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Yeast diversity in the vineyard: how it is defined, measured and influenced by fungicides

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This review focuses on the considerable amount of research directed at defining microbial diversity in the vineyard and the subsequent contribution to uninoculated fermentations, with an emphasis on the effect of fungicide applications. From this research it is clear that there are many factors affecting diversity in the vineyard including: sprays, climate, location and grape derived parameters. With their increasing affordability, Next Generation Sequencing methods to measure diversity in environmental samples are now being adopted for studies of the grapevine microbiome. We bring together the results of these studies, discuss how diversity is measured and consider the potential applications of current knowledge. An in-depth analysis of how fungicides affect yeast diversity in the vineyard and the mode of action of different fungicide groups are also discussed. Finally, we report on alternative treatments to maintain vineyard health and reduce fungicide applications in the future.
Introduction

Grapes and wine are not produced in a sterile environment and by extension of this we can infer that winemaking does not involve a microbial monoculture. Not only does the vineyard contain a range of yeast, bacteria, and fungi (Bokulich et al. 2014, Morrison-Whittle and Goddard 2018), but so do the wineries in which the grapes are fermented (Bokulich et al. 2013, Varela et al. 2017). During winemaking, grape must is converted to wine via an alcoholic fermentation (AF) carried out by one or more strains of yeast (Fleet 1990). If inoculated with yeast, typically Saccharomyces cerevisiae is used, or if un-inoculated a range of non-Saccharomyces yeasts usually begin the fermentation prior to the eventual dominance of S. cerevisiae (Beltran et al. 2002, Albergaria and Arneborg 2016). After the alcoholic or primary fermentation, a secondary fermentation by lactic acid bacteria (LAB), known as malolactic fermentation (MLF), is often undertaken, depending on the style of wine that the winemaker seeks to achieve (Sumby et al. 2019).

In the vineyard, the soil and rhizosphere microbiome make an important contribution to grapevine health and performance boosting yields (Belda et al. 2017a). Mechanisms include phosphorus solubilisation and, when vines are associated to a Leguminosae cover crop, nitrogen fixation (Misra et al. 2017, Schütz et al. 2018). Arbuscular mycorrhiza fungi can enhance vine resistance to drought by creating an extended root system for them (Dodd and Ruiz-Lozano 2012). Moreover, when beneficial soil microbiota dominate, soil-borne pathogens are less likely to thrive, enhancing overall plant health (Vukicevich et al. 2016). Leaves and grapes share common species with bark and soil indicating a possible role of soil in defining the whole plant microbiome (Martins et al. 2013). Additionally, the soil is a microbial pool that may also be the source for endophytes colonising internal tissues (Zarraonaïndia et al. 2015). These can affect plant growth, and resistance to herbivores, pathogens, or environmental factors (Bakker et al. 2012). Last, phyllospheric microorganisms, those living on the aboveground tissues, including yeasts contribute to vineyard health because of their biocontrol abilities (Sipiczki 2006, Raspor et al. 2010, Nally et al. 2012, Carmichael et al. 2019).

The vineyard microbiome is not only important for vine health but will also contribute to fermentation and the final product (Liu et al. 2016, Padilla et al. 2016a, Belda et al. 2017a Morrison-Whittle and Goddard 2018). Therefore, the contribution of the microbiome is important for winemakers because consumers influence wine style preferences. For example, market trends show that consumers are more willing to pay for more complex wines, where aromas and flavours play a key role (Malherbe et al. 2013, Pagliarini et al. 2013, Danner et al. 2016), especially if they are produced under environmentally friendly and sustainable systems (Vecchio 2013). So-called ‘natural wines’, those produced without preservatives such as sulfur dioxide (SO₂), are also highly appreciated by some consumers. Young, Italian consumers demonstrated an increased willingness to pay for natural wine because of an interest in natural
products and label information (winemaking techniques, sensory attributes) (Galati et al. 2019). In addition, Italian and Spanish consumers that linked sulfites with headaches were willing to pay premium prices for wines labelled with ‘No-added sulfites’ (Amato et al. 2017), a trend borne out through interviews with American consumers (Costanigro et al. 2014). Thus in the context of a consumer move towards more complex wines and natural ‘wild ferments’, the non-
Saccharomyces yeasts contained within a diverse microbiome will be desirable for increasing aromatic complexity (Capozzi et al. 2015, Whitener et al. 2015, 2017, Padilla et al. 2016b, Varela et al. 2016, Lin et al. 2020) and potentially lower alcohol concentration wines (Contreras et al. 2014).

Both biotic and abiotic factors will affect yeast diversity in the vineyard and by default the overall microbiome. In this review, we examine the current literature with regard to the diversity of yeast on wine-grapes, discuss what diversity is and how it is measured, and take a deeper look at one factor that may influence diversity, fungicides. The mode of action of different fungicides and an overview of at times conflicting results is discussed. Finally, fungicide alternatives such as biocontrol agents are explored.

What is diversity and is it important?
Diversity in the context of grape and wine microbiology is seen as many different fungal and bacterial species existing together either in the soil or the vine phyllosphere, that is grape berries, leaves and bark (Gilbert et al. 2014, Perazzolli et al. 2014, Mezzasalma et al. 2017, Wei et al. 2018). Therefore, when discussing fungal diversity, this review is referring to species richness, that is the number of species present in a particular environment/microbiome. The microbiome of humans and plants has garnered increasing attention in recent years and it is generally accepted that the microbiome of a given system, be it human gut (Cénit et al. 2014, Yang et al. 2016) or plant (Compant et al. 2019), plays an important role in the overall health of the organism with which it is associated. It is also possible that microbial diversity is not only affected by its environment but responds to it and, in the case of wine, ultimately sculpts final wine aroma characteristics.

Much research attempts to define the unique terroir of a region, and whether microbial diversity of the vineyard is contributing to this. Whilst diversity is important, however, not all species are desirable. Certainly, some can be important for grapevine and berry health, potentially being used as indicators of good plant health. Aureobasidium pullulans, the predominant species on sound grape surfaces from conventional, organic, and biodynamic vineyards (Setati et al. 2012) has biocontrol effects against Botrytis cinerea (Bozoudi and Tsaltas 2018). Moreover, A. pullulans can stop growth of Diplodia seriata, a grapevine pathogenic fungus that causes significant economic losses every year (Pinto et al. 2018). Other
species cause undesirable effects if present during/after fermentation. For example, Dekkera bruxellensis and Zygosaccharomyces bailii are strictly considered spoilage yeasts (Loureiro and Malfeito-Ferreira 2003), with S. cerevisiae also causing spoilage by refermenting sweet wines. D. bruxellensis is common in rot-infected berries and may be transported to the winery (Loureiro and Malfeito-Ferreira 2003). In addition, insects such as Drosophila, can be a vector (Christiaens et al. 2014, Steensels et al. 2015). Being resistant to ethanol and carbon dioxide, D. bruxellensis can develop after fermentation has finished during wine maturation in oak barrels (Howell 2016). This yeast generates phenolic off flavours (POF), which cannot be readily removed from wine, and thereby produce high economic losses worldwide. A maximum threshold of 620 µg/L has been cited at which POF becomes unpleasant and spoils wine (Chatonnet et al. 1992, 1993, Loureiro and Malfeito-Ferreira 2003). Like D. bruxellensis, Pichia guilliermondii converts p-coumaric acid into 4-ethyl phenol but cannot grow in wine (Loureiro and Malfeito-Ferreira 2003).

Zygosaccharomyces bailii, a well-recognised spoiler of wine, is often isolated from wine fermentations (Kuanyshew et al. 2017). It has a high osmotolerance (Martorell et al. 2007) and thrives under high ethanol concentration, low pH, and elevated sulfur dioxide (Thomas and Davenport 1985). This species can colonise finished sweet wines and through refermentation produce CO₂ that risks the bottle exploding (Leyva et al. 1999, Zuehlke et al. 2013). Zygosaccharomyces bailii grows only in sour-rot infected berries in the vineyard (Loureiro and Malfeito-Ferreira 2003, Zuehlke et al. 2013), but has been isolated from winery equipment.

Diversity in the vineyard will carry into winemaking where autochthonous yeasts aid in the creation of unique wines (Liu et al. 2016, Padilla et al. 2016a, Belda et al. 2017a, Morrison-Whittle and Goddard 2018, Gupta et al. 2019, Liu et al. 2019a). Avoidance of sulfur dioxide or starter cultures allows the impact of these yeasts to be greater, even though they typically do not persist until the end of fermentation (Wang et al. 2016). Wines from such uninoculated fermentations are seen as a way of maintaining or expressing the ‘microbial terroir’ (microbial biogeography) of the region (Belda et al. 2017b, Liu et al. 2019b). Representing up to 99% of species richness and diversity, non-Saccharomyces yeast originating from grapes are therefore of particular interest (Carrau et al. 2015). Those often found in uninoculated fermentations include, Kluyveromyces marxianus, Pichia kluveri, Torulaspora delbrueckii, Lachancea thermotolerans, Kazachstania spp., Starmerella bacillaris, and Metschnikowia pulcherrima. Many contribute positively to wine quality by modifying aroma and/or mouthfeel and, in some cases, result in a reduction in ethanol concentration (Anfang et al. 2009, Gobbi et al. 2013, Jolly et al. 2014, Loira et al. 2015, Varela et al. 2017, Whitener et al. 2017, Benito 2018, Hranilovic et al. 2018, Rollero et al. 2018, Ruiz et al. 2018, Lin et al. 2020). In addition to species diversity, non-Saccharomyces present a high level of strain variability that can also be exploited in obtaining unique products (Capozzi et al. 2015).
Whilst the above studies report that the microflora present in a given fermentation is extremely important for the quality of the final product, one question that remains is how much diversity is needed in order to create unique wines. It is also suggested that the ratio of *Saccharomyces* to non-*Saccharomyces* may be the main driver of sensory quality of the finished wine (Capozzi et al. 2015). For example, when Chardonnay was fermented using two different techniques, uninoculated vs co-inoculated with *Hanseniaspora vineae*, aromatic profiles were richer when compared to the fermentation by a monoculture of a conventional yeast (Carras et al. 2015). Esters and higher alcohols are the main compounds resulting from yeast secondary metabolite production during fermentation (Rapp and Versini 1995, Romano et al. 2003, Sumby et al. 2010). Chardonnay ferments using a *M. pulcherrima* starter followed by *S. cerevisiae* (sequential inoculation) produced wines with a higher total concentration of esters and higher alcohols (Contreras et al. 2014). *Kazachstania spp.* used in sequentially inoculated Viognier ferments yielded elevated phenylethyl and isoamyl acetate/alcohol concentration (Lin et al 2020). *Pichia fermentans* used as a pure culture or sequentially with *S. cerevisiae*, increased acetaldehyde and higher alcohols in Macabeo wines (Clemente-Jimenez et al. 2005). The maximum acetaldehyde concentration was 350 mg/L, which exceeds the 125 mg/L where negative bruised-apple characters are seen (The Australian Wine Research Institute 2020a). Other non-*Saccharomyces* species reported to show higher ester concentration in Bobal musts are *Hanseniaspora guilliermondii* and *Pichia anomala*, inoculated as a 10:1 mixed culture with *S. cerevisiae* (Rojas et al. 2003). Thus even though autochthonous yeasts may require an *S. cerevisiae* inoculum to complete fermentation, they offer a useful palette of interesting properties, something that is also being explored for biofuel and distillate production, cheese making, and in biocontrol (Varela and Borneman 2017).

Yet to be fully investigated is the creation of artificial diversity of that seen in juices to recreate uninoculated fermentation. Such creations offer the potential to eliminate undesirable yeast and influences, whilst tailoring the beneficial ones. In addition, while species interactions are important in the wine itself (Bartle et al. 2019), the nature and significance of such interactions before winemaking on the grapes has not been defined. Microbial diversity is considered to be the regional imprint of the place where grapes are grown (Knight et al. 2015, Liu et al. 2019b), whereas cultivar and farming practices can also have a driving effect (Martins et al. 2012, Bokulich et al. 2014, Gilbert et al. 2014, Wang et al. 2015). In this context, application of chemicals in the vineyard will shift populations of these naturally occurring yeast species, depending on their sensitivity to treatments, and thereby influence wine sensory attributes.

**Factors that influence fungal diversity on grapes**
One motivator for detailed studies of the grapevine-associated microbiome is that they may lead to the identification of autochthonous strains of oenological value, which enhance regional characteristics in wine (Lin et al. 2020). Certainly, it is hypothesised that the grapevine-associated microbiome, or even the main subset of it, could be used to identify a vineyard, thereby linking wine characteristics specifically to the ‘terroir’ or environment (e.g. Knight et al. 2015). There are several factors that are thought to influence this microbiome including: grape cultivar, insect activity, berry physiology, species-species interactions, geographical location, climate, soil, terrain and farming and harvesting procedures including herbicide and fungicide spray use and canopy management (Figure 1) (Barata et al. 2012, Capozzi et al. 2015, Kioroglou et al. 2019). To date, however, several reports on grapevine microflora do not detail any spray regime utilised in the tested vineyard.

As discussed above many individual factors affect fungal diversity in the vineyard. The study of vineyard diversity is compounded by the likely interaction of the above factors with each other (Figure 1). The grapes themselves naturally provide a variable environment with differences in: skin thickness (i.e. microbial access to nutrients), bunch architecture, berry phenolic substances and flavonoids. Grape temperature also affects the types of species present on grapes, with *Hanseniaspora* spp. more commonly found in warm climates and *Kloeckera* spp. in cooler climates (Villa and Longo 1996). Add to these geographical features, such as location, altitude, sunlight hours, and rainfall, and a complex matrix of effects is quickly developed. It has been shown that when rain falls around harvest *Metschnikowia* and *Hanseniaspora* communities tend to increase, potentially due to the high RH (Jara et al. 2016), whereas basidiomycete yeast numbers decline (Perazzolli et al. 2014). Such studies did not consider, however, the likelihood that wet conditions also demand an increased use of fungicides, which themselves may influence yeast diversity and numbers. Drumonde-Neves and co-workers (2016) suggested that abandoned vineyards, which do not receive fungicide applications, provide a means of teasing apart fungicide vs rain effects, wherein less-abundant yeast populations would result more specifically from heavy rainfall patterns.

During ripening and depending on vintage, climatic conditions, and any chemical treatments used, microbial diversity generally drops (Pinto et al. 2014). The predominance of specific yeasts is linked to the phenological phase of the vines. Generally, the poor fermenters *Cryptococcus* and *Rhodotorula* exist during the early stages of berry development, but as berries ripen, *Hanseniaspora*, *Candida*, and *Metschnikowia* ascomycetes appear on the berry surface (Rosini et al. 1982, Combina et al. 2005, Raspor et al. 2006). But farming practices including fungicide and herbicide sprays, and canopy management can have a large effect on fungal populations, and potentially mask other influences (Martins et al. 2012, Bokulich et al. 2014, Gilbert et al. 2014, Wang et al. 2015, Morrison-Whittle et al. 2017, Chou et al. 2018, Mandl et al. 2018, Carneiro et al. 2019, Vincent and Lasnier 2020). As discussed above, rainfall
and its timing may alter fungal populations, but rainfall will also impact berry physiology, itself an important determinant of fungal populations, and potentially also wash sprays from grapevines. Thus, the study of the grapevine fungal microbiome has many unanswered questions and further careful investigation that looks at vineyard condition more holistically is warranted.

Methods to measure diversity

Microbial diversity can be explored on different tissues (barks, leaves, grapes), and soils, at a specific developmental stage or as a continuum through time to have a better understanding of fungal community behaviour (Martins et al. 2013, Bokulich et al. 2014, Gilbert et al. 2014, Belda et al. 2017b, Morrison-Whittle et al. 2017, Liu et al. 2019a). Choosing the right approach for data acquisition is key to research success, as is defining the most suitable statistical analysis for it. The first step towards untangling the possible interactive effects and complexity of the grapevine microbiome is the establishment of consistent methods to not only measure diversity, but statistically analyse these data. Species diversity itself has two separate components: (i) the number of species present (species richness); and (ii) their relative abundance (termed dominance or evenness). Therefore, whilst vineyard microbial diversity is of great interest to the wine industry, quantifying species diversity of different vineyards is complicated as it requires careful planning and a multidisciplinary approach. Many authors used both culture dependent and independent methods (Milanović et al. 2013, Martins et al. 2104, Cordero-Bueso et al. 2014, Grangeteau et al. 2017b, Escribano-Viana et al. 2018, Agarbari et al. 2019a, Agarbari et al. 2019b, Cachón et al. 2019, Anguita-Maes et al. 2020) with a range of statistical techniques being applied (Figure 2, Table 1). Genome analyses start with DNA extraction from the samples followed by quantitation and purification ahead of downstream analysis, perhaps by quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE) or next generation sequencing (NGS). Culture-independent studies are especially valuable when dealing with non-culturable or difficult-to-cultivate microorganisms (Zapka et al. 2017). Moreover, the utilisation of multi-omics techniques makes it possible to quantify these communities, at both a taxonomic and functional level (Zhang et al. 2010, Franzosa et al. 2015, Bokulich et al. 2016, Malla et al. 2018).

Several issues need to be addressed in such work, including; statistical sampling methods, the arbitrary nature of delineating an ecological community, and the difficulty of positively identifying all of the species present. Next generation sequencing technology has an advantage over culture-dependent techniques in that slower growing or less abundant species have a greater chance of being detected. Identification of the species present is, however, only the first step. From that information it is necessary to investigate the proportionality and role of
each species. As a result, many different measures (or indices) of biodiversity have been
developed. Microbiome diversity is traditionally assessed by means of a large list of metrics
that account for the richness (S), reported as the number of operational taxonomic units (OTUs)
or amplicon sequence variants (ASVs), and evenness (how homogeneously distributed these
species are) at different scales (α, β, γ diversities) (Whittaker 1972).

Alpha diversity (α) measures richness and evenness at the ecosystem scale (i.e. within
a sample). Traditional metrics include, Shannon diversity index (H) (Shannon 1948), Simpson
diversity Index (D) (Simpson 1949), Simpson’s evenness (E, (DeBenedectis 1973, Morris et
al. 2014) and Berger-Parker dominance (BP, Berger and Parker 1970). H includes both richness
and evenness, so it measures how uniformly microbial taxa are distributed amongst samples. It
is based on a logarithmic calculation and ranges between 1.5 and 3.5, increasing when richness
and evenness increase (Shannon 1948). D focuses on species evenness and is calculated from
the sum of squared proportions. It ranges between 0 and 1, the higher the value of D, the lower
the diversity (Simpson 1949). E can be calculated by taking Simpson's index (D) and expressing
it as a proportion of the maximum value that D could assume if individuals in the community
were completely evenly distributed, in which case the use of this index might not be useful as
there is a mathematical correlation between both (DeBenedectis 1973, Morris et al. 2014).
Finally, Berger-Parker incorporates proportional abundance of species to the diversity analysis,
estimating dominance of the most abundant species (Berger and Parker 1970, Morris et al.
2014). If phylogenetic distances are also considered, the phylogenetic diversity index must be
included in the analysis (Lozupone and Knight 2008). Additionally, it is necessary to take into
account that identification of fungi by sequencing based on internal transcribed spacer (ITS) ()
is more accurate compared to 18S rRNA sequencing approaches (Halwachs et al. 2017).
However, because of the high intraspecific variability of ITS, read alignment can be
problematic and, therefore, phylogenetic trees derived from it may not be definitive (Fouquier

Beta diversity (β) establishes how different two environments (samples) are. The main
metrics employed are: Bray–Curtis dissimilarity index, which takes the number of OTUs
measured in two samples, compares them and depending on how different they are, ranges
between 0 (exact same species at the same abundance) and 1 (completely different species and
their abundances) (Bray and Curtis 1957). The Jaccard distance is based on species presence or
absence, and ranges between 0 and 1 (Jaccard 1912). UniFrac adds information on phylogenetic
relationships between organisms (Lozupone and Knight 2005, Lozupone et al. 2011). This
measurement can include only the distance between OTUs (unweighted), or weight branches
by abundance information (weighted). Gamma diversity can be considered as an overall
measurement of how different a set of samples is, and it considers both α and β diversities.
All of the changes in the yeast species present on grapes must be taken with the preverbal ‘grain of salt’. Whilst it is rarely acknowledged, count data generated by NGS exist as compositions for which the abundance of each component (i.e. ITS gene sequences and OTUs) is only coherently interpretable relative to other components within that sample (Quinn 2018). This is because the number of counts recorded for each sample is constrained by an arbitrary total sum (i.e. library size). Therefore, without normalisation or transformation, many conventional analyses, including distance measures, correlation coefficients and multivariate statistical models cannot be used. Whilst several studies report differences observed with NGS in yeast diversity based on various chemical (e.g. sprayed and unsprayed grapes) and environmental factors (Chou et al. 2018, Agarbati et al. 2019a), many do not consider the actual abundance of species (i.e. CFU/mL). It may be that changes in the balance of particular genera/species are going unnoticed by being masked by changes in other genera/species. With this in mind, it is important to note that changes in the presence of a single species might also be explained by correlated changes in all of the other species.

Therefore, if alpha and beta diversity indices are used in isolation a seemingly static image of microbial assemblages might be observed, where no information on underlying relationships between these communities is provided. New data techniques are needed to analyse the complexity of the information and provide a more complete overview of diversity. In addition, there are cases where the complexity of the analysis leads to the need for alternative or complementary data interpretation (Morris et al. 2015, Morton et al. 2017). Using these metrics, a path model of ‘hypothesised relationships between organism/traits’ was reported by Morris and collaborators (2014). They investigated how the relationships between different traits, in this case aboveground arthropods, arbuscular mycorrhizal fungi, land use intensity and *Plantago lanceolata* chemical and molecular diversity, affected diversity indices (species richness (S), Shannon’s diversity (H’), Simpson’s dominance (D1), Simpson’s evenness (E), and Berger–Parker dominance (BP)). This study served as a community interactions model, from which it could be concluded which traits and relationships had positive or negatives effects, which were significant, and which were not. This highlights the need of considering the system globally, and where obtaining additional data can become vital for understanding microbial community evolution (Figure 2).

More recently it has been recommended that looking at the balance of species might be more useful than examining changes in the proportion of species (Morton et al. 2017). Taking the approach of focusing on balance in a system and the transition of dominance between these species might avoid the error of inferring absolute decreases or increases in their abundance (Morton et al. 2017). The concept of log-ratio balance, which turns out to be more dynamic, has been introduced as a novel approach for microbial diversity understanding (Morton et al. 2017, Kioroglou et al. 2019). The former group relied on balance to ‘infer
meaningful properties of subcommunities, rather than properties of individual species’, thereby helping to separate niches and underpin the types of relationships being held between these taxa. Kioroglou and co-workers (2019) found how specific genera changes affected the fungal community structure, which was not obvious when traditional OTUs/relative abundance type data analysis was undertaken. The authors use the species data to build a bifurcating tree (where the tree reflects a series of branching processes in which one lineage splits into two descendant lineages) relating microbial taxa to each other by using the criteria of interest. Balances can be calculated on the internal nodes of the tree from the geometric means of the corresponding subtrees (Morton et al. 2017). All NGS abundance data are compositional because sequencers sample only a portion of the total input material. The benefit of analysing the data this way is that due to their scale invariant nature balance trees correct for differences in sequencing depth without requiring rarefaction, therefore avoiding many of the limitations associated with this procedure. Additionally, balances are sub compositionally coherent, which means that changes in non-overlapping subcommunities do not impact each other. These examples break our paradigm of having to measure diversity in a particular and strict way. There is no one valid approach. Indeed, it might be that some cases require a mix of methods to get the most informative results.

Alternate indicators also take in account the phylogenetic association between microbial communities (Lozupone and Knight 2008). Simpler analysis, such as those only based on alpha and/or beta diversity, might use any of the available parameters without altering results too much, but in more complex situations, parameters must be chosen carefully. Moreover, special considerations such as a rarefaction step should be considered in some cases. Bias can arise from sampling size or, in sequencing data, from library sizes altering final results and their interpretation (Willis 2019). It is important to remember that the presence of a particular species does not describe either their function or if they are viable/active. Some questions that we could look to answer with more in-depth approaches include: are environmental factors having an effect that masks the true trend? What is the effect of interactions between more resistant fungi and those undergoing recovery? What is also needed in this space is for researchers to deposit diversity data into a central database following publication to enable other researchers to mine for information. Whilst traditionally sequencing data such as purified ITS sequences from single colony isolates, and whole genome sequences are regularly deposited into databases such as NCBI, there currently appears to be no requirement to do so with diversity profiling sequences. Examples of such databases that do contain this information include: Human Microbiome Project Data Portal (https://portal.hmpdacc.org/), MicrobiomeDB (https://microbiomedb.org/mbio/app/), Human Oral Microbiome Database, (http://www.homd.org/index.php), and Genomic Features of Bacterial Adaptation to Plants (http://labs.bio.unc.edu/Dangl/Resources/gfobap_website/).
Access to the sequencing data of others would increase the ability of the research community to compare studies and generate a more holistic view of particular microbiomes. Having discussed the importance of diversity and how it is measured the following sections take an in-depth look at the role that fungicide applications play on yeast diversity.

**Fungicides and grapevine protection**

Grapevines often need to be treated with fungicides in order to prevent or cure the wide range of fungal and oomycete-caused diseases affecting soil, vine and grape health. The first attempts at human intervention and active protection of wine grapes began in 1847 in England, and 3 years later in France (Lamy 1992). Powdery mildew (*Erysiphe necator*) destroyed most of the 1854 harvest, reaching a record low level of 2.82 hL/ha (Chevet et al. 2011). Due to the high economic importance of controlling this disease, the French Government and the Société d'Encouragement pour l'Industrie Nationale (Society for National Industry Encouragement) organised a competition in 1855 to inspire the rise of treatment ideas (Lamy 1992); with sulfur treatments being successful. Some years later, when downy mildew (*Plasmopara viticola*, an oomycete) was responsible for a new production crisis (Gianessi and Williams 2011), the discovery of the ‘Bordeaux mixture’ (copper, lime, and water) in Médoc (France) by Alexis Millardet and Ulysse Gayon gave growers hope (Roudié 1997). Consequently, copper and sulfur-based products have been marketed and used for more than 150 years.

Unfortunately, many other diseases threaten crops every year, and chemical companies have developed a vast list of different active ingredients (Fungicide Resistance Action Committee 2020), to help growers fight economic and quality loses due to fungal infection. Many of these new compounds are used at lower doses, which is beneficial from an environmental point of view. Whilst some of them have succeeded and persist in the market, others whose efficacy was destroyed because of resistance mechanisms were simply abandoned or reformulated (Morton and Staub 2008). This is often the case with products with a single action mechanism or in the same family (Sharpe et al. 2017).

Different vineyard management approaches are used in the field: conventional, organic, and biodynamic. No matter the approach taken, by the end of the season, all have released chemicals into the environment, potentially polluting water bodies and soils (Komárek et al. 2009). Additionally, this represents a high economic cost to growers. Conventional systems use a range of products as either preventive treatments or as curatives. Some fungicides are systemic, which means they enter the vine tissue and move around the plant, stopping fungal growth in all tissues. Organic management systems rely only on copper and sulfur, however, formulations containing live microbes can be also applied in certain cases (Pylak et al. 2019). The main difference between organic and biodynamic systems is the incorporation of the ‘moon
cycle’ and special composting techniques (preparations) by the latter (Diver 1999). Consumers consider organic practices to be more environmentally friendly, sustainable, and healthy, and thus are willing to pay extra for organic wines (Vecchio 2013). As a result, growers feel encouraged to certify their organic vineyards, and/or sometimes also apply biodynamic procedures. Australia is the country with the most extensive organic-certified surface, being 4.5% of the world vineyard surface managed organically (Castellini et al. 2017).

Regardless of management approach, fungicide overuse is undesirable because it can lead to pest resistance development (Hahn 2014), environmental pollution (Zubrod et al. 2019), human health issues and economic loss to growers (Pimentel and Burgess 2012). One way to combat this is to grow/develop plants that are naturally resistant. To that end, breeding has recently resulted in new grape cultivars with resistance to fungal and oomycete pathogens (Holzapfel et al. 2020). Until these are in more common use, however, fungicide applications remain necessary during the growing season. Many fungicides will have a wide spectrum of activity, which is useful to prevent resistance, however, this will also potentially affect non-target microorganisms and thus their application has the potential to affect the vineyard microbiome. Additionally, fungicides are often applied several times throughout the season in order to control a range of pathogens including, but not limited to, powdery and downy mildew. It is generally recognised that fungicide treatments have the potential to influence both the health and natural balance of the grapevine microbiome and, as a consequence, wine quality especially from uninoculated fermentations.
Effect of fungicide application on yeast diversity

Whilst attention has been directed towards the effect of fungicide sprays on bacteria and fungi (including yeast) using both culture-dependent and independent approaches, there are many contradictory reports of the actual effect (Table 1). The effect that the type of vineyard protection has on yeast populations is summarised in Tables 1 and 2. Contradictory reports may be due to several factors, including the wide range of vineyard protection practices, such as conventional, organic, biodynamic, integrated, differences in cultivar or location, sampling differences, experimental design (i.e. treatments in the vineyard prior to the experiment), method used to detect yeast species and downstream analysis of the effect on biodiversity (Figure 2). This is likely the basis for inconsistencies such as Milanović et al. (2013) finding the yeast-like fungus *A. pullulans* linked to conventional vineyards, whilst Martins et al. (2014) found the same species more frequently associated with organic vineyards. Both research teams, however, reached the conclusion that copper-based fungicides had a detrimental effect on fungi. In contrast, some authors suggested that anti-fungal sprays do not have a significant effect on yeast communities (Čadež et al. 2010). Other authors have also reported lower yeast biodiversity in vineyards using organic management and a shift in yeast populations towards *A. pullulans* (Comitini and Ciani 2008). Organic production has also shown a greater richness of minor species (Cachón et al. 2019).

While *Aureobasidium* sp. is one of the most commonly reported fungal species on grapes, many reports fail to mention enough detail, for example, about farming practices and climate (e.g. see Wei et al. 2018) making it more difficult to interpret what is influencing diversity differences. It is not even possible to speculate that *A. pullulans* is present on all organic grapes as this is true for some studies (Martins et al. 2014, Cachón et al. 2019, Rantsiou et al. 2020) but not others (Milanovic et al. 2013). This may just be because it was not reported or was deliberately not selected based on the method used to look at diversity, for example culture-dependent methods (Cordero-Bueso et al. 2014). Additionally, it has been hypothesised that the presence of some filamentous fungi might inhibit certain yeast genera (Grangeteau et al. 2017b). This is most likely to be a problem when filamentous fungi become resistant to the fungicides applied allowing them to outcompete other yeast and fungi.

It has been reported that synthetic fungicides inhibit fermentative yeast species to a greater degree than oxidative species. For example, *S. cerevisiae* is quite sensitive to these fungicides but *Cryptococcus* spp. and *Rhodotorula* spp. less so (Villa and Longo 1996, Oliva et al. 2020). Agarbatì and coworkers (2019b) found that *H. uvum* was abundant in Montelpuciano and Verdicchio samples, but fungicide treatments influenced its relative abundance. Organic treatments enhanced oxidative colonization by *Cryptococcus* spp., whereas conventional treatments had the same effect on *A. pullulans*. A wider survey is still needed in order to be definitive. For example, Cordero-Bueso et al. (2014) reported that while *S.
cerevisiae had the highest resistance to sulfur (along with other fermenting species) it was the
most sensitive to penconazole (FRAC 3). It is therefore important to understand the role that
fungicide application plays in the larger picture of a vineyard microbiome.

A consistent observation is that within a given vineyard, when multiple protection
systems are studied, the fungal microbiome is affected by the farming system used (Milanovic
et al. 2013, Cordero-Bueso et al. 2014, Cachón et al. 2019). What is still unclear is whether
inconsistencies between studies arise because most are single time-point, that is just a snapshot
in time. Longitudinal studies have the potential to help unravel these effects. One study
measuring diversity over three seasons reported that grapes sprayed only twice with sulfur
showed an increase in diversity over 3 years compared to those that were sprayed multiple
times, those with no treatment and those treated with penconazole (FRAC 3) (Cordero-Bueso
et al. 2014). It is possible that sulfur sprays are selecting for fermenting yeast over other fungi.
The limitation of this study is the analysis techniques used; random selection of up to 30
colonies from YPD agar plates. Bias may have been unintentionally introduced due to the
differential ability of isolates to grow on this laboratory medium. Of interest would be a
longitudinal study (up to 5–10 years) in multiple locations with care taken to avoid spray drift
and utilising a metagenomics approach to achieve a more holistic view of the vineyard
microbiome with different treatments. Finally, it is also possible that unreported insecticides,
miticides and herbicides are indirectly adding to the differences in conventional vineyards by
affecting insect vectors or their habitats.

Recent reports investigating the impact of chemical and biological fungicides on grape
microbial diversity have used a wide range of techniques making it difficult to compare between
studies. For example, Escribano-Viana et al (2018) utilised both culture-dependent and non-
culture-based (PCR-DGGE) techniques, however, whilst they report the species present the
relative abundance of those species was not reported. The two species common to all treatments
were A. pullulans and H. osmophila and whilst the authors reported that the microbial
community was not significantly modified after fungicide application, no information was
given on spray regimes in previous years, which could potentially affect results due to lower
background diversity (Escribano-Viana et al. 2018).

There is also a large amount of variation in relative abundance of yeast species between
replicates within the same treatment (Agarbati et al. 2019b) and the cause of this variation
deserves further investigation. For example, are differences due to sampling strategies, berry
heterogeneity, or sequencing errors? While it is clear that fungicides will have an effect on the
fungal microflora, this is yet to be comprehensively defined. There is need for a rigorous study
looking at these effects. As expected, the use of NGS technology consistently identifies more
species that culture-dependent techniques, however, the most abundant species are usually
detected by both methods (Agarbati et al. 2019b).
Mode of action of fungicides and how these might affect pathways in yeast

Commercial fungicides belong to several classes and affect several cellular functions. Fungicides can be described by their mode of action (MOA), or their chemical class and with a large range of products on the market this can be confusing. The available fungicides are too numerous to list in this review, however, there are several online resources available to help navigate the choice. These sites include the Pesticide Properties Database (Lewis et al. 2016), viticulture spray guides from The Department of Primary Industries in Australia (Department of Primary Industries and Regional Development 2020), and The Australian Wine Research Institute (The Australian Wine Research Institute 2020b).

To avoid the development of fungicide resistance, it is necessary to know how a particular fungicide works. Most agrochemicals (fungicides, herbicides, insecticides and miticides) are assigned an ‘activity group’ based on their mode of action and this FRAC (Fungicide Resistance Action Committee 2020) code now appears on the product label. The mode of action of a given fungicide will vary depending on the chemical class to which it belongs (Figure 3). It is often recommended that fungicides with different modes of action are alternated to reduce the risk of fungi becoming resistant. Often when resistance does develop to one chemical in a group, fungi are resistant to other chemicals in the same group.

The mode of action can be described in general or specific terms, that is a fungicide with broad-spectrum activity is effective against a large variety of pathogenic fungi. Examples of broad-spectrum fungicides include the multisite inhibitor group, M for example captan (M4), sulfur (M2) and mancozeb (M3). Other fungicides have a narrow spectrum of activity, for example Mefenoxam (FRAC 4), which is effective only against downy mildew and must be used in mixtures (The Australian Wine Research Institute 2020b). The problem with fungicide use, however, is that indirect non-target effects are likely and difficult to predict. Microorganisms exist in a community, often either functionally or nutritionally connected with each other. Therefore, a decrease in the population of sensitive yeast may affect the structure of the whole community. The mode of action of several fungicide groups and how they might affect autochthonous fungi populations in the vineyard is reported in Figure 3.

Although fungicides are sprayed on grapevines to reduce unwanted fungi and oomycetes such as powdery mildew and downy mildew, there are many ways fungicides might also affect yeast present on the grapevines. Fungicides can be divided into two groups: those that are permitted in organic production or synthetic fungicides which cannot. The main fungicides used in organic production are sulfur and copper. Copper is a broad-spectrum antifungal and works by causing plasma membrane damage (Ohsumi et al. 1988, Avery et al. 1996), whereas the MOA of sulfur as a fungicide is not fully understood. Sulfur may inhibit spore germination and mycelium growth in filamentous fungi and part of its MOA is also likely
related to oxidation of sulphydryl groups in mitochondrial respiratory enzymes (Williams and Cooper 2004, Fungicide Resistance Action Committee 2020). Sulfur residues can be toxic to autochthonous yeast found on grapes, however, sulfur is not thought to be toxic to strains of S. cerevisiae (Boudreau et al. 2017). It has also been suggested that there might be a synergistic effect of copper and sulfur, and of the non-Saccharomyces yeast tested so far, only A. pullulans is able to withstand both products (Grangeteau et al. 2017a). The many synthetic fungicides on the market can be grouped based on their chemistry and MOA (Figure 3).

The potential MOA of a fungicide is currently classed into 11 categories. These include those that target nucleic acid metabolism, cytoskeleton and motor proteins, respiration, amino acid and protein synthesis, signal transduction, lipid synthesis or transport, sterol biosynthesis, cell wall biosynthesis and cell membrane integrity. Additionally, there are several fungicides whose mode of action remains unknown (MOA = U), while a newer group that utilises the plants natural defence mechanisms are in group P (Fungicide Resistance Action Committee 2020). The main target of many fungicides is the cell wall, which has a characteristic structure in fungi being composed mainly of glucans, chitin and glycoproteins (Figure 3). The cell wall is arranged in different layers where the innermost layer is a more conserved structure on which the remaining layers are deposited and can vary between different species of fungi.

Several fungicides have been studied for their effect on various fungi. Many that have been in long-term use have multisite activity (M) indicating that these molecules affect several different fungal structural components and or metabolic pathways (Lukens 1971). For example, dithiocarbamates (e.g. mancozeb, thiram, ziram; FRAC M3) interfere with membrane organisation and embedded transport systems. This induces intracellular acidification and oxidative stress leading to inactivation of cellular thiol groups (Dias et al. 2010), and possibly apoptosis (Scariot et al. 2016). Other studies indicate that mancozeb interferes with respiration and is therefore more inhibitory towards respiring rather than fermenting yeast (Casalone et al. 2010). Another fungicide group in long-term use is phthalimides (e.g. captan, captanfol, folpet; FRAC M4), which interferes with cellular respiration and glycolysis. Captan is a broad-spectrum fungicide that can affect non-target microorganisms including wine yeast (Scariot et al. 2016).

Fungicides with more site-specific actions targeting certain functions of the fungal cell include: dicarboximides, which interfere with membrane function (FRAC 2) and ergosterol biosynthesis inhibitors (FRAC 3, including triazole-based fungicides). Triazole-based fungicides contain compounds that are demethylation and sterol biosynthesis inhibitors (Trzaskos et al, 1989), inhibiting three steps in ergosterol biosynthesis (Figure 3). More recently, Katragkou et al. (2016) showed that the biosynthesis of amino acids, including glycine, proline, tryptophan, asparagine, aminoisobutanoate (thiamine catabolism product), and products of purine metabolism, represented by guanine, were decreased in the presence of
fluconazole, suggesting that the mode of action of these fungicides is possibly more complex than so far reported. Non-*Saccharomyces* yeast are also susceptible to these fungicides with *Metschnikowia* spp. reported to be susceptible to the azole antifungals (FRAC 3), ketoconazole, epoxiconazole and to a lesser extent imazalil (Álvarez-Pérez et al. 2016). It is hypothesised that the lower sensitivity to the latter compound may be linked to its extensive use in agriculture since the 1970s (Álvarez-Pérez et al. 2016).

One way to look at how fungicides affect yeast is to investigate their metabolism in the presence of various fungicides. To this end, the BacTiter-Glo microbial cell viability assay, which determines the number of viable microbial cells by quantifying the ATP present has been useful (Kosel et al. 2019). Several authors have also investigated how fungicides might affect gene expression and/or aroma compounds in the final wine. Effects will obviously depend on the fungicide tested, for example pyrimethanil (FRAC 9) has been shown to alter gene expression in a manner dependent on the dosage tested. Genes differentially expressed include those involved in biosynthesis of arginine and sulfur amino acid metabolism, energy conservation, antioxidant response and multi-drug transport (Gil et al. 2014). Whereas tetraconazole (FRAC 3) was reported to alter the activity of enzymes involved in methionine and ergosterol biosynthesis (Sieiro-Sampedro et al. 2020). Interestingly, there were also differences when purified antifungal agent was tested alongside the commercial product. It is possible that other components of the commercial products are affecting biosynthesis (Sieiro-Sampedro et al. 2020), something that will need to be taken into account in future analysis of fungicide effects.

It is important to note that other plant protection procedures, such as herbicides and insecticides, also have the ability to affect the vineyard microbiome, either directly or indirectly. This is outside the scope of this review and the reader is referred to recent publications that address this (Chou et al. 2018, Mandl et al. 2018, Carneiro et al. 2019, Vincent and Lasnier 2020).

**In vitro fungicide resistance of non-*Saccharomyces* yeasts**

Researchers have begun to examine the effect of fungicides on desired non-*Saccharomyces* yeast. So far, it appears that fungicides reduce the viability of desirable non-*Saccharomyces* yeast and could potentially promote the growth of spoilage yeast (Kosel et al. 2019). This is obviously an undesirable side-effect of crop protection and in the case of winemaking it may be one with a negative impact on wine aroma by favouring spoilage organisms. Agarbarati et al. (2019a) suggested that *A. pullulans* and *Cryptococcus* spp. are favoured by conventional and organic treatments, respectively, as neither of them were significant on untreated samples. This is hypothesised to be due to reduced competition from susceptible yeasts and their ability to detoxify CuSO₄ (Schmid et al. 2011). Resistance of *A. pullulans* to both copper and sulfur...
reveals this organism can oxidise inorganic sulfur into sulfate ions (Killham et al. 1981). Additionally, *A. pullulans* can differentiate into melanin pigmented chlamydomspores and hyphal filaments, which allows for biosorption of copper (Gadd and Griffiths 1980a, Gadd 1983, Gadd and de Rome 1988, Fogarty et al. 1996). Resistant strains take up less metal than sensitive strains (Gadd and Griffiths 1980b). Copper had an inhibitory effect on the cultivable yeast population from two Bordeaux vineyards, and was found at higher levels in conventionally managed vineyards (Martins et al. 2014). With regards to *S. cerevisiae*, Adamo et al. (2012) reported a sevenfold amplification of CUP1 expression in a copper resistant isolate. This suggests that CUP1, encoding a copper-binding protein, plays a role in protecting *S. cerevisiae* cells against copper toxicity. This type of resistance could also be engineered in *Pichia pastoris* (Koller et al. 2000) and *Kluyveromyces lactis* (Macreadie et al. 1991).

A recent study testing 21 isolates of *Aureobasidium pullulans*, nine *Hansensiaspora guilliermondii*, 13 *Hansensiaspora uvarum*, 63 Metschnikowia spp., eight *Pichia membranifaciens*, 41 *Starmella bacillaris* and one isolate of *S. cerevisiae* for their sensitivity to copper and sulfur reported a high degree of intraspecies variability (Grangeteau et al. 2017a). In this study, *A. pullulans* and *St. bacillaris* were the most resistant to copper and *A. pullulans*, *H. guilliermondii* and *Metschnikowia* spp. to sulfur. Only isolates of *A. pullulans* had high resistance to both antifungal agents (Grangeteau et al, 2017a). In the vineyard, copper and sulfur are often applied simultaneously or sequentially and future work should therefore include analysis of the combined effect of the two products and the mechanisms behind intraspecies variation in resistance. Authors who have reported fungal abundance on untreated grapes report that *A. pullulans* is only a minor part of the whole yeast population under those conditions (Agarbat et al 2019a).

It is possible that some yeasts are dominant in sprayed vineyards because of an increase in the resistance of the particular population to a fungicide that may have been applied over several years. Yeast fungicide resistance was tested in a recent study which investigated the minimal inhibitory concentration of different fungicide treatments (iprodione, pyrimethanil, and fludioxonil + cyprodinil) for 109 grape associated yeasts. Species such as *S. cerevisiae*, *Naganishia adeliensis*, *Papiliotrema flavescens*, *Meyerozyma guilliermondii*, *P. membranifaciens* and *Pseudozyma prolific* were not susceptible to any of the tested fungicides (Kosel et al. 2019). Both viability and growth of many beneficial isolates, however, were inhibited by fungicides at a residue concentration below the maximum permitted residue limits including isolates of *M. pulcherrima* (three strains, iprodione; three strains, both iprodione and fludioxonil + cyprodinil), *P. kluyveri* (four strains iprodione and pyrimethanil), and *H. uvarum* (seven strains pyrimethanil and fludioxonil + cyprodinil). Furthermore, isolates of spoilage yeasts, for example *D. bruxellensis*, were found to be tolerant of a concentration of fungicides greater than that recommended for application by the suppliers (Kosel et al. 2019). Further
work is therefore required to model fungicide application and diversity in the vineyard to improve our understanding of the impact of sprays towards yeast.

The effect of fungicide residues on fermentation

The level of fungicide in must will of course be related to the nature and amount of the specific fungicide applied in the vineyard. For example, a comprehensive review of the concentration of fungicides in wines and must throughout the 1990s reported that azoxystrobin (FRAC 11) and pyrimethanil (FRAC 9) residues in the must were equivalent to that on the grapes (Cabras and Angioni 2000). In all other cases, residues in the must were lower than on the grapes, and in some cases no residues [myclobutanil (FRAC 3) and tetraconazole (FRAC 3)] were present in the must (Cabras and Angioni 2000). As for non-Saccharomyces, a wide range of methods have been utilised to study the effect of fungicide residues. For example, Conner (1983) used the paper-disc agar diffusion technique to demonstrate that fungicides varied markedly in their toxicity to Saccharomyces wine yeasts. This, however, is not likely to be an accurate reflection of the impacts in fermentation, since juice/wine have features that could influence the effect of fungicides, including a low pH, increasing concentration of ethanol and other stresses. It can be hypothesised that anything that also impacts the target of the fungicide, such as the cell wall, may enhance the sensitivity of the yeast to the fungicide. Such interactions may well be additive, if not synergistic and thereby affect fermentation progress.

Interestingly, when Noguerol-Pato et al. (2014) tested ten new generation fungicides added to must at the maximum permitted residue level, they found that the prior filtration of the must had a strong influence on fermentative activity. Specifically, when S. cerevisiae was grown in red pasteurised must enriched with sugar and addition of various fungicides, only three fungicides showed an effect. These were ametoctradin (FRAC 45, MOA = C), dimethomorph (FRAC 40, MOA = H) and mepanipyrim (FRAC 09, MOA = D) (see Figure 3 for MOA definitions and FRAC codes). The same was observed in filtered Tempranillo must, but when the must was tested unfiltered, no effect was observed (Noguerol-Pato et al. 2014). Calhelha et al. (2006) also reported that while fungicides had a negative effect on in vitro yeast growth, laboratory-scale red wine fermentations spiked with benomyl (FRAC 1) and dichlofluanid (FRAC M6) had a limited effect on wine chemical and sensory properties when compared to a control without fungicides. These findings imply that the complex matrix that is unfiltered grape must offers protection to yeast, perhaps by absorption of fungicide residues onto particles in the must. Other factors such as a difference in pH can also impact fungicide inhibition, with low pH values enhancing fungicide disassociation and thereby producing different MIC values compared to tests at a neutral pH (Scariot et al. 2016).

The presence of antifungal residues and grape must can seriously affect progress of alcoholic fermentation (Bizaj et al. 2014). For example, the fungicides pyrimethanil (FRAC 9)
and fenhexamid (FRAC 17) negatively affect fermentation kinetics in a strain dependent manner, when various industrial strains were inoculated into spiked grape juice (Bizaj et al. 2014). Additionally, both the aromatic and basic composition of the resulting wines were affected, albeit it differently, depending on the fungicide added, but with no increase in desirable compounds and an increase in undesirable ones (Bizaj et al. 2014). Yeast cells treated with captan exhibited altered membrane integrity, reduction of thiol compounds and an increase in intracellular reactive oxygen species. Concentration of 2.5 µmol/L of captan completely inhibit fermentation with a dose-dependent delay when a lower concentration was tested (Scariot et al. 2016, 2017).

More recently Sieiro-Sampedro et al. (2020) reported that when tetraconazole (FRAC 3) was added into Garnacha wine, to mimic residual fungicide concentration, the volatile profile ranged between 23 and 145% of the control, mainly due to changes in ethyl esters derived from medium-chain fatty acids. Proteomic analysis of the yeast was also carried out and when fungal residues were present there were changes in the abundance of enzymes involved in the methionine and ergosterol biosynthesis pathways (Sieiro-Sampedro et al. 2020).

Therefore, in impacting yeast populations on grapes, in the fermentation as well as the metabolism of these, fungicides have the potential to not only interfere with fermentation progress but also the final aroma profile. The formation, release or degradation of sensorially important compounds could conceivably be altered either through a metabolic response to fungicides or because of the altered yeast population profile and metabolic interactions that result. Certainly, it is well established that different combinations and proportions of yeast result in a different wine composition, including aroma compounds (e.g. Capozzi et al. 2015, Padilla et al. 2016b). This is a potentially important determinant of winemaking outcome that is perhaps not often considered before the application of fungicide sprays to the vineyard.

**Alternative treatments**

Due to high potential yield losses, agriculture relies on the application of chemical treatments to protect against bacteria, fungi, viruses, weeds and insects. Unfortunately, a large proportion of these products do not arrive at their site of action and a large quantity of spray is needed to ensure coverage. For example, pesticide losses ranging between 63–74% were reported when testing different nozzle and pressure levels in lemon and tangerine orchards (Soheilifard et al. 2020). This potentially causes pollution of soil, water bodies and air, as well as economic losses to growers. Additionally, these chemical compounds can negatively impact human health, affect non-target microorganisms and may also encourage pest/disease resistance (Worrall et al. 2018). It is therefore imperative to find new, safer ways of protecting crops. This next section
describes and evaluates alternative ways to control pathogenic fungi in the vineyard, with a view towards finding less harsh treatments that enable us to nurture the grapevine microbiome.

Is there a vineyard version of probiotics?

Previous studies in banana trees and maize (Marcano et al. 2016, Youseif 2018) show that the application of plant growth promoters (PGP) as probiotics in grapevines is feasible but is yet to be investigated. In order to achieve this, we hypothesise that it would be necessary to isolate and purify bacterial probiotics from the roots and rhizosphere of healthy grapevines for this technique. The soil and rhizosphere microbiome contain species that are cultivar specific due to the chemical composition of root exudates varying among plant genotypes, thereby representing a selective force defining host-microbiome interaction (Bakker et al. 2012). In addition, soil physical properties such as particle size and chemical characteristics, such as pH, nutrient, water and oxygen concentration and texture, also affect microbial community development and activity (Gilbert et al. 2014). We suggest that research should prioritise local and culturable PGP strains to guarantee growth and reproduction of these PGP following transplant. Also, those PGP species that look promising in in vitro screenings, need to be tested in the field before further conclusions about their efficacy can be made.

Insect vectors to improve diversity

Vineyards are complex agroecosystems with intricate relationships occurring between all their components even if grapevines are the predominant species. Throughout this review we have emphasised how important yeast diversity is to ensure vineyard health. Yeast diversity must be considered as a part of the overall vineyard biodiversity. Plant, insects and microbes will enhance vine adaptation and resilience (Retallack 2011). In previous sections we have discussed how yeast diversity could be affected by fungicides. It is also important, however, to consider the potential unwanted side effects of other pesticides such as insecticides, which should be mindfully chosen as they can affect beneficial insects as well. Yeasts rely on insects to move around the vineyard and also from the vineyard to the winery (Madden et al. 2018, Liu et al. 2019b). In addition, practices such as adding vegetal species, native or exotic, around the vineyard (vineyard scaping), planting flowering species that beneficial insects feed from, and creating shelter for them can improve not only pest management, but yeast diversity (Retallack 2011). Finally, the use of grasses and *Leguminosae* species as cover crops improves the carbon/nitrogen relationship in the soil, enhancing soil structure and microflora diversity around the roots (Vukicevich et al. 2016).

Bioprotectants
Instead of using of fungicides to control undesirable fungi it is possible that antagonistic yeast and/or bacterial species could be propagated in the vineyard and used as ‘bioprotectants’ (Bleve et al. 2006). Indeed, many isolates that are sensitive to residue levels of fungicides could potentially be used against the species that the fungicides are ultimately designed to eradicate. The introduction of bacteria such as the lactic acid bacterium *Lactobacillus plantarum* (Gobbi et al. 2020) or yeast strains with biocontrol activity is a ‘hot-topic’ and a promising alternative to traditional methods. Indeed, grape-derived epiphytic yeast species such as *Issatchenkia terricola* have antagonistic activity towards *Aspergillus* spp. in vitro (Bleve et al. 2006). Raspor et al. (2010) tested several wine yeast species for their potential against the growth of *Botrytis cinerea* with *A. pullulans* and *M. pulcherrima* having the highest potential biocontrol activity. A more recent study comparing a range of available biological controls against *Botrytis bunch rot*, (*Bacillus subtilis*, *B. amyloliquefaciens*, *A. pullulans*, *Ulocladium oudemansii*, and *Candida sake*) along with six experimental bacterial and two fungal biological control strains found that treatments based on *C. sake* and *B. subtilis* QST713, achieved the highest reduction in disease severity rates (45 and 54%, respectively), but the effect was dependant on the season (year tested) and the grape cultivar (Calvo-Garrido et al. 2019).

*Aureobasidium pullulans* is also a good biocontrol agent able to limit the development of *B. cinerea*, *Rhizopus stolonifer*, *Aspergillus niger* and *A. carbonarius* on grape berries (Schena et al. 1999, 2003, Dimakopoulou et al. 2008). These capacities combined with a resistance to copper and sulfur (used in organic viticulture) make *A. pullulans* an interesting choice as an organic biological control agent. The *A. pullulans* strain Fito_F278 significantly reduced the mycelium growth of the botryosphaeria dieback agent *Diplodia seriata*, via direct antagonism under in vitro conditions (Pinto et al. 2018). No significant reduction, however, of disease lesions and relative frequency were found in cutting plants, reinforcing that the antagonistic activities of this strain are dependent on a direct interaction with the phytopathogen (Pinto et al. 2018). Thus, further studies using direct application treatments are required. However, in order to measure the impact and interaction of these strains on the resident microbial community, studies based on analyses of the microbiome in vineyards are necessary. Many epiphytic yeast isolates that have been tested in vitro have shown antagonistic activity towards a range of grape pathogens (Bleve et al. 2006, Cordero-Bueso et al. 2017). But when tested in vivo (outdoor conditions) they are so far ineffective (Perazzolli et al. 2014). Therefore, there is still much work to be done to maximise this antagonistic activity in the field.

Looking to the future, many agrochemical manufacturers are using techniques such as RNAi or CRISPR-Cas9 to generate microbes able to produce biopesticides (Borel 2017). Even so, more work is needed as the success of these products depends on producing large quantities of organisms with a long-shelf life. In addition, since microbiome changes are expected amongst regions, crops or climatic conditions, variable that may also alter the efficacy of
candidate agrochemicals, manufacturers are faced with significant challenge given their preference to produce a treatment with a standard or limited formulation(s) (Parnell et al. 2016, Schütz et al. 2018). Moreover, when we introduce microbes into a foreign environment, there will be competition between the indigenous and the inoculated microflora (Ambrosini et al. 2016), which also needs to be taken into consideration. Last, there are some incompatibilities between inputs (e.g. fertilisers) or agricultural practices (e.g. tillage), whose application may alter soil microbial communities (Lupwayi et al. 2010).

Other novel anti-fungal treatments

Other potential biological controls include; clay for powdery mildew treatments (Sholberg et al. 2006), vegetable or mineral oil, or potassium silicate or more recently, nanoparticles. Nanoparticles can act either as protectants themselves (gold, silver, chitosan, copper, titanium dioxide) or be the carriers for other compounds (Worrall et al. 2018). Practically speaking they have improved efficacy, over a longer period of time, which potentially translates to reduced need to spray. For example, nanoparticle-delivered tebuconazole had a similar decay amount when used at 10% of the original dose in wood treatments (Liu et al. 2002). Additionally, decreased phytotoxicity of carbendazim when loaded onto nanoparticles, improved germination rates and root growth in cucumber, tomato and corn (Kumar et al. 2017). Pyrimethanil is a specific fungicide for the treatment of Botrytis on grapes (Bayer Crop Science Australia 2020), but if applied after 80% capfall, traces will still be detectable in finished wines, which can lead to export issues (The Australian Wine Research Institute 2020b). Zhao et al. (2017) working with pyrimethanil loaded onto mesoporous silica nanoparticles (MSN) concluded that this formulation minimised the risk of the fungicide accumulating in cucumber. If applied to grapevines, a similar strategy may improve both grape health and the safety of finished wines for human consumption. Another pathogen-specific nanoparticle delivery system has also been shown to be effective with azoxystrobin, pyraclostrobin, tebuconazole, and boscalid, to treat Phaeomoniella chlamydospora and P. minimum (ESCA) in Vitis vinifera cv. Portugieser. But despite showing great promise, the effect of this technology on indigenous microflora is to yet be defined, therefore demanding further research.

Enhancing plant immune systems

A newer approach to plant protection is being developed by seeking to enhance the response to pathogens by means of ‘antibodies’ collected from symptomatically affected plants. This method, utilising NDM (Natural Defence Messengers), transforms a sensitive plant into a resistant one (Gabel 2019) and depends on the plant’s phenology and its sensitivity to infections. This process begins with an infected plant from which material is collected and antibodies are extracted from affected tissues using organic solvents (Gabel 2019). In contrast
with the human immune system in which defence responses are specific to a particular microbe, the effects of priming in plants are broad-spectrum, protecting the plant against a wide range of diseases and insect pests. For a review and more information on the potential use of the grapevine defence response (by the use of elicitors) as an alternative to fungicide treatment please see Delaunois et al. (2014). Again, further research is needed to improve our understanding of the molecular mechanisms behind NDM but priming a plants natural defence mechanism could well be a valuable tool in sustainable agriculture.

Conclusion

This review has highlighted the complex nature of investigating yeast diversity in the vineyard. The factors that determine diversity inevitably form a complex matrix of interactions and we have chosen to focus on fungicides. In doing so we have highlighted the large amount of work that has been undertaken in this area of research. Unfortunately, due to the inherently complex nature of investigating yeast diversity under many variable conditions, it is difficult to achieve standardised methods. Perhaps a microbiome database where sequences from such studies are deposited along with as much available information about sprays, climate and geographical location as possible would enable other researchers to make comparisons between studies.

The microbial population on the grapes will have an effect on both inoculated and uninoculated fermentations. It is likely, however, that many vigneron and winemakers do not consider the impacts of fungicides on their fermentations. There are several different modes of action for each fungicide, and yeast may have differing ability to adapt to each MOA, for example Saccharomyces yeast are more resistant to copper than non-Saccharomyces yeast. Finally, as we have reported there are several novel crop protection strategies being studied to help protect crops and increase diversity. Alternative treatments, such as plant growth promoters and bioprotectants, show great progress. It may also be after more longitudinal studies that we discover better management techniques to enhance microbial diversity whilst still protecting the vineyard.

Acknowledgements

Financial support for this work was provided by The Australian Research Council Training Centre for Innovative Wine Production [www.ARCwinecentre.org.au; IC170100008], which is funded by the Australian Government with additional support from Wine Australia and industry partners. The University of Adelaide is a member of the Wine Innovation Cluster (http://www.thewaite.org.waite-partners/wine-innovation-cluster/).
References


reduces *Aspergillus carbonarius* (sour rot) incidence in wine-producing vineyards in Greece. Biological Control 46, 158-165.


Lukens, R.J. (1971) Chemistry of fungicidal action (Springer-Verlag: Berlin, Germany)


ecological applications from the German Biodiversity Exploratories. Ecology and Evolution 4, 3514-3524.


Tetraconazole alters the methionine and ergosterol biosynthesis pathways in Saccharomyces yeasts promoting changes on volatile derived compounds. Food Research International 130, 108930.


Table 1. Reported trend of yeast diversity with regard to spray treatment applied in the
vineyard.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Organic spray</th>
<th>Conventional spray</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diversity trend†</td>
<td>Diversity index‡</td>
</tr>
<tr>
<td>Cordero-Bueso et al. (2011)</td>
<td>↑</td>
<td>✔% ‡‡</td>
</tr>
<tr>
<td>Setati et al. (2012)</td>
<td>↑</td>
<td>✔§</td>
</tr>
<tr>
<td>Milanovic et al. (2013)</td>
<td>↓</td>
<td>×</td>
</tr>
<tr>
<td>Cordero-Bueso et al. (2014)</td>
<td>↑</td>
<td>✔% ‡‡</td>
</tr>
<tr>
<td>Martins et al. (2014)</td>
<td>↑</td>
<td>✔§</td>
</tr>
<tr>
<td>Grangeteau et al. (2017)</td>
<td>↓</td>
<td>✔§</td>
</tr>
<tr>
<td>Agarbatì et al. (2019a, b)</td>
<td>↑</td>
<td>×</td>
</tr>
<tr>
<td>Cachón et al. (2019)</td>
<td>↑</td>
<td>✔% ‡‡</td>
</tr>
</tbody>
</table>

†↑ diversity increased, ↓ diversity decreased; † Diversity index reports whether diversity indexes were calculated (✔) or not (×); §Shannon diversity index; ‡Simpsons diversity index; ‡‡Shannon-Wiener index.
Table 2. Fungal diversity as a function of grape cultivar and fungicide treatment. Relative abundances are reported, in decreasing order of abundance, when available.

<table>
<thead>
<tr>
<th>Fungicide(s) (number of applications)</th>
<th>FRAC Code (Fungicide group)</th>
<th>Grape cultivar tested</th>
<th>Detection method</th>
<th>Diversity method</th>
<th>Most abundant fungal species reported (100–10%)</th>
<th>Low abundance species reported (&lt;10%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Verdicchio</td>
<td>Culture dependent</td>
<td>Relative abundance (count/total)</td>
<td><em>H. uvarum</em>, <em>St. bacillaris</em>, <em>M. pulcherrima</em>, <em>P. fermentans</em></td>
<td><em>A. pullulans</em>, <em>C. californica</em>, <em>P. fermentans</em></td>
<td>Agarbati et al. (2019a)</td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Montepulciano</td>
<td>Culture dependent</td>
<td>Relative abundance (count/total)</td>
<td><em>H. uvarum</em></td>
<td><em>A. pullulans</em>, <em>I. terricola</em>, <em>St. bacillaris</em>, <em>Z. meyerae</em></td>
<td>Agarbati et al. (2019a), Agarbati et al. (2019b)</td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Montepulciano</td>
<td>Culture independent (NGS)</td>
<td>Relative abundance (count/total)</td>
<td><em>A. pullulans</em>, <em>H. uvarum</em></td>
<td><em>St. bacillaris</em>, <em>Z. meyerae</em>, <em>Rh. nothofagi</em>, <em>M. pulcherrima</em> (filamentous fungi reported), <em>Bot. caroliniana</em>, <em>Alternaria sp.</em>, <em>Cl. ramotenellum</em>, <em>Cl. Delicatulum</em></td>
<td>Agarbati et al. (2019b)</td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Tempranillo</td>
<td>Culture dependent</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td><em>A. pullulans</em>, <em>H. osmophila</em>, <em>L. thermotolerans</em>, <em>R. babjevae</em>, <em>R. nothofagi</em>, <em>S. cerevisiae</em> (Relative abundance not reported)</td>
<td><em>A. pullulans</em></td>
<td>N/A</td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Tempranillo</td>
<td>PCR-DGGE</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td><em>A. pullulans</em></td>
<td>N/A</td>
<td>Escribano-Viana et al. (2018)</td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>Passerina, Malvasia, Montepulciano</td>
<td>Culture dependent</td>
<td>Cell count only</td>
<td><em>H. uvarum</em>, <em>M. pulcherrima</em>, <em>Cry. macerans</em></td>
<td><em>A. pullulans</em>, <em>T. delbrueckii</em>, <em>C. kruis</em></td>
<td>Comitini et al. (2008)</td>
</tr>
<tr>
<td>Fungicide(s) (number of applications)</td>
<td>FRAC Code (Fungicide Group)</td>
<td>Grape variety tested</td>
<td>Detection method</td>
<td>Diversity method</td>
<td>Most abundant fungal species reported (100-10%)</td>
<td>Low abundance species reported (&lt;10%)</td>
<td>Reference</td>
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<tr>
<td><strong>Bio-fungicide†</strong></td>
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<tr>
<td>2 (21 days and 3 days before harvest)</td>
<td>P6, BM2</td>
<td>Tempranillo†</td>
<td>Culture dependent</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>A. pullulans, H. osmophila, Hyp. pseudoburtonii, R. babjevae, R. glutini, R. nothofagii, S. cerevisiae, A. pullulans</td>
<td>N/A</td>
<td>Escribano-Viana et al. (2018)</td>
</tr>
<tr>
<td>2 (21 days and 3 days before harvest)</td>
<td>P6, BM2</td>
<td>Tempranillo†</td>
<td>PCR-DGGE</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>A. pullulans, Cryptococcus spp., H. uvarum, St. bacillaris</td>
<td>N/A</td>
<td>Escribano-Viana et al. (2018)</td>
</tr>
<tr>
<td><strong>Fungicide(s) (number of applications)</strong></td>
<td><strong>FRAC Code (Fungicide Group)</strong></td>
<td><strong>Grape variety tested</strong></td>
<td><strong>Detection method</strong></td>
<td><strong>Diversity method</strong></td>
<td><strong>Most abundant fungal species reported (100-10%)</strong></td>
<td><strong>Low abundance species reported (&lt;10%)</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td><strong>Organic fungicides‡</strong></td>
<td></td>
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</tr>
<tr>
<td>15</td>
<td>M (multi-site inhibitors)</td>
<td>Verdicchio†‡</td>
<td>Culture dependant</td>
<td>Relative abundance (count/total)</td>
<td>A. pullulans, Cryptococcus spp., H. uvarum, St. bacillaris</td>
<td>M. pulcherrima, D. hansenii, P. membranifaciens, I. terricola</td>
<td>Agarbati et al. (2019a)</td>
</tr>
<tr>
<td>Location</td>
<td>Region</td>
<td>Year</td>
<td>Initials</td>
<td>Culture</td>
<td>Method</td>
<td>Organisms Reported</td>
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<tr>
<td>Montepulciano</td>
<td>Italy</td>
<td>2019</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
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<tr>
<td>Chardonnay</td>
<td>Italy</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture independent (NGS)</td>
<td>A. pullulans, H. uvarum, St. bacillaris</td>
<td></td>
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<tr>
<td>Chardonnay</td>
<td>Italy</td>
<td>2013</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture independent (NGS)</td>
<td>A. pullulans, H. uvarum, St. bacillaris</td>
<td></td>
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<tr>
<td>Chardonnay</td>
<td>Italy</td>
<td>2014</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture independent (NGS)</td>
<td>A. pullulans, H. uvarum, St. bacillaris</td>
<td></td>
</tr>
<tr>
<td>Verdicchio</td>
<td>Italy</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
<td></td>
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<tr>
<td>Treixadura</td>
<td>Portugal</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
<td></td>
</tr>
<tr>
<td>Mencía</td>
<td>Spain</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
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<td>Brancellao</td>
<td>Spain</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
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<tr>
<td>Not reported</td>
<td>Portugal</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
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<tr>
<td>Not reported</td>
<td>Spain</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
<td></td>
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<tr>
<td>Not reported</td>
<td>Spain</td>
<td>2012</td>
<td>M (multi-site inhibitors)</td>
<td>Montepulciano</td>
<td>Culture dependant</td>
<td>A. pullulans, St. bacillaris, H. uvarum, I. terricola</td>
<td></td>
</tr>
</tbody>
</table>

*Filamentous fungi reported: Bot. caroliniana, Alternaria sp., Cl. ramotenellum, Cl. delicatulum

**References:**
- Cachón et al. (2019)
- Agarbari et al. (2019a)
- Agarbari et al. (2019b)
- Milanović et al. (2013)
<p>| Not reported | M (multi-site inhibitors) | Treixadura(^{1,2,3}) (location 2) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>Aureobasidium</em> sp., <em>Cry. victoriae</em> | <em>Cry. carnescens, Cry. af. victoriae, R. graminis</em> | Cachón et al. (2019) |
| Not reported | M (multi-site inhibitors) | Mencía(^{1,2,3}) (location 3) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>H. uvarum, Metschnikowia</em> sp., <em>P. kluveri</em> | <em>Aureobasidium</em> sp., <em>Cry. af. victoriae</em>, <em>Cyst. macerans</em>, <em>Zygos. Bisporus</em> | Cachón et al. (2019) |
| Not reported | M (multi-site inhibitors) | Albariño(^{1,2,3}) (location 4) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>Aureobasidium</em> sp., <em>H. uvarum</em>, <em>Cry. terrestris</em> | <em>D. hansenii, Metschnikowia</em> sp., <em>R. nothofagi</em> | Cachón et al. (2019) |
| Not reported | M (multi-site inhibitors) | Treixadura(^{1,2,3}) (location 4) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>D. hansenii, H. uvarum</em> | <em>Cry. stepposus, Metschnikowia</em> sp., <em>Aureobasidium</em> sp., <em>Cry. af. victoriae</em>, <em>C. terricola</em>, <em>C. carncens, I. terricola</em>, <em>C. oleophila</em>, <em>P. kluveri</em> | Cachón et al. (2019) |
| 6 | M (multi-site inhibitors) | Passerina, Malvasia, Montepulciano and Sangiovese(^{2,4}) <em>individual variety differences were not reported</em> | Culture dependent | Cell count only | <em>H. uvarum, M. pulcherrima, Cry. macerans</em> | <em>A. pullulans, Tri. pullulans</em> | Comitini et al. (2008) |
| 4 | M (multi-site inhibitors) | Temperanillo(^2) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>R. mucilaginosa, S. cerevisiae, L. thermotolerans</em> | <em>W. anomalus, H. guilliermondii, M. pulcherrima, C. sorbose, T. delbrueckii</em> | Cordero-Bueso et al. (2014) |
| 2 | M (multi-site inhibitors) | Temperanillo(^2) | Culture dependent | Shannon-Wiener index and Simpson’s diversity index | <em>R. mucilaginosa, L. thermotolerans, S. cerevisiae</em> | <em>T. delbrueckii, M. pulcherrima, H. guilliermondii, W. anomalus</em> | Cordero-Bueso et al. (2014) |
| Not reported | M | Merlot(^{2,5,6}) | Culture dependent | Shannon-diversity index | <em>Aureobasidium</em> sp. | <em>Phoma</em> sp., <em>Cryptococcus</em> sp. | Martins et al. (2014) |</p>
<table>
<thead>
<tr>
<th>Fungicide(s)</th>
<th>FRAC Code (Fungicide Group)</th>
<th>Grape variety tested</th>
<th>Detection method</th>
<th>Diversity method</th>
<th>Most abundant fungal species reported (100-10%)</th>
<th>Low abundance species reported (&lt;10%)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Synthetic fungicides%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>12 (Also used organic fungicides%)</td>
<td>M1, M2, 40, 46, P7, other</td>
<td>Verdicchio%</td>
<td>Culture dependant</td>
<td>Relative abundance (count/total)</td>
<td>A. pullulans, H. uvarum, St. bacillaris</td>
<td>Cryptococcus sp., C. californica</td>
<td>Agarbati et al. (2019a)</td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>Variety</td>
<td>Vintage</td>
<td>Sampling Method</td>
<td>Culture Independence</td>
<td>Relative Abundance (count/total)</td>
<td>Species/Genus</td>
</tr>
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<tr>
<td>Esribano-Viana et al. (2018)</td>
<td>17</td>
<td>Tempranillo</td>
<td>2019</td>
<td>PCR-DGGE</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>A. pullulans, Bot. cinerea</td>
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<td>Esribano-Viana et al. (2018)</td>
<td>17</td>
<td>Tempranillo</td>
<td>2019</td>
<td>PCR-DGGE</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>A. pullulans, Bot. cinerea</td>
<td>N/A</td>
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<td>Agarbat et al. (2019b)</td>
<td>9</td>
<td>Montepulciano</td>
<td>2019</td>
<td>Culture independent (NGS)</td>
<td>A. pullulans, H. uvarum</td>
<td>L. thermotolerans, St. bacillaris, P. terricola, *filamentous fungi reported; Bot. caroliniana, Alternaria sp., Cl. ramotellum, Cl. delicatulum</td>
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<tr>
<td>Grangeteau et al. (2017b)</td>
<td>8</td>
<td>Chardonnay</td>
<td>2014 vintage</td>
<td>Culture independent (454)</td>
<td>Aureobasidium sp., Cryptococcus sp., Hanseniaspora sp., Saccharomyces sp.</td>
<td>Metschnikowia sp., Saccharomyces sp.</td>
<td></td>
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<tr>
<td>7</td>
<td>Managed with a dose reduction and/or with a reduced number of treatments compared to conventional treatment - above</td>
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</tr>
<tr>
<td>M1, M2, M3, M4, M9, 3, 5, 7, 11, 21, 27, 40, 43, 45, 50, P3</td>
<td>Chardonnay (2012 vintage)</td>
<td>Culture independent biodiversity index</td>
<td><em>Aureobasidium</em> sp., <em>Botryotinia</em> sp., <em>Cladosporium</em> sp., <em>Erysiphe</em> sp., <em>Alternaria</em> sp., <em>Phoma</em> sp., <em>Metschnikowia</em> sp., <em>Saccharomyces</em> sp., <em>Soporidiobolus</em> sp.</td>
<td>Grangeteau et al. (2017b)</td>
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</tr>
<tr>
<td>8</td>
<td>(managed with a dose reduction and/or with a reduced number of treatments compared to conventional treatment - above)</td>
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</tr>
<tr>
<td>M1, M2, M3, M4, M9, 3, 5, 7, 11, 21, 27, 40, 43, 45, 50, P3</td>
<td>Chardonnay (2013 vintage)</td>
<td>Culture independent biodiversity index</td>
<td><em>Aureobasidium</em> sp., <em>Cryptococcus</em> sp., <em>Saccharomyces</em> sp., <em>Monilinia</em> sp., <em>Hanseniaspora</em> sp., <em>Soporidiobolus</em> sp.</td>
<td>Grangeteau et al. (2017b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(managed with a dose reduction and/or with a reduced number of treatments compared to conventional treatment - above)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1, M2, M3, M4, M9, 3, 5, 7, 11, 21, 27, 40, 43, 45, 50, P3</td>
<td>Chardonnay (2014 vintage)</td>
<td>Culture independent biodiversity index</td>
<td><em>Hanseniaspora</em> sp., <em>Cryptococcus</em> sp., <em>Saccharomyces</em> sp., <em>Aureobasidium</em> sp., <em>Meyerozyma</em> sp., <em>Itersonilia</em> sp.</td>
<td>Grangeteau et al. (2017b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-8 (Also used organic fungicides 1,2)</td>
<td>Verdicchio 1,6,7,9,11, 12,13,14,15</td>
<td>Culture dependant</td>
<td><em>H. uvarum</em>, <em>C. zemplinina</em>, <em>M. pulcherrima</em>, <em>R. nothofagi</em>, <em>R. glutinis</em>, <em>A. pullulans</em>, <em>R. babjevae</em>, <em>P. fermentans</em>, <em>Cryptococcus</em> sp., <em>Candida</em> sp.</td>
<td>Milanović et al. (2013)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not reported</td>
<td>Not reported</td>
<td>Culture dependant</td>
<td>None reported</td>
<td>Cachón et al. (2019)</td>
<td></td>
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</tbody>
</table>

1. Culture independent (454) Shannon biodiversity index
2. ANOVA and Duncan test (diversity index not reported) Shannon-Wiener index and Simpson’s diversity index
3. Names not reported

**Note:** The page number is 51.
<table>
<thead>
<tr>
<th>Location</th>
<th>Spray Name</th>
<th>Crop Name</th>
<th>Fungicide Application</th>
<th>Culture Dependent</th>
<th>Fungal Diversity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mencía</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Culture dependent</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td><em>Aureobasidium</em> sp., <em>Cry. stepposus, S. cerevisiae</em></td>
</tr>
<tr>
<td>2</td>
<td>Brancellao</td>
<td>Not reported</td>
<td>Not reported</td>
<td>None reported</td>
<td><em>Aureobasidium</em> sp., <em>D. hansenii</em></td>
<td>None reported</td>
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<tr>
<td>2</td>
<td>Treixadura</td>
<td>Not reported</td>
<td>Not reported</td>
<td>None reported</td>
<td><em>Aureobasidium</em> sp., <em>Cry. laurentii</em></td>
<td><em>Metschnikowia</em> sp.</td>
</tr>
<tr>
<td>3</td>
<td>Mencía</td>
<td>Not reported</td>
<td>Not reported</td>
<td>None reported</td>
<td><em>Metschnikowia</em> sp., <em>Cry. terrestris, H. uvarum,</em></td>
<td><em>Z. hellenicus/meyerae</em></td>
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<td>4</td>
<td>Albairino</td>
<td>Not reported</td>
<td>Not reported</td>
<td>None reported</td>
<td><em>Aureobasidium</em> sp., <em>Cry. stepposus, Metschnikowia</em> sp.</td>
<td><em>R. graminis</em></td>
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<tr>
<td>4</td>
<td>Treixadura</td>
<td>Not reported</td>
<td>Not reported</td>
<td>None reported</td>
<td><em>H. uvarum, Aureobasidium</em> sp.</td>
<td><em>Metschnikowia</em> sp., <em>R. graminis, S. ruberrimus</em></td>
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<tr>
<td>5</td>
<td>Verdicchio</td>
<td>9 and 12</td>
<td>Culture dependent</td>
<td>Cell count only</td>
<td><em>A. pullulans, Cry. albidus, Cry. humicolus</em></td>
<td><em>Tri. pullulans, H. uvarum, R. aurantiaca</em></td>
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<tr>
<td>6</td>
<td>Temperanillo</td>
<td>3</td>
<td>Culture dependent</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td><em>R. mucilaginosa, L. thermotolerans</em></td>
<td><em>M. pulcherrima, T. delbrueckii, W. anomalus, S. cerevisiae</em></td>
</tr>
<tr>
<td>7</td>
<td>Temperanillo</td>
<td>3</td>
<td>Culture dependent</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td><em>R. mucilaginosa, L. thermotolerans, S. cerevisiae</em></td>
<td><em>T. delbrueckii, W. anomalus, M. pulcherrima</em></td>
</tr>
<tr>
<td>Not reported (Also used organic fungicides⁸)</td>
<td>M3, M4, 4, 22, 27, P7</td>
<td>Merlot¹⁷, 18, 19, 20</td>
<td>Culture dependent</td>
<td>Shannon-diversity index</td>
<td>Sporidiobolus sp., Rhodotorula sp., Cladosporium sp., Aureobasidium sp.</td>
<td>Cryptococcus sp., Epicoccum sp.</td>
</tr>
<tr>
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</tr>
<tr>
<td>8 (Also used organic fungicides³)</td>
<td>M2, M3, M4, 3, 13, 40, P7</td>
<td>Cabernet Sauvignon³², 35, 37, 38, 39, 40</td>
<td>Culture dependent</td>
<td>Relative abundance (count/total) &amp; Shannon diversity index</td>
<td>A. pullulans, Cryptococcus spp.</td>
<td>Sporobolomyces roseus, Rhodotorula slooffiae, Bullera dendrophila, Candida sp., Issatchenka terricola, Rhodotorula nothofagi, Blastobotrys nivea* (*only 87% identity)</td>
</tr>
<tr>
<td>8 (Integrated management system, also used organic fungicides³)</td>
<td>M2, M3, 11, 13, 27, 40, P7</td>
<td>Cabernet Sauvignon¹⁹, 32, 37, 38, 39, 41</td>
<td>Culture dependent</td>
<td>Relative abundance (count/total) &amp; Shannon diversity index</td>
<td>A. pullulans, Cryptococcus spp.</td>
<td>Rhodotorula glutinis, Issatchenka terricola, Sporobolomyces roseus</td>
</tr>
<tr>
<td>Not reported</td>
<td>3</td>
<td>Shiraz¹²</td>
<td>Culture dependent RFLP and PCR-RAPD analysis</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>S. cerevisiae, K. thermotolerans (now L. delbrueckii) P. anomala</td>
<td>P. toletana, C. sorbose, T. delbrueckii</td>
</tr>
<tr>
<td>Not reported</td>
<td>3</td>
<td>Grenache¹²</td>
<td>Culture dependent RFLP and PCR-RAPD analysis</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>K. thermotolerans, H. guilliermondii</td>
<td>None reported</td>
</tr>
<tr>
<td>Not reported</td>
<td>3</td>
<td>Barbera¹²</td>
<td>Culture dependent RFLP and PCR-RAPD analysis</td>
<td>Shannon-Wiener index and Simpson’s diversity index</td>
<td>C. stellata, T. delbrueckii, K. thermotolerans</td>
<td>None reported</td>
</tr>
</tbody>
</table>
Bio-fungicides include \(^1\)Bacillus subtilis QST 713 (Serenade\(^\text{Ô}\)); Organic fungicides include: \(^1\)Bordeaux mixture (copper (II) sulfate and calcium hydroxide), \(^2\)sulfur, \(^3\)pyrethrins, \(^4\)copper sulphate, \(^5\)copper hydroxide, \(^6\)cuprous oxide, \(^7\)chitosan (striker); \(^8\)Synthetic fungicides include: \(^1\)copper-oxychloride, \(^2\)cyclohexanol + 1,2- propanediol + abamectin + 2,6-diterbutylp-cresol, \(^3\)iprovalicarb + copper oxychloride, \(^4\)sulfur (selenium free) + terpene alcohols + sodium salt of an aromatic polymer, \(^5\)phosphorus pentoxide + potassium oxide, \(^6\)spiroxamina, \(^7\)metalaxyl-M14+ copper-oxychloride, \(^8\)quin oxyfen+ myclobutanil+coformulants, \(^9\)fenhexamid based (Teldor\(^\text{Ô}\)), \(^10\)fosetyl-A1+ copper sulfate, \(^11\)mandipropamid, \(^12\)penconazole, \(^13\)iprovalicarb + copper hydroxide, \(^14\)quinoxyfen, \(^15\)cymoxanyl + famoxadone, \(^16\)ciprodynil and fludioxonil, \(^17\)metalaxyl-M mancozeb, \(^18\)zoxamide + mancozebe, \(^19\)cytoconazole, folpet, fosetyl, \(^20\)folpet, fosetyl, \(^21\)benzamides, \(^22\)pyridinyl-ethyl-benzamides, \(^23\)pyridine-carboxamides, \(^24\)oximino-acetates, \(^25\)cyano-imidazole, \(^26\)triazolo-pyrimidylamine, \(^27\)triazoles, \(^28\)spiroketal-amines, \(^29\)cinnamic acid amides, \(^30\)mandelic acid amides, \(^31\)cyanoacetamide-oxime, \(^32\)phosphonates, \(^33\)benzophenone, \(^34\)dithiocarbamates, \(^35\)phthalimides, \(^36\)quinones, \(^37\)dimethomorph, \(^38\)proquinazid, \(^39\)mancozeb, \(^40\)propiconazole, \(^41\)kresoxim-methyl; \(^\ddagger\)Yeast abbreviations: \(^1\)A. (Aureobasidium), \(^2\)C. (Candida), \(^3\)Cry. (Cryptococcus), \(^4\)Cyst. (Cystofilobasidium) D. (Debaryomyces), \(^5\)H. (Hanseniaspora), \(^6\)Hyp. (Hypopichia), \(^7\)I. (Issatchenkia), \(^8\)K. (Kluyveromyces), \(^9\)L. (Lachancea), \(^10\)M. (Metschnikowia), \(^11\)P. (Pichia), \(^12\)R. (Rhodotorula), \(^13\)S. (Saccharomyces), \(^14\)Sp. (Sporobolomyces), \(^15\)St. (Starmerella), \(^16\)Tri. (Trichosporon) T. (Torulaspora), \(^17\)W. (Wickerhamomyces), \(^18\)Z. (Zygosaccharomyces). Other fungi abbreviations: \(^1\)Bot. (Botrytis), \(^2\)Cl. (Cladosporium). 454, pyrosequencing; L/A, low abundance; NGS, next generation sequencing (Illumina paired end); PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis); PCR-RAPD (Polymerase Chain Reaction-Random Amplified Polymorphic DNA); RFLP (Restriction Fragment Length Polymorphism).
Figure 1. Factors to consider when investigating yeast biodiversity on grapes. These factors are not mutually exclusive and interactions between all these factors will determine both fungal diversity and the species present.
Figure 3: Mode of action (MOA) of antifungals. *Saccharomyces cerevisiae* is used as an example, but different yeast species will have different cell wall structure and may be more or less resistant to inhibition from fungicides because of this. Groups based on MOA: A, nucleic acid metabolism; B, cytoskeleton and motor protein; C, respiration; D, amino acids and protein synthesis; E, signal transduction; F, lipid synthesis or transport, membrane integrity or function; G, membrane sterol biosynthesis; H, cell wall biosynthesis; I, cell wall melanin synthesis; U, Unknown mode of action (Fungicide Resistance Action Committee 2020). In addition, FRAC codes are used to distinguish the fungicide groups according to their cross-resistance behaviour and define the GROUP Number on product labels (Fungicide Resistance Action Committee 2020).