International Journal of Food Microbiology 153 (2012) 243–259

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Review

The microbial ecology of wine grape berries

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ARTICLE INFO

Article history:
Received 8 July 2011
Received in revised form 28 October 2011
Accepted 27 November 2011
Available online 2 December 2011

Keywords:
Microbial ecology
Grapes
Damaged grapes
Spoilage yeasts
Acetic acid bacteria
Lactic acid bacteria

ABSTRACT

Grapes have a complex microbial ecology including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects upon wine production. Some species are only found in grapes, such as parasitic fungi and environmental bacteria, while others have the ability to survive and grow in wines, constituting the wine microbial consortium. This consortium covers yeast species, lactic acid bacteria and acetic acid bacteria. The proportion of these microorganisms depends on the grape ripening stage and on the availability of nutrients. Grape berries are susceptible to fungal parasites until *véraison* after which the microbiota of truly intact berries is similar to that of plant leaves, which is dominated by basidiomycetous yeasts (e.g. *Cryptococcus* spp., *Rhodotorula* spp. *Sporobolomyces* spp.) and the yeast-like fungus *Aureobasidium pullulans*. The cuticle of visually intact berries may bear microfissures and softens with ripening, increasing nutrient availability and explaining the possible dominance by the oxidative or weakly fermentative ascomycetous populations (e.g. *Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp., *Pichia* spp.) approaching harvest time. When grape skin is clearly damaged, the availability of high sugar concentrations on the berry surface favours the increase of ascomycetes with higher fermentative activity like *Pichia* spp. and *Zygoascus hellenicus*, including dangerous wine spoilage yeasts (e.g. *Zygosaccharomyces* spp., *Torulaspora* spp.), and of acetic acid bacteria (e.g. *Gluconobacter* spp., *Acetobacter* spp.). The sugar fermenting species *Saccharomyces cerevisiae* is rarely found on unblemished berries, being favoured by grape damage. Lactic acid bacteria are minor partners of grape microbiota and while being the typical agent of malolactic fermentation, *Oenococcus oeni* has been seldom isolated from grapes in the vineyard. Environmental ubiquitous bacteria of the genus *Enterobacter* spp., *Enterococcus* spp., *Bacillus* spp., *Burkholderia* spp., *Serratia* spp., *Staphylococcus* spp., among others, have been isolated from grapes but do not have the ability to grow in wines. Saprophytic moulds, like *Botrytis cinerea*, causing grey rot, or *Aspergillus* spp., possibly producing ochratoxin, are only active in the vineyard, although their metabolites may affect wine quality during grape processing.

The impact of damaged grapes in yeast ecology has been underestimated mostly because of inaccurate grape sampling. Injured berries hidden in apparently sound bunches explain the recovery of a higher number of species when whole bunches are picked. Grape health status is the main factor affecting the microbial ecology of grapes, increasing both microbial numbers and species diversity. Therefore, the influence of abiotic (e.g. climate, rain, hail), biotic (e.g. insects, birds, phytopathogenic and saprophytic moulds) and viticultural (e.g. fungicides) factors is dependent on their primary damaging effect.

Contents

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doi:10.1016/j.ijfoodmicro.2011.11.025

1. Introduction

Grapes used for making wine have been studied for several decades. The earlier work of Louis de Pasteur, by the last quarter of the XIXth century, already showed that the microorganisms responsible for wine fermentations are yeasts present on the grapes. Since then a huge amount of information has been gathered on yeast dissemination in wine associated environments but ecological relationships are still to be fully understood. The prime focus has been the study of *Saccharomyces cerevisiae* given its relevance to winemaking. Earlier isolations using grape juice fermentations lead to the conclusion that this species was frequent in grapes (as reviewed by Vaughan-Martini and Martini, 1995). However, the use of direct isolation techniques showed a much different picture, revealing that *Saccharomyces* spp. is an absent or rare contaminant of grapes (Davenport, 1973, 1974). This fact has lead to a still lively debate on the origin of the yeasts responsible for wine fermentation, being hypothesised that *S. cerevisiae* may be regarded as the first domesticated microbe (Martini, 1993).

Pioneering works on grape microbial ecology describe the main yeast species isolated from grapes and their environs (as reviewed by Fleet et al., 2002), while bacteria have been less studied. The recent increase in the number of grape-related yeast ecological studies did not contribute to a significant body of new knowledge. On the other hand, other areas of yeast ecology, like those related with plants, insects, soil or extreme habitats, have witnessed a significant development (Rosa and Péter, 2006) which provide useful guidance to study grape microbiota. In fact, most recent grape-related works are mostly dissemination studies. Microbial ecology is a wider concept, studying the ecosystems with their microbial interactions, microbial vectors, sources and sinks of microorganisms (Boddy and Wimpenny, 1992). Herman Phaff, the pioneer of yeast ecology, described ecology as "where microbes live and why they live in one habitat and how yeasts interact with other microorganisms" (Lachance, 2003). This global approach has been scarcely adopted to study the vineyard environment (Fleet et al., 2002). There are large gaps in the knowledge of the diversity and significance of yeast–yeast, yeast–fungi and yeast–bacteria interactions on grapes (Fleet, 2003). This may be because the microorganisms relevant to vine and grapes diseases (phytopathogenic parasitic and saprophytic fungi) do not survive and grow in wine, while the species of the wine microbial consortium (WMC: yeasts, acetic acid bacteria and lactic acid bacteria) are not responsible for those diseases. The apparent rising of sour rot incidence, where the agents are part of the WMC (Barata et al., 2008a, in press), justify a reappraisal of the microbial ecology of grapes.

In this review we will attempt to make an update of the current awareness on the microbial ecology of grape surfaces, describing the species diversity, the factors affecting the species balance and the interactions with other microorganisms and vectors. We will also evaluate the current methods for microbial recovery and identification, trying to explain the contradictory results often found in literature. Special attention will be paid to the distribution of WMC species responsible for wine spoilage and to the effect of grape diseases on wine quality, given their technological importance. The question of the origin and dissemination of the WMC in the environment will be discussed aiming to provide future research directions.

1.1. Technological significance of microbial grape contaminants

The microbial species recovered from grapes may be divided into several groups according to their technological significance in grape and wine production. Under this view, microorganisms are characterised as a function of their effect on grape and wine quality.

The vine plant and grapes may be affected by a series of diseases of which the most well known are downy mildew (*Plamospara viticola*), powdery mildew (*Erysiphe necator*) and grey rot (*Botrytis cinerea*), which are mostly prevented by phytochemical application. In addition, grapes may also bear saprophytic moulds (*e.g.* *Cladosporium* spp., *Aspergillus* spp., *Penicillium* spp.) responsible for several grape rots or mycotoxin production. However, these fungi do not have the ability to grow in wines and their effect on wine quality is due to grape damage. Contrarily, the microorganisms of the WMC are able to survive or grow on wine, depending on the efficiency of adequate processing measures. Thus, based on the concept already suggested by the authors (Loureiro and Malfeito-Ferreira, 2003; Malfeito-Ferreira, 2011), the species in this consortium may be grouped into: (i) easily controllable or innocent species, without the ability to

spoil wine when good manufacturing practises (GMP's) are applied; (ii) fermenting species, responsible for sugar and malic acid conversion; and (iii) spoilage *sensu stricto* species responsible for wine alteration even when GMP's are believed to be applied. Table 1 lists the most relevant microorganisms of referred groups, including recognised empirical denominations.

Concerning yeasts, the innocent group includes basidiomycetous species which are regarded as irrelevant to winemaking due to their inability to ferment juice sugars or to survive in wines. The ascomycetous dimorphic fungus, *Aureobasidium pullulans* (also called black yeast), a common yeast-like species, is also technologically irrelevant. The oxidative, weakly fermentative or fermentative ascomycetous species (*Candida* spp., *Kloeckera apiculata*/*Hanseniaspora uvarum*, *Metschnikowia* spp., *Pichia* spp.) are present in pre-fermentation steps or at the beginning of fermentation. The fermentation ability is not a well defined taxonomic feature and several species may be regarded as weakly fermentative or not. Among these, apiculate yeasts are determined by their microscopical shape and some strains may produce off-flavours in juices before or during fermentation (Romano, 2002). Film-forming yeasts (e. g. *Pichia*) owe their denomination to the ability of forming pellicles on the surface of bulk wines, being common contaminants of grapes, juices and wines, with the ability to produce off-flavours. Both apiculate and film-forming yeasts are regarded as contaminants because good manufacturing practises prevent their activity. Fermentative yeasts include those responsible for wine fermentation, where *S. cerevisiae* is the most important, but other species (*S. bayanus*, *S. pastorianus* and *S. paradoxus*) may also conduct or participate in the process (Skelin et al., 2008; Arroyo-López et al., 2010). These species may also be seen as wine spoilers if their activity persists beyond the fermentative steps of wine or sparkling wine production. The exception is that of *Saccharomyces* spp. forming desirable films on the surface of particular oxidative ageing processes of Sherry-like wines (Farris et al., 2002). Additionally, fermentative species comprise the spoilage *sensu stricto* yeasts which are technologically relevant due to their ability to spoil wines, either by off-flavour production (e. g. *Dekkera bruxellensis*) or sediment and cloudiness formation (e. g. *Zygosaccharomyces bailii*), under conditions following the GMP rules.

Concerning bacterial species, acetic acid bacteria are regarded as innocent because they are easily controllable by GMP's in the winery, although the exaggerated production of acetic acid during grape sour rot is a serious threat to wine quality. The physiological diversity of lactic acid bacteria does not allow a precise assessment of their technological significance. The typical agent of malolactic fermentation is *O. oeni* while *Lactobacillus* spp. and *Pediococcus* spp. may be responsible for spontaneous fermentations (Lerm et al., 2010). These species may spoil wine when their activity goes beyond malic conversion, particularly in high pH wines, producing off-flavours or biogenic amines (Arena et al., 2011; Capozzi et al., 2011; Pan et al., 2011). Grapes also bear a wide diversity of bacterial species common in nature or in other food related environments (Table 1). However, they should not be integrated in the WMC because they do not influence wine quality.

2. Methods to recover and identify grape microbial species

Several reviews on the analytical approaches to study overall yeast ecology have been published elsewhere (Boundy-Mills, 2006; Ciani et al., 2002; Kurtzman et al., 2011). We will try to extend the discussion to bacterial species and to the issues related to grape analysis, taking in consideration the advice of Lachance (2003) to obtain ecologically meaningful conclusions: (i) adequate sample size, (ii) correct identification, (iii) habitat characterisation, (iv) substrate sampling, that is far more important than the sampling procedure, and (v) sample replication, which is more important than serial dilutions and plate counts.

2.1. Sampling schemes

Yeast populations in nature suffer spatial and temporal fluctuations (Fonseca and Inácio, 2006) that must be taken into account when devising sampling schemes. Ecological conclusions must be based on extensive sampling. In fact, different bunches of grapes bear different populations and it is more informative to analyse several smaller samples than to blend several bunches into a larger one (Barata et al., 2008b). Samples should be taken in several locations in the vineyard so that spatial fluctuations dictated by the uneven microbial distribution are minimised. Repeated sampling over the years is also a sound practise to understand the behaviour of natural microbial populations and avoid unsupported conclusions.

2.2. Sample picking and treatment

Once sampling schemes are defined, the first step in grape analysis concerns the choice of the method to pick the grapes. The main concern should be to aseptically separate sound berries from damaged ones given their completely different microbial load. This cannot be achieved if whole bunches are analysed because damaged berries may be hidden inside. As an example, a sound bunch containing 50 sound berries with an average of 10^3 CFU/berry would contain 2×10^4 CFU/berry if only one damaged berry with 10^6 CFU/berry blended together with the sound berries. Further, the number of species of the "sound" bunches would reflect the diversity of the damaged berry and not of the whole sound bunch. Berries may be picked separately in the vineyard, or grape bunches are collected in the field and the berries are separated in the laboratory to give a 300 g sample. When bunches are picked and taken to the laboratory, sound berries should be chosen from fully sound bunches after visual inspection because sound berries from partially damaged bunches bear higher yeast numbers (Barata et al., 2008a).

The isolation from grape juices obtained in the winery (industrial or experimental) may only be regarded as an approximation of the natural grape microbiota. In fact, when grape juice is only sampled in the winery, even at the beginning of fermentation, a different picture of yeasts species may be obtained, due to bulk transport, crushing or pressing in the winery (Barata et al., in press; Sturm et al., 2006). Therefore, it is mandatory to isolate yeasts from grapes aseptically collected in the vineyard and they must be processed as quickly as possible because grapes damaged during transport to laboratory may accumulate higher numbers of yeasts (Yanagida et al., 1992).

The method for yeast dislodgement from grapes is also of importance. Belin (1972) discussed the efficiency of recovery methods, saying that simple washings with shaking may not be enough. Later, Martini et al. (1980) presented evidence of the importance of strong disruptive methods followed by enrichment cultures to obtain an exhaustive picture of the yeast flora. Direct enrichment gave lower number of species, than washing and sonication, but enrichment may be the only way to recover fermenting species. These authors advised enrichment in one sample, and agitation or percolation followed by sonication in another sample, but they did not try grape blending. This method was performed by Combina et al. (2005) who found higher results with grape blending in plastic bags, than with jet streaming and shaking. Prakitchaiwattana et al. (2004) evaluated recovery during 4 successive rinsings for 10 min each. Rinsing released 80% of total yeasts in the first wash and 96% in damaged grapes. Differences in species diversity through all steps were not apparent or were due to a dilution effect. Therefore, the classical food sample suspension in peptone or saline solutions followed by stomaching and serially diluting is a reliable option.

2.3. Enrichment cultures

When juice is obtained after grape blending or when suspensions are recovered after grape or single berry washing, the following step

Table 1
Dissemination and technological significance of microbial species isolated from the vineyard and winery environment.
Data compiled from Loureiro and Malfeito-Ferreira, 2003; Malfeito-Ferreira, 2011, and Loureiro et al., in press).

Group	Metabolism	Genus	Relevant species	Technological significance	Main Sources	Occasional sources
Filamentous fungi	Obligate parasites		*Plamospara viticola*	Downy mildew	Vine tissues	
			Erysiphe necator	Powdery mildew	Vine tissues	
	Saprophytic moulds	*Botrytis*	*B. cinerea*	Grey rot, Noble rot	Ubiquitous	
		Aspergillus	*A. alliaceus*, *A. carbonarius*, *A. niger aggregate*, *A. ochraceus*	*Aspergillus* rot, ochratoxin A producers	Ubiquitous	
		Penicillium	*P. expansum*	Green mould, patulin producer	Ubiquitous	
		Cladosporium	*C. herbarum*	*Cladosporium* rot	Ubiquitous	
		Colletotrichum	*C. acutatum*	Ripe rot	Ubiquitous	
		Greeneria	*G. uvicola*	Bitter rot	Ubiquitous	
Yeasts						
Basidiomycetous	Oxidative	*Filobasidium*, *Cryptococcus*, *Rhodotorula* (pink yeast)		Absent/unknown	Soil, bark, leaf, grape	Grape juice
Ascomycetous	Oxidative	*Aureobasidium*	*A. pullulans* (yeast-like fungi, black yeast)	Absent/unknown	Soil, bark, leaf, grape	Grape juice
	Oxidative or weakly fermentative	*Hanseniaspora*/*Kloeckera* (apiculate yeast)	*H. uvarum*/*K. apiculata*	Contamination/spoilage	Grape, grape juice, fermentation	Soil
		Candida (film-forming yeast)		Contamination	Grape, grape juice, fermentation, wine	Bark, soil, insects
			C. stellata or *C. zemplinina*	Contamination	Grape, grape juice, fermentation, wine	
			Zygoascus hellenicus /*C. steatolytica*	Contamination	Grape, grape juices	Fermentation, insects
		Metschnikowia	*M. pulcherrima*	Contamination	Grape, fermentation	
		Pichia (film-forming yeast)	*P. anomala*	Contamination/spoilage	Grape, fermentation	Insects
			P. membranifaciens	Contamination/spoilage	Grape, fermentation, wine	
			P. guilliermondii	Contamination/spoilage	Grape, fermentation, wine	
		Debaryomyces	*D. hansenii*	Contamination	Grape, fermentation	
		Lachancea (ex Kluyveromyces)	*L. thermotolerans*	Contamination	Grape, fermentation	
			L. fermentati (ex *Z. fermentati*)		Grape, Fermentation	
	Fermentative	*Torulaspora*	*T. delbrueckii*	Spoilage	Wine, concentrated grape juices	Grape, fermentation
		Zygosaccharomyces	*Z. bailii*	Spoilage	Wine	Grape, fermentation
			Z. bisporus	Spoilage	Wine	Grape, fermentation
			Z. rouxii	Spoilage	Concentrated grape juices	
		Dekkera/*Brettanomyces*	*D. bruxellensis*	Spoilage	Wine	Grape, insects
		Saccharomyces	*S. cerevisiae*	Fermenting/spoilage	Fermentation, wine	Soil, Grape
			S. bayanus	Fermenting/spoilage	Fermentation, wine	Grape
			S. paradoxus		Fermentation, wine	Grape
			S. pastorianus		Fermentation, wine	Grape
		Schizosaccharomyces	*Sc. pombe*	Spoilage	Wine	Grape, fermentation
		Saccharomycodes	*S. ludwigii*	Spoilage	Wine	Grape, fermentation
Bacteria						
Acetic acid bacteria	Aerobic	*Gluconobacter* spp., *Acetobacter* spp., *Gluconoacetobacter* spp.,		Wine spoilage, vinegar production	Grape, wine	Insects
Lactic acid bacteria	Anaerobic, semi-anaerobic	*Oenoccocus* sp., *Lactobacillus* spp., *Pediococcus* spp., *Weissella* spp.		Malolactic fermentation or wine spoilage	Grape, wine	
Several bacterial species		*Acinetobacter* spp., *Curtobacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Enterobacter* spp., *Enterococcus* spp., *Bacillus* spp., *Staphylococcus* spp.		Innocuous contaminants	Grapes	

is plating or to continue using enrichment cultures. If juice is allowed to ferment spontaneously this corresponds to an auto-enrichment step. True spontaneous fermentations are performed in the absence of sulphur dioxide additions. If this is added, sensitive species are affected. The enrichment may also be done with culture media where single berries, diluted suspensions or juices are introduced.

The enrichment step elicits the recovery of minority species which would not be detected by plating. The typical example is the wine fermenting *S. cerevisiae*, as mentioned before, but the recovery of other technologically relevant species also requires this approach. Renouf and Lonvaud-Funel (2007) only detected the dangerous spoilage yeast *D. bruxellensis* by using a selective medium as an enrichment step. Washing solutions of grapes did not reveal this species. The enrichment step also enabled the detection of *S. cerevisiae* and other fermentative species (*C. cantarelli* and *P. fermentans*) while oxidative basidiomycetous species (*Cryptococcus laurentii*, *Rhodotorula mucilaginosa* and *Sporidiobolus pararoseus*) were detected by these authors in the washing solution. The isolation of the malolactic agent *O. oeni* also required this type of approach using washing solutions (Renouf et al., 2005) or enrichment broths (Renouf et al., 2007). The matter of appropriate culture media may be required to isolate *O. oeni* but using enrichment broths Bae et al. (2006) did not manage to recover this species. On the contrary, these authors detected species of *Lactobacillus*, *Enterococcus*, *Lactococcus* and *Weissella* using enrichments broths.

2.4. Culture media

The choice of culture media is critical for characterising complex microbial populations (Loureiro et al., 2004). The utilisation of general purpose culture media directly from grape juice or dilutions only enables the recovery of the most frequent and faster growing species. Spread plates using a 0.1 ml sample are commonly preferred and colonies of the most representative species occupy the medium surface and cover minority colonies, leaving undetectable those representing less than about 1% of the population (Fleet et al., 2002).

The recovery of minority species and/or slow growers requires the use of selective media, possibly following sample enrichment. If the enrichment step is done with selective media then a general purpose medium may be used to isolate growing species because the overall populations were already restricted. Wine spoilage species are typically minority species and so it is advised to use selective media. Several reports mention selective media for *Schizosaccharomyces pombe* (Florenzano et al., 1977), *Z. bailii* (Schuller et al., 2000), *D. bruxellensis* (Rodrigues et al., 2001) and *S. cerevisiae* (Kish et al., 1983). Lactic acid bacteria are also minor constituents of berry microbiota and profit from the utilisation of specific media supplemented with ethanol or tomato juice (Bae et al., 2006).

The use of antifungal compounds is advisable to restrict the growth of filamentous fungi, although some yeast species may be affected (Loureiro et al., 2004). Further, the selection of adequate antibiotics is essential to detect separately yeasts, lactic acid and acetic acid bacteria. When moulds or environmental bacteria are numerous and a quantification of WMC species is wanted it is advisable to use the Most Probable Number (MPN) technique (Loureiro et al., 2004). With appropriate choice of culture media, antibiotics and incubation conditions, it is possible to inhibit, at least partially, the background microbiota and obtain numbers of the selected species (Barata et al., in press).

2.5. Incubation conditions and colony selection

After surface inoculation plates should be kept upright when yeasts are to be recovered, as opposed to bacteria (Deák and Beuchat, 1996). Incubation temperature is usually set at 25–30 °C, but lower temperatures (10 °C) are essential to recover *S. uvarum* (Sampaio and Gonçalves, 2008). Aerobic conditions are preferred but anaerobiosis was advised for *Sc. pombe* (Florenzano et al., 1977) and lactic acid bacteria (Barata et al., in press). In liquid media, the availability of oxygen may also be varied by using different shaking rates. Another important issue is to use long incubation periods, up to 14 days, specially when wine spoilage yeasts are screened (Florenzano et al., 1977; Rodrigues et al., 2001; Schuller et al., 2000).

After incubation, strain choice by colony observation must be done by experienced technicians, small differences are frequently overlooked, being also indispensable to perform microscopical examinations. Frequently, same colony morphologies belong to different species, being a good practise to choose more than one colony for each morphological type.

2.6. Identification methods

Accurate identification is obviously crucial for the establishment of microbial communities. It is accepted that classical identification techniques based on morphological, biochemical and physiological criteria (Barnett et al., 1990; Kreger-van Rij, 1984; Krieg and Holt, 1984–1989) may have provided incorrect results in the past or could not reach species definition due to heterogeneous phenotypical results. This is particularly true for species of the genus poorly defined by conventional methodologies (e. g. *Candida* spp., *Pichia* spp.), and so it is not surprising that the number of their species is increasing in recent surveys. Moreover, the reproducibility of these techniques is somewhat questionable, since in many cases they depend on the physiological state of the cells. Molecular biological techniques circumvent these difficulties by allowing direct analysis of the genome, irrespective of the physiological state of the cell, providing more precise identifications.

2.6.1. Molecular methods for yeast species identification

The principles underlying yeast identification by molecular techniques have been previously addressed (Giudici and Pulvirenti, 2002). This theme is subjected to a permanent evolution and more recent reviews provide an adequate update of the available methodologies (Cocolin et al., 2011a; Fernández-Espinar et al., 2011; Querol and Fleet, 2006). The main technical alternatives for species identification are briefly described below, while approaches required for accurate studies of source tracking or evolutionary assessments are available elsewhere (Fernández-Espinar et al., 2011).

2.6.1.1. Sequencing of ribosomal DNA. Yeast species can be identified by comparison of nucleotide sequences from rDNA regions. The two most commonly used regions are the D1 and D2 regions encoding the 26S (Kurtzman and Robnett, 1998) and 18S (James et al., 1997) ribosomal subunits. The availability of sequences in DNA databases, particularly for the D1/D2 region of the 26S gene, makes this technique useful for assigning unknown yeast to a specific species when the homology of the sequence is greater than 99% (Kurtzman and Robnett, 1998).

2.6.1.2. Restriction analysis of ribosomal DNA (rDNA). Simpler methods have been designed based on PCR amplification of rDNA regions followed by restriction analysis of the amplified products. A very useful rDNA region that can be used to differentiate between species is that containing the 5.8S gene and the adjacent intergenic regions ITS1 and ITS2. This technique was used by Guillamón et al. (1998) for the rapid identification of wine yeasts, and was later extended to yeasts associated with foodstuffs and beverages (de Llanos et al., 2004; Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000). The amplified fragments and restriction profiles for these species with *Hae*III, *Hinf*I, *Cfo*I and *Dde*I are available online at http://yeast-id.com/. In the absence of restriction profiles the solution is to sequence the ribosomal

DNA as described before. When restriction profiles are coincident, identification may also rely on the utilisation of classical biochemical tests (Barata et al., 2008a, 2008b).

2.6.1.3. Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE). This genetic fingerprinting technique based on PCR amplification and denaturing gradient gel electrophoresis (DGGE) has been introduced into microbial ecology by Muyzer et al. (1993). This technique allows DNA fragments of the same length to be separated on the basis of sequence differences. DNA migration is retarded when the DNA strands dissociate at a specific concentration of denaturing agent. A related technique is temperature gradient gel electrophoresis (TGGE), which is based on a linear temperature gradient for separation of DNA molecules (Fernández-Espinar et al., 2011). The DGGE and TGGE methods have only recently been used, for yeast identification in wine fermentations (Andorrà et al., 2008; Cocolin et al., 2000; Di Maro et al., 2007; Prakitchaiwattana et al., 2004; Renouf et al., 2007; Urso et al., 2008). These techniques are directly applied to the sample but their low sensitivity is a major drawback to study minority populations.

2.6.2. Molecular methods for LAB species identification

A wide variety of molecular techniques have been used to characterise LAB from wine. These methods were recently reviewed by Munõz et al. (2011). Amplified rDNA restriction analysis (ARDRA) has been used as a rapid, reliable method of identifying the main LAB involved in winemaking (Rodas et al., 2003; Ventura et al., 2000).

Amplified rDNA fragment analysis via PCR followed by DGGE has also been used to compare diversity and monitor changes in populations of LAB during the winemaking process (Lopez et al., 2003). More recently. Rodas et al. (2005) concluded that the randomly amplified polymorphic DNA (RAPD) and ribotyping are useful for identifying and classifying LAB, whereas ARDRA is useful only for identification purposes and the restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE) is useful for distinguishing between different LAB strains of the same species (Rodas et al., 2003).

2.6.3. Molecular techniques for the rapid identification of AAB

Sequencing is one of the methods proposed for the identification of AAB and generally involves ribosomal genes or the region between the 16S and 23S ribosomal genes (Yamada and Yukphan, 2008). The latter involves intergenic regions known as internal transcribed spacers (ITSs). New approaches were recently developed to detect and group different microorganisms using simple, rapid, and inexpensive techniques in order to reduce the cost of DNA sequencing in descriptive ecological studies evolving hundreds of samples (Guillamón and Mas, 2011).

Several molecular techniques have been developed for the rapid and reliable identification of most AAB species, particularly those present in grapes, wine, and vinegars. The first method involves restriction analysis of the 16S ribosomal gene following amplification by PCR using a protocol known as 16S-ARDRA (Poblet et al., 2000; Ruiz et al., 2000). More recently González et al. (2006a) proposed a method based on the RFLP-PCR of the 16S rRNA and 16S-23S ITS regions, involving the sequential use of different restriction enzymes to allow the grouping and distinguishing of the currently described AAB species. This approach has been used for the identification of AAB present in grapes (Prieto et al., 2007), wines (González et al., 2004, 2005) and vinegars (Gullo et al., 2006; Ilabaca et al., 2008).

Real-time quantitative PCR methods circumvents the problem of culturing AAB, which affects the previous culture depending techniques and have been successfully used for the identification and quantification of AAB in grapes, wine and vinegars (González et al., 2006b; Torija et al., 2010).

Finally, DGGE and TGGE can also be used for the identification of AAB in combination with PCR to separate amplified fragments of the 16S gene according to small sequence differences (Guillamón and Mas, 2011). DGGE (Andorrà et al., 2008; De Vero et al., 2006; Gullo et al., 2009; Lopez et al., 2003) and TGGE (Ilabaca et al., 2008) methods also circumvent the problem of culturing AAB and have been successfully used for the identification AAB in grapes, wine and vinegars.

2.6.4. Other molecular and instrumental techniques

Metagenomics is an increasingly used alternative, which does not require cloning or PCR amplification, and can produce huge numbers of DNA readings to study uncultured organisms (Huson et al., 2007). Its application to grape musts evidenced the expected microbiota diversity and the unexpected presence of some species unusual in wines, such as *Enterobacter* spp. (Nisiotou et al., 2011) or the green algae *Dunaliella tertiolecta* (Gomes et al., 2009).

Fourier transformed infrared spectroscopy has been reported as a powerful technique to identify yeasts, moulds and bacteria (Wenning et al., 2002, 2010; Santos et al., 2010) but we are not aware of reports concerning grape berry microbiota.

In conclusion, according to our experience, grape sampling is the critical step to obtain sound ecological data. The evaluation of the factors affecting the microbial ecology of grapes depends on berry selection according to its health status and on adequate experiment repetition during more than 2 annual cycles. In addition, species diversity is enhanced by the utilisation of selective media and enrichment techniques, particularly to recover minor populations. Identification can be achieved by molecular techniques chosen according to the targeted species. At present, direct molecular identification is only suitable for the dominant populations, being difficult to apply when samples are contaminated by a wide variety of moulds, yeasts and bacteria.

3. Characterisation of grape microbial populations

3.1. Population quantification

The yeast populations of grapes are roughly comprised between 10^2 and 10^4 cells/g (Fleet et al., 2002), but higher values have also been reported (Table 2). This wide range of values may be explained, at least partially, by bunch sampling without accurate separation of damaged berries, as explained before. When this is done, smaller variations are found (Barata et al., 2008a). Table 3 shows a range of total counts provided by surveys where samples were separated according to health status. As a rule, damage induced at least an increase of one log cycle. The availability of high sugar concentrations explains the higher populations on damaged grape berries. The exception is related to damage by honeydew produced by mealybugs, where counts are similar in sound and affected berries, probably because of antimicrobial compounds and high sugar concentration, as in honey produced by bees (Barata et al., 2008b).

Bacterial populations are usually several orders of magnitude lower than those of yeasts in sound grapes. Lactic acid bacteria have counts lower than 10^2 CFU/g (Barata et al., in press; Francesca et al., 2011), explaining the use of enrichment cultures for their current detection (Bae et al., 2006; Renouf et al., 2007), which do not seem to increase significantly on damaged grapes (Barata et al., in press).

Contrarily, acetic acid bacteria are stimulated by berry damage, increasing from less than 10 CFU/g to 10^6 CFU/g on rotten grapes (Barata et al., in press; Barbe et al., 2001). On the contrary, Nisiotou et al. (2011) found comparable acetic acid bacterial counts in sound and grey rotten grapes, ranging from 10^5 to about 10^6 CFU/mL.

3.2. Species diversity

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

Table 2
Yeast and yeast-like species isolated from "sound" grapes or berries at harvest (data collected from selected surveys, the symbol + indicates relative proportion of the detected species).

Species	France	France	Italy	Spain	Spain	Portugal	Greece	Slovenia	Canada	Brazil	Argentina	Japan	Australia	India	China
Basidiomycetes[a]	+++	+++	++	+++		+++		+++	+++	+	+	+++	++		+++
Aureobasidium pullulans	+		+++	+++	+		+	++	+++	+			+++		
Hanseniaspora spp.		++			+++		++					+		+++	+
H. uvarum	+++		++	+	+++	++	+++	++	++	+++	+++	+++		+	+++
Metschnikowia spp.	+		++		+	+		+			+++				+
Candida spp.		+++	+	++		+					+	++		++	++
C. stellata/ zemplinina						+	++				+				+
Debaryomyces spp.		+						+						+	
Issatchenkia spp.		+			++		+			+++	+			++	+
Kluyveromyces spp./*Lachancea* spp.		+			++										
Pichia spp.		+	+		+	+		+			++			++	++
Brettanomyces spp.			+												
Saccharomyces spp.		+	+												
S. cerevisiae					++									++	
Saccharomycopsis spp.			++												
Saccharomycodes ludwigii											+				
Torulaspora spp.					+	+									
Zygosaccharomyces spp.			+												+
Z. bailii					+										+
Sampling[b]	Be	Bu	Be	Be	Bu	Be	Bu	Bu	Bu	Be, Bu	Bu	Bu	Bu	Bu	Bu
Culture media[c]	G	G	G	G	G,A	G,S	G,S	G	G	G	G	–	G	G	G,S
Count range (log)	4–6/mL	3–5/berry	0.1–4/g	–	–	2–6/g	3–4/g	3–6	–	–	5–6/g	0.7–3/mL	2–4/g	–	3–5/g
References	Barnett et al. (1972)	Renouf et al. (2005)	Guerzoni and Marchetti (1987)	Sabate et al. (2002)	Clavijo et al. (2010)	Barata et al. (2008a, 2008b)	Nisiotou and Nychas (2007)	Raspor et al. (2006)	Subden et al. (2003)	Baffi et al. (2011)	Combina et al. (2005)	Yanagida et al. (1992)	Prakitchaiwattana et al. (2004)	Chavan et al. (2009)	Li et al. (2010)

[a] *Cryptococcus* spp., *Bulleromyces* spp., *Sporidiobolus* spp., *Sporobolomyces* spp., *Rhodotorula* spp., *Trichosporon* spp.
[b] Be, berry, Bu, bunch.
[c] G, general purpose, A, autoenrichment, S, selective media.

Table 3
Yeast and yeast-like counts (range (x–y) or mean ± standard deviation (z ± t) of log CFU/g or log CFU/berry) and species frequency found in sound and damaged grapes at harvest.

Damage	Sound grapes	Species (frequency)	Damaged grapes	Species (frequency)	Reference
Undefined[a]	4.4–4.4 (2)[b]	*Aureobasidium pullulans* (95–100) *Cyptococcus victoriae* (0–4) *Rhodotorula laryngis* (0–1)	5.9–7.2 (2)[b]	*Metschnikowia* spp. (64–75) *Hanseniaspora* spp. (0–36) *A. pullulans* (0–25)	Prakitchaiwattana (2004)[c]
Diffuse powdery mildew	2.3–4.0 (4)	*Aureobasidium* spp. (65–66) *Hanseniaspora* spp. (22–23)	4.0–6.0 (4)	*Hanseniaspora* spp. (67–80) *Metschnikowia* spp. (3–19) *Aureobasidium* spp. (2–5) *Candida stellata* (4–10)	Gadoury et al. (2007)[c]
Sour rot	3.2–3.9 (5)	*A. pullulans* (19) *Saccharamycopsis vini* (15) *Metschnikowia pulcherrima* (14) *Rhodotorula* (11) *H. uvarum* (11) *Pichia membranifaciens* (8) *Zygosaccharomyces* spp. (6) *C. krusei* (4)	6.6–6.8 (5)	*C. krusei* (33)a *Sac. vini* (19) *M. pulcherrima* (15) *Hanseniaspora uvarum* (11) *C. steatolytica* (10) *Zygosaccharomyces* spp. (3) *C. sorbosa* (3) *P. membranifaciens* (1) *A. pullulans* (<1)	Guerzoni and Marchetti (1987)[d]
	2.0–2.8 (2)	Basidiomycetes (0–100) *C. vanderwaltii* (0–11)	6.8–7.2 (2)	*C. vanderwaltii* (60–74) *H. guilliermondii* (3–24) *Lachancea thermotolerans* (2–6) *P. membranifaciens* (0.3–16) Basidiomycetes (0.1–0.3) *H. uvarum* (0–12) *Zygoascus hellenicus* (0 ≤ 0.1) *Candida* spp. (0–3) *Issatchenkia* spp. (0–1)	Barata et al. (2008a)[c]
Grey rot	4.3 ± 0.4 (3)	*H. uvarum* (67) *C. zemplinina* (22) *H. opuntiae* (6.5) *A. pullulans* (4.5)	5.7 ± 0.6 (3)	*H. uvarum* (76) *C. zemplinina* (12) *Issatchenkia occidentalis* (5) *I. terricola* (3.5) *H. opuntiae* (3.5)	Nisiotou and Nychas (2007)[c]
Noble rot	3.3 ± 0.7 (3)	*H. uvarum* (77) *H. guilliermondii* (9) *H. opuntiae* (9) *I. terricola* (5)	6.8 ± 0.8 (3)	*H. uvarum* (75) *M. pulcherrima* (7) *H. opuntiae* (5.5) *H. guilliermondii* (3.5) *Z. bailii* (3.5) *C. zemplinina* (3.5) *I. terricola* (2)	Nisiotou and Nychas (2007)[c]
Honeydew	3.7–5.9 (19)	Basidiomycetes (62)[b] *H. uvarum* (23) *M. pulcherrima* (6) *Candida* spp. (3) *C. stellata* (2) *C. vanderwaltii* (2) *Torulaspora pretoriensis* (2)	3.3–6.5 (26)	Basidiomycetes (68)[b] *Z. hellenicus* (10) *H. uvarum* (7) *C. vanderwaltii* (6) *Pichia* spp. (4) *T. pretoriensis* (2) *L. thermotolerans* (2) *M. pulcherrima* (1) *Sac. vini* (1) *Z. bisporus* (1)	Barata et al. (2008b)[d]

[a] Analysis of intact berries or berries physically damaged, shrivelled, broken skin, mouldy (seldom) or discoloured.
[b] Number of samples analysed given within brackets.
[c] Frequency expressed as a percentage of specific colonies relative to total colony number on plate media.
[d] Frequency expressed as a percentage of positive samples in relation to total samples analysed.

explanation (Table 2). However, this variety may be reduced to relatively few groups of similar physiological characteristics. For instance, the ubiquitous *Candida* spp. and *Pichia* spp. are highly heterogeneous, and new species are likely to be found in each new survey because the accuracy of molecular identifications is constantly increasing (Rao et al., 2007). In addition, these genera are ubiquitous and given the strong influence of the vineyard surroundings, it is also likely that a wide diversity of their species will be found. For instance, (Chavan et al. (2009) were the first to isolate *C. azyma* from grapes. This species was characteristic of the sugarcane formerly planted in the area of the studied vineyard which is likely to be the explanation for this outcome. Therefore, we propose to systematise the WMC microbiota of grape berries into three main yeast groups, characterised by similar behaviour on grape berries:

(i) oligotrophic, oxidative basidiomycetous yeasts, the yeast-like fungi *A. pullulans*, and lactic acid bacteria (*Lactobacillus* spp., *Oenococcus oeni*);
(ii) copiothrophic, oxidative ascomycetes (several *Candida* spp.); weakly fermentative apiculate (*Hanseniaspora* spp.), film-forming (*Pichia* spp.), fermentative (*C. zemplinina*, *Metschnikowia* spp.) yeasts;
(iii) copiotrophic strongly fermentative yeasts (*Saccharomyces* spp., *Torulaspora* spp., *Zygosaccharomyces* spp., *Lachancea* spp. and *Pichia* spp.) and the obligate aerobic acetic acid bacteria (*Gluconobacter* spp., *Gluconoacetobacter* spp., *Acetobacter* spp.).

The balance among these groups, after *véraison*, is particularly dependent on nutrient availability on berry surface, as described below.

The first group is composed by species favoured by the nutrient poor environment of truly sound berries. The awareness that basidiomycetes are dominant on grape surfaces is relatively recent (Yanagida et al., 1992) and reflects the use of direct analysis of grapes before juice fermentation (Davenport, 1973, 1974). This predominance, similar to that of plant leaves (Fonseca and Inácio, 2006; Sabate et al., 2002), may be explained by their oligotrophy required

to survive in the harsh environment of the unblemished cuticle. These species are ubiquitous, spread all over the environment. In vineyards, soil, leaves and bark also characterised by the dominance of basidiomycetous oxidative yeasts and the yeast-like fungi *A. pullulans* (Davenport, 1974; Poulard et al., 1981; Sabate et al., 2002).

The increase in the proportion of oxidative or weakly fermentative ascomycetous species (*Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia* spp.), may occur during ripening. The mechanisms underlying this succession are not clear, species interaction may occur (Fleet, 2003), but the main factor should be related to nutrient availability. In fact, when approaching maturity, berries begin to behave differently from plant leaves, probably because of cuticle softening and release of volatile organic compounds (VOC's). The emergence of these species is probably the result of juice release, even in visually intact berries, as suggested by the effect of diffuse powdery mildew which injury is invisible to the naked eye (Table 3). This hypothesis has not been tested but the fact that mature sound berries may only harbour basidiomycetes and *A. pullulans* at harvest time (Barata et al., in press), suggests that some nutrient release should occur to enable growth of the copiotrophic biota. Moreover, we believe that many of the apparently contradictory results may be explained by using grape bunches without separating damaged berries. This has been demonstrated recently by Čadež et al. (2010). As expected, these authors found that basidiomycetes were predominant on sound berries, contrary to the observed predominance of *C. zemplinina* and *H. uvarum* when grape whole bunches were analysed. In particular, the apiculate *H. uvarum/K. apiculata* appears to be the most common grape berry species worldwide (Table 2) which is consistent with its predominance in the beginning of spontaneous must fermentations.

The proliferation of the third yeast group is explained by the high nutrient availability resulting from grape damage. Damaged grapes possess, besides much higher cell counts, wider species diversity than sound grapes (Table 3). Basidiomycetes may be still present in numbers similar to those of sound grapes but their proportion is strongly decreased by the proliferation of ascomycetous species (Barata et al., 2008a, 2008b, in press). *H. uvarum* and *C. zemplinina* may be present in higher numbers but their relative proportion also decreases in favour of the fermentative yeasts (e. g. *Pichia* spp., *Zygosaccharomyces* spp., *Zygoascus* spp., *Torulaspora* spp.), which may occasionally dominate the overall microbiota (Barata et al., 2008a, in press). Although regarded as oxidative or weakly fermentative, the physiological heterogeneity of *Candida* spp. and *Pichia* spp. explains the recovery of strongly fermentative species of these genera in damaged grapes or fermented juices (Barata et al., 2008b). In particular, with sour rot and honeydew, Barata et al. (2008a, 2008b, in press) proposed that *Zygoascus hellenicus*/*Candida steatolytica*, *P. terricola* and *P. kudriavzevii* (the two latter were named as *Issatchenkia* spp.) may be regarded as zymological indicators of these types of damage. *C. zemplinina* (formerly identified as *C. stellata*) is a typical contaminant of botrytised juice fermentations (Sipiczki, 2003) but its dissemination is also spread to sound grapes (Barata et al., 2008a, in press).

The proposed grouping is based on yeast species diversity. While acetic acid bacteria are clearly favoured by damage, it is not so for lactic acid bacteria and their grouping may be questionable. Grape lactic acid bacteria include *Lactobacillus* spp. and *Pediococcus* spp. mostly recovered after enrichment (Table 4). In particular, *O. oeni* has only

Table 4
Bacterial species detected in both sound and damaged grapes.

Bacterial groups	Species	References [a, b]
Lactic acid	*Lactobacillus plantarum*	Bae et al. (2006); Lafon-Lafourcade et al. (1983); Renouf et al. (2005, 2007)
	L. hilgardii	Lafon-Lafourcade et al. (1983)
	L. casei, *L. sanfranciscansis*	Renouf et al. (2005, 2007)
	L. lindneri, *L. kunkeei*	Bae et al. (2006)
	L. kefiri [c], *L. mali* [c], *L. plantarum* [c]	Bae et al. (2006)
	L. brevis	Prieto et al. (2007)
	Lactococcus lactis	Bae et al. (2006); Francesca et al. (2010)
	Leuconostoc fallax	Francesca et al. (2010)
	Lc. mesenteroides	Renouf et al. (2005, 2007)
	Oenococcus oeni[a]	Renouf et al. (2005, 2007)
	Pediococcus parvulus, *P. damnosus*, *P. acidilactici*	Renouf et al. (2005, 2007)
	Weissella paramesenteroides	Bae et al. (2006); Renouf et al. (2005, 2007)
Acetic acid	*Acetobacter aceti*	Barbe et al. (2001); González et al. (2005); Joyeux et al. (1984)
	A. pasteurianus	Barbe et al. (2001); Joyeux et al. (1984)
	A. cerevisiae	Prieto et al. (2007)
	A. orleanensis [c], *A. syzygii* [c]	Barata et al. (in press)
	Gluconobacter oxydans	Barata et al. (in press); Barbe et al. (2001); González et al. (2005); Joyeux et al. (1984); Prieto et al. (2007); Renouf et al. (2005, 2007) Barata et al. (in press)
	G. cerinus	Nisiotou et al. (2011)
	Gluconoacetobacter hansenii	Barata et al. (in press); González et al. (2005)
	Gl. saccharivorans, *Gl. intermedius* [c]	Barata et al. (in press)
Other species	*Burkholderia vietnamiensis*, *Pseudomonas jessenii*, *Serratia rubidae*, *Enterobacter gergoviae*, *Leifsonia xyli*, *Enterococcus faecium*, *Bacillus mycoides*	Renouf et al. (2005, 2007)
	Enterococus durans, *E. faecium*, *E. avium*	Bae et al. (2006)
	E. hermaniensis [c]	Bae et al. (2006)
	Acinetobacter spp., *Curtobacterium* spp., *Enterobacter* spp., *Stenotrophomonas maltophila Serratia* spp., *Staphylococcus* spp.	Prieto et al. (2007)
	Enterococcus durans, *Kocuria kristinae*, *Staphylococcus saprophyticus*	Barata et al. (in press); Nisiotou et al. (2011)
	Klebsiella oxytoca, *Bacillus subtilis* [c], *Erwinia* spp., *Pantoea dispersa*, *Enterobacter ludwigii c*, *Tatumella ptyseos*, *Providencia rettgeri* [c], *Serratia marcescens* [c], *Citrobacter freundii* [c]	Nisiotou et al. (2011)

[a] Microbial detection after culture enrichment (Bae et al., 2006; Prieto et al., 2007; Renouf et al., 2005, 2007).
[b] Microbial detection after plating (Barbe et al., 2001; Francesca et al., 2010; González et al., 2005; Joyeux et al., 1984; Lafon-Lafourcade et al., 1983; Renouf et al., 2005, 2007).
[c] Recovery from damaged grapes only.

been recovered from grapes using methodologies adequate to elicit minority populations (Renouf et al., 2005, 2007). Then, the justification to include lactic acid bacteria in the first group is related with their rare isolation either from sound or damaged berry surfaces. In fact, these bacteria are regarded as anaerobic (or semi-anaerobic) and nutritively fastidious, probably unable to compete with yeasts and acetic acid bacteria under the aerobic conditions of the grape berry.

Acetic acid bacteria, dominated by *Gluconobacter* spp., but also *Acetobacter* spp., have been recovered from sound grapes (Table 4), and are particularly frequent in grey rotten grapes (Barbe et al., 2001). Barata et al. (in press) found that *Acetobacter* spp. are the dominant acetic acid bacteria in the final stage of sour rot, while *Gluconoacetobacter* spp. and *Gluconobacter* spp. were mostly isolated from sound berries.

3.3. The case of *Saccharomyces* spp. and wine spoilage yeasts

The *Saccharomyces* spp. and wine spoilage species are mostly present in low numbers and in low frequencies, even in damaged grapes. Davenport (1973, 1974), studying English vineyards in 1965, carried out a thorough ecological study where the ecosystem was divided in atmosphere, phyllosphere and rhizosphere. *Schizosaccharomyces* spp. was found in rhizosphere (soil and leaf litter). *Z. bailli* was found in mummified fruit (grapes) in soil. *Z. rouxii* was found in mummified pears in soil from an adjacent orchard and from wild flowers. This author, by direct techniques, only found *S. cerevisiae* in one sample of acid soil, mushroom compost and vine flowers but not from mature fruit. The isolation of this species from grapes was only achieved by enrichment cultures. Subsequent works have confirmed this assumption (De la Torre et al., 1999; Rementeria et al., 2003; Renouf et al., 2005). Moreover, the frequency of occurrence of *S. cerevisiae* was found about 0.05 to 0.1% in sound berries and 25% in damaged berries, usually with numbers of about 10^5–10^6/berry (Mortimer and Polsinelli, 1999). Interestingly, Schuller et al. (personal communication) recovered *S. cerevisiae* from auto-enrichment cultures using whole bunches with a frequency higher in Portuguese regions more prone to grape rot. The fact that *S. cerevisiae* was absent from all ferments of a hot and dry region (Alentejo) corroborates the importance of damage on its proliferation.

Wine spoilage species should behave similarly to *S. cerevisiae*, given their physiological similarities. Jolly et al. (2003), analysing bunches, reported *Z. bailii* as the predominant strain (>50%) in one site over 12, but no mention to damage was made. The genera *Zygosaccharomyces* spp. and *Torulaspora* spp. were detected at higher frequencies in grapes affected by noble rot, sour rot and honeydew, suggesting their adaption to conditions of reduced water activity and presence of weak organic acids (Barata et al., 2008a, 2008b; Nisiotou and Nychas, 2007). However, even in sour rot berries, they are rarely the dominant population probably because the aerobic conditions favour the fast growing populations of *Candida* spp., *Hanseniaspora* spp. and *Pichia* spp..

Concerning other spoilage species, their isolation from grapes is very rare. *Dekkera* spp. has been recovered from both sound and sour rot berries, apparently with higher numbers in rotten berries (Guerzoni and Marchetti, 1987), and *D. bruxellensis* from grapes after enrichment (Renouf and Lonvaud-Funel, 2007). In addition to the reports of Davenport (1973, 1974), *Sc. pombe* has been mentioned by Florenzano et al. (1977) and *Saccharomycodes ludwigii* by Combina et al. (2005), but no relation to grape health was established. Future works with appropriate sampling plans are required to definitely assess their dissemination in the vineyard environment and track the sources of wine spoilage species.

3.4. Habitat shared by other microbial species

The microbiota of grapes also includes fungi (see Table 1) which may dominate under favourable weather conditions accompanied by inefficient phytochemical utilisation. Fungal obligate parasites are able to penetrate through the intact grape skin by their own biochemical and mechanical activities and are responsible for high economic losses. The main species are the oomycete *Plasmopara viticola*, responsible for downy mildew, and the ascomycetes *Erysiphe necator* (powdery mildew), *Elsinoë ampelina* (anthracnose), *Guignardia bidwellii* (black rot) and *Pseudopezicula tracheiphila* (rotbrenner). Their biology and epidemiology, well-known by phytopathologists, are strongly dependent on weather conditions (mainly temperature and humidity). Berry susceptibility to these diseases decreases from berry set until *véraison*, after which development of ontogenic resistance explains the absence of parasite attacks, even in the absence of phytochemical treatments (Kennelly et al., 2005). Therefore, these populations are absent when the components of the WMC dominate the berry microbiota, but the induced damage may stimulate their growth as shown by the effect of diffuse powdery mildew (Kennelly et al., 2005).

Saprophytic moulds, inducing grape rots, include *B. cinerea* (grey rot), and other ubiquitous genera (e. g. *Cladosporium* spp., *Aspergillus* and *Penicillium* spp.) (Serra and Peterson, 2007), are especially visible when berries are injured, either within tight bunches or on bunch surfaces, competing with the WMC microbiota. The spores of these moulds are spread all over vine tissues and germinate when temperature and humidity are appropriate. Studies are required to enlighten possible interactions among overall grape microbiota and explain their succession and predominance during grape ripening. Probably, the emergence of sour rot against grey rot, besides better phytochemical efficiency against the latter, is related with higher average temperatures during ripening and harvest.

Ubiquitous bacterial species have also been reported on grapes as a result of contamination from the surrounding environment or, hypothetically, as constituents of endophytic populations (Table 4). The genus *Burkholderia* spp. (Renouf et al., 2005) is a representative of the latter while *Bacillus thuringiensis*, used as a biopesticide, may be predominant at harvest (Fleet, 2003). A recent report, revealed the presence of *Enterobacter ludwigii* after laboratorial must fermentation justifying further studies to understand its possible effect on wine quality (Nisiotou et al., 2011). On the contrary, Barata et al. (in press) did not find these environmental bacterial species on winery fermenting musts or wines.

4. Factors influencing species diversity

The microbial communities on grapes may be affected by a large number of factors as described for other fields of microbial ecology (Boddy and Wimpenny, 1992). In vineyards and winemaking, Pretorius et al. (1999) listed a series of variables that may affect the ecology of grape yeasts. Many of those variables are not independent and may be gathered in broad groups of effects, as described below. Moreover, all factors commonly described as influencing grape microbiota (rainfall, wind, temperature, diseases, pests, viticultural practises, etc.), affect primarily skin integrity and so their impact will be discussed further taking into account the expected changes induced by berry damage.

4.1. Climatic conditions

The climatic and microclimatic conditions include the effect of temperature, UV exposure, rainfall, sunlight and winds. Several studies mention that diversity and quantity of microbial populations are dependent on these conditions. However, results are often unclear because it is not easy to apply the scientific method. For instance, rainy vintages lead to higher use of phytochemicals, higher fungal proliferation and higher berry damage, in conjunction with lower UV irradiation. Concerning total yeast counts, Combina et al. (2005) found that years with increased rainfall yielded higher counts,

probably due to increase in berry volume allowing release of juice in joint areas such as the area between the pedicel and the berry, and higher exosmosis leading to nutrient release on the grape surface. With careful sound berry sampling, Čadež et al. (2010) also found that colder harvests with higher rainfall lead to higher yeasts counts. On the contrary, Comitini and Ciani (2006) found 10 times less total counts in years with high rainfall. Rementeria et al. (2003) also found higher numbers in warmer and drier years.

The reports on species diversity are also not conclusive. Some works state that oxidative yeast, like *M. pulcherrima* increase in years with increased rainfall (Combina et al., 2005; Longo et al., 1991; Poulard et al., 1981). Yanagida et al. (1992) found higher proportions of basidiomycetous yeasts in late crops from chilly climatic conditions while *K. apiculata was* more frequent in mild climates. Rementeria et al. (2003) isolated mostly *K. apiculata* in the warmer years but De La Torre (1999), in a hot region, never detected *K. apiculata* on grapes. Ganga and Martínez (2004) detected different proportions of non-*Saccharomyces* in two different vintages and in two producing regions. One harvest had higher rainfall and greater species variability was reported, but only in one region. The other showed less diversity that was explained by the use of fungicides against *B. cinerea*. Without noticeable fungicide effect, Čadež et al. (2010) found dominance by *A. pullulans* and lower species diversity in the warmer and drier vintage.

Large scale works do not demonstrate any relation between climatic conditions and yeast diversity. Jolly et al. (2003), in South Africa, during 3 vintages and in 4 different regions, did not find any pattern linking non-*Saccharomyces* species to climatic zones. Schuller et al. (2000) and Valero et al. (2005), in Northwest Portugal, from 2001 to 2003, using sensitive molecular and statistical techniques, found no correlation between *S. cerevisiae* and regional climatic conditions. Van der Westhuizen et al. (2000b) (1995 to 1998) and Khan et al. (2000) found different populations of *S. cerevisiae* in coastal and inner warmer regions of South Africa. These authors stated that, when a large number of vintages are analysed, appearance/reappearance cycles of certain strains have no obvious explanation.

4.2. Vineyard treatments

The main vineyard treatment studied is related with the use of pesticide treatments, mainly those against fungi (downy mildew, powdery mildew and grey rot). The studies are either based on analysing grapes after vineyard treatment, which do not exclude the influence of other factors, or from auto-enrichment fermentations which cannot be correctly extrapolated to evaluate the variations on berry microbiota.

Viviani-Nauer et al. (1995) found that pesticides decreased yeast population and diversity in fermenting musts. Cabras et al. (1999) reported the absence of effect on fermentation of *S. cerevisiae* by 6 different fungicides while fermentation by *K. apiculata* was stimulated. Guerra et al. (1999) concluded that pesticide affect diversity and frequency of *S. cerevisiae* and other species by comparing two different groups of pesticide application and one abandoned vineyard. However, careful analyses of results do not allow this conclusion. For instance, i) the abandoned vineyard had a diversity similar to one of the treated groups; ii) biodiversity was determined after fermentation and so any effect if existent should be on biodiversity of species enduring fermentation and not on grape contamination yeasts; and iii) in two other vintages, distribution of *Saccharomyces* was different for one grapevine variety and not for the other, perhaps due to its more resistant skin.

Ganga and Martínez (2004) detected less diversity of non-*Saccharomyces* species, which was explained by the use of fungicides against *B. cinerea*. However, while Ganga and Martínez (2004) did not find reduced *S. cerevisiae* numbers after fungicide use, Regueiro et al. (1993) and Valero et al. (2007) recovered lower numbers of this species. Van der Westhuizen et al. (2000b), from 1995 to 1998, found a lower incidence of *S. cerevisiae* in 1997 where fungal infestation and chemical use were higher due to high rainfall, but clearly stated that a direct relation could not be made.

Longo et al. (1991) found that the number of oxidative yeasts increased in years with high rainfall, with high fungal proliferation, higher fungicide treatments and higher berry damage. Combina et al. (2005) found that oxidative yeasts were higher in years with increased rainfall (*M. pulcherrima* and *C. stellata* higher than *K. apiculata*) which were not attributed to chemical treatments because they were identical in both years. Čadež et al. (2010), with careful berry selection, showed that, after the safety interval, fungicides against *Botrytis* had a minor impact on the composition of grape berry microbiota and untreated grapes were less contaminated. Further, Oliva et al. (2007) did not find any adverse effect on yeast numbers and must fermentation, of several fungicides even when applied in the day of harvest.

When studying phytochemicals, attention should be paid to the product adjuvants as well. Rogiers et al. (2005) found that the spray adjuvants used to increase the effect of pesticides affect the epicuticular wax and favour *B. cinerea* infection and lower the contamination yeasts on grape surface. On the contrary, Čadež et al. (2010) attributed higher yeast counts on iprodione (an anti-*Botrytis* product) treated grapes to the presence of such adjuvants.

More recent works concern the differences from organic and conventional farming systems and it is tempting to conclude that organic farming leads to higher biodiversity, both in *S. cerevisiae* and in non-*Saccharomyces* yeasts (Cordero-Bueso et al., 2011). However, these authors recovered yeasts after blending 2 kg of grapes and using the juice as auto-enrichment medium that makes the use of the reported ecological indexes questionable. Moreover, their results may be explained by the effect of grape damage. In fact, *S. cerevisiae* was not detected in the ferments of two out of the 3 grape varieties in the 3 analysed vintages when pesticides were used, while it was always found in all samples from organic farming. Additionally, some of the recovered species from both farming systems (*C. sorbosa*/*H. occidentalis*, *C. stellata*, *K. thermotolerans*, *T. delbrueckii*) are consistent with those recovered from damaged grapes (see Table **2**). Then, if winemakers want to obtain grapes with biodiversity similar to that of organic farming, the solution might be to leave some grapes rotting and use it in the fermentations.

4.3. Biotic factors

4.3.1. Microbial vectors

The association between yeasts and invertebrates is well-known in general ecological studies (review of Ganter, 2006). Miller and Phaff (1962) showed that figs, infected by wasps and *Drosophila* (in external body parts), carry apiculate yeasts and *C. stellata*. Few data available from vineyards showed that bees and wasps carry yeasts for the grapes (Davenport, 1973, 1974; Stevic, 1962). Fermaud et al. (2000), studying sour rot, associated *Drosophila* flies with *K. apiculata*, *C. stellata*, *P. membranifaciens*, *M. pulcherrima*, *C. krusei* and acetic acid bacteria. Moreover, yeasts and acetic acid bacteria, carried by *Drosophila* flies were found essential to trigger sour rot (Barata et al., accepted for publication). Lactic acid bacteria, mostly *L. plantarum*, were isolated from the intestinal tract of *Drosophila simulans* collected from a winery (Groenewald et al., 2006).

Regarding birds, a recent survey evidenced that swabs of bird beaks and the initial part of the digestive tract are reservoirs of several grape contamination species, like *H. uvarum* (Francesca et al., 2010).

4.3.2. Microbial interactions and enzymatic activities

There are few reports on the interactions between microbial populations that possibly include killer toxin, antibiotic and quorum sensing mechanisms (Fleet, 2003; Golubev, 2006). The interactions

between resident populations may affect diversity but there is no clear explanation. Yeast–yeast interactions are mostly studied regarding the killer effect, but this activity is probably not relevant in natural populations (Sangorrín et al., 2001). In particular, Van der Westhuizen et al. (2000b) found that killer activity did not affect the distribution of *S. cerevisiae*. The yeast like fungi *A. pullulans* seems to reduce basidiomycete diversity but results require confirmation (Čadež et al., 2010). Research directed to table grape storage showing inhibition of moulds by particular yeast species may provide some clues to future works on natural environments (Golubev, 2006).

Enzymatic activities may favour the survival on grape surface but the real effect remains to be elucidated. Moore et al. (1988) found lipolytic activity in all strains studied, suggesting a competitive advantage on the cutin of grape surfaces. Khan et al. (2000) also observed that all grape isolates exhibited identical results in the hydrolysis of various compounds: cellulose negative, starch negative, poligalacturonase negative, cellobiose positive, β-glucan negative and arbutin positive. Perhaps, these traits are common to most grape contaminants and so do not constitute competitive advantages among yeast species. Gognies et al. (2006) showed that *S. cerevisiae* is a phytopathogen to grapevine plantlets *in vitro*. The mechanisms depend on the filamentous growth process and the endopolygalacturonase activity alone was not responsible for invasion of plant tissues. This ability to form pseudohyphae by *S. cerevisiae* may enable the penetration in tissues and allow persistence (Khan et al., 2000), but results have not been validated under field conditions.

4.4. Geographic location and vineyard factors: age, size, grape variety and vintage year

There is a broad line of thought considering that the grape microflora is dependent on the vineyard location, grape variety and other vineyard related factors. The wish for this belief is that wine tipicity may be, at least partially, dependent on the specific grape microbiota of the producing region. The ultimate goal is to find yeasts according to each wine "terroir". We believe that the available data do not allow conclusions on the existence of "terroir" species/strains, as described below. Works concluding otherwise are probably based on insufficient sample numbers, not accounting for variability of microbial populations in space and time.

With sound grapes, Guerzoni and Marchetti (1987) could not explain the variability within the same year for different varieties and within the same variety for different years. Poulard et al. (1981) concluded that the grape variety did not influence microbiota composition. De La Torre et al. (1999) found that the diversity varied with the vintage year. Sabate et al. (2002) found in one grape variety mostly basidiomycetous (similar to that of soil, bark and leaves) and, in the other, *H. uvarum* and *C. zeylanoides* predominated, but the same grape variety in the same vineyard showed a different species proportion in two different vintages. Zahavi et al. (2002) found lower numbers in one cultivar but the diversity was not dependent on the cultivar. Rementeria et al. (2003) found no correlation between grape variety or origin of grapes and number of yeasts. Chamberlain et al. (1997), in icewine grapes, found a lack of yeast species reproducibility in samples taken at different times or different parts of the same vineyard.

The most thorough studies of yeast distribution in vineyards concern *S. cerevisiae* and the conclusion may be conceivably extended to wine spoilage species. Van der Westhuizen et al. (2000a), Khan et al. (2000), Schuller et al. (2005), Schuller and Casal (2007) and Valero et al. (2005, 2007), on different hemispheres, found essentially the same results. They observed that indigenous *S. cerevisiae* populations on grapes were subjected to natural fluctuations of periodical appearance/disappearance, but no attempt to define "terroir" strains was made because no strain common to all sites, in one region, was found. Furthermore, the studies of Schuller et al. (2005), Schuller and Casal (2007) and Valero et al. (2005, 2007) also concluded that *S. cerevisiae* from commercial starters were only detected near water running off the winery and that there was no influence of starter utilisation in the biodiversity of *S. cerevisiae* populations in the vineyards. Pretorius et al. (1999), describing close vineyards with equal climate, stated that intra-annual variations should be attributed to other vineyard factors like age and size. The vineyard age and size were not evaluated by Valero et al. (2007) but, from 2001 to 2003, found greater biodiversity in larger and older wine regions.

The saprophyte mould *B. cinerea* is known to overwinter in vine tissues (Gabler et al., 2003) and wine spoilage yeasts, also of saprophytic nature, could conceivably behave in the same manner. However, this hypothesis has not been demonstrated. Therefore, yeasts must be vectored from the surroundings to berry surface and be able to thrive afterwards, mainly during the period between *véraison* and harvest (about 2 months long) when the cuticle is thinner and VOCs attracting insects are produced. Colonisation should be driven by vector dissemination and further growth, upon inoculation, depends on the nutrient availability on berry skin. As a result, the grape berry is under the overwhelming influence of the surrounding environment from which vectors bring all kinds of yeast species. If the berry is sound, the usual plant colonisers, basidiomycetes and *A. pullulans*, predominate. If berries are damaged, the influence of environment is overcome and the predominance is diverted to yeast species rarely found in nature, as described before. Under this view, it is difficult to accept that the microbiota of a certain grapevine may be restrained by regional boundaries coincident with a particular "terroir".

5. Influence of grape damage or overripeness on grape juice and wine quality

The detrimental influence of damaged grapes on quality is long known by enologists, being widely accepted that when damaged grapes arrive at the winery the resulting wine will be of lower rate. Surprisingly, the mechanisms explaining the detrimental effect of grape damage are not well understood. Apparently grape juice composition is affected in a negative way giving rise to unbalanced wines, but the origin of molecules responsible for the disorder or the role of fermentative microbiota are not fully understood. However, available data highlight the main role of fungal pathogens or saprophytes on wine depreciation while yeasts and bacteria are mostly regarded as innocuous contaminants. The two most famous exceptions to this quality depreciation are the wines produced from dried grapes—e.g. *Passito* wines, *vins de paille*—and the highly valued late harvest wines, produced with grapes affected by the noble rot mould *Botrytis cinerea*—e.g. Tokay, Sauternes.

5.1. Dried or overripe grapes

Grapes are naturally or artificially dried to obtain certain types of wine (*Passito*, *vin de paille*) or become dried due to unwanted overmaturation. In this kind of grapes it is expectable to find osmotolerant species. Davenport (1974) found *Z. bailii and Z. rouxii* in overripe grapes. Caridi and Audino (1997) isolated few *Z. bailii* from dried grapes that were not present in immature or mature grapes, being *H. guilliermondii*, the most frequent species in all 3 ripening stages. In mature and dried grapes *S. cerevisiae* appeared, but strains (former races *aceti*, *capensis*, *globosus*) were considered as having not good enological properties. Dried grapes had more diversity, because *Candida* spp. also appeared (Caridi and Audino, 1997). In other studies, no particular differences between overripe and sound grapes were observed (Prakitchaiwattana et al., 2004). Knowing that this particular type of wine requires dried grapes it is expected that this grape microbiota has no harmful effect on quality. Possible concerns are related with the occurrence of stuck fermentations due to high initial sugar content. When fungal growth accompanies berry dehydration

changes may occur in aroma composition (Noble-rot) or in the concentration of mycotoxins (Malfeito-Ferreira et al., 2009).

5.2. Noble-rot grapes

Noble-rot or late-harvest wines owe their quality to particular climate conditions that enable the drying process to be accompanied by desirable *B. cinerea* activities. These grapes bear higher constant microbial numbers (yeast and bacteria) during 1 week before maturation than healthy grapes (Barbe et al., 2001). Magyar and Bene (2006) listed the species isolated from these grapes during 5 years, showing that *C. pulcherrima*, *H. uvarum* and basidiomycetous yeasts (*Sporidiobolus*, *Rhodotorula*, *Cryptococcus* and *Filobasidium* spp.) were the most frequent species. There was only one isolate from grapes belonging to *Brettanomyces nanus* or to *Zygosaccharomyces* spp. The species dominant in the winery was *C. zemplinina*, already detected in other studies (Mills et al., 2002; Sipiczki, 2003), but without clear technological significance. It seems to be able to decrease volatile acidity (Cocolin et al., 2011b) that is a favourable ability.

The volatile compounds identified in sweet botrytised wines (e. g. Tokay, Fiano, Sauternes) belong to several chemical classes (e.g. lactones, terpenes, aldehides, ketones, furanones) (Genovese et al., 2007; Sarrazin et al., 2007 and references cited therein).

The mould affects grapes by a smooth drying process, but the major benefit seems to be the alteration of grape metabolism by *Botrytis* that stimulates the production of a S-glutathione conjugate which gives S-3-(hexan-1-ol)cysteine (P-3SH) in musts. This molecule is the cysteinylated precursor of the most abundant volatile thiol in wine (3-sulfanylhexanol, 3SH) (Thibon et al., 2009). Therefore, yeasts should play a minor role in the particular aromatic features of these wines, if any.

5.3. Grey rot

The most common and feared grape rot—grey rot—is due to *B. cinerea* growing on grapes favoured by high rainfall before or during vintage. The fungal strong oxidasic and esterasic activities are responsible for deterioration of phenolic compounds (anthocyanins, hydroxycinnamic acids and flavanols), for transformation of terpenes into less odourous compounds and for hydrolysis of ethyl esters of fatty acids (La Guerche et al., 2006). Moreover, several studies have shown that grape rot, due to the association of *B. cinerea* with other, less visible, fungi (*Penicillium* spp., *Rhizopus* spp.) frequently leads to the development of organoleptic defects in grapes and wines. These compounds have been identified as 2-methylisoborneol, (−)-geosmin, 1-octen-3-one, 1-octen-3-ol, 2-octen-1-ol, and 2-heptanol (La Guerche et al., 2006 and references therein). This mould also induces the production of a pathogenesis-related (PR) protein causing haziness in white wines (Girbau et al., 2004).

Yeasts and bacteria have been isolated from grey rot grapes (see Table **2**) but their role in decreasing wine quality should be irrelevant when compared with the detrimental effects of *B. cinerea*.

5.4. Powdery mildew

Powdery mildew (oidium) due to the fungus *Uncinula necator* is an important disease for the vineyard affecting grape yield, juice and wine quality (Calonnec et al., 2004), titratable acidity (TA), total phenolics, hydroxycinnamates and flavonoids (Stummer et al., 2005). It leads to the occurrence of a very characteristic and sometimes intense mushroom-type odour default (Darriet et al., 2002; Stummer et al., 2005). Strongly odourant compounds were identified as 1-octen-3-one (mushroom odour), (Z)-1,5-octadien-3-one (geranium-leaf odour). During alcoholic fermentation, the enzymatic reduction of 1-octen-3-one and (Z)-1,5-octadien-3-one to much less odourant compounds, namely 3-octanone and (Z)-5-octen-3-one, was observed (Darriet et al., 2002). Those results explain to some extent the disappearance of the fungal aroma specific to powdery mildew grapes during alcoholic fermentation. The influence on wine odour is very effective being noticed in juice and wines with as little as 1–5% of rotten bunches (Stummer et al., 2005), but the partners of the wine microbial consortium have no noticeable effect. The oidium also induces the formation of PR proteins, as mentioned before concerning grey rot (Girbau et al., 2004).

5.5. Diffuse powdery mildew (DPM)

Diffuse powdery mildew (DPM) is the initial stage of powdery mildew when the damage is not visible to the naked eye. It induces strong changes in microbiota composition. The difference between healthy and affected grapes is between a total count of 10^2–10^4 to about 10^4–10^7/ml, including yeast, lactic and acetic bacteria (Gadoury et al., 2007). These microorganisms increase volatiles (ethanol, ethyl acetate, acetic acid) that attract insects (sap beetles, ants, wasps, yellow jackets) which damage berry and promote bunch rot severity by creating entry points to spoilage organisms. It is conceivable that grey or sour rot may follow the initial infection by DPM, but it is difficult to evaluate the detrimental effect of this initial step of powdery mildew *per si.*

5.6. Sour rot

The description of sour rot disease is somewhat unclear perhaps because it occurs simultaneously with other diseases, particularly when mould growth is evident. Formerly, it was regarded as the final stage of grey rot given the observation of *Botrytis* in affected berries (Bisiach et al., 1986). Other authors related it to the activity of saprohytic fungi like *Aspergillus carbonarius* (Dimakopoulou et al., 2008) or *Colletotrichum acutatum* (Meunier and Steel, 2009), and to *Acetobacter* spp. (Oliva et al., 1999). The diseases named as bunch or ripe rot may be taken as synonyms, given the analogy to the sour rot symptoms (Loinger et al., 1977; Meunier and Steel, 2009). The massive presence of yeasts, in the absence of moulds or bacteria, is considered as a requirement for the development of the disease (Marchetti et al., 1984). Blancard et al. (2000) found that the most effective agents, inducing symptoms *in vitro* and *in vivo* were the yeasts *K. apiculata* and *C. stellata* and the acetic acid bacteria *Gluconobacter* spp. and *A. pasteurianus*. It seems that, without acetic acid bacteria, disease symptoms are minor (Barata et al., accepted for publication). Probably, the best way to describe the microbial effects of sour rot is as the result of a spontaneous fermentation of juice, released on berry surface, by a natural mixed culture of yeasts and acetic acid bacteria, where the major final product with impact in wine quality is acetic acid (Barata et al., accepted for publication).

The effect on winemaking has been scarcely studied, being responsible for yield losses compensated by higher initial sugar and colour concentration (Loinger et al., 1977). However, colour is not stable and wine shows higher levels of volatile acidity and residual sugars (Barata et al., 2011b).

Guerzoni and Marchetti (1982) regarded acetic acid as the chemical indicator of the disease. Marchetti et al. (1984) studied the volatiles produced by the yeasts and observed that species *M. pulcherrima* and *H. uvarum* were the single species yeasts providing volatiles (mainly ethanol and ethyl acetate) similar to those of rotten grapes and absent from sound or *B. cinerea* infected grapes. According to these authors *Endomycopsella* spp. (now *Saccharomycopsis* spp.) enabled tissue penetration, but did not produce volatile acidity or ethyl acetate, then *M. pulcherrima* and *H. uvarum*, produce it and spoil the grape. The wine spoilage genera *Brettanomyces* spp. and *Zygosaccharomyces* spp. were found in both grapes, but more frequently in sour rotten samples.

Recently, phenylacetic acid and ethyl phenylacetate were found as key aroma components of grapes affected by sour rot (Barata et al., 2011a). These authors speculated that phenylacetic acid is produced on berry skin tissue as a plant defence mechanism, being esterified to ethyl phenylacetate during fermentation and storage. Furthermore, they observed a loss in the fatty acids and their ethyl esters which was attributed to the consumption of precursor amino acids by the contaminant microflora on damaged grapes.

5.7. Other forms of damages

Wine quality may be affected by other forms of damage which have received little attention in the literature. Recently, the effect of *Cladosporium* rot was reported in delayed harvests in Chile (Briceño et al., 2009). This type of rot reduced colour, aroma, and flavour in Cabernet Sauvignon and Carménère wines but there was no mention of yeast and bacterial activity.

Colletotrichum acutatum and *Greeneria uvicola* have been associated with ripe rot and bitter rot, respectively, in sub-tropical regions (Steel et al., 2007), decreasing grape quality and imparting wine taints (Meunier and Steel, 2009). Their emergence, with a concurrent decrease of grey rot, is probably related with climate changes (Steel et al., 2011).

Grape infection with *Aspergillus carbonarius* can increase the sugar and the citric acid concentrations (Leong et al., 2006) possibly affecting the perceptions of astringency and bitterness (Gawel, 1998; Noble, 1998).

Another two cases, not originally associated to grape health or yeast activity, may be the result of grape damage. One is attributed to the untypical "sweet-like" off-odour of Italian Aglianico del Vulture wines, due to ethyl phenylacetate, associated to late-ripening and empirically connected to some cryptogamic diseases (Tat et al., 2007). The other, is the so-called "premature red wine ageing", where the key compound was 3-methyl-2,4-nonanedione originated from a furan fatty acid (FFA) (Pons et al., 2008). This molecule is a widespread component of plant lipids (Sigrist et al., 2001), present in cutin (Velíšek and Cejpek, 2006) and related with plant defence mechanisms (Batna and Spiteller, 1994). It is conceivable that FFA and phenylacetic acid are produced in grape berry as a response to berry injury, as mentioned for sour rot. Increased levels of these aroma precursors in grape juices originate higher levels of aromatic compounds—3-methyl-2,4-nonanedione and ethyl phenylacetate—that affect wine flavour during storage.

Other concerns are related with microbial metabolites affecting human health. The agents are mostly saprophytic moulds of the genus *Aspergillus* producing ochratoxin A (as reviewed by Malfeito-Ferreira et al., 2009). Recently, fumonisin B2 produced by *A. niger* was reported as an additional potential risk in wine production (Logrieco et al., 2011). As far as we are aware wine sensory attributes are not affected by these mycotoxins.

6. Future prospects

The knowledge of microbial ecology in the vineyard environment has many gaps and it is possible to suggest several subjects of future research.

The first issue is concerned with the origin and dissemination of the main agents of wine fermentation—*S. cerevisiae* and *O. oeni*—and of the other components of the WMC. Despite all information available, it is not yet possible to state where these microorganisms come from and their persistence in the environment during all year is not clarified. Lachance (2003) speculated that wild *S. cerevisiae* are associated with an interface involving *Drosophila* spp., oaks and the surrounding soil. Probably, wine spoilage and bacterial species share the same sites. The microbial vectors (e. g. insects, birds, rain, dusts) are also poorly studied. Which is their relative importance in berry colonisation? Which factors determine their attraction to the sources of microorganisms and to grape berries? Where do microorganisms overwinter? All these questions can only be solved when the overall environment gathering vine plants, microorganisms and vectors will be studied by multidisciplinary research teams, including vine physiologists, entomologists and microbiologists.

Acknowledgements

A. Barata was a recipient of a fellowship support by the Portuguese research foundation FCT (grant SFRH/BD/28451/2006). We are grateful to Dr. Sally Burr for revising the English language.

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