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Microbial Assemblage in Grapevine's Phyllosphere: Who is the Driver?

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Microbial Assemblage in Grapevine's Phyllosphere: Who is the Driver?

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Je déclare par la présente que cette thèse est mon travail original et qu'il a été écrit par moi en son intégralité. J'ai dûment reconnu toutes les sources d'information qui ont été utilisé dans la thèse.

Cette thèse n'a pas non plus été soumise dans aucune université auparavant.

Prashant Singh

12 December, 2018

Dedicated to

Phyllosphere, a generous niche supporting the microbial life, for their contributions to plants and mankind

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Contents

Thesis Summary

Vitis vinifera subsp. vinifera L., the main grape species are grown for fruit and wine production over the world is a natural host of a wide variety of prokaryotic and eukaryotic microorganisms that interact with grapevine, having either beneficial or phytopathogenic effects. They could also play a major role in fruit yield, grape quality, plant protection and, ultimately, in the pattern of grape fermentation and wine production. Phyllosphere (consists of the aerial parts of the plant) is one of the most prevalent microbial habitats on earth and is quite a neglected milieu, especially in grapevines and many questions related to this microbial habitat, are still unanswered.

This thesis is an effort to answer a very fundamental question in microbial ecology- what are the drivers that shape the microbiome in the grapevine's phyllosphere? The phyllosphere microbial communities (PMCs) live at the plant-climate interface and its ability to establish, thrive and reproduce on the leaf or fruit surface depends on several microbial functional traits, such as the ability to attach to the cuticle and to use the foliar nutrients as well as well as to the prevailing climatic conditions like temperature, air humidity and rain. Leaf or fruit chemistry, physiology, and morphological structure differ among plant genotype and species as all these traits have a genetic basis, and this variation may lead to a different combination of PMCs assemblage among plant genotypes. Hence, the first objective of our work was to assess the impacts of grapevine cultivars (varieties of *Vitis vinifera L*) and grapevine species (entirely different *Vitis* species) on microbiome assemblage in the phyllosphere at a particular geographic location (to minimize the environmental effects). Later on, impacts of some commercially important grapevine cultivars and terroirs (represented by three French climate zones) were also assessed and compared. Impacts of the season and exterior plant organs (leaf and berries) on microbial taxa structuring in the phyllosphere was also assessed and presented in this work. Furthermore, species-specific impacts on phyllosphere microbiome were also tested and represented.

Overall our study assessed and compared the many facets of the factors that may influence the microbiome structure in the phyllosphere with a special focus on relative selection pressure exerted by grapevine genotype and its interaction with different climatic conditions (or terroir), which may improve our chances to find genes that controls PMCs on phyllosphere, and simultaneously increase our confidence that those genes are actually important in realistic environments and probably those genes would give us new insights for breeding new and healthy grape varieties displaying better traits on their phyllosphere. Moreover, considering that the plant PMCs plays a crucial role in plant health and fitness as it can modulate leaf susceptibility to infection, this study could also be helpful to develop innovative and natural biocontrol methods phytostimulation against grapevine pathogens or rethink breeding schemes for the creation of

innovative resistant varieties.

Résumé de la thèse

Vitis vinifera subsp. vinifera L., les principales espèces de raisins sont cultivées pour la production de fruits et la production de vin dans le monde est un hôte naturel d'une grande variété de micro-organismes procaryotes et eucaryotes qui interagissent avec la vigne, ayant des effets bénéfiques ou phytopathogènes. Ils pourraient également jouer un rôle majeur dans le rendement des fruits, la qualité du raisin, la protection des plantes et, finalement, dans le modèle de la fermentation du raisin et la production de vin. La phyllosphère (constituée des parties aériennes de la plante) est l'un des habitats microbiens les plus répandus sur terre et est un milieu assez négligé, en particulier dans les vignes et de nombreuses questions liées à cet habitat microbien sont toujours sans réponse.

Cette thèse est un effort pour répondre à une question fondamentale en écologie microbienne: quels sont les facteurs qui déterminent le microbiome dans la phyllosphère de la vigne? Les communautés microbiennes de la phyllosphère (PMCs) vivent à l'interface plante-climat et sa capacité à s'établir, prospérer et se reproduire sur la surface des feuilles ou des fruits dépend de plusieurs caractéristiques fonctionnelles microbiennes, comme la capacité de se fixer sur la cuticule et d'utiliser la foliaire. nutriments ainsi que les conditions climatiques dominantes comme la température, l'humidité de l'air et la pluie. La chimie des feuilles ou des fruits, la physiologie et la structure morphologique diffèrent selon le génotype et l'espèce puisque tous ces traits ont une base génétique, et cette variation peut mener à une combinaison différente d'assemblage de PMC parmi les génotypes de plantes. Ainsi, le premier objectif de notre travail était d'évaluer les impacts des cultivars de vigne (variétés de *Vitis vinifera L*) et des espèces de vigne (espèces Vitis entièrement différentes) sur l'assemblage du microbiome dans la phyllosphère à un endroit géographique particulier (pour minimiser les effets environnementaux) . Plus tard, les impacts de certains cultivars et terroirs de vigne commercialement importants (représentés par trois zones climatiques françaises) ont également été évalués et comparés. Les impacts de la saison et des organes extérieurs de la plante (feuilles et baies) sur la structuration des taxons microbiens dans la phyllosphère ont également été évalués et présentés dans ce travail. De plus, des impacts spécifiques à l'espèce sur le microbiome de la phyllosphère ont également été testés et représentés.

Dans l'ensemble, notre étude a évalué et comparé les nombreuses facettes des facteurs qui peuvent influencer structure du microbiome dans la phyllosphère avec un accent particulier sur la pression de sélection relative exercée par le génotype de la vigne et son interaction avec différentes conditions climatiques (ou terroir), ce qui peut améliorer nos chances de trouver des gènes contrôlant les PMCs sur la phyllosphère. les gènes sont réellement importants dans des

environnements réalistes et probablement ces gènes nous donneraient de nouvelles idées pour la sélection de nouveaux cépages sains présentant de meilleurs caractères sur leur phyllosphère.

De plus, considérant que les PMC végétales jouent un rôle crucial dans la santé et la forme des plantes car elles peuvent moduler la susceptibilité foliaire aux infections, cette étude pourrait également être utile pour développer des méthodes de biocontrôle innovantes et naturelles ou phytostimulation contre les pathogènes de la vigne. de variétés résistantes innovantes.

CHAPTER 1

INTRODUCTION & STATE OF THE ART

CHAPTER SUMMARY:

- This chapter deals with the basic introduction about the grape species, grapevines and plant habitats for microorganisms.
- State of the art for characterizing plant-associated microbiome has also been presented.

Chapter 1

Introduction

1.1. Grapevines

The subgenus ***Vitis*** is a collection of a group of 79 species of vining plants in the flowering plant family *Vitaceae* and the phylogenetic relationships among species of this grape family (*Vitaceae*) has been described recently [1-3]. ***Vitis vinifera L*** (also known as grapevines) is the most common cultivated species (native to Eurasia) of this family, whose domestication began nearly 6000–8000 years ago and cultivars then found their way to most European, Northern African and Eastern countries through different routes [4,5]. A large number of diverse cultivars (*V. vinifera subsp. vinifera*) are used for fruit and juice consumption (table grape) and/or wine production (wine grape). Worldwide, 73.7 million tonnes of grapes were produced in 2014, and wine trade represented a gross value of 25.6 billion euros [6].

Due to the fast rate of climate change, many researchers have predicted that by 2050, most major wine-producing regions could become unsuitable for currently grown cultivars [7,8]. In addition, viticulture is required to reduce pesticides use, grapevine being one of the most intensively treated crops. It is, therefore, crucial to rapidly breed new adapted and resistant cultivars. In this perennial species with a long juvenile period, breeding is still a slow process although knowledge of the genetic determinism of agronomic traits is just emerging to speed up breeding through marker-assisted selection [9-13].

At Institut National de la Recherche Agronomique (INRA, Montpellier, France) we have maintained the largest grapevine collection worldwide at Vassal. Using 20 simple sequence repeat (SSR) markers, we constructed a diversity panel of 279 grapevine cultivars representing three genetic pools (western Europe, WW; from eastern Europe, WE; and table grape, TE). This panel adequately captured most of the genetic and phenotypic diversity existing within the entire Vassal collection (Fig 1A, B) while minimizing relatedness and retaining the main founders of modern cultivated grapevine to optimize the genetic diversity [14]. This constitutes a new, highly valuable resource for genetic association studies in grapevine, and deserves dissemination to the diverse field and greenhouse trials to gain more insight into the genetic control of many agronomic traits and their interaction with the microbes and environment.

A **B**

Fig 1. Schematic representation of the method used to design (A) the association panel of three genetic pools and (B) PCA analysis based on 20 SSRs for comparing the association panel with the whole Vassal collection by ***Nicolas et al 2016*** [14].

1.2. Plant associated Microbiome

The word ‘microbiome’ was first used by Joshua Lederberg as the “ecological community of commensal microorganisms, symbionts or pathogens, that literally occupy a space in our body” [15]. Hence, the human body is a great reservoir of microbes, recently studied by the Human Microbiome Project, which linked several features of the host to the presence of specific sets of microbial groups [16, The Human Microbiome Project Consortium, 2012]. More recently, this term has been broadly applied to different sets of microbes found in specific hosts or inhabiting a given environment [17,18].

There are several reports published in the recent past, which also described the plant-microbe interactions and some micro-organisms that have shown an association with the different tissues have been represented (Fig 2B). Most microbes inhabiting plant-related niches have neutral or beneficial roles in plant health and development [18,19]. Considering the microbiome as an active component of the host, being also responsive to changes in environmental (biotic and abiotic) conditions, a better understanding of the most important drivers of the composition of plant microbiomes is very important. In order to understand the factors that influence this microbial

assembly and the dynamics from a phylogenetic and functional perspective, recent studies have targeted different fractions of the plant microbiome separately. Partitioning the plant microbiome considers three major compartments (Figure 2A) where microbial cells can establish and develop: the so-called rhizosphere, endosphere and phyllosphere [20,21].

1.2.1 Rhizosphere

The rhizosphere is defined as the soil region under the influence of the roots [19,23]. This soil matrix is the major reservoir of microbes that interact with plants, being described as the most biodiverse ecosystem on Earth and often reported to influence plant-associated microbial communities, also in grapevines [24,25]. Primary selection of microbial communities in rhizosphere depends on various chemical components released in the vicinity of the plant roots (carbohydrates, proteins, lipids, phenolic compounds, organic acids and other cellular components). Few of them (commonly metabolized by most of the soil organisms, e.g., glucose) are related to the activation of major fractions of microbial communities, whereas few others (related to signaling and chemotaxis, e.g.- flavonoids. [26,27]) can also activate those microbial groups related to signaling and chemotaxis, e.g.- flavonoids. [26,27]. Plants have evolved to allow the rhizosphere to attract and harbour specific microbes, which support the promotion of plant growth [28,29].

1.2.2 Endosphere

Endosphere (space inside plant tissues) is another microbial habitat. A detailed analysis of endophytes has divided the endophytic communities into subgroups, named ‘obligate’ or ‘facultative’ by the researchers [21]. Endophytes are likely to be involved in controlling plant pathogens and promoting plant growth. It has been shown that the ability of endophytic *Burkholderia* spp. to control the growth of the pathogen *Fusarium moniliforme* [30]. Another report observed that the endophytic diazotrophs from sugarcane roots are able to produce plant growth-promoting substances and to secrete higher amounts of amino acids that might facilitate plant nutrition [31].

In a few grapevine cultivars, endophytic microbiome has also been extensively characterized [30] but their functional characterization is still an ongoing process.

1.2.3 Phyllosphere

A third component of the plant microbiome consists of micro-organisms colonizing the external area of aerial plant tissues, the phyllosphere. Although this term can be used for any external surface of plants, it is mostly applied when describing the leaf surface but sometime fruit surface is called carposphere [33].

The microbial communities found in the phyllosphere have critical roles in processes related to plant development, for example, performing nitrogen fixation, protecting plants against invading pathogens and biosynthesizing phytohormones [34-37]. It makes them quite important for global processes, such as carbon sequestration [38], and they can be used as potential sources for the development of sustainable agricultural practices. Microbes living in phyllosphere are able to thrive under particular and harsh environmental conditions, characterized as an oligotrophic environment, where there is a limited availability of nutrients and variable conditions of humidity, UV radiation, pH, and temperature [39]. Although less studied as compared to other niches, phyllosphere of the grapevines or other plants have been reported to be composed of fungi (filamentous and yeasts), bacteria, algae, and, at lower frequencies, protozoa and nematodes [40,41].

Another issue of this theme is the origin of the microbes that make up the microbial communities in the phyllosphere. Recent shreds of evidence suggested that air and its aerosols, soil, water biogeography of a specific location are the most important sources for microbial cells that make up the communities in the phyllosphere [38-41].

Fig 2. Schematic representation of the major sources for microbes that compose the plant-associated communities: the rhizosphere, endosphere, and phyllosphere and the contribution of environmental sources for the composition of microbial communities in these niches.

Fig 3. Diagrammatic representation of some of characteristic bacteria and fungi known to show associations with the different tissues of *Vitis vinifera* by ***Gilbert et al, 2014*** [93].

1.3 Characterisation of plant associated microbiome

1.3.1 Culturable vs unculturable

The conversion of grape juice into wine was first confirmed to be the result of a microbial process by Louis Pasteur in the middle of the nineteenth century. Since then, the diversity of the vineyard, grape and wine microbiota has been extensively investigated using traditional microbiological methods involving microscopy, cultivation on different agar media and biochemical characteristics. However, the arrival of DNA-based molecular techniques such as polymerase chain reaction (PCR) and the identification of evolutionarily stable molecular marker genes such as 16S ribosomal RNA (rRNA) genes improved our ability to identify microbial species with better resolution and reliability [42-44]. To date, more than 40 yeast species [45], 50 bacterial species [46] and ~70 genera of filamentous fungi [47] associated with grapevine and wine fermentation processes have been isolated and identified using traditional culture-based methods. These methods are however extremely laborious, time-consuming and often inconsistent and biased [48,49]. In addition, only species that are able to grow on the culture media and under the cultivation conditions used can be isolated and identified, while species that are in low abundance, those species for which the prevailing cultivation conditions are not conducive, as well as viable but unculturable cells, are often overlooked [44]. These limitations in culture-based methods, as well as the difference between culturable and in situ diversity, increased the importance of research into culture-independent molecular approaches [50].

Introduction of PCR-based methods created new opportunities for the development and improvement of several techniques in grapevine's molecular ecology. The application of molecular techniques allowed researchers to study microbes, not on the basis of their ability to grow on certain culture media but rather use nucleic acids polymorphisms for detection and identification. Such methods, mostly use DNA extracted directly from the environment as a template for PCR, followed by separation and detection for microbial community profiling. These methods include single-strand conformational polymorphisms (SSCP), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (T-RFLP), and automated ribosomal intergenic spacer analysis (ARISA)[42,44,51-54]. The PCR-DGGE technique is often employed in combination with culture-dependent methods and has allowed researchers to decipher the complexity and evolution of microbial populations, during berry ripening and throughout the fermentation process [55-58]. Although PCR-DGGE is typically thought to be appropriate for the analysis of less species-rich environments such as grape must, it has low sensitivity and is unable to detect populations that are present at a relative abundance of <1% of the population [48, 55] but it is critical to monitor such populations as they can influence wine quality.

More recently, SSCP, T-RFLP, and ARISA [49,59-60] have been employed to profile the wine microbial diversity, but these methods have some limitations too (Table 1).

TABLE 1 | A summary of the advantages and disadvantages of PCR-based culture-independent microbial community fingerprinting methods [54, 66].

Methods	Advantages	Disadvantages
Single-strand conformational polymorphisms (SSCP)	1. Distinct bands can be isolated and sequenced 2. No clamped primers and Restriction Enzymes required	A high rate of re-annealing of single strands with high DNA concentrations
Denaturing gradient gel electrophoresis (DGGE) + Real-time quantitative PCR (qPCR)	1. Ability to target both RNA and DNA 2. Can be applied to RNA and therefore measures viable population	Only intense and well-separated bands can be sequenced and require species-specific primers.
Terminal restriction fragment length polymorphisms (T-RFLP)	1. Easily applicable to large sample numbers 2. Web-based tools allow in silico prediction of TRFs	Incomplete and non-specific digestion leads to overestimation of diversity and poor resolution of complex communities
Automated ribosomal intergenic spacer analysis (ARISA)	1. Less labor intensive 2. Allows detection of dominant species. 3. Allows high resolution of subtle differences.	Preferential amplification of shorter templates

1.3.2 Next Generation Sequencing (NGS)

Improvements in DNA sequencing broadened the ability of researchers to study the microbial community structure and function with a higher resolution by employing metagenomic approaches. Metagenomics can be defined as the direct genetic analysis of the collection of genomes within an environmental sample, this can be achieved either through whole metagenome sequencing or amplicon-based sequencing [61,62]. The innovations in high-throughput, short-amplicon sequencing are revolutionary in a way that they can describe the microbial diversity within and across complex biomes [63]. Although high throughput methods have been widely used to investigate the microbial ecology of various environments [44,64-65], their application in grapevine and wine fermentation microbial ecology is relatively recent, and their contribution to the field has not been much explored. Until recently, the 454 pyrosequencing and Illumina platforms were the most commonly used platforms for grapevine ecology surveys. At least 48% of the published data on the vineyard, grapevine and wine microbiome is derived from 454 pyrosequencing while the remaining 52% is derived from Illumina sequencing [67]. Both platforms work on a sequencing by synthesis approach but differ in their chemistries. Bridge amplification of adaptor-ligated DNA

fragments on the surface of a glass is the core process of Illumina sequencing [68]. Afterward, bases are determined using a cyclic reversible termination technique, which sequences the template strand, a single nucleotide at a time through progressive rounds of base incorporation, washing, scanning, and cleaning. In this method, labeled dNTPs are used to stop the polymerization reaction, allowing the removal of unincorporated bases. The fluorescent dye is captured to identify the bases added, and then cleaved so that the next nucleotide can be added, this is then repeated [68-70]. Earlier Illumina analysis generated at least 1 Gb of sequences with reads averaging 35 bp and the duration of 2–3 days. However, the introduction of HiSeq and MiSeq machines altered the duration time to ∼4 days and 24–30 h, and increased the read length to 250–300 bp, respectively with error rates of below 1%, with substitution the most occurring issue [71,72].

In 454 pyrosequencing an emulsion PCR is used for bridge amplification of adaptor-ligated DNA fragments on the surface of a bead. The beads are thereafter distributed and fixed into 44 µm wells, where the sequencing by synthesis occurs. After the nucleotide bases are incorporated as an enzymatic luciferase coupled reaction occurs, allowing for the identification of bases, which is measured using a charged couple device [66-68]. The 454 pyrosequencing technique was reported in 2008, as the most published NGS platform, however, the technology has since been discontinued, and has therefore been surpassed by Illumina [69,70].

NGS has been widely used for the comprehensive evaluation of the vineyard or grape microbiome, and typically two key questions were addressed. Firstly, which microorganisms are present in the environment, and secondly the role of the individual species [73]. To understand the role of the identified species, in grape or wine microbiome requires that standard microbiological methods be applied to isolate the strains and then evaluate them for their potential contribution to grape or wine quality by assessing their phenotypic and genotypic properties and thereafter they will be evaluated in different wine matrices to assess their growth and metabolic profile. To this effect, several species retrieved using culture-dependent methods and have been shown to contribute positively in the winemaking process. For instance, some strains of *Wickerhamomyces anomalus*, *Candida pyralidae*, *T. delbrueckii*, and *Kluyveromyces wickerhamii* were shown to suppress the growth of *B. bruxellensis* [74], a wine spoilage yeast; *M. pulcherrima* was highlighted as a desirable co-inoculant for the reduction of ethanol [75], while others such as *Hanseniaspora vineae*, *Starmerella bacillaris*, *L. thermotolerans*, *P. kluyveri*, and *T. delbrueckii* present various desirable aroma signatures [72,74].

1.3.3 Target genes

The target marker genes are universally present in all species evaluated and contain both highly conserved fragments that facilitate the design of PCR primers targeting all members of a community and variable regions that allow for the discrimination of different species within the community [43,45]. Both bacteria and fungi have ribosomal RNA genes as suitable target genes. In bacteria, the 16S rRNA is typically targeted while in fungi the internal transcribed regions (ITS1-5.8S rRNA-ITS2), as well as the 26S rRNA, are the target molecules for high throughput amplicon sequencing and microbiome analyses (Fig 3A,B). The nine hypervariable regions (V1–V9) of bacteria (Fig 3A) have all been targeted for the estimation of vineyard bacterial diversity [24,32,41,77-79]. Depending on the region sequenced the data might be similar or differ significantly. For example, in a study comparing the V4 and V5 region [80], found that the regions resulted in almost a similar bacterial composition with minor variation in the lower taxa; although the V4 region provided greater taxonomic depth for certain Proteobacteria and lactic acid bacteria (LAB) species. In contrast, another study targeted the V3–V4 and V5–V6 regions and evaluated the bacterial community associated with grape must. A total of 89 genera were identified, however, only 31 of these were common in both target regions evaluated [81].

The fungal ITS regions (ITS1 or ITS2) are the most commonly targeted region for fungal diversity estimation. The classification of general fungi and arbuscular mycorrhizae (AMF) has been accomplished by targeting the ITS region [32,82].

Using these regions, most of the studies (including grapevine research) have reached the phylogenetic level of “Phyla” or “Family” [25,32,63] in characterizing the plant-associated microbial communities but probably with greater sequencing depth and better bioinformatic pipelines, “genus” or “Species” level could be reached.

A

B

Fig 4. (A) Reference map of the 16S rRNA gene. The map shows variable regions V1-V9 (above chart) and the locations of common primers and (B) Relative binding positions for primers within ITS region.

1.3.4 Bioinformatic Data Analysis: A powerful tool to unravel microbial diversity

High throughput sequencing techniques usually generate large amounts of sequence data, and the only viable option to handle such information is via automated approaches. There are currently several open source pipelines (described below) and most of these pipelines provide the tools for basic data analysis steps such as data cleaning, sequence clustering, functional annotation, and taxonomic assignments (Fig 5).

1.3.4.1 Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST):

It is one of the biggest repositories for metagenomic data and an open source web application server that suggests automatic phylogenetic and functional analysis of metagenomes [83]. Using a combination of several bioinformatics tools, the MG-RAST offers automated quality control, annotation, comparative analysis and archiving service of metagenomic and amplicon sequence datasets. The application supports amplicon 16S, 18S, and ITS sequences and metatranscriptome (RNA-seq) sequences processing [84] and the profiles for the metagenomes can be visualized and compared by using bar charts, trees, spreadsheet-like tables, heatmaps, PCoA, rarefaction plots, circular recruitment plot, and KEGG maps.

Apart from metagenome analysis, MG-RAST can also be used for data discovery. The visualization or comparison of metagenomes profiles and data sets can be implemented in a wide variety of modes; the web interface allows to select data based on criteria like composition, sequences quality, functionality or sample type and offers several ways to compute statistical inferences and ecological analyses [83,84].

1.3.4.2 Quantitative Insights Into Microbial Ecology (QIIME):

QIIME is another bioinformatic pipeline designated for the task of analyzing microbial communities that were sampled through a marker gene (e.g., 16S or 18S rRNA genes) amplicon sequencing. In its heart, the pipeline includes the steps of quality control over the input sequencing reads, clustering the marker gene nucleotide sequences at a requested phylogenetic level (e.g. 97% for species level) into OTUs (operational taxonomic units) and taxonomically annotates the OTUs by looking for sequences similar to them on a reference taxonomic database [85]. "OTU" is the common term used to refer the clusters of uncultivated or unknown microorganisms, grouped by DNA sequence similarity [61] of a specific taxonomic marker gene(e.g, 16S or ITS). In other words, OTUs are pragmatic proxies for microbial "species" at different taxonomic levels, in the absence of traditional systems of biological classification as are available for macroscopic organisms.

The main output from the QIIME pipeline is the OTU table, which describes the microbial OTUs and their abundances in each of the samples. Additional tools like including rarefaction, beta diversity assessment, principal coordinates analysis (PCoA), that are relevant to ecological aspects of the samples being investigated are also provided within the pipeline [85]. QIIME is under active development since its release in 2010.

1.3.4.3 MOTHUR

MOTHUR is an open source software package for NGS data processing. The package is frequently used in the analysis of DNA from uncultured microbes and is capable of processing data generated from several DNA sequencing methods including 454 pyrosequencing, Illumina HiSeq and MiSeq, Sanger, PacBio, and IonTorrent. It is a comprehensive software package that builds upon previous tools to provide a flexible and powerful software package for analyzing sequencing data. The algorithm includes the steps to trim, screen, and align sequences; calculate distances; assign sequences to operational taxonomic units, and describes the diversity of samples characterized by pyrosequencing of 16S rRNA gene fragments [86].

1.3.4.4 UPARSE: highly accurate OTU sequences from microbial amplicon reads

The UPARSE pipeline reports operational taxonomic unit (OTU) sequences with ≤1% incorrect bases in artificial microbial community tests, compared with >3% incorrect bases commonly reported by other methods. The improved accuracy results in far fewer OTUs, consistently closer to the expected number of species in a community [87]. UPARSE works by quality-filtering reads, trimming them to a fixed length, optionally discarding singleton reads and then clustering the remaining reads. Unlike QIIME, MOTHUR and UPARSE do not require technology- or gene-specific parameters (such as an OTU size cutoff), which makes it highly robust with respect to variations in the input data and suggests that UPARSE could be successfully applied to a wide range of marker genes and sequencing technologies [87]. It uses USEARCH pipeline for clustering (http://www.drive5.com/) that are often orders of magnitude faster than BLAST. USEARCH combines many different algorithms into a single package that cuts your learning curve, reduces the number of steps you need to take for a given task, and slashes computing times.

1.3.4.5 Divisive Amplicon Denoising Algorithm (DADA):

Errors in Illumina sequenced amplicon data are currently addressed by quality-filtering and construction of OTUs: cluster sequence that differs less than a fixed dissimilarity threshold (typically ~ 3%). All clustering algorithms typically interpreted sequences within an OTU as a taxonomic grouping, without specifying whether the variation within an OTU represents errors or the real diversity. Hence, when probing finer scale diversity, OTU methods have intrinsically high false positive and false negative rates and they usually overestimate diversity (e.g- QIIME and MOTHUR), when there exist errors larger than OTU defining cutoff and cannot resolve real diversity at a scale finer than that arbitrary cutoff [88].

DADA addresses this issue, it starts with a parametric statistical model of substitution error (derived from data- unsupervised learning). Later, it incorporates this error model into a divisive hierarchical clustering algorithm that groups error containing reads into the cluster. Finally, it couples this clustering algorithm with the inference of error parameters from the clustered data, and perform each step in alternation until both converge [88]. Other clustering approaches does not have error model and causes estimates of diversity to strongly dependent on experimental variables such as size of the datasets, length of the region sequenced and the details of the PCR chemistry and these issues are not amenable to simple fixes, and it is not possible to separate real diversity and error without using DADA [88,89]. Latest version; the DADA2 has been shown to work much better than existing methods of UPARSE, QIIME or MOTHUR. It is good in separating error from real diversity and more targetted to the particular task of producing conservative estimates of diversity from noisy sequence data, with much less false positives [89].

Fig 5. Schematic representation of bioinformatic data analyis procedure of 16S/ITS amplicon sequence data.

1.4 Scope of the thesis

1.4.1 Objectives

Biotic and abiotic factors, plant developmental stages, plant genotype, environmental conditions, and agricultural practices have been shown as drivers for plant-associated microbial communities in various plants or crops including grapevines [19,32,90-92]. The grapevine's phyllosphere is relatively less explored subject especially in the context of microbial assemblage on various cultivars grown in the Mediterranean region or in different climatic conditions. Therefore, this thesis aims to explore the microbial diversity present on the phyllosphere of grapevine cultivars, grape species and to identify the potential drivers for it.

Major objectives of the current study are-

- To identify and characterize the phyllosphere microbiome of grapevine cultivars (randomly selected first and later selected on the basis of genetic distances; more distant cultivars were selected within the three genetic pools) grown in the Mediterranean.
- To asses the impacts of genetic diversity of these cultivars over microbial assemblage in the phyllosphere.
- To asses the impacts of grape-species (five species were selected from *Vitaceae* family) on its phyllosphere microbiome assembly at a particular environment and location and to compare differential taxa abundance using recently developed statistical methods.
- To compare the impacts of genotype and French climate zones (or terroir) on grapevine's phyllosphere microbiome structure and establish a potential driver for it.

1.4.2 Organization

This thesis is organized into five chapters. The **Chapter 1**, which includes the current section of introduction and describes the state of the art related to the characterization of grapevines and is associated microbiomes. A detailed systems biology approach to handle metagenomic data and obtain meaningful summaries out of it has also been discussed.

Chapter 2 provides the details of the characterization of the phyllosphere microbial structure of 27 cultivars of *Vitis vinifera* (9 cultivars from each genetic pool) and assessed the impacts of their genetic diversity on microbial assemblage. It also discusses the optimized protocols to obtain phyllosphere microbial communities from leaf and berry surface and further processing from DNA extraction to sequence data generation. A detailed data processing and statistical analysis procedure were also provided to sustain the findings.

Chapter 3 provides the comparative study of the two strong drivers of the phyllosphere microbiome structure, i.e grapevine varieties, and environmental conditions at three different

geographic locations of France on its impacts on grapevine's phyllosphere microbiome assemblage. We also explore the impacts of seasons and exterior plant organs.

Chapter 4 provides the detailed study of phyllosphere microbiome structure of 5 different grape-species of *Vitaceae family* (represents higher genetic distances than that occurring within V. vinifera at the cultivar level) and also assesses the impact of grape-species in shaping microbiome assemblage in the phyllosphere as well as the comparison of the few data normalisation methods.

Chapter 5 deals with the conclusion and future application of grapevine microbiomes, especially improving plant health using Biocontrol agents (BCA), breeding new and innovative resistant cultivars and the use of microbiome engineering for healthier plants, which are able to show better traits.

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CHAPTER 2

Assessing the Impact of Plant Genetic Diversity in Shaping the Microbial Community Structure of *Vitis vinifera* Phyllosphere in the Mediterranean

CHAPTER SUMMARY:

- Published manuscript
- Provides phylum level characterization of microbial structures present in leaf and fruit surface (Phyllosphere)
- Describes the impacts of genetic diversity of grapevines in microbial assemblage in the phyllosphere

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Assessing the impact of plant genetic diversity in shaping the microbial community structure of *Vitis vinifera* phyllosphere in the Mediterranean

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ABSTRACT

The aerial surface of the plant (phyllosphere) is the habitat of complex microbial communities and the structure of this microbiome may be dependent on plant genetic factors, local environment or interactions between them. In this study, we explored the microbial diversity present in the phyllosphere of a very diverse set of grapevine cultivars representing the three genetic pools of the species, grown on an experimental plot at Montpellier (French Mediterranean region). We assessed microbiome variation in the phyllosphere using amplicon sequencing of the 16S rRNA gene and of the internal transcribed spacer (ITS), according to the grapevine genetic pools or cultivars, and organs (i.e. leaves and grape berries). The observed microbiome was complex; out of 542 bacterial genera; *Pseudomonas*, *Pantoea*, *Sphingomonas*, and *Acinetobacter* were the most abundant and almost ubiquitously present across the samples, and out of 267 fungal genera; *Aureobasidium*, *Alternaria*, *Mycosphaerella* and *Aspergillus* were most represented. Our results illustrated that the microbial taxa were almost uniformly distributed among the genetic pools and only a few cultivar or genetic pool level differences were found, but a very clear differential taxa abundance was found between the leaf and berry samples. Some genus level associations were also observed with certain genetic pools.

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Genetic diversity; grapevine; microbiome; phyllosphere

Introduction

Vitis vinifera (subsp. *vinifera L.*), is the main grape species grown for fruit and wine production over the world. It is a natural host of a wide variety of prokaryotic and eukaryotic microorganisms that interact with grapevine, having either beneficial or phytopathogenic effects (Schulz et al. 1999). These microbes also play a major role in fruit yield, grape quality and, ultimately, in the pattern of grape fermentation and wine production (Compant et al. 2011; Bokulich et al. 2016; Belda et al. 2017).

The grapevine phyllosphere is rather less extensively studied as compared to the rhizosphere and endosphere (Vorholt 2012). The phyllosphere (in general) also harbors complex microbial communities involved in many crucial functions such as nitrogen fixation (Jones 1970), carbon sequestration (Bringel and Couée 2015), degradation of pesticides and organic pollutants (Brandl et al. 2001; Kishore et al. 2005; Bulgarelli et al. 2013). It is a significant and ubiquitous habitat for microorganisms and also an open system that microbes can invade by migration from the atmosphere, soil, other plants and insects (Lugtenberg et al. 2002; Williams et al. 2013). But microbial populations on phyllosphere are also known to live and thrive under harsh environmental factors such as UV radiation, air pollution, temperature fluctuations, water and nutrient availability (Andrews and Harris. 2000; Lugtenberg et al. 2002; Müller and Ruppel 2014).

A very fundamental question in microbial ecology is what drivers shape the microbiome on phyllosphere? Environmental conditions at the particular location and biotic factors such as leaf age have been identified as important drivers (Kadivar and Stapleton 2003; Ikeda et al. 2011, Copeland et al. 2015). Some reports on grapevine phyllosphere also suggested that the bacterial and fungal communities of the phyllosphere are minimally affected by the chemical and biological

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All of the data are provided fully in the result section of this paper and the sequence data for 16S and ITS sequences are available at institutional server http://agap-ng6.supagro.inra.fr/inra and can be obtained upon reasonable request to authors.

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treatments tested, and they mainly differed according to the grapevine location (Gu et al. 2010; Bokulich et al. 2014; Perazzolli et al. 2014). Few authors suggested that in the tropical and temperate forests, the plant genotype is a major driver of the composition of the bacterial communities in the phyllosphere (Lambais et al. 2006; Redford et al. 2010). Another study on *Arabidopsis thaliana* also illustrated that the plant genetic factors may influence the community composition of the phyllosphere (Bodenhausen et al. 2014).

Until recently, there have been no scientific reports available, analyzing the effect of grapevine genetic factors on the microbiome structure of the phyllosphere. Considering that the microbial diversity present in phyllosphere could be relevant for plant health (Lugtenberg et al. 2002; Compant et al. 2005; Vorholt 2012), a better understanding of how the microbiota associated with grapevine phyllosphere is structured according to the grapevine genetic diversity available at a particular geographic location may provide unexpected opportunities to develop innovative and natural biocontrol methods or phytostimulators against plant pathogen or new breeding scheme for the creation of innovative resistant cultivars. As a first step towards this goal, we explored the bacterial and fungal diversity in the phyllosphere of leaf and berry samples from a set of rather diverse grapevine cultivars that belongs to the three genetic pools of the cultivated grapevine (Nicolas et al. 2016), in the French Mediterranean region. These experiments led us to address two major questions: (i) What microbial diversity is present in the phyllosphere of our Mediterranean vineyards and (ii) how this microbiome structure itself according to the grapevine genetic diversity and plant organs.

Materials and methods

Sample collection and DNA extraction

A total of 279 grapevine cultivars were grown in a completely randomized block design at Le Chapitre INRA Villeneneuve-Les-Maguelonne field station near Montpellier (French Mediterranean region). A panel of cultivars representing three genetic pools (western Europe, WW; from eastern Europe, WE; and table grape, TE) was constructed for genome-wide association studies while minimizing relatedness and retaining the main founders of modern cultivated grapevine to optimize the genetic diversity (Nicolas et al. 2016). Nine cultivars were randomly selected from each genetic pool and leaf (with sizes +1 to +4) samples were taken from four to five plants of each cultivar. Leaf samples were taken before spraying of pesticides; each plant had the same age. We collected the leaf samples in the Spring (mid-May 2016) and at the beginning of harvesting season, we also collected samples of berries from the same cultivars. A metadata table containing all the information about the samples and replicates can be downloaded from the GitHub repository (https://github.com/PrashINRA/MetaData_GrapevinePhyllo.git). All samples were washed with an isotonic solution of sodium chloride (0.15 M) containing 0.01% Tween 20 using a horizontal shaker for 1hr at 100 RPM. Afterward, samples were given an ultrasonic bath for 7–10 min using Ultrasonic Cleaner (Branson 5510) for maximum recovery of microbes from the sample surface. The remaining solution was centrifuged at 4,000 g and microbial pellets obtained in a 2-ml Eppendorf tube were collected and stored at −20°C. DNA was extracted from the pellets using the Meta-G-Nome Isolation Kit (Epicentre, Illumina) following the manufacturer's instructions.

PCR amplifications and MiSeQ library preparation.

To access bacterial communities, the V4 region of the 16S ribosomal gene was amplified using primers 515F and 806R (Caporaso et al. 2011). Fungal community diversity and abundance were accessed using modified ITS9 and ITS4 primers targeting the ITS2 region (Blaadid et al. 2013; Lundberg et al. 2013). Two-step PCR was performed to prepare sequencing libraries. PCR1 was designed to perform amplification of the target regions and to add Illumina Nextera transposase sequence to the amplicons. Primers from Illumina kit for dual indexing of the amplicons was used in PCR2. Both forward and reverse primers for PCR1 were amended with frameshift (FS) sequences in their 5′ overhang to improve sequence diversity and overall read quality (de Souza et al. 2016). PCR1 was performed in 20 μL reactions with 30 ng of sample DNA using the Advantage 2 PCR kit (Clontech, 639206). PNA PCR clamps were also used to reduce host organelle contamination (de Souza et al. 2016). The same PCR1 was performed for ITS amplification except for the step of PNA annealing. Amplicon replicates were pooled, purified using Agencourt AMPure XP beads (Beckman Coulter) at a

bead-to-DNA ratio of 0.7:1, resuspended in 30 μL MilliQ water and evaluated in agarose gels. In PCR2, each cleaned PCR1 product within the same sample received a unique combination of forward and reverse primers (respectively, N7 and S5 Illumina dual index oligos). Afterward, samples were again cleaned using AmPure XP magnetic beads, pooled in equimolar concentrations and sequenced using 2 × 250 bp MiSeq v2 sequencing (Illumina Inc., San Diego, CA, USA).

Data processing and analysis

All RAW data files were imported and processed in the R-environment (R Core Team, 2017) using various codes and inbuilt functions available in different R-packages. The whole dataset for 16S and ITS amplicon sequences were uploaded and available at the institutional server http://agap-ng6.supagro.inra.fr/inra. Data processing and further analysis were done in two phases. In phase-I, raw data files from both the datasets were filtered and trimmed using the fastqPairedFilter() function of the *dada2* package (Callahan et al. 2016) and bases with low-quality scores were discarded. These filtered files were then processed using the Divisive Amplicon Denoising Algorithm (DADA) pipeline which included the steps of dereplication, core denoising algorithm and merging of the base pairs. Merging function provided global ends-free alignment between paired forward and reverse reads, and merged them together if they overlapped exactly and a table for ribosomal sequence variants (RSVs, a higher analog of operational taxonomic units-OTUs) was constructed, which records the number of times each amplicon sequence variant was observed in each sample. DADA infers sample sequences exactly and resolves differences of as little as one nucleotide (Callahan et al. 2016). Chimeras were removed using the removeBimeraDenovo() function of the *dada2* package. OTU sequences were assigned a taxonomy using the RDP classifier and the UNITE database (Wang et al. 2007; Abarenkov et al. 2010) with assignTaxonomy() function of the same *dada2* package for 16S and ITS sequences, respectively. Then, at the end of phase-I data processing, a phyloseq data object was created to initiate phase-II data analysis.

In phase-II, a phylogenetic tree for the taxa was constructed using the R-package a*pe* (Paradis et al. 2004) and merged with the phyloseq data object of phase-I. Unassigned taxa and singletons were also removed using the subset_taxa() and prune_taxa() functions of the *phyloseq* package in R (McMurdie and Holmes 2013). This data object was then used to calculate microbial abundances, α, β diversity analysis and for other statistical tests using various functions in the *phyloseq* and *vegan* packages (McMurdie and Holmes 2013; Oksanen et al. 2017).

Prevalence plot for taxa abundances was made using ggplot() function of the *ggplot2* package (Wickham 2009) using the entire 16S and ITS data-sets. Chao1 estimates of α diversity (Chao 1987) was measured within sample categories using estimate_richness() function of the *phyloseq* package. Relative abundances of microbial genera were also plotted using the *ggplot2* package (Wickham 2009) on the above data, which were also rarified to even depth of 5,000 reads per sample.

Multidimensional scaling (MDS, also known as principal coordinate analysis; PCoA) was performed using Bray-Curtis dissimilarity matrix (Beals 1984) between samples and visualized by using their base functions in the *phyloseq* package (McMurdie and Holmes 2013).

Statistical analysis

We analyzed all the data from 16S and ITS amplifications separately in R version 3.3.4 using the *dada2*, *phyloseq* and *vegan* packages. CRAN packages *plyr* and *ggplot2* (Wickham 2009; Wickham 2011) were also used to draw the figures. We assessed the statistical significance ($P < 0.05$) throughout and whenever necessary, we adjusted *P*-values for multiple comparisons according to the Benjamini and Hochberg method to control False Discovery Rate (Benjamini and Hochberg 1995) while performing multiple testing on taxa abundance according to sample categories. We performed an analysis of variance (ANOVA) among sample categories while measuring the Chao1 estimates of α-diversity. Stratified permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted on all principal coordinates obtained during PCoA with the adonis() function of the *vegan* package, to observe the statistical significance of clusters according to the sample categories.

Linear regression (parametric test), and Wilcoxon (Non-parametric) test (Hollander and Wolfe 1973) were performed on taxa abundances against genetic

pools using their base functions in R (Myles and Douglas 1973; Bauer 1972).

Results

Quality assessment of the data

Raw demultiplexed sequence data files were generated using high-throughput amplicon sequencing of 16S and ITS ribosomal RNA genes and the number of reads per sample has been taken into account to obtain the depth of the sequencing. Rarefaction curves (number of reads vs number of OTUs) from both the datasets (Figure 1(A and B)) began to level off for most of the samples suggesting a good quality and coverage of both the data-sets and thus we can assume that the microbial communities were reasonably characterized with the sampling effort.

Microbial diversity in the phyllosphere

A total of 5,772,135 16S and 3,807,033 ITS amplicon sequences were generated from 80 samples covering

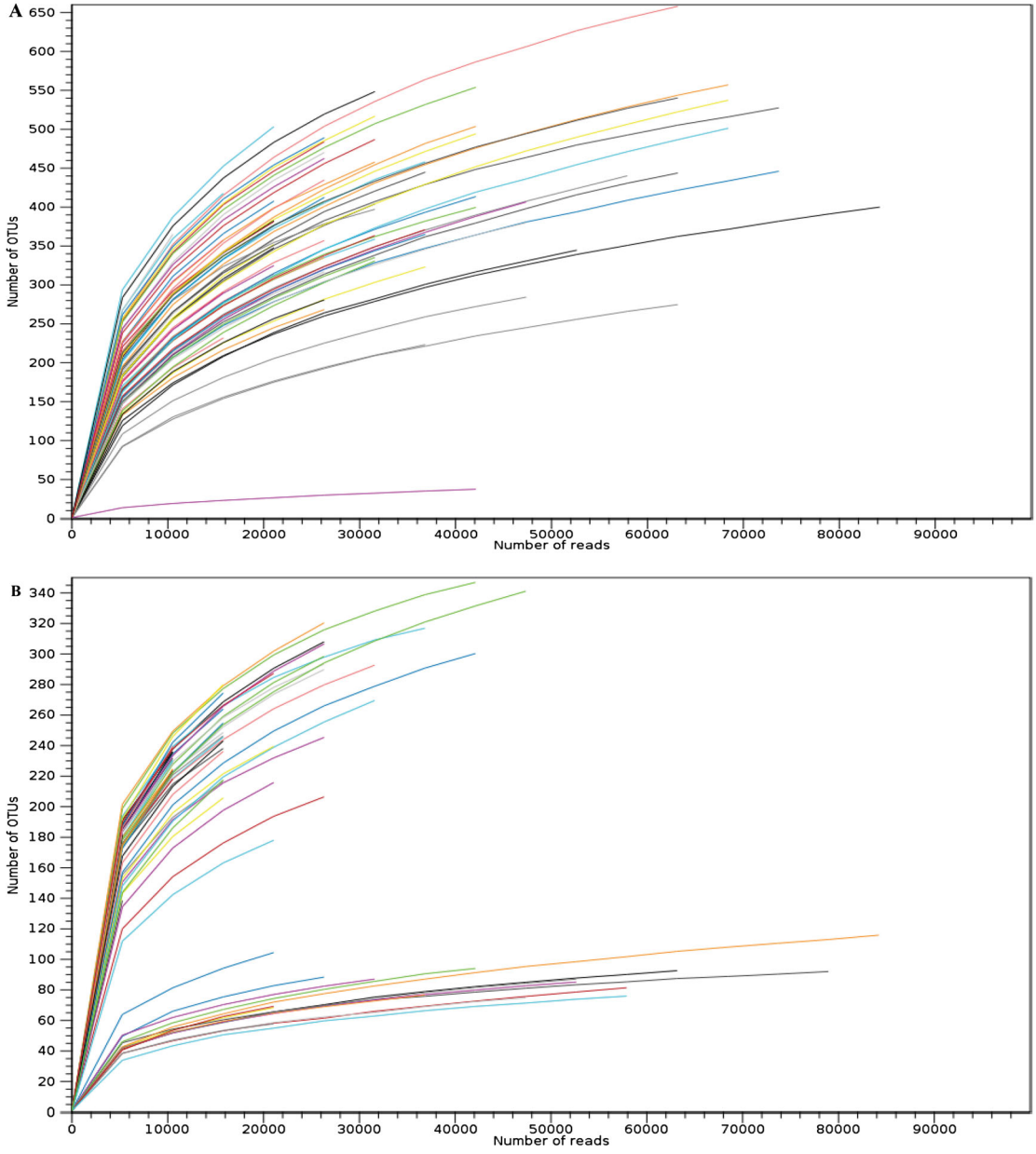

Figure 1. Rarefaction curves for (A) bacterial and (B) fungal datasets based on sequencing reads, describing the observed number of OTUs as a function of the sequencing reads per samples. Each color represents the sample ($n = 80$). Saturation of the curves represents the good coverage and quality of the data-sets.

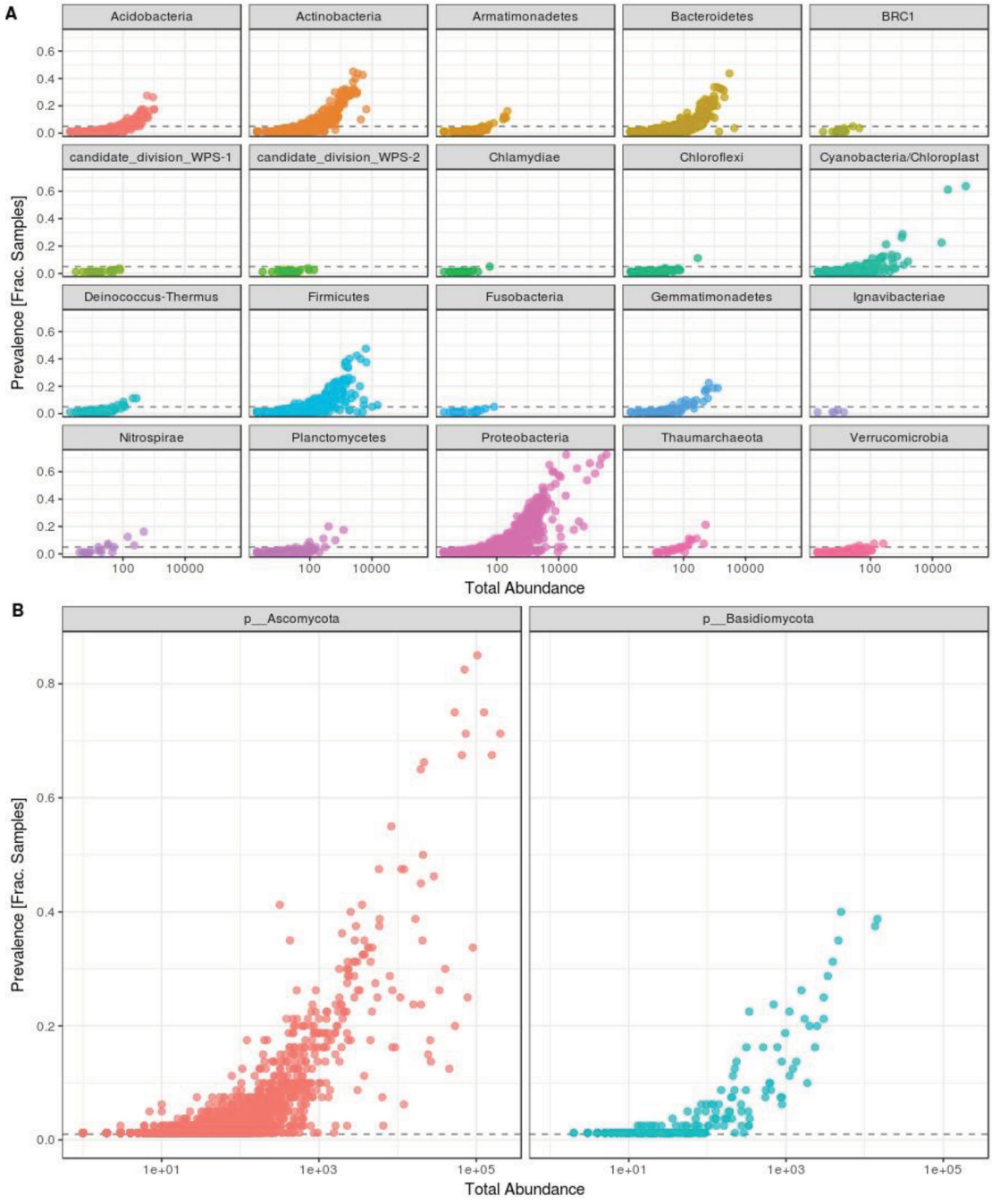

Figure 2. Prevalence plot (taxa prevalence versus total count) for (A) bacterial and (B) fungal taxa representing the phylum level diversity across samples. Each point corresponds to a different or unique taxon. The *y*-axis represents the fraction of samples, these taxa are present.

two sample types (or organ types) and three genetic pools, respectively. We identified 12,875 unique bacterial and 3,413 unique fungal OTUs, in our phyllosphere samples. After removal of unassigned taxa (genus level assignment) and singletons, 6017 unique bacterial and 2075 unique fungal OTUs belonging to 542 bacterial and 267 fungal genera were recovered. Phylum level classification of bacterial and fungal communities was also identified (Figure 2(A and B)) using the feature prevalence of entire16S and ITS data-sets, which is the number of samples in which a taxon appeared at least once. For example, the phylum *Ignavibacteriae* had only five unique taxa with the cumulative abundance of thirty-eight and its presence is observed in less than 10% of the samples. Bacterial and fungal communities were heavily dominated by phylum *Proteobacteria* (relative abundance $>$ 55%) and *Ascomycota* ($>$ 65%) respectively.

Effects of genetic diversity on microbial communities in the phyllosphere

Multiple testing on each of bacterial 6017 and fungal 2075 OTUs (with adjusted *p-values:adjp* and controlling false discovery rates) was performed according to cultivars and genetic pools and apart from two bacterial taxon (OTU1309, genus: *Gemmatimonas, adjp* = 0.0209, *FDR* = 0.06017 and OTU120, genus: *Hymenobacter*, *adjp* = 0.036, *FDR* = 0.05), and one fungal taxon (OTU63, genus: *Penicillium*, *adjp* = 0.02, *FDR* = 0.028), which were differentially abundant between WW, and WE, we did not recover any taxa whose abundance is significantly different (statistically) among the genetic pools.

Relative abundances for the twenty most abundant genera were plotted as well for each cultivar within their genetic pools (Figure 3(A and B)) and microbial genera were quite uniformly abundant among the three genetic pools. This pattern was also the same when we analyzed the abundances in leaf and berry samples individually within these three genetic pools (Figure 4(A and B)), except for few cultivar level differences (e.g. bacterial genus *Vagococcus* in the cultivars of TE and fungal genus *Pichia* in the cultivars of WW genetic pool). To test the association of these genera with genetic pools, we performed a linear regression for abundances of these genera against genetic pools (parametric test). As the *Pichia* also seems more abundant in the phyllosphere of berries (Figure 4(B)), we also added this as confounders to the regression and the results indicated a highly significant association of these genera to TE and WW genetic pools, respectively (Tables 1 and 2). We also observed that the abundance data for these genera were not normally distributed and therefore performed a nonparametric test (Wilcoxon rank sum test), that confirmed the association.

The Chao1 estimator of alpha diversity was also measured and plotted according to the genetic pools (Figure 3(C and D)) and again we did not observe a very significant genetic pool wise differences in these estimates (ANOVA, for 16S data: Chao1, $P = 0.033$; for ITS data: Chao1, $P = 0.041$).

Microbial community structure assemblages among the three genetic pools were also compared using PCoA to look for the genetic pool wise patterns of microbiota present in the phyllosphere. Taxa in both the PCoA plot (Figure 3(E and F)) were clustered together (PERMANOVA, for 16S data: at $F = 0.971$, $R^2 = 0.285$, $P = 0.408$; for ITS data: at $F = 0.991$, $R^2 = 0.172$, $P = 0.394$), which also indicated the impact of genetic diversity is less evident. Results were the same when PCoA was performed on the data-sets grouped within 27 grapevine cultivars (Supplementary data S1).

Effect of organs on phyllosphere microbiome

Multiple testing on taxa abundances in the phyllosphere of leaves and berries gave 17 bacterial and 33 fungal OTUs whose abundance was significantly different between these two organs. The data revealed the organ-specific patterns of phyllosphere microbiota in these grapevine cultivars. Tables 3 and 4 are provided for 16S and ITS data respectively to display various bacterial and fungal OTUs (along with their respective genera) with their false discovery rates (FDRs) and adjusted *p*-values. According to the corrected *p*-values and FDRs, 5 bacterial (e.g. *Pseudomonas* and *Pantoea*. adjusted *P*-value; *adjp* = 0.0038 & FDR = 0.00118) and 31 fungal genera (e.g. *Aspergillus* and *Mycosphaerella*. *adjp* = 0.0005 & FDR = 0.000129) were most differed between leaf and berries were, respectively.

Relative microbial abundances for top twenty taxa was also calculated on leaf and berry samples (also grouped in genetic pools; Figure 4(A and B)) and differential abundances on both sample type were clearly visible. Leaf phyllosphere was heavily occupied by bacterial and fungal genera of *Pseudomonas* and *Pantoea* & *Aureobasidium*, *Mycosphaerella*, respectively. On the other hand, berry surfaces mainly comprised of bacterial genera of *Acinetobacter* and *Sphingomonas* & with fungal genera of *Aureobasidium*, *Aspergillus* and *Pichia*.

To investigate the influence of leaves and berries, we also compared Chao1 estimates of alpha diversity between leaf and berry samples (Figure 4(C and D)) and these estimates were also significantly different (ANOVA, for 16S data: Chao1, $P = 0.007$; for ITS data: Chao1, $P = 4.53e\text{-}08$).

PCoA also indicated the same as it identified clear, separate clusters (Figure 4(E and F)) corresponding to both organs (PERMANOVA; for 16S data: at $F = 45.384$, $R^2 = 4.121$, $P = 0.001$; for ITS data: at $F = 48.306$, $R^2 = 2.539$, $P = 0.001$).

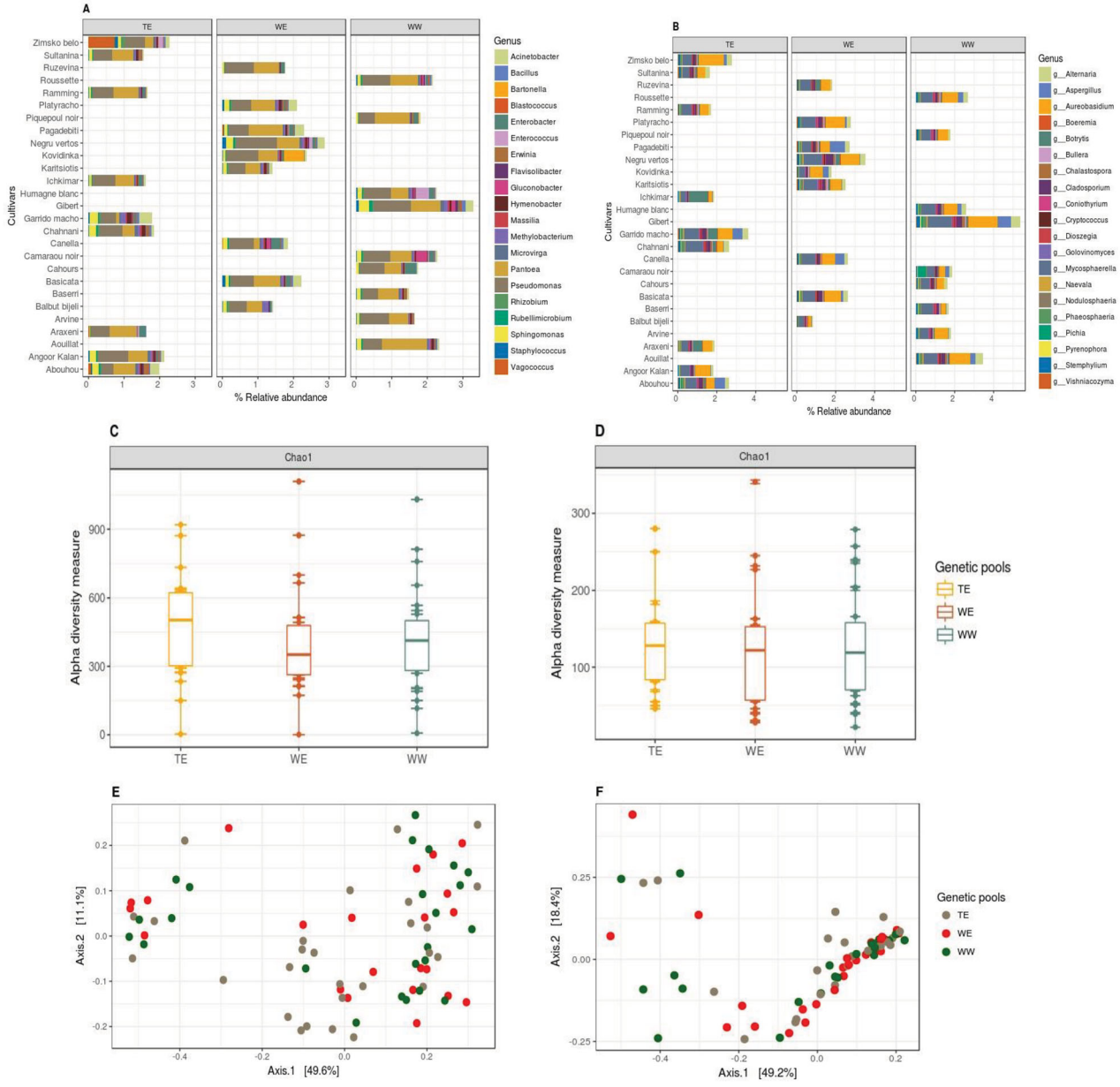

Figure 3. Relative abundances of (A) bacterial and (B) fungal genera present on each cultivar, grouped within their genetic pools (9 cultivars per genetic pool, top 20 taxa, characterized to the genus level and datasets were rarified to 5000 sequence reads per sample). Chao1 estimates of α-diversity for (C) bacterial and (D) fungal data-sets for three genetic pools. PCoA plots using Bray-Curtis distance between samples for (E) bacterial and (F) fungal data-sets among three genetic pools, explaining $>$ 60% variations with first two axes (taxa with variance $<$ 1e-05 were trimmed).

Discussion

Our analysis based on high throughput 16S and ITS profiling identifies the presence of complex microbiota in the phyllosphere of leaves and fruits (berries) of grapevine cultivars grown in our Mediterranean vineyard and it is dominated by bacterial genera of *Pseudomonas, Sphingomonas, Enterobacter* and the fungal genera of *Aureobasidium, Alternaria, Cladosporium*, respectively which is concordant with the findings of other grapevine related studies (Zarraonaindia et al. 2015; Zhang et al. 2017). High relative abundances of some other microbial genera such as *Pantoea* and *Mycosphaerella* have also been identified in this study, which has been reported in few grapevine cultivars before as endophytes (Bell et al. 1995; Baldan et al. 2015). This is not uncommon as epiphytes and endophytes are separated by a thin boundary between their habitats and due to vertical and horizontal microbial

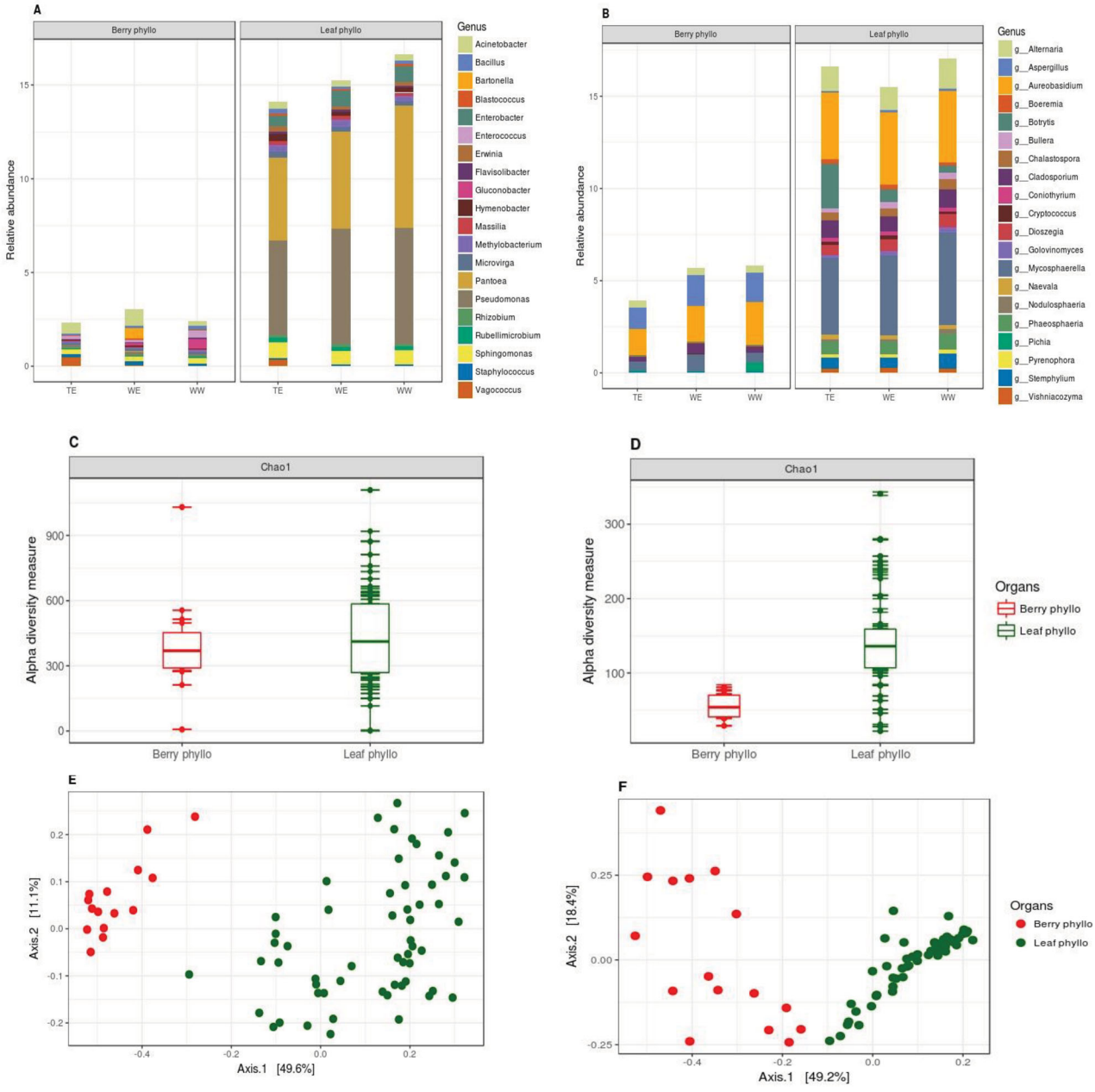

Figure 4. Relative abundances of (A) bacterial and (B) fungal genera present on leaf and berry samples, also grouped within their genetic pools (top 20 taxa, characterized to the genus level, datasets were rarified to 5000 sequence reads per sample). Chao1 estimates of α-diversity for (C) bacterial and (D) fungal data-sets for both the organ types. PCoA plots using Bray-Curtis distance between samples for (E) bacterial and (F) fungal data-sets as per leaf and berry samples based on Bray-Curtis distance matrices, explaining $>$ 60% variations with first two axes (taxa with variance $<$ 1e-05 were trimmed).

transfers (Frank et al. 2017) sharing of the major chunk of OTUs are inevitable (Bodenhausen et al. 2013). The bacterial genus *Vagococcus* has also not been widely reported in plants by research communities except rhizosphere and phyllosphere of Rice (Mwajita et al. 2013). Hence, the specific abundance of this genus must be further identified to have the preliminary view of its functionality in the Mediterranean vineyards. The reason for its abundance could be the interaction between plant genetic factors and environmental conditions at this specific geographic location of the vineyard. Epiphytes (the phyllosphere microbes) associated with grapevine have been suggested to originate from soil, but are distinct from those in the rhizosphere microbiome (Zarraonaindia et al. 2015); this is most likely a consequence of the physio-chemical composition and surrounding environment which strongly modulates microbial

Table 1. Summary of association tests for bacterial genus *Vagococcus* against three genetic pools.

Statistical tests	Genetic pools	*P-values*
Linear regression(Parametric test)	**TE**	**0.000985**
	WE	0.06004
	WW	0.125
Wilcoxon Rank Sum test(Non-parametric test)	**TE**	**0.0008407**
	WE	0.06324
	WW	0.1072

Table 2. Summary of association tests for fungal genus *Pichia* against three genetic pools.

Statistical tests	Genetic Pools	*P-values*
Linear regression(Parametric test)	TE	0.1932
	WE	0.2294
	WW	**0.01239**
Wilcoxon Rank Sum test(Non-parametric test)	TE	0.0732
	WE	0.3316
	WW	**0.005286**

Table 3. Bacterial OTUs with differential abundance between leaf and berry samples with their respective genera, *adjusted p-values*, and *FDRs*.

OTUs	Genus	adj *p*-values	FDRs
OTU5	*Pseudomonas*	0.0038	0.00118
OTU1	*Pantoea*	0.0038	0.00118
OTU25	*Erwinia*	0.0038	0.00118
OTU12	*Enterobacter*	0.0038	0.00118
OTU70	*Gluconobacter*	0.0038	0.00118
OTU81	*Cloacibacterium*	0.007	0.001475
OTU184	*Comamonas*	0.007	0.001475
OTU112	*Carnobacterium*	0.007	0.001475
OTU150	*Bacillus*	0.0104	0.001475
OTU389	*Stenotrophomonas*	0.0104	0.001475
OTU44	*Staphylococcus*	0.0104	0.001475
OTU149	*Glucononacetobacter*	0.0104	0.001475
OTU136	*Duganella*	0.0205	0.00317
OTU37	*Orbus*	0.0259	0.00379
OTU16	*Acinetobacter*	0.0273	0.00393
OTU92	*Nocardioides*	0.0355	0.00479
OTU29	*Bartonella*	0.0405	0.00520

Table 4. Fungal OTUs with differential abundance between leaf and berry samples with their respective genera, *adjusted p-values,* and *FDRs.*

OTUs	Genus	adj *p*-values	FDRs
OTU29	*Phaeosphaeria*	0.0005	0.000129
OTU27	*Dioszegia*	0.0005	0.000129
OTU20	*Stemphylium*	0.0005	0.000129
OTU46	*Nodulosphaeria*	0.0005	0.000129
OTU78	*Golovinomyces*	0.0005	0.000129
OTU51	*Pyrenophora*	0.0005	0.000129
OTU36	*Coniothyrium*	0.0005	0.000129
OTU81	*Ramularia*	0.0005	0.000129
OTU24	*Chalastospora*	0.0005	0.000129
OTU54	*Naevala*	0.0005	0.000129
OTU53	*Bullera*	0.0005	0.000129
OTU45	*Vishniacozyma*	0.0005	0.000129
OTU124	*Blumera*	0.0005	0.000129
OTU59	*Cryptovalsa*	0.0005	0.000129
OTU71	*Lachnum*	0.0005	0.000129
OTU57	*Hormonema*	0.0005	0.000129
OTU43	*Boeremia*	0.0005	0.000129
OTU91	*Cryptococcus*	0.0005	0.000129
OTU111	*Phoma*	0.0005	0.000129
OTU88	*Sydowia*	0.0005	0.000129
OTU2	*Mycosphaerella*	0.0005	0.000129
OTU95	*Angustimassarina*	0.0005	0.000129
OTU5	*Aspergillus*	0.0005	0.000129
OTU117	*Sigarispora*	0.0005	0.000129
OTU89	*Diplodia*	0.0005	0.000129
OTU126	*Hortaea*	0.0005	0.000129
OTU15	*Botrytis*	0.0005	0.000129
OTU275	*Diaporthe*	0.0005	0.000129
OTU96	*Acaromyces*	0.0005	0.000129
OTU120	*Candida*	0.0005	0.000129
OTU18	*Pichia*	0.0005	0.000129
OTU84	*Metschnikowia*	0.001	0.00025
OTU1	*Aureobasidium*	0.0195	0.00606

community structure and its dynamics (Wagner et al. 2016). Other reports also evidenced that both environment and the plant genotype could be the major drivers for epiphytic community structuring (Redford et al. 2010; Turner et al. 2013).

Our preliminary study based on random sampling of 27 cultivars (from three genetic pools) also indicated that there is probably an impact of grapevine genetic diversity over the microbial composition in the phyllosphere, but it is not quite evident as we found only a few microbial OTUs were differentially abundant among genetic pools. Sampling from cultivars (among these genetic pools) which are more distant in the context of their genetic relatedness must be done in the future to further explore the impact of this genetic diversity. Few genera were also found specifically associated with some cultivars of certain genetic pools (e.g. *Vagococcus* with the genetic pool TE) and this association (if further confirmed with above mentioned sampling strategies), should be taken into account in developing new selective breeding strategies in order to have the putative beneficial role of the phyllosphere as a performance trait of the cultivars. Moreover, environmental control in shaping microbiome in these genetically diverse grapevine cultivars should also be further investigated by sampling at different geographic locations displaying variable climatic conditions.

On the other hand, leaf and berry samples clearly displayed very distinct microbial patterns. Type of genera present and their taxa abundances was significantly different in both the organs. The physical features of berry surface like the number of waxy layers (or bloom, which prevent water loss from the skin) and their thicknesses are cultivar-specific (Knoche and Lang 2017). These physical features could influence

the contact and permeability of the grape berry cuticle to different microorganisms as observed for some pathogens, such as *Botrytis cinerea* (Herzog et al. 2015) and could be the reason for organ-specific microbiome differences and deserve further investigations.

This finding is also consistent with the few other findings, in which organ-specific microbial patterns have been reported in sugarcane (de Souza et al. 2016) and in some commercially important grapevine cultivars (Bokulich et al. 2014). Our leaf samples were majorly occupied by the bacterial and fungal genus *Pseudomonas*, *Pantoea* and *Sphingomonas* & *Aureobasidium*, *Mycosphaerella* and *Cladosporium*. At the other end, berry surfaces displayed higher abundances of bacterial genus like *Acinetobacter*, *Gluconobacter*, *Enterobacter*, but major fungal abundances were similar in both leaf and fruit surfaces except the genus of *Aspergillus* and *Pichia*. *Pichia* (a yeast, family *Saccharomycetaceae*) was also found specifically abundant in berries of grapevine cultivars of the genetic pool WW. Taxonomy of *Pichia* is not fully resolved, and thus, a large diversity of roles in winemaking may be expected within this genus with some species inducing potential faults in winemaking (Fugelsang and Edwards 2010). Therefore the information regarding its association with certain genotypes should further be investigated in the context of wine fermentation.

A richly diverse fungal component of the grapevine microbiome has also been uncovered in this work and it could also be particularly significant because there is not sufficient information on the potential risks or benefits of plant-fungi associations. Grapevine associated microbial communities are relevant to industrial fermentation processes for wine production. Based on our results it can be assumed that grape juice used for wine production harbors a diverse bacterial and fungal community originating from its phyllosphere as well (e.g. *Pantoea* and *Aspergillus*).

Some species of most abundant microbial genera we found (e.g. *Pseudomonas* and *Mycosphaerella*) have been previously reported for acting as biocontrol agents or BCAs (Kurose et al. 2016; Jousset et al. 2006). An interesting question would be to evaluate how to integrate microbial community studies into traditional biocontrol approaches? This integration could provide a better understanding of how microbial communities are interacting with each other, with the host plant, pathogen or with BCAs, which would be definitely helpful for designing a novel biocontrol method. This also suggests that there is an open field for further studies of the possible role of bacterial and fungal colonizers in plant growth, development and response to biotic and abiotic stress.

A whole-genome shotgun sequencing followed by metagenomic analysis (Qin et al 2010) can add a more detailed layer of information to the taxonomical characterization of a wide variety of grapevine samples, by generating information on the gene composition of the bacteria and fungi present. This information can, in turn, be used to discover new genes and to formulate putative functional pathways and modules, thus could provide insight into functional and genetic microbiome variability. Apart from metagenomics, the use of additional tools such as RNA-Seq (for meta-transcriptomics) may offer a more informative perspective as it can reveal details about populations that are transcriptionally active and not just identify the taxa and genetic content of microbial populations. Moreover, the integration of different omic approaches (e.g. meta-transcriptomics & meta-proteomics) may open a window into discovering the regulatory mechanisms orchestrating observed gene expressions, thereby uncovering how host-microbe and microbe-microbe interactions that regulate microbiome activity.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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CHAPTER 3

Genotype-Environment Interaction Shapes the Microbial Assemblage in Grapevine's Phyllosphere and Carposphere: An NGS Approach

CHAPTER SUMMARY:

- Published Article
- Provides genus level characterization of the grapevine's phyllosphere microbiome
- Describes relative selection pressure exerted by genotype and environment to shape phyllosphere microbiome and establishes the major driver
- Discusses the impacts of season and plant organs in shaping microbial structure in the phyllosphere

microorganisms

MDPI

Article

Genotype-Environment Interaction Shapes the Microbial Assemblage in Grapevine's Phyllosphere and Carposphere: An NGS Approach

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Abstract: Plant surface or phyllosphere is the habitat of hyperdiverse microbial communities and it is always exposed to the fluctuating environmental factors, which is thought to be one of the potential drivers of microbial community structuring. Impact of grapevine genotypes in variable environmental factors (i.e., at different geographic locations) on the phyllosphere has never been studied and is the main objective of this report. Using high throughput short amplicon sequencing of 16S rRNA genes and internal transcribed spacer (ITS), we analyzed the impacts of genotypes of *Vitis Vinifera* (coming from three genetic pool), on the microbial (bacterial and fungal) assemblage in the phyllosphere. First, we performed the analysis of the phyllosphere microbiome while using fifteen genotypes that were chosen to maximize intra-specific diversity and grown in two Mediterranean vineyards. Then, the same analysis was performed on five commercially important varieties of *Vitis vinifera* that were sampled from three different French agro-climatic zones (or terroir: a combination of climate, soils, and human practices). Our study revealed that, at a particular geographic location, genotypes have an impact on microbial assemblage in the phyllosphere and carposphere of leaf and fruit (or berries), respectively, which is more prominent on the carposphere but the effect of terroir was much stronger than the genotype when the leaf phyllosphere of five grapevine varieties grown in different agro-climatic zones was compared. Impacts of the season and exterior plant organs (leaf and berries) on microbial taxa structuring in the phyllosphere was also assessed and presented in this report.

Keywords: agro-climate zones; genotype; grapevine; microbiome; phyllosphere; PMCs; terroir

1. Introduction

The phyllosphere consists of the aerial parts of the plant and it is one of the most prevalent microbial habitats on earth [1]. Its heterogeneous environment harbors a myriad of microorganisms, like yeast, bacteria, and filamentous fungi and many uncultured organisms [1,2]. The phyllosphere or carposphere microbial communities (PMCs) live at the plant-climate interface and its ability to establish, thrive and reproduce on the leaf or fruit surface depends on several microbial functional traits, such as the ability to attach to the cuticle and to use the foliar nutrients, as well as to the prevailing climatic conditions, like temperature, air humidity, and rain [3–5]. Leaf or fruit chemistry, physiology, and morphological structure differ among plant genotype or species, and as all of these traits have a genetic basis, these variations may lead to a different combination of PMCs among plant genotypes [6,7].

The plant genotype may exert selection pressure on PMCs, as often reported in A. thaliana [7,8]. In the literature, impacts of climatic stressors have received much more attention, especially on soil

communities than on the PMCs. Nevertheless, phyllosphere faces constant direct exposure to the outside conditions and available pieces of evidence suggest that PMCs significantly alters in response to the climatic stressors like heat, rain or drought [9–12]. Air pollutants (e.g., oxides of nitrogen and sulfur and particulate matters) that are produced by human activities can alter foliar traits, including cuticle properties [13], leaf chemistry, and phenology [14,15] may also affect the structure of PMCs. Moreover, some of the pollutants can be used as a carbon source by PMCs [16].

The PMCs that are associated with *Vitis vinifera* L., the major crop for fruit and wine production in the world, is less extensively studied when compared to the other habitats (e.g., soil, rhizosphere, and endosphere), especially in relation with the genotypes and the variable climatic conditions or geographic locations. One study suggested that the leaf PMCs are minimally affected by the chemical and biological treatments tested on the plant, but mainly differed according to the grapevine location [17,18]. Berry surfaces also exhibit a huge bacterial and fungal diversity and that can have an impact on grapevine health and wine qualities [19].

In this study, we assessed both the effect of grapevine genotype and environmental factors on the diversity and structure of phyllosphere and carposphere microbiome. When considering that the PMCs on leaf and berry surface plays a crucial role in plant health and fitness as it can modulate leaf or fruit susceptibility to infection [19–21], this study could bring new insights to develop innovative and natural biocontrol methods or phytostimulators against grapevine pathogens or rethink breeding schemes for the creation of innovative resistant varieties.

2. Materials and Methods

2.1. Sample Preparation for PMCs and DNA Extraction

Samples were collected in two sets. In Set1, A total of 279 grapevine cultivars was grown in two vineyards, Chapitre (Supagro field station, Villeneuve-les-Maguelone, Hérault, France) and Vassal (INRA Experimental Unit, Marseillan-Plage, France) near Montpellier (French Mediterranean region). A panel of cultivars representing three genetic pools (western Europe, WW; from eastern Europe, WE; and table grape, TE) was constructed for genome-wide association studies while minimizing relatedness and retaining the main founders of modern cultivated grapevine to optimize the genetic diversity [22]. Five cultivars from each genetic pool, which are far apart based on their distances on PCoA map shown by Nicolas et al. 2016 [22], were selected (Table 1) to maximize the distance between genetic pools. Leaf or berry samples were taken from four to five plants of each cultivar at Spring season (mid of May 2017, before spraying of the fungicides) and harvesting season of (September 2017). Berries were also collected from eleven of these varieties during the harvest season.

Table 1. Schematic representation of 15 grapevine cultivars (grouped in three genetic pools) that were sampled in Set1.

	Genetic Pools		
Cultivars of *Vitis Vinifera*	**WW**	**WE**	**TE**
	Donzelinho	Basicata	Ichkimar
	Petit Verdot	Negru Vertos	Khoussaïné blanc
	Camaraou Noir	Alba Imputotato	Sourkhak Biley
	Courbu	Gros Bourgogne	Abouhu
	Savagnin Blanc	Koilliniatico	Dabouki

In Set2, leaf samples from five commercially important varieties (Cabernet Sauvignon, Chardonnay, Syrah, Grenache, Sauvignon Blanc) were taken from three different geographic locations, (INRA field stations from Bordeaux, Montpellier, and Colmar) within France, representing the three agro-climate zones (Oceanic, Mediterranean, and Continental) of France or different terroirs at the mid of spring season (before spraying of fungicides).

All the samples from both of the sets were washed with an isotonic solution of sodium chloride (0.15 M) containing 0.01% Tween 20 in 50 mL propylene tubes (2–3 leaves and 50–80 g of berries were washed per tube) while using a horizontal shaker for 1h at 100 RPM. Afterward, samples were given an ultrasonic bath for 7–10 min while using Ultrasonic Cleaner (Branson 5510, Marshall Scientific, Hampton, NH, USA) for maximum recovery of microbes from the sample surface. The remaining solution was centrifuged at 4000×g and microbial pellets containing PMCs were transferred in a 2 mL Eppendorf tube and were collected and stored at −20 °C. PMCs from two of these tubes were mixed to make one biological replicate of a single variety and a total of three biological replicates were made for each variety per vineyard. DNA was extracted from each sample by using the ZymoBiomics DNA MicroPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions.

2.2. DNA Amplification and Amplicon Sequence Library Preparation

To access bacterial communities, the V4 region of the 16S ribosomal gene was amplified using primers 515F and 806R and fungal community diversity and abundance were accessed using modified ITS9 and ITS4 primers targeting the ITS2 region [23,24]. Two-step PCR was performed to prepare sequencing libraries. PCR1 was designed to perform amplification of the target regions and to add Illumina Nextera transposase sequence to the amplicons. Both forward and reverse primers for PCR1 were amended with frameshift (FS) sequences in their 5′ overhang to improve sequence diversity and overall read quality [25]. PCR1 was performed in 25 µL reactions with 30 ng of sample DNA while using the KAPA HiFi HotStart (KAPA Biosystems, Wilmington, MA, USA) PCR mix (Initial denaturing at 95 °C followed by 30 cycles of denaturing at 95 °C for 30 s, primer annealing at 57 °C for 60 s, and primer extension at 68 °C for 60 s). Amplicons were purified while using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) at a bead-to-DNA ratio of 0.7:1, resuspended in 30 µL MilliQ water, and evaluated in agarose gels. In PCR2, Primers from Illumina kit for dual indexing of the amplicons was used. Each cleaned PCR1 product within the same sample received a unique combination of forward and reverse primers (respectively, N7 and S5 Illumina dual index oligos). Afterward, samples were again cleaned while using AmPure XP magnetic beads, pooled in equimolar concentrations, and sequenced using 2×250 bp MiSeq v2 sequencing (Illumina Inc., San Diego, CA, USA).

2.3. Data Processing and Analysis

Demultiplexed RAW data files covering all of the samples were imported into the R-environment, (R Core Team, Vienna, Austria). The entire amplicon sequences data was uploaded to the institutional server (http://agap-ng6.supagro.inra.fr/inra). Paired forward and reverse reads from raw data files were trimmed (primer removal) and filtered (base quality) while using the fastqPairedFilter function of the *dada2* package [26] and bases with low-quality scores (<11) were discarded. These filtered files were then processed using Divisive Amplicon Denoising Algorithm (DADA) pipeline which included the steps of dereplication, core denoising algorithm (that models and corrects Illumina-sequenced amplicon errors) and the merging of the base pairs. Merging function provided global ends-free alignment between paired forward and reverse reads and merged them together if they overlapped exactly and a table for amplicon sequence variants (ASVs, a higher analog of operational taxonomic units—OTUs) was constructed. It records the number of times each amplicon sequence variant is observed in each sample. DADA infers sample sequences exactly and resolves differences of as little as one nucleotide [26]. Chimeras were removed using the removeBimeraDenovo function of the same *dada2* package (Table 2 represents the total number of reads available during these steps). ASVs or OTU sequences were assigned a taxonomy using the RDP classifier [27,28] with k-mer size 8 and 100 bootstrap replicates. Afterward, a phyloseq data object was created using phyloseq function of the *phyloseq* package in R [29]. Unassigned taxa and singletons were removed and this data object was then used to calculate microbial abundances, α, β diversity analysis and for other statistical tests using various functions in the *phyloseq* and *vegan* packages [29,30].

Estimates of observed α-diversity [31] were measured within sample categories using estimate_richness function of the *phyloseq* package. Relative abundances of microbial genera and phylum were plotted using the *ggplot2* package [32] after transforming abundance data into relative abundances. Multidimensional scaling (MDS, also known as principal coordinate analysis; PCoA) was performed while using the Bray-Curtis dissimilarity matrix between samples and visualized by using their base functions in the *phyloseq* package.

2.4. Statistical Analysis

We analyzed all of the amplicon sequences in R version 3.3.4 using above mentioned Bioconductor packages. CRAN packages *plyr* and *ggplot2* [32,33] were also used to draw the figures. We assessed the statistical significance ($p < 0.05$) throughout and whenever necessary, we adjusted *p*-values for multiple comparisons according to the Benjamini and Hochberg method to control False Discovery Rate [34], while performing multiple testing on taxa abundance according to sample categories. We performed an analysis of variance or ANOVA [35] among sample categories while measuring the *Observed* estimates of α-diversity (richness of unique OTUs). Stratified permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted on all principal coordinates that were obtained during PCoA with the adonis function of the *vegan* package, to observe the statistical significance of clusters according to the sample categories.

3. Results

16S and ITS Amplicon sequencing of all the samples from both sets gave millions of reads. Table 2 describes the total number of reads that were obtained during the processing steps. 30–35% of the reads were trimmed due to the filtering parameters and chimera removal in both 16S and ITS datasets.

A total of 13521 + 4581 bacterial and 10162 + 3164 fungal OTUs were recovered from 213 + 45 samples of both sets (Table 2) and after phylum level assignment 9516 + 3755 bacterial and 6749 + 1800 fungal OTUs were retained and used for further analysis.

Table 2. Total number of reads during each step of microbiome data (16S/ITS) analysis.

Data	Number of Samples	Input Reads	Filtered Reads	Denoised and Merged	OTUs
16S data					
Set1	213	16113978	10874688	7795650	13521
Set2	45	7460569	5294234	3866297	4581
ITS data					
Set1	213	14780926	13600570	9900482	10162
Set2	45	6683219	4564572	2450315	3164

3.1. Seasonal Shifts in Leaf Microbiome Structure

PCoA analysis on leaf data (from spring and harvest season) showed fluctuation in taxonomic structuring (Figure 1A,B) between two seasons (PERMANOVA for 16S data: at $F = 5.285$, $p < 0.001$; for ITS data: at $F = 99.057$, $p < 0.001$), but the *Observed* α-diversity estimates (Figure 1C, for bacterial data) indicated that the richness for unique bacterial OTUs did not change between seasons (ANOVA, at $F = 2.973$, $p > 0.085$). On the contrary, *Observed* α-diversity estimates for fungal data (Figure 1D) displayed significant differences in richness of unique fungal OTUs (ANOVA, at $F = 47.958$, $p < 1.2 \times 10^{-10}$). In combination, our results indicated a compositional dissimilarity for bacterial populations between two seasons, but the uniqueness of the composition (or bacterial diversity) did not change, which was further confirmed by the relative abundance analysis (Figure 1E). From spring to harvest season, leaf microbiota loose significant amount of Cyanobacteria (79.5%) and gained an ample amount of Proteobacteria (28%), which was probably the cause of the seasonal drift obtained. On the other hand, there was a strong impact of season on fungal composition as well as diversity (Figure 1F) in the phyllosphere, which was more evident at the genus or species level (Supplementary Figure S1).

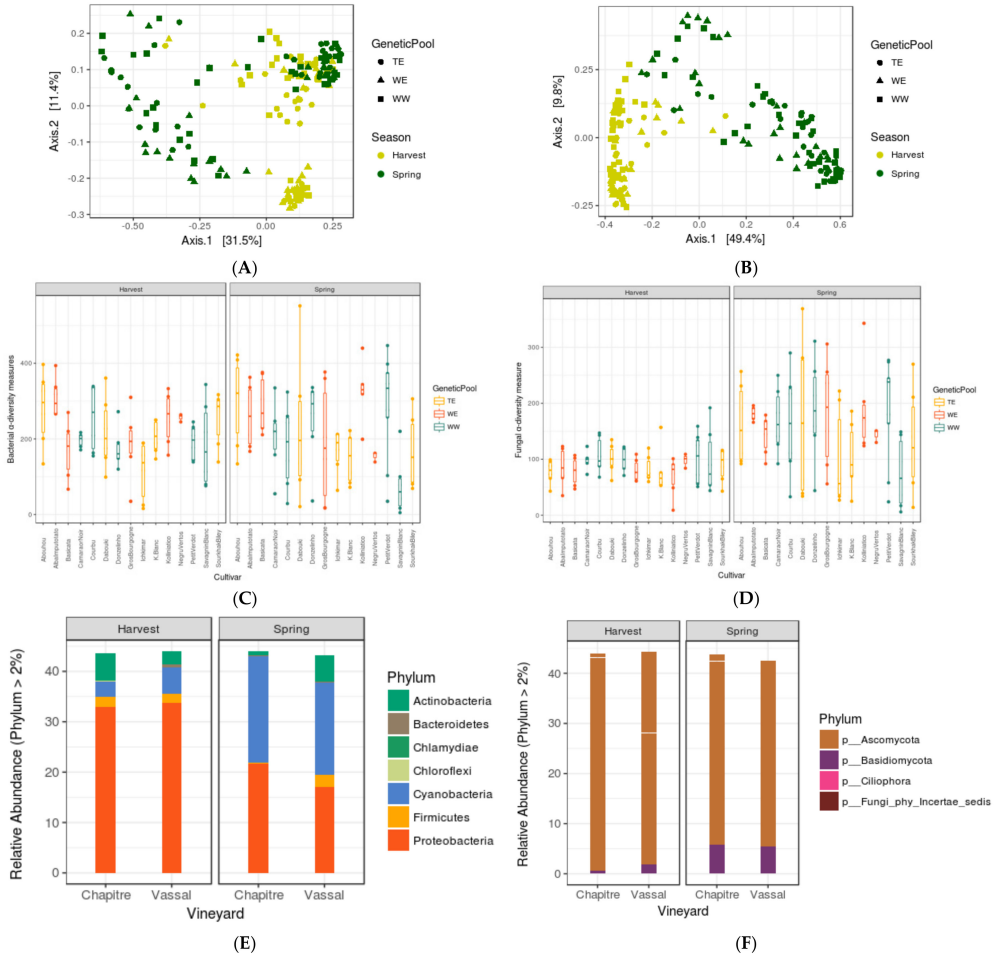


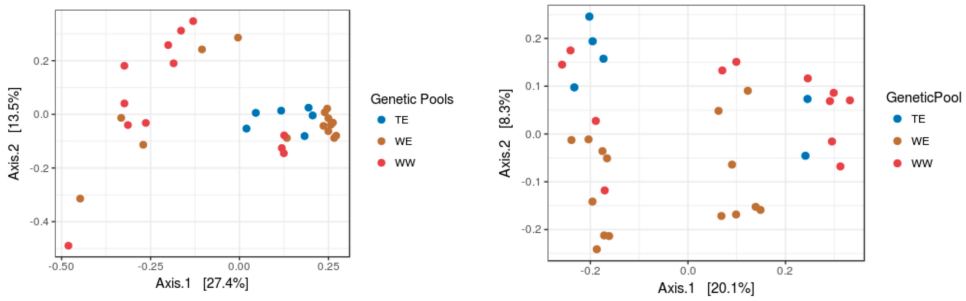
Figure 1. PCoA analysis on leaf data (**A**,**B**); PCoA plots representing the compositional dissimilarity in leaf communities (both axis covered >40% of the variation) and *Observed* (**C**) bacterial and (**D**) fungal α-diversity measures of each variety (X-axis) grouped in two season and relative abundances of (**E**) bacterial and (**F**) fungal Phylum during spring and harvest season. $n = 180$.

3.2. Assessing the Impacts of Grapevine Cultivars and Genetic Pools

By PCoA analysis on leaf microbiome data over two seasons, we did not observe genetic pool wise variation (Figure 1A,B Shape represent genetic pools) on taxonomic structuring (PERMANOVA, at $F = 2.018$, $p = 0.083$) in the phyllosphere. However, at each individual season, we observed some significant differences in α-diversity measures (Figure 1C,D) and PCoA clusters, according to grapevine cultivars and genetic pools (Table 3). On the other hand, PCoA analysis of berry microbiome data displayed stronger effects (Figure 2A,B) of both the factors (Table 4) on PMC structuring.

3.3. Impact of Organs

Comparisons of PMCs on leaves and berries (samples from Set1, collected at harvest season, Figure 3 revealed a very clear differentiation of microbiome communities on both organs. PCoA revealed a clear difference in taxonomic structuring (Figure 3A,B; PERMANOVA for 16S data: $F = 14.6$, $p = 0.001$; for ITS data: $F = 45.738$, $p = 0.001$), while the α-diversity estimates displayed very significant differences in OTU richness (Figure 3C,D) between the leaves and berries (ANOVA for 16S data: $F = 7.17$, $p = 6.95 \times 10^{-14}$; for ITS data: $F = 4.575$, $p = 0.000143$), multiple testing on taxa abundance between the two organs revealed 20 bacterial and 26 fungal genera, differentially abundant (Supplementary Table S1).



(**A**) (**B**)

Figure 2. PCoA on (**A**) bacterial and (**B**) fungal microbiome data of berry displaying the impact of genetic pools on taxa structuring on the surface (both axis covered >25% of the variation in data). $n = 33$.

Table 3. Factors predicting the impacts of grapevine varieties and genetic pools on the leaf bacterial communities at each season.

	Spring		Harvest	
Factors	**ANOVA (on α-Diversity Measures)**	**PERMANOVA on PCoA Clusters**	**ANOVA (on α-Diversity Measures)**	**PERMANOVA on PCoA Clusters**
16S data				
Cultivars	$F = 2.361$, $p = 0.0009$	$F = 1.129$, $p = 0.002$	$F = 2.837$, $p = 0.002$	$F = 2.737$, $p = 0.001$
Genetic Pool	$F = 1.54$, $p = 0.221$	$F = 1.178$, $p = 0.082$	$F = 1.189$, $p = 0.308$	$F = 2.617$, $p = 0.001$
ITS data				
Cultivars	$F = 1.17$, $p = 0.315$	$F = 1.583$, $p = 0.006$	$F = 0.752$, $p = 0.715$	$F = 2.098$, $p = 0.001$
Genetic Pool	$F = 1.384$, $p = 0.255$	$F = 2.218$, $p = 0.015$	$F = 3.368$, $p = 0.038$	$F = 2.764$, $p = 0.001$

Table 4. Factors predicting the impacts of grapevine varieties and genetic pools on bacterial communities on berry surface at Harvest season.

Factors	**ANOVA (on α-Diversity Measures)**	**PERMANOVA on PCoA Clusters**
16S data		
Cultivars	$F = 2.546$, $p = 0.002$	$F = 2.598$, $p = 0.001$
Genetic Pool	$F = 4.261$, $p = 0.023$	$F = 4.612$, $p = 0.001$
ITS data		
Cultivars	$F = 4.575$, $p = 0.00142$	$F = 3.169$, $p = 0.001$
Genetic Pool	$F = 2.739$, $p = 0.07$	$F = 4.612$, $p = 0.003$

3.4. Impact of Agro-Climate Zones (or Terroir) and Genotype

Analysis of the microbiome of leaf phyllosphere on the 5 grapevine cultivars of set2 in the three very diverse French regions revealed a strong effect of terroir. A very clear differentiation of the samples collected in the three regions was observed on PCoA plots for bacterial (Figure 4A,B). Leaf PMCs for the five cultivars indeed clustered only according to grapevine locations (PERMANOVA for 16S data: $F = 12.98$, $p = 0.001$; for ITS data: $F = 6.094$, $p = 0.001$). The α-diversity estimates also indicated very significant differences in OTU richness (Figure 4C,D) between the three regions (ANOVA for 16S data: $F = 25.73$, $p = 3.11 \times 10^{-7}$; for ITS data: at $F = 26.329$, $p = 2.5 \times 10^{-7}$). In combination, these results illustrated that French agro-climatic zones have very strong impacts in shaping the microbial assembly in the leaf phyllosphere. In addition, it has also suggested that there is not only a region-wise difference in taxonomic compositions, but each region (or agro-climate zone) has a unique microbial signature (Figure 4E,F). Multiple testing (with corrected p-values to control false discovery rates) on taxa abundance gave 31 bacterial and 21 fungal genera, which were differentially abundant among these three regions representing different environment (Supplementary Table S2).

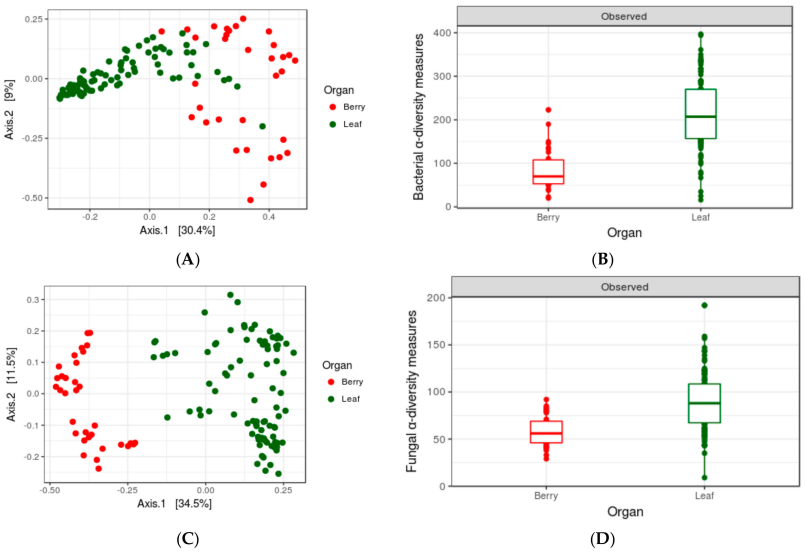


Figure 3. PCoA plot representing compositional dissimilarity for (**A**) bacterial and (**B**) fungal population between leaf and berry samples (both axis covered ~40% of the variation) and *Observed* α-diversity measures for (**C**) bacteria and (**D**) fungi for two organs. n = 123.

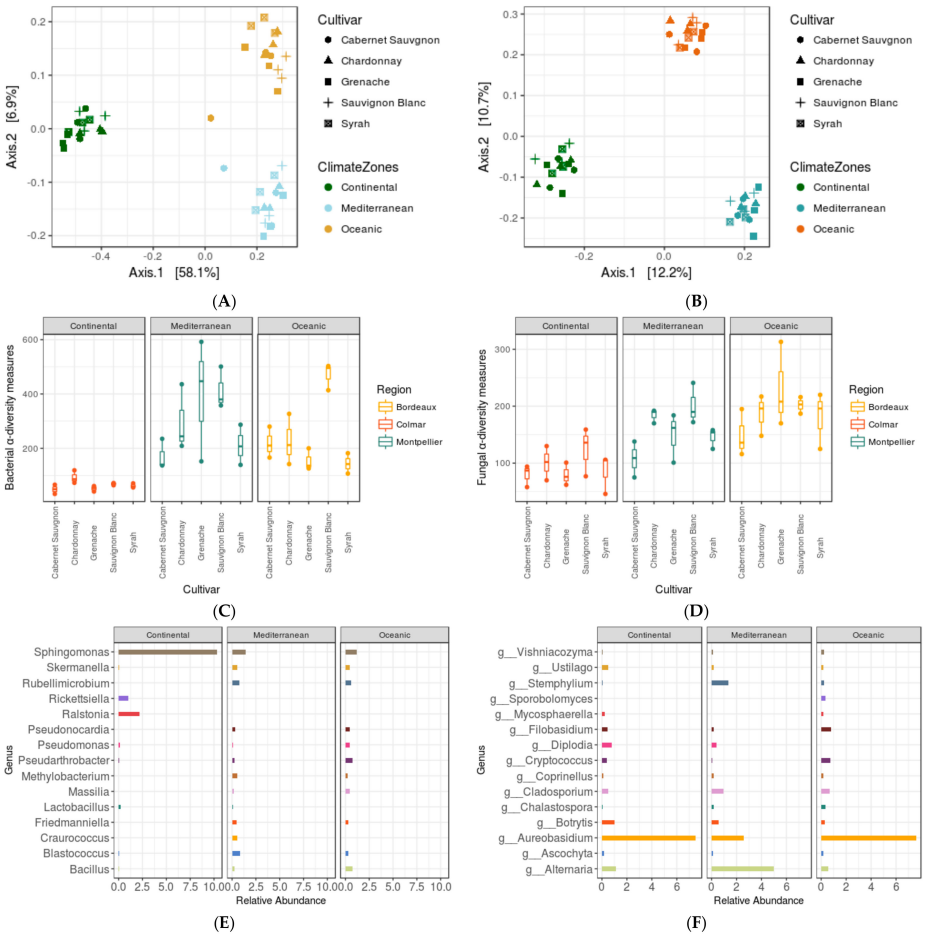


Figure 4. Set2 microbiome data. PCoA plots displaying strong (**A**) bacterial and (**B**) fungal compositional dissimilarity among agro-climate zones and *Observed* (**C**) bacterial and (**D**) fungal α-diversity measures of each variety (X-axis) grouped in three agro-climate zones and relative abundance plot for (**E**) bacterial and (**F**) fungal genera displaying differential abundance of few genera among three agro-climate zones (or region). n = 45.

A lower but significant cultivar level differences on Observed α-diversity estimates (ANOVA for 16S data: $F = 7.18$, $p = 0.00022$; for ITS data: $F = 3.798$, $p = 0.013$) was however also observed (Figure 4C,D). Within a specified region, genotype had also an effect on the diversity in both microbial and fungal communities even if PCoA analysis did not reveal any differentiation according to the cultivar (PERMANOVA for 16S data: $F = 0.893$, $p = 0.675$; for ITS data: $F = 1.171$, $p = 0.851$).

4. Discussion

Phyllosphere of the grapevines is quite a neglected milieu and many questions related to this microbial habitat are still unanswered, especially the relative impacts of potential factors that could play key roles in shaping the microbial community structure in the phyllosphere. A better understanding of the principal factors affecting community structure and multitrophic interactions in the phyllosphere will be the key to develop new strategies for grapevine protection. The better we understand the role of these stressors and PMCs that they affect, the better we would be able to predict and protect grapevine against pathogen infection.

In this study, we first explored the microbial communities present in the Mediterranean, Continental and Oceanic vineyards. Major bacterial and fungal taxa (at genus level) were *Pseudomonas*, *Sphingomonas*, *Pantoea*, *Skermanella* & *Aureobasidium*, *Filobasidium*, *Alternaria*, and *Stemphylium*, respectively. Differences in relative abundances of major taxa were quite visible according to agro-climate zones (or growing region) as compared to cultivars (grouped in three genetic pool), growing in the Mediterranean (Supplementary Figures S2 and S3). We mainly investigated the impacts of grapevine genotypes (or cultivars) and of terroir on the assemblage of PMCs using a culture-independent method. In the Mediterranean vineyards, grapevine cultivars, and their genetic pools had a significant impact on leaf and berry microbiome and the impact is stronger on the berry surface. Assuming that the PMCs on berries would also be present on wine must this result is in line with reports, suggesting that the microbiota exhibits varietal level differences in wine musts of Chardonnay and Cabernet Sauvignon [36,37].

While comparing the impacts of climatic stressors and cultivars at three different locations, we observed a very strong impact of French agro-climate zones or terroirs. Although the impact of genetic factors was significant but much lower in comparison with terroir, which suggests that genotype-by-environment interactions contributed to the complexity of microbiome assembly. Such interactions also represent the cumulative influence of a potentially large number of environmental factors can be involved: soil type, for example, was different in the locations tested. Since the epiphytes (PMCs) that are associated with grapevine could originate from soil [38], leaf communities could be influenced by soil chemistry or other abiotic factors of the regions where plants are grown, leading to these region-specific unique microbial signatures.

Few strains of Sphingomonas, which was found quite abundant in all three regions (Figure 4E) were recently reported in plant protection against a bacterial pathogen (*P. syringae* DC3000) in *A. thaliana* model system [39]. Although, the molecular basis of pathogen reduction is unknown, but available evidence suggests that several traits contribute to the outcome of plant protection [40]. Differential abundance of Sphingomonas in grapevine grown in different regions should thus be studied in future in relation to plant traits to assess its impacts on grapevine health. Similarly, a fungal genera *Aureobasidium* was also quite abundant in all three regions (Figure 4F) and this prevalence of *Aureobasidium* was due to the presence of *A. pullulans* (relative abundance >12%, Supplementary Figure S1). *A. pullulans* have an antagonistic activity for Botrytis molds and for certain bacteria like Bacillus [28,41], which probably explains the lower prevalence of Bacillus and Botrytis in our data (Figure 4E,F).

Seasonal shifts in phyllosphere microbiome structure and the impacts of plant organs were also observed. At a particular location, the microbial composition of few bacterial phyla might change while bacterial diversity does not change during season shift. For example, cyanobacteria (photosynthetic bacteria) change its abundance from high to lower due to season change from spring to harvest. Lower daylight presence in harvest season probably explains these changes. These results are coherent with

another grapevine (Tempranillo) related study [38]. On contrary, fungal community diversity and their relative abundances, both were significantly impacted by season. Apart from genotype and terroir, the vineyard management practices could also be the possible reason for these differences [9]. Although a significant fraction of the members of PMCs were shared between plant organs (leaves and berries), we observed distinct assemblage patterns between both organs, which is also in accordance with recently published reports [25,37]. These differences among organs do not only reflect the compositional differences (or difference in the relative abundance of shared OTUs), but also the diversity in taxa present.

5. Conclusions

Our present study assessed the major microbial diversity present over French agro-climate zones and compared the many facets of factors that may influence the microbiome structure in the phyllosphere, with special focus on relative selection pressure that is exerted by grapevine genotype and its interaction with different climatic conditions (or terroir represented by French agro-climate zones), which may improve our chances to find genes that control PMCs on phyllosphere, and simultaneously increase our confidence that those genes are actually important in realistic environments, and probably those genes would give us new insights for breeding new and healthy grape varieties displaying better traits.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/6/4/96/s1, Table S1a: Differentially abundant bacterial genera between Leaf and Berries, Table S1b: Differentially abundant fungal genera between Leaf and Berries, Table S2a: Differentially abundant bacterial genera among three climate zones, Table S2b: Differentially abundant fungal genera among three climate zones, Figure S1: Relative Abundance major species between two seasons, displaying the uniqueness of the fungal microbiome structure at Spring and Harvest season, Figure S2: Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three genetic pools). Set1. n = 213, Figure S3: Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three geographic locations). Set2. n = 45.

Author Contributions: P.S., J.-P.P. and P.T. designed the research; P.S. and S.S. performed the lab experiments; P.S. analyzed the data; P.S., J.-P.P. and P.T. wrote the paper.

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Supplementary Table S1a: Differentially abundant bacterial genera between Leaf and Berries

OTUs	Genus	Corrected P-values	FDRs
OTU94	*Geodermatophilus*	0.0060	0.00066
OTU428	*Kineococcus*	0.0060	0.00066
OTU903	*Hymenobacter*	0.0060	0.00066
OTU152	*Arthrobacter*	0.0060	0.00066
OTU39	*Paenisporosarcina*	0.0060	0.00066
OTU288	*Paenibacillus*	0.0060	0.00066
OTU101	*Microbacterium*	0.0060	0.00066
OTU460	*Planifilum*	0.0060	0.00066
OTU200	*Methylobacterium*	0.0060	0.00066
OTU72	*Cutibacterium*	0.0060	0.00066
OTU134	*Nocardioides*	0.0060	0.00066
OTU415	*Noviherbaspirillum*	0.0060	0.00066
OTU56	*Massilia*	0.0060	0.00066
OTU374	*Adhaeribacter*	0.0060	0.00066
OTU26	*Gluconobacter*	0.0060	0.00066
OTU451	*Roseomonas*	0.0117	0.0012375
OTU897	*Streptococcus*	0.0172	0.001747059
OTU193	*Domibacillus*	0.0278	0.00275
OTU849	*Tumebacillus*	0.0329	0.003126316
OTU211	*Actinomycetospora*	0.0426	0.00396

Supplementary Table S1b: Differentially abundant fungal genera between Leaf and Berries

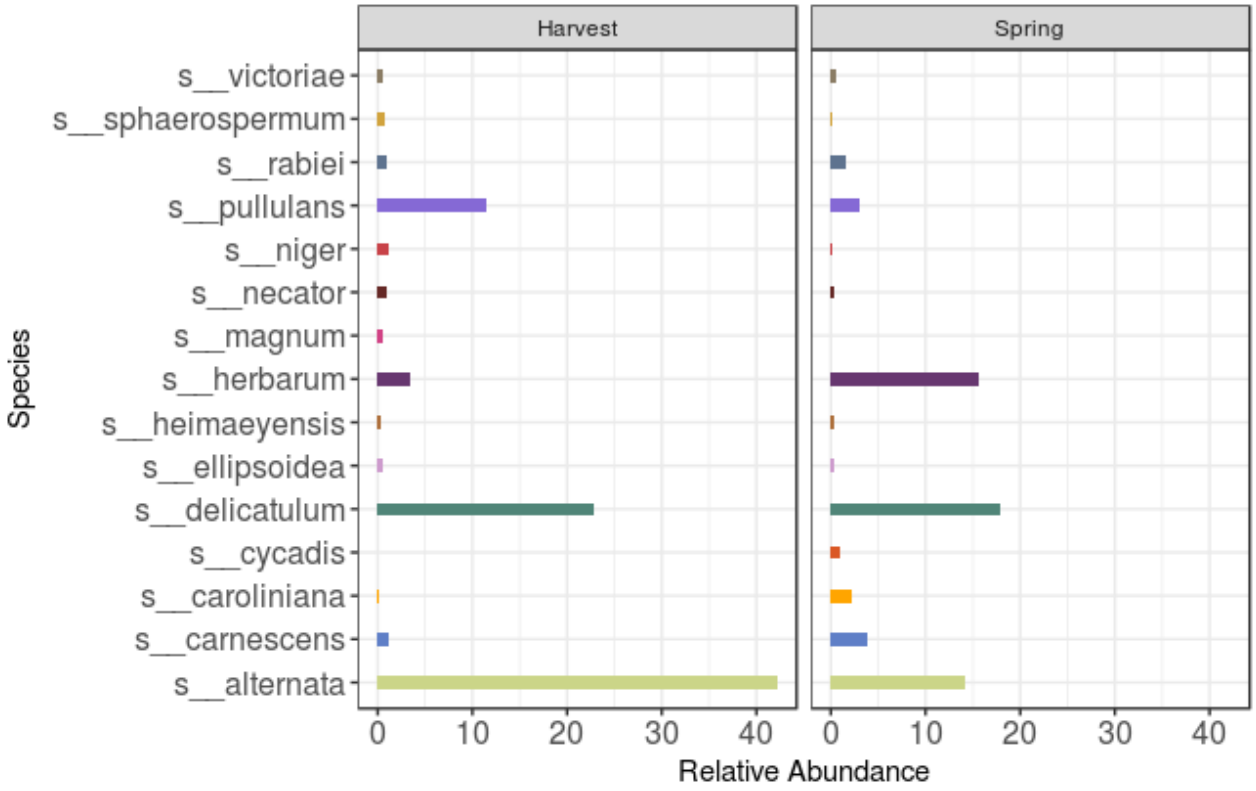
OTUs	Genus	Species	Corrected P-values	FDRs
OTU2	*g__Alternaria*	*s__alternata*	0.0021	0.0003764706
OTU4	*g__Cladosporium*	*s__delicatulum*	0.0021	0.0003764706
OTU33	*g__Sporobolomyces*	*s__roseus*	0.0021	0.0003764706
OTU18	*g__Sporobolomyces*	*s__roseus*	0.0021	0.0003764706
OTU62	*g__Aureobasidium*	*s__pullulans*	0.0021	0.0003764706
OTU49	*g__Rhodotorula*	*s__babjevae*	0.0021	0.0003764706
OTU11	*g__Filobasidium*	*s__magnum*	0.0021	0.0003764706
OTU12	*g__Alternaria*	*s__alternata*	0.0021	0.0003764706
OTU32	*g__Sporormia*	*NA*	0.0021	0.0003764706
OTU19	*g__Alternaria*	*s__alternata*	0.0021	0.0003764706
OTU79	*g__Rhodotorula*	*s__babjevae*	0.0021	0.0003764706
OTU61	*g__Quambalaria*	*s__cyanescens*	0.0021	0.0003764706
OTU83	*g__Metschnikowia*	*s__pulcherrima*	0.0021	0.0003764706
OTU73	*g__Metschnikowia*	*s__pulcherrima*	0.0021	0.0003764706
OTU96	*g__Metschnikowia*	*s__pulcherrima*	0.0021	0.0003764706
OTU35	*g__Citeromyces*	*s__matritensis*	0.0021	0.0003764706
OTU70	*g__Acaromyces*	*s__ingoldii*	0.0021	0.0003764706
OTU5	*g__Aureobasidium*	*s__pullulans*	0.0041	0.0006736842
OTU97	*g__Metschnikowia*	*s__pulcherrima*	0.0041	0.0006736842
OTU136	*g__Cryptococcus*	*s__heimaeyensis*	0.0123	0.00192
OTU71	*g__Filobasidium*	*s__stepposum*	0.0154	0.0024380952
OTU108	*g__Metschnikowia*	*s__pulcherrima*	0.0176	0.0026181818
OTU51	*g__Filobasidium*	*s__magnum*	0.0187	0.0027826087
OTU85	*g Saccharomycopsis*	*s vini*	0.0243	0.0034666667
OTU34	*g__Botrytis*	*s__caroliniana*	0.0337	0.0044307692
OTU52	*g__Rhizopus*	*s__arrhizus*	0.0337	0.0044307692

Supplementary Table S2a: Differentially abundant bacterial genera among three climate zones

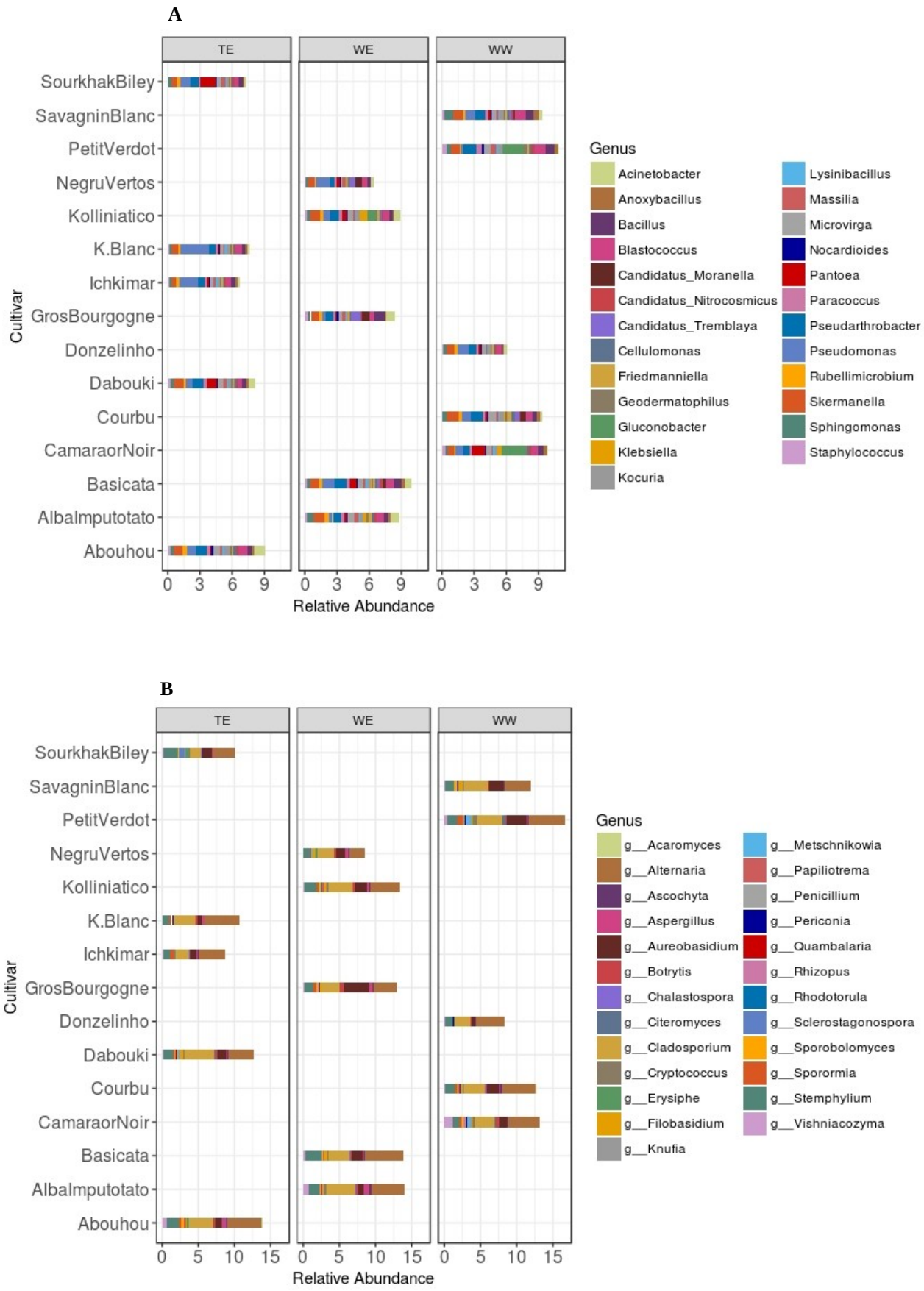
OTUs	Genus	Corrected *P*-values	FDRs
OTU29	*Modestobacter*	0.0032	0.003833333
OTU87	*Microvirga*	0.0032	0.003833333
OTU114	*Rubellimicrobium*	0.0032	0.003833333
OTU66	*Friedmanniella*	0.0032	0.003833333
OTU161	*Craurococcus*	0.0032	0.003833333
OTU271	*Neorhizobium*	0.0032	0.003833333
OTU55	*Paracoccus*	0.0063	0.0046
OTU50	*Blastococcus*	0.0063	0.0046
OTU61	*Kocuria*	0.0063	0.0046
OTU113	*Rathayibacter*	0.0063	0.0046
OTU1	*Sphingomonas*	0.0114	0.008363636
OTU218	*Paenisporosarcina*	0.0143	0.009583333
OTU7	*Ralstonia*	0.0246	0.012266667
OTU124	*Kocuria*	0.0246	0.012266667
OTU250	*Deinococcus*	0.0246	0.012266667
OTU279	*Devosia*	0.0269	0.0129375
OTU45	*Methylobacterium*	0.029	0.013529412
OTU11	*Rickettsiella*	0.0358	0.016611111
OTU14	*Pseudoarthobacter*	0.0032	0.00328
OTU171	*Acinetobacter*	0.0032	0.00328
OTU262	*Chamaesiphon_PCC-7430*	0.0032	0.00328
OTU102	*Rubellimicrobium*	0.0032	0.003285714
OTU152	*Pleurocapsa_PCC-7319*	0.0058	0.00575
OTU333	*Aliterella_CENA595*	0.0111	0.010222222
OTU57	*Methylobacterium*	0.0033	0.003833333
OTU30	*Skermanella*	0.0054	0.00575
OTU42	*1174-901-12*	0.012	0.0115
OTU72	*Staphylococcus*	0.012	0.0115
OTU95	*Snodgrassella*	0.0112	0.012545455
OTU29	*Modestobacter*	0.019	0.013416667
OTU55	*Paracoccus*	0.0237	0.015923077

Supplementary Table S2b: Differentially abundant fungal genera among three climate zones

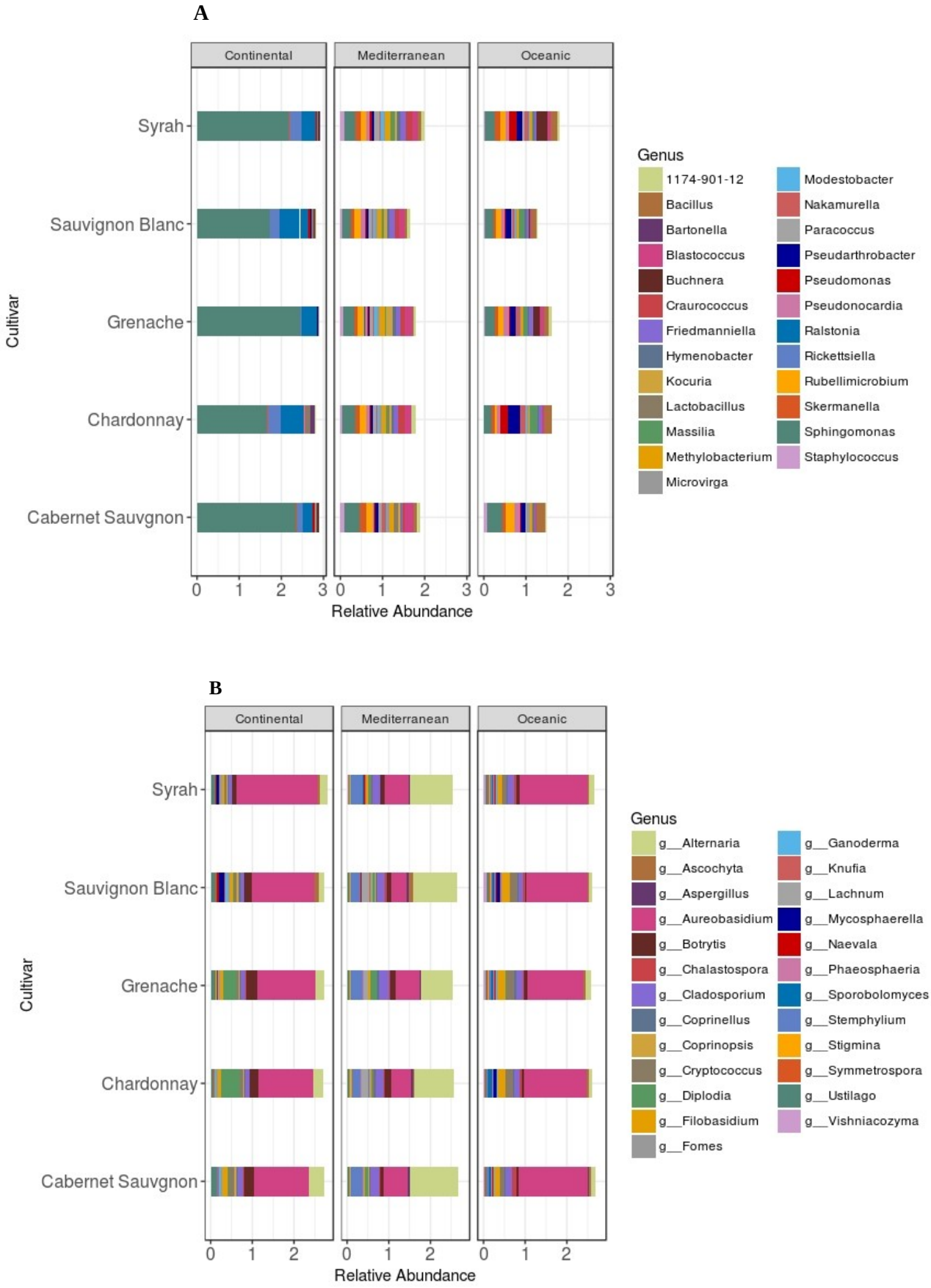
OTUs	Genus	Corrected P-values	FDRs
OTU30	*g__Sporobolomyces*	0.0019	0.00158
OTU3	*g__Alternaria*	0.0019	0.00158
OTU54	*g__Sporobolomyces*	0.0019	0.00158
OTU34	*g__Ascochyta*	0.0019	0.00158
OTU9	*g__Cryptococcus*	0.0019	0.00158
OTU66	*g__Hyperphyscia*	0.0084	0.005266667
OTU1	*g__Aureobasidium*	0.0166	0.0079
OTU8	*g__Stemphylium*	0.0231	0.009875
OTU37	*g__Filobasidium*	0.0285	0.011411111
OTU61	*g__Ganoderma*	0.0313	0.01185
OTU9	*g__Cryptococcus*	0.0014	0.0009875
OTU31	*g__Stigmina*	0.0014	0.0009875
OTU6	*g__Cladosporium*	0.0014	0.0009875
OTU73	*g__Aureobasidium*	0.0014	0.0009875
OTU39	*g__Symmetrospora*	0.0047	0.002633333
OTU56	*g__Coprinopsis*	0.0097	0.004253846
OTU62	*g__Aspergillus*	0.0138	0.004253846
OTU115	*g__Exophiala*	0.0138	0.004253846
OTU21	*g_Ustilago*	0.0165	0.005078571
OTU16	*g__Alternaria*	0.0159	0.0069125
OTU17	*g__Chalastospora*	0.0286	0.010772727



Supplementary Figure S1. Relative Abundance major species between two season, displaying the uniqueness of the fungal microbiome structure at Spring and Harvest season.



Supplementary Figure S2. Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three genetic pools). Set1. N = 213.



Supplementary Figure S3. Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three geographic locations). Set2. N = 45.

CHAPTER 4

Understanding the Phyllosphere Microbiome Assemblage in Grape-species with Amplicon Sequence Data Structures

CHAPTER SUMMARY:

- Submitted Article
- Describes the relative impacts of grape species (of *Vitaceae* family) and growing-year on phyllosphere microbiome structure.
- Compares the recent data normalization methods for microbiome data-sets

Understanding the phyllosphere microbiome assemblage in grape-species with amplicon sequence data structures

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Keywords: Grape Species, Growing year, Microbiome, Phyllosphere, Data Normalization

Abstract

The phyllosphere consists of the aerial parts of plants, and primarily the set of photosynthetic leaves is one of the most prevalent microbial habitats on earth. Phyllosphere microbiome can have profound impacts on host plant health and their performance traits. Impacts of plant genotype on phyllosphere within *Vitis vinifera* cultivars have been studied but the impact of grape species (of *Vitaceae* family) have been completely ignored. We performed 16S and ITS profiling to obtain genus level characterization of bacterial and fungal populations present in the leaf phyllosphere of the five grape species (genetically distant), sampled from the experimental plot in the Mediterranean. Secondly, we performed α and β-diversity analysis with robust statistical estimates to test the impacts of grape species and growing year, over a two-year period. At last, we normalized our high-throughput data with some recent but different normalization methods and compared the results of differentially abundant genera according to grape species and growing year. Our results indicated the presence of complex microbial diversity in the phyllosphere and very significant impact of the growing year in shaping this diversity (especially in fungi assemblage) has been observed. A reasonable impact of grape species was also noticed. Differential taxa abundance analysis using various data normalization methods also suggested the need for more robust normalization methods to study the differential abundance of taxa among groups in microbiome research.

Introduction

Plant phyllosphere harbors hyperdiverse microbial communities of prokaryotes, eukaryotes, and viruses that interact with each other and with the host plant[1]. Because of limited nutrient availability and fluctuating climatic conditions, it is a stressful and dynamic habitat for its microbial colonizers[2,3] and the knowledge of these colonizers and drivers that may affect their assemblage can reveal the mechanisms that govern processes at the interface between plants, microorganisms, and the atmosphere. However, in most of the grape related microbiome studies,

phyllosphere has been overlooked for a long time and studies were mainly focused on rhizosphere (root and soil colonizers) and endosphere (inside tissue colonizers)[4-9]. Also, all of these researches including few in phyllosphere related microbiome studies[10,11], suggested that the environmental conditions at different geographic location (or terroir) and season impacts microbial assemblage the most in the phyllosphere. Cultivars or genotype interaction with environment also seems to play a role in the assemblage of phyllosphere colonizers[10], but these studies have always taken samples from cultivars of only one grape species; *Vitis vinifera* (most widely cultivated fruit crops for wine and raisins), therefore, the genetic diversity among samples may not be strong enough to assess the genotype effect on microbial community dynamics in the phyllosphere in the natural environment. As microbiome selection could lead to next-generation plant breeding strategies[12], identifying microbiome differences among grape species could be an interesting opportunity for new grape breeding schemes to develop resistant, healthy and more productive varieties. Moreover, grape associated epiphytes have been recently established as promising biocontrol agents (BCA) against *Vitis vinifera* fungal pathogens[13], the species-level variation could provide us hints of new potential players for BCA. In this research, we sampled epiphytes from five different species in the grape family *Vitaceae*[14,15] in order to identify major microbial colonizers in the phyllosphere and also compared the impact of growing year and grape species. To mitigate the effect of environmental conditions at different geographic locations (or terroir), we sampled all five grape species (*Vitis vinifera cv*. *Cabernet-Sauvignon*, *Muscadinia rotundifolia*, *Parthenocissus quinquefolia*, *Vitis pentagona*, and *Vitis riparia*) from the same experimental plot in the Mediterranean.

From a methodological point of view, we also compared the differential abundance of microbial genera using three separate data normalization methods to predict the better normalization approach for grape related or other microbiome data. Normalization is the data transformation process enabling the accurate comparison of statistics from different measurements by eliminating artifactual biases in the original measurements. Existing literature of many microbiome studies (including grapevine related works[9,21]) still uses most standard statistical methods for differential abundance analysis without testing the data distribution and transformation. Rarefying samples to even sequencing depth is standard normalization method in microbial ecology but is not an ideal one, as it potentially reduces statistical power depending upon how much data is removed and does not address the challenge of compositional data[16]. The log ratio transformation methodology was also widely accepted by statisticians and researchers in various high dimensional studies[17,18]. Here, we are using few recently published data transformation methods of cumulative sum scaling (CSS)[19], DESeq2[20] and log ratio to normalize our zero-inflated taxa abundance data and compared estimates of differentially abundant genera

between two growing years and grape species.

Results

Phyllosphere exhibits diverse bacterial and fungal communities. Millions of amplicon reads were processed from both data-sets (16S and ITS) and 10825 bacterial and 5252 fungal amplicon sequence variants (or operational taxonomic units-OTUs) were obtained (Table 1). After assigning the OTUs to phylum level more than 73% bacterial OTUs and ~ 95% of fungal OTUs were assigned to phylum level. Unknown sequences corresponded to ~ 27% and ~ 5% in the case of bacterial and fungal data respectively, meaning that these sequences were not assigned to any microorganism during the assignment process. Proteobacteria (relative abundance ~ 15%) and Cyanobacteria (~ 14.8%) were the most dominated phylum across the samples followed by Firmicutes (~ 3%) and Actinobacteria (~ 1.3%). On the other hand, samples were heavily dominated by fungal phylum of Ascomycota (~ 91%) followed by Basidiomycota (~ 9%). After gloming of these OTUs at the genus level, 677 bacterial and 434 fungal genera were recovered. Out of these, *Sphingomonas*, *Methylobacterium*, *Rubelimicrobium*, *Blastococcus* and *Alternaria*, *Aureobasidium*, *Cladosporium*, *Lachnum* were most abundant bacterial and fungal genera, respectively (Fig 1A, B).

Microbial communities clustered distinctly with year and grape species. Performing multidimensional scaling (or Principal Coordinate Analysis; PCoA) on microbial abundance data showed that the samples from each year clustered together and distinctly from each other (Fig 2A, B), confirmed the very significant impact of the growing year on microbial community structuring in the phyllosphere. Clustering among grape species was not prominent (Fig 2A, B, shape represents grape species) but performing PCoA on the subset of the data (i.e. separate data for each year) displayed a lower but significant impact of grape species in shaping phyllosphere microbiome, especially the fungal microbiome (Fig 3). Permutational analysis of variance (PERMANOVA) statistics according to Year, Grape Species and the interaction term (Year × Grape Species), further confirmed the hypothesis (Table 2) that the environment and the host genotype interaction could be held responsible for microbial community structuring in the phyllosphere.

Furthermore, *Observed* α-diversity estimates of bacterial and fungal OTUs of each grape species (within each growing year, Fig 4) revealed that the unique OTU-richness in the phyllosphere of each grape species differed significantly (ANOVA, $P < 0.05$) between the years (Table 2) reconfirmed the major impact of growing year in shaping phyllosphere microbial assemblage. Although OTU-richness estimates didn't vary according to grape species ($P \sim 0.05$), the

interaction term (Year × Grape Species) showed strong differences in the richness of unique OTUs (Table 2).

Comparing normalization methods. Most of the microbiome data doesn't follow a normal distribution for taxa abundance across the samples and our data-sets were not the exceptions. Using three separate data transformation methods (square root, logratio, and CSS) we were unable to achieve proper normalization of our data-sets, and even after transformations, most of the distribution followed negative binomial distribution except CSS which performed better than other two methods (Fig 5). We used these transformed data for differential abundance analysis of each taxon (at genus level) according to the grape species and growing year (DESeq2 was applied to square root transformed data to handle negative binomial distribution).

Performing multiple testing with FDR corrected P-values (adj-P value < 0.05) gave nine bacterial genera for DESeq2 as compared to two and three genera for log and CSS transformed data between the two years. Similarly, 45 fungal genera were obtained for DESeq2 as compared to 11 and 13 genera for other methods between the two years (Fig 6, 7). The same testing was performed on data-sets of Spring 2017 and Spring 2018 separately to identify differential taxa abundance among five grape species and the results (Table 4) were similar. DESeq2 gave higher number of genera as compared to other methods (probably an overestimation). Combining normalization performance on taxa abundance across samples and identification of differentially abundant genera, indicated that CSS normalization methods worked better and were statistically more robust, i.e. fewer false positives and lesser false discovery rates, as compared to other methods.

Discussion

Endosphere and Rhizosphere of grapevines (cultivars of *Vitis vinifera*) have been studied before quite extensively[4,8,9]. Recently grapevines phyllosphere has also been explored by researchers[10,11], but these habitats were not explored in the context of different grape species of *Vitaceae* family until recently. Our study, at first, explored the phyllosphere of five different grape species using the culture-independent method of 16S and ITS profiling. Out of the complex microbial diversity, *Sphingomonas* and *Methylobacterium* were the most dominant bacterial genera and the fungal community was dominated by *Aureobasidium*, *Cladosporium* and *Alternaria*. These results are in line with the previous phyllosphere related works[22-24], which suggests that the phyllosphere is generally dominated by these genera because of their important functions, for example, *Methylobacterium* is often quite abundant in the phyllosphere and can benefit the plant by promoting its growth[25]. Biocontrol potential of some of these genera (e.g- *Sphingomonas* and *Aureobasidium*) have been explored previously[26-28] and should be explored in future in the

context of grapes and in collaboration with other other taxa (with major or minor abundances) as a community. As the microbial communities in the phyllosphere can modulate leaf susceptibility to infection, it can protect the plant from foliar diseases[29,30]. It can thus regulate the fitness of their host plant, depending on the microbial taxa involved and the local environmental conditions. Major challenges are to understand the properties of these microbial communities like taxonomic diversity, functional diversity, and microbial network structure that will be beneficial under future environmental conditions to foster properties of biocontrol and better fitness in grape plants to sustain the productivity and resilience of agricultural systems. Among the diverse and complex communities found in the phyllosphere the genus, *Massilia* is also noteworthy as its presence was quite consistent during the two years and it is a major contaminant of an aerosol with applications in agriculture[31,32] and could be an indicator of agricultural practices in the Mediterranean. The genus *Rubelimicrobium* was also one of the major dominant genus found as a leaf epiphyte. Few species of this genus were isolated from soil[33], and this could be an evidence to support the claim that the soil microbiota may influence the epiphyte compositions[9].

Secondly, we assessed the relative impacts of grape species and the growing year to shape microbial community assemblage in the phyllosphere. Leaf chemistry, physiology, and morphological structure differ among grape genotype or species as all these traits have a genetic basis, and this variation may lead to very different combinations of phyllosphere community structure[34,35] but our analysis indicated that the growing year had much stronger impacts than grape species in microbial community structuring. At each individual growing year, grape species also showed some influence on shaping this assemblage (especially for fungal assemblage) but it is much lower in comparison with the growing year. Statistical estimates of α and β-diversity suggest that the plausible hypothesis could be the genotype-year interaction is responsible for recruiting microbes on the leaf surface of different grape species. Temperature, humidity, rain, vineyard management may change the edaphic factors each year and in combination with grape genotype could be studied in future to better understand the influence of this interaction on phyllosphere microbiome.

At last, we analyzed the impacts of a few data normalization methods to detect the differential taxa abundance among different groups. Data in microbiome studies, such as microbiome taxonomy reads or OTU counts from amplicon sequencing experiments or differential expression (RNA-Seq) data are often overdispersed and have many zeros. In order to fit the microbiome count data with overdispersion and excess zeros, typically, the negative binomial (NB) is often applied[20,39,41]. For example, an NB model was fitted in a few microbiome studies, used to analyze gut microbiome in Parkinson's disease and effect of edible cricket consumption[36-38]. An NB model developed in recent past[39] was used to test for assessing differences in sequence tag

abundance and used for detecting differentially abundant features in clinical metagenomic samples[40]. In 2013, Zero-inflated Gaussian (ZIG) mixture model was proposed[19] and it uses the novel cumulative sum scaling (CSS) normalization technique to correct the bias in the assessment of differential abundance introduced by total sum normalization. This model directly estimates the probability that an observed zero is generated from the detection distribution due to undersampling or from the count distribution (absence of the taxonomic feature in the microbial community). We evaluated one NB method (DESeq2), CSS method and log transformation of the data to generate normalized counts and performed multiple testing on differentia taxa abundance. Our results predicted that the CSS method worked better than other methods in obtaining the normalized counts and gave statistically robust estimates of differential taxa abundance. However, the method needs to be further evaluated with a sufficient amount of other microbiome studies.

Material and Methods

Sampling, isolation of phyllosphere microbes and DNA extraction. Leaf samples (from 5 grape species) were collected from an experimental vineyard of INRA field station at Montpellier, France (Mediterranean). Leaf samples were taken from four to five plants of each species at Spring season (mid of May 2017 and 2018, before spraying of the fungicides). All samples were washed with an isotonic solution of sodium chloride (0.15M) containing 0.01% Tween 20 in 50 ml propylene tubes (2-3 leaves were washed per tube) using a horizontal shaker for 1hr at 100 RPM. Afterward, samples were given an ultrasonic bath for 7-10 minute using Ultrasonic Cleaner (Branson 5510) for maximum recovery of microbial pellets. The remaining solution was centrifuged at 4,000g and microbial pellets were transferred in a 2-ml Eppendorf tube and stored at -20°C. Pellets from the two of these tubes were mixed to make one biological replicate of a single grape-species and a total of three biological replicates were made for each species. DNA was extracted from each sample by using the ZymoBiomics DNA MicroPrep Kit (Zymo Research, USA) following the manufacturer's instructions.

PCR amplification and amplicon sequence library preparation. V4 region of the 16S ribosomal gene was amplified using primers 515F and 806R to characterize bacterial communities and fungal community diversity and abundance were accessed using modified ITS9 and ITS4 primers targeting the ITS2 region[42,43]. Two-step PCR was performed to prepare sequencing libraries. PCR1 was designed to perform amplification of the target regions and to add Illumina Nextera transposase sequence to the amplicons. Both forward and reverse primers for PCR1 were amended with frameshift (FS) sequences in their 5' overhang to improve

sequence diversity and overall read quality[44]. PCR1 was performed in 25µL reactions with 30ng of sample DNA using the KAPA HiFi HotStart (KAPA Biosystems) PCR mix (Initial denaturing at 95°C followed by 30 cycles of denaturing at 95°C for 30s, primer annealing at 57°C for 60s and primer extension at 68°C for 60s). Amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter) at a bead-to-DNA ratio of 0.7:1, resuspended in 30µL MilliQ water and evaluated in agarose gels. In PCR2, Primers from Illumina kit for dual indexing of the amplicons was used. Each cleaned PCR1 product within the same sample received a unique combination of forward and reverse primers (respectively, N7 and S5 Illumina dual index oligos). Afterward, samples were again cleaned using AmPure XP magnetic beads, pooled in equimolar concentrations and sequenced using 2x250bp MiSeq v2 sequencing (Illumina Inc., San Diego, CA, USA).

Data trimming and analysis. Paired-end sequence reads from 16S and ITS sequences were filtered, trimmed and processed with the *dada2* (R Bioconductor package)[45]. Primers were removed from each sequence using the fastqPairedFilter function of the *dada2* and bases with low-quality scores (< 11) were also discarded. These filtered files were then processed using the core Divisive Amplicon Denoising Algorithm (DADA) and amplicon sequence variants (or OTUs) were inferred[10,45]. Chimeras were removed using the removeBimeraDenovo function of the same *dada2* package (Table 1 represents the total number of reads available during these steps). Bacterial and fungal OTU sequences were assigned a taxonomy using the RDP classifier[46] and UNITE data base[47] respectively with k-mer size 8 and 100 bootstrap replicates.
Further analysis of α and β-diversity estimates were performed using functions of the *phyloseq* package[48]. Estimates of observed α-diversity were measured within sample categories using the estimate_richness function of the phyloseq package. Relative abundances of microbial genera were plotted using the *ggplot2* package[49] after gloming the data at the genus level (using the tax_glom function of the *phyloseq* package) and transforming genus abundance data into relative counts. PCoA ordination was performed on log-transformed data (for variance stabilization) using Bray-Curtis dissimilarity matrix between samples and visualized by using their base functions in the *phyloseq* package.

Data Normalization and Statistical analysis. Square root transformation of the data was performed on taxa counts using sqrt(1+x) function. Log transformation was done using log(1+x) function on taxa counts (Pseudo count of 1 was added to avoid log or the square root of Zeros). DESeq2 normalization was done using the phyloseq_to_deseq2 function of the *DESeq2* package[20]. CSS normalization was done using the *metagenomeSeq* package[19]. Multiple testing

was performed using the mt function of the *phyloseq* package after any desired data transformations to identify differentially abundant taxa between groups.

We performed all the analysis in R-environment (R3.3.4, R Core Team, 2017) and assessed the statistical significance ($P < 0.05$) throughout and whenever necessary, we adjusted P-values for multiple comparisons according to the Benjamini and Hochberg method to control False Discovery Rate[50] (e.g- while performing multiple testing on taxa abundance according to sample categories). We performed an analysis of variance or ANOVA[51] among sample categories while measuring the Observed estimates of α-diversity. Stratified permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations was conducted (at $\alpha = 0.05$) on all principal coordinates obtained during PCoA with the *adonis* command (with appropriate model matrix) of the vegan package[52], to observe the statistical significance of clusters according to the sample categories.

Data availability. All of the data are provided fully in the result section of this paper and the sequence data is available at institutional server http://agap-ng6.supagro.inra.fr/inra and can be obtained upon reasonable request to authors.

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Author Contribution

Prashant Singh, Jean-Pierre Péros and Patrice This designed the research; Prashant Singh, Sylvain Santoni & Audrey Weber performed the lab experiments; Prashant Singh analyzed the data; Prashant Singh, Jean-Pierre Péros and Patrice This wrote the paper.

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Table 1 : Total number of reads during each step of bacterial microbiome data (16S/ITS) processing

Number of Samples	Input reads	Filtered reads	Denoised & Merged	Chimera removal	OTUs
16S Data					
30	5,568,565	4,538,503	4,139,738	3,9763,42	10,825
ITS Data					
30	2,7429,06	2,674,944	2,411,474	2,407,048	5,252

Table 2: Factors predicting the impacts of Year and Grape Species on the leaf phyllosphere communities

Data	ANOVA (on α-diversity measures)	PERMANOVA (on PCoA clusters)
16S		
Year	At F = 5.725, P = 0.0076	R^2 = 0.269, F = 1.811, P = 1e-04
Year × Grape Species	At F =9.022, P = 0.00138	R^2 = 0.154, F = 1.737, P = 1e-04
Grape Species (Spring 2017)	At F = 3.752, P = 0.041	R^2 = 0.379, F = 1.525, P = 1e-04
Grape Species (Spring 2018)	At F = 1.743, P = 0.217	R^2 = 0.304, F = 1.134, P = 0.031
ITS		
Year	P = 1.24e-07, F = 49.261	R^2 = 0.101, F = 3.532, P = 1e-04
Year × Grape Species	P = 2.71e-07, F = 57.340	R^2 = 0.112, F = 3.767, P = 1e-04
Grape Species (Spring 2017)	F = 2.843, P = 0.08	R^2 = 0.325, F = 1.206, P = 0.0038
Grape Species (Spring 2018)	F = 1.274, P = 0.34	R^2 = 0.334, F = 1.257, P = 1e-04

Table 3: Differential taxa abundance (bacterial taxa, genus level) among Grape Species at each year.

Method	Genus	Adj P-value	FDR
Spring 2017			
CSS	*Cutibacterium*	0.033	0.001
	Rubelimicrobium	0.0171	0.001
	Sphingomonas	0.034	0.018
Log	*Cutibacterium*	0.038	0.017
	Rubelimicrobium	0.168	0.001
DESeq2	*Cutibacterium*	0.038	0.128
	Rubelimicrobium	0.0192	0.212
	Sphingomonas	0.042	0.201
	Microvirga	0.022	0.113
	Rhodococcus	0.022	0.113
Spring 2018			
CSS	*Hymenobacter*	0.036	0.061
	Rubelimicrobium	0.036	0.061
Log	*Hymenobacter*	0.034	0.045
	Rubelimicrobium	0.034	0.045
	Methylobacterium	0.039	0.045
DESeq2	*Hymenobacter*	0.027	0.118
	Rubelimicrobium	0.027	0.118

	Paracoccus	0.027	0.118
	Methylobacterium	0.038	0.221
	Pedobacter	0.038	0.221
	Kineococcus	0.038	0.221
	Spirosoma	0.043	0.312
	Blastococcus	0.043	0.312

Table 4: Differential fungal taxa abundance (fungal taxa, genus level) among Grape Species at each year.

Method	Genus	Adj P-value	FDR
Spring 2017			
CSS	*Aspergillus*	0.0011	0.022
	Alternaria	0.0171	0.033
Log	*Aspergillus*	0.0012	0.017
	Alternaria	0.0012	0.017
	Botrytis	0.0131	0.112
DESeq2	*Hyphodontia*	0.006	0.081
	Aspergillus	0.026	0.119
	Alternaria	0.026	0.119
	Botrytis	0.026	0.119
Spring 2018			
CSS	*Alternaria*	0.024	0.038
	Sclerostagonospora	0.024	0.038
	Truncatella	0.032	0.048
Log	*Alternaria*	0.027	0.0199
	Sclerostagonospora	0.027	0.0199
	Truncatella	0.034	0.0231
DESeq2	*Knulfa*	0.021	0.132
	Alternaria	0.021	0.132
	Sclerostagonospora	0.021	0.132
	Truncatella	0.037	0.172
	Botrytis	0.037	0.172

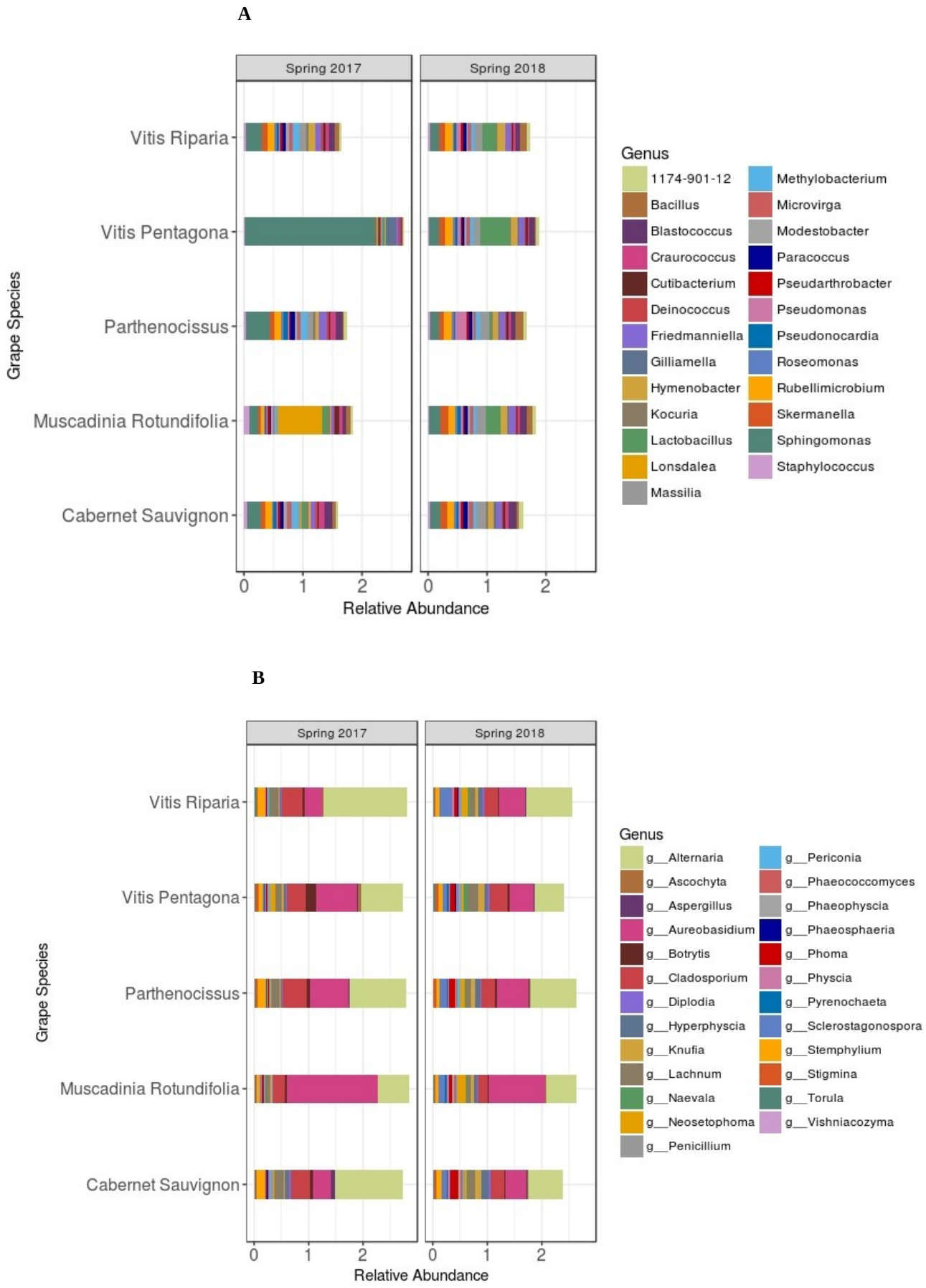

Fig 1. Genus level characterization of Bacteria and Fungai present on leaf phyllosphere of 5 grape-species (relative abundance of top 25 genus).

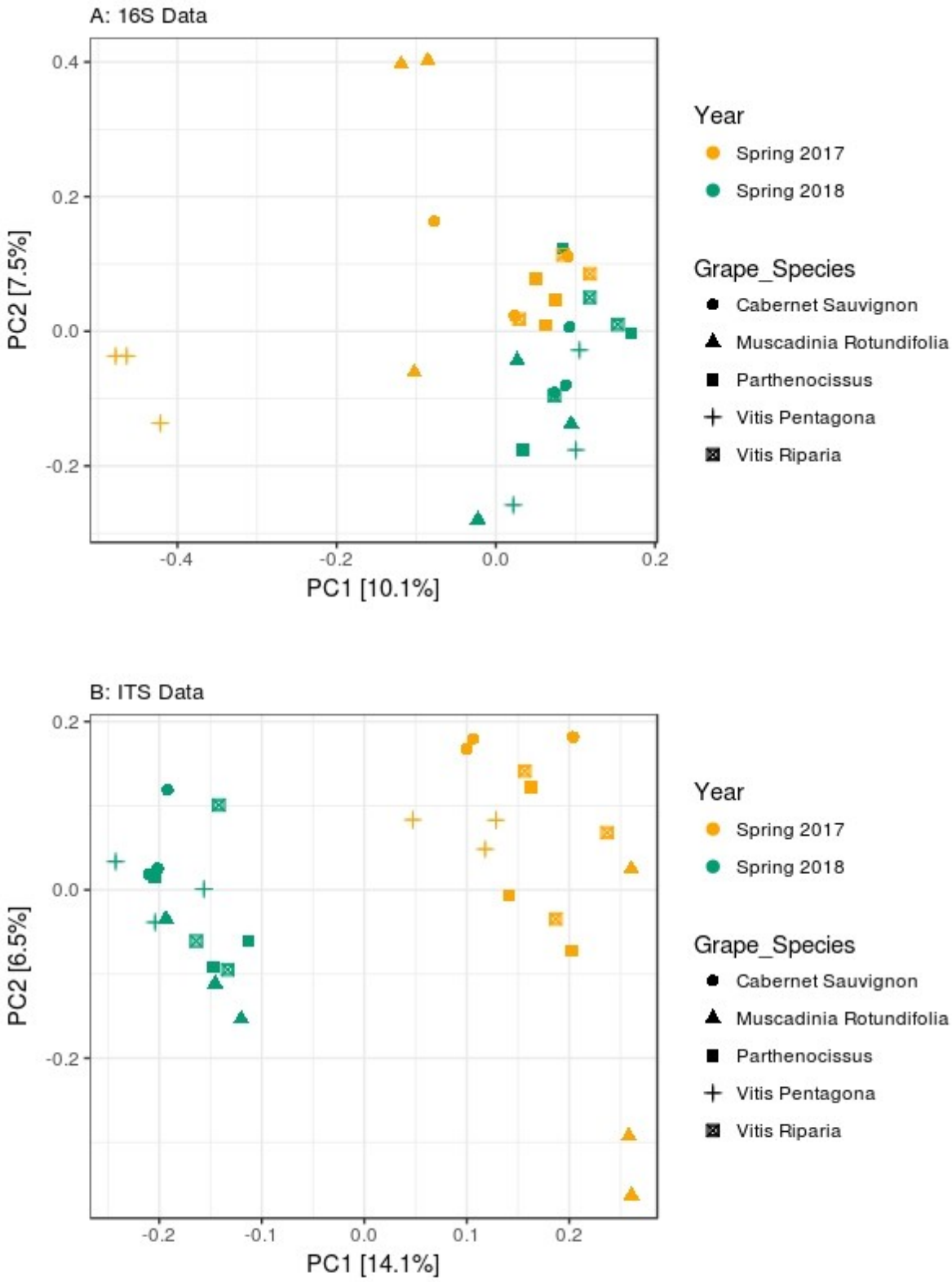

Fig 2. PCoA ordinations of (A) bacterial and (B) fungal communities derived from leaf phyllosphere at two growing years, using Bray-Curtis distance matrix. Both the axis explains ~ 20% of variations (Shape represents grape species). N = 30.

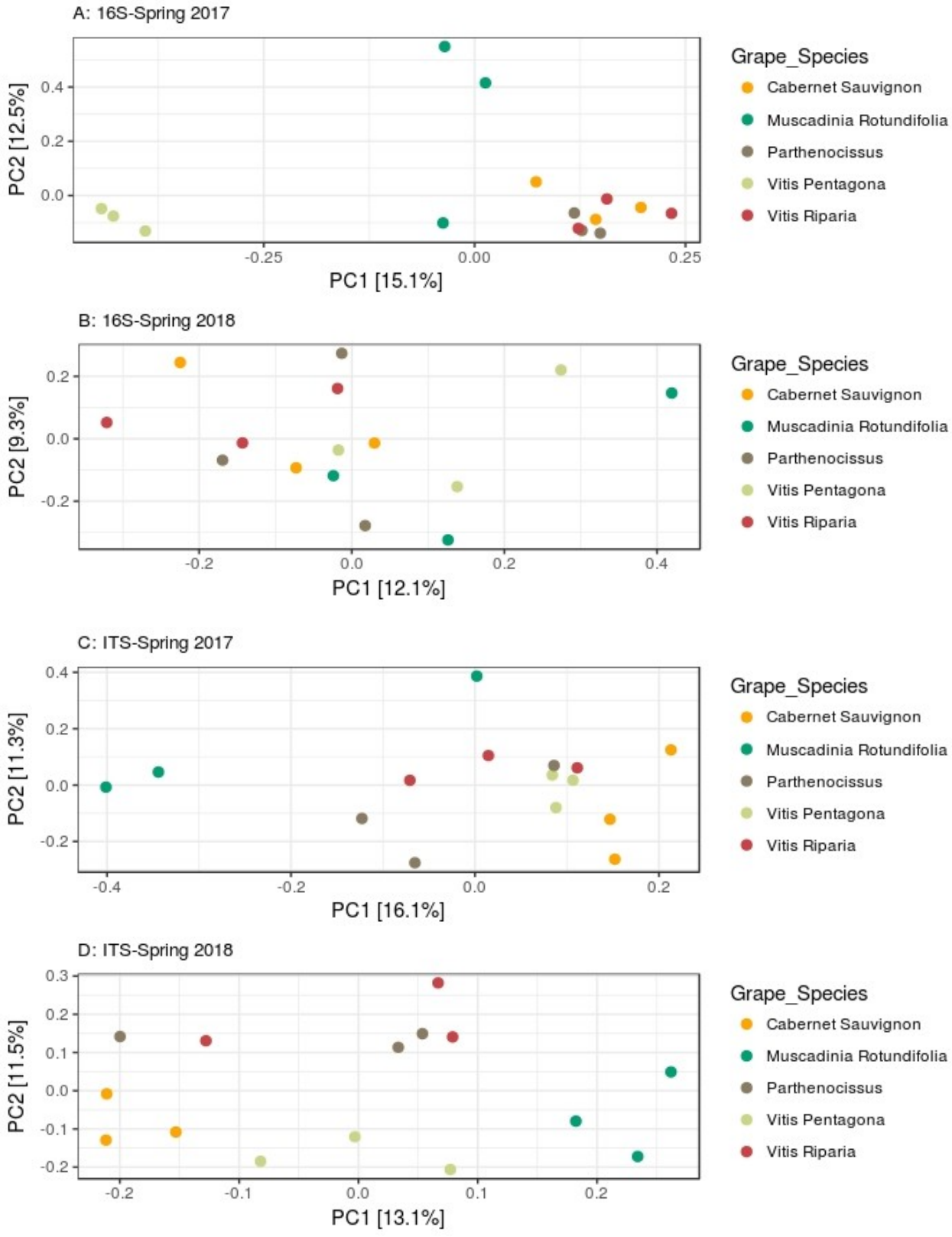

Fig 3. PCoA ordinations of bacterial (A, B) and fungal (C, D) communities derived from leaf phyllosphere at spring 2017 and spring 2018 separately, using Bray-Curtis distance matrix.

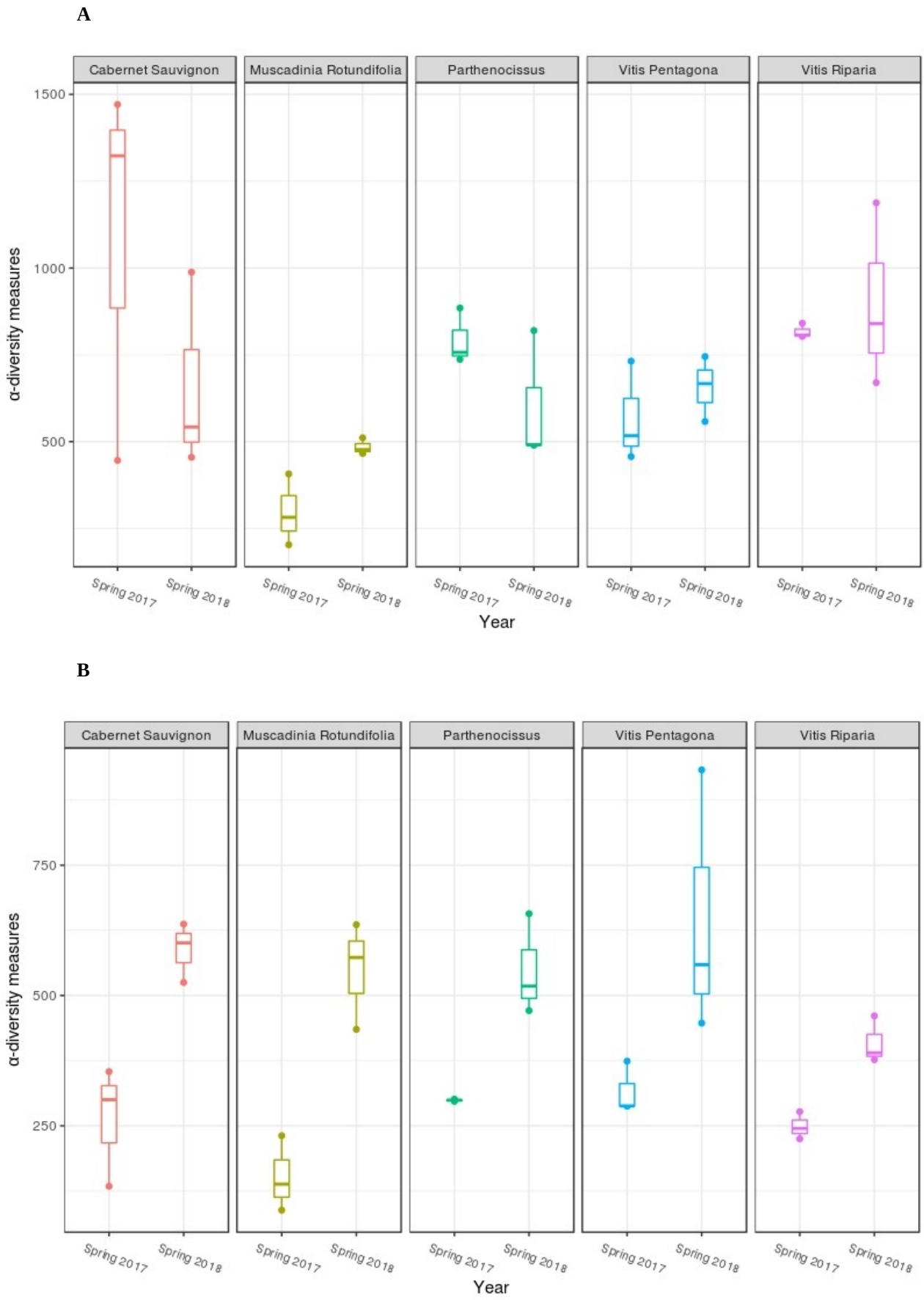

Fig 4. *Observed* α-diversity measures for (A) bacterial and (B) fungal data for each grape species in Spring of 2017 and 2018. N = 30.

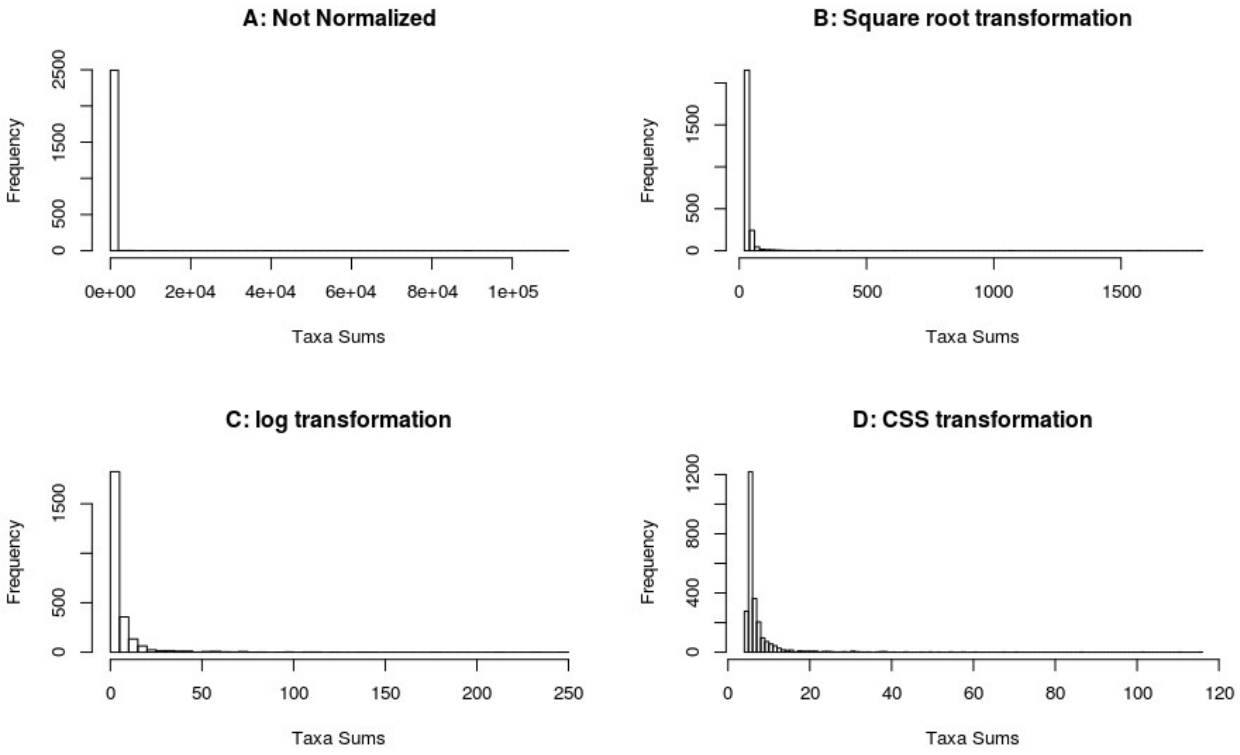

Fig 5. Histograms of the distribution of taxa sums across samples using (A) no normalization (B) Square root transformation (C) log transformation and (D) CSS transformations.

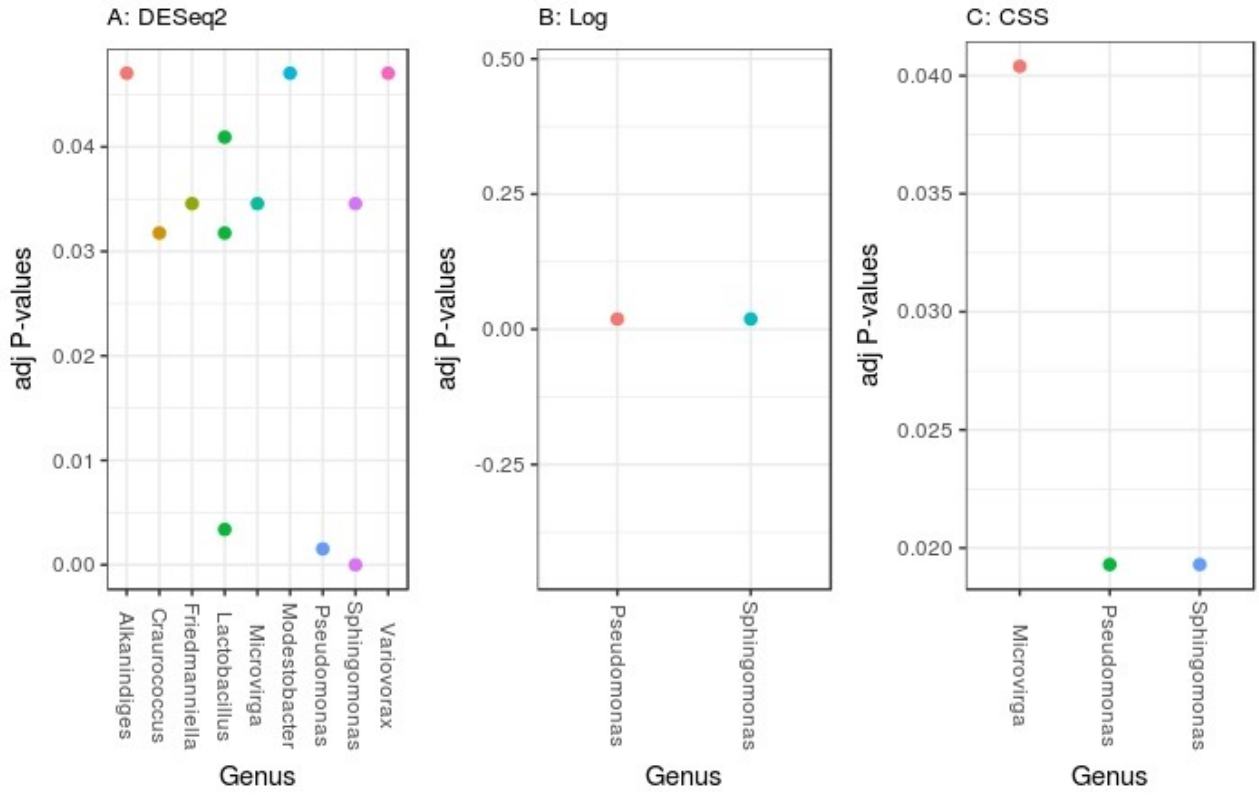

Fig 6. Different normalization methods identified several bacterial taxa that significantly contributed (adj P < 0.05) to differences between two growing years.

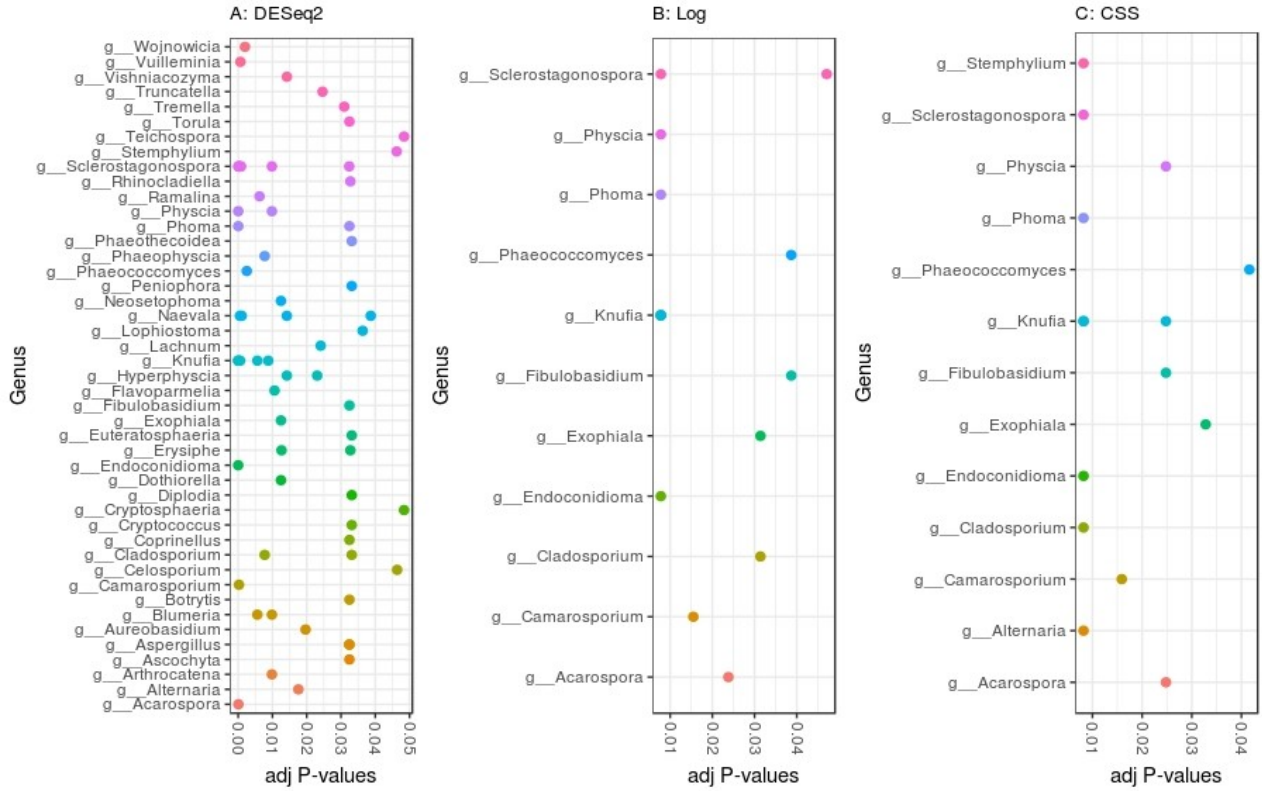

Fig 7. Different normalization methods identified several fungal taxa that significantly contributed (adj P < 0.05) to differences between two growing years.

CHAPTER 5

Conclusion & Perspectives

CHAPTER SUMMARY:

- This chapter talks about the conclusive summary of the thesis and also discusses the future prospects of microbiome as Bio-control agents, improving plant performance traits using microbiome engineering and quality improvement of wines by selecting novel group of microbes for fermentation.

Chapter 5

Conclusion & Perspectives

5.1. Concluding remarks

Phyllosphere or carposhere microbial community diversity, assemblage and it's drivers are less studied research areas especially in the context of grapevines or different grape species. There is also a lack of knowledge about the relative contributions of various potential drivers (e.g.- genotype, terroir etc.) from different sources in developing the phyllosphere community structure, and this makes it difficult to draw a general conclusion about who is the major driver? At present, the drivers that determine the interactions between epiphytes and ultimately shape microbial communities are poorly elucidated.

This thesis is an attempt to answer the questions related microbial diversity present in leaf and fruit surfaces and assessment of the relative selection pressure applied by potential drivers (grapevine cultivars, grape-species of *Vitaceae* family and terroir) to shape phyllosphere and carposhere microbial structure (using NGS followed by subsequent data analysis and robust statistical methods), which establishes the terroir as a major factor in shaping the microbial assemblage. We first selected at random nine grapevine cultivars for each of the three main genetic pool existing in cultivated *V. vinifera* and estimated the microbial diversity in the phyllosphere of leaf and berry in the French Mediterranean region. We observed a minor impact of grapevine genetic diversity in shaping microbial community structure in the phyllosphere (Chapter 2) with some associations of few bacterial and fungal genera to a particular genetic pool.

Secondly, we repeated the same analysis using the five of the most genetically distant cultivars in each genetic pool (sampled from two vineyards within the Mediterranean) and again revealed a weak impact of genotype although on the fruit surface genotypic impacts appeared more prominent (Chapter 3). Five commercially important varieties of *Vitis vinifera* that were sampled from three different French agro-climatic zones and showed that the effect of terroir was much stronger than the genotype (Chapter 3).

Finally, we maximized the genetic diversity among samples by sampling leaves from five different species of grapes of the *Vitaceae* family and again explored the microbial diversity present among five grape species (Chapter 4). Using the culture-independent method, a diverse and complex microbial community was observed. A Significant impact of grape-species was observed in shaping the microbial assemblage (especially fungal assemblage) but the interaction of growing year and grape-species was statistically more robust in defining phyllosphere community structure. These results reconfirmed our hypothesis in Chapter 3 that terroir is the major driver but also genotype and environment interaction may shape the microbial assemblage in the phyllosphere. An improved

knowledge of the principal factors of community structure and multitrophic interactions in the phyllosphere will be the key to developing new ideas for plant protection. Biocontrol applications in future will benefit from fundamental research; in other words, the better we understand the roles and importance of indigenous microorganisms, the better we will be able to predict and protect against pathogen infection.

On a very different note, we analyzed the impacts of fairly recent data normalization methods to detect the differential taxa abundance among different groups and predicted the better data normalization methods for microbiome data-sets. This could be useful for grape related or other microbiome studies to produce statistically more robust estimates for their hypothesis.

5.2 Phyllosphere Microbiome as Biocontrol Agent (BCA)

In the human gut, several microbes were previously considered commensals are now regarded as beneficial symbionts because of their contributions to host metabolism and immunity [1]. The thought-provoking statement by Janzen [2], that “Plants wear their guts on the outside”, suggests that similar questions concerning host benefit and microbe-microbe interactions should be asked about plant-associated communities.

Complex and multipartite interactions are expected to occur in the phyllosphere between various microorganisms as well as between microorganisms and host plants (e.g- parasitic, commensal and mutualistic interactions) [3]. Benefits of these interactions to microbes are obvious and include a supply of nutrients, but the advantages provided by phyllosphere inhabitants to their host plants are not quite clear. It has been suggested that plants benefit from the microbial production of plant hormones, such as cytokinins and indole 3-acetic acid (IAA); however, there is a lack of sufficient reports in this area. On the other hand, there are few reports suggesting the induced systemic resistance against pathogen in plant by plant-associated microorganisms [4] and indigenous microbial populations might affect the outcome of plant-pathogen interactions in the phyllosphere [5,6]. However, the mechanisms that provide plants with resistance to a wide range of pathogens and is induced by beneficial microorganisms are not yet well established for phyllosphere microbes. Likewise, functional studies like metatranscriptomics with their integration with metagenomic studies might uncover the potential of microorganisms to assist in plant defense are yet to be carried out for the phyllosphere. Recently, Sphingomonas spp. isolates were identified to be involved in plant protection against bacterial pathogens in *A. thaliana* model system [7].

Although the molecular basis for reduced pathogen growth in the presence of *Sphingomonas spp.* is not yet understood, the available evidence suggests that several traits contribute to the outcome of plant protection [8]. Another report [9] suggested that the grape associated yeasts are promising biocontrol agents against *Vitis vinifera* fungal pathogens (*Botrytis cinerea*). In general, the

interactions that prevail in the plant phyllosphere are far from being understood, and it will be important to attain more system-level insights into the complex interactions that govern outcomes among community members in the context of the plant host. Furthermore, application of phyllosphere microbiome structure in improving plant health (specially grapevines) to increase biomass production cannot be ignored. As many foliar bacterial pathogens colonize plant surfaces before infection, and the size of the final populations is often correlated with disease severity. This suggests that a reduction in pathogen numbers would lead to plant protection [10-12]. Possible mechanisms to suppress pathogen proliferation may include exploiting competition for nutrients and space, antibiosis and stimulating systemic host responses [6,13]. Proposed alternative biocontrol strategies use single protective strain or strain combinations [14-16].

The use of chemical fungicides has been restricted by the European Union because of the ever-increasing level of hazardous residues in the environment and food chains. New, natural antagonistic phyllosphere microorganisms against bacterial or fungal diseases could serve the agricultural production to reduce pre- and post-harvest losses, to boost safer practices for workers and to protect the consumer's health. The advent of next-generation sequencing (NGS) technologies is now driving a paradigm change that allows researchers to integrate microbial community studies into the traditional biocontrol approach. This integration could answer relevant scientific questions and will develop new biocontrol hypotheses using phyllosphere microorganisms.

Another potential application area for phyllosphere inhabitants would be the phytoremediation (can also be called phylloremediation) using phyllosphere microorganisms to remove volatile pollutants such as phenol or benzene from the air [17,18].

5.3 Microbiome Engineering

It employs basic principles of quantitative genetics and community ecology and consists of experimental methods that improve host performance by artificially selecting for microbial communities with specific effects on host performance or fitness [19]. Host performance can include any trait that is biologically, medically, or economically important (e.g., growth rate or disease resistance).

The artificial selection on microbiomes is applied over multiple generations and in an indirect manner, meaning that the host traits are used to direct whether the host's microbiome gets to ‘reproduce’ via experimental passage to the next generation of hosts Microbiome engineering applies multigenerational, artificial selection upon hosts that vary in the microbiome content affecting the host trait [19]. Both animal and plant microbiomes encompass habitats for diverse microbial communities and these microbiomes enhance host functions, contributing to host health and fitness. A novel approach to improve plant or animal fitness is to artificially select upon

microbiomes, thus engineering evolved microbiomes with specific effects on host fitness and this process is called host-mediated microbiome selection (HMMS). A preliminary research goal in microbiome studies is to elucidate microbiome functions that alter host performance. Several complementary approaches have emerged to differentiate between beneficial, neutral, and detrimental effects on host fitness [20,21]. Principle approaches to investigate microbiome functions includes correlational analyses, Single-cell Genomics, Whole-Community Metagenomics, Metaproteomics, and Experimental Manipulations [22-25]. With any of these approaches, it remains a daunting task to elucidate specific functional roles of the microbiome in shaping host performance traits (e.g., growth, health, antibiosis, and overall fitness). Central to this challenge is the complexity of microbiome properties, which can be driven by interactions among taxa within the microbiome community and which can vary with both the host genotype, environment or interaction between them [26,27].

HMMS is a new and innovative method and has been used successfully to engineer rhizosphere of microbiomes of *Arabidopsis thaliana* host plants [28,29]. After sterilization of initial soil, plantlets were inoculated with a starter soil-community. At the end of each growth cycle (or generation), a host trait (e.g., plant-shoot biomass, or flowering time) was measured for each replicate; then soils of the best-performing (or poorest-performing) replicates were chosen to inoculate the next generation of sterilized soils of the respective High- and Low-Line (Fig 1). This scheme propagated all viable microorganisms (e.g., viruses, bacteria, fungi, nematodes, and mites) from a parental community to the next generation, whereas the host plants did not evolve between generations because all seeds were taken from the stock of an inbred Arabidopsis line. Ten rounds of selection on soil-microbiomes produced significantly different plant phenotypes between the microbiome selection lines in both studies [28,29].

Another variant of HMMS is to propagate only a portion of the host-associated community, for example by filtering out microbial community members with larger cells (e.g., fungi) while retaining for co-propagation only smaller community members (e.g., bacteria and viruses).

Despite the time-consuming filtering step, elucidating changes in the co-propagated microbiomes is simplified because only small-celled microbes (e.g., bacterial communities) have to be analyzed. Sub-microbiome selection will be more useful to engineer some microbiomes (e.g., rhizosphere/phyllosphere microbiomes), but less so for microbiomes with strongly interacting fungal and bacterial components (e.g., endophyte microbiomes of leaves). Probably the mechanism of functioning of host-mediated microbiome engineering works at the initiation of a host-microbe interaction, host control occurs via partner choice or screening, in which the host selectively alters the subset of microbes that are allowed to colonize or persist in association with the host (e.g., via resistance, immunity, and genotypic specificity [30,31].

Using HMMS or microbiome engineering, novel and improved microbiome functions can be selected upon without any prior knowledge of the microbiome composition, or of their synergistic interactions, but how does host-mediated selection alter the Makeup of the Microbiome? Probably the microbiome engineering can alter microbiomes through ecological and evolutionary processes [19]. The ecological processes include changes in community evenness and diversity, relative species abundances, and the structure of microbe-microbe and host-microbe interaction networks. The evolutionary processes include extinction of some microbiota in the community, changes in allele frequencies, mutation, and horizontal gene transfer that restructure microbial genomes. Both ecological and evolutionary changes can be tracked with NGS methods that infer taxon presence-absence and abundance, active microbial functions that are being expressed, and permit mechanistic inferences of microbiome functions. Host-mediated microbiome engineering is thus a powerful method to manipulate microbiomes and understand their functions [19].

It has been predicted that host-mediated microbiome engineering will often be more efficient using wild hosts rather than hosts that have experienced domestication, or adaptation to microbially deprived laboratory environments. This is because genes that enable hosts-microbes interactions, which may have been lost during domestication [32,33], and agricultural soil microbiomes likely varied greatly between successive plant generations in the absence of host-microbiome co-propagation. Host-mediated microbiome engineering has diverse applications, particularly in agricultural research aiming to enhance plant productivity, including drought and salt tolerance, and disease resistance and must be tested for phyllosphere/rhizosphere microbiomes in grapevines with a large number of design criteria for host-mediated microbiome engineering that may explore the possibilities to obtain desired plant traits in grapevines.

Fig 1. Artificial selection on microbiomes in the plant rhizosphere. At the start of each experiment, microbiomes differ in community composition between hosts; host–microbiome associations are allowed to mature (Step 1), then are phenotyped for the trait used as a direct target for indirect selection on microbiomes (Step 2), then microbiomes are chosen for transmission to the next generation of hosts (Steps 3 and 4). The most extreme host phenotypes are chosen to identify microbiomes for propagation (microbiomes from the largest plant) [*Muller & Sachs*, 19].

5.4 Phyllosphere microbiome for wine quality improvement

Wine contains a complex mixture of bio-molecules and aroma producing compounds and microorganisms plays a critical role in it. Nowadays, it is a widely known fact that wine fermentation is not a single-species process and the role of the different wine related microbes in wine production is in the spotlight of worldwide research [34]. Wine grape microbiome could be related to the sensorial properties of wines [35] and this microbiome, which comes mainly from vineyards, is dependent on the geographical location, grape variety, climatic conditions, and agronomical practices (or terroir) suggest that the microbial profile of grapes could predict the composition and abundance of certain wine impact metabolites [35-37]. Future studies could be on training phyllosphere microbiota efficiently to predict wine metabolite profiles which may provide actionable information to winemakers to improve wine characteristics (e.g- aroma and flavors) or mitigate fermentation problems such as unwanted sulfur removal. However, there are many microbial species (both fermentative and dominant grape epiphytes) with the potential incidence in wine flavor [37] but whose specific role in wine fermentation is still poorly understood and could be explored in future studies. Once we better understand which microbial species (epiphytes or wine must microbes) influence the flavor of wine or the health and productivity of grapevines, we can begin to identify how some of these microorganisms might be selectively applied to this crop.

Alcoholic fermentation is mainly achieved by *Saccharomyces cerevisiae*, leads to the formation of many alcohols and esters [38]. As a rule, most of the fermentative aroma compounds have high sensory thresholds and therefore do not individually contribute, in a significant way, to the distinctive aroma of wines. However, their combination establishes the basic matrix of wine aroma. Lactic acid bacteria (LAB) are one of the most relevant groups of microorganisms in winemaking and are responsible for the decarboxylation of malic acid to lactic acid in a process known as malolactic the fermentation that provides additional advantages, like microbial stability and improved aroma complexity [39]. The use of different yeast or bacterial strains for wine fermentation could contribute to significant variations in higher alcohol levels in wine [40,41]. *Lachancea thermotolerans*, *Hanseniaspora uvarum*, *Candida zemplinina*, *Saccharomycodes ludwigii* and *Pichia anomala* have been described as high fusel alcohols producers when used in single inoculations and in mixed fermentations with *S. cerevisiae*, generally with independency of the inocula ratio used. On the other hand, *Candida stellata* and *Zygosaccharomyces fermentati* species have been reported as low producers, when they were used as sole inocula and in co-inoculation with S. cerevisiae [42].

Presence of undesirable sulfur compounds like hydrogen sulphide (H_2S) in wine is an important problem for the wine industry because it imparts undesirable off-flavors like sulfurous or rotten egg aromas as well as due to its low perception threshold (10 to 80 μg/L)[43].

Wine yeast strongly affects the H_2S production during wine fermentation as the major production occurs during the biosynthesis of sulfur-containing amino acids like cysteine and methionine from inorganic and organic sulfur sources [44,45].

One of the few strategies to reduce H_2S production is the application of unconventional yeasts in winemaking. Sulfite reductase activity is one of the main enzymatic activities responsible for H_2S production and is a rare feature among the majority of non-Saccharomyces species [46] since only species from *Hanseniaspora* genus (mainly *H. osmophila* and *H. opuntiae)* had a quite high sulfite reductase activity among the 15 species tested. In addition, some *T. delbrueckii* strains, apart from *S. cerevisiae*, had certain H_2S production ability. However, as occurs in *S. cerevisiae*, a great strain-dependent behavior exists in other wine-related yeast species, such as *Dekkera*, *Lachancea*, *Hanseniaspora*, and *Metschnikowia.* The future efforts should be made in identifying carposphere strains or group of strains, which can reduce the sulfite reductase activity in winemaking process to provide better quality wines.

Chapitre 5 (en français)

Conclusion et perspectives

5.1. Remarques finales

La diversité des communautés microbiennes phyllosphériques ou carposhériques, leur assemblage et ses moteurs sont des domaines de recherche moins étudiés, en particulier dans le contexte des vignes ou de différentes espèces de raisins. Il existe également un manque de connaissances sur les contributions relatives de divers facteurs potentiels (génotype, terroir, etc.) de différentes sources dans le développement de la structure de la communauté de la phyllosphère. Il est donc difficile de tirer une conclusion générale quant à savoir qui est le principal facteur. ? À l'heure actuelle, les facteurs qui déterminent les interactions entre les épiphytes et façonnent les communautés microbiennes sont mal élucidés.

Cette thèse tente de répondre aux questions relatives à la diversité microbienne liée à la surface des feuilles et des fruits et à l'évaluation de la pression de sélection relative exercée par les facteurs potentiels (cultivars de vigne, espèces de vigne de la famille des Vitaceae et terroir) afin de façonner la structure microbienne de la phyllosphère et de la carposher (utilisant des NGS suivies d'analyses de données et de méthodes statistiques robustes), qui fait du terroir un facteur majeur dans la formation de l'assemblage microbien. Nous avons d'abord sélectionné au hasard neuf cultivars de vigne pour chacun des trois principaux pools génétiques existant chez *V. vinifera* cultivé et avons estimé la diversité microbienne dans la phyllosphère de feuilles et de baies dans la région méditerranéenne française. Nous avons observé un impact mineur de la diversité génétique de la vigne dans la formation de la structure de la communauté microbienne dans la phyllosphère (chapitre 2), avec quelques associations de quelques genres bactériens et fongiques à un pool génétique particulier.

Deuxièmement, nous avons répété la même analyse en utilisant les cinq cultivars les plus éloignés génétiquement de chaque pool génétique (échantillonnés dans deux vignobles de la Méditerranée) et avons de nouveau révélé un faible impact du génotype bien que les impacts génotypiques à la surface des fruits semblaient plus importants (Chapitre 3).). Cinq variétés de Vitis vinifera d'importance commerciale, échantillonnées dans trois zones agro-climatiques françaises différentes, ont montré que l'effet du terroir était bien plus fort que celui du génotype (chapitre 3).

Enfin, nous avons maximisé la diversité génétique des échantillons en échantillonnant les feuilles de cinq espèces de raisins appartenant à la famille des vitacées et avons à nouveau exploré la diversité microbienne présente parmi cinq espèces de raisins (chapitre 4). En utilisant la méthode indépendante de la culture, une communauté microbienne diverse et complexe a été observée.

Un impact significatif des espèces de raisin a été observé dans la formation de l'assemblage microbien (en particulier l'assemblage de champignons), mais l'interaction de l'année de croissance et de l'espèce de raisin était statistiquement plus robuste pour définir la structure de la communauté de phyllosphère.

Ces résultats ont confirmé notre hypothèse au chapitre 3 selon laquelle le terroir est le principal facteur mais que les interactions génotype et environnement peuvent également façonner l'assemblage microbien dans la phyllosphère. Une meilleure connaissance des principaux facteurs de la structure de la communauté et des interactions multitrophiques dans la phyllosphère sera la clé du développement de nouvelles idées pour la protection des plantes. Les applications futures de Biocontrol bénéficieront de la recherche fondamentale; autrement dit, mieux nous comprendrons les rôles et l'importance des microorganismes indigènes, mieux nous pourrons prévoir et protéger contre les infections par des agents pathogènes.

Sur une note très différente, nous avons analysé les impacts de méthodes de normalisation de données relativement récentes pour détecter l'abondance de taxons différentiels entre différents groupes et avons prédit les meilleures méthodes de normalisation de données pour les ensembles de données de microbiome. Cela pourrait être utile pour les études portant sur le raisin ou d'autres microbiomes afin de produire des estimations statistiquement plus robustes pour leur hypothèse.

5.2 Le microbiome de la phyllosphère en tant qu'agent de lutte biologique (BCA).

Dans l'intestin humain, plusieurs microbes précédemment considérés comme commensaux sont maintenant considérés comme des symbiotes bénéfiques en raison de leurs contributions au métabolisme et à l'immunité de l'hôte [1]. La déclaration incitative de Janzen [2], selon laquelle «les plantes portent les tripes à l'extérieur», suggère que des questions similaires concernant les avantages pour l'hôte et les interactions microbes-microbes devraient être posées au sujet des communautés associées à des plantes.

Des interactions complexes et multipartites devraient se produire dans la phyllosphère entre divers microorganismes, ainsi qu'entre ceux-ci et les plantes hôtes (interactions parasitaires, commensales et mutualistes, par exemple) [3]. Les avantages de ces interactions pour les microbes sont évidents et incluent un apport de nutriments, mais les avantages fournis par les habitants de la phyllosphère aux plantes hôtes ne sont pas tout à fait clairs. Il a été suggéré que les plantes tiraient profit de la production microbienne d'hormones végétales, telles que les cytokinines et l'acide indole 3-acétique (IAA); Cependant, il y a un manque de rapports suffisants dans ce domaine. Par ailleurs, il existe peu de rapports suggérant une résistance systémique induite par des microorganismes associés à des plantes contre les agents pathogènes dans les plantes [4] et les populations microbiennes indigènes pourraient affecter le résultat des interactions plantes-agents pathogènes dans la phyllosphère [5,6].

Cependant, les mécanismes qui fournissent aux plantes une résistance à un large éventail d'agents pathogènes et qui sont induites par des micro-organismes bénéfiques ne sont pas encore bien établis pour les microbes de la phyllosphère. De même, des études fonctionnelles telles que la métatranscriptomique et leur intégration à des études métagénomiques pourraient révéler le potentiel des microorganismes pour aider à la défense des plantes doivent encore être réalisées pour la phyllosphère. Récemment, Sphingomonas spp. Les isolats ont été identifiés comme étant impliqués dans la protection des plantes contre les bactéries pathogènes dans le système modèle *A. thaliana* [7].

Bien que la base moléculaire de la croissance réduite de l'agent pathogène en présence de Sphingomonas spp. n'est pas encore comprise, les preuves disponibles suggèrent que plusieurs traits contribuent au résultat de la protection des plantes [8]. Un autre rapport [9] a suggéré que les levures associées au raisin sont des agents de biocontrôle prometteurs contre les agents pathogènes fongiques de *Vitis vinifera* (*Botrytis cinerea*). En général, les interactions qui prévalent dans la phyllosphère végétale sont loin d'être comprises et il importera d'obtenir une meilleure compréhension au niveau du système des interactions complexes qui régissent les résultats des membres de la communauté dans le contexte de la plante hôte. De plus, l'application de la structure du microbiome de la phyllosphère pour améliorer la santé des plantes (spécialement les vignes) afin d'augmenter la production de biomasse ne peut être ignorée. Autant d'agents pathogènes bactériens foliaires colonisent la surface des plantes avant l'infection, et la taille des populations finales est souvent corrélée à la gravité de la maladie. Ceci suggère qu'une réduction du nombre d'agents pathogènes conduirait à la protection des plantes [10-12]. Les mécanismes possibles pour supprimer la prolifération des agents pathogènes peuvent inclure l'exploitation de la concurrence pour les nutriments et de l'espace, l'antibiose et la stimulation des réponses systémiques de l'hôte [6,13]. Les stratégies de biocontrôle alternatives proposées utilisent une seule souche protectrice ou une combinaison de souches [14-16].

L'Union européenne a restreint l'utilisation de fongicides chimiques en raison du nombre toujours croissant de résidus dangereux dans l'environnement et les chaînes alimentaires. De nouveaux microorganismes phyllosphériques antagonistes naturels contre les maladies bactériennes ou fongiques pourraient aider la production agricole à réduire les pertes avant et après la récolte, à renforcer les pratiques plus sûres pour les travailleurs et à protéger la santé du consommateur.

L'avènement des technologies de séquençage de prochaine génération (NGS) est en train de conduire un changement de paradigme qui permet aux chercheurs d'intégrer les études sur les communautés microbiennes à l'approche traditionnelle du biocontrôle. Cette intégration pourrait répondre à des questions scientifiques pertinentes et développera de nouvelles hypothèses de biocontrôle utilisant des microorganismes phyllosphériques.

Un autre domaine d'application potentiel pour les habitants de la phyllosphère serait la phytoremédiation (peut également être appelée phylloremédiation) utilisant des microorganismes de la phyllosphère pour éliminer les polluants volatils tels que le phénol ou le benzène [17,18].

5.3 Ingénierie du microbiome

Il utilise les principes de base de la génétique quantitative et de l'écologie des communautés et consiste en des méthodes expérimentales qui améliorent les performances de l'hôte en sélectionnant artificiellement des communautés microbiennes ayant des effets spécifiques sur les performances ou la forme de l'hôte [19]. Les performances de l'hôte peuvent inclure tout trait de caractère important sur le plan biologique, médical ou économique (par exemple, le taux de croissance ou la résistance aux maladies). La sélection artificielle sur les microbiomes est appliquée sur plusieurs générations et de manière indirecte, ce qui signifie que les traits de l'hôte permettent de déterminer si le microbiome de l'hôte parvient à «se reproduire» via le passage expérimental à la prochaine génération d'hôtes. L'ingénierie du microbiome applique une sélection artificielle multigénérationnelle sur des hôtes dont le contenu du microbiome varie selon le trait de l'hôte [19]. Les microbiomes d'animaux et de plantes englobent des habitats pour diverses communautés microbiennes et ces microbiomes renforcent les fonctions de l'hôte, contribuant ainsi à la santé et à la forme physique de l'hôte. Une nouvelle approche pour améliorer la condition physique des plantes ou des animaux consiste à sélectionner artificiellement les microbiomes, ce qui permet de concevoir des microbiomes évolués ayant des effets spécifiques sur la forme physique de l'hôte. Ce processus est appelé sélection du microbiome par l'hôte (HMMS). Un objectif de recherche préliminaire dans les études sur le microbiome est d'élucider les fonctions du microbiome qui altèrent les performances de l'hôte. Plusieurs approches complémentaires ont permis de différencier les effets bénéfiques, neutres et néfastes de la forme physique de l'hôte [20,21]. Les principales approches pour étudier les fonctions du microbiome comprennent les analyses de corrélation, la génomique unicellulaire, la métagénomique globale, la métaprotéomique et les manipulations expérimentales [22-25]. Quelles que soient ces approches, il reste difficile d’élucider les rôles fonctionnels spécifiques du microbiome dans la définition des caractéristiques de performance de l’hôte (par exemple, la croissance, la santé, l’antibiose et l’aptitude générale). La complexité des propriétés du microbiome, qui peut être dictée par les interactions entre les taxons au sein de la communauté du microbiome, peut varier en fonction du génotype de l’hôte, de son environnement ou de ses interactions [26,27].

HMMS est une méthode nouvelle et innovante qui a été utilisée avec succès pour la rhizosphère de microbiomes de plantes hôtes d'Arabidopsis thaliana [28,29]. Après stérilisation du sol initial, les plantules ont été inoculées avec une communauté de sol de départ. À la fin de chaque cycle de

croissance (ou génération), un trait de l'hôte (par exemple, la biomasse des pousses de plantes ou le temps de floraison) a été mesuré pour chaque réplicat; ensuite, les sols des répliques les plus performantes (ou les moins performantes) ont été choisis pour ensemencer la prochaine génération de sols stérilisés des lignées haute et basse respectives (figure 1). Ce schéma a propagé tous les micro-organismes viables (par exemple, virus, bactéries, champignons, nématodes et acariens) d'une communauté parentale à la génération suivante, alors que les plantes hôtes n'ont pas évolué d'une génération à l'autre, car toutes les graines ont été prélevées dans le stock d'Arabidopsis. ligne. Dix cycles de sélection sur des microbiomes du sol ont produit des phénotypes de plantes très différents entre les lignées de sélection du microbiome dans les deux études [28,29].

Une autre variante de HMMS consiste à ne propager qu'une partie de la communauté associée à l'hôte, par exemple en filtrant les membres de la communauté microbienne avec des cellules plus grosses (par exemple, les champignons) tout en ne retenant pour la co-propagation que les membres plus petits de la communauté (par exemple, bactéries et virus). Malgré l'étape de filtrage qui prend beaucoup de temps, élucider les changements dans les microbiomes co-propagés est simplifiée, car seuls les microbes à petites cellules (par exemple, les communautés bactériennes) doivent être analysés. La sélection dans le sous-microbiome sera plus utile pour concevoir certains microbiomes (microbiomes de rhizosphère / phyllosphère, par exemple), mais moins pour les microbiomes dont les composants fongiques et bactériens interagissent fortement (microbiomes de feuilles endophytes). Le mécanisme de fonctionnement de l'ingénierie du microbiome par l'hôte fonctionne probablement au début de l'interaction hôte-microbe. Le contrôle de l'hôte s'effectue via le choix du partenaire ou le dépistage, dans lequel l'hôte modifie de manière sélective le sous-ensemble de microbes autorisés à coloniser ou à rester associés. avec l'hôte (par exemple via la résistance, l'immunité et la spécificité génotypique [30,31].

En utilisant HMMS ou l'ingénierie du microbiome, des fonctions nouvelles et améliorées du microbiome peuvent être sélectionnées sans aucune connaissance préalable de la composition du microbiome, ni de leurs interactions synergiques, mais en quoi la sélection médiée par l'hôte modifie-t-elle la composition du microbiome? L'ingénierie du microbiome peut probablement altérer les microbiomes par le biais de processus écologiques et évolutifs [19].

Les processus écologiques comprennent les modifications de l'uniformité et de la diversité des communautés, l'abondance relative des espèces et la structure des réseaux d'interactions microbes-microbes et hôtes-microbes. Les processus évolutifs comprennent l'extinction de certains microbiotes dans la communauté, des modifications de la fréquence des allèles, des mutations et un transfert de gène horizontal qui restructure les génomes microbiens. Les changements écologiques et évolutifs peuvent être suivis avec les méthodes NGS qui déduisent la présence, l'absence et l'abondance des taxons, les fonctions microbiennes actives qui sont exprimées et permettent des

inférences mécanistiques des fonctions du microbiome. L'ingénierie du microbiome par l'hôte est donc une méthode puissante pour manipuler les microbiomes et comprendre leurs fonctions [19]. Il a été prédit que l'ingénierie du microbiome par l'hôte serait souvent plus efficace en utilisant des hôtes sauvages plutôt que des hôtes ayant subi une domestication ou une adaptation à des environnements de laboratoire dépourvus de bactéries microbiennes. En effet, les gènes qui permettent les interactions hôtes-microbes, qui ont pu être perdus lors de la domestication [32,33], et les microbiomes des sols agricoles variaient probablement beaucoup entre les générations de plantes successives en l'absence de co-propagation hôte-microbiome. L'ingénierie du microbiome à médiation hôte a diverses applications, en particulier dans la recherche agricole visant à améliorer la productivité des plantes, notamment la résistance à la sécheresse et au sel, et la résistance aux maladies; elle doit être testée pour les microbiomes de phyllosphère/rhizosphère dans les vignes selon un grand nombre de critères de conception. l'ingénierie du microbiome qui pourrait explorer les possibilités d'obtenir les caractéristiques souhaitées chez la vigne.

5.4 Microbiome Phyllosphere pour l'amélioration de la qualité du vin

Le vin contient un mélange complexe de biomolécules et de composés producteurs d'arômes et les micro-organismes y jouent un rôle essentiel. De nos jours, il est de notoriété publique que la fermentation du vin n'est pas un processus impliquant une seule espèce et que le rôle des différents microbes liés au vin dans la production de vin est mis à l'honneur dans les recherches mondiales [34]. Le microbiome du raisin viticole pourrait être lié aux propriétés sensorielles des vins [35] et ce microbiome, qui provient principalement de vignobles, dépend de la situation géographique, du cépage, des conditions climatiques et des pratiques agronomiques (ou du terroir) suggèrent que le profil microbien de raisins pourraient prédire la composition et l'abondance de certains métabolites de l'impact du vin [35-37]. Les futures études pourraient porter sur la formation efficace du microbiote de phyllosphère afin de prédire les profils de métabolites du vin, ce qui pourrait fournir des informations exploitables aux vignerons pour améliorer les caractéristiques du vin (arômes et saveurs par exemple) ou atténuer les problèmes de fermentation tels que l'élimination du soufre. Cependant, il existe de nombreuses espèces microbiennes (épiphytes du raisin fermentatives et dominantes) pouvant avoir une incidence sur le goût du vin [37], mais dont le rôle spécifique dans la fermentation du vin est encore mal compris et pourrait être exploré dans de futures études. Une fois que nous aurons mieux compris quelles espèces microbiennes (épiphytes ou microbes du vin indispensables) ont une influence sur la saveur du vin ou sur la santé et la productivité de la vigne, nous pouvons commencer à déterminer comment certains de ces microorganismes pourraient être appliqués de manière sélective à cette culture.

La fermentation alcoolique est principalement réalisée par Saccharomyces cerevisiae, conduit à la

formation de nombreux alcools et esters [38]. En règle générale, la plupart des composés d'arôme de fermentation ont des seuils sensoriels élevés et ne contribuent donc pas de manière significative, individuellement, à l'arôme distinctif des vins. Cependant, leur combinaison constitue la matrice de base de l'arôme du vin. Les bactéries lactiques (LAB) sont l'un des groupes de microorganismes les plus importants dans la vinification et sont responsables de la décarboxylation de l'acide malique en acide lactique dans un processus connu comme la fermentation malolactique qui offre des avantages supplémentaires, comme la stabilité microbienne et la complexité accrue de l'arôme [39].
L'utilisation de différentes levures ou souches bactériennes pour la fermentation du vin pourrait contribuer à des variations significatives des niveaux d'alcool plus élevés dans le vin [40,41]. Lachancea thermotolerans, Hanseniaspora uvarum, Candida zemplinina, Saccharomycodes ludwigii et Pichia anomala ont été décrits comme des producteurs d'alcool à haute teneur en fusel lorsqu'ils sont utilisés dans des fermentations mixtes avec S. cerevisiae, généralement avec indépendance par rapport aux inoculums utilisés. Par ailleurs, les espèces de Candida stellata et de Zygosaccharomyces fermentati ont été signalées comme étant de faibles producteurs, lorsqu'ils étaient utilisés comme inoculums uniques et en co-inoculation avec S. cerevisiae [42].
La présence de composés soufrés indésirables tels que l'hydrogène sulfuré (H_2S) dans le vin est un problème important pour l'industrie vinicole, car elle confère des arômes indésirables tels que des arômes de soufre ou d'œufs pourris, ainsi qu'en raison de son seuil de perception bas (10 à 80 µg / L).) [43]. La levure viticole affecte fortement la production de H_2S pendant la fermentation du vin, la production principale se produisant lors de la biosynthèse d'acides aminés soufrés tels que la cystéine et la méthionine à partir de sources de soufre inorganiques et organiques [44,45].
L'une des rares stratégies visant à réduire la production de H_2S est l'application de levures non conventionnelles dans la vinification. L'activité sulfite réductase est l'une des principales activités enzymatiques responsables de la production de H_2S et est une caractéristique rare de la majorité des espèces non Saccharomyces [46], étant donné que seules les espèces du genre *Hanseniaspora* (principalement H. osmophila et H. opuntiae) activité sulfite réductase parmi les 15 espèces testées. En outre, certaines souches de *T. delbrueckii*, à l'exception de S. cerevisiae, avaient une certaine capacité de production de H_2S. Cependant, comme chez S. cerevisiae, un autre comportement dépendant de la souche existe chez d'autres espèces de levure liées au vin, telles que *Dekkera*, *Lachancea*, *Hanseniaspora* et *Metschnikowia*. Les efforts futurs devraient viser à identifier les souches de carposphère ou les groupes de souches susceptibles de réduire l'activité de la sulfite réductase dans le processus de vinification afin de fournir des vins de meilleure qualité.

5.5 References

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ANNEXES

ANNEXES

List of abbreviations

AMF	Arbuscular Mycorrhizae Fungi
ANOVA	Analysis of Variance
ARISA	Automated Ribosomal Intergenic Spacer Analysis
ASV	Amplocon sequence variants
BCA	Biocontrol Agents
CSS	Cumulative Sum Scaling
cv	Cultivar
DADA	Divisive Amplicon Denoising Algorithm
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxy Ribonucleic Acid
FDR	False Discovery Rate
FS	Frameshift
ITS	Internal Transcribed Regions
KEGG	Kyoto Encyclopedia of Genes and Genomes
WE	Wine East
WW	Wine West
µm	Micro Meter
LAB	Lactic Acid Bacteria
MDS	Multi Dimensional Scaling
MG-RAST	Metagenomic Rapid Annotations using Subsystesm Technology
NB	Negative Binomial
NCBI	National Centre for Bioinformatics
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Units
PERMANOVA	Permutational Analysis of Variance
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
PMCs	Phyllosphere Microbial Communities
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RSV	Ribosomal Sequence Variants

SSCP	Single-strand Conformational Polymorphisms
SSR	Simple Sequence Repeat
Spp.	Species
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative PCR
TE	Table East
T-RFLP	Terminal Restriction Fragment length polymorphisms
UNITE	User-friendly Nordic ITS Ectomycorrhiza Database
UV	Ultra Violet
ZIG	Zero-inflated Gaussian

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unique taxon. The y-axis represents the fraction of samples, these taxa are present.

Figure 3. Relative abundances of (A) bacterial and (B) fungal genera present on each cultivar, grouped within their genetic pools (9 cultivars per genetic pool, top 20 taxa, characterized to the genus level and datasets were rarified to 5,000 sequence reads per sample). Chao1 estimates of α-diversity for (C) bacterial and (D) fungal data-sets for three genetic pools. PCoA plots using Bray-Curtis distance between samples for (E) bacterial and (F) fungal data-sets among three genetic pools, explaining >60% variations with first two axes (taxa with variance < 1e-05 were trimmed).

Figure 4. Relative abundances of (A) bacterial and (B) fungal genera present on leaf and berry samples, also grouped within their genetic pools (top 20 taxa, characterized to the genus level, datasets were rarified to 5,000 sequence reads per sample). Chao1 estimates of α-diversity for (C) bacterial and (D) fungal data-sets for both the organ types. PcoA plots using Bray-Curtis distance between samples for (E) bacterial and (F) fungal data-sets as per leaf and berry samples based on Bray-Curtis distance matrices, explaining >60% variations with the first two axes (taxa with variance < 1e-05were trimmed).

Supplementary figure S1. PCoA plots using Bray-Curtis distance between samples for (A) bacterial and (B) fungal data-sets as per samples from 27 cultivars based on Bray-Curtis distance matrices, explaining >60% variations with the first two axes (taxa with variance < 1e-05 were trimmed).

Chapter 3

Figure 1. PCoA analysis on leaf data (A, B); PCoA plots representing the compositional dissimilarity in leaf communities (both axis covered >40% of the variation) and Observed (C) bacterial and (D) fungal α-diversity measures of each variety (X-axis) grouped in two season and relative abundances of (E) bacterial and (F) fungal Phylum during spring and harvest season. N = 180.

Figure 2. PCoA on (A) bacterial and (B) fungal microbiome data of berry displaying the impact of genetic pools on taxa structuring on the surface (both axis covered >25% of the variation in data). N = 33.

Figure 3. PCoA plot representing compositional dissimilarity for (A) bacterial and (B) fungal population between leaf and berry samples (both axis covered ~40% of the variation) and Observed α-diversity measures for (C) bacteria and (D) fungi for two organs. N = 123.

Figure 4. Set2 microbiome data. PCoA plots displaying strong (A) bacterial and (B) fungal compositional dissimilarity among agro-climate zones and Observed (C) bacterial and (D) fungal α-diversity measures of each variety (X-axis) grouped in three agro-climate zones and relative abundance plot for (E) bacterial and (F) fungal genera displaying differential abundance of few

genera among three agro-climate zones (or region). N = 45.

Supplementary Figure S1. Relative Abundance major species between two season, displaying the uniqueness of the fungal microbiome structure at Spring and Harvest season.

Supplementary Figure S2. Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three genetic pools). Set1. N = 213.

Supplementary Fig S3. Relative abundances of major (A) bacterial and (B) fungal taxa (top 25, at genus level) of each cultivar (grouped in three geographic locations). Set2. N = 45.

Chapter 4

Figure 1. Genus level characterization of Bacterial and Fungal diversity present on leaf phyllosphere of 5 grape-species (relative abundance of top 25 genus).

Figure 2. PCoA ordinations of (A) bacterial and (B) fungal communities derived from leaf phyllosphere at two growing years, using Bray-Curtis distance matrix. Both the axis explains ~ 20% of variations (Shape represents grape species). N = 30.

Figure 3. PCoA ordinations of bacterial (A, B) and fungal (C, D) communities derived from leaf phyllosphere at spring 2017 and spring 2018 separately, using Bray-Curtis distance matrix.

Figure 4. Observed α-diversity measures for (A) bacterial and (D) fungal data for each grape species in Spring of 2017 and 2018. N = 30.

Figure 5. Histograms of the distribution of taxa sums across samples using (A) no normalization (B) Square root transformation (C) log transformation and (D) CSS transformations.

Figure 6. Different normalization methods identified several bacterial taxa that significantly contributed (adj $P < 0.05$) to differences between two growing years.

Figure 7. Different normalization methods identified several fungal taxa that significantly contributed (adj $P < 0.05$) to differences between two growing years.

Chapter 5

Fig 1. Artificial selection on microbiomes in the plant rhizosphere. At the start of each experiment, microbiomes differ in community composition between hosts; host–microbiome associations are allowed to mature (Step 1), then are phenotyped for the trait used as a direct target for indirect selection on microbiomes (Step 2), then microbiomes are chosen for transmission to the next generation of hosts (Steps 3 and 4). The most extreme host phenotypes are chosen to identify microbiomes for propagation (microbiomes from the largest plant) [Muller & Sachs, 19].