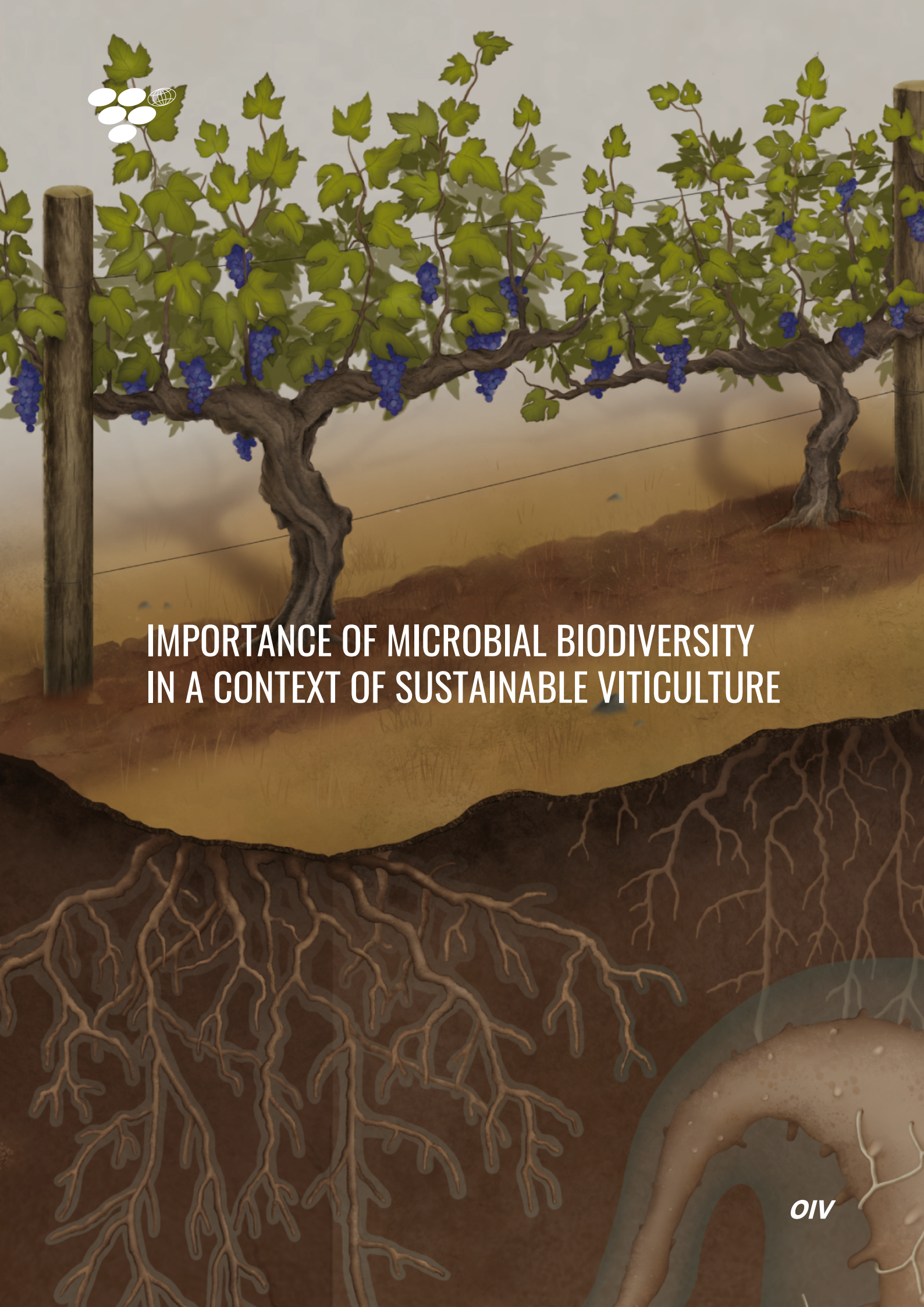

IMPORTANCE OF MICROBIAL BIODIVERSITY IN A CONTEXT OF SUSTAINABLE VITICULTURE

OIV

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1 • SCOPE

According to the principles of sustainable vitiviniculture adopted by the OIV-CST 518-2016 RESOLUTION (OIV, 2016), and the guide for their implementation adopted by the OIV-VITI 641-2020 RESOLUTION (OIV, 2020), the protection of soils, water, air, biodiversity, and landscapes is particularly relevant in the vitivinicultural field. Therefore, a sound planning is required before planting new vineyards or establishing other vitivinicultural facilities, using well-established ecological principles and the optimum management of existing and new assets.

The biodiversity in living organisms is considered an important functional element for the implementation of the principles of sustainable vitiviniculture and this sense, the OIV-VITI 655-2021 RESOLUTION (OIV, 2021) established the recommendations about valuation and importance of microbial biodiversity in a sustainable vitiviniculture context.

An overview about the microbial diversity in vineyard is presented in this Expertise Collective Document, by illustrating the major traits of fungi and bacteria populating the viticultural environment, such as their roles and mechanisms of interaction, and how these interactions can be beneficial from the vineyard to the winery. The publication of this document is aimed at encouraging and supporting the application of the recommendations established in the OIV-VITI 655-2021 RESOLUTION (OIV, 2021).

2 • INTRODUCTION TO SOIL MICROBIAL PROCESSES OF INTEREST FOR VINEYARD HEALTH AND NUTRITION

2.1 • UNDERSTANDING THE INTIMATE VINE-MICROBE INTERACTIONS (RHIZOSPHERE AND PHYLOSPHERE)

Like any other plant, grapevines live in close association with microorganisms. These microorganisms are structured in the form of complex communities of highly interacting species. These microorganisms, including bacteria, fungi, protists, and viruses, can have beneficial, detrimental, or neutral effects on vines, thrive in different parts of the plant (both outside and inside) and, together, constitute the grapevine microbiome. Microbes thriving outside the plant can be naturally found inhabiting in the rhizosphere, the thin layer of soil around fine roots, and on the leaves (phyllosphere), trunk (lignosphere), stem (caulosphere), flowers (anthosphere), and berries (carposphere). Despite this diversity of habitats, the ultimate origin of all these microorganisms is typically the surrounding vineyard soils, while trunks play an important role as perennial reservoirs of microbial biodiversity. Microorganisms found in the rhizosphere and phyllosphere are particularly important for the metabolism of vines due to their role in nutrient cycling and plant nutrition and immunity and, hence, in the functioning of vineyards. In contrast, microbes living on the aerial parts, specially yeast communities inhabiting on berries, are particularly important for the protection of grapes to diseases and later on in the winery, both in terms of wine fermentation and spoilage. Microorganisms found within plant tissues are called endophytes, most of which are fungi that can play a key role in plant immune response and nutrition. Considering the role that microorganisms have in defining the health of plants, grapevines and their associated microorganisms can be considered together as a supraorganism or holobiont.

Plants can greatly benefit from their interaction with microorganisms, but microorganisms also benefit from their interactions with plants. For example, apart from providing adequate microhabitats for a myriad of microbial species to thrive, plants release a high proportion of their carbon fixed through photosynthesis (typically between 10-20%) through the roots in the form of root exudates. These exudates, also known as rhizodeposits, include low molecular weight carbon-rich compounds such as carbohydrates, aminoacids, organic acids, and more complex secondary metabolites like flavonoids and glucosinolates which together contribute to feed the rhizosphere microorganisms, thus priming their metabolism. Plants also invest an important portion of their photosynthetically fixed carbon to feed associated organisms such as the arbuscular mycorrhizal fungi colonizing their roots. This is why high soluble solids (°Brix) readings in sap can be considered as an adequate proxy for healthy plants and plant-soil interactions. Mycorrhizal fungal hyphae colonizing plant roots can effectively increase the surface area explored by plant roots by one thousand times. These fungal networks can act as highways for various mineral and organic compounds, including nutrients and water, and can thus be seen as a true extension of the plant root system.

2.2 • ROLE OF MICROBES IN NUTRIENT/MINERAL ELEMENTS CYCLING AND MOBILIZATION

Plants need a set of at least 17 chemical elements to carry out their normal functions. Some of these elements can be extracted from the atmosphere (e.g., carbon) or from water (hydrogen and oxygen). However, in the absence of external inputs like mineral fertilizers, other elements such as phosphorus, magnesium, calcium, potassium, sulphur, and trace elements such as iron, copper, manganese, cobalt, zinc or molybdenum, can only be supplied from the weathering of soils and the recycling of organic matter. Other elements like nitrogen can be both mineralised or fixed by certain groups of bacteria and archaea (the so-called nitrogen fixers). Some of these organisms can live in close association with plant roots (e.g., rhizobia in nodules of legumes), while some are free-living. Both symbiotic and free-living nitrogen fixers use an enzyme complex called nitrogenase to convert the inert atmospheric dinitrogen into usable reactive nitrogen forms. Grapevines do not form symbiotic associations with nodule-forming rhizobacteria, but some herbaceous leguminous plants that can be used as cover crops in vineyards can do it. Actually, legumes such as lucerne and lupine have been long used as cover crops in vineyards to improve their nitrogen nutrition, making nitrogen-fixing bacteria potentially highly relevant for the functioning of low-input vineyards.

THE MICROBIOME OF THE GRAPEVINE

Microorganisms, such as bacteria, archaea, yeasts, fungi, protists, and small invertebrates, live in close association with the vegetative parts (like leaves, trunk and roots) and reproductive organs (berries and flowers) of grapevines, both outside and inside, configuring their microbiome.

While the vineyard soil represents the main reservoir for the vineyard microbiome, the vast majority of microorganisms inhabit the rhizosphere, the portion of soil that is in close contact with roots, where nutrient exchanges and specific physicochemical processes occur.

Dominant microbial groups *Arranged from small to large*

Bacteria · Archaea · Yeasts · Fungi · Protists · Nematodes

Leaves

Microbial functions:

- Plant protection
- Nutrition

Berries and flowers

Microbial functions:

- Fermentation
- Plant protection

Trunk

Microbial functions:

- Microbial reservoir

Rizhosphere

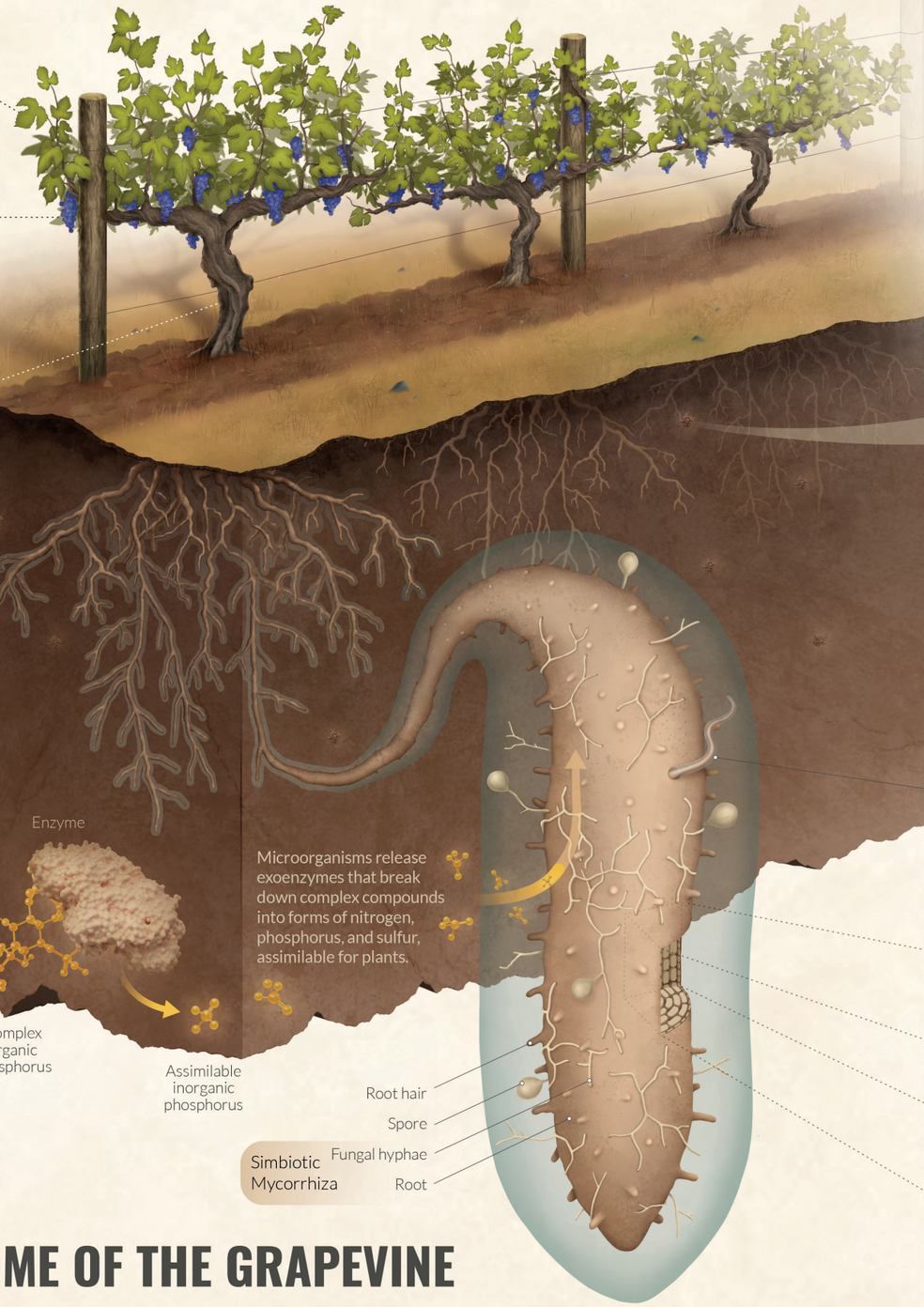
Microbial functions:

- Nutrition
- Increased root exploration and exploitation surface
- Increased resistance to disturbance
- Enhance plant immunity
- Nutrient cycling

Bulk soil

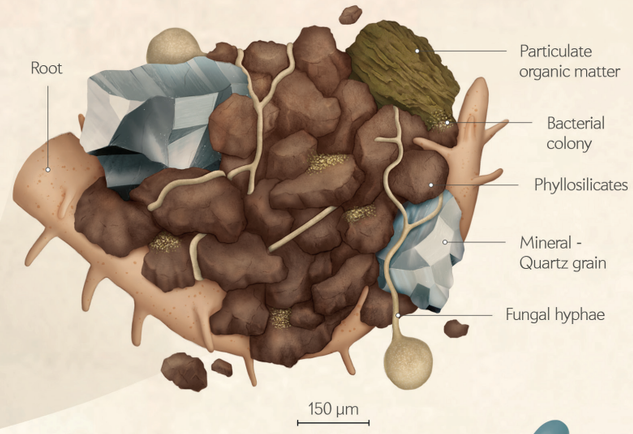
Microbial functions:

- Microbial reservoir
- Nutrient cycling
- Carbon sequestration
- Soil formation

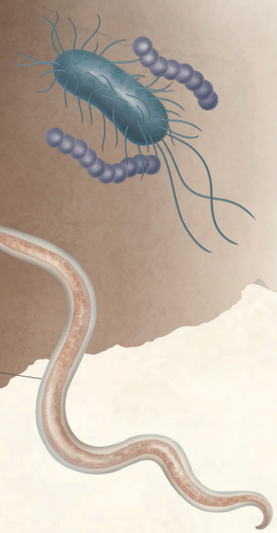


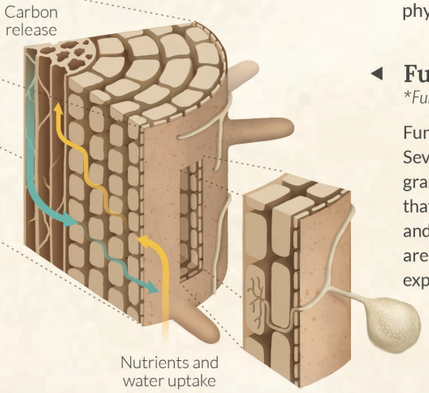
How do microbes live in the soil?

Soil aggregates are formed by mineral particles glued to distinct amounts of carbon compounds. They are formed and colonized by different soil organisms and are hotspots of microbial interactions. The greatest microbial diversity is typically found in microaggregates. The formation of aggregates is greatly favored by the presence of plant roots.



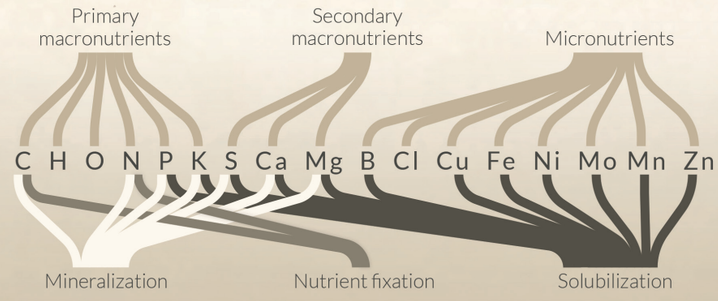
Formation of soil aggregates



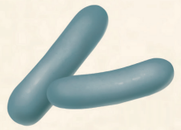


Soil nutrient bioavailability

Soil microorganisms can affect soil nutrient bioavailability, and thus plant nutrition, via: decomposition and mineralization of organic matter, nutrient fixation, and solubilization of unavailable nutrient forms.



Microbes in the vineyard



◀ Bacteria Size: 0.1–10µm

Bacteria are the most diverse and abundant organisms in soil. They play crucial roles in the biogeochemical cycling of nutrients, making them available for plants. Most can release hydrolytic enzymes to degrade organic matter and some can fix atmospheric nitrogen. Some are symbiotic and get sugars exuded from plants in exchange.

◀ Nematodes Size: 500µm–10cm

These non-segmented worms play different roles in food webs: predators, herbivores, bacterivores, and fungivores. They enhance organic matter decomposition and nutrient cycling by grazing bacterial and fungal colonies, and by spreading these to newly available organic residues. Some of them can also act as vectors of harmful microbial phytopathogens.

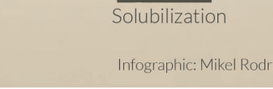
◀ Fungi Size: 2–20µm*

**Fungal mycelia can reach several meters in size.*

Fungi tend to grow in the form of hyphae. Several species cause plant diseases and grape rots. However, they release enzymes that contribute to organic matter degradation and nutrient bioavailability. Some species are symbiotic with roots and increase the exploration capacity of the root system.

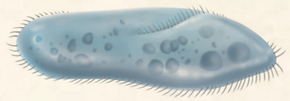
Archaea Size: 0.1–10µm ▲

They tend to dominate in extreme environments. Many are decomposers and release enzymes, and some species play a role in the cycling of nitrogen –they transform ammonium into nitrate and, through fixation of atmospheric nitrogen, they increase the availability of this nutrient–.



Yeasts Size: 1–10µm ▲

Yeasts are unicellular fungi specialized in colonizing sugar-rich environments. Although they play a minor role in soil processes, the soil acts as a highly relevant reservoir of autochthonous fermentative yeasts in vineyards.



Protists Size: 30µm–1mm ▲

Protists are the most diverse eukaryotes in soils. Despite being highly understudied as compared to other soil microorganisms, they are fundamental elements in the soil food web, performing key functions such as microbial predation.

Infographic: Mikel Rodríguez

Microorganisms can, thus, be seen as the engine that keeps entire ecosystems running by driving the chemical weathering and the processing (i.e., breaking up, decomposition, and mineralization) of organic matter in soils, and by contributing to nutrient fixation. This organic matter can be in the form of fresh inputs (either in the form of rhizodeposits or as litter), or as soil organic matter in various stages of decomposition and associations with mineral particles. The first stage of organic matter processing is the breaking up by soil organisms. Although this process is typically carried out by larger eukaryotic organisms (meso- and macrofauna such as collembolans, mites, nematodes, earthworms, etc.), some microorganisms such as certain protists can also play a role here. This contributes to increasing the surface of organic matter that can then be attacked by microbial enzymes. Microorganisms, particularly fungi and bacteria, are known to release a battery of extracellular enzymes that contribute to mineralise shredded organic matter by accelerating the breakup of specific molecular bonds. Depending on the type of bonds being broken, these enzymes can be categorised as being linked to different nutrient cycles, including the carbon (e.g., glucosidades, xylosidades, etc.), nitrogen (e.g., amonipeptidases, glucosaminidases, etc.), phosphorus (e.g., phosphatases), and sulphur (e.g., sulphatases) cycles. Such as enzymes can also be broadly categorised as hydrolytic enzymes (released by both bacteria and fungi, including all the enzymes previously mentioned), and oxidative enzymes (released by fungi, including phenoloxidases, ligninases, and peroxidases). The release of phosphorus by phosphorus-solubilising bacteria is particularly relevant for grapevine nutrition. Some metallic elements such as copper, zinc, molybdenum, and cobalt, in turn provided in adequate amounts in the absence of fertilization through the activity of soil microorganisms, such as mycorrhizae, are particularly important for the synthesis of plant enzymes.

Complex soil microbial foodwebs are also critical for the nutrition of plants, including those in agroecosystems such as vineyards, with predatory protists playing a particularly important role here. Non-predatory microorganisms also play a key role by acting as prey to larger macroorganisms such as nematodes, thus supporting the adequate functioning of soil networks. For example, when predatory protists prey on bacteria and fungi, part of their intracellular compounds is released to the external medium, thus representing a highly valuable source of readily usable nutrients for plants. This suggests the importance of maintaining complex communities of highly interacting microbial species within vineyards to ensure their sustainability. In this sense, it has been suggested that maintaining a balanced microbial community is key to plant health. Conversely, microbial community imbalances (or dysbiosis) can lead to a decline in plant health. Ensuring an adequate and reliable source of carbon compounds belowground, either through rhizodeposition by healthy plants or the addition of compost, the use of cover crops, and the reintroduction of animals like sheep within the portfolio of available management practices, seems critical to the integrity and balance of soil foodwebs in vineyards.

2.3 • ROLE OF MICROBES IN RESISTANCE TO BIOTIC (PATHOGENS) AND ABIOTIC (E.G. DROUGHT) STRESSES

Vineyards are constantly exposed to both biotic and abiotic stresses, with important economic consequences. Beneficial microorganisms are known to play a critical role in regulating their response to these stresses, including in terms of their resilience and resistance. Biotic stresses include microbial pathogens such as viruses (e.g., leaf roll disease virus), bacteria (e.g., Xylella fastidiosa), fungi (e.g., Botrytis cinerea), and protists (e.g., Plasmopara viticola) as well as animal pests (e.g., Lobesia botrana). There are different ways by which the response of vines to pathogens and pests can be mediated by soil microorganisms. First of all, by allowing plants to have access to a better nutrition (e.g., mycorrhizal fungi), microorganisms can enhance the health status of plants, thus increasing their ability to defend themselves against the attack. Similarly, rhizosphere, phyllosphere, and endophytic microorganisms can also supply plants with compounds that are critical to their physiology and immune response such as vitamins (e.g., B vitamins), hormones (e.g., indole acetic acid, gibberellins, and cytokinins), and antioxidants (e.g., catalase), thus helping plants to keep pathogens and pests under control. These microorganisms, including plant growth-promoting rhizobacteria (PGPRs), are typically said to have biostimulating effects. Another way by which microbes can prevent the infection by pathogens is through competitive exclusion of some of the harmful bacterial and fungal species, thus allowing some level of biocontrol. This can happen because a thriving community of beneficial microbes growing on the surface of grapevines, many of which have antimicrobial capabilities, leaves no space for potentially harmful microbial species to colonise and grow, thus outcompeting them. Moreover, some of these microorganisms can synthesize chemical compounds that have insecticidal effects (e.g., Bacillus thuringiensis). Similarly, it has been shown that mycorrhizal fungi proliferating around vine roots can create a physical barrier impeding the infestation by root-feeding nematodes such as Meloidogyne spp. and Xiphinema spp., while some oomycetes have been described as parasites of grapevine pathogens (Bettenfeld et al. 2021).

Vineyards are also frequently exposed to abiotic stresses, including lack of water (e.g., drought), low and high temperatures (e.g., frost and heatwaves), and anomalous soil conditions (e.g., salinity). The role of climatic stresses is particularly important to consider given that climate change is predicted to increase their frequency and severity. In all cases, an adequate supply of nutrients, water, and organic compounds such as hormones and vitamins via functional plant-microbial interactions can improve the response of grapevines to these stresses. The role of mycorrhizae is particularly important in the response of vines to abiotic stress, including water stress, iron deficiency, and salinity. For example, a highly developed network of mycorrhizal mycelium can contribute to water retention by favouring soil micro-aggregation and structure (Bettenfeld et al. 2021). Considering a predicted scenario in which more than 20% of the terrestrial surface will cross, at least, one non-reversible threshold of aridity by 2100 (Berdugo et al., 2020), and knowing that global warming increases the proportion of soil-borne plant pathogens (Delgado-Baquerizo et al., 2020), we anticipate that the vineyard microbiome will increasingly become a keystone aspect to consider when designing strategies towards the adaptation and mitigation of the effects of climate change in vitiviniculture.

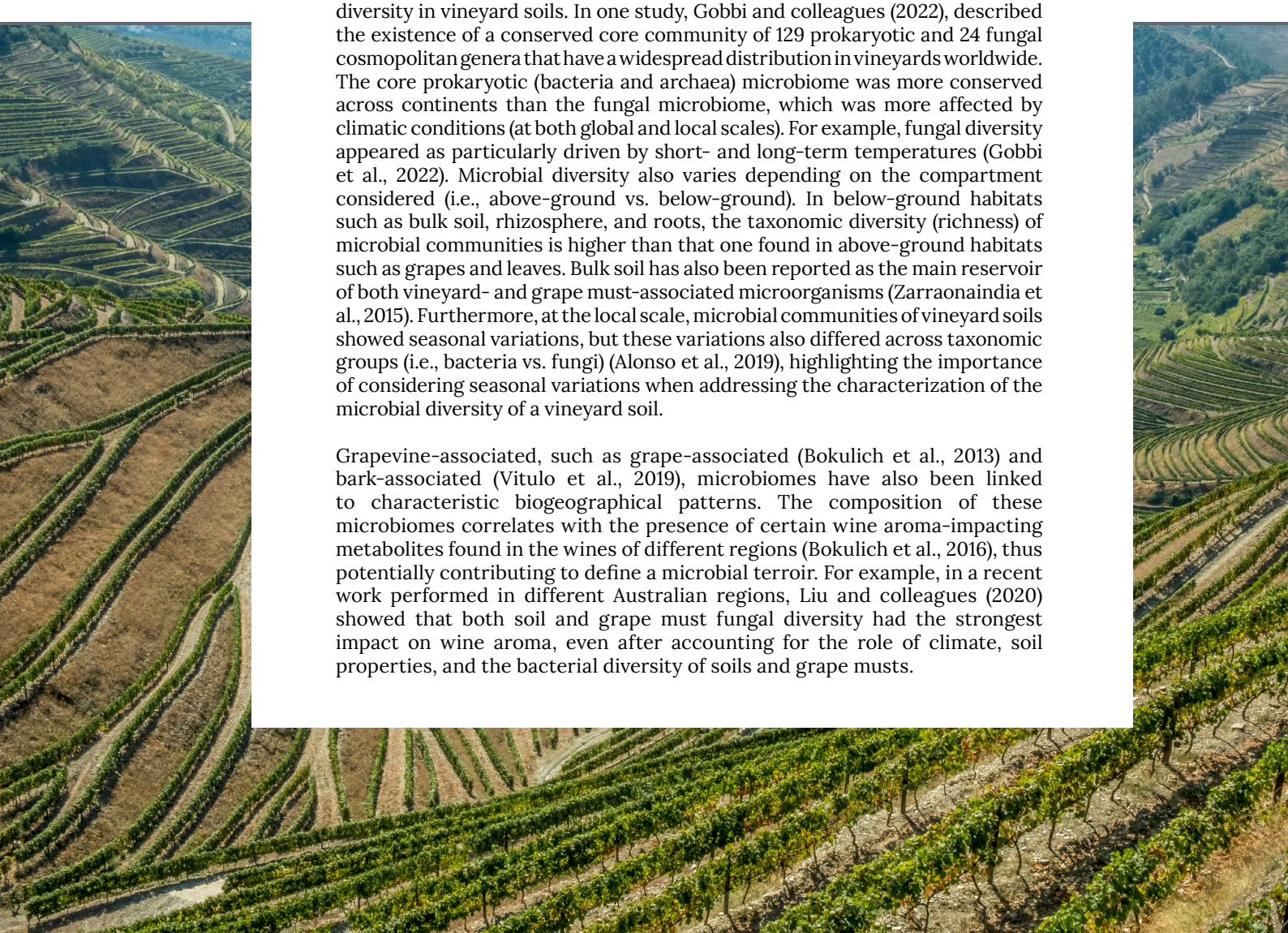
3 • MICROBIAL DIVERSITY IN THE VINEYARD (FROM LOCAL TO GLOBAL BIOGEOGRAPHICAL PATTERNS)

3.1 • IMPACT OF ABIOTIC FACTORS ON VINEYARD MICROBIAL DIVERSITY (EDAPHOCLIMATIC CONDITIONS)

The physical-chemical properties of soils and the environmental characteristics of a specific geographic location, including short- and long-term climatic conditions, are major drivers of the composition and structure of plant-associated microbiomes. Although different plants can filter those microbes inhabiting the rhizosphere, the microbiome of bulk soils is strongly defined by abiotic factors (including edaphoclimatic conditions), and geographical dispersal limitations, meaning that any biotic or anthropogenic factor such as the type of management will always affect the vineyard-associated microbiome within the context of pre-existing microbial patterns found in a particular region. It has been shown that several abiotic factors can have a higher importance in structuring plant-associated soil microbial communities than plant species or the type of cultivar (Fierer, 2017). Soil pH and the carbon:nitrogen ratio are the main edaphic drivers of vineyard soil microbial community composition, but other parameters such as soil organic matter content, soil temperature, moisture, and nitrogen concentration can also have a significant impact (Zarraonaindia et al., 2015).

Previous studies have evaluated the global biogeography of bacterial and fungal diversity in vineyard soils. In one study, Gobbi and colleagues (2022), described the existence of a conserved core community of 129 prokaryotic and 24 fungal cosmopolitan genera that have a widespread distribution in vineyards worldwide. The core prokaryotic (bacteria and archaea) microbiome was more conserved across continents than the fungal microbiome, which was more affected by climatic conditions (at both global and local scales). For example, fungal diversity appeared as particularly driven by short- and long-term temperatures (Gobbi et al., 2022). Microbial diversity also varies depending on the compartment considered (i.e., above-ground vs. below-ground). In below-ground habitats such as bulk soil, rhizosphere, and roots, the taxonomic diversity (richness) of microbial communities is higher than that one found in above-ground habitats such as grapes and leaves. Bulk soil has also been reported as the main reservoir of both vineyard- and grape must-associated microorganisms (Zarraonaindia et al., 2015). Furthermore, at the local scale, microbial communities of vineyard soils showed seasonal variations, but these variations also differed across taxonomic groups (i.e., bacteria vs. fungi) (Alonso et al., 2019), highlighting the importance of considering seasonal variations when addressing the characterization of the microbial diversity of a vineyard soil.

Grapevine-associated, such as grape-associated (Bokulich et al., 2013) and bark-associated (Vitulo et al., 2019), microbiomes have also been linked to characteristic biogeographical patterns. The composition of these microbiomes correlates with the presence of certain wine aroma-impacting metabolites found in the wines of different regions (Bokulich et al., 2016), thus potentially contributing to define a microbial terroir. For example, in a recent work performed in different Australian regions, Liu and colleagues (2020) showed that both soil and grape must fungal diversity had the strongest impact on wine aroma, even after accounting for the role of climate, soil properties, and the bacterial diversity of soils and grape musts.



3.2 • IMPACT OF FARMING PRACTICES ON VINEYARD MICROBIAL DIVERSITY

As mentioned above, plant-associated microbiomes are integral to viticulture and winemaking, where diverse fungi and bacteria can exert positive, negative, and neutral effects on vine health and wine quality. Therefore, the sources and persistence of wine-relevant microbiota in vineyards critically impact the final product quality. Moreover, it is well known that human intervention can affect the vineyard microbiome through several different direct and indirect itineraries (Bettenfeld et al., 2021; Griggs et al., 2021), with potential effects on microbial terroirs (OIV, 2010) (Gilbert et al., 2014).

3.2.1 • CONVENTIONAL VS ORGANIC AND BIODYNAMIC MANAGEMENT

Conventionally managed vineyards typically rely on high external inputs of synthetic chemicals such as pesticides, fungicides, and herbicides, as well as on frequent tilling to keep pests, diseases, and adventitious plants at bay. As a general trend, studies comparing vineyards under conventional, organic, and biodynamic agriculture showed that soil microbial communities differ depending on management (Coller et al., 2019; Ortiz-Álvarez et al., 2021). In most cases, vineyards cultivated under organic and biodynamic farming, as compared with conventional farming, showed greater specific fungal and bacterial richness and diversity, particularly in the soil, which is the most studied vineyard compartment (Bettenfeld et al., 2021; Burns et al., 2016; Hernandez and Menéndez, 2019; Likar et al., 2017). Some studies have also shown that microbial community composition can significantly differ between organic and biodynamic management, but with species richness remaining unaffected (Hendgen et al., 2018). Although the diversity of yeasts in grape musts can be also affected by vineyard management strategy, with higher species richness found in organic grape musts, Grangeteau et al. (2017) found that the addition of SO2 at the early stages of wine fermentation had a critical impact in reducing the diversity of fermenting yeasts, independently of the treatments previously applied to the grapes in the vineyard. Moreover, changes in the phyllosphere-associated endophytic microbial communities in response to chemical treatments appear to be transient over time (Perazzolli et al., 2014).

The type of fertilizer used (e.g., inorganic vs. organic amendments) can also affect the soil microbiota of vineyards (Canfora et al., 2017) (Bettenfeld et al., 2021). As a general rule, organic and biodynamic vineyards receive greater inputs of organic amendments such as compost, animal manure, or green manure, while conventional vineyards rely more on chemical and mineral inputs. One study, (Longa et al., 2017) showed that green manure resulted in greater bacterial richness of taxa involved in the soil nitrogen cycle such as Microvirga sp., Pontibacter sp. and Nitrospira sp., regardless of the type of management (organic vs. biodynamic). In another study, Burns et al., (2016) showed that soil bacterial communities were more diverse in vineyards that were tilled less recently, were farmed following biodynamic practices, and had compost application. Regarding the use of chemical fertilizers, a 30-yr experiment showed that soil microbial communities were only slightly modified in response to additions of 150 kg N ha^{-1} yr^{-1}, and that these effects were minor when compared with the effects of tillage. (Pingel et al., 2019). Despite the available information regarding the impacts of other types of fertilizers such as sulfur and copper is little by little increasing (Colautti et al., 2023), a comprehensive knowledge regarding their effects on the vineyard-associated microbiota, both in plants and soils, is still hard to achieve, also considering that, many of these products are also added as fungicides.

3.2.2 • COVER CROPS

Cover cropping, which consists in the cultivation of herbaceous plants in the inter-rows of vineyards, is considered as one of the most effective solutions for enhancing the supply of multiple ecosystem services in agriculture, including reducing soil erosion, and enhancing carbon sequestration. The use of cover crops is generally opposed to the frequent tillage of the soil. The soil microbiome is known to be highly impacted by tilling. Tilling not only results in the loss of soil structure and the break-up of soil aggregates, but also in the absence of plant roots, thus reducing the amount of carbon entering the soil, and thus feeding the microbes, with negative consequences for soil carbon stabilization in the long-term (Kim et al., 2020; Novara et al., 2020). A meta-analysis conducted across different orchards, including vineyards, showed that, overall, cover cropping significantly increased parameters of soil microbial abundance, activity, and diversity, compared to bare soils. Nevertheless, cover cropping effect sizes varied by agricultural practices and environmental conditions, suggesting a context-dependent effect of cover cropping on soil microbes. For example, effects of cover crops were less pronounced under continental climatic conditions (Kim et al., 2020). Another study showed that fungal diversity was slightly enhanced under conventional tillage, while bacterial diversity increased under cover cropping (Novara et al., 2020). However, whether cover crop-induced changes in terms of soil microbial composition are also coupled with alterations in the microbial composition of other vineyard compartments and plant organs is not so well known, although this is highly likely, despite some studies that found the lack of corresponding shifts in the fruit microbiome (Chou et al., 2018).

Even though it is extremely difficult to draw overall conclusions about the general impact of agronomic practices on the vineyard microbiome, as different plots under the same management (i.e., conventional, organic, or biodynamic) can receive completely different treatments, some general trends can be inferred. Overall, all the above mentioned studies showed a high resilience of the vineyard microbiota, also suggesting that indigenous microbial communities are highly adapted to environmental and biotic factors in the areas where the grapevines are grown (Perazzolli et al., 2014). Therefore, the existing data support the existence of a complex vineyard-associated microbiome that, despite evolving according to the local environmental conditions and responding to agronomical inputs, is highly conserved within the context of a particular region (Guzzon et al., 2022).

4 • DIVERSITY AND TAXONOMY OF THE FUNCTIONAL MICROBIOTA IN VITI-VINICULTURE

4.1 • THE VINEYARD - PATHOGENS, BIOCONTROL AGENTS, OTHER POSITIVE-IMPACTING MICROBES

4.1.1 • PATHOGENS

Grapevines can be attacked by many pathogenic microorganisms, including bacteria, fungi, protists like oomycetes (or water molds) and viruses, which can cause plant diseases (Wilcox et al., 2015; Armijo et al., 2016). In general, pathogens can infect the plant in pre- and/or post-harvest periods and their uncontrolled proliferation has a direct effect on the productive sector, reducing yield, and fruit quality and safety, ultimately causing economic losses and potential risks on the health of the consumers and of the environment.

Pathogenic microorganisms can be classified according to their lifecycle and infection/evasion mechanisms as necrotrophic, biotrophic, and hemibiotrophic agents. Necrotrophic pathogens feed on dead tissue, secreting lytic enzymes and phytotoxins to promote cell death into the host plant. Biotrophic pathogens on the other hand, feed on living tissue, developing structures to invade the cell and obtain metabolism products. Finally, hemibiotrophic pathogens start with a biotrophic infection phase and then turn to a final necrotrophic phase, killing its host at the end of the infection cycle (Glazebrook, 2005). Apart from the microbial species causing Grapevine Trunk Diseases, which are explained in detail in the OIV document by Fontaine et al. (OIV, 2016), the most significant biological threats for V. vinifera are the grey mold, powdery mildew, and downy mildew, caused by Botrytis cinerea, Erysiphe necator and Plasmopara viticola, respectively (Wilcox et al., 2015; Armijo et al., 2016).

Botrytis cinerea, also known as grey mold, is a necrotrophic fungus. Since it can live as a parasite in green tissues and as a saprophyte in dead or decaying ones, it shows a wide distribution in nature and host unspecificity (Wilcox et al., 2015; Armijo et al., 2016). After Botrytis penetrates the plant barriers, infection expands to surrounding cells by degradation of the cell wall, using the nutrients resulting from the process, and continuing until plant defences have been broken down. Grey mold is considered as the most outstanding of all plant pathogenic fungi of the grapevine, causing economic losses of 10 to 100 billion USD worldwide (Roca-Couso et al., 2021).

Erysiphe necator, the etiologic agent of the grapevine powdery mildew, is an obligate biotrophic fungus that spreads through the air by conidial sporulation (Armijo et al., 2016). Powdery mildew easily infects all green tissues (leading to chlorosis and premature aging and shedding of leaves and forming white or grey powdery bloom on green stems), inflorescences, and berries. Fruit infection leads to uneven ripening, wrinkling, or cracking of the berries, resulting in fruit rotting, reduced yields, and deterioration of the wine quality. Fruit quality deteriorates greatly, acidity rises, and anthocyanins and sugar levels decrease.

Plasmopara viticola, the causal agent of grapevine downy mildew, is an obligate biotrophic oomycete and one of the most important pathogens affecting grapevine production worldwide. All cultivated European V. vinifera cultivars are susceptible to P. viticola. The disease causes significant yield losses, especially

in years with humid climatic conditions. The pathogen is able to spread to many parts of the plant including petioles, shoot tips, berries and seeds, and in favourable climatic conditions, it causes the flowers and clusters to dry (Wilcox et al., 2015; Armijo et al., 2016).

Other pathogenic microorganisms affecting grapevine include: (i) Agrobacterium vitis, a biotrophic bacteria causing the grapevine crown gall. It maintains a parasitic relationship with living tissues of their host to complete its life cycle; (ii) Xylella fastidiosa, a biotrophic bacteria that does not kill the host tissue until later stages of its life cycle, is transmitted by insect vectors. It is a xylem-limited bacterium and the causal agent of Pierce's disease in V. vinifera; (iii) Nearly 70 virus species have been identified to date that are able to infect the plants in the Vitis genus, accounting for at least 25 different diseases in grapevine. Viruses are phloem limited microorganisms, whose infections can cause different systemic symptoms in the host. From an economic point of view, the most important grapevine viruses are those who cause the leafroll diseases, known as Grapevine Leaf Roll associated Viruses (Wilcox et al., 2015; Armijo et al., 2016).

For a complete list of grapevine pathogenic microbial agents and diseases see Wayne et al. (2015).

4.1.2 • MICROBIAL BIOCONTROL AGENTS

The use of chemical pesticides and inorganic substances in viticulture (mainly sulphur), to protect crops against pathogenic microorganisms contributes to soil, water and air pollution, affecting non-target organisms and thus contributing to the ongoing global-scale biodiversity loss. Despite only accounting for 0.005% of the world's arable land, approximately 35% of all pesticides are used for viticulture (OIV, 2019). However, populations of pathogens rapidly develop resistance to fungicides (Ma and Michailides, 2005). This makes the mitigation of fungicide impact in viticulture a priority; indeed, one of the main objectives of the "Farm to Fork Strategy" for a fair, healthy and eco-friendly food system in Europe, is to reduce the overall use of chemical pesticides by 50% in 2030. Thus, there is a growing interest of modern viticulture in searching for new alternative and more environmentally friendly methods of protection against pathogens.

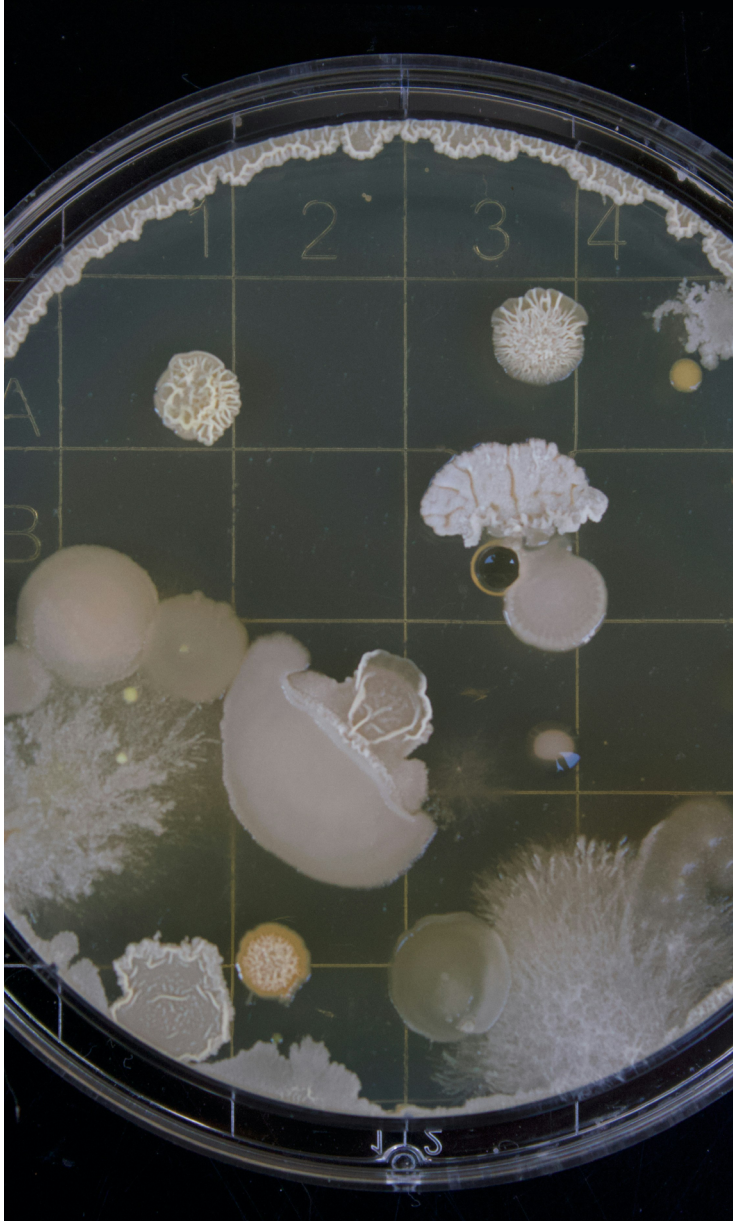
The term "biological control" includes practically all pest control measures except the application of chemicals. In particular, it usually refers to the use of selected microorganisms, or biocontrol agents (BCAs), with antagonistic activity against other pathogenic microorganisms. Using BCAs typically aims to reduce the use of pesticides and boosting food quality and safety. According to the definition of biocontrol in

the agri-food sector, this approach includes a set of emerging strategies that are alternatives to the use of chemicals for combatting fruit and vegetable diseases. Moreover, biocontrol also extends to food production and preservation (Smith, 1919; Baker and Cook, 1974; Di Canito et al., 2021). Ideally, BCAs should be: (i) genetically stable, (ii) effective at a low concentration, (iii) not fastidious in its nutritional requirements, (iv) capable of surviving under adverse environmental conditions, (v) effective against a wide range of pathogens and different harvested commodities, (vi) resistant to pesticides, (vii) non-producer of metabolites harmful to humans, (viii) non-pathogenic to the host, (ix) storable, and (x) compatible with other chemical and physical treatments. In addition, (xi) a microbial antagonist should have an adaptive advantage over specific pathogens (Wilson and Wisniewski, 1989; Sharma et al., 2009; Ab Rahman et al., 2018). The most studied biocontrol mechanisms of action of BCAs include competition for space and nutrients, iron competition, biofilm formation, and production of elicitors, volatile organic compounds, and killer toxins (Cordero-Bueso et al., 2017; Di Canito et al., 2021). The advent of high-throughput sequencing technologies is now driving a paradigm change, allowing researchers to integrate microbial community studies into the traditional biocontrol approach (Droby et al., 2009).

4.1.3 • OTHER BENEFICIAL MICROBES

Some mutualistic interactions among the grapevine-associate microorganisms, such as bacteria and fungi responsible for growth promotion, pathogen inhibition, and resistance induction against biotic stresses, can be beneficial to the host (Garbeva and Weisskopf, 2020). In particular, plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) play such a positive role (Bettenfeld et al. 2021).

Plant growth-promoting bacteria can provide nutrients to the plant (biofertilization) or release phytohormones that promote plant growth and help plants face biotic and abiotic stresses (biostimulation). Arbuscular mycorrhizal fungi, in turn, form associations with plant roots called mycorrhiza, a non-disease-producing interaction in which the fungal hyphae are interconnected with plant roots. Arbuscular mycorrhizal fungi increase the exploitation of the soil and supply additional water and nutrients to their host. Moreover, mycorrhiza-induced resistance promotes a better tolerance of mycorrhized plants to abiotic and biotic stresses, including biotrophic and necrotrophic pathogens and nematodes (Bettenfeld et al. 2021). In grapevine, PGPB, AMF, and endophytic microorganisms, can also affect the release of volatile organic compound (VOC) (volatile diterpenes, monoterpenes, sesquiterpenes), which can help grapevines to cope with biotic and abiotic



stresses. In addition, microbial VOCs synthesized by grapevine-associated microorganisms are also key mediators of plant-microbe communications, supporting the plant by direct growth inhibition of phytopathogens or by induction of plant resistance (Lazazzara et al., 2022). Finally, new strategies using associated rhizospheric organisms, predominantly AMF, to increase the phytoremediation efficiency of plants are also under study (Kullu et al., 2020).

4.2 • THE WINERY: FERMENTATIVE AND SPOILAGE MICROBIOTA

In addition to natural habitats such as the vineyard soil, vegetative organs, and grapes, a relevant and consistent microbial community has found its habitat in the winery. During the winemaking process, the grape juice and wine come into contact with the equipment surfaces present in the winery, which become important reservoirs of microorganisms that influence and contribute to the final composition of wines. Transfer of microorganisms between different surfaces can occur by direct contact, air flow and bioaerosols, with consequent transport of microbial cells, spores, biopolymers, plant debris, and decaying biomass (Theisinger et al 2017). Although the main source for the winery microbiota is grape material, microorganisms found in the winery equipment can also originate from humans or other environmental sources (Doyle et al. 2017). Therefore, the equipment of the winery, such as crush/press equipment, valves, collectors, and barrels, which are difficult to clean, represent favourable sites for microbial adsorption and multiplication (Pretorius et al. 1999; Bokulich et al. 2013; Varela et al. 2021). Moreover, the winery microbiome changes across both time and space, reflecting both the seasonality of the process and the functional specialization of different equipment and surfaces within the winery (Bokulich et al. 2013). The microorganisms residing in the winery do not only determine the microbiota during fermentation, but also alter the appearance, aroma, and flavour of the wine. In addition, the resident microbiota of the winery can also contribute to shape the microbial populations present in the surrounding vineyards, mainly the yeasts, accentuating the specific contribution of certain strains to the sensory characteristics of the wines of a given winery (de Celis et al., 2019). It should be noted, however, that the microbial consortia of processing surfaces most likely depend on facility design, age, surface material, sanitation regimens, and processing decisions. Thus, the microbial community cannot be generalized across all winemaking scenarios, as each winemaking facility may present certain unique conditions.

The Table 1 includes the names of a number of microbial genera that have been detected as particularly associated with different habitats within the winery or specific moments during the winemaking process. In the winery there may be numerous genera of filamentous fungi which, by inhabiting the vineyard, enter the winery environment in association with the grapes or the must (Bokulich et al. 2013; Grangeteau et al. 2016; Ocon et al. 2010). These fungi colonize all surfaces, from the floors and walls of the winery, to the barrels, fermentation tanks and the rest of the machinery that, at some point, has direct contact with the grapes, grape musts or wines.

The yeasts present in the winery can be classified in two categories: non-enological yeasts (yeast genera without an active role in the winemaking process) and wine-associated yeasts (yeast genera already described in the winemaking process). Regarding non-enological yeasts, different genera are detected at the arrival of the first harvest on all wine-related equipment. Several of these genera are usually found in natural ecosystems like plants, soil, water, and decaying wood material (Landell et al. 2014; Summerbell 1983), and can be thus qualified as ubiquitous genera. Although the total diversity of yeasts in the winery is remarkably high, certain environments are usually dominated by a few genera, for instance, Abdo et al. (2020) found that only four genera (Aureobasidium, Candida, Cystobasidium, and Wickerhamomyces) can represent more than 65% of the total yeasts, found in winery floor. The used equipment is dominated by the genera Meyerozyma and Aureobasidium; and genus Naganishia is dominant on all the winery surfaces and is found ubiquitously before, during and after winemaking (Abdo et al. 2020; Bokulich et al. 2013; Varela et al. 2021), being also the most prevalent yeast genera in vineyard soil fungal communities (Gobbi et al., 2022). Strain patterns within Saccharomyces change dramatically as soon as commercial or winery-made starter cultures are added to grape must to avoid lagging fermentations and wine defects.

Before harvest, yeast genera of enological interest, such as Hanseniaspora, Candida, Pichia, Cryptococcus, Debaryomyces, Starmerella, and Saccharomyces are found on winery walls, and particularly in areas that are difficult to clean (Varela et al. 2021). This is due to transfer via grape berries and/or must from previous years (Cordero-Bueso et al. 2013). When harvest begins, the winery becomes inundated with grapes and fermenting grape juice and the absolute abundance of yeast and bacteria cells increases significantly on all grape processing equipment (grape elevator, crusher, press) and fermentation tank surfaces compared to pre-harvest levels (Bokulich et al. 2013). It is therefore not surprising that the yeast Saccharomyces spreads more in the environment, especially around fermentation tanks.

Regarding wine-associated yeasts, the yeast communities of winery surfaces are largely dominated by Saccharomyces cerevisiae and other fermentative yeasts, mainly Hanseniaspora uvarum (Ocon et al. 2010), which plays an important role in the early stages of wine fermentation (Fleet 2003). While this later species is typically present on grapes (Barata et al. 2012), winery surface establishment may ensure that the same strains are introduced to successive batches and vintages of wine. Regarding Saccharomyces, its wide colonization of winery surfaces is an important source of this yeast in wine fermentations, particularly in non-inoculated grape juice (Bokulich et al. 2013). The constant presence of S. cerevisiae in the winery yeast biota identifies this species as dominant on winery surfaces at pre-harvest time (Ocón et al. 2010), accounting for 30-40% of the total yeast population. Investigations found that specific Saccharomyces strains become established on winery surfaces, which resulted in repeatable detection over multiple years in uninoculated wines (Santamaría et al. 2008; Blanco et al. 2011; Ciani et al. 2004; Mercado et al. 2007). These findings support the role of the winery as a man-made niche of S. cerevisiae and a possible source of reproducibility, as well as regionality, of wine sensory characteristics produced at a given

winery. Recently, an extensive catalogue of the yeast diversity detectable in wine fermentations was published, estimating global prevalence and relative abundance figures for 242 fungal and yeast genera in wine samples (de Celis et al. 2022). In addition to species of oenological interest, also spoilage yeasts (i.e. Brettanomyces), can persist in the winery over consecutive vintages (Doyle et al. 2017). Consequently, these yeasts can inoculate the fresh grape must in the winery, dominating the grape yeast biota and thus actively participating in the wine fermentation process (Gerhards et al 2016; Santamaria et al. 2005).

Regarding bacterial communities, their abundance and diversity are usually higher before vintage (Varela et al. 2021), with winery surfaces dominated by aerobic, non-fermentative bacteria, primarily Pseudomonas, Comamonadaceae, Flavobacterium, Enterobacteraceae, Brevundimonas, and Bacillus. At harvest time the fermenter and fermentation-related surfaces have significant populations of Sphingomonas, Methylobacterium, and Nakamurellaceae (Bokulich et al. 2013). The bacterial genera Leuconostoc and Oenococcus, which are associated with winemaking, show very low abundance. In particular, Oenococcus is associated with the different fermentation areas depending on sampling time, whereas Leuconostoc is found only after vintage (Varela et al. 2021).

The bacterial communities inhabiting barrel surfaces mirror those found elsewhere in the winery, characterized by prevalent species such as Pseudomonas, Comamonadaceae, Brevundimonas, and Flavobacterium. Notably, Pseudomonas populations in here exhibit a marked elevation in comparison to most other surfaces within the winery environment. During grape harvest, spoilage bacteria (i.e. Lactobacillus, Acetobacter, and Gluconobacter), are frequently encountered. Each of these microorganisms tends to be associated with distinct areas. Prior to vintage, Lactobacillus predominates as the most prevalent spoilage bacterium. However, following the harvest season, the relative abundance of these bacteria notably diminishes (Varela et al., 2021). Acetobacter and Lactobacillus are commonly observed on healthy, intact grape berries but are found in substantially higher concentrations on damaged ones and are often detected on crushing equipment (Barata et al., 2012). Anyway, the ecology of microbial population at the winery can be modified strongly depending on the treatments, maintenance, clean up, aeration and inoculation programs for the fermentation.

TABLE 1. MICROBIAL GENERA FOUND AS NOTABLY PRESENT IN WINERY ENVIRONMENTS AND SURFACES

	Filamentous fungi	References	Yeasts	References	Bacteria	References
FLOOR AND WALLS	*Aspergillus*, *Penicillium*, *Exophiala*, *Pyrenochaeta*, *Didymella*	Abdo et al. 2020; Bokulich et al. 2013; Varela et al. 2021	*Aureobasidium* *Bullera* *Candida* *Cystobasidium* *Exophiala* *Hannaella* *Leucosporidium* *Naganishia* *Saccharomyces* *Wickerhamomyces*	Abdo et al. 2020; Bokulich et al. 2013; Varela et al. 2021; Ocón et al. 2010; Doyle et al., 2017	*Bacillus* *Brevundimonas* *Comamonadaceae* *Enterobacteraceae* *Exigiobacterium* *Flavobacterium* *Micrococcaceae* *Pseudomonas*	Varela et al. 2021 Bokulich et al. 2013; Barata et al. 2012
BARRELS, TANKS AND OTHER EQUIPMENT	*Aspergillus conicus*, *A. restrictus*		*Aureobasidium* *Brettanomyces* *Hanseniaspora* *Meyerozyma* *Saccharomyces* *Zygosaccharomyces*		*Acetobacter* *Lactobacillus* *Methylobacterium* *Nakamurellaceae* *Sphingomonas*	
CLIMATE- OR SEASON-DEPENDENT	*Cladosporium and Penicillium (increased with rainfall and temperature)*	Barata et al. 2012	**Pre-harvest:** *Candida* *Cryptococcus* *Debaryomyces* *Hanseniaspora* *Pichia* *Starmerella* *Saccharomyces*	Barata et al. 2012; Varela et al. 2021; Santamaría et al. 2008; Blanco et al. 2011; Ciani et al. 2004; Mercado et al. 2007		
			Harvest: *Cryptococcus* *Hanseniaspora* *Metschnikowia* *Rhodotorula* *Starmerella* *Wickerhamomyces*			
			Post-harvest: *Cryptococcus* *Rhodotorula* *Torulaspora*			

5 • ANALYTICAL METHODS FOR THE CULTURE-INDEPENDENT STUDY OF VINEYARD'S MICROBIOTA

Classic microbiology studies focused on the role of specific bacterial or fungal species in ecosystem functioning (i.e., plant health or fermentation performance). This approach is still used and useful when trying to isolate and deliberately use microbial strains with interesting properties as biocontrol agents, plant growth promoters, or wine ferments. However, when trying to understand the ecology of the complex microbial communities, such as the vineyard microbiome, it is essential to study them through the conceptual and methodological framework of community ecology. Furthermore, it is crucial to consider a comprehensive set of mandatory, strongly recommended, and optional variables that ought to be measured or annotated as metadata linked to the vineyard samples collected for subsequent microbiome analysis. This approach facilitates the comprehension of the environmental and agronomic factors influencing microbiome composition and functionality (refer to Annex I for further details).

Culture-independent approaches such as those based on DNA extraction and amplicon sequencing are useful to describe the diversity patterns of complex communities; but rather than using them to only obtain a list of microbial taxa and their relative abundance patterns, they should be used to describe alpha- (taxa richness and evenness), beta- (spatial and/or temporal compositional patterns across samples) and gamma-diversity (overall taxa diversity within a region) patterns. These metrics are essential descriptors of diversity patterns of complex microbial communities. These metrics can also be calculated at different taxonomical resolution levels, from species to phyla.

With the development of high-throughput sequencing technologies (also named as: next-generation sequencing (NGS)), the possibility to evaluate the microbial diversity in various environments has become reality based on a metagenomics approach. Briefly, metagenomics is a culture-independent methodology that allows the classification of all microbes of a given environment through the direct genetic analysis of genomes in the sample. This tool includes a set of genetic and bioinformatic techniques in which the extraction of DNA, the construction of a library, the sequencing and the data analysis steps play a critical role for the success of the approach.

5.1 • LABORATORY METHODS: FROM SAMPLES COLLECTION TO DNA EXTRACTION AND SEQUENCING

The evaluation of microbial community structure across vineyard, wine and cellar habitats starts with the collection of representative environmental samples that allow the following step of DNA extraction. Regarding the sampling approach, different vineyard sampling strategies have been proposed, including the selection of specific sites in the vineyard or by following a sampling grid obtained by applying a randomized complete block design.

5.1.1 • SAMPLE COLLECTION

Regarding soil sampling, at least 3 vineyard sites are usually analysed and from a minimum of 3 to 10 soil cores (replicates) per site are collected and pooled in a composite sample. Depending on the sampling design, soil cores can be collected from 50 cm from the plant trunk up to the centre of the vineyard alleyways.

Samplings can be executed collecting up to 45 cm of soil in depth, 5-7 cm diameter core. After removing the first 5 cm of the soil layer of the sample, the soil can be sieved (< 2 mm particles), dried (air-dried or lyophilised), cleaned from any non-soil particles and frozen until further analysis (Burns et al., 2016; Morrison-Whittle et al. 2017; Longa et al. 2017; Canfora et al. 2018; Chou et al., 2018; Coller et al. 2019; Novara et al., 2020). Part of the vineyard environment is also the rhizosphere, bringing the soil microorganisms associated to the plant root (root microbiome). It can be recovered as a 3 cm root section (>2 mm root diameter) and analysed (Azevedo-Silva et al. 2021).

Different plant tissues have been studied at the level of microbial community so far. Leaves (1.5-2 cm2 section of a single leaf) can be picked from mixed position along the shoot (in either the top, middle or bottom part) and bark samples from at least 30 cm above the soil. The analysis of grape-associated microorganisms can be carried out from: (i) washing solution of the berries, collected from the middle and the bottom of the grape cluster, which allows to recover the epiphytic population of the grapes; ii) or the grape must, obtained by manually collecting and pressing undamaged grapes in sterile containers, including endophytic microorganisms in the analysis as well (Morrison-Whittle et al. 2017; Vituo et al., 2019; Gobbi et al., 2020; Azevedo-Silva et al. 2021; Guzzon et al., 2023). The sampling of the winery environment (i.e. equipment, walls, floor) is also reported in the literature. In this case, cotton-tipped swabs moistened with physiological water solution or useful media are used to assay all the cellar surfaces by streaking over a 10 cm2 area a specific area (Bokulich et al. 2013; Abdo et al., 2020; Varela et al., 2021).

5.1.2 • DNA EXTRACTION AND LIBRARY CONSTRUCTION

To obtain high purity and suitable DNA for further metagenomic approach, a useful sample size must be considered; indeed, the amount of sample to be treated depends on microbial concentration. Several studies report that the metagenomic DNA can be efficiently obtained from 0.25 to 10 g dried soil, about 4 g bark and at least 5 leaves. Concerning grape samples, approximatively 50 berries (Vitulo et al., 2019) per sample and 50 mL of must, obtained from 5 to 10 kg of grape (Tronchoni et al., 2022) (of,grape to study the epiphytic or the total microbial populations, respectively.

For the direct extraction of DNA from samples, commercial kits are nowadays available. However, they rely on protocols which often differ in the strategy for disrupting microbial cells; enzymatic treatment, thermal disruption and/or mechanical lysis are likely to influence and select certain microbial populations. In general, the DNA extracted should be representative of all cells present in the sample and enough high-quality nucleic acids must be obtained for subsequent library production and sequencing. Indeed, library production for most sequencing technologies require high nanograms or micrograms amounts of DNA, and hence amplification of starting material might be required (Thomas et al., 2012). In case the further sequencing step exploits an amplicon library obtained from the amplification of the V1 and V2, V3 and V4, V4, and V4 and V5 hypervariable regions of the 16S rRNA, 18S rRNA–5.8S rRNA internal spacer (ITS) or D1/D2 domain of 26S rRNA and 18S rRNA the term “metataxonomics” (also named as metabarcoding or amplicon sequencing) is used. These are taxonomically relevant regions within the ribosomal DNA of prokaryotes (bacteria and archaea), fungi, and other eukaryotes (protists), respectively.

5.1.3 • DNA SEQUENCING TECHNOLOGIES

The introduction of NGS technologies together with the development of a variety of novel sequencing platforms such as short-read sequencers (Roche 454 sequencing, Illumina sequencing, Ion Torrent Personal Genome Machine) and long-read sequencers (Oxford Nanopore Technologies MinION and Pacific Biosciences Sequel II) have increased the metagenomic research. Short-read sequencers have been the mainstay of NGS, but they have problems such as poor reads of repetitive sequences and GC-rich sequences, and the presence of gaps. Long-read sequencers are expected to solve these problems and are expected to be useful in the near future. The main current high-throughput sequencing platforms are listed below (Thomas et al., 2012; Meslier et al., 2022, Nakamura and Komatsu, 2023):

- 454/Roche system applies emulsion polymerase chain reaction to clonally amplify random DNA fragments, which are attached to microscopic beads that are deposited into the wells of a picotitre plate and then individually and in parallel pyrosequenced. This technology produces an average read length between 600-800 bp.

- Illumina/Solexa technology immobilizes random DNA fragments on a surface and then performs solid surface PCR amplification, resulting in clusters of identical DNA fragments. These are then sequenced with reversible terminators in a sequencing-by-synthesis process. HiSeq3000 instrument produces read length approaching 150 bp.

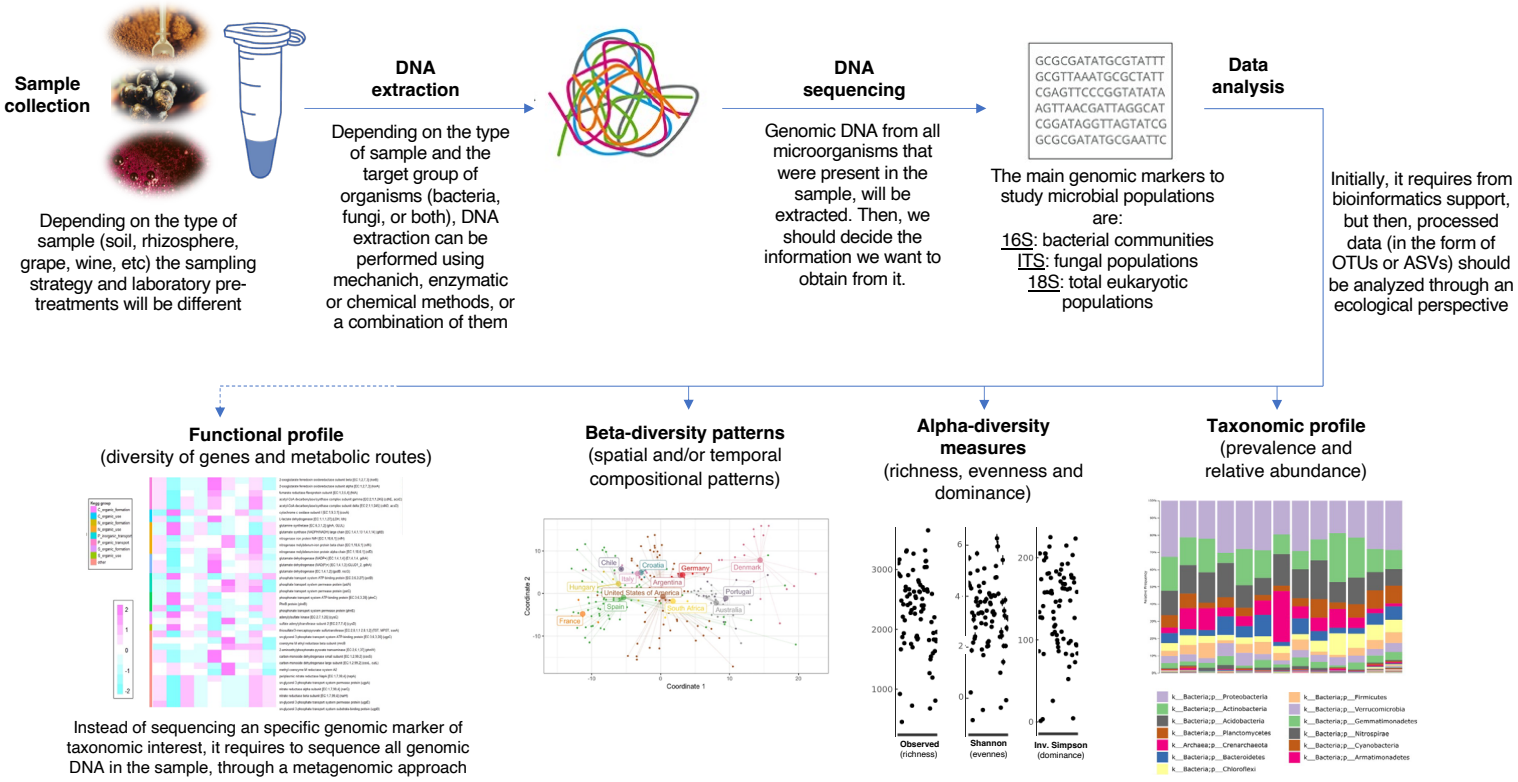
- Ion Torrent (Ion Proton P1 and Ion S5) is another emerging technology and is based on the principle that protons released during DNA polymerization can detect nucleotide incorporation. This system produces read lengths of 350-370 bp and throughput on the order of magnitude of the 454/ Roche sequencing systems.

- ONT MinION and Pacific Biosciences (PacBio) have released a sequencing technology based on single molecule, real-time detection in zero-mode waveguide wells. Theoretically, these technology platforms should provide much greater read lengths (> 60 and 40 Kbp, respectively) than the other technologies mentioned, which would facilitate annotation and assembly.

5.2 • DATA ANALYSIS FOR AN ECOLOGICAL PERSPECTIVE OF VINEYARD MICROBIOTA

After sequencing the diversity of amplicons (amplified sequences) present in the total DNA extracted from a natural sample (vineyard soil, vine tissue or wine samples), DNA sequences should be checked and bioinformatically treated to ensure the quality and reliability of any further analysis. Then, DNA sequences can be used for ecological analysis both directly (in the form of Amplicon Sequence Variants-ASVs or Operational Taxonomic Units-OTUs) or after taxonomic assignment. The taxonomic assignment of DNA sequences from amplicon sequences is based on the phylogenetic distance between taxa, and its accuracy and reliability depend on several factors, including the resolution power of the genetic marker (it means, how different it is between phylogenetically close taxa), and the quality of the reference database used to assign a specific DNA sequence to a specific species. At this moment, the main public databases used for the taxonomic assignment of DNA reads are: SILVA (specially for 16S and 18S amplicon strategies; https://www.arb-silva.de) and UNITE (specially for ITS amplicon strategies; https://unite.ut.ee). The length of DNA sequences obtained in most amplicon sequencing protocols limits the precision with which they can be assigned at high levels of taxonomic resolution; that is, its ability to differentiate between species and, even more so, between strains. Thus, alpha- and beta-diversity patterns are recommended to be explored attending to the sequences (ASVs or OTUs) detected, since trying to do it using the diversity of detected taxa, at any resolution level, will imply a notable loss of information, for example, derived from the loss of all those sequences that cannot be correctly assigned to the selected taxonomic level. Therefore, the use of data obtained by DNA sequencing is more appropriate to perform comparative studies (both spatial and temporal studies studying; for example, the effect of biogeography and climate at different scales or the effect of a certain viticulture intervention in changing the microbiota of a certain vineyard, respectively) rather than descriptive studies (microbial taxonomy profiling) of one or a few samples aiming to detect specific species.

Workflow to obtain microbiome-based ecologically-relevant information from vitivinicultural samples



6 • CONCLUSION

In this document, we have outlined the main ways by which microbial biodiversity impacts the functioning of the vineyard, both positively and negatively. We have also described the presence of microbes in the winery, especially on its surfaces and machineries as important habitats acting as reservoirs for microbes with potential impact in wine fermentation processes. Increasing knowledge in the role of microbial communities in vitiviniculture will help the sector to develop strategies for a better preservation of the functional biodiversity in the vineyard, and to harness the great potential of microbes to mitigate the effects of climate change in wine production.

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8 • ANNEX I

List of biogeographic, edaphic, climatic and viticulture-related data to be considered when studying the composition and functioning of the vineyard microbiome.

Type of variable	Importance	Reason	Equipment needed	Additional information
BASIC INFORMATION				
Date of sampling	mandatory	Microbial communities are highly dynamic		
Person carrying out the sampling	mandatory			
GEOGRAPHY				
Latitude	mandatory	Microbes are spatially structured	Portable GPS	Important to provide reference system
Longitude	mandatory	Microbes are spatially structured	Portable GPS	Important to provide reference system
CLIMATE				
Mean annual rainfall	strongly recommended	Climate is a main regulator of microbial growth and group dominance	Computer	This can be extracted from climatic models like worldclim - use most recent 30 years average
Mean annual Temperature	strongly recommended	Climate is a main regulator of microbial growth and group dominance	Computer	This can be extracted from climatic models like worldclim - use most recent 30 years average
Weather during sampling	strongly recommended	This can affect the samples	Notebook, computer	Personal annotations, local weather stations
Air temperature	strongly recommended	Microbial communities are highly dynamic	Computer	This may be extracted from local weather stations - daily air temperature 30 days prior to sampling
Rainfall	strongly recommended	Microbial communities are highly dynamic	Computer	This may be extracted from local weather stations - daily rainfall 30 days prior to sampling*

Air humidity	optional	Microbial communities are highly dynamic	Computer	This may be extracted from local weather stations - daily air humidity 30 days prior to sampling
Other bioclimatic variables	optional	Climate is a main regulator of microbial growth and group dominance	Computer	This can be extracted from climatic models like worldclim
*It is highly recommended not to collect samples for microbiome analysis until 7 days after the last rain				
SOIL				
Sampling depth	mandatory	Soil properties vary exponentially with depth	Tape measure and stick	Recommended: 0-20 cm in interrows; 0-20 cm next to trunk (0-40cm may be required in case of not finding fine roots in the first 20cm)
Distance from trunk	mandatory	Plans are major drivers of microbial community composition	Tape measure	
Texture	strongly recommended	Main driver of soil microbial communities	Sieves and calgon	Two information needed: i) % gravel (> 2mm) versus % fine earth; and ii) on fine earth, % sand, silt and clay
pH	strongly recommended	Main driver of soil microbial communities	pH-meter	
CEC	strongly recommended	Main driver of soil microbial communities	Conductivimeter	
Salinity	strongly recommended	Main driver of soil microbial communities	Conductivimeter	
Soil organic matter	strongly recommended	Main driver of soil microbial communities	Furnace/elemental analyser/NIR	Another precise option is to analyse organic carbon (OC) by the dichromate oxidation method; then Organic Matter = OC*1.72

Soil C and N	strongly recommended	Main driver of soil microbial communities	Elemental analyser/ digestors/NIR	
Redox potential	strongly recommended	Main driver of soil microbial communities	ORP-meter	
Total CaCO3	strongly recommended	Presence of lime have a big impact on soil microbial communities	classical soil analysis	If not possible to carry out classical soil analysis, the presence of CaCO3 can be assessed with HCl in the field
Elemental content	optional	Microbes can be adapted to mine specific nutrients	X-ray equipment, ICP-OES	Macro and micronutrients, plus potentially phytotoxic elements. K, Mg, Ca and Cu are especially advised
SOIL FUNCTIONING				
Soil enzymes	optional	Indicator of metabolic potential of a soil. Soil health indicator	Microplate reader	Hydrolytic/oxidative enzymes can be assayed through colorimetric or fluorometric methods
Basal respiration	optional	Indicator of metabolic potential of a soil. Soil health indicator	Microplate reader	
Microbial biomass	optional	Microbial biomass is a main driver of soil functioning	Microplate reader	Fumigation-extraction method, SIR, etc.

PLANT	STRONGLY RECOMMENDED			
Phenological state at sampling	mandatory	Microbial communities are highly dynamic	Direct observation	
Position and maturity stage of plant parts collected	mandatory	There is within plant variability	Direct observation	
Variety (scion)	strongly recommended	Vine physiology can control the associated microbiome	Interview to farmer	
Rootstock	strongly recommended	Rootstock physiology and morphology can control the associated microbiome	Interview to farmer	
Age	strongly recommended	Communities develop over time	Direct observation - Interview to farmer	
Type of pruning/ training system	strongly recommended	This can have direct effect on the local microenvironment	Direct observation	
MANAGEMENT				
Type of farming	mandatory	This has major effects on all aspects of the operation	Interview to farmer	Organic, conventional, biodynamic, regenerative, integrated
Irrigation	mandatory	Water is usually a limiting factor	Interview to farmer	Y/N. If Y, specify regime ((total amount of irrigation in mm/ year, frequency of water supply, drip irrigation / flooding / sprinklers)
Mineral fertilization	strongly recommended	Nutrients can have major effects	Interview to farmer	Y/N. If Y, specify regime (kg N, P, K per year)
Organic fertilization	strongly recommended	Nutrients can have major effects	Interview to farmer	Y/N. If Y, specify regime (amount in tons/year; composition of the fertilizer, in particular C/N ratio)

Ploughing	strongly recommended	Breaking the soil alters soil networks	Interview to farmer	Y/N. If Y, specify regime
Chemical protection measures	strongly recommended	Disrupts the beneficial microbiota	Interview to farmer	Y/N. If Y, specify describe the products that are used and the frequency
Cover crops	strongly recommended	Plant-soil interactions	Interview to farmer	Y/N
Plantation frame	strongly recommended	Plant-soil interactions and microclimatic effects	Tape measure	Measure interrow distance and intervine distance
% of soil covered by cover crops	optional	Plant-soil interactions	Interview to farmer	
Type of cover crops	optional	Plant-soil interactions	Interview to farmer	Permanent/ transient; spontaneous/ seeded; What are the dominant species/ functional groups?
LANDSCAPE				
Size of vineyard	optional		Computer/ Interview to farmer	This can be extracted from GIS
Type of surrounding matrix	optional		Computer	Homogeneous/ heterogeneous; natural/agricultural/ urban; this can be extracted from GIS
% of surrounding land covered by forest	optional		Computer	This can be extracted from GIS;
% of surrounding land covered by agriculture	optional		Computer	This can be extracted from GIS
% of surrounding land covered by urban areas	optional		Computer	This can be extracted from GIS
Presence of ecological infrastructures nearby	optional		Direct observation/ interview to farmer	Y/N. If Y, make detailed list

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