Metagenomics has revolutionized the agricultural microbial ecology. In particular, the adoption of such technique in viticulture dates back to 10 years, where the microbial diversity and composition of grapevine and wine environment have been studied.
The aim of this study is to characterize the bacterial diversity and composition of the soil and *H. zuri* grape variety all over the 3 Designation of Origin of txakoli in the Basque Country, using high-throughput sequencing. This research shows that soil and grape bacteriome differs significantly among vineyards, but no DO-specific bacterial profile was identified, suggesting that the Txakoli viticultural region of the Basque Country represents a single bacterial terroir. Yeast and fungal studies that track microorganisms in the field, cellar and in winemaking processes are needed to unravel whether the final wine has a microbial terroir and at what step differentiation begins. Soil samples bacterial communities are driven by pH, while grape bacteriome is influenced by rootstock genotype. Interestingly both soil and grape microbiota were shown to be affected by distance from the sea or the influence of sea breeze. Further analyses should be conducted to gain more knowledge on the influence of microclimatic factors on grape microbial ecology, so that wine growers can use this information to improve grape quality and wine characteristics.

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

2021

Txakoli vineyard's microbiome: an omic perspective

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

Txakoli vineyard's microbiome: an omic perspective

PhD Thesis

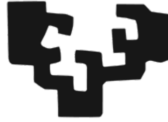
By

Igor Baroja Careaga

Supervised by **Andone Estonba Rekalde** and **Iratxe Zarraonaindia Martínez**

Universidad del País Vasco UPV/EHU
2021

eman ta zabal zazu



Universidad del País Vasco — Euskal Herriko Unibertsitatea

Txakoli vineyard’s microbiome: an omic perspective

PhD Thesis

By

Igor Baroja Careaga

Supervised by **Andone Estomba Recalde** and **Iratxe Zarraonaindia Martínez**

Universidad del País Vasco UPV/EHU

2021

(c) 2021 Igor Baroja Careaga

Metagenomics has revolutionized the agricultural microbial ecology field by making progress on our knowledge about microbe/environment, microbe/vine and microbe/microbe interactions in a more holistic view, shedding light into the role of different players in plant productivity and disease resistance. In particular, the adoption of such technique in viticulture dates back to 10 years, where the microbial diversity and composition of grapevine and wine environment have been studied, such as the microbial diversity in vineyard and wine fermentation, terroir markers, the influence of viticultural practices in these communities, etc.

The aim of this study is to characterize the bacterial diversity and composition of the soil and *H. zuri* grape variety all over the 3 Designation of Origin of txakoli in the Basque Country, using high-throughput sequencing. This research shows that soil and grape bacteriome differs significantly among vineyards, but no DO-specific bacterial profile was identified, suggesting that the Txakoli viticultural region of the Basque Country represents a single bacterial terroir. Yeast and fungal studies that track microorganisms in the field, cellar and in winemaking processes are needed to unravel whether the final wine has a microbial terroir and at what step differentiation begins. Soil samples bacterial communities are driven by pH, while grape bacteriome is influenced by rootstock genotype. Interestingly both soil and grape microbiota were shown to be affected by distance from the sea or the influence of sea breeze. Further analyses should be conducted to gain more knowledge on the influence of microclimatic factors on grape microbial ecology, so that wine growers can use this information to improve grape quality and wine characteristics.

Acknowledgment

Lehenik eta behin, ikerketa hau ahalbidetu duten bi pertsonei eskertu nahi diet, nire zuzendariak izan diren Andone eta Iratxeri. Zuengatik ez balitz doktorego hau ez litzateke aurrera eramango, eta urteotan ikasi dudan guztiak ez du presiorik. Bereziki Iratxeri eskertu nahi diot, haurdunaldi bat pasa badu ere, beti egon delako hor, nire ondoan edozertan laguntzen.

My sincere thanks also goes to Dr. Dario Cantu, for offering me the internship opportunity in his UC Davis group and helping me with my project. And of course, to everybody in the Cantu Lab, thanks you for doing my stay unforgettable. Mireia, Jadran, Daniela, Noe, thank you for everything.

Lankideen artean sortutako adiskide berriei ere eskerrak eman behar dizkizuet, Abereak taldeko denei, Sofia, June, Iratxe A. eskerrik asko sortutako lan giro paregabeagatik. Marta, estuviste en mis comienzos con la Bioinformatica y todo ese aprendizaje no hubiera sido lo mismo sin ti. Melanie siempre dispuesta a ayudar como sea, gracias por todo. Y Jorge, aunque algunas veces me has puesto de los nervios, siempre has podido ayudarme con lo que sea, gracias. Eta noski, Irati eta Fer, eskerrik asko nire sekuentziazio guztiekin laguntzearren, ikerketa ez litzateke aurrera joango zuengatik ez balitz. Y también a Elena García, que ha sido la que me ha ayudado con todas las dudas enológicas.

Genetikako lankideetaz ere ezin nahiz ahaztu, Endika, Alejandro, Martin, plazer bat izan da zuen alboan lan egitea. JonVa, Anne, Gartze, JonLa zuek izan zinaten nire posizioan zeundeten lehenak eta zuen ikerketak aurrera zihoazela ikustea, inspirazio bat izan da niretzat. Eskerrik asko baita ere 11etako pintxo galezinegatik. Naiara, jada laborategian ez bazaude ere, zu izan zara laborategia erakutsi zenidana, eta asko ikasi dut zuregatik. Eta nola ez, Mery eta Bea, batera hasi genuela aventura hau, oidik gogoan dakat gabonetako bazkarian beka eman ziguten momentua. Bea, zuk ikerketarekin jarraituz aparte, ume bat ekarri duzu mundura, increible!!!. Ta Mery, ze ingo nun nik zu gabe, dena batera egin dugula. Doktoretza hau ez zen izango berdina zu gabe, eskerrik asko bene-benetan.

Eta noski, familia eta lagunei ere, soportau behar izan duzuen guztiagatik, Gure Kai, Kulunka, Top 4, kuadrillakuak, nik uste nire Tesiaren gora-berak aurrera eramaten lagundu diazuen psikologuak izan zeratela, beraz, eskerrik asko.

Eta bukatzeko, Aita eta Ama, eskerrik asko emandako guztiagatik.

INDEX

INTRODUCTION - 1 -

1.1 GENERAL ASPECTS - 3 -
1.2 VINEYARD ASSOCIATED MICROBIAL ECOLOGY - 5 -
1.3 NEXT GENERATION SEQUENCING IN GRAPEVINE AND WINE ENVIRONMENTS - 6 -
1.4 FACTORS INVOLVED IN VINE-ASSOCIATED MICROBIOTA DISTRIBUTION - 9 -
1.5 TXAKOLI: THE CASE STUDY - 12 -

OBJECTIVES - 15 -

MATERIALS AND METHODS - 19 -

3.1 SAMPLES STUDIED - 21 -
3.1.1 SOIL SAMPLE COLLECTION - 22 -
3.1.1.1 Soil chemical analysis - 23 -
3.1.2 GRAPE SAMPLES COLLECTION - 23 -
3.1.2.1 Must chemical analysis - 23 -
3.2 DNA EXTRACTION, LIBRARY PREPARATION AND SEQUENCING - 24 -
3.2.1 SOIL SAMPLES - 24 -
3.2.2 GRAPE SAMPLES - 24 -
3.3 SEQUENCE PROCESSING - 25 -
3.4 STATISTICAL ANALYSIS - 26 -
3.5 METHODOLOGICAL LIMITATIONS - 27 -

RESULTS AND DISCUSSION - 31 -

4.1 CHAPTER 1: SOIL MICROBIAL BIOGEOGRAPHY IN TXAKOLI - 33 -
4.1.1 RESULTS - 33 -
4.1.1.1 Sequencing performance - 33 -
4.1.1.2 Bacterial richness differs between vineyards and correlates with pH - 34 -
4.1.1.3 Soil bacterial communities' dissimilarity at different biogeographical scales - 36 -
4.1.1.3.1 Between viticultural regions: Basque Country vs. two locations in the USA - 36 -
4.1.1.3.2 Within the Basque Country: 41 vineyards - 39 -

4.1.2 DISCUSSION - 48 -
4.2 CHAPTER 2: *H. ZURI* GRAPE MICROBIAL BIOGEOGRAPHY - 55 -
4.2.1 RESULTS - 55 -
4.2.1.1 Sequencing performance - 55 -
4.2.1.2 Bacterial richness and composition differ between vineyards and rootstock genotype - 55 -
4.2.1.3 Differentially abundant OTUs responding to Rootstock genotype - 61 -
4.2.2 DISCUSSION - 65 -

GENERAL DISCUSSION - 71 -

CONCLUSION - 77 -

SUPPLEMENTARY DATA - 81 -

DATA AVAILABILITY STATEMENT - 91 -

REFERENCES - 95 -

INTRODUCTION

1 Introduction

1.1 General aspects

Grapevine (*Vitis vinifera* L.) is one of the oldest and most economically important fruit species in the world. It is mainly used for wine and spirit production but also for fresh fruits, raisins, juice and jams, etc. (Bouquet, 2011; Soneji and Nageswara-Rao, 2011). Wine is an integral part of food culture in Mediterranean Europe, from where its consumption has spread to many other regions of the world. France, Italy and Spain are the largest European wine producing countries representing altogether more than a half of the world wine production (http://www.oiv.int/). Spain is first in the ranking of planted surface area; third in wine production in the 2020 campaign (after Italy and France), and the second exporter in terms of volume in 2020 (after Italy), third in terms of value. Wine has become an extremely important sector in Spain not only for its importance in economics, but also in social and environmental terms, as well as the importance of wine as an image of the country worldwide.

There are currently 96 Protected Designations of Origin (PDO) for wine in Spain, 70 Designations of Origin (DO) among them (Figure 1). One of the most important Spanish wine regions is Rioja, with a lot of tradition in the cultivation of the vine and the elaboration of wines of quality and also was the first brand recognized as DO in Spain.

Until the 1980s in the Basque Country, a home-made wine was elaborated, named Txakoli. It was almost in danger of dying out towards the middle of the 19th century (Barreda, 2001; Pauls and Facaros, 2003), but 1989 onward, quality, spread and appeal of the Txakoli wines began to emerge. Some varieties managed to achieve certification, and this way, the Txakoli gained acceptance from the consumer and the society in general. Nowadays, the Basque Country holds 3 Designations of Origin (DO) of Txakoli, one per province.

INTRODUCTION

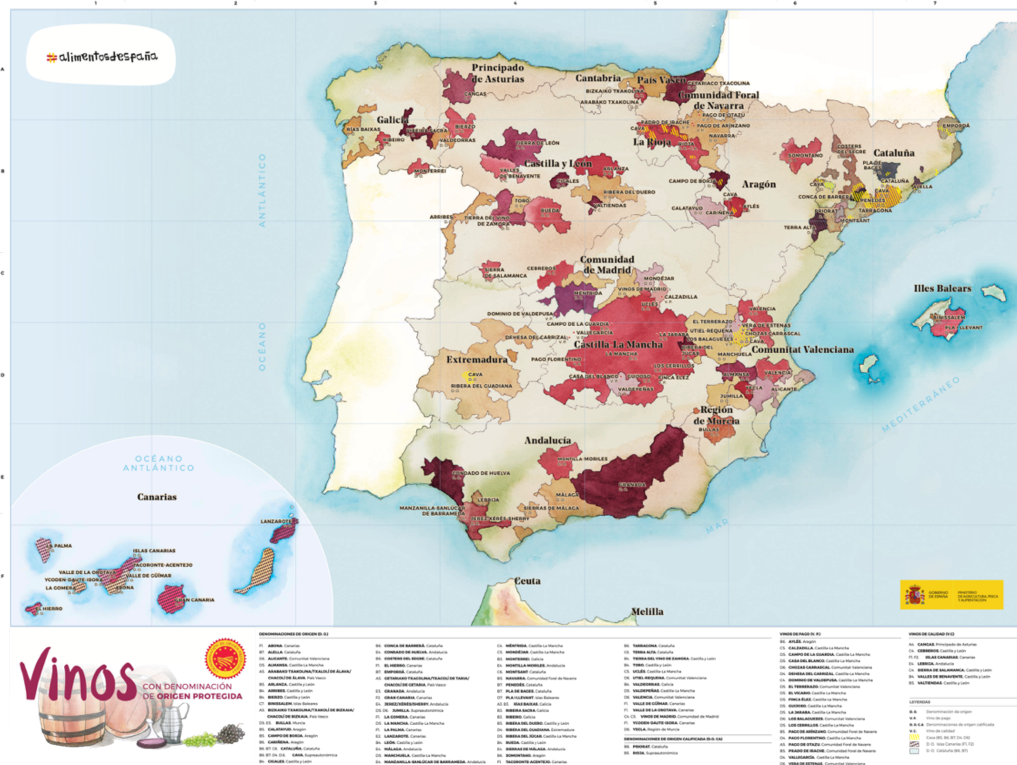


Figure 1: Map with the Designations of Origin (DO) in Spain nowadays.

Vine's environment (soil, climate), species and varieties, viticultural practices and enological practices are fundamental factors that influence grape and wine quality (van Leeuwen, 2010). In addition, the plant microbiota emerges as a novel trait that extends the capacity of plants to adapt to the environment (Bulgarelli et al., 2013) and impact grape and wine quality (Bokulich et al., 2012a, 2014, 2016; Gilbert et al., 2014). The microbiota associated to soil and plant is involved in plant nutrition and resistance to biotic / abiotic stress, therefore it plays an important role in the growth and survival of the plant offering indirect pathogen protection (Lugtenberg and Kamilova, 2009; Bhattacharyya and Jha, 2012). In addition, vineyards harbor a wide variety of microorganisms that play a pivotal role in pre- and post-harvest grape quality and will contribute significantly to the final aromatic properties of wine (Nisiotou et al., 2011;

Bokulich et al., 2012a, 2016; Setati et al., 2012). Therefore, further knowledge of the vineyard and wine associated microbiota would help facing viticulture challenges related with the improvement on productivity, grapevine health and quality of wine.

1.2 Vineyard associated microbial ecology

Historically, microbial surveillance of the vine and wine environment has been done based on culture-based methods, while most of the studies were focused in the fermentation process of wines, studies from soil and grape were less extended. The traditional culture-based methods have allowed isolating and identifying more than 40 yeast species (Jolly et al., 2014), 50 bacterial species (Barata et al., 2012) and 70 genera of filamentous fungi (Rousseaux et al., 2014) associated with grapevine and wine fermentation processes. The most widely known cultivable bacteria from vineyard and winery environment are acetic acid bacteria (AAB; e.g., *Acetobacter* and *Gluconacetobacter*) and lactic acid bacteria (LAB; e.g., *Lactobacillus, Oenococcus,* and *Pediococcus*). Among yeasts, Saccharomyces members are known to be main fermentation agents commonly used as inoculant (e.g., *Saccharomyces cerevisiae, S. bayanus, S. pastorianus,* and *S. paradoxus* among others), and wine spoilers (e.g., *Brettanomyces/Dekkera, Issatchenkia, Zygoascus,* and *Zygosaccharomyces*) have received most of the attention (Loureiro, 2003; Malfeito-Ferreira, 2011). Barata and colleagues (Barata et al., 2012) divided those species into 3 big groups: (i) easily controllable or innocent species, without the ability to damage wine when good manufacturing practices (GMP's) are applied; (ii) fermenting species, responsible for sugar and malic acid conversion; and (iii) spoilage *sensu stricto* species responsible for wine alteration, even when GMP's are believed to be applied.

However, cultivation-based methods are time consuming and laborious (Andorrà et al., 2008; Sun and Liu, 2014). Importantly, it only detects species that are able to grow on the culture media and under the cultivation conditions, which is estimated by environmental microbiologists to be less than 2% of the total community (Wade, 2002). In addition, the high concentration of ethanol, the addition of SO_2 etc. makes the wine a stressful environment where some of the bacteria and yeast enter in a Viable but Non-Culturable

state (VBNC) to survive (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). Those microorganisms (e.g. *Candida stellata, Brettanomyces bruxellensis, S. cerevisiae, Zygosaccharomyces bacilli,* etc.) will not grow on culture media (Salma et al., 2013) but still affect the fermentation performance and flavor, and yet they go undetected by traditional culture-based methods (Belda et al., 2017). The mentioned limitations fostered the search for culture-independent molecular approaches (Nocker et al., 2007) associated to vine and winemaking.

The introduction of molecular methods that allowed the exploration of microbial community structure without the need to cultivate enhanced the understanding of the microbial dynamics throughout grape berry ripening and wine fermentation. While quantitative real time PCR (qPCR) has been the most adopted strategy to detect and quantify wine spoilers and VBNC strains e.g., *Brettanomyces spp.* (Tofalo et al., 2012), DNA-based community fingerprinting methods such as SSCP, T-RFLP, ARISA and mostly DGGE, arose as quick and non-expensive approach to provide a community view (rather than targeting particular species). Despite the advantages, those approaches suffer from some drawbacks, as they biased species richness estimates often underestimating it (David et al., 2014), and show low sensitivity to detect low abundance species (Andorrà et al., 2010; Neilson et al., 2013). This is crucial for instance when studying diverse environmental samples (for example soil or rhizosphere).

1.3 Next generation sequencing in grapevine and wine environments

The identification of evolutionarily stable molecular marker genes, such as ribosomal RNA (rRNA) genes, improved our ability to identify microbial species with better resolution and reliability (Juste et al., 2008; Solieri and Giudici, 2008; Cocolin et al., 2013; Sun and Liu, 2014; Wang et al., 2014; Abbasian et al., 2015). Furthermore, the recent advances in massively parallel sequencing revolutionized the microbial ecology field (Humblot and Guyot, 2009; Roh et al., 2010; Alegría et al., 2012; Bokulich et al., 2012b, 2012a, 2013; Bokulich and Mills, 2013a; De Filippis et al., 2013). Amplicon sequencing, often called metagenomics, metabarcoding or environmental genomics, is a culture-

independent approach for taxonomic, phylogenetic, or functional profiling of microbial communities, directly from environmental DNA without prior enrichment or cultivation of the target population (Franzosa et al., 2015).The approach shows great resolution capacity that allows the simultaneous detection of thousands of taxa, as well as the simultaneous analysis of a large number of samples at a relatively low cost (Gilbert and Dupont, 2011). This methodology is showing a greater microbial complexity than the previously described associated to both the vine (Leveau and Tech, 2011; Pinto et al., 2014; Zarraonaindia et al., 2015) and the must and fermentation (Bokulich et al., 2012a; Piao et al., 2015; Portillo and Mas, 2016; Stefanini et al., 2016). However, the vast majority of the microorganisms detected by metagenomics have previously gone undetectable by traditional techniques and their role in vine health and grape quality is to be still resolved.

In amplicon sequencing DNA is first extracted directly from the biomass of the original sample (soil, root, grape, etc.) without culturing or cloning the microorganisms contained within it. Then, a phylogenetic gene is amplified through PCR with barcoded primers, enabling pooling high number of samples together. The target marker genes contain conserved fragments to facilitate targeting all members of a community and variable regions to allow for the discrimination of different species within the community (Juste et al., 2008; Cocolin et al., 2013; Sun and Liu, 2014; Wang et al., 2014). While the 9 hypervariable regions (V1–V9) of 16S rRNA have all been targeted for the estimation of vineyard bacterial diversity (Leveau and Tech, 2011; Campisano et al., 2014; Perazzolli et al., 2014; Bokulich et al., 2015, 2016; Burns et al., 2015; Calleja-Cervantes et al., 2015; Piao et al., 2015; Pinto et al., 2015; Zarraonaindia et al., 2015; Holland et al., 2016; Marzano et al., 2016; Portillo et al., 2016), most of the studies used the V4 region as it has been proven to have greater taxonomic depth for certain Proteobacteria and lactic acid bacteria (LAB) species (Bokulich et al., 2012a). For the estimation of fungal diversity, the ITS regions are the most commonly targeted (Bokulich et al., 2013, 2015, 2016; Setati et al., 2015; Bouffaud et al., 2016; Holland et al., 2016; Kecskeméti et al., 2016; Marzano et al., 2016; Stefanini et al., 2016), although D1–D2 regions of the 26S rRNA (Holland et

al., 2014; Taylor et al., 2014) and the partial 18S rRNA gene have also been studied (Lumini et al., 2009; David et al., 2014; Holland et al., 2016; De Filippis et al., 2017; Grangeteau et al., 2017). Overall, the ITS1 locus appears to be the most promising target for a complete overview of the microbial populations in ecological studies, demonstrating higher levels of taxonomic classification accuracy (species and genus), the smallest difference between Ascomycota and Basidiomycota amplicon lengths, as well as a maximized sequence coverage (Morgan et al., 2017).

Fragments originated after the PCR amplification of the targeted gene are then sequenced in a next generation sequencer. In 2006, Roche's 454 pyrosequencing (Margulies et al., 2005) became the first high-throughput sequencing technology to be successfully applied for biodiversity analysis and was key to uncovering the 'rare biosphere' (Sogin et al., 2006). However, Illumina sequencers, despite having short read lengths (150-300 bp), became the preferable choice due its reduced per base costs and comparatively high sequencing depth (Caporaso et al., 2012).

However, different biases have been described in multiple steps of the process; 1) different DNA extraction methods can produce different results (Keisam et al., 2016). 2) The choice of primers and targeted variable region will bias identification and quantification (Soergel et al., 2012; Bokulich and Mills, 2013b), where members of a microbial community may be omitted, or misrepresented, typically due to primer mismatches or PCR biases (Acinas et al., 2005; Hong et al., 2009; Lee et al., 2012; Pinto and Raskin, 2012; Logares et al., 2014), while other primers might favor certain species amplification (Baker et al., 2003; Sipos et al., 2007; Klindworth et al., 2013). 3) The taxonomic assignment of the sequences relies on the completeness of public databases. For certain groups a reliable identification farther down to genera level is not usually possible (Belda et al., 2017).

Besides these pitfalls, metagenomics has revolutionized the agricultural microbial ecology field by making progress on our knowledge about microbe/environment,

microbe/vine and microbe/microbe interactions in a more holistic view, shedding light into the role of different players in plant productivity and disease resistance. In particular, the adoption of such technique in viticulture dates back to 10 years, where the microbial diversity and composition of grapevine and wine environment have been studied, such as the interference of microorganisms in plant physiology (Compant et al., 2010; Martins et al., 2013), microbial diversity in vineyard (Pinto et al., 2014; Zarraonaindia et al., 2015), microbial diversity in wine fermentation (Bokulich et al., 2012b; Piao et al., 2015; Pinto et al., 2015; Stefanini et al., 2016), microbial contribution in wine chemistry (Burns et al., 2016; Grangeteau et al., 2017), terroir markers (Bokulich et al., 2014; Burns et al., 2015, 2016) the influence of viticultural practices in these communities (Setati et al., 2015; Kecskeméti et al., 2016; Marzano et al., 2016).

1.4 Factors involved in vine-associated microbiota distribution

Plant-associated bacteria colonize both exterior and interior surfaces, being the soil surrounding plants the most likely origin for many of these organisms (Zarraonaindia et al., 2015; Morrison-Whittle and Goddard, 2018). Although, it is generally assumed that many bacterial endophyte communities are the product of a colonizing process initiated in the root zone (van Peer et al., 1990; McInroy and Kloepper, 1995; Sturz et al., 2000; Welbaum et al., 2004), they may also originate from other source than the rhizosphere, such as the phyllosphere (Hallmann et al., 1997). Bulgarelli and colleagues (Bulgarelli et al., 2013) proposed a model of root selection for soil microbiota whereby the composition of the soil microbiome is defined by edaphic factors, followed by substrate-driven community selection within the rhizosphere, and finally by host genotype-dependent tuning of endophytic bacteria that colonize roots and, eventually, leaves and reproductive structures.

Phylloxera

The European grapevine is a particularly interesting crop model system to explore this concept. In the late 19th century, the European wine industry was devastated by the spread of phylloxera (Daktulosphaira vitifoliae), an aphid-like insect introduced from North America. To stop the destruction of European viticulture, the grafting of European grapevine cultivars onto American rootstocks was encouraged in Europe. This was also a strategy adopted in the Txakoli vineyards, although some ungrafted vineyards still remain. Grafting creates a composite plant by surgically attaching the roots from one plant (the rootstock) to the shoot (the scion) of another, joining their vascular and cambial systems. Grafting was originally implemented for easier clonal propagation, but today this method achieves a variety of agricultural goals, including drought tolerance, dwarfing, and disease resistance. Beyond its practical implications, grafting offers a unique opportunity to independently manipulate parts of the plant to under-stand how roots impact shoots, and *vice versa.*

In the model proposed by Bulgarelli et al., (2013) bulk soil microbiota would be governed mainly by edaphic conditions, while the rhizosphere, root and aerial organs (leaves, grape, flower) would have species-specific associations that change little with geographic location (Redford et al., 2010). In accordance, it has been demonstrated that the composition of soil bacterial communities is primarily driven by soil pH (Fierer and Jackson, 2006; Lauber et al., 2009; Qi et al., 2018; Tan et al., 2020), which is believed to be due to the relatively narrow range in pH growth tolerance of bacterial groups (Rousk et al., 2010). Soil pH changes with moisture (Zárate-Valdez et al., 2006), land-use (Tilston et al., 2010) or even with the plant species (Wang et al., 2020) and alters many soil properties, together with soil aggregation and soil nutrients availability that ultimately influences elements uptake of plants (Yang et al., 2010). Interestingly, Zarraonaindia et al., (2015) hypothesized the soil and its associated microbiota to be indirectly involved in wine characteristics. First, according to these authors' studies, the aboveground bacterial community was significantly influenced by soil edaphic factors such as total carbon, moisture and soil temperature, which would ultimately impact the quality of grapes due to changes in nutrient availability for the plant. Second, soil bacterial communities differed between the sampled vineyards in Long Island, and those differences were

reflected in the microbial composition of roots. These root endophytes can shape the microbial assemblages of aboveground organs by changing the endophytic microbial loads in grapes. Third, a significant input of soil microorganisms to grapes through epiphytic migration during harvest was suggested. Those results led Zarraonaindia and colleagues to propose that those soil derived microorganisms could have a greater role than previously anticipated in wine, as they will ultimately end up in the fermentation tanks. Interestingly, Burns and colleagues (Burns et al., 2015) reported that vineyard soil microorganisms are affected by winegrowing region, climate and topography, as mediated in part by soil properties, like pH and soil organic matter, management practices and production systems. Interestingly, distinguishable microbial communities have been shown in viticultural soils defining AVAs (American Viticultural Areas), therefore supporting the evidence of the role of soil-borne microbiota in the Terroir; considered as a physiological response of the vines to soil type, climatic conditions, and vineyard characteristics and management, that gives particular organoleptic properties to the wine (Bokulich et al., 2016).

Regarding aboveground microbiota, epiphytic communities are mostly selected by nutrient-poor conditions and highly variable conditions of temperature, humidity, and UV radiation intensity (Lindow and Brandl, 2003; Whipps et al., 2008), showing differences with the plant genotype, developmental stage... Studies in grape microbiota also revealed the impact of farming practices on the microbial community, highlighting the impact of copper-based pesticides in grape-berry bacterial communities (Martins et al., 2012). Interestingly intra-vineyard microclimate differences have been evidenced to lead to high within block microbial heterogeneity, sometimes shown to be higher than the between vineyards microbial differences. Studies on grape microbial ecology showed there are cultivar specific microbial profiles (Bokulich et al., 2014; Portillo et al., 2016). For instance, Bokulich et al., (2014) evidenced community differences among grape varieties (*Cabernet Sauvignon, Zinfandel* and *Chardonnay*). However, Zarraonaindia and coauthors did not show differences on merlot clonal varieties. Similar to the soil bacterial terroir, Bokulich et al., (2014) showed that Cabernet Sauvignon must from different

growing regions in California could be distinguished based on the abundance of several key fungal and bacterial taxa and therefore suggested evidences for region specific microorganisms "the microbial terroir". Interestingly, those authors showed that those taxa could possibly survive the fermentation process (Setati et al., 2012; Bokulich et al., 2016).

1.5 Txakoli: the case study

The Txakoli, a white wine from the Basque Country has recently gained popularity due to the wine quality improvements, increasing in both acreages of production and wine consumption. However, aside from the study of the causative pathogen agents of particular diseases the microbial diversity and composition associated to this variety is still unknown.

Txakoli vineyards are grown in coastal and non-coastal regions of the three Basque provinces, representing three Txakoli Designations of Origin (DO) Protected Designation of Origin (PDO).

Getariako Txakolina was the first variety of txakoli to receive the DO certification in 1989, mostly cultivated in Getaria area, and in 2007 it expanded its geographical area to the whole province of Gipuzkoa. The cultivated area covers around 433 hectares of devoted vineyards. There are now 32 wineries registered within the DO (Figure 2), 90% located on coastal areas. Annually some 900,000 liters (240,000 U.S. gal) are produced in this area.

Figure 2: 32 wineries belonging to Getariako Txakolina Designation of Origin.

Bizkaiko Txakolina DO was the second txakoli to receive the DO certification in 1994 (Garaizabal Pildain, 2002). Vineyards are present throughout the entire geography near the coastline, in interior valleys, or in the mountain sides at medium altitude. Within Bizkaia the vineyards have been divided into 6 districts, being Uribe and Urdaibai districts (Figure 3, 2nd and 3rd) the ones with highest number of vineyards and wineries approximately 150 ha are grown by 85 villages and towns producing some 700,000 liters of txakoli annually.

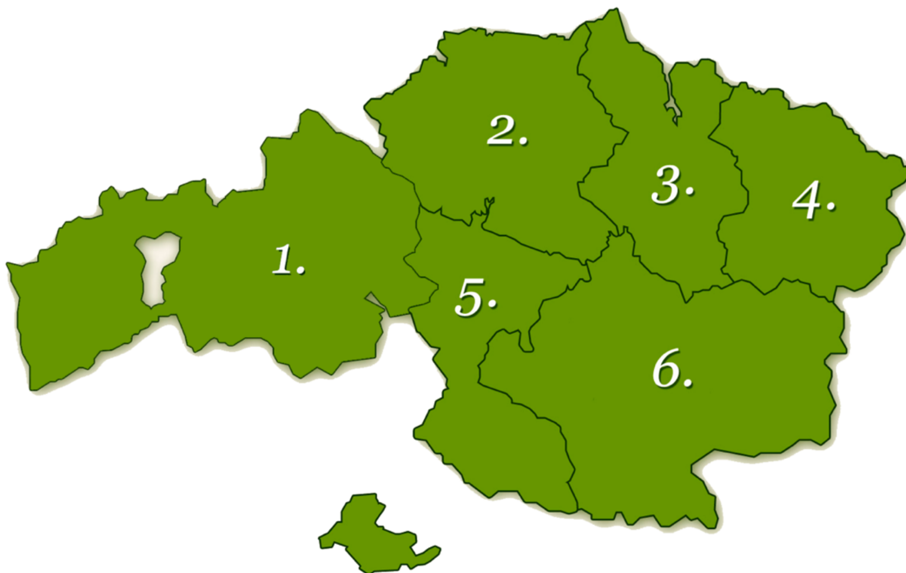


Figure 3: The division of six districts in Bizkaiko Txakolina. 1st Encartaciones; 2nd Uribe; 3rd Urdaibai; 4th Lea Artibai; 5th Nervión and 6th Duranguesado.

Arabako Txakolina DO is the youngest of the three DO varieties of txakoli, having gained certification in 2001. It is grown over some 55 ha of land around the towns of Aiara, Amurrio, Artziniega, Laudio and Okondo. In the late 19th century, grapes were grown on more than 500 ha of land, declining to 5ha in the late 20th century before the recent revival (Garaizabal Pildain, 2002).

Table 1: Summary table of some important characteristics of each DO.

	Getariako Txakolina	**Bizkaiko Txakolina**	**Arabako Txakolina**
Year of certification	1989	1994	2001
Nº of wineries	32	36	7
Registered Ha	443	403	60
Main areas	Getaria	Uribe, Urdaibai	Ayala
Max. authorized yield kg/Ha	13,000	13,000	12,500
Min. alcohol content	9.5%-11%	11.5 (white) – 12% (red)	9.5%

In all of the three DOs, *Hondarrabi zuri* is the main cultivated variety among whites, while *H. beltza* is used for other mono-varietal wines or sometimes mixing it with *H. zuri* to produce red or rosé wines. As other European cultivars, this grapevine is grafted onto phylloxera resistant rootstock, mostly SO4 and 3309. The vineyards from the different regions show variation on their soil characteristics, where coastal and inland vineyards show some differences. While all three regions are under the influence of the Atlantic climate, they differ on their soil edaphology, rainfall and temperatures. Soils near the coast are predominantly clay while further inland it is more varied. The shallow soils are neutral or acidic and rich in organic matter. Interestingly, due to those climatic and training system differences, those regions differ in their susceptibility to different pathogens. For instance, in 2007 while mildew affected the 15-30% of vineyards from Arabako Txakolina, vineyards at Getariako Txakolina DO were able to contain the disease and achieved normal production (Soto, 2007).

OBJECTIVES

2 Objectives

Vineyards representing the Basque Country viticultural region for Txakoli wine are spatially distributed into 3 DOs covering the three provinces and cover heterogeneous locations, including inland and nearshore areas. The *Hondarrabi zuri* variety, which is widespread in the region, is being successfully grown on highly variable soil types, different rootstock genotypes and on broad management strategies that differ in their fertilization regimes and phytosanitary treatments. All the above makes the Basque Country Txakoli viticultural area a suitable system to study potential factors responsible for differences within and between vineyards microbial diversity and composition, and importantly, to study the existence of region-specific communities that might define a terroir. This PhD study objective is to characterize the bacterial diversity and composition of soil and *H. zuri* grape variety from the Basque Country viticultural region, which is currently an explored research field, using high throughput sequencing methods. A deeper understanding of *H. zuri* microbiota could ultimately provide Txakoli winegrowers with information that could help on decision making related to the specific needs of their vineyards to promote their sanitary status, as well as enhancing the typicity of their wines.

The specific objectives of the study are:

1) To generate an in-depth bacterial inventory of soil and grape environments for *H. zuri* vineyards
2) To determine the main factors behind the shifts on bacterial distribution and abundances in soil and grape communities
3) To determine the degree of distinctiveness on bacterial profiles between plots/wineries/vintages/DOs
4) To explore the existence of a microbial terroir associated to each of the DOs

In the following sections of this PhD thesis, a general overview on vineyard sample collection for soil and grape sample types will be given, and the methods for 16S rRNA

high throughput sequencing as well as the analysis pipeline conducted will be described. Afterwards the results and discussion obtained by each of the sample types studied will be presented into two chapters: Chapter 1 (Results and Discussion for soil bacterial profiles) and Chapter 2 (Results and Discussion for grape samples). To finalize, a "General Discussion" including main results and limitations encountered will be described.

MATERIALS AND METHODS

3 Materials and methods

3.1 Samples studied

Samples were collected in private properties, under the supervision and acceptance of the owners. No additional specific permissions were required because the study did not involve endangered or protected species nor areas. The sampling was conducted in near-shore and inland vineyards located in the Basque Country (North of Spain) in 2016 and 2017. Twenty-two wineries were selected (covering an approximate area of 100 kms); these comprised 41 vineyard plots that grow *H. zuri* within the 3 DOs (Figure 4).

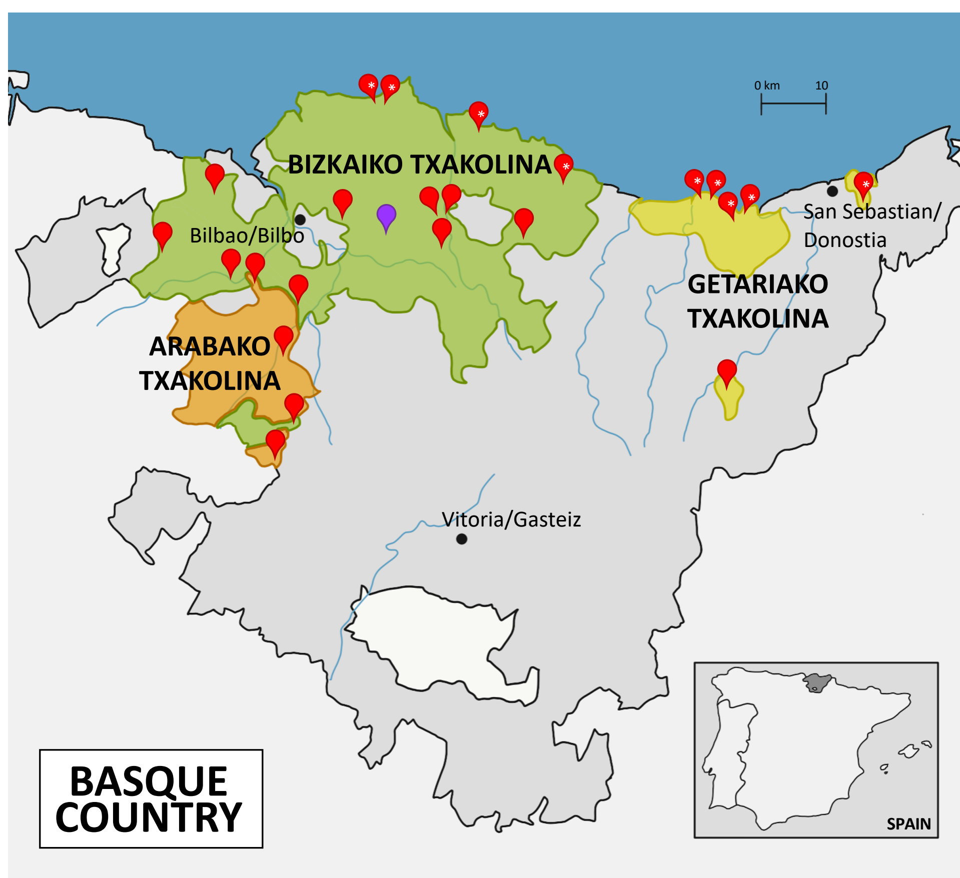


Figure 4: Txakoli wineries' location. The 22 wineries collaborating in the project are represented with red markers and the unestablished vineyard location is represented with a purple marker. The 3 Designations of Origin (DO) (Bizkaiko, Getariako and Arabako Txakolina) are referred in the map. Near-shore vineyards are identified with a white (*), while inland vineyards are represented without.

Samples associated metadata (Supplementary Table 1) was recovered at the time of the sampling and included:

1. Experimental factors: DO, vineyard and year of collection. For privacy purposes winery names were hidden and a number followed by a letter was established.
2. Vineyard associated factors: slope (inclination of the land in degrees), orientation (vines rows angle degrees from the north-south line), rootstock (SO4, 3309, Gravessac, ungrafted, 41B), geographical coordinates (latitude, longitude and altitude), near-shore effect and total accumulated temperature and precipitations. Total temperature and precipitations were calculated using the available data from each nearest meteorological station ($TTemp = \Sigma(T_m - 10)$) (http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos).
3. Soil edaphic and chemical factors: moisture, pH, conductivity, organic matter and nitrogen content.
4. Grape chemical factors: pH, total acidity (TA), Yeast-assimilable nitrogen (YAN) and potential alcohol (PA).
5. Historical land use changes for each of the vineyard plots were obtained from the Spanish national geology institution (https://www.ign.es/iberpix2/visor/).

3.1.1 Soil sample collection

Within each vineyard plot, soil samples were collected randomly with a soil corer, covering the whole vineyard block and were pooled in a bag to assure the within vineyard heterogeneity was covered. Two replicates per plot were generated. Samples were preserved in pre-labelled zip bags in the field and kept in portable coolers at 0-4 ºC during their transportation to the laboratory. Once in the laboratory, they were stored in darkness at -20 ºC until processing. In total, 168 samples were gathered: 82 in 2016 and 86 in 2017.

In addition, soil samples from a land intended for viticulture within the Bizkaia DO were collected for comparison purposes (Figure 4, purple marker). In this land, *H. zuri* grapevines were planted in 2015 and samples were collected a month before and after the

plantation. A vineyard needs at least 4 years to be productive and develop quality grapes, therefore we referred to this plot as "unestablished vineyard". Sample collection and storage was the same as the one conducted with the Txakoli vineyard soils. Part of the soil was stored at -20 and used for microbial analysis while the other section was used for chemical analysis.

3.1.1.1 Soil chemical analysis

Moisture was calculated by drying the soil sample in an oven at 80 ºC and calculating the difference of weight; pH and conductivity were measured creating a dissolution of 1:2.5 proportion of dried soil to distilled water, and mixing them for 15 min. Organic matter was calculated by digestion (PEC/EN/A-068) and total nitrogen was measured by electrothermic combustion (PEC/EN/A-215) by Neiker.

3.1.2 Grape samples collection

10-15 grape bunches were randomly collected from different vines across each of the plots in triplicate and were transported on ice to the laboratory to be processed upon arrival. Grapes were destemmed (approx. 500 g) and 100 mL of PBS were added to the sample. After 2 minutes mixing in a shaker, grapes were crushed using a Masticator Homogenizer (30 seg in sterile bags containing membrane filters of 0.2 um). Part of the resultant must be used for chemical analysis, while the other part was stored in a – 20 °C freezer until DNA extraction.

3.1.2.1 Must chemical analysis

After ensuring that there were no significant differences among the replicas, one, two or three replicas of grape must sample per plot and year was used for chemical analysis, depending on the amount of sample. pH was measured with a pH-meter. Total Acidity (TA) was determined by titration (g/L). Yeast-assimilable nitrogen (YAN), that refers to the organic and inorganic available nitrogen, was calculated using a spectrometry (mg/L). The potential alcohol (PA) was measured by

densitometry at 20 °C (CCE Nº2679/90, 1990). The mentioned analytical determinations were conducted by ARDOATEK S.L.

3.2 DNA extraction, library preparation and sequencing

3.2.1 Soil Samples

The Power Lyzer Power Soil kit from MoBio laboratories was used to extract the total genomic DNA from the collected soil samples. The V4 domain of bacterial 16S rRNA gene was amplified using the primers 515F and 926R (Quince et al., 2011; Parada et al., 2016) with the forward primer modified to contain a unique 12-nt and a 2-nt linker sequence. Each PCR reaction contained 12.5 µl of KAPAHifi HotStart ReadyMix, 1µl of Golay Barcode Tagged Forward Primer (5 µM concentration, 200 pM final), 1 µl Reverse Primer (5 µM concentration, 200 pM final) and 1 µl of template DNA. The amplification conditions used were a hot start of 94 ºC for 4 min, followed by 35 cycles of 95 ºC for 15s, 50 ºC for 30s, 72 ºC for 30s. PCR amplifications were checked by electrophoresis in an agarose gel (1.5 %) and different volumes of each of the products were pooled into a single tube so that each sample was represented equally. After that, samples were cleaned using the UltraClean® PCR Clean-Up Kit (MoBio), and quantified with Qubit (Invitrogen). Finally, the molarity of the pool was determined, and denatured for sequencing on the Illumina MiSeq platform. The txakoli vineyard soils were sequenced at the Sequencing and Genotyping unit of the University of the Basque Country (SGIKER). The first run contained soils from the 2016 harvest campaign and the second run contained soils from 2017 harvest and several samples from 2016 that were already sequenced in the previous run (to be able to test for sequencing biases). Soil samples from the unestablished vineyard were sequenced at Argonne National Laboratory (USA).

3.2.2 Grape samples

All three replicas of must samples, per plot and year, plus two technical replicas (total N = 246) were centrifuged at 4000 rpm for 10 min and the supernatant was discarded. The pellet was dissolved in 1 mL of PBS (1X, pH 7.4), and centrifuged in a 2 mL Eppendorf

tube, and this cleaning step was repeated 3 times. DNA extraction was done using the QIAAmp DNA Mini Kit (Qiagen, Inc.) by adding a homogenizing step in PowerBead Tubes (glass 0.1 mm) (in a Precellys® 24 – Precellys, 3 x 30 s at 6.800 rpm) after the incubation step.

PCR amplification of the V4 hypervariable region of the 16S rRNA gene was performed using the universal barcoded primer pair 515F and 806R, following the Earth Microbiome Project protocols (Caporaso et al., 2012). PNA PCR clamps were used to reduce plant cell amplification. The PCR was conducted in 25 µL reactions, containing 5 µL Colorless GoTaq® Reaction Buffer, 2 µL of $MgCl_2$ and DNTPs, 0.5 µL of each primer (5 µM) and 0.3 µL GoTaq® DNA Polymerase. PCR amplification consist of 3 min at 94 °C; 35 cycles of 15 s at 95 °C, 10 s at 78 °C, 30 s at 50 °C and 30 s at 72 °C. Samples were pooled equimolarly and cleaned with UltraClean® PCR Clean-Up Kit (MoBio). Finally, the pool was denatured for sequencing in an Illumina MiSeq platform (2 x 150 bp, 300 cycles) at the Sequencing and Genotyping unit of the University of the Basque Country (SGIKER). Two sequencing runs were conducted, one for the 2016 harvest campaign and another for the grapes collected in 2017.

3.3 Sequence processing

All FASTQ files were quality filtered and trimmed using Trimmomatic (v0.38 for soil and v 0.39 for grape) (Bolger et al., 2014), retaining sequences with quality > Q20. Sequences were merged with Pear v 0.09.10 (with a 15 bp overlap) and were demultiplexed with QIIME v1.9 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were identified by sequence homology using the SILVA 132 database using an open reference approach; reads that failed to match any reference sequence were subsequently clustered *de novo* by their 97 % similarity level using Uclust (Edgar et al., 2011). OTUs taxonomy was assigned using SILVA 132 database (Quast et al., 2013). OTUs with less than 10 sequences were removed, as well as the sequences assigned to chloroplast and mitochondria.

To avoid biases related to differences in sequencing depth/sample, the OTU table of soil samples was rarified to 22,897 sequences/sample while the OUT table of grape sample was rarified to 14,848 sequences/sample. The rarefaction threshold was selected based on rarefaction curves, and those values verify that most of the diversity was covered for each of the sample types.

3.4 Statistical analysis

Alpha-diversity (samples richness and evenness) was calculated by means of observed-OTUs and Shannon metrics. Rarefaction curves and bar plots were generated with alpha diversity indexes to visualize the results. Correlations between alpha diversity and metadata factors (Experimental, Vineyard, Soil associated and must chemical factors, as defined in 3.1 section) were determined using linear correlation in Vegan's R package. In addition, pairwise comparison between samples grouped by the metadata factors studied were computed and its significance tested by kruskall-wallis in QIIME2.

Beta-diversity (between-samples community dissimilarity) analysis was calculated using Bray-curtis dissimilarity distances. Each environmental factor's impact on the microbial community composition was tested using non-parametric permutational multivariate analysis of variance, ADONIS, with 999 permutations (Vegan's R) and permanova pairwise comparisons were also computed in QIIME2. An ordistep stepwise analysis was carried out to find the factors mostly related to the between sample bacterial dissimilarity and to build the model best explaining the community differences. The variables selected in this model were then chosen to constrain the canonical Correspondence Analysis (CCA) plots (Vegan R package (RStudio Team, 2016; Oksanen et al., 2017)).

Linear Discriminant analysis of effect size (LEfSe) (https://huttenhower.sph.harvard.edu/galaxy/) was used to identify the taxa whose abundances significantly differed ((Kruskal-wallis Bonferroni p value < 0.05, Wilcoxon test $p < 0.01$ and Linear Discriminant Analysis > 2) between sample grouping of factors identified to be of interest according to the ordistep model. In addition, a deeper analysis

into the taxonomic assignment and distribution of viticulturally important bacteria was undertaken, by retrieving the representative sequences belonging to particular taxa (e.g. *agrobacterium* and *lactobacillus*) to search for their homology to NCBI database sequences using BlastN and generating a phylogenetic tree.

3.5 Methodological limitations

There is no doubt that sequencing the conserved 16S rRNA gene has improved our understanding of the biodiversity and drives of microbial distribution in vineyards. Those studies are critical for elucidating the impact of low-abundant community members on plant health and diseases. However, the characterization of microbial communities has been hampered by inherent differences generated in community profiles when sequencing different hypervariable regions, the chosen primers and the taxonomic classification difficulties due to the incompleteness of databases and the limited resolution to distinguish between closely related species.

In this study two sets of primers of the 16S rRNA gene were used, V4-V5 (515F-926R) and V4 (515F-806R), described within the Earth Microbiome Project (https://earthmicrobiome.org/). Screening longer sequences was expected to return a higher taxonomic resolution and so, soil and grape samples were studied first with 515F-926R primers. However, an unexpected result was observed for grape sample type, as an unspecific longer band was obtained in the PCR amplification (Figure 5). Our attempts to get rid of this nonspecificity were not successful and we finally extracted the band from the gel and sanger sequenced it. The sequences were contrasted with NCBI databases and showed to match with the 18S ribosomal RNA gene of fungi: Sclerotiniaceae family (with an E value of 3e-154), Leotiomycetes class (1e-153) or even *Botrytis cinerea*, (1e-153). Therefore, this led to the conclusion that while 515F-926R primers are efficient for soil type environments (bulk soil, rhizosphere, etc.) they are not suitable for grapes or any related environment (must and ferments).

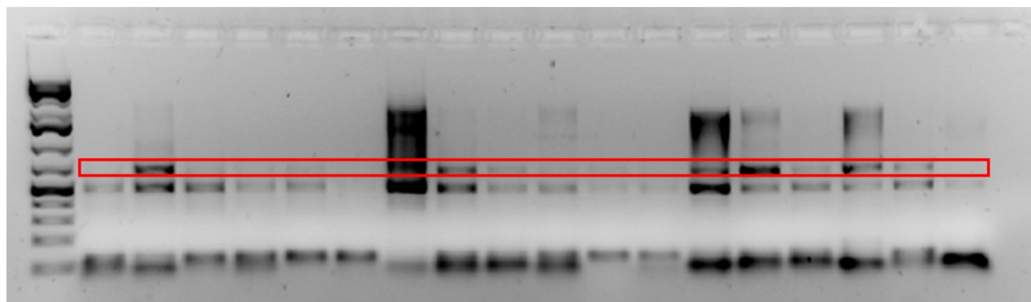


Figure 5: Agarose gel photo of amplified 16S rRNA V4-V5 region. The first column has the 100 bp DNA ladder marker and the other columns belong to the amplification of grapes for the V4-V5 region. The band around 500 bp is the desired amplicon, while the band with approx. 600 bp band (in red) is the result of a non-specific amplification product.

Sequencing bias was found to be a problematic aspect of this methodology, which could preclude drawing firm conclusions. A total of 4 runs have been conducted in this study, two for soil samples and two for grape samples. Some samples from the first sequencing run were also included in the second run to check for sequencing bias (Figure 6). Results evidenced that the direct comparison of two independent runs is controversial and could lead to erroneous conclusions of finding significant community differences when there are none.

In soil samples the sequencing bias was not so pronounced and a strategy to solve the problem was found. In our sequence processing method, the sequences were clustered into Operational Taxonomic Units OTUs (according to their 97% of similarity) and then those OTUs were classified taxonomically. Some OTUs could be assigned to the same species, so a new cluster unit was proposed, the species level (L7) cluster unit. The grouping of OTUs into species was methodologically positive, since the conversion, the samples that appear separated only because they were analyzed in different runs, now they become more similar. Unfortunately, the problem was not solved for grape samples.

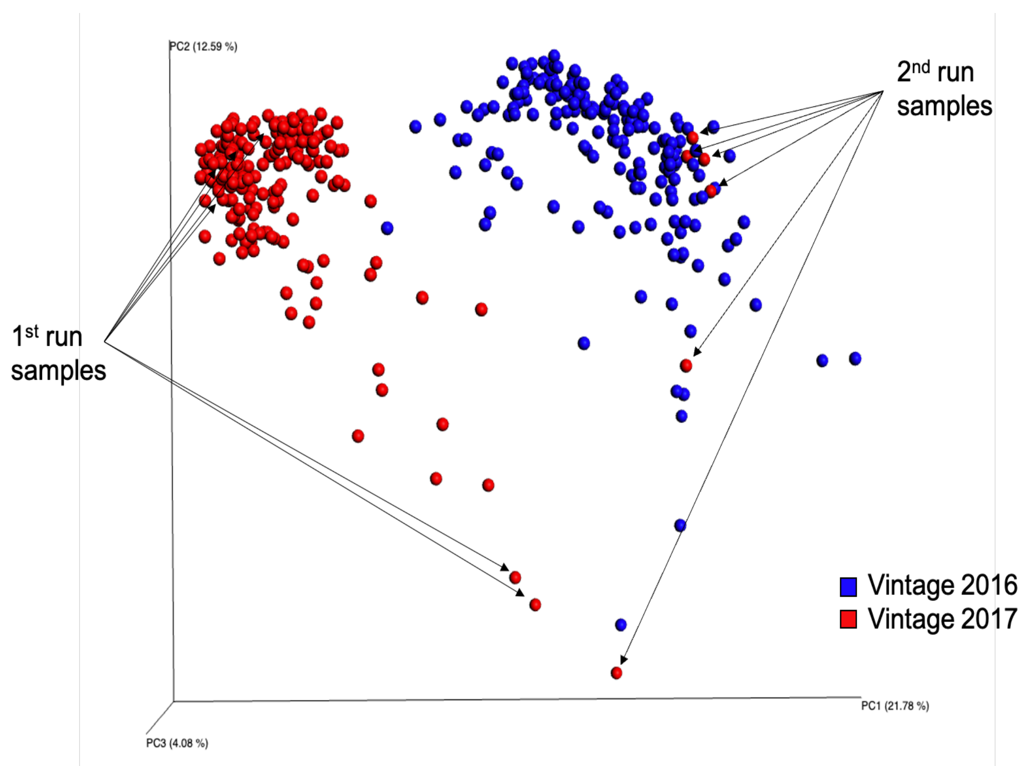


Figure 6: Grape sample's PCoA based on Bray-curtis distance. Two independent runs were performed: one with samples collected in 2016 (red dots) and a second run with samples from 2017 (in blue). The six samples from 2016 identified with arrows in the figure were included in both of the runs, and their microbial composition differs significantly between sequencings. In the second run, those samples are clustered together with samples from 2017.

Another known limitation of the method is the lack of resolution for certain taxonomic groups. This is of particular relevance in grape and must like environments which are low in taxonomic diversity (being mainly dominated by two genera *Sphingomonas* and *Methylobacterium*) but where the strain level heterogeneity is expected to be high. A good example is the Lactobacillus family. While some Lactobacillus are known for their role in the malolactic fermentation, other members are considered wine spoilers (Bartowsky, 2009). Members of this group have very few nucleotide differences within the 16S rRNA gene, making it complicated to determine the exact taxon the sequence belongs to (O'Callaghan et al., 2021). Other studies (Bokulich et al., 2012a) compared the resolution of the different variable regions of 16s rRNA (V4 or V5) for must and wine, concluding that the V4 region is the most suited for Lactobacillus and *Pediococcus*. However, in order

to advance in our understanding of wine associated microbial ecology and its relationship with wine characteristics, the improvement of databases, the development of additional primers or the screening of a different gene or a combination of genes are necessary.

RESULTS AND DISCUSSION

4 Results and Discussion

4.1 CHAPTER 1: Soil microbial biogeography in txakoli

The Txakoli viticultural region, in the Basque Country, characterized by growing an endemic grape variety, the *Hondarrabi zuri*, is an unexplored viticultural area in terms of its microbiota. Next generation sequencing technology has proven to be a valuable approach to acquire a broader picture of vineyard microbial ecology (Cocolin et al., 2011; Quigley et al., 2011; Bokulich et al., 2016; Cao et al., 2017).

Soil microorganisms are critical to the maintenance of soil functions, helping with plant pathogen suppression (Garbeva et al., 2004), contributing to nitrogen, carbon, phosphorus, and sulfur cycles (Elsas et al., 2006), and preserving soil organic matter (KoÈgel-Knabner, 2002; Grandy and Neff, 2008; Kuzyakov et al., 2009; Plaza et al., 2013). Growing evidence indicates that soil microbiota can, directly and indirectly, interact with the plants, improving their fitness and health (Sapkota et al., 2015).

In this study, we sequenced the 16S rRNA gene from the soils of 41 vineyard plots to gain knowledge on soil microbiota diversity and distribution at an intra-vineyard, inter-vineyard and regional scale.

4.1.1 Results

4.1.1.1 Sequencing performance

From the 41 Txakoli vineyards where soil samples were collected in two consecutive years (N = 168 samples), we obtained a total of 25,383,570 sequences after quality trimming and demultiplexing (10,367,318 and 15,016,252, from 2016 and 2017, respectively). After removing chloroplast sequences and OTUs with lower than 10 sequences 19,648,408 sequences remained. The sample with the lowest number of sequences (22,897 counts) was used as a depth for rarefaction. A total of 64,716 OTUs were identified, ranging from 3,537 to 6,362 OTUs per sample, clustered into 375 to 558 different species/sample.

We obtained a total number of 1,566,608 filtered sequences from the samples collected at an unestablished vineyard (a plot located in Bizkaia province a month before and after *H. zuri* was planted). In these samples, we identified 10,642 OTUs, ranging from 2,853 to 5,680 OTUs per sample, representing 318 to 467 species/sample.

4.1.1.2 Bacterial richness differs between vineyards and correlates with pH

The number of Observed Species significantly differed between the 41 plots studied. For instance, the richness of the unestablished vineyard soils was significantly lower compared to the rest of Txakoli vineyards (well established and productive vineyards over 15 years old) located in the same region. The pairwise comparisons of the unestablished vs Txakoli lands were significant in 20 out of the 41 comparisons (Student T test $p < 0.05$).

Within the Txakoli vineyard soils, the number of Observed Species significantly differed (T test, $p < 0.05$, Supplementary Table 2). In particular, two vineyards showed significantly lower richness than the rest of the plots (A2b and B9e in Figure 7). On the contrary, significantly higher richness was reported for the B1a plot (Figure 7).

The Linear correlation analysis of Observed Species Index and the Experimental factors (year, vineyard, winery, DO), vineyard factors (slope, orientation, altitude and geographical coordinates as latitude and longitude) and soil physicochemical factors (pH, conductivity and moisture) revealed that "vineyard" was the factor with the highest correlation ($r^2 = 0.557$, $p = 1.976 \cdot 10^{-15}$). In addition, pH was positively correlated, being a significant driver of the richness differences found between the samples ($r^2 = 0.1565$, $p = 7.96 \cdot 10^{-8}$) (Table 2). "Latitude" and "Conductivity" also significantly correlated with richness ($p < 0.05$) but with low r^2 values ($r^2 = 0.0334$ and $r^2 = 0.0246$). Interestingly, no significant richness differences were found between DOs nor vintages.

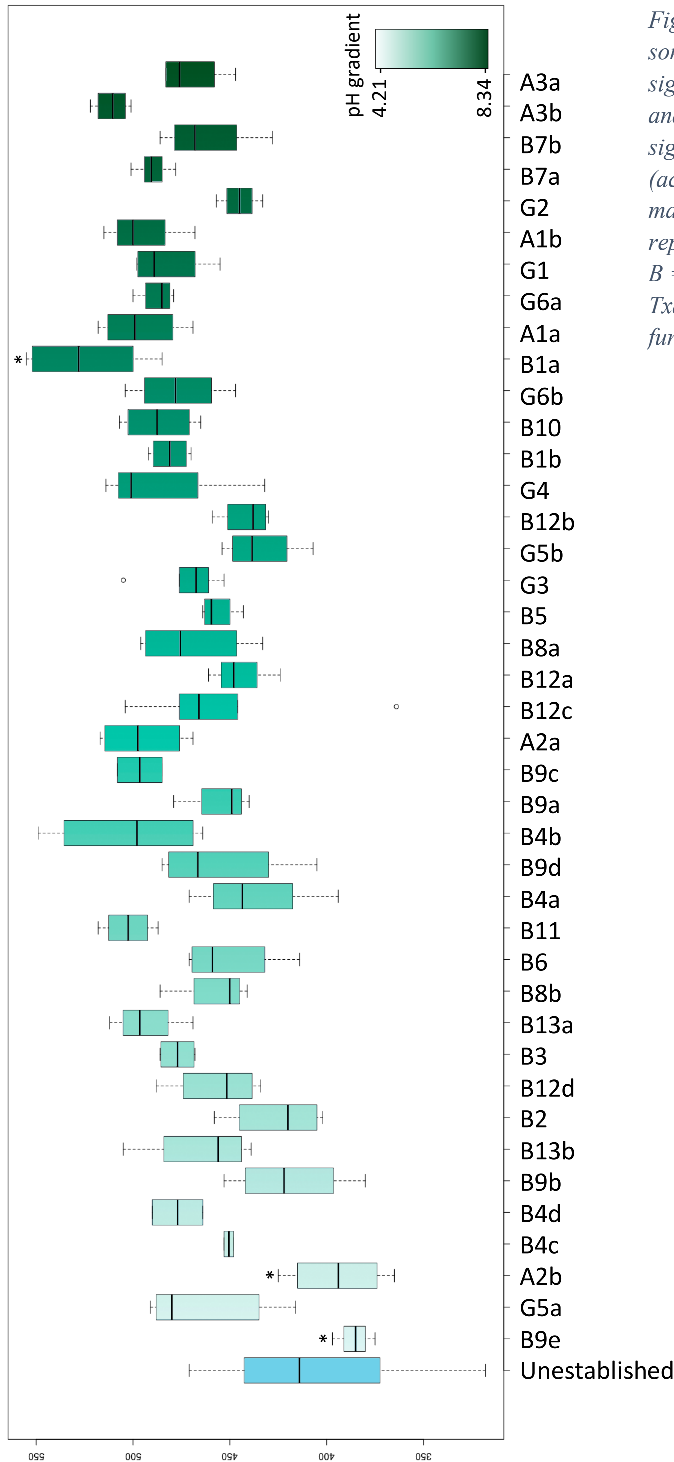


Figure 7: Vineyard's richness boxplot sorted by pH gradient. A positive and significant correlation between richness and pH is observed. The vineyards having significantly lower/higher richness (according to Pairwise t-test < 0.05) are marked with (*). Vineyard names first letter represents the DO (A = Arabako Txakolina, B = Bizkaiko Txakolina and G = Getariako Txakolina) (See Supplementary Table 1 for further information of the samples).

Similarly, the evenness of the samples was mainly correlated to "vineyard", "winery" and "pH" ($r^2 = 0.644$, $p = 2,20 \cdot 10^{-16}$; $r^2 = 0.366$, $p = 1.31 \cdot 10^{-10}$ and $r^2 = 0.131$, $p = 9.47 \cdot 10^{-7}$, respectively), and to a lower extent to "DO" and "conductivity", while the rest of variables did not show significant results (Table 2).

Table 2: Richness and evenness linear correlation with experimental, soil physicochemical and vineyard factors. p <0.05, p <0.01 ** and p < 0.001***.*

		Richness		Evenness	
		r^2	**p-value**	**r^2**	**p-value**
Experimental factors	**DO**	0.002	0.303	0.057	0.003 **
	Winery	0.270	< 0.001 ***	0.366	< 0.001 ***
	Vineyard	0.557	< 0.001 ***	0.644	< 0.001 ***
	Year	0.005	0.182	-0.006	0.885
Soil physicochemical factors	**Moisture**	0.006	0.160	0.006	0.161
	pH	0.156	< 0.001 ***	0.131	< 0.001 ***
	Conductivity	0.025	0.024 *	0.040	0.006 **
Vineyard factors	**Altitude**	0.003	0.224	-0.001	0.345
	Latitude	0.033	0.010 *	0.015	0.060
	Longitude	-0.003	0.510	0.096	0.300
	Orientation	0.009	0.120	0.006	0.165
	Slope	0.019	0.043	-0.001	0.339

4.1.1.3 Soil bacterial communities' dissimilarity at different biogeographical scales

4.1.1.3.1 Between viticultural regions: Basque Country vs. two locations in the USA

The soils collected in the Basque Country belonging to Txakoli vineyards and an unestablished vineyard were studied together with sequences from samples from Long Island (Zarraonaindia et al., 2015) (downloaded from https://qiita.ucsd.edu/study/description/1024) and from Napa Valley (Burns et al., 2015) (retrieved from https://qiita.ucsd.edu/study/description/10082). To avoid differences in sampling effort between the projects, 30 samples were randomly selected per study. The

most prevalent bacterial species were compared by selecting only the sequences present in 80 % of the samples within each project.

The bacterial community structure of Txakoli soils from the Basque Country showed lower mean Bray-Curtis distances with vineyard soils from distant viticultural regions located in a different continent (California and Long Island) than to the soils collected in the unestablished vineyard, located in the same region few kms away (Figures 8 and 9).

LEfSe analysis determined that the unestablished vineyards were characterized by an enrichment of Gemantimonadetes (3 times higher 6,04% vs 1-2%), Chloroflexi, Chlorobi or Armatimonadetes, these latter groups being absent in the established/productive Txakoli, Long Island, and Napa Valley's vineyards.

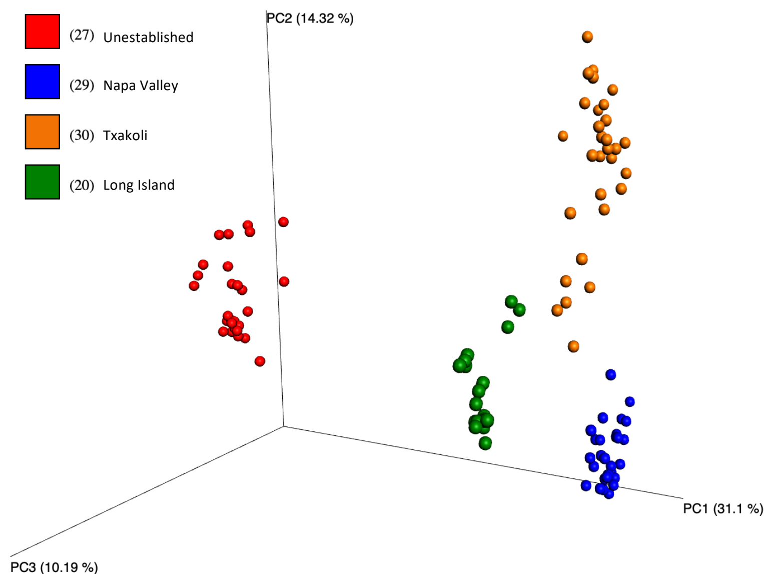


Figure 8: Bray-curtis dissimilarities within and between Viticultural regions. PCoA plot representation with samples colored by viticultural region.

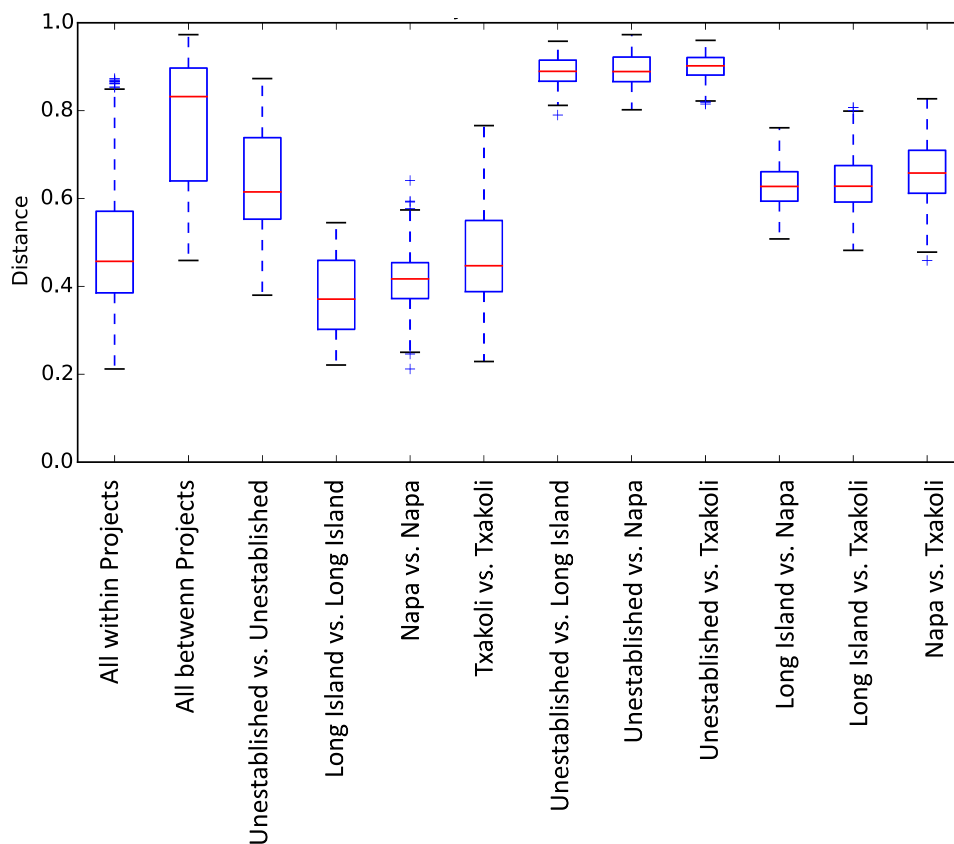


Figure 9: Bray-curtis dissimilarities within and between Viticultural regions. Boxplot representation of beta diversity distances. The center-line of the boxplots show the medians, and the bottom and upper limits indicate the 25 and 75th percentiles, respectively.

Overall, all soils compared in this study were dominated by Proteobacteria phyla (ranging from 32 % to 46 % of relative abundances) (Table 3). Acidobacteria was the second most abundant phylum in all three mature vineyards (Napa Valley with a 23.7 %, Long Island with 24.78 % and Txakoli vineyards with a 21.11 % of abundances). On the contrary, Verrucomicrobia was ranked second in the “unestablished vineyard” (16.48 %), being Acidobacteria the third most abundant with a mean value of 12.3 %.

Table 3: Mean relative abundances of prokaryotes in the different viticultural regions studied.

	Unestablished (n=28)	Napa Valley (n=30)	Long Island (n=30)	Txakoli (n=30)
Unassigned	0.22	0.00	0.00	0.00
Archaea, Crenarchaeota	0.03	1.36	0.94	1.38
Bacteria, AD3	0.04	0.00	0.00	0.00
Bacteria, Acidobacteria	12.30	23.70	24.78	21.11
Bacteria, Actinobacteria	3.05	16.54	2.93	4.62
Bacteria, Armatimonadetes	0.06	0.00	0.00	0.00
Bacteria, BRC1	0.01	0.14	0.17	0.03
Bacteria, Bacteroidetes	8.78	10.09	13.59	3.43
Bacteria, Chlorobi	0.14	0.00	0.00	0.00
Bacteria, Chloroflexi	2.90	1.32	0.58	1.34
Bacteria, Elusimicrobia	0.03	0.00	0.00	0.00
Bacteria, FBP	0.02	0.00	0.00	0.00
Bacteria, Fibrobacteres	0.04	0.12	0.26	0.10
Bacteria, Firmicutes	0.30	1.24	1.29	1.07
Bacteria, Gemmatimonadetes	6.04	1.05	2.77	1.04
Bacteria, Nitrospirae	1.14	0.31	3.99	2.88
Bacteria, OP3	0.01	0.00	0.00	0.00
Bacteria, Planctomycetes	1.47	1.51	3.69	2.98
Bacteria, Proteobacteria	46.60	35.06	32.71	44.25
Bacteria, Verrucomicrobia	16.48	7.57	12.30	15.79
Bacteria, WS3	0.35	0.00	0.00	0.00

4.1.1.3.2 Within the Basque Country: 41 vineyards

Within the Txakoli viticultural region, Bray-Curtis distances ranged from 0.063 up to 0.707 between the soil samples studied. Samples collected at the same vineyard, even in different years, had a very similar microbial community composition, with Bray-Curtis indexes ranging from 0.06 to 0.25 and grouping close to each other in the NMDS (Figure 10). However, significant compositional changes were observed between vineyards, being the "vineyard" factor the mayor explanatory variable of the community structure dissimilarities (Adonis test $R^2 = 0.812$, $p < 0.001$, Table 4). In addition, as much as 40.18% of the species significantly differed in their abundances between vineyards (Kruskal-Wallis test, Bonferroni corrected $p < 0.05$).

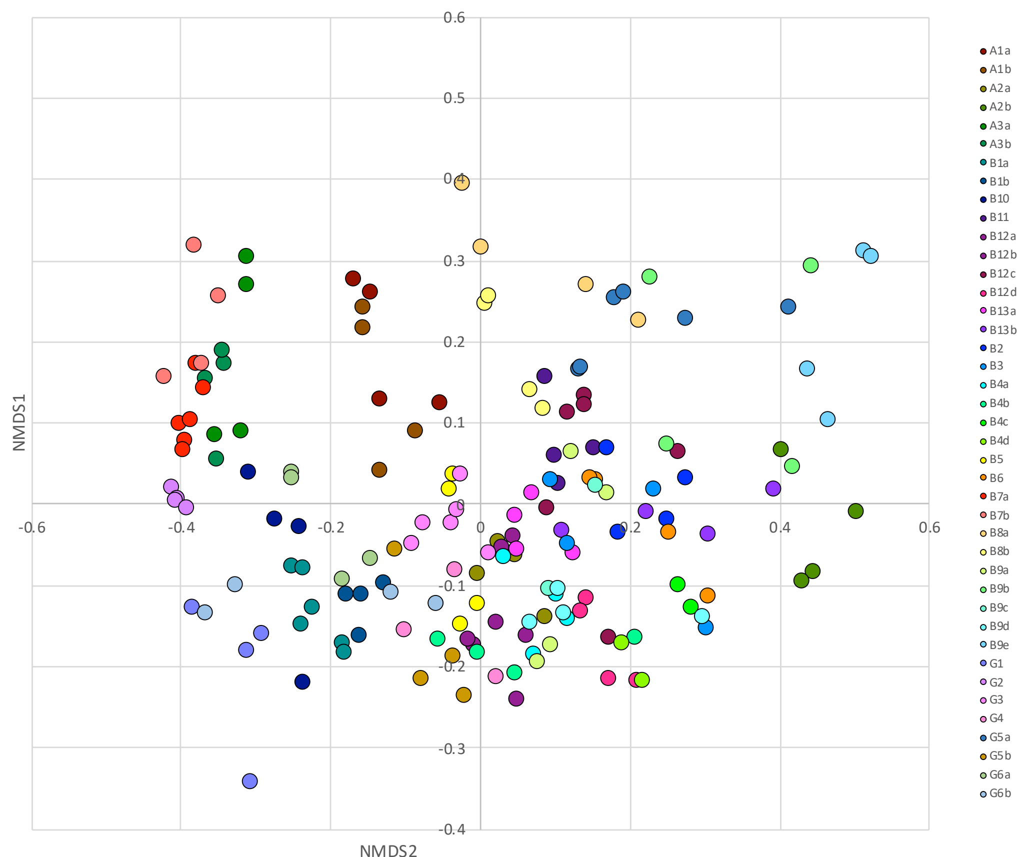


Figure 10:Txakoli soil's bacterial communities non-metric multidimensional scaling plot (NMDS) colored by "vineyard" based on Bray-Curtis distances.

Metagenomi
Aplicaciones

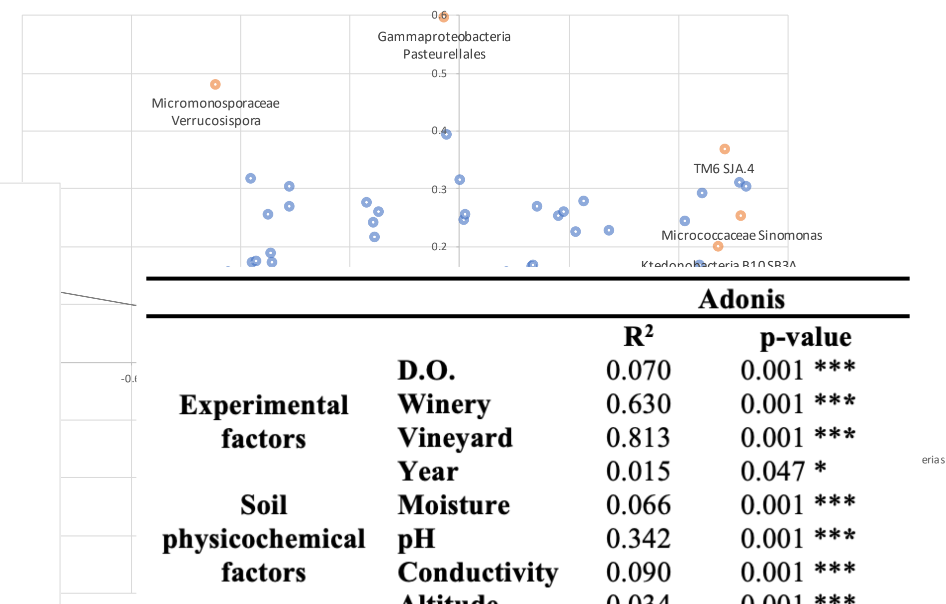
Bacteria community structure was influenced by pH and while significant shifts were detected for other factors (e.g., conductivity, moisture or latitude) according to Adonis test (Table 4), the latter were statistically less robust. pH was the variable with maximum correlation with the community dissimilarities among the quantitative variables tested in the study (Adonis test, $R^2 = 0.341$, $p < 0.001$) and the factor showing the largest vector size in the NMDS (Env-fit $R^2 = 0.798$, $p < 0.05$) (Figure 11). In addition, samples grouped according to the soil pH gradient in the PCoA (Figure 12).

PCoA
Parallel

*Table 4: Adonis test (999 permutations) values of exploratory factor's effects on microbial diversity patterns (p-value significance: p < 0.05 *, p < 0.01 ** and p < 0.001***).*

		Adonis	
		r^2	**p-value**
Experimental factors	**DO**	0.070	0.001 ***
	Winery	0.630	0.001 ***
	Vineyard	0.813	0.001 ***
	Year	0.015	0.047 *
Soil physicochemical factors	**Moisture**	0.066	0.001 ***
	pH	0.342	0.001 ***
	Conductivity	0.090	0.001 ***
Vineyard factors	**Altitude**	0.034	0.001 ***
	Latitude	0.123	0.001 ***
	Longitude	0.041	0.002 ***
	Orientation	0.013	0.068
	Slope	0.041	0.001 ***

Chloroflexi phylum showed a strong correlation with pH according to the Spearman test (Supplementary Dataset 1). Within this phylum, Anaerolineae, Gitt-Gs-136, Thermomicrobia, and S085 classes showed a positive correlation while the abundance of SHA-26, C0119, and Ktedonobacteria significantly decreased with pH. On the contrary, all classes of Actinobacteria (Nitriliruptoria, Rubrobacteria, Acidimicrobia, TakashiAC-B11 and Thermoleiphilia) were positively correlated. Among Proteobacteria, the dominant group in the soils studied, most classes, except Alphaproteobacteria, were positively correlated with pH, including Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria and TA18.



		Adonis	
		R^2	p-value
	D.O.	0.070	0.001 ***
Experimental factors	Winery	0.630	0.001 ***
	Vineyard	0.813	0.001 ***
	Year	0.015	0.047 *
Soil physicochemical factors	Moisture	0.066	0.001 ***
	pH	0.342	0.001 ***
	Conductivity	0.090	0.001 ***
Vineyard factors	Altitude	0.034	0.001 ***
	Latitude	0.123	0.001 ***
	Longitude	0.041	0.002 ***
	Orientation	0.013	0.068
	Slope	0.041	0.001 ***

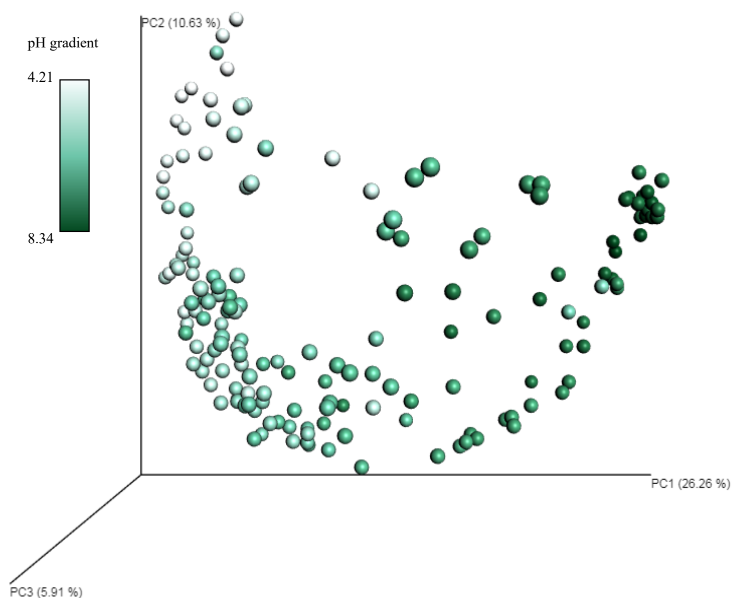


Figure 12: Soil bacterial community dissimilarities PCoA plot, colored by pH gradient (4.21 - 8.34).

A stepwise selection model identified “vineyard”, “pH” and “year” as the most significant explanatory variables for the soil bacterial community beta diversity distribution in a CCA ordination (CCA model ANOVA $p = 0.001$). An UPGMA tree constrained by those 3 factors clustered the soil samples into two main branches regardless of the DO, and those were divided into 5 subclusters (Branch 1: A and B subgroups; Branch 2: C, D and E Subgroups, in Figure 13). The two main clusters showed marked bacterial abundance differences according to the LEfSe test (Figure 14, Supplementary Dataset 2).

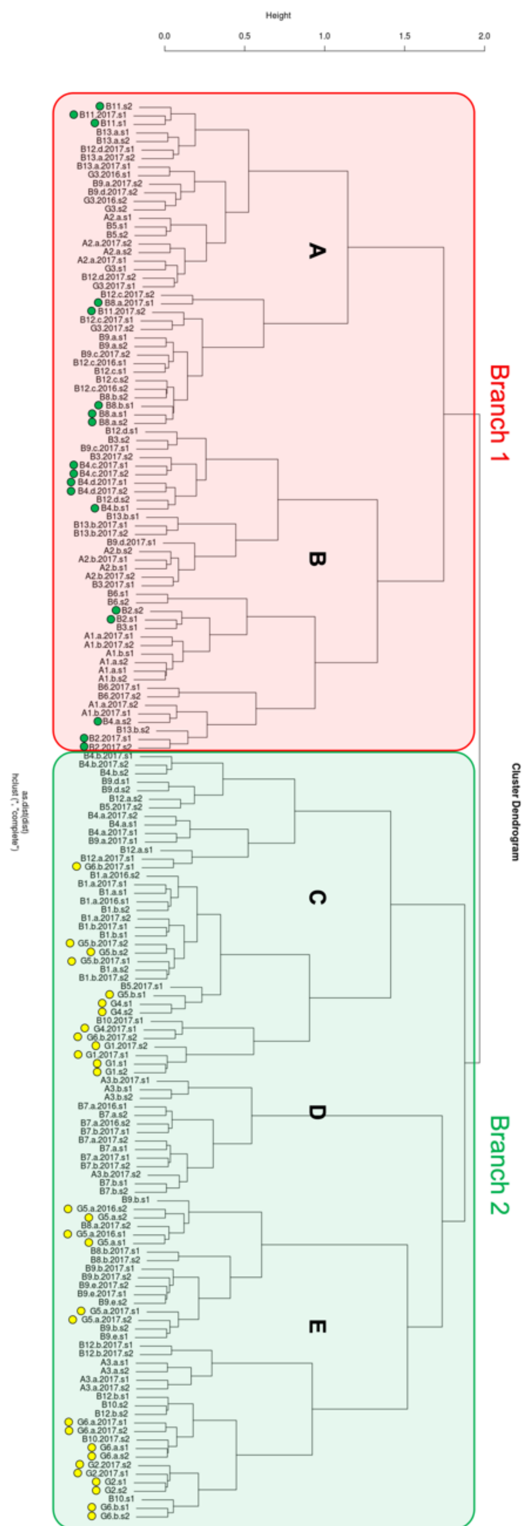


Figure 13: UPGMA tree. UPGMA tree based on CCA distances constrained by the significant factors resultant from a forward selection analysis (vineyard, year and pH). Samples clustered into two main branches and 5 subgroups were identified within them (A, B, C, D and E). Near-shore vineyards belonging to Bizkaiko Txakolina DO are highlighted in green, while those belonging to Getariako Txakolina are in yellow.

Samples grouped in branch 1 were enriched in Elusimicrobia and TM7, while Chlorobi was significantly more abundant in branch 2. Among Acidobateria and Actinobacteria*,* *Candidatus Koribacter, Candidatus Solibacter* and *Streptomyces* genera were relatively more abundant in branch 1, while *Agromyces* and *Rubrobacter* genera were more representative in branch 2. Members of Chloroflexi, such as Roseiflexales, were significantly more abundant in branch 2. Regarding Firmicutes, different genera within Clostridia and Bacilli classes were identified to be differentially abundant according to the branch: branch 1 was enriched in the Clostridiales *Clostridium, Oxobacter* and *Oscillospira* and the Bacillales *Alicyclobacillus, Bacillus* and *Paenibacillus*; branch 2 was enriched in the Clostridiales *Clostridium neonatale* and *Alkalibacter*, but no Bacillales. In addition, branch 1 showed significantly more abundant *Planctomyces* and *Gemmata* (Planctomycetes), while branch 2 showed an enrichment of *Pirellula* genus and of the Nitrospira clade, from class to genera. Within Proteobacteria, some taxonomic genera showed significant differences according to the cluster. In branch 1 *Bradyrhizobium, Devosia, Hyphomicrobium, Rhodoplanes, Methylosinun, Agrobacterium, Rhizobium, Labrys* (Rhizobiales order), *Burkholderia, Candidatus Glomeribacter, Methylibium* and *Polaromonas* (Burkholderiales order), *Bdellovibrio,* Myxococcales and *Geobacter* (Deltaproteobacteria class) were comparably increased. Besides, in branch 2 *Balneimonas, Phyllobacterium* and *Afifella* (Rhizobiales order), *Sphingomonas* (Sphingomonadales), *Plesiocystis* (Myxococcales) and *Steroidobacter* (Xanthomonadales) dominated.

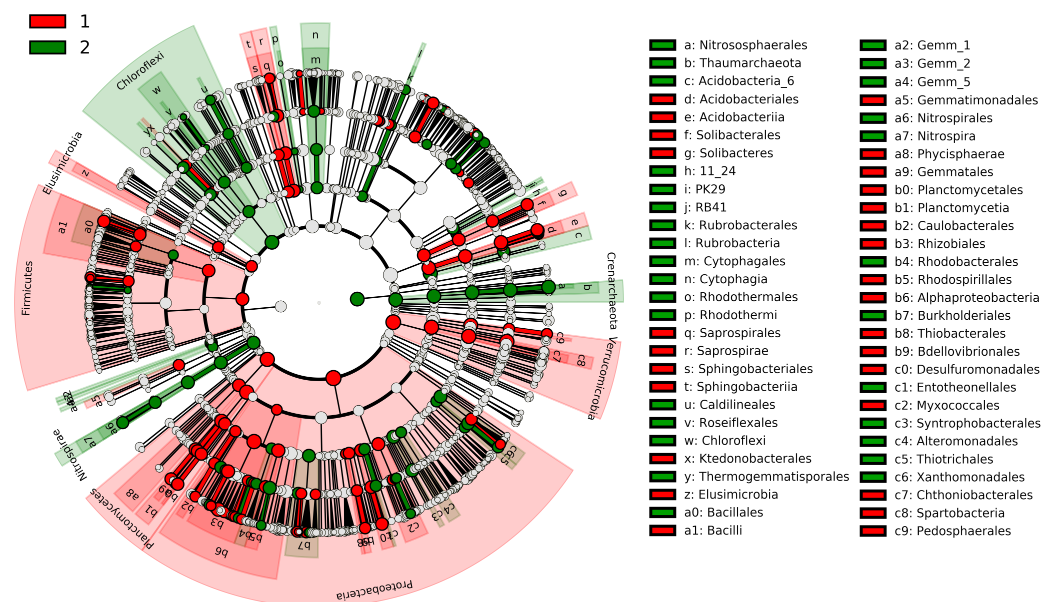


Figure 14: Cladogram representation of statistically and biologically consistent differences between UPGMA's branch one and two soils. Taxonomic groups enriched (linear discriminant analysis (LDA) effect size (LEfSe) analysis based on Kruskal–Wallis p < 0.05, and LDA scores Log10 > 2) in branch one soils samples are represented with red dots, while green dots represent samples from branch 2. The legend indicates the differentially abundant classes and orders per branch. Genera or species level information and the LDA score values for each group are available in Supplementary Dataset 2.

Biogeographical trends could be distinguished in particular cases. While vineyard soils located inland were distributed across both of the main UPGMA branches regardless of the DO, near-shore vineyards showed a different pattern: those from Getariako Txakolina exclusively clustered in branch 2 while the ones from Bizkaiko Txakolina clustered in branch 1 (Figure 13). In addition, some geographically close vineyards (despite belonging to different wineries, differing in topography, orientation, slope etc. and possibly having received different management practices) showed similar microbial community structures. For instance, the soil samples collected in the A3 winery (A3b vineyard, from Arabako Txakolina DO) and B7 winery (B7a and B7b, Bizkaiko Txakolina DO) located 3 kms apart in the same town, clustered within the same group (branch 2, group D), despite belonging to different DOs. These two wineries' samples were particularly abundant in Opitutaceae (Verrucomicrobia) and Beijerincjiaceae (Alphaproteobacteria). The

complete opposite situation was also observed, where in several cases, different vineyards belonging to the same winery and located geographically close to each other (approximately 200 m) clustered in different groups. This is for instance the case of the B12 winery, with vineyards clustering in group A (Branch 1) and E (Branch 2). Furthermore, in some specific cases, marked intra-vineyard bacterial differences were found, whereby soils collected within the same vineyard plot separated by few meters clustered within different groups, or even at different branches. This is for instance the case of plots B9a, B9b and B9e within B9 winery (clustering within A and E group, respectively) and B4b, B4c and B4d plots within B4 winery (that clustered in B and C groups, respectively). Satellite photos of the Spanish National Geology Institution revealed that the vegetation type was different for those particular areas within the vineyards in the past, differentiating soils historically supporting grassland vegetation from those supporting forests or Pine grove (Figure 15).

Figure 15: Satellite photos retrieved from the Spanish National Geology Institution. Land vegetation comparison between 2005 and 2017 for A) B9 winery, and B) B4 winery. The letters a, b, c, d and e refer to the nomenclature of plots studied within each winery.

4.1.2 Discussion

The microbial community composition of the studied Txakoli vineyards resembled those found in other distant viticultural regions, and significantly differed from soils from a yet not productive vineyard (the "unestablished vineyard") collected few meters away. These findings suggest that land use and management have a stronger influence than climate or vintage even at a continental scale. For instance, Proteobacteria was the phylum dominating Txakoli soils (30.27 %), similar to previous studies conducted in other viticultural regions (Lauber et al., 2009; Burns et al., 2015; Zarraonaindia et al., 2015; Gupta et al., 2019). The other well represented bacteria phyla were Acidobacteria (16.8%), Verrucomicrobia (16.5%) and Planctomycetes (9.2%). Trivedi et al., (2016) suggested that Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomyces, and Gemmatimonadetes are phyla with the fastest response to human agricultural practices. On the other hand, the unestablished vineyard soils were depleted in Acidobacteria, Fibrobacteria and Firmicutes (12.3% vs 23% on average in productive vineyards) and enriched in Chloroflexi and Verrucomicrobia. The greater abundance of Gemmatimonadetes in the unestablished vineyard was remarkable. Gemmatimonadetes include slow-growing heterotrophic bacteria with versatile metabolism that have a cosmopolitan distribution in terrestrial systems, being present in different soil ecosystems, including grassland, prairie pasture soil, agricultural soil, forest soil and contaminated soils. Studies on their spatial and temporal abundances across five land treatments however demonstrated that their relative abundances were not related to land management but were inversely correlated to soil moisture, suggesting an adaptation to drier soils (DeBruyn et al., 2011).

Prokaryote's alpha- and beta-diversities of Txakoli vineyard's soils varied across the samples studied. Higher richness is generally considered to improve ecosystem resilience (Giller et al., 1997). Most organisms are functionally redundant (Andrén and Balandreau, 1999), however some groups are considered essential species for ecosystem functioning,

such as Cyanobacteria, Armantimonadetes and Fibrobacteres (Chen et al., 2020). Amongst the vineyards with significantly lowest diversity, B9e and A2b, lacked 9 families of the Gammaproteobacterias involved in organic matter release during plant growth (Hou et al., 2017). In addition, some genera within Myxococcales, that plays a role in the iron-reduction during the anaerobic degradation of organic matter (Treude et al., 2003) were also absent, as well as certain families belonging to Cyanobacteria, such as Nostocaceaae. Cyanobacteria represent a very interesting taxon from an agronomic point of view, due to their implication in N_2 fixation, their contribution to improving the soil physicochemical characteristics, the protection against diseases, and the stimulation of plant growth (Singh et al., 2016). Particularly, A2b vineyard showed low vine productivity and poor-quality grapes at harvest, which could in part be due to its lower bacterial richness, essential for maintaining stable processes in changing environments (Loreau, 2001).

Previous studies evidenced that pH is the main soil parameter that determines microbial diversity and composition (Fierer and Jackson, 2006; Ge et al., 2008; Liang et al., 2019). In concordance, pH was a significant driver of the between sample richness differences found in the present study. The pH in Txakoli soils ranged from 4.21 to 8.34; the lowest values for richness were detected in soils with pH around 4, while the highest values were detected in soils with pH around 6.6-7, decreasing slightly afterwards, suggesting a positive correlation (Figure 7) between the richness and pH, similar to Rousk et al., (2010). This is consistent with the optimum soil pH range for grapevine productivity, which ranges between 6.0 - 7.0 (Browde et al., 2006), while for *H. zuri* optimal pH is recommended between 6.5 – 7.0 (JI. Agirretxe -agricultural technical engineer- personal communication, 18-dic 2020). Despite grapevines being able to grow between 4.5 and 8.5 pH, their productivity suffers outside of the optimum range where below 5.5 pH, aluminum and manganese can become toxic, and above 7.0, the availability of metallic micronutrients and phosphorus suffers (Penn and Camberato, 2019).

A strong correlation between microbial community composition and pH was observed too. Interestingly, Chloroflexi phylum was the group showing strongest correlations but

the response to pH was not uniform within the group, with Cladilineae, Anaerolineae, Gitt-GS-136, Ardenticatenia and S085 classes positively correlating, while Ktedonobacteria, JG37-AG-4 and SHA-26 had a negative correlation. The correlation of Anaerolineae with alkaline soils was also observed by Bartram et al., (2014), while Russo et al., (2012) reported it in clay loam acidic soils. Previous studies documented that Proteobacteria, Actinobacteria and Acidobacteria (Jones et al., 2009; Rousk et al., 2010; Fierer et al., 2012; Shen et al., 2013; Bartram et al., 2014) are groups whose relative abundances are affected by pH. Lauber et al., (2009) found that the relative abundance of Acidobacteria decreased with pH, while Actinobacteria and Bacteroidetes positively correlated with soil pH. Other studies document positive correlations for Proteobacteria (Rousk et al., 2010; Bartram et al., 2014). In the Txakoli vineyard soils studied here, Betaproteobacteria and Deltaproteobacteria positively correlated with pH, while Alphaproteobacteria showed a negative relation, similar to what Yun et al., (2016) reported. Bartram et al., (2014) detected particular genera within alphaproteobacteria (*Devosia, Roseomonas, Labrys, Methylosinus, Fulvimarina, Filomicrobium, Rhodobacter, Hyphomicrobium, Bartonella,* and *Mesorhizobium*) to be associated with high pH values in an experimental farm soil with pH gradient ranging 4.5-7.5, similarly to this study. From those genera, in this study, *Rhodobacter* was the only positively correlated, the others were negatively or not correlated. As previously reported [96], [99], [142], Actinobacteria clearly correlated with pH, with the abundance of all its classes positively correlating, such as, TakashiAC-B11 and Thermoleophilia. Concerning Acidobacteria, Rousk and colleagues (Rousk et al., 2010) reported that particular classes correlate with pH either positively (subgroup 5, 6 and 7) or negatively (subgroup 1, 2 and 3). In our study, Subgrup 6 and Acidobacteriia classes were the only significantly correlating, positively and negatively, respectively.

Interestingly, besides pH, other local scale factors determined the community structure of Txakoli soils, such as “vineyard” and “year”. This is in agreement with Zarraonaindia et al., (2015) that reported that “vineyard”, “pH” and “year” were the most significant variables (after sample type) explaining the bacterial community dissimilarities of the

studied vineyards in Long Island. When constraining for those variables, Txakoli soil samples clustered into two big branches that differed in their relative abundances of bacteria involved in N and Carbon cycling and those with antimicrobial activity. For instance, higher numbers of nitrogen fixing bacteria were enriched in branch 1. Nitrifying bacteria can stimulate nitrate transport systems and increase the intake of nitrogen by the plant (Mantelin and Touraine, 2003). In branch 1 Nitrososphaeraceae, Frankiaceae and Blattabacteriaceae families, as well as, *Bradyrhizobium, Devosia, Hyphomicrobium, Methylosinus, Rhizobium, Burkholderia* or *Geobacter* genera dominated. However, branch 2 held higher relative abundances of Nitrospira and *Candidatus Nitrososphaera*, the denitrifiers Steroidobacter and Sphingomonas, and families Saprospisaceae, Beijerinckiaceae, and Sinobacteraceae. The relative abundances of bacteria involved in the carbon cycle, particularly those playing an important role in carbon degradation of complex polymers and CO oxidation improving plant growth (Baker et al., 2009), such as the genera *Candidatus koribacter* and *Candidatus solibacter* (Acidobacteria), were higher also in branch 1. Those two genera are considered to be adapted to nutrient-limited/low resource availability environments (Fierer et al., 2007) and higher acidity (Jones et al., 2009), which is in agreement with the present work that identified those genera to be inversely correlated to pH (Spearman correlation = 0.718, Bonferroni corrected $p < 0.01$). They have been suggested to be associated with conventional farming systems that rely on chemical fertilizers as nutrient input, while in contrast, Cloracidobacteria (enriched in branch 2) have been reported to be more abundant in soils solely receiving organic fertilizers (Hartmann et al., 2015). The fertilization regimes of the vineyards studied in the present work encompass a wide range of practices within organic and conventional agriculture. Farming practice has been documented to significantly affect fungal and bacterial communities in grapes (Martins et al., 2014; Morrison-Whittle and Goddard, 2018), as well as in viticultural soils (Burns et al., 2016; Morrison-Whittle and Goddard, 2018). However, in this study it is difficult to disentangle the intersection between pH or soil properties and management at a regional scale, as they are intrinsically related (Burns et al., 2016) and also, because there are differences between compounds used by Txakoli growers in relations to pesticides, fungicides, and

herbicides even within DOs, as well as on training, pruning, trellising and canopy methodologies, that ultimately will affect soil microbial diversity and composition.

Bacteria with antimicrobial or pathogen inhibitor capacities, as well as capable of degrading xenobiotic compounds, were preferably accumulated in branch 1. These included the Myxococcaceae, a family who's some members produce extracellular anti-fungal metabolites and antibiotics (Daoud and Foster, 1993), and the Bacillus and Paenibacillus genera (Sarker, D. et al., 2010; Grady et al., 2016) known to produce antibiotics or even toxins able to kill insects. The Rhodocyclaceae family, commonly used in bioremediation (Rosenberg et al., 2014), was also more abundant in branch 1. Few bacterial groups, possibly containing pathogenic members, were identified in both branches. *Agrobacterium* genera were found to be distributed along the vineyards, but was significantly enriched in branch 1. However, we were not able to taxonomically assign those OTUs further than genera. Grapevine crown gall disease causing *A. vitis* (Gelvin, 2010) was detected although in low numbers.

According to Fierer (2008), from an oversimplified perspective, there are two drivers of microbial biogeographical distribution: environmental heterogeneity (contemporary environmental conditions such as climate and soil properties) and dispersal limitation (including past environmental conditions) (Baas Becking, 1934; de Wit and Bouvier, 2006; Martiny et al., 2006). Burns and colleagues (Burns et al., 2015), found that American Viticultural Areas (AVAs) with different climate and soil properties had distinctive soil communities. On the contrary, despite the vineyards being dispersed at a similar distance scale, the general distribution of Txakoli soils microbiome did not follow a correlation with the 3 DOs that are established for Txakoli wines. This is in agreement with the idea that large-scale geographic and climatic features significantly affect microbial communities, but that at smaller scales these differences may not be that apparent (Liu et al., 2019). While in the Txakoli viticultural region the climate differences between the 3 DOs are minor, particular trends had been observed in near-shore vineyards which are influenced by microclimate conditions where the sea breeze tempers the

climate, making it less extreme than in inland areas. Inland samples were heterogeneously distributed between the identified two main branches, however near-shore samples from Bizkaiko Txakolina and Getariako Txakolina were exclusive to branch 1 and branch 2, respectively. While the differential clustering of near-shore vineyards is likely due to the combined influence of several factors, differences in management practices could be behind the separation. For example, the amendment of soils with dolomitic lime to raise the pH of acid soils, that commonly leads to accumulation of Mg and Calcium carbonate, is frequent in Getariako Txakolina DO but not in the near-shore vineyards of Bizkaiko Txakolina DO, which might influence microbial composition.

At a smaller local scale, high variability in bacterial composition was observed at an intra-vineyard level in particular cases, for example in B9 and B4 wineries. Setati et al., (2012) demonstrated that fungal populations in grapes had greater intra-vineyard variation than inter-vineyard suggesting that microclimate may play an important role in structuring fungal communities aboveground. However, in general, in viticultural soils microbial communities' variation seem to be higher at an inter-vineyard level than intra-vineyard (Bokulich et al., 2014; Zarraonaindia et al., 2015). Besides, within specific vineyards significant community structure differences were observed, despite the plots studied being located only meters away and therefore having the same climatic conditions and receiving similar management practices. Such spatial variability might likely be a product of past historical events (Martiny et al., 2006) governing those zones. In this regard, the information obtained from satellite photos of the Spanish National Geology Institution revealed that the plots differed in their land usage in the past, supporting different vegetation that is likely behind the significant microbial community variation observed at such a small spatial scale.

4.2 CHAPTER 2: *H. zuri* grape microbial biogeography

The interaction of the grapevine with its environment has been a research topic for a long time now, seeking to understand the basics that would lead to high quality grapes and desired wine characteristics. Indeed, the role of grapes associated microbiota (lactic and acetic bacteria, as well as fermentative fungi and yeast) in the wine production has been long acknowledged, but technological advances such as Next Generation Sequencing have demonstrated microorganisms' interplay in further processes, such as grapevine health. In this study, we sequenced the 16S rRNA gene from 368 grape samples collected in 41 vineyard plots covering the 3 DOs to gain knowledge on microbial terroir and factors governing grape bacterial richness and distribution.

4.2.1 Results

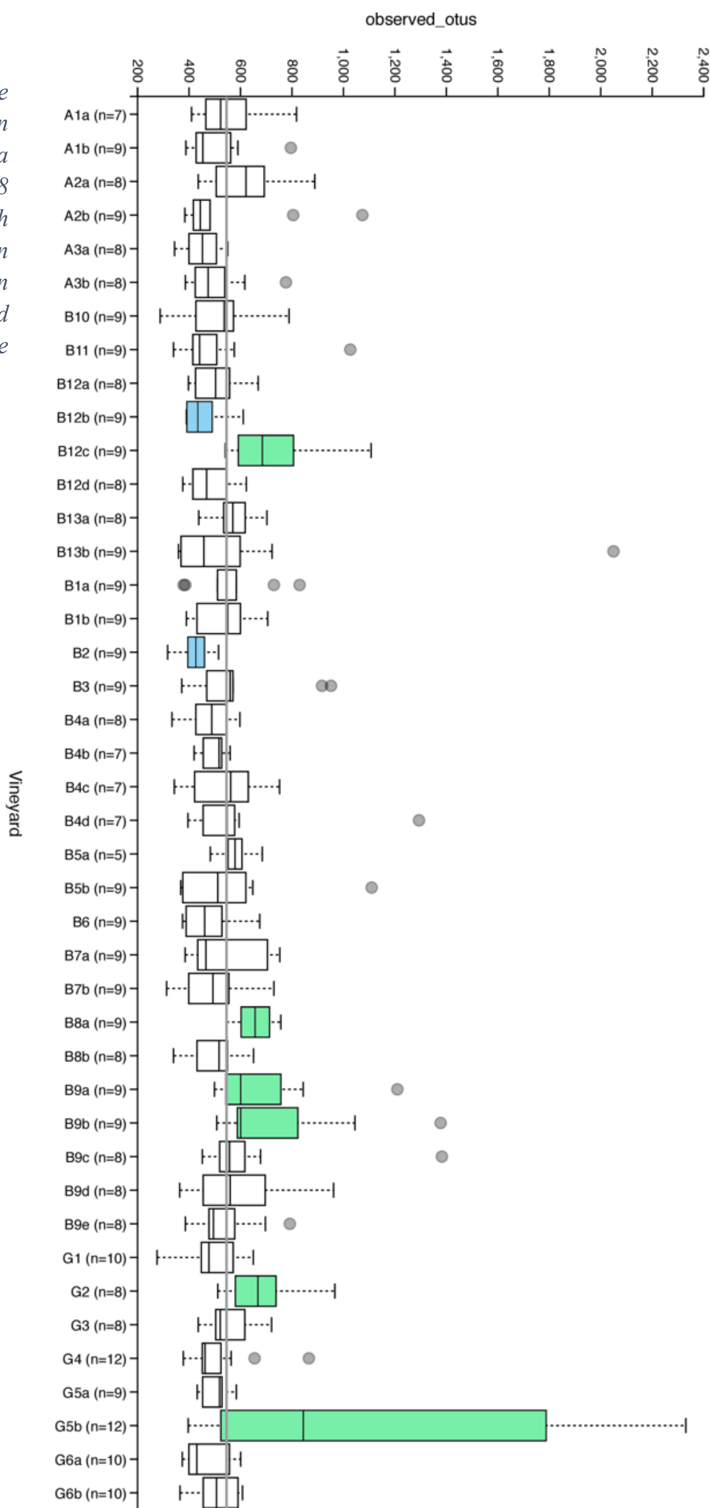
4.2.1.1 Sequencing performance

A total number of 27,490,278 reads were generated in two sequencing runs. After quality trimming (Q20) length filtering (253bp), removing sequences assigned to plants and OTUs with less than 10 sequences, the final OTU table comprised a total number of 16,382,137 sequences in 362 samples.

4.2.1.2 Bacterial richness and composition differ between vineyards and rootstock genotype

Observed OTUs slightly varied between vineyards (Figure 16). Eight vineyard plots out of the 41 studied, (G5b, B12c, B8a, b9a, b9b, B2, B12b, G2) belonging to either Bizkaiko or Getariako Txakolina DO, showed to have a significant difference in richness values with more than 12 other plots (Kruskal-wallis pairwise $p < 0.05$). Those vineyards are therefore the main contributors to the significant correlation found between "vineyard" factor and richness (kruskal-wallis $H = 87.896$ and $p = 0.000028$, and Linear correlation $R^2 = 0.125$ and p-value < 0.001 for 2016 and $R^2 = 0.212$ and p-value < 0.001 for 2017, Table 5). In overall, vineyards richness was higher in 2017 vintage for most of the plots but we cannot rule out that this result is not due to sequencing bias, therefore 2016 and

Figure 16: Vineyard's grape richness barplot. The mean value had been indicated with a horizontal grey line at 558 observed OTUs. Vineyards with significant differences in richness values with more than 12 other plots had been colored in green (higher) and blue (lower).



2017 dataset were not directly comparable and were analyzed separately. The Rootstock genotype grapes were grafted on was the second most strongly correlating factor with the alpha diversity metric in both years dataset (mean R^2 values between 2016 and 2017 samples of 0.135 and 0.212 with a p-value < 0.005, Table 5). Kruskal-Wallis pairwise comparison of samples for 2016 vintage showed that grapes growing in SO4 Rootstock had significantly lower richness, while in 2017, 41B and the ungrafted vines had significantly higher and lower observed OTUs values, respectively (Kruskal-Wallis $p < 0.05$).

Other variables showed subtle influence on the differences on the number of observed OTUs found between samples, with lower R^2 values < 0.056. However, their correlation was not consistent for both vintages. For instance, “Total Temperature”, “Longitude” and “Soil Organic matter” showed to be smoothly correlated with must grape richness in 2016, while “grape pH”, “Training System” (spur or pergola) and “shore effect” (plots located close to the sea or under the influence of sea breeze, and plots located inland) slightly correlated in 2017 vintage.

Table 5: Richness and evenness linear correlation with experimental, vineyard factors, grape chemical factors and soil physicochemical. p <0.05, p <0.01 ** and p < 0.001***.*

	Richness (Observed OTU)				Evenness (Shannon)			
	2016		2017		2016		2017	
	R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value
DO	0.005	0.239	0.009	0.174	-0.001	0.387	-3.6E-04	0.381
Winery	0.074	0.041 *	0.151	0.001 ***	0.078	0.040 *	0.182	1.1E-04 ***
Vineyard	0.170	0.004 **	0.302	2.9E-06 ***	0.209	0.001 **	0.249	7.4E-05 ***
pH	0.016	0.059	0.025	0.021 *	0.018	0.053	0.029	0.014 *
Nitrogen	0.005	0.183	-0.005	0.683	0.006	0.165	-0.005	0.734
TotalAcid	0.005	0.174	-0.004	0.577	0.007	0.162	0.002	0.238
Alcohol	0.010	0.101	-0.005	0.693	-0.001	0.344	-0.005	0.655
Rootstock	0.125	2.7E-05 ***	0.212	6.1E-09 ***	0.093	0.001 ***	0.219	2.8E-09 ***
TTemp	0.024	0.023 *	-0.005	0.700	0.003	0.222	-0.006	0.930
Tprec	-0.005	0.784	0.013	0.069	-0.007	0.970	0.035	0.008 **
Training System	-0.006	0.925	0.021	0.033 *	-0.007	0.866	0.034	0.008 **
Orientation	-0.002	0.414	-0.001	0.375	-0.006	0.686	0.002	0.262
Slope	0.005	0.166	0.004	0.194	-0.005	0.596	0.004	0.200
Altitude	0.006	0.148	-0.004	0.617	-0.005	0.650	-0.045	0.632
Latitude	0.004	0.202	-0.005	0.686	-0.006	0.829	0.016	0.053

Longitude	0.027	0.018 *	-0.002	0.424	0.009	0.120	0.000	0.307
SoilMoisture	0.006	0.156	-0.005	0.660	0.023	0.029 *	-0.002	0.423
SoilpH	-4.9E-05	0.321	0.003	0.210	-4.7E-04	0.339	-0.005	0.658
SoilConductivity	0.017	0.050	0.007	0.142	0.013	0.078	-0.006	0.873
SoilOrganicMatter	0.056	0.001 ***	-0.006	0.945	0.052	0.002 **	-0.006	0.950
SoilNitrogen	0.014	0.069	-0.006	0.784	0.028	0.016 *	-0.003	0.491
Near-shore	0.002	0.242	0.028	0.017 *	-0.006	0.900	0.060	0.001 ***

Bray-Curtis distances ranged between 0.091 to 0.951 values (mean 0.379) among the samples studied. Intra-vineyard beta diversity values (replicas comparisons) were in particular cases similar to inter-vineyard distances, reflecting within plot microbial heterogeneity. Besides, Adonis test revealed significant ($P < 0.05$) compositional differences across Vineyards, this factor explaining the 33 and 27% of the variation for 2016 and 2017 samples, respectively (Table 6). Between vineyards Pairwise Permanova test demonstrated that particular plots had significant composition differences: plots B5a, B9a and B2 from Bizkaiko Txakolina DO, as well as plots G5b, G6a and G6b from Getariako Txakolina DO, showed significant differences for most of the comparisons they were involved in ($p < 0.05$).

*Table 6: Adonis test (999 permutations) values of exploratory factor's effects on microbial structure patterns (p-value significance: p < 0.05 *, p < 0.01 ** and p < 0.001***).*

		2016		**2017**	
		R^2	**p-value**	**R^2**	**p-value**
Experimental factors	DO	0.034	0.001 ***	0.019	0.003 **
	Winery	0.200	0.001 ***	0.158	0.001 ***
	Vineyard	0.327	0.001 ***	0.269	0.001 ***
Grape chemical factor	pH	0.029	0.001 ***	0.010	0.021 *
	Nitrogen	0.008	0.166	0.005	0.528
	TotalAcid	0.020	0.003 **	0.006	0.310
	Alcohol	0.009	0.119	0.005	0.528
Vineyard factors	Rootstock	0.111	0.001 ***	0.077	0.001 ***
	TTemp	0.015	0.003 **	0.006	0.305
	Tprec	0.010	0.052	0.012	0.011 *
	Training system	0.006	0.312	0.008	0.106
	Orientation	0.008	0.146	0.056	0.330
	Slope	0.009	0.078	0.012	0.009
	Near-shore	0.022	0.009 **	0.015	0.002 **
	Altitude	0.008	0.099	0.006	0.321
	Latitude	0.006	0.290	0.010	0.021 *

	Longitude	0.018	0.003 **	0.010	0.013 *
Soil chemical factors	SoilMoisture	0.017	0.005 **	0.017	0.018 *
	SoilpH	0.005	0.493	0.006	0.367
	SoilConductivity	0.009	0.082	0.005	0.616
	SoilOrganicMatter	0.017	0.002 **	0.012	0.081
	SoilNitrogen	0.014	0.017 *	0.030	0.001

Interestingly, Adonis test (Table 6) revealed that beta diversity variation was significantly described by "rootstock" genotype, this factor accounting for the second highest R^2 value (in 2016 $R^2 = 0.111$, p = 0.001 and in 2017 $R^2 = 0.077$, p = 0.001). This result was then confirmed by a stepwise selection Anova model for CCA analysis (Table 7), which identified "rootstock" and "grape pH" (for 2016 dataset) and "rootstock" (for 2017) as the main factors best explaining the variances of *Hondarrabi Zuri* bacterial composition across the two vintages studied.

Pairwise Permanova comparisons evidenced that 41B rootstock and ungrafted vines showed a significantly different bacterial composition in grapes when compared to the other genotypes, while SO4, Gravessac and 3309 composition was more similar (Table 8).

Table 7: Stepwise Anova model selection for CCA analysis of two years. The underlined factor of each step is the one selected to be constrained in the next step.

2016								
Step1			**Step2**			Step3		
	F Pr	**(>F)**		**F Pr**	**(>F)**		**F Pr**	**(>F)**
Rootstock	1.9077	0.005 **	**pH**	1.4915	0.005 **	**Alcohol**	1.0762	0.165
pH	1.6571	0.005 **	**DO**	1.1849	0.015 *	**DO**	1.0639	0.190
TotalAcid	1.3379	0.005 **	**TotalAcid**	1.1422	0.030 *	**TotalAcid**	1.0365	0.265
Vineyard	1.0481	0.005 **	**Longitude**	1.1394	0.065	**Nitrogen**	1.0651	0.280
DO	1.1775	0.020 *	**Slope**	1.0745	0.180	**Management**	0.9394	0.625
Longitude	1.1378	0.095	**Vineyard**	1.015	0.180	**TTemp**	0.9073	0.800
Altitude	1.0609	0.155	**Nitrogen**	1.0727	0.215	**Orientation**	0.9163	0.825
Slope	1.0444	0.225	**Alcohol**	1.0499	0.305	**Altitude**	0.9349	0.840
Orientation	1.0452	0.265	**Management**	1.0215	0.310	**Slope**	0.8712	0.940
Alcohol	1.0248	0.315	**Orientation**	0.9466	0.725	**Longitude**	0.8617	0.950
Nitrogen	1.0414	0.350	**Altitude**	0.9532	0.730	**Tprec**	0.8148	0.990
Management	0.9463	0.550	**TTemp**	0.9199	0.755	**Winery**	0.9261	0.990
Tprec	0.9618	0.585	**Winery**	0.964	0.855	**Latitude**	0.8087	0.995
TTemp	0.9204	0.720	**Tprec**	0.9124	0.865	**Vineyard**	0.9333	0.995
Latitude	0.8976	0.870	**Latitude**	0.8769	0.940			
Winery	0.9497	0.910						

2017					
Step1			**Step2**		
	F	**Pr(>F)**		**F**	**Pr(>F)**
Rootstock	1.1489	0.040 *	**Winery**	1.0419	0.123
Winery	1.0419	0.075	**Longitude**	1.0888	0.195
Longitude	1.0941	0.075	**DO**	1.0599	0.200
DO	1.0958	0.095	**TTemp**	1.076	0.215
Alcohol	1.0747	0.150	**Altitude**	1.0329	0.255
TTemp	1.0125	0.390	**Alcohol**	1.0006	0.435
Nitrogen	0.9993	0.445	**Tprec**	0.9947	0.495
pH	0.9902	0.460	**pH**	0.9691	0.550
Latitude	0.9871	0.485	**Latitude**	0.9736	0.600
Tprec	0.9875	0.590	**Nitrogen**	0.9536	0.715
Vineyard	0.9972	0.670	**Slope**	0.9355	0.810
Altitude	0.9616	0.710	**TotalAcid**	0.9281	0.825
TotalAcid	0.9445	0.720	**Orientation**	0.9121	0.930
Slope	0.9575	0.725	**Vineyard**	0.9722	0.930
Orientation	0.9546	0.780	**Management**	0.7784	0.995
Management	0.8149	0.960			

*Table 8: Pairwise permanova comparison. Different rootstock's bacterial communities' comparison to identify the significantly different bacteriome (p-value significance: p < 0.05 *, p < 0.01 ** and p < 0.001***).*

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
41B	**3309**	89	5.453	**0.001 *** **	**0.007 ** **
41B	**SO4**	213	9.700	**0.001 *** **	**0.007 ** **
41B	**Gravessac**	28	2.867	**0.014 * **	**0.037 * **
41B	**Ungrafted**	51	7.576	**0.001 *** **	**0.007 ** **
SO4	**Ungrafted**	240	2.547	**0.012 * **	**0.036 * **
3309	**Ungrafted**	116	2.603	**0.008 ** **	**0.028 * **
Gravessac	**Ungrafted**	55	2.075	**0.031 * **	0.065
Gravessac	**SO4**	217	1.726	0.065	0.105
Gravessac	**3309**	93	1.150	0.253	0.266
3309	**SO4**	278	1.354	0.122	0.167

Several other factors, for instance grape pH, Nearshore category, longitude, and soil N and moisture showed to have less explanatory power with R^2 values < 0.03, but their significance was consistent across vintages.

4.2.1.3 Differentially abundant OTUs responding to Rootstock genotype

Abundance differences of several viticulturally important taxa were found to be associated to the rootstock genotype *H. zuri* grape was growing on (Figure 17, Supplementary Dataset 3). The group 41B and the ungrafted grapes were the samples with the highest number of bioindicative taxa in each of the years (75 and 154 in 41B, and 45 and 9 in the ungrafted berries, for 2016 and 2017 respectively). Nitrogen fixing bacteria such as envOPS12 (Choroflexi), vadinHA49 class (Plactomycetes), *LCP_6* genera (Nitrospira), Puniceicoccaceae family (Verrucomicrobia) and MBGB class (Crenarchaeota) were consistently more abundant in 41B rootstock grapes across both vintages. Acetobacteraceae family was also augmented in both years but the enrichment was due to different genera, *Gluconobacter* in 2016 and *Gluconacetobacter* in 2017. Other bacterial groups showed to be just significantly augmented in one of the years. For instance, *Pseudomonas viridiflava* (Gammaproteobacteria) and *Lewinella persicus* (Saprospirae) were significantly more abundant for 41B rootstock in 2016. Similarly,

2017 41B samples had particular genera augmented, mainly belonging to Proteobacteria phyla. Within Alphaproteobacteria, higher abundances of *Asticcacaulis (*Caulobacterales order), *Hyphomicrobium, Kaistia* and *Labrys* (Rhizobiales order), and *Kaistobacter* (Sphingomonadales) were found. Likewise, members of Deltaproteobacteria were also particularly augmented, such as *Desulfofaba* (Desulfobacterales), *Geobacter* (Desulfuromonadales) and *Rickettsiella* (Legionellales).

Ungrafted *H. zuri* were characterized by the enrichment of several genus related with wine making (Paenibacillus, Oenococcus, Streptococcus, Acetobacteraceae, Sphingomonas, Mycobacterium, Staphylococcus, Anaerococcus, Aggregatibacter) and diseases (*Agrobacterium*). However, none of them was consistent with both vintages. A deeper analysis of the sequences classified as Agrobacterium evidenced that, according to NCBI blast, the here identified sequences belonged to *Agrobacterium larrymoorei*, *A. vitis, A. tumefaciens, A. salinitolerance, A. rhizogenes, A. rubi* and some non-classified. Among them, *Agrobacterium larrymoorei* was identified to be generally more abundant on ungrafted *H. zuri* grapes (Figure 18), although some particular plots grafted on SO4 showed also high values.

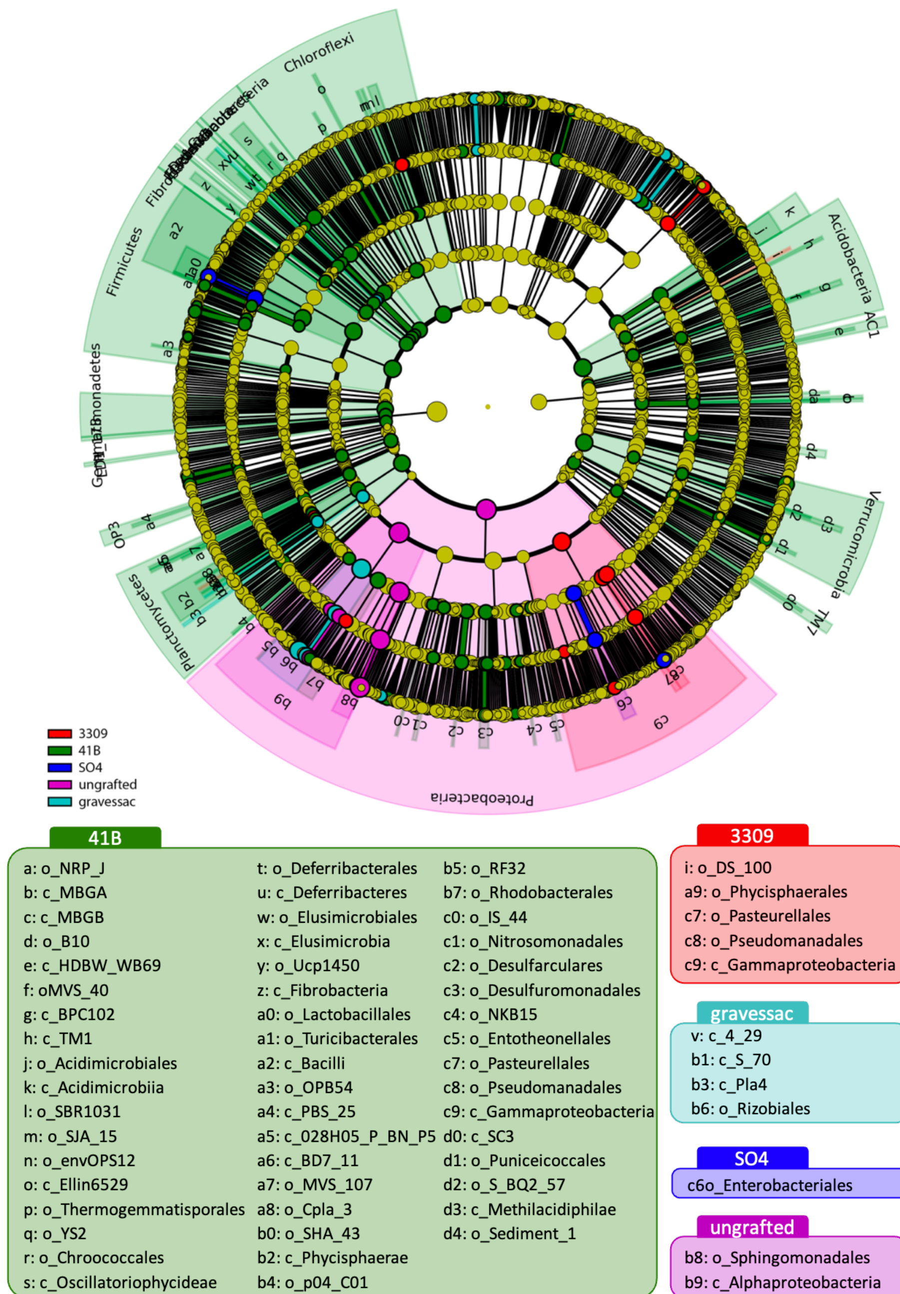


Figure 17: Cladogram representation of statistically bacterial differences between grapes grown in different rootstock. Taxonomic groups enriched (linear discriminant analysis (LDA) effect size (LEfSe) analysis based on Kruskal–Wallis $p < 0.05$, and LDA scores Log10 > 2) in each rootstock are represented with colored dots. The legend indicates the differentially abundant classes and orders per genotype. Genera or species level information and the LDA score values for each group are available in Supplementary Dataset 3.

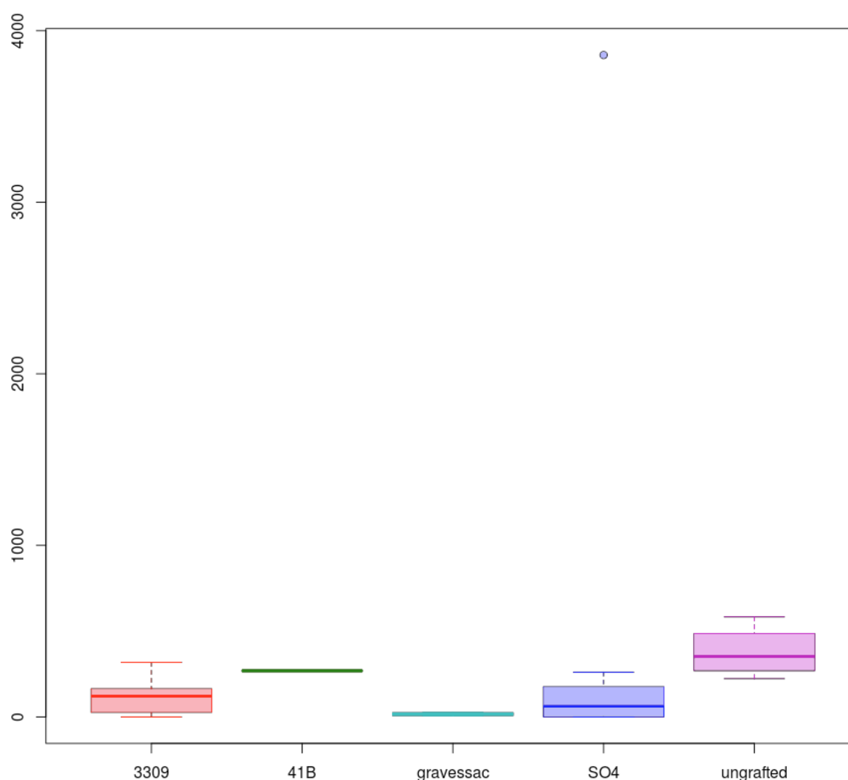


Figure 18: Agrobacterium larrymoorei abundance on grapes on top of different rootstock. Ungrafted grape samples showed the highest abundances of this harmful species. A particular vineyard on top of SO4 showed an exceptionally high abundance which coincides with the poor state of health the vineyard was in.

Grapes growing on top of 3309 rootstock exhibited higher abundances of the fungal antagonist *Curtobacterium* in both years. In addition, members of Pseudomonadales order (Pseudomadaceae in 2016 and Moraxellaceae families in 2017) were also increased. SO4 samples had the plant promoting *Acinetobacter rhizosphaera* consistently augmented, while Lactobacillus genera was enriched just in 2017 samples. Finally, samples grafted on Gravessac rootstock were enriched for the *Methylobacterium* and *Phyllobacterium* epiphytic genus. In addition, other viticulturally important groups, such as Leuconostocaceae family and *Acetobacter* genera were differentially abundant (Figure 17).

4.2.2 Discussion

The microbial distribution associated with vineyards is governed by local environmental factors such as microclimate, soil management, etc. (Martins et al., 2012; Bokulich et al., 2014) giving rise to the so-called "microbial terroir", which is key in determining the regional wines/AVAS (Bokulich et al., 2014, 2016; King et al., 2014; Taylor et al., 2014; Burns et al., 2015; Liu et al., 2019).

In the present study bacterial biogeographical trends were observed at a small geographical scale (7,234 km^2) in a local grape variety (*Hondarrabi zuri*) at a single agriculture region, the Basque Country. Despite this viticultural zone holding 3 Designations of Origin, DO factor was not associated with microbial richness nor with the beta diversity patterns. This indicates there are no differences in bacterial diversity and composition among the DOs. Therefore, the differences in organoleptic perception that are associated with the Txakoli coming from different DOs would not be determined by the existence of DO specific bacteria. The study of the fungal and yeast microbial fraction, together with the metabolic profile of each DO grape will be necessary to elucidate where and when do the perceptible differences between wines originate. The process followed in the cellars and technical aspects during wine-making are likely one of the main sources for the differentiation of DOs. In fact, while each winery has developed its own style to adjust to consumer preferences, there are certain regional technical commonalities that go beyond the microbial profiles, as described below.

The biogeographical trends observed in *H. zuri*'s bacterial community were driven by vineyard localization, being longitude and distance to the shore two determinant factors. Geography was also suggested to be of particular relevance in the fungal community composition associated with Chardonnay grapes from New Zealand (Taylor et al., 2014). Similarly, in the case of Sauvignon Blanc grapes, six genetically distinct zonal populations of *S. cerevisiae* were also found within the same region (Knight and Goddard, 2015). Furthermore, the community similarity of Chardonnay must from California was

shown to follow a geographical axis across the coastline (Bokulich et al., 2014). Interestingly, we also found that *Hondarrabi zuri* microbiota is responsive to the particular microclimate conditions generated by the sea breeze, as plots located close to the coast, or being under the influence of the winds coming from the Atlantic Sea, showed a distinguishable bacterial composition in comparison to that of inland locations. Indeed, plots located nearshore had lower pH values on average, which coincides with the perception of viticulturist and enologist that berries from coastal or intermediate locations have a later maturation and higher acidity than inland ones (personal communication of Elena Garcia from ARDOATEK S.L., and master thesis of Asier Garcia Diez). Berry maturation is linked to climatic conditions (Martin et al., 2016), accordingly in the present study we found that several environmental parameters correlated with *H. zuri* grape microbiota, such as total temperature, total precipitation and vineyard orientation. However, more in-depth research on grape microclimate would be necessary to better understand the influence of the coastal winds on grape maturation and microbiota. In this contest, it would be of interest to look more deeply into factors such as, the total amount and distribution of vine leaf area through defoliation, pruning or even training systems (spur or pergola), that alters the entrance of coastal winds into the branches modifying the temperature and humidity within grape clusters (Reynolds and Heuvel, 2009).

Previous studies have reflected that soil is a bacterial reservoir for the plant (Martins et al., 2013; Zarraonaindia et al., 2015). Soil properties affect not only soil and belowground (rhizosphere and root) microbiota but also aboveground compartments. In this sense, Zarraonaindia et al., (2015) showed that total soil carbon, and soil moisture and temperature influenced the canopy organs microbiota. In this study, soil C did not have a significant impact on *H. zuri* grape microbiota, but N and soil moisture content did, which could be explained by differences in fertilization dynamics and historical land use in the vineyard plots studied. Moreover, in addition to soil properties, plants themselves might also play a role in grape microbiota diversity/composition. Indeed, plants interact with their surrounding soil by rhizodeposition through the roots. These exudates vary with the development of plants, among species and even among genotypes within same species

(İnceoğlu et al., 2010; Philippot et al., 2013; Gilbert et al., 2014; Bazghaleh et al., 2015; Hacquard, 2016; Wagner et al., 2016; Lemanceau et al., 2017; Qiao et al., 2017). Elucidating the effect of grapevine genotypes on the composition of the bacterial and fungal microbiota in grape has its intricacies in *Vitis vinifera*, because most European plants are grafted onto rootstocks of other *Vitis* species. Its ultimate goal is to confer resistance against phylloxera (*Phylloxera vastratix* or *Daktulosphaira vitifoliae* nowadays) and other pathogens, in addition to enhance their adaptability to several environmental stressors (Reynolds and Wardle, 2001). Anyway, grafting connects the genotype of the scion of one grapevine species (young shoot, branch, or bud) with the genotype of the root of another species/hybrid and the impact of this process in the grapevine microbiome is poorly understood. Recent studies demonstrate that the rootstock genotype alters root and rhizosphere bacterial and fungal diversity/composition (D'Amico et al., 2018; Marasco et al., 2018; Berlanas et al., 2019; Dries et al., 2021). However, rootstock genotype influence on the microbiota in the compartments of the canopy, such as grapes, has been less investigated. In this sense, previous studies showed that rootstock influences grapevine physiology, having implications for the plant vigor, yield, and fruit and wine qualities (Warschefsky et al., 2016). Therefore, it seems likely that the grape microbiome would be shaped by the rootstock. Yet, in a recent study, Swift and colleagues (Swift et al., 2021) observed that rootstock genotype did not significantly influence bacterial diversity or composition of leaves and grapes, while the fungal community showed subtle differences. However, abundance of certain bacteria such as *Acetobacterales* and *Saccharomycetes* did show to be significantly different between rootstock. In our study, bacterial taxa particularly important for grapevine health and growth, and implicated in the fermentation process and wine quality were also associated with the different rootstock genotypes.

H. zuri grape microbial composition observed in this study correlated with the disease resistance/susceptibility patterns described for each of the rootstock analyzed. Grapes on top of SO4 rootstock (*V. Riparia* x *V. Berlandieri*), the most popular rootstock for *H. zuri* vines in the Basque Country, hold a more similar bacterial composition to those grafted

on 3309C (*V. Riparia* x *V. Rupestris*) or Gravessac (*V. Berlandieri* x *V. Riparia* x *V. Rupestris*). SO4, 3309 and Gravessac grafted grapes showed an enhancement of several beneficial groups, and interestingly all three rootstocks are known for being resistant to phylloxera, and most importantly, to downy mildew (Catalogue des Varietes et Clones de Vigne cultives en France), a frequent pathogen in the region. For instance, SO4 rootstock grapes were enriched with the plant-growth promoting rhizobacteria (PGPR) *Acinetobacter rhizosphaerae*. The fact that this group abundance appears to be rootstock-mediated demonstrates that, as previously suggested by other author (Bulgarelli et al., 2013), the root genotype is key for selecting and facilitating the passage of soil borne/rhizosphere bacteria through the roots, and from there, to aerial organs (Compant et al., 2019). Likewise, 3309 rootstock exhibited higher abundances of the PGPR *Pseudomonas* and the fungal antagonist *Curtobacterium*. Similarly, grapes onto Gravessac rootstock were enriched in Methylobacterium, which stimulates plant development through phytohormone productions (Kutschera, 2007) and Phyllobacterium, studied of the bio-efficacy of growth regulators to improve fruit quality of grape (Flores-Félix et al., 2015). Phyllobacterium has been found to be a contributor to wine final characteristics. On the other hand, the enrichment of *Agrobacterium* genera in ungrafted grapevines, highly susceptible to phylloxera and nematodes, evidenced that as expected, own-rooted vines have higher susceptibility to the development of crown-gall disease (Burr et al., 1998) than grafted vines. A closer look at the sequences suggested that *Agrobacterium larrymorei*, renamed as *Rhizobium larrymorei* (Young et al., 2003) was significantly more abundant on ungrafted grapes. In good agreement with the susceptibility to *Agrobacterium vitis* of 41B rootstock (*Vitis Vinifera* x *V. Chasselas* x *V. Berlandieri*), grapes grafted onto 41B were the ones presenting the second most abundant values of *Rhizobium larrymorei*. Furthermore, *Pseudomonas viridiflava*, pathogenic bacteria causing foliar and stem necrotic lesions, as well as root rots, were also significantly enriched in 41 B grapes, suggesting that grapevines grafted on such rootstock have an increased risk of having abnormal fruit development in diseased plants.

Certain bacteria particularly interesting from the winemaking point of view were also differentially abundant according to the rootstock. In this regard, 41B rootstock grapes showed significantly higher abundances of *Gluconobacter* and *Gluconacetobacter*. Both acetic acid bacteria are commonly isolated from grapes. While once considered to be spoilage microorganism, several studies demonstrated they acquire high proportions during spontaneous fermentation (Portillo and Mas, 2016). *Gluconobacter* and *Gluconacetobacter* enhancement coincides with the particularities of 41B rootstock, which are known to yield grapes less rich in sugar and slightly more acidic. In this sense, *Gluconobacter* has been negatively associated with must pH in "Chardonnay" from California (Bokulich et al., 2016). Moreover, several Lactic acid bacteria, for instance, *Oenococcus, Lactobacillus, Leuconostaceae, Enterococcus* and *Sphingomonas*, together with acetic acid bacteria (mainly members of Acetobacteraceae family) showed to be differentially distributed between rootstock. Considering that those groups are important players in the wine fermentation process, this demonstrates that the rootstock genotype, aside from interfering in grapevine growth and health, has implications for determining wine characteristics by shaping grape bacterial richness and composition of key bacterial groups.

GENERAL DISCUSSION

5 General Discussion

Vineyards microbial biogeography is the result of the joint influence of countless of factors that impact at different spatial scales, which limits in part our ability to acquire general microbial biogeographic trends, but allows us to inquire on the ecological role of the bacterial community differences and their correlations with environmental factors.

We characterized the profile of bacterial communities in soils (Chapter 1) and grapes (Chapter 2) of multiple vineyards from the Txakoli viticultural area, and we studied the drivers of the observed microbial diversity and distribution patterns. In this regard, 16S rRNA Illumina amplicon sequencing was applied to study 41 vineyard´s soil and grape samples covering the three Designation of Origin (DO) for the Txakoli wine production area.

Given the differential organoleptic characteristics of the tree DOs, one might think of the concept of terroir, and therefore look for differences in the bacterial communities of the three DOs. However, no DO specific bacterial profiles were found neither in soils nor in grapes. Those results suggest that at least from a bacterial point of view, the Basque Country viticulture region for txakoli contains a unique bacterial terroir. However, enologists do find specific regional characteristics within Txakoli wines, with the origin of these particularities of the DOs, possibly being differences inherent to the winemaking procedure. DOs are divided based on geographic political provinces, rather than scientific bases, and each DO follows its own regulations and rules. For instance, in all three provinces, for a bottle of Txakoli to have a DO seal, at least 85% of the juice must come from the *H. zuri* variety. But the three DOs vary as to the rest of the varieties allowed. In fact, Getariako Txakolina can blend their must with *Petit Courbu, Gros Manseng, Riesling* and *Chardonnay* varieties while in Bizkaiko Txakolina *Folle Blanche, Petit Manseng* and *Sauvignon Blanc* are also permitted. This certainly has implications for the organoleptic characteristics of the wine and therefore in wine’s typicity, as it is known that metabolites and aromatic compounds, as well as the microbial profiles, significantly

differ from one grape variety to another (Bokulich et al., 2014). In addition, the minimum alcohol degree for Getariako Txakolina wines is 9.5, while it is established at 10 degrees for Bizkaiko Txakolina. Similarly, in Getariako Txakolina the volatile acid value of the wine is placed at a maximum of 0.8 g/L, but in Bizkaiko txakolina the threshold is placed at 0.6 g/L (BOE-A-2010-123, BOE-A-2010-124). Additionally, wines from Getariako Txakolina are sparkling as they have adopted the inclusion of carbon dioxide in their wines, which is not a common technique in Bizkaiko or Arabako Txakolina. In short, all of the above will promote regional enological differences and suggests that, if DO specific bacterial profiles do exist in Txakoli, they originate at the cellar and during the winemaking process rather than at the vineyard. Even so, the study of the fungal and yeast microbial fraction, together with the metabolic profile of each DO grape, must and ferments will be necessary to elucidate where and when do the perceptible differences between wines from the three DOs originate.

In any event, significant bacterial profile differences were found between and within the studied vineyards that point to a biogeographic trend. On the one hand, the microbial community composition of the studied Txakoli vineyards soils resembled those found in other distant viticultural regions, but interestingly, they differ significantly from nearby soils, i.e., from a still unproductive vineyard a few meters away. This suggested to us that land use was the most determinant factor at continental scale for bacterial profiling, overpassing growing region, climate or year.

On the other hand, at a local scale we appreciated that historical anthropogenic modifications in land use are relevant to understanding bacterial variation. In fact, certain samples collected within the same plot, a few meters apart, showed greater bacterial differences than those collected in vineyards located km-s apart. Upon inquiry, we learned that those parts of the plot with large bacterial differences had very different vegetation prior to vineyard planting, i.e., one part of the plot was once a grassland, while the other one was part of a forest. Little is known about bacterial community successions as land use changes, but we know, based on this study, that historical land use and vegetation are

important factors shaping microbial community composition, in addition to current soil characteristics, such as pH or nutrient status. Indeed, within the Basque Country, soil pH significantly correlated with differences in bacterial diversity and composition, and together with “year” and “vineyard” grouped the 41 vineyard soils into 2 clusters. The clusters differed in their relative abundances of bacteria associated with N and Carbon cycling and antimicrobial activity. Further research should be dedicated to understanding soil processes and the impacts of soil borne microbial community shifts on nutrient dynamics in vineyards, as these processes have implications for grapevine productivity and grape quality and health. The identification of subzones within the vineyards would allow conducting more targeted management adjusted to the needs of its patch. For instance, it would lead to more efficient applications of pesticides and fertilizers, as well as a greater control of the fermentation and the quality of the final product, in line with precision viticulture technology.

Interestingly, in concordance to Bulgarelli et al., (2013), while soil microbes were shaped by edaphic factors, grape microbial diversity and distribution were more strongly linked to the rootstock genotype. A differential recruitment of several plant growth promoting bacteria was evidenced among the 4 grafted grapevines types (SO4 (*V. riparia* x *V. berlandieri*), 41b (*V. vinifera* x *V. chasselas* x *V. berlandieri*), 3309 (*V. riparia* x *V. rupestris*), and Gravessac (*V. berlandieri* x *V. riparia* x *V. rupestris*)) and the ungrafted vines. In addition, various bacteria particularly interesting from the winemaking point of view (known fermentative and wine spoilers, members of lactic acid and acetic acid bacteria) were also differentially abundant according to the rootstock which has a tremendous importance due to its implication to wine quality. Studies conducted by Compant and colleagues (Compant et al., 2010) in grapevines evidenced that soil borne bacteria are transported through the roots, and from there, to aerial organs. While the rootstock genotype having an impact on the rhizosphere and soil in the vicinity of roots is a known fact (Bulgarelli et al., 2013; Berlanas et al., 2019; Dries et al., 2021), the influence of the rootstock on aerial parts is yet hardly known. Our study demonstrates that root genotype is key for selecting and facilitating the passage of soil borne bacteria

through the roots to the grapes. However, bulk soil samples from the exact same vineyards did not show rootstock genotype dependency and were mainly driven by pH. The explanation behind likely relies on the fact that while plants interact with the belowground by the release of secondary compounds that modify the metabolic activity of the microorganisms, this impact is only perceptible in the soil closely in touch with the root (rhizosphere and rhizoplane).

Interestingly, similar to what we found in the soil, *Hondarrabi zuri* grape microbiota is perceptible to the particular microclimate conditions generated by the sea breeze, as grapes from plots located close to the coast, or being under the influence of the winds coming from the Atlantic Sea, showed a distinguishable bacterial composition from that of inland locations. On average lower grape pH values were found in plots located nearshore which coincides with viticulturist and enologist perception that state berries in coastal or intermediate locations have a delayed maturation and higher acidity than inland ones (personal communication of Elena Garcia from ARDOATEK S.L., and Final master thesis of Asier Garcia Diez). Berry maturation is linked to climate conditions (Martin et al., 2016). More in-depth research on microclimate conditions, such as canopy temperature/humidity and vines physiology (photosynthetic activity, etc.) in different training systems would be necessary to better understand the particularities of the influence of the coast on grape maturation and microbiota.

CONCLUSION

6 Conclusions

1. High throughput 16S rRNA sequencing of the V4 region is effective in unraveling the diversity of both the dominant and rare microbial fraction of Txakoli vineyards, making it a powerful tool to describe the bacterial communities. In fact, it allowed us to generate an in-depth bacterial inventory of *H. zuri* soil and grape environments by identifying ample plant growth-promoting bacteria, antagonists, pathogens, wine spoilers and bacteria associated with must fermentation.

2. Regarding the variance shown by the bacterial distribution, in the soil samples we were able to find factors explaining 81% of the variance, while in the berry samples only 30% was explained. This leads us to consider that, although a comprehensive sampling was carried out covering vineyards throughout the region and taking into account a large number of potential factors, other important factors such as fertilization practices, phytosanitary treatments, trellising strategies, etc., should be included to acquire a broader picture of the factors determining the bacterial biogeography of grapes.

3. In Txakoli vineyards, the diversity and distribution of soil bacteria depend mainly on soil pH, so that although Txakoli vineyards showed the ability to grow in a wide range of soil pH (4.21 to 8.34), soils with pH values between 6.5 -7 showed the greatest bacterial richness and, therefore, the greatest resilience and functional adaptation.

4. In terms of microbial communities within vineyards, significant differences linked to historical land use were appreciated. The ability of the used approach to identify microbial differences on such a small scale opens the possibility of including 16S rRNA sequencing as an additional tool in precision viticulture.

5. The bacterial communities of *H. zuri* grapes are highly dependent on the rootstock. In detail, the relative abundance of antagonists, plant growth-promoting bacteria and fermenting bacteria are of particular importance, which greatly inform the resistance/susceptibility of the grapevine to diseases and its organoleptic potential. This information could provide guidance for more in-depth decisions when choosing the appropriate rootstock for new plantings.

6. Among the rootstocks, ungrafted and 41B are the most susceptible genotypes to *Rhizobium larrymorei* and *Rhizobium vitis*, responsible for crown-gall disease.

7. In *H. zuri* grapes, the bacterial community's distribution and abundance are also dependent on soil N and moisture, evidencing the great interplay between the grapevine and the soil in which it grows.

8. Soil and grape bacterial diversity and composition of *H. zuri* differ significantly among vineyards, but no DO-specific bacterial profile was identified, suggesting that the Txakoli viticultural region of the Basque Country represents a single bacterial terroir. Yeast and fungal studies that track microorganisms in the field, cellar and in winemaking processes are needed to unravel whether the final wine has a microbial terroir and at what step differentiation begins.

9. Both soil and grape microbiota were shown to be affected by distance from the sea or the influence of sea breeze. Further analyses should be conducted to gain more knowledge on the influence of microclimatic factors on grape microbial ecology, so that wine growers can use this information to improve grape quality and wine characteristics.

SUPPLEMENTARY DATA

Supplementary Table 1: Samples studied and exploratory factors analyzed. Experimental factors (DO, winery, vineyard and year), soil edaphic factors (moisture, pH and conductivity), and vineyard associate attributes (slope, orientation, near-shore effect, altitude and geographical coordinate) of the soil samples collected in the Txakoli viticultural region.

DO	Winery	Vineyard	Sample	Year	Orientation	Slope	Moisture	pH	Conductivity	Near-shore	Altitude	Latitude	Longitude
Araba	A1	A1a	A1.a.2017.s1	2017	15	13.4	22.2322639	6.6	0.412	no	198	43.15527778	-3.037222222
			A1.a.2017.s2	2017	15	13.4	20.3374778	6.55	0.428	no	198	43.15527778	-3.037222222
			A1.a.s1	2016	15	13.4	14.09465021	6.73	0.24	no	198	43.15527778	-3.037222222
			A1.a.s2	2016	15	13.4	16.51785714	6.82	0.279	no	198	43.15527778	-3.037222222
		A1b	A1.b.2017.s1	2017	28	17	24.58888019	7.15	0.836	no	179	43.15611111	-3.035277778
			A1.b.2017.s2	2017	28	17	25.27075812	7.12	0.713	no	179	43.15611111	-3.035277778
			A1.b.s1	2016	28	17	15.11226252	6.81	0.2	no	179	43.15611111	-3.035277778
			A1.b.s2	2016	28	17	17.73109244	6.97	0.283	no	179	43.15611111	-3.035277778
	A2	A2a	A2.a.2017.s1	2017	48	18.4	18.97810219	5.66	0.296	no	334	43.12944444	-2.988333333
			A2.a.2017.s2	2017	48	18.4	20.58414465	5.48	0.208	no	334	43.12944444	-2.988333333
			A2.a.s1	2016	48	18.4	14.15789474	5.94	0.176	no	334	43.12944444	-2.988333333
			A2.a.s2	2016	48	18.4	14.01574803	5.92	0.165	no	334	43.12944444	-2.988333333
		A2b	A2.b.2017.s1	2017	25	28	11.65821868	4.76	0.177	no	317	43.13027778	-2.9875
			A2.b.2017.s2	2017	25	28	11.56069364	4.69	0.162	no	317	43.13027778	-2.9875
			A2.b.s1	2016	25	28	13.72891216	5.11	0.098	no	317	43.13027778	-2.9875
			A2.b.s2	2016	25	28	15.54404145	5.29	0.076	no	317	43.13027778	-2.9875
	A3	A3a	A3.a.2017.s1	2017	37	23.4	7.261592301	8.32	0.449	no	381	42.98611111	-2.978333333
			A3.a.2017.s2	2017	37	23.4	9.807010634	8.34	0.427	no	381	42.98611111	-2.978333333
			A3.a.s1	2016	37	23.4	9.591642925	7.23	0.268	no	381	42.98611111	-2.978333333
			A3.a.s2	2016	37	23.4	8.226037196	7.18	0.23	no	381	42.98611111	-2.978333333
		A3b	A3.b.2017.s1	2017	65	5.5	12.45376079	8.28	0.501	no	323	42.98333333	-2.984722222
			A3.b.2017.s2	2017	65	5.5	12.63581489	8.33	0.459	no	323	42.98333333	-2.984722222
			A3.b.s1	2016	65	5.5	9.267431598	7.15	0.234	no	323	42.98333333	-2.984722222
			A3.b.s2	2016	65	5.5	9.557109557	7.17	0.229	no	323	42.98333333	-2.984722222
	B1	B1a	B1.a.2016.s1	2016	39	6.5	20.19950125	6.58	0.197	no	75	43.27694444	-2.498888889
			B1.a.2016.s2	2016	39	6.5	21.62615256	6.8	0.236	no	75	43.27694444	-2.498888889
			B1.a.2017.s1	2017	39	6.5	23.54551677	6.55	0.485	no	75	43.27694444	-2.498888889
			B1.a.2017.s2	2017	39	6.5	25.73710074	6.63	0.511	no	75	43.27694444	-2.498888889
			B1.a.s1	2016	39	6.5	20.19950125	6.58	0.197	no	75	43.27694444	-2.498888889
			B1.a.s2	2016	39	6.5	21.62615256	6.8	0.236	no	75	43.27694444	-2.498888889

	B1b	B1.b.2017.s1	2017	40	11.5	22.07233065	6.36	0.285	no	74	43.27777778	-2.498333333
		B1.b.2017.s2	2017	40	11.5	22.70898525	6.43	0.342	no	74	43.27777778	-2.498333333
		B1.b.s1	2016	40	11.5	21.03533279	6.48	0.189	no	74	43.27777778	-2.498333333
		B1.b.s2	2016	40	11.5	20.15334064	6.47	0.197	no	74	43.27777778	-2.498333333
B10	B10	B10.2017.s1	2017	12	13.4	20.10331468	5.81	0.212	no	56	43.30083333	-2.875277778
		B10.2017.s2	2017	12	13.4	24.78909799	5.87	0.485	no	56	43.30083333	-2.875277778
		B10.s1	2016	12	13.4	17.10526316	7.2	0.273	no	56	43.30083333	-2.875277778
		B10.s2	2016	12	13.4	19.7172619	6.93	0.317	no	56	43.30083333	-2.875277778
B11	B11	B11.2017.s1	2017	12	7.3	26.30806846	5.4	0.325	yes	208	43.38361111	-2.621944444
		B11.2017.s2	2017	12	7.3	26.89994533	5.29	0.281	yes	208	43.38361111	-2.621944444
		B11.s1	2016	12	7.3	18.59177215	5.83	0.11	yes	208	43.38361111	-2.621944444
		B11.s2	2016	12	7.3	18.05135952	5.83	0.088	yes	208	43.38361111	-2.621944444
B12	B12a	B12.a.2017.s1	2017	53	14.4	23.9292365	5.77	0.179	no	97	43.26	-2.789166667
		B12.a.s1	2016	53	14.4	21.09660574	5.96	0.112	no	97	43.26	-2.789166667
		B12.a.s2	2016	53	14.4	19.64696853	6.01	0.141	no	97	43.26	-2.789166667
	B12b	B12.b.2017.s1	2017	70	17.3	23.56202356	5.72	0.377	no	229	43.30388889	-2.768611111
		B12.b.2017.s2	2017	70	17.3	25.02078138	6.04	0.355	no	229	43.30388889	-2.768611111
		B12.b.s1	2016	70	17.3	21.15384615	6.18	0.137	no	229	43.30388889	-2.768611111
		B12.b.s2	2016	70	17.3	21.26436782	6.53	0.129	no	229	43.30388889	-2.768611111
	B12c	B12.c.2016.s1	2016	38	12.7	13.02190988	6.14	0.091	no	105	43.2575	-2.785833333
		B12.c.2016.s2	2016	38	12.7	15.15007146	5.92	0.082	no	105	43.2575	-2.785833333
		B12.c.2017.s1	2017	38	12.7	21.85338866	5.28	0.366	no	105	43.2575	-2.785833333
		B12.c.2017.s2	2017	38	12.7	19.70286523	5.45	0.141	no	105	43.2575	-2.785833333
		B12.c.s1	2016	38	12.7	13.02190988	6.14	0.091	no	105	43.2575	-2.785833333
		B12.c.s2	2016	38	12.7	15.15007146	5.92	0.082	no	105	43.2575	-2.785833333
	B12d	B12.d.2017.s1	2017	57	16	27.48473071	5.29	0.193	no	240	43.29083333	-2.754444444
		B12.d.2017.s2	2017	57	16	25.06839945	5.15	0.219	no	240	43.29083333	-2.754444444
		B12.d.s1	2016	57	16	20.00943841	5.46	0.097	no	240	43.29083333	-2.754444444
		B12.d.s2	2016	57	16	22.71186441	5.4	0.102	no	240	43.29083333	-2.754444444
B13	B13a	B13.a.2017.s1	2017	22	17.1	19.57627119	5.06	0.446	no	190	43.16666667	-3.044444444
		B13.a.2017.s2	2017	22	17.1	19.45003353	5.29	0.512	no	190	43.16666667	-3.044444444
		B13.a.s1	2016	22	17.1	15.88021779	5.73	0.187	no	190	43.16666667	-3.044444444
		B13.a.s2	2016	22	17.1	14.62217861	5.81	0.205	no	190	43.16666667	-3.044444444
	B13b	B13.b.2017.s1	2017	22	23.3	23.40951204	4.75	0.219	no	205	43.1675	-3.046666667
		B13.b.2017.s2	2017	22	23.3	22.47191011	4.89	0.263	no	205	43.1675	-3.046666667
		B13.b.s1	2016	22	23.3	16.49709302	5.69	0.173	no	205	43.1675	-3.046666667
		B13.b.s2	2016	22	23.3	14.88833747	5.55	0.133	no	205	43.1675	-3.046666667

Bizkaia	B2	B2	B2.2017.s1	2017	26	29.9	21.80451128	5.13	0.235	yes	54	43.42	-2.821388889
			B2.2017.s2	2017	26	29.9	25.69444444	5.33	0.269	yes	54	43.42	-2.821388889
			B2.s1	2016	26	29.9	16.87725632	5.39	0.11	yes	54	43.42	-2.821388889
			B2.s2	2016	26	29.9	16.95464363	5.27	0.137	yes	54	43.42	-2.821388889
	B3	B3	B3.2017.s1	2017	4	25	21.24688279	4.97	0.223	no	225	43.20222222	-3.186666667
			B3.2017.s2	2017	4	25	20	5.02	0.173	no	225	43.20222222	-3.186666667
			B3.s1	2016	4	25	13.59223301	5.89	0.108	no	225	43.20222222	-3.186666667
			B3.s2	2016	4	25	12.85604311	5.95	0.129	no	225	43.20222222	-3.186666667
	B4	B4a	B4.a.2017.s1	2017	20	13.5	32.4742268	5.7	0.429	yes	102	43.41138889	-2.823611111
			B4.a.2017.s2	2017	20	13.5	29.8488665	5.46	0.544	yes	102	43.41138889	-2.823611111
			B4.a.s1	2016	20	13.5	23.08743169	5.57	0.112	yes	102	43.41138889	-2.823611111
			B4.a.s2	2016	20	13.5	24.89244007	5.69	0.16	yes	102	43.41138889	-2.823611111
		B4b	B4.b.2017.s1	2017	60	16.3	25.03355705	5.56	0.267	yes	105	43.41277778	-2.824166667
			B4.b.2017.s2	2017	60	16.3	25.92592593	5.65	0.242	yes	105	43.41277778	-2.824166667
			B4.b.s1	2016	60	16.3	19.75806452	6.16	0.113	yes	102	43.41138889	-2.823611111
			B4.b.s2	2016	60	16.3	17.94871795	5.27	0.096	yes	102	43.41138889	-2.823611111
		B4c	B4.c.2017.s1	2017	37	16.3	25.77251808	4.84	0.219	yes	111	43.41222222	-2.824722222
			B4.c.2017.s2	2017	37	16.3	30.77851539	5.13	0.38	yes	111	43.41222222	-2.824722222
		B4d	B4.d.2017.s1	2017	65	16.3	24.64678179	4.92	0.217	yes	117	43.41166667	-2.825555556
			B4.d.2017.s2	2017	65	16.3	29.19826652	5.05	0.459	yes	117	43.41166667	-2.825555556
	B5	B5	B5.2017.s1	2017	20	3.3	25.15406162	5.86	0.348	no	100	43.20666667	-3.145277778
			B5.2017.s2	2017	20	3.3	24.70664928	5.85	0.385	no	100	43.20666667	-3.145277778
			B5.s1	2016	20	3.3	17.37288136	6.1	0.218	no	100	43.20666667	-3.145277778
			B5.s2	2016	20	3.3	16.68351871	5.98	0.24	no	100	43.20666667	-3.145277778
	B6	B6	B6.2017.s1	2017	20	19.5	30.25147929	5.1	0.232	no	234	43.25472222	-3.129722222
			B6.2017.s2	2017	20	19.5	32.29954614	5.07	0.238	no	234	43.25472222	-3.129722222
			B6.s1	2016	20	19.5	21.35416667	5.97	0.157	no	234	43.25472222	-3.129722222
			B6.s2	2016	20	19.5	23.4123948	6	0.141	no	234	43.25472222	-3.129722222
	B7	B7a	B7.a.2016.s1	2016	45	2.5	14.60587326	7.04	0.21	no	271	42.99805556	-3.000277778
			B7.a.2016.s2	2016	45	2.5	14.83286908	7.04	0.254	no	271	42.99805556	-3.000277778
			B7.a.2017.s1	2017	45	2.5	16.40986133	8.22	0.514	no	271	42.99805556	-3.000277778
			B7.a.2017.s2	2017	45	2.5	13.95027624	8.18	0.57	no	271	42.99805556	-3.000277778
			B7.a.s1	2016	45	2.5	10.26077098	7.34	0.242	no	271	42.99805556	-3.000277778
			B7.a.s2	2016	45	2.5	9.516214779	7.37	0.245	no	271	42.99805556	-3.000277778
		B7b	B7.b.2017.s1	2017	18	6.1	10.13774105	8.21	0.527	no	305	42.99027778	-2.988611111
			B7.b.2017.s2	2017	18	6.1	8.481901675	8.2	0.542	no	305	42.99055556	-2.988611111
			B7.b.s1	2016	18	6.1	14.60587326	7.04	0.21	no	305	42.98972222	-2.988611111
			B7.b.s2	2016	18	6.1	14.83286908	7.04	0.254	no	305	42.98972222	-2.988611111

B8	B8a	B8.a.2017.s1	2017	60	21.7	19.48579161	5.75	0.478	yes	204	43.33944444	-2.478888889
		B8.a.2017.s2	2017	60	21.7	18.01003838	5.21	0.164	yes	204	43.33944444	-2.478888889
		B8.a.s1	2016	60	21.7	10.94339623	6.35	0.117	yes	204	43.33944444	-2.478888889
		B8.a.s2	2016	60	21.7	11.20923913	6.37	0.119	yes	204	43.33944444	-2.478888889
	B8b	B8.b.2017.s1	2017	32	22.4	16.44004944	4.88	0.157	yes	138	43.34277778	-2.475277778
		B8.b.2017.s2	2017	32	22.4	15.41755889	4.99	0.182	yes	138	43.34277778	-2.475277778
		B8.b.s1	2016	32	22.4	9.130434783	6.14	0.108	yes	138	43.34277778	-2.475277778
		B8.b.s2	2016	32	22.4	9.826152683	5.99	0.102	yes	138	43.34277778	-2.475277778
B9	B9a	B9.a.2017.s1	2017	15	8	24.38790206	5.48	0.262	no	51	43.345	-2.865
		B9.a.2017.s2	2017	15	8	24.95288353	5.52	0.31	no	51	43.345	-2.865
		B9.a.s1	2016	15	8	12.1488764	5.91	0.13	no	51	43.345	-2.865
		B9.a.s2	2016	15	8	11.85520362	5.73	0.118	no	51	43.345	-2.865
	B9b	B9.b.2017.s1	2017	15	8	24.49936089	4.86	0.161	no	56	43.34583333	-2.864722222
		B9.b.2017.s2	2017	15	8	24.63768116	4.53	0.147	no	56	43.34583333	-2.864722222
		B9.b.s1	2016	15	8	14.73533619	4.96	0.106	no	56	43.34583333	-2.864722222
		B9.b.s2	2016	15	8	13.14713896	5.72	0.114	no	56	43.34583333	-2.864722222
	B9c	B9.c.2017.s1	2017	8	15.3	24.91824722	5.88	0.366	no	53	43.29805556	-2.690833333
		B9.c.2017.s2	2017	8	15.3	24.92227979	5.52	0.314	no	53	43.29805556	-2.690833333
	B9d	B9.d.2017.s1	2017	83	14.3	25.11711712	5.5	0.217	no	233	43.29527778	-2.756388889
		B9.d.2017.s2	2017	83	14.3	25.02034174	5.62	0.226	no	233	43.29527778	-2.756388889
		B9.d.s1	2016	83	14.3	20.62328139	5.71	0.146	no	233	43.29527778	-2.756388889
		B9.d.s2	2016	83	14.3	20.31403337	5.71	0.181	no	233	43.29527778	-2.756388889
	B9e	B9.e.2017.s1	2017	15	11.2	23.81158168	4.3	0.163	no	47	43.34416667	-2.865833333
		B9.e.2017.s2	2017	15	11.2	23.78318584	4.49	0.133	no	47	43.34416667	-2.865833333
		B9.e.s1	2016	15	11.2	17.10296684	4.54	0.126	no	47	43.34416667	-2.865833333
		B9.e.s2	2016	15	11.2	16.18435155	4.82	0.126	no	47	43.34416667	-2.865833333
G1	G1	G1.2017.s1	2017	64	9	21.22905028	7.14	0.358	yes	68	43.30222222	-2.210277778
		G1.2017.s2	2017	64	9	21.0744946	7.13	0.352	yes	68	43.30222222	-2.210277778
		G1.s1	2016	64	9	18.419033	6.84	0.195	yes	68	43.30222222	-2.210277778
		G1.s2	2016	64	9	19.63001028	6.91	0.185	yes	68	43.30222222	-2.210277778
G2	G2	G2.2017.s1	2017	19	17.3	31.12203112	7.95	0.972	yes	104	43.28333333	-2.145277778
		G2.2017.s2	2017	19	17.3	23.23669407	8.14	0.73	yes	104	43.28333333	-2.145277778
		G2.s1	2016	19	17.3	21.1829436	6.86	0.252	yes	104	43.28333333	-2.145277778
		G2.s2	2016	19	17.3	22.91021672	6.8	0.255	yes	104	43.28333333	-2.145277778

Gipuzkoa	G3	G3	G3.2016.s1	2016	27	9	16.38448935	6.09	0.205	no	227	43.03694444	-2.210277778
			G3.2016.s2	2016	27	9	17.48781808	6.11	0.232	no	227	43.03694444	-2.210277778
			G3.2017.s1	2017	27	9	21.85828877	5.8	0.356	no	227	43.03694444	-2.210277778
			G3.2017.s2	2017	27	9	22.93080054	5.82	0.354	no	227	43.03694444	-2.210277778
			G3.s1	2016	27	9	16.38448935	6.09	0.205	no	227	43.03694444	-2.210277778
			G3.s2	2016	27	9	17.48781808	6.11	0.232	no	227	43.03694444	-2.210277778
	G4	G4	G4.2017.s1	2017	14	18.3	22.1669627	6.99	0.492	yes	22	43.34888889	-1.824166667
			G4.s1	2016	14	18.3	17.0166546	6.02	0.149	yes	22	43.34888889	-1.824166667
			G4.s2	2016	14	18.3	15.56141673	6.08	0.149	yes	22	43.34888889	-1.824166667
	G5	G5a	G5.a.2016.s1	2016	60	34.3	13.6307311	5.1	0.135	yes	118	43.2875	-2.142222222
			G5.a.2016.s2	2016	60	34.3	12.27665706	5.38	0.138	yes	118	43.2875	-2.142222222
			G5.a.2017.s1	2017	60	34.3	19.30715935	4.59	0.143	yes	118	43.2875	-2.142222222
			G5.a.2017.s2	2017	60	34.3	18.00369686	4.21	0.145	yes	118	43.2875	-2.142222222
			G5.a.s1	2016	60	34.3	13.6307311	5.1	0.135	yes	118	43.2875	-2.142222222
			G5.a.s2	2016	60	34.3	12.27665706	5.38	0.138	yes	118	43.2875	-2.142222222
		G5b	G5.b.2017.s1	2017	22	30.4	20.58058925	5.92	0.193	yes	105	43.28861111	-2.145
			G5.b.2017.s2	2017	22	30.4	21.93608439	6.05	0.209	yes	105	43.28861111	-2.145
			G5.b.s1	2016	22	30.4	16.40161405	6.29	0.143	yes	105	43.28861111	-2.145
			G5.b.s2	2016	22	30.4	18.08510638	6.16	0.219	yes	105	43.28861111	-2.145
	G6	G6a	G6.a.2017.s1	2017	0	48.7	21.63299663	7.11	0.342	yes	67	43.29611111	-2.194166667
			G6.a.2017.s2	2017	0	48.7	20.68965517	7.23	0.294	yes	67	43.29611111	-2.194166667
			G6.a.s1	2016	0	48.7	17.34762224	6.61	0.175	yes	67	43.29611111	-2.194166667
			G6.a.s2	2016	0	48.7	16.61698957	6.45	0.146	yes	67	43.29611111	-2.194166667
		G6b	G6.b.2017.s1	2017	60	30.7	24.07600208	6.38	0.24	yes	70	43.29527778	-2.194444444
			G6.b.2017.s2	2017	60	30.7	24.74315068	6.02	0.253	yes	70	43.29527778	-2.194444444
			G6.b.s1	2016	60	30.7	20.73097663	7.03	0.236	yes	70	43.29527778	-2.194444444
			G6.b.s2	2016	60	30.7	20.24793388	7.15	0.21	yes	70	43.29527778	-2.194444444

SUPPLEMENTARY DATA

Supplementary Table 2: Pairwise comparison between vineyard's richness. P- values for the Multiple t-test comparison of the observed OTUs between all samples grouped by vineyard. The three vineyards showing a higher number of significant comparisons are highlighted in grey.

	A1a	A1b	A2a	A2b	A3a	A3b	B10	B11	B12a	B12b	B12c	B12d	B13a	B13b	B1a	B1b	B2	B3	B4a
A1b	1,000																		
A2a	1,000	1,000																	
A2b	0,000	0,000	0,000																
A3a	1,000	1,000	1,000	0,016															
A3b	1,000	1,000	1,000	0,000	1,000														
B10	1,000	1,000	1,000	0,000	1,000	1,000													
B11	1,000	1,000	1,000	0,000	1,000	1,000	1,000												
B12a	1,000	1,000	1,000	1,000	1,000	0,366	1,000	1,000											
B12b	1,000	1,000	1,000	1,000	1,000	0,066	1,000	0,403	1,000										
B12c	1,000	1,000	1,000	0,193	1,000	0,293	1,000	1,000	1,000	1,000									
B12d	1,000	1,000	1,000	0,364	1,000	1,000	1,000	1,000	1,000	1,000	1,000								
B13a	1,000	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000							
B13b	1,000	1,000	1,000	0,070	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000						
B1a	1,000	1,000	1,000	0,000	0,591	1,000	1,000	1,000	0,006	0,000	0,001	0,020	1,000	0,136					
B1b	1,000	1,000	1,000	0,001	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000				
B2	0,047	0,053	0,059	1,000	1,000	0,001	0,364	0,011	1,000	1,000	1,000	1,000	0,087	1,000	0,000	1,000			
B3	1,000	1,000	1,000	0,003	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000		
B4a	0,733	0,808	0,890	1,000	1,000	0,032	1,000	0,206	1,000	1,000	1,000	1,000	1,000	1,000	0,000	1,000	1,000	1,000	
B4b	1,000	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	0,424	1,000	1,000	1,000	1,000	1,000	1,000	0,012	1,000	0,217
B4c	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,218	1,000	1,000	1,000	1,000
B4d	1,000	1,000	1,000	0,112	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
B5	1,000	1,000	1,000	0,346	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,022	1,000	1,000	1,000	1,000
B6	1,000	1,000	1,000	1,000	1,000	0,493	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,005	1,000	1,000	1,000	1,000
B7a	1,000	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,050	1,000	0,974
B7b	1,000	1,000	1,000	0,097	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,095	1,000	1,000	1,000	1,000
B8a	1,000	1,000	1,000	0,018	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,530	1,000	1,000	1,000	1,000
B8b	1,000	1,000	1,000	0,329	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,023	1,000	1,000	1,000	1,000
B9a	1,000	1,000	1,000	0,545	1,000	0,980	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,012	1,000	1,000	1,000	1,000
B9b	0,013	0,014	0,016	1,000	1,000	0,000	0,109	0,003	1,000	1,000	1,000	1,000	0,024	1,000	0,000	0,364	1,000	0,808	1,000
B9c	1,000	1,000	1,000	0,003	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,714	1,000	1,000
B9d	1,000	1,000	1,000	0,403	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,018	1,000	1,000	1,000	1,000
B9e	0,000	0,000	0,000	1,000	0,002	0,000	0,000	0,000	1,000	1,000	0,022	0,053	0,000	0,009	0,000	0,000	1,000	0,000	1,000
G1	1,000	1,000	1,000	0,001	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,808	1,000	1,000
G2	1,000	1,000	1,000	1,000	1,000	0,150	1,000	0,848	1,000	1,000	1,000	1,000	1,000	1,000	0,001	1,000	1,000	1,000	1,000
G3	1,000	1,000	1,000	0,002	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,189	1,000	1,000	1,000	1,000
G4	1,000	1,000	1,000	0,004	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
G5a	1,000	1,000	1,000	0,012	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,031	1,000	1,000	1,000	1,000
G5b	0,364	0,403	0,446	1,000	1,000	0,014	1,000	0,097	1,000	1,000	1,000	1,000	0,632	1,000	0,000	1,000	1,000	1,000	1,000
G6a	1,000	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,329	1,000	1,000
G6b	1,000	1,000	1,000	0,004	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000

B4b	B4c	B4d	B5	B6	B7a	B7b	B8a	B8b	B9a	B9b	B9c	B9d	B9e	G1	G2	G3	G4	G5a	G5b	G6a
1,000																				
1,000	1,000																			
1,000	1,000	1,000																		
1,000	1,000	1,000	1,000																	
1,000	1,000	1,000	1,000	1,000																
1,000	1,000	1,000	1,000	1,000	1,000															
1,000	1,000	1,000	1,000	1,000	1,000	1,000														
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000													
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000												
0,003	1,000	1,000	1,000	1,000	0,012	1,000	1,000	1,000	1,000											
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,277										
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000									
0,000	1,000	0,022	0,050	0,176	0,000	0,013	0,002	0,047	0,083	1,000	0,000	0,059								
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,254	1,000	1,000	0,000							
0,890	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,573	1,000						
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,861	1,000	1,000	0,000	1,000	1,000					
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,711	1,000	1,000	0,001	1,000	1,000	1,000				
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,001	1,000	1,000	1,000	1,000			
0,103	1,000	1,000	1,000	1,000	0,458	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000		
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,097	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	1,000	
1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	0,848	1,000	1,000	0,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000

DATA AVAILABILITY STATEMENT

7 Data Availability Statement

The sequences and experimental factors information for the vineyard's soils and grapes analyzed in the present study are available on Qiita portal under ID 13591 (https://qiita.ucsd.edu/study/description/13591) and ID 13635 (https://qiita.ucsd.edu/study/description/13635).
The Supplementary Dataset 1, 2 and 3 are available in the next link and QR code. (https://drive.google.com/drive/folders/1UY4jerYLBqNuVEy09YGDZvsLX_l1Sgah?usp=sharing)

Supplementary Dataset 1: Spearman test to measure pH correlated taxa in soil samples.
Supplementary Dataset 2: Raw data of LEfSe (Linear discriminant analysis Effect Size) analysis of soil bacterial taxa, to determine the microorganisms most likely to explain differences between Branch 1 and Branch 2.
Supplementary Dataset 3: Raw data of LEfSe analysis from grape bacterial taxa, to determine the microorganisms significantly explaining differences between grapes growing onto different rootstock genotype.

REFERENCES

8 References

Abbasian, F., Lockington, R., Megharaj, M., and Naidu, R. (2015). The integration of sequencing and bioinformatics in metagenomics. *Rev Environ Sci Biotechnol* 14, 357–383. doi:10.1007/s11157-015-9365-7.

Acinas, S. G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., and Polz, M. F. (2005). PCR-Induced Sequence Artifacts and Bias: Insights from Comparison of Two 16S rRNA Clone Libraries Constructed from the Same Sample. *Appl Environ Microbiol* 71, 8966–8969. doi:10.1128/AEM.71.12.8966-8969.2005.

Alegría, Á., Szczesny, P., Mayo, B., Bardowski, J., and Kowalczyk, M. (2012). Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. *Appl Environ Microbiol* 78, 1890–1898. doi:10.1128/AEM.06081-11.

Andorrà, I., Landi, S., Mas, A., Esteve-Zarzoso, B., and Guillamón, J. M. (2010). Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. *Food Research International* 43, 773–779. doi:10.1016/j.foodres.2009.11.014.

Andorrà, I., Landi, S., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiology* 25, 849–856. doi:10.1016/j.fm.2008.05.005.

Andrén, O., and Balandreau, J. (1999). Biodiversity and soil functioning-from black box to can of worms? *Applied Soil Ecology*, 4.

Baas Becking, L. G. M., 1895-1963 (1934). *Geobiologie of inleiding tot de milieukunde*. Den Haag : Van Stockum Available at: http://lib.ugent.be/catalog/rug01:001271403.

Baker, G. C., Smith, J. J., and Cowan, D. A. (2003). Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods* 55, 541–555. doi:10.1016/j.mimet.2003.08.009.

Baker, K. L., Langenheder, S., Nicol, G. W., Ricketts, D., Killham, K., Campbell, C. D., et al. (2009). Environmental and spatial characterisation of bacterial community composition in soil to inform sampling strategies. *Soil Biology and Biochemistry* 41, 2292–2298. doi:10.1016/j.soilbio.2009.08.010.

REFERENCES

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *International Journal of Food Microbiology* 153, 243–259. doi:10.1016/j.ijfoodmicro.2011.11.025.

Barreda, F. (2001). *El Chacolí santanderino en los siglos XIII al XIX*. Valladolid.

Bartowsky, E. J. (2009). Bacterial spoilage of wine and approaches to minimize it. *Letters in Applied Microbiology* 48, 149–156. doi:10.1111/j.1472-765X.2008.02505.x.

Bartram, A. K., Jiang, X., Lynch, M. D. J., Masella, A. P., Nicol, G. W., Dushoff, J., et al. (2014). Exploring links between pH and bacterial community composition in soils from the Craibstone Experimental Farm. *FEMS Microbiol Ecol* 87, 403–415. doi:10.1111/1574-6941.12231.

Bazghaleh, N., Hamel, C., Gan, Y., Tar'an, B., and Knight, J. D. (2015). Genotype-Specific Variation in the Structure of Root Fungal Communities Is Related to Chickpea Plant Productivity. *Appl Environ Microbiol* 81, 2368–2377. doi:10.1128/AEM.03692-14.

Belda, I., Zarraonaindia, I., Perisin, M., Palacios, A., and Acedo, A. (2017). From Vineyard Soil to Wine Fermentation: Microbiome Approximations to Explain the "terroir" Concept. *Front. Microbiol.* 8, 821. doi:10.3389/fmicb.2017.00821.

Berlanas, C., Berbegal, M., Elena, G., Laidani, M., Cibriain, J. F., Sagües, A., et al. (2019). The fungal and bacterial rhizosphere microbiome associated with grapevine rootstock genotypes in mature and young vineyards. *Front. Microbiol.* 10, 1142. doi:10.3389/fmicb.2019.01142.

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J Microbiol Biotechnol* 28, 1327–1350. doi:10.1007/s11274-011-0979-9.

Bokulich, N. a, Joseph, C. M. L., Allen, G., Benson, A. K., and Mills, D. a (2012a). Next-generation sequencing reveals significant bacterial diversity of botrytized wine. *PloS one* 7, e36357. doi:10.1371/journal.pone.0036357.

Bokulich, N. A., Bamforth, C. W., and Mills, D. A. (2012b). Brewhouse-Resident Microbiota Are Responsible for Multi-Stage Fermentation of American Coolship Ale. *PLoS ONE* 7, e35507. doi:10.1371/journal.pone.0035507.

Bokulich, N. A., Collins, T. S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S. E., et al. (2016). Associations among Wine Grape Microbiome, Metabolome, and Fermentation Behavior Suggest Microbial Contribution to Regional Wine Characteristics. *mBio* 7, e00631-16, /mbio/7/3/e00631-16.atom. doi:10.1128/mBio.00631-16.

Bokulich, N. A., and Mills, D. A. (2013a). Facility-Specific “House” Microbiome Drives Microbial Landscapes of Artisan Cheesemaking Plants. *Appl. Environ. Microbiol.* 79, 5214–5223. doi:10.1128/AEM.00934-13.

Bokulich, N. A., and Mills, D. A. (2013b). Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities. *Appl Environ Microbiol* 79, 2519–2526. doi:10.1128/AEM.03870-12.

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring Seasonal Changes in Winery-Resident Microbiota. *PLoS ONE* 8, e66437. doi:10.1371/journal.pone.0066437.

Bokulich, N. A., Swadener, M., Sakamoto, K., Mills, D. A., and Bisson, L. F. (2015). Sulfur Dioxide Treatment Alters Wine Microbial Diversity and Fermentation Progression in a Dose-Dependent Fashion. *Am. J. Enol. Vitic.* 66, 73–79. doi:10.5344/ajev.2014.14096.

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *PNAS* 111, E139–E148. doi:10.1073/pnas.1317377110.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi:10.1093/bioinformatics/btu170.

Bouffaud, M.-L., Bernaud, E., Colombet, A., Van Tuinen, D., Wipf, D., and Redecker, D. (2016). Regional-scale analysis of arbuscular mycorrhizal fungi: the case of Burgundy vineyards. *OENO One* 50, 1. doi:10.20870/oeno-one.2016.50.1.49.

Bouquet, A. (2011). “Grapevines and Viticulture,” in *Genetics, Genomics, and Breeding of Grapes* (Florida, USA), 1–29.

Browde, J., Garn, J., Jordan, A., Ross, K., and Thrupp, L. A. (2006). *California Code of Sustainable Winegrowing Workbook (2nd ed)*. 2nd Edition. California Sustainable Winegrowing Alliance, Wine Institute, and California Association of Winegrape Growers.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., and Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi:10.1146/annurev-arplant-050312-120106.

Burns, K. N., Bokulich, N. A., Cantu, D., Greenhut, R. F., Kluepfel, D. A., O’Geen, A. T., et al. (2016). Vineyard soil bacterial diversity and composition revealed by

16S rRNA genes: Differentiation by vineyard management. *Soil Biology and Biochemistry* 103, 337–348. doi:10.1016/j.soilbio.2016.09.007.

Burns, K. N., Kluepfel, D. A., Strauss, S. L., Bokulich, N. A., Cantu, D., and Steenwerth, K. L. (2015). Vineyard soil bacterial diversity and composition revealed by 16S rRNA genes: Differentiation by geographic features. *Soil Biology and Biochemistry* 91, 232–247. doi:10.1016/j.soilbio.2015.09.002.

Burr, T. J., Bazzi, C., Süle, S., and Otten, L. (1998). Crown Gall of Grape: Biology of *Agrobacterium vitis* and the Development of Disease Control Strategies. *Plant Disease* 82, 1288–1297. doi:10.1094/PDIS.1998.82.12.1288.

Calleja-Cervantes, M. E., Menéndez, S., Fernández-González, A. J., Irigoyen, I., Cibriáin-Sabalza, J. F., Toro, N., et al. (2015). Changes in soil nutrient content and bacterial community after 12 years of organic amendment application to a vineyard: Compost changes soil's mineral content and microbial community. *Eur J Soil Sci* 66, 802–812. doi:10.1111/ejss.12261.

Campisano, A., Antonielli, L., Pancher, M., Yousaf, S., Pindo, M., and Pertot, I. (2014). Bacterial Endophytic Communities in the Grapevine Depend on Pest Management. *PLoS ONE* 9, e112763. doi:10.1371/journal.pone.0112763.

Cao, Y., Fanning, S., Proos, S., Jordan, K., and Srikumar, S. (2017). A Review on the Applications of Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies. *Front. Microbiol.* 8, 1829. doi:10.3389/fmicb.2017.01829.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7, 335–336. doi:10.1038/nmeth.f.303.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6, 1621–1624. doi:10.1038/ismej.2012.8.

Catalogue des Varietes et Clones de Vigne cultives en France 2nd Edition. Le Grau-du-Roi: ITV SERVICE PUBLICATIONS.

Chen, Q.-L., Ding, J., Zhu, Y.-G., He, J.-Z., and Hu, H.-W. (2020). Soil bacterial taxonomic diversity is critical to maintaining the plant productivity. *Environment International* 140, 105766. doi:10.1016/j.envint.2020.105766.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., and Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during

food fermentation. *International Journal of Food Microbiology* 167, 29–43. doi:10.1016/j.ijfoodmicro.2013.05.008.

Cocolin, L., Dolci, P., and Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: The contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science* 89, 296–302. doi:10.1016/j.meatsci.2011.04.011.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* 42, 669–678. doi:10.1016/j.soilbio.2009.11.024.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research* 19, 29–37. doi:10.1016/j.jare.2019.03.004.

D'Amico, F., Candela, M., Turroni, S., Biagi, E., Brigidi, P., Bega, A., et al. (2018). The Rootstock Regulates Microbiome Diversity in Root and Rhizosphere Compartments of Vitis vinifera Cultivar Lambrusco. *Front. Microbiol.* 9, 2240. doi:10.3389/fmicb.2018.02240.

Daoud, N. N., and Foster, H. A. (1993). Antifungal activity of Myxococcus species 1 production, physicochemical and biological properties of antibiotics from Myxococcus fulvus S110 (Myxobacterales). *Microbios* 73, 173–184.

David, V., Terrat, S., Herzine, K., Claisse, O., Rousseaux, S., Tourdot-Maréchal, R., et al. (2014). High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. *Journal of Industrial Microbiology and Biotechnology* 41, 811–821. doi:10.1007/s10295-014-1427-2.

De Filippis, F., La Storia, A., and Blaiotta, G. (2017). Monitoring the mycobiota during Greco di Tufo and Aglianico wine fermentation by 18S rRNA gene sequencing. *Food Microbiology* 63, 117–122. doi:10.1016/j.fm.2016.11.010.

De Filippis, F., La Storia, A., Villani, F., and Ercolini, D. (2013). Exploring the Sources of Bacterial Spoilers in Beefsteaks by Culture-Independent High-Throughput Sequencing. *PLoS ONE* 8, e70222. doi:10.1371/journal.pone.0070222.

de Wit, R., and Bouvier, T. (2006). "Everything is everywhere, but, the environment selects"; what did Baas Becking and Beijerinck really say? *Environ Microbiol* 8, 755–758. doi:10.1111/j.1462-2920.2006.01017.x.

DeBruyn, J. M., Nixon, L. T., Fawaz, M. N., Johnson, A. M., and Radosevich, M. (2011). Global Biogeography and Quantitative Seasonal Dynamics of

Gemmatimonadetes in Soil. *Appl. Environ. Microbiol.* 77, 6295–6300. doi:10.1128/AEM.05005-11.

Divol, B., and Lonvaud-Funel, A. (2005). Evidence for viable but nonculturable yeasts in botrytis-affected wine. *Journal of Applied Microbiology* 99, 85–93. doi:10.1111/j.1365-2672.2005.02578.x.

Dries, L., Hendgen, M., Schnell, S., Löhnertz, O., and Vortkamp, A. (2021). Rhizosphere engineering: leading towards a sustainable viticulture? *OENO One* 55, 353–363. doi:10.20870/oeno-one.2021.55.2.4534.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi:10.1093/bioinformatics/btr381.

Elsas, J. D., Trevors, J. T., Jansson, J., and Nannipieri, P. (2006). *Modern soil microbiology*.

Fierer, N. (2008). “Microbial Biogeography: Patterns in Microbial Diversity across Space and Time,” in *Accessing Uncultivated Microorganisms*, ed. K. Zengler (Washington, DC, USA: ASM Press), 95–115. doi:10.1128/9781555815509.ch6.

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). TOWARD AN ECOLOGICAL CLASSIFICATION OF SOIL BACTERIA. *Ecology* 88, 1354–1364. doi:10.1890/05-1839.

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences* 103, 626–631. doi:10.1073/pnas.0507535103.

Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., et al. (2012). Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences* 109, 21390–21395. doi:10.1073/pnas.1215210110.

Flores-Félix, J. D., Silva, L. R., Rivera, L. P., Marcos-García, M., García-Fraile, P., Martínez-Molina, E., et al. (2015). Plants Probiotics as a Tool to Produce Highly Functional Fruits: The Case of Phyllobacterium and Vitamin C in Strawberries. *PLoS ONE* 10, e0122281. doi:10.1371/journal.pone.0122281.

Franzosa, E. A., Hsu, T., Sirota-Madi, A., Shafquat, A., Abu-Ali, G., Morgan, X. C., et al. (2015). Sequencing and beyond: integrating molecular “omics” for microbial community profiling. *Nat Rev Microbiol* 13, 360–372. doi:10.1038/nrmicro3451.

Garaizabal Pildain, M. (2002). *Euskal Herriko Ardoak*. Elorrio: Ardoxka Gastronomi Elkartea.

Garbeva, P., van Veen, J. A., and van Elsas, J. D. (2004). MICROBIAL DIVERSITY IN SOIL: Selection of Microbial Populations by Plant and Soil Type and Implications for Disease Suppressiveness. *Annu. Rev. Phytopathol.* 42, 243–270. doi:10.1146/annurev.phyto.42.012604.135455.

Ge, Y., He, J., Zhu, Y., Zhang, J., Xu, Z., Zhang, L., et al. (2008). Differences in soil bacterial diversity: driven by contemporary disturbances or historical contingencies? *ISME J* 2, 254–264. doi:10.1038/ismej.2008.2.

Gelvin, S. B. (2010). Plant Proteins Involved in *Agrobacterium* -Mediated Genetic Transformation. *Annu. Rev. Phytopathol.* 48, 45–68. doi:10.1146/annurev-phyto-080508-081852.

Gilbert, J. A., and Dupont, C. L. (2011). Microbial Metagenomics: Beyond the Genome. *Annu. Rev. Mar. Sci.* 3, 347–371. doi:10.1146/annurev-marine-120709-142811.

Gilbert, J. A., van der Lelie, D., and Zarraonaindia, I. (2014). Microbial *terroir* for wine grapes. *Proc Natl Acad Sci USA* 111, 5–6. doi:10.1073/pnas.1320471110.

Giller, K. E., Beare, M. H., Lavelle, P., Izac, A.-M. N., and Swift, M. J. (1997). Agricultural intensification, soil biodiversity and agroecosystem function. *Applied Soil Ecology* 6, 3–16. doi:10.1016/S0929-1393(96)00149-7.

Grady, E. N., MacDonald, J., Liu, L., Richman, A., and Yuan, Z.-C. (2016). Current knowledge and perspectives of Paenibacillus: a review. *Microb Cell Fact* 15, 203. doi:10.1186/s12934-016-0603-7.

Grandy, A. S., and Neff, J. C. (2008). Molecular C dynamics downstream: The biochemical decomposition sequence and its impact on soil organic matter structure and function. *Science of The Total Environment* 404, 297–307. doi:10.1016/j.scitotenv.2007.11.013.

Grangeteau, C., Roullier-Gall, C., Rousseaux, S., Gougeon, R. D., Schmitt-Kopplin, P., Alexandre, H., et al. (2017). Wine microbiology is driven by vineyard and winery anthropogenic factors. *Microb. Biotechnol.* 10, 354–370. doi:10.1111/1751-7915.12428.

Gupta, V. V. S. R., Bramley, R. G. V., Greenfield, P., Yu, J., and Herderich, M. J. (2019). Vineyard Soil Microbiome Composition Related to Rotundone Concentration in Australian Cool Climate 'Peppery' Shiraz Grapes. *Front. Microbiol.* 10, 1607. doi:10.3389/fmicb.2019.01607.

REFERENCES

Hacquard, S. (2016). Disentangling the factors shaping microbiota composition across the plant holobiont. *New Phytol* 209, 454–457. doi:10.1111/nph.13760.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43, 895–914. doi:10.1139/m97-131.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., and Widmer, F. (2015). Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J* 9, 1177–1194. doi:10.1038/ismej.2014.210.

Holland, T. C., Bowen, P. A., Bogdanoff, C. P., Lowery, T. D., Shaposhnikova, O., Smith, S., et al. (2016). Evaluating the diversity of soil microbial communities in vineyards relative to adjacent native ecosystems. *Applied Soil Ecology* 100, 91–103. doi:10.1016/j.apsoil.2015.12.001.

Holland, T. C., Bowen, P., Bogdanoff, C., and Hart, M. (2014). Arbuscular Mycorrhizal Fungal Communities Associated with Vitis vinifera Vines under Different Frequencies of Irrigation. *American Journal of Enology and Viticulture* 65, 222–229. doi:10.5344/ajev.2014.13101.

Hong, S., Bunge, J., Leslin, C., Jeon, S., and Epstein, S. S. (2009). Polymerase chain reaction primers miss half of rRNA microbial diversity. *ISME J* 3, 1365–1373. doi:10.1038/ismej.2009.89.

Hou, J., Li, M., Mao, X., Hao, Y., Ding, J., Liu, D., et al. (2017). Response of microbial community of organic-matter-impoverished arable soil to long-term application of soil conditioner derived from dynamic rapid fermentation of food waste. *PLoS ONE* 12, e0175715. doi:10.1371/journal.pone.0175715.

Humblot, C., and Guyot, J.-P. (2009). Pyrosequencing of Tagged 16S rRNA Gene Amplicons for Rapid Deciphering of the Microbiomes of Fermented Foods Such as Pearl Millet Slurries. *Appl Environ Microbiol* 75, 4354–4361. doi:10.1128/AEM.00451-09.

İnceoğlu, Ö., Salles, J. F., van Overbeek, L., and van Elsas, J. D. (2010). Effects of Plant Genotype and Growth Stage on the Betaproteobacterial Communities Associated with Different Potato Cultivars in Two Fields. *Appl Environ Microbiol* 76, 3675–3684. doi:10.1128/AEM.00040-10.

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* 14, 215–237. doi:10.1111/1567-1364.12111.

Jones, R. T., Robeson, M. S., Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J* 3, 442–453. doi:10.1038/ismej.2008.127.

Juste, A., Thomma, B., and Lievens, B. (2008). Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiology* 25, 745–761. doi:10.1016/j.fm.2008.04.009.

Kecskeméti, E., Berkelmann-Löhnertz, B., and Reineke, A. (2016). Are Epiphytic Microbial Communities in the Carposphere of Ripening Grape Clusters (Vitis vinifera L.) Different between Conventional, Organic, and Biodynamic Grapes? *PLoS ONE* 11, e0160852. doi:10.1371/journal.pone.0160852.

Keisam, S., Romi, W., Ahmed, G., and Jeyaram, K. (2016). Quantifying the biases in metagenome mining for realistic assessment of microbial ecology of naturally fermented foods. *Sci Rep* 6, 34155. doi:10.1038/srep34155.

King, P. D., Smart, R. E., and McClellan, D. J. (2014). Within-vineyard variability in vine vegetative growth, yield, and fruit and wine composition of Cabernet Sauvignon in Hawke's Bay, New Zealand: Vigour effects on Cabernet Sauvignon vine growth. *Australian Journal of Grape and Wine Research* 20, 234–246. doi:10.1111/ajgw.12080.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* 41, e1–e1. doi:10.1093/nar/gks808.

Knight, S., and Goddard, M. R. (2015). Quantifying separation and similarity in a Saccharomyces cerevisiae metapopulation. *ISME J* 9, 361–370. doi:10.1038/ismej.2014.132.

KoÈgel-Knabner, I. (2002). The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology*, 24.

Kutschera, U. (2007). Plant-Associated Methylobacteria as Co-Evolved Phytosymbionts: A Hypothesis. *Plant Signaling & Behavior* 2, 74–78. doi:10.4161/psb.2.2.4073.

Kuzyakov, Y., Subbotina, I., Chen, H., Bogomolova, I., and Xu, X. (2009). Black carbon decomposition and incorporation into soil microbial biomass estimated by 14C labeling. *Soil Biology and Biochemistry* 41, 210–219. doi:10.1016/j.soilbio.2008.10.016.

REFERENCES

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. *AEM* 75, 5111–5120. doi:10.1128/AEM.00335-09.

Lee, C. K., Herbold, C. W., Polson, S. W., Wommack, K. E., Williamson, S. J., McDonald, I. R., et al. (2012). Groundtruthing Next-Gen Sequencing for Microbial Ecology–Biases and Errors in Community Structure Estimates from PCR Amplicon Pyrosequencing. *PLoS ONE* 7, e44224. doi:10.1371/journal.pone.0044224.

Lemanceau, P., Barret, M., Mazurier, S., Mondy, S., Pivato, B., Fort, T., et al. (2017). "Plant Communication With Associated Microbiota in the Spermosphere, Rhizosphere and Phyllosphere," in *Advances in Botanical Research* (Elsevier), 101–133. doi:10.1016/bs.abr.2016.10.007.

Leveau, J. H. J., and Tech, J. J. (2011). Grapevine Microbiomics: Bacterial Diversity on Grape Leaves and Berries Revealed by High-Throughput Sequence Analysis of 16S rRNA Amplicons. *Acta Hortic.*, 31–42. doi:10.17660/ActaHortic.2011.905.2.

Liang, Y., He, X., Chen, X., Hu, Y., and Su, Y. (2019). Nitrogen-Fixing Bacterial Communities ExhibitStrong Spatial Structure along a Slope Gradientin Karst Shrublands Ecosystem. *Pol. J. Environ. Stud.* 28, 3369–3376. doi:10.15244/pjoes/94383.

Lindow, S. E., and Brandl, M. T. (2003). Microbiology of the Phyllosphere. *APPL. ENVIRON. MICROBIOL.* 69, 9.

Liu, D., Zhang, P., Chen, D., and Howell, K. (2019). From the Vineyard to the Winery: How Microbial Ecology Drives Regional Distinctiveness of Wine. *Front. Microbiol.* 10, 2679. doi:10.3389/fmicb.2019.02679.

Logares, R., Sunagawa, S., Salazar, G., Cornejo-Castillo, F. M., Ferrera, I., Sarmento, H., et al. (2014). Metagenomic 16S rDNA Illumina tags are a powerful alternative to amplicon sequencing to explore diversity and structure of microbial communities: Using *mi tag* s to explore microbial communities. *Environ Microbiol* 16, 2659–2671. doi:10.1111/1462-2920.12250.

Loreau, M. (2001). Biodiversity and Ecosystem Functioning: Current Knowledge and Future Challenges. *Science* 294, 804–808. doi:10.1126/science.1064088.

Loureiro, V. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology* 86, 23–50. doi:10.1016/S0168-1605(03)00246-0.

Lugtenberg, B., and Kamilova, F. (2009). Plant-Growth-Promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi:10.1146/annurev.micro.62.081307.162918.

Lumini, E., Orgiazzi, A., Borriello, R., Bonfante, P., and Bianciotto, V. (2009). Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. *Environmental Microbiology*. doi:10.1111/j.1462-2920.2009.02099.x.

Malfeito-Ferreira, M. (2011). Yeasts and wine off-flavours: a technological perspective. *Ann Microbiol* 61, 95–102. doi:10.1007/s13213-010-0098-0.

Mantelin, S., and Touraine, B. (2003). Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. *Journal of Experimental Botany* 55, 27–34. doi:10.1093/jxb/erh010.

Marasco, R., Rolli, E., Fusi, M., Michoud, G., and Daffonchio, D. (2018). Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality. *Microbiome* 6, 3. doi:10.1186/s40168-017-0391-2.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. doi:10.1038/nature03959.

Martin, D., Grose, C., Fedrizzi, B., Stuart, L., Albright, A., and McLachlan, A. (2016). Grape cluster microclimate influences the aroma composition of Sauvignon blanc wine. *Food Chemistry* 210, 640–647. doi:10.1016/j.foodchem.2016.05.010.

Martins, G., Lauga, B., Miot-Sertier, C., Mercier, A., Lonvaud, A., Soulas, M.-L., et al. (2013). Characterization of Epiphytic Bacterial Communities from Grapes, Leaves, Bark and Soil of Grapevine Plants Grown, and Their Relations. *PLoS ONE* 8, e73013. doi:10.1371/journal.pone.0073013.

Martins, G., Miot-Sertier, C., Lauga, B., Claisse, O., Lonvaud-Funel, A., Soulas, G., et al. (2012). Grape berry bacterial microbiota: Impact of the ripening process and the farming system. *International Journal of Food Microbiology* 158, 93–100. doi:10.1016/j.ijfoodmicro.2012.06.013.

Martins, G., Vallance, J., Mercier, A., Albertin, W., Stamatopoulos, P., Rey, P., et al. (2014). Influence of the farming system on the epiphytic yeasts and yeast-like fungi colonizing grape berries during the ripening process. *International Journal of Food Microbiology* 177, 21–28. doi:10.1016/j.ijfoodmicro.2014.02.002.

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., et al. (2006). Microbial biogeography: putting microorganisms on the map. *Nature Reviews Microbiology* 4, 102–112. doi:10.1038/nrmicro1341.

REFERENCES

Marzano, M., Fosso, B., Manzari, C., Grieco, F., Intranuovo, M., Cozzi, G., et al. (2016). Complexity and Dynamics of the Winemaking Bacterial Communities in Berries, Musts, and Wines from Apulian Grape Cultivars through Time and Space. *PLoS ONE* 11, e0157383. doi:10.1371/journal.pone.0157383.

McInroy, J. A., and Kloepper, J. W. (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Can. J. Microbiol.* 41, 895–901. doi:10.1139/m95-123.

Millet, V., and Lonvaud-Funel, A. (2000). The viable but non-culturable state of wine micro-organisms during storage. *Lett Appl Microbiol* 30, 136–141. doi:10.1046/j.1472-765x.2000.00684.x.

Morgan, H. H., du Toit, M., and Setati, M. E. (2017). The Grapevine and Wine Microbiome: Insights from High-Throughput Amplicon Sequencing. *Front. Microbiol.* 8, 820. doi:10.3389/fmicb.2017.00820.

Morrison-Whittle, P., and Goddard, M. R. (2018). From vineyard to winery: a source map of microbial diversity driving wine fermentation: The origins of microbial diversity in wine. *Environ Microbiol* 20, 75–84. doi:10.1111/1462-2920.13960.

Neilson, J. W., Jordan, F. L., and Maier, R. M. (2013). Analysis of artifacts suggests DGGE should not be used for quantitative diversity analysis. *Journal of Microbiological Methods* 92, 256–263. doi:10.1016/j.mimet.2012.12.021.

Nisiotou, A. A., Rantsiou, K., Iliopoulos, V., Cocolin, L., and Nychas, G.-J. E. (2011). Bacterial species associated with sound and Botrytis-infected grapes from a Greek vineyard. *International Journal of Food Microbiology* 145, 432–436. doi:10.1016/j.ijfoodmicro.2011.01.017.

Nocker, A., Burr, M., and Camper, A. K. (2007). Genotypic Microbial Community Profiling: A Critical Technical Review. *Microb Ecol* 54, 276–289. doi:10.1007/s00248-006-9199-5.

O'Callaghan, J. L., Willner, D., Buttini, M., Huygens, F., and Pelzer, E. S. (2021). Limitations of 16S rRNA Gene Sequencing to Characterize Lactobacillus Species in the Upper Genital Tract. *Front. Cell Dev. Biol.* 9, 641921. doi:10.3389/fcell.2021.641921.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2017). *vegan: Community Ecology Package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. Version 2.4-3. URL https://CRAN.R-project.org/package=vegan*.

Pauls, M., and Facaros, D. (2003). *Bilbao & the Basque Lands*. 2nd ed. Cadogan Guides.

Penn, C., and Camberato, J. (2019). A Critical Review on Soil Chemical Processes that Control How Soil pH Affects Phosphorus Availability to Plants. *Agriculture* 9, 120. doi:10.3390/agriculture9060120.

Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., et al. (2014). Resilience of the Natural Phyllosphere Microbiota of the Grapevine to Chemical and Biological Pesticides. *Appl. Environ. Microbiol.* 80, 3585–3596. doi:10.1128/AEM.00415-14.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat Rev Microbiol* 11, 789–799. doi:10.1038/nrmicro3109.

Piao, H., Hawley, E., Kopf, S., DeScenzo, R., Sealock, S., Henick-Kling, T., et al. (2015). Insights into the bacterial community and its temporal succession during the fermentation of wine grapes. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00809.

Pinto, A. J., and Raskin, L. (2012). PCR Biases Distort Bacterial and Archaeal Community Structure in Pyrosequencing Datasets. *PLoS ONE* 7, e43093. doi:10.1371/journal.pone.0043093.

Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., et al. (2015). Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00905.

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and C. Gomes, A. (2014). Unravelling the Diversity of Grapevine Microbiome. *PLoS ONE* 9, e85622. doi:10.1371/journal.pone.0085622.

Plaza, C., Courtier-Murias, D., Fernández, J. M., Polo, A., and Simpson, A. J. (2013). Physical, chemical, and biochemical mechanisms of soil organic matter stabilization under conservation tillage systems: A central role for microbes and microbial by-products in C sequestration. *Soil Biology and Biochemistry* 57, 124–134. doi:10.1016/j.soilbio.2012.07.026.

Portillo, M. del C., Franquès, J., Araque, I., Reguant, C., and Bordons, A. (2016). Bacterial diversity of Grenache and Carignan grape surface from different vineyards at Priorat wine region (Catalonia, Spain). *International Journal of Food Microbiology* 219, 56–63. doi:10.1016/j.ijfoodmicro.2015.12.002.

Portillo, M. del C., and Mas, A. (2016). Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT - Food Science and Technology* 72, 317–321. doi:10.1016/j.lwt.2016.05.009.

REFERENCES

Qi, D., Wieneke, X., Tao, J., Zhou, X., and Desilva, U. (2018). Soil pH Is the Primary Factor Correlating With Soil Microbiome in Karst Rocky Desertification Regions in the Wushan County, Chongqing, China. *Front. Microbiol.* 9, 1027. doi:10.3389/fmicb.2018.01027.

Qiao, Q., Wang, F., Zhang, J., Chen, Y., Zhang, C., Liu, G., et al. (2017). The Variation in the Rhizosphere Microbiome of Cotton with Soil Type, Genotype and Developmental Stage. *Sci Rep* 7, 3940. doi:10.1038/s41598-017-04213-7.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41, D590–D596. doi:10.1093/nar/gks1219.

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., and Cotter, P. D. (2011). Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology* 150, 81–94. doi:10.1016/j.ijfoodmicro.2011.08.001.

Redford, A. J., Bowers, R. M., Knight, R., Linhart, Y., and Fierer, N. (2010). The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves: Biogeography of phyllosphere bacterial communities. *Environmental Microbiology* 12, 2885–2893. doi:10.1111/j.1462-2920.2010.02258.x.

Reynolds, A. G., and Heuvel, J. E. V. (2009). Influence of Grapevine Training Systems on Vine Growth and Fruit Composition: A Review. 18.

Reynolds, A. G., and Wardle, D. A. (2001). Evaluation of Minimal Pruning upon Vine Performance and Berry Composition of Chancellor. 4.

Roh, S. W., Kim, K.-H., Nam, Y.-D., Chang, H.-W., Park, E.-J., and Bae, J.-W. (2010). Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *ISME J* 4, 1–16. doi:10.1038/ismej.2009.83.

Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., and Thompson, F. eds. (2014). *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*. Berlin, Heidelberg: Springer Berlin Heidelberg doi:10.1007/978-3-642-30197-1.

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., et al. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J* 4, 1340–1351. doi:10.1038/ismej.2010.58.

Rousseaux, S., Diguta, C. F., Radoï-Matei, F., Alexandre, H., and Guilloux-Bénatier, M. (2014). Non-Botrytis grape-rotting fungi responsible for earthy and moldy off-

flavors and mycotoxins. *Food Microbiology* 38, 104–121. doi:10.1016/j.fm.2013.08.013.

RStudio Team (2016). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc. Available at: http://www.rstudio.com/.

Russo, S. E., Legge, R., Weber, K. A., Brodie, E. L., Goldfarb, K. C., Benson, A. K., et al. (2012). Bacterial community structure of contrasting soils underlying Bornean rain forests: Inferences from microarray and next-generation sequencing methods. *Soil Biology and Biochemistry* 55, 48–59. doi:10.1016/j.soilbio.2012.05.021.

Salma, M., Rousseaux, S., Sequeira-Le Grand, A., Divol, B., and Alexandre, H. (2013). Characterization of the Viable but Nonculturable (VBNC) State in Saccharomyces cerevisiae. *PLoS ONE* 8, e77600. doi:10.1371/journal.pone.0077600.

Sapkota, R., Knorr, K., Jørgensen, L. N., O’Hanlon, K. A., and Nicolaisen, M. (2015). Host genotype is an important determinant of the cereal phyllosphere mycobiome. *New Phytol* 207, 1134–1144. doi:10.1111/nph.13418.

Sarker, D., Roy, N., and Yeasmin, T. (2010). Isolation and antibiotic sensitivity of Bacillus thuringinesis strain from dump soil. *MJM*. doi:10.21161/mjm.19909.

Setati, M. E., Jacobson, D., Andong, U.-C., and Bauer, F. (2012). The Vineyard Yeast Microbiome, a Mixed Model Microbial Map. *PLoS ONE* 7, e52609. doi:10.1371/journal.pone.0052609.

Setati, M. E., Jacobson, D., and Bauer, F. F. (2015). Sequence-based Analysis of the Vitis vinifera L. cv Cabernet Sauvignon Grape Must Mycobiome in Three South African Vineyards Employing Distinct Agronomic Systems. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.01358.

Shen, C., Xiong, J., Zhang, H., Feng, Y., Lin, X., Li, X., et al. (2013). Soil pH drives the spatial distribution of bacterial communities along elevation on Changbai Mountain. *Soil Biology and Biochemistry* 57, 204–211. doi:10.1016/j.soilbio.2012.07.013.

Singh, J. S., Kumar, A., Rai, A. N., and Singh, D. P. (2016). Cyanobacteria: A Precious Bio-resource in Agriculture, Ecosystem, and Environmental Sustainability. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.00529.

Sipos, R., SzÃ©kely, A. J., Palatinszky, M., RÃ©vÃ©sz, S., MÃ¡rialigeti, K., and Nikolausz, M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis: PCR

parameters influencing quantitative bias. *FEMS Microbiology Ecology* 60, 341–350. doi:10.1111/j.1574-6941.2007.00283.x.

Soergel, D. A. W., Dey, N., Knight, R., and Brenner, S. E. (2012). Selection of primers for optimal taxonomic classification of environmental 16S rRNA gene sequences. *ISME J* 6, 1440–1444. doi:10.1038/ismej.2011.208.

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored '"rare biosphere."' 6.

Solieri, L., and Giudici, P. (2008). Yeasts associated to Traditional Balsamic Vinegar: Ecological and technological features. *International Journal of Food Microbiology* 125, 36–45. doi:10.1016/j.ijfoodmicro.2007.06.022.

Soneji, J. R., and Nageswara-Rao, M. (2011). "Chapiter 11: Vitis," in *Wild Crop Relatives: Genomic and Breeding Resources*, ed. C. Kole (Berlin, Heidelberg: Springer Berlin Heidelberg). doi:10.1007/978-3-642-16057-8.

Soto, M. (2007). El txakoli de Getaria se libra del hongo 'mildiu. *EL DIARIO VASCO*.

Stefanini, I., Albanese, D., Cavazza, A., Franciosi, E., De Filippo, C., Donati, C., et al. (2016). Dynamic changes in microbiota and mycobiota during spontaneous ' V ino S anto T rentino' fermentation. *Microbial Biotechnology* 9, 195–208. doi:10.1111/1751-7915.12337.

Sturz, A. V., Christie, B. R., and Nowak, J. (2000). Bacterial Endophytes: Potential Role in Developing Sustainable Systems of Crop Production. *Critical Reviews in Plant Sciences* 19, 1–30. doi:10.1080/07352680091139169.

Sun, Y., and Liu, Y. (2014). Investigating of yeast species in wine fermentation using terminal restriction fragment length polymorphism method. *Food Microbiology* 38, 201–207. doi:10.1016/j.fm.2013.09.001.

Swift, J. F., Hall, M. E., Harris, Z. N., Kwasniewski, M. T., and Miller, A. J. (2021). Grapevine Microbiota Reflect Diversity among Compartments and Complex Interactions within and among Root and Shoot Systems. *Microorganisms* 9, 92. doi:10.3390/microorganisms9010092.

Tan, W., Wang, J., Bai, W., Qi, J., and Chen, W. (2020). Soil bacterial diversity correlates with precipitation and soil pH in long-term maize cropping systems. *Sci Rep* 10, 6012. doi:10.1038/s41598-020-62919-7.

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ Microbiol* 16, 2848–2858. doi:10.1111/1462-2920.12456.

Tilston, E. L., Sizmur, T., Dixon, G. R., Otten, W., and Harris, J. A. (2010). "The Impact of Land-Use Practices on Soil Microbes," in *Soil Microbiology and Sustainable Crop Production*, eds. G. R. Dixon and E. L. Tilston (Dordrecht: Springer Netherlands), 273–295. doi:10.1007/978-90-481-9479-7_7.

Tofalo, R., Schirone, M., Corsetti, A., and Suzzi, G. (2012). Detection of *Brettanomyces* spp. in Red Wines Using Real-Time PCR. *Journal of Food Science* 77, M545–M549. doi:10.1111/j.1750-3841.2012.02871.x.

Treude, N., Rosencrantz, D., Liesack, W., and Schnell, S. (2003). Strain FAc12, a dissimilatory iron-reducing member of the Anaeromyxobacter subgroup of Myxococcales. *FEMS Microbiology Ecology* 44, 261–269. doi:10.1016/S0168-6496(03)00048-5.

Trivedi, P., Delgado-Baquerizo, M., Anderson, I. C., and Singh, B. K. (2016). Response of Soil Properties and Microbial Communities to Agriculture: Implications for Primary Productivity and Soil Health Indicators. *Front. Plant Sci.* 7. doi:10.3389/fpls.2016.00990.

van Leeuwen, C. (2010). "Terroir: the effect of the physical environment on vine growth, grape ripening and wine sensory attributes," in *Managing Wine Quality* (Elsevier), 273–315. doi:10.1533/9781845699284.3.273.

van Peer, R., Punte, H. L. M., de Weger, L. A., and Schippers, B. (1990). Characterization of Root Surface and Endorhizosphere Pseudomonads in Relation to Their Colonization of Roots. *Appl Environ Microbiol* 56, 2462–2470. doi:10.1128/aem.56.8.2462-2470.1990.

Wade, W. (2002). Unculturable bacteriaÐthe uncharacterized organisms that cause oral infections. 95, 3.

Wagner, M. R., Lundberg, D. S., del Rio, T. G., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nat Commun* 7, 12151. doi:10.1038/ncomms12151.

Wang, C. H., Wu, L., Wang, Z., Alabady, M. S., Parson, D., Molumo, Z., et al. (2020). Characterizing changes in soil microbiome abundance and diversity due to different cover crop techniques. *PLoS ONE* 15, e0232453. doi:10.1371/journal.pone.0232453.

REFERENCES

Wang, Z. K., Yang, Y. S., Stefka, A. T., Sun, G., and Peng, L. H. (2014). Review article: fungal microbiota and digestive diseases. *Aliment Pharmacol Ther* 39, 751–766. doi:10.1111/apt.12665.

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., von Wettberg, E. J. B., et al. (2016). Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. *Trends in Plant Science* 21, 418–437. doi:10.1016/j.tplants.2015.11.008.

Welbaum, G. E., Sturz, A. V., Dong, Z., and Nowak, J. (2004). Managing Soil Microorganisms to Improve Productivity of Agro-Ecosystems. *Critical Reviews in Plant Sciences* 23, 175–193. doi:10.1080/07352680490433295.

Whipps, J. M., Hand, P., Pink, D., and Bending, G. D. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of Applied Microbiology* 105, 1744–1755. doi:10.1111/j.1365-2672.2008.03906.x.

Yang, S.-X., Liao, B., Li, J., Guo, T., and Shu, W.-S. (2010). Acidification, heavy metal mobility and nutrient accumulation in the soil–plant system of a revegetated acid mine wasteland. *Chemosphere* 80, 852–859. doi:10.1016/j.chemosphere.2010.05.055.

Young, J. M., Kuykendall, L. D., Martínez-Romero, E., Kerr, A., and Sawada, H. (2003). Classification and nomenclature of Agrobacterium and Rhizobium – a reply to Farrand et al. (2003). *International Journal of Systematic and Evolutionary Microbiology* 53, 1689–1695. doi:10.1099/ijs.0.02762-0.

Yun, Y., Wang, H., Man, B., Xiang, X., Zhou, J., Qiu, X., et al. (2016). The Relationship between pH and Bacterial Communities in a Single Karst Ecosystem and Its Implication for Soil Acidification. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.01955.

Zárate-Valdez, J. L., Zasoski, R. J., and Läuchli, A. E. (2006). Short-term effects of moisture content on soil solution pH and soil Eh. *Soil Science* Vol. 171, 423–431. doi:10.1097/01.ss.0000222887.13383.08.

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., et al. (2015). The Soil Microbiome Influences Grapevine-Associated Microbiota. *mBio* 6, e02527-14. doi:10.1128/mBio.02527-14.