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Grape Microbiome: Potential and Opportunities as a Source of Starter Cultures

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Abstract

Grape microbiome is the source of a vastly diverse pool of filamentous fungi, yeast and bacteria that will play a coordinated role for the quality of the produced wines. In recent times, the significance of this pool of microorganisms with a long list of studies of the microbial ecology of grape berries of different geographical origin, cultural practices, grape varieties and climatic conditions has been acknowledged. Similarly, the ongoing microbial evolution of must fermentations has been fully uncovered. All these ecology studies, along with detailed metabolic studies and sensorial characterisations of the produced wines, led to the suggestion of the microbial *terroir*. These new concepts are today leading worldwide research efforts to the production of unique wines, preserving their historical identity and verifying their quality and geographical origin. This chapter is a quick but thorough and up-to-date review of how autochthonous microbiota highlight the *terroir* in wines, a comparison of commercial and wild yeast strains and how this biodiversity has been explored. Moreover, technological, physiological and oenological selection criteria will be under consideration. At the end, the positive and negative aspects of wild vinifications, the technological problems of wild strains and some suggestions for the future in starter cultures will be presented.

Keywords: grape microbiome, autochthonous yeasts, microbial *terroir*, *Saccharomyces*, starter culture

1. Introduction

Traditionally, wine making process relies on spontaneous fermentation without the addition of any chemical compounds or externally added microbes to begin the fermentation. Under

these conditions, the biodiversity of the fermenting microorganisms, mainly yeasts and lactic acid bacteria (LAB), as well as the final quality of the resulting wine, is considered to be quite unpredictable. However, several works have shown the positive effects of spontaneous fermentations on the organoleptic complexity of wine as a consequence of the growth of different species and/or strains together, while commercial starter culture driven fermentations show “universally flatten” characteristics [1, 2].

On the other hand, modern oenological practices commonly use commercial starter cultures in order to ensure a controlled fermentation. Although starter cultures are subjected to strict selection for their technological properties for fermentation, they may not be able to compete with the indigenous microbiota of a certain must and for this reason, they cannot dominate the vinification process. The addition of sulphites is usually beneficial in that direction. Recently, the request for wines with unique style is on the increase as well as the demand of special wines, such as Marsala, Madeira, Sherry and Commandaria [3–6].

Several kinds of microorganisms, i.e., yeasts, bacteria and filamentous fungi, are responsible for turning the grape juice into wine, throughout the fermentation. During this process, some species are replaced by others, mostly due to antagonistic actions in order to gain access to nutrients and as a result, eventually, dominate. The substitution of species normally takes place because of the changes that occur in the must matrix turning into wine. Yeasts, such as *Hanseniaspora* (Kloeckera), *Torulaspora*, *Candida* and *Zygosaccharomyces*, are commonly present on the surface of grapes. Although grape cultivar and cultivation provide the foundations of wine flavour, microorganisms, and especially yeasts, impact on the subtlety and individuality of the flavour response. Generally, species of *Hanseniaspora* (Kloeckera), *Candida* and *Metschnikowia*, initiate the fermentation. Sometimes, species of *Pichia*, *Issatchenkia* and *Kluyveromyces* may also grow at this stage. The survival of non-*Saccharomyces* species during the fermentation process is regulated by ethanol production as the main *Saccharomyces cerevisiae* metabolic product. Specific species of *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia* and *Issatchenkia* isolated from grapes and must are sensitive in high ethanol concentrations (more than 5–7%), and that is probably the reason for their decline and finally their death, as the fermentation progresses [7]. Surviving indigenous microorganisms seem to be better adapted to the environmental conditions of a given wine producing area, as well as to the cellar where the winemaking process takes place. Function-targeted ecology studies, also referred as metagenomics, are at the time among the most reliable approaches to analyse the microbiota of fermented products (i.e. wine) and is expected to reveal astonishing results helping to understand the undergoing functions of many times unknown microorganisms in certain substrates [8].

This chapter aims to review in a thorough but concise way all latest literature in the scope of helping connect basic research with application. From the race to define originality of different types of wine worldwide, to basic scientific questions and technological obstacles of oenology, we are reviewing the most current literature in an effort to offer to the reader a conclusive opinion on modern wine microbiology.

2. Autochthonous microbiota in order to highlight *terroir*

Terroir is defined as high complex ecosystem in which the vine interacts with the environmental factors (i.e. soil, climate, humans, etc.) affecting the quality and typicality of the wine produced in a particular location. The biogeography model presented by the uniqueness of the wine grapes, including the microbial heterogeneity at the different viticultural areas, is important in order to preserve and sustain this biodiversity. Additionally, the product quality is enhanced, and as a result the consumer acceptance, as well. Therefore, a financial benefit for both the consumer and the producer is being established. On their journey from the vineyard to the wine bottle, grapes are transformed to wine through microbial activity, which determines a wide range of the wine quality parameters. Wine grapes harbour a wide range of microorganisms originating from the vine, the soil, the fauna, and the humans, many of which are recognised for their role in vine and grapes health and therefore, the wine quality. Nevertheless, the factors affecting the specific region wine characteristics have not been acknowledged, but are frequently assumed to originate from viticultural practices. It has been shown that these microbial aggregations are correlated to specific regional factors, suggesting a link between vineyard environmental conditions and microbial distribution. Bukolich et al. [9] reported that these factors taken together shape a unique microbial fingerprint to regional wines, setting the existence of non-random “microbial *terroir*” as a determining factor in regional variation among wine grapes [9].

Currently, there is a continuously rising interest for autochthonous yeast starters, which are potentially adapted to a specific grape must and reflect the biodiversity of a particular area, which support the idea that indigenous yeast strains can be associated with a “*terroir*” [9–11]. The composition of yeast communities on grapes had been shown to be dependent on several factors, including the geographical location of the vineyard, the type of soil, the age of the vineyard, the grape variety, the harvesting technique, the degree of grape maturation and the grape sanity [12]. Furthermore, it has been demonstrated that certain yeast strains are fully adapted to a specific climatic environment and/or substrate [13]. Some good results have been obtained when selected yeast starters from the micro-area where wines are produced were used for must fermentation [11]. It is quite obvious that in a given wild fermented wine, most of the yeasts derive from the vineyard environment. Further studies are needed in order to better understand the factors influencing yeast diversity in vineyards towards facilitating the selection process.

So far, studies on grape microbiota biogeography are mostly focused on the distribution of yeasts, where *S. cerevisiae* populations vary in respect of their presence or absence at the different regions, often affected by climate and vineyard age and size [14]. Setati et al. [15] interpreted their findings of higher yeast heterogeneity on grape samples collected at different sites inside individual vineyards due to the many microclimates existing even because of differential shading by leaves and grape bunch structure. In this study, fewer differences in the spatial distribution of fungal microbial communities were found between vineyards with very contrasting farming strategies. Bokulich et al. [9] proved that differentiation between regions increases dramatically at the biogeography within a grape variety of vintage. These

findings suggest that factors such as host genotype and of course the vintage also play a significant role.

Introducing microbial ecology into agriculture, observations by farmers and viticulturists can be now better understood. This practice can be used to help improve the wine *terroir* or even reproduce those *terroirs* in sites a priori unsuitable for generating a wine with such characteristics. Upon till recently, the contribution of, and link between, microbes and differential geographic phenotypes (or *terroirs*), of agricultural products has not been objectively verified. It was the work of Knight et al. [16] that performed the first empirical test for whether there is a microbial aspect to *terroir*. The researchers conducted a crucial next step experimentation testing whether the genetic variance in microbial populations correlates with altered crop phenotypes. Their results show a quantifiable microbial aspect to *terroir*.

Generally, only few native *S. cerevisiae* strains are able to dominate the final phases of the process. Some predominant *S. cerevisiae* strains, recovered from spontaneous fermentation in the same winery, could occur over year, assuming that might be some correlation between strain and winery environment. Additionally, some *S. cerevisiae* strains isolated from different wineries located in the same region could be very similar, highlighting a correlation between strains and oenological region [17]. Studies based on genetic and microbiological analyses suggest that a significant part of the mechanisms that generate this genetic polymorphism in this yeast, occur during the vegetative phase of its growth cycle, where meiosis is an infrequent event [18]. This means that, once yeasts reproduce clonally and they are constantly adapting to a specific habitat, there must be a link between the genetic similarity of the strains and their ecological/geographic origin. Geographic or ecological isolation is one of the mechanisms involved in the species differentiation, as it is an obstacle for genetic flow. Thus, strains originating from the same microenvironment will be more alike to each other than with those from other geographic origins [19].

The selection and the employment of autochthonous microorganisms could be a powerful instrument to improve the organoleptic and sensory characteristics of wine produced from indigenous grape cultivars. In fact, autochthonous yeasts are the microorganisms better adapted to a specific must, which detain characteristics determined by the variety of the grapes and the *terroir* and therefore, they are able to exalt the peculiarities (aromas, structure, and colour) of the wine.

3. Commercial versus wild yeast strains

The importance of molecularly determining the autochthonous character of strains collected in strain selection programs for fermentation, is shown by the detection of commercial yeasts from the isolation of wild-type strains [19]. This is in spite of the studies which suggest that the continual use of commercial yeasts on the autochthonous yeast populations has a limited influence [20]. Therefore, there is a possibility that commercial strains used disseminate in the wine cellar and the vineyard of the same or neighbouring vineyards. This is due to oenological

practices that facilitate the dispersion of these yeasts, allowing commercial strains to be erroneously recollected and selected as native strains.

Two main practices are usually used by oenologists. The first is to inoculate the must with commercial dry yeasts according to the manufacturer's instructions. The second one is to let the must ferments spontaneously. This last practice gives quite questionable results, since annual variations on quality and quantity of the dominant autochthonous microbiota, have been observed. However, there might be pitfalls in production also when using commercial dry yeasts, since commercial starter culture driven fermentations show "universally flatten" characteristics. Recently, Orlic et al. [21] used indigenous *Saccharomyces paradoxus* strains in order to study their influence in the aromatic profile of regional wine [21]. The inoculation of musts with *S. cerevisiae* strains selected from indigenous populations, at concentrations allowing the development of wild yeasts, can control the alcoholic fermentation better than commercial yeasts, as well as contribute to the production of more balanced wines [22].

Although there are plenty industrial yeast strains on the market promising to give special sensorial features to the produced wine, they do not possess the necessary metabolic pathways to enhance the typicity of local wines, as the indigenous yeasts has been proved to do so [23]. In a recent study by Borneman et al. [24], the results suggest that many commercial strains from multiple suppliers are nearly genetically identical, suggesting that the limits of effective gene variation within this genetically narrow group may be approaching saturation. They propose that, future strain development efforts should be introgressing new variation from outside of the wine yeast clade into these commercial yeasts, in order to enhance their genetic diversity and as a result their phenotypic one. Obviously, this work also reinforces the point that genetic homogeneity equals to genotypic homogeneity and therefore, to wine homogeneity.

4. Wine yeasts diversity, phylogeny and genomics

As the different strains of *S. cerevisiae* encompass different fermentation properties, their identification is a fundamental process which includes phenotypic, genotypic and karyotypic characterisation and can be applied with several molecular methods such as Polymerase Chain Reaction (PCR) amplification, capillary electrophoresis and fluorescence-based techniques. As more than one *Saccharomyces* strain is involved and interacts with the other strains during fermentation, their identification is of great importance. Despite the fact that non-*Saccharomyces* species that grow during fermentation have a low fermentative capacity, they play a key role in wine flavour as they produce flavour compounds such as esters, higher alcohols, acetic acid and acetaldehyde. Therefore, the identification and differentiation between *S. cerevisiae* and non-*Saccharomyces* species allows the creation of specific mixtures which are used to improve the sensory quality of the wine [7]. The discrimination of different strains is also of ecological interest. New studies aim to exploit the interactions between *S. cerevisiae* wild strains and their environment which highly affects the fermentation products [25]. Characterisation of the strains at a molecular level with high discrimination power techniques, such as multilocus

sequence typing (MLST), helps to understand their biodiversity and dynamics during fermentation and also helps the detection of possible spoiling agents [26].

S. cerevisiae was the first eukaryote whose genome was completely sequenced [27]. Since then, several *S. cerevisiae* industrial strains and particularly wine yeast strains have also been sequenced [28]. Genomics in an industrial context has the potential to provide valuable information for strain development programs and for mapping of quantitative trait loci (QTL) of yeast phenotypic characteristics relevant to a particular process [24, 28].

Likewise, the availability of non-*Saccharomyces* genome sequences will help in the characterisation of commercially relevant strains in order to select useful strains in the future.

The majority of the non-*Saccharomyces* genomes that have been sequenced are type strains, and not strains that are found in the respective must. Notwithstanding, useful information for commercial strains will be provided, especially for the wine yeasts strains. The yeast strains *Lachancea kluyveri*, *Lachancea thermotolerans*, *Debaryomyces hansenii*, *Millerozyma farinosa*, *Candida glabrata*, *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, and *Zygosaccharomyces rouxii* have been fully sequenced, while several have been submitted to NCBI database [29].

The microbial ecosystem of grapes is composed of highly diverse groups of microorganisms which may include the genera *Kloeckera*, *Candida*, *Brettanomyces*, *Cryptococcus*, *Pichia*, and *Rhodotorula* and accompany the grapes into the fermentation vats. These species are of great interest for the wine industry because of their potential for use in mixed starters together with *S. cerevisiae* [30] and for their contribution to the organoleptic characteristics of wine [31]. Recently, SAU-PCR (the name of this technique comes from the restriction endonuclease Sau3AI, used to fragment genomic DNA) and Repetitive Element Palindromic PCR (Rep-PCR) have been used to molecularly characterise *Starmerella bacillaris* strains, and it was proved that isolates from different grapevine cultivars were grouped together [32].

The presence of these yeasts on grape berries are determined by different factors, such as geographical location, climatic conditions, grape variety and maturity, and viticulture practices [14]. Accurate species identification is crucial for ecological studies. Classical identification techniques based on morphological, biochemical, and physiological characteristics may incorrectly identify species because of heterogeneous phenotypic expression of these traits. Development of molecular methods has enabled rapid description of the microbial ecology [14]. Many authors have therefore adopted these methods to study diverse yeast populations. Furthermore, in order to detect populations that are numerically less abundant or in a stressed condition, culture independent methods also provide an important contribution to the study of grape ecology [33].

Cocolin et al. [34] used Denaturing Gradient Gel Electrophoresis PCR (DGGE-PCR) in the field of wine microbiology to validate the identification of yeast isolates from grapes, musts and wine. Since then, the use of PCR-DGGE for studying wine species increased [30, 35]. Alessandria et al. [35] used culture-independent molecular techniques to study the wild mycobiota on Barbera grapes and proved that a fast characterisation of the grape ecology was possible in every stage of the winemaking process. On the other hand, the characterisation of autochthonous *S. cerevisiae* strains is an important step towards the conservation and employment of

microbial biodiversity. The 5.8S rRNA gene flanking the internal transcribed spacers 1 and 2 as a culture-dependent technique has been widely used to identify grape and must yeasts [36, 37]. Cluster analysis employing the use of Random Amplified Polymorphic DNA (RAPD), delta sequences, Restriction Fragment Length Polymorphism (RFLP) and Pulsed Field Gel Electrophoresis (PFGE) have been successfully used to study the molecular polymorphism of wild strains [19, 38] in order to select appropriate starters for winemaking. Also, differentiation of wild *S. cerevisiae* strains in natural fermentations has been achieved by using mtDNA [20, 39] as well as to discriminate strains belonging to different species [40]. mtDNA-RFLP and RAPD-PCR has been used to distinguish between *S. cerevisiae* strains with the first to have better discriminating ability [41]. In addition, Amplified Fragment Length Polymorphism (AFLP) over RAPD genotyping lies in the possibility to amplify much more loci per genome suggesting the suitability of this method for intraspecies discrimination. Employing these techniques, significant diversity of *Saccharomyces* and non-*Saccharomyces* yeasts originating from spontaneously fermented grape musts in Austria has been reported [42]. Using mitochondrial DNA restriction analysis on Chilean non-*Saccharomyces* yeast populations, Ganga and Martinez [43] found that their biodiversity is lower in industrialised zones than in the artisan ones. On the other hand, Schuller et al. [39], using mitochondrial DNA restriction analysis to characterise *S. cerevisiae* yeast populations in Portugal, did not detect a lower diversity of yeasts in areas where commercial strains are of common use, nor did they find commercial strains scattered in the vineyards. However, a subsequent study, using microsatellite analysis carried out on the same yeast populations, detected slight changes on the population structure of strains isolated from areas near cellars [44]. Reports of commercial yeast isolation in areas adjacent to cellars have been published [20].

5. Technological, physiological and oenological selection criteria

It is important to select yeasts that are proper for each kind of wine, territory, vinification techniques and even vineyard, since the role of yeasts in wine production has become complex and strongly associated with the quality of the produced wine. Resistance to high ethanol content and SO₂, the high sugar tolerance, the presence of killer factor, as well as the enzymatic features (proteolytic, lipolytic, β -glucosidase and esterase activity) able to improve the sensorial quality of the product, are the main technological properties yeast strains must possess. Wine quality is also affected by the enzyme activity before, during and after must fermentation. Even though *S. cerevisiae* is the principal wine yeast, it has low enzymatic activity and generally produces wines with ordinary and plain aromatic profiles [23].

On the other hand, non-*Saccharomyces* species have shown great enzymatic activities and especially a great protease and β -glucosidase activity. Moreover, it is well known that the enzymes secreted by non-*Saccharomyces* yeasts have the ability to transform compounds coming from the grapes to various aromatic precursors which are positively influencing the sensorial profile of the produced wines [45]. More specifically, during wine fermentation different non-*Saccharomyces* species secrete significant amounts of proteases which produce odorous compounds such as terpenes, C13-norisoprenoids, esters and ketones and affect the

aromatic quality of the produced wine [7]. Therefore, by screening and measuring the proteolytic activity of non-*Saccharomyces* strains, suitable mixtures of *Saccharomyces* and non-*Saccharomyces* strains can be created in order to facilitate the production of wines with improved aroma and flavour [46]. The proteolytic activity can be tested in media containing gelatin or casein and can be measured by different methods including the determination of the optical density of the solution containing the preferred mixture employing the Cd-ninhydrin method [47]. For the above reasons the ascertainment of the potential of non-*Saccharomyces* species for producing enzymes which can improve the quality of the wine is of major concern for the wine industry. At the same time, those strains can be successfully employed as parental stains in yeast improvement programmes [48]. **Figures 1 and 2** are presenting in a concise way the properties of *Saccharomyces cerevisiae* and the most commonly tested ones respectively.

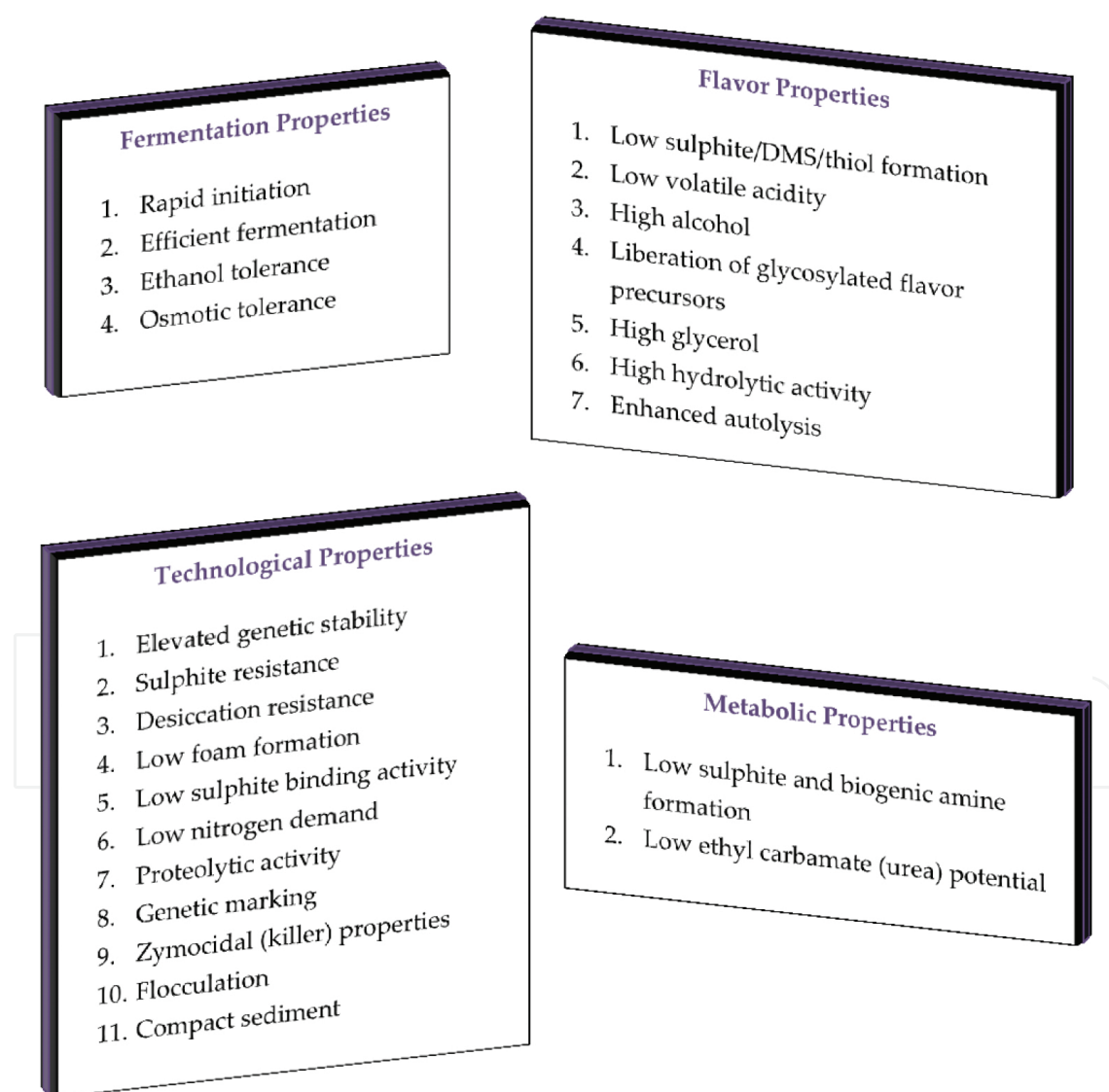


Figure 1. *Saccharomyces cerevisiae* oenological properties as described by Pretorius [49].

The autolytic ability is another very important trait of wine fermentation yeasts. *Saccharomyces cerevisiae* differs among strains and was independent of the degree of flocculation, presenting a great biodiversity that could be useful for starter strains selection in order to improve sparkling wine production [50]. Finally, autochthonous yeasts having the killer factor are much desired in spontaneous must fermentation, especially at high numbers, in order nutrient limitation to be avoided. Any of these chance occurrences, may retard or even stop the fermentation process, decreasing the quality of the resulting product [51].

On the other hand, wines may contain toxic or even carcinogenic molecules, i.e. histamine, ochratoxin A (OTA) and ethyl carbamate, all deriving by microbial enzymatic activity [52]. Different approaches have been conceived to remove OTA in wines, since OTA can be adsorbed by some yeast and bacteria strains [53].

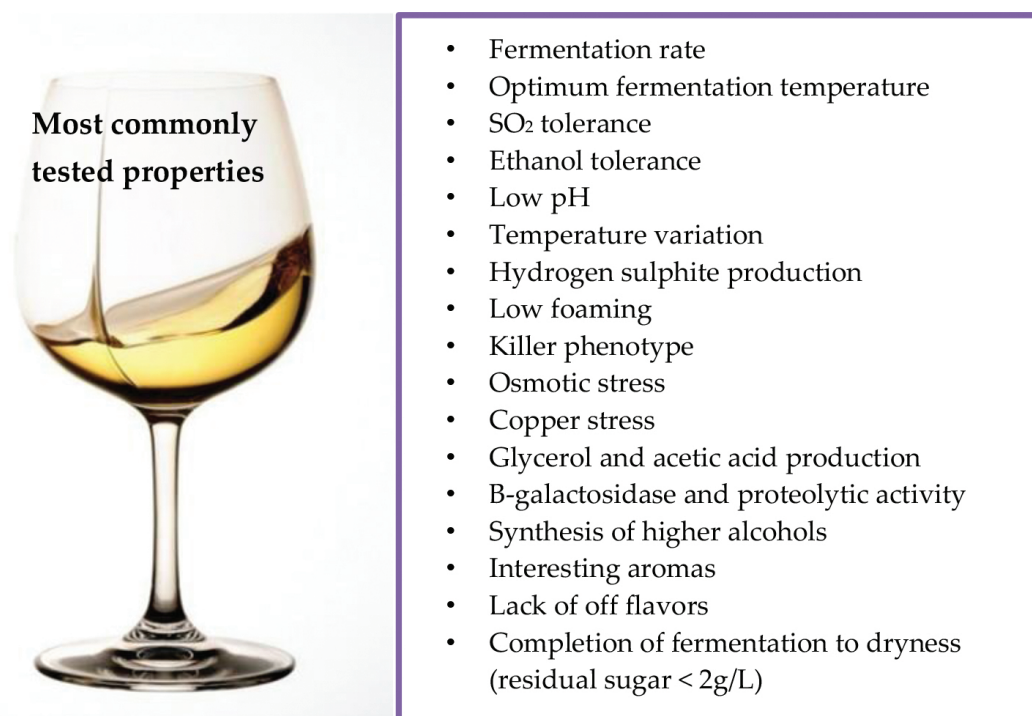


Figure 2. List of most commonly tested properties for *Saccharomyces* and non-*Saccharomyces* isolates.

6. Grape microbiota worldwide ecology

The increase in the worldwide wine market has meant that new countries are now becoming important wine producers. At the same time, the “old” wine producers are looking into new vinification techniques that will enhance their product identity in a highly competitive market. Wine with distinct characteristics has promoted strain selection programs in several countries [19, 35, 38, 54–56]. From the first classic microbial ecology work of Louis Pasteur to today’s microbiome analysis with next generation sequencing (NGS) tools, we now have a good

understanding that a plethora of microorganisms that colonise the grape skin and internal tissues, take place. These microorganisms are primarily yeasts and bacteria and modern studies show that their presence is significantly influenced by the grape varieties, the agricultural practices and the microenvironment [14]. Verginer et al. [57] had shown the role of the autochthonous microbiota on volatile organic compounds. The researchers showed that three single grape associated isolates of *Paenibacillus* sp., *Sporobolomyces roseus*, and *Aureobasidium pullulans* emit typical, well-known flavour components of red wine (i.e. 2-methylbutanoic acid, 3-methyl-1-butanol and ethyl octanoate). It is not yet reported if endophytic microorganisms have a role on grape aromatic compounds but grapevine endophyte studies have progressed [58] and it is very likely to identify such interplay in the near future.

The microbiome consists of yeasts from the basidiomycetous species, that are not able to ferment the juice sugars and are therefore, of non-importance to winemaking and ascomycetous fungi, such as *Aureobasidium pullulans* (also technologically not useful) and the fermentative *Candida* spp., *Hanseniaspora uvarum*/*Klockera apiculata*, *Metschnikowia* spp. and *Pichia* spp. [59]. More fermentative yeasts will follow their dominant presence when alcohol levels exceed 4–5%. These are of the *Saccharomyces* genus, with *S. cerevisiae* as the most prominent, followed by *Saccharomyces bayanus*, *Saccharomyces pastorianus* and *S. paradoxus*. Unfortunately, fermentative yeasts including the species *Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* can spoil wine with off flavours and sediment/cloudiness formation, respectively.

Bacterial species common in grape microbiota are acetic acid bacteria (AAB) and LAB although their control is relatively easy to be succeeded by good manufacturing practices. In addition, *Oenococcus oeni* is of high interest in recent years due to its worldwide appreciation for malolactic fermentation.

Populations of yeasts on grapes surface are 10^2 – 10^4 cells/g although higher counts have been observed while bacteria are usually lower at maximum 10^2 cfu/g. These numbers though vary significantly depending on sampling methods and more importantly on berries conditions. In an exhaustive review by Barata et al. [14] the authors propose a very simple but applicable systematic for the wine microbial consortia on grape berries. The following three main groups characterised by similar behaviour on the berries are particularly dependent on nutrient availability on berry skins:

1. Oligotrophic, oxidative basidiomycetous yeasts, the yeast-like fungi *A. pullulans*, and LAB (*Lactobacillus* spp., *O. oeni*). Species favoured on poor environments-intact berries.
2. Copiotrophic, oxidative ascomycetes (several *Candida* spp.); weakly fermentative apiculate (*Hanseniaspora* spp.), film-forming (*Pichia* spp.), fermentative (*Candida zemplinina*, *Metschnikowia* spp.) yeasts. The emergence of these species is likely a result of juice and volatile organic compounds release as berries initiate their ripening process and cuticle is softened releasing these compounds.
3. Copiotrophic strongly fermentative yeasts (*Saccharomyces* spp., *Torulaspora* spp., *Zygosaccharomyces* spp., *Lachancea* spp. and *Pichia* spp.) and the obligate aerobic acetic acid bacteria (*Gluconobacter* spp., *Gluconacetobacter* spp., *Acetobacter* spp.). This group may be explained by the high nutrient availability as a result of berries damage.

[illegible]

Species	France	Italy	Spain	Greece	Portu- gal	Slov- enia	Austria	Can- da	Bra- zil	Argen- tina	Ja- pan	Aus- tra- lia	In- dia	Chi- na	South Africa	Hun- gary	Chile-Peru- Uruguay	New Zea- land
<i>Kregervanrija fluxuum</i>							+											
<i>Lachancea thermotolerans</i>							+											
<i>Metschnikowia</i> spp.	+	++	+			+	+++			+++				+				
<i>M. fructicola</i>		++																
<i>Metschnikowia pulcherrima</i>	++														+++			
<i>Metschnikowia viticola</i>							+											
<i>Penicillium brevicompactum</i>															+			
<i>P. corylophilum</i>															+			
<i>P. glabrum</i>															+			
<i>Pleospora herbarum</i>															+			
<i>Pichia</i> spp.	+	+	+		+	+				++			++	++				
<i>Pichia terricola</i>					+													
<i>Pichia fermentans</i>		+																
<i>Pichia kluyveri</i>							++											
<i>Pichia kudriavzevii</i>					+													
<i>Rhodotorula glutinis</i>		+																+++
<i>Saccharomyces</i> spp.	+	+																
<i>S. uvarum</i>																+		
<i>S. bayanus</i>							++									+		
<i>S. cerevisiae</i>	++	++	++				+++						++			+++	+++	

Species	France	Italy	Spain	Greece	Portu- gal	Sloven- ia	Aus- tria	Cana- da	Bra- zil	Argen- tina	Ja- pan	Aus- tra- lia	In- dia	Chi- na	South Africa	Hun- gary	Chile-Pe- ru-Uru- guay	New Zea- land
<i>Saccharomycopsis</i> spp.		++																
<i>Saccharomyces ludwigii</i>										+								
<i>Sporobolomyces roseus</i>	++																	
<i>Starmerella bacillaris</i>					+++										+			
<i>Pichia manshurica</i>					+													
<i>Torulaspora</i> spp.			+		+													
<i>T. delbrueckii</i>	+	+																
<i>Zygoascus hellenicus</i>					+		+											
<i>Zygosaccharomyces</i> spp.		+												+				
<i>Z. bisporus</i>					+													
<i>Z. bailii</i>			+		+									+				
Sampling	Be	Be	Be, Bu	Bu	Be	Bu	Bu	Bu	Be, Bu	Bu	Bu	Bu	Bu	Bu	Be	Bu	Be	Be
Culture media	G	G	G, A	G,S	G,S	G	A, S	G	G	G	–	G	G	G,S	NGS	G, S	G	G
References	[60–62]	[63, 64]	[65, 66]	[67]	[38, 68, 69]	[70]	[42]	[71]	[72]	[73]	[74]	[12]	[75]	[76]	[77]	[78]	[19]	[63]
Yeast and yeast like species isolated from sound grapes or berries at harvest. The data are collected from published surveys (see references) and “+” in dicates relative proportion of the detected species Be: berry, Bu: bunch G: general purpose, A: autoenrichment, S: selective media NGS: Next Generation Sequencing (metagenomic approach)																		

Table 1. Yeast and yeast like species isolated from sound grapes or berries at harvest.

Worldwide surveys seem to indicate that apparently sound grapes are colonised by a wide variety of yeast species without any obvious explanation.

Table 1 summarises the most indicative surveys on yeast species found in the respective countries.

7. Vinification examples with autochthonous starter cultures: pros and cons

The last four decades, wine industries worldwide try to exploit new indigenous strains of *S. cerevisiae* in order to produce wines with specific characteristics resulting from the biodiversity of each different area. Studies done on Debina must, a white-wine producing variety, in Zitsa (Epirus, Greece) have shown that a specific indigenous strain was the most predominant and responsible for a variety of aromas in the produced wine [25]. Another interesting example of application of indigenous *S. cerevisiae* strains in winemaking is that of Negroamaro wines, where selected strains are used to produce Negroamaro wines in Salento (Apulia, Italy) and share interesting volatile profiles that are associated with their geographical origin [79]. The application of combined mixtures of *S. cerevisiae* and non-*Saccharomyces* strains has widely been used, in cases such as the production of Italian passito wines, where studies have shown that the combination of *Botrytis cinerea* strains (non-*Saccharomyces* species with great esterase, glucosidase and protease activities) with *S. cerevisiae* strains can result to the production of highly improved passito varieties [1]. Moreover, studies on Italian Amarone wine have shown that mixtures of *S. cerevisiae* and *S. bayanus* strains, which are used during fermentation in different wineries in Valpolicella area (Italy), all produce specific amounts of isobutanol and amylic alcohols and therefore contribute to the production of traditional varieties with desired aromatic and flavour features [2]. An indigenous *S. cerevisiae* strain can be used in both primary and secondary fermentations which are needed for the production of Champenoise sparkling wine, as it responds perfectly to the stressful conditions presented in both fermentations such as low nitrogen content and increased accumulation of toxic by-products [80]. Moreover, Aponte and Blaiotta [81], used a selected *S. cerevisiae* autochthonous strain as starter culture in the production of “Moscato di Saracena”, a southern Italy passito wine, and suggested that the physicochemical traits obtained, showed better characteristics compared to those obtained by spontaneous fermentation. Finally, various studies were undertaken in order to develop region-specific starter cultures, such as wines in ‘El Penedes’ area of Spain [13] and sweet white wine in Tokaj area of Hungary [82]. They demonstrated that native selected strains may be better adapted to fermentation conditions than commercial strains, and selected inoculated strains were found to play an important role in the resulting wine.

As the importance of *S. cerevisiae* role in winemaking has long been established, the use of the commercial strains of these yeast cultures in fermentation is an ordinary practice in order to ensure a reproducible product and to reduce the risk of wine spoilage. However, this approach can cause a progressive substitution of local microflora and a consequent reduction of microbial biodiversity. Indeed, knowledge of the autochthonous yeast strains will help to preserve and employ the most representative strains which will enhance the quality charac-

teristics and retain the product typicity. The selection and the employment of autochthonous microorganisms could be a powerful tool in order to improve the organoleptic and sensory characteristics of wine produced from indigenous grape cultivars.

8. Problems with wild strains

Wine obtained with pure culture fermentation of non-*Saccharomyces* yeast may show several problems, due to their fermentative behaviour or metabolite compounds production. Non-*Saccharomyces* yeasts can produce several secondary compounds, such as acetic acid, acetaldehyde, acetoin and ethyl acetate, compounds which are undesirable even at low concentrations. They also cause the presence of off-odours, such as ethyl and vinyl phenols, generally produced by *Brettanomyces* spp. and/or *Dekkera* spp. [83]. In addition, the majority of the non-*Saccharomyces* strains lack of good fermentative parameters, i.e., poor SO₂ resistance, low power and rate of fermentation. Nevertheless, some negative traits of non-*Saccharomyces* yeasts may not be expressed or could be modified during multi-starters fermentations in the presence of *S. cerevisiae* strains [31].

Similarly, spoilage species of LAB, AAB and, occasionally, *Bacillus* and *Clostridium* species may grow in wines during storage in the cellar and after bottling [59]. Their growth is probably encouraged by nutrients released by autolysis of wine Yeasts, as well as *O. oeni* strains [84]. Fornachon [85] reported that the spoilage yeasts, *Pichia* spp., *Saccharomycodes ludwigii* and *Candida pulcherrima*, showed an inhibitory activity towards spoilage LAB (i.e. *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*) which could be caused by the toxic concentrations of sulphur dioxide produced by the above mentioned yeasts.

Moreover, studies concluded that besides LAB, some yeasts such as *S. cerevisiae* and *Brettanomyces bruxellensis* are also responsible for biogenic amine formation. Various histaminogenic abilities of the yeasts have been confirmed in fermentation tests [86]. However, the relation between the concentrations of the biogenic amines and their precursor amino acids during fermentation depend on the yeast strain involved in the fermentation. Together with the decarboxylating aptitude of the starter cultures, the presence and relative activity of amino-oxidases (or amino-acid oxidases) should be considered as an important factor in the selection of starter cultures for wine production. Inoculation with species and strains of LAB with none or low forming capacities of biogenic amines reduces their occurrence in wine [87].

9. Future perspectives

Several studies undertaken in different countries attributed an important contribution of non-*Saccharomyces* species to yeast growth dynamics during wine fermentations [88, 89]. Hence, non-*Saccharomyces* yeast species supply a factor of diversity that requires specific studies to avoid any negative consequences, and to exploit their beneficial contributions [88]. Yeasts populations on grapes and in must, the effect of winemaking practices on these yeasts, as well

as how their metabolites interact with each other and with LAB, must be known [48]. In addition, during the last years, the improvement and application of molecular approaches for the analysis of yeast populations have shown that, together with species variability, spontaneous fermentation is characterised by a significant intraspecific biodiversity [34], as well as by a high genetic polymorphism observed in the population of *S. cerevisiae* present during spontaneous fermentation. That is to say, the population of yeasts correlated to wild wine fermentation is composed of genotypically different strains with most likely different phenotype and therefore, potentially capable of influencing, in proportion to their relative abundance, the flavour profile of the resulting wine [90].

As the demand for high quality wines is emerging worldwide, the need for discovering new strains and new innovative techniques for their application in wine production is increasing. An example of the effort given by wine industry to implement new techniques is the management of nutrient availability and uptake before and during fermentation which has the potential to increase the biomass production by *S. cerevisiae* [91]. The same nutrient demands should be explored for the non-*Saccharomyces* species as well. Another aspect of interest is the understanding of the kinetic and metabolic behaviour developed by mixtures of *Saccharomyces* and non-*Saccharomyces* strains, as it can contribute to the production of wine yeasts with improved technological characteristics which can be used for the production of improved quality wines [8, 92]. In addition, studies done on experimental hybrids of different *Saccharomyces* species like *S. cerevisiae* and *S. bayanus* exploit the production of new yeasts through a variety of evolutionary programmes [29]. Moreover, as *S. cerevisiae* is a stable microorganism that can survive under the unfavourable conditions during the winemaking process, studies on recombinant yeast strains aim to the creation of yeasts with excellent fermentative behaviour and improved oenological characteristics [93]. Also an interesting case is the one of the application of auxotrophic strains of *S. cerevisiae* which have the ability to produce large quantities of high quality fermentation products at very low growth rate [94].

Saccharomyces cerevisiae is by far the most widely used yeast in oenology. However, many studies of wine fermentation ecology have shown that several other yeast species participate in the phenomenon and can positively impact wine quality. *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Lachancea thermotolerans*, *Hanseniaspora uvarum*, *Starmerella bacillaris* are now proposed as starter cultures in mixed fermentations with *S. cerevisiae*. The knowledge of these non-conventional yeasts is increasing because of the advancement in genomic and proteomic analysis tools. The next step lies on the development of selection programs and/or genetic improvement of these non-conventional species. In addition, next generation sequencing is for seeing to help the efforts in wine differentiation based on the biological/genetic fingerprint [95].

The scientific community should enhance its efforts studying microbial genetic fingerprint and metabolic footprints, resulting from biodiversity and microbial activity, respectively, in order to preserve food heritage and support the typicality and authenticity of traditional fermented products.

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