Microbial ecology of wine: Modeling the Microbial Ecology

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CHAPTER 28
Microbial ecology of wine

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28.1 Introduction

Wine quality depends on many factors, the microorganism's activity being one of the more important. A wide diversity of microorganisms, yeasts, and bacteria are involved in winemaking and, thus, determination of the composition and evolution of the different species present during this process would clearly help to increase the quality of a wine.

After confirmation of the role of microorganisms as responsible for the alcoholic fermentation by Pasteur in the nineteenth century, the predominant role of the genus *Saccharomyces*, mainly the species *S. cerevisiae* and *S. bayanus*, on the complete fermentation of the grape sugars became clear. However, nowadays it is known that the grape-wine ecological habitat has a much more complex microbial biodiversity.

Several highly specialized species of yeast and bacteria are active in different phases of the fermentation and contribute to the transformation of grape juice to wine. During winemaking, the biodiversity initially present on the grape surface and in the early stages of fermentation tends to decrease as the ethanol content, the main limiting factor during fermentation, increases. Generally, at the end of the fermentation, exclusively *S. cerevisiae* and *S. bayanus*, the best adapted species to high ethanol content in the medium, are found. Despite this, the contribution of the so-called non-*Saccharomyces* species present at the start of fermentation and their concentration variation until the final dominance of *S. cerevisiae* can be crucial in determining the quality of the wine (Fleet, 2003; Jolly *et al.*, 2006).

Malolactic fermentation (MLF) is a biochemical transformation conducted by lactic acid bacteria (LAB), which usually takes place after alcoholic fermentation during winemaking, and is generally desirable in the production of red wines, as well as in some white wines. MLF produces a biological deacidification of wine by transforming malic acid in lactic acid, with a consequent increase in the pH; it also contributes to the microbiological stability of wine and leads to the production of many secondary compounds that induce changes in the organoleptic properties of the wine (Davis *et al.*, 1986; Lonvaud-Funel, 1999; Ugliano *et al.*, 2003). This process can be conducted by LAB belonging to the genera *Oenococcus, Lactobacillus, and Pediococcus* (Wibowo *et al.*, 1988); however, the main agent of MLF is *Oenococcus oeni*, because of its ability to grow in the particular conditions of wine, which are a high ethanol content, low pH, and the presence of SO_2 (Wibowo *et al.*, 1988; Davis *et al.*, 1988; Kunkee, 1991).

28.2 Biodiversity of grape microorganisms

Several studies over the past 10 years have shown that the biodiversity and the quantity of the microorganisms present on the surface of the grape berry is highly dependent on many

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factors, including the health state of the grapes, the climate temperature, and the pesticide treatments. Recent works seems to confirm that the "terroir" idea should be extended to the microbiological aspect, that is, that the geographical distribution of the organisms associated with grapes is not randomly dispersed but is dependent on the cultivar, the location of the vineyard, and the vintage (Bokulich *et al.*, 2014).

Recently, it has been reported that while yeast counts fluctuated between 10^2 and 10^5 in healthy grapes, it is two orders of magnitude higher in damaged berries (Barata *et al.*, 2012). The number and type of species of yeasts are strongly influenced by the grapes health, as it influences the access of microorganisms to the nutrients of the juice.

Three groups of yeast can be distinguished on the grape surfaces (Barata *et al.*, 2012):

1 An oligotrophic group with oxidative metabolism represented by *Basidiomycetes*, such as *Cryptococcus* spp. and *Rhodotorula* spp., which dominate the surface poor in nutrients of the sound berry.
2 A group of ascomycetes with an oxidative, or weakly fermentative, metabolism represented by *Metschnikowia pulcherrima* and some species of the genus *Candida, Hanseniaspora*, and *Pichia*, being *H. uvarum*, the yeast most frequently detected. These species increase their presence and become dominant during ripening.
3 A strongly fermentative group is detected on the surface of damaged berries, which, as commented above, used to have a greater number of yeast cells. Although basidiomycetes and ascomycetes oxidative yeasts are also present, usually, under this condition, the predominating species are strong fermenting yeasts, such as *Zygosaccharomyces* spp. and some species of *Candida* and *Torulaspora*. Interestingly, it has been described that some yeasts are frequently associated to fungal grape diseases. For instance, *M. pulcherrima* and *Candida zemplinina* are recurrently found in botrytized grapes and juices (Sipiczki, 2003, 2006).

S.cerevisiae, which predominates during alcoholic fermentation, is rarely found on the surface of the berry. This fact supports the view that the winery environment, and not the vineyard, represents the natural habitat of this yeast (Martini, 2003).

On the other hand, and since LAB are minor partners of the grape microbiota (the initial LAB population in wine grapes is low, around 10^2 cfu/g, Bae *et al.*, 2006), few studies have focused on the bacteria associated with grapes. For example, *Lactobacillus plantarum, L. casei, L. brevis, L. hilgardii, L. curvatus, L. buchneri, Leuconostoc dextranicum*, and *Leuconostoc mesenteroides* were inconsistently isolated from several grape varieties harvested from vineyards in Spain (Sieiro *et al.*, 1990; Suàrez *et al.*, 1994), France (Lafon-Lafourcade *et al.*, 1983), and Germany (Weiller and Radler, 1970). Moreover, most studies have failed to detect *O. oeni* in grapes or vineyards (Bae *et al.*, 2006; Lafon-Lafourcade *et al.*, 1983; Renouf *et al.*, 2007), despite its frequent isolation from winery environments after fermentation (Edwards *et al.*, 1991; Garijo *et al.*, 2009).

One of the few common enological bacteria detected on grape skins is *Gluconobacter oxydans* (Renouf *et al.*, 2007), an acetic acid bacteria (AAB), an important group of bacteria in the food and beverage industry, which can oxidize ethanol to acetic acid. AAB are ubiquitous and have also been found on grapes (Bartowsky, 2008; Bartowsky and Henschke, 2008; González *et al.*, 2005; Valera *et al.*, 2011). Up to 10^6 cell/g of AAB, mainly *Acetobacter*, can be found in damaged grapes (Joyeux *et al.*, 1984).

28.3 Microorganism ecology in winemaking

The microbial composition of grape must after crushing reflect the composition on the berry surface at harvest. Later, the availability of nutrients and the high concentration of sugar promote the growth of fermentative species, while the others tend to succumb or be inactivated.

Numerous variables define the ecological mosaic of fermentation and several works have analyzed the microbial development under different fermentation conditions (Fleet, 1993; Torija *et al.*, 2001; Beltran *et al.*, 2002; Van Keulen *et al.*, 2003; Di Maro *et al.*, 2007; Bezerra-Bussoli *et al.*, 2013).

28.3.1 Yeast ecology

Taking into account a spontaneous fermentation of grapes, that is, without the massive inoculation of selected strains of *S. cerevisiae* that upsets the natural ecology in winemaking (whose advantages and disadvantages are currently subject of discussion), the following ecological phases can be outlined:

Phase 1: Crushing. The microbial composition in crushed, and eventually in pressed grapes, reflects that of the grapes they come from. Among these species, those able to grow in the grape must be able to do so because of their fermentative metabolism and are relatively few in number; the most frequently found are *M. pulcherrima*, some *Candida* species, including *C. stellata*, and *H. uvarum*. *S. cerevisiae* is usually present at very low concentrations in the must.

Phase 2: Initial phase of alcoholic fermentation. In this step the growth of non-*Saccharomyces* species is favored by their initial concentration and fermentative metabolism, and their persistence is dependent on the ethanol resistance of single species. For instances, *M. pulcherrima* succumbs above 2–3% ethanol, while *Candida* and, especially, *K. apiculata* can survive at up to 6–8%. During this stage *S. cerevisiae* grows quickly until it gradually becomes the dominant species.

Phase 3: Prevalence of *Saccharomyces*. Although some species such as *C. stellata* can resist high concentrations of ethanol, the exponential growth of *S. cerevisiae* tends to dominate the fermentation, being usually the only species detected by the end of the process. The concentration of *S. cerevisiae* can reach in the order of 10^8 in 5–6 generations, and it can complete the fermentation of grape musts under sugar concentrations greater than 250 g/l. Even though, at this stage, a great biodiversity of *S. cerevisiae* species are present (Valero *et al.*, 2007; Schuller *et al.*, 2005), the fermentation is mainly conducted by a small number of strains. (Versavaud *et al.*, 1995).

Numerous factors can affect both the number and the charge of the species present during the winemaking process, especially among the non-*Saccharomyces* yeasts.

The harvesting system, manual or mechanical, can determine major or minor damage to the berries and modify the composition of the initial microbial load in the must, especially if the time interval between harvesting and crushing is delayed (Boulton *et al.*, 1996). Some antifungals used against *Botrytis* seem to favor the growth of *Metschnikowia pulcherrima* in must (Regueiro *et al.*, 1993). Cold settlement of must in the prefermentative step and low fermentation temperatures commonly represent growth advantage for non-*Saccharomyces*, such as *Candida* spp. and *Hanseniaspora uvarum*, while high temperatures promote *S. cerevisiae* growth (Hierro *et al.*, 2006).

During fermentation, ethanol concentration is the most prominent variable that determines the temporal sequence of yeast species predominance; only a few can grow above 10% v/v, such as some species of *Candida, Torulaspora, Zygosaccharomyces*, and *Schizosaccharomyces pombe*.

The sulfur dioxide content affects the amount of *Basidiomycetes* present after crushing, but has less influence on the fermentative yeasts, with S. *cerevisiae* usually being less sensitive than the non-*Saccharomyces* spp. (Rementeria *et al.*, 2003).

Another important factor is the period at which fermentation takes place. When conducted at the beginning of the harvest time the influence of the vineyard microflora is greater than at the end, since, as *S. cerevisiae* is strongly implanted in the winery (on tanks and equipment surfaces), it quickly overcomes the non-*Saccharomyces* species (Ribéreau-Gayon *et al.*, 2004).

28.3.2 LAB ecology

Grape must frequently contains bacterial species, 10^2–10^3 cfu/ml, of the genera *Lactobacillus, Pediococcus, Leuconostoc,* and *Oenococcus* (Costello *et al.*, 1983; Fleet *et al.*, 1984; Pardo and Zuniga, 1992; Fleet, 1993; Fugelsang and Edwards, 1997).

During the first days of alcoholic fermentation, LAB can increase to a maximum of 10^4 cfu/ml, and then decline due to the presence of SO_2, the ethanol content, and the competition with yeasts (Fugelsang and Edwards, 1997). At the end of the alcoholic fermentation, the bacteria population increases again, up to the 10^6 cfu/ml necessary to start MLF.

A crucial factor in this phase is the pH, as it determines the species of LAB present in wine; usually *O. oeni* is the only one present in wines having pH below 3.5, while a higher pH promotes the growth of *Lactobacillus and Pediococcus* spp. (Davis *et al.*, 1986). In fact, a recent study by Juega *et al.* (2014) showed that bacteria isolated in Albariño and Caiño wines, with a pH of about 3.6, were *Pediococcus* spp., which successfully perform MLF on the wine without negative effects.

28.4 Microorganism ecology during aging

After alcoholic and malolactic fermentations, the reduction of nutrients and fermentable compounds, together with the racking, fining, and filtration operations and storage during the aging of wine, tend to drastically reduce the load of microorganisms in wine. After these two fermentations, with a few exceptions related to the particular typologies of some wines, any residual microorganism should be considered a contaminant and should ideally be absent. Even *S. cerevisiae* must be considered as a spoilage yeast, for example, in the elaboration of sweet dessert wines, due to its capability to ferment residual sugar and, thus, to alter the quality of the wine. Normally, in the case of dry wines with alcohol contents greater than 12–13%, few microorganisms are able to survive and to be active. In the undesirable case of tanks not properly dried after being washed, some species of *Candida* spp. and *Pichia* spp. are able to form films that, if not eliminated, can produce high amounts of acetic acid and other substances with a negative sensorial impact. Yeast of the genera *Zygosaccharomyces*, particularly *Z. bailii* and the specie *Saccharomycodes ludwigii*, can cause refermentations in wines with residual sugar contents due to their resistance to high concentrations of ethanol and SO_2 (Loureiro and Malfeito-Ferreira, 2003).

The genus *Brettanomyces/Dekkera* is probably considered the most detrimental contaminant microorganism for the quality of red wine. Several studies conducted worldwide (Chatonnet *et al.*, 1995; Gerbaux *et al.*, 2000; Suàrez *et al.*, 2005) have shown that the presence of this microorganism in the wine field is a stringent problem. Currently, the genus *Brettanomyces*, anamorph of *Dekkera*, includes five species, with *Brettanomyces bruxellensis* being the most common in wine (Henick–Kling *et al.*, 2000; Kurtzman *et al.*, 2011).

Brettanomyces were isolated in equipment, walls, floors, and oak barrels used in the winery. The contamination by this yeast is usually manifested after alcoholic and malolactic fermentations, mostly during aging in barrels (Chatonnet, 2000). These yeasts have the capability of growing under very wide ranges of temperature, acidity, sulfur dioxide, and ethanol (Gerós *et al.*, 2000; Silva *et al.*, 2004), frequently remaining in a latent state, ready to grow when the conditions become favorable and reaching concentrations of 10^4–10^5 cells/ml. From the sensorial point of view, *Brettanomyces* growth is detrimental for the wine's quality, due to the appearance of strong, unpleasant odors, described as horse sweat, band aid, or burnt plastic. These sensorial faults are mainly caused by volatile phenols produced by this yeast, starting from phenol precursors present in wine.

Moreover, poor management during bottling and storage of red wine can also give rise to spoilage by *Acetobacter pasteurianus* (Bartowsky

and Henschke, 2004, 2008), with the undesirable production of acetic acid. Du Toit *et al.* (2005) isolated AAB in tanks and barrels, thus showing that these bacteria can survive in quite low oxygen availability. Different authors (Waters *et al.*, 1996; Caloghiris *et al.*, 1997; Bartowsky and Henschke, 2008) suggested that the oxygen permeation of the natural cork can facilitate the formation of the neck ring deposit in the bottles.

In addition to AAB, some LAB can also cause spoilage in wine and give some faults to wine; for example, some species of *Pediococcus damnosus* and *Pediococcus pentosaceus* can produce exopolysaccharides, molecules implicated in ropiness (Lonvaud-Funel *et al.*, 1993; Fugelsang and Edwards, 1997), while some *Lactobacillus* can, for example, degrade the glycerol with the subsequent production of acrolein, implicated in the development of bitterness in wine.

28.5 Microbial identification by classical methods

The adoption of appropriate methods of microbial identification is essential to study the presence and evolution of the microbial species during the winemaking process. Classical identification techniques based on morphological, physiological, and biochemical essays (Barnett *et al.*, 1990; Kurtzmann *et al.*, 2011) have been largely overcome in the last two decades by molecular analysis based on the microorganism's genome. Major criticisms of the traditional methodologies are the time needed to get results and their reliability and reproducibility. In fact, phenotypical assessments based on morphological and biochemical traits are influenced by the physiological state of the cells.

However, some classical methods are still used, allowing the rapid determination of the total number of microorganisms in must and wine, and the rapid identification of some of them. Microscope observation, supported by counting the chamber cells (e.g., the Bürker chamber) and methylene blue staining, gives rapid information on total cells, viability, and, in a few cases, allows identification of the genus. For example, genus *Hanseniaspora* and *Saccharomycodes* (*Saccharomycodes ludwigii*) can be easily identified by their characteristic lemon-shape morphology and distinguished by their size, with *Saccharomycodes* being much larger than *Hanseniaspora* (8–10 and 15–20 μm, respectively). *Schizosaccharomyces* is also recognized due to their fission reproduction. However, overall, the other wine-related species have the anonymous ovoid shape with a budding reproduction and are indistinguishable from microscope observations. Figure 28.1 shows some species observed using the optical microscope.

Most of the bacteria grown in wine can be isolated by traditional microbiological techniques using nutrient agar media. The most common used medium for LAB is MRS (deMan Rogosa Sharpe broth), sometimes added with 20% tomato (or apple or grape) juice.

Information on the morphological and physiological characteristics and on the evolution of taxonomic and systematic information related to yeasts can be followed in the constantly updated editions of *The Yeast: A Taxonomic Study* (Kurtzman *et al.*, 2011) and for bacteria in *Bergey's Manual of Systematic Bacteriology* (Vos *et al.*, 2009).

The main characteristics of the genera (yeast and bacteria) found in wine are displayed in Table 28.1.

28.6 Microbial identification by molecular methods

In the last two decades, DNA-based diagnostic techniques have revolutionized the study of microorganisms, and new methods are continuously being developed for the molecular identification and characterization of yeast and bacteria (reviewed in Ruiz *et al.*, 2000; Pozo-Bayón *et al.*, 2009; Fernandez-Espinar *et al.*,

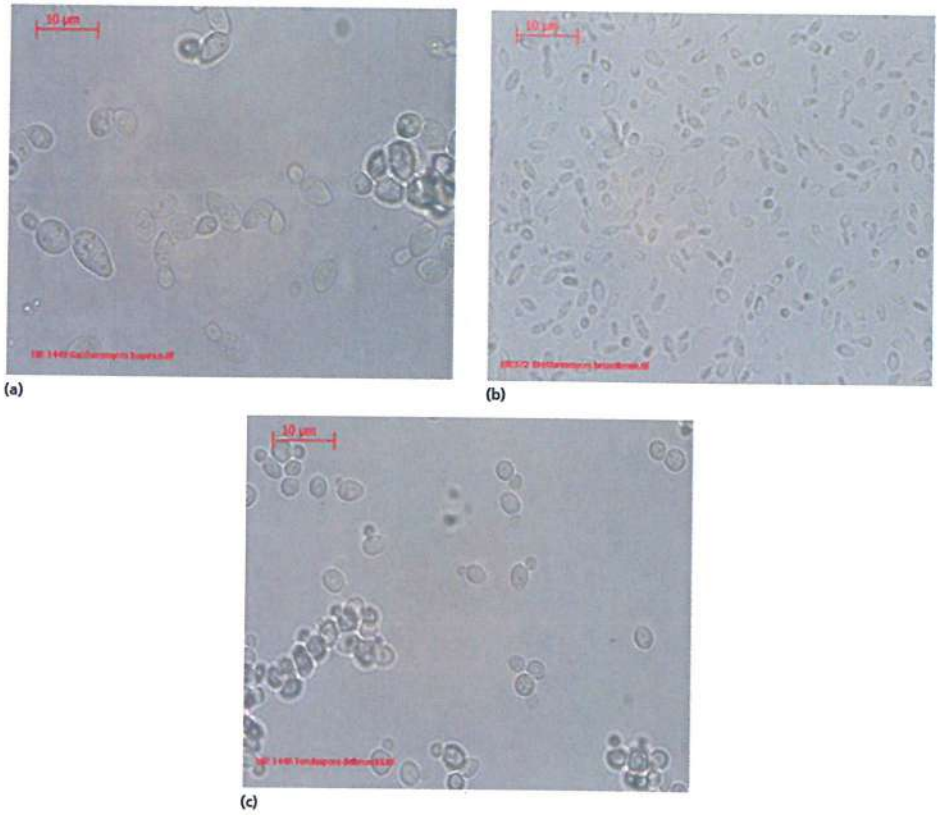

Figure 28.1 Yeast species observed under microscope: (a) *Saccharomyces bayanus*, (b) *Dekkera bruxellensis*, (c) *Torulaspora delbrueckii*.

2011; Ivey and Phister, 2011). Table 28.2 displays the main molecular identification techniques for microorganisms of wine.

Molecular methods can be direct or indirect. In the latter, the microorganisms have to be cultivated on agar media, before the species and their frequency can be subsequently determined by molecular analysis of DNA extracted from a random sample of the colonies grown. This allows an accurate determination at the species level and sometimes a subspecific characterization; however, it has the drawbacks of the time consumed and its incapability to detect viable but non-cultivatable (VBNC) cells (Millet and Lonvaud-Funel, 2000).

In direct methods, molecular analysis is performed on the sample (must or wine) without prior cultivation, reducing the time needed and allowing the detection of non-cultivatable species. One drawback is the difficulties in differentiating viable from dead cells, as the target DNA (and in some case also RNA) persists after the death of the microorganisms (Hierro *et al.*, 2006).

Recently the metagenomic approach has been applied to the study of microbial communities in ecosystems, providing a great insight into the processes responsible for microbial diversity; for example, it has been shown that the microbial population is strongly related to climatic conditions, grape variety, and vineyard environmental conditions (Bokulich *et al.*, 2014). These authors concluded that there is a unique microbial pattern that influences the

Table 28.1 Main species and characteristics of must/wine related species: (A) yeasts; (B) bacteria.

(A) Yeasts

Genus	Morphology	Cell size (μm)	Main species in wine
Saccharomyces	Spherical Elongated	5–10 × 5–12	*S. cerevisiae* *S. bayanus* *S. exiguus* *S. pastorianus* *S. paradoxus*
Candida	Ellipsoidal Elongated	2.2–3.0 × 3.0–5.2	*C. stellata* *C. vini* *C. vinaria* *C. membranifacies* *C. zemplinina*
Debaryomyces	Spherical Short oval	2–7 × 2.4–8.5	*D. hansenii*
Hanseniaspora	Lemon shape, elongated	1.5–5 × 2.5–11.5	*H. guillermondii* *H. osmophila* *H. uvarum* *H. vinae*
Kluyveromyces	Ellipsoidal Spheroidal	3–6.5 × 5.5–8	*K. lactis* *K. marxianus* *K. thermotollerans*
Metschnikowia	Globose Elongated	2.5–5 × 4–7	*M. pulcherrima*
Pichia	Ovoidal Elongated	1.8–4.5 × 2.5–17	*P. anomala* *P. fermentans* *P. membranifaciens*
Saccharomycodes	Elongated Lemon shaped	4–7 × 8–23	*S. ludwigii*
Schizosaccharomyces	Globose Ellipsoidal	3–5 × 5–15–24	*S. pombae* *S. japonicus*
Torulaspora	Ellipsoidal	2.5–6.5 × 2.5–7	*T. delbrueckii*
Zygosaccharomyces	Ovoidal Ellipsoidal	3.5–7 × 5.5–14	*Z. balii* *Z. bisporus* *Z. rouxii*
Brettanomyces	Ellipsoidal Elongated	2–7 × 3.5–18	*B. bruxellensis* *B. anomalus*

(B) Bacteria

Genus	Morphology		Cell size (μm)	Main species in wine
Lactobacillus	Rods	Single, pair chains	0.5–0.7 × 1–10	*L. delbrueckii* *L. casei* *L. plantarum* *L. hilgardii* *L. brevis* *L. buchneri* *L. fermentum*

(Continued)

Table 28.1 (Continued)

Oenococcus	Coccus	Pairs chains	0.5–0.6×0.7–0.8	*O. oeni*
Pediococcus	Coccus	Tetrads pairs	0.5×1.1	*P. damnosus* *P. parvulus* *P. pentosaceus*
Leuconostoc	Coccus		0.5–0.7×0.7–1.2	*Leuconostoc mesenteroides*
Acetobacter	Ellipsoidal Rod-shaped	Single pair	0.6–1×2–4	*A. aceti* *A. pasteurianus*
Gluconobacter	Ovoid	Single pair	0.5–0.8×0.9–4.2	*G. oxydans*

Table 28.2 Molecular methods used to identify wine microorganisms.

Technique	Level of identification	Microorganism	Reference
PCR- species specific	Species	*Oenococcus oeni*	Zapparoli *et al.*, 1998
		Saccharomyces cerevisiae, S. bayanus	Josepa *et al.*, 2000
		Zygosaccharomyces	Harrison *et al.*, 2011
		Brettanomyces	Phister and Mills, 2003
			Cocolin *et al.*, 2004
RFLP	Species	Yeasts	Esteve-Zarzoso *et al.*, 1999
	Species	Lactic acid bacteria	Claisse *et al.*, 2007
	Species	Acetic acid bacteria	Gonzáles *et al.*, 2004
DGGE	species	yeasts	Cocolin *et al.*, 2001
		lactic acid bacteria	Renouf *et al.*, 2006
			Lopez *et al.*, 2003
		acetic acid bacteria	De Vero *et al.*, 2006
PFGE	strain	*Oenococcus oeni*	Larisika *et al.*, 2008
		Brettanomyces	Miot-Sertier and Lonvaud-Funnel, 2007
		Saccharomyces	Vaugan-Martini *et al.*, 1993
microsatellite	strain	*S. cerevisiae*	Legras *et al.*, 2005
	strain	*S. cerevisiae*	Vaudano and Garcia-Moruno, 2008
interdelta region	strain	*S. cerevisiae*	Legras and Karst, 2003
mtDNA	strain	*S. cerevisiae*	Guillamón *et al.*, 1994
RAPD	species	*Lactobacillus*	Du Plessis and Dicks, 1995
	species	yeasts	Quesada and Cenis, 1995
	strain	*Oenococcus oeni*	Reguant and Bordous, 2003
	strain	*S. cerevisiae*	Xufre *et al.*, 2000

wine quality and asserts the existence of non-random "microbial terroir".

In conclusion, yeasts and bacteria growth is characterized by specific metabolic activities, which determine the final organoleptic characteristics of wine. Therefore, the possibility of knowing the grape and wine biodiversity can help to have better control of the fermentation processes and also offers a tool to detect unwanted yeasts or bacteria, which can depreciate the wine.

The development of molecular techniques for the identification of species and strains are providing strong support for microbiologists and winemakers.

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