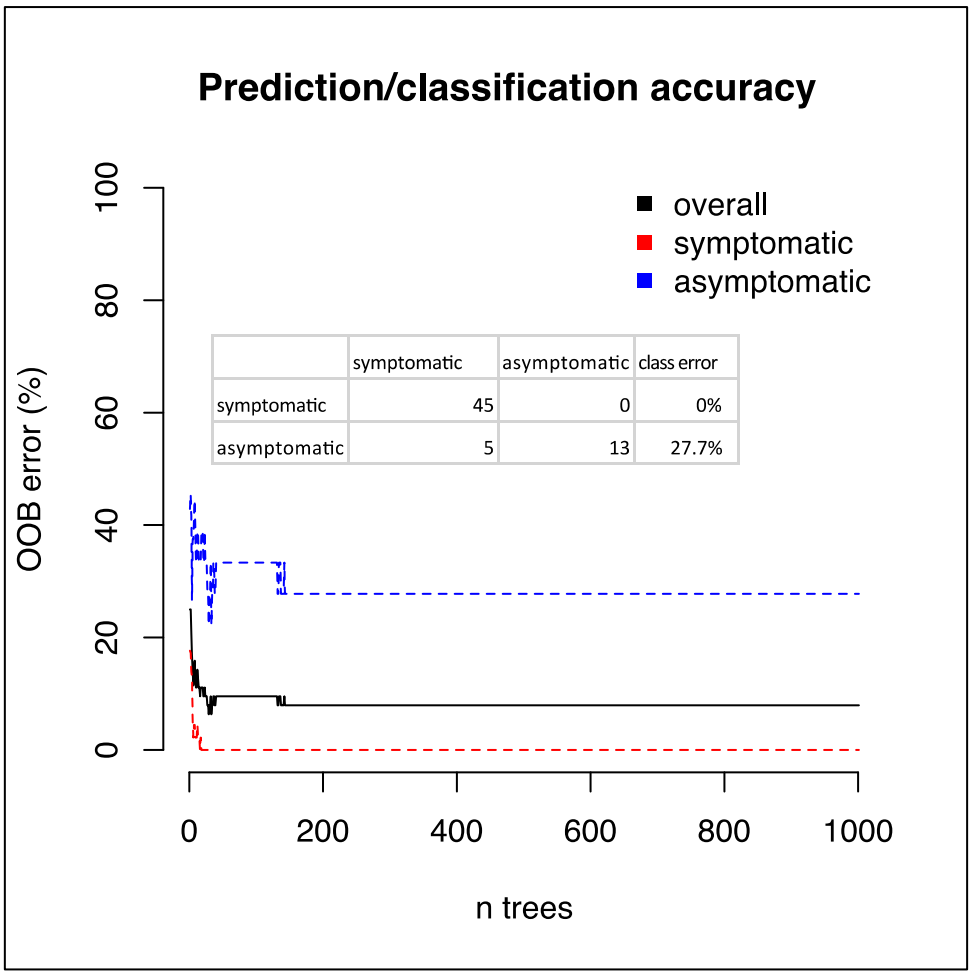
Supplementary Figure S4. Fungal ASVs phylogenetically assigned to taxa previously identified as causal agents of GTDs that showed differential abundance in samples collected from symptomatic and asymptomatic vines. Data are presented collectively for all geographic locations/cultivars (a) and for each variety separately (b) Agiorgitiko (c) Xinomavro (d) Vidiano. ASV014: *Phaeomoniella chlamydospora*, ASV068: *Kalmusia variispora*, ASV13: *Seimatosporium vitis*, ASV52: *Fomitiporia* spp., ASV338: *Seimatosporium vitis*, ASV005: *Phaeomoniella chlamydospora*, ASV008: *Phaeoacremonium iranianum*, ASV75: *Neosetophoma* spp., ASV159: *Neosetophoma* spp., ASV074: *Diaporthe* spp., (Signif. Differences: '***' 0.001 '**' 0.01 '*' 0.05).



Supplementary Figure S5. Stacked bar plots showing the composition of the bacterial community (at phylum taxonomic level and for the class Proteobacteria) in wood samples collected from GTP asymptomatic and symptomatic vines of the cultivars Agiorgitiko (a), Xinomavro (b), Vidiano (c) each located in a distinct viticultural zone in Greece.



Supplementary Figure S6. Network analysis of the fungal wood microbiome (a,b) and of the GTD-associated fungal genera with the wood bacterial microbiome (c,d) in symptomatic (a,c) and asymptomatic (b,d) vines, regardless of cultivar/viticultural zones. Regarding the wood fungal and bacterial microbiome analysis, only fungal and bacterial genera which showed a relative abundance >1% in 10% of the samples analyzed were considered in the analysis. Blue and green bubbles designate fungal and bacterial genera respectively, while the size of each bubble indicates the relative abundance of each microbial genera. Green and red links signify significant positive and negative co-occurrence patterns between the linked microorganisms while the width of the line is a measure of the level of the co-occurrence correlation between the linked microorganisms (the higher the width of the line the higher the correlation between the co-occurrence of the microorganisms).



	symptomatic	asymptomatic	class error
symptomatic	45	0	0%
asymptomatic	5	13	27.7%

Supplementary Figure S7. Out of bag (OOB) error rates of the Random Forest model parameter states next to the overall model error rates (7.94% shown with the black line) for each one of the 1000 model trees. The confusion matrix is also provided on the plot.

Supplementary Table S1: A list of the wood samples analyzed with all relevant information regarding vine cultivar and viticultural zone, GTDs symptoms presence or absence, plant part sampled, vineyard code, plant code and geographic location.

Sample No	Cultivar – Viticultural Zone	GTD Condition	Plant part	Vineyard Code Number	Plant Code Number	Geographic location
1	Agiorgitiko-Nemea	Symptomatic	Cordon	No3I	No1	Peloponnese
2	Agiorgitiko-Nemea	Symptomatic	Trunk	No3I	No1	Peloponnese
3	Agiorgitiko-Nemea	Symptomatic	Trunk	No3I	No2	Peloponnese
4	Agiorgitiko-Nemea	Symptomatic	Cordon	No3I	No3	Peloponnese
5	Agiorgitiko-Nemea	Symptomatic	Cordon	No1G	No1	Peloponnese
6	Agiorgitiko-Nemea	Symptomatic	Trunk	No1G	No1	Peloponnese
7	Agiorgitiko-Nemea	Symptomatic	Cordon	No1G	No2	Peloponnese
8	Agiorgitiko-Nemea	Symptomatic	Trunk	No1G	No2	Peloponnese
9	Agiorgitiko-Nemea	Symptomatic	Cordon	No1G	No3	Peloponnese
10	Agiorgitiko-Nemea	Symptomatic	Trunk	No1G	No3	Peloponnese
11	Agiorgitiko-Nemea	Symptomatic	Cordon	No2H	No1	Peloponnese
12	Agiorgitiko-Nemea	Symptomatic	Trunk	No2H	No1	Peloponnese
13	Agiorgitiko-Nemea	Symptomatic	Cordon	No2H	No2	Peloponnese
14	Agiorgitiko-Nemea	Symptomatic	Trunk	No2H	No2	Peloponnese
15	Agiorgitiko-Nemea	Symptomatic	Cordon	No2H	No3	Peloponnese
16	Agiorgitiko-Nemea	Symptomatic	Trunk	No2H	No3	Peloponnese
17	Agiorgitiko-Nemea	Asymptomatic	Trunk	No1G	No3	Peloponnese
18	Agiorgitiko-Nemea	Asymptomatic	Trunk	No2H	No2	Peloponnese
19	Agiorgitiko-Nemea	Asymptomatic	Trunk	No2H	No3	Peloponnese
20	Agiorgitiko-Nemea	Asymptomatic	Trunk	No3I	No1	Peloponnese
21	Xinomavro-Amyntaio	Symptomatic	Trunk	No4E	No1	North-West Greece
22	Xinomavro-Amyntaio	Symptomatic	Trunk	No4E	No3	North-West Greece
23	Xinomavro-Amyntaio	Symptomatic	Trunk	No4E	No2	North-West Greece

24	Xinomavro-Amyntaio	Symptomatic	Cordon	No4E	No2	North-West Greece
25	Xinomavro-Amyntaio	Symptomatic	Cordon	No4E	No1	North-West Greece
26	Xinomavro-Amyntaio	Symptomatic	Trunk	No4E	No3	North-West Greece
27	Xinomavro-Amyntaio	Symptomatic	Trunk	No5D	No1	North-West Greece
28	Xinomavro-Amyntaio	Symptomatic	Cordon	No5D	No1	North-West Greece
29	Xinomavro-Amyntaio	Symptomatic	Trunk	No5D	No2	North-West Greece
30	Xinomavro-Amyntaio	Symptomatic	Trunk	No5D	No3	North-West Greece
31	Xinomavro-Amyntaio	Symptomatic	Cordon	No5D	No2	North-West Greece
32	Xinomavro-Amyntaio	Symptomatic	Trunk	No5D	No4	North-West Greece
33	Xinomavro-Amyntaio	Symptomatic	Cordon	No5D	No3	North-West Greece
34	Xinomavro-Amyntaio	Symptomatic	Cordon	No5D	No4	North-West Greece
35	Xinomavro-Amyntaio	Symptomatic	Trunk	No7F	No2	North-West Greece
36	Xinomavro-Amyntaio	Symptomatic	Trunk	No7F	No3	North-West Greece
37	Xinomavro-Amyntaio	Symptomatic	Cordon	No7F	No1	North-West Greece
38	Xinomavro-Amyntaio	Symptomatic	Trunk	No7F	No1	North-West Greece
39	Xinomavro-Amyntaio	Symptomatic	Cordon	No7F	No3	North-West Greece
40	Xinomavro-Amyntaio	Asymptomatic	Trunk	No4E	No1	North-West Greece
41	Xinomavro-Amyntaio	Asymptomatic	Cordon	No4E	No1	North-West Greece
42	Xinomavro-Amyntaio	Asymptomatic	Trunk	No4E	No2	North-West Greece
43	Xinomavro-Amyntaio	Asymptomatic	Cordon	No4E	No2	North-West Greece
44	Xinomavro-Amyntaio	Asymptomatic	Trunk	No4E	No3	North-West Greece
45	Xinomavro-Amyntaio	Asymptomatic	Cordon	No4E	No3	North-West Greece
46	Xinomavro-Amyntaio	Asymptomatic	Trunk	No5D	No1	North-West Greece
47	Xinomavro-Amyntaio	Asymptomatic	Cordon	No5D	No1	North-West Greece
48	Xinomavro-Amyntaio	Asymptomatic	Cordon	No5D	No2	North-West Greece
49	Xinomavro-Amyntaio	Asymptomatic	Trunk	No5D	No3	North-West Greece
50	Xinomavro-Amyntaio	Asymptomatic	Cordon	No5D	No3	North-West Greece

51	Vidiano-Crete	Symptomatic	Trunk	No2A	No1	South Greece
52	Vidiano-Crete	Symptomatic	Cordon	No2A	No1	South Greece
53	Vidiano-Crete	Symptomatic	Trunk	No2A	No2	South Greece
54	Vidiano-Crete	Symptomatic	Cordon	No2A	No2	South Greece
55	Vidiano-Crete	Symptomatic	Trunk	No2A	No3	South Greece
56	Vidiano-Crete	Symptomatic	Cordon	No2A	No3	South Greece
57	Vidiano-Crete	Symptomatic	Trunk	No6B	No1	South Greece
58	Vidiano-Crete	Symptomatic	Cordon	No6B	No1	South Greece
59	Vidiano-Crete	Symptomatic	Trunk	No6B	No2	South Greece
60	Vidiano-Crete	Symptomatic	Cordon	No6B	No2	South Greece
61	Vidiano-Crete	Symptomatic	Trunk	No6B	No3	South Greece
62	Vidiano-Crete	Symptomatic	Trunk	No7C	No1	South Greece
63	Vidiano-Crete	Symptomatic	Cordon	No7C	No1	South Greece
64	Vidiano-Crete	Symptomatic	Trunk	No7C	No2	South Greece
65	Vidiano-Crete	Symptomatic	Cordon	No7C	No2	South Greece
66	Vidiano-Crete	Symptomatic	Trunk	No7C	No3	South Greece
67	Vidiano-Crete	Symptomatic	Cordon	No7C	No3	South Greece
68	Vidiano-Crete	Asymptomatic	Trunk	No2A	No1	South Greece
69	Vidiano-Crete	Asymptomatic	Trunk	No6B	No1	South Greece
70	Vidiano-Crete	Asymptomatic	Cordon	No2A	No1	South Greece
71	Vidiano-Crete	Asymptomatic	Cordon	No6B	No1	South Greece
72	Vidiano-Crete	Asymptomatic	Trunk	No6B	No2	South Greece
73	Vidiano-Crete	Asymptomatic	Cordon	No6B	No2	South Greece
74	Vidiano-Crete	Asymptomatic	Trunk	No6B	No3	South Greece
75	Vidiano-Crete	Asymptomatic	Cordon	No6B	No3	South Greece
76	Vidiano-Crete	Asymptomatic	Trunk	No7C	No1	South Greece
77	Vidiano-Crete	Asymptomatic	Cordon	No7C	No1	South Greece

78	Vidiano-Crete	Asymptomatic	Trunk	No2A	No2	South Greece
79	Vidiano-Crete	Asymptomatic	Trunk	No7C	No2	South Greece
80	Vidiano-Crete	Asymptomatic	Cordon	No7C	No2	South Greece
81	Vidiano-Crete	Asymptomatic	Trunk	No7C	No3	South Greece
82	Vidiano-Crete	Asymptomatic	Cordon	No7C	No3	South Greece
83	Vidiano-Crete	Asymptomatic	Cordon	No2A	No2	South Greece
84	Vidiano-Crete	Asymptomatic	Trunk	No2A	No3	South Greece
85	Vidiano-Crete	Asymptomatic	Cordon	No2A	No3	South Greece

Supplementary Table S2. Primers used for amplicon sequencing analysis. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step, which are composed of the sequence of the universal primers 515f (bacteria) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a TT (linker) sequence at the 5' end of each primer.

Kingdom	PCR step	Primers	Gene target	Amplicon size (bp)	Primer sequences (5'-3')	References
Bacteria	1st	515F 806R	16S rRNA	~290	GTGCCAGCMGCCGCGGTAA GGACTACHVHHHTWTCTAAT	Parada et al., (2016)
	2nd	Indexed (n=85)	B0001-515f		TTCTTCTTCGT**GTGYCAGCMGCCGCGGTAA**	This study
			B0002-515f		TTCTCAATGGT**GTGYCAGCMGCCGCGGTAA**	
			B0003-515f		TTCAGTTCAGT**GTGYCAGCMGCCGCGGTAA**	
			B0004-515f		TTCGAATCAGT**GTGYCAGCMGCCGCGGTAA**	
			B0005-515f		TTGTCAGGTGT**GTGYCAGCMGCCGCGGTAA**	
Fungi	1st	ITS7F ITS4R	ITS2	~310	GTGARTCATCGAATCTTTG TCCTCCGCTTATTGATATGC	Ihrmark et al., (2012) White et al., (1990)
	2nd	Indexed (n=85)	FI0001-ITS4r		TTAACCTTGGA**TCCTCCGCTTATTGATATGC**	This study
			FI0002-ITS4r		TTAACCGAAGA**TCCTCCGCTTATTGATATGC**	
			FI0003-ITS4r		TTAACGACAGA**TCCTCCGCTTATTGATATGC**	
			FI0004-ITS4r		TTACTTACGGA**TCCTCCGCTTATTGATATGC**	
			FI0005-ITS4r		TTACTTGTCGA**TCCTCCGCTTATTGATATGC**	

Supplementary Table S3. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	**Volume (μl)**	**Concentration**	**Comments**
Primer F	1	0.5 μM	
Primer R	1	0.5 μM	
BSA	0.4	0.4 μg/μl	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH_2O	5.6		
DNA	2	0.2 ng/μl	
Total	20		
PCR conditions			
Step	**Temperature (°C)**	**Time**	**Number of Cycles**
Initial Denaturation	98	30 sec	28 in the first amplification step / 7 in the second amplification step
Denaturation	98	10 sec	
Annealing	50 for bacteria/ 55 for fungi	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	

Supplementary Table S4. PERMANOVA analysis of the fungal and bacterial wood microbiome (Signif. codes: 0.001 '***' 0.01 '**' 0.05 '*')

Factor	Degrees of Freedom	Sum of squares	F. Model	R^2 (%)	P-value (>F)
Fungi – All varieties					
GTDs condition	1	1.221	3.5576	3.5	0.001***
Variety/Geographic Location	8	7.902	7.2183	22.7	0.001***
Residuals	75	25.75	0.73838		
Fungi – Agiorgitiko					
GTDs condition	1	0.4414	1.8848	8.4	0.012*
Vineyard	2	1.0777	2.3008	20.5	0.001***
Residuals	16	3.7473	0.71154		
Fungi – Xinomavro					
GTDs condition	1	1.5707	4.6644	13.9	0.001***
Vineyard	2	0.9678	1.437	8.6	0.038*
Residuals	26	8.7552	0.77523		
Fungi – Vidiano					
GTDs condition	1	0.6186	1.6121	4.2	0.054
Vineyard	2	2.2099	2.8793	15	0.001***
Residuals	31	11.8962	0.80791		
Bacteria – All varieties					
GTDs condition	1	1.863	5.5443	5.2	0.001***
Variety/Geographic Location	8	9.169	10.3873	25.5	0.001***
Residuals	74	24.863	0.69267		
Bacteria – Agiorgitiko					
GTDs condition	1	0.3776	1.0526	5.3	0.263

Vineyard	2	0.9631	1.3423	13.6	0.001***
Residuals	16	5.7399	0.81065		
Bacteria - Xinomavro					
GTDs condition	1	2.2788	6.7866	19.7	0.001***
Vineyard	2	0.8801	1.3105	7.6	0.079 .
Residuals	25	8.3943	0.72658		
Bacteria - Vidiano					
GTDs condition	1	0.6553	2.2694	6	0.007**
Vineyard	2	1.3166	2.2799	12.1	0.006**
Residuals	31	8.951	0.81947		

Supplementary Table S5. ASVs detected in the wood microbiome of the vines studied that are considered as putative pathogens involved in GTDs. Each ASV was phylogenetically assigned to the closest verified taxonomic level (genus or species)

ASV	Cultivar	State	%	Phylum	Class	Order	Family	Genus	Species
ASV005	Agiorgitiko	Asymptomatic	8.61	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV005	Agiorgitiko	Symptomatic	7.78	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV005	Xinomavro	Symptomatic	1.80	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV005	Vidiano	Asymptomatic	12.90	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV005	Vidiano	Symptomatic	6.85	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV008	Agiorgitiko	Asymptomatic	4.57	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV008	Agiorgitiko	Symptomatic	7.46	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV008	Xinomavro	Asymptomatic	0.03	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV008	Xinomavro	Symptomatic	3.91	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV008	Vidiano	Asymptomatic	5.15	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV008	Vidiano	Symptomatic	3.43	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV009	Agiorgitiko	Asymptomatic	1.10	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV009	Agiorgitiko	Symptomatic	3.87	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV009	Xinomavro	Asymptomatic	11.99	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV009	Xinomavro	Symptomatic	6.88	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV009	Vidiano	Asymptomatic	1.73	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV009	Vidiano	Symptomatic	1.26	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV011	Agiorgitiko	Symptomatic	6.02	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV011	Xinomavro	Asymptomatic	0.02	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV011	Xinomavro	Symptomatic	2.09	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV011	Vidiano	Asymptomatic	0.51	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV011	Vidiano	Symptomatic	5.26	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV013	Agiorgitiko	Asymptomatic	7.64	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV013	Agiorgitiko	Symptomatic	1.99	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis

ASV013	Xinomavro	Asymptomatic	1.59	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV013	Xinomavro	Symptomatic	1.19	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV013	Vidiano	Asymptomatic	6.64	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV013	Vidiano	Symptomatic	2.07	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV014	Agiorgitiko	Symptomatic	2.98	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV014	Xinomavro	Symptomatic	0.65	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV014	Vidiano	Asymptomatic	4.03	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV014	Vidiano	Symptomatic	6.23	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	chlamydospora
ASV015	Xinomavro	Symptomatic	7.47	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Lasiodiplodia	spp.
ASV020	Agiorgitiko	Symptomatic	0.55	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV020	Xinomavro	Symptomatic	3.38	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV020	Vidiano	Asymptomatic	0.68	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV021	Agiorgitiko	Symptomatic	3.82	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV024	Xinomavro	Asymptomatic	1.61	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Botryosphaeria	spp.
ASV024	Xinomavro	Symptomatic	1.33	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Botryosphaeria	spp.
ASV024	Vidiano	Asymptomatic	0.51	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Botryosphaeria	spp.
ASV024	Vidiano	Symptomatic	10.41	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Botryosphaeria	spp.
ASV026	Agiorgitiko	Asymptomatic	0.16	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Neofusicoccum	spp.
ASV026	Agiorgitiko	Symptomatic	0.25	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Neofusicoccum	spp.
ASV026	Vidiano	Asymptomatic	1.56	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Neofusicoccum	spp.
ASV026	Vidiano	Symptomatic	5.84	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Neofusicoccum	spp.
ASV027	Xinomavro	Symptomatic	3.18	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Acremonium	alternatum
ASV027	Vidiano	Symptomatic	1.81	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Acremonium	alternatum
ASV029	Agiorgitiko	Symptomatic	2.13	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV029	Vidiano	Asymptomatic	0.53	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV032	Xinomavro	Asymptomatic	6.98	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae		
ASV040	Agiorgitiko	Symptomatic	1.72	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.

ASV041	Agiorgitiko	Symptomatic	2.08	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Phellinus	rhamni
ASV042	Xinomavro	Symptomatic	1.52	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV045	Vidiano	Asymptomatic	3.74	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	sicilianum
ASV045	Vidiano	Symptomatic	0.84	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	sicilianum
ASV046	Agiorgitiko	Asymptomatic	0.16	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	rosarum
ASV046	Agiorgitiko	Symptomatic	0.45	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	rosarum
ASV046	Vidiano	Asymptomatic	1.34	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	rosarum
ASV046	Vidiano	Symptomatic	1.46	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	rosarum
ASV052	Agiorgitiko	Symptomatic	1.63	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV053	Agiorgitiko	Symptomatic	1.02	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV058	Agiorgitiko	Asymptomatic	0.12	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV058	Vidiano	Asymptomatic	2.06	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV058	Vidiano	Symptomatic	1.03	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV063	Agiorgitiko	Symptomatic	0.55	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV063	Xinomavro	Symptomatic	1.12	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV065	Xinomavro	Symptomatic	0.26	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	spp.
ASV065	Vidiano	Symptomatic	1.11	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	spp.
ASV068	Agiorgitiko	Asymptomatic	0.15	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Kalmusia	variispora
ASV068	Agiorgitiko	Symptomatic	0.19	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Kalmusia	variispora
ASV068	Xinomavro	Symptomatic	0.18	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Kalmusia	variispora
ASV068	Vidiano	Symptomatic	1.07	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Kalmusia	variispora
ASV072	Xinomavro	Symptomatic	1.61	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	spp.
ASV073	Agiorgitiko	Symptomatic	0.48	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV073	Vidiano	Symptomatic	1.22	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV074	Vidiano	Symptomatic	2.13	Ascomycota	Sordariomycetes	Diaporthales	Diaporthaceae	Diaporthe	spp.
ASV075	Xinomavro	Asymptomatic	0.08	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV075	Xinomavro	Symptomatic	1.39	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.

ASV075	Vidiano	Symptomatic	0.14	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV076	Xinomavro	Symptomatic	0.30	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Acremonium	sordidulum
ASV076	Vidiano	Symptomatic	0.79	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Acremonium	sordidulum
ASV091	Agiorgitiko	Asymptomatic	0.70	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	spp.
ASV091	Agiorgitiko	Symptomatic	0.25	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	spp.
ASV093	Vidiano	Symptomatic	2.09	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV098	Vidiano	Asymptomatic	1.22	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	spp.
ASV103	Agiorgitiko	Symptomatic	0.45	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Phellinus	rhamni
ASV104	Xinomavro	Asymptomatic	0.19	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Lasiodiplodia	spp.
ASV104	Xinomavro	Symptomatic	0.78	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Lasiodiplodia	spp.
ASV118	Vidiano	Asymptomatic	1.14	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV131	Xinomavro	Symptomatic	0.71	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV134	Xinomavro	Symptomatic	1.33	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV159	Xinomavro	Asymptomatic	0.03	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV159	Xinomavro	Symptomatic	0.47	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV163	Xinomavro	Symptomatic	0.37	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV168	Xinomavro	Symptomatic	0.51	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV179	Agiorgitiko	Asymptomatic	0.41	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	spp.
ASV202	Vidiano	Asymptomatic	0.65	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Fomitiporia	spp.
ASV210	Agiorgitiko	Asymptomatic	0.30	Ascomycota	Eurotiomycetes	Phaeomoniellales	Phaeomoniellaceae	Phaeomoniella	spp.
ASV216	Agiorgitiko	Symptomatic	0.18	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Dothiorella	rosulata
ASV223	Vidiano	Asymptomatic	0.27	Ascomycota	Sordariomycetes	Togniniales	Togniniaceae	Phaeoacremonium	iranianum
ASV232	Xinomavro	Symptomatic	0.29	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV270	Vidiano	Symptomatic	0.20	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Diplodia	spp.
ASV278	Agiorgitiko	Asymptomatic	0.10	Ascomycota	Sordariomycetes	Xylariales	Diatrypaceae	Eutypa	spp.
ASV278	Vidiano	Symptomatic	0.12	Ascomycota	Sordariomycetes	Xylariales	Diatrypaceae	Eutypa	spp.
ASV282	Vidiano	Symptomatic	0.24	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Neofusicoccum	spp.

ASV319	Xinomavro	Asymptomatic	0.03	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae		
ASV325	Vidiano	Symptomatic	0.19	Ascomycota	Sordariomycetes	Hypocreales	Hypocreales_fam_Incertae	Acremonium	fusidioides
ASV338	Agiorgitiko	Asymptomatic	0.21	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV339	Vidiano	Asymptomatic	0.63	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV341	Xinomavro	Symptomatic	0.18	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium	vitis
ASV552	Xinomavro	Symptomatic	0.22	Ascomycota	Sordariomycetes	Xylariales	Diatrypaceae	Eutypa	crustata
ASV659	Vidiano	Asymptomatic	0.15	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Didymosphaeria	futilis
ASV677	Vidiano	Symptomatic	0.37	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	spp.
ASV803	Vidiano	Asymptomatic	0.12	Ascomycota	Eurotiomycetes	Phaeomoniellales			
ASV805	Vidiano	Asymptomatic	0.12	Ascomycota	Sordariomycetes	Xylariales	Diatrypaceae	Eutypa	tetragona
ASV806	Vidiano	Asymptomatic	0.09	Ascomycota	Dothideomycetes	Botryosphaeriales			

Chapter 3

Different factors are operative in shaping the epiphyticgrapevine microbiome across different geographical scales:Biogeography, cultivar or vintage?

The work presented in **Chapter 3** is included in the following article:

Elena Papadopoulou, Fotios Bekris, Sotirios Vasileiadis, Kalliope K. Papadopoulou and Dimitrios G. Karpouzas (2022). Different factors are operative in shaping the epiphytic grapevine microbiome across different geographical scales: Biogeography, cultivar or vintage?

J Sustain Agric Environ. 2022;1:287–301. DOI: 10.1002/sae2.12030

3.1 Introduction

Grapevine (*Vitis vinifera*) supports a diverse microbial community, which modulates its health, growth and productivity (Rolli et al., 2017). The composition of the grapevine microbiome has attracted much attention in recent years due to its role in the establishment of the unique metabolic profile of wine, the product of grape fermentation (Liu et al., 2019). Recent studies using high-throughput sequencing verified the role of the indigenous grapevine microbiota in the establishment of the unique organoleptic profile of wine and identified certain microorganisms as producers of desirable aromatic metabolites (Bokulich et al., 2016; Liu et al., 2020). The composition of the grapevine microbiome differs between above-and belowground plant parts, with rhizosphere soil and roots supporting more diverse and distinct microbial communities compared to leaves and grapes (Deyett & Rolshausen, 2020; Zarraonaindia et al., 2015). Still, soil is the major reservoir of the grape microbiome (Liu et al., 2020; Morrison-Whittle & Goddard, 2018). The grapevine microbiome, from soil to endophytic and to grape must, follows nonrandom assembly mechanisms with biogeography, cultivar and vintage being the most important determinants (Bokulich et al., 2014; Miura et al., 2017; Portillo et al., 2016). The influence of these factors along with soil physicochemical properties, climatic conditions (Liu et al., 2020) and viticultural management practices (Morrison-Whittle et al., 2017) are collectively described as terroir (Leeuwen & Gerard, 2006). In this frame, Gilbert et al. (2014) suggested that the regional distinctiveness of a vineyard and its grapes is to a large extent associated with the grapevine microbiota and coined the term 'microbial terroir' to explain the particular composition of the grapevine microbiome.

Most previous studies have explored 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., 2018; Zarraonaindia et al., 2015), while the epiphytic grapevine microbiome(surface of leaves and berries) has attracted less attention (Portillo et al., 2016). In a pioneering study, Bokulich et al. (2014) showed that biogeography was the stronger factor affecting must microbiome, while cultivar and vintage were less important confounding factors affecting microbial composition.

Regardless of the grapevine tissue explored, most studies have tried to disentangle the effects of one (e.g., biogeography for a single cultivar or cultivar in different regions) (Liu et al., 2020; Zhang et al., 2020) or two of these factors (e.g., biogeography and cultivar) (Mezzasalma et al., 2018; Vitulo et al., 2019), while the combinatory effects of all three factors in the formation of the grapevine microbiome has not been explored in depth. The effect of biogeography has been studied at different scales: vineyard scale (Bokulich et al., 2016), viticultural zone scale (Knapp et al., 2021), national scale (Liu et al., 2020; Vitulo et al., 2019) and recently global scale for vineyard soils (Gobbi et al., 2022). All these studies agree that the degree of similarity of microbial community composition decays with increasing geographic distance and this implies that microbial community assemblages are influenced by spatial factors. Most of these studies have been performed 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 or150 km), where different terroir units are present, although this was not considered in the selection of samples (Bokulich et al., 2016; Liu et al., 2020) (iii) at the national level where the microbiome of a single or more cultivar found in different viticultural zones (>150 km) was determined (Chalvantzi et al., 2021; Li et al., 2022; Vitulo et al., 2019). Hence, we are still missing knowledge of which factors are operative at different geographical scales and shape the grapevine microbiota. This is particularly true for the epiphytic grapevine microbiota expected to be most affected by environmental conditions.

Other studies have highlighted the role of cultivars in shaping the grape microbiome. These effects were mostly attributed to the distinctive morphological and physiological features of the berries of the different grapevine cultivars (Bokulich et al., 2014; Portillo et al., 2016). Zhang et al. (2019) showed that, in the absence of spatial factors, grapevine genotype has a strong influence on the composition of the bacterial communities of leaf samples. Considering the great fluctuation in climatic conditions at the annual level, which is expected to magnify in the forthcoming years with climate change, vintage will play a major role on the assembly of the grapevine microbiome. Previous studies have highlighted the influence of vintage in shaping the

grapevine microbiome, although the magnitude of its effect could vary between fungi and bacteria (Bokulich et al., 2014; Liu et al., 2020).

We aimed to disentangle the role of biogeography, cultivar and vintage on the composition of the epiphytic grapevine microbiome when operated at different experimental scales (a) at the national level across different viticultural zones and (b) at a viticultural zone level across different terroir units. The main scientific questions set were: (i) how the influence and relative importance of bio-geography, cultivar and vintage on epiphytic grapevine micro-biome vary at different geographical scales, (ii) do fungal and bacterial epiphytic communities follow similar assembly mechanisms and do they respond differently to the factors shaping the epiphytic grapevine microbiome. To verify our hypotheses we determined, via amplicon sequencing, the bacterial and fungal communities on the surface of leaves and grapes of four important Greek grapevine cultivars (i) Vidiano and Agiorgitiko, each collected from vineyards located in three distinct viticultural zones in Greece (ii) Roditis and Sideritis, each collected from vineyards located in different terroir units of the same viticultural zone. Finally, we determined the core microbiome of the two studied cultivars in the viticultural zone of Aigialeia aiming to identify microorganisms which might carry specific beneficial traits for the local cultivars Roditis and Sideritis.

3.2 Materials and Methods

3.2.1. Grapevine samples

Leaves and grapes were collected during September and October (depending on the maturation time of each cultivar) of 2019 and 2020 from selected vineyards organised at two different geographic scales:(a) National geographical scale, which included four distinct viticultural zones in Greece, north (Thessaloniki), central (Athens), south (Crete) or south-west (Nemea, Peloponnese) and (b) Viticultural zone scale involving the viticultural zone of Aigialeia located in north Peloponnese, Western Greece (Supplementary Figure S1). Different cultivars were studied at different scales as dictated by their national and regional distribution. At the national scale, we sampled the cultivars Agiorgitiko and Vidiano, characterised by broader distribute on but also their availability in germplasm collections. At viticultural zones cale, we sampled the cultivars Roditis and Sideritis, which are of particular importance for the viticultural zone of Aigialeia, which is the highest altitude viticultural zone of Greece (862 m).

Grapes and leaves from the cultivars Agiorgitiko and Vidiano were sampled from the national germplasm collection of the Hellenic Agricultural Organization—Demeter in Thessaloniki, Thermi and Athens, Lykovrisi. In those collections, 10 grapevines of both cultivars are cultivated in two separate rows of the same vineyard, with a distance of 50–100m between them. Further samples were collected for Vidiano and Agiorgitiko from commercial vineyards in Herakleio, Crete and in Nemea, Peloponnese, respectively. All fields sampled were under organic cultivation and no pesticides were applied. Regarding Roditis and Siderits, samples were collected from two commercial vineyards per cultivar, each located in a different terroir unit. For Roditis, we sampled (i) a vineyard in the region Koutsoura representing terroir A. The vineyard is located at 800m altitude, being influenced by the sea climate (ii) a vineyard in the region Petsakoi representing terroir B. The vineyard is located at 530m altitude, being under the influence of the continental climate. For Sideritis, we collected samples from two commercial vineyards located: (i) in south Achaia, designated as terroir A and (ii) in the region of Diakofto, designated as terroir B. The details of the location of the vineyards sampled and the physicochemical properties of the soils of the different vineyards are given in Supplementary Table S1. The climatic characteristics of the different viticultural zones considered in the study are given in Supplementary Table S2. In each vineyard, six grapevines spatially distributed in the field were sampled in both years. From each grapevine, 10 well-developed and healthy leaves and two healthy grape bunches were collected with a sterile blade on the day of harvest. All samples were placed in sterilised plastic bags and immediately stored at −20°C until downstream analysis.

3.2.2. DNA extraction

The DNA from the epiphytic microorganisms colonising the leaves and berries of grapevines was done as described before (Katsoula et al., 2021). Briefly, 3–4 g of leaves and berries were submerged in isotonic phosphate buffered saline 1× solution and the cells of the epiphytic microorganisms were detached from the plant tissue surface through intermittent sonication in an ultrasonic bath for 8 min and vortexing every 2 min for 10 s. Subsequently, plant tissues were removed and the solution was centrifuged for 15 min at 9000 rpm and 4°C. The supernatant was removed and the obtained microbial pellet was resuspended in the extraction buffer of the DNeasy Power soil DNA isolation kit (QIAGEN), which was further used for DNA extraction.

The integrity of the extracted DNA was assessed via agarose gel (1%) electrophoresis and it was quantified using the Quant-iT kit with a Qubit Fluorometric device (Invitrogen).

3.2.3. Amplicon sequencing analysis

The bacterial and fungal communities of leaves and berries were determined via amplicon sequencing of the V4 region of the 16S rRNA gene and the ITS2 region, respectively, using a HiSeq Illumina platform in Rapid Mode for obtaining 2 × 250 bp paired-end reads of the amplicons, performed at Admera Health. Amplification, facilitating sample-wise multiplexing during sequencing, was based on our inhouse protocol described in detail in Vasileiadis et al. (2015). Briefly, extracted DNA was diluted to a final concentration of 1 ng μl−1 for fungi and 0.2 ng μl−1 for bacteria. Then a two-step amplification process was followed, which allowed the sample-wise amplicon indexing. In the first step, the bacterial 16S rRNA gene was amplified with the updated version of the primers 515f-806r (Lorenzini et al., 2019) following the protocol of the earth microbiome project (Lozupone et al., 2007). Regarding fungi, a first amplification step of the ITS2 region was performed with primers ITS7F-ITS4R (Martins et al., 2013). The first amplification step included 28 cycles using the primers mentioned above and was followed by a second polymerase chain reactions (PCR), of 7 cycles, using the same primers with sample-specific 5′ overhangs. All PCR were conducted with the use of Q5® High-Fidelity DNA Polymerase (NEB). Primers, PCR conditions and reagents can be found in Supplementary Tables S3 and S4, respectively.

3.2.4. Bioinformatic analysis

Amplicon libraries were cleaned up with the use of NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel) according to manufacturer instructions and they were sent for sequencing databases for bacteria and fungi, respectively. ASVs not classified at Kingdom and Phylum level were excluded from downstream analysis, whereas ASVs were classified to the lowest possible taxonomical rank having at least 80% annotation probability score. The sequencing data were submitted to the Sequence Read Archive of NCBI with BioProject accession number (ID: PRJNA834626).

3.2.5. Statistical analysis

The ASV matrices of bacteria and fungi were used to calculate α- diversity indices like 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) using the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. The parametric analysis of variance (ANOVA) followed by the Tukey's post hoc analysis or the nonparametric Kruskal–Wallis followed by the Wilcoxon rank-sum post hoc test (if the ANOVA conditions were not met), were used to assess the differences of α-diversity indices. Pairwise comparisons were performed using the Student's t-test or its non-parametric analog, the Wilcoxon rank sum test.

The ASV matrices of bacteria and fungi were also used to determine the effects of biogeography (or terroir units at viticultural zone scale), cultivar and vintage on the β-diversity of the epiphytic grapevine microbiome. Effects of the different factors on the β-diversity of bacteria and fungi were determined via nonmetric multidimensional scaling (NMDS) and permutational multivariate ANOVA (PERMANOVA). NMDS was performed with dissimilarity matrices using the Bray–Curtis algorithm, while the association of microbiome composition with the covariates of interest, the determination of the % of variance explained by those factors and the variation partitioning between factors were accessed with PERMANOVA using 999 permutations as implemented in the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. We employed distance-decay relationship analysis to determine the impact of geographical distance between sampling sites on the composition of the fungal and bacterial communities. The comparison between the microbial community Bray–Curtis dissimilarities and the physical distances was performed with Mantel tests (9999 permutations) using the Pearson correlation algorithm. Further, we constructed random forest models to reflect the contribution of each factor in the composition of the grapevine epiphytic microbiome as described by Bekris et al. (2021). Briefly, the data matrix was subsampled with replacement to equal read numbers. Then, random forest supervised classification models were performed by the pime v0.1.0 R-software package (Roesch et al., 2020), which relies on the randomForest v4.6.14 R-software package (Liaw & Wiener, 2002). Each random

forest model was run with 1000 trees, using 66% of the samples for building the model and 33% of the samples as out-of-bag (OOB) samples for the model error assessment. Differential abundance (DA) tests using the Kruskal–Wallis and the Wilcoxon rank-sum post hoc tests, were employed to determine bacterial and fungal taxa that were significantly associated with specific viticultural zones, cultivars or terroir units. p Value adjustment for multiple hypothesis testing was conducted with the false discovery rate (FDR) algorithm with implemented adjusted p value cut-off of 0.05. Heatmaps were produced to depict ASVs whose relative abundance was positively correlated with viticultural zones, cultivars or terroir units according to the DA tests. We also determined the core microbiome for the cultivars of Roditis and Sideritis in the viticultural zone of Aigialeia. We filtered microbial taxa (for each community) and retained those occurring in at least 60% of the samples in each cultivar at a consistent detection threshold of 0.1% relative abundance to reduce noise.

All diagrams were produced with the R package ggplot2 v3.3.3 while core heatmaps were constructed using the microbiome package (Shetty & Lahti, 2019). The full analysis code can be found in the Github repository in the link https://github.com/ELEPAP/ Grapevine_epiphytic_microbiome.

3.3.Results

3.3.1. The grapevine fungal community and factors shaping its structure at different geographical scales

3.3.1.1. The composition of the fungal community

In total, 2,541,923 nonchimeric quality sequences for fungi (3188–52,356) were obtained and they were assigned to 6699 ASVs. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the fungal diversity (Supplementary Figure S2a). Overall, the fungal phyllosphere and carposphere communities were composed of *Ascomycota* and *Basidiomycota*. Regardless of cultivars and regions, *Dothideomycetes* dominated the epiphytic fungal communities, while other fungal classes become important at different viticultural zones, cultivars or vintages (Supplementary Figure S3). For example, we noted a clearly higher relative abundance of *Agaricomycetes* in the carposphere and phyllosphere of Roditis, unlike the samples of Sideritis where *Leotiomycetes* and *Microbotryomycetes* showed an increase in their relative abundance in both years.

3.3.1.2. Factors affecting the α-diversity of the epiphytic fungal community of grapevine

When looking across viticultural zones biogeography and vintage had a significant effect on the α-diversity of fungi. Specifically, significantly higher values ($p < 0.05$) of richness (ACE and Observed) were evident in the samples from Athens compared to Thessaloniki and for all samples of 2019 compared to 2020 (Supplementary Figure S4). Within the viticultural zone of Aigialeia, we noted significantly higher values ($p < 0.05$) of all diversity indices in the cultivar Roditis compared to Sideritis, with terroir A of the former and terroir B of the latter showing significantly higher values ($p < 0.05$) between the two terroir units per cultivar (Supplementary Figure S5).

3.3.1.3. Factors affecting the β-diversity of the epiphytic fungal community of grapevine at different geographical scales

Preliminary analysis of the amplicon sequencing data suggested limited effect of grapevine tissue (leaves or berries) ($p > 0.05$) on the composition of the fungal community regardless of the regional scale studied. Hence, samples of leaves and berries were considered together (as a single sample type collectively identified as the epiphytic grapevine microbiome) in the analysis of the β-diversity. We first looked at the effect of different factors across viticultural zones. NMDS analysis grouped samples according to viticultural zone and vintage (Figure 1), while cultivar showed a weak clustering effect (Supplementary Figure S6). PERMANOVA-based variation partitioning analysis verified the stronger effect of the viticultural zone and vintage on the fungal community contributing 21.1% and 11.7% of the overall variance, respectively ($p < 0.001$), while cultivar explained only 2% of the variance ($p < 0.002$) (Table 1). Random forest analysis also showed that biogeography (viticultural zone) and vintage were the best explanatory variables with low OOB error values (1.25% and 0%, respectively), compared to cultivar with OOB error value of 20.64% (Supplementary Figure S7a).

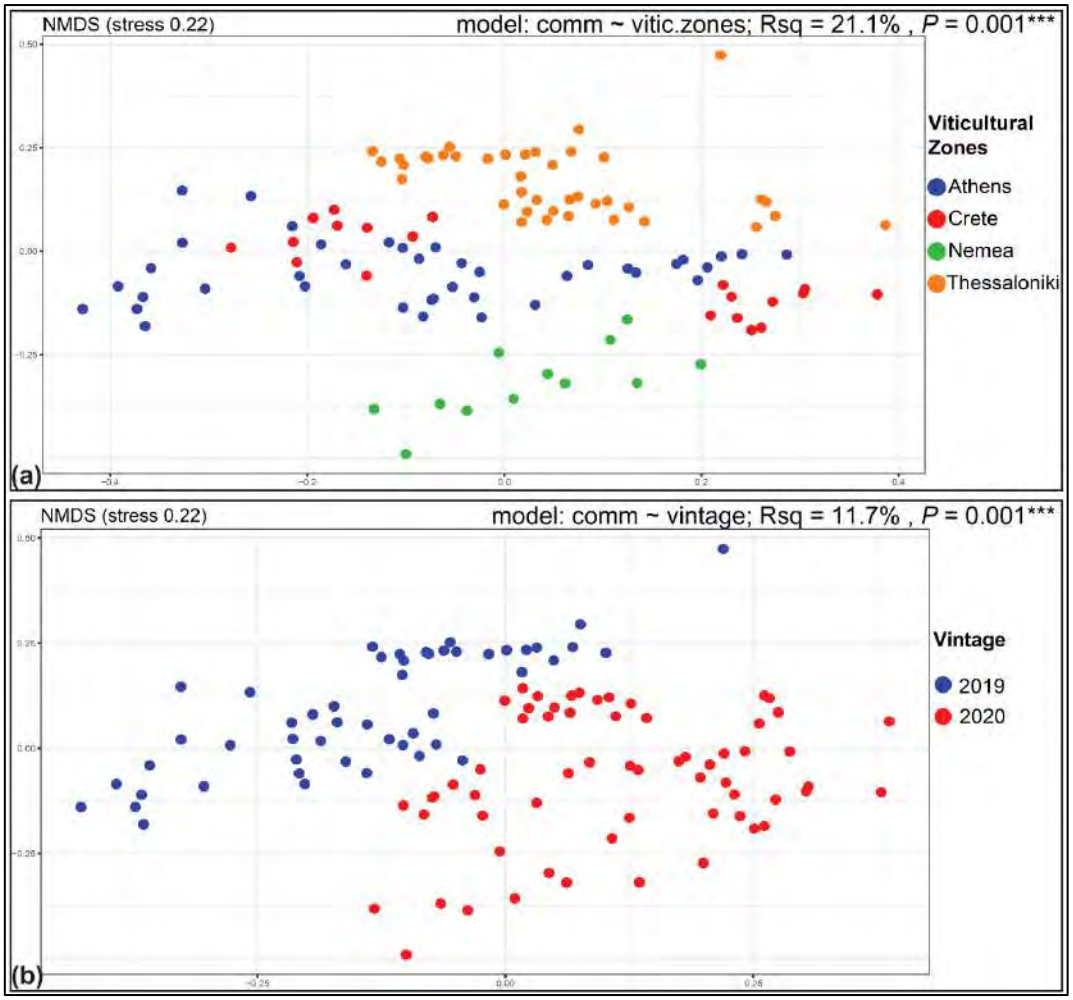


Fig.1. NMDS ordination of the fungal microbiome in grapevine samples collected from the cultivars Agiorgitiko and Vidiano located invineyards from different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea). Samples are ordinated according to viticultural zone(a)and vintage (b). Variance partitioning and thepvalue for the factor studied are given in each graph. NMDS, nonmetric multidimensional scaling.

When the data were analysed at the viticultural zone scale, we noted a clear grouping of samples according to cultivar (Figure 2a), while vintage seemed to have a weaker clustering effect (Supplementary Figure S8c). PERMANOVA verified the stronger effect of cultivar (21.3% of the variance) compared to vintage (4.9% of the variance, $p < 0.001$) (Table 1). The terroir unit was identified as the most important contributor in the variance of the fungal data set (26.9%, $p < 0.001$). Further analysis of the β-diversity separately for each cultivar showed a clear clustering of the samples of the different terroir units sampled per cultivar (Figure 2b,c), while vintage seemed to contribute to sample grouping within each cultivar (Supplementary Figure S8a,b). PERMANOVA verified the strong effect of terroir unit in structuring the fungal

community per cultivar with the effect varying between cultivars, being higher for Sideritis (54.5%, $p < 0.001$) compared to Roditis (20.9%, $p < 0.001$) (Table 1). Random forest analysis also showed that the explanatory variables studied (terroir unit, cultivar and vintage) showed particularly low OOB error values (1.04%, 0% and 0%, respectively) (Supplementary Figure S7b).

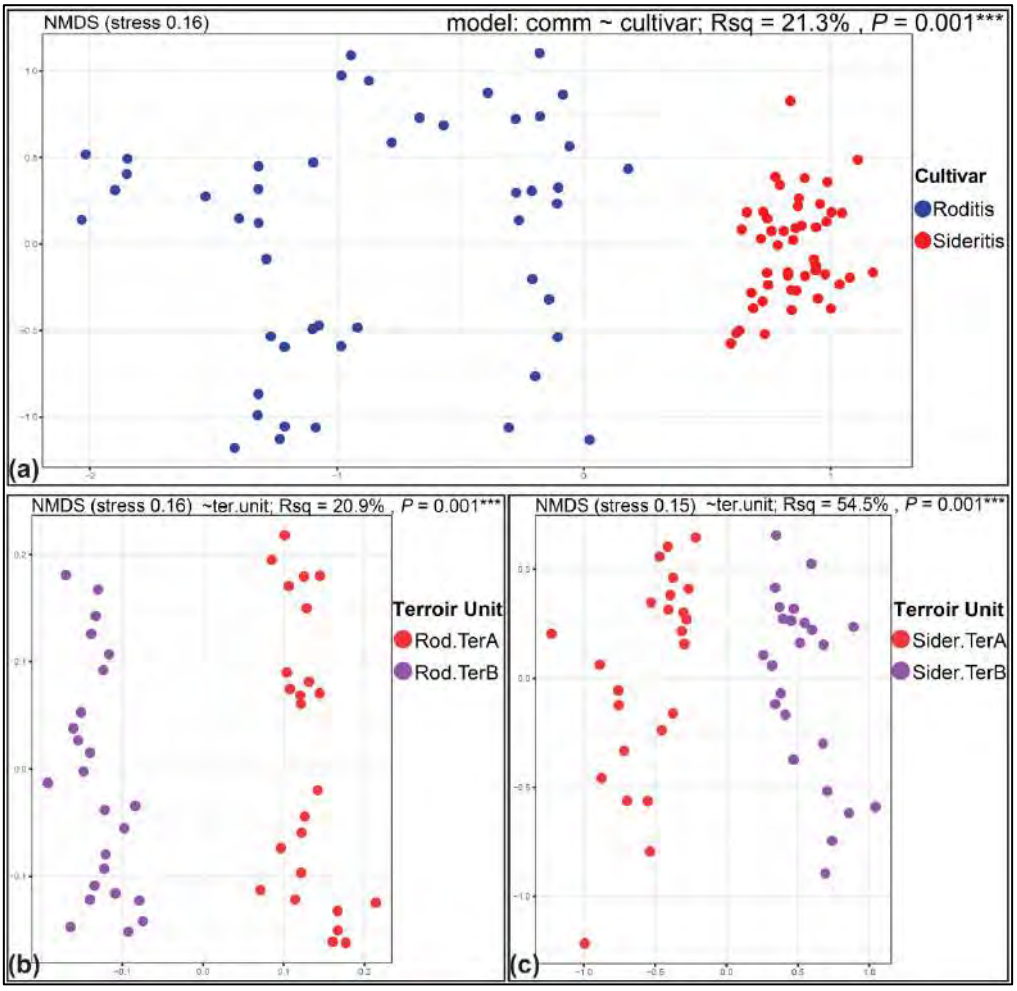


Fig.2. NMDS ordination of the fungal microbiome in grapevine samples collected from the cultivars Roditis and Sideritis located in vineyards from different terroir units in the viticultural zone of Aigialeia. Samples are ordinated according to the cultivar (a). Further, samples of the cultivar Roditis (b) and Sideritis (c) were separately analysed and ordinated according to terroir units. Variance partitioning and the p value for the factor studied are given in each graph. NMDS, nonmetric multidimensional scaling.

Distance-decay relationship analysis for the two datasets showed significant correlations between the dissimilarity index values and the geographical distances (a) within the viticultural zone of Aigialeia (0–60 km, Mantel test r = 0.43, p < 0.0001) with linear regression analysis r2 = 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 r2 = 8.6% and p < 2.2e−16 (Supplementary Figure S9).

3.3.1.4. Fungal ASVs as signatures of viticultural zones at national geographical scale

We further identified members of the fungal community whose relative abundance was associated with a particular region. Highly abundant *Alternaria* (e.g., 00001, 00032, 00018, 00019) and *Nigrospora* (00027) ASVs were associated with more than one viticultural zone (Figure 3a). ASVs belonging to *Aureobasidium*, *Pyronema*, *Ustillago* were associated with the viticultural zone of Nemea, and *Cladosporium*, *Devriesia* were correlated with Crete. ASVs belonging to *Didymellaceae*, *Periconia*, *Ustilaginaceae*, *Pseudopithomyces*, *Curvularia* and *Xylariales* were positively correlated with Thessaloniki, while a *Ganoderma* ASV showed a positive correlation with Athens.

3.3.1.5. Fungal ASVs as signatures of cultivars Roditis and Siderits at viticultural zone scale

We noted a strong positive association of the relative abundance of ASVs belonging to *Helotiales*, *Aureobasidium*, *Ganoderma*, *Peniophora* with the cultivar Roditis and *Nigrospora*, *Sporobolomyces*, *Sporidiobolaceae*, *Vischniacozyma* with the cultivar Sideritis (Figure 3b). We also determined the core microbiome of the cultivars Roditis and Sideritis, which was composed of (i) dominant ASVs (*Alternaria*, *Aureobasidium*, *Cladosporium*, *Capnodiales*, *Pleosporaceae*, *Vishniacozyma*), that were shared between the two cultivars and represented cumulatively >60% of the total fungal community and (ii) several less abundant ASVs which were unique per cultivar as shown in Supplementary Figure S10 and Table S5.

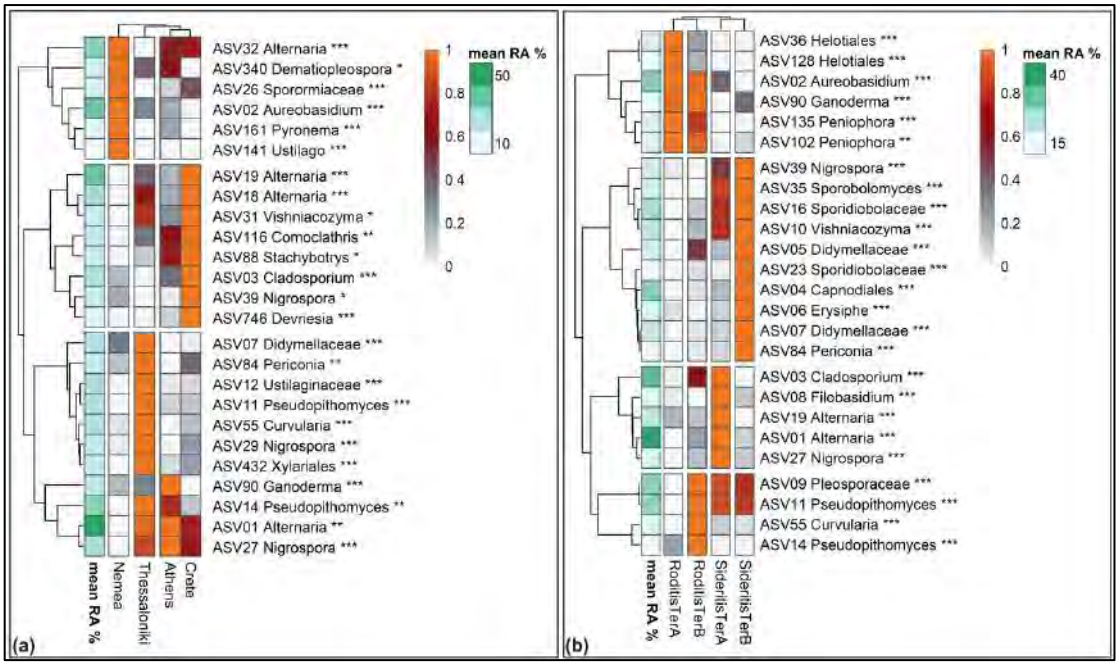


Fig.3. DA heatmaps showing fungal ASVs whose relative abundance showed a significant positive correlation ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***) with specific viticultural zones (Athens, Thessaloniki, Nemea/Crete) (a) or specific cultivars (Roditis/Sideritis) in the viticultural zone of Aigialeia (b). ASVs, amplicon sequence variants; DA, differential abundance.

3.3.2. The grapevine bacterial community and factors shaping its structure at different geographical scales

3.3.2.1. The composition of the bacterial community

In total, 3,641,387 quality sequences for bacteria (902–90,893) were obtained and they were assigned to 36,187 ASVs. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the bacterial diversity (Supplementary Figure S2b). The grapevine bacterial community was dominated by *Proteobacteria* (53.8% average relative abundance), *Actinobacteriota* (18.46%), *Firmicutes* (12.7%), *Bacteroidota* (5.54%), *Myxococcota* (2.49%) and *Cyanobacteria* (1.97%) (Supplementary Figure S11).

3.3.2.2. Factors affecting the α-diversity of the epiphytic bacteria community of grapevine

Across viticultural zone we noted a significantly higher α-diversity ($p < 0.05$) of the epiphytic bacterial community in grapevines in the regions of Thessaloniki and Athens compared to Crete, whereas cultivar and vintage showed no significant effects (Supplementary Figure S12). When looking within the viticultural zone of Aigialeia,

none of the factors studied had a significant effect on the α-diversity of the epiphytic bacterial community (Supplementary Figure S13).

3.3.2.3. Factors affecting the β-diversity of the epiphytic bacterial community at different geographical scales

As with the fungal microbiome, the carposphere and phyllosphere supported similar bacterial communities and hence samples from leaves and berries we considered together in our analysis. We first looked at the bacterial assemblages in the different viticultural zones. NMDS analysis showed a weak clustering of the samples according to viticultural zone and vintage (Figure 4), whereas no clustering of the samples according to the cultivar was evident (Supplementary Figure S14). PERMANOVA-based variation partitioning analysis verified the weak but still statistically significant contribution of biogeography (6.7%, $p < 0.001$) and vintage (3.5%, $p < 0.001$) to the data set variance, while cultivar showed an even weaker explanatory power (1.0%, $p < 0.044$) (Table 1). Random forest analysis verified the weak explanatory strength of all variables with OOB error values varying from 6.03% for vintage to 33.96% and 41.15% for viticultural zone and cultivar, respectively (Supplementary Figure S7c).

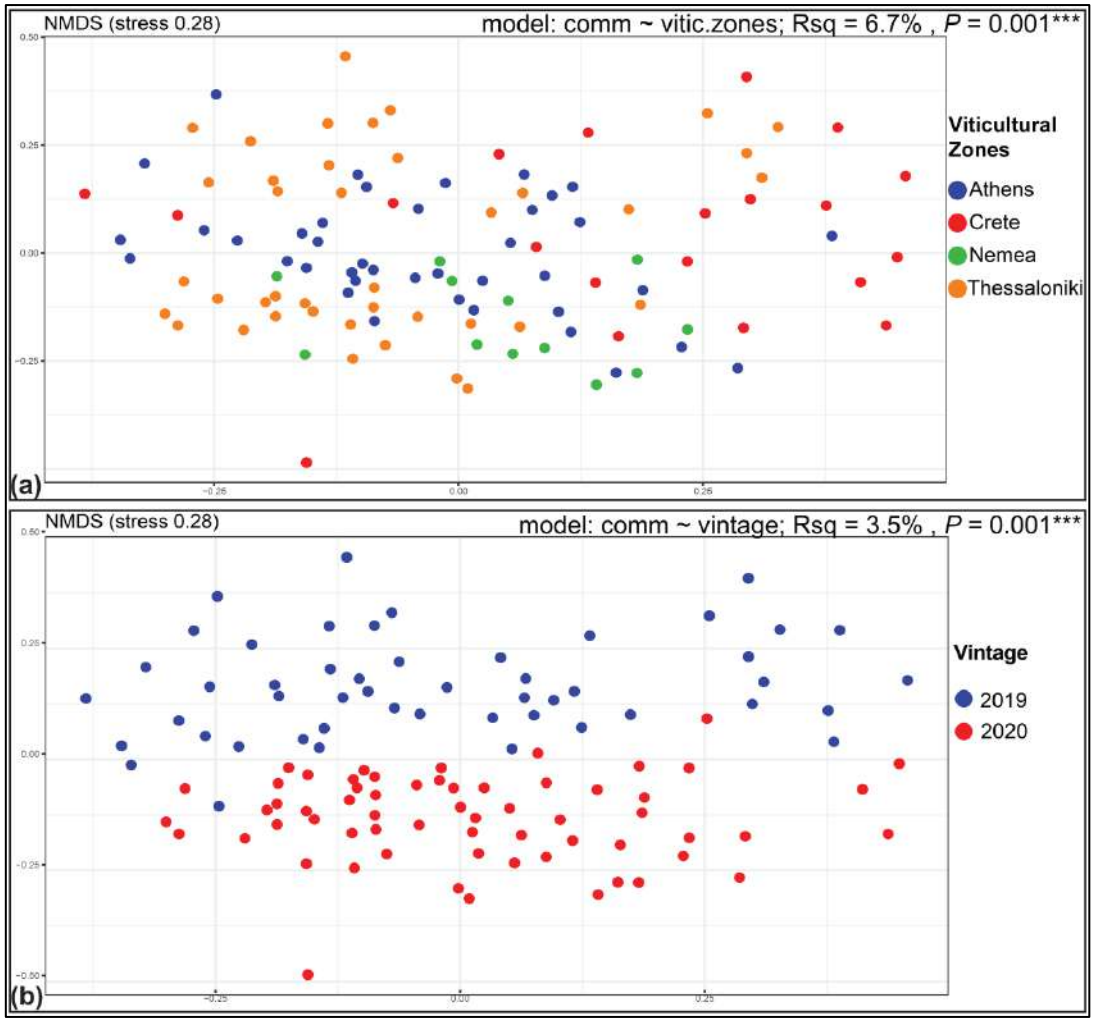


Fig.4. NMDS ordination of the bacterial microbiome in grapevine samples collected from the cultivars Agiorgitiko and Vidiano located in vineyards from different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea). Samples are ordinated according to viticultural zone (a) and vintage (b). Variance partitioning and the p value for the factor studied are given in each graph. NMDS, nonmetric multidimensional scaling.

When looking at the factors shaping the epiphytic bacterial community at a single viticultural zone, in Aigialeia, NMDS analysis revealed a significant clustering of samples primarily according to cultivar and secondly based on vintage (Figure 5). PERMANOVA verified the stronger effect of these two factors on the β-diversity of the epiphytic bacterial community with cultivar explaining 6.1% ($p < 0.001$) and vintage 4.7% ($p < 0.001$) of the total variance. Beyond these, we noted that terroir unit significantly affected the composition of the epiphytic bacterial community explaining 4.8% ($p < 0.001$) of the variance (Table 1). When looking the effect of terroir unit (and vintage) within each cultivar, we observed a better clustering of the samples according to the vintage rather along the terroir unit in the case of Roditis, while a weaker grouping of the Sideritis samples between cultivars. In the case of Roditis

vintage showed a higher explanatory strength (8.6%, $p < 0.001$) compared to terroir units (3.2%, $p < 0.009$), unlike Sideritis where both vintage and terroir unit had equivalent explanatory strength (6.9% and 7.4%, respectively, $p < 0.001$) (Table 1). Random forest analysis further highlighted the weak explanatory strength of all variables with OOB error values varying from 11.46% for vintage and 12.5% for cultivar to 34.38% for terroir unit (Supplementary Figure S7d). according to the terroir unit and vintage was evident (Supplementary Figure S15). PERMANOVA showed that the explanatory strength of vintage and terroir unit for the bacterial community varied Distance-decay relationship analysis for the epiphytic bacterial communities showed a weak effect of geographical distances at viticultural zone scale (Mantel test $r = 0.06$, $p < 0.05$) with linear regression analysis r2 of 0.3% and $p < 9.2e{-}5$ (Supporting Information: Figure S16). In contrast, when looked across viticultural zones we noted a stronger correlation of the community dissimilarities with increasing geographical distances (Mantel test $r = 0.30$, $p < 0.0001$, linear regression r2 = 8% and $p < 2.2e{-}16$).

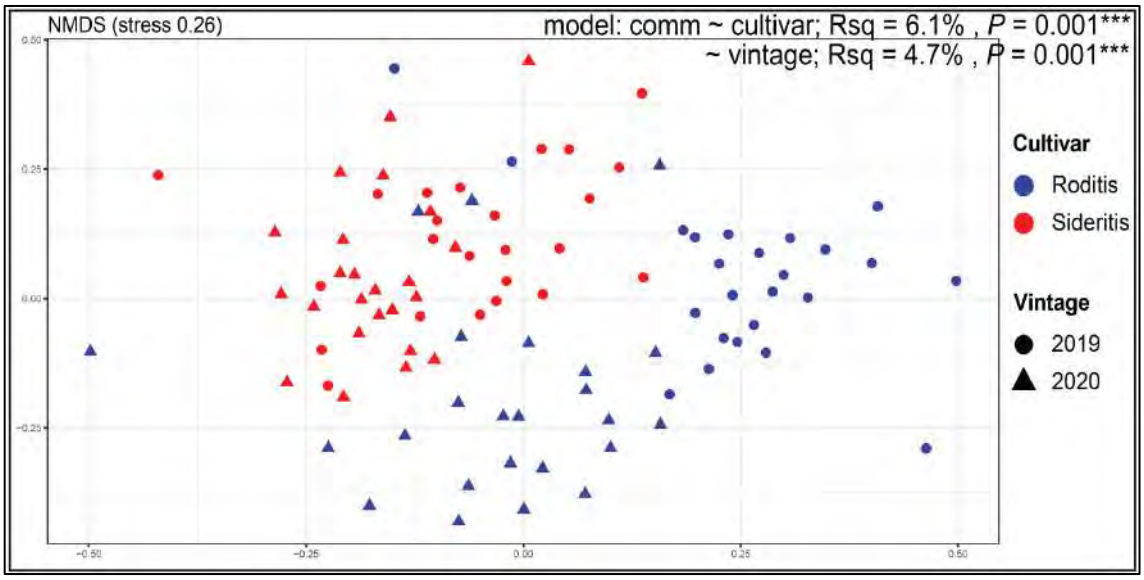


Fig.5. NMDS ordination of the bacterial microbiome in grapevine samples collected from cultivars Roditis and Sideritis located in vineyards from different terroir units in the viticultural zone of Aigialeia, Greece. Samples are ordinated according to the cultivar (colour code) or vintage (caption code). Variance partitioning and the p value for the factor studied are given in each graph. NMDS, nonmetric multidimensional scaling.

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	18.9616	11.7	0.001***
Cultivar	1	0.3058	3.2609	2.0	0.002**
Viticultural zone	3	3.2153	11.4289	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***
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: Statistical significance levels: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.
Abbreviation: PERMANOVA, permutational multivariate analysis of variance.

3.3.2.4. Bacterial ASVs as signatures of viticultural zones at national geographical scale

Skermanella and *Massilia* ASVs were positively associated with more than one region (Figure 6a). ASVs belonging to *Sphingomonadaceae* were positively associated with Nemea. ASVs belonging to *Staphylococcus* and *Streptococcus* were associated with Crete, while ASVs belonging to *Enterobacterales* were associated with Athens and Thessaloniki (Figure 6a)

3.3.2.5. Bacterial ASVs as signatures of cultivars Roditis and Sideritis at viticultural zone scale

ASVs belonging to *Streptococcus* (2) and *Staphylococcus* (1) were positively associated with Roditis, while several ASVs belonging to *Sphingomonas* (7) and *Methylobacterium* (7) were positively associated with Sideritis (Figure 6b). The epiphytic core bacterial microbiome of the two cultivars was composed of unique to each cultivar low abundance ASVs. The core bacterial microbiome of Roditis was composed of *Sphingomonas*, *Staphylococcus*, *Masillia*, *Methylobacterium*, *Acinetobacter*, *Streptococcus*, *Corynebacterium*, *Hymenobacter*, *Skermanella* and *Acetobacteraceae* (Supplementary Figure S17 and Table S6), whereas the core bacterial microbiome of Sideritis encompassed ASVs assigned to *Blastococcus*, *Skermanella*, *Staphylococcus*, *Methylobacterium*, *Masillia*, *Sphingomonadaceae*, *Microbacteriaceae* and *Hymenobacter*.

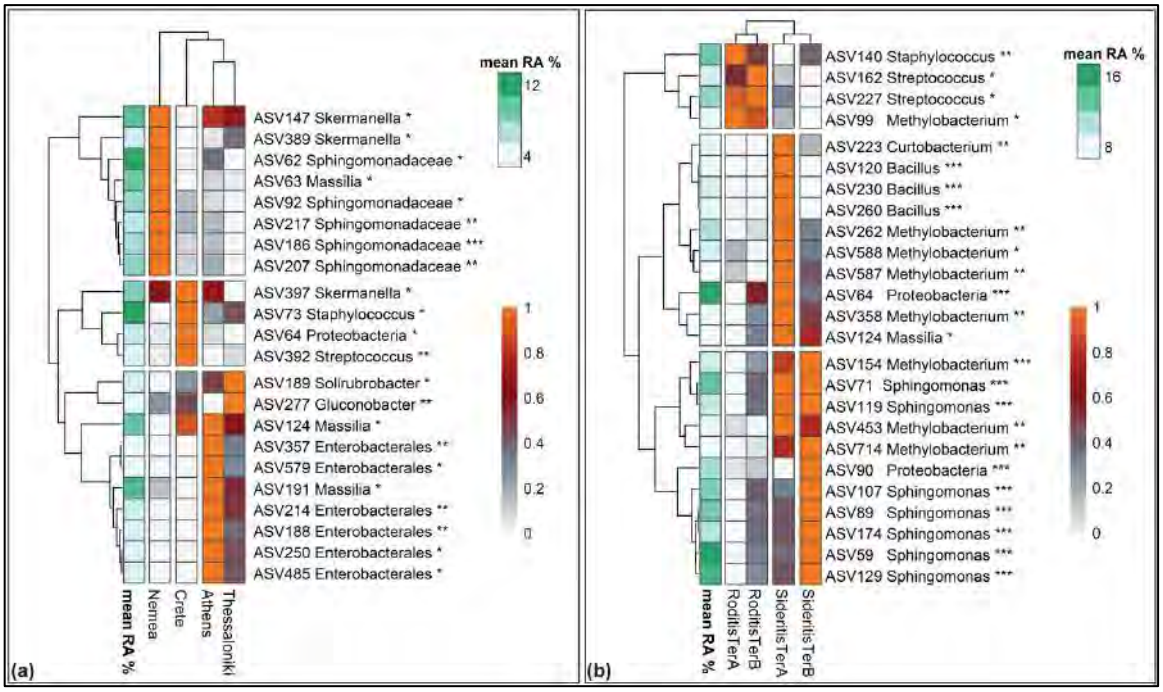


Fig.6. DA heatmaps showing bacterial ASVs whose relative abundance showed a significant positive correlation ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***) with specific viticultural zones (Athens, Thessaloniki, Nemea/Crete) (a) or specific cultivars (Roditis/Sideritis) in the viticultural zone of Aigialeia (b). ASVs, amplicon sequence variants; DA, differential abundance.

3.4. Discussion

The grapevine microbiome is the main reservoir of microorganisms affecting the quality and chemical profile of wine. We focused on the epiphytic phyllospheric and carpospheric fungal and bacterial communities colonizing the interface between plant surface and the atmosphere in selected Greek grapevine cultivars at different geographical scales.

3.4.1. Factors shaping the epiphytic fungal community in grapevine

We first asked the question if different factors are operative in shaping the epiphytic grapevine microbiome at different geographical scales. While analysing the grapevine microbiome across different viticultural regions (distances 100–>600 km) we noted that geographic location was the strongest factor shaping the epiphytic fungal grapevine communities, followed by vintage, whereas cultivar had a weak effect as depicted by variation partitioning and verified by random forest analysis. Only a few studies have explored the factors shaping the epiphytic fungal community of grapevine at large geographical scales. Kioroglou et al. (2019) reported strong regional signatures in the carposphere fungal community of Shiraz and Cabernet Sauvignon cultivars located in two geographically distant viticultural zones in Australia, while cultivars and vintage showed a less important effect. Previous studies on the must fungal microbiome at similar geographical scales also reported strong regional patterns in the composition of the fungal community 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). When the effect of other confounding factors like vintage and cultivar was investigated at the national geographical scale, only vintage seemed to have an appreciable effect. Bokulich et al. (2014) reported a weak vintage effect on the composition of the fungal must community, which further varied along the studied cultivars. When compared with the must microbiome, the epiphytic fungal community is more heavily exposed to environmental and climatic conditions, hence the influence of vintage on the latter is expected to be more prominent.

To provide a comprehensive answer to our initial hypothesis, we further investigated the epiphytic fungal community at a viticultural zone scale, where environmental condition variations and distances between vineyards were <100 km.

Unlike most studies which have explored grapevine microbiome assemblage mechanisms at regional level in randomly selected vineyards, we studied the epiphytic microbiome of the two cultivars in vineyards assigned to distinct terroir units of the viticultural zone of Aigialeia. We noted that at regional scale, the cultivar becomes the most important determinant of the composition of the epiphytic fungal community as depicted by variation partitioning analysis and verified by random forest analysis. Singh et al. (2018) also showed that within the same viticultural zone cultivar appears to be the main determinant of the grape fungal community. Cultivar-specific traits like the colour and the thickness of the skin of berries and bunch features have been identified as having a major influence on the carposphere microbiota (Bokulich et al., 2014; Mezzasalma et al., 2018). In our study, both cultivars produce white to pink coloured berries but they differ in their other characteristics: The berries of Sideritis are big and thick-skinned grown in particularly large but not very dense bunches, unlike Roditis, which is a small-berried cultivar with semithick skin that is characterised by not very big and dense bunches. These differences may explain the strong cultivar filtering effect on the epiphytic grapevine microbiome of Roditis and Sideritis.

Interestingly within each cultivar, the terroir unit, seems to be the most important factor shaping the fungal community with its effect being more pronounced for the cultivar Siderits. This was most probably a function of the largely different microclimatic conditions, landscape and longer geographical distance between the two terroir units of Sideritis compared to Roditis. Indeed, Miura et al. (2017) showed that the assembly of epiphytic fungal communities follow distance-decay patterns, which are still operative even at the viticultural zone scale, in line with our distance–decay relationship analysis for the fungal community in the viticultural zone of Aigialeia. Overall, these results support our initial hypothesis and suggest that the epiphytic fungal community is structured by different factors at different geographical scales: biogeography at large geographical scales (across viticultural zones) and cultivar at the viticultural zone level.

3.4.2. Factors shaping the epiphytic bacterial communities in grapevine

We extended our study to the bacterial epiphytic microbiome to answer our second scientific question if the fungal and bacterial epiphytic grapevine communities are subject to different assemblage mechanisms. At national geographic scale, the

bacterial community followed the same structural patterns as fungi with biogeography being the most important determinant, followed by vintage and cultivar, which showed marginal effects. Similar structural patterns were also evident at the viticultural zone scale, where cultivar and secondly vintage become important determinants of the epiphytic bacterial microbiome. For each cultivar, terroir unit seems to influence the bacterial community with its effect being more prominent for Sideritis, a result attributed, as explained above, to the longer distance between the two studied terroir units of this cultivar. Despite the largely similar structural patterns of the bacterial and the fungal grapevine communities, the effect of the studied factors (biogeography, cultivar, terroir unit and vintage) on the bacterial communities was weaker as demonstrated by variance partitioning analysis and reinforced by random forest analysis. Distance–decay relationship analysis verified the weak effect of geographical distance on epiphytic bacterial communities at viticultural zone scale, which becomes significant only across viticultural zones. Previous studies have also showed that the epiphytic and must bacterial communities are less affected by distance compared to fungi (Bokulich et al., 2014; Liu et al., 2020; Miura et al., 2017). The dispersal limitations of fungi compared to bacteria, attributed to the large size propagules of the former (1–30 μm diameter) compared to the latter (0.25–8 μm diameter), prevent long-distance dispersal in the atmosphere (Smets et al., 2016; Wilkinson et al., 2012). Our findings suggest that the composition of the epiphytic bacterial grapevine community is determined by the same factors as the fungal community, although their effects are much weaker, suggesting the contribution of other variables that were not considered in our study.

3.4.3. Members of the epiphytic grapevine microbiome showing regional signatures

We further looked in the epiphytic grapevine microbiome for members that were associated with specific regions (across viticultural zones) or cultivars (within viticultural zone). Regardless of the geographical scale, the epiphytic fungal community was dominated by *Alternaria*, *Aureobasidium*, *Cladosporium* and *Nigrospora*. *Aureobasidium* is a ubiquitous nonfermentative yeast that has been reported (*A. pullulans*) to act antagonistically against grapevine plant pathogenic fungi (Pantelides et al., 2015). *Alternaria* and *Cladosporium* are cosmopolitan fungi identified as saprobes, plant pathogens and plant beneficial microorganisms (Del Frari et al., 2019; Lorenzini & Zapparoli, 2014). *Nigrospora* could act as regulators of

grapevine metabolism (Yang et al., 2016). An interesting observation was the clear association of *Pyronema*, known for their capacity to degrade pyrolyzed organic matter (Fischer et al., 2021), with the Nemea region, most probably a result of the recent fires in the Nemea viticultural zone in 2017. The epiphytic grapevine bacterial community was characterised by the ubiquitous presence of *Skermanella* and *Massilia* in more than one geographical location. Members of these genera have been reported as members of the epiphytic grapevine microbiome (Leveau & Tech, 2011; Mezzasalma et al., 2018), although their potential function is unknown.

3.4.4. The core epiphytic microbiome of the cultivars Roditis and Sideritis

We further focused on the Aigialeia region and we determined the core microbiome of the cultivars Roditis and Sideritis, showing strong filtering effects on the epiphytic grapevine microbiome. The core fungal microbiome was dominated by a few highly abundant ASVs belonging to *Alternaria*, *Aureobasidium*, *Cladosporium* and *Capnodiales* that were shared by the two cultivars. On the contrary, the bacterial core microbiome was composed of unique and not numerically dominant members per cultivar with the most abundant belonging to *Sphingomonas*, *Masillia* and *Methylobacterium*. Bacteria of these genera are dominant members of the carposphere and phyllosphere of grapevine (Martins et al., 2013). *Sphingomonas* and *Methylobacterium* are known as biological control agents (Innerebner et al., 2011) or plant growth promoters, respectively (Kutschera, 2007). Bokulich et al. (2012) showed that Sphingomonas and *Methylobacterium* could survive the vinification process, although their oenological contribution remains unknown (Bokulich et al., 2012). On going core microbiome-driven culturomics will aim to isolate epiphytic microorganisms from the grapevine microbiome and assess their capacities as plant growth promoters or biological control agents.

3.5 Conclusions

We analysed the epiphytic grapevine microbiome in four emblematic Greek cultivars at different geographical scales to identify the factors shaping the epiphytic grapevine microbiome at the national and regional scale levels. We showed that different factors are operative at different geographical scales: biogeography was the major determinant of the epiphytic grapevine microbiome across viticultural zones, whereas cultivar becomes the most important determinant at the viticultural zone scale. The

epiphytic fungal grapevine community was more responsive to the studied structural factors and exhibited clear distance–decay patterns regarding community similarity at both geographic scales, unlike the bacterial community whose composition showed a weaker response to the confounding factors being most probably driven by factors not considered in the current study.

3.6 References

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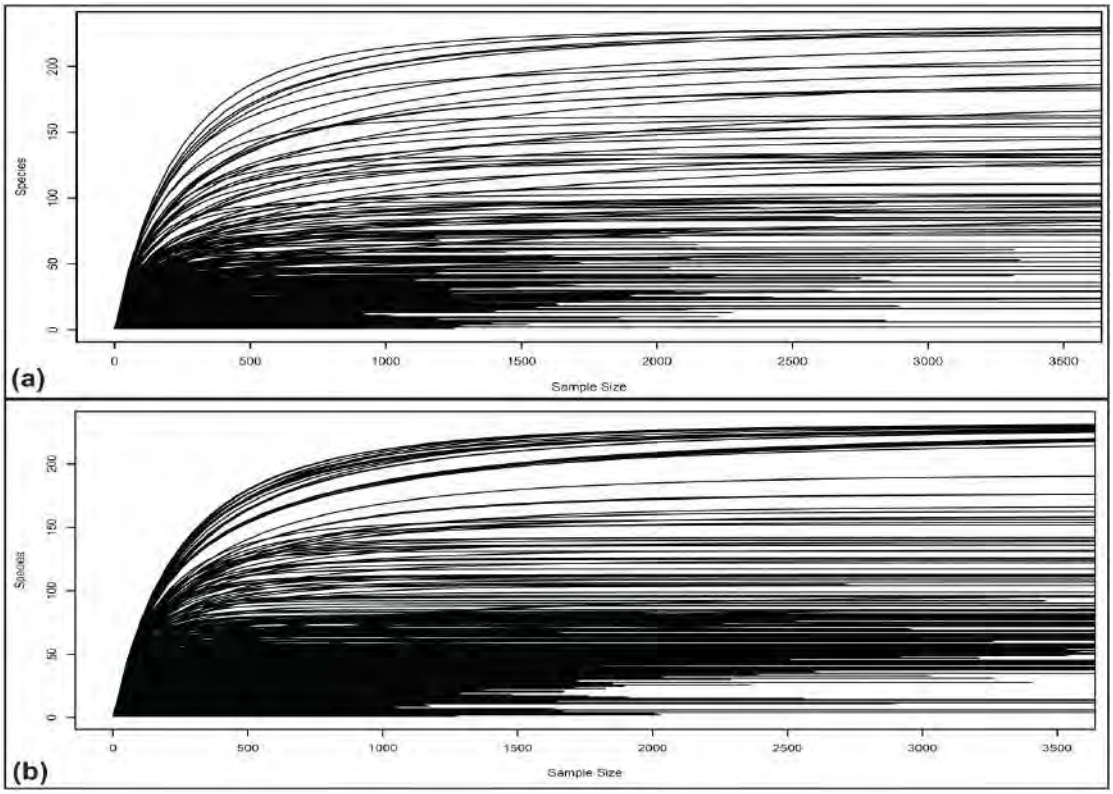
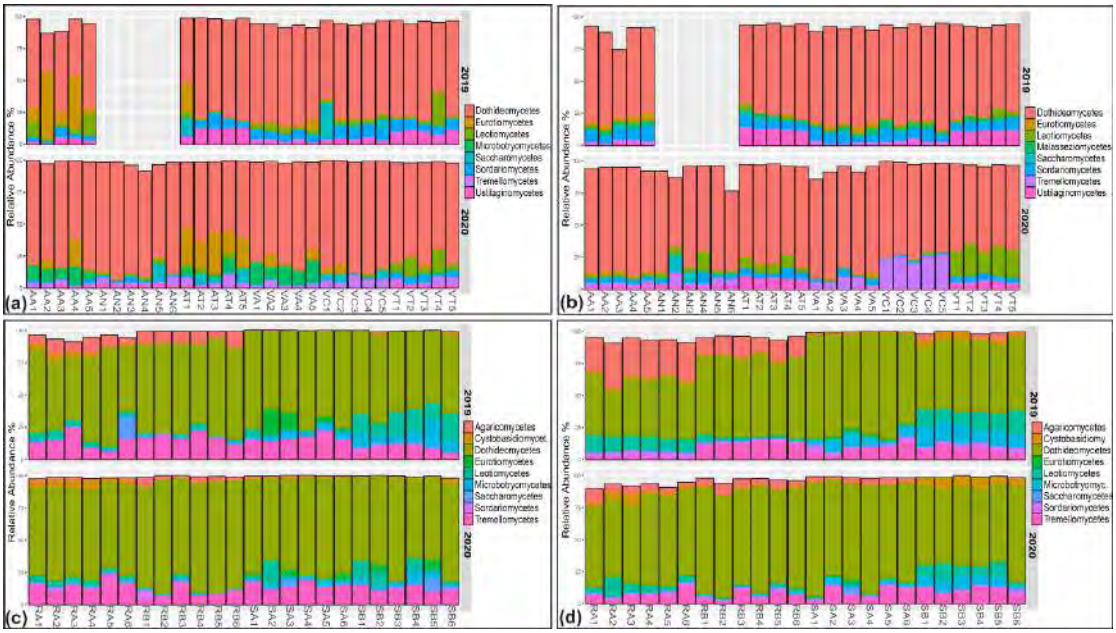
3.7. Annex II – Supplementary data

Supplementary Figure S1. A map of Greece showing the location of the vineyards that were sampled in the different region in Greece, while in the inserted map we show the location of the vineyards cultivated with Roditis and Sideritis in the Aigialeia viticultural zone.

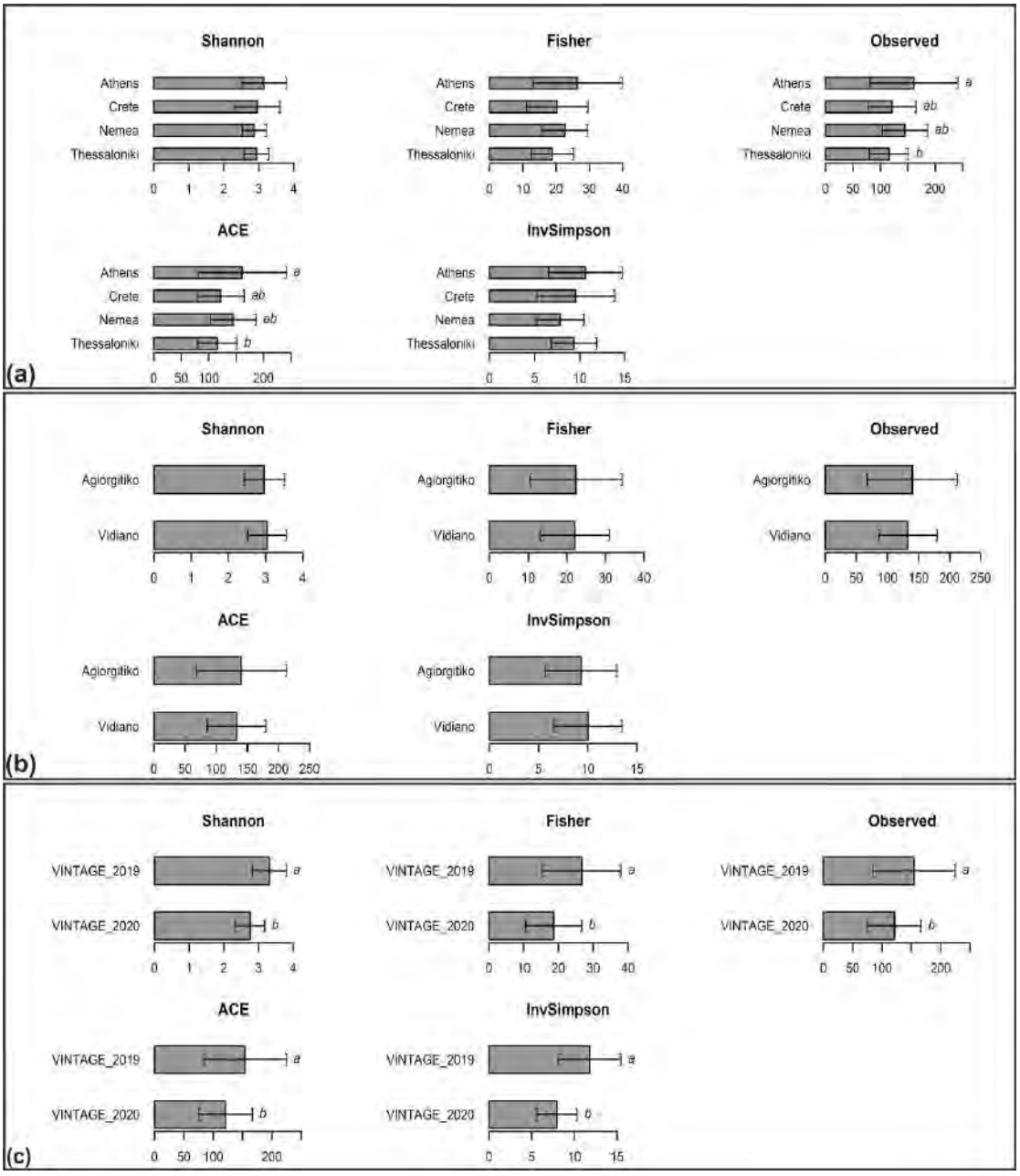


Supplementary Figure S2. Rarefaction curves for berry and leaf samples analyzed for their fungal (a) and bacterial microbiome (b).

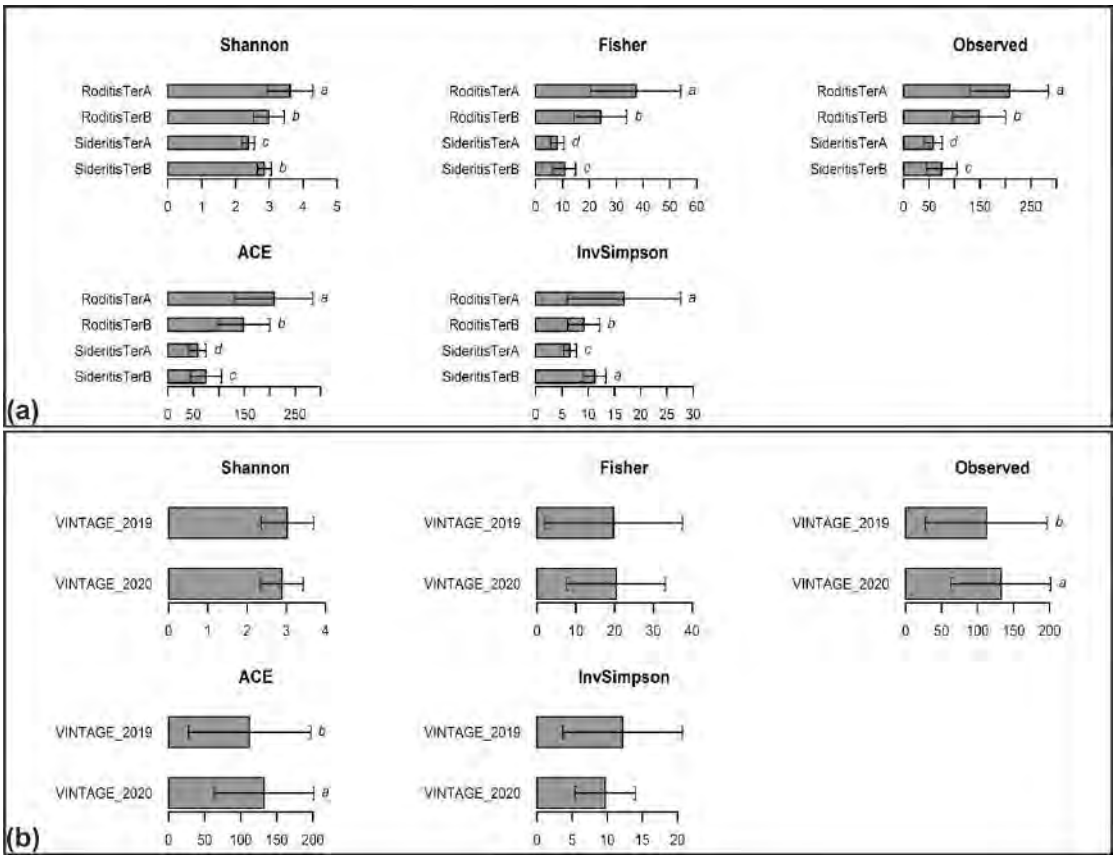


Supplementary Figure S3. Stacked bar plots showing the composition of the fungal microbiome (Class taxonomic level) on the surface of berries (a, c) and leaves (b, d) of the cultivars Agiorgitiko and Vidano (a, b) cultivated at different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea) or of the cultivars Roditis and Sideritis (c, d) cultivated in different terroir units in the viticultural zone of Aigialeia. Codes of samples: (i) first letter represents the cultivar A: Agiorgitiko, V: Vidiano, R: Roditis and S: Sideritis (ii) second letter

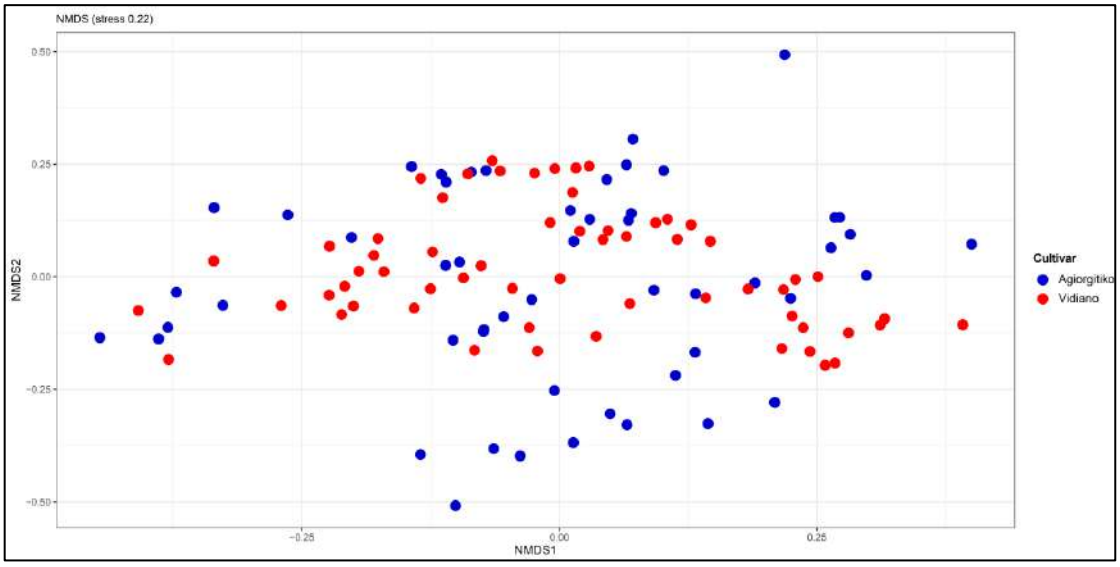
represents the geographic region A: Athens, T: Thessaloniki, C: Crete, N: Nemea or Terroir Unit: A or B (iii) Number indicates replicate number.



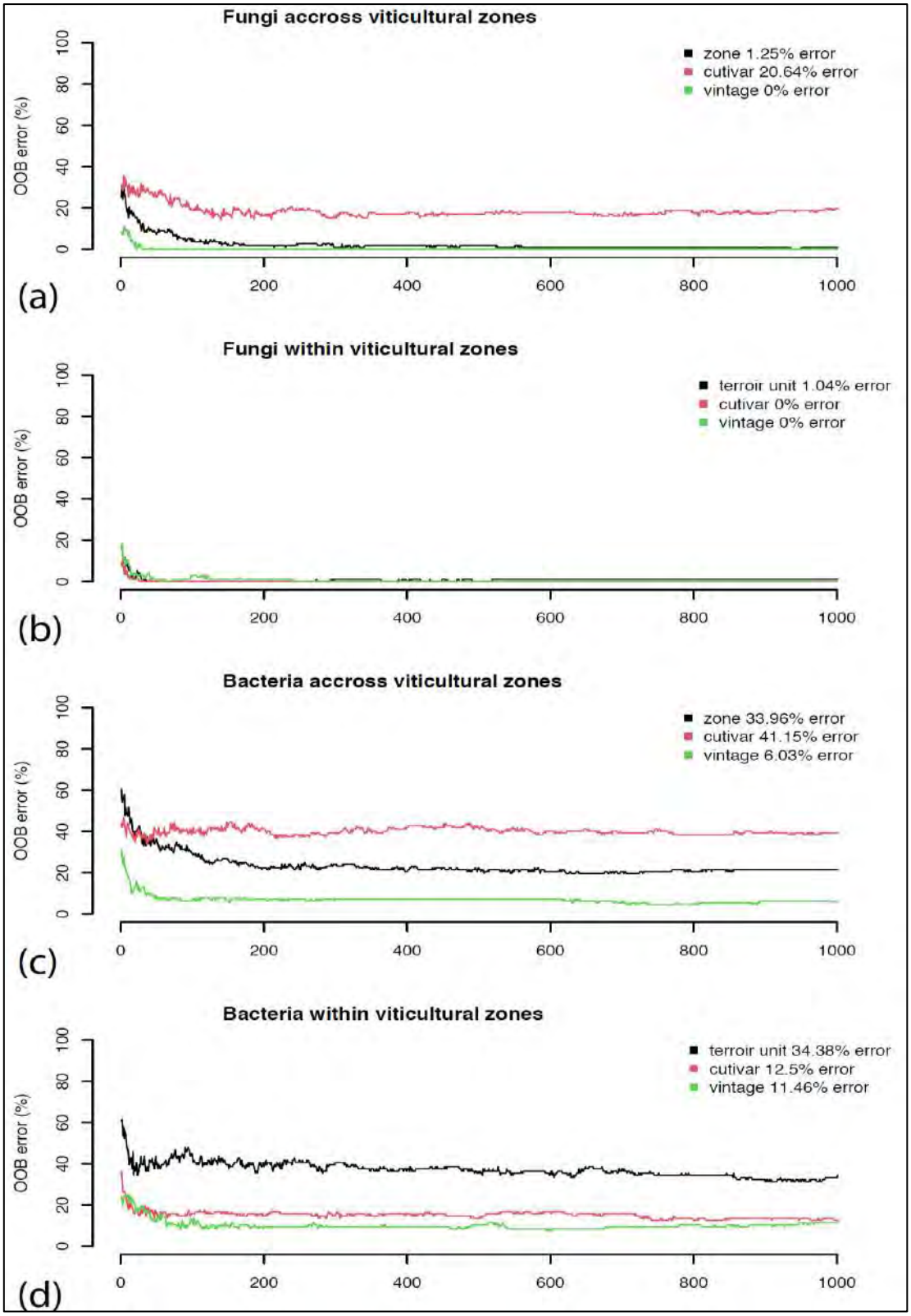
Supplementary Figure S4. The α-diversity indices calculated for the epiphytic fungal community in grapevine samples collected from different viticultural zones. Data are presented according to (a) viticultural zones (b) cultivar and (c) vintage (α 0.05 for performed statistical tests).



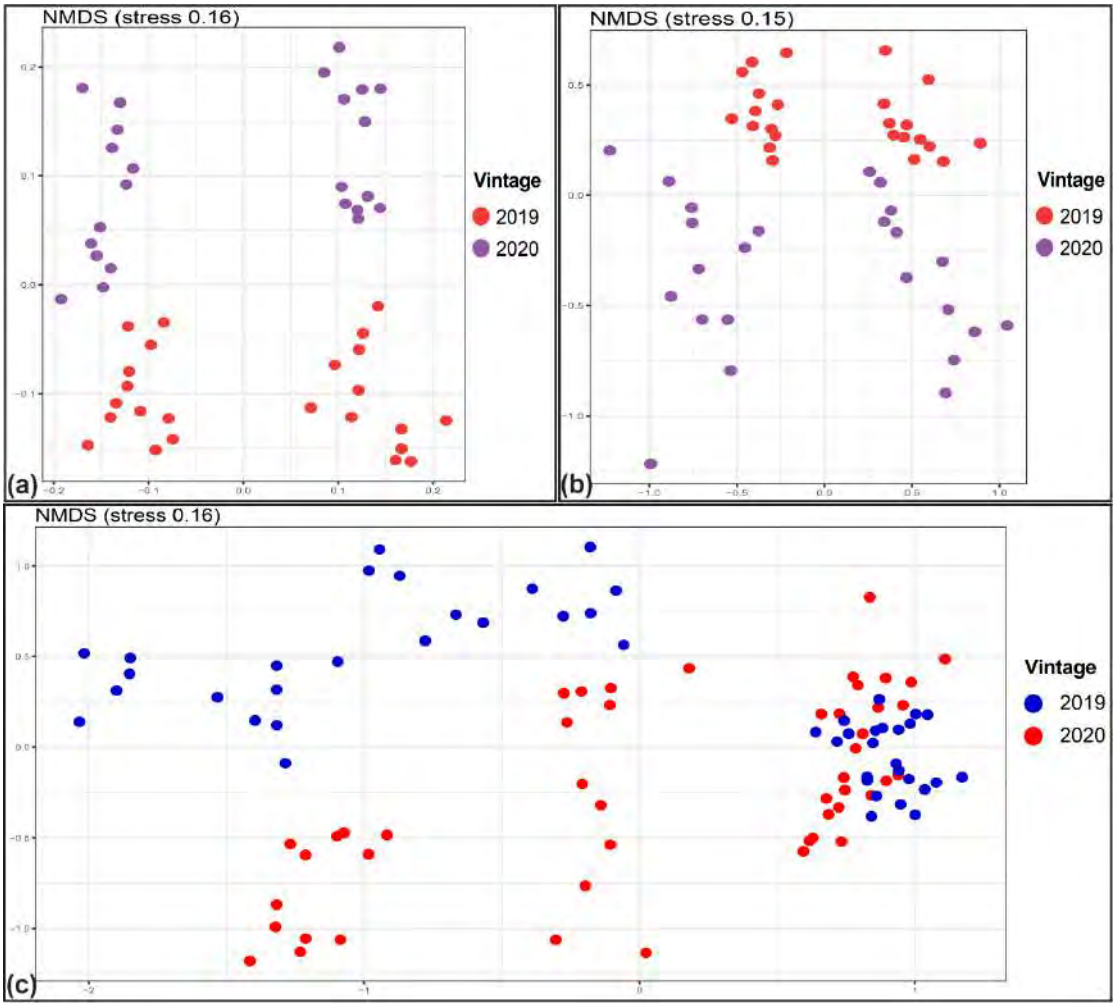
Supplementary Figure S5. The α-diversity indices calculated for the epiphytic fungal community in grapevine samples collected from the viticultural zone of Aigialeia. Data are presented according to (a) cultivar and terroir unit (b) vintage (α 0.05 for performed statistical tests).



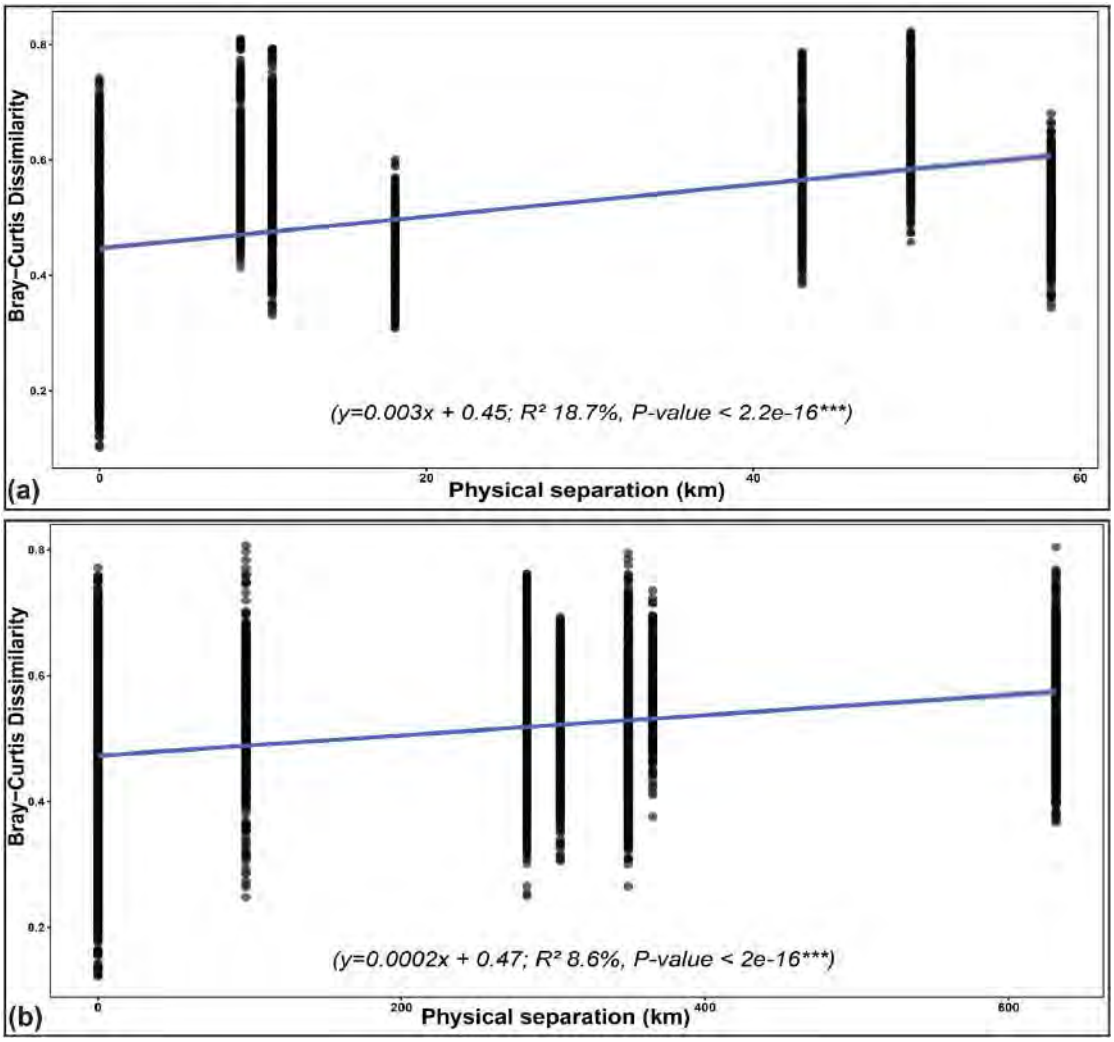
Supplementary Figure S6. NMDS ordination of the fungal microbiome in grapevine samples collected from vineyards with the cultivars Agiorgitiko and Vidiano located at different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea). Samples are ordinated according to their cultivar.



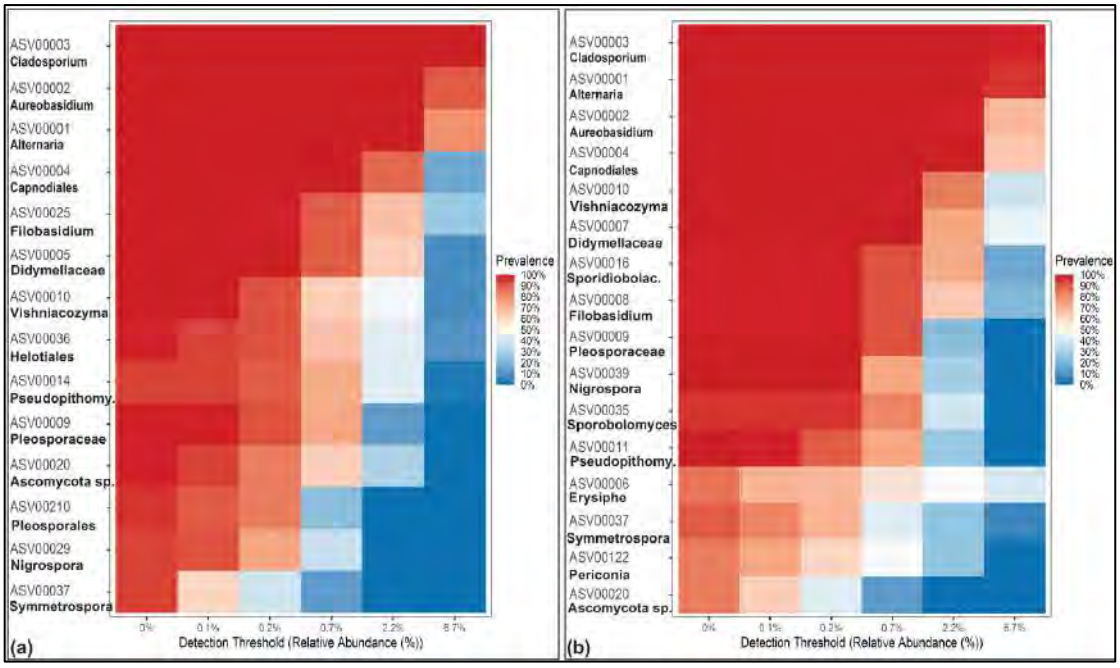
Supplementary Figure S7. Prediction accuracy of the tested experimental parameters of viticultural zone / *terroir* unit, cultivar, and vintage for the fungal or bacterial datasets across and within viticultural zones (a-d as provided in the panel titles). The out of bag (OOB) error rates for each tested independent variable (see color keys) are provided along the Y-axis, for each cumulative tree of the 1001 total trees (along the X-axis) used for building the models.



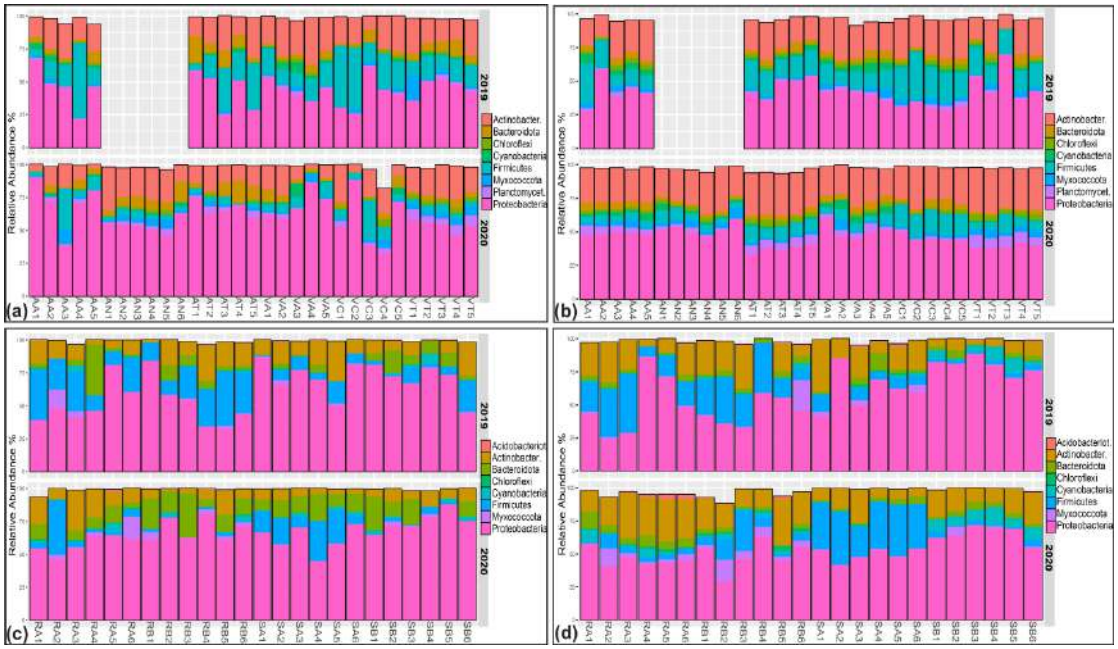
Supplementary Figure S8. NMDS ordination of the fungal microbiome in grapevine samples collected from the cultivars (a) Roditis, (b) Sideritis, (c) Roditis and Sideritis grown in different *terroir* units in the viticultural zone of Aigialeia. Samples are ordinated according to vintage.



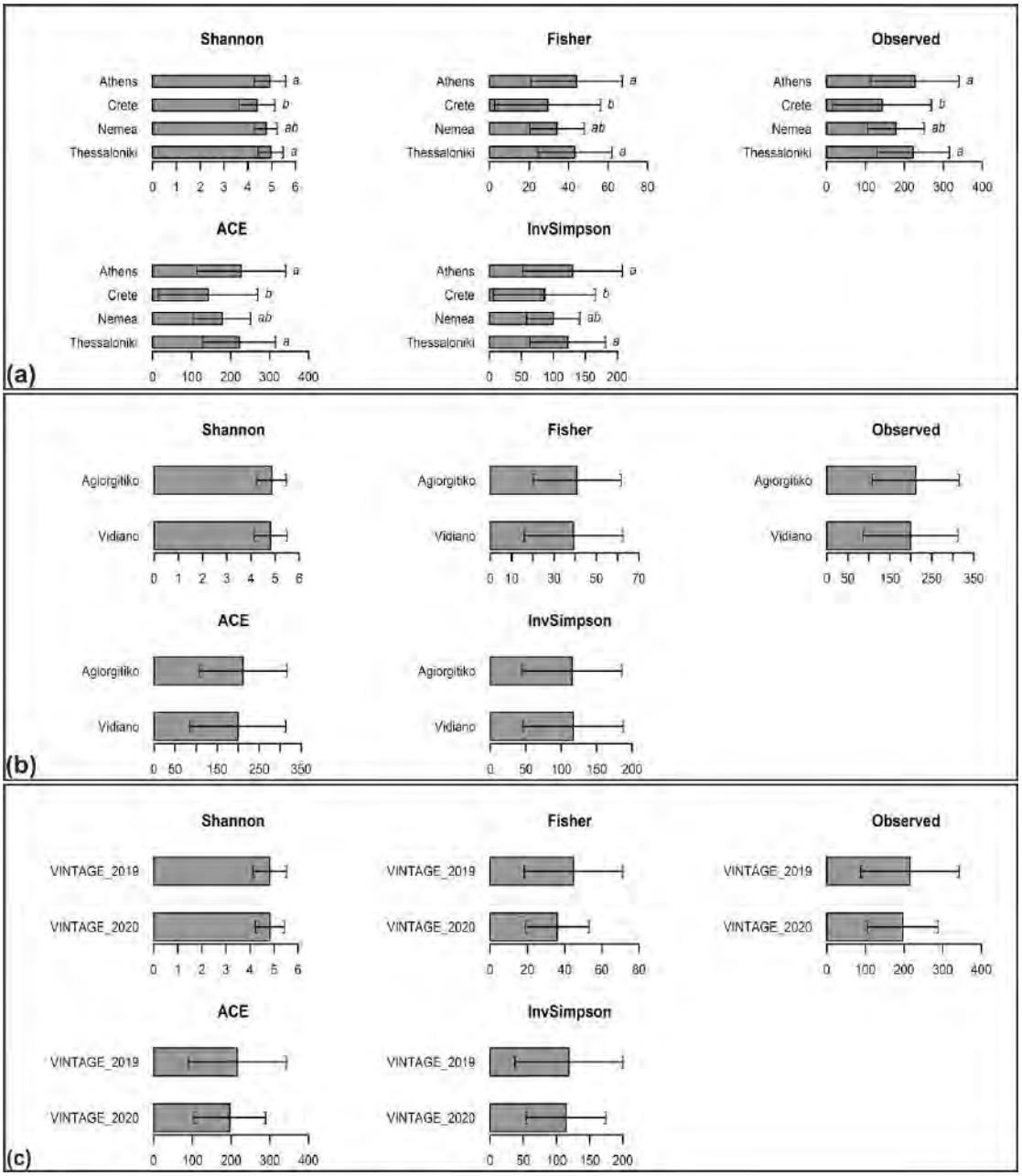
Supplementary Figure S9. Relation between physical distance and Bray-Curtis dissimilarity index illustrating the increase of fungal community dissimilarity with increasing geographic distance (a) within viticultural zones for the cultivars Roditis and Sideritis (Mantel test, r=0.43, p<0.0001) and (b) across viticultural zones for the cultivars Agiorgitiko and Vidiano cultivars (Mantel test, r=0.30, p<0.001). Linear regression analysis is also presented at the bottom of each graph (regression formula, R^2 and P values).



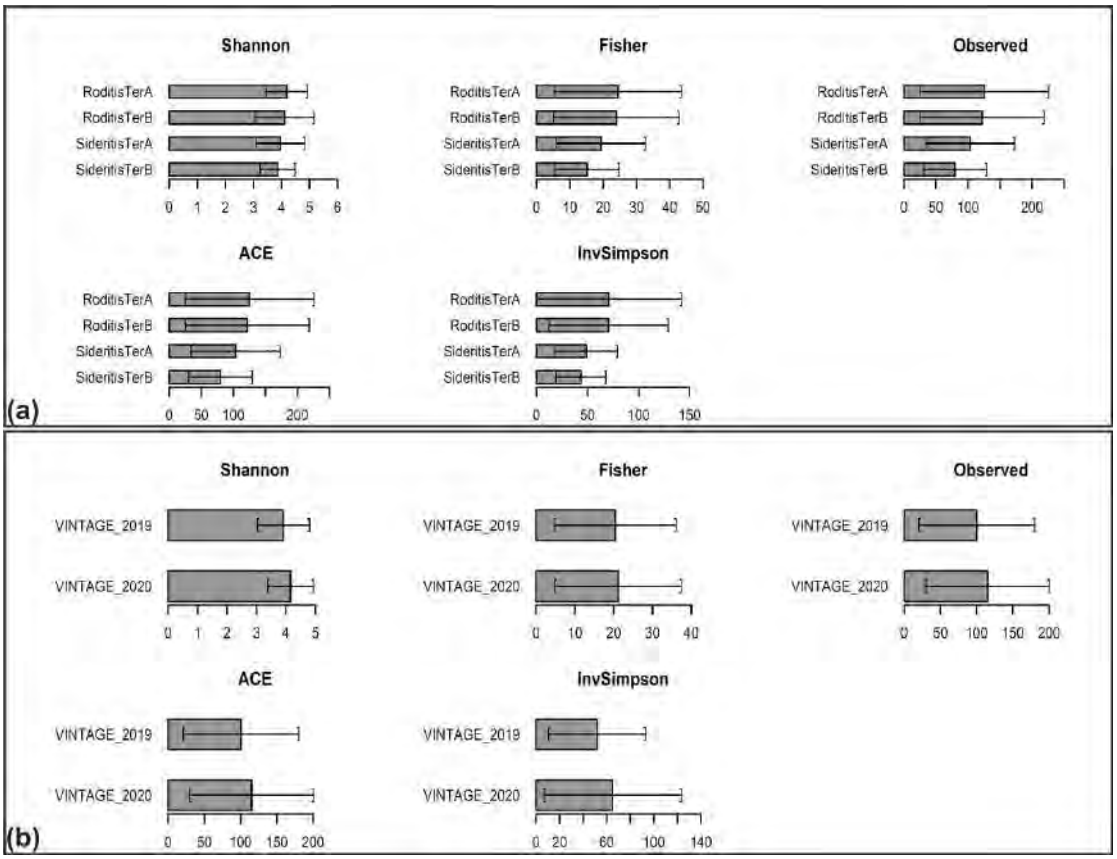
Supplementary Figure S10. The core fungal microbiome of the cultivars Roditis (a) and Sideritis (b) in the viticultural zone of Aigialeia.



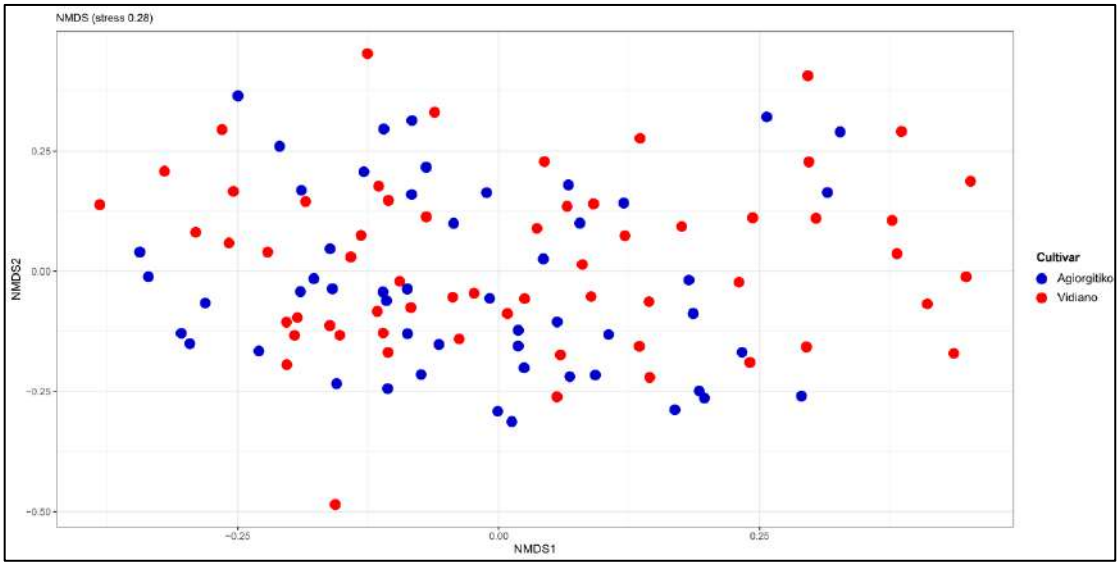
Supplementary Figure S11. Stacked bar plots showing the composition of the bacterial microbiome (Phylum taxonomic level) on the surface of berries (a, c) and leaves (b, d) of the cultivars Agiorgitiko and Vidano (a, b) cultivated at different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea) or of the cultivars Roditis and Sideritis (c, d) cultivated in different terroir units in the viticultural zone of Aigialeia. Codes of samples: (i) first letter represents the cultivar A: Agiorgitiko, V: Vidiano, R: Roditis and S: Sideritis (ii) second letter represents the geographic region A: Athens, T: Thessaloniki, C: Crete, N: Nemea or Terroir Unit: A or B (iii) Number indicates replicate number.



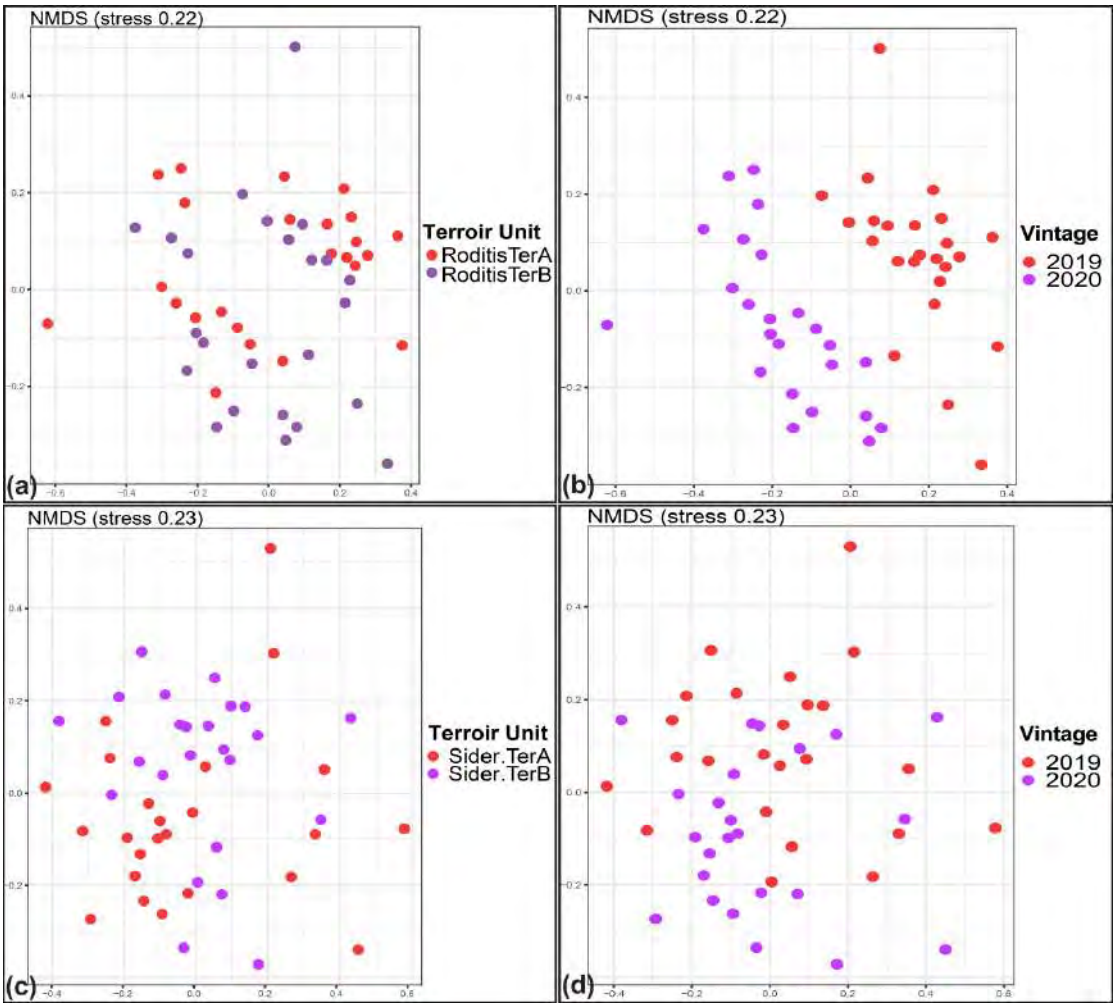
Supplementary Figure S12. The α-diversity indices calculated for the epiphytic bacterial community in grapevine samples collected from different viticultural zones. Data are presented according to (a) viticultural zone (b) cultivar and (c) vintage (α 0.05 for performed statistical tests).



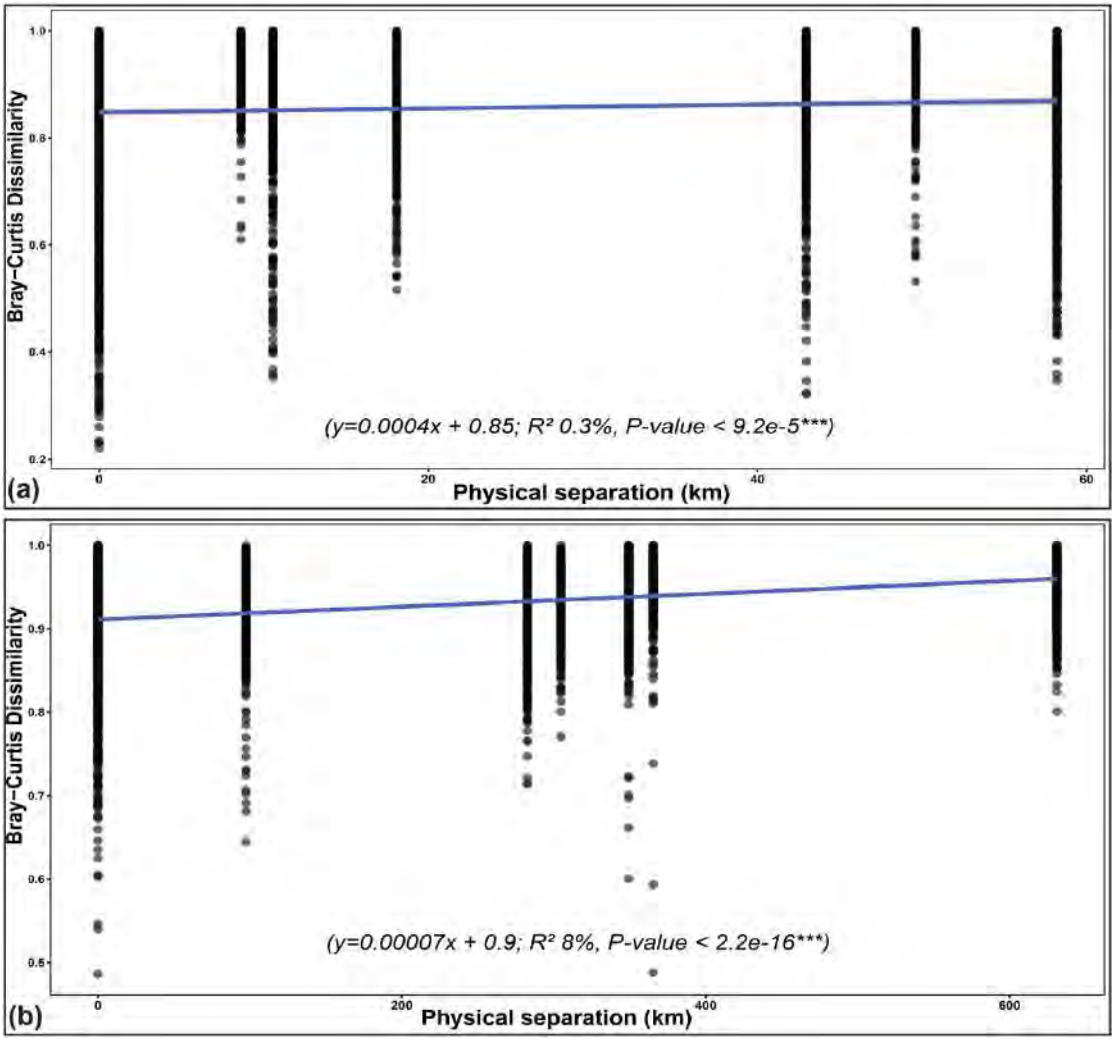
Supplementary Figure S13. The α-diversity indices calculated for the epiphytic bacterial community in grapevine samples collected from the viticultural zone of Aigialeia. Data are presented according to (a) cultivar and terroir unit (b) vintage (α 0.05 for performed statistical tests).



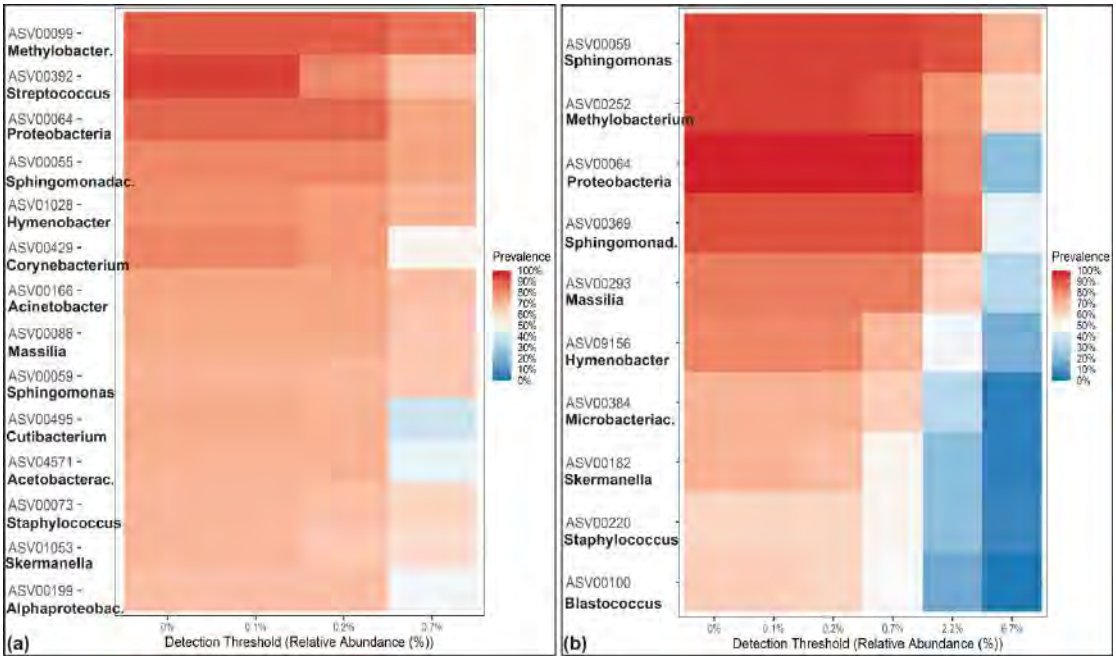
Supplementary Figure S14. NMDS ordination of the bacterial microbiome in grapevine samples collected from vineyards with the cultivars Agiorgitiko and Vidiano located at different viticultural zones in Greece (Thessaloniki, Athens, Crete/Nemea). Samples are ordinated according to their cultivar.



Supplementary Figure S15. NMDS ordination of the bacterial microbiome in grapevine samples collected from the cultivars Roditis (a, b) and Sideritis (c, d) grown in different terroir units in the viticultural zone of Aigialeia. Samples are ordinated according to terroir units (a, c) and vintage (b and d).



Supplementary Figure S16. Relation between physical distance and Bray-Curtis dissimilarity index illustrating the increase of bacterial community dissimilarity with increasing geographic distance (a) within viticultural zones for Roditis and Sideritis cultivars (Mantel test, r=0.06, *p*=0.05) and (b) across viticultural zones for Agiorgitiko and Vidiano cultivars (Mantel test, r=0.30, *p*=0.0001). Linear regression analysis is also presented at the bottom within brackets (regression formula, R^2 and *P* values).



Supplementary Figure S17. The core bacterial microbiome of the cultivars Roditis (a) and Sideritis (b) in the viticultural zone of Aigialeia.

Supplementary Table S1: A list of the vineyards sampled during the study.

Location	Coordinates	Cultivar	Vineyard	Altitude (m)	Soil physicochemical properties					
					Soil texture[a]	pH[b]	Organic Matter (%)[c]	Electrical Conductivity (ds/m)[b]	CEC (meq/100g)	Total N (mg/kg)[d]
Thessaloniki	40° 32' 11.58" N 23° 0' 26.262" E	Agiorgitiko, Vidiano	Genotype collection	21	Sandy loam	7.55	2.15	0.933	27.4	0.23
Athens	38°4' 10.6284" N 23°46' 30.4068" E	Agiorgitiko, Vidiano	Genotype collection	199	Sandy Clay loam	7.40	1.07	0.89	13.1	0.13
Crete	35°5'33.2736"N 25°0'52.0128"E	Vidiano	Commercial	365	Clay loam	6.50	1.25	0.99	30.5	0.29
Nemea	37°48'28.65"N 22°42'34.36"E	Agiorgitiko	Commercial	800	Clay loam	7.48	1.35	0.32	30.9	0.42
Koutsoura	38º10'20.09"N 22º5'22.99"E	Roditis	Commercial	800	Clay loam	4.52	1.20	1.51	36.78	0.37
Petsakoi	38°5'43.76"N 22°1'6.71"E	Roditis	Commercial	530	Clay	7.20	3.40	1.45	44.00	0.47
Karya (South Achaia)	38°11'29.44" N 22° 11' 53.38" E	Sideritis	Commercial	400	Sandy Loam	5.96	2.48	1.51	23.85	0.33
Diakofto (Aigialeia)	38°06'38.58" N 21° 31' 42.04" E	Sideritis	Commercial	250	Clay	7.43	2.65	1.81	43.26	0.33

Supplementary Table S2. Annual meteorological data from the viticultural zones sampled during the study.

Geographic region	Sampling Location	Cultivar	Mean Annual temperature (^{0}C)	Mean annual maximum temperature (^{0}C)	Mean annual minimum temperature (^{0}C)	Mean annual relative humidity (%)	Cumulative Annual Precipitation (mm)
Central Greece	Athens	Agiorgitiko, Vidiano	17.7	22.3	14.0	62	378
Central Macedonia	Thessaloniki	Agiorgitiko, Vidiano	15.6	20.4	9.5	67	460
Crete	Heraklion	Vidiano	17.8	21.9	14.0	63	464
Peloponnese	Nemea	Agiorgitiko	16.3	22.5	8.7	63	611.3
Western Greece	Aigialeia	Roditis, Sideritis	17.9	22.0	12.6	66	678

Supplementary Table S3. Primers used for amplicon sequencing analysis. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step, which are composed of the sequence of the universal primers 515f (bacteria) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a TT (linker) sequence at the 5' end of each primer.

Kingdom	PCR step	Primers	Gene target	Amplicon size (bp)	Primer sequences (5'-3')	References
Bacteria	1st	515F 806R	16S rRNA	~290	GTGCCAGCMGCCGCGGTAA GGACTACHVHHHTWTCTAAT	Parada et al., (2016)
	2nd	Indexed (n=85)	B0001-515f		TTCTTCTTCGT**GTGYCAGCMGCCGCGGTAA**	This study
			B0002-515f		TTCTCAATGGT**GTGYCAGCMGCCGCGGTAA**	
			B0003-515f		TTCAGTTCAGT**GTGYCAGCMGCCGCGGTAA**	
			B0004-515f		TTCGAATCAGT**GTGYCAGCMGCCGCGGTAA**	
			B0005-515f		TTGTCAGGTGT**GTGYCAGCMGCCGCGGTAA**	
Fungi	1st	ITS7F ITS4R	ITS2	~310	GTGARTCATCGAATCTTTG TCCTCCGCTTATTGATATGC	Ihrmark et al., (2012) White et al., (1990)
	2nd	Indexed (n=85)	FI0001-ITS4r		TTAACCTTGGA**TCCTCCGCTTATTGATATGC**	This study
			FI0002-ITS4r		TTAACCGAAGA**TCCTCCGCTTATTGATATGC**	
			FI0003-ITS4r		TTAACGACAGA**TCCTCCGCTTATTGATATGC**	
			FI0004-ITS4r		TTACTTACGGA**TCCTCCGCTTATTGATATGC**	
			FI0005-ITS4r		TTACTTGTCGA**TCCTCCGCTTATTGATATGC**	

Supplementary Table S4. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	**Volume (μl)**	**Concentration**	**Comments**
Primer F	1	0.5 μM	
Primer R	1	0.5 μM	
BSA	0.4	0.4 μg/μl	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH_2O	5.6		
DNA	2	0.2 ng/μl	
Total	20		
PCR conditions			
Step	**Temperature (°C)**	**Time**	**Number of Cycles**
Initial Denaturation	98	30 sec	28 in the first amplification step / 7 in the second amplification step
Denaturation	98	10 sec	
Annealing	50 for bacteria/ 55 for fungi	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	

Supplementary Table S5. The main members (ASVs with relative abundance ≥ 0.1%) of the core fungal epiphytic microbiome of the cultivars Roditis and Sideritis in the viticultural zone of Aigialeia.

Phylogenetic assignment	**% Relative abundance (± standard deviation)**	
	Roditis	**Sideritis**
Alternaria, Ascomycota (ASV1)	10.66 (±0.58)	14.32 (±0.89)
Aureobasidium, Ascomycota (ASV2)	20.46 (±1.49)	11.35 (±0.90)
Cladosporium, Ascomycota (ASV3)	16.99 (±1.01)	20.19 (±1.36)
Capnodiales, Ascomycota (ASV4)	4.82 (±0.34)	11.10 (±0.86)
Didymellaceae, Ascomycota (ASV5)	3.47 (±0.36)	
Erysiphe, Ascomycota (ASV6)		5.95 (±0.97)
Didymellaceae, Ascomycota (ASV7)		6.18 (±0.60)
Filobasidium, Basidiomycota (ASV8)		4.31 (±0.51)
Pleosporaceae, Ascomycota (ASV9)	1.27 (±0.13)	1.59 (±0.12)
Vishniacozyma, Basidiomycota (ASV10)	2.61 (±0.42)	5.87 (±0.46)
Pseudopithomyces, Ascomycota (ASV11)		1.53 (±0.15)
Pseudopithomyces, Ascomycota (ASV14)	2.20 (±0.29)	
*Sporidiobolaceae, Basidiomycota (*ASV16)		4.00 (±0.41)
Ascomycota, Ascomycota (ASV20)	1.72 (±0.24)	0.31 (±0.05)
Filobasidium, Basidiomycota (ASV25)	4.54 (±0.52)	
Nigrospora, Ascomycota (ASV29)	0.67 (±0.08)	
*Sporidiobolomyces, Basidiomycota (*ASV35)		1.98 (±0.20)
Helotiales, Ascomycota (ASV36)	2.68 (±0.38)	
Symmetrospora, Basidiomycota (ASV37)	0.33 (±0.06)	1.70 (±0.34)
Nigrospora, Ascomycota (ASV39)		1.70 (±0.17)
*Periconia, Ascomycota (*ASV122)		1.29 (±0.22)

Supplementary Table S6. The main members (ASVs with relative abundance ≥ 0.1%) of the core bacterial epiphytic microbiome of the cultivars Roditis and Sideritis in the viticultural zone of Aigialeia.

Phylogenetic assignment	**% Relative abundance (± standard deviation)**	
	Roditis	**Sideritis**
Sphingomonadaceae, Proteobacteria (ASV55)	4.08 (±0.65)	
Sphingomonas, Proteobacteria (ASV59)	4.40 (±1.50)	16.40 (±1.88)
Proteobacteria, Proteobacteria (ASV64)	2.35 (±0.37)	4.95 (±0.5)
Staphylococcus, Firmicutes (ASV73)	5.63 (±0.95)	
Massilia, Proteobacteria (ASV86)	4.45 (±1.27)	
Methylobacterium-Methylorubrum, Proteobacteria (ASV99)	4.1 (±0.6)	
Blastococcus, Actinobacteriota (ASV100)		1.22 (±0.27)
Acinetobacter, Proteobacteria (ASV166)	2.27 (±0.41)	
Skermanella, Proteobacteria (ASV182)		1.53 (±0.30)
Alphaproteobacteria, Proteobacteria (ASV199)	1.11 (±0.24)	
Staphylococcus, Firmicutes (ASV220)		1.62 (±0.39)
Methylobacterium-Methylorubrum, Proteobacteria (ASV252)		9.12 (±0.97)
Massilia, Proteobacteria (ASV293)		6.60 (±1.10)
Sphingomonadaceae, Proteobacteria (ASV369)		7.65 (±0.92)
Microbacteriaceae, Actinobacteriota (ASV384)		2.00 (±0.33)
Streptococcus, Firmicutes (ASV392)	2.39 (±0.4)	
Corynebacterium, Actinobacteriota (ASV429)	2.26 (±0.39)	
Cutibacterium, Actinobacteriota (ASV495)	1.73 (±0.42)	
Hymenobacter, Bacteroidota (ASV1028)	4.25 (±1.09)	
Skermanella, Proteobacteria (ASV1053)	2.30 (±0.48)	
Acetobacteraceae, Proteobacteria (ASV4571)	1.06 (±0.22)	

Hymenobacter, Bacteroidota (ASV9156)		4.28 (±0.81)

Chapter 4

Vineyard-mediated factors are still operative in spontaneous and commercial fermentations shaping the vinification microbial community and affecting the antioxidant and anticancer properties of wines

The work presented in **Chapter 4** is included in the following article:

Elena Papadopoulou, Fotios Bekris, Sotirios Vasileiadis , Afroditi Krokida, Theodora Rouvali, Aristidis S. Veskoukis, Kalliopi Liadaki, Demetrios Kouretas, Dimitrios G. Karpouzas (2023). Vineyard-mediated factors are still operative in spontaneous and commercial fermentations shaping the vinification microbial community and affecting the antioxidant and anticancer properties of wines

Food Research International, Volume 173, Part 2, Nov. 2023, 113359.
https://doi.org/10.1016/j.foodres.2023.113359

4.1. Introduction

Wine is the resulting product of a biodynamic fermentation process where the sugars of the grape are microbially transformed to ethanol (Beckner Whitener et al., 2016). Typically, vinification is performed under highly controlled conditions with the addition of preservatives like SO_2 and commercial Saccharomyces inocula that reduce the risk of wine spoilage, ensure consistency in wine quality but restrict native yeast diversity and aroma complexity (Beltran et al., 2002). In recent years there is a growing interest in the use of spontaneous vinification processes (Galati et al., 2019) which rely on the indigenous grapevine microbiota for must fermentation, reinforcing the regional character of he produced wine (Wei et al., 2022). The microbial community of the vinification process is mainly comprised of yeasts, filamentous fungi and bacteria (Barata et al., 2012; Piao et al., 2015; Maicas, 2021) whose origin varies. Soil is a major reservoir of the vinification microbiota (Zarraonaindia & Gilbert, 2015; Liu et al., 2020), while grapevine tissues like grapes, leaves and bark (Vitulo et al., 2019; Morrison-Whittle & Goddard, 2018) as well as winery surfaces (Bokulich et al., 2013) contribute to vinification microbiota to a great extent.

Yeasts are considered the most important members of the vinification microbiota and can be distinguished into Saccharomyces and nonSaccharomyces (NS) (Settanni et al., 2012). The NS yeasts are usually dominant in the early stages of fermentation, but their abundance declines as ethanol levels increase (Bagheri et al., 2020). They were initially considered of low significance or even spoilage microorganisms. However, the interest on NS yeasts increased in recent years due to their verified contribution in improving sensory quality, stability and wine freshness (Morata et al., 2019; Velenosi et al., 2021), reflecting grape origin (Padilla et al., 2016). On the contrary, Saccharomyces strains are minor members of the grape microbiota but become dominant at the later stages of fermentation (Settanni et al., 2012; Hu et al., 2018).

Regarding prokaryotes, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), are important players in the vinification process. The former are considered desirable components of the vinification microbiota, although undesirable effects in wine quality by LAB cannot be not excluded. In contrast, AAB are considered undesirable components of the wine microbiota. LAB like *Oenococcus spp*. (Dimopoulou et al., 2018), *Lactobacillus spp*. and *Leuconostoc spp*. (Takase et al., 2018), are responsible for malolactic fermentation (MLF) (Filimon et al., 2022;

Bubeck et al., 2020; Brizuela et al., 2017), which involves the conversion of L-malic acid to L-lactic acid and the reciprocal production of volatile secondary compounds (Cappello et al., 2017). AAB are associated with wine spoilage by converting ethanol to acetic acid (Bartowsky & Henschke, 2008). The most common AAB detected in vinification are *Gluconobacter*, *Acetobacter*, and *Komagataeibacter* and their presence is associated with wine acidification (Andres-Barrao et al., 2013; Gomes et al., 2018). Filamentous fungi like *Cladosporium*, *Aspergillus*, *Alternaria* (Bokulich et al., 2016) and bacteria like *Pseudomonas*, *Methylobacterium* and *Sphingomonas* (Bokulich et al., 2012; Piao et al., 2015) are also present, and often persist, in the vinification process. Although their role in the vinification is largely unknown, recent studies have suggested their potential involvement in the production of desirable wine metabolites (Bokulich et al., 2016).

Several studies to date have investigated the factors shaping the grapevine and must microbiota (Bokulich et al., 2014; Zarraonaindia and Gilbert, 2015; Miura et al., 2017; Vitulo et al., 2019; Papadopoulou et al., 2022). Nevertheless, only a few of them have followed the dynamics of the microbial community during the vinification process using either amplicon sequencing (Bokulich et al., 2016; Bohmer et al., 2020; Wang et al., 2021; Ding et al., 2023), shotgun metagenomics (Sternes et al., 2017) and recently meta-transcriptomics (Reiter et al., 2021). These studies mostly followed microbial succession and did not determine the influence of factors associated with the grapevine environment (e.g., cultivar, terroir, vintage) on the composition of the microbial community at contrasting vinification conditions (spontaneous vs commercial). Such knowledge would enable a microbiota-driven optimization of the vinification process supporting the production of high-quality wines with typified regional character.

Wine distinctiveness is driven by its chemical metabolic profile which is configured during the fermentation. Recent studies have shown a direct association of must and vinification microbiota with specific wine metabolites and aromatic compounds (Bokulich et al., 2016; Wei et al., 2022). Based on this Bokulich et al. (2016) showed that must microbiota could act as a predictor of the chemical composition of the produced wine. Moreover, the significant antioxidant properties of wine (Tekos et al., 2021, Tzanova et al., 2020), attributed to its high polyphenolic content (Snopek et al., 2018), along with its antimutagenic potential have been acknowledged by the relevant literature (Di Sotto et al., 2013). However, the role of

the members of the vinification microbiota in the establishment of the antioxidant and anticancer potential of wines remains unknown.

In this frame, we aimed to determine how factors associated with the vineyard environment such as cultivar, vintage and terroir could influence, under different vinification strategies reflecting current wine making practices, (a) the microbial succession during fermentation and (b) the antioxidant, antimutagenic and anticancer properties of the produced wines. To achieve this we followed, via amplicon sequencing, the dynamics of the bacterial and fungal communities at five distinct stages along three different vinification processes (spontaneous V1, spontaneous with addition of SO_2 V2, commercial with addition of SO_2 and Saccharomyces inoculum V3) performed separately for two Greek local cultivars (Roditis and Sideritis), two terroir units and two vintages (2019 and 2020) at semi full-scale fermenters established and operated in local commercial wineries. We further examined the produced wines for their potential antioxidant scavenging ability and their capacity to inhibit cancer cell proliferation and explored the association of members of the vinification microbiota with the antioxidant and anticancer activity profiles of the produced wines.

4.2. Materials and methods

4.2.1. Grape samples and vineyards topography

Grape samples from the cultivars Roditis and Sideritis were collected at harvest in two consecutive years, 2019 and 2020, from two distinct terroir units per cultivar from the viticultural zone of Aigialeia, north Peloponnese, Greece (Supplementary Fig. S1). Aigialeia is the highest altitude viticultural zone in Greece (250–1050 m) covering 5000 ha, of which 900 ha are devoted to Roditis, while Sideritis is an old and re-emerging local cultivar. Details about the edaphoclimatic conditions of the vineyards are given in Papadopoulou et al. (2022). Grape samples were collected from each terroir unit per cultivar at harvest (September and October for Roditis and Sideritis respectively) and they were immediately transferred to the wineries for vinification

4.2.2. Vineyard harvest and fermentations samples

When in wineries the whole clusters of fresh ripe grapes were destemmed, and mechanically crushed to obtain the must that was used in the vinification process. For

each cultivar and terroir combination three different vinification processes were employed (180 L total volume) in 200 L fermenters by two local wineries (Rouvalis Winery for Roditis and Krokidas Winery for Sideritis) representing realistic wine-making practices in commercial wineries: (V1) spontaneous vinification (allowing the indigenous microbiota of grape must to ferment the sugars in the absence of preservatives), (V2) vinification with the addition of preservatives (Potassium metabisulfite 100 g t^{-1} Laffort company (Bordeaux Cedex, France)), and (V3) a commercial-type vinification with the addition of preservatives (Potassium metabisulfite as in V2), pectolytic enzymes for rapid clarification of must at 100 g t^{-1} Lafazym CL, Laffort company (Bordeaux Cedex, France), nutrients for yeast growth at 200 g t^{-1} Nutristart, Laffort company (Bordeaux Cedex, France) and a commercial S. cerevisiae inoculum at 200 g t^{-1} , Fervens GN from Dalcin company (Milan,Italy) for Roditis and Zymaflore XPURE, from Laffort company (Bordeaux Cedex, France) for Sideritis. Triplicate samples (50 ml) were collected from every vinification at the following stages: (i) crushing (S1), (ii) must descaling (S2), (iii) start of fermentation (S3), (iv) mid fermentation (S4), (v) end of fermentation (S5). The progress of the fermentation and the vinification stages which were selected for the study were defined based on regular monitoring of the must density/specific gravity separately in each different vinification. Samples were locally stored at − 20 ◦C and they were transferred to the laboratory on dry ice before DNA extraction and downstream activities. The wines produced by the different vinification processes were stored and used for characterization of their antioxidant, antimutagenic and anticancer activity as described below. A schematic representation of the vinification processes and the different treatment employed, as well as our experimental design can be seen in Supplementary Fig. S2.

4.2.3. Analysis of the composition of the vinification microbiota

4.2.3.1. DNA extraction

Must samples were thawed and centrifuged at 8500 rpm at 4 ◦C for 15 min. The microbial pellet was washed twice with ice cold 1xPBS, centrifuged and after washing it was subjected to DNA extraction with the DNeasy Power soil DNA isolation kit from Qiagen company (Hilden, Germany). DNA was quantified by the Quant-iT kit with a Qubit Fluorometric device from Thermo Fisher Scientific (Waltham, MA, USA).

4.2.3.2. Amplicon sequencing analysis of the vinification microbiota

The succession of the fungal and bacterial communities during vinification was determined via amplicon sequencing of the ITS2 and the V4 region of the 16S rRNA gene in a HiSeq Illumina platform in Rapid Mode 2x250, paired-end in Admera Health company (South Plainfield, New Jersey, USA). An in-house protocol (details in Vasileiadis et al., 2015) was used for amplicons preparation whereby must DNA was subjected to a two-step amplification process enabling indexing of samples. DNA was initially amplified with primers ITS7F-ITS4R for fungi (Martins et al., 2013; Martiny et al., 2006) and the updated version of primers 515f-806r for bacteria (Lorenzini et al., 2019) according to the earth microbiome project (Lozupone et al., 2007). The first amplification stage was run for 28 cycles and the product obtained was subjected to a second PCR of 7 cycles, using the same primers but this time containing sample-specific 5′ overhangs for sample indexing. For all amplifications the Q5® High-Fidelity DNA Polymerase from NEB company (Ipswich, Massachusetts, USA) was used. The amplicon sequencing libraries were cleaned up with the NucleoMag NGS Clean-up and the Size Select Kit from Macherey-Nagel company (Duren, Germany) and sequenced. Primers, PCR conditions and reagents are given in Supplementary Tables S1 and S2.

4.2.3.3. Bioinformatic analysis of amplicon sequencing data

Flexbar v3.0.3 (Martiny et al., 2011) was used for demultiplexing amplicon sequences to their samples of origin, having no barcode mismatches, while dada2 v1.14.1 (Callahan et al., 2016) package the R software v.4.0.2 (R Core Team, n.d.) supported the sequences qualitytrimming/filtering and checking for chimerical sequences. The reference databases UNITE ITS v.8.2 (04.02.2020) (Morrison-Whittle et al., 2017) and Silva v.138 (Yilmaz et al., 2014) were used to perform the fungal and bacterial amplicon sequence variants (ASVs) assemblage and taxonomical assignment. ASVs not assigned at Kingdom and Phylum level were excluded and all remained ASVs were classified to the lowest possible taxonomical rank, having at least 80% annotation probability score. The sequencing data were submitted to sequence Read Archive of NCBI with BioProject accession number (ID: PRJNA924015).

4.2.3.4. Statistical analysis of amplicon sequencing data

ASV matrices were used to define the influence of different factors on the composition and dynamics of the bacterial and fungal vinification microbiota. In addition, ASV matrices were used to calculate α-diversity (Shannon index) for bacteria and fungi along the different vinification regimes using the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. Non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) were used to depict the impact of cultivar, vintage, terroir, vinification type and stage on the fungal and bacterial β-diversity of the vinification. The NMDS analysis was performed using the dissimilarity matrices by means of Bray-Curtis algorithm and PERMANOVA (Lozupone et al., 2007) as implemented in the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. Differential abundance (DA) tests using the Kruskal-Wallis and the Wilcoxon rank-sum post-hoc tests were applied to determine fungal and bacterial taxa associated with specific cultivar, terroir units and vintage combinations. P-value adjustment was conducted with false discovery rate (FDR) algorithm with implemented adjusted P-value cutoff values of 0.05. All diagrams were produced using the R package ggplot2 v3.3.3 (Wickham, 2009). The full analysis code is available in the Github repository: https://github.com/Fotisbs/Grapevine_Vinifi cation_Microbiome.

4.2.4. Assessment of the antioxidant, antimutagenic and anticancer activity of wines

4.2.4.1. Antioxidant and antimutagenic activity assays

The evaluation of the antioxidant and antimutagenic properties of the wines produced was based on the measurement of a battery of biomarkers that are widely used in the in vitro screening of compounds of plant origin (Veskoukis et al., 2019). All experiments were carried out in triplicate and in two separate occasions. In detail, the ability of the tested wine samples to reduce (a) DPPH•, (b) $ABTS^{\bullet+}$, (c) superoxide ($O2^{\bullet-}$) and hydroxyl (OH•) radicals and (e) Fe^{3+} to Fe^{2+} using the reducing power assay (RPA), as described by Veskoukis et al. (2012, 2019). The antimutagenic activity of the produced wines was determined based on plasmid relaxation assay (PRA) via measurement of the inhibition of the plasmid DNA strand scissions induced by peroxyl radical (ROO•) (Kerasioti et al., 2014). A more detailed description of the above protocols can be found in the section Materials and Methods of Annex III Supplementary Data.

4.2.4.2. Anticancer activity assays

Wine samples were initially converted into dry extracts before administered directly to cultured cells due to their high alcohol content. In the process of extract preparation, 200 ml of wine sample provided 20 g of dried extract. This was amended with maltodextrin DE-18 at a ratio of 20% to the solid components and the samples underwent freezedrying. All extracts were stored at 4 ◦C and were diluted in ddH_20 before use. These dry extracts, in serial dilutions, were tested on cultures of human breast adenocarcinoma cell line MDA-MB-231 and human hepatocellular liver carcinoma cell line HepG2. All cell cultures were routinely maintained in DMEM (Dulbecco's modified Eagle medium) (containing 4.500 mg/L D-glucose, 110 mg/L sodium pyruvate, Lglutamine) supplemented with 10% heat-inactivated fetal bovine serum (FBS) from Thermo Fisher Scientific (Waltham, MA, USA) and antibiotics (PS: 100 units/mL of penicillin G, 100 μg/mL of streptomycin sulfate), in a humidified chamber at 37 ◦C with 5% CO_2. The inhibition of cell proliferation was measured using the XTT assay method as previously described (Tselepi et al., 2011).

4.2.4.3. Statistical analysis of antioxidant, antimutagenic and anticancer assays

The obtained data were analyzed through two-way analysis of variance (ANOVA, $p < 0.05$) and Bonferroni t-test ($p < 0.025$) for pairwise comparisons. The results are expressed as Mean ± Standard Error (SE). For the statistical analysis, the Statistical Package for the Social Sciences (SPSS, v.22) was used.

4.3. Results

4.3.1. Vinification fungal community

4.3.1.1. The composition and succession of the fungal community

In total, 7,887,083 high quality sequences for fungi were obtained and they were assigned to 5454 ASVs. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the fungal diversity (Supplementary Fig. S3a). All vinification samples were analyzed collectively to assess the fungal microbial community succession during vinification. Regardless of cultivar and vinification protocol, we noted a similar microbial succession pattern (Fig. 1). At the early stages of fermentation (S1, S2) the fungal community showed a higher phylogenetic diversity composed of non-oenologically relevant fungal ASVs of *Aureobasidium*, *Cladosporium*, *Lachancea*, *Sclerotiniaceae*, while *Alternaria*,

Aspergillus and *Torulaspora* were also present to a lower extent (Fig. 1). From the third stage of the vinification process (S3) and onwards, the fungal community landscape changes and ASVs of *Saccharomyces* dominate both in spontaneous and commercial vinifications. It is noteworthy that, regardless of cultivar or vintage, the spontaneous vinifications (V1, V2) were dominated by the same *Saccharomyces* ASV (ASV01) in contrast to the commercial vinifications (V3), where, as expected, an ASV02 corresponding to the commercial inoculum of S. cerevisiae dominated the process (Fig. 1). This fungal succession pattern was reflected in the α-diversity measures which showed a rather universal pattern of gradual and significant reduction of Shannon diversity index along the vinification process (Supplementary Fig. S4a).

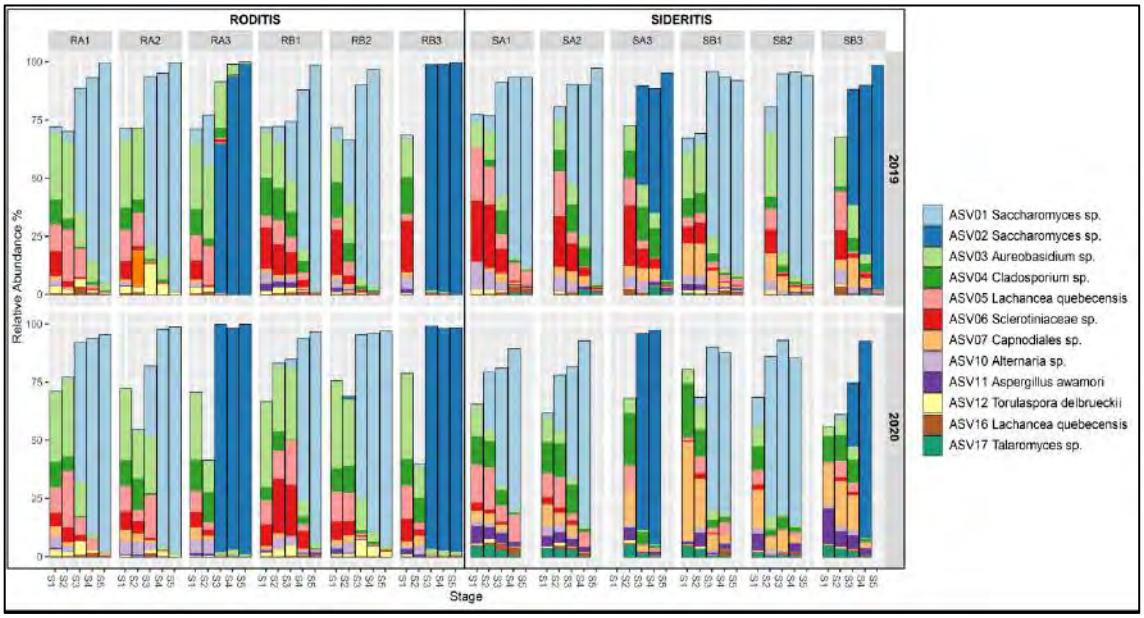


Fig. 1 Stacked bar plots showing the succession of the fungal microbiome (taxonomic level of genus) across five vinification stages in the three different vinifications employed for each cultivar (Roditis and Sideritis) by terroir combinations. Codes of samples: (i) first letter represents the cultivar R: Roditis, S: Sideritis, (ii) second letter represents the terroir unit A: terroir A, B: terroir B, (iii) Number indicates vinification type 1: spontaneous/without preservatives, 2: spontaneous/with preservatives, 3: commercial with Saccharomyces cerevisiae inoculum, (iv) Vinification stages: S1, S2, S3, S4, S5.

4.3.1.2. Factors affecting the β-diversity of the fungal microbial community

We then aimed to disentangle the key drivers of the vinification microbial community like cultivar and vintage. NMDS analysis showed a clear clustering of samples according to cultivar (Fig. 2a) and vintage (Fig. 2b) as confirmed by PERMANOVA-based variation partitioning analysis ($p < 0.001$) (Supplementary Table S3). We further explored, for each cultivar separately, the effect of terroir, vinification type and

stage on the fungal community. Terroir was less important in shaping the vinification fungal community of Roditis ($p > 0.05$) than of Sideritis ($p < 0.05$) (Fig. 3a and 3c). When we assessed the effect of terroir separately at the early (S1, S2) and late (S4, S5) stages of vinification we noted a clear terroir effect early in the vinification which becomes non-significant at the later vinification stages (Supplementary Fig. S5). The type of vinification process followed seemed to be the major determinant of the fungal community for both cultivars (Fig. 3b and 3d) explaining 33.1% ($p < 0.001$) and 28.3% ($p < 0.001$) of the variance for Roditis and Sideritis, respectively (Supplementary Table S3). For both cultivars the commercial vinification process supported a significantly different fungal community compared to the two spontaneous vinifications (V1 and V2) ($p < 0.01$), which did not significantly differ between them. Finally, the vinification stages seemed to have a strong effect on the fungal community with its composition at stages S1, S2, S3, significantly differing from the following stages S4 and S5 ($p < 0.001$; Supplementary Fig. S6a and S6b).

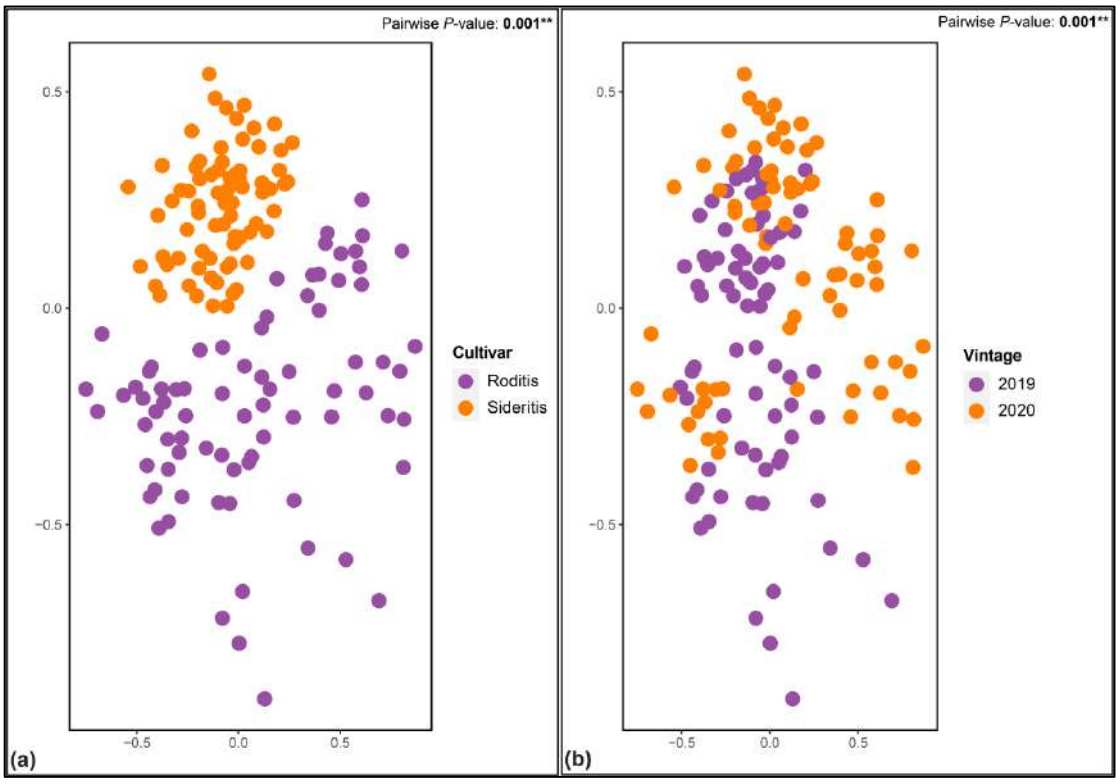


Fig. 2. NMDS analysis of the fungal vinification microbiome. Samples are ordinated according to their (a) cultivar (Roditis and Sideritis) and (b) vintage (2019 and 2020).

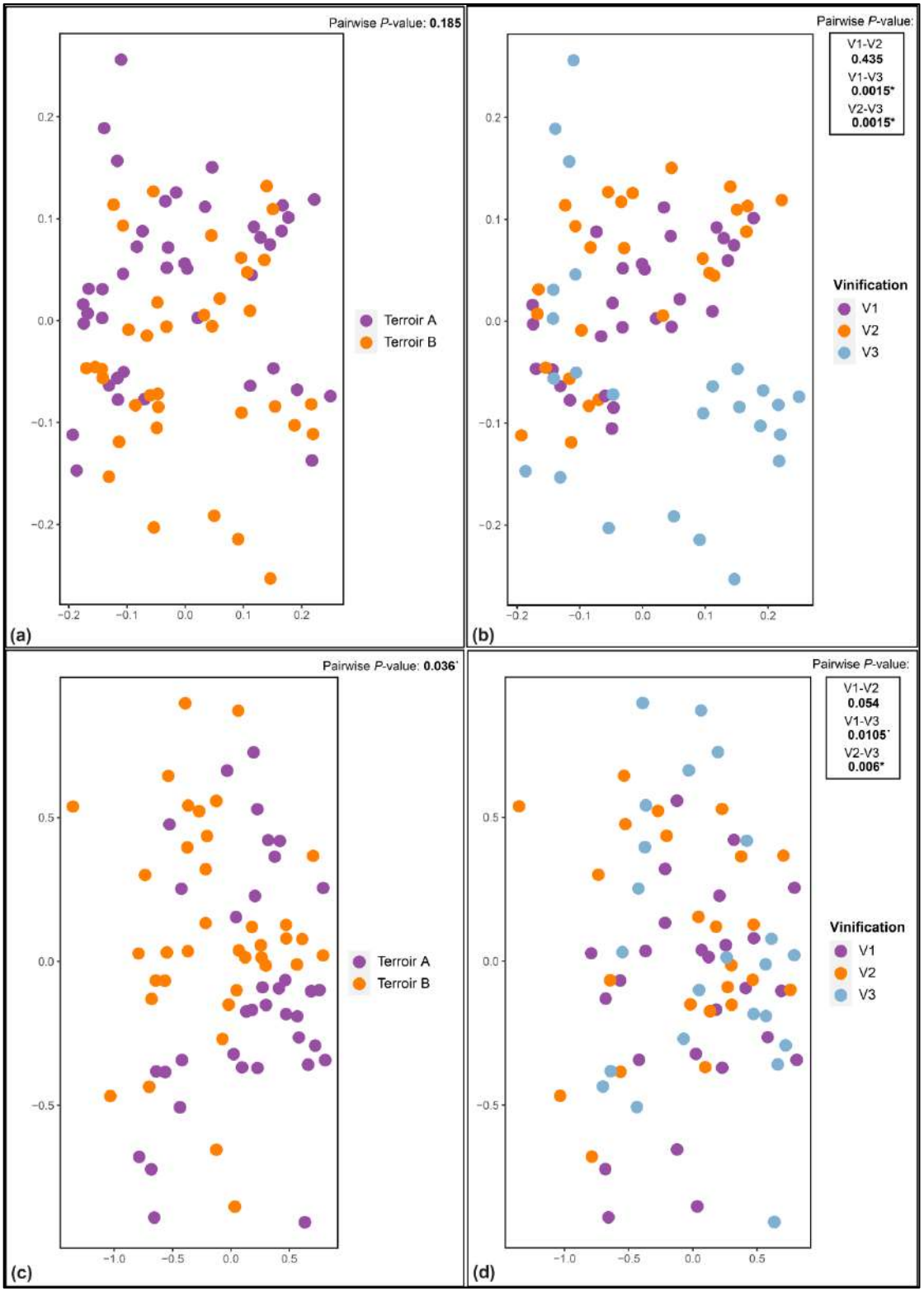


Fig. 3. NMDS ordination of the fungal microbiome in vinification samples analyzed separately for the two cultivars Roditis (a, b) and Sideritis (c, d). Samples are ordinated according to their (a and c) *terroir* (A and B), and vinification type (b and d) (V1 – spontaneous no preservatives, V2 – spontaneous with preservatives, V3 – commercial).

4.3.2. Vinification bacterial community

4.3.2.1. The composition and succession of the bacterial community

In total, 16,392,811 high quality sequences for bacteria were obtained and they were assigned to 36883 ASVs. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the bacterial diversity (Supplementary Fig. S3b). Unlike fungi, the bacterial community followed more variable succession patterns according to cultivar, vintage and the vinification process employed. This was reflected in the temporal patterns of the α-diversity measures where the Shannon diversity index showed either non-significant changes along the vinification process (i.e. Roditis), or their patterns varied for the different vinifications without any clear motif (i.e. Sideritis) (Supplementary Fig. S4b).

Regarding Roditis, we noted a gradual domination of *Oenococcus* at the latter stages of fermentation in V1 (spontaneous without preservatives) at both years (Fig. 4). This was complemented by a gradual reduction in the relative abundance of *Gluconobacter, Enterobacterales, Lactobacillus*, which dominated at the earlier stages. A rather similar pattern was evident in V2 (spontaneous with preservatives) in both years with the sole exception of RA2 (Roditis terroir A) where the composition of the bacterial community was stable during fermentation. The bacterial succession patterns in the commercial vinification of Roditis (V3) varied between years. In 2019 for both terroir units we noted a gradual establishment of a *Prevtoleaceae*-rich bacterial community along with *Gluconobacter* and *Lactobacillus*. An interesting observation in the vinifications of Roditis in 2020 was the strong presence of *Tatumella*. Similarly to Roditis, we noted a gradual establishment of an *Oenococcus*-rich bacterial community in V1 in both years and terroirs for Sideritis, while this time the same pattern was also evident in V2 and particularly in V3 in 2019 (Fig. 4). *Oenococcus* seemed to establish at the latter two stages gradually replacing *Lactobacillus*, *Gluconobacter*, *Acetobacter* and *Komagataeibacter*. In contrast, the composition of the bacterial community in V2 and V3 in 2020 did not change substantially along the vinification process composed of *Bacillus*, *Komagataeibacter*, *Acetobacter*, *Gluconobacter* supplemented by a subtle increase in *Oenococcus* at S5.

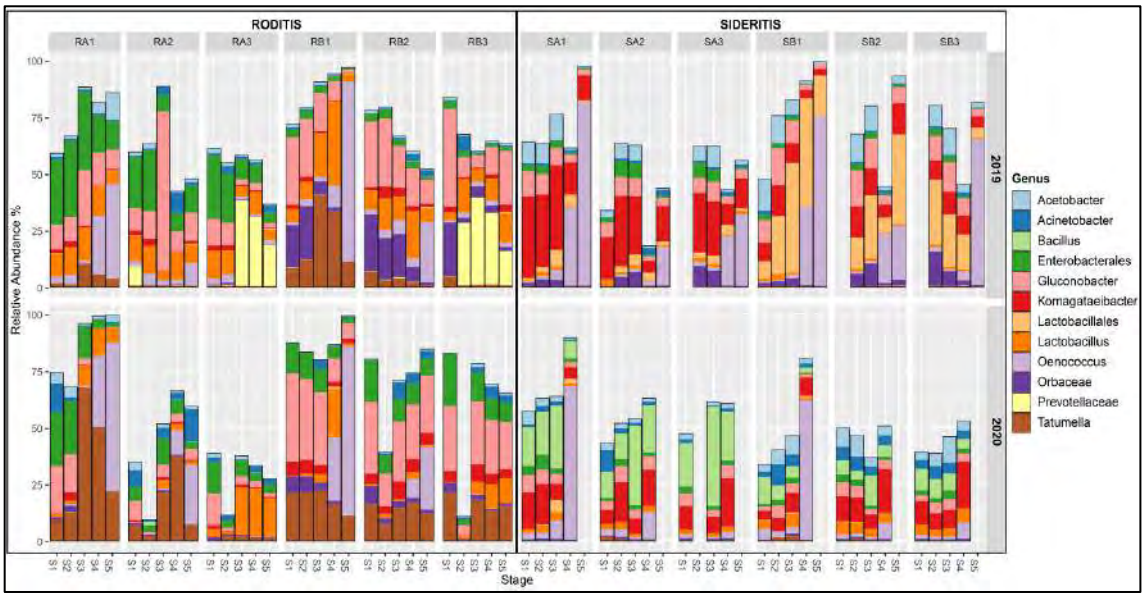


Fig. 4. Stacked bar plots showing the succession of the bacterial microbiome (taxonomic level of genus) across five vinification stages in the three different vinifications employed for each cultivar (Roditis and Sideritis) by *terroir* combination. Codes of samples: (i) first letter represents the cultivar R: Roditis, S: Sideritis, (ii) second letter represents the terroir unit A: terroir A, B: terroir B, (iii) Number indicates vinification type 1: spontaneous/without preservatives, 2: spontaneous/with preservatives, 3: commercial with *Saccharomyces cerevisiae* inoculum, (iv) Vinification stages: S1, S2, S3, S4, S5

4.3.2.2 Factors affecting the β-diversity of the bacterial community of vinification

NMDS analysis of the bacterial community revealed a strong clustering based on cultivar (Fig. 5a) and vintage (Fig. 5b) which contributed 16.7% and 6.2% of the variance respectively ($p < 0.001$) (Supplementary Table S3). We further examined the influence of terroir, vinification type and stage separately for each cultivar. For both cultivars, terroir (Fig. 6a and 6c) showed a significant clustering effect ($p < 0.001$) (Supplementary Table S3). However, when looking at the effect of terroir along the vinification process, we noted, similarly to the results obtained with fungi, that geographic signatures were evident only at the early fermentation stages (S1, S2) and become non-significant as the fermentation progresses (S4, S5) (Supplementary Fig. S7). The bacterial community of the vinification of the two cultivars showed diverse dynamics in the different vinification processes. Hence, for Roditis all three vinification processes supported significantly different bacterial communities ($p < 0.01$, 11.3% of the variance), unlike Sideritis whose bacterial community varied less in the different vinification processes employed with V2 and V3 not significantly differing ($p > 0.05$, 4.6% of the variance) (Fig. 6b and 6d). The bacterial community of both cultivars seemed to diversify on the same way along the different vinification

stages, with S1, S2, S3 supporting a significantly different bacterial community compared to S4 and S5 (Supplementary Fig. S6c and S6d).

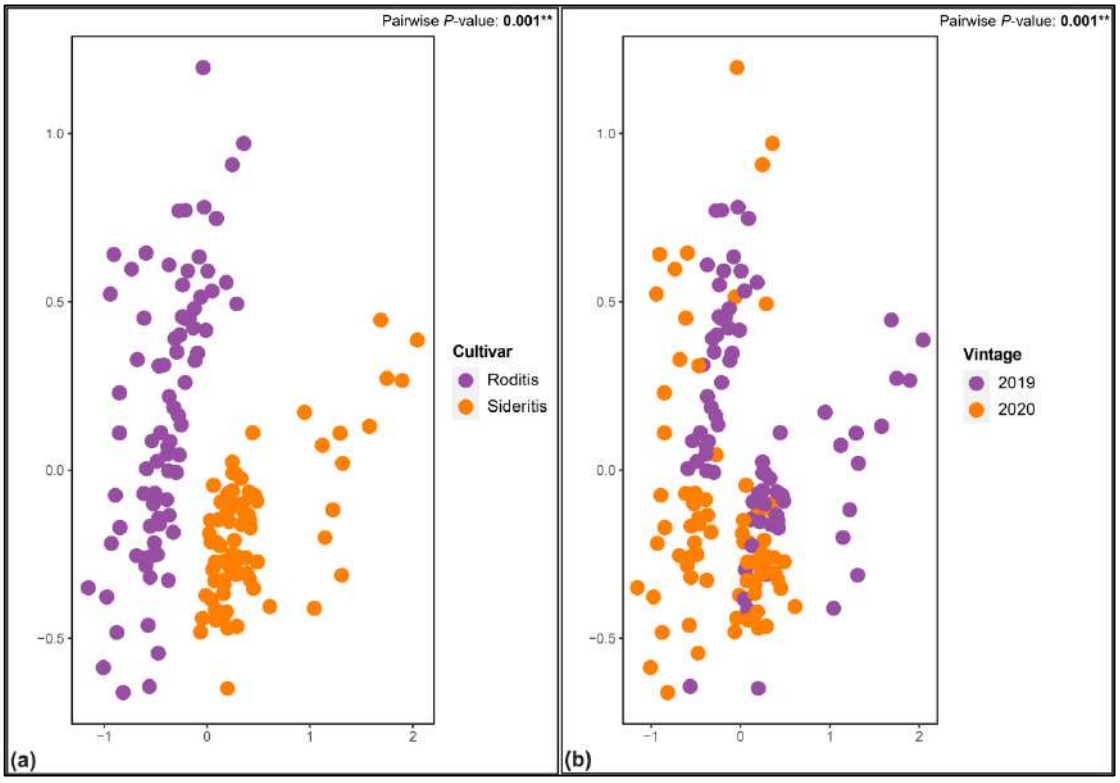


Fig. 5. NMDS analysis of the bacterial vinification microbiome. Samples are ordinated according to their (a) cultivar (Roditis and Sideritis) and (b) vintage (2019 and 2020).

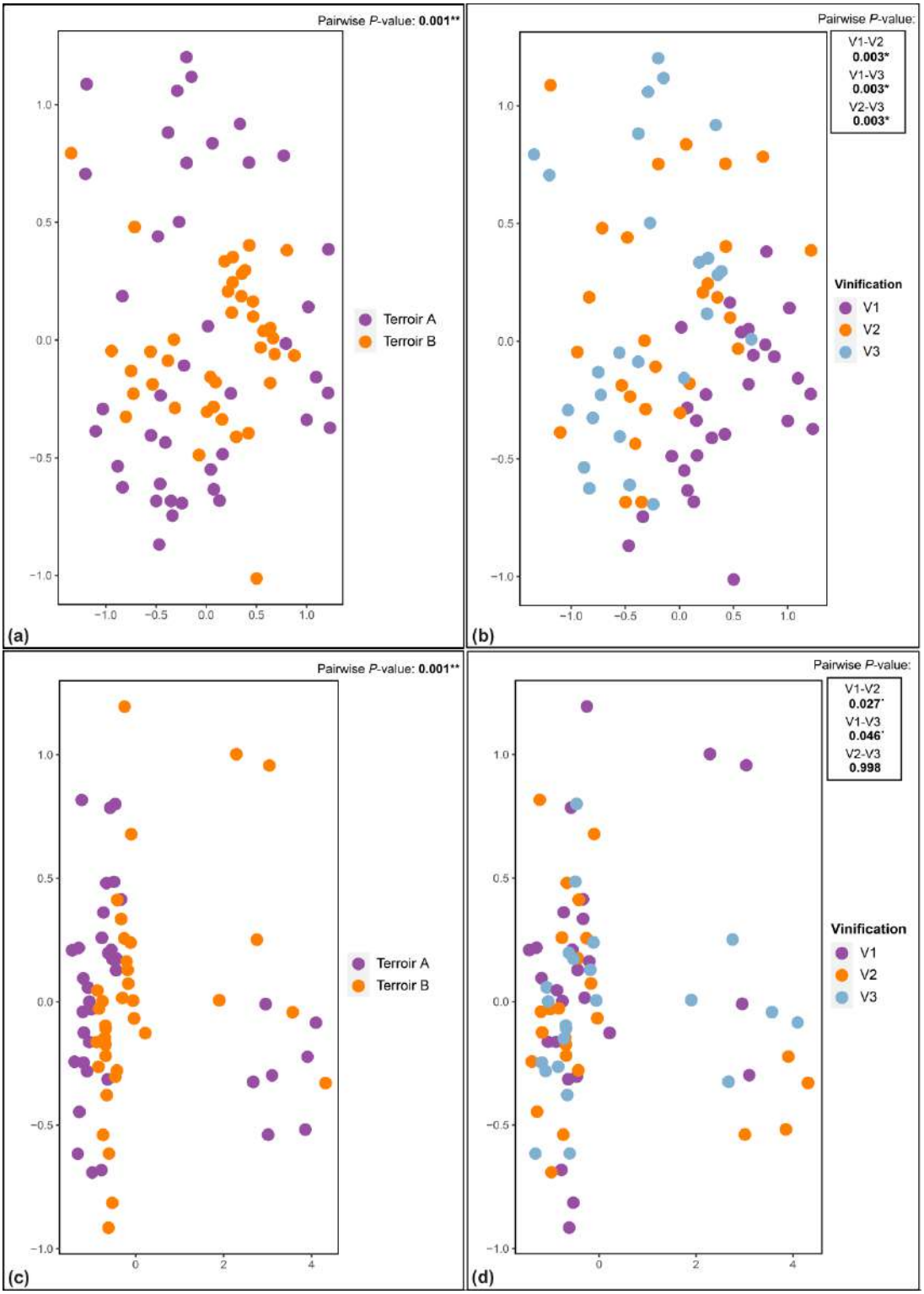


Fig. 6. NMDS ordination of the bacterial microbiome in vinification samples analyzed separately for the two cultivars Roditis (a, b) and Sideritis (c, d). Samples are ordinated according to their (a and c) *terroir* (A and B), and vinification type (b and d) (V1 – spontaneous no preservatives, V2 – spontaneous with preservatives, V3 – commercial).

4.3.3. Antioxidant and antimutagenic activity of wine samples

A main effect of vintage on the superoxide radical assay ($p < 0.05$) was observed (Table1), with the samples of 2019 showing significantly lower IC50 values. Regarding the reducing power assay, a main effect of vinification was found. The samples produced from V2 and V3 showed significantly lower IC50 values compared to V1 ($p < 0.05$) (Fig. 7). Concerning the effects of the different factors per cultivar, for Roditis, significant effects of vinification and vintage were evident for the RPA ($p < 0.05$) and the superoxide radical ($p < 0.05$) assays, respectively. pattern of significantly lower IC50 values ($p < 0.05$) of the samples from V2 and particularly from V3 compared to V1 was observed for ABTS, DPPH and RPA in both years and terroirs (Fig. 7). For Sideritis, we noted significant main effects of (i) vintage in the DPPH ($p < 0.01$) and ABTS ($p < 0.05$) assays with samples of 2019 showing significantly lower IC50 values, (ii) vinification in the RPA ($p < 0.05$) with V3 samples showing significantly lower IC50 values ($p < 0.05$) compared to V1 and (iii) terroir unit in the PRA ($p < 0.05$) and superoxide radical ($p < 0.01$) assays with terroir A showing significantly lower IC50 values (Table1, Fig. 7). For the plasmid relaxation assay, a measure of the antimutagenic activity of the samples, significant main effects of vintage ($p < 0.05$) were observed with the samples of 2020 showing lower IC50 values compared to 2019 (Table1). Significant interactions between terroir × vintage were also evident ($p < 0.01$). In particular, we observed a clear pattern of significantly lower IC50 values in the samples of Roditis from V2 and V3 compared to V1 in 2020, a pattern which was replicated in 2019 only for terroir B (Fig. 7). Regarding Sideritis, significantly lower IC50 values ($p < 0.05$) were observed in 2020 compared to 2019 samples in terroir A.

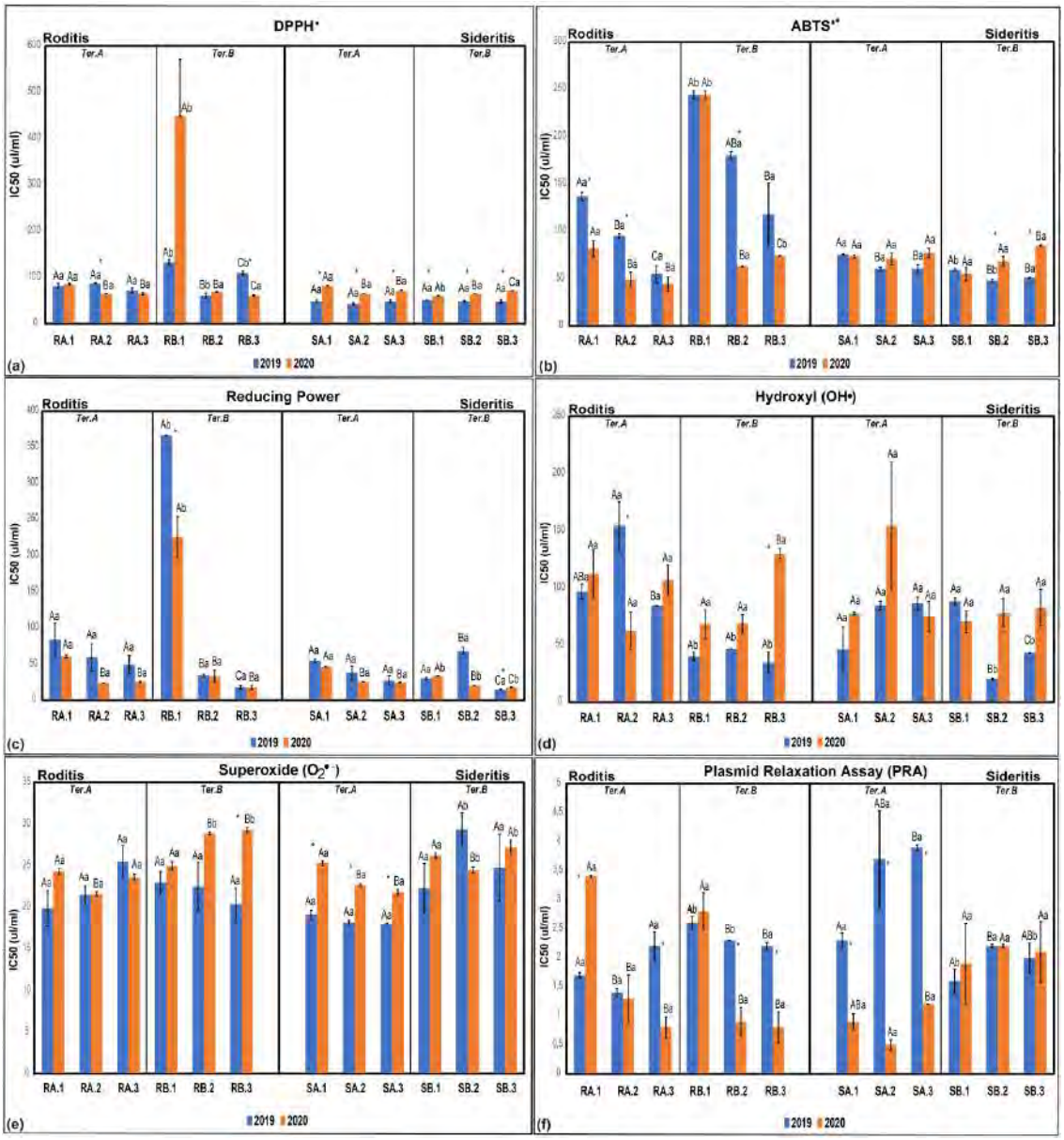


Fig.7. The IC_{50} values for a range of antioxidant (ABTS, DPPH, Hydroxyl (OH), Reducing Power, Superoxide (O_2)) and antimutagenic (Plasmid Relaxation assay) assays performed on wine samples obtained upon vinification of grape samples from the cultivars Roditis and Sideritis collected in 2019 and 2020. Index for samples: First letter: Cultivar (R: Roditis, S: Sideritis); Second Letter: terroir unit (A and B); Number: Type of vinification process followed (1: Spontaneous without preservatives; 2: Spontaneous with preservatives; 3: Commercial). Each value is the mean of three replicate $\pm$ the standard error. Bars designated with the same capital letter indicate non-significant differences between different vinification processes (5% level) within each cultivar, terroir and vintage combination. Bars designated with the same lower-case letter indicate non-significant differences between different terroir units (5% level) within each cultivar, vintage and vinification process combination. Finally asterisk indicates significant differences (5% level) between vintages for each cultivar, terroir unit and vinification process combination.

4.3.4. Anticancer activity of wine samples

The anticancer activity of wine samples was evaluated as inhibition of proliferation of hepatic (HepG2) and breast cancer (MDA-MB-231) cell lines. A significant main effect of vintage was observed ($p < 0.001$) for both cell lines with the 2019 wine

samples exhibiting higher anticancer activity (Table1). The significant main effect of vintage was still evident when the samples from the two cultivars were analyzed separately (Table1). Based on the significant interactions between terroir and vintage, major patterns in the anticancer activity of the wine samples were further explored. Significantly higher anticancer activity on HepG2 cells was measured for the Roditis and Sideritis samples from terroir A vs terroir B in 2019, but this was not verified in 2020 (Fig. 8b). For the Roditis wine samples the different vinification processes did not significantly affect the activity on HepG2 cells at both years (Fig. 8b). In the case of Sideritis, wine samples from 2019 from both terroirs derived from V3 showed significantly higher anticancer activity compared to V1.

Regarding the activity on MDA-MB-231 cells, the Roditis wine samples did not significantly differ across different vinification processes in 2019, unlike 2020 where a significantly higher activity of the V3 samples compared to V1 was measured (Fig. 8a). Across the terroirs of Roditis, a significantly higher activity on the samples from terroir A vs terroir B was noted in 2019, an observation which was reversed in 2020 (Fig. 8a). The Sideritis samples derived from V1 showed significantly lower activity compared to V2 and V3 in terroir A at both years, whereas no consistent pattern of effects of the vinification process in the two study years was evident in the samples from terroir B. Finally, wine samples from Sideritis terroir A exhibited significantly higher activity compared to terroir B in 2019, but this pattern was not observed in 2020 (Fig. 8a).

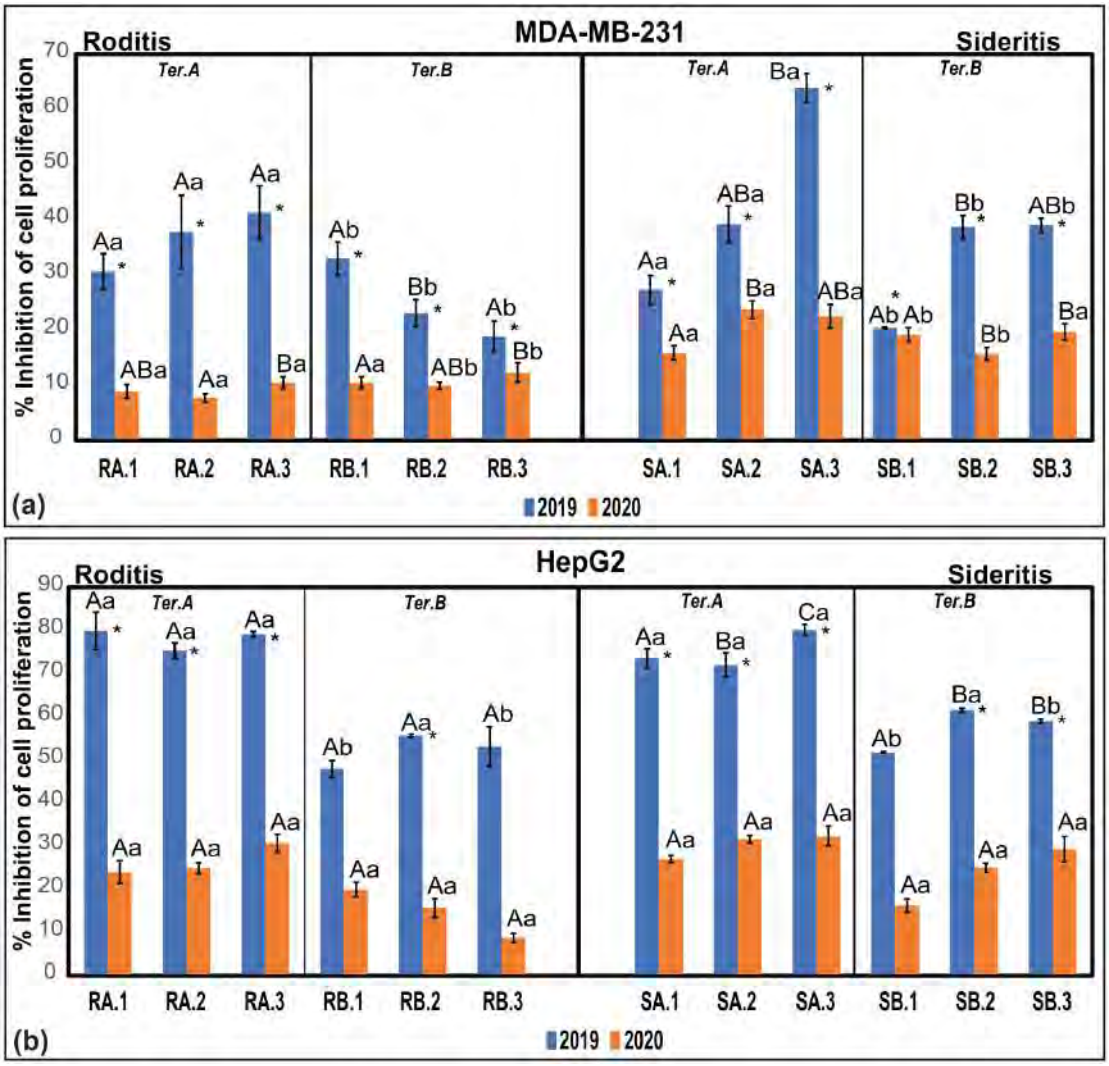


Fig.8. The % inhibition observed in cancer cell lines HepG2 and MDA-231 by dry extracts of wine samples obtained upon vinification of grape samples from the cultivars Roditis and Sideritis collected in 2019 and 2020. Index for samples: First letter: Cultivar (R: Roditis, S: Sideritis); Second Letter: terroir unit (A and B); Number: Type of vinification process followed (1: Spontaneous without preservatives; 2: Spontaneous with preservatives; 3: Commercial). Each value is the mean of three replicate $\pm$ the standard error. Bars designated with the same capital letter indicate non-significant differences between different vinification processes (5% level) within each cultivar, terroir and vintage combination. Bars designated with the same lower-case letter indicate non-significant differences between different terroir units (5% level) within each cultivar, vintage and vinification process combination. Finally asterisk indicates significant differences (5% level) between vintages for each cultivar, terroir unit and vinification process combination

Table 1. Summary of ANOVA/Kruskal-Wallis test p-values between Antioxidant, Anticancer properties and Factors tested (Cultivar, Vinification, Vintage and Terroir Unit) for Roditis and Sideritis (combined and separate datasets).

	Antioxidant						Anticancer	
	DPPH	ABTS	Reducing Power	Hydroxyl Radical	Superoxide Radical	PlasmidRelaxation Assay	HepG2	MDA-MB-231
	Roditis and Sideritis							
Cultivar (Roditis, Sideritis)	0.11	0.07 ˙	0.09 ˙	0.7966	0.72	0.76	0.14	0.7
Vinification (V1, V2, V3)	0.25	0.16	0.04 *	0.65	0.91	0.66	0.54	0.95
Vintage (2019, 2020)	0.36	0.53	0.54	0.2	0.02 *	0.02 *	0.0002 ***	0.00003 ***
Cultivar:Vinification	0.14	0.12	0.01 *	0.9	0.98	0.42	0.46	0.1
Cultivar:Vintage	0.32	0.07 ˙	0.34	0.6	0.15	0.08 ˙	0.0001 ***	0.0004 ***
Vinification:Vintage	0.39	0.53	0.23	0.6	0.33	0.06 ˙	0.002 **	0.000002 ***
Cultivar:Vinification:Vintage	0.06 ˙	0.12	0.11	0.8	0.75	0.12	0.05 *	0.06 ˙
	Roditis							
Terroir Unit (Ter.A, Ter.B)	0.28	0.08 ˙	0.3	0.07 ˙	0.24	0.8	0.94	0.15
Vinification (V1, V2, V3)	0.25	0.09 ˙	0.02 *	0.94	0.77	0.08 ˙	0.99	0.99
Vintage (2019, 2020)	0.54	0.32	0.56	0.5	0.05 *	0.44	0.004 **	0.004 **
Terroir Unit:Vinification	0.31	0.17	0.09 ˙	0.6	0.68	0.37	0.96	0.82
Terroir Unit:Vintage	0.56	0.21	0.73	0.08 ˙	0.03 *	0.71	0.0002 ***	0.0000002 ***
Vinification:Vintage	0.23	0.2	0.1	0.57	0.47	0.08 ˙	0.1	0.14
	Sideritis							
Terroir Unit (Ter.A, Ter.B)	0.79	0.19	0.05 *	0.38	0.01 *	0.75	0.44	0.36
Vinification (V1, V2, V3)	0.81	0.67	0.03 *	0.33	0.96	0.72	0.32	0.89
Vintage (2019, 2020)	0.004 **	0.05 *	0.69	0.23	0.22	0.02 *	0.01 *	0.004 **
Terroir Unit:Vinification	0.98	0.49	0.07 ˙	0.49	0.27	0.99	0.6	0.8
Terroir Unit:Vintage	0.001 **	0.09 ˙	0.23	0.48	0.009 **	0.003 **	0.07 ˙	0.000004 ***
Vinification:Vintage	0.12	0.23	0.24	0.83	0.68	0.22	0.12	0.1

4.3.5. Correlations of the antioxidant, antimutagenic and anticancer activity of wines with members of the vinification microbiota

We further explored potential significant correlations between members of the vinification microbiota and the antioxidant, antimutagenic and anticancer properties of the wines produced (Supplementary Figs. S8 and S9). Fungi belonging to *Didymellaceae, Lambartella,* and bacteria of *Lactobacillus, Frauteria* showed significant negative correlation with the IC50 values of the PRA (antimutagenic activity) for the Roditis samples (Supplementary Fig. S8a and S8c). The abundance of fungi belonging to *Torulaspora debrueckii, Saccharomyces,* and bacteria of *Shingomonas, Streptomyces, Paenibacillus, Komagataeibacter, Methylobacterium, Clostridium, Lactococcus,* were negatively correlated with the IC50 values of the PRA for the Sideritis samples (Supplementary Fig. S8b and S8d). We further looked for fungal and bacterial genera that seem to be associated with the high antioxidant activity of the wine samples. We noted significant correlations of *Saccharomyces, Cladosporium, Rodotorula, Peniophora, Quambalaria cyanescens, Flavobacterium, Kozakia, Clostridium, Lactobacillus, Frauteria* with the high antioxidant activity of Roditis wines as depicted by the ABTS, DPPH and RPA assays (Supplementary Fig. S8a and S8c). Similarly, *Aspergillus, Papillotrema terrestris, Udeniomyces puniceus, T. delbrueckii, Lachancea quebecensis,* were associated with the high antioxidant activity of Roditis wines as depicted by the superoxide and hydroxyl radical assays (Supplementary Fig. S8b and S8d). Regarding Sideritis, we observed significant associations of (i) *Botryosphaeria, Stenotrophomonas, Burkholderia, Anaerocolumna*

with the high antioxidant activity of wines as depicted by the ABTS, DPPH, hydroxyl radical and superoxide radical assays and of (ii) *Aspergillus awamori, Saccharomyces, Cladosporium, L. quebecensis, Acinetobacter, Lysinibacillus, Lactobacillus, Clostridium* with the high antioxidant activity of wines as depicted by the RPA (Supplementary Fig. S8b and S8d). The anticancer activity of Roditis wines was positively correlated with *Aspergillus, P. terrestris, U. puniceus, T. delbrueckii, L. quebecensis* (Supplementary Fig. S9a and S9c) and of Sideritis *with T. delbrueckii, Saccharomyces, Sphingomonas, Streptomyces, Paenibacillus, Komagataeibacter, Methylobacterium, Clostridium* (Supplementary Fig. S9b and S9d).

4.4. Discussion

We first aimed to identify the main factors shaping the composition and the succession of the fungal and bacterial communities along the vinification. In this frame we posit the question if vineyard-related factors like cultivar, vintage and terroir, all previously shown to be strong determinants of the grape and must microbiota (Bokulich et al 2014; Chalvantzi et al., 2021; Hu et al. 2022; Papadopoulou et al., 2022), remain important determinants of the composition of the microbial communities along the vinification process. This was investigated under different vinification processes, employed at semi full-scale fermenters, reflecting realistic and current vinification practices in wine production. Our data suggested that under practical vinification conditions the fungal and bacterial communities exhibit strong cultivar and vintage signatures along the fermentation process, although their importance was higher for bacteria than fungi. Previous studies have highlighted the role of vintage on structuring the bacterial community during vinification (Rivas et al., 2021), and the presence of strong cultivar signatures in the microbiota during the fermentation process (Marzano et al., 2016; Kamilari et al., 2021).

Our experimental planning allowed us to further disentangle the influence of terroir for the two cultivars studied within the same viticultural zone of Aigialeia, Greece. We noted significant terroir signatures in the fungal and bacterial communities which disappear at the later stages of the fermentation. This was further highlighted when we included in the analysis data of the epiphytic microbiota of the same grapes used for fermentation at harvest (Papadopoulou et al., 2022). We noted a gradual loss of terroir signatures which are particularly evident at vineyard level

(field) but become weaker at the initial fermentation stages (early) and finally disappear at the latter fermentation stages (Supplementary Fig. S10). Several previous studies have also showed that initial strong geographic signatures at the early vinification stages become less important along the vinification process (Pinto et al. 2015; MorrisonWhittle & Goddard, 2018; Bokulich et al., 2016).

Beyond vineyard-mediated factors, we explored the influence of the vinification process employed on the vinification microbial communities. We compared the bacterial and fungal communities under three different vinification conditions reflecting current trends in wine making; (i) spontaneous vinifications with (V2) or without SO_2 addition (V1), which promote the geographical identity of wines but their management and quality assurance are challenging (Liu et al., 2017), (ii) commercial vinification inoculated with a commercial S. cerevisiae strain and amended with SO_2 that ensure standardized quality of produced wines but suppressed geographic identity (Galati et al., 2019). We noted that the vinification process employed was the stronger determinant of the fungal vinification microbiota for both cultivars, with spontaneous vinification supporting different fungal communities compared to the commercial vinification. The addition of SO_2 during the spontaneous fermentation (V2) did not exert a strong effect. In contrast, the effect of the vinification process employed on the bacterial community varied between cultivars. All three different vinifications supported distinct bacterial communities in Roditis, unlike Sideritis where the fully spontaneous fermentation (V1) supported different bacterial communities compared to the other two vinification processes characterized by addition of preservatives and/or inoculum. These results point to a stronger effect of preservatives on the bacterial compared to the fungal microbiota. Although SO_2 is generally used to inhibit bacteria and undesirable yeasts (Takahashi et al., 2014), its clearly stronger effect over the whole bacterial community is reported for the first time and worth to be further explored towards optimization of more traditional wine making processes. Bohmer et al. (2020) studied microbial succession under just fully spontaneous and commercial vinifications of Pinot Blanc and showed that both bacterial and fungal communities varied substantially between the two processes, in line with our findings. In a complementary study Wang et al. (2021) studied microbial community composition in spontaneous vinifications supplemented or not with SO_2

and noted a reduction in yeast richness but at levels of SO_2 (800 ppm), which are much higher than the ones used in this study.

Based on these observations we determined the composition of the bacterial and fungal communities along the vinification process aiming to identify key microbial succession patterns that might differentiate according to the different vinification process followed. The fungal communities showed a rather universal succession pattern across the vinification processes employed where NS yeasts (*Aureobasidium*, *L. quebecensis*, *T. delbrueckii*) and filamentous fungi (*Cladosporium*, *Alternaria*, *Aspergillus*), known as common dwellers of the grapevine microbiota (Deyett & Rolshausen, 2020; Liu & Howell, 2021; Papadopoulou et al., 2022), dominate at the early vinification stages and act as starters of fermentation contributing special regional and grapevine cultivar features (Padilla et al., 2016; Anfang et al., 2009; Kuchen et al., 2019; Escribano et al., 2017). This is drastically altered as the fermentation process initiates and towards stages characterized by high alcohol content where indigenous in spontaneous or the inoculated Saccharomyces strain in the commercial fermentation dominates the fungal community. Similar patterns of fungal succession have been reported in previous studies (Bokulich et al., 2016; Wei et al., 2022). In our study, *L. quebecensis* and particularly *T. delbrueckii* were the two fermentative NS yeasts that were present in the initial stages of the vinification process but declined along the fermentation process, in line with their sensitivity to high ethanol content (Morgan et al., 2017). Strains of *T. delbrueckii* are used as mixed inocula with *S. cerevisiae*, implanted either successively or simultaneously in must, to improve certain characteristics of wine like glycerol and acetaldehyde content, acidity and aromatic complexity (Benito, 2018; Sgouros et al., 2023). Little is known about the role of *L. quebecensis* in vinification. Members of another *Lachancea* species, *L. thermotolerans*, are common in spontaneous wine fermentations (Morata et al., 2018). In addition, they are used in wine making as mixed inocula with *S. cerevisiae* based on their capacity to reduce ethanol content, produce lactic acid, ethyl lactate, a fruity-like flavor compound (Vilela, 2018) and ethyl isobutyrate, a strawberry flavor compound (Sgouros et al., 2020).

In contrast, the bacterial community did not show a universal succession in the different vinification process employed. In all spontaneous fermentations of Roditis, *Gluconobacter*, *Lactobacillus* and *Enterobacterales* dominated early in the fermentation but they were gradually displaced by the LAB *Oenococcus sp*.,

especially in the absence of SO_2 addition. This pattern was replicated in all fermentations of Sideritis in 2019 and in the fully spontaneous fermentations of the same cultivar in 2020, where *Oenococcus sp.,* displaced *Lactobacillus*, *Gluconobacter*, *Acetobacter* and *Komagataeibacter*, in accord with other studies (Marzano et al., 2016). *Oenococcus* is the most important LAB performing the MLF that prevents the undesirable acidification of wine (Bartowsky et al., 2015), and contributes to the production of volatile aromatic compounds (Bokulich et al., 2016; Capello et al., 2017). Its tolerance to low pH, high ethanol levels and scarcity of nutrients explain the domination by *Oenococcus* at the last vinification stages. On the other hand, *Gluconobacter*, *Acetobacter*, and *Komagataeibacter*, dominating the community at the early fermentation stages, are common dwellers of grapes (Barata et al., 2012). Their persistence in vinification beyond the first stages is a sign of wine spoilage due to their capacity to transform ethanol to acetic acid, hence their classification as AAB (Bartowsky 2009). In addition, *Gluconobacter* and *Acetobacter* are often competitive to *Saccharomyces* with negative effects on fermentation rates (Bokulich et al., 2016; Berbegal et al., 2019). Hence, the bacterial succession in all these vinifications suggests an efficient MLF performed by *Oenococcus*. However, we also noted other bacterial succession patterns like (a) in the commercial vinification of Roditis in 2019 where a gradual establishment of *Prevotelaceae* was noted. This family includes strong fermenting bacteria of the gut microbiome, that were recently identified as key component of the fermentation of Xiaoqu liquor being associated with different flavor compounds (Su et al., 2020), (b) the consistent presence of *Tatumella* in the vinifications of Roditis in 2020. *Tatumella* is a common encounter of vinification often persisting until the later stages of fermentation (Pinto et al., 2015; Nisiotou et al., 2011; Steenwerth et al., 2021). They have been associated with reduced wine acidity (Bubeck et al., 2020; Wei et al., 2022), although their exact role and interaction with other key microbial members of the vinification remain unknown.

The influence of the same factors was also examined on important biological properties of the wine samples, namely their antioxidant, antimutagenic and anticancer activities. Vintage and the vinification process employed were the main determinants of the antioxidant activity of the produced wines from both cultivars. In particular, the commercial vinification (V3) produced wines with an overall higher antioxidant and antimutagenic activity compared to the fully spontaneous vinification

(V1). These results highlight the key role of the fermentation process which select for microorganisms that influence, qualitatively and quantitatively the phenolic content of wines (Septembre-Malaterre et al., 2018). Regarding the anticancer activity of wines, vintage was its stronger determinant. The vinification process and terroir also affected the anticancer activity of the wine samples. However, no consistent pattern across cultivars was evident, apart from the consistently higher anticancer activity of the samples produced from the commercial compared to the fully spontaneous vinification for the two cultivars in alternate years. Fermentation appears to be a process that indirectly influences the anticancer activity of food-derived products (Tasdemir & Sanlier, 2020). Fermented products help to maintain a healthy gut microbiome favoring the establishment of bacteria that produce bacteriocins, vitamins and specific enzymes that exert anticancer properties (Sharma et al., 2018; Stanton et al., 2005).

Finally, we identified members of the fungal and bacterial microbiota that were positively associated with the antoxidant, antimutagenic and anticancer activity of the wines produced suggesting potential direct or indirect involvement in the establishment of these desirable properties. *Lactobacillus* and *Frauteria*, identified as LAB and AAB respectively (Lisdiyanti et al., 2003), were positively associated with the antioxidant and the antimutagenic activity of the Roditis wines, while *Saccharomyces* and *Clostridium* were associated with the antioxidant, antimutagenic and anticancer activity of the Sideritis wines. Previous studies have suggested the involvement of *S. cerevisiae* and *Lactobacillus* in the increased content of Tempranillo wines in flavonoids and phenolic acids, known to contribute to the antioxidant and anticancer activity of wines (Hernandez et al., 2007). We also noted a consistent association of *T. debrueckii* and *L. quebecensis* with the anticancer and antioxidant activity of wines, respectively, across cultivars. The capacity of these yeasts to alter the composition and total concentration of phenolics in wine has been reported before and suggests a potential role in enhancing the antioxidant and anticancer potential of wine (Minnaar et al., 2018; Zhang et al., 2021). Previous studies have identified correlations between members of the vinification microbiota and metabolites or aromatic compounds of wine (Bokulich et al., 2016; Wei et al., 2022), while it is the first time that associations between members of the vinification microbiota and the antioxidant, anticancer and antimutagenic potential of wines are shown. Although we acknowledge that these results do not prove causality, they

constitute first indication for the possible involvement of the microorganisms in the production or activation of compounds that exhibit antioxidant (Romanet et al., 2021) and anticancer activity in wines (Duan et al., 2021).

4.5. Conclusion

The composition and the dynamics of the vinification microbiota constitutes fundamental knowledge for optimization of the wine making process. We showed that under realistic scale-wise and process-wise wine making practices, vineyard-mediated factors, such as cultivar and vintage, remain operative along the vinification process, unlike terroir whose effect, although significant at start, becomes less important as fermentation progresses. The type of vinification process employed, namely spontaneous or commercial, strongly influenced the composition of the vinification microbiota with the effect varying between fungi and bacteria. Fungi showed a universal pattern with NS yeasts and filamentous fungi dominating earlier in fermentation being displaced by indigenous or inoculated *Saccharomyces* strains. Whereas bacterial succession varied, with the most common pattern mirrored in an initially rich in AAB bacterial community replaced by *Oenococcus* at the end of fermentation. Vintage and secondly vinification type were also major determinants of the antioxidant, antimutagenic and anticancer potential of the produced wines. Our study went a step ahead and identified strong associations between certain members of the vinification microbiota and the antioxidant, antimutagenic and anticancer potential of the produced wines. This information could be exploited, along with similar information on wine volatiles and aromatic metabolites, towards a microbiota-mediated optimization of the produced wines.

4.6. References

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4.7 Annex III – Supplementary data

Materials and Methods

Assessment of the antioxidant's activity of wines

DPPH• reduction assay

The assay was based on a previously described protocol. In brief, the volume of the reaction mixture was equal to 1 ml and consisted of a freshly made methanolic solution of DPPH• (100 μM) and the tested wine samples at a wide volume range (100-50-25-12.5-6.25μl/ml). The contents were mixed, incubated in the dark at room temperature (RT) for 20 min and the absorbance values were monitored at 517 nm. In each experiment, the tested wine sample alone served as the blank and DPPH• alone in methanol was the control. The percentage of radical scavenging capacity (RSC) of the tested wine samples was calculated according to the following equation:

% DPPH• radical scavenging activity = [(Abscontrol − Abssample)/Abscontrol] ×100,

where Abscontrol and Abssample are the absorbance values of the control and the tested sample, respectively. Finally, the IC50 values showing the volume of the samples that reduced DPPH• at 50% was estimated.

$ABTS^{\bullet+}$ radical reduction assay

Briefly, the reaction was carried out in 1 ml and the mixture contained $ABTS^{\bullet+}$ (1 mM), H_2O_2 (30 μM) and horseradish peroxidase (HRP) (6 μM) (Cano et al., 1998; Veskoukis et al., 2012). The samples were vigorously mixed and incubated in the dark at RT for 45 min. Subsequently, the wine samples were added to the reaction mixture at a wide volume range (100-50-25-12.5-6.25 μl/ml) and the absorbance at 730 nm was monitored. In each experiment, the HRP free tested sample containing $ABTS^{\bullet+}$ and H_2O_2 was used as the blank, whereas the control sample contained the $ABTS^{\bullet+}$ solution, H_2O and HRP. The percentage of RSC and the IC50 values of the wine samples were determined similarly to the DPPH• assay.

Superoxide radical ($O_2^{\bullet-}$) reduction assay

The superoxide radical scavenging capacity of the samples was evaluated as previously described (Kerasioti et al., 2014). Briefly, 50 ml of each wine sample at a wide volume range (100-50-25-12.5-6.25-3.125 μl/ml) was added to 625 μl of

trishydroxymethylaminomethane hydrochloride (Tris-HCl, 16 mM, pH=8), 125 μl of nitro blue tetrazolium chloride (NBT, 300 μM) and 125 μl of NADH (60 μM). Then, the samples were incubated in the dark at RT for 5 min and the absorbance was monitored at 560 nm. The percentage of RSC and the IC50 values were determined similarly to the DPPH• assay.

Hydroxyl radical (OH•) reduction assay

The hydroxyl radical scavenging capacity of the samples was evaluated as previously described (Kerasioti et al., 2014). According to the protocol, 400 μl of each wine sample at a wide volume range (100-50-25-12.5-6.25-3.125 μl/ml) was added to 225 μl of phosphate buffer (0.2 M, pH=7.4), 75 μl of 2-deoxyribose (10 mM), 250 μl of FeSO4-EDTA (10 mM), 250 μl of H_2O_2 (10 mM) and 250 μl of dH_2O. The samples were incubated in the dark at 37˚C for 1 h. Subsequently, 375 μl of TCA (2.8%) and 375 μl of 2-thiobarbituric acid (1% w/v) were added and the samples were incubated at 100˚C for 10 min. Then, the samples were cooled on ice for 5 min, centrifuged (5,000 g, 10 min, 25˚C) and the absorbance was monitored at 520 nm. The samples without H_2O_2 were used as the blank and the samples without protein were used as the control. The percentage of RSC and the IC50 values were determined similarly to the DPPH• assay.

Reducing power assay (RPA, Fe^{3+} to Fe^{2+})

Reducing power of the samples was determined as previously described (Kerasioti et al., 2014). In brief, 250 μl of each sample was added to 250 μl of potassium ferricyanide (1% w/v) and 250 μl of phosphate buffer (0.2 M, pH=6.6) and incubated at 50˚C for 20 min. The samples were cooled on ice for 5 min. Then, 250 μl of trichloroacetic acid (TCA, 10%) was added to the mixture and the samples were centrifuged (5,000 g, 10 min, 25˚C). Then, 250 μl of dH_2O and 500 μl of ferric chloride (0.1% w/v) were added to 700 μl of the supernatant, the samples were incubated in the dark at RT for 10 min and the absorbance was monitored at 700 nm. The reducing power of the extracts is also expressed as IC50.

Plasmid Relaxation Assay (PRA)

The assay was performed as previously described (Veskoukis et al., 2012; Kerasioti et al., 2014). The protective activity of the tested wine samples against ROO•-induced

DNA oxidation is based on the inhibition of the conversion of the normal supercoiled form of DNA to the open circular, which is an indication of single-stranded DNA scissions due to oxidative modifications. The reaction mixture (10 μl) containing Bluescript-SK+ plasmid DNA (1 μg), various volumes of the tested wines (3-1.5-0.75-0.375-0.19-0.094-0.047-0.023-0.012-0.006 μl/10μl) and 22,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH, 570 mM) in phosphate buffered saline was incubated for 45 min at 37˚C. The reaction was terminated by the addition of loading buffer (3 μl, 0.25% bromophenol blue and 30% glycerol) and analyzed in 0.8% agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide (0.5 μg/ml), distained with dH_2O, photographed by UV transillumination using the Vilber Lourmat photodocumentation system (DP-001.FDC, Torcy, France) and analyzed with the Gel-Pro Analyzer version 3.0 (Media Cybernetics, Silver Spring, Rockville, MD, USA). The percentage inhibition was calculated using the following equation: % inhibition=[(S − So)/(Scontrol − So)] ×100, where Scontrol is the percentage of the supercoiled DNA of the negative control sample (plasmid DNA alone), So is the percentage of the super-coiled plasmid DNA of the positive control sample (without the tested wine samples, but in the presence of the radical initiating factor) and S is the percentage of the supercoiled plasmid DNA of the sample with the tested wines and the radical initiating factor. The IC50 values were determined similarly to the $DPPH^{\bullet}$ assay.

Assessment of the anticancer activity of the produced wines

Wine samples were initially converted into dry extracts before administered directly to cultured cells due to their high alcohol content. In the process of extract preparation, 200 ml of wine sample provided 20 g of dried extract. This was amended with maltodextrin DE-18 at a ratio of 20% to the solid components and the samples underwent freeze-drying. All extracts were stored at 4°C and were diluted in ddH_20 before use. These dry extracts, in serial dilutions, were tested on cultures of human breast adenocarcinoma cell line MDA-MB-231 and human hepatocellular liver carcinoma cell line HepG2. All cell cultures were routinely maintained in DMEM (Dulbecco's modified Eagle medium) (containing 4.500 mg/L D-glucose, 110 mg/L sodium pyruvate, L-glutamine) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Gibco) and antibiotics (PS: 100 units/mL of penicillin G, 100 μg/mL of streptomycin sulfate), in a humidified chamber at 37 °C with 5% CO_2. The inhibition

of cell proliferation was measured using the XTT assay method, as previously described (Tselepi et al., 2011). More details about the protocol used can be found in the Supplementary data. XTT is a tetrazolium salt which is reduced only in metabolically active cells to orange-colored formazan compounds, which absorb at 450 nm. In brief, seeding density for MDA-MB-231 and HepG2 was 10^4 and 2.5×10^4 cells/well, respectively, in culture medium (DMEM−FBS−PS) in a 96-well flat-bottomed microplate. After 24 h incubation at 37 °C under humidified 5% CO_2, the extracts diluted at a concentration of 20 mg/ml in DMEM-PS medium were added to the cells and were incubated for additional 24 h. Cultures containing only DMEM−PS (without extracts) were used as control samples. Cell cultures were additionally incubated for 6 h with the XTT reagent (Roche), and the optical density (OD) was measured using an EL808 microplate reader (Biotek Instruments, Inc.), at 450 nm with a reference wavelength at 650 nm. For each extract, the percentage of inhibition of cell proliferation compared to the control sample was calculated as follows: % Inhibition of proliferation = $[(OD_{control} - OD_{sample})/OD_{control}] \times 100$. In each experiment all samples were tested in duplicates. At least 3 independent experiments were performed.

Supplementary References

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Supplementary Figures

Fig. S1. Map showing the location of the cultivated vineyards of Roditis and Sideritis in the Aigialeia viticultural zone (Peloponnese) and the locations of collaborating wineries.

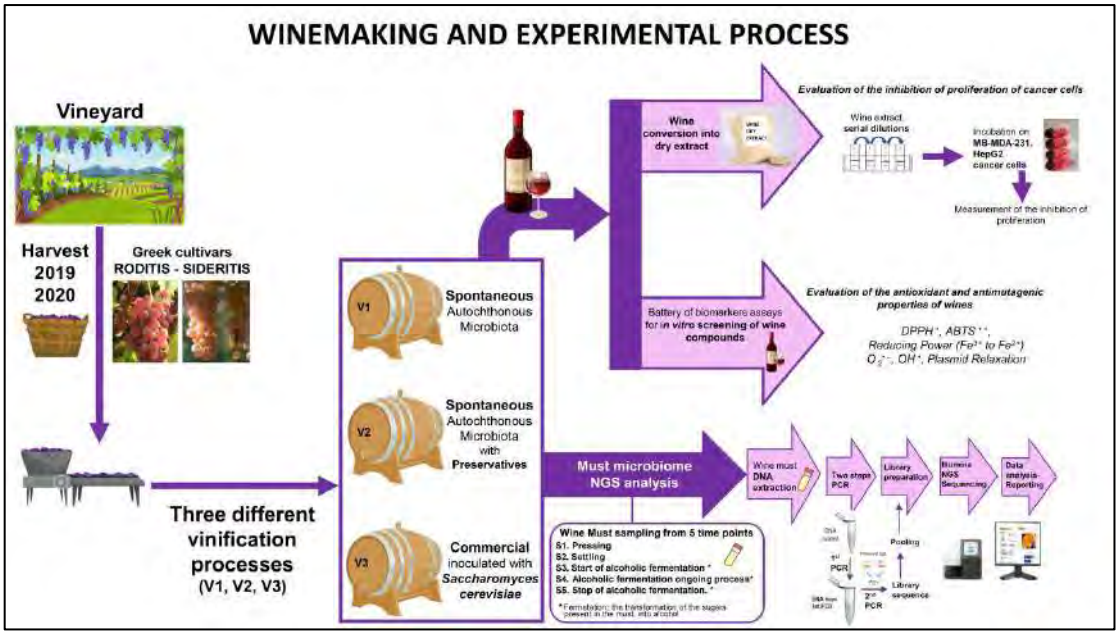

Fig. S2. A schematic representation of the vinification process and the different treatment employed.

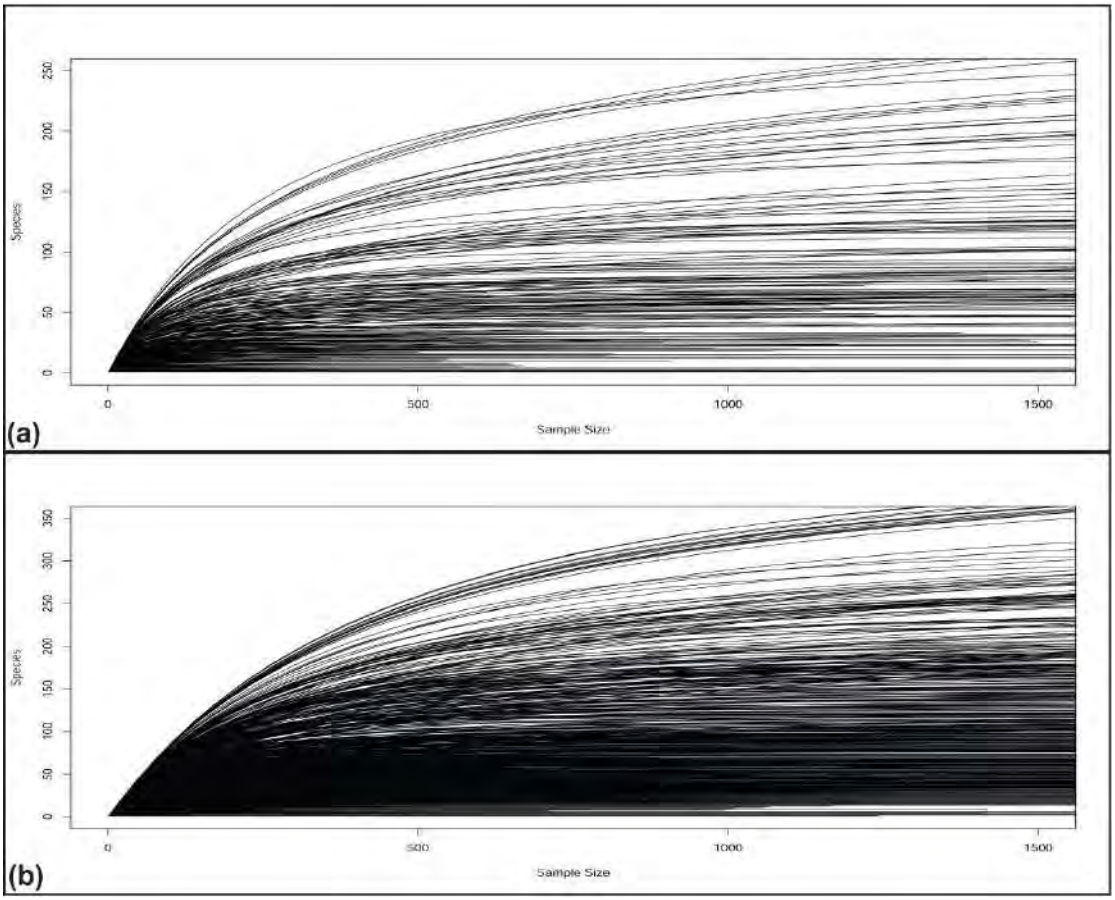

Figure S3. Rarefaction curves constructed based on amplicon sequencing analysis of the fungal (a) and the bacterial community (b) in grape must samples.

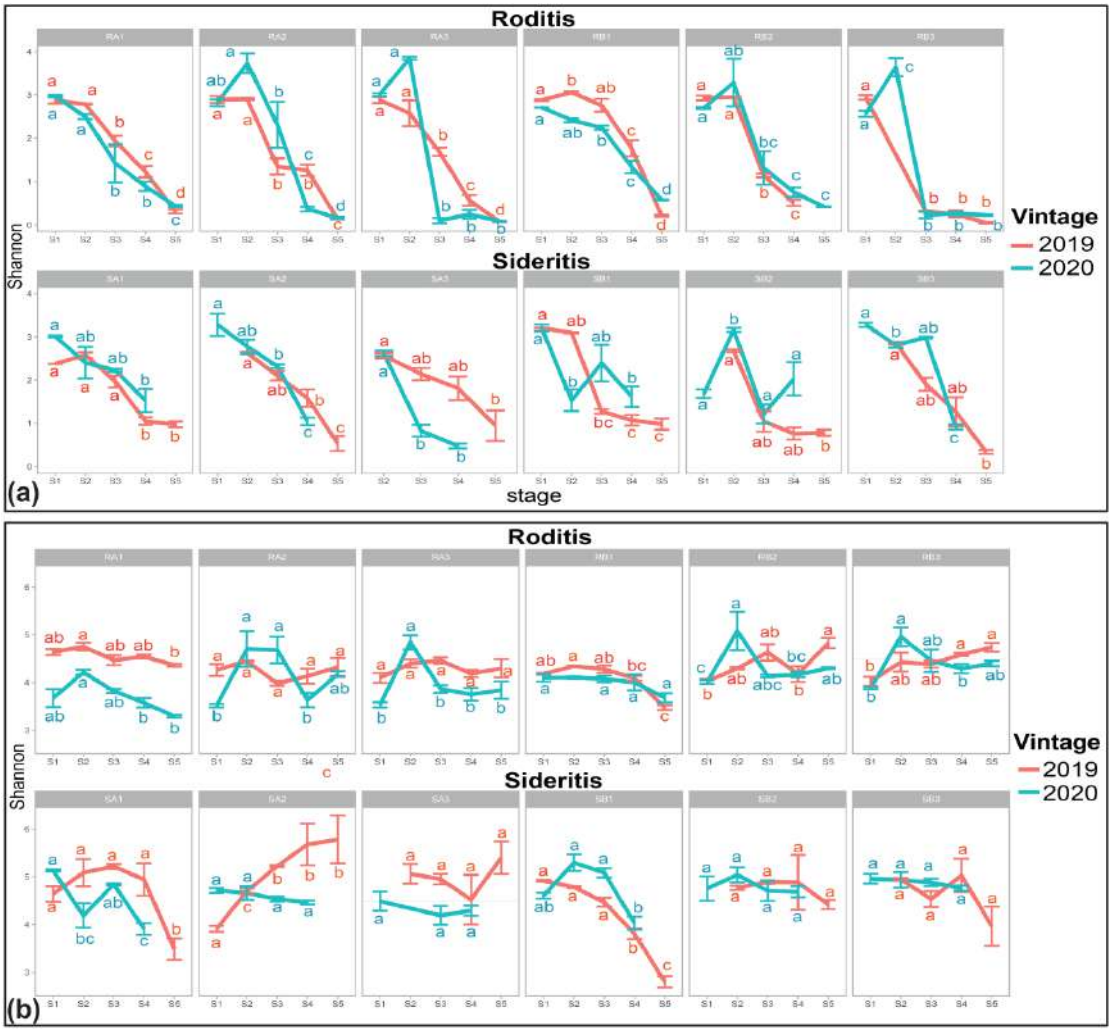

Figure S4. The α-diversity Shannon index for the (a) fungal and (b) bacterial community calculated for each vinification performed with grapes of the cultivars Roditis and Sideritis collected at 2019 and 2020. Within each vinification values designated by different letters indicate significant differences along the vinification process. Codes of samples: (i) first letter indicates the cultivar R: Roditis, S: Sideritis, (ii) second letter indicates the terroir unit A: terroir A, B: terroir B, (iii) Number indicates vinification type 1: spontaneous/without preservatives, 2: spontaneous/with preservatives, 3: commercial with Saccharomyces cerevisiae inoculum, (iv) Vinification stages: S1, S2, S3, S4, S5.

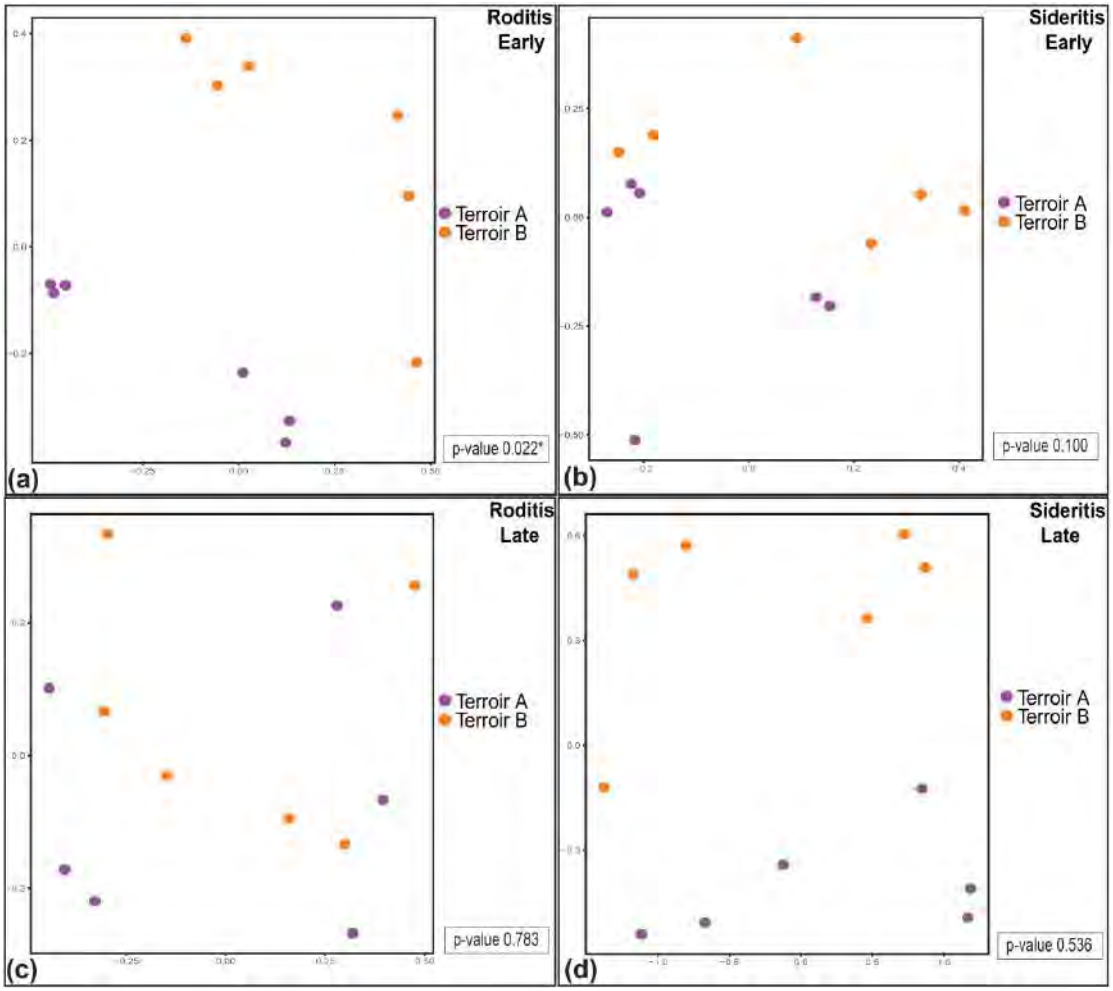

Figure S5. NMDS ordination of the fungal community according to *terroir* unit for samples of the cultivars Roditis (a and c) and Sideritis (b and d) collected either at early (S1, S2) and late (S4, S5) stages of the vinification. P-values <0.05 indicate significant differences between *terroir* units.

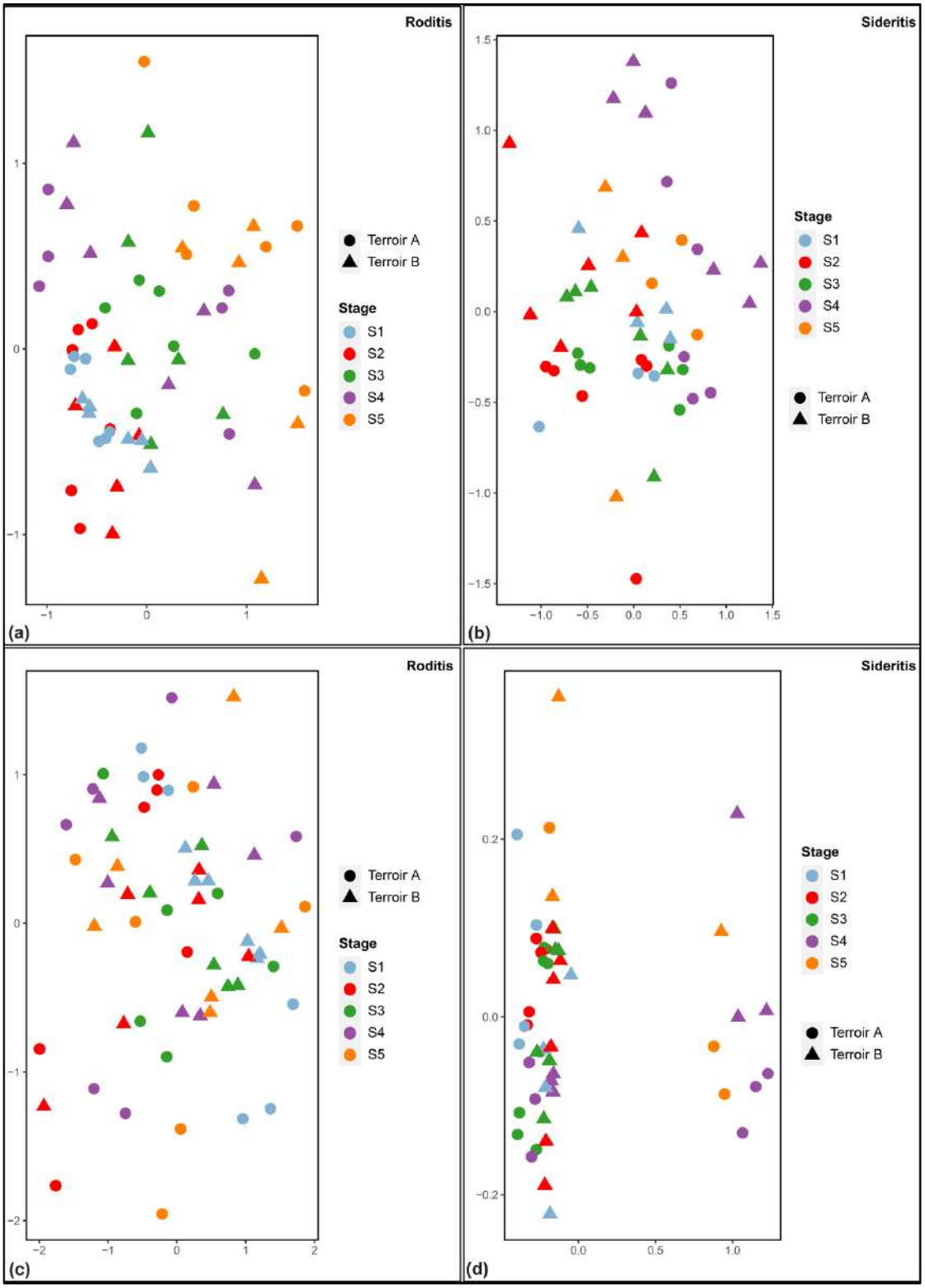

Figure S6. NMDS ordination of the fungal (a, b) and bacterial (c, d) communities in vinification samples collected from three different vinification processes performed with grapes collected in 2019 and 2020 from the cultivars (a, c) Roditis and (b, d) Sideritis from two different terroir units (A and B). Samples are ordinated according to their vinification stage.

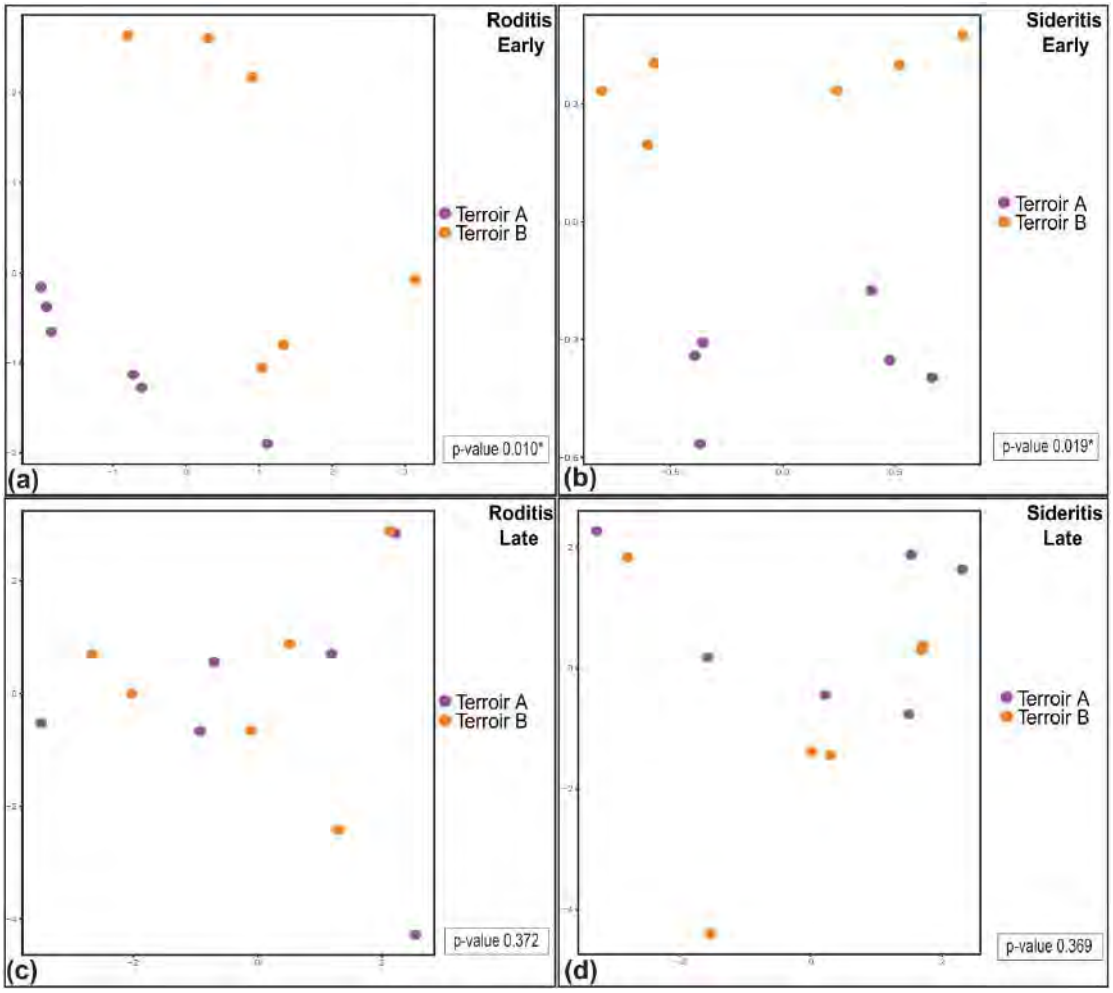

Figure S7. NMDS ordination of the bacterial community according to *terroir* unit for samples of the cultivars Roditis (a and c) and Sideritis (b and d) collected either at early (S1, S2) and late (S4, S5) stages of the vinification. P-values <0.05 indicate significant differences between *terroir* units.

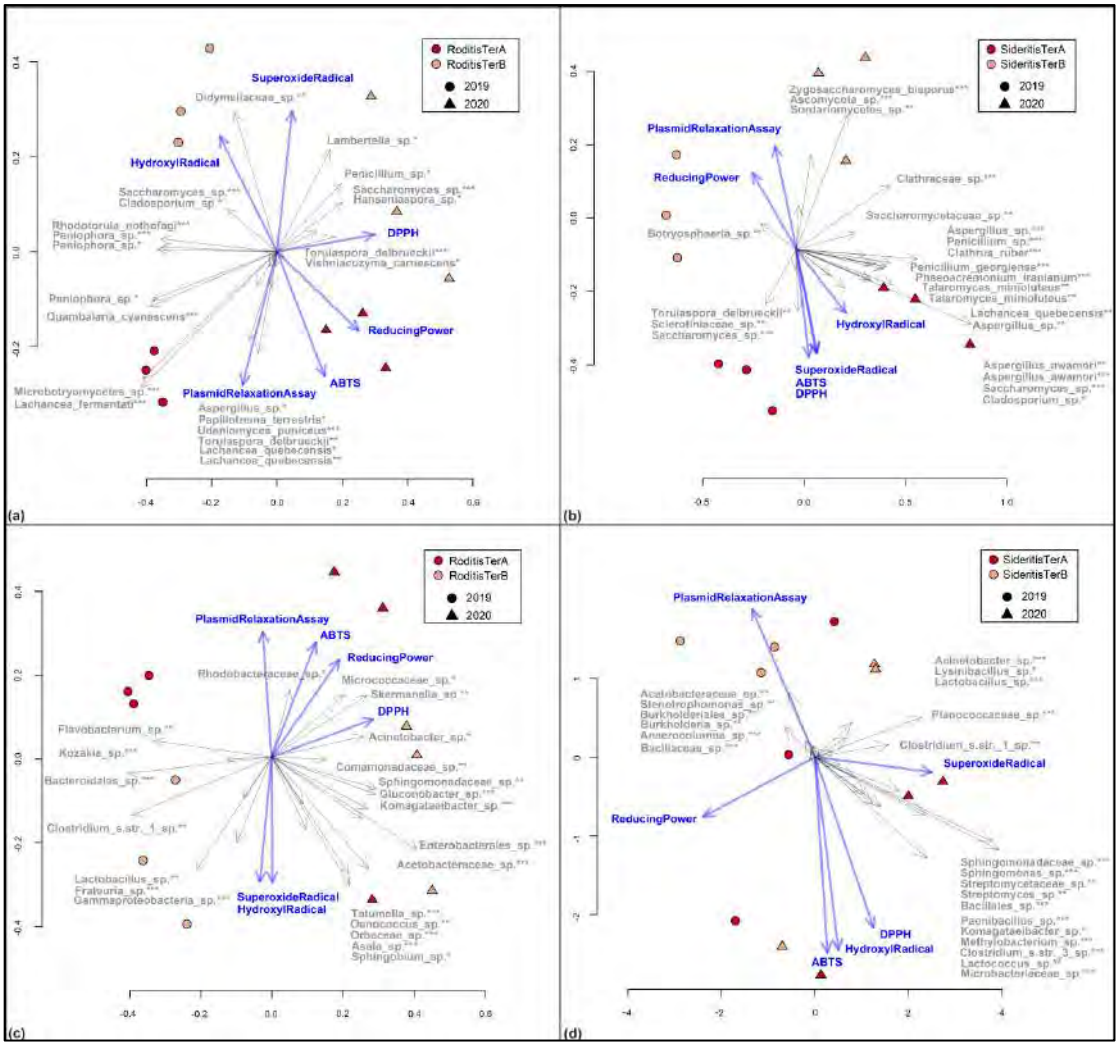

Fig. S8. Ordination triplot correlation matrix between members of the fungal (a, b) and bacterial (c, d) microbiota and the antioxidant properties of the wine samples of Roditis (a, c) and Sideritis (b, d) depicted by the different assays like the DPPH• radical reduction capacity, the ABTS•+ radical reduction capacity, the Reducing Power Assay (RPA), the Superoxide radical (O2•–) reduction capacity, the hydroxyl radical (OH•) capacity and the Plasmid Relaxation Assay (PRA).

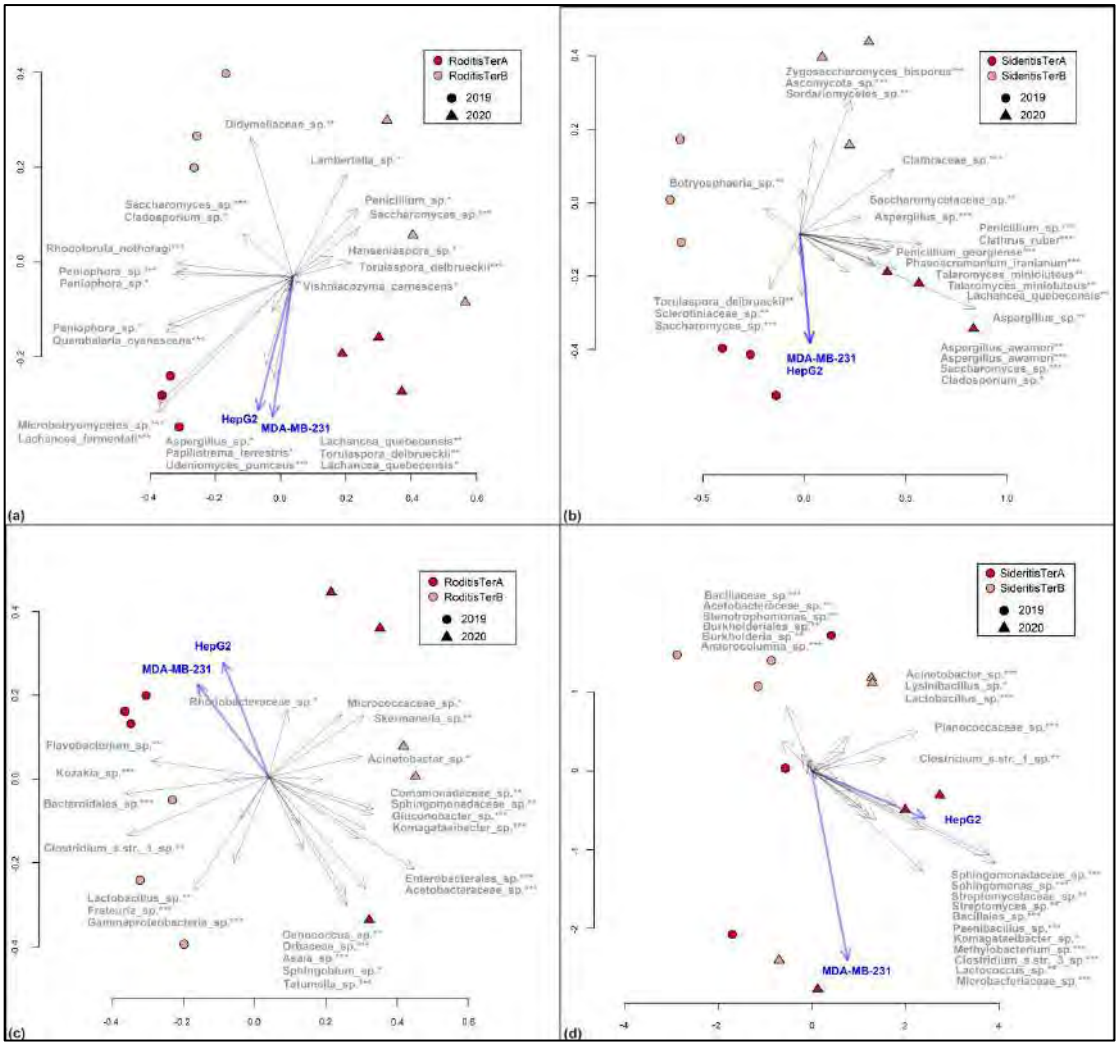

Fig. S9. Ordination triplot correlation matrix between members of the fungal (a, b) and bacterial (c, d) microbiota and the anticancer properties of the wine samples of Roditis (a, c) and Sideritis (b, d) depicted by assays with HepG2 and MDA-MB-231 cancer cell lines.

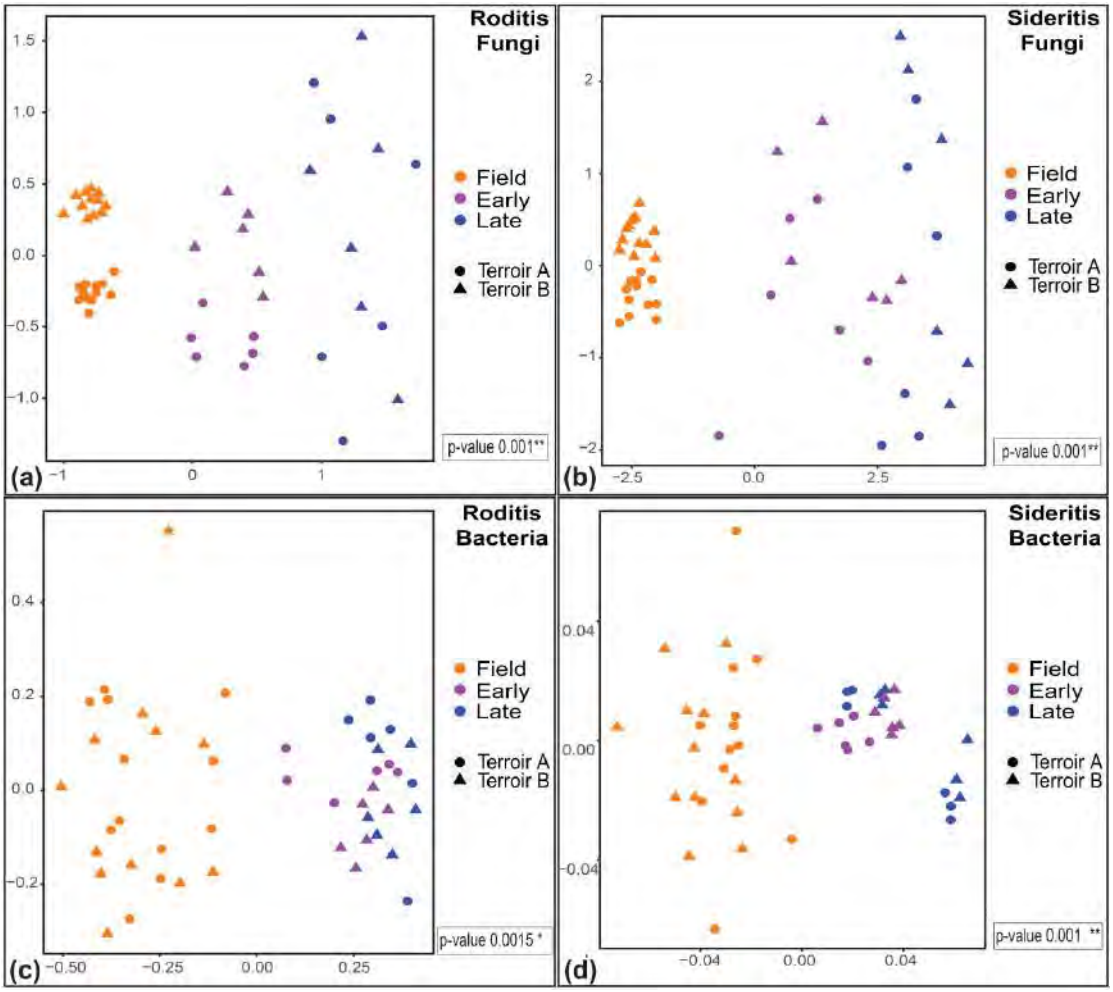

Figure S10. NMDS analysis of the fungal (a, b) and bacterial (c, d) communities of cultivars Roditis and Sideritis from the field (epiphytic grape microbiome, data obtained from Papadopoulou et al., 2022), to early stages of fermentation (S1, S2) and late stages of fermentation (S4, S5). At each stage (field, early, late) data are ordinated according to terroir. Data from all vinifications and vintages per cultivar were merged.

Supplemental Table S1. Primers used for amplicon sequencing analysis. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step, which are composed of the sequence of the universal primers 515f (bacteria) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a 2bp linker sequence in between.

Kingdom	PCR step	Primers	Gene target	Amplicon size (bp)	Primer sequences (5'-3')	References
Bacteria	1st	515F 806R	16S rRNA	~290	GTGYCAGCMGCCGCGGTAA GGACTACHVHHHTWTCTAAT	Parada et al., (2016)
	2nd	Indexed (n=96)	B0001-515f		NNNNNNNNNGT**GTGYCAGCMGCCGCGGTAA**	Katsoula et al., (2021)
Fungi	1st	ITS7F ITS4R	ITS2	~310	GTGARTCATCGAATCTTTG TCCTCCGCTTATTGATATGC	Ihrmark et al., (2012) White et al., (1990)
	2nd	Indexed (n=96)	FI0001-ITS4r		NNNNNNNNNGA**TCCTCCGCTTATTGATATGC**	Katsoula et al., (2021)

Supplemental Table S2. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	**Volume (μl)**	**Concentration**	**Comments**
Primer F	1	0.5 μM	
Primer R	1	0.5 μM	
BSA	0.4	0.4 μg/μl	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH_2O	5.6		
DNA	2	0.2 ng/μl	
Total	20		
PCR conditions			
Step	**Temperature (°C)**	**Time**	**Number of Cycles**
Initial Denaturation	98	30 sec	28 in the first amplification step / 7 in the second amplification step
Denaturation	98	10 sec	
Annealing	50 for bacteria/ 55 for fungi	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	

Supplemental Table S3: PERMANOVA-based variation partitioning analysis of the fungal and bacterial vinification microbiota. (Signif. codes: 0.001 '***' 0.01 '**' 0.05 '*').

Factor	Degrees of Freedom	Sum of squares	F. Model	R^2 (%)	P-value (>F)
Fungi of vinifications (Roditis, Sideritis)					
Cultivar	1	1.683	12.600	4.1	0.001 ***
Vintage	1	1.577	11.808	3.8	0.001 ***
Terroir Unit	3	1.013	3.791	2.5	0.003 **
Stage	6	11.411	14.237	27.6	0.001 ***
Vinification	2	7.211	26.992	17.4	0.001 ***
Residuals	138	18.435		44.6	
Fungi Roditis					
Vintage	1	0.448	3.640	1.9	0.023*
Terroir Unit	1	0.412	3.348	1.8	0.020*
Stage	6	7.657	10.363	33.1	0.001 ***
Vinification	2	6.352	25.791	27.5	0.001 ***
Residuals	67	8.251		35.7	
Fungi Sideritis					
Vintage	1	2.103	18.141	12.7	0.001 ***
Terroir Unit	1	0.609	5.253	3.7	0.002 **
Stage	6	4.684	6.734	28.3	0.001 ***
Vinification	2	1.939	8.362	11.7	0.001 ***
Residuals	62	7.187		43.5	
Bacteria of vinifications (Roditis, Sideritis)					
Cultivar	1	8.973	41.425	16.7	0.001 ***
Vintage	1	3.339	15.415	6.2	0.001 ***
Terroir Unit	3	3.403	7.855	6.3	0.001 ***
Stage	6	4.962	3.818	9.2	0.001 ***
Vinification	2	2.466	5.693	4.6	0.001 ***
Residuals	141	30.541		56.9	
Bacteria Roditis					
Vintage	1	3.084	15.349	12.5	0.001 ***
Terroir Unit	1	1.924	9.572	7.8	0.001 ***
Stage	6	2.952	2.448	12	0.001 ***
Vinification	2	2.791	6.945	11.3	0.001 ***
Residuals	69	13.866		56.3	
Bacteria Sideritis					
Vintage	1	3.488	20.949	17.3	0.001 ***
Terroir Unit	1	1.472	8.839	7.3	0.001 ***
Stage	6	3.737	3.741	18.6	0.001 ***
Vinification	2	0.931	2.795	4.6	0.003 **
Residuals	63	103.882		51.6	

Chapter 5

Wine must metatranscriptomic analysis reveals metabolic activities and functions mobilized by the microbiota in spontaneous and inoculated vinifications

Elena Papadopoulou, Sotirios Vasileiadis, Fotios Bekris, Giorgos Kotseridis, Dimitrios G. Karpouzas

5.1 Introduction

Fermentation constitutes one of the oldest biotechnological processes (Anagnostopoulos and Tsaltas, 2019). It comprises several successive biochemical reactions driven by the growth and metabolic activity of microorganisms which breakdown organic substances or other components into simpler compounds (Terefe, 2016). In winemaking, fermentation for wine production involves mainly the conversion of grape sugars (glucose, fructose, etc.) to ethanol and carbon dioxide, driven by a diversity of microorganisms, many of which are derived from grapes. Grape–berries constitute a rich reservoir of microorganisms like yeasts, filamentous fungi, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), with different physiological attributes that interact and reflect the origin of grapes (Barata et al., 2012; Martins et al., 2012; Ding et al., 2021; Ding et al., 2023).

Spontaneous fermentation, a wine-making process driven purely by the indigenous grapes microbiota leads to the production of wines with increasing aroma complexity and enhanced regional identity (Belda et al., 2020; Liu et al., 2019; Luzzini et al., 2021), although it often suffers from repeatability and spoilage. As a result, current winemaking practices still prefer the use of commercial active dry yeasts to control vinification results and reduce the risk of spoilage. Wine fermentation is a combination of alcoholic fermentation (AF) and malolactic fermentation (MLF), implicating a mixture of different microorganisms. The AF is conducted by yeasts with the capability of fermenting sugars to ethanol, the so-called fermentative yeasts (Ciani and Comitini, 2019). *Saccharomyces cerevisiae* is regarded as the predominant fermentative yeast, characterized by a high capacity to degrade glucose and fructose to ethanol. Fermentative yeasts can develop an oxygen-limited environment during fermentation (Merico et al., 2007). Apart from *S. cerevisiae*, non-Saccharomyces (NS) yeasts participate also in AF, but mostly at the early stages, since they cannot tolerate high ethanol levels or efficiently compete for nutrients. This leads to *S. cerevisiae* dominating and completing the fermentation process (Liu et al., 2015; Ciani and Comitini, 2006; Brandam et al., 2013; Jolly et al., 2014; Taillandier et al., 2014). Despite their presence mostly at the first stages of fermentation, NS yeasts such as *Lachancea thermotolerans*, *Hanseniaspora uvarum, Kloeckera spp., Torulaspora delbrueckii, Starmerella, Pichia, Metschnikowia, Vishniacozyma,* are known to contribute to the production of desirable chemosensory attributes, like fruity

and flowery aroma, of wine, while they can also successfully co-ferment with *S. cerevisiae* (Fazio et al., 2023; Joran et al., 2022; Ciani et al., 2016; Ramirez and Velazquez, 2018; Jolly et al., 2014; Padilla et al., 2016).

MLF usually follows AF and it leads to increasing acidity (pH), contributes to the stability of wine by malic acid removal and modifies wine aroma (Ines and Falco, 2018; Lerm et al., 2010). MLF results from the metabolic activity of LAB, that converts L-malic acid to L-lactic acid. Known LAB are *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*, of which *Oenococcus* is the most resistant to ethanol and SO_2 preservatives, while the other LAB contribute mostly to the aroma and flavour of wine (Maitre et al., 2014; Bubeck et al., 2020; Rivas et al., 2022; Cappello et al., 2017). Additionally, AAB like *Acetobacter*, *Gluconobacter*, *Komagataeibacter,* are frequent encounters of vinification, associated often with wine spoilage due to their contribution to the formation of high acetic acid levels (Guillamon and Mas, 2011; Gomes et al., 2018; Bartowsky and Henschke, 2008).

Although the metabolic activity of *S. cerevisiae* is a predominant feature of the AF and strongly affects the quality of the final product, the contribution of the other members of the vinification microbiota to the quality of the final wine product should not be overlooked (Querol et al., 2003; Pfeiffer and Morley, 2014; Ghosh et al., 2015; Bagheri et al., 2015). Today most studies have used correlation testing to shed light into the contribution of members of the vinification microbiota to the production of specific metabolites and sensory attributes of wine (Bokulich et al., 2016; Martin et al., 2018; Morata et al., 2018), while direct evidence regarding the metabolic pathways and functions activated by the microorganisms during fermentation are lacking. Determination of the metatranscriptome of the vinification microbiome along the vinification process combined with the use of appropriate bioinformatic tools would identify the microorganisms that are active during fermentation and provide direct evidence for the nature of the metabolic pathways mobilized by the vinification microbiota to develop the chemical profile of the produced wine.

We aimed to monitor microbial succession during vinification and explore the active fraction and the metabolic pathways and functions employed by the vinification microbiota during fermentation. In this frame we conducted lab-scale vinifications, spontaneous and inoculated (both though subjected to preservatives addition), with grapes of the Greek cultivar Vidiano and explored the variations in microbial succession during the different vinification strategies through amplicon sequencing

analysis. We further employed metatranscriptomic analysis at different vinification stages to define the active fraction of the vinification microbiota and its activated functions/pathways during fermentation.

5.2 Materials and Methods

5.2.1. Samples collection and preparation.

Grape-berries from the Greek cultivar Vidiano collected at 2019 were used for fermentation. After collection, the fresh grapes were destemmed and mechanically crushed and the generated must was then used for the vinification process. Right after crushing, potassium metabisulfite (PMB, $K_2S_2O_5$, 4gr/hL) and ascorbic acid (100gr/tn) were added in the must. Two strategies of microscale fermentation were followed: (a) a spontaneous vinification supplemented with the chemical additives used in commercial fermentations and (b) a commercial vinification which involved inoculation with a commercial strain of *S. cerevisiae* and addition of all additives. Throughout vinification, must samples were collected in duplicates (50 ml) at six time points: (i) at fresh grape-must generation (*S0*); (ii) after the addition of commercial yeast inoculum (in commercial-type must) (*S1*); (iii) at the beginning of AF (*S2*); (iv) at mid-stage of AF (*S3*); (v) at the end of AF (*S4*) and (vi) after the final addition of potassium metabisulfite (*S5*). The progress of the fermentation was monitored through measurements of sugars density and alcohol levels every two days.

The grape must was treated with tannins (50 g/tn) and clarifying enzymes (40 g/tn) at S0. Commercially fermented must was inoculated (S1) with "SafŒno™ GV S107" and "SpringFerm™" (Fermentis by Lesaffre, Marquette-lez-Lille – France), following manufacturers' instructions, whilst at the middle of the fermentation (S2-S3) both spontaneous and inoculated fermentations were treated with diammonium phosphate (DAP) 100 mg/L. Finally, after the end of the fermentation (S5) potassium metabisulfite (10 g/hL) was added. Must samples were stored immediately at -80^{o}C until DNA and RNA extraction. It should be noted that the vinifications were performed in the Laboratory of Oenology, Agricultural University of Athens.

Table 1. Vinification samples of Vidiano collected along spontaneous and commercial vinification.

Vinification types	**Spontaneous (V1)**	**Commercial (V2)**
Vinification stages (sampling time points)	**S0:** Fresh must generation **S1:** Inoculation (*commercial*) **S2:** Fermentation Start **S3:** Fermentation Middle **S4:** Fermentation End **S5:** Vinification completion	
Six stages: S0, S1, S2, S3, S4, S5 **1 replicate per stage** from duplicate vinifications **DNA extraction** ↓	**Three stages: S2, S3, S4** **2 replicates per stage** from duplicate vinifications **RNA extraction** ↓	
12 DNA samples **12 RNA samples**		

5.2.2 Analysis of the composition of the vinification microbiota

5.2.2.1. DNA and RNA extraction

Must samples were thawed and centrifuged at 8500 rpm at 4°C for 15 min. The microbial pellet was washed twice with ice cold 1xPBS, centrifuged and after washing it was subjected to DNA or DNA/RNA extraction. Samples from stages S0, S1, S5 were subjected to DNA extraction with the DNeasy Power soil DNA isolation kit (Qiagen, Hilden, Germany). Samples from stages S2, S3 S4 were subjected to both RNA and DNA extraction with the Rneasy PowerSoil DNA Elution Kit (Qiagen, Hilden, Germany), while during the RNA extraction process two (2) technical replicates of each stage were obtained. DNA and RNA were quantified by the Quant-iT kit with a Qubit Fluorometric device (Thermo Fisher Scientific, Waltham, MA, USA), as has previously described by Papadopoulou et al. (2023). The extracted DNA was stored at -20^{o}C, while RNA was stored at −80°C until sequencing.

5.2.2.2. Amplicon sequencing analysis of the vinification microbiota

The succession of the fungal and bacterial communities during vinification was determined via amplicon sequencing of the ITS2 and the V4 region of the 16S rRNA

gene respectively, in a HiSeq Illumina instrument in Rapid mode (Illumina, San Deigo, CA, USA) generating 250 bp paired-end reads, performed by Admera Health (South Plainfield, New Jersey, USA). Amplicons were prepared according to an in-house protocol (Vasileiadis et al., 2015) whereby the DNA must samples were subjected to a two-step amplification process, by indexing the samples. At the first amplification step, DNA was amplified using the primers ITS7F-ITS4R for fungi (Martins et al., 2013; Martiny et al., 2006) and the updated version of primers 515f-806r for bacteria (Lorenzini et al., 2019) according to the earth microbiome project (Lozupone et al., 2007). This first amplification stage comprised 28 cycles and the generated product was subjected to a second PCR of 7 cycles, using the same described primers, but providing this time sample-specific 5′ overhangs for sample indexing. To obtain all these amplifications the Q5® High-Fidelity DNA Polymerase from NEB company (Ipswich, Massachusetts, USA) was used. The generated amplicon sequencing libraries were cleaned up using the NucleoMag NGS Clean-up and the Size Select Kit from Macherey-Nagel company (Duren, Germany) and then sequenced. All primers, PCR conditions and the rest reagents are given in Supplementary Tables S1 and S2.

5.2.2.3. Bioinformatic analysis of amplicon sequencing data

To assess and demultiplex the amplicon sequences to their samples of origin, Flexbar v3.0.3 (Martiny et al., 2011) was used, having no barcode mismatches, while the sequence quality trimming/filtering, the removal of chimeric sequences, and the amplicon sequence variant (ASV) calling was performed with the dada2 v1.14.1 (Callahan et al., 2016) package the R software v.4.0.2 (R Core Team, n.d.). The taxonomic annotations of the resulting fungal and bacterial ASVs were performed using the UNITE ITS v.8.2 (04.02.2020) (Morrison-Whittle et al., 2017) and the Silva v.138 (Yilmaz et al., 2014) databases as references respectively. ASVs not assigned at Kingdom and Phylum levels were excluded from downstream analysis and the rest assigned ASVs were classified to the lowest possible taxonomical rank, with an at least 80% obtained annotation probability score. The sequencing data will be submitted to sequence Read Archive of NCBI.

5.2.2.4. Statistical analysis of amplicon sequencing data

The assessment and evaluation of the impact of different factors affecting the composition of the fungal and bacterial communities along the vinification, was based on ASV matrices. Non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) were used to visualize the influence of vinification type and vinification stage on the fungal and bacterial β-diversity of the vinification. The NMDS analysis was performed using the dissimilarity matrices by means of Bray-Curtis algorithm and PERMANOVA (Lozupone et al., 2007) as implemented in the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. Differential abundance (DA) tests using the Kruskal-Wallis and the Wilcoxon rank-sum post-hoc tests were applied to determine fungal and bacterial taxa associated with specific vinification strategies performed. *P*-value adjustment was conducted with the false discovery rate (FDR) algorithm with cutoff values of 0.05. All diagrams were produced using the R package ggplot2 v3.4.3 (Wickham, 2008).

5.2.3. Metatranscriptomic analysis

5.2.3.1. Bioinformatics

In total, 12 must RNA samples were used for preparing directed sequencing libraries with the TruSeq Stranded with RiboZero plus kit (Illumina, San Deigo, CA, USA) with prokaryotic/eukaryotic rRNA depletion, and were sequenced (two replicates per fermentation stage by two different fermentations) by Admera Health at a HiSeq 250 Illumina instrument run at Rapid mode generating 250 bp paired-end reads as described above. The raw sequencing data will be submitted to sequence Read Archive of NCBI. The retrieved sequence reads were processed with the SAMSA2 v2.2.0 pipeline (Westreich et al., 2018) for phylogenetic and functional annotation. Briefly, Trimmomatic v0.39 (Bolger et al., 2014) was used for quality controlling/filtering the reads, followed by read-pair assembly performance with PEAR v0.9.10 (Zhang et al., 2014). The off target rRNA sequence remnants were identified with SortMeRNA v2.1b (Kopylova et al., 2012) and were removed from the resulting reads. These reads were contrasted against the RefSeq (O'Leary et al., 2016) and the SEED Subsystems (Overbeek et al., 2014) databases with the Diamond v2.0.11.149 (Buchfink et al., 2014) BLASTx algorithm. The resulting taxonomy and functional gene transcript count tables were statistically processed as follows.

5.2.3.2. Statistical analysis of differential expression data

In order to focus the analysis on functions associated with the different fermentation protocols employed and the different fermentation stages, each two groups of different conditions were compared to generate contrasts. Based on these contrasts, the annotated gene_id count data were provided as input to DESeq2 v1.24.0 (Love et al., 2014) in R (v4.3.1) and were normalized according to the DESeq2 approach. Then the datasets tested for significant differential expression between the different conditions studied. The false discovery rate (FDR) *p*-value adjustment method (Benjamini and Hochberg, 1995) was used as main criterion for identifying differentially expressed genes (DEGs), using the 0.05 value as threshold. Volcano plots were prepared for the visualization of differences in gene expression between the fermentation protocols performed, indicating significantly different gene expressed functions by applying the filters p-value < 0.05 and Logarithm of fold change ratio (log2FC) > 0.1 for up- and (log2FC) < 0.1 for down-regulated genes. NMDS and CCA analysis were performed by dissimilarity matrices resulted of Bray-Curtis algorithm and PERMANOVA (Lozupone et al., 2007) as implemented in the Vegan v2.5-7 package (Oksanen et al., 2019) of the R software. All figures were created using the ggplot2 (version 3.4.3) R package (Wickham, 2009). All statistical analyses were carried out using R software 4.3.1.

5.3 Results

5.3.1 Vinification fungal microbiome

5.3.1.1 The composition of the fungal microbiome and community succession.

In total 595478 high quality sequences for fungi were obtained and they were assigned to 178 ASVs. Vinification samples were analyzed all together to estimate the succession of the fungal community during vinification. Distinct patterns were obvious according to vinification protocols at the earlier stages of fermentation, while microbial community patterns tend to become less dissimilar at completion of vinification (Fig. 1). In spontaneous vinification, at the early stages of fermentation (S0, S1, S2) we observed a higher phylogenetic diversity with ASVs of *Capnodiales*, *Malassezia*, *Aureobasidium*, *Cladosporium*, *Alternaria* and *Saccharomyces*

dominating in the fungal community. On the other hand in the commercial vinification we noted a quite diverse fungal community at S0 composed of *Sclerotiriaceae, Aureobasidium, Cladosporium, Lachancea, Saccharomyces, Peniophora* and *Torulaspora.* The higher phylogenetic diversity observed at earlier stages in both vinifications was not evident anymore from S3 and onwards in the spontaneous vinification and from S1 onwards (right after inoculation) in the inoculated vinification with a notable dominance of *Saccharomyces* (Fig.1).

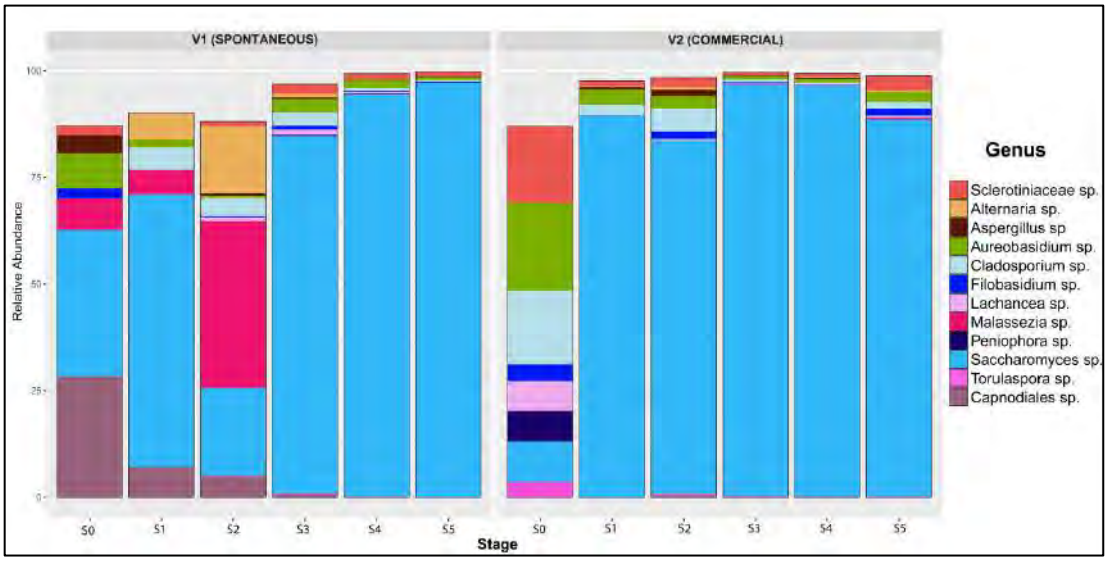

Fig. 1. Stacked bar plots showing the succession of the fungal microbiota (taxonomic level of genus) across six vinification stages in the two different vinification processes employed with grapes from the cultivar Vidiano. V1: Spontaneous vinification, V2: Commercial vinification; Vinification stages: S0, S1, S2, S3, S4, S5.

5.3.1.2. Factors affecting the β-diversity of the fungal microbial community

We further assessed the effect of vinification type and stage on the β-diversity of the fungal vinification microbiome. NMDS analysis showed a grouping of samples according to the stage of vinification while vinification strategy showed a weaker clustering effect on the fungal microbiome ($p > 0.2$) (Fig. 2b). PERMANOVA verified that the main determinant of the composition of the fungal community was the stage of vinification, explaining 55.84% of the variation ($p < 0.2$), compared to the type of vinification which represented 8.98% ($p > 0.2$) (Table 2). In addition we observed a strong positive association of the relative abundance of ASVs belonging to *Capnodiales* (Supplementary Fig. S1a) with the spontaneous vinification and of *Naganishia* with the commercial vinification (Supplementary Fig. S1a).

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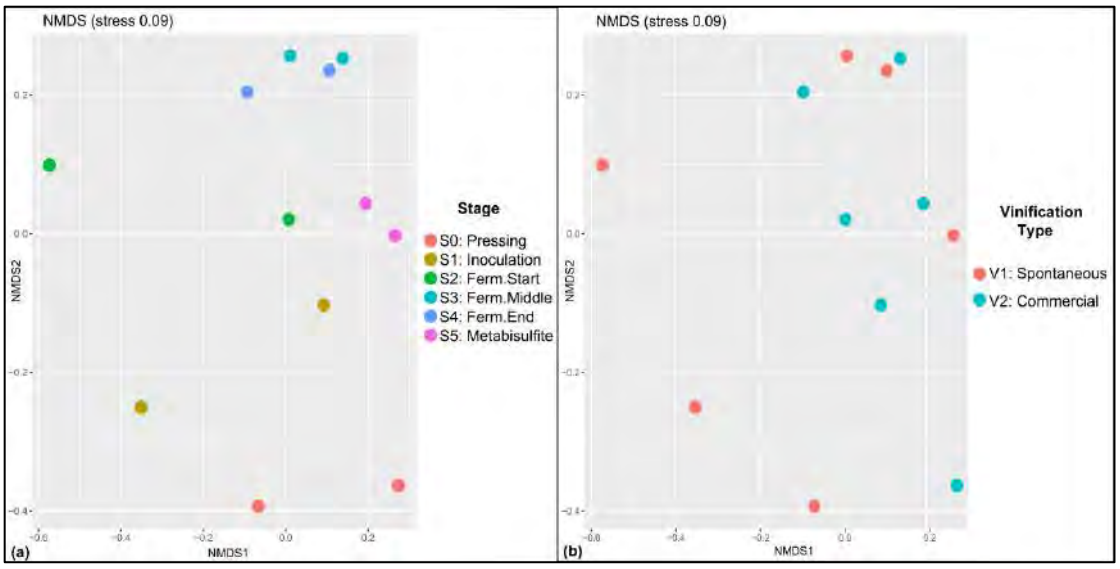

Fig.2 NMDS ordination of vinification samples, analyzed for their fungal microbiome, according to their stage of vinification (a) and the type of vinification (b).

5.3.2. Vinification bacterial microbiome

5.3.2.1. The composition of the bacterial microbiome and community succession

In total, 616235 high quality sequences for bacteria were obtained and they were assigned to 197 ASVs. A more universal pattern across vinifications was evident for the bacterial microbiome. In both vinifications we noted a high phylogenetic diversity that was evident from S0 and it was preserved until the end of fermentation (S5), with minor fluctuations (Fig. 3). This pattern included ASVs belonging to *Acinetobacter*, *Gluconobacter*, *Lactobacillus*, *Oenococcus* and to a much lower extent to *Komagataeibacter*, *Kozakia*, *Massilia*, *Tatumella*, *Pseudomonas* and *Prevotellaceae*. At the same time, all stages were characterized by the presence of ASVs of *Enterobacterales* and *Clostridiaceae* (Fig. 3). The main difference between the two vinification strategies was the emergence of *Escherichia/Shigella* ASV at the beginning of the spontaneous vinification (S0, S1) (Fig. 3).

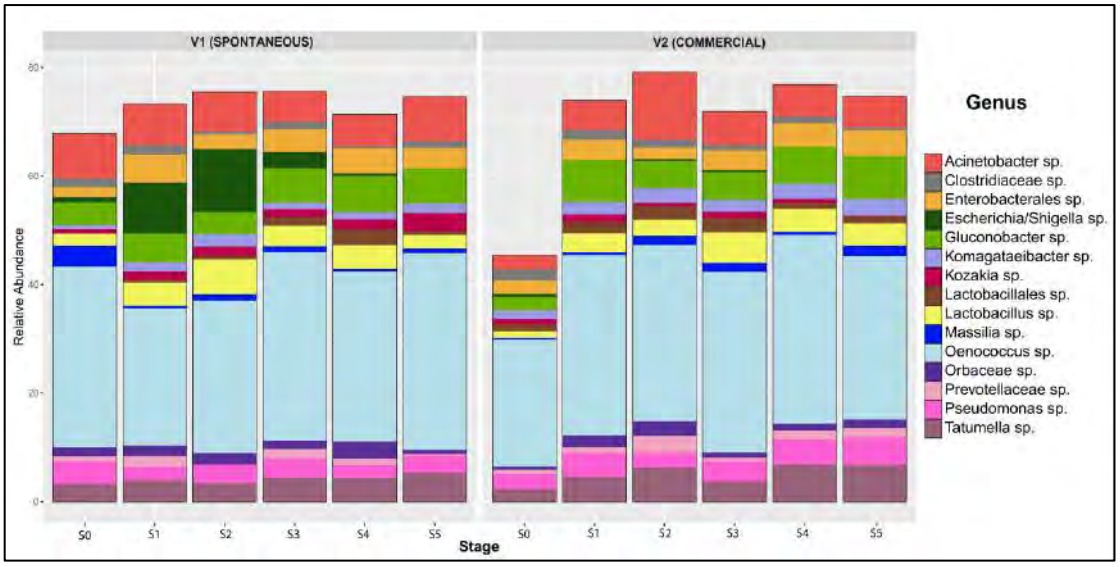

Fig. 3 Stacked bar plots showing the succession of the bacterial microbiome (taxonomic level of genus) across six vinification stages in the two different vinifications employed for cultivar Vidiano. i) V1: Spontaneous vinification, V2: Commercial vinification, ii) Vinification stages: S0, S1, S2, S3, S4, S5

5.3.2.2. Factors affecting the β-diversity of the bacterial community of vinification

When looking at the factors affecting the bacterial β-diversity, NMDS analysis revealed not clear clustering of samples according to vinification stage or vinification protocol ($p > 0.3$) (Table 2). Between these two factors, a relatively higher contribution of the vinification stage (44.9%) and a lower contribution of the vinification type (9.6%) was evident by PERMANOVA (Table 2). When looking at ASVs associated with different vinification types, we identified a positive association of ASVs of *Cutibacterium*, *Kozakia*, *Bulkholderiales* and *Enhydrobacter* with the spontaneous vinification and of ASVs of *Komagataeibacter*, *Pseudomonas* and *Oxalobacteraceae* with the commercial vinification (Supplementary Fig. S1b).

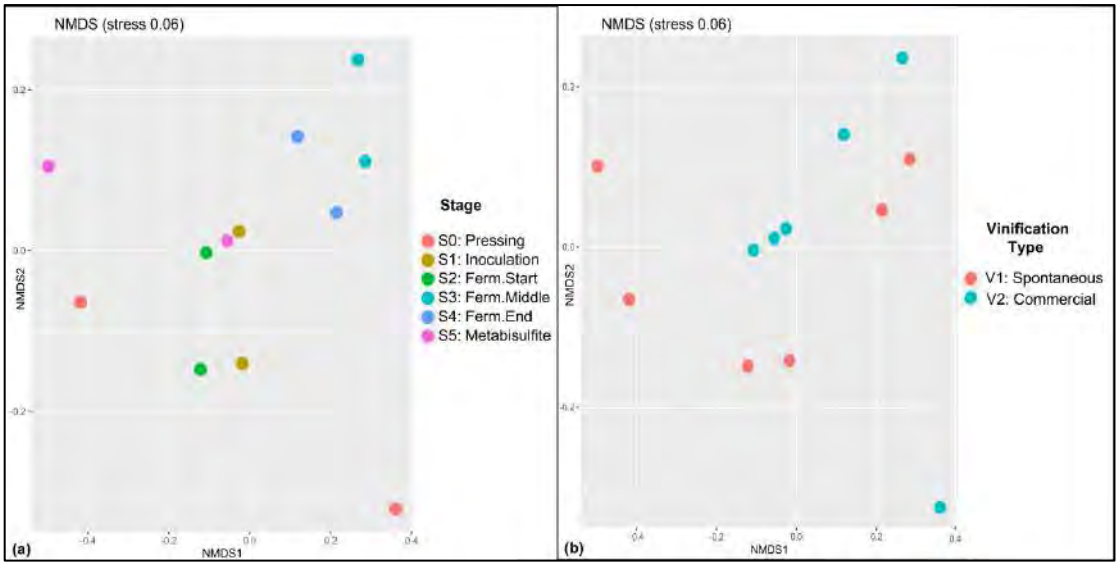

Fig.4 NMDS ordination of vinification samples, analyzed for their bacterial microbiome, according to their stage of vinification (a) and the type of vinification (b).

Table 2. PERMANOVA-based variation partitioning analysis of the fungal and bacterial vinification microbiota. (Signif. codes: 0.001 '***' 0.01 '**' 0.05 '*').

Factor	Degrees of Freedom	Sum of squares	F. Model	R^2 (%)	P-value (>F)
Fungi					
Vinification	1	0.13249	1.2744	9	0.249
Stage	5	0.82467	1.5865	55.8	0.166
Residuals	5	0.51981		35.20	
Total	11	1.47697		100	
Bacteria					
Vinification	1	0.13627	1.0497	9.6	0.325
Stage	5	0.63889	0.9843	44.9	0.554
Residuals	5	0.64909		45.6	
Total	11	1.42425		100	

5.3.3 Metatranscriptomic analysis

5.3.3.1. Factors affecting functional gene-expression across vinifications

Through metatranscriptomic analysis we tried to disentangle the influence of different factors on the metabolic activity and functioning of the vinification microbiota along the fermentation process. The impact of different fermentation strategies and of the stage of fermentation were analysed via CCA (Fig. 5) and NMDS (Supplementary Fig S2). Both of the studied factors showed no significant effect on the vinification functions based on gene expression (PERMANOVA vinification, $P > 0.1$; stage, $P > 0.2$, Supplementary Table S3).

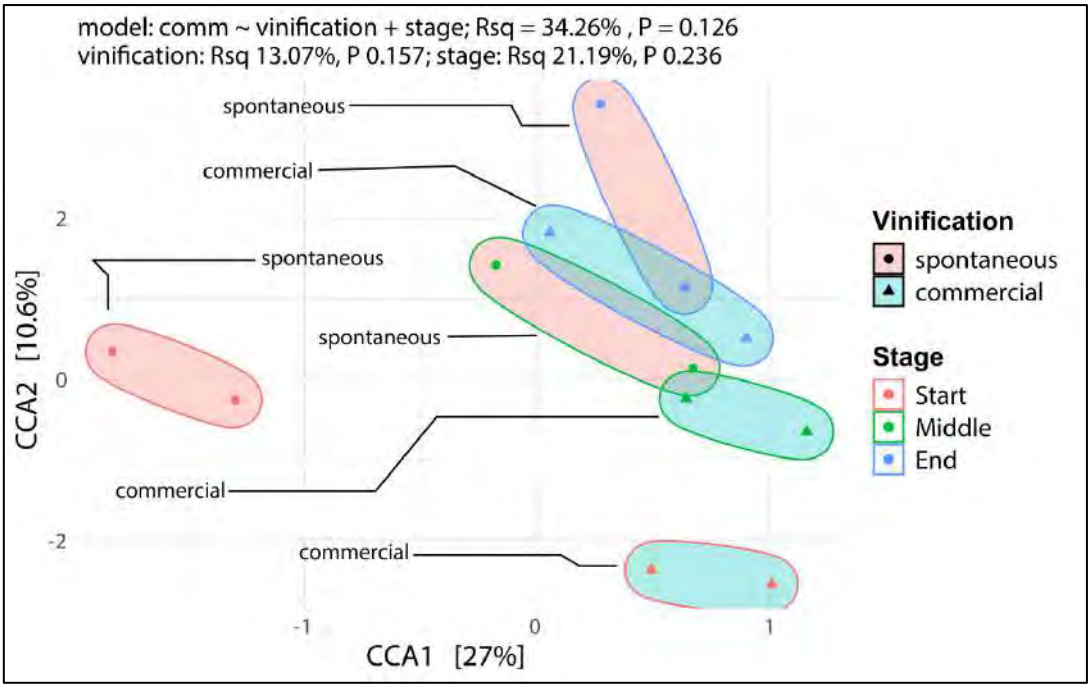

Fig.5 Canonical correspondence analyses (CCA) plot of gene-expressed functions in spontaneous and commercial fermentation and their three different fermentation stages (start, middle, end). Data have been variance stabilized with negative binomial transformation in DESeq2. Red-filled groups include samples of spontaneous, while green-filled groups include samples of commercial fermentation, indicated by the respective different shape. Different sample color indicates different stage of vinification.

5.3.3.2. Differential gene-expression between the different vinifications

We further explored the profile of differentially expressed genes of the different fermentation processes employed. Spontaneous and commercial fermentation exhibited distinguishable RNA expression profiles, depicted as the change in transcript abundance for each gene. These differences in gene expression between the two studied fermentations are visualized in the Volcano plot in Fig. 6. From a total of 1095 differentially expressed genes, 17 genes were significantly down-regulated. Among these genes we identified in the spontaneous fermentation DEHA2B13156p, encoding for a protein of the yeast *Debaryomyces hansenii*, an IS256 family transposase and a sugar ABC transporter permease. On the other hand, we identified 219 genes that were significantly up-regulated in the commercial fermentation including *Saccharomyces*-derived genes with non-characterized function like *YGL088W* and *YGR079*, and genes encoding for the 50S Ribosomal protein L2, cytosine deaminase, Sip18p, Rlp11b, Qrc6, Om45, the hypothetical protein KLMA 20782, associated with the aerobic yeast *Kluyveromyces marxianus*, and NDAI 0A03600 associated with *Naumovozyma dairenensis* (or *Saccharomyces dairenensis*).

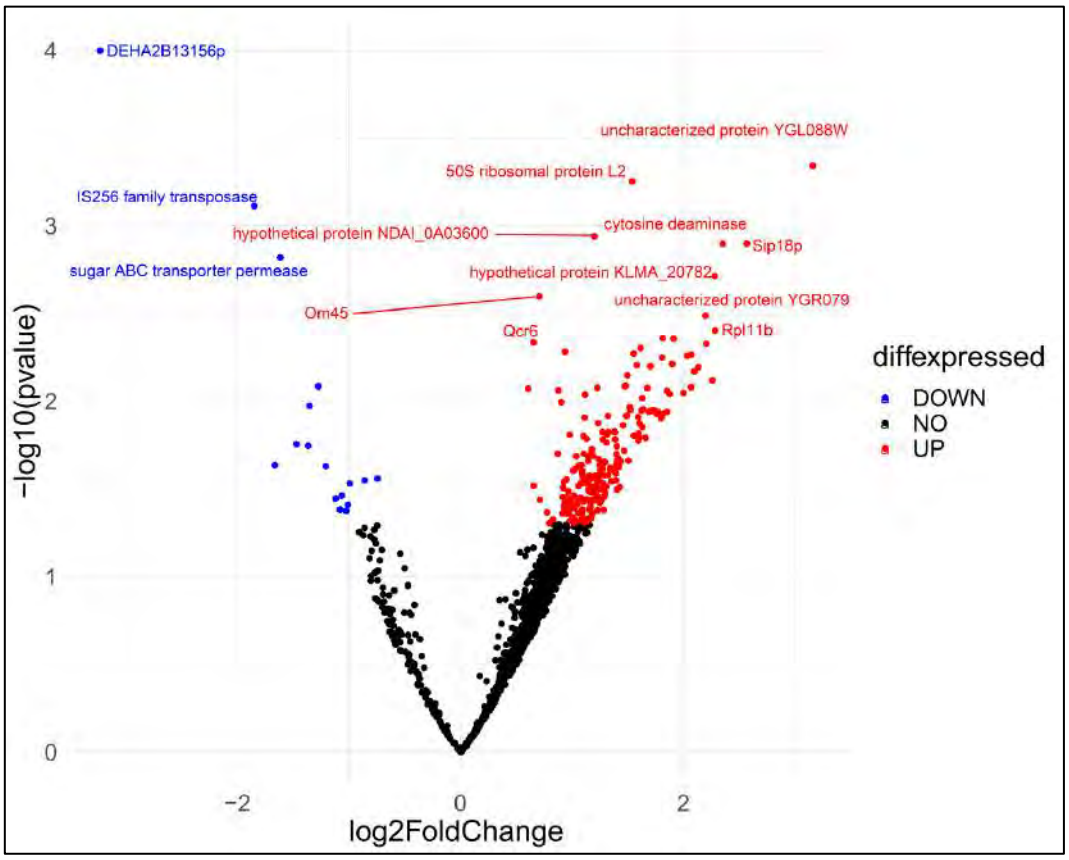

Fig.6 Volcano plot for the differentially expressed genes obtained from DESeq2 analysis of grape-must fermentation samples from cultivar Vidiano, derived from two fermentation strategies (spontaneous vs. commercial). Genes with adj. p-value < 0.05 are marked with blue and red dots and genes with adj. p-value >0.05 are marked with black dots. The up-regulated genes ($log_2FC > 0.1$) are shown with red dots, while the down-regulated genes ($log_2FC < 0.1$) are shown with blue dots.

5.3.3.3. Differential gene-expression in each stage between the different fermentations

We finally identified genes exhibiting differential expression at each vinification stages across spontaneous and inoculated fermentation. DA heatmaps were constructed collectively for the two fermentations, but separately for each stage of fermentation. At the start and the middle of vinification different transcripts were associated with each fermentation, but at the final vinification stage, the two vinifications shared similar transcription products. When both fermentations were considered together at the first stage, four gene transcripts showed significant association with the commercial fermentation, while all the other genes were associated with spontaneous fermentation. Hence genes encoding for redox-related protein, Hbt1p and Sed1, were upregulated in the commercial fermentation, while

genes encoding for Tdh2, PDC-like protein, phosphopyruvate hydratase ENO2, Hxt6, Hsp30, MFS transporter, ABC transporter ATP-binding protein and DEHA2B13156p were upregulated in the spontaneous fermentation. At the mid fermentation stage we observed an upregulation of a gene encoding for phosphorpyruvate hydratase ERR3 in the commercial fermentation, whereas genes encoding for alcohol dehydrogenase Adh, a PGK1-like protein, Eno2, MFS transporter and an ABC transporter ATP-binding protein were upregulated in the spontaneous vinification. At the last fermentation stage, in both vinification we noted an upregulation of genes encoding for alcohol dehydrogenase (Adh) and 1,3-beta-glucan synthetase GSC2. Genes encoding for ABC transporters, acyltransferase, 3-hydroxyacyl-CoA dehydrogenase, (R,R)-butanediol-dehydrogenase, adenyl-nucleotide exchange factor SEE2 were highly expressed solely in the commercial vinification, whereas genes encoding for aconitate hydratase was associated with the spontaneous fermentation.

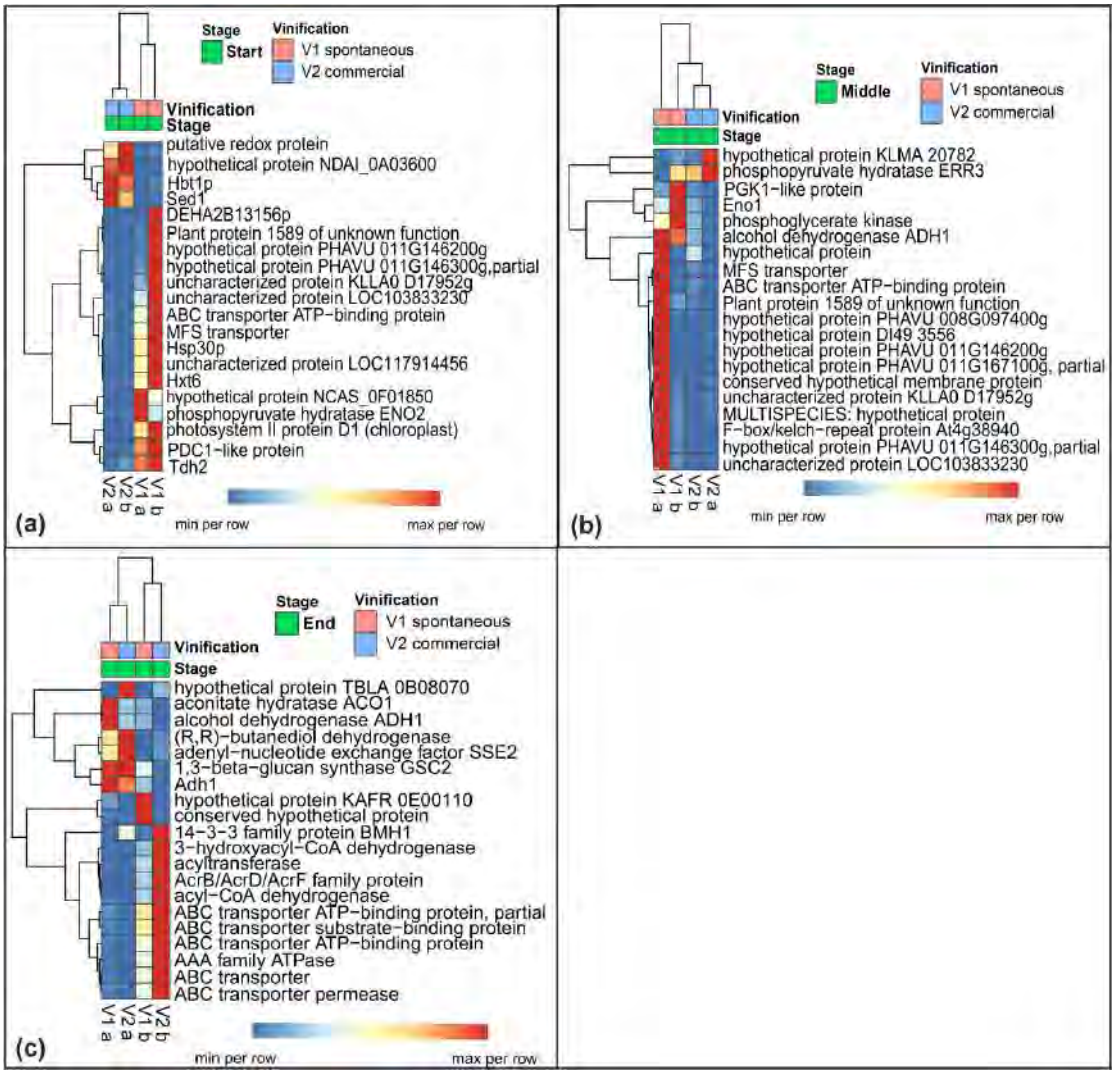

Fig.7 Heatmaps obtained from DESeq2 analysis of grape-must fermentation samples from cultivar Vidiano, deriving from two different fermentation strategies (spontaneous vs. commercial). Each of the heatmaps represent the differential expression associated with fermentation Start stage (a), fermentation Middle stage (b), fermentation End stage (c). Columns in the heatmap represent must samples of spontaneous and commercial fermentation, showing the intensity of expression profile per gene.

5.4 Discussion

We initially investigated the microbial landscape of vinification at specific time points along two different vinification regimes: (i) a spontaneous vinification, under minor interventions of preservatives (SO_2) and additives (enzymes, nitrogen sources), and (ii) a commercial vinification, under standard oenological practices that include both preservatives, additives and the use of commercial *S. cerevisiae* inoculum commonly used in the winemaking sector. Vinification – related factors like the stage of fermentation and the type of vinification strategy have already been described as significant contributors on the structure of microbial communities of vinification (Pinto et al., 2015**;** Böhmer et al., 2020**;** Papadopoulou-Bekris et al., 2023). Our results come in terms with these findings for the fungal microbiome, indicating mainly stage-related patterns, while the bacterial microbiome was not much influenced by the different stages and vinification strategies.

Albeit in both vinifications at the initial stage (S0) the grape must supported a diverse fungal community composed of microorganisms originated from the vineyard environment like the non-fermentative *Filobasidium* and *Aureobasidium*, filamentous fungi like *Cladosporium, Alternaria* and *Aspergillus* (Prendes et al., 2021; Rodríguez-Andrade et al., 2020; Knapp et al., 2021; Papadopoulou et al., 2022; Awad et al., 2023) and NS yeasts like *Lachancea* and *Torulaspora* (Benito S. et al., 2018, 2020; Benito Á. et al. 2019). This diverse fungal community was displaced by *S. cerevisiae* upon its inoculation in the commercial vinification. On the contrary, in the spontaneous vinification the initial high phylogenetic diversity was preserved until the start of fermentation when *S. cerevisiae* starts becoming dominant until the end of fermentation. *S. cerevisiae* became predominant immediately in commercial vinification, after inoculation, unlike in spontaneous where it became the most prevalent member of the microbiota from the middle of fermentation and onwards. However, in both vinifications, spontaneous and inoculated, *S. cerevisiae* presence was evident right from the first vinification stage (pressing-crushing), a result of its presence on grapes or on the room where vinification was employed (Ciani et al.,

2004). *S. cerevisiae* are often present on grape-berries at low levels but they are favored by damaged or cracked grape-skin which was the case most probably in our study (Watanabe and Hashimoto, 2023). Furthermore, the addition of several chemicals in spontaneous vinification, following the exact same routine as in the inoculated vinification, has probably resulted in the early flourish of *S. cerevisiae* which is tolerant to SO_2, in comparison to the other members of the vinification microbiota (García-Ríos and Guillamón, 2019).

In contrast to what we observed for fungi, the composition of the bacterial community was not significantly altered along the fermentation process in both vinifications. This might have been the result of the oenological preservation practices that were employed in both vinification strategies. The conditions generated right from the first stage by the addition of SO_2, might have favored the establishment and proliferation of *Oenococcus*, given its high tolerance to those preservatives (Onetto et al., 2021). The bacterial community was composed of LAB like *Oenococcus* and *Lactobacillus*, responsible for MLF (Capello et al., 2017; Takase et al., 2018**;** Michlmayr et al., 2010), AAB like *Gluconobacter*, *Komagataeibacter* and *Kozakia* known for their high capability to oxidize ethanol to acetic acid (Gomes et al., 2018**;** Guillamón and Mas, 2011), the common dweller of vinification *Tatumella* (Bubeck et al., 2020; Steenwerth et al., 2021), other bacteria of unknown role in the vinification process like *Pseudomonas*, *Massilia* and *Acinetobacter* (Martins et al., 2013**;** Ding et al., 2021; Singh et al., 2018) and *Enterobacterales* found on grapevine phyllosphere (Papadopoulou et al., 2022).

We further examined the metabolic activity of the vinification microbiome at the different stages of the spontaneous and the inoculated fermentations via metatranscriptomics. We noted that fermentation stage was a most important factor differentiating gene transcription patterns compared to the vinification type. When comparing gene expression between the two vinification strategies we identified genes encoding for Sip18p, and the hypothetical proteins YGL088W, YGR079, KLMA_20782 and NDAI 0A03600 showing higher expression levels in the inoculated fermentation. The early predominance of *S. cerevisiae* in commercial fermentation, can be associated with the upregulation of the hypothetical genes YGR079 and YGL088W, which are both found in the genomes of *Saccharomyces* strains and the latter is generally conserved across *S. cerevisiae* strains (Song et al., 2016). The hypothetical gene product KLMA_20782 is associated with *Kluveromyces*

marxianus, a yeast originally isolated from grapes known to excrete hydrolyzing enzymes for pectic substances found on grape cell wall (Wimborne and Rickard 1978). The hypothetical gene product NDAI0A03600 is associated with *Naumovozyma dairensis*, a Saccharomyces-related strain often detected in milky products fermentation, but with limited sugars fermenting ability (Ilikkan et al., 2021). *Sip18* encodes for hydrophilins, a family of proteins (Rodriguez-Porrata et al., 2011; Rodriguez-Porrata et al., 2012), acting as antioxidants and stabilizers of cellular membrane and other proteins, pivotal in survival under water deficient conditions (Tunnacliffe & Wise, 2007; Rodríguez-Porrata et al., 2012**;** López-Martínez et al., 2013)**.** Their upregulation in the inoculated fermentation could be linked with possible dehydration of the cells of the inoculated *S. cerevisiae* strain (Câmara Jr. et al., 2020). On the other hand, we noted a significant enrichment in gene transcripts encoding for DEHA2B13156p and a IS256 family transportase in the spontaneous fermentation. DEHA2B13156p is ascribed to *Debaryomyces hansenii*, one of the most important NS yeasts in winemaking. *D. hansenii* is known to be present at the early fermentation stage and can tolerate up to 15% (vol/vol) ethanol levels (Wrent et al., 2014). The IS256 family transportases constitute a widespread insertion sequence among bacterial genomes, particularly prevalent in *Enterococcus* and *Staphylococcus* (Byrne et al., 1989) conferring resistance to metals and antibiotics (Maki et al., 1997**,** Hennig and Ziebuhr, 2010).

As a final step of our metatranscriptomic analysis, we determined differentially expressed genes between the two vinification strategies separately at each vinification stage. The early fermentation stage was characterized by high glycolytic activity associated with sugar metabolism. In commercial fermentation, *S. cerevisiae* mobilizes its fermentative tolerance to the produced alcohol, as suggested by the higher expression levels of *sed1*, a gene encoding a stress-induced glycosyl phosphatidylinositol (GPI)-cell wall glycoprotein (Samakkarn et al., 2021; Udom et al., 2019). In addition we found increasing expression of a putative redox protein which could be mobilized by *S. cerevisiae* to maintain its redox homeostasis during ethanol and CO_2 production (Duncan et al., 2023). Generally, the metabolism of *S. cerevisiae* during fermentation can be separated in two distinct phases, exponential and stationary. It starts with an initial exponential phase, characterized by rapid cell proliferation imposed by the fermentation of glucose to ethanol. It then follows an intermediate adjustment phase where cell growth is paused under lower glucose

levels, and then a pre-phase of reduced cell growing rate as a result of the lower availability of glucose supplements and the higher ethanol content. Finally, the yeast gets into a stationary phase where cells cease dividing as a result of the limitation of essential nutrients and the high ethanol levels (Busti et al., 2010). *Hbt1* was one of the highly upregulated genes during the stationary phase. This gene has been associated with the long-term survival of yeasts under adverse environmental conditions (Martinez et al., 2004; Mendes-Ferreira et al., 2007). At the beginning of the spontaneous fermentation, *S. cerevisiae* co-exists with other yeasts and fungi by co-fermenting and competing for glucose consumption. Under those conditions we noted an upregulation of (i) *hsp30* encoding a stress-induced heat-shock protein, (ii) *hxt6* a hexose transporter required at the alcoholic fermentation and (iii) *eno2* encoding for a phosphopyruvate hydratase, an ubiquitous and key glycolytic enzyme in the cytoplasm of prokaryotic and eukaryotic cells, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis (Fukano and Kimura, 2014). The upregulation of all these genes at the early stages of the spontaneous fermentation could be associated with the high phylogenetic diversity of the fungal community which forces high competition of *Saccharomyces* with the other members of the vinification microbiota for nutrients.

The highly induced glycolytic processes were still prevalent at the mid fermentation stage in both vinifications. In the inoculated fermentation, dominated by *S. cerevisiae*, we noted an upregulation of *err3*, encoding a phosphopyruvate hydratase involved in glycolytic processes. In the spontaneous fermentation, we identified an upregulation of genes encoding a phosphoglycerate kinase, conserved enzymes among prokaryotes and eukaryotes with essential role in glycolysis by catalysing a phospho-transfer with ATP production (Watson et al., 1982). In addition, we noted an upregulation of genes encoding for MFS and ABC transporters. These are ubiquitous membrane transporters mediating the transport of variable substrates in and out of cell, while a large number of MFS sugar transporters expressed by *S. cerevisiae* enable its growth over various glucose concentrations (Leandro et al., 2009). Similarly the upregulation of *adh1,* a gene enconding alcohol dehydrogenase which is directly involved in ethanol production from sugars (Raj et al., 2014) is in line with the release of ethanol during fermentation.

In the final stage of fermentation, the upregulation, especially in spontaneous fermentation, of membrane resistance transporters of the ATP-Binding Cassette

(ABC) superfamily, known as tolerance response mechanism towards the elevated alcohol levels, might be mobilized by *S. cerevisiae* as the predominant member of the microbiota at this stage (Jungwirth and Kuchler, 2006; Sa´-Correia et al., 2009; Teixeira et al., 2012). Following up from the mid-fermentation stage, *adh1* was still upregulated in both fermentations as a result of ethanol production. Finally we noted an upregulation of *aco1* in spontaneous fermentation. This encodes for aconitase participating at the first steps of the citric acid cycle. Its upregulation might be associated with the establishment of mitochondrial aerobic respiration (Chen et al., 2007) denoting the entrance of *S. cerevisae* to the stationary phase and the end of fermentation.

5.5 Conclusions

Good knowledge of the microbial community composition and its succession patterns during vinification could be particularly useful for enhancing wine quality. In our study we showed that the most important structural factor of the vinification microbiota was vinification stage while the vinification type had a weaker influence most probably due to the addition of preservatives in both inoculated and spontaneous fermentation. The fungal community showed variable patterns between vinifications only at the earlier stages with the spontaneous fermentation being characterized by high phylogenetic diversity up until the start of fermentation followed by the proliferation and dominance of *Saccharomyces*. Whereas the inoculated vinification was dominated by the inoculated *S. cerevisiae* strain from inoculation onwards. On the other hand the bacterial community was composed of LAB and AAB and it was characterized by a gradual growth of *Oenococcus* along vinification in both fermentations. We went a step further and tried to identify gene expression patterns along the vinification that could point to key microorganisms and the metabolic pathways they mobilize during fermentation. Gene expression patterns varied in the two vinifications up until the mid-fermentation stage, while at the final vinification stage the two fermentations shared a similar transcription profile, a result of the predominance of *S. cerevisiae* at this stage. In general, we noted upregulation of genes mostly originated from *S. cerevisiae* and other yeasts which were associated with stress response mechanisms, transportation, glycolysis and ethanol production in line with the biochemical environment of fermentations. Overall, our study provides first

evidence for the activation of metabolic pathways that the vinification microbiota mobilizes to cope with the conditions prevailing during fermentation.

5.6 References

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5.7 Annex IV –Supplementary data

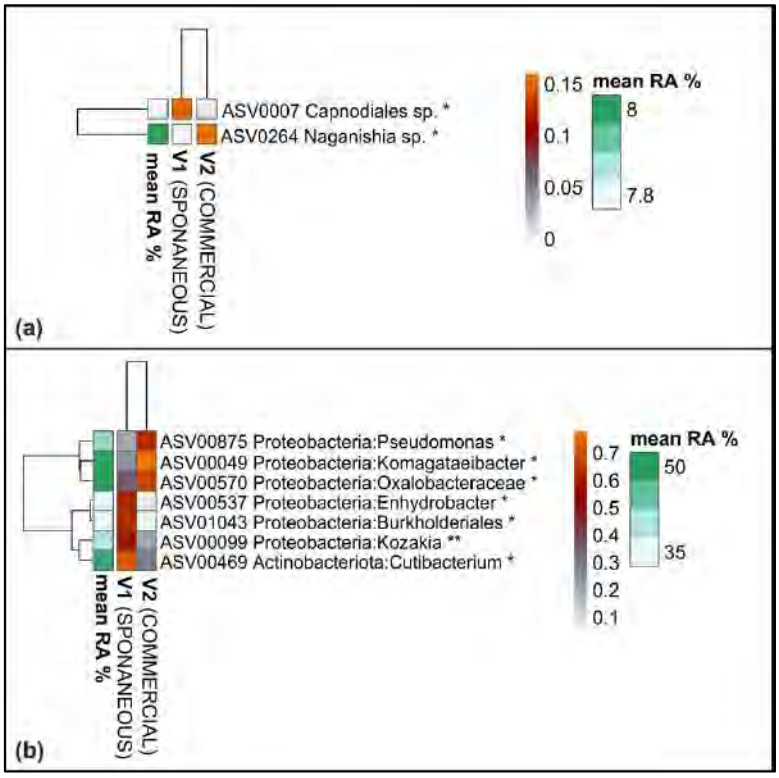

Fig. S1 DA heatmaps showing fungal ASVs at genus taxonomic level (a) and bacterial ASVs at genus taxonomic level (b) whose relative abundance showed a significant positive correlation ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***) with a specific vinification protocol (V1/Spontaneous, V2/Commercial). ASVs, amplicon sequence variants; DA, differential abundance.

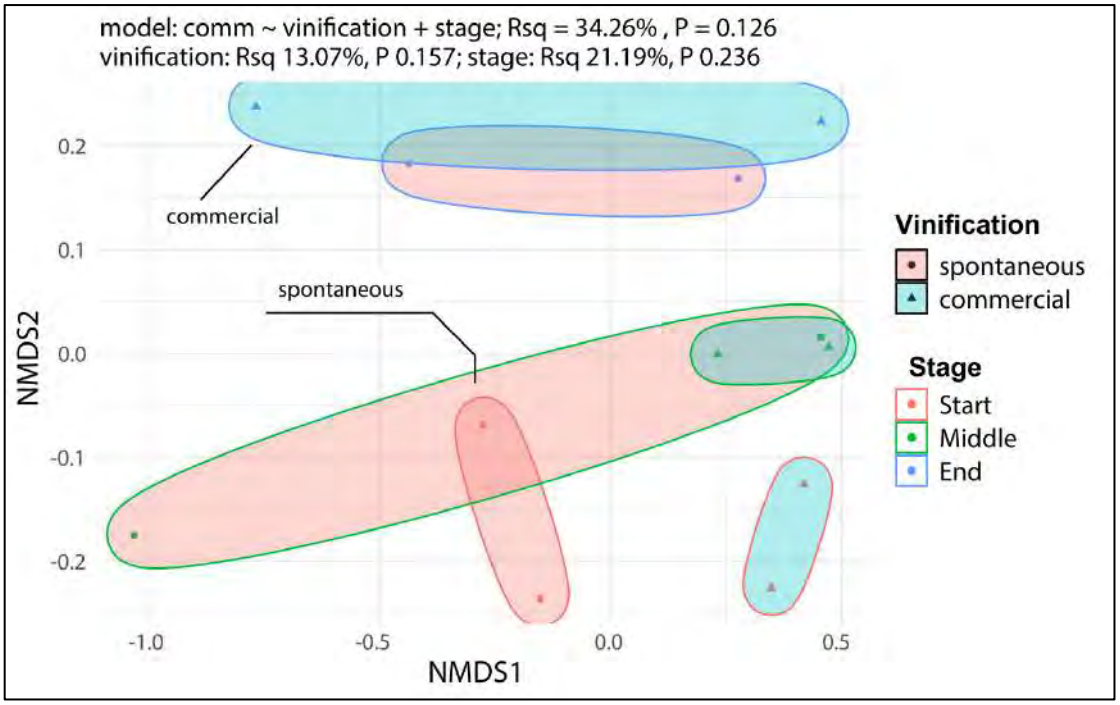

Fig. S2 Non-metric multidimensional scaling (NMDS) plot ordination of gene-expressed functions in spontaneous and commercial fermentations at three different fermentation stages (start, middle, end). Ordinations were based on DESeq2-normalized counts. Red-filled groups include samples of spontaneous, while green-filled groups include samples of commercial fermentation, indicated by the respective different shape. Different sample color indicates stage of vinification

Supplemental Table S1. Primers used for amplicon sequencing analysis. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step, which are composed of the sequence of the universal primers 515f (bacteria) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a 2bp linker sequence in between.

Kingdom	PCR step	Primers	Gene target	Amplicon size (bp)	Primer sequences (5'-3')	References
Bacteria	1st	515F 806R	16S rRNA	~290	GTGYCAGCMGCCGCGGTAA GGACTACHVHHHTWTCTAAT	Parada et al., (2016)
	2nd	Indexed (n=96)	B0001-515f		NNNNNNNNNGT**GTGYCAGCMGCCGCGGTAA**	Katsoula et al., (2021)
Fungi	1st	ITS7F ITS4R	ITS2	~310	GTGARTCATCGAATCTTTG TCCTCCGCTTATTGATATGC	Ihrmark et al., (2012) White et al., (1990)
	2nd	Indexed (n=96)	FI0001-ITS4r		NNNNNNNNNGA**TCCTCCGCTTATTGATATGC**	Katsoula et al., (2021)

Supplemental Table S2. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	**Volume (μl)**	**Concentration**	**Comments**
Primer F	1	0.5 μM	
Primer R	1	0.5 μM	
BSA	0.4	0.4 μg/μl	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH_2O	5.6		
DNA	2	0.2 ng/μl	
Total	20		
PCR conditions			
Step	**Temperature (°C)**	**Time**	**Number of Cycles**
Initial Denaturation	98	30 sec	28 in the first amplification step / 7 in the second amplification step
Denaturation	98	10 sec	
Annealing	50 for bacteria/ 55 for fungi	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	

Table S3. PERMANOVA-based variation partitioning analysis of the gene-expressed functions vinification microbiota. (Signif. codes: 0.001 '***' 0.01 '**' 0.05 '*').

Factor	Degrees of Freedom	Sum of squares	F. Model	R^2 (%)	P-value (>F)
Gene-expressed functions					
Vinification	1	0.308623389430355	1.59066061115917	13.07	0.157
Stage	2	0.500320920317173	1.28933970681284	21.2	0.236
Residuals	8	1.55217718859814		65.74	
Total	11	2.36112149834566		100	

Chapter 6

General Discussion and Future Perspectives

6.1 General discussion

Grapevine as a plant offers a range of different compartment as niches for microorganisms. Of those the upper ground plant parts, encompassing the phyllosphere and carposphere, are exposed to environmental conditions and support a microbial community which is affected by host-plant interactions, agricultural practices and environmental conditions (Vorholt, 2012; Perreault and Laforest-Lapointe, 2022). The epiphytic grapevine microbiota has a decisive role on supporting the growth and productivity of grapevines (Bettenfeld et al., 2022; Liu et al., 2020). At the same time the carpospheric microbiota is implicated in grape must vinification and contributes heavily local vineyard signatures on wine sensorial traits, with valuable socio-economic reflections (Mezzasalma et al., 2017; Liu et al., 2019). Despite the importance of the indigenous grape microbiota on wine production, wine industry relies mostly on the use of commercial strains for wine production instead of the indigenous microbiota, as a strategy to ensure stable quality, reduced chance of spoilage and homogenous wine profiles. In this frame, a great research interest has been put on studying the composition and succession of the microbial communities of grapevines (Vidal et al., 2023) in order to achieve high wine quality with enhanced regional features (Bokulich et al., 2016). In the present thesis, we aimed to evaluate the grapevine microbiota in selected Greek grapevine cultivars, determine its role in grapevine trunk diseases (GTDs), explore the factors controling its composition and investigate the role of these vineyard-mediated but also vinifcation-mediated factors on its succession and activity along the vinification process. In the first leg of this thesis (presented in Chapter 2), we assessed the grapevine wood microbiome, aiming to discover fungal pathogens involved in GTDs (Diaz et al., 2023), potential factors affecting their presence and members of the fungal microbiota that could be used as early indicators of GTDs. In the second leg of this thesis (described in Chapter 3), we determined the composition of the epiphytic fungal and bacterial microbiome on grapes and leaves and the influence of vineyard-related factors on its structure at different geographical scales: across and within viticultural zones. In the third phase of this thesis (described in Chapter 4), we moved our attention from vineyards to vinification and we explored the effect of different vineyard-mediated factors and vinification-mediated practices on microbial communities and microbial succession along vinification but also on the antioxidant, antimutagenic and anticancer properties

of the produced wines. At the final part of this thesis (described in Chapter 5), we explored the vinification microbial composition and succession in spontaneous and inoculated fermentations, both subjected to preservatives addition, and further looked into the metabolic activity and functions of the active vinification microbiota during vinification via metatranscriptomic analysis.

In **Chapter 2** we used amplicon sequencing, bioinformatic and statistical tools to define the composition of the fungal and bacterial wood grapevine microbiome in GTDs-symptomatic and asymptomatic vines, from three Greek cultivars, each from different viticultural zone (Agiorgikiko/Nemea, Xinomavro/Amyntaio, Vidiano/Crete). Regardless of cultivar/location, the fungal wood microbiome was dominated by Ascomycota, in line with previous studies (Bruez et al. 2020; Del Frari et al., 2019a; Deyett and Rolshausen, 2020) and less by Basidiomycota. We observed a strong effect of biogeography/cultivar, as a lumped factor on the composition of the fungal wood microbiome as also reported in earlier studies (Bokulich et al., 2014; Mezzasalma et al., 2018). The GTD status of grapevine (symptomatic vs asymptomatic) was significant in differentiating the wood microbiome only in one of the three cultivars tested (cv. Xinomavro). These observations might be the associated with strong variations of the climatic conditions in the study regions and also in the differential susceptibility of the tested cultivars on GTDs. Symptomatic vines were collectively characterized by the consistent presence of *Fomitiporia* spp., *Phaemoniella chlamydospore* and *Kalmusia variispora* all being reported as wood pathogens (Bruez et al., 2014; Ntasiou et al., 2021; Cimmino et al., 2021), while random forest analysis identified *P. chlamydospora, K. variispora, Alternaria alternata* and *Cladosporium* sp. as early and accurate predictors of GTDs. Network analysis revealed in asymptomatic vines a negative co-occurrence pattern of the grapevine wood fungus *Alternaria* (Hofstetter et al., 2012; Del Frari et al., 2019b) with GTD-related fungal genera (e.g. *Phaemomoniella* and *Phaeoacromonium)*, suggesting its potential involvement in the suppression of GTDs. Regarding the bacterial wood microbiome in grapevines, it was composed of *Proteobacteria*, mainly *α-* and *γ-proteobacteria, Actinobacteria*, *Bacteroidetes* and members of *Sphingomonadaceae* and *Bacillaceae*. The wood bacterial community showed similar patterns with the fungal community, with strong biogeography/cultivar (lumped factor) signatures and weak effects of the GTD status, in line with earlier studies for biogeography (Bokulich et al., 2014; Coller et al., 2019; Gobbi et al., 2020; Vitulo et

al., 2019) and GTD status (Bruez et al., 2020; Bruez et al., 2015). Co-occurrence network analysis in asymptomatic grapevines revealed strong and consistent negative co-occurrence relationships between *Bacillus, Streptomyces* and GTD-related fungi like *Phaeoacremonium*, *Phaeomoniella* and *Seimatosporium* suggesting a potential role of members of these bacterial genera in the suppression of the GTD fungal pathobiome, as previously suggested in other studies in grapevine (Rezgui et al., 2016; Haidar et al., 2016; Alvarez-Perez et al., 2017).

In the next step (**Chapter 3**), we focused on another grapevine compartment, the carposphere and phyllosphere, which is known to influence wine quality. Hence we studied the effect of different factors that can affect the epiphytic grapevine microbiota at different geographic scales: from across to within viticultural zones. To explore this we studied the epiphytic fungal and bacterial microbiome of the phyllosphere and carposphere in four selected Greek cultivars (Agiorgitiko, Vidiano , Roditis and Sideritis) at different scales: (i) at a national scale in different viticultural zones (vineyards distances 100→600km) and (ii) at regional scale within the viticultural zone of Aigialeia (vineyards distances <100 km). Across viticultural zones we noted a strong biogeography effect on the composition of the fungal microbiota, followed by vintage while weak cultivar signature were evident (for cv. Agiorgitiko and Vidiano). Interestingly all these factors showed much weaker effects on the epiphytic bacterial microbiota. Similar regional signatures for the epiphytic fungal microbiota have been reported before by Kioroglou et al (2019) at similar geographic scales, while others have also reported strong regional signatures in grape musts (Li et al., 2022; Bokulich et al., 2014; Chalvantzi et al., 2021; Kamilari et al., 2021; Morrison-Whittle & Goddard, 2018). When moving in smaller geographical scales, within the viticultural zone of Aigialeia, we noted strong cultivar (cv. Roditis and Sideritis) and *terroir* signatures on the fungal community while vintage was a weaker structural factor. When analyzing effects separately for each of the two cultivars, we observed stronger *terroir* signatures for Sideritis compared to Roditis which might be associated with the longer distance of the *terroir* units for the former compared to the latter cultivar. Regarding the bacterial community, we observed strong cultivar and secondly vintage effects, but when analyzing the data separately for the two cultivars a strong clustering effect of vintage rather than of terroir unit became evident. The particular morphological features of the grapes and leaves of the two cultivars, Roditis and Siderits, could be associated with the strong cultivar signatures in the epiphytic

fungal and bacterial communities within the viticultural zone of Aigialeia. Although the bacterial community showed weaker distance-decay relationships compared to fungi, as also confirmed by Miura et al.(2017), generally similar structural patterns were evident for both fungal and bacterial communities with biogeography being the main structural factor at large scale (across different viticultural zones) and cultivar at regional scale. We further identified fungi and bacteria that were universal dwellers of the phyllosphere and carposphere of grapevines. The epiphytic fungal community across viticultural zones was dominated by *Alternaria*, *Aureobasidium*, *Cladosporium*, *Nigrospora*, common dwellers of plants phyllosphere. Similarly the epiphytic bacterial microbiome was represented by *Skermanella* and *Massilia*. In the viticultural zone of Aigeialeia, a core fungal microbiome shared by the two cultivars was composed of *Alternaria*, *Aureobasidium, Cladosporium* and *Capnodiales* while the bacterial core epiphytic microbiome consisted of *Sphingomonas*, *Masillia* and *Methylobacterium*. We overall showed that different structural factors are operative at different geographical scales: biogeography was the most important determinant of the epiphytic grapevine microbiome across viticultural zones, while cultivar becomes the stronger determinant with viticultural zone, with the fungal community being more responsive to the confounding factors compared to the bacterial community.

In **Chapter 4** we monitored microbial succession during vinification of grapes of the same cultivars (Rodits and Sideritis) and *terroir* units described in Chapter 3. In particular we assessed the effect of the same vineyard-mediated factors (cultivar, vintage, terroir unit) on the composition and succession of the fungal and bacterial communities along different vinification regimes, relevant to modern wine making, like spontaneous, spontaneous with preservatives and inoculated. Both cultivar and vintage seemed to impose strong and long-lasting effects on the fungal and the bacterial community, unlike *terroir* whose effect becomes weaker from vineyard, to the start of the vinification and eventually disappears at the late fermentation stages. Besides vineyard mediated factor, the type of vinification employed was the most important determinant of the composition of the fungal community, with the inoculated vinifications supporting different microbiota compared to the two spontaneous vinification regimes. Regarding bacteria, we noted cultivar-dependent patterns with the three vinification types supporting largely different communities for Roditis, unlike Sideritis where clear differences between the fully spontaneous and the other two vinifications were evident, a result that might be associated with the

presence of SO_2 in those last two vinifications. The fungal community showed universal succession patterns dominated at the early stages by NS yeasts (*Aureobasidium, L. quebecensis, T. delbrueckii*) and filamentous fungi (*Cladosporium*, *Alternaria*, *Aspergillus)*, as starters of the fermentation derived from grapes (Deyett & Rolshausen, 2019; Liu & Howell, 2021). These were displaced as the fermentation progresses by indigenous or commercial *Saccharomyces* in line with their higher tolerance to the increasing alcohol levels. Regarding bacteria, we noted an early presence of AAB like *Gluconobacter, Acetobacter, Komagataeibacter* in most vinifications which were followed by late establishment and proliferation of the LAB *Oenococcus*, related to its participation in the MLF at the later fermentation stages.

We went a step ahead and we determined the effects of vineyard- and vinification-mediated factors on the antioxidant, antimutagenic and anticancer properties of the produced wines. The antioxidant profile of wines from both cultivars was mostly affected by vintage and the vinification process employed with the highest antioxidant and antimutagenic properties being observed in the wines produced by the inoculated vinification. For the anticancer properties of wines, vintage was the stronger determinant with the wines produced through the inoculated vinification exhibiting a stronger anticancer activity compared to the spontaneous fermentation. Evidence from studies that have analysed the composition of wine metabolome, have suggested positive associations of wine detected compounds with common vinification members activity (Wei et al., 2022; Bokulich et al., 2016). However, no correlations between the tested antioxidant and anticancer activity of wines with vinification microbiota have reported yet. Hence, in the last part of this Chapter we attempted to identify fungal and bacterial members of the vinification microbiota that are positively associated with the anticancer, antimutagenic and antioxidant properties of wines. We identified strong and universal across cultivars positive associations of the abundance of *Torulaspora debrueckii* and *Lachancea quebecensis* with the antioxidant and anticancer activity of the produced wines. This finding is particularly important considering the suggested role of these NS yeasts in the production of phenolics during AF that might be associated with the anticancer and antioxidant properties of wine.

Finally, in **Chapter 5**, taking into account the strong effect of the vinification type on the microbial community, we aimed to identify the influence of two types of vinifications on the composition and dynamics of the bacterial and fungal community.

However, this time we further explored the gene expression patterns of the microbiota along the vinification process using meta-transcriptomic analysis. We explored microbial dynamics in a spontaneous (not inoculated) and a commercial vinification (inoculated with a *S. cerevisiae* strain) \with grapes of the cultivar Vidiano, while this time both vinifications were subjected to the addition of industrial vinification treatments of SO_2 and other additives. Vinification stage was the stronger determinant of the fungal community, followed by the vinification process, while the bacterial community showed rather universal patterns across vinifications and stages. Similarly with Chapter 4, we noted a wide fungal phylogenetic diversity at the first stages of fermentation with the fungal community composed of filamentous fungi, NS yeasts (*Aureobasidium, Cladosporium, Alternaria, Filobasidium, Lachancea, Torulaspora, Aspergilus, Sclerotiniaceae, Malassezia)* along with *Saccharomyces*. The latter became dominant immediately after inoculation in the commercial vinification and at the start and mid-stage of fermentation in the spontaneous fermentation in line with the capacity of these strains to tolerate high ethanol and SO_2 levels (Merico et al., 2007; Drygas et al., 2017; Riles and Fay, 2019). Bacterial community succession was characterized, in both vinifications, by the same phylogenetic diversity of LAB, AAB and grapevine relevant members (*Acinetobacter, Enterobacterales, Escharichia/Shigella, Kozakia, Massilia, Orbaceae, Prevotellaceae, Pseudomonas, Tatumella)* with a gradual increase in the presence of *Oenococcus* as the fermentation progresses, in accord with its reported high tolerance under harsh fermentation conditions (Onetto et al., 2021). Metatranscriptomic analysis revealed that the vinification stage was the most important determinant of the transcriptional profile of vinification microbiome, while differences between vinifications were less evident. By looking at the transcriptomes between the two vinifications at each fermentation stage we noted, besides a universal upregulation of the glycolytic pathway, differences at the start and mid-stages of fermentation. Hence in the spontaneous fermentation we noted at start an upregulation of stress response related proteins and glycolytic enzymes which were assigned to different NS yeasts and *Saccharomyces*, while at the mid-stage we identified upregulation of a *Saccharomyces*-related alcohol dehydrogenase implicated in the production of ethanol. On the other hand in the commercial fermentation we noted at the onset of fermentation an upregulation of stress related proteins associated with *Saccharomyces*, while at the mid-fermentation stage we noted upregulation of glycolytic enzymes. Finally at the end of fermentation

we observed a universal upregulation of alcohol dehydrogenase associated with the elevated ethanol production (Raj et al., 2014). Overall transcriptomic analysis revealed an upregulation of genes mostly originated from *S. cerevisiae* and other yeasts which were associated with stress response mechanisms, transportation, glycolysis and ethanol production in line with the biochemical environment of fermentations.

6.2. Conclusions

Overall, our findings identified the key factors shaping grapevine and vinification microbiome along different Greek grapevine cultivars, identified early microbiome predictors of GTDs, provided first evidence for the association of the grapevine and vinification microbiota with desirable properties of the produced wine and highlighted metabolic pathways that are mobilized by the vinification microbiota during the vinification process. Overall the main conclusions of this thesis are the following:

- Biogeography and/or cultivar were the most important driver of the wood grapevine microbiome with *P. chlamydospora, K. variispora, Fomitiporia* spp. and *Diaporthe* spp., being the key components of the GTD pathobiome and *P. chlamydospora, K. variispora* along with *Cladosporium* and *A. alternata* identified as accurate predictors of GTDs appearance.

- Different factors are operative in shaping the epiphytic grapevine microbiome at different geographical scales: Biogeography was the key determinant of the epiphytic microbiome across different viticultural zones whereas cultivar becomes the dominant factor shaping the epiphytic microbiome within viticultural zones. Still when cultivar-dependent effects were excluded, the local *terroir* seems to be particularly important at regional scale with fungi showing stronger distance-decay responses compared to bacteria.

- Under nearly full-scale fermentation conditions and regardless of the vinification process employed (spontaneous or inoculated), vineyard-mediated factors, such as cultivar and vintage, remain operative and affect microbial succession along the vinification process, unlike *terroir* whose effect, although

significant at vineyard level and at the start of fermentation, becomes less important as vinification progresses.

- The type of vinification process employed (spontaneous, spontaneous with preservatives and commercial) strongly affected the composition of the fungal and bacterial communities under full-scale fermentation conditions (Chapter 4) but had a weaker effect in smaller-scale fermentations when both spontaneous and inoculated vinifications were amended with preservatives and additives (Chapter 5).

- In all vinification the fungal communities showed rather universal successional patterns with NS yeast and filamentous fungi dominating at early fermentation stages getting displaced by *Saccharomyce*s strains as the fermentation progress. In contrast bacteria showed less universal successional patterns with *Oenococcus* gradual establishment along fermentations being the sole common pattern observed.

- *S. cerevisiae* and other NS yeasts, as revealed by metatranscriptomic analysis, mobilize a repertoire of functions associated with stress response mechanisms, transportation, glycolysis and ethanol production in line with the biochemical conditions prevailing during alcoholic fermentation.

6.3. Future perspectives

The findings will open new horizons in the investigation of the role of the grapevine and vinification microbiota on the health of grapevine and the quality of wines:

- Combination of metagenomic, metatranscriptomic and metabolomic analysis in spontaneous vinifications will provide for the first-time direct evidence for the exact contribution of the different microbiota members to the metabolic profile and sensory attributes of the produced wines. This could subsequently lead to the directed isolation of microorganisms associated with the beneficial metabolic traits of wines towards the construction of Synthetic Microbial

Communities that would lead to the production of high-quality wines with enhanced regional signatures

- Targeted isolation of *Bacillus* and *Streptomyces* members of the grapevine wood microbiome of GTD asymptomatic grapevines which have shown clear negative co-occurrence networking with the GTD-associated fungal pathobiome

- Validation of the application of the early fungal predictors of the GTD status of grapevines using similar datasets from different viticultural zones or from wider geographical areas. The optimization and implementation of this tool will facilitate the early diagnosis of GTDs appearance and enable the implementation of measures to minimize its spread in the vineyard

6.4. References

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