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Instituto Superior de Agronomia
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Microbial ecology of sour rotten grapes and their influence on chemical and sensorial wine quality

Tese apresentada para obtenção do grau de Doutor em

Engenharia Alimentar

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Aos meus pais e ao meu querido irmão.

“As pessoas influenciam-nos,
as vozes comovem-nos,
os livros convencem-nos,
os feitos entusiasmam-nos”

John Henry Newman

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ABSTRACT

The grape berry microbiota is deeply influenced by the sanitary status of grapes. In sound grapes the most frequent yeast species were basidiomycetes, *Aureobasidium pullulans*, *Candida zemplinina* and *Hanseniaspora uvarum*. Sour rotten grapes were mostly colonised by *C. zemplinina*, *H. uvarum*, *Issatchenkia* spp. and *Zygoascus hellenicus*. The presence of minoritary populations of *Zygosaccharomyces bailii* was only found in damaged grapes. The acetic acid bacteria *Gluconobacter* spp. and *Gluconoacetobacter* spp. were isolated from healthy and injured grapes, while *Acetobacter* spp. was only associated with sour rot.

Sour rot induced an increase of the sugar content, volatile acidity, colour and total phenols of grape juices. Wines with sour rot up to 30% showed equal overall quality after one year of bottle storage. However, wines made with sour rotten grapes showed a distinctive chemical and honey-like aroma. Phenylacetic acid and ethyl phenylacetate, both exhibiting honey-sweet like aromas, emerged as key-aroma compounds of sour rotten wines.

The causal agents of sour rot were acetic acid bacteria disseminated by the insect *Drosophila* spp.. In the absence of these flies, putative plant defense mechanisms, probably mediated by the accumulation of phenylacetic acid, led to skin healing, preventing sour rot spreading.

Keywords: Sour rot, damaged grapes, grape ecology, yeasts, spoilage yeasts, acetic acid bacteria, wine, aroma, phenylacetic acid, *Drosophila* spp.

TÍTULO DA TESE: **Ecologia microbiana de uvas afectadas por podridão ácida e sua influência na qualidade química e sensorial do vinho**

RESUMO

A microbiota da uva é fortemente influenciada pelo seu estado sanitário. A flora de leveduras de uvas sãs foi dominada por basidiomicetas, *Aureobasidium pullulans*, *Candida zemplinina* e *Hanseniaspora uvarum,* enquanto que uvas afectadas por podridão ácida foram principalmente colonizadas por *C. zemplinina*, *H. uvarum*, *Issatchenkia* spp. e *Zygoascus hellenicus*. Populações minoritárias de *Zygosaccharomyces bailii* foram apenas encontradas em uvas afectadas. Bactérias acéticas dos géneros *Gluconobacter* spp. e *Gluconoacetobacter* spp. foram isoladas de uvas sãs e afectadas, enquanto que o género *Acetobacter* spp. foi apenas associado à doença. A podridão ácida induziu um aumento do conteúdo em açúcar, acidez volátil, cor e fenóis totais nos mostos. A vinificação contendo até 30% de uvas afectadas não modificou significativamente a qualidade global dos vinhos. No entanto, estes vinhos foram associados aos descritores aromáticos "químico" e "mel". O ácido fenilacético e o etilfenilacetato, ambos associados ao descritor "mel", foram considerados como marcadores aromáticos dos vinhos com podridão ácida. Os agentes causadores da doença foram bactérias acéticas, sendo disseminadas na vinha pelo insecto *Drosophila* spp. Na ausência deste vector, um possível mecanismo de defesa da planta mediado pela acumulação de ácido fenilacético, conduziu à cicatrização das fissuras dos bagos, prevenindo a disseminação da doença.

Palavras-chave: Podridão ácida, uvas podres, ecologia das uvas, leveduras, leveduras de alteração, bactérias acéticas, vinho, aroma, ácido fenilacético, *Drosophila* spp.

PREAMBLE

Wine quality is deeply dependant on the sanitary state of the harvest and chemical characteristics of the grapes. Winemakers commonly accept that the vinification of damaged grapes is associated to the production of low quality wines with weak storage/aging potential. Sour rot, is becoming a frequent grape vine disease, causing heavy crop losses and reduce vintage quality on several wine-growing regions all over the world.

To date, the microbial ecology of sour rotten grapes has been poorly studied and it is still not clear what are the main causative agents of this disease and the real contribution of sour rot for the final wine quality. Moreover, fruit flies *Drosophila* spp., which are constantly present around grape bunches infected by sour rot, are empirically regarded as the most important vectors of sour rot agents. However, the contribution of *Drosophila* flies on the grape decay process has not been studied in depth.

The evaluation of chemical, microbiological and sensorial changes during the aging of wine made with sour rotten grapes has been little studied.

In addition, to our knowledge, no work focused on the aroma profile of wines affected by sour rot to date, which means that the number and nature of the odorants associated to this grape disease are not known.

Therefore, for all the above, the following objectives were defined in the present work:

1. Characterize the dynamics of the overall grape contaminants including yeasts, acetic acid bacteria and lactic acid bacteria populations present on healthy and sour rotten berries surfaces, during different stages of grape ripening.

2. Find the main agent of sour rot disease and study the importance of the fruit fly *Drosophila* spp. in the development of the disease.

3. Elaborate wines produced from grapes with different levels of sour rot and evaluate the impact of this disease on the winemaking process and on the chemical and sensorial wine quality.

4. Identify and characterize the aroma profile of wines elaborated from sour rot infected grapes with the aim of pointing out the potentially relevant odor volatiles associated to this disease, combining both chemical quantitative and sensory descriptive analyses in order to understand the role of sour rot in the odor nuances of these wines.

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CHAPTER 1 General Introduction

Barata, A., Malfeito-Ferreira, M. and Loureiro, V. (2011). The microbial ecology of wine grape berry. *International Journal of Food Microbiology* (Review article, submitted for publication)

The microbial ecology of wine grape berry

Barata, A., Malfeito-Ferreira, M. and Loureiro, V.

Abstract

The microbial ecology of grapes is complex including filamentous fungi, yeasts and bacteria with different physiological characteristics and significances concerning 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 gathers yeast species, lactic acid 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, dominated by basidiomycetous yeasts (e.g. *Cryptococcus* spp., *Rhodotorula* spp. *Sporobolomyces* spp.) and the yeast-like fungi *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 berry surface favours the increase of ascomycetes with higher fermentative activity like *Issatchenkia* 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 while 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 gray rot, or *Aspergillus* spp., possibly producing ochratoxin, are only active in the vineyard, although their metabolites may affect wine quality during grape processing.

In this review we hope to demonstrate that 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. We support that 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 conditioned by their primary effect on grape damage.

Keywords: microbial ecology, grape berry, damaged grapes, rot, spoilage yeasts, acetic acid bacteria, lactic acid bacteria.

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1. Introduction

Grapes used for making wine have been studied for several decades. The earlier works 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 wine making. Earlier isolations using grape juice fermentations lead to the conclusion that this species was frequent in grapes (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), being bacteria little studied. The recent increase in the number of grape related yeast ecological studies did not originate a significant body of new knowledge. On the contrary, 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 on wine, while the species of the wine microbial consortium (WMC: yeasts, acetic acid and lactic acid bacteria) are not responsible for those diseases. The apparent rising of sour rot incidence, where the agents are partners of the WMC (Barata *et al.*, 2008a; 2011b), 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 their balances 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 in 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 where the most well known are downy mildew (*Plamospara viticola*), powdery mildew (*Erysiphe necator*) and gray 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 have not the ability to grow in wines and their effect on wine quality is due to grape damage. Contrarily, the microorganisms of the WMC, do not damage grapes (except in sour

rot) but 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 partners of this consortium may be grouped in: (i) easily controllable or innocent species, without ability to spoil wine when good manufacturing practices (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*, is a common yeast-like species, commonly mentioned as black yeast, being also technologically irrelevant. The oxidative or weakly fermentative species ascomycetous species (*Candida* spp., *Kloeckera apiculata/Hanseniaspora uvarum*, *Metschnikowia* spp., *Pichia* spp.) are present in pre-fermentation steps or at the beginning of fermentation with debatable activities (see section 1.2). 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 its microscopical shape and are usually regarded as unwanted microorganisms due to the production of 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 practices prevent its 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. 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 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. oenii*, available as starter cultures, while *Lactobacillus* spp. and *Pediococcus* spp. may be responsible for spontaneous fermentations (Lerm *et al.*, 2010).

Wine spoilage may be due to the same species when their activity goes beyond malic conversion, particularly in high pH wines, producing off-flavours or biogenic amines (Capozzi *et al.*, 2011; Lerm *et al.*, 2010). 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 have no role in wine technology.

1.2 Beneficial or detrimental activities of grape contaminant yeasts

The most frequent grape contaminant species are basidiomycetous, oxidative or weakly fermentative species, regarded as innocuous or easily controllable yeasts. Their inability to grow, or their limited growth, in musts and wines explains their recognised irrelevance technological terms. In the worst scenario, apiculate, oxidative or film-forming yeasts have been traditionally regarded as detrimental to wine quality due to the production of unwanted volatiles or to the induction of stuck fermentations, which may be prevented by appropriate technological measures (Malfeito-Ferreira, 2011). However, some reports have mentioned the possible beneficial role of contamination yeasts due to volatile production imparting peculiar flavour characteristics, dependent or not on enzymatic activities releasing aroma precursors or hydrolysing pectolytic molecules (Fernández *et al.*, 2000), or to factors (mouthfeel, body) independent of ester production (Jolly *et al.*, 2006). Even basidiomycetes (*Sporobolomyces roseus*) *and A. pullulans* were found to produce wine flavour compounds after grape inoculation *in vitro* (Verginer *et al.*, 2010), but results lack from validation under technological conditions.

Must co-inoculation with non-*Saccharomyces* yeasts has been used to benefit wine quality, such as decrease in volatile acidity or improvement of mouthfeel sensations (Ciani *et al.*, 2002).

Table 1. Dissemination and technological significance of yeast species isolated from the vineyard and winery environment.

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 cinerea*	Grey rot, Noble rot	Ubiquitous	-
		Aspergillus	*Aspergillus alliaceus, A. carbonarius, A. niger aggregate, A. ochraceus*	*Aspergillus* rot, ochratoxin A producers	Ubiquitous	
		Penicillium	*Penicillium expansum*	Green mold, patulin producer	Ubiquitous	
		Cladosporium	*Cladosporium herbarum*	*Cladosporium* 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
			C. stellata or *C. zemplinina*	Contamination	Grape, grape juice, fermentation, wine	
			Zygoascus hellenicus /C. steatolytica	Contamination	Grape, grape juices	Fermentation
		Metschnikowia	*M. pulcherrima*	Contamination	Grape, fermentation	
		Pichia (film-forming yeast)	*P. anomala*	Contamination /Spoilage	Grape, fermentation	
			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	

Table 1. (continued) Dissemination and technological significance of yeast species isolated from the vineyard and winery environment.

Group	Metabolism	Genus	Relevant species	Technological significance	Main Sources	Occasional sources
Ascomycetous	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	*S. 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	
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	

More recently, fermentation by mixed cultures has been claimed to play a role in wine tipicity and in the so-called “terroir” effect, being regarded as the solution to avoid wine standardisation due to extensive use of *S. cerevisiae* starters (Cordero-Bueso *et al.*, 2011). The results are often contradictory. In fact, the spontaneous/natural or co-inoculated fermentations may: (i) show higher acetic acid, ethyl acetate, acetaldehyde, acetoin, methionol and other undesirable flavour active compounds (Garde-Cerdán and Ancín-Azpilicueta, 2006; Jolly *et al.*, 2006; Lopes *et al.*, 2007; Moreira *et al.*, 2005; Rojas *et al.*, 2003; Romano *et al.*, 2003; Schütz and Gafner, 1993; Zohre and Erten, 2002); (ii) induce stuck or problematic fermentations (Gutiérrez *et al.*, 1999); and (iii) decrease fermentation efficiency (Velázquez *et al.*, 1991). If these non-*Saccharomyces* yeasts are to be used, the main technological questions are how to: (i) balance desirable flavour production with unwanted excessive levels of other compounds (Rojas *et al.*, 2003); (ii) limit the production of detrimental molecules that at low concentration contribute favourably to wine complexity (Clemente-Jimenez *et al.*, 2005); and (iii) co-inoculate with *S. cerevisiae* assuring fermentation completion (Jolly *et al.*, 2006). Therefore, at winery level caution should be taken before applying these non-*Saccharomyces* species because the results are not predictable.

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 and Fell, 1998). We will try to extend the discussion to bacterial species and to the issues related to grape analysis, taking in consideration the advices 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, (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, neighbour grapes bear different populations being more significant to analyse several smaller samples than to blend several

samples 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 practice 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 separate aseptically 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 103 CFU/berry would contain 2x104 CFU/berry if only one damaged berry with 106 CFU/berry is blended together. 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. When bunches are picked and taken to 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.*, 2011b; Sturm *et al.*, 2006). Therefore, isolation from grapes collected aseptically in the vineyard is mandatory and processed as quickly as possible because higher numbers of yeasts in grapes damaged during transport to laboratory may be obtained (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 bag, than with jet streaming and shaking. Prakitchwattana *et al.* (2004) evaluated recovery during 4 successive rinsing for 10 min each. Rinsing released 80% total yeasts in first operation 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 diluent is recovered after grape or single berry washing, the following step 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).

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 most frequent and faster growing species. Spread plates using 0.1 ml sample are commonly preferred and colonies of the most

representative species occupy the medium surface and cover minority colonies, remaining 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).

The use of antibiotics 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.*, 2011b).

2.5 Incubation conditions and colony selection

After surface inoculation plates should be kept upright when yeasts are to be recovered, contrarily to bacteria (Deák and Beuchat, 1996). Incubation temperature is usually set at 25-30 ºC, but lower temperatures (10 ºC) are essential to recover *S. uvarum* (Sampaio and Gonçalves, 2008). Aerobic conditions are preferred but anaerobiosis was advised for *S. pombe* (Florenzano *et al.*, 1977) and lactic acid bacteria (Barata *et al.*, 2011b). 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 practice to choose more than one colony for each morphological type.

2.6 Identification methods

The accuracy of 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).

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).

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).

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 characterize 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 distinguish 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.*, 2005; González *et al.*, 2004) 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 cultured 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 circumvents the problem of culturing AAB and have been successfully used for the identification AAB in grapes, wine and vinegars.

3. Characterisation of vineyard and grape environments

3.1 Vineyard habitat

The vineyards are part of a larger environment and subjected to the global changes occurring therein, mainly when the area occupied is relatively small. Yeasts and bacteria are not motile and so spread in nature depends on transport by vectors, aerosols and water. Furthermore, grapes are only present in a small part of the annual plant cycle, being understandable that their microbial ecology is strongly dependent on surrounding environmental factors (Deak, 2006). Thus, the understanding of grape microbial ecology depends on the knowledge of their possible sources (e.g. soil, trees, decaying material, flowers and fruits) and vehicles of dissemination that enable grape surface colonisation after berry formation. The vicinity of wineries also drives the microbial dissemination but only to neighbour vineyards, especially when there is water streams running off (Valero *et al.*, 2005).

3.2 The berry *niche*

Grape berries may be considered as a *niche*, suffering the effect of particular physical, chemical and biotic factors (Lachance and Starmer, 1998), possibly different from those affecting the vine plant or the vineyard. The understanding of changes occurring on berry surface is essential to explain how the different yeast species colonise grape surface and their populations evolve during ripening until harvest, or decay.

3.2.1 Berry cuticle

The aerial organs of all plants are covered by a thin, conspicuous lipophilic layer, the cuticle, at the interface between plant tissue and environment, which defines their boundaries and provides protection against biotic and abiotic stresses (Jeffree, 2006). Although it is not completely impermeable (Burghardt and Riederer, 2006), it provides a critical and efficient barrier against solute and water loss. Increasing evidence highlights that the cuticle cannot be regarded as a multifunctional passive physical barrier on the plant surface. Its function as a source of signals for invading fungal pathogens and for the induction of innate immune responses in plants must also be taken into account (Chassot and Métraux 2005).

The hydrophobic nature of the cuticle may as well dissolve, and thus retain some isoprenoid volatile organic compounds (VOCs) released by grapes – a potential nutrient carbon source for the berry microbiota and an attraction factor for vector insects (Engelberth *et al.*, 2004).

In grape berry cuticle, the major component is cutin, constituted by C_{16} and C_{18} fatty acid esters (Walton and Kolattukudy, 1972). The capacity of certain microorganisms to metabolise cutin and cuticular waxes as well as pectic and hemicellulosic components may explain why certain species of fungi and aerobic yeasts, with high metabolic diversity, are so frequent on the sound berry surface. Also, the composition of the cuticular waxes changes during the period from flowering to maturity and significant modifications occur on the wax surface morphology. A decrease in the thickness of the primary cuticle is detected from berry set to harvest, partly justifying the well-known augmented susceptibility of ripe grapes to *Botrytis cinerea* infection. In tightly closed bunches abrasion between skins weakens cuticle thickness and creates conditions to the loss of pellicle integrity (Casado and Heredia, 2001).

The morphology of berry surface presents several natural or artificial points of entry. In berries, as in leaves, stomata are natural pores bordered by two guard cells which regulate transpiration and gas exchanges. Stomata also constitute natural openings providing direct access to leaf tissue for numerous microbes. With time, functional stomata turn into wax-occluded lenticels, occasionally surrounded by crevices which may be perforated by hyphae of parasitic fungi (Rogiers *et al.*, 2005).

3.2.2 The evolution of grape berry from anthesis to maturity

Grape berry development consists of two distinct but successive sigmoidal growth periods separated by a lag phase. The first stage is the *berry formation*, also termed herbaceous or green phase, lasting from bloom to approximately 60 days afterwards. During this stage, it is expected for the grape skin to behave much like a leaf. The berry is formed and expands in volume as solutes accumulate (Deytieux *et al.*, 2007). An intermediate stage appears afterwards, named *lag phase* or *véraison*. It occurs between 8 and 14 weeks after flowering (Sarry *et al.*, 2008). The second stage is *berry ripening*. After *véraison* berries typically accumulate sugar, soften, and undergo skin coloration which is most notorious in the case of red varieties due to the accumulation of reddish anthocyanins (Sarry *et al.*, 2008). Overall, the berry approximately doubles in size between the beginning of the second growth period and harvest (Kennedy, 2002).

The plant goal during the second period of growth is to make the berry as appealing as possible to birds and mammals so that seed dispersal may occur (Kennedy, 2002). The overall modifications of cutin during grape ripening and the production of a large arrays of defensive compounds contribute to the change in susceptibility to phytopathogenic fungi, due to the so-called “ontogenic resistance” which explains the absence of powdery and downy mildew after *véraison* (Ferreira *et al.*, 2006; Kennelly *et al.*, 2005).

3.2.3 Nutrient availability on berry surface

Intact grape surfaces are part of the phylloplane, considered in the sense proposed by Fonseca and Inácio (2006). From a microbe perspective, the phylloplane is a continuously fluctuating physical environment both spatially and temporally, which must not be considered as an extreme environment, but as a harsh environment due to the frequent, repeated and rapid changes in the microclimate factors which occur in different time scales, from a few minutes to months. The nature and size of epiphytic microbial populations are shaped by the availability of nutrients (Fonseca and Inácio, 2006).

The oligotrophic nature of the phylloplane (Andrews and Harris, 2000) derives from the multiple origins of its nutrients, which may be exogenous, such as compounds contained in debris, pollen and honeydew, or derived from other organisms (microbes or insects), or endogenous. In addition, grape berries have been reported to exudate a variety of compounds through the cuticle and the epicuticular wax layer onto their surface, including phenols, sugars, lipids, malic acid, potassium and sodium (Padgett and Morrison, 1990).

The content in sugars, organic acids and amino acids removed from sound berry surfaces by washing with water, increases smoothly during berry development (Prakitchaiwattana, 2004). The scenario changes dramatically when access is provided to berry pulp, due to loss of skin integrity. The content in sugars, organic acids and amino acids are about 100 times higher in visibly damaged berries than in sound berries (Prakitchaiwattana, 2004).

The conditions of low pH (3.0 to 3.8), high sugar concentration (*ca.* 200 g/L) and oxygen availability on grape surface are particularly suited to the proliferation of a copiotrophic microbiota, constituted by saprophytic moulds and by the partners of the WMC, as described below.

4. Characterisation of grape microbial populations

4.1 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.*, 2011b; 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.*, 2011b). 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.*, 2011b; Barbe *et al.*, 2001). On the contrary, Nisiotou *et al* (2011) found comparable acetic acid bacterial counts in sound and gray rotten grapes, ranging from 10^5 to about 10^6 CFU/mL.

4.1.1 Evolution during ripening

Yeast numbers usually increase with maturation, especially after *véraison* (Fleet *et al.*, 2002; Rosini *et al.*, 1982). Numbers may increase from 10-10^2 up to 10^6 cfu/g (Jolly *et al.*, 2003; Raspor *et al.*, 2006; Renouf *et al.*, 2005), but the increase period varies. Last stage of grape maturation appears to favour yeast and bacterial proliferation (Renouf *et al.*, 2005; Renouf *et al.*, 2007; Schuller *et al.*, 2005; Valero *et al.*, 2007). Barbe *et al.* (2001) detected a 10^4 increase in only 7 days for yeasts and acetic acid bacteria. Increase was thought to be due to

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		+			++										
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)	Prakitchaiw attana 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) *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]

[a] Analysis of intact berries or berries physically damaged, shriveled, broken skin, mouldy (seldom) or discolored.

[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.

Table 3. (continued) 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
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) *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) *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, shriveled, broken skin, mouldy (seldom) or discolored.

[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.

sugar diffusion from pulp through the skin, but obvious yeast and bacterial growth during sound grape ripening may not be observed (Barata *et al.*, 2008a; Barata *et al.*, 2011b). Therefore, significant increases in microbial numbers after *véraison* are probably a consequence of grape damage.

4.1.2 Yeast spatial distribution on grapes

The distribution of yeast cells on grapes has been scarcely studied. Belin (1972) described the distribution of yeasts on grape surface by electron scanning microscopy. Yeasts are mainly located where juice excretion might appear like peduncle lenticels, on the stomata and at stylar remnants. Under pseudomycelium form they penetrate to the inner tissues by lenticel and microfissures, but proliferation is limited on grape surface. In fact, yeasts are not surrounded by the waxy cutin layer, being free from the pruine, and proliferate much more on the secretions (Belin, 1972). Rosini *et al.* (1982) showed that higher numbers are near the stem. The berries close to the peduncle had 10 to 100 times higher populations than central and lower parts of the bunch which may be attributed to juice leakage. These observations suggest that even little juice release on grape surface trigger yeast multiplication, reinforcing the effect of damage on berry microbiota.

4.2 Species diversity

Worldwide surveys seem to indicate that apparently sound grapes are colonised by a wide variety of yeast species without any obvious 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, being expectable to find new species in each new survey because the accurateness of molecular identifications is constantly increasing (Rao *et al.*, 2007). In addition, these genera are ubiquitous and given the strong influence of the environment surrounding vineyards, it is expectable to find a wide diversity of their species. 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;

(ii) copiotrophic, oxidative ascomycetes (*Candida* spp. and *Metschnikowia* spp.); weakly fermentative apiculate (*Hanseniaspora* spp.) or film-forming (*Pichia* spp.) yeasts;

(iii) copiotrophic strongly fermentative yeasts (*Saccharomyces* spp., *Torulaspora* spp., *Zygosacharomyces* spp, some *Candida* 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 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 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.*, 2011b), 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, contrarily to the observed predominance of *C. zemplinina* and *H. uvarum* when grape whole

bunches were analysed. In particular, the apiculate *H. uvarum/Kloeckera 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 uprising 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 uprising of ascomycetous species (Barata *et al.*, 2008a, 2008b, 2011b). *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. *Issatchenkia* spp., *Zygosaccharomyces* spp., *Zygoascus* spp., *Torulaspora* spp.), which may occasionally dominate the overall microbiota (Barata *et al.*, 2008a; 2011b). 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, 2011b) proposed that *Zygoascus hellenicus/Candida steatolytica* and *Issatchenkia* spp. (e.g. *I. orientalis/Candida krusei*) 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; 2011b).

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 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 microaerophilic) and nutritively fastidious, probably unable to compete with yeasts and acetic acid bacteria under the aerobic conditions of the grape berry.

Acetic bacteria, dominated by *Gluconobacter* spp., but also *Acetobacter* spp., have been recovered from sound grapes (Table 4), being particularly frequent in gray rotten grapes (Barbe *et al.*, 2001). Barata *et al.* (2011b) 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.

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.* (1983a); Renouf *et al.* (2005, 2007)
	L. hilgardii	Lafon-Lafourcade *et al.* (1983a)
	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.* (2011b)
	Gluconobacter oxydans	Barata *et al.* (2011b); Barbe *et al.* (2001); González *et al.* (2005); Joyeux *et al.* (1984); Prieto *et al.* (2007); Renouf *et al.* (2005, 2007) Barata *et al.* (2011b)
	G. cerinus	Nisiotou *et al.* (2011)
	Gluconoacetobacter hansenii	Barata *et al.* (2011b); González *et al.* (2005)
	Ga. saccharivorans, *Ga. intermedius* [c]	Barata *et al.* (2011b)
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.* (2011b); 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; Renouf *et al.*, 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.*, 1983a; Renouf *et al.*, 2005, 2007).
[c] Recovery from damaged grapes only.

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

The *Sacharomyces* 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.* (personnal 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 rotten 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 (1073, 1974), *S. 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 methodologies are required to definitely assess

their dissemination in the vineyard environment and track the sources of wine spoilage species.

4.4 Habitat shared by other microbial species

The microbiota of grapes is also composed by 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 partners 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* (gray 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 gray 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 thurigiensis*, used as a biopesticide, may be predominant at harvest (Fleet, 2003). The advent of direct molecular methods and of metagenomics (Huson *et al.*, 2009; Nisiotou *et al.*, 2011) is revealing the presence of DNA from an increasing the number of bacterial species on grapes, most of them without enological significance (Table

4). 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.* (2011b) did not find these environmental bacterial species on winery fermenting musts or wines.

5. 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 practices, etc.), affect primarily skin integrity and so their impact will be discussed further taking into account the expectable changes induced by berry damage.

5.1 Berry damage

Grape damage depends directly on the resistance of berry skin to injuries inflicted by a wide variety of agents. Grapevine genetic factors determine skin integrity and bunch morphology. There is a positive correlation between skin thickness and pellicle elasticity on one hand and berry resistance to rot on the other. In fact, winemakers are aware that grape varieties with thin skin and tight bunches are more prone to rot, particularly when climatic conditions are favourable. Skin may be damaged by several abiotic factors, mostly associated with weather or climatic conditions, such as hail, rain or strong winds, late frosts, wide thermal amplitudes and intense radiation. In our experience, the most frequent situations arise from early rain in late summer which, occurring after a long drought period, may originate an abrupt grape volume increment (which may reach 30% of the berry weight). Such conditions lead to pellicle rupture, particularly in the case of fragile skin varieties, as mentioned before. Biotic factors may also assume an important role in grape skin damaging. Thus, for example, the grape moths *Lobesia botrana* and *Eupoecilia ambiguella*, whose larvae penetrate the intact berry skin, explain the positive correlation between their presence and gray mould or sour rot infections. Wasps which, unlike bees, possess mouthparts capable of piercing the peel play also an active role in this process, as do many bird or mammalian (e.g. rabbits, dogs) species. Viticultural practices include either mechanical damage by careless operations or

utilization of phytochemicals directed to control vine diseases. Particularly, in this last case, it is difficult to separate the influence of the pesticide application from the influence of damage.

In practical situations it is practically impossible to evaluate each factor *per si* because all may be simultaneously effective. Then, studies of grape berry microbiota must account for this complexity to prevent unsupported conclusions. Moreover, we believe that many of the apparently contradictory results described below may be explained either by not individuating factors and/or by using grape bunches without separating damaged berries, as explained before.

5.2 Climatic conditions

The climatic and microclimatic conditions include the effect of temperature, UV exposition, 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 rainy years yielded higher counts, probably due to increase in berry volume allowing release of juice in joint areas like between pedicel and berry, and higher exosmosis leading to nutrient release on grape surface. With careful sound berry sampling, Čadež *et al.* (2010) also found that rainier and colder harvests lead to higher yeasts counts. On the contrary, Comitini and Ciani (2006) found 10 times less total counts in rainy vintage. Rementeria *et al.* (2003) also found higher numbers in warmer and drier vintage.

The reports on species diversity are also not conclusive. Some works refer that oxidative yeast, like *M. pulcherrima* increase in rainy years (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 vintage 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 was more rainy leading to greater species variability 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 large number of vintages are analysed, appearance/reappearance cycles of certain strains have no obvious explanation.

5.3 Vineyard treatments

The main vineyard treatment studied is related with the use of pesticide treatments, mainly those against fungi (downy mildew, powdery mildew and gray 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 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; iii) in two other vintages, distribution of *Sacharomyces* 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*, which was explained by the use of fungicides against *B. cinerea*. However, they did not find reduced *S. cerevisiae* numbers after fungicide use, as observed by Regueiro *et al.* (1993) and Valero *et al.* (2007). Van der Westhuizen *et al.* (2000b), from 1995 to 1998, found lower incidence of *S. cerevisiae* in 1997 where fungal infestation and chemical use were higher due to rainy season, but clearly stated that a direct relation could not be made.

Longo *et al.* (1991) found that the number of oxidative yeasts increased in rainy vintage, with high fungal proliferation, higher fungicide treatments and higher berry damage. Combina *et al.* (2005) found that oxidative yeasts were higher in rainy years (*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 being tempting to conclude that organic farming leads to higher biodiversity, both in *S. cerevisiae* and in non-*Saccharomyces* yeasts (Cordero-Bueso *et al.*, 2011). These authors recovered yeasts after blending two kg of grapes and using the juice as auto-enrichment medium that makes the use of the reported ecological indexes questionable. The awareness of damaged grapes was not evidenced, but the results could be explained on that basis. In conventional farming pesticides were used and so damage should be reduced. In fact, *S. cerevisiae* was not detected in the ferments of two out of the 3 grape varieties in the 3 analysed vintages, while it was always found in all samples from organic farming. Moreover, 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.

5.4 Ripening stage and degree of maturity at harvest

Véraison is considered as the key stage of berry ripening concerning the resistance to phytopathogenic fungi, as mentioned before. Afterwards, saprophytic microbiota includes moulds responsible for several rots, and a diversity of yeast and bacterial species.

Renouf *et al.* (2005) studied the microbial diversity during grape development. *A. pullulans*, the most frequent species, was detected until *véraison* but not in harvest. *C. stellata* was the main species at harvest while *S. cerevisiae* and *S. boulardii* were only detected at harvest. On the contrary, De La Torre *et al.* (1999) found mostly basidiomycetes (*Sp. roseus* and *Cr. albidus)* on grapes during ripening and at harvest, but did not find *K. apiculata* at any stage. In a review, Fleet (2003) concluded that basidiomycetes give place to ascomycetes (oxidative/weakly fermentative and apiculate) as maturity approaches. Barata *et al.* (2008a; 2011b) provided similar results for sound grapes. However, sour rot damage, since *véraison*, was shown to be more important than the ripening stage in the variability of species diversity and quantity. Moreover, even at harvest, microbiota may only be constituted by basidiomycetous species. These results support our view that nutrient availability is the main driving force altering berry microbiota. If berries are unblemished through ripening it is expectable that they show constant numbers and species diversity, or that the variability is similar to that found on the surface of other parts of the phylloplane, as described by Fonseca and Inácio (2006).

5.5 Biotic factors

5.5.1 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.*, 2011d).

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

5.5.2 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 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 lack of validation under field conditions.

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

There is a broad line of thought considering that 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 concluding 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 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 utilization 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. These factors were not evaluated by Valero *et al.* (2007) that, 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 two months long) when cuticle is thinner and VOC's 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, 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”.

6. 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.

6.1 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 has 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 (Fleet *et al.*, 2002). Knowing that this particular type of wine requires dried grapes it is expectable 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).

6.2 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 one 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. Few isolates from grapes belong to *Brettanomyces nanus* (one isolate) and *Zygosaccharomyces* spp. (one isolate). 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.

6.3 Gray Rot

The most common and feared grape rot – gray 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 odorous 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).

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

6.4 Powdery mildew

Powdery mildew 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 odor default (Darriet *et al.*, 2002; Stummer *et al.*, 2005). Strongly odorant compounds were identified as 1-octen-3-one (mushroom odor), (Z)-1,5-octadien-3-one (geranium-leaf odor). During alcoholic fermentation, the enzymatic reduction of 1-octen-3-one and (Z)-1,5-octadien-3-one to much less odorant 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.

6.5 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 gray or sour rot may follow the initial infection by DPM, being difficult to evaluate the detrimental effect of this initial step of powdery mildew *per si.*

6.6 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 gray rot given the observation of *Botrytis* in affected berries (Bisiach, 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 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.*, 2011d). 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.*, 2011d).

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.*, 2011c).

Guerzoni and Marchetti (1982) regarded acetic acid as the chemical indicator of the disease. Marchetii *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 aminoacids by the contaminant microflora on damaged grapes.

6.7 Other damages

Wine quality may be affected by other damages which have received little attention in literature. Recently, the effect of *Cladosporium* rot was reported in delayed harvests in Chile (Briceño *et al.*, 2009). This type of rot reduced color, aroma, and flavour in Cabernet Sauvignon and Carménère wines but there was no mention to 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, in detriment of gray rot, is probably related with climate changes (Steel *et al.*, 2011).

Other 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-odor 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 aging”, 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 defense 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 flavor during storage.

7. Final remarks

The knowledge of microbial ecology in the vineyard environment has many gaps being possible to advance several hypotheses as future research subjects, as described below.

Grapes are specially adapted to colonisation by the wine microbial consortium from *véraison* to harvest, lasting about two months each year. The species diversity depends on the integrity of the skin. In unblemished berries microbiota is dominated by oxidative

basidiomycetous yeasts and the yeast-like fungi *A. pullulans*, like in any other plant leaves. The rising of the weakly fermentative yeast genera *Hanseniaspora/Kloeckera* spp. and several *Candida* spp. is probably explained by the increase in nutrient ability on visually intact berry surfaces during ripening. When visible damage occur total microbial numbers and species diversity markedly increase. Fermentative species and acetic acid bacteria become easily recovered turning berries into the primary source of wine spoilage yeasts, like *Zygosaccharomyces* spp., or bacteria, like *Acetobacter* spp.. Therefore, grape injury appears as the main ecological shaping force. The influence of other biotic or abiotic factors depends primarily on their impact on damage.

The typical agents of wine fermentations – *S. cerevisiae* and *O. oeni* - are rarely found on grape surfaces. 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. From these sources dissemination begins through several vectors (e. g. insects, birds, rain, dusts) with undetermined relative importance in distance and in time, and not restricted by on regional boundaries. Yeasts and bacteria should be first transmitted to rich nutrient substrates such as flower pollens and ripe sound or damaged fruits. Then, insects should have a prominent role in grape colonisation, being attracted by volatiles released during ripening especially after berry damage. The annual cycle is closed by grape harvesting or decay on the ground. The overall environment gathering vine plants, microorganisms and vectors deserves to be studied by multidisciplinary research teams, including vine physiologists, entomologists and microbiologists.

In technological terms, the detrimental activity of the WMC species is not a concern during grape ripening, except for acetic acid bacteria in sour rot. In fact, grape quality is badly affected by fungal diseases (e. g. powdery mildew and gray rot) but these are not active in wine. Grape contaminant yeasts do not seem to play a significant role in any grape disease, having at most a secondary effect in sour rot. These contaminant yeasts may occasionally pose a problem after grape processing if pre-fermentative growth is not prevented and stuck fermentations occur. Moreover, species now claimed to have a beneficial role during fermentation (*Torulaspora* spp., *C. zemplinina)* are common contaminants of damaged grapes. Under our view, the main hazard related with yeasts associated with grapes occurs in the winery and not in the vineyard. In fact, most common dangerous spoilage species (*D. bruxellensis*, *S. cerevisiae* and *Zygosaccharomyces* spp.) may enter the winery, especially when damaged grapes are processed, and, once inside the winery, may contaminate and spoil wine during storage and bottling.

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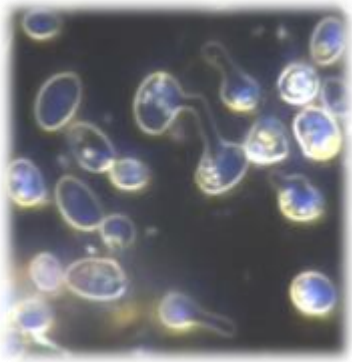

CHAPTER 2

Sour rot damaged grapes are sources of wine spoilage

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MINIREVIEW

Sour rot-damaged grapes are sources of wine spoilage yeasts

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Keywords
damaged grapes; sour rot; spoilage yeasts; selective culture media; PCR-RFLP; *Zygosaccharomyces* spp.

Abstract

Yeast species of sound and sour rot-damaged grapes were analysed during fermentation and grape ripening in the vineyard, using general and selective culture media. During 2003 and 2004 vintages, microvinifications were carried out with sound grapes to which different amounts of grapes with sour rot were added. The wine spoilage species *Zygosaccharomyces bailii* was only recovered during fermentations with sour rot, reaching 5.00 log CFU mL^{-1} (2003) and 2.48 log CFU mL^{-1} (2004) at the end of fermentation. The study of yeast populations during the sour rot ripening process (2005 vintage) showed that the veraison-damaged grapes always exhibited higher total yeast counts and a much greater diversity of species. From a total of 22 ascomycetous species, 17 were present only in damaged grapes. The most frequent species were *Issatchenkia occidentalis* and *Zygoascus hellenicus*. The spoilage species *Z. bailii* and *Zygosaccharomyces bisporus* were consistently isolated exclusively from damaged grapes. This work demonstrates that one of the most dangerous wine spoilage species, *Z. bailii*, is strongly associated with sour rot grapes and survives during fermentation with *Saccharomyces cerevisiae*. The use of selective media provides a more accurate characterization of grape contamination species.

Introduction

The effect of non-*Saccharomyces* yeasts on fermentation and wine quality is one of the principal issues currently studied in countries with an oenological tradition (Jolly *et al.*, 2003). Many ecological studies in different wine regions of the world have identified the major yeast species that develop during fermentation (Povhe-Jemec *et al.*, 2001; Jolly *et al.*, 2003). However, the study of the microbial succession during grape juice fermentations is usually applied when sound grapes are the raw material for wine production (Fleet, 2003).

The yeasts associated with wine may be classified in empirical groups according to their technological properties (Loureiro & Malfeito-Ferreira, 2003). Oxidative basidiomycetous yeasts, without any oenological interest, such as *Sporobolomyces*, *Cryptococcus*, *Rhodotorula*, *Filobasidium* spp. and *Aureobasidium pullulans*, are mostly prevalent in the vineyard environment (soil, bark, leaves, grapes) (Davenport, 1974; Sabate *et al.*, 2002; Subden *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2005). Among ascomycetes, apiculate weakly fermentative yeasts (*Hanseniaspora* and *Kloeckera* spp.) and oxidative yeasts (mostly *Candida*, *Pichia* and *Metschnikowia* spp.) are predominant on ripe sound grapes (Davenport, 1974; Sabate *et al.*, 2002; Jolly *et al.*, 2003; Subden *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2005). The last group includes fermentative species, which are rare contaminants of grapes but represent the most dangerous wine spoilers, such as *Saccharomyces cerevisiae*, *Torulaspora* spp., *Zygosaccharomyces* spp. and *Dekkera* spp. (Fleet *et al.*, 2002).

Significant growth of non-*Saccharomyces* yeasts in fermentations has been associated with off-character production in wines and/or stuck and sluggish fermentations (Bisson, 1999). In addition, there is little work on the survival of yeast species dangerous to bottled wine stability during fermentation. In fact, wine spoilage yeasts such as *Dekkera/Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* are usually studied for their activities during storage and after bottling stages (Loureiro & Malfeito-Ferreira, 2003).

The occurrence of fermentative species, such as *S. cerevisiae*, is higher when grape skin is damaged and juice escapes onto the grape surface (Mortimer & Polsinelli, 1999). Bearing in mind that wine spoilage species are also

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Significant growth of non-*Saccharomyces* yeasts in fermentations has been associated with off-character production in wines and/or stuck and sluggish fermentations (Bisson, 1999). In addition, there is little work on the survival of yeast species dangerous to bottled wine stability during fermentation. In fact, wine spoilage yeasts like *Dekkera/Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* are usually studied for their activities during storage and after bottling stages (Loureiro & Malfeito-Ferreira, 2003).

The occurrence of fermentative species, such as *S. cerevisiae*, is higher when grape skin is damaged and juice escapes onto the grape surface (Mortimer & Polsinelli, 1999). Bearing in mind that wine spoilage species are also fermentative, Loureiro & Malfeito-Ferreira (2003) hypothetised that the knowledge of their dissemination could greatly improve if more attention was given to the microbiology of damaged grapes. The microbial ecology of damaged grapes is poorly studied and it is not clear if damaged grapes are significant vehicles of dangerous spoilage microorganisms (Loureiro & Malfeito-Ferreira, 2003). Damaged grapes include those attacked by several types of rot, insects, hail and/or heavy

rains. In particular, sour rot is a disease characterized by grape pulp browning, disagregation of the internal tissues, detachment of the rotten berry from the pedicel, grape dropping and strong smell to ethyl acetate (Bisiach *et al.*, 1982, Guerzoni & Marchetti, 1987). It may affect a large proportion of the crop and is frequently the main cause for grape damage. The most frequently isolated yeast species from sour rot damaged grapes are *Hanseniaspora uvarum, Candida stellata*, *Metshnikowia pulcherrima, Candida krusei* and *Kloeckera apiculata* (Bisiach *et al.*, 1986, Guerzoni & Marchetti, 1987).

Until now, studies of the microbial communities of sound and damaged grapes were carried out using general purpose culture media leading to the underestimation of wine spoiling species. Spoilage species usually grow slower in general purpose culture media and so tend to be underestimated in samples heavily contaminated by other microorganisms (Loureiro *et al.*, 2004). Barata *et al.* (2008) showed that the use of selective media was crucial to characterize the yeast ecology of honeydew excreted by mealybugs on grape surfaces and preliminary results demonstrated the occasional presence of *Z. bailii* in sour rot damaged grapes.

The purpose of this work was to evaluate the occurrence of the wine spoilage species *Z. bailii* in sour rotten grapes and to check its survival during grape must fermentation.

2. Material and Methods

2.1 Sample collection and wine fermentations

During the 2003 and 2004 vintages, healthy and sour rot affected bunches (Trincadeira Preta red grape variety) were collected at the time of harvest from a experimental vineyard of Instituto Superior de Agronomia (Lisbon, Portugal). Grapes were transported to the laboratory stored in sterile bags at 5 ºC. Three sets of microvinifications (red style fermentation with grape skin contact) were carried out in 2-L Schott flasks at 25 ºC using sound grapes to which different amounts of sour rot damaged grapes were added: (1) 100% (p/p) of sound grapes (control); (2) 10% (p/p) of damaged grapes; and (3) 30% (p/p) of damaged grapes. After addition of 80 mg L^{-1} potassium metabissulphite to emulate winery conditions, grape musts were inoculated with 10^6 cells/mL of a commercial *S. cerevisiae* (Fermivin®, DSM, Delft, The Netherlands), and fermentations were carried out until ºBrix determinations indicated the end of fermentation.

2.2 Yeast isolation during fermentations

Samples from the fermenting musts were taken during the beginning, middle and end of fermentations and yeasts were recovered after decimal dilutions in peptone water (Merck, Darmstadt, Germany). The criteria to define the middle and end of fermentation were based on sugar consumption. The isolation and enumeration of yeasts was carried out spreading onto plates of several culture media: GYP (20 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 20 g L^{-1} agar) and MRS (52 g L^{-1} Mann Rogosa Sharpe, Oxoid) general purpose media in order to isolate *Saccharomyces* and non-*Saccharomyces* species and DBDM (Rodrigues *et al.*, 2001) and ZDM (Schuller *et al.*, 2000) selective/differential media for the isolation of *Dekkera/Brettanomyces* and *Zygosaccharomyces* species, respectively. To avoid growth of bacteria, chloramphenicol (Sigma) was added to the media at 100 mg L^{-1} The plates were incubated at 25 ºC for 7 (GYP and MRS) and 20 days (DBDM and ZDM). Plates containing between 30 and 300 yeast colonies were examined, and all colony morphological types were counted, isolated and purified by streaking on GYP plates.

The yeast isolates were preserved on YM agar slants (3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose, 20 g L^{-1} agar) until their later analysis, stored at 4 ºC and subcultured every 3 months.

2.3 Analysis of natural yeast community from sound and sour rotten grapes

To quantify the yeast community of healthy and sour rot affected grapes, 100-g samples of sound and damaged berries were aseptically removed from single sound and sour rot affected bunches during the 2004 vintage. The berries were placed in a sterile stomacher bag and crushed in a stomacher blender (IUL, Barcelona, Spain). The grape musts were subsequently diluted in peptone water and the natural yeast community was analysed by plating (duplicate) onto GYP, DBDM and ZDM plates, as described previously.

2.4 Yeast community analysis during sour rot ripening process

In the 2005 vintage, two sets of three vines from the same red grape variety and experimental vineyard were selected for taking samples of sound or sour rot affected bunches at three different ripening phases: (1) after veraison; (2) middle of ripening; and (3) before the harvest. A total of three bunches from each vine set were collected aseptically and transported to the laboratory. Individual grape berries were randomly and aseptically removed from the bunches and combined to give 300-g samples. The berries were crushed

in a stomacher blender, and grape musts were directly diluted in 10-fold series in peptone water. The yeasts recovered from grape samples were analysed by spreading (duplicates) 0.1 mL of the appropriate dilutions onto GYP, DBDM and ZDM plates. Colonies were counted after incubation at 25 ºC for 7 days (GYP) and 20 days (DBDM and ZDM), and yeast isolation was performed as described previously.

2.5 Biochemical characterisation and identification

The yeast isolates during the 3-years study were characterized previously according to the urease reaction. Only the urease-negative strains (ascomycetes) were identified by PCR amplification of the 5.8S-internal transcribed spacer (ITS) rRNA gene region and subsequent restriction analysis (Esteve-Zarzoso *et al.*, 1999). This region was amplified using the primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). Yeast cells were picked from a fresh colony (48-h-old) and resuspended directly in the PCR mixture. PCR reactions were performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) termocycler as follows: a first step at 95 ºC for 15 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min, with a final extension cycle at 72 ºC for 10 min. Two units of DNA polymerase (Biotools, Madrid, Spain) were added to each reaction. For restriction reactions of the 5.8S-ITS region, 10 μl or 0.5-10 μg of amplified PCR product was incubated for 5 h at 37 ºC with 10 U of *Cfo*I, *Hae*III and *Hin*fI (Roche Molecular Biochemicals, Mannheim, Germany) restriction endonucleases. PCR products and their corresponding restriction fragments were separated on 1.4% and 3% agarose gels, respectively, detected by ethidium bromide staining, and photographed under UV light with a GelDoc system (Bio-Rad, California, USA). Fragment sizes were estimated by comparing their mobility against a 100 bp DNA Ladder (Bioron, GmbH, Ludwigshafen, Germany).

The strains of the *Lachancea* clade (*Zygosaccharomyces fermentati*, *Zygosaccharomyces cidri*, *Kluyveromyces thermotolerans* and *Kluyveromyces waltii*) with similar restriction profiles, and the yeast isolates with misidentified restriction profiles were identified by sequencing of the D1/D2 variable domains of the large subunit rRNA gene. The D1 and D2 domains of the 26S rRNA gene were amplified using the external primers NL-1 (5´-GCATATCAATAAGCGGAGGAAAAG-3´) and NL-4 (5´-GGTCCGTGTTTCAAGACGG-3´) (O'Donnell, 1993). PCR reactions were performed as follows: a first denaturation step at 95 ºC for 5 min, followed by 40 cycles of 94 ºC for 40 s, 55 ºC for 40 s and 72 ºC for 30 s, with a final extension of 10 min at 72 ºC. The PCR products were purified using a GFX PCR

and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions and then sequenced directly. Primers NL-1 and NL-4 were used in the sequencing reactions to read both DNA strands of D1 and D2 domains of the 26S rRNA gene. Sequences of the D1/D2 26S rRNA gene were edited and assembled using BIOEDIT SEQUENCE ALIGNMENT EDITOR version 7.0.1 software (Hall, 1999), and then subjected to a GenBank BLASTN search to retrieve sequences of closely related taxa. Distinction of species with identical restriction profiles or similar 26S rRNA gene sequences was carried out using biochemical reactions (Kurtzman & Fell, 1998) and the DBDM medium. *Hanseniaspora uvarum* was differentiated from *Hanseniaspora guilliermondii* and *Dekkera anomala* by growth on GYP agar plates incubated at 37 ºC and growth on DBDM plates. *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii* were differentiated by growth on GYP agar plates incubated at 37 ºC and 40 ºC, and growth with D-galactose and 0.01% cycloheximide.

3. Results

3.1 Identification of yeast isolates

The isolates recovered from grape and must samples were first tested with the urease reaction to estimate the proportion of ascomycetes (urease negative) and basidiomycetes (urease positive). Because basidiomycetous yeasts are not regarded as relevant to winemaking (Loureiro and Malfeito-Ferreira, 2003) no further identification analysis was accomplished for these strains.

A total of 426 yeast strains were isolated from grape or grape must samples in 2003, 2004 and 2005 vintages. A total of 188 and 125 yeast strains were isolated from Trincadeira Preta sound and sour rot contaminated grape musts fermentations during 2003 and 2004 vintages, respectively. In 2005 vintage, 113 yeast isolates were recovered from samples of sound or sour rot affected bunches in three different ripening phases. The phenotypic urease activity test allowed the distinction of 390 ascomycetes yeast strains, which were analysed by performing a PCR-restriction fragment length polymorphism (RFLP) analysis of the 5.8S-ITS rRNA gene region (restriction fragments not shown). Using *Cfo*I, *Hae*III and *Hin*fI restriction endonucleases, 23 different restriction patterns were generated, from which it was possible to assign 18 species according to the restriction patterns in the Yeast-id database (http://www.yeast-id.com). These corresponded to *Candida amapae*, *Candida diversa*,

Candida ethanolica, *Candida stellata/zemplinina*, *Candida tropicalis*, *Candida vanderwaltii*, *Hanseniaspora osmophila*, *Issatchenkia occidentalis*, *Issatchenkia orientalis*, *Issatchenkia terricola*, *Pichia galeiformis*, *Pichia guilliermondii*, *Pichia kluyveri*, *Pichia membranifaciens*, *S. cerevisiae*, *Zygoascus hellenicus*, *Z. bailii* and *Zygosaccharomyces bisporus*. However, this method could not identify species when the restriction bands differed by < 50 bp, necessitating further characterisation by sequencing or biochemical tests. One group included *H. guilliermondii*, *H. uvarum* and *D. anomala.* Their distinction was made based on the observed growth on agar plates incubated at 37 ºC and by the absence of growth on plates of DBDM, indicating the presence of *H. guilliermondii*. Absence of growth on DBDM plates and agar plates at 37 ºC, indicated the presence of *H. uvarum* or its anamorph *K. apiculata*. Another group of similar restriction profiles included the former species *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii*. The recent genus *Lachancea* includes the above-mentioned four species with similar restriction profiles. All the isolates with these similar restriction profiles displayed positive growth with D-galactose and at 37 ºC, and did not grow under 40 ºC and 0.01 % cycloheximide. These physiological results are consistent with the old species *K. thermotolerans* and not with *Z. cidri, Z. fermentati* or *K. waltii*. Moreover their 26S rRNA gene sequence results (EU541357 GenBank accession number) yielded 99% similarity with *Lachancea* spp. CBS 6924 (EF463105) as the closest relatives. Therefore we assigned those strains to *Lachancea thermotolerans*.

Because of the absence of species assignment of their restriction patterns in the Yeast-id database, *Candida oleophila* (EU541359) and *Hanseniaspora occidentalis* (EU541358) were identitied by sequence analysis of the 26S rRNA gene D1/D2 domain, yielding 100% similarity with *C. oleophila* CBS 2219 (AY528671), and 99% with *H. occidentalis* var. *citrica* CBS 6783 (AJ973092), respectively.

3.2 Yeast community dynamics during alcoholic fermentation

Yeast species populations recovered from three sets of microvinifications with different proportions of sour rot damaged grapes at the beginning, the middle and the end of fermentation are presented in Table 1. The most frequent species was *S. cerevisiae* as expected because of starter addition. However, the identification method by RFLP analysis is not adequate for intraspecific typing; hence, we cannot guarantee that all identified strains belong to the *S. cerevisiae* starter. In the control set (100% sound grapes), besides *S. cerevisiae*, only basidiomycetous yeasts, *H. uvarum* and *C. diversa* strains were isolated at the initial the stages of fermentation during 2004 vintage, while in 2003 vintage only *S. cerevisiae* was isolated.

The alteration of the ecological balance was particularly evident from the great population diversity of non-*Saccharomyces* yeast species isolated in the two sets of vinifications with sour rot affected grapes.

Table 1. *Saccharomyces* and non-*Saccharomyces* populations (log_{10} CFU mL^{-1}) in fermentation of sound grapes (control set) and sound grapes contaminated with different amounts of sour rotten grapes (2-years study).

Vintage	Yeast group	Microvinification set								
		C			SR10			SR30		
		BF	MF	EF	BF	MF	EF	BF	MF	EF
2003	*S. cerevisiae*	5.20	7.64	8.19	4.54	8.36	8.05	4.40	8.94	6.64
	Oxidative or weakly fermentative*				1.53	3.52		1.96	3.39	0.82
	Issatchenkia terricola				2.40			2.49		
	Issatchenkia occidentalis								2.76	
	Zygoascus hellenicus				2.04			2.26		
	Zygosaccharomyces bailii				1.60	2.30		2.78	3.15	5.00
	Zygosaccharomyces bisporus								2.18	
	Total yeast counts	5.20	7.64	8.19	4.55	8.36	8.05	4.42	8.94	6.65
2004	*S.cerevisiae*	5.95	8.25	8.01	5.88	8.26	8.23	5.81	8.32	8.10
	Oxidative or weakly fermentative	1.70	4.09		5.82	6.25		5.99	7.16	
	Issatchenkia occidentalis				3.48	3.98		3.40	3.00	
	Zygoascus hellenicus				2.78	2.52		3.59	4.33	
	Zygosaccharomyces bailii				4.72	3.93		4.38	4.16	2.48
	Basidiomycetes	3.38	3.04		3.45	2.48		3.38	2.83	
	Total yeast counts	5.96	8.25	8.01	6.17	8.26	8.23	6.22	8.35	8.10

* Oxidative yeasts include: *Candida stellata/zemplinina*, *Candida diversa*, *Candida vanderwaltii*, *Pichia galeiformis* and *Pichia membranifaciens.* Weakly fermentative yeasts includes: *Hanseniaspora osmophila* and *Hanseniaspora uvarum.*

BF, beginning fermentation; MF, middle fermentation; EF, end fermentation; C, control set (100% sound grapes); SR10, 10% (p/p) sour rot grapes; SR30, 30% (p/p) sour rot grapes.

At the beginning of vinification, sets with 10% (p/p) and 30% (p/p) sour rot grapes (SR10 and SR30), seven non-*Saccharomyces* species were detected (2003 and 2004 vintages). *Zygoascus hellenicus*, *Issatchenkia* spp. and *Z. bailii* were always isolated in both vintages. During the initial and middle fermentation phases, in the 2004 vintage, the oxidative or weakly fermentative yeasts *C. stellata/zemplinina* and *H. uvarum* were the most frequent yeast species isolated from SR10 and SR30 microvinification sets. In middle fermentation, a higher number of species was detected in the trial with a greater proportion of affected grapes in 2003. Considering basidiomycetous yeasts, in 2004, they were recovered in numbers ranging from 2.48 to 3.38 log CFU g^{-1} from beginning and middle fermentations. In both vintage years, only *Z. bailii* persisted in all stages of sour rot affected fermentations.

At the end of fermentations, *S. cerevisiae* was the sole recovered species from the trial with 10% sour rot grapes, while *Z. bailii* reached 5.00 log CFU mL^{-1} in 2003, and 2.48 log CFU mL^{-1} in 2004, with 30% of affected grapes. The species *P. galeiformis* was only detected during the middle and end of fermentation in the 2003 vintage, with 30% of grapes affected by sour rot.

3.3 Natural yeast flora from sound and sour rotten grapes at harvest time in 2004

Table 2 shows the ascomycetous species recovered from one sample of 100 g of healthy and sour rotten berries from the 2004 harvest, as well as the health status and culture media utilized. Significant differences in the health of grapes between yeast populations were found. The mean total yeast population counts and species diversity in sour rotten grapes were higher than those of healthy grapes. In sound grapes, *C. stellata/zemplinina*, *H. uvarum* and *L. thermotolerans* populations were identified, with *L. thermotolerans* being the most frequent species. These three ascomycetous yeasts were also isolated in damaged grapes. The species *Z. bailii* (7.65 log CFU g^{-1}), *I. terricola* (6.34 log CFU g^{-1}) and *Z. hellenicus* (3.82 log CFU g^{-1}) were only recovered from 100% sour rotten berries sample. The latter species was only recovered in DBDM selective culture media. Surprisingly, no basidiomicetous yeasts were detected from the healthy grape samples, while 3.86 log CFU g^{-1} were detected from sour rot damaged grape samples.

Concerning culture media, the highest counts were obtained with GYP for all species, except *Z. bailii*. The ZDM selective medium provided higher counts for *Z. bailii* than GYP in sour rotten grapes.

Table 2. *Ascomycetes* enumeration (log CFU g^{-1}) in 100 g samples of sound and sour rot damaged grapes at harvest time (2004 vintage) as a function of isolation culture media.

Yeast species	Sound grapes		Sour rot grapes	
	Medium	Count	Medium	Count
Basidiomycetes	—	ND	DBDM	3.86
Candida stellata/zemplinina	GYP	4.00	GYP	6.34
Hanseniaspora uvarum	DBDM	3.70	GYP	6.26
			DBDM	4.75
Issatchenkia terricola	—	ND	GYP	6.62
			ZDM	4.88
Lachancea thermotolerans	GYP	4.54	GYP	6.34
Zygoascus hellenicus	—	ND	DBDM	3.82
Zygosaccharomyces bailii	—	ND	GYP	7.00
			ZDM	7.65
Total yeasts	GYP	4.65	GYP	7.31
	DBDM	3.70	DBDM	4.80
			ZDM	7.65

ND, not detected.

3.4 Yeast population dynamics during the sour rot ripening process

In 2005, the study of yeast population dynamics during the grape-ripening period was performed in samples of sound and sour rot affected bunches collected from the same vine sets during three different phases, from veraison until harvest time. Table 3 shows the results of the different yeast populations isolated in the three ripening phases. Since the beginning of the rotting process, sour rot damaged grapes samples always exhibited higher total yeast counts and a much greater diversity of yeast species.

In sound grape samples, during the entire ripening period, basidiomycetous species dominated the yeast populations. A total of four ascomycetous yeast species were isolated from sound grapes at the middle of ripening phase (vine set1 and vine set2) and before the harvest (vine set1), in counts up to 2.70 log CFU g^{-1}. C*. stellata/zemplinina* (2.61 log CFU g^{-1} in vine set1 and 1.70 log CFU g^{-1} in vine set2), *H. uvarum* (1.88 log CFU g^{-1} in vine set1), and *P. membranifaciens* (1.18 log CFU g^{-1} in vine set1 and 1.48 log CFU g^{-1} in vine set2) were recovered from sound grapes at the middle phase and *C. vanderwaltii* (1.00 log CFU g^{-1} in vine set1) at the harvest time (Table 3).

Concerning sour rot samples, 19 ascomycetous yeast species were isolated including the four species present in sound grapes. The 15 yeast species only recovered from rotten grapes during all sampling dates were: *C. amapae, C. diversa, C. ethanolica, C. tropicalis, C. oleophila, H. guilliermondii, H. occidentalis, P. guilliermondii, P. kluyveri, I. occidentalis, I. orientalis, I. terricola, L. thermotolerans, Z. hellenicus and Z. bisporus* (Table 3). Higher yeast species diversity was detected at the middle of ripening phase, in which 12 (vine set1) and 11 (vine set2) ascomycetous species were isolated. In this phase *C. vanderwaltii* (7.49 log CFU g^{-1} in vine set2) and *H. uvarum* (7.49 log CFU g^{-1} in vine set2) were the dominant yeast species. The isolation of spoilage yeasts *P. guilliermondii* (3.78 log CFU g^{-1} in vine set1) and *Z. bisporus* (4.51 log CFU g^{-1} in vine set1) was only achieved during middle ripening in vine set1 (Table 3). In this vintage year, *Z. bailii* was not recovered in any grape-ripening phase.

Despite the increased species diversity since the beginning of the rotting process, the yeast diversity was slightly lower at harvest time (five ascomycetous species in vine set1 and nine ascomycetous yeast species in vine set2) which suggests that the final stage provides a more restrictive environment. In addition, results show that the diversity of isolated species was dependent on the vine set (Table 3). For example, if results from both vine sets are pooled, 14 different species were isolated during the first ripening phase (after veraison). However, the diversity reduced to seven species (vine set1) or 11 species (vine set2) if only one vine set was analysed.

Table 3. Yeast dynamics during grape ripening (2005 vintage)

Yeasts	Grape ripening phase											
	After veraison				Middle of ripening				Before the harvest			
	Vine set 1		Vine set 2		Vine set 1		Vine set 2		Vine set 1		Vine set 2	
	S	SR	S	SR	S	SR	S	SR	S	SR	S	SR
Basidiomycetes	2.81	5.54	2.38	5.70	2.11	5.01	1.90	5.23	1.90	4.21	2.80	4.39
Candida amapae				5.70		3.95		4.92				5.65
C. diversa				5.70								
C. ethanolica				4.11								
C. oleophila								2.08				
C. stellata/C. zemplinina		5.00			2.61		1.70					
C. tropicalis												2.90
C. vanderwaltii				6.74		6.82		7.49	1.00	6.58		7.11
Hanseniaspora uvarum				6.60	1.88	6.08		7.08				6.30
H. guilliermondii		6.08		6.08		4.68		4.76		6.18		5.70
H. occidentalis		5.30										
Pichia guilliermondii						3.78						
P. kluyveri		2.48		3.04		4.11		5.08				
P. membranifaciens		6.18			1.18	5.48	1.48	5.70		5.95		4.70
Issatchenkia occidentalis						1.88		3.72				
I. orientalis				6.48		5.30						5.30
I. terricola				6.00				4.40				3.00
Lachancea thermotolerans		4.70		6.00		5.30		6.00		5.00		6.00
Zygoascus hellenicus		3.61		1.40		3.58		4.28		1.90		
Zygosaccharomyces bisporus						4.51						
Total *Ascomycetes*		6.48		7.22	2.70	6.94	1.90	7.65	1.00	6.80		7.24
Total yeast counts	2.81	6.53	2.38	7.24	2.80	6.94	2.20	7.65	1.95	6.80	2.80	7.24

Results reported as log CFU g^{-1};
S, sound grapes; SR, sour rot damaged grapes.

3.5 Overall yeast species dissemination in sound and sour rotten grapes

In ecological terms, dissemination studies should include a large number of samples, over several years. Furthermore, the detection and isolation of a species is more ecologically relevant than its quantification (Lachance, 2003). Therefore, the overall species identified over a 3-year period is presented in Table 4. In this study, *C.stellata/zemplinina*, *I. occidentalis* and *Z. hellenicus* were isolated during 2003, 2004 and 2005 vintages. *C. stellata/zemplinina* was recovered from both healthy and sour rot grapes, while *I. occidentalis* and *Z. hellenicus* were only isolated from sour rotten grape samples.

Beyond the presence of basidiomycetous yeasts, only five ascomycetous species (*C.stellata/zemplinina*, *C. vanderwaltii*, *H. uvarum, P. membranifaciens* and *L. thermotolerans*) were isolated from sound grape samples and always recovered with GYP general-purpose media (Table 4). Regarding sour rot grapes, a much higher diversity was found. A total of 22 different ascomycetes were isolated in this ecological study, from which only 17 species were present in these grapes. Therefore, this observation indicates that the typical grape yeast flora is greatly changed when grapes are damaged by sour rot. In our study, these different species were *C. amapae, C. diversa, C. ethanolica, C. tropicalis, C. oleophila, H. guilliermondii, H. occidentalis, H. osmophila*, *P. galeiformis*, *P. guilliermondii, P. kluyveri, I. occidentalis, I. orientalis, I. terricola, Z. hellenicus, Z. bisporus* and *Z. bailii*. This greater diversity was particularly evidenced by the use of several culture media. The general-purpose medium GYP enabled the recovery of the highest number of ascomycetous species (10). However, the additional three culture media provided 10 other ascomycetous species. In particular, ZDM selective culture media was crucial for the isolation of tecnhologically relevant *Z. bailii* and *Z. bisporus* species. The isolation of *C. oleophila*, *C. tropicalis*, *P. guilliermondii* and *Z. hellenicus* was only possible because of the use of DBDM selective culture media (Table 4).

Table 4. Non-*Saccharomyces* species populations on healthy and sour rot grapes and influence of culture media on species recovery (3-year study).

Yeast Group	Yeasts	Vintage year	Sound grapes	Sour rot grapes	Culture media			
					GYP	MRS	DBDM	ZDM
Basidiomycetes		2003, 2004, 2005	+	+	+		+	
Oxidative or weakly fermentative	*Candida amapae*	2005		+	+		+	
	C. diversa	2004, 2005		+				+
	C. ethanolica	2005		+				+
	C. oleophila	2005		+			+	
	C. stellata/C. zemplinina	2003, 2004, 2005	+	+	+			
	C. tropicalis	2005		+			+	
	C. vanderwaltii	2003, 2005	+	+	+			
	Hanseniaspora uvarum	2004, 2005	+	+	+		+	
	H. guilliermondii	2005		+	+		+	
	H. occidentalis	2005		+	+			
	H. osmophila	2003		+		+		
	Pichia galeiformis	2003		+				+
	P. guilliermondii	2005		+			+	
	P. kluyveri	2005		+				+
	P. membranifaciens	2003, 2005	+	+				+
Issatchenkia spp.	*I. occidentalis*	2003, 2004, 2005		+	+			+
	I. orientalis	2005		+	+			+
	I. terricola	2003, 2005		+	+	+		+
Zygoascus hellenicus		2003, 2004, 2005		+			+	
Zygosaccharomyces related species	*Lachancea thermotolerans*	2004, 2005	+	+	+			
	Zygosaccharomyces bailii	2003, 2004		+	+			+
	Z. bisporus	2003, 2005		+				+
Total ascomycetous species			6	23	11	2	8	10

4. Discussion

The impact of damaged grapes on yeast diversity and their influence on yeasts dynamics during alcoholic fermentation were studied during three consecutive vintages. Our results are in accordance with the literature showing the predominance of basidiomycetous, apiculate and oxidative or weakly fermentative species in grapes (Pretorius, 2000, Fleet, 2003). The influence of grape health on yeast ecology demonstrated increased yeast numbers and diversity in damaged grapes and in their respective fermentations. During the three vintages, five ascomycetous species were isolated from sound grapes while 17 ascomycetous species were present only in damaged samples.

In technological terms, our most significative result was the isolation of *Z. bisporus* (2003 and 2005 vintages) and the osmotolerant and acidophilic species *Z. bailii* (2003 and 2004 vintages), which is regarded as one of the most dangerous wine spoilage species (Loureiro & Malfeito-Ferreira, 2003). The frequency of isolation of *Z. bisporus* from foods is much lower than that of *Z. bailii,* although it has a similar capacity to cause food spoilage and is preservative resistant (Pitt & Hocking, 1999). Jolly *et al.* (2003) reported the rare isolation of *Z. bailii* and *Zygosaccharomyces* spp. from sound Chardonnay grapes, while Nisiotou *et al.* (2007) isolated *Z. bailii* from fermentations of *Botrytis* affected grapes. González *et. al* (2007) isolated *Z. bisporus* at the end of only one wine fermentation set from a total of 14 red grape must fermentations in Tenerife. *Z. bisporus*, isolated from sherry film, was shown to resist to sorbic acid and sulphur dioxide (Splitstoesser *et al.*, 1978) and to produce odorous acyloins in sherry wines (Neuser *et al.*, 2000). However, it is not usually regarded as a dangerous wine spoilage yeast (Loureiro & Malfeito-Ferreira, 2003).

Other species, less relevant in technological terms than *Zygosaccharomyces* spp., were also detected. *Lachancea thermotolerans* was isolated from sound and damaged grapes. The *Lachancea* genus has been classified recently as a result of closely related DNA sequences between the species *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii* (Kurtzman, 2003). Baleiras-Couto *et al.* (2005) reported the isolation of *K. thermotolerans* species at the middle of several red musts fermentations and Xufre *et al.* (2006) showed its isolation from white grape juices. *Lachancea thermotolerans* has been detected in grapes affected by honeydew (Barata *et al.,* 2008) and from *Botrytis*-affected grape musts (Nisiotou *et al.,* 2007). *Zygosaccharomyces fermentati* (or *Z. cidri*) has been reported in the initial step of sherry fermentation at winery level (Esteve-Zarzoso *et al.*, 2001) and at the end of laboratory grape juice fermentation obtained by winery crushing of grapes (Romano & Suzzi, 1993). This species has the ability to produce volatile compounds in synthetic sherry-like medium (Freeman *et al.*, 1977). To the best of our knowledge there are no reported wine spoilage

outbreaks because of the *Lachancea* clade. However, the afinity with *Zygosaccharomyces* spp. justifies the future investigation of any possible spoilage ability. Another species, *Z. hellenicus*, or its anamorph, *C. steatolytica* (Smith *et al.*, 2005) has seldom been described as a grape contaminant. It has been isolated from Riesling grapes in Ontario (Holloway *et al.*, 1990) without any particular technological significance (Chamberlain *et al.*, 1997). Guerzoni and Marchetti (1987) sporadically isolated this species from sour rot damaged grapes and not from sound grapes. Because of the utilization of the DBDM selective media, we have shown its frequent isolation previously (Barata *et al.*, 2008). We are not aware of wine spoilage provoked by this species, but it would be interesting to understand why it is commonly isolated in damaged grapes.

The recovery of *P. guilliermondii* was also only achieved using DBDM medium. This species (or its anamorph *C. guilliermondii*) is regarded as a rare contaminant of grapes and musts (Jolly *et al.*, 2003). We have already isolated it from grapes in the vineyard, grape musts, grape stems, wines and insects using DBDM (Dias *et al.*, 2003, Martorell *et al.*, 2006, Barata *et al.*, 2008). Recently, *P. guilliermondii* was isolated from the beginning and end of healthy fermenting musts from Greece (Nisiotou *et al.*, 2007). Therefore, this species seems a common inhabitant of winemaking environments, although with low frequency. It has the ability to produce 4-ethylphenol in grape musts before fermentation but does not spoil wines by volatile phenol production (Barata *et al.*, 2006).

The yeast dynamics study performed during grape ripening (2005) showed that in healthy grapes, basidiomycetes and *C. stellata/zemplinina* were the most frequent species, while in sour rotten grapes *H. uvarum* and *C. vanderwaltii* dominate the yeast populations in all three grape-ripening phases. Other common isolates belong to the genus *Candida* and *Pichia*, which are present in both types of health states. These species are common contaminants of grapes (Fleet, 2003), so their isolation was not unexpected. The distinction between *C. stellata* and *C. zemplinina* has been described recently (Sipiczki, 2003, Sipiczki *et al.,* 2005) and was not performed because we could not recover the strains from the maintenance slants, probably because of acetic acid production (Sipiczki, 2004). Probably, our strains were also *C. zemplinina* because it was more frequently found than *C. stellata* in damaged grapes used for botrytized wines (Csoma and Sipiczki, 2008). These genera are not regarded as dangerous wine spoilers (Loureiro & Malfeito-Ferreira, 2003).

In the microvinification trials, results showed that several yeast species were present during the beginning and middle fermentations phases, emphasizing the diversity of microorganisms that constitute the fermentation environment. A greater diversity of yeast species at initial fermentations phases was clearly observed when sour rot damaged grapes

were introduced in the vinification. Most of the recovered species from microvinifications with sour rotten grapes are regarded as innocuous to wine stability and do not survive fermentation with the addition of *S. cerevisiae*. The most relevant result of our work was the consistent recovery of the species *Z. bailii* during all fermentation steps with 30% (p/p) sour rot damaged grapes (2003 and 2004 vintages). This demonstrates that *Z. bailii* from sour rot grapes is an important contamination source of wineries and wines. If technological preventive measures fail, this species may contaminate winery surfaces and be hazardous to bottled wine stability.

The current application of direct molecular methods for ecological analysis has revealed a great diversity of microorganisms in various habitats that were not detected previously by plating methods (Pace, 1997). Recently developed direct molecular techniques are useful to detect the presence of different species (Prakitchaiwattana *et al.*, 2004). However, these are not sensitive enough to recover minority species and are therefore not able to detect minor but technologically significant differences in the ecology of damaged grapes. The results of this work, and those of Barata *et al.* (2008), show that in order to ascertain the extent of the impact of damaged grapes on yeast diversity, the use of selective media directed to the recovery of minority species is required, as hypothesized by Loureiro *et al.* (2004). This fact was already demonstrated in early ecological works on wine environments (Van der Walt and van Kerken, 1961; Davenport, 1974; Florenzano *et al.*, 1977) but has not been taken into account in recent studies which rely on the utilisation of general-purpose media, followed by species identification by molecular methods.

In conclusion, our results strongly indicate that grape damage constitutes the main factor determining the yeast ecology of grape surfaces. The alteration of the ecological balance is particularly evidentiated by the high frequency of isolation of *Z. hellenicus* and *Issatchenkia spp*., only from sour rotten grape samples. Furthermore, the scarce but technologically relevant isolation of *Z. bisporus* and *Z. bailii* shows the importance of using selective culture media. The latter species, carried into the winery by damaged grapes, endure fermentation and may contaminate wine during the next stages of winemaking.

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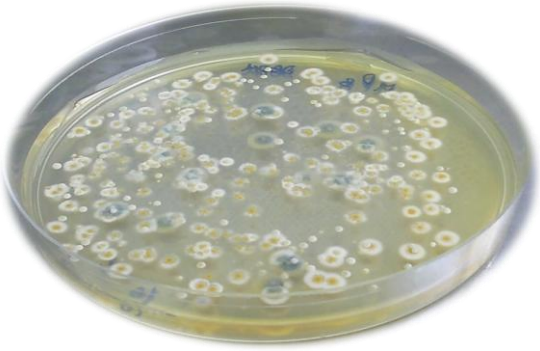
CHAPTER 3

Dynamics of sour rotten grape berry microbiota during ripening and wine fermentation



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Dynamics of sour rotten grape berry microbiota during ripening and wine fermentation

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Abstract

The present study investigates the dynamics of the overall grape berry microbiota involved in sour rot grape bunch disease. The microbial populations of yeasts, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) were studied on sound and sour rotten berries during ripening and also throughout experimental wine fermentations of sound and sour rot grape musts. A careful sampling strategy with separation of berries or bunches according to their health state was performed, followed by isolation and enumeration of the dominant and minority species. Yeast isolates were identified by RFLP of the 5.8S-ITS rDNA and sequencing of D1/D2 domains of 26S rRNA gene, AAB by RFLP of the 16S and 16S-23S ITS rRNA and sequencing of both regions and LAB by 16S-ARDRA combined with sequencing of 16S rRNA gene. The alteration of the ecological balance induced by sour rot was particularly evidentiated by the unequivocal increase of yeast and AAB counts on rotten grapes, since the beginning of ripening. Yeast and AAB species diversity in rotten grape samples were much higher to those found in sound grapes. LAB populations were absent from both healthy and sour rotten grapes. The yeast species *Issatchenkia* spp., *Zygoascus hellenicus* and *Zygosaccharomyces bailii* and the AAB species *Gluconacetobacter hansenii, Gluconacetobacter intermedius, Acetobacter malorum, Acetobacter orleaniensis* and *Acetobacter syzygii* were only isolated from damaged grapes. To our best knowledge, this is the first time that these AAB species are isolated in the vineyard from sour rotten grapes. Wine fermentation inoculated with *Saccharomyces cerevisiae* induced a strong reduction on the microbial diversity. After malolactic fermentation, racking and sulphur dioxide addition the only remaining species were *Trigonopsis cantarelli* and *S. cerevisiae*, independently of the grape health status.

Keywords: damaged grapes, sour rot, yeasts, acetic acid bacteria, lactic acid bacteria, wine fermentation

1. Introduction

Grape quality may be affected by a wide range of rots. Among them, sour rot is an emergent grapevine disease affecting late ripening cultivars with tightly-packed, thin-skinned and dense bunches close to harvesting, causing heavy crop losses and being detrimental to juice and wine quality (Bisiach, 1986; Wolf *et al.*, 1990; Zoecklein *et al.*, 1992). Rotten bunches have a strong and pungent odor of vinegar as a result of the production of high levels of acetic acid, accompanied by high concentrations of glycerol, ethyl acetate, ethanol, acetaldehyde and galacturonic and gluconic acids (Marchetti *et al.*, 1984; Zoecklein *et al.*, 2001). These products are the result of a mixed population of yeasts and acetic acid bacteria (AAB) (*Acetobacter* spp. and *Gluconobacter* spp.) (Bisiach, 1986; Blancard *et al.*, 2000; Gravot *et al.*, 2001).

Berry rupture is associated with a sudden increase in yeast load up to about $10^6 - 10^8$ CFU/g and deep alterations in species diversity occur when compared with sound grapes (Barata *et al.*, 2008a; 2008b). The main yeast species recovered from sour rotten grapes are *Candida krusei*, *Issatchenkia orientalis*, *Kloeckera apiculata*/*Hanseniaspora uvarum*, *Saccharomycopsis vini*, *Candida steatolytica* (syn. *Zygoascus hellenicus*), *Torulaspora delbrueckii*, *Issatchenkia terricola* and *Zygosaccharomyces bailii* (Barata *et al.*, 2008a; 2008b; Bisiach, 1982, 1986; Guerzoni and Marchetti, 1987; Guerzoni and Marchetti, 1982; Marchetti *et al.*, 1984). Other fungi, such as *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Cladosporium* spp., *Rhizopus* spp., *Mucor* spp. and *Phomopsis viticola*, though isolated from grapes with sour rot, cannot induce the disease (Bisiach, 1986). The population size of AAB on healthy grapes is typically low ($10^2 – 10^3$ cells/g) and *Gluconobacter oxydans* is the species most represented, while in grapes damaged by *Botrytis cinerea* (gray rot), AAB populations can reach up to $10^5 – 10^6$ cells/g, comprising *Gluconobacter* spp. and *Acetobacter* spp., mainly *A. aceti* and *A. pasteurianus* (Barbe *et al.*, 2001). Undamaged grapes contains low populations of LAB, not exceeding 10^3 CFU/g and the initial titer in must is low (Bae *et al.*, 2006; Fugelsang, 1997; Lafon-Lafourcade *et al.*, 1983). Only a few LAB species of the genera *Lactobacillus* spp. (*Lb.*), *Leuconostoc* spp. (*Lc.*), *Pediococcus* spp. (*P.*), *Oenococcus* spp. (*O.*) and *Weissella* spp. (*W.*) can grow in must and wine (König and Fröhlich, 2009). The species *Oenococcus oeni*, regarded as the main agent of malolactic fermentation (Henick-Kling, 1993; Lonvaud-Funel, 1995, 1999) has been seldom isolated from grapes in the vineyard (Renouf *et al.*, 2007). Several other bacterial species have also been isolated from sound and gray rotten grapes (Nisiotou *et al.*, 2011). However, these species have no technological

significance in winemaking, being probably only a result of environmental contamination of berry surfaces.

In the past, one of the biggest hurdles in ecological studies has been the lack of rapid and reliable methods for species identification, which have been performed according to morphological, physiological and biochemical tests, leading frequently to imprecise results. On the last decade, quick and easy molecular biology techniques based on DNA analysis have been developed and are now available for the classification and identification of yeasts, AAB and LAB differentiating at the genera, species and strain levels. In case of yeasts, the most definitive and universal assay determines the sequence of the D1/D2 domain of the 26S ribosomal DNA, which have sufficient variation to allow differentiation of most ascomycetous (Kurtzman and Robnett, 1998, 2003) and basidiomycetous (Fell *et al.*, 2000; Scorzetti *et al.*, 2002) yeast species. On the other hand restriction fragment length polymorphism (RFLP) of the 5.8S-ITS rDNA region proved to be one the most usefull, rapid and easy method for yeast identification (Esteve-Zarzoso *et al.*, 1999; Guillamón *et al.*, 1998). The 16S rDNA gene sequence has been widely used as a molecular method to estimate phylogenetic relationships among bacteria. Ribosomal genes are compared in most taxonomical studies of bacteria (Guillamón and Mas, 2009). In particular, the 16S rRNA gene is a highly conserved region, but with some variable zones that can be used for identification purposes. PCR-RFLP of the 16S rDNA is appropriate for differentiating and grouping AAB (Poblet *et al.*, 2000; Ruiz *et al.*, 2000). Moreover, it is known that DNA sequences in the 16S-23S ITS region exhibit a great deal of sequence and length variation and conserved sequences with functional roles (Kretova and Grones, 2005; Prieto *et al.*, 2007; Ruiz *et al.*, 2000; Sievers *et al.*, 1996; Trček and Teuber, 2002). Combining PCR-RFLP of both 16S rDNA and 16S-23S-ITS regions, the resulting restriction patterns obtained with several restriction enzymes are characteristic of every species and make it possible to characterize almost all AAB species (González *et al.*, 2006; Guillamón and Mas, 2009). Concerning LAB identification, the amplified 16S rDNA restriction analysis (16S-ARDRA) generates species-specific patterns allowing the identification of the majority wine LAB species (Rodas *et al.*, 2003; Rodas *et al.*, 2005; Sato *et al.*, 2001), but it is not useful for typing purposes because 16S rRNA gene sequence is highly conserved at the species level (Rodas *et al.*, 2005).

Previous work from our laboratory, based on accurate berry sampling, has shown that grape damage by sour rot induced dramatic increases on yeast counts and number of species since the beginning of grape ripening (Barata *et al.*, 2008a; 2008b). However, the knowledge of the complex microbial dynamics that lead to sour rot disease has not

yet been completed and clearly understood, particularly in regard to the role and evolution of bacterial populations. Therefore, the purposes of this work were: (1) to investigate the dynamics of the overall grape contaminants including yeasts, AAB and LAB populations present on healthy and sour rotten berries surfaces during different stages of ripening, and (2) to study the fate of these populations during winemaking at winery level. For that, a careful berry sampling followed by isolation and enumeration on both general purpose media and selective and differential media, together with identification by molecular methods were performed.

2. Material and Methods

2.1 Grape Samples

During the 2007 vintage, healthy and sour rot affected bunches of *Vitis vinifera* L. cv. Trincadeira red grape variety were selected from a experimental vineyard of Instituto Superior de Agronomia, located in Tapada da Ajuda, Lisbon, Portugal (latitude 38º 42´31.57´´ N and longitude 9º 11´14.01´´ W). Just after the veraison period, the Trincadeira parcels were visually inspected and vines containing bunches with sour rot symptoms were marked. Two sets of three different vines (duplicates) were selected for taking samples of sound and sour rot affected bunches at three different ripening phases: i) after veraison (10 August 2007); ii) middle of ripening (22 August 2007); iii) harvest time (7 September 2007). A total of three bunches from each vines set were aseptically collected and transported to the laboratory in sterile bags at 5 ºC. Individual grape berries were randomly and aseptically removed from the bunches and combined to give a total of 100 sound berries plus 100 damaged berries.

The berries were placed in a sterile stomacher bag and crushed in a stomacher blender (IUL, Barcelona, Spain). The obtained grape juices from each phase were subsequently used for the microbiological analyses and 50 mL samples were collected in sterile 50 mL labeled plastic tubes and stored frozen at -20 ºC until chemical analysis by Fourier Transform Infrared (FTIR) Spectroscopy.

2.2 Experimental wines

Sound and sour rotten Trincadeira grape bunches were hand-harvested at time of full maturation in the same experimental vineyard, carefully separated in different 20 kg plastic boxes and transported to the experimental cellar of Instituto Superior de Agronomia, located 700 m distance from the vineyard (latitude 38º 42´26.36´´ N and longitude 9º 10´57.39´´ W).

Microvinification sets of 100% sound Trincadeira grapes (control sets) and Trincadeira musts containing 30% of sour rotten grapes were performed in 50 kg capacity stainless steel vats (30 kg of grapes per set). Firstly, sound bunches were combined to give a total of 60 kg of grapes and later sound and sour rotten bunches were weighed and mixed in order to obtain a total of 60 kg of a grape mixture containing 30% sour rotten grapes. Rotten bunches were visually inspected and only the rotten parts of the bunch were selected for the mixture. Afterwards, both grape blends were mechanically destemmed and crushed on a commercial grape destemmer-crusher. The equipment was disinfected between operations. The resulting grape musts were divided into different vats (30 kg each).

A 50 mL sample of both 100% sound and 30% sour rotten grape musts were collected and transported for the laboratory for immediate microbiological analyses.

In order to evaluate the effect of the initial vatting sulfur dioxide (SO_2) concentration on the must microbial flora and further wine microbial stability, both grape musts sets were added with 30 mg/kg and 100 mg/kg, using an aqueous solution of SO_2 (6% w/v). A pre-fermentative maceration period (18 hours) were performed at cellar temperature (18 $\pm$ 2 ºC), followed by inoculation of musts with 10^6 cells/mL of selected commercial Saccharomyces cerevisiae yeast (Fermivin®, DSM, Delft, The Netherlands). Alcoholic fermentations took place at cellar temperature and the cap was punched down twice a day until it remained submerged. After completion of fermentation, the resulting wines (free run and press wines obtained from pomace pressing) were transferred to clean 20L glass carboys, where it underwent spontaneous malolactic fermentation (MLF) at cellar temperature. Degradation of malic acid was monitored through paper chromatography (Ribéreau-Gayon *et al.*, 1972). Once MLF was completed, wines were racked (lees were removed) and free SO_2 levels were adjusted to 40 mg/L. After one week, wines were bottled without filtration in 0.75 L glass bottles capped with natural corks and kept at cellar temperature.

Microbiological analyses were performed in three different stages of winemaking: i) vatting (initial grape must); ii) just after alcoholic fermentation; iii) after MLF.

2.3 Chemical analysis of grape musts during ripening process

Sound and sour rotten grape musts samples were analyzed by Fourier Transform Infrared (FTIR) Spectroscopy in a WineScan FT120 spectrometer (FOSS, Hilleroed, Denmark, http://www.foss.dk), that employs a Michelson interferometer to obtain the FTIR spectra. The numbers of scans generated per sample, the selection of wavenumbers, and the processing of spectra have been fixed by the manufacturer (FOSS, 2000) and are not accessible to change by the user. Partial least squares regression (PLS), available within the software package of the WineScan FT 120, was the multivariate method that carried out calibrations. Statistical data analysis has been set by the manufacturer and is not accessible to change by the user.

The FOSS-supplied GrapeScan Calibration was used. This calibration was designed for analyzing the quality of the incoming grapes by means of the sanitary state of the grapes. Grape musts sanitary results are based on four different parameters within the following range: gray rot index (0-60; 0=excellent grapes; 10=good grapes; 20=slight attack; 30=critical attack; 40=serious attack; 50=severe attack; 60=total rotten grapes), sour rot index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe attack), yeast activity index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe attack) and lactic bacteria activity index (0-2; 0=good grapes, 1=medium attack, 2=severe attack) (FOSS, 2002). Additionally, the following analytical parameters were also determined: Glucose-Fructose content, Brix degree, total acidity, volatile acidity, pH, tartaric acid, malic acid, total assimilable nitrogen, anthocyanins, total phenols (Folin-Ciocalteau index) and color intensity.

2.4 Microbiological analyses

2.4.1 Enumeration and isolation of yeasts from grapes

The microbial populations of yeasts were studied on grape musts samples obtained from sound and sour rotten grapes during ripening. Just after berries crushing (100 berries sample), grape musts were decimally diluted (10^{-1} to 10^{-6}) in peptone water (Merck, Darmstadt, Germany). The enumeration and isolation of yeasts was carried out by surface plating (0.1 mL) of the adequate dilutions onto plates (duplicates) of several

culture media: i) general purpose medium GYP agar, composed by 20 g/L glucose (Merck), 5 g/L yeast extract (Oxoid, Hampshire, UK), 5 g/L peptone (Difco Laboratories, Detroit, USA), 20 g/L agar, pH 6.0, supplemented with 100 mg/L of chloramphenicol (Sigma, Steinheim, Germany) and 0.5 g/L of biphenyl (Fluka, Buchs, Switzerland) to avoid growth of bacteria and moulds, respectively; ii) selective/differential media DBDM (Rodrigues *et al.*, 2001) and ZDM (Schuller *et al.*, 2000) for the isolation of *Dekkera/Brettanomyces* and *Zygosaccharomyces* species, respectively, or other non-*Saccharomyces* species as previously reported by (Barata *et al.*, 2008a; 2008b). Total yeasts enumeration was obtained from the GYP plates, recording the number of colony forming units (CFU) counts.

In case of *Dekkera/Brettanomyces* species, a secondary procedure was performed using the enrichment medium EBB (Renouf and Lonvaud-Funel, 2007) in order to increase the changes of recovering these particular wine spoilage yeasts species from grapes. Fifty berries randomly selected from each grape sample were placed in a 500 mL erlenmeyer flask containing 170 mL EBB broth. Berries were incubated at 30 ºC in a orbital shaker (Agitorb 200IC, Aralab, Lisbon, Portugal) at 80 rpm for 10 days. After the enrichment step, the medium was decimally diluted (10^{-1} to 10^{-6}) in peptone water and yeasts were recovered by spreading 0.1 mL (duplicates) of the appropriate dilutions onto GYP, DBDM and ZDM plates. Plates were incubated at 25 ºC during 7 days (GYP) and up to 15 days (DBDM and ZDM).

Following incubation, all media plates containing countable yeast colonies (1 to 300) were examined, all different colony morphological types were registered, differentially counted and representative isolates of each morphology were selected for identification. All isolates were streaked and purified on GYP plates. After purification, yeast isolates were preserved on YM agar slants (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 20 g/L agar) until their later analysis, stored at 4 ºC and subcultured every month.

2.4.2 Enumeration and isolation of AAB and LAB from grapes

The enumeration of AAB and LAB was carried out using the Most Probable Number (MPN) technique (OIV, 2010). Briefly, grape musts samples were decimal diluted until extinction (10^{-1} to 10^{-7}), and 1-mL portion of must (10^{0}) and 1-mL of each dilution, were inoculated into three tubes containing 3 mL of GY [50 g/L glucose, 10 g/L yeast extract, and 13 g/L agar, 200 mg/L Delvocid® (DSM, Delft, Netherlands) (100 mg/L natamycin),

3000 U/L penicillin (Sigma), pH 4.5] and 5 mL of MRS [52 g/L MRS (Oxoid), 200 mg/L Delvocid®, 0.5 g/L L−cysteine (Sigma)] broths, for enumeration of AAB and LAB, respectively. Delvocid® and penicillin were used to inhibit the growth of yeasts and LAB, respectively. Inoculated tubes were incubated at 28 ºC during 8 days (up to 10 days), under aerobic conditions, for AAB, and under microaerophilic conditions, by addition of 500 µL sterile paraffin oil (Sigma), for LAB, making periodic observations up to the last day of incubation. All those tubes that showed a microbial development leading to the formation of a more or less evident whitish deposit and/or disturbance were considered as positive. Results were confirmed by observation through a microscope.

Bacterial isolates were obtained by streaking an aliquot (10 µL) of all tubes with visible growth in agar plates containing the respective isolation medium. Plates were incubated at 28 ºC up to 8 days. The different colony/cell morphologies found in each positive tubes of each dilution were registered and representative isolates of each morphology were purified, by re-streaking on the respective GY or MRS plates and selected for molecular identification. For long term storage, purified isolates were maintained frozen at -80 ºC in vials containing the isolation liquid medium with 15% (v/v) glycerol (Merck), until their later analysis. After identification, enumeration (MPN/mL) of each recovered species was obtained by using the statistical tables and rules presented in OIV 206/2010 Resolution (OIV, 2010). Gram staining and catalase production tests were conducted for all bacterial isolates.

2.4.3 Yeasts, AAB and LAB enumeration and isolation during wine fermentations

This study was also conducted for the vinification samples (initial vatting grape musts and final wines) on each previously described winemaking phase. Initial vatting grape musts and wine samples were decimally diluted (10^{-1} to 10^{-7}) in peptone water (Merck). The enumeration and isolation of yeasts was carried out by surface plating (0.1 mL) of the adequate dilutions onto plates (duplicates) of GYP, DBDM and ZDM, as described previously. In case of wine samples just after alcoholic fermentation and MLF, additional GYPC plates [20 g/L glucose (Merck), 5 g/L yeast extract (Oxoid), 5 g/L peptone (Difco), 20 g/L agar, 10 mg/L of cycloheximide (Sigma), pH 6.0] were also used in order to inhibit the growth of *S. cerevisiae* species. Plates were incubated at 25 ºC during 7 days (GYP and GYPC) and up to 15 days (DBDM and ZDM). Likewise, the quantification and isolation of AAB and LAB during wine fermentations, were performed by the MPN technique as described before.

2.5 Identification of yeasts isolates

Yeast isolates were identified by restriction fragment length polymorphism (RFLP) analysis of the 5.8S-ITS rDNA region (Esteve-Zarzoso *et al.*, 1999). For the majority isolates, yeast cells were picked from a fresh colony (48-h-old) and resuspended directly in the PCR mixture. Isolates for which direct amplification from colony could not be performed, the yeast genomic DNA for PCR amplification was isolated as described in Cryer *et al.* (1975) after a previous enzymatic treatment with liticase (5 mg/mL).

PCR reactions were performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) termocycler using the primers (ITS1 and ITS4) and conditions described in Esteve-Zarzoso *et al.* (1999). Two units of Go Taq® DNA polymerase (Promega, Madison, Wisconsin, USA) were added to each reaction. For restriction reactions of the 5.8S-ITS region, 10 μl of amplified product was incubated for 5 h at 37 ºC with 10 U of *Cfo*I, *Hae*III and *Hin*fI (Bioron, GmbH, Ludwigshafen, Germany) restriction endonucleases. PCR products and restriction fragments were detected and separated by electrophoresis on 1.4% (w/v) and 3% (w/v) agarose gels in 1× TAE buffer, respectively. Gels were stained with ethidium bromide (0.05 μg/mL), visualized and photographed under UV light using the Bio-Rad Gel Doc 2000 system (BioRad, California, USA). Lenghts of amplification products and restriction fragments were determined by comparison with 100-bp DNA ladder (Bioron) using the Quantity One 1-D analysis software (BioRad).

Restriction patterns generated were recorded and compared with those contained in the Yeast-id database at www.yeast-id.com (Valencia University and CSIC, Spain).

The isolates sharing similar restriction patterns or with misidentified patterns, were grouped and only representative isolates of each case were identified by sequencing the D1/D2 domains of the 26S rRNA gene. This region was amplified using the primers NL− 1 and NL− 4 (O'Donnell, 1993) and PCR reactions were performed as defined by Kurtzman and Robnett (1998). The amplified products were purified by GFX PCR and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions and then directly sequenced. Primers NL− 1 and NL− 4 were used in the sequencing reactions to read both DNA strands of D1/D2 26S rRNA gene. Sequences were edited and assembled using BioEdit Sequence Alignment Editor version 7.0.1 software (Hall, 1999), and then subjected to the GenBank BLASTN search tool of the NCBI database (http://ncbi.nlm.nih.gov/blast) to retrieve sequences of closely related taxa.

The cluster *Hanseniaspora uvarum/H. guilliermondii/H. opuntiae* was differentiated by growth on GYP agar plates incubated at 37 ºC (Smith, 1998) and by restriction of 5.8S-ITS region with *Dde*I and *Dra*I (Bioron) endonuclease in accordance with Cadez *et al.* (2002) and Nisiotou and Nychas (2007), respectively. The *Lachance*a clade former species (*L. fermentati*, *L. cidri*, *L. thermotolerans* and *L. waltii)* were differentiated by growth on GYP agar plates incubated at 37 ºC and 40 ºC, and growth with D-galactose and 0.01% cycloheximide (Kurtzman, 2003; Kurtzman and Fell, 1998). *Pichia deserticola* was differentiated from *Candida pseudolambica* by growth on GYP agar plates incubated at 37 ºC and growth with 50% D-Glucose (Kurtzman and Fell, 1998). *Candida stellata* was distinguished from *C. zemplinina* by digestion of the 5.8S-ITS region with *Dra*I (Bioron) endonuclease in accordance with Sipiczki (2004).

2.6 Identification of acetic acid bacteria isolates

Gram-negative and Gram variable, catalase positive isolates recovered from GY broth tubes were considered as putative AAB and selected for identification. The isolates were identified by RFLP of PCR-amplified 16S rRNA gene and RFLP of PCR-amplified 16S-23S ITS region according to González *et al.* (2006). DNA extraction was carried out using the method described by Ruiz *et al.* (2000) with the following modifications: fresh cultures were grown on GY plates and 3 to 5 bacterial colonies were picked and ressuspended in 50 μL of lysis solution (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and incubated at 100 °C for 15 min (boiling water bath). The suspension was cooled at room temperature, centrifugated (5000 *g*, 4 ºC, 5 min) and the supernatant containing the released DNA was diluted 100-fold with sterile miliQ water and used as template for PCR amplification. The 16S rRNA gene and 16S-23S ITS regions were amplified using the primers and thermocycling conditions designed by Ruiz *et al.* (2000). PCR amplifications were carried out in 50 μL reaction volume, consisting of 2 μL bacterial DNA lysate and 48 μL amplification mixture, containing 10μL of 5× amplification buffer Green Go Taq Flexi (Promega), 200 μM of dNTPs mixture, 2 mM $MgCl_2$, 0.3 μM of each primer and 2.5 U Go Taq® (Promega). For the restriction reactions of the 16S rRNA gene-amplified products, 5 μL of each PCR product was digested with 10 U of *Alu*I (Fermentas, Thermo Fisher Scientific Inc., Leicestershire, UK), *Cfo*I, *Hinf*I, *Hae*III, *Tru9*I (Bioron) and *Taq*I (Metabion, Martinsried, Germany) restriction endonucleases, as recommended by the manufacturers.

Isolates inconclusively identified by RFLP of 16S rRNA gene as *Gluconacetobacter europaeus*/*Ga. xylinus/Ga. intermedius/Ga. saccharivorans* and *Acetobacter cerevisae*/*A. orleaniensis*/*A. malorum*, were resolved by RFLP of 16S-23S ITS region and by sequencing of both 16S rRNA gene and 16S-23S ITS region. In case of the *Gluconacetobacter* species the amplified 16S-23S ITS product (5 μL) was digested with *Pvu*II (Fermentas) endonuclease, as described by Sievers *et al.* (1996), while the three *Acetobacter* species were separated using *Alu*I and *Cfo*I endonucleases (González *et al.*, 2006).

The PCR products and restriction fragments were detected and separated by 1.0% (w/v) and 3.5% (w/v) agarose gel electrophoresis in 1× TBE buffer, respectively. Restriction patterns were obtained after estimation of fragment sizes by comparing their mobility against a 100-bp DNA Ladder (Bioron), as described above.

Sequencing reactions were performed with the same direct and forward primers used for PCR amplification of both regions. The obtained sequences were corrected and later aligned in the Genbank database using the BLASTn algorithm, as previously described.

2.7 Identification of lactic acid bacteria isolates

Isolates obtained from positive growing MRS tubes were firstly assigned as presumed LAB on the basis of their character Gram + and catalase negative. Those isolates were initially characterized and grouped in terms of the molecular patterns obtained by 16S-ARDRA (Amplified Ribosomal DNA Restriction Analysis) for presumptive identification at species level, as described by Rodas *et al.* (2003).

Prior to PCR amplification, the isolates were grown on MRS plates supplemented with L-cysteine (0.5 g/L) at 28 ºC for 2-3 days, and 2 to 3 fresh colonies were picked and suspended in 50 μL of lysis solution following the same strategy previously described for DNA extraction of AAB isolates. The 16S-rDNA was amplified using the primers (pA and pH) and PCR conditions described in Rodas *et al.* (2003). DNA amplification was carried out in 50 μL PCR mixture containing 10 μL of 5× amplification buffer Green Go Taq Flexi (Promega), 200 μM of dNTPs mixture, 2 mM $MgCl_2$, 0.5 μM of each primer and 2.5 U Go Taq® (Promega). Restriction of the amplified 16S-rRNA gene was carried out using *FspB*I and *Mse*I (Fermentas) endonucleases, selected as the most discriminative enzymes in accordance to Rodas *et al.* (2003). PCR products (5 μL)

were digested overnight with 10 U of each enzyme and the obtained restriction fragments were separated and analysed following the same procedure described for the AAB identification. Representative isolates from each restriction pattern were then selected for sequencing of partial 16S rDNA, using pA and pH primers, in order to confirm the restriction analysis results and to stablish the LAB isolates final identity at species level. The amplified 16S rDNA fragments were purified, sequenced and the final corrected sequences were submitted to the BLASTn network service.

2.8 Nucleotide sequences accession numbers

Nucleotide sequences were deposited in the NCBI GenBank database library under the accession numbers JF749205-JF749219, JF718412-JF718428 and JF718430-JF718436.

3. Results and Discussion

3.1 Sour rot effect on grape must chemical composition during ripening

The evolution of the oenological parameters of sound and sour rotten grapes during three different ripening phases, determined by FTIR analysis are shown in Figure 1.

Results showed that the rotting process exerts significant effects on several parameters. Since veraison, sour rotten grape samples had always higher glucose-fructose and Brix degree content than sound grapes. In the harvest, significant (p<0.05) higher sugar concentrations were found in rotten grapes. This result could be explained as a consequence of the typical dehydration process occurring in rotten berries, which leads to an obvious concentration in sugar content. Therefore, higher alcohol contents shoud be expected in wines produced from fermentations containing higher percentages of rotten grapes.

At the harvest period, sour rot had a significant (p<0.01) rising effect on total acidity accompanied with a significant (p<0.05) drop in pH values (Figure 1). Despite this clear increase in total acidity, the tartaric and malic acid content in sour rotten grapes and its evolution during maturation was not significantly different from those of sound grapes in each ripening phase.

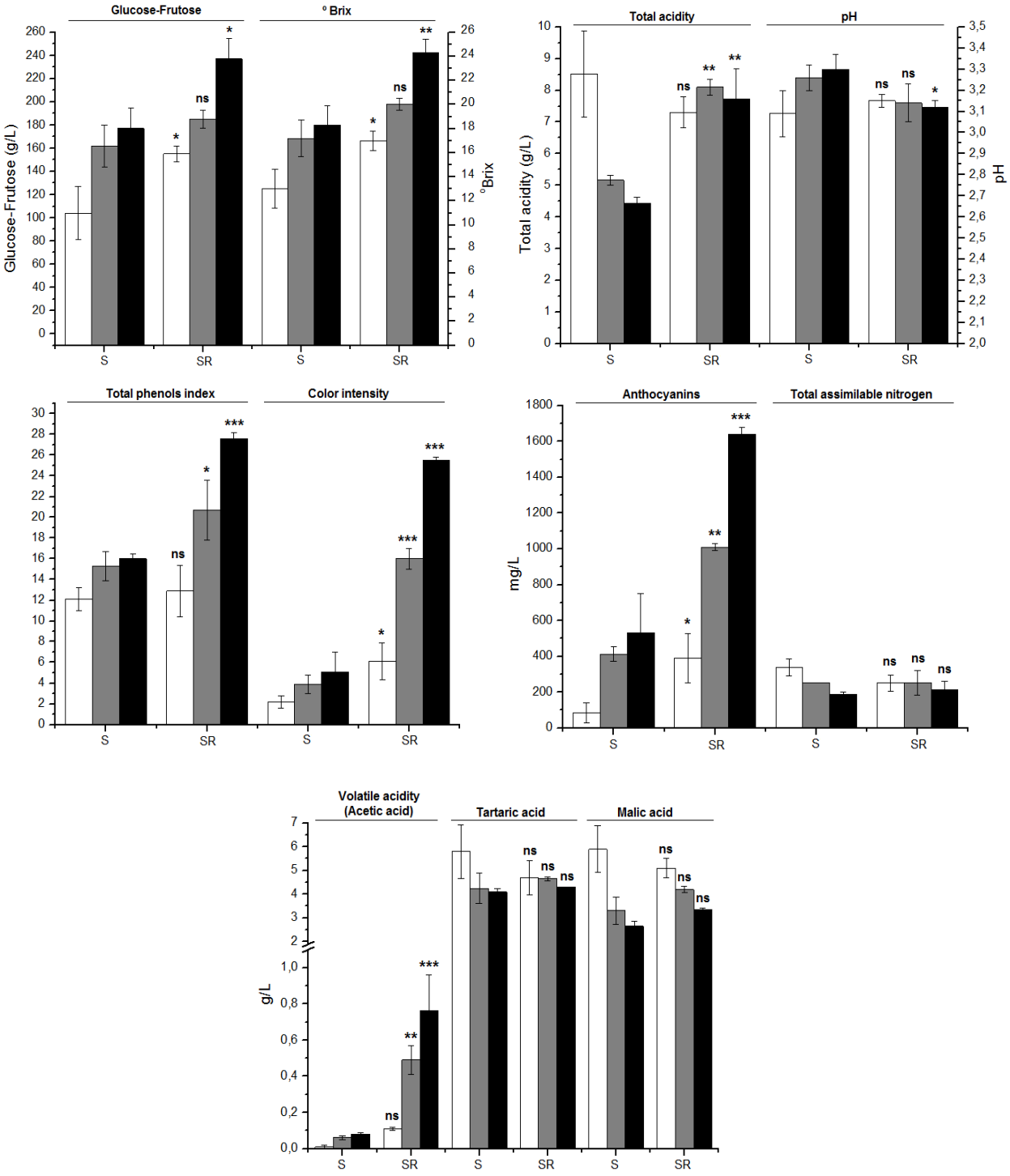

Fig. 1. Chemical composition of sound (S) and sour rotten (SR) grape must samples (100 berries) analysed by FTIR during three ripening phases (☐ = After Veraison, ■ = Middle of ripening; ■ = Harvest). Values are the average of two independent samples. Errors bars represent the standard deviation. For each parameter, significance (*p*-value) of sour rot factor was measured by ANOVA by comparing sound and sour rotten samples of each ripening phase. Notations at *, **, *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively; ns indicates no significant difference.

These results confirmed the typical evolution profile of both acids during grape maturation: tartaric acid concentration remained relatively constant during ripening, while malic acid followed the decrease in total acidity (Ribéreau-Gayon *et al.*, 2006). The unambiguous and highly significant increase (p<0.001) of volatile acidity present in rotten musts at harvest time (Figure 1), was most likely due to the activity of AAB. This result explains the strongest association between volatile acidity in wine and AAB when grapes are physically damaged or infected with fungal diseases, especially *B. cinerea* (Drysdale and Fleet, 1988; Sponholz, 1993). *Acetobacter* spp. are well known for the ability to oxidize the ethanol producing high amounts of acetic acid, while *Gluconobacter* spp. is characterized by a high oxidation activity of glucose accompanied by the accumulation of high gluconic acid concentrations (Guillamón and Mas, 2009). Moreover, the possible accumulation of other sugar degradation products such as gluconic, galacturonic, lactic, propionic, and butyric acids (Ribéreau-Gayon *et al.*, 2006), could also explain the significant drop in pH values found in rotten grapes.

FTIR results evidenced a clear effect of sour rot on raising the color intensity and the total phenols and anthocyanins grape musts contents. Independently of the ripening phase, rotten grapes showed always higher values of these parameters when compared with sound grapes. However, it was especially noteworthy the significant higher concentrations (p<0.001) present in rotten grapes at final of maturation.

At this stage, the dehydration degree of rotten berries and the probable higher skin/free juice ratio, could contribute to such a marked effect. Sour rot did not induce significant differences in total assimilable nitrogen content.

The FTIR analysis also allowed the evaluation of the sanitary quality of the grape samples throughout the maturation, by means of gray rot, sour rot, yeast activity and lactic bacteria indexes (Table 1).

Results of the sour rot index showed a suitable correlation with the sanitary state of grape must samples and the growing severity of sour rot attack throughout maturation. Sound samples maintained a constant low value of sour rot index, and were always classified as “good grapes” during ripening.

Sour rotten grapes showed significant higher values than those of sound grapes, being classified as “slight attack”, just after veraison, and as “serious attack” in the middle ripening and at harvest time.

Table 1. Evolution of sanitary state indexes during ripening for the sound and sour rotten grape samples (100 berries) determined by FTIR analysis.

Ripening phase	Gray Rot [a] (0-60)			Sour Rot [a] (0-30)			Yeast Activity [a] (0-30)			Lactic Bacteria [a] (0-2)		
	S	SR	p[b]	S	SR	p	S	SR	p	S	SR	p
After Veraison	22±9	43±1	**	4±1	8±0	*	6±0	6±0	ns	0±0	0±0	ns
Middle	16±9	48±2	**	4±0	12±1	**	6±0	7±0	ns	1±1	0±0	ns
Harvest	16±2	49±0	***	4±0	17±4	***	6±0	7±0	ns	1±1	0±0	ns

[a] Values are the average content of two independent samples (average±sandard deviation), S: sound grapes, SR: sour rotten grapes; [b]Significance of sour rot effect estimated for each ripening phase by ANOVA by comparing sound and sour rotten samples. Notations at *, **, *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively; ns indicates no significant difference.

The gray rot index did not prove to be an accurate measure of *Botrytis cinerea* contamination, since sound grape must samples (obtained from berries carefully selected and without visual signs of *Botrytis* infection) were classified as “good grapes to slight attack” during ripening. In case of sour rotten grapes, apparently without signs of *Botrytis cinerea* activity, significant higher values of gray rot index were obtained, resulting in a classification of “serious attack to severe attack”. These results seem to demonstrate that FTIR gray rot index may overestimate the contamination degree of *Botrytis cinerea* when sour rot is the dominant disease. The yeast and lactic bacteria activity indexes did not show significant differences between sound and rotten samples in each ripening phase.

3.2 Isolation and identification of yeasts, AAB and LAB

Concerning yeast isolation strategy, several culture media (GYP, DBDM and ZDM) were used for the isolation and enumeration of dominant and minority yeast species. An additional approach using a pre-enrichment step in EBB broth was used in order to attempt the recovery of *Brettanomyces* species from grapes. Similar strategy, using GYP, GYPC, DBDM and ZDM media, was followed for the analyses of grape musts and wine samples collected during winemaking. From countable plates of each culture media, yeasts colonies were morphologically classified into groups, and representative isolates were further subjected to identification by PCR-RFLP of the 5.8S-ITS rDNA analysis. A total of 203 yeasts isolates, recovered from sound and rotten grape samples during the three ripening phases, and 97 yeast isolates obtained from grape musts and wine samples throughout the three winemaking phases, were selected for identification. These isolates were gathered into 29 groups, on the basis of the restriction patterns generated by *Cfo*I, *Hae*III and *Hinf*I restriction endonucleases. From

the 29 different restriction patterns, 15 were characteristic of a single species and successfully identified by comparison with the restriction profiles contained in the Yeast-id database. Three other patterns were shared by more than one species (*C. zemplinina*/*C. stellata*, *H. uvarum/H. guilliermondii*/*H. opuntiae* and *Pichia deserticola*/*Candida pseudolambica*), and one pattern matched with the four yeast species included on the *Lachancea* clade (*L. fermentati*, *L. cidri*, *L. thermotolerans* and *L. waltii*). The remaining 10 groups were identified by sequencing of D1/D2 domains of the large subunit 26S rRNA gene. All identified yeast species, their respective restriction fragments and GenBank accession numbers of the D1/D2 domains sequences are listed in Table 2.

The AAB and LAB populations were recovered using the MPN technique, which is an advisable approach for the quantification of wine microbial consortium, in samples where moulds or environmental bacteria are numerous (Loureiro *et al.*, 2004). By using GY and MRS broths, supplemented with appropriate antibiotics, it was possible to inhibit, at least partially, the background microbiota and quantify the AAB and LAB species, respectively. A total of 76 and 74 AAB isolates, representative of the diversity found in all positive GY tubes, were obtained throughout grapes maturation and during the three winemaking phases, respectively.

The digestion of the PCR amplified 16S rRNA with six restriction enzymes (*Alu*I, *Cfo*I, *Hinf*I, *Hae*III, *Tru9*I and *Taq*I) yielded 5 different restriction patterns (I,II, VI, VII and XI) (Table 3) characteristic of a single species, which were successfully associated with *Acetobacter aceti*, *A. syzygii*, *A. tropicalis*, *G. oxydans* and *Gluconacetobacter hansenii* as described by González *et al.* (2006). These identifications were confirmed by sequencing of the 16S rRNA gene. Two other patterns were initially inconclusively identified, since they are characteristic of two groups of very closely related AAB species: *A. cerevisiae/A. orleaniensis /A. malorum* and *Ga. europaeus/Ga. xylinus/Ga. intermedius/Ga. saccharivorans*. Therefore, these species were differentiated combining the results obtained by PCR-RFLP of the 16S-23S ITS rRNA gene and sequencing of both 16S and 16S-23S ITS rRNA regions. Restriction patterns of the 16S-23S ITS rRNA region were obtained using *Alu*I and *Cfo*I endonucleases for the first group (González *et al.*, 2006) and *Pvu*II (Sievers *et al.*, 1996) for the second group.

Table 2. Yeast species identified by PCR-RFLP of 5.8S-ITS region of the rRNA using five restriction endonucleases (*Cfo*I, *Hae*III, *Hinf*I, *Dde*I and *Dra*I) and sequencing of the D1/D2 region of the 26S rRNA gene.

Yeast species	Restriction patterns						D1/D2 Sequencing	
	AP[a]	*Cfo*I	*Hae*III	*Hinf*I	*Dde*I	*Dra*I	BLASTn [b]	Acession no[c]
Aureobasidium pullulans	590	190-180-95-95	450-150	280-180-130				
Candida amapae	680	580-80	650	320-320				
Candida diversa	420	160-100-100	420	220-200				
Candida frutus	390	220-90-90	280-120	200-130-80				
Candida stellimalicola	540	210-160-130	540	295-240			EF550286 (100)	JF749207
							EF550286 (100)	JF749211
Candida zemplinina	480	210-110-110-60	480	240-240		310-120-50		
Candida apicola	450	210-90	420	230-120-120			FM201316 (99)	JF749210
Candida pomicola	650	590	570-80	320-320			EF460576 (99)	JF749218
Cryptococcus laurentii	530	280-260	350-120-60	270-270				
Cryptococcus magnus	630	360-280	490-80	260-240-140				
Dekkera bruxellensis	490	250-140-80-50	380-105	270-220				
Hanseniaspora guilliermondii	775	320-310-120	775	350-200-170	380-180-95-80	430-160-130		
Hanseniaspora uvarum	775	320-310-120	775	350-200-170	300-170-90-90			
Issatchenkia occidentalis	480	220-105-70-70	300-90-70	250-110-100				
Issatchenkia orientalis	550	220-180-90-70	400-100-50	230-170-50			FM180548 (100)	JF749209
							FM180548 (100)	JF749212
Issatchenkia terricola	450	130-95-95-80	290-130	230-110-110				
Lachancea thermotolerans	680	310-290-70	300-220-80-80	340-340				
Metschnikowia pulcherrima	400	210-100-95	290-100	210-190				
Pichia burtonii	450	440	440	240-200				
Pichia deserticola	450	150-150-100-50	280-100-80	250-210				
Pichia guilliermondii	630	300-270	400-120-80	320-300				
Pichia sporocuriosa	450	150-120-100-80	320-150	260-230			EF550232 (100)	JF749216
							EF550232 (100)	JF749219
Saccharomyces cerevisiae	850	380-330-140	310-230-170-130	380-370-120				
Saccharomycopsis crataegensis	590	530-60	580	310-270			HQ149313 (99)	JF749215
Saccharomycopsis vini	600	550	380-160-90	290-195 -140			FM212446 (100)	JF749205
Sporobolomyces roseus	600	295-220-90	595	280-190-160-120-80			GU585220 (100)	JF749213
Trigonopsis cantarellii	580	280-230-90	495-85	300-280			GU080055 (99)	JF749206
							GU080055 (100)	JF749217
Zygoascus hellenicus	650	320-320	630	340-170-130				
Zygoascus meyerae	450	140-120-90-80	290-150	240-210			HM450997 (100)	JF749208
Zygosaccharomyces bailii	780	320-270-95-95	690-90	340-220-160				

[a] Size of the amplified PCR product in base pairs; [b] GenBank accession number of the closely related taxa after alignment of the submitted sequence using the BLASTn algorithm. Percentage of similarity between brackets; [c] GenBank accession number of the sequence deposited in NCBI GenBank database library.

Table 3. AAB and LAB identified using PCR-RFLP of 16S and 16S-23S ITS rDNA, 16S-ARDRA and sequencing of both 16S and 16S-23S ITS rDNA regions.

		PCR-RFLP									16S ARDRA		Sequencing			
		16S rDNA						16S-23S ITS rDNA					16S		16S-23S ITS	
		*Alu*I	*Cfo*I	*Hinf*I	*Hae*III	*Tru*9I	*Taq*I	*Alu*I	*Cfo*I	*Pvu*II	*Fsp*BI	*Mse*I	BLASTn[a]	GenBank Acession no.	BLASTn	GenBank Acession no.
	AAB species															
I	*A. aceti*	Al1	Cf1	Hi1									D30768 (99)	JF718428		
II	*A. syzygii*	Al2	Cf2	Hi2			Tq1						DQ523496 (100)	JF718419		
III	*A. cerevisiae*	Al3	Cf3	Hi3		Tr1	Tq2	ITS_Al1	ITS_Cf1				NR_025512 (100)	JF718424		
IV	*A. malorum*	Al3	Cf3	Hi3		Tr1	Tq2	ITS_Al2	ITS_Cf2				FJ831444 (100)	JF718421		
V	*A. orleaniensis*	Al3	Cf3	Hi3		Tr1	Tq2	ITS_Al3	ITS_Cf3							
VI	*A. tropicalis*	Al3	Cf4	Hi3		Tr2	Tq3						DQ523494 (100)	JF718427		
VII	*G. oxydans*	Al4	Cf5	Hi4									AB178424 (100)	JF718426		
VIII	*Ga. europaeus*	Al5	Cf6	Hi5	Ha1					ITS_Pv1			FJ715623 (100)	JF718422	X85406 (92)	JF718423
IX	*Ga. intermedius*	Al5	Cf6	Hi5	Ha1					ITS_Pv2			AB099297 (100)	JF718417	FR716493 (99)	JF718418
X	*Ga. saccharivorans*	Al5	Cf6	Hi5	Ha1					ITS_Pv3			AB166741 (99)	JF718412	FR71649 (99)	JF718413
XI	*Ga. hansenii*	Al6	Cf6	Hi6	Ha2								AB166736 (100)	JF718415		
	LAB species															
XII	*E. durans*										Fs1	Ms1	HM462407(100)	JF718431		
XIII	*Enterococcus sp.*										Fs2	Ms1	FJ514018 (100)	JF718432		
XIV	*L. brevis*										Fs3	Ms2	HM058385 (100)	JF718436		
XV	*O. oeni*										Fs4	Ms3	AJ831548(100)	JF718434		
	Other Gram+ bacteria															
	S. saprophyticus												AP008934 (100)	JF718435		
	K. kristinae												FR682682 (99)	JF718436		

[a] Results of the comparison of the sequenced 16S and 16S-23S ITS regions with those present in GenBank from NCBI. Percentage of similarity between brackets.

These molecular approaches allowed the obtention of 6 different restriction patterns (III, IV, V, VIII, IX and X) associated to the species *A. cerevisiae, A. malorum*, *A. orleaniensis, Ga.europaeus, Ga. intermedius* and *Ga. saccharivorans* (Table 3).

Regarding LAB analysis, a total of 78 isolates representative of the diversity found in all positive MRS tubes were obtained. Within those set of isolates, 40 were considered as putative LAB (Gram positive and catalase negative) and 38 as non-LAB (Gram positive and catalase positive). Four molecular patterns were obtained by 16S-ARDRA using *FspB*I and *Mse*I endonucleases. Two of them (XIV and XV) matched with the characteristic patterns of *Lactobacillus brevis* and *Oenococcus oen*i, respectively, as described by Rodas *et al.* (2003), while the other two were misidentified (XII and XIII). Sequencing of the 16S rRNA gene PCR products were performed for representative isolates of each pattern. Results confirmed the identification of patterns XIV and XV, and revealed 100% homology with *Enterococcus durans*, for the pattern XII, and 99% homology with *Enterococcus* spp. for the pattern XIII (Table 3).

The set of non-LAB isolates were separated in two groups in function of its cell and colony morphology, and representative isolates were selected for sequencing of 16S rDNA, which allowed the identification of the species *Staphylococcus saprophyticus* and *Kokuria kristinae* (Table 3). Table 4 shows the patterns obtained with each restriction endonuclease for both PCR-RFLP of the 16S and 16S-23S ITS rRNA regions and 16-ARDRA required to identify the recovered AAB and LAB isolates.

3.3 Sour rot effect on microbial populations during grape ripening

The population dynamics of yeasts, AAB and LAB were analysed since the end of veraison until harvest time on healthy and sour rot grapes. Samples of 100 representative berries of sound and sour rotten grapes, were carefully selected from a set of three different bunches collected from three different vines, and analysed in duplicate at each ripening phase.

Table 4. Electroforetic restriction fragments sizes (bp) of the amplified 16S and 16S-23S ITS rDNA of the AAB and LAB isolates.

AAB identification						LAB identification		
PCR-RFLP of 16S rDNA			PCR-RFLP of 16S-23S ITS rDNA			16S-ARDRA		
Pattern		Restriction fragments	Pattern		Restriction fragments	Pattern		Restriction fragments
*Alu*I	Al1	330-280-250-210-200-120	AluI	ITS_Al1	480-190-120	*Fsp*BI	Fs1	790-270-200-150
	Al2	450-310-280-190-120		ITS_Al2	480-210-120		Fs2	790-270-200-155-120-90
	Al3	320-280-240-210-190-120-50		ITS_Al3	480-280-120		Fs3	780-590-195
	Al4	550-280-200-190-120-70					Fs4	530-500-200-145-90
	Al5	790-490-120						
	Al6	780-490-120						
*Cfo*I	Cf1	500-420-210-150-140	*Cfo*I	ITS_Cf1	400-190-170	*Mse*I	Ms1	620-280-270-200-145-90
	Cf2	540-350-220-160-150		ITS_Cf2	400-210-170-70		Ms1	620-280-270-200-145-90
	Cf3	520-350-210-150-140-70		ITS_Cf3	700-170		Ms2	490-350-350-190-140-110-90
	Cf4	650-350-220-140-70					Ms3	610-240-210-50
	Cf5	430-340-180-160-140-110						
	Cf6	430-220-190-170-150-140						
*Hinf*I	Hi1	950-200	*Pvu*II	ITS_Pv1	470-310			
	Hi2	980-210		ITS_Pv2	450-320			
	Hi3	980-200-200-80		ITS_Pv3	460-340			
	Hi4	950-270-190						
	Hi5	980-270-190						
	Hi6	980-210-170-130						
*Hae*III	Ha1	550-210-180-160						
	Ha2	500-270-180-160						
*Tru*9I	Tr1	530-340-320-150-120						
	Tr2	530-350-350-140-110						
*Taq*I	Tq1	520-370-220-180-170						
	Tq2	900-370-230						
	Tq3	710-380-220-180						

3.3.1 Yeast population dynamics during grape ripening

A total of 22 yeast species belonging to 10 different genera were identified from healthy and rotten grape samples, of which 3 belong to basidiomycetous yeasts (Table 5).

In healthy grapes, seven ascomycetous and three basidiomycetous yeasts species were recovered during the three maturation phases. Regarding basidiomycetes, the red to pinkish ballistoconidia-forming *Sporobolomyces roseus* species was isolated since veraison until harvest, while the non pigmented *Cryptococcus laurentii* and *Cr. magnus* were only recovered at the middle of ripening. These species are typical phylloplane colonists (Fonseca and Inácio, 2006; Nakase, 2000). In particular, *S. roseus* and *Cr. laurentii* are the dominant species of the epiphytous complex, i.e. the yeasts that occur on living, green aboveground plant parts (mainly the phylloplane), and regarded as non-geographic (similar species composition in different zones) (Babjeva and Chernov, 1995; De la Torre *et al.*, 1999; Maksimova and Chernov, 2004; Yanagida *et al.*, 1992). This dominance of *S. roseus* was already described by De la Torre *et al.* (1999), which showed the occurrence of these yeast species at all maturation physiological stages, in two Spanish *Vitis vinifera* grape varieties, during a three years study. Prakitchaiwattana (2004) detected various species of *Cryptococcus* and *Sporobolomyces* at most stages throughout maturation of sound grapes from Shiraz and Chardonnay grape varieties.

Their populations were quite low ($10^1 - 10^3$ CFU/g), with *Cr. laurentii* and *Cr. magnus* being the most prevalent species. More recently Čadež *et al.* (2010) described the dominance of *Cr. magnus* (>50% frequency of occurrence) in healthy grape berries collected at harvest time. Seven ascomycetous yeast species were also recovered from sound grape samples during maturation. The most obvious trend that emerges from the analysis of Table 5 is the clear prevalence of *Aureobasidium pullulans* during grape maturation, since it was the unique species isolated in every ripening phases and in all independent vine sets sampling, recovered in numbers ranging from 1.00 to 2.70 log CFU/g. This ascomycetous yeast-like fungus is a common inhabitan of the phylloplane (McGrath and Andrews, 2006) and one of the most widespread and well-adapted saprophytes in the phyllosphere (Andrews *et al.*, 1994).

Our results are in agreement with Prakitchaiwattana *et al.* (2004), which refers to *A. pullulans* as the main species isolated from immature, mature, and both damaged and undamaged grapes, persisting until harvest with populations ranging between $10^2 - 10^3$ CFU/g.

Table 5. Yeast dynamic populations on healthy and sour rot grapes during ripening. Results reported as log CFU/g and the isolation culture media is shown between brackets.

Sanitary state	Yeast	Species	After Veraison				Middle				Harvest			
			Vine Set A		Vine Set B		Vine Set A		Vine Set B		Vine Set A		Vine Set B	
Sound	Basidiomycetes	*Cryptococcus laurentii*							1.90	(GYP)				
		Cryptococcus magnus					1.7	(GYP)						
		Sporobolomyces roseus	1.18	(GYP)	1.18	(GYP)	2.18	(GYP)	1.30	(GYP)	0.70	(GYP)	2.54	(GYP)
	Ascomycetes	*Aureobasidium pullulans*	2.70	(GYP)	1.60	(GYP)	2.65	(GYP)	1.00	(GYP)	1.00	(GYP)	2.00	(GYP)
		Candida stellimalicola	na[a]	(EBB)	na	(EBB)								
		Candida zemplinina	na	(EBB)	1.78	(GYP)			3.02	(GYP)	2.19	(GYP)	2.00	(GYP)
		Hanseniaspora guilliermondii					na	(EBB)						
		Hanseniaspora uvarum			3.14	(GYP)	na	(EBB)	2.74	(GYP)	1.00	(DBDM)		
		Pichia burtonii					1.48	(GYP)						
		Saccharomycopsis vini			1.00	(GYP)					1.18	(DBDM)		
		Total yeast counts	2.70		3.19		2.88		3.23		2.23		2.74	
Sour rot	Ascomycetes	*Aureobasidium pullulans*					4.7	(GYP)						
		Candida amapae			2.74	(DBDM)								
		Candida diversa	3.90	(ZDM)	3.60	(GYP)								
		Candida frutus							4.00	(GYP)				
		Candida stellimalicola			na	(EBB)	na	(EBB)						
		Candida zemplinina	5.71	(GYP)	5.16	(GYP)	6.41	(GYP)	6.78	(GYP)	6.95	(GYP)	6.76	(GYP)
		Hanseniaspora guilliermondii	na	(EBB)					na	(EBB)				
		Hanseniaspora uvarum	6.11	(GYP)	5.11	(GYP)	6.26	(GYP)	6.48	(GYP)	6.70	(GYP)	6.34	(GYP)
		Issatchenkia terricola	5.16	(GYP)	3.88	(GYP)	5.65	(GYP)	6.30	(GYP)	5.81	(GYP)	5.00	(GYP)
		Issatchenkia orientalis	na	(EBB)	na	(EBB)	na	(EBB)	2.74	(ZDM)	na	(EBB)		
		Issatchenkia occidentalis					na	(EBB)	na	(EBB)	4.55	(ZDM)	2.78	(ZDM)
		Pichia deserticola									3.48	(ZDM)	3.84	(ZDM)
		Pichia guilliermondii							na	(EBB)	4.53	(DBDM)		
		Saccharomycopsis crataegensis											3.70	(DBDM)
		Saccharomycopsis vini	4.98	(GYP)	3.18	(GYP)	5.40	(GYP)	5.30	(GYP)			6.00	(GYP)
		Zygosaccharomyces bailii					na	(EBB)			na	(EBB)	na	(EBB)
		Zygoascus hellenicus			1.60	(DBDM)	4.67	(DBDM)	4.93	(DBDM)	3.81	(DBDM)	3.40	(DBDM)
		Zygoascus meyerae					4.27	(ZDM)	4.52	(ZDM)				
		Total yeast counts	6.34		5.46		6.71		7.04		7.15		6.98	

[a] na – not applicable (isolated after enrichment step).

Candida zemplinina and *H. uvarum* were also isolated in the three ripening phases. However, our results seem to demonstrate that these species may have a less homogeneus distribution on grape surfaces, since were not detected in some vine sets. In terms of the global yeast population, the ascomycetous *C. zemplinina* (ranging from 1.78 to 3.02 log CFU/g) and *H. uvarum* (ranging from 1.00 to 3.14 log CFU/g) prevailed over the basidiomycetous *Cr. laurenti* (1.90 log CFU/g), *Cr. magnus* (1.70 log CFU/g) and *S. roseus* (ranging from 0.70 to 2.54 log CFU/g). The predominance of basidiomycetous *Rhodotorula* spp., *Sporobolomyces* spp. and *Cryptococcus* spp., along with *A. pullulans*, *C. zemplinina* and *H. uvarum* was also reported by Raspor *et al.* (2006) and Čadež *et al.* (2010). Our results also revealed the existence of low populations of *Candida stellimalicola*, *H. guilliermondii* and *Pichia burtoni*, detected in immature sound grapes and *Saccharomycopsis vini,* in both immature and mature grapes (Table 5). The first two ascomycetous species should be minor species on grape surfaces, since were recovered only after a pre-enrichment step in EBB medium. Minor species may account for a significant fraction of species richness on the phylloplane especially in samples with large yeast counts, as demonstrated in other studies (Inácio *et al.*, 2002).

In the case of sour rot damaged grapes, yeast dynamic populations changed markedly since the initial phase of the rotting process. Total yeast counts and yeast diversity species in rotten grape samples were much higher to those found in sound grapes. Indeed, the first observations about the effect of sour rot on grape yeast communities were the increment in total yeast counts between 2.5 and 4 log CFU/g, and the absence of basidiomycetes recovered from rotten berries since the first signs of the sour rot development (just after veraison) (Table 5). Total yeasts counts increased slightly during grape maturation, ranging from 5.9 log CFU/g, just after veraison, to 7.06 log CFU/g in harvest (average values of both vine sets). Taking into account the total of yeast species recovered in both independent sampling sets, results showed that the highest diversity of yeasts was found at the middle of ripening phase. At this stage, 14 different ascomycetous species were isolated, in contrast with the 10 and 11 species found just after veraison and harvest time, respectively, suggesting that final maturation stages provides a more restrictive environment. In global terms, a total of 18 ascomycetous yeast species were isolated from rotten grapes during maturation, including the species present in sound grapes, with the exception of *P. burtonii*, which was, along with three species of basidiomycetes, only found in sound grapes. Thus, 12 ascomycetous yeast species were only recovered from rotten grapes: *C. amapae, C. diversa, C. frutus, I. occidentalis, I. orientalis, I. terricola, P. deserticola, P.*

guilliermondii, S. crataegensis, Z. bailii, Z. hellenicus and *Z. meyerae.* Concerning the isolation frequency, 6 species were isolated in all ripening phases: *C. zemplinina, H. uvarum, I. terricola, I. orientalis, S. vini* and *Z. hellenicus*. From this group, the first 3 species were consistently isolated in all grapes sampling sets and registered the highest counts (average of both vine sets): *C. zemplinina* was the major dominant species (6.29 log CFU/g), followed by *H. uvarum* (6.17 log CFU/g) and *I. terricola* (5.25 log CFU/g). *S. vini* (4.97 log CFU/g) and *Z. hellenicus* (3.69 log CFU/g) showed also a high isolation frequency and relatively high populations in rotten grapes.
The isolation approach followed in this study, using several culture media in order to maximize the recovery of both major and minority species, was successfully apllied. Indeed, *Z. hellenicus* and *Z. meyerae*, were only recovered in DBDM and ZDM media, while the minority species *C. stellimalicola*, *H. guilliermondii* and the tecnhological relevant and dangerous wine spoilage yeast *Z. bailii* (Loureiro and Malfeito-Ferreira, 2003) were isolated only after enrichment phase in EBB medium.

The recovery of *P. guilliermondii* was achieved using DBDM medium and after enrichment in EBB. This species is regarded as a rare contaminant of grapes and musts (Jolly *et al.*, 2003). We have already isolated it from sound, sour rotten and honeydew damaged grapes in the vineyard (Barata *et al.*, 2008a; 2008b), grape musts, grape stems, wines and insects using DBDM (Dias *et al.*, 2003b; Martorell *et al.*, 2006) and Nisiotou *et al.* (2007) isolated it from the beginning and middle of healthy fermenting musts. In spite of its low isolation frequency in common winemaking environments, the hight counts of *P. guilliermondii* found at the harvest period (4.53 log CFU/g in vine set A) (Table 5) seems to indicate that sour rotten grapes surfaces may be a great niche for the growth of this species. *P. guillermondii* has the ability to produce 4-ethylphenol in grape musts before fermentation but do not spoil wines by volatile phenol production (Barata *et al.*, 2006).

Z. hellenicus and *Issatchenkia* spp. have been consistently isolated only from sour rotten grapes in a 4-years study (2002 to 2005 vintages) recently performed by Barata *et al.* (2008a; 2008b). The results described here confirm the high frequency of isolation of *Z. hellenicus* and *Issatchenkia* spp. only from affected samples, which clearly suggest that these yeasts should be considered as zymological markers of sour rotten grapes. We are not aware of wine spoilage provoked by these species but it would be interesting to understand why they are so commonly isolated in damaged grapes. Interestingly, Nisiotou and Nychas (2007) in a survey of yeast populations residing on healthy and *Botrytis*-infected grapes, found that the gray rotten grape samples had higher yeast populations and were also associated to the presence of *I.*

occidentalis, I. terricola and *Z. bailii*. Albeit with different isolation frequencies, the species *I. terricola*, *I. occidentalis*, *I. orientalis* had been often isolated in several other surveys (Beltran *et al.*, 2002; Combina *et al.*, 2005; Di Maro *et al.*, 2007; González *et al.*, 2007; Hierro *et al.*, 2006; Pallmann *et al.*, 2001; Romancino *et al.*, 2008; Sabate *et al.*, 2002). However, all these studies have in common the fact that wine fermentations were conducted in high volumes (100L up to 20000L) where no concern on grapes sanitary state was observed and without separation of rotten bunches or possible rotten spots on apparently sound bunches.

The occurrence of *S. cerevisiae* is higher when grape skin is damaged (Mortimer and Polsinelli, 1999). However, we could not isolate *S. cerevisiae* from any sample, which confirms, for one hand, the extreme rarity of *S. cerevisiae* in grapes (Pretorius, 2000), and for the other hand, the difficult in prove its existence by spreading out diluted must on a solid medium (Martini *et al.*, 1996; Mortimer and Polsinelli, 1999).

3.3.2 AAB and LAB dynamic populations during ripening

The alteration of the ecological balance induced by sour rot was particularly evidentiated by the unequivocal increase of AAB counts on rotten grapes.

Table 6 shows that sound grape samples harbour very low numbers of AAB, which were only detected at the middle of ripening (1.18 log MPN/mL) and on mature grapes (0.49 log MPN/mL) (average of both vine sets). On the contrary, sour rotten grapes had very high numbers of AAB populations right from the onset of sour rot symptoms (4.18 log MPN/mL), reaching up to 7.13 log MPN/mL at the middle of ripening and decreasing to 5.83 MPN/mL, in average, at harvest. These results are consistent with previous studies, showing that the population size of AAB is typically small ($10^2 - 10^3$ cells/g) on sound berries but can exceed 10^6 cells/g on damaged grapes (Drysdale and Fleet, 1988; Joyeux *et al.*, 1984; Lafon-Lafourcade and Joyeux, 1981). More recently, González *et al.* (2005) analysed the AAB populations on red Grenache grapes surfaces during a vintage characterised by high incidence of grape spoilage and damage caused by unusual rainfall. Similar results were obtained by the authors, which detected 8 and 3 days before harvest, 4.88 log CFU/mL and 6.06 log CFU/mL counts, respectively, and 4.11 log CFU/mL on the harvest day.

Only two AAB species were isolated from the surface of healthy grapes: *G. oxydans* was detected at the middle of ripening while mature grapes harboured a low population of *Ga. saccharivorans.* This scenario has changed drastically in damaged grapes

samples, for which 7 different ABB species were recovered throughout maturation. The diversity of species increased progressively during ripening as the extent of sour rot was rising, and a clear microbial succession was observed: *Ga. saccharivorans* was the single species found in the early phase of maturation, followed by the presence of *Ga. hansenii* and *G. oxydans* at the middle of ripening, and ending with the emergence of *Ga. intermedius* and three different *Acetobacter* species (*A. malorum, A. orleaniensis* and *A. syzygii*). At the harvest period, *G. oxydans* (6.04 log MPN/mL in vine set B) was the dominant species in rotten grapes, being notable the high number the *Acetobacter* species, ranging from 3.38 to 4.88 log MPN/mL (Table 6).

Generally, previous studies correlate spoiled grapes with increased populations of *Acetobacter* species, mainly *A. aceti* and *A. pasteurianus* (Barbe *et al.*, 2001; Joyeux *et al.*, 1984), whereas healthy grapes show low populations of *G. oxydans*, which has been found on the grape surface in both spoiled and sound grapes in almost all the studies made up to date (Du Toit and Lambrechts, 2002; González *et al.*, 2005; Joyeux *et al.*, 1984; Prieto *et al.*, 2007; Renouf *et al.*, 2005).

G. oxydans prefers a sugar-rich environment, while *Acetobacter* species and *Gluconacetobacter* species prefer ethanol as carbon source (De Ley *et al.*, 1984; Drysdale and Fleet, 1988). Thus, in rotten grapes glucose should be first oxidised to gluconic acid by *Gluconobacter* (Guillamón and Mas, 2009) and to ethanol by yeasts, which is further oxidised to acetic acid by the *Acetobacter* species (Matsushita *et al.*, 1994). This evolution of AAB should be the explanation for the highly significant increase of volatile acidity (see Figure 1) and sour rot sanitary state index values (see Table 1), during ripening.

To our best knowledge, this is the first time that the recently new proposed *Ga. saccharivorans* (Lisdiyanti *et al.*, 2006) species is isolated from sound grapes in the vineyard and *A. malorum, A. orleanensis, A. syzygii, Ga. hansenii, Ga. intermedius* are isolated from sour rotten grapes. Interestingly, *A. malorum* and *Ga. hansenii* have been recently isolated from cooked grape must for traditional balsamic vinegar production (Gullo and Giudici, 2006) and *A. intermedius*, transferred to the genus *Gluconacetobacter* as *Ga. intermedius* (Yamada, 2000) is a well known AAB isolated from industrial vinegar fermentations (Boesch *et al.*, 1998).

Moreover, *A. malorum* was also isolated from rotting apple by Cleenwerck *et al.* (2002). *A. orleanensis* was isolated from rice wine and *A. syzygii* from vinegar in Asia (Lisdiyanti *et al.*, 2003).

Table 6. Bacterial dynamic populations on healthy and sour rot grapes during ripening. Results reported as log MPN/mL.

Sanitary state	Bacteria	Species	After Veraison		Middle		Harvest	
			Vine Set A	Vine Set B	Vine Set A	Vine Set B	Vine Set A	Vine Set B
Sound	AAB	*Gluconacetobacter saccharivorans*					0.63	0.36
		Gluconobacter oxydans			0.97	1.38		
		Total AAB counts	nd[a]	nd	0.97	1.38	0.63	0.36
	LAB	*Enterococcus durans*			1.38	0.36		
		Enterococcus sp.						0.63
		Total LAB counts	nd	nd	1.38	0.36	nd	0.63
	Other Gram+	*Staphylococcus saprophyticus*					2.18	
		Kocuria kristinae	0.63	0.97			0.36	1.38
		Total counts	0.63	0.97	nd	nd	2.18	1.38
Sour rot	AAB	*Acetobacter malorum*					4.04	3.38
		Acetobacter orleaniensis					4.66	
		Acetobacter syzygii					4.88	
		Gluconacetobacter hansenii			5.66	7.04		
		Gluconacetobacter intermedius					5.18	4.66
		Gluconacetobacter saccharivorans	4.18	4.18	5.66	6.38	5.04	
		Gluconobacter oxydans				2.97		6.04
		Total AAB counts	4.18	4.18	5.96	7.13	5.59	6.06
	LAB	*Enterococcus durans*			1.18			
		Enterococcus sp.				1.66	2.04	1.38
		Total LAB counts	nd	nd	1.18	1.66	2.04	1.38
	Other Gram+	*Kocuria kristinae*	2.66	4.66	3.18	2.97	4.66	4.04
		Total counts	2.66	4.66	3.18	2.97	4.66	4.04

[a] nd – not detected in 1 mL of grape must.

Taking into account the isolation sources found in the literature for *A. malorum, A. orleanensis, A. syzygii, Ga. hansenii, Ga. intermedius*, it is not surprising their isolation from sour rotten grape samples.

The incidence and populations of LAB on grapes were very low on both healthy and sour rotten grapes. Detectable populations were only found from the middle of ripening phase: 0.87 and 1.42 MPN/mL (average of both vine sets), in case of sound and rotten grapes, respectively (Table 6). However, at the harvest time, results showed a slight trend for higher counts in damaged grapes, increasing from 0.63 MPN/mL (vine set B) to 1.71 MPN/mL (average of both vine sets). In this study we were only able to isolate *Enterococcus* species from the surface of both sound and rotten grapes even after using enrichment cultures. Likewise, *E. durans* were one of the most frequently isolated species in a broad study on the bacteria associated with wine grapes cultivated in Australia (Bae *et al.*, 2006), and other authors have already described the isolation of *E. faecium* from surface of grape berries at harvest (Renouf *et al.*, 2005) or grape must (Marcobal *et al.*, 2004). In fact, the association of *Enterococcus* species with the surface of plants, soil, and agricultural environments is well documented (Chen *et al.*, 2005). The usual LAB of wine (*Oenococcus oeni*, *Lactobacillus* spp., *Pediococcus* spp.) were not detected. Other reports showed their rarity in sound or damaged grapes, with counts usually lower than 10^2 CFU/g (Bae *et al.*, 2006; Francesca *et al.*, 2011), which justifies the use of enrichment cultures for their current detection (Bae *et al.*, 2006; Renouf *et al.*, 2005; Renouf *et al.*, 2007). LAB are regarded as anaerobic and nutritively fastidious (Hammes and Hertel, 2009), probably unable to compete with yeasts and acetic acid bacteria under the aerobic conditions of the grape berry.

3.4 Sour rot effect on microbial populations during wine fermentation

Wine fermentations were performed to emulate common industrial winemaking practices. In particular, SO_2 addition and yeast inoculation are both well established practices in winemaking for restricting the growth of indigenous yeasts and bacterial populations. The evolution of yeasts, AAB and LAB populations were studied throughout fermentations of sound and 30% sour rot grape musts in three different winemaking stages.

3.4.1 Yeasts dynamic populations during wine fermentation

The overall results of this survey are listed in Table 7. The first important observation emerged from the results of total yeast count and species diversity found in the "apparently" sound grape musts. Similar total yeast counts were observed both in healthy or 30% rotten grape must samples. Contrary to the previous low yeast populations sizes found on healthy mature grape samples (2.49 log CFU/g in average) (see Table 5), the freshly sound grape must sample harboured a total yeast population of 6.12 log CFU/g after crushing in cellar equipment, which represent and increment of around 3.6 log CFU/g (Table 7). This difference should be due to the contamination of injured berries hidden in apparently sound bunches, explaining the recovery of a higher number of species when whole bunches are crushed and analyzed. This result highlight the importance of sampling schemes in grapes ecology studies, since the main concern should be to separate sound berries from damaged ones given their completely different microbial load. 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 (Sturm *et al.*, 2006).

A total of 9 ascomycetous yeast species were recovered from the sound must sample (VAT) (Table7). Five of them (*A. pullulans, C. zemplinina, H. guilliermondii, H. uvarum* and *S. vini*) had been previously detected on the surface of mature healthy grapes (Table 5). However, relative high counts of *C. diversa, L. thermotolerans, M. pulcherrima* and *P. sporocuriosa* were also detected. Similar populations of these last four species were also found on the must sample with 30% sour rot (Table 7), which seems to confirm the occurrence of cross-contamination from damaged berries, possibly hidden inside of some apparently healthy bunches.

In case of grape must containing 30% rotten grapes, a total of 12 ascomycetous yeast species were isolated, from which 4 of them (*C. apicola, I. occidentalis, Z. hellenicus* and *Z. bailii*) were not found on sound musts.

Table 7. Evolution of yeasts, acid bacteria (AAB) and lactic acid bacteria (LAB) populations, during wine fermentations containing sound and 30% rotten grapes.

Organism	Species	Medium	100% sound grapes vinification[a]					30% Sour rot vinification[a]				
			VAT	AF		MLF		VAT	AF		MLF	
				(30)	(100)	(30)	(100)		(30)	(100)	(30)	(100)
Yeasts	*Aureobasidium pullulans*	GYP	4.00					4.18				
	Candida apicola	GYP						5.85				
	Candida diversa	GYP	4.85					5.30				
	Candida pomicola	GYPC		1.00								
	Candida zemplinina	GYP	6.00					6.49				
	Dekkera bruxellensis	DBDM		1.00	3.31	1.00			1.00			
	Hanseniaspora guilliermondii	DBDM	1.48									
	Hanseniaspora uvarum	GYP	5.23					5.81				
	Issatchenkia occidentalis	GYP						4.40		1.78		
	Lachancea thermotolerans	GYP	4.30					5.00				
	Metschnikowia pulcherrima	GYP	4.00					3.30				
	Pichia sporocuriosa	ZDM	3.20					3.75				
	Saccharomyces cerevisiae	GYP		8.04	8.00	4.89	4.16		8.09	8.00	5.72	2.81
	Saccharomycopsis vini	GYP	3.74					4.06				
	Trigonopsis cantarellii	DBDM		3.79	3.77	1.90	1.88		3.35	3.46	1.98	1.30
	Zygoascus hellenicus	DBDM						4.60				
	Zygosaccharomyces bailii	ZDM						4.18				
	Total Yeasts counts (CFU/g)	GYP	6.12	8.04	8.00	4.89	4.16	6.68	8.09	8.00	5.72	2.83
AAB	*Acetobacter aceti*	GY		2.04					2.04	0.36		
	Acetobacter cerevisiae	GY						5.04	1.38			
	Acetobacter malorum	GY	1.66					4.66				
	Acetobacter tropicalis	GY							1.38			
	Gluconacetobacter europaeus	GY						4.04				
	Gluconacetobacter hansenii	GY	2.38					6.04				
	Gluconacetobacter intermedius	GY						3.04				
	Gluconacetobacter saccharivorans	GY	2.66					5.66				
	Gluconobacter oxydans	GY	3.38	2.38	3.04			3.66	2.38	2.04		
	Total AAB counts (log MPN/mL)	GY	3.50	2.54	3.04	nd[b]	nd	6.24	2.60	2.05	nd	nd
LAB	*Enterococcus durans*	MRS	1.38					2.66				
	Enterococcus sp.	MRS	2.04					1.38				
	Lactobacillus brevis	MRS								0.36		
	Oenococcus oeni	MRS		2.38	2.66				1.66	0.97		
	Total LAB counts (log MPN/mL)	MRS	2.13	2.38	2.66	nd	nd	2.68	1.66	1.06	nd	nd

[a] VAT: Vatting grape must after grapes crushing. Analysis performed prior to commercial yeast and initial SO_2 addition; AF: just after alcoholic fermentation; MLF: just after malolactic fermentation and after free SO_2 levels adjusted to 40 mg/L; Initial SO_2 concentration (mg/kg) added on initial vatting phase between brackets; [b] nd – not detected in 1 mL of wine sample

It should be highlighted the high population of the dangerous spoilage yeast *Z. bailii* (4.18 log CFU/g). These results confirm our observations in previous studies (Barata *et al.*, 2008a; 2008b). The association of *Z. bailii* with rotten grapes was likewise clearly verified by Nisiotou *et al.* (2007), which isolated this spoilage species from fermentations of *Botrytis* affected grapes.

There are few studies concerning the role of *Issatchenkia* species in wine fermentation. *I. terricola* has been associated with low fermentative capacity and high production of ethyl acetate, which gives unpleasant off-flavours (Clemente-Jimenez *et al.*, 2004).

Grape must samples were dominated by *C. zemplinina* and *H. uvarum* ascomycetes species in agreement with several other surveys (Beltran *et al.*, 2002; Combina *et al.*, 2005; Di Maro *et al.*, 2007; González *et al.*, 2007; Hierro *et al.*, 2006; Magyar and Bene, 2006; Nisiotou *et al.*, 2007; Pardo *et al.*, 1989; Romancino *et al.*, 2008). The genus *Hanseniaspora* has been reported to be the major non-*Saccharomyces* yeast in grape must at the beginning of fermentation (Pretorius, 2000).

Initial grape musts populations were also characterized by hight counts of *L. thermotolerans* and *M. pulcherrima* species, independently of the sanitary state of grapes. Many studies have found *M. pulcherrima* as a predominant yeast species on wine grapes at the time of harvest (Fleet *et al.*, 2002) and freshly crushed must (Combina *et al.*, 2005; González *et al.*, 2007; Pardo *et al.*, 1989; Pramateftaki *et al.*, 2000). However, Prakitchaiwattana *et al.* (2004) reported the predominance of *M. pulcherrima* (10^5 – 10^7 CFU/g) in damaged grape berries sampled at the time of harvest and similar results were obtained by Magyar and Bene (2006), which identified *M. pulcherrima* as the most frequent species of the mycobiota of noble rotted grapes.

L. thermotolerans has been previously detected in grapes affected by honeydew (Barata *et al.*, 2008b) and sour rot (Barata *et al.*, 2008a) and from *Botrytis* affected grape musts (Nisiotou *et al.*, 2007). Baleiras-Couto *et al.* (2005) also reported the isolation of *L. thermotolerans* species at the middle of several red musts fermentations also characterized by the presence of *Z. bailii.*

We were not able to obtain basidiomycetous yeast isolates from both sound and 30% rotten grape musts samples. Basidiomycetes are typically present at low populations (Prakitchaiwattana, 2004; Yanagida *et al.*, 1992) even in grape musts containing up to 30% rotten grapes (Barata *et al.*, 2008a). Having used the direct agar plating method, these minority basidiomycetous species could hardly be isolated from samples heavily dominated by the ascomycetous species.

As expected, after alcoholic fermentation, most non-*Saccharomyces* yeasts present on the initial musts disappeared and *S. cerevisiae* populations grew to around 10^8 cells/mL. Since any intra-specific typing strain characterization was performed, we can not guarantee the predominance of the inoculated commercial yeast strain.

The presence of 30% sour rotten grapes on wine fermentation and the use of higher SO_2 concentration (100 mg/kg) at the initial vatting phase, did not induce significant changes at wine microbiological stability level after alcoholic fermentation and MLF. Besides *S. cerevisiae*, 3 ascomycetous yeasts species were isolated at the end of sound musts fermentations initially sulphited with 30 mg/kg SO_2: *C. pomicola, D. bruxellensis* and *Trigonopsis cantarellii* (syn. *Candida cantarellii*) (Table 7). These species were not isolated from grapes surfaces or freshly grape musts and thus were probably originated from winery environment/equipment contaminations. Low counts of *D. bruxellenis* and *I. occidentalis* were detected after fermentation of 30% rotten musts, however after MLF only viable populations of *T. cantarellii* were observed in all wines. Strains of *C. cantarellii* sporadically isolated from wine-related environments have been reported as producing traces of 4-ethylphenol in synthetic media (Dias *et al.*, 2003a).On the other hand, Toro and Vazquez (2002) used *C. cantarellii* in mixed cultures with *S. cerevisiae* and obtained wines with 40% more glycerol than those fermented only with *S. cerevisiae*. Nevertheless, the role of these species during winemaking needs further investigation.

3.4.2 AAB and LAB dynamic populations during wine fermentation

Low populations of AAB (3.50 log MPN/mL) were found on the freshly sound must sample, characterized by the dominance of *G. oxydans* and *Ga. saccharivorans* (Table 7). However, low counts of *Ga. hansenii* and *A. malorum* were also obtained. Given that only *G. oxydans* and *Ga. saccharivorans* were recovered from the surface of healthy berries during ripening, we believe that the presence of *Ga. hansenii* and *A. malorum* was probably due to cross contamination originated by rotten berries hidden on some healthy bunches. Indeed, total AAB counts were significantly higher in the must sample containing 30% rotten grapes (6.24 log MPN/mL) and 7 different AAB species were found: *G. oxydans, Ga. saccharivorans, Ga. hansenii, Ga. intermedius, Ga. europaeus, A. cerevisiae* and *A. malorum.* Results show that *Ga. hansenii* was the dominant species (6.04 log MPN/mL) in the rotten must.

The isolation of *A. cerevisiae* was associated to Chilean valleys by Prieto *et al.* (2007) who speculated that this species may be absent from European vineyards. Our results refute this hypothesis and report for the first time the isolation of this species in musts obtained from grapes collected in European vineyards. Moreover, it is also the first time that *Ga. europaeus* was found on grape must samples which has been associated, up to date, only to industrial (Sievers *et al.*, 1992) and traditional wine (Vegas *et al.*, 2010) and balsamic (Gullo *et al.*, 2009) vinegars sources.

Alcoholic fermentation induced a strong reduction in AAB populations, which fell to around 2.5 log MPN/mL (average of the four trials), confirming the general trend commonly described (Drysdale and Fleet, 1989; Du Toit and Lambrechts, 2002). It appears that the anaerobic conditions due to the vigorous production of carbon dioxide by yeasts during fermentation restrict the growth of AAB and the ethanol increasing concentration select the most resistant species (González *et al.*, 2005).

Table 7 shows that none of the *Gluconacetobacter* species survived after fermentation and that *G. oxydans* and *A. aceti* were the dominant species in the wine obtained from both sound and 30% rotten grape musts added with 30 mg/kg SO_2. In case of rotten wines low populations of *A. cerevisiae* and *A. tropicalis* were also found. The addition of 100 mg/kg SO_2 on both freshly musts did not allow the inactivation of *G. oxydans* and *A. aceti*. These results confirm, on one hand, that *A. aceti* is better adapted to higher ethanol content and tend to colonise the wine rather than grape must (Bartowsky and Henschke, 2008) and on the other hand that *G. oxydans* can survive on fully fermented wine as described by González *et al.* (2004).

Low LAB populations were observed in the initial must samples, and the presence of sour rot grapes did not led to a significant increase in LAB counts. Once again, *O. oeni* was not isolated from freshly grape musts, and only *Enterococcus* spp. were found (Table 7) in agreement with Bae *et al.* (2006). Moreover, these authors showed that *E. durans* and other *Enterococcus* species were the most frequently isolated LAB species from grapes. Just after fermentation, *Enterococcus* species disappeared and *O. oeni* become the dominant LAB species. Low *L. brevis* populations were detected after fermentation of rotten musts added with 100 mg/kg SO_2, which show that LAB were not inhibited by higher SO_2 concentrations. Du Toit and Lambrechts (2002) highlighted the advantages of high SO_2 concentration to control unwanted growth of AAB in musts and the importance for the maintenance of low temperatures during the process of cold soaking before fermentation. After the completion of MLF and subsequent adjustment

of the free SO_2 levels to 40 mg/L, no viable populations of AAB and LAB were detected in one mL of all analysed wine samples (Table 7).

4. Conclusions

In conclusion, the results obtained in this work support our view that grape damage is the main driving force altering berry microbiota. Grape damage increase sugar accessibility and create opportunities for new species to become established. Moreover, we believe that many of the apparently contradictory results found on literature regarding the factors affecting yeast diversity on grapes, may be explained by using grape bunches without separating damaged berries. Undoubtedly, deep changes on the yeast microbiota and on the AAB populations were detected right from the earliest phases of the rotting process, mainly characterized by a noteworthy increase in species diversity and population sizes. To our best knowledge, several AAB species were isolated for the first time from grapes and were clearly associated to sour rotten damaged grapes.

The results obtained during wine fermentations in the presence of sour rotten grapes seems to demonstrate that the impact of sour rot on wine microbiological stability could be minimized if suitable oenological practices are taken, such as: i) ensure a rapid onset of alcoholic fermentation by using high populations of a commercial yeast; ii) addition of adequate SO_2 concentrations just after grape crushing and the lowering of the maceration temperature to 10 – 15 ºC, whenever skin maceration is used prior to yeast inoculation; iii) maintenance of recommended free SO_2 levels just after MLF and minimize the winemaking practices which could introduce oxygen into the wine during storage.

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CHAPTER 4

Influence of sour rotten grapes on the chemical composition and quality of grape must and wine

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ORIGINAL PAPER

Influence of sour rotten grapes on the chemical composition and quality of grape must and wine

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Abstract This study evaluated the effect of grape sour rot on wine fermentation and characterized the chemical composition and the sensory changes in wines produced from rotten musts. Microvinifications were performed during two vintages using healthy Trincadeira and Cabernet Sauvignon red grape varieties to which were added grapes affected by sour rot. Increasing sour rot percentages, up to 50%, contributed to a clear decrease in free run must and final wine yields and induced significant changes in grape must chemical composition expressed by the increase in sugar content, total acidity, volatile acidity, anthocyanins, total phenols, and color intensity. After malolactic fermentation, wines from rotten grapes showed higher values of alcohol content, dry extract, reducing sugar content, total and volatile acidity, anthocyanins, total phenols, and color intensity. Despite the higher levels of reducing sugars, the microbial stability was similar to that of healthy wines. The sensorial evaluation, after malolactic fermentation, showed that both types of wine were not statistically different regarding color, aroma, taste, and overall quality. During 6–8 month storage, wines from rotten grapes showed a significant higher percentage of color loss, suggesting that sour rot is responsible for the decrease in color stability. Nevertheless, the results of sensorial analysis demonstrated that the fermentation of grape musts containing up to 30% sour rot yields wines with similar or even higher-quality scores than wines made with healthy grapes.

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Keywords Grape sour rot · Wine · FTIR · Aroma · Taste · Sensory analysis

Introduction

Wine quality is primarily dependent on the sanitary state of the grapes. Winemakers are aware that damaged fruit leads to poor quality wine, and their first concern is to obtain grapes at the optimum of ripening and health. Among possible injuries, the several types of rot are some of the most common. In particular, gray rot, due to the activity of the mold *Botrytis cinerea*, is a well-known disease all over wine-producing countries. Another type of rot, sour rot, is becoming more frequent, probably due either to better gray rot prevention or to climate changes that favor the growth of other microorganisms [1]. Sour rot may cause heavy crop losses and reduce vintage quality, mainly when heavy rainfalls during fruit maturation [2] and often determines harvest date in warm and humid regions [3].

The initial stages of sour rot are difficult to distinguish and are not altogether different from gray rot, as they both show a similar color change, usually beginning at the point of pedicel attachment to the berry. Later, however, and especially nearing harvest time, sour rot shows distinct and clearly recognizable symptoms: grape pulp browning, disaggregation of the internal tissues and detachment of the rotten berry from the pedicel, juice leaking from higher to lower berries in the cluster, release of a pungent odor of acetic acid (vinegar) and ethyl acetate from the rotten berries, and the presence of *Drosophila* fruit flies, all around and on rotten bunches [4–6]. In the final stages, the berries are completely empty and the skins are dried out as a result of the complete fermentative breakdown of the pulp. On the other hand, in berries infected by *B. cinerea*,

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Papo and Vermes, 1961; Ribéreau-Gayon *et al.*, 2006). Climate changes have been associated to increase in sour rot occurrence in comparison to gray rot given the lower optimum growth temperature for *Botrytis cinerea* (Ribéreau-Gayon *et al.*, 2006).

Sour rot is troublesome with tightly packed, thin-skinned cultivars, and dense bunches close to harvesting, even when produced with low canopy densities (Bisiach, 1986; Wolf *et al.*, 1990; Zoecklein *et al.*, 1992). In particular, Trincadeira, a vigorous and productive red *Vitis vinifera* L. variety widely planted in Portugal, is known to be extremely sensitive to sour rot, since it is characterized by medium-sized, compact bunches with round medium berries, epidermis of medium thickness, short pedicel that can be easily separated and soft and juicy pulp (Magalhães, 2008).

The complex group of organisms involved in sour rot is known to change fruit composition as a result of the production of high levels of a wide range of undesirable metabolites including acetic acid, glycerol, ethyl acetate, ethanol, acetaldehyde, galacturonic acid, and gluconic acid (Marchetti *et al.*, 1984; Zoecklein *et al.*, 2001). Therefore, it would be advisable to select the grapes according to its health status before fermentation. In small wineries it is current practice to separate damaged grapes manually, but in large wineries, this is not feasible. In this case, crop quality may be visually evaluated but automation is an effective alternative using Fourier transform infrared (FTIR) spectroscopy (Eichinger *et al.*, 2004; Gishen *et al.*, 2005; Kupina and Shrikhande, 2003; Patz *et al.*, 2004; Soriano *et al.*, 2007). The FOSS WineScan FTIR spectrometer has been approved for wine analysis since 1996 with ready-to-use must and wine calibrations provided by the manufacturer (Patz *et al.*, 2004). FTIR spectroscopy in the mid-infrared range in combination with partial least squares (PLS) regression is nowadays an established procedure for the multi-component screening in wine analysis and is able to simultaneously determine several common wine analytes in less than 1 min per sample (Arzberger and Lachenmeier, 2008). In particular, this instrument provides several indicators of grape health, including gray rot, sour rot and yeast and lactic acid bacteria activities (FOSS, 2002).

The real contribution of sour rot damaged grapes for the final wine quality, and the evaluation of chemical, microbiological and sensorial changes during aging has been little studied (Loinger *et al.*, 1977; Zoecklein *et al.*, 2001). Empirical experiences are contradictory, diminishing, or exaggerating the effect of sour rot on wine quality. Therefore, the purpose of this work was to characterize wines produced from grapes with different levels of sour rot in order to evaluate the impact of this disease on winemaking and wine quality.

2. Materials and methods

2.1 Grape Samples

Vitis vinifera L. cv. Trincadeira and Cabernet Sauvignon grapes grown during the 2007 and 2008 harvest seasons on the vineyards of the Tapada da Ajuda at the Instituto Superior de Agronomia (ISA), located in Lisbon (Portugal, latitude 38º 42´31.57´´ N and longitude 9º 11´14.01´´ W) were used in this study. Healthy grapes from the two cultivars and Trincadeira bunches affected by sour rot were manually harvested at their technological maturity and transported in 20-kg plastic boxes to the experimental cellar of Instituto Superior de Agronomia, where wine fermentations were performed.

2.2 Wine fermentations and wine bottling

Microvinification sets (classic red style fermentation with maceration) were carried out in 50-kg capacity stainless steel vats, using sound grapes to which were added different amounts of sour rot damaged grapes. During 2007 vintage, microvinification sets of monovarietal Trincadeira grape musts containing 30% of sour rotten grapes and 100% healthy Trincadeira grapes (control sets) were carried out. In order to evaluate the effect of the initial vatting SO_2 concentration, each grape must were added with 30 and 100 mg/kg (Table 1), using an aqueous solution of sulfur dioxide (6% w/v).

During 2008 vintage, five microvinification sets were carried out: two sets of monovarietal Trincadeira grape variety with 30% (T_{30}) and 50% (T_{50}) sour rotten grapes and one set of 70% sound Cabernet Sauvignon blended with 30% sour rotten Trincadeira grapes (CT_{30}). Two additional control sets composed, respectively, of 100% sound Trincadeira grapes (T_0) and 70% sound Cabernet Sauvignon blended with 30% sound Trincadeira grape cultivars (CT_0) were also carried out (Table 1).

In both vintages, the grapes (30 kg per set) were mechanically destemmed and crushed with a commercial grape destemmer-crusher and collected into 50-L stainless steel wine vats, followed by SO_2 addition and 18 hours of pre-fermentative maceration at room temperature. After maceration period, the grape musts were inoculated with 10^6 cells/mL of selected commercial *Saccharomyces cerevisiae* yeast (Fermivin®, DSM, Delft, The Netherlands). Fermentation occurred at cellar temperature, and the cap was punched down twice a day until it remained submerged. The juice density was

measured daily, and fermentations were carried out until density determinations indicated the end of fermentation. Once the fermentation was completed, the pomace was pressed and the resulting wine (free run and press wine) was transferred to another vat, where it underwent spontaneous malolactic fermentation (MLF). Degradation of malic acid was monitored through paper chromatography (Ribéreau-Gayon *et al.*, 1972). Once MLF was completed, lees were removed, free SO_2 levels were corrected to 40 mg/L, and wines were bottled without filtration in 0.75-L glass bottles capped with natural corks and kept at cellar temperature. Four different stages of winemaking were analyzed: vatting (initial grape must), just after wine fermentation, after MLF and after of bottle aging in cellar (8 months for 2007 wines and 6 months for 2008 wines). Samples of each phase were collected in 250-mL labeled glass bottles and stored frozen at -20 ºC until chemical analysis.

Table 1. Characterization of grape must composition used in the microvinification trials performed during the 2-year study and yield parameters calculated during the 2008 vintage.

Vintage	Trial Code[a]	Grapes % (w/w)[b]			Vatting SO_2 (mg/kg)
		Trincadeira		Cabernet Sauvignon	
		(S)	(SR)	(S)	
2007	$T_0(30)$[c]	100	–	–	30
	$T_{30}(30)$	70	30	–	30
	$T_0(100)$[c]	100	–	–	100
	$T_{30}(100)$	70	30	–	100
2008	T_0[c]	100	–	–	50
	T_{30}	70	30	–	50
	T_{50}	50	50	–	50
	CT_0[c]	30	–	70	50
	CT_{30}	–	30	70	50

Yield parameter	2008 Vinification sets				
	T_0	T_{30}	T_{50}	CT_0	CT_{30}
Sour Rot %	0	30	50	0	30
Total grapes weight (kg)	30	30	30	30	30
Free run grape must (mL)	5200	4525	2850	4800	3600
Loss in free run must (%)	0	13.0	45.2	0	25.0
Stems weight (g)	1178	1400	1559	1195	1291
Increase in stem weight (%)	0	18.8	32.3	0	8.0
Wine after pressing (mL)	20380	20000	19325	20550	19805
Lees (mL)	540	970	1740	1000	1385
Final wine (mL)	19840	19030	17585	19550	18420
Wine yield (%)	66.1	63.4	58.6	65.2	61.4

[a] Subscript numbers indicate the % of sour rotten Trincadeira grapes. In the case of 2007 wines, initial vatting SO_2 concentration is given between brackets
[b] (S): sound grapes; (SR): sour rotten grapes
[c] Control set vinifications

2.3 Chemical analysis

2.3.1 Fourier Transform Infrared (FTIR) Spectroscopy

Grape musts and wine samples were analyzed in a WineScan FT120 spectrometer (FOSS, Hilleroed, Denmark, http://www.foss.dk) that employs a Michelson interferometer to obtain the FTIR spectra. Samples were scanned from 926 to 5012 cm^{-1} at 4 cm^{-1} intervals (FOSS, 2000). The numbers of scans generated per sample, the selection of wavenumbers, and the processing of spectra have been fixed by the manufacturer and are not accessible to change by the user. Partial least squares regression (PLS), available within the software package of the WineScan FT 120, was the multivariate method that carried out calibrations. Statistical data analysis has been set by the manufacturer and is not accessible to change by the user.

For grape must samples, the FOSS-supplied GrapeScan Calibration was used. This calibration was designed for analyzing the quality of the incoming grapes by means of the sanitary state of the grapes and has been established based on a wide range of grapes from several countries and representing grapes from healthy grapes to extreme poor quality. Grape musts' sanitary results are based on 4 different parameters within the following range: gray rot index (0-60; 0=excellent grapes; 10=good grapes; 20=slight attack; 30=critical attack; 40=serious attack; 50=severe attack; 60=total rotten grapes), sour rot index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe attack), yeast activity index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe attack) and lactic bacteria activity index (0-2; 0=good grapes, 1=medium attack, 2=severe attack) (FOSS, 2002).

Additionally, the following analytical parameters were also determined: glucose-fructose content, Brix degree, density, total acidity, volatile acidity, pH, tartaric acid, malic acid, total assimilable nitrogen, anthocyanins, total phenols (Folin C index), and color intensity. Concerning wine samples, the ready-to-use wine calibration provided by the manufacture included the following chemical parameters: alcohol content, dry extract, residual sugars, total acidity, volatile acidity, pH, malic acid and lactic acid. All samples were analyzed by the Analysis Service of the Adega Cooperativa de Borba winery.

2.3.2 Analysis of conventional oenological parameters

Chemical analyses were also performed using reference laboratory methods as a complement of FTIR analysis. Alcohol content , pH, density, total and volatile acidities, and free and total sulphur dioxide were measured according to Organisation International de la Vigne et du Vin methods (OIV, 2006). Color intensity was calculated as the sum of absorbance at 420, 520 and 620 nm; total phenol content and anthocyanins (mg/L) were determined following the method described by Ribéreau-Gayon *et al.*, (1972).

2.4 Microbiological analysis

The effect of sour rotten grapes on must and wine microbiological stability was tested during the 2-years study. Total yeasts, acetic acid bacteria (AAB), and lactic acid bacteria (LAB) counts were performed in four different winemaking stages: (1) initial grape musts, before SO_2 addition; (2) after wine fermentation; (3) after MLF and (4) after 6 months of bottle aging (2008 wines).

Samples from initial grape musts and wines from the different winemaking stages were decimally diluted in peptone water (Merck, Darmstadt, Germany), and the enumeration of total yeasts was determined by surface plating 0.1 mL of the adequate dilutions onto GYP agar [20 g/L glucose (Merck), 5 g/L yeast extract (Oxoid, Hampshire, UK), 5 g/L peptone (Difco Laboratories, Detroit, USA), 20 g/L agar, pH 6.0] plates (duplicates). To avoid growth of bacteria and moulds, 100 mg/L of chloramphenicol (Sigma, Steinheim, Germany) and 0.5 g/L of biphenyl (Fluka, Buchs, Switzerland) were incorporated into the medium, respectively. Total counts were obtained after incubation at 25 ºC during 3–5 days.

The enumeration of AAB and LAB was carried out using the most probable number (MPN) technique according to the procedures described in OIV (2010). Briefly, grape musts and wine samples were decimal diluted until extinction (10^0–10^{-7}), and 1-mL portion of must/wine and 1-mL of each dilution, was inoculated into three tubes containing 3 mL of GY [50 g/L glucose, 10 g/L yeast extract, and 13 g/L agar, 200 mg/L Delvocid® (DSM, Delft, Netherlands) (100 mg/L natamycin) and 3000 U/L penicillin (Sigma), pH 4.5] and 5 mL of MRS [52 g/l MRS (Oxoid), 200 mg/L Delvocid®, and 0.5 g/L L-cysteine (Sigma)] broths, for the enumeration of AAB and LAB, respectively. Delvocid® and penicillin were used in order to inhibit the growth of yeasts and LAB,

respectively. Inoculated tubes were incubated at 28 ºC during 8 days (up to 10 days), under aerobic conditions, for AAB, and under microaerophilic conditions, by addition of 500-µL sterile paraffin oil (Sigma), for LAB, making periodic observations up to the last day of incubation. All those tubes that showed a microbial development leading to the formation of a more or less evident whitish deposit and/or disturbance were considered as positive. Results were confirmed by observation through a microscope. The MPN was obtained by using the tables and rules presented in OIV 206/2010 Resolution (OIV, 2010).

2.5 Sensory analysis

Two sensory analysis sessions of the experimental wines were carried out to determine whether there were sensory differences between sound grape-produced and sour rotten grape-produced wines.

In a first session (Session 1), 2007 wines (12 months of bottle aging) and 2008 wines (just after MLF) were tasted separately. The sensory panel was composed of 3 women, and 5 men (n=8; 23–56 years of age, mean=42), all of them belonging to the panelist of the Enology Laboratory of ISA, which includes enology students, researchers, wine technicians, and winemakers with substantial wine and sensorial background and experience in descriptive profiling of experimental wines. The 2008 wines were tasted again after 6 months of bottle aging, in a second sensory analysis session (Session 2). The sensory panel was composed by 15 judges (5 women, 10 men; 24-59, years of age, mean=36) from the same laboratory panellist.

In both sessions, experimental wines were evaluated on a prescribed evaluation sheet by scoring the wines for the following sensory attributes: color (intensity); aroma (intensity, quality and equilibrium); taste (intensity, quality, body, equilibrium, persistence and final taste) and overall quality. Judges scored the color intensity attribute using a five-point scale (1=absent; 2=weakly intense; 3=intense; 4=fairly intense; 5=very intense), and the attributes related to aroma and taste were ranked from 1 to 5 (1=poor; 2=satisfactory; 3=good; 4=very good and 5=excellent). The overall quality was evaluated with a 20-point hedonic scale (1=very bad; 5=bad; 10=acceptable; 15= good; 20=excellent).

Participants were not informed about the nature of wine samples. In all cases, 30-mL wine samples were served at room temperature and presented to the panel in individual tasting booths, using transparent International Standards Organization (ISO)

wine-tasting glasses, coded with three-digit random numbers. Sample order was randomized across all judges.

2.5.1 Statistical Analysis

In order to check for significant differences between wines, a one-way (wine) analysis of variance (ANOVA, post hoc Fisher LSD test) was performed separately for 2007 and 2008 wine samples on the individual attributes intensity scores obtained in the two sensory analysis sessions. Moreover, for the 2008 wines, monovarietal Trincadeira and blends of Cabernet Sauvignon with Trincadeira wines were analysed separately in both sessions. In order to check for differences during wine aging, an additional ANOVA was performed between 2008 wines tasted just after MLF (Session 1) and after 6 months of bottle aging (Session 2). These analyses were performed using the Statistica software (version 7.0; StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1 Effect of sour rot on yield parameters

Table 1 illustrates the influence of sour rot on the change of several wine yield parameters. Results obtained during the 2008 vintage showed that the increase of sour rot percentage contributed to a large loss in free run must after crushing of grapes. A loss of 45% of fresh grape juice was observed for the vinification trial containing 50% of rotten grapes (T_{50}). On the other hand, the measurements of the total stems weight showed that all sets with sour rotten grapes had higher quantity of stems, when compared with the respective control sets. This fact results from the addition of more clusters to obtain the same amount of grapes in each vessel (30 kg/set), increasing consequently the percentage of dry parts (skins, stems, and seeds) for the same grape volume and leading to a higher concentration of extractable compounds. These observations are consequence of the typical berry dehydration and loss of berry juice in affected grapes, as result of the pulp breakdown caused by the sour rot microbiota.

After pomace pressing, a slight decrease of wine volume and a clear increase in lees volume was observed after MLF as a function of the proportion of sour rotten grapes. Thus, sour rot contributes to a marked decrease in the final wine yield.

3.2 Effect of sour rot on grape must chemical analysis

Table 2 summarizes the characterization of enological parameters of grape musts used on 2007 and 2008 vinifications. Results obtained by FTIR analysis and classical reference methods, showed that sour rotten grapes were responsible for changes in chemical grape must composition. Sour rot increased the Brix and glucose-fructose content and thus the potential alcohol content. Results showed an average increase of around 0.73% (v/v) of potential alcohol content for grape musts containing 30% of rotten grapes, while grape must with 50% of rotten grapes (T_{50}) showed an increase of 1.7% (v/v) in comparison with the respective total sound vinification sets (Table 2).

The growth of AAB (*Gluconobacter* spp. and *Acetobacter* spp.) and oxidative yeasts associated with sour rot are believed to degrade only a small amount of sugar (Ribéreau-Gayon *et al.*, 2006). Therefore, the observed increase in sugar was likely due to the process of concentration as a consequence of berry dehydration. Parallel with the increase in sugar, FTIR analysis showed that sour rot had a rise effect on total acidity accompanied with an increase in volatile acidity and a drop in pH values. The increase in total acidity was moderate up to 30% rot (0.74 g/L of average increase) and large up to 50% (1.46 g/L) (Table 2). These results could probably be explained by the berry dehydration and/or acetic acid and gluconic acid production, as result of the activity of oxidative yeasts (*Candida* spp., *Kloeckera* spp., *Hanseniaspora* spp.) associated with AAB that typically colonize sour rotten berries. In particular, *Acetobacter* spp. is responsible for the oxidation of ethanol into acetic acid with a high transformation yield and *Gluconobacter* spp. is characterized by a high oxidation activity of glucose that is accompanied by the accumulation of high gluconic acid concentrations. Moreover, AAB could also lead to a probable accumulation of other sugar degradation products such as lactic, propionic, and butyric acids (Ribéreau-Gayon *et al.*, 2006), resulting in lower pH values.

Although the clear increase in total acidity was induced by sour rot, the 2008 FTIR results showed that the tartaric acid concentration decreased and malic acid content remained relatively constant with the increase of sour rot (Table 2). These results emphasize the probable contribution of gluconic and acetic acid accumulation for the change in titrable acidity values. Zoecklein *et al.* (2001), in rotten white Riesling grapes, showed higher levels of gluconic acid in rotten grapes, although acetic acid did not vary significantly between sound and damaged grapes.

Regarding color intensity, anthocyanins and phenols quantification, results evidenced the effect of sour rot on raising the concentration of these parameters (Table 2), as a

probable consequence of berry dehydration process and higher skin and stems proportion relative to free juice (see Table 1).

Sour rot had an important effect on the grape musts' free SO_2 content. This effect was evident in 2008 grape must samples (all treated with an initial 50 mg/kg SO_2 concentration) for which the free SO_2 content decreased considerably with the increase in sour rot (Table 2). These results could be explained by the activity of *Gluconobacter* spp., since these bacteria oxidize gluconic acid, leading to the formation of keto-5 gluconic, keto-2 gluconic and diketo-2,5 gluconic acids, which energetically bind to SO_2 (Ribéreau-Gayon, 2006).

Table 2. Effect of sour rot on chemical composition of grape musts used on the 2007 and 2008 vinification sets.

Oenological parameters	Vatting grape musts								
	2007				2008				
	T_0 (30)	T_{30} (30)	T_0 (100)	T_{30} (100)	T_0	T_{30}	T_{50}	CT_0	CT_{30}
Glucose-Fructose (g/L)[a]	185	193	190	195	214	223	235	208	223
°Brix[a]	18.7	19.9	19.2	19.8	21.4	22.4	23.8	21.3	22.7
Potential alcohol content (% v/v)	10.5	11.3	10.8	11.3	12.3	13.0	14.0	12.3	13.2
Volumic mass (g/L)[a]	1088	1092	1090	1093	1102	1104	1108	1100	1106
Total acidity (g/L)[a]	3.23	4.50	3.11	4.10	4.26	4.54	5.72	3.94	4.35
Volatile acidity (g/L acetic acid)[a]	0.07	0.22	0.04	0.20	0.00	0.10	0.33	0.00	0.07
pH[a]	3.33	3.34	3.33	3.38	3.52	3.29	3.19	3.39	3.31
Tartaric acid (g/L)[a]	2.9	3.7	2.5	3.3	4.7	2.4	1.8	2.8	1.8
Malic acid (g/L)[a]	2.2	2.5	2.1	2.5	3.4	3.2	3.3	3.3	3.2
Total assimilable nitrogen (mg/L)[a]	211	198	169	183	253	230	270	223	206
Anthocyanins (mg/L)[b]	628	1044	574	739	314	357	390	297	361
Total phenols[b]	12.8	17.7	19.8	21.9	18.6	20.2	23.9	14.8	18.4
Color intensity[b]	4.8	12.0	5.2	8.4	1.56	5.85	9.09	0.92	1.52
Free SO_2 (mg/L)[c]	14	8	64	42	49	13	6	52	17
Total SO_2 (mg/L)[c]	130	75	173	155	110	98	104	108	102
Sanitary state indexes[a]									
Gray rot index (0-60)	15	28	14	29	29	45	48	31	47
Sour rot index (0-30)	2	6	2	6	3	6	13	4	6
Yeast activity index (0-30)	6	6	6	6	6	6	7	6	6
Lactic bacteria activity index (0-2)	0	1	0	1	2	1	2	1	2

[a] FTIR analysis; [b] Calculated by FTIR analysis for 2007 wines and by classical method (Ribéreau-Gayon *et al.*, 1972) for 2008 wines; [c] Calculated by classical method (OIV 2006).

The FTIR sour rot index showed a satisfactory correlation with the sanitary state of grape must samples. Control set musts containing sound grapes were classified as "good grapes", while must with 30 and 50% sour rot showed increasing index values, being classified as "good grapes to slight attack" and "slight attack to severe attack", respectively (Table 2). Higher percentages of sour rot were also characterized with greater gray rot index values in both vintage trials. Taking into account the FTIR classification scale, the 2007 control sets T_0 (30) and T_0 (100) were classified as "good

grapes to slight attack" and the sets with 30% rotten grapes T_{30} (30) and T_{30} (100) as "slight attack to critical attack". Concerning 2008 samples, no differences were found between samples with 30% (T_{30} and CT_{30}) and 50% (T_{50}) sour rot, which were all classified as "serious to severe attack". Despite the higher gray rot indexes of rotten musts, control sets showed an average index value of 30 (T_0 and CT_0), equivalent to a "slight attack to critical attack". These results suggest that gray rot indexes may not be adequate when sour rot is the dominant disease, because no signs of *Botrytis* infection were visually detected in rotten grapes. Yeast and lactic bacteria activity indexes did not show significant differences between sound and sour rotten grape musts.

3.3 Sour rot effect on wine chemical composition

Tables 3 and 4 summarize the analytical results of 2007 and 2008 wines during three different winemaking stages. The chemical analyses indicated a clear effect of rot percentage on wine chemical composition, and similar changes were found in both vintages. To better understand the most significant changes, results from both vintages were pooled and average differences and variations between sound and rotten wines are shown in Table 5.

Concerning ethanol content, wines produced from musts containing 30% rotten grapes had an average increase of 0.9% (v/v), while the rise to 50% rot contributed in average for wines with more 2.4% (v/v) ethanol. This increase results from higher sugar concentration in rotten musts due to berry dehydration that may also explain the increase in the dry extract of rotten wines, reaching around 44% increment in wines containing 50% infected grapes. To the increase in dry extract also contributed the higher proportion of skins and stems and the higher residual sugar content of rotten wines. After MLF, reducing sugars represent a critical oenological parameter in terms of future microbiological wine stability, and wines are considered as "dry" when sugar is below 2 g/L. This effect was particularly enhanced in 2008 monovarietal Trincadeira wines with 30% and 50% rotten grapes, which both registered 4.3 g/L residual sugar content just after wine fermentation (Table 4). This study shows that wines produced with 30 and 50% rotten grapes should have, in average, 1.2 and 2.5 g/L additional reducing sugar content, respectively (Table 5).

Table 3. Effect of sour rot on chemical composition of wines produced during 2007 vintage, analyzed along three different winemaking stages.

Oenological parameters	2007 Wines											
	After wine fermentation				After MLF				After 8 months bottle aging			
	T_0 (30)	T_{30} (30)	T_0 (100)	T_{30} (100)	T_0 (30)	T_{30} (30)	T_0 (100)	T_{30} (100)	T_0 (30)	T_{30} (30)	T_0 (100)	T_{30} (100)
Alcohol (% v/v)[a]	10.6	11.0	10.7	11.0	11.4	11.7	11.3	11.7	10.5	11.0	10.6	11.2
Dry extract (g/L)[a]	23.0	26.9	23.6	26.9	21.8	24.4	21.6	25.0	19.8	23.2	19.3	23.7
Reducing sugars (g/L)[a]	1.3	2.3	1.4	2.2	1.2	2.2	1.3	2.3	0.72	1.15	0.62	1.15
Total acidity (g/L)[a]	4.50	5.20	4.59	4.98	4.16	4.43	3.92	4.34	3.98	4.12	3.78	4.06
Volatile acidity (g/L)[a]	0.07	0.12	0.07	0.11	0.17	0.23	0.15	0.21	0.39	0.39	0.31	0.31
pH[a]	3.38	3.38	3.39	3.39	3.54	3.53	3.56	3.57	3.52	3.54	3.54	3.55
Anthocyanins (mg/L)[b]	ND	ND	ND	ND	ND	ND	ND	ND	425	534	409	528
Total phenols[b]	14.4	20.0	15.8	17.9	20.3	20.8	17.8	20.4	28.5	37.9	28.2	39.5
Color intensity[b]	21.1	27.8	21.9	28.8	22.6	26.1	20.6	27.6	13.5	15.7	13.1	14.5
L (%)									-36.2	-43.7	-40.0	-49.6
Malic acid (g/L)[a]	1.6	1.7	1.7	1.8	0.3	0.2	0.3	0.3	0.2	0.2	0.2	0.3
Lactic acid (g/L)[a]	0.0	0.0	0.0	0.0	0.8	0.9	0.7	0.8	0.8	0.9	0.8	0.8
Free SO_2 (mg/L)[c]	10	16	16	16	8	9	9	9	36	42	39	39
Total SO_2 (mg/L)[c]	57	70	77	93	45	51	61	80	94	78	91	91

ND, not determined; L (%) – percentage of color loss relative to the respective wine sample just after wine fermentation
[a] FTIR analysis
[b] Calculated by classical method (Ribéreau-Gayon *et al.* 1972)
[c] Calculated by classical method (OIV 2006)

Table 4. Effect of sour rot on chemical composition of wines produced during 2008 vintage, analyzed along three different winemaking stages.

Oenological parameters	2008 Wines														
	After wine fermentation					After MLF					After 6 months bottle aging				
	T_0	T_{30}	T_{50}	CT_0	CT_{30}	T_0	T_{30}	T_{50}	CT_0	CT_{30}	T_0	T_{30}	T_{50}	CT_0	CT_{30}
Alcohol (% v/v)[a]	12.5	13.5	14.9	13.0	14.3	12.3	14.1	14.6	12.3	13.8	12.1	13.7	14.6	12.3	13.8
Dry extract (g/L)[a]	23.6	30.2	33.1	25.7	30.4	21.7	28.7	31.6	22.1	27.0	20.3	25.5	29.9	22.6	26.3
Reducing sugars (g/L)[a]	1.7	4.3	4.3	1.2	2.4	1.6	3.2	4.1	0.7	2.0	1.4	2.6	3.8	0.6	1.9
Total acidity (g/L)[a]	5.12	6.26	7.00	5.95	6.60	3.96	5.30	5.94	4.32	5.25	3.70	4.85	5.53	4.47	5.02
Volatile acidity (g/L)[a]	0.00	0.21	0.40	0.05	0.21	0.10	0.36	0.45	0.11	0.33	0.12	0.30	0.46	0.15	0.33
pH[a]	3.38	3.3	3.35	3.34	3.32	3.63	3.54	3.54	3.57	3.53	3.62	3.52	3.54	3.57	3.55
Anthocyanins (mg/L)[b]	466	478	510	473	475	nd	nd	nd	nd	nd	432	458	488	389	406
Total phenols[b]	50.2	51.5	57.4	48.6	50.5	nd	nd	nd	nd	nd	42.9	45.7	53.8	39.1	42.6
Color intensity[b]	18.3	18.6	20.4	16.9	17.3	nd	nd	nd	nd	nd	16.1	15.3	16.0	15.4	14.6
L (%)											-11.7	-17.7	-21.6	-8.9	-15.6
Malic acid (g/L)[a]	2.0	2.1	2.2	2.4	2.3	0.2	0.2	0.4	0.2	0.3	0.1	0.2	0.3	0.2	0.3
Lactic acid (g/L)[a]	0.0	0.0	0.0	0.0	0.0	0.8	0.7	0.7	0.8	0.7	0.8	0.7	0.6	0.9	0.7
Free SO_2 (mg/L)[c]	16	23	19	23	23	10	16	10	13	13	29	32	32	32	42
Total SO_2 (mg/L)[c]	78	78	98	88	88	43	41	54	57	57	112	90	128	83	109

ND, not determined; L (%) – percentage of color loss relative to the respective wine sample just after wine fermentation
[a] FTIR analysis
[b] Calculated by classical method (Ribéreau-Gayon *et al.* 1972)
[c] Calculated by classical method (OIV 2006)

At the end of MLF, the volatile acidity was systematically slightly higher in all samples from rotten grapes. Nevertheless, the higher volatile acidity of wines from rot grapes may be ascribed to the higher initial volatile acidity of musts, because the increase during fermentation was similar in all trials. Despite this initial effect, the analysis performed in 2007 wines after 8 months did not show significant differences between wines from rotten and sound grapes. In 2008, both types of wines showed constant volatile acidity between the end of MLF and the 6-month storage.

Regarding total acidity, the chemical analyses indicated a clear effect of rot percentage only when the level reached 50%, for which was observed an increment of around 45% comparatively with the respective control wines. The increase of total and volatile acidities with sour rot did not influence significantly the pH values, since no significant changes were found between sound and sour rotten produced wines. These findings are in accordance with the results reported by Loinger *et al.* (1977).

Our research also showed that sour rot influenced the levels of anthocyanins, total phenols and wine color intensity, probably as a consequence of higher skin plus stems/free juice ratio. These values were higher in wines just after fermentation resulting from higher concentrations in grape juices. Wines produced from infected grapes had higher values of total phenols, which showed an average enrichment in total phenols of about 13 and 19%, when 30 and 50% rotten grapes were used, respectively. Moreover, an average increment of about 12% in the anthocyanin content was observed in wines produced up to 50% rotten grapes (Table 5).

Table 5. Overall chemical composition differences between wines produced with sour rotten grapes and their respective control wines, calculated during the 2-year study.

Parameter	30% Sour Rot		50% Sour Rot	
	Aver Diff[a]	Var (%)[b]	Aver Diff	Var (%)
Alcohol content (% v/v)	0.9	7.7	2.4	19.9
Dry extract (g/L)	4.4	20.0	9.7	44.2
Residual sugars (g/L)	1.2	101.6	2.5	159.6
Total acidity (g/L)	0.7	15.2	1.9	44.5
Volatile acidity (g/L)	0.1	84.0	0.4	495.5
Anthocyanins (mg/L)	47.5	11.0	55.0	12.2
Total phenols	4.1	13.4	9.0	19.4

[a] Average difference between wines with rotten grapes and the corresponding control wines with healthy grapes
[b] Variation (%) relative to the average content of the parameter in the control wines

Regarding color stability, wines produced with rotten grapes showed slightly higher color intensity after fermentation. However, during storage, results obtained in both vintages showed a clear trend for a higher loss of color intensity with sour rot. After 8 months of bottle aging, 2007 wines with 30% rotten grapes showed an average color loss of about 47%, in contrast to the 38% in control wines (Table 3). Likewise, a similar trend was observed for 2008 wines. Control sound wines (T_0 and CT_0) lost in average 10% of color intensity, while wines containing 30% of rotten grapes (T_{30} and CT_{30}) lost about 17%. The maximum decrease (22%) was observed for the wine sample produced with 50% infected grapes (T_{50}) (Table 4).

3.4 Fermentation duration and microbial counts

The duration of must fermentations was not affected by the proportion of sour rotten grapes in 2007 (6 days) and in the Trincadeira trials in 2008 (7–8 days). The Cabernet-Trincadeira fermentations with 30% sour rot increased the duration from 8 to 11 days (results not shown). On the contrary, malolactic fermentations were markedly delayed by the addition of sour rotten grapes, increasing 7 days in 2007 and 24 (30% rot) to 66 days (50% rot) in 2008. The duration of malolactic fermentations of Cabernet-Trincadeira in 2008, differed only by 2 days (results not shown).

The evaluation of total yeasts, AAB, and LAB performed during the 2007 vintage, showed that the introduction of 30% sour rot was accompanied by higher total counts of these three microbiological parameters on the initial vatting grape musts (Table 6). In particular, initial rotten grape juices T_{30} (30) and T_{30} (100) showed two orders of magnitude more AAB than healthy juices T_0 (30) and T_0 (100), confirming that rotten grapes contributes for the introduction of AAB populations able to produce volatile acidity in musts contaminated with sour rot (Table 2). Low microbial counts in bottled wines were due to the maintenance of free sulphur dioxide concentrations between 30 and 40 mg/L. The higher residual sugar of rotten wines did not appear to induce higher susceptibility to microbial growth or activity, in accordance with the stable results of volatile acidity, previously described.

Table 6. Total yeast counts and most probable number (MPN) enumeration of acetic acid bacteria (AAB) and lactic acid bacteria (LAB) in different winemaking stages.

Microbial analysis	Stage[a]	2007 wines				2008 wines				
		T_0 (30)	T_{30} (30)	T_0 (100)	T_{30} (100)	T_0	T_{30}	T_{50}	CT_0	CT_{30}
Total Yeasts (CFU/mL)	G	1.6×10^6	1.2×10^7	1.6×10^6	1.2×10^7	nd[c]	nd	nd	nd	nd
	AF	1.1×10^8	1.2×10^8	9.9×10^7	9.9×10^7	nd	nd	nd	nd	nd
	MLF	7.8×10^4	5.3×10^5	1.4×10^4	6.7×10^2	1.3×10^5	7.8×10^3	6.2×10^3	8.5×10^2	5.4×10^3
	BOT[b]	ND	ND	ND	ND	384	<1	<1	31	120
AAB (MPN/mL)	G	1.1×10^4	1.1×10^6	1.1×10^4	1.1×10^6	nd	nd	nd	nd	nd
	AF	2.4×10^2	2.4×10^2	1.1×10^3	1.1×10^2	nd	nd	nd	nd	nd
	MLF	2.3	2.3	<0.3	<0.3	25	25	2.5	25	2.5
	BOT	ND	ND	ND	ND	<0.3	25	<0.3	<0.3	<0.3
LAB (MPN/mL)	G	9.3×10^1	4.6×10^2	9.3×10^1	4.6×10^2	nd	nd	nd	nd	nd
	AF	2.4×10^2	4.6×10^1	4.6×10^2	9.3	nd	nd	nd	nd	nd
	MLF	2.3	2.3	2.3	2.3	45	1.1×10^3	1.4×10^4	1.1×10^4	1.4×10^4
	BOT	ND	ND	ND	ND	2.5	2.5	2.5	40	110

ND, not determined

[a] G, Vatting grape must (before SO_2 and commercial *S. cerevisiae* yeast addition); AF, after wine fermentation (just after pomace pressing); MLF, after malolactic fermentation; BOT, after 6 months of bottle aging (correction to 40 mg/L free SO_2 after MLF)

[b] CFU/100 mL

3.5 Sensory analysis

Table 7 shows the ANOVA results obtained for the sensory attributes for the 2007 and 2008 wines evaluated in the two formal sessions. In the first session, three groups of wine samples were analyzed by different ANOVA: (1) 2007 wines tasted after 12 months of bottle aging; (2) 2008 monovarietal Trincadeira wines tasted just after MLF; and (3) blends of Trincadeira and Cabernet Sauvignon tasted just after MLF.

Results showed that in case of 2007 wines, from the 10 attributes examined, only color intensity and over-all quality changed significantly (p<0.05). The comparison of the mean intensity scores associated to this 2 attributes are present in Fig.1A. No significant differences were found between the Trincadeira rotten wine with initial 30 mg/kg SO_2 vatting concentration [T_{30} (30)] and the respective control wine with sound grapes [T_0 (30)]. On the contrary, when the initial SO_2 vatting concentration was increased to 100 mg/kg, a significant decrease (p<0.05) in color intensity and overall quality was found for Trincadeira rotten wine [T_{30} (100)]. Therefore, lower overall quality scores were associated to wines with lower color intensities, which can be a result of the present consumer preference for red wines with deeper color (Kontoudakis *et al.*, 2011). Indeed, the influence of SO_2 concentration was also observed when the two rotten samples were compared: the wine with initial 100 mg/kg of SO_2 [T_{30} (100)]

showed significant lower scores of color intensity and overall quality than the wine with initial 30 mg/kg [T_{30} (30)]. This observation is consistent with the possible effect of SO_2 on the decrease of color intensity during aging (Sacchi *et al.*, 2005).

For the second and third groups, ANOVA results performed within each group showed that no significant differences were found between all young 2008 wines just after MLF, for all sensory attributes.

In the second tasting session, the 2008 wines were again tasted after 6 months of bottle aging (BOT). Again, monovarietal Trincadeira wines (T_0, T_{30} and T_{50}) and blends with Cabernet Sauvignon (CT_0 and CT_{30}) were analyzed separately in two different ANOVA. Table 7 show that in case of Trincadeira wines, the presence of 30% and 50% sour rot did not induce significant effects (p<0.05) on the 10 sensorial attributes studied. Regarding the Trincadeira and Cabernet Sauvignon wine blends, ANOVA results showed that sour rot only induced a significant effect (p<0.05) on color intensity attribute. Indeed, a significant decrease in color intensity was observed in the wine blend with 30% sour rot (CT_{30}), comparing with their respective control wine (Fig. 1B). However, contrarily to 2007 wines, the decrease on color intensity was not reflected by a statistically significant decrease of the overall quality between sound and rotten wines (see Table 7).

3.5.1 Effect of sour rot during wine aging

Two additional ANOVAs were performed separately for the 2008 monovarietal and blend wines, using the combined results obtained in Session 1 and 2, in order to check for significant differences during aging. As expected, color intensity decreased significantly in all wine samples after 6 months of bottle aging (Fig. 1B). However significant higher decreases were found in all wines with increasing sour rot proportions. These results are in agreement with the previous chemical analyses data (see Tables 3 and 4).

Concerning aroma and overall quality, no significant differences were found for the attributes intensity and quality for both monovarietal and blend wines. The presence of sour rot only induced significant changes in intensity-, quality-, and equilibrium taste-related attributes (Table 7).

Table 7. Sensory attributes: F statistics and *p* values of each ANOVA.

Sensory attributes[a]	Session 1						Session 2				2008 Wine aging			
	2007 Wines		2008 Wines (MLF)				2008 Wines (BOT)				(MLF vs BOT)			
			T		CT		T		CT		T		CT	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Color														
Intensity	**5.13**	**0.0059**	1.14	0.3390	0.23	0.6420	2.24	0.1193	**6.40**	**0.0173**	**8.49**	**<0.0001**	**14.04**	**<0.0001**
Aroma														
Intensity	2.04	0.1304	0.09	0.9101	0.47	0.5057	0.31	0.7378	1.41	0.2448	0.17	0.9742	0.85	0.4722
Quality	0.06	0.9808	1.06	0.3649	0.52	0.4833	1.21	0.3073	0.00	1.0000	0.91	0.4796	0.74	0.5357
Taste														
Intensity	0.46	0.7150	1.17	0.3308	0.64	0.4384	0.48	0.6238	0.00	1.0000	**3.48**	**0.0077**	0.33	0.8050
Quality	0.78	0.5129	0.93	0.4107	0.94	0.3487	2.53	0.0914	0.97	0.3342	**3.85**	**0.0041**	0.67	0.5768
Body	2.08	0.1256	0.26	0.7720	2.33	0.1489	0.75	0.4803	0.14	0.7069	0.39	0.8571	0.78	0.5094
Equilibrium	1.47	0.2437	1.48	0.2513	0.25	0.6217	1.95	0.1550	2.23	0.1468	**2.67**	**0.0299**	1.24	0.3064
Persistence	0.69	0.5671	0.62	0.5464	0.06	0.8087	0.82	0.4469	1.00	0.3259	0.63	0.6807	0.33	0.8006
Final taste	0.67	0.5756	0.27	0.7666	0.84	0.3761	1.43	0.2505	0.06	0.8161	0.69	0.6347	0.47	0.7076
Over-all quality	**3.05**	**0.0451**	0.52	0.6004	0.50	0.4928	1.19	0.3148	0.10	0.7559	0.77	0.5768	1.48	0.2328

[a]Significant effects ($p<0.05$) in bold; [b] T- Monovarietal Trincadeira wines. CT – Cabernet Sauvignon and Trincadeira blend wines.

The comparison of the mean intensity scores obtained for these attributes showed that only the monovarietal Trincadeira wine with 30% rot (T_{30}) increased significantly the quality and equilibrium taste attributes. Furthermore, this sample did not change significantly its taste intensity, contrary to the samples T_0 and T_{50}, which both decreased significantly this attribute after 6 months of bottle aging (Fig. 1B).

To date, and to our best knowledge, only the work of Loinger *et al.* (1977) mentions the sensory evaluation of wines produced with different sour rot percentages (ranging from 5 to 80%). These authors stated that 40% rot should be regarded as the threshold above which wines become depreciated.

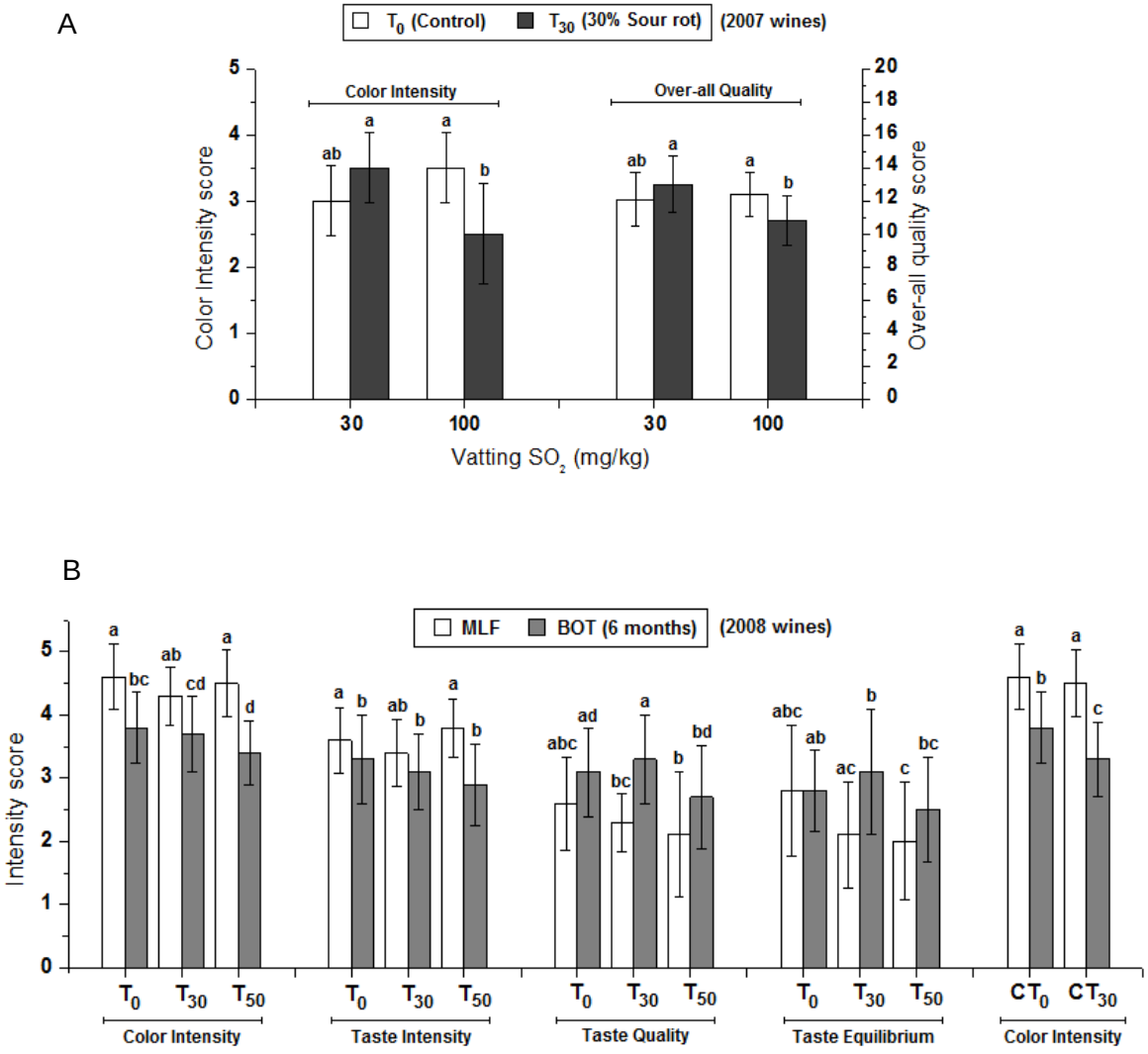

Fig. 1 Mean intensity scores of 2007 (A) and 2008 (B) wines for the sensory attributes that changed significantly (p<0.05) in each ANOVA and groupings after means comparisons within each sensory attribute (post hoc Fisher LSD test). Different letters indicate significant differences (p<0.05).

Our research suggests that the presence of 30% rotten grapes should not be regarded as responsible for a significant decrease on wine aroma and taste characteristics. Interestingly, even the presence of 50% sour rot grapes is not responsible for a remarkable overall wine quality decrease effect, contrary to what might be empirically expected. Nevertheless, and taking into account the significant decreases in taste intensity and color intensity, observed in T_{50} wine sample after 6 months of bottle aging, we think that this percentage should be regarded as detrimental to wine quality. Therefore, our results agree with the sour rot limit percentage given by Loinger *et al.* (1977).

4. Conclusions

This study demonstrates that grape sour rot is responsible for serious crop losses in the vineyard and contributes for a decrease in final wine yield in the winery, resulting in important economic losses for winegrowers and winemakers. The use of increasing grape sour rot percentages was associated with important changes on the initial chemical grape must composition, expressed by the increase in sugar content, total acidity, volatile acidity, color intensity, anthocyanins, total phenols and by SO_2 binding power. The FTIR sour rot index showed to be a reliable tool to evaluate sour rot grading of the incoming grapes.

The overall chemical composition of rotten wines were distinguished from control wines by its higher values of alcohol content, dry extract, reducing sugar content, total and volatile acidity, anthocyanins, total phenols, and color intensity, after fermentation and MLF. At the sensorial point of view, after the MLF, rotten wines were not statistically different in terms of color, aroma, taste and overall quality. This overall similarity was not maintained during aging for the wines made with 50% sour rotten grapes, given their higher losses in color and in taste intensity sensorial attribute. However, the presence of 30% rot yielded wines with similar or better sensorial scores than the control wines up to one year of storage. In addition, our results showed that the winery practice of increasing the initial SO_2 levels when gray rot is present is not required for sour rotten grapes.

Although no significant differences were found in the aroma attributes of rotten and sound wines, future work should be carried out in order to study more in depth the volatile composition of sour rotten wines, to understand the role of the disease in the odor nuances of these particular red wines.

Acknowledgments

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CHAPTER 5

Analytical and Sensorial Characterization of the Aroma of Wines Produced with Sour Rotten Grapes Using GC-O and GC-MS: Identification of Key Aroma Compounds

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Analytical and Sensorial Characterization of the Aroma of Wines Produced with Sour Rotten Grapes Using GC-O and GC-MS: Identification of Key Aroma Compounds

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ABSTRACT: In the present work, the aroma profiles of wines elaborated from sound and sour rot-infected grapes as raw material have been studied by sensory analysis, gas chromatography−olfactometry (GC-O), and gas chromatography−mass spectrometry (GC-MS), with the aim of determining the odor volatiles most likely associated with this disease. The effect of sour rot was tested in monovarietal wines produced with the Portuguese red grape variety Trincadeira and in blends of Cabernet Sauvignon and sour rotten Trincadeira grapes. Wines produced from damaged berries exhibited clear honey-like notes not evoked by healthy samples. Ethyl phenylacetate (EPhA) and phenylacetic acid (PAA), both exhibiting sweet honey-like aromas, emerged as key aroma compounds of sour rotten wines. Their levels were 1 order of magnitude above those found in controls and reached 304 and 1668 μg L^{-1} of EPhA and PAA, respectively, well above the corresponding odor thresholds. Levels of γ-nonalactone also increased by a factor 3 in sour rot samples. Results also suggest that sour rot exerts a great effect on the secondary metabolism of yeast, decreasing the levels of volatiles related to fatty acids and amino acid synthesis. The highest levels of γ-decalactone of up to 405 μg L^{-1} were also found in all of the samples, suggesting that this could be a relevant aroma compound in Trincadeira wine aroma.

KEYWORDS: sour rot, wine, aroma, flavor, Trincadeira, GC-O, GC-GC, ethyl phenylacetate, phenylacetic acid, γ-lactones

INTRODUCTION

Fruit rots often determine harvest date and influence grape quality in warm and humid grape-growing regions.[1] The cause of grape sour rot, appearing 3−4 weeks before ripening, is the combined activity of three groups of factors: (a) primary factors (insects, birds, diseases such as mildews, grape berry moth attacks (*Lobesia botrana*), mechanical and physiological injuries), which damage the berry skin; (b) secondary microorganisms (bacteria, yeasts, and other fungi) that penetrate the broken skins of the injured berries; and (c) secondary insects (*Drosophila* flies and beetles) quickly attracted by the rotting and fermenting grapes, enhancing the process and accelerating its spread throughout the entire cluster.[2,3] Sour rot is characterized by the main role of yeasts in the rotting process. The most frequently isolated yeast species from sour rot-damaged grapes are *Hanseniaspora uvarum*, *Candida stellata*, *Metshnikowia pulcherrima*, *Candida krusei*, and *Kloeckera apiculata*,[4,5] and *Zygoascus hellenicus* and *Issatchenkia occidentalis*.[6] Other microorganisms, especially the bacteria *Acetobacter*, are involved in sour rot infection. Among the transformations carried out by acetic acid bacteria, the most important is the oxidation of ethanol into acetic acid. Indeed, sour rot owes its name to the strong and pungent odor of acetic acid (vinegar) present on rotten grapes and is characterized by grape pulp browning, disaggregation of the internal tissues, detachment of the rotten berry from the pedicel, and grape dropping.[5,7] In hot climates, *Botrytis cinerea* has occasionally been isolated from grape sour rot.[8] However, sour rot development is not dependent on *B. cinerea* infection,[9] in contrast to the predominant role of this fungal pathogen on bunch rot in regions with relatively cool fruit-ripening conditions.

Sour rot affects both crop yield and wine quality. It is commonly accepted between winemakers that the vinification of damaged grapes is associated with the production of low-quality wines with weak storage/aging potential.

The diverse group of organisms involved in sour rot is known to alter fruit composition as a result of the production of high levels of a wide range of metabolites including acetic acid, glycerol, ethyl acetate, ethanol, acetaldehyde, and galacturonic and gluconic acids.[10−12] In the late 1970s, Loinger et al.[13] tried to clarify for the first time the consequences of sour rot on wine quality, investigating the effect of the disease on the sensorial characteristics of wines from the Semillon grape variety. This study showed that clusters with 20−40% rotten berries resulted in a clear and marked reduction in wine quality, whereas wines from clusters with 80% rot were totally rejected. Later, Zoecklein et al.[10] determined the influence of sour rot on white Riesling must monoterpenic composition. Sour rot had no influence on free or bound volatile terpenes, but did alter the terpene profile by reducing monoterpene alcohols, whereas monoterpene oxides increased. Trincadeira is a neutral *Vitis vinifera* L. red native grape variety widely planted in Portugal,[14] highly susceptible to

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André Barata, Eva Campo, Manuel Malfeito-Ferreira, Virgílio Loureiro, Juan Cacho, Vicente Ferreira

Abstract

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Keywords: Sour rot, Wine, Aroma, Flavour, Trincadeira, GC-O, GC-GC, Ethyl phenylacetate, Phenylacetic acid, γ-lactones.

1. Introduction

Fruit rots often determine harvest date and influence grape quality in warm and humid grape-growing regions (Wolf *et al.*, 1990). The cause of grape sour rot, appearing 3–4 weeks before ripening, is the combined activity of three groups of factors: (a) primary factors (insects, birds, diseases such as mildews, grape berry moth attacks (*Lobesia botrana*), mechanical and physiological injuries), which damage the berries skin; (b) secondary microorganisms (bacteria, yeasts and other fungi) that penetrate the broken skins of the injured berries; and (c) secondary insects (*Drosophila* flies and beetles) quickly attracted by the rotting and fermenting grapes, enhancing the process and accelerating its spread throughout the entire cluster (Berlinger, 1970; Papo and Vermes, 1961). Sour rot is characterized by the main role of yeasts in the rotting process. The most frequently isolated yeast species from sour rot-damaged grapes are *Hanseniaspora uvarum*, *Candida stellata*, *Metshnikowia pulcherrima*, *Candida krusei* and *Kloeckera apiculata* (Bisiach, 1986; Guerzoni and Marchetti, 1987), *Zygoascus hellenicus* and *Issatchenkia occidentalis* (Barata *et al.*, 2008). Other microorganisms, especially the bacteria *Acetobacter*, are involved in sour rot infection. Among the transformations carried out by acetic acid bacteria, the most important is the oxidation of ethanol into acetic acid. Indeed, sour rot owes its name to the strong and pungent odor of acetic acid (vinegar) present on rotten grapes and is characterized by grape pulp browning, disaggregation of the internal tissues, detachment of the rotten berry from the pedicel, and grape dropping (Bisiach, 1982; Guerzoni and Marchetti, 1987). In hot climates, *Botrytis cinerea* has occasionally been isolated from grape sour rot (Zoecklein *et al.*, 1992). However, sour rot development is not dependent on *B. cinerea* infection (Marchetti *et al.*, 1984), in contrast to the predominant role of this fungal pathogen on bunch rot in regions with relatively cool fruit-ripening conditions.

Sour rot affects both crop yield and wine quality. It is commonly accepted between winemakers that the vinification of damaged grapes is associated to the production of low quality wines with weak storage/aging potential.

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with 20-40% rotten berries resulted in a clear and marked reduction in wine quality, whereas wines from clusters with 80% rot were totally rejected. Later, Zoecklein *et al.* (2001) determined the influence of sour rot on white Riesling must monoterpenic composition. Sour rot had no influence on free or bound volatile terpenes, but did alter the terpene profile by reducing monoterpene alcohols, whereas monoterpene oxides increased. Trincadeira is a neutral *Vitis vinifera* L. red native grape variety widely planted in Portugal (Botelho *et al.*, 2008), highly susceptible to cryptogamic diseases, and particularly sensitive to sour rot (Magalhães, 2008). Concerning the aroma characterization of Trincadeira wines, Cabrita *et al.* (2006) studied the composition of the glycosidic flavour precursors extracted from Trincadeira grape samples, as well as the fermentative compounds and glycoconjugated aroma compounds (monoterpenes, norisoprenoids) present in the respective wines (Cabrita *et al.*, 2007). Botelho *et al.* (2008) investigated the differences in odor-active compounds of Trincadeira wines obtained from five different certified clones, using gas chromatography-olfatometry (GC-O).

Previous studies performed by Escudero *et al.* (2007) and more recently by San-Juan *et al.* (2010) has evidenced that, by using a dynamic headspace technique, based on purge-and-trap headspace-solid-phase extraction (HS-SPE) system in the preparation of wine extracts for GC-O, it is possible to obtain relatively simple and clean olfactograms and to establish a hierarchy of the most important odorants according to their potential sensory impact. The GC-O technique strongly contributed in the past decade to the overall identification of odor-active compounds in white and red musts and/or young and aged wines. In fact, GC-O analysis has been widely used to identify odor-active compounds in wines made from Chardonnay (Buettner, 2004; Moio *et al.*, 1994), Riesling (Chisholm *et al.*, 1994), Gewurztraminer (Guth, 1997a,b; Ong and Acree, 1999), Merlot and Cabernet Sauvignon (Gürbüz *et al.*, 2006; Kotseridis and Baumes, 2000; Lopez *et al.*, 1999), Grenache and Tempranillo (Ferreira *et al.*, 1998, 2000; Lopez *et al.*, 1999, 2004; Marti *et al.*, 2003), Zalema (Gómez-Míguez *et al.*, 2007), Palomino Fino (Campo *et al.*, 2006), Touriga Nacional (Falco, 2004), Aragonez (Botelho *et al.*, 2007, 2010), and Trincadeira (Botelho *et al.*, 2008) grape cultivars. This technique has also been applied to screen the odor volatile composition of sweet wines obtained from overripe berries affected by *B. cinerea*. These works have been especially devoted to noble rotten wines from Sauternes (France) region (Bailly *et al.*, 2009; Campo *et al.*, 2008; Sarrazin *et al.*, 2007), although some other examples such as Fiano wines from Amarone (Italy) region can be found in the literature (Genovese *et al.*, 2007). Despite the existing investigations on botrytized wines (noble rot), to our

knowledge, no work focused on the aroma profile of wines affected by sour rot has been published to date, which means that the number and nature of the odorants associated to this grape disease are not known.

Therefore, the main goal of this research was to compare the volatile composition and sensory profiles of wines produced with sound and sour rot affected grapes. For this purpose, the methodological approach combines the GC-O and chemical quantitative analysis of major and minor compounds, together with sensory descriptive analysis, to understand the role of sour rot in the odor nuances of these wines.

2. Materials and methods

2.1 Wines

During the 2008 vintage, healthy (sound) and sour rot affected bunches (Trincadeira and Cabernet Sauvignon grape varieties) were collected at the time of harvest from an experimental vineyard at the Instituto Superior de Agronomia (Lisbon, Portugal, latitude 38º 42´31.57´´ N and longitude 9º 11´14.01´´ W) and transported to the experimental cellar in 20 kg plastic boxes. Infected bunches were collected at the same stage of infection. Five microvinification sets (red style fermentation with grape skin contact) were carried out in 50 kg capacity stainless steel tanks using sound grapes to which different amounts of sour rot damaged grapes were added: two sets of monovarietal Trincadeira grape variety with 30 and 50% sour rotten grapes and one set of 70% sound Cabernet Sauvignon blended with 30% sour rotten Trincadeira grapes. Two additional control sets composed, respectively, by 100% sound Trincadeira grapes and 70% sound Cabernet Sauvignon blended with 30% sound Trincadeira grape cultivars were performed (Table 1). The grapes (40 kg per set) were mechanically destemmed and crushed on a commercial grape destemmer-crusher, followed by SO_2 addition (50 mg/Kg) to emulate winery conditions and 18 h of pre-fermentative maceration at room temperature. Musts were inoculated with 10^6 cells/mL of selected commercial *Saccharomyces cerevisiae* yeast (Fermivin, DSM, Delft, The Netherlands). Fermentation temperature varied from 22 to 27 ºC, and grape skins were submerged twice a day. Once malolactic fermentation (MLF) was complete, free SO_2 levels were corrected to 40 mg/L and wines were bottled without filtration in 0.75 L glass bottles capped with natural corks and kept at cellar temperature (18±2 ºC). For each produced wine, two different phases within aging were analyzed: just after MLF and after 16 months of bottle aging in cellar (BOT). Wine samples of each phase were stored at

-20 ºC until analysis. The detailed information of all wine samples analyzed in the experiment is shown in Table 1.

Table 1. Wines analyzed in the experiment including grape varieties, healthy state, wine production phase, some basic compositional parameters determined by FTIR and analyses performed for each wine samples.

Trial	Wine Sample	Grape % (w/w)[a]			Wine phase	Alcohol (% v/v)	pH	Volatile acidity[b] (g L^{-1})	Residual Sugar (g L^{-1})	TPI[c]	CI[d]	Analyses
		S (T)	S (CS)	SR (T)								
T0[e]	T0 MLF	100			MLF[f]	12.3	3.63	0.10	1.6	33.5	17.0	GC-O; Chemical[g]; Sensory
	T0 BOT	100			BW[h]	11.4	3.62	0.12	1.4	30.8	14.1	GC-O; Chemical; Sensory
T30	T30 MLF	70		30	MLF	14.1	3.54	0.36	3.2	35.9	15.9	Sensory
	T30 BOT	70		30	BW	13.3	3.52	0.30	2.6	29.6	12.6	Sensory
T50	T50 MLF	50		50	MLF	14.6	3.54	0.45	4.1	39.4	20.1	GC-O; Chemical
	T50 BOT	50		50	BW	13.9	3.54	0.46	3.8	39.0	14.8	GC-O; Chemical
CT0[e]	CT0 MLF	30	70		MLF	11.9	3.57	0.11	0.7	36.5	16.8	GC-O; Chemical; Sensory
	CT0 BOT	30	70		BW	12.1	3.57	0.15	0.6	37.8	18.3	GC-O; Chemical; Sensory
CT30	CT30 MLF		70	30	MLF	13.3	3.53	0.33	2.0	39.7	17.5	GC-O; Chemical; Sensory
	CT30 BOT		70	30	BW	12.8	3.55	0.33	1.9	39.4	14.8	GC-O; Chemical; Sensory

[a] S(T), sound Trincadeira. S(CS), sound Cabernet Sauvignon; SR(T), sour rot Trincadeira. [b] Expressed as acetic acid. [c] TPI, total phenols index, expressed in absorbance ($A_{280\ nm}$) ×100. [d] CI, color intensity, expressed as ($A_{420\ nm}$ + $A_{520\ nm}$ +$A_{620\ nm}$) ×5. [e] Control set vinifications. [f] MLF, after malolactic fermentation. [g] Major (liquid-liquid microextraction and GC-FID) and minor (SPE and GC-Ion Trap-MS) compounds. [h] BW, bottled wine (after 16 months bottle aging).

2.2 Reagents and standards

The chemical standards were supplied by Aldrich (Gillingham, UK), Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA), Lancaster (Strasbourg, France), PolyScience (Niles, USA), Chemservice (West Chester, USA), Interchim (Monluçon, France), International Express Service (Allauch, France) and Firmenich (Geneva, Switzerland). LiChrolut EN resins (ethylvinylbenzenedivinylbenzene) and polypropylene cartridges were obtained from Merck (Darmstadt, Germany). Dichloromethane and methanol of LiChrosolv quality were from Merck (Darmstadt, Germany); absolute

ethanol, and ammonium sulfate were from Panreac (Barcelona, Spain) and all of them were ARG quality; pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Semi automated Solid Phase Extraction (SPE) was carried out with a VAC ELUT 20 station from Varian (Walnut Creek, CA, USA).

2.3 Enological parameters

All wines were analyzed by Fourier Transform Infrared Spectroscopy (FTIR), in a WineScan FT120 (Foss, Hillerød, Denmark) equipment. The following enological parameters were determined: ethanol content (% v/v), pH, volatile acidity, residual sugar, total phenol index (TPI) and color intensity (CI).

2.4 Sensory descriptive analysis

The sensory panel was composed of 7 females and 7 males, 24–70 years of age (mean = 36), all belonging to the laboratory staff and with a long experience in sensory analysis. Four specific 1 h training sessions were carried out. In the first one, judges generated descriptive terms to define the wines of the study. In sessions two and three, different aroma standards were presented and discussed by the panel. From these discussions, 12 aroma attributes were retained as the most appropriate to describe the wine samples: fresh fruit, ripe fruit, vegetal/earthy, floral, spicy, honey, toasted/vanilla, butter, animal, chemical, reduction, and oxidized. The aroma reference standards used to define each of these attributes are presented in Table 2. In training session four, panelists scored the intensity of each attribute using a seven-point scale (0 = non detected, 1 = weak, hardly recognizable note, 2 = clear but not intense note, 3 = intense note); half values were allowed. After the training period, the eight wine samples were evaluated once during a formal session. Judges were asked to evaluate wines orthonasally and to rate the intensity of each attribute listed in Table 2 according to the 7-point scaled used during training. Sessions took place in individual booths and lasted approximately 45 min. A 10 min break was enforced in the middle of each session to limit judge fatigue. Samples were presented according to a William Latin-square arrangement. The data were processed to calculate the “modified frequency” parameter proposed by Dravnieks (1985): $MF(\%) = \sqrt{F(\%) \times I(\%)}$, where *F* (%) is the detection frequency of an aromatic attribute expressed as percentage of total

number of judges, and *I* (%) is the average intensity expressed as percentage of the maximum intensity.

Table 2. Aroma attributes selected for descriptive sensory analysis and composition of the corresponding reference standards.

Attribute	Reference Standard[a]
Fresh fruit	3 mL each of pear, banana, strawberry and blueberry juice
Ripe fruit	1mL of prune juice + 5mL of brine from canned figs
Vegetal/earthy	5g of sliced bell pepper soaked for 15 min + 5mL of brine from canned green beans
Toasted	1 drop each of samples 48 (toast) and 49 (roasted almonds) of "Le Nez du Vin" Jean Lenoir
Oxydized	5 mL of brine from canned potatoes
Honey	Half teaspoon of honey
Floral	1 drop of phenylethyl alcohol
Chemical	2 mL of 95% ethanol + 1.5 mL vinegar
Spicy	Pinch of black pepper + 2 cloves
Butter	Half teaspoon of butter
Animal	100 mg of shoe polish + 1 drop of sample 45 (leather) of "Le Nez du Vin" Jean Lenoir
Reduction	1 drop each of samples 6 (sulphur) and 9 (cabbage) of "Le Nez du Vin" Jean Lenoir

[a] Quantities specified are those added to 40 mL of neutral red wine.

2.5 GC-O study

Wine extracts were obtained by a dynamic headspace sampling technique. A standard SPE cartridge (0.8 cm internal diameter, 3 mL internal volume) filled with 400 mg of LiChrolut EN resins was first washed with 20 mL of dichloromethane and then dried by letting air pass through (negative pressure of 0.6 bar, 10 min). The cartridge was placed on the top of a flask containing 80 mL of wine kept at room temperature (approximately 21 ºC). A stream of nitrogen (500 mL min^{-1}) was applied onto the surface of the liquid, purging the volatile compounds of the headspace. The volatile wine constituents released were trapped in the cartridge containing the sorbent. After 100 min, the cartridge was removed and dried by letting N_2 pass through; then, analytes were eluted with 3.2 mL of dichloromethane with 5% methanol. After this, the extract was concentrated under a stream of pure N_2 to a final volume of 200 µL.

GC-O analysis was carried out in a gas chromatograph Trace GC (Termoquest, Milan, Italy*)* with a flame ionisation detector (FID) and olfactometric port ODO-I from SGE

(Ringwood, Australia). This instrument was equipped with a capillary column DB-WAX (polyethylene glycol) from J&W Scientific (Folsom, CA), 30 m × 0.32 mm i.d., 0.5 µm film thickness; and a pre-column (3 m × 0.32 mm i.d.) from Supelco (Bellefonte, PA). Chromatographic conditions were as follows: hydrogen as the carrier gas (3.5 mL min^{-1}); splitless injection (splitless time 60 s); injection volume, 1 µL; injector temperature, 250 °C; detector temperature, 250 °C. The oven temperature program was the following: 40 ºC for 5 min, then raised at 4 ºC min^{-1} up to 100 ºC and at 6 ºC min^{-1} up to 220 ºC, followed by 20 min at 220 ºC. The sniffing port was equipped with a humidified air makeup and sequentially heated using a laboratory-made rheostat to prevent condensation of high-boiling compounds.

A panel of six judges (two women and four men, ranging from 23 to 32 years of age), carried out the sniffing of the extracts. Prior to GC-O analysis, panelist followed a training period as described in Ferreira *et al.* (2003). Sniffing time was approximately 40 min, and each judge carried out one session per day. The panelists were asked to provide a descriptor to characterize the eluted odor and to rate its intensity using a 7-point category scale (0 = no odour; 1 = weak odour, low intensity; 2 = clear perception of odour, strong intensity; 3 = extremely strong intensity of odour; intermediate values of 0.5, 1.5, and 2.5 were allowed). The quantitative capacity of this technique has been demonstrated elsewhere (Ferreira *et al.*, 2003). As a large number of odorants are at concentrations near the threshold in the headspace extracts, the data processed were a mixture of the intensity and the frequency of detection of an odorant. This parameter is labeled as “modified frequency” (MF) and is calculated with the formula proposed by Dravnieks (1985): $MF(\%) = \sqrt{F(\%) \times I(\%)}$, where *F* (%) is the detection frequency of an aromatic odorant expressed as percentage of total number of judges, and *I* (%) is the average intensity expressed as percentage of the maximum intensity. The odorants were identified by comparison of their odors and chromatographic retention index in DB-WAX column with those of pure reference compounds.

2.5.1 Identification of unknown odorants

The isolation of seven unknown odorants detected in the GC-O experiment and for which the identity could not be achieved by the standard procedure was carried out by means of a multidimensional GC technique (GC-GC). Basically, in GC–GC a fraction of effluent from a primary column is isolated and reanalyzed by a second column of different stationary phase selectivity. We employed a dual GC-GC-MS system

composed of two independent chromatographs interconnected by means of a Deans valve and a thermoregulated transfer line kept at 200 ºC. The first chromatograph was equipped with DB-WAX column (polyethylene glycol), a FID, and an olfactometric port, and the second one with FactorFour VF-5MS column (polymethylsiloxane-5% diphenyl) from Varian (Walnut Creek, CA), a mass spectrometry (MS) detector, and a second olfactometric port. The complete description of the system is given in Campo *et al.* (2007). Concentrated wine extracts were injected on this dual system until a satisfactory mass spectrum for the target odorant could be obtained. The identity was finally confirmed by the injection of the pure reference standard in the GC-GC-MS system.

2.6 Chemical quantitative analysis

2.6.1 Major Compounds (Liquid-Liquid Microextraction and GC-FID Analysis)

The quantification of major compounds was carried out using the method proposed and validated by Ortega *et al.* (2001). The extract was prepared in accordance with this method with the following adjustments: in 15 mL screw-capped centrifuge tubes, containing 4.1 g of ammonium sulphate, were added 2.7 mL of wine, 6.3 mL of water, 20 µl of internal standard solution (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, heptanoic acid and 2-octanol at 200 µg/mL in ethanol), and 0.25 mL of dichloromethane. The tubes were shaken for 90 min and then centrifuged at 2500 rpm for 10 min. Once the phases had been separated, the dichloromethane phase was recovered with a 0.5 mL syringe and transferred to a 0.3 mL vial. The extract was then analyzed by GC in a Hewlett-Packard 5890 Series II gas chromatograph with FID. This instrument was equipped with a capillary column DB-WAX (30 m × 0.32 mm i.d. and 0.5 µm film thickness) from J&W Scientific preceded by a 2 m × 0.53 mm uncoated precolumn. Chromatographic conditions were as follows: hydrogen as the carrier gas (2.2 mL min^{-1}); split injection mode (1:10 split relation) with 3 µL injection volume; injector temperature at 250 °C and detector temperature at 250 °C. The initial column temperature was 40 ºC for 2 min, heated up to 200 ºC at 2ºC min^{-1}, and remaining at that temperature for 30 min. Quantitative data were obtained by interpolation of relative peak areas in the calibration graphs built by the analysis of synthetic wines containing known amounts of the analytes.

2.6.2 Minor Compounds (SPE and GC-Ion Trap-MS Analysis)

This analysis was carried out using the method proposed and validated by Lopez *et al.* (2002) with the following changes in the previous procedure: standard SPE cartridges (1 mL total volume) filled with 50 mg of LiChrolut EN resins were placed in the vacuum manifold extraction system, and the sorbent was conditioned by rinsing the cartridges with 6 mL of dichloromethane, 2 mL of methanol, and, finally, 2 mL of a water-ethanol mixture (12%, v/v). The cartridges were then loaded with 15 mL of wine sample and 10 μl of a surrogate standards solution containing 3-octanone, β-damascone and heptanoic acid (all at 200 μg/g of ethanol). This mixture was passed through the SPE cartridges (2 mL min^{-1}), followed by a wash step using 5 mL of 40% water-methanol solution. The resins were then dried by letting air pass through (negative pressure of 0.6 bar, 10 min). Analytes were recovered in a 2 mL vial, by elution with 0.6 mL of dichloromethane. Twelve microliters of an internal standard solution (300 mg L^{-1} of 4-hydroxy-4methyl-2-pentanone and 2-octanol) was added to the eluted sample. The extract was then analyzed by GC with ion trap MS detection. A CP-3800 gas chromatograph fitted to Saturn 2200 ion trap MS from Varian was used. Chromatographic analyses were performed under the conditions described in Lopez *et al.* (2002).

2.7 Statistical Analysis

For the data obtained in the sensory descriptive analysis, a two-factor (wines and subjects) ANOVA was performed on the attributes' intensity scores derived from the four Trincadeira wines and the four blends of Trincadeira/Cabernet Sauvignon. To look for discriminant odorants, a two-factor (wine and subjects) analysis of variance (ANOVA) was performed on the individual intensity GC-O scores. For the chemical quantitative data, a two-factor ANOVA was carried out to check for significance of the factors "sour rot" and "time". These analyses were performed using the SPSS software (version 15.0) from SPSS Inc. (Chicago, IL).

3. Results and Discussion

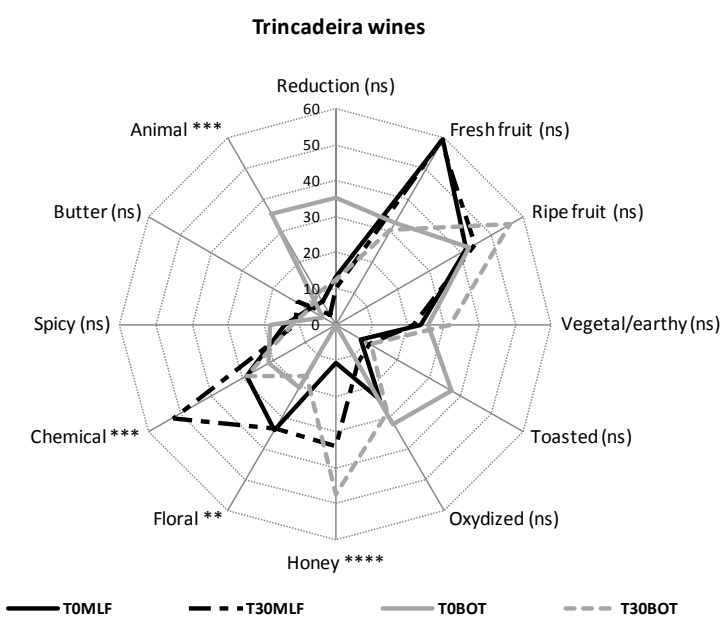
3.1 Enological parameters

Table 1 shows the enological parameters of the different wines analyzed. The parameters most influenced by sour rot infection are alcoholic degree, volatile acidity, and residual sugar content. Wines produced with damaged berries tended to have higher values of alcohol (between 12.8 and 14.6 % v/v) and residual sugars (between 1.9 and 4.1 g L^{-1}), in accordance with previous results (Loinger *et al.*, 1977). This fact is probably a consequence of berry dehydratation in rotten grapes, which leads to an increase of the fermentable sugar concentration. The volatile acidity was also systematically higher in samples from rotten grapes. In samples with 30% of damaged berries this parameter was around 0.33 g L^{-1}, a value 3 times higher than the one observed for healthy samples (around 0.12 g L^{-1}). The increase of volatile acidity can be attributed to the activity of acetic acid bacteria, the principal property of which is to oxidize ethanol into acetic acid. The capacity of the sour rot associated organisms *Acetobacter* spp., *Gluconobacter* spp., and yeast growing on rotten grapes to produce high levels of volatile acidity has been well established on the literature (Guerzoni and Marchetti, 1987; Nelson and Ough, 1966). However, the higher volatile acidity of wines from rot grapes may be ascribed to the higher initial volatile acidity of musts, because the increase during fermentation was similar in all trials (results not shown).

3.2 Sensory descriptive analysis

The aromatic characterization of the 8 wines considered in this study was performed by a sensory panel using the 12 sensory descriptors given in Table 2. For the sake of simplicity, the results, expressed as the modified frequency (MF (%)) value, are shown separately for monovarietals of Trincadeira (Figure 1a) and for blends of Trincadeira/Cabernet Sauvignon (Figure 1b). As shown in Figure 1a, the aroma of monovarietal Trincadeira wines just after the malolactic fermentation (T0 MLF and T30 MLF) were mainly characterized by fresh fruit notes, which decreased significantly to values around 30% MF in the aged samples (T0 BOT and T30 BOT). This sensory pattern was not so clear for blends of Cabernet Sauvignon and Trincadeira (Figure 1b), as for these wines the fresh fruit attribute had a similar value (between 45 and 55% MF) in all the four samples.

(a)



(b)

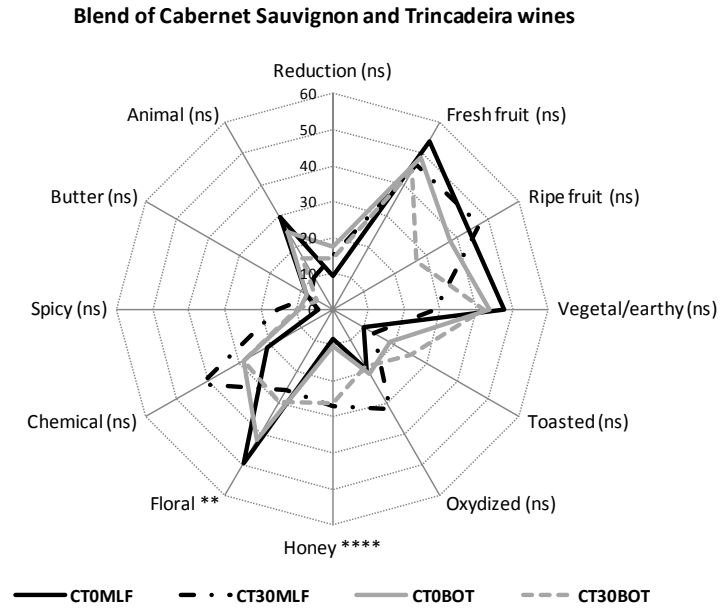


Fig. 1. Graph of the mean sensory ratings MF (%) of the four Trincadeira (a) and Trincadeira/Cabernet Sauvignon (b) wines studied. Notations of *, **, *** and **** indicate significance at $p<0.1$, $p<0.05$, $p<0.01$ and $p<0.001$, respectively; ns indicates no significant difference.

This could be a consequence of the vegetal/earthy character introduced by the Cabernet Sauvignon variety, which represents 70% of these blends. The attribute “chemical”, which was defined as an acetic/alcoholic note, was especially high in the two samples elaborated with sour rot grapes just after the MLF, independently of the variety type (samples T30 MLF and CT30 MLF). This result can be attributed to the

relatively high values of both the alcoholic degree and the volatile acidity observed in these samples (see Table 1). The honey attribute was the single one varying significantly in both sets of samples (Trincadeira and blends of Trincadeira/Cabernet Sauvignon). In both cases, the two samples elaborated from 30% infected berries presented significantly higher values of the honey attribute than blends from entirely healthy grapes. Concerning Trincadeira wines, T30 MLF and T30 BOT reached, respectively, 26 and 47% (MF) values of the honey attribute. With respect to blends of Trincadeira/Cabernet Sauvignon, CT30 MLF and CT30 BOT exhibited values around 30%. In all cases, wine samples from healthy grapes were relatively poor in the honey attribute, with MF (%) around 10. These results suggest that independently of the grape variety blend, the presence of 30% of rotten berries in the must submitted to fermentation is able to induce a honey character to the final wine. The other attribute varying significantly between samples was “floral”, which presented relatively high scores in both samples of Cabernet Sauvignon and healthy Trincadeira (CT0 MLF and CT0 BOT).

3.3 Gas Chromatography-Olfactometry

The major goal of this section is to screen the odor profile of wines elaborated from healthy and from sour rotten grapes to determine the odorants that are more specific to wines elaborated with infected grapes.

The GC-O experiment was carried out on extracts obtained in a dynamic headspace system (San-Juan *et al.*, 2010). For the sake of simplicity, those odorants not reaching a maximum GC-O score (MF) of 25% in any of the eight studied wines were eliminated and considered as noise. After this operation, the number of odorants present in this set of wine extracts was 37. Table 3 summarizes the results of the GC-O study. The identification of three compounds (2-heptanol, dimethyl trisulfide, and ethyl phenylacetate) was achieved by means of heart-cutting GC techniques, which allowed us to compare the retention indices in two columns of different phases (DB-WAX and FactorFour) as well as to contrast aromatic qualities and mass spectra with those of pure standards. On the contrary, three other odorants (ni 1010, ni 1216, and ni 1767) could not be successfully identified even if the same heart-cutting strategy was employed.

Table 3. Odorants found by GC-O in the eight studied wines[a].

LRI[b]	LRI[c]	Odor description	Identity	T0 MLF	T50 MLF	T0 BOT	T50 BOT	CT0 MLF	CT30 MLF	CT0 BOT	CT30 BOT	Average (SD)	max-min
956		butter, cream	2,3-Butanedione (Diacetyl)[d]	91	83	88	83	83	73	78	75	82 (6.2)	18
1010	912	solvent, alcoholic	ni[e] 1010	0	0	10	41	10	31	29	26	18 (15.4)	41
1034		fruity, strawberry	Ethyl butyrate[d]	67	50	53	55	60	65	60	58	59 (5.8)	17
1049		fruity, strawberry	Ethyl 2-methylbutyrate[d]	65	55	75	71	71	63	76	73	69 (7.1)	21
1069		fruity, strawberry	Ethyl 3-methylbutyrate[d]	68	61	73	71	76	59	75	71	69 (6.3)	17
1099		fusel	Isobutanol [d]	19	29	41	20	26	45	41	41	33 (10.5)	26
1125		banana	Isoamyl acetate [d]	54	33	35	55	65	68	43	33	48 (14.2)	35
1196		fruity, strawberry	Ethyl 4-methylpentanoate [d]	29	19	30	0	48	22	29	19	25 (13.6)	48
1204		gas	(E)-2-hexenal [d]	0	0	22	10	0	0	33	7	9 (12.4)	33
1216	914	geranium, green	ni[e] 1216	58	24	27	47	0	0	0	0	20 (23.4)	58
1220		fusel	Isoamyl alcohol [d]	90	85	88	85	90	90	93	87	89 (2.8)	8
1239		fruity, strawberry	Ethyl hexanoate [d]	80	67	73	55	75	69	69	58	68 (8.4)	25
1302		mushroom	1-octen-3-one [d]	0	31	24	22	19	19	30	19	21 (9.6)	31
1334	982	coconut, sweet	2-heptanol [f]	0	0	24	17	26	12	36	0	14 (13.8)	36
1371		green, grass	1-hexanol [d]	0	14	0	30	0	0	0	0	6 (11.0)	30
1401	1010	rotten food, garlic, onion	Dimethyl trisulfide [f]	0	0	0	25	0	0	0	0	3 (8.5)	24
1449		baked potatoes	Methional [d]	33	0	31	22	31	19	12	14	20 (11.4)	33
1452		vinegar	Acetic acid [d]	45	49	50	53	63	49	55	49	52 (5.5)	18
1503		aldehydic, rancidity	(Z)-2-nonenal [d]	0	0	31	12	0	33	12	19	13 (13.5)	33
1530		pepper, earthy	3-isobutyl-2-methoxypyrazine [d]	0	0	0	0	27	0	36	10	9 (14.4)	36
1632		cheese	Butyric acid [d]	19	36	24	29	24	26	17	19	24 (6.2)	19
1675		cheese	Isovaleric acid [d]	63	61	51	47	82	71	85	62	65 (13.5)	38
1718		baked vegetables	Methionol [d]	0	0	0	0	33	29	17	10	11 (13.8)	33
1767	1041	geranium, green	ni[e] 1767	24	43	22	49	61	24	12	19	32 (17.1)	49
1782	1262	honey, sweet	Ethyl phenylacetate [f]	0	0	0	47	0	7	0	10	8 (16.2)	47
1803		coconut, sweet, flowers	β-phenylethyl acetate [f]	43	45	43	49	10	22	12	22	31 (15.9)	39
1813		baked apple	β-damascenone [d]	41	43	29	24	33	31	41	17	32 (9.1)	26
1856		medicinal	2-methoxyphenol (Guaiacol) [d]	29	19	26	24	24	29	12	36	25 (7.2)	24
1880		floral	Ethyl dihydrocinnamate [d]	45	10	12	10	43	33	58	17	29 (18.8)	48
1909		rose	β-phenylethanol [d]	80	51	80	63	65	80	71	80	71 (10.8)	29
2040		burnt, caramel	2,5-dimethyl-4-hydroxy-3(2H)-furanone (Furaneol) [d]	20	26	12	10	17	0	14	19	15 (7.8)	26
2084		animal, stable	p-cresol[d]	59	59	43	55	43	38	46	38	48 (8.8)	21
2128		floral, fruity	Ethyl cinnamate [d]	50	24	26	38	41	31	50	33	37 (10.0)	26
2182		animal	4-ethylphenol [d]	17	30	24	41	19	24	17	19	24 (8.2)	24
2192		toasted, spicy	4.5-dimethyl-3-hydroxy-2-(5H)-furanone (Sotolon) [d]	47	53	31	49	50	38	41	45	44 (7.2)	22
2555		vanilla, spicy	Vanillin [d]	53	38	10	47	65	31	10	26	35 (19.7)	55
2633		sweet, honey	Phenylacetic acid [d]	14	26	12	31	10	33	0	33	20 (12.5)	33

[a] Gas chromatographic retention data, olfactory description, chemical identity, modified frequency percentage (MF(%)), average MF(%) and standard deviation (SD, between parenthesis), Max-Min MF score among wines and significance of the factor "wine". [b] Linear retention index on polar capillary column (DB-WAX). [c] Linear retention index on nonpolar capillary column (FactorFour-VF-5MS). [d] Identification based on the coincidence of gas chromatographic retention data and on the similarity of odor with standars in the laboratory. [e] Not identified. The compound did not produce any clear signal in the mass spectrometer. [f] Identification based on the coincidence of gas chromatographic retention indices in both columns and mass spectrometric data with those of the pure standards available in the laboratory.

Several odorants listed in Table 3 exhibited an average MF (%) value higher than 50: 2,3-butanedione (diacetyl); the ethyl esters of butyric, 2-methylbutyric, 3-methylbutyric and hexanoic acids; isoamyl and β-phenylethyl alcohols; and acetic and isovaleric acids. All of the compounds in this group, for which the standard deviation (SD) of the olfactometric signal was below 10, are byproducts of alcoholic fermentation and constitute the base of wine aroma (Rapp and Mandery, 1986). The rest of compounds present in the table are well-known wine aroma components and have been detected in previous GC-O studies, although there are some remarkable differences between samples that are worth commenting on. For example, 3-isobutyl-2-methoxypyrazine, a well-known marker of Cabernet Sauvignon varietals (Ferreira *et al.*, 2000), and methionol were only detected by the panel in blends of Cabernet Sauvignon and Trincadeira. On the contrary, the unknown compound n.i. 1216 with a characteristic geranium-green odor was only perceived in the extracts of monovarietals of Trincadeira, reaching a MF score near 60% in sample T0 MLF. This odorant could be successfully transferred to the second column in the GC-GC system (LRI=914) and clearly detected by all the panelists. Despite this, the identification could not be achieved as in this column the odorant coeluted with a major compound in wine, isoamyl alcohol, the presence of which did not allow a MS spectra of the target compound to be obtained. This compound could be a varietal marker of Trincadeira, although it could not be detected in its blends with Cabernet Sauvignon.

To assess the effects caused by sour rot on the aromatic profile, a paired *t* test was carried out between samples infected and their corresponding healthy controls. The results of this test are given in Table 4. As can be seen, sour rot seems to exert a significant effect on at least eight odorants (in two cases confidence level was <0.1). In the cases of ethyl hexanoate, diacetyl, ethyl 2-methylbutyrate, 4-methylpentanoate and isoamyl alcohol, the presence of the rot in the grape brings about a decrease on the odorant. This result suggests a strong effect of the rot on the must amino acid profile, because, leaving aside ethyl hexanoate, the other compounds are known to be influenced by such factor (Hernandez-Orte *et al.*, 2002, 2005). The likely most relevant changes from the sensory point of view are, however, the significant increments caused by rot on the GC-O levels of phenylacetic acid, β-phenylethyl acetate, and 4-ethylphenol. The increment of phenylacetic acid is particularly noteworthy. In relation to this, it is worth commenting that the ethyl ester of this acid reaches a high GC-O score on the T50 BOT sample.

Table 4. Effects of the presence of sour rot in grapes on the GC-O aroma profile of the corresponding wines[a].

Compound	Av. Diff.[b]	*t*	*p(t)*
Phenylacetic acid	**21.75**	**4.96**	**0.016**
Ethyl hexanoate	-12	-4.83	0.017
2,3-Butanedione (Diacetyl)	-6.5	-4.18	0.025
Ethyl 2-methylbutyrate	-6.25	-3.78	0.032
Ethyl 4-methylpentanoate	-19	-3.61	0.036
β-phenylethyl acetate	**7.5**	**3.38**	**0.043**
4-ethylphenol	9.25	2.66	0.076
Isoamyl alcohol	-3.5	-2.65	0.077

[a] Results of a paired *t* test between wines from infected samples and their corresponding controls made with healthy grapes. A two-tailed *t* distribution with 3 degrees of freedom was used as reference distribution.
[b] Average difference between the GC-O olfactometric score obtained for wines made with infected grapes and that obtained in the corresponding control.

3.4 Chemical quantitative analysis

A total of 78 major and minor volatile compounds were quantified. Results are presented in Table 5, where compounds have been arranged into 11 chemical families. Results in the table show that these wines are rather poor in varietal compounds (terpenes, norisoprenoids, volatile phenols, and vanillin and benzene derivatives), but that the wines contain surprisingly high levels of γ-decalactone. This odorant is present at levels as high as 185-405 μg L^{-1} in all of the tested wines, and its level significantly increase (p<0.05) during bottle aging, which suggests that the recently fermented wines should contain large amounts of γ-hydroxydecanoic acid from which the lactone would be slowly formed by internal esterification. To the best of our knowledge, these levels are the highest ever reported in wine, much higher than those reported in young (Ferreira *et al.*, 2000) or aged (Jarauta *et al.*, 2006) Spanish reds, Australian reds (Cooke *et al.*, 2009) or even Uruguaian tannats (Escudero *et al.*, 2007) for which maxima levels of 75 μg L^{-1} were found. For this compound thresholds of 88 μg L^{-1} (Etiévant, 1991) and 790 μg L^{-1} (Jarauta *et al.*, 2006) have been reported, and a synergic action with the other γ-lactones has also been found (Jarauta *et al.*, 2006), which would indicate that γ-decalactone can be an important aroma compound in Trincadeira wines.

Table 5. Volatile compounds composition of the eight studied wines, and significance (*p* value) of factors "sour rot (SR)" and "Bottle aging (BA)" measured by ANOVA[a].

COMPOUND	T0 MLF	T0 BOT	T50 MLF	T50 BOT	CT0 MLF	CT0 BOT	CT30 MLF	CT30 BOT	SR effect	BA effect
CARBONYL COMPOUNDS										
Acetoin	5254	6625	9434	9315	6958	8120	8021	8411	0.043	ns[b]
Diacetyl (2,3-butanedione)	2436	2867	2563	2756	3074	3315	4025	3142	ns	ns
Phenylacetaldehyde	3.12	1.57	3.06	2.56	1.79	5.78	5.75	1.80	ns	ns
ESTERS										
Ethyl butyrate	54	55	44	46	76	57	47	46	0.057	ns
Ethyl hexanoate	202	183	94	78	453	154	135	122	0.096	ns
Ethyl lactate	18405	65661	16442	86077	22496	83775	21916	83775	ns	0.000
Ethyl octanoate	180	92	21	36	339	97	69	47	0.033	ns
Ethyl decanoate	34	30	7.6	17	35	29	42	20	ns	ns
Diethyl succinate	5830	12325	962	2918	20231	16438	1114	3178	0.036	ns
ACETATES										
Isoamyl acetate	234	129	150	112	427	156	254	168	ns	ns
2-Phenylethyl acetate	31	25	30	32	43	35	59	46	ns	ns
Ethyl phenylacetate	nd[c]	nd	130	304	8.6	18	60	138	0.029	ns
ACIDS										
Acetic acid	167359	174938	449830	447897	134050	172290	243417	272953	0.048	ns
2-methylbutyric acid	76	42	66	31	54	64	64	68	ns	ns
3-methylbutyric acid	114	62	92	43	75	88	87	94	ns	ns
2-ethyl-hexanoic acid	nd	nd	nd	3.5	1.3	nd	nd	nd	ns	ns
Benzoic acid	57	59	61	209	43	48	48	98	ns	ns
Isobutyric acid	2252	2531	2184	2309	2210	2720	1967	2291	0.030	0.013
Butyric acid	504	460	344	334	485	519	384	395	0.006	ns
Isovaleric acid	2542	2510	1611	1223	2292	2862	2612	2684	ns	ns
Hexanoic acid	1672	1775	766	701	1308	1852	1067	1160	0.012	ns
Octanoic acid	796	1013	346	286	532	982	369	582	0.012	ns
Decanoic acid	102	117	98	92	64	92	72	221	ns	ns
Phenylacetic acid	102	107	1668	1557	103	85	638	645	0.041	ns
ALCOHOLS										
Isobutanol	63978	64842	61147	61645	59794	59332	57702	57792	ns	ns
1-butanol	491	496	663	675	570	565	743	736	0.009	ns
Isoamyl alcohol	350705	359139	202823	215213	409590	397690	341419	315347	0.075	ns
1-Hexanol	507	536	441	414	847	850	748	732	ns	ns
(Z)-3-Hexen-1-ol	2.9	2.4	3.7	2.9	7.5	8.9	10.9	9.7	ns	ns
(E)-2-Hexen-1-ol	4.8	6.2	4.2	3.2	3.4	7.1	nd	2.6	ns	ns
Benzyl alcohol	626	894	3664	3341	284	518	364	1997	ns	ns
β-phenylethanol	123291	141723	87450	62564	98309	156535	150184	134014	ns	ns
Methionol	6806	8651	4441	3321	4062	7506	4586	5223	0.055	ns
TERPENES										
(Z)-linalol oxide	nd	nd	nd	nd	nd	2.0	nd	1.9	ns	ns
(E)-linalol oxide	1.2	1.8	1.3	2.4	0.5	2.0	1.5	1.8	ns	0.025
Linalool	nd	nd	nd	nd	nd	4.5	4.9	nd	ns	ns
α-terpineol	1.1	4.5	2.2	12	1.3	10	1.7	14.1	ns	0.005
β-citronellol	6.4	3.5	8.0	5.1	4.2	2.8	6.1	4.257	ns	0.046
Geraniol	nd	nd	nd	nd	nd	nd	4.0	0.870	ns	ns
Farnesol	nd	nd	nd	nd	nd	12	nd	nd	ns	ns
Terpinen-4-ol	0.15	0.13	0.42	0.63	0.074	0.56	0.24	0.45	ns	ns
2,6-dimethyl-1,7-Octadien-3,6-diol	nd	nd	nd	nd	nd	nd	nd	0.21	ns	ns
Neric acid	6.8	6.2	6.4	6.6	3.2	3.1	3.4	3.23	ns	ns
NORISOPRENOIDS										
β-damascenone	nd	nd	nd	nd	1.23	1.06	0.54	nd	ns	ns
β-ionone	0.27	0.24	0.33	0.24	0.26	nd	0.31	nd	ns	ns
Vitispirane A	0.44	1.8	0.613	2.97	0.49	2.8	0.39	2.6	ns	0.002
Vitispirane B	0.71	1.3	1.09	2.48	0.67	2.6	0.82	2.8	ns	0.012
Riesling acetal	nd	nd	nd	nd	nd	2.9	nd	2.2	ns	ns
TDN	0.15	0.78	0.23	1.20	nd	0.12	nd	nd	ns	ns
TPB	nd	nd	nd	nd	nd	0.77	nd	nd	ns	ns
3-Oxo-β-ionone	nd	2.8	nd	4.2	nd	7.4	nd	7.9	ns	0.019
Actinidols	1.1	4.2	1.7	5.9	2.1	11	12	13	ns	ns
3-oxo-α-ionol	21	23	16	17	19	24	16	19	0.002	0.013

[a] Compounds showing significance ($p<0.1$) for any of the factors are highlighted in bold. Quantitative data expressed as μg L^{-1}. [b] ns, not significant; [c] nd, not detected.

Table 5. (continued).

COMPOUND	T0 MLF	T0 BOT	T50 MLF	T50 BOT	CT0 MLF	CT0 BOT	CT30 MLF	CT30 BOT	SR effect	BA effect
VOLATILE PHENOLS										
Guaiacol	1.3	2.0	1.9	2.5	0.65	1.24	1.15	1.5	ns	ns
o-cresol	1.2	1.1	1.1	1.3	0.99	1.08	1.09	1.0	ns	ns
4-ethylguaiacol	nd	nd	0.35	0.52	1.78	1.95	2.38	0.31	ns	ns
Eugenol	2.41	2.53	2.94	3.59	0.81	1.17	1.3	1.75	ns	ns
4-ethylphenol	0.46	0.45	0.67	0.74	0.53	0.56	0.45	0.47	ns	ns
4-vinylguaiacol	3.9	6.2	3.9	6.6	3.4	3.6	4.5	4.6	ns	ns
2,6-dimethoxyphenol	8.9	20	7.9	15	4.9	14	4.9	11	ns	0.016
(E)-isoeugenol	2.3	1.8	4.0	3.1	1.5	0.82	2.9	1.9	ns	ns
4-vinylphenol	40	143	13	33	13	39	7.6	18	ns	ns
VANILLIN DERIVATES										
Vanillin	5.6	13	5.3	12	3.6	12	2.8	9.2	ns	0.003
Methyl vanillate	17	18	18	21	17	17	18	14	ns	ns
Ethyl vanillate	167	354	256	596	95	162	109	197	ns	ns
Acetovanillone	79	81	76	78	32	34	29	32	ns	ns
Zingerone	26	28	19	14	nd	7.4	6.9	6.8	ns	ns
Ácido homovanillínico	32	nd	nd	nd	nd	52	34	8.7	ns	ns
Homovanillil alcohol	3.5	nd	nd	nd	nd	nd	nd	nd	ns	ns
BENZENE DERIVATES										
Benzaldehyde	2.3	4.1	29	25	2.5	3.5	8.1	7.8	ns	ns
2-phenoxyethanol	0.99	0.78	1.47	1.12	1.10	1.38	0.91	nd	ns	ns
Dihydromethyl-eugenol	14	15	21	22	10	11	13	14	ns	ns
Ethyl dihydrocinnamate	nd	nd	nd	nd	nd	0.085	0.19	0.21	ns	ns
Ethyl cinnamate	0.81	0.82	0.83	1.3	0.65	0.68	0.50	0.64	ns	ns
LACTONES										
δ-octalactone	4.04	3.2	8.8	8.9	2.5	2.9	4.3	4.9	ns	ns
γ-nonalactone	10	9.7	37	37	3.2	4.0	16	19	0.049	ns
γ-decalactone	203	302	280	405	202	297	185	319	ns	0.025
δ-decalactone	5.0	8.1	4.7	8.8	3.7	5.7	3.7	4.5	ns	ns
γ -butyrolactone	5802	12232	12223	14932	5040	8947	7475	10701	ns	ns

[a] Compounds showing significance (p<0.1) for any of the factors are highlighted in bold. Quantitative data expressed as μg L^{-1}. [b] ns, not significant; [c] nd, not detected.

Results of the ANOVA tests carried out on those data to assess the effects of sour rot are summarized in Table 6, which mostly confirm GC-O observations. As shown in the table, the presence of sour rotten grapes exerts a deep effect on the levels of fatty acids and their ethyl esters formed by yeast, which decreased nearly by a 2 factor on average. The levels of some volatiles related to the synthesis and catabolism of amino acids, such as isobutyric acid, methionol, and isoamyl alcohol, were also significantly reduced. These changes suggest that the presence of sour rot exerts a deep impact on the secondary metabolism of yeast with a likely relevant impact on the wine sensory properties. It is remarkable, that only one purely varietal compound, the norisoprenoid 3-oxo-α-ionol, was significantly affected by sour rot.

On the other hand, six compounds (1-butanol, ethyl phenylacetate (EPhA), phenylacetic acid (PAA), acetoin, acetic acid and γ-nonalactone) present significantly higher concentrations (p<0.05) in wines made from sour rotten grapes. As seen in the

table, increases in the cases of 1-butanol and acetoin are not very large, quantitatively, and most likely will not cause any relevant sensory impact.

Table 6. Effects of the presence of sour rot in grapes on the levels of volatile compounds of the corresponding wines[a].

Compound	Av. Diff.[b]	inc(%)[c]	*p*(SR)
3-oxo-α-ionol	-4.75	-21.8	0.002
Butyric acid	-128	-26.0	0.006
1-butanol	**174**	**32.8**	**0.009**
Hexanoic acid	-728	-44.1	0.012
Octanoic acid	-435	-52.4	0.012
Ethyl phenylacetate	**149**	**1627**	**0.029**
Isobutyric acid	-240	-9.9	0.030
Ethyl octanoate	-134	-75.6	0.033
Diethyl succinate	-11663	-85.1	0.036
Phenylacetic acid	**1028**	**1035**	**0.041**
Acetoin	**2056**	**30.5**	**0.043**
Acetic acid	**191365**	**118**	**0.048**
γ-nonalactone	**20.5**	**305**	**0.049**
Methionol	-2363	-35.0	0.055
Ethyl butyrate	-14.7	-24.4	0.057
Isoamyl alcohol	-110580	-29.2	0.075
Ethyl hexanoate	-141	-56.8	0.096

[a] Results of ANOVA test between wines from infected samples and their corresponding controls made with healthy grapes; [b] Average difference between the levels (in μg L^{-1}) obtained in wines made with infected grapes and those obtained in the corresponding controls; [c] Increase (%) with respect to the average content of the compound in the control wines.

In the case of acetic acid, the increment is relatively important in quantitative terms (118% in average), although the final levels on the wines still can be considered normal. In the case of γ-nonalactone, the increments are really notable, and the levels finally produced in the T50 samples are above the 30 μg L^{-1} threshold for this compound (Nakamura *et al.*, 1988). However, the highest effects are noted on the levels of EPhA and PAA, which increase by factors in average above 1 magnitude of order as a consequence of the presence of sour rot. In the case of monovarietals of Trincadeira (50% of sour rotten grapes), the concentration of EPhA in sour rotten samples reached 130 and 304 μgL^{-1} in wines after malolactic fermentation and bottle aging, respectively. Blends of Trincadeira/Cabernet Sauvignon exhibited slightly

smaller concentration levels (60 and 138 μgL^{-1}), as only 30% of sour rotten grapes were employed. The same pattern was observed for PAA (p<0.05), the direct precursor from which the acetate is formed by esterification.

In both set of wines, the concentration of both EPhA and PAA in sour rotten wines were above their corresponding odor threshold: 73 $\mu g\ L^{-1}$ for the ester (Tat *et al.*, 2007) and 1000 $\mu g\ L^{-1}$ for the acid (Maga and Lorenz, 1973). Considering that these two compounds are associated to a clear honey-like and sweet aroma, EPhA and PAA could be considered as responsible of the honey-sweet aroma associated to wines elaborated from sour rot infected grapes.

PAA has already been identified as the main odorant in sweet Fiano wines (Moio, 2002), which are produced with grapes in an advanced state of maturation, and dried on racks for sugar concentration. Genovese *et al.* (2007) found 181 $\mu g\ L^{-1}$ of EPhA in those Fiano wines. More recently, Tat *et al.* (2007) attributed the untypical "sweet-like" off-odor developed in some samples (empirically connected with some cryptogamic diseases) of Italian Aglianico del Vulture wine, also produced with a late-ripening variety, to this compound. It should be noted that the maximum levels of EPhA in those defective samples were 150 $\mu g\ L^{-1}$, a figure well below the 304 $\mu g\ L^{-1}$ found in our T50BOT sample.

The biosynthetic pathways that lead to the formation of EPhA in wines are not clearly understood, although this compound could be produced by esterification of PAA during alcoholic fermentation (Tat *et al.*, 2007). PAA is known for its weak auxin activity in a range of crop plants (Wightman and Lighty, 1982), working as a plant growth regulator, and may be involved in plant defence mechanisms (Somers *et al.*, 2005). Moreover, Ziauddin *et al* (1992) showed a striking response in plant regeneration from wheat anther and on barley anther/microspore culture using PAA in the induction medium. In light of these studies, our results, and the empirical observation that the honey-like smell is not present in Aglianico wines produced without skin contact (Tat *et al.*, 2007), our hypothesis is that an alteration of berry surface could activate a plant response mechanism that lead to the production of high concentrations of PAA. This compound could be extracted to the must if a maceration process occurs, and be further converted to the ester (EPhA) during fermentation or later during bottle aging.

In summary, this study shows that sour rot infection clearly affects both the chemical and the sensory profile of wines elaborated with blends containing between 30 and 50% of damaged grapes. Our research strongly indicates that EPhA and PAA may be

taken as chemical markers of sour rot infection in grapes and that both compounds are most likely responsible for the characteristic honey note evoked by these products. Sour rot also increases the levels of γ-nonalactone in wine. Results also suggest that sour rot exerts a great effect on the secondary metabolism of yeast, decreasing the levels of volatiles related to fatty acids and amino acids synthesis. Finally, the high levels of γ-decalactone found in all of the samples, suggest that this compound may be a relevant odorant of Trincadeira wines.

More research is needed to understand the biochemical pathways that lead to the synthesis of PAA and EPhA in damaged berries.

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CHAPTER 6

The role of the insect *Drosophila* sp. and of the grape berry microbiota in the development of sour rot disease

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The role of the insect *Drosophila* spp. and of the grape berry microbiota in the development of sour rot disease

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Abstract

The sour rot disease affecting grape berries results from a complex mechanism involving microorganisms (yeasts and bacteria), insects (*Drosophila* spp.) and a vine plant response to injury. Without berry damage, sour rot does not occur because microorganisms do not have access to the sugar rich pulp tissues. Berry damage provokes a clear increase in total counts and a shift in yeast populations from basidiomycetes to ascomycetous fermentative species. The latter are mostly *Pichia terricola*, *Hanseniaspora uvarum*, *Candida zemplinina* and *Zygoascus hellenicus*. However, these species do not produce the metabolites characteristic of sour rot (gluconic and acetic acids) in significant amounts. These acids result from the activity of acetic acid bacteria (*Gluconobacter* spp., *Gluconoacetobacter* and *Acetobacter* spp.). This microbial consortium is transported by drosophilid insects which disseminate sour rot among damaged berries. In the absence of these insects, plant defense mechanisms, probably mediated by the production of phenylacetic acid, are effective and lead to skin healing, preventing disease spread.

Keywords: *Drosophila* spp., damaged grapes, sour rot, yeasts, acetic acid bacteria

1. Introduction

Insects are probably the most significant source of yeasts colonizing grape berries (Mortimer and Polsinelli, 1999). There is a wide diversity of insects including honey bees, syrphid flies, wasps, moths, mites, mealybugs, weevils, cicadas, locusts, spiders, earwigs and grasshoppers that visit, infect and attack grape vines at various stages of their annual development (Buchanan and Amos, 1992). It is most likely that these "visitors" contribute to the yeast flora of grapes. In particular, drosopholid fruit flies, mainly *Drosophila melanogaster* (Diptera: Drosophilidae) commonly visit grapes to feed on the yeasts associated with grape berries and identical species can be recovered from both the fruit and the crop of the insect (Phaff and Starmer, 1987).

The mutualistic association between *Drosophila* flies and yeasts occurs mainly on rotting fruits. Yeasts are the major nutritional source, providing proteins, vitamins and other nutrients for *Drosophila* adults and their larvae while the flies disperse the yeasts among the different microhabitats (Gilbert, 1980; Phaff and Starmer, 1987; Starmer and Fogleman, 1986). The role of bacteria in *Drosophila* ecology has received less attention than that of yeasts. Recent research in microbe-insect symbiosis has shown that acetic acid bacteria (AAB) establish symbiotic relationships with several insects of the orders Diptera, Hymenoptera, Hemiptera, and Homoptera, all relying on sugar-based diets, such as nectars, fruit sugars, or phloem sap (Crotti *et al.*, 2010). The fruit flies *Drosophila melanogaster* hosts a rich AAB microbiome and have been found to be associated with the bacterial genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, and the novel genus *Commensalibacter* (Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Ren *et al.*, 2007; Roh *et al.*, 2008; Ryu *et al.*, 2008). AAB establish symbiotic associations with the insect midgut, a niche characterized by the availability of diet-derived carbohydrates and oxygen and by an acidic pH, selective factors that support their growth. AAB have been shown to actively colonize different insect tissues and organs, such as the epithelia of male and female reproductive organs and the salivary glands (Crotti *et al.*, 2010).

Fruit flies (*Drosophila* spp.) are constantly present around grape bunches infected by sour rot, in every state of development (eggs, larvae, pupae and adults) and are empirically regarded as the most important vectors of sour rot agents (Bisiach, 1986; Cantoni, 1984). This emergent grape disease affects late ripening cultivars with tightly-packed, thin-skinned and dense bunches close to harvesting, causing heavy crop losses and is detrimental to juice and wine quality (Barata *et al.*, 2011a, 2011b; Wolf *et al.*, 1990; Zoecklein *et al.*, 1992). Sour rot is mainly induced by yeasts (Barata *et al.*,

2008a, 2008b) associated with acetic acid bacteria (AAB) (*Acetobacter* spp. and *Gluconobacter* spp.) (Bisiach, 1986; Blancard *et al.*, 2000; Gravot *et al.*, 2001). Fruit flies captured in vineyards affected by sour rot are carriers of yeasts (*Kloeckera apiculata*, *Candida stellata*, *Pichia membranifiaciens*, *Metschnikowia pulcherrima*, *C. krusei*) and AAB (*Gluconobacter* spp. and *Acetobacter pasteurianus*), which are supposed to be responsible for the syndrome (Fermaud *et al.*, 2002).

Despite this knowledge, the role of yeasts and AAB on the development of sour rot and the contribution of *Drosophila* flies on the grape decay process has not been properly investigated. Therefore, the purpose of this work was to find the main microbial agent of sour rot and the importance of the insects in the development of the disease.

2. Material and Methods

2.1 Vineyard experimental trials

During the 2008 vintage, the parcels of *Vitis vinifera* L. cv. Trincadeira red grape variety from the experimental vineyard of Instituto Superior de Agronomia, located in Tapada da Ajuda, Lisbon, Portugal (latitude 38º 42´31.57´´ N and longitude 9º 11´14.01´´ W), were visually inspected just after *veráison* (beginning of ripening), in order to detect bunches with initial symptoms of sour rot. At this phase, two sets of three different vines (duplicates) containing bunches with the first disease symptoms were selected and marked. The same number of vines containing only healthy bunches were used as control vine sets. The microbial yeast population present on both sound and with initial sour rot symptoms bunches was studied at this grape ripening phase (19 August 2008).

In order to study the effect of *Drosophila* flies on disease development, two types of bunches were studied: i) free bunches without impediments for the action of any vector and ii) bunches protected inside a handmade plastic structure covered by a transparent nylon cloth with a pore size sufficiently small to prevent the entrance of *Drosophila* flies and other insects. The plastic structure was adjusted to the size of each selected bunch and the nylon cloth assured air circulation and sunlight entry. A total of six different experimental modalities were analyzed: i) free sound bunches (S_F); ii) protected sound bunches (S_P); iii) free sound bunches with induced wounds (Sw_F) using a sterile scalpel blade; iv) protected sound bunch with induced wounds (Sw_P); v)

free bunches with sour rot symptoms (SR_F) and vi) protected bunches with sour rot symptoms (SR_P) (see Figure S1 in supplementary material). The selected bunches were left in the vineyard during the ripening period and subsequently collected at the time of harvest (11 September 2008) and used for the analyses of the microbial yeast flora and chemical composition of their respective grape musts.

2.2 Microbial populations of yeasts from grape samples

Just after the end of *veráison*, a total of three bunches (one per vine) of both sound and affected grapes were collected from each vine set. The same grape sampling was used for the before mentioned six trial modalities, analyzed at the harvest time. Duplicates were performed for all grape samples. Bunches were aseptically collected from vines and transported to the laboratory in sterile bags.

Representative grape berries were carefully removed from each set of three bunches and combined to give a total of 300 g sample. Berries were then placed in a sterile stomacher bag and crushed in a stomacher blender (IUL, Barcelona, Spain) during 5 minutes, and the obtained grape juices were subsequently decimally diluted (10^{-1} to 10^{-6}) in peptone water (Merck, Darmstadt, Germany).

The yeast flora recovered from each grape musts sample was analyzed by surface plating (0.1 mL) of the adequate dilutions onto plates (duplicates) of several culture media: i) general purpose medium GYP agar (Barata *et al.*, 2011b) and ii) selective/differential media DBDM (Rodrigues *et al.*, 2001) and ZDM (Schuller *et al.*, 2000) for the isolation of *Dekkera/Brettanomyces* and *Zygosaccharomyces* species, respectively, or other non-*Saccharomyces* species as previously reported (Barata *et al.*, 2008a; 2008b). Plates were incubated at 25 ºC up to 5 days in case of GYP plates and 12 days for DBDM and ZDM media. Total yeast counts were obtained from the GYP plates and reported as log CFU g^{-1}.

For each analyzed berry sample, plates with countable number of yeast colonies (1 to 300) were examined and the different colony morphological types found were registered and differentially counted. Representative isolates of each colony type were isolated and selected for identification. All isolates were streaked and purified on GYP plates. For long term storage, purified isolates were maintained frozen at -80 ºC in vials containing the GYP broth with 15% (v/v) glycerol (Merck), until their later analysis.

2.3 Capture of wild *Drosophila* flies in the vineyard and yeast flora analysis

One week before harvest, fruit-fly traps were placed in the vineyard surrounding the sour rotten bunches present in the selected infected vines. Sour rotten berries were used as bait and placed in the bottom of small uncapped plastic bottles. The lower and higher bottles parts were separated by a fine nylon net layer, used as a physical barrier in order to avoid the contact between flies and the rotten grapes. Once inside the traps, flies remained captured after capping the higher part of bottles. Traps were transported to the laboratory and flies were killed by freezing.

In order to analyze the yeasts carried predominantly on the external surface of flies, sets of 10 intact *Drosophila* bodies were directly transferred to flasks containing 50 mL of GYP and DBDM enrichment broth (duplicate). Additionally, other sets of 10 flies were surface sterilized by immersion in ethanol 70% for 1 min and then transferred for a sterile Petri dishes lid containing 2 mL of sterile peptone water. Flies were dissected with the aid of a sterile scalpel blade, to analyze and isolate the yeast species from their digestive tract. The obtained crop mixtures were transferred for flasks with 50 mL GYP and DBDM broths. Flasks were incubated at 25 ºC in an orbital shaker (Agitorb 200IC, Aralab, Lisbon, Portugal) at 120 rpm up to 5 and 12 days in case of GYP and DBDM broths, respectively. After enrichment, yeast isolates were obtained by streaking an aliquot (10 µL) of all media with visible growth in GYP agar plates incubated at 25 ºC up to 5 days. Representative isolates of each colony type found were purified by streaking on GYP plates and stored at -80 ºC until identification.

2.4 Chemical analysis of grape musts at harvest time

Grape must samples of the six studied modalities were analyzed by Fourier Transform Infrared (FTIR) Spectroscopy in a WineScan FT120 spectrometer (FOSS, Hilleroed, Denmark, http://www.foss.dk) using the FOSS-supplied GrapeScan Calibration (FOSS, 2002) as described in Barata *et al.* (2011b). The following oenological and sanitary parameters were determined: glucose-fructose content, total acidity, pH, tartaric acid, malic acid, volatile acidity and grey rot, sour rot, yeast activity and lactic bacteria indexes. The results of the sanitary indexes were based on the following ranges: grey rot index (0-60; 0=excellent grapes; 10=good grapes; 20=slight attack; 30=critical attack; 40=serious attack; 50=severe attack; 60=total rotten grapes), sour rot index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe attack), yeast activity index (0-30; 0=good grapes; 10=slight attack; 20=serious attack; 30=severe

attack) and lactic bacteria activity index (0-2; 0=good grapes, 1=medium attack, 2=severe attack).

2.5 *In-vitro* sour rot development tests

A laboratory method was developed in order to study the role of the fruit fly *Drosophila* spp. on development of sour rot grape disease. Commercial white table grapes (Italia grape variety) and laboratory-reared *Drosophila* spp. were used on the *in vitro* sour rotting assays. Healthy grape berries (with pedicel) were removed from the bunches and inoculated separately with yeast strains belonging to ten different species, one acetic acid bacteria (AAB) species and one lactic acid bacteria (LAB) species, after a previous phase of rinsing, surface disinfection, and washing. The detailed procedure is shown in Figure S2 (see supplementary material). With the exception for *Saccharomyces cerevisiae* ISA 1000 strain (Malfeito-Ferreira *et al.*, 1997), used as control yeast, the remaining selected strains belong to the typical species consortium isolated from the surface of sour rotten grapes or rotten grape musts which have been isolated in our laboratory in previous works (Barata *et al.*, 2008a, 2008b, 2011b). The inoculated strains and their origins are listed in Table 1.

Table 1. Origin of yeasts, acetic acid bacteria (AAB), and lactic acid bacteria (LAB) strains used in the *in-vitro* sour rot development survey.

Species	Strain[a]	Source	Reference
Yeasts			
Candia zemplinina	ISA 2339	Sour rot grapes	Barata *et al.* (2008b)
Hanseniaspora uvarum	ISA 2294	Grape must with 10% sour rot	Barata *et al.* (2008b)
Issatchenkia occidentalis	ISA 2384	Sour rotten grapes	Barata *et al.* (2008b)
Issatchenkia orientalis	ISA 2381	Sour rotten grapes	Barata *et al.* (2008b)
Issatchenkia terricola	ISA 2322	Sour rotten grapes	Barata *et al.* (2008b)
Lachancea thermotolerans	ISA 2380	Grape must with 30% sour rot	Barata *et al.* (2008b)
Saccharomyces cerevisiae	ISA 1000	Commercial starter (Fermivin®)[b]	Malfeito-Ferreira *et al.* (1997)
Zygoascus hellenicus	ISA 2324	Sour rotten grapes	Barata *et al.* (2008b)
Zygosaccharomyces bailii	ISA 2295	Grape must with 10% sour rot	Barata *et al.* (2008b)
Zygosaccharomyces bisporus	ISA 2336	Sour rotten grapes	Barata *et al.* (2008b)
Acetic acid bacteria			
Gluconacetobacter saccharivorans	ISA 4244	Sour rotten grapes	Barata *et al.* (2011) [c]
Lactic acid bacteria			
Enterococcus durans	ISA 4288	Sour rotten grapes	Barata *et al.* (2011) [c]

[a] ISA, Instituto Superior de Agronomia, Lisbon, Portugal; [b] Fermivin® (DSM, Delft, Netherlands);
[c] Unpublished data.

After surface sterilization and washing steps, each berry set was immersed in a yeast or bacterial cellular suspension (10^7 cells mL^{-1}) prepared in 300 mL of sterile peptone

water. For that, an inoculum of each tested strain was prepared from a loopful of fresh culture (24-48 h) inoculated in 50 mL GYP culture broth and incubated at 25 ºC with orbital shaking (120 rpm). Growth was followed by measurement of the absorbance at 640 nm and when OD was about 1.0 unit, the appropriate inoculum volume was centrifuged (10000 rpm, 5 min), cells were washed twice with peptone water and finally resuspended in 300 mL of peptone water.

For each inoculated strain, four types of grape samples were analyzed: i) sound berries (control set) (C); ii) sound berries in the presence of *Drosophila* spp. flies (D); iii) wounded berries (W) and iv) wounded berries in the presence of *Drosophila* spp. flies (WD) (see Figure S3 in supplementary material). Wounds were performed prior to inoculation using a sterile scalpel blade. After inoculation, grape berries sets were placed inside a sterile 1L glass cup and in case of sets D and WD, 10 reared adult *Drosophila* flies (5 males and 5 females) previously anesthetized were placed inside the cups. Finally, cups were sealed with nylon cloth. Evaluation of *Drosophila* role was based on sour rot symptoms development (berries browning, dehydration and disaggregation of the internal tissues and release of a pungent odor of acetic acid) after 12 days of incubation at 25 ºC in a climate-controlled room.

2.5.1 Laboratory-reared Drosophila spp. *fly stocks and growth conditions*

The *Drosophila* flies used in the *in vitro* sour rot developing assays were reared from initial wild *Drosophila* spp. adult flies captured during the summer of 2009. Several fly-traps similar to those used on the previously described 2008 experimental trial performed on the vineyard were placed in an airy room of our laboratory. Inside the traps, rotten grapes with a strong vinegar odor were used as bait to attract the flies. Once inside the traps, flies were anesthetized with ether and transferred to 23×75 mm sterile disposable polystyrene vials (Sarstedt, Nümbrecht, Germany) with cotton closure, containing nutrient *Drosophila* medium (NDM) composed by 20 g L^{-1} yeast extract (Oxoid, Hampshire, UK), 70 g L^{-1} commercial corn flour, 75 g L^{-1} sucrose (JTBaker, Deventer, Holland), 45 g L^{-1} malt extract (Oxoid) and 10 g L^{-1} agar, supplemented with 25 mL L^{-1} of the antiseptic Nipagin-M (José M. Vaz Pereira, S.A., Benavente, Portugal) 10% in ethanol 96% solution (methyl 4-hydroxybenzoate). Stock flies were reared and maintained for several generations on NDM vials (10 pairs/vial) incubated at room temperature (20-25 °C) in the laboratory.

2.5.2 Enumeration and isolation of yeasts and bacteria during in vitro assays

The microbial populations of yeasts and bacteria present at grape surfaces were determined initially (just after inoculation of each tested strain) and after 12 days of berries incubation, whenever sour rot development was observed. Berries were crushed in a sterile stomacher bag and grape musts were analyzed by surface plating (0.1 mL) of the adequate dilutions onto GYP agar plates supplemented with 100 mg L^{-1} chloramphenicol (Sigma, Steinheim, Germany) and TSA plates (Oxoid) supplemented with 200 mg L^{-1} Delvocid® (DSM, Delft, Netherlands) (100 mg L^{-1} natamycin), for the enumeration of total yeasts and bacteria, respectively (see Figure S2). Plates were incubated up to 5 days at 25 and 28 ºC for GYP and TSA plates, respectively.

For the grape sets inoculated with *Enterococcus durans* strain ISA 4315, initial and final bacterial enumeration were carried out using MRS plates [52 g L^{-1} MRS (Oxoid), 200 mg L^{-1} Delvocid®, 0.5 g L^{-1} L–cysteine (Sigma), 20 g L^{-1} agar].

The countable plates used in the final microbial analysis were visually analyzed. Colonies showing different morphological characteristics than those of the inoculated strain were registered and representative isolates of those colonies were isolated, purified by streaking on GYP and TSA agar plates, in case of yeasts or bacteria, respectively, and stored at -80 ºC until identification.

2.5.3 Yeasts and AAB flora of Drosophila spp. *reared flies*

A total of 50 reared *Drosophila* spp. stock flies (25 males and 25 females) were anesthetized with ether and macerated in 5 mL of sterile peptone water using a sterile mortar and pestle.

Afterwards, aliquots of 1 mL of the macerate were transferred for flasks containing 50 mL of GYP supplemented with 100 mg L^{-1} chloramphenicol and DBDM broths, both for the isolation of yeasts species and GY broth [50 g L^{-1} glucose, 10 g L^{-1} yeast extract, and 13 g L^{-1} agar, 200 mg L^{-1} Delvocid® (DSM, Delft, Netherlands) (100 mg L^{-1} natamycin), 3000 U L^{-1} penicillin (Sigma), pH 4.5], used as a specific enrichment media for AAB. Flasks were incubated in an orbital shaker (Aralab) at 120 rpm up to 5 and 12 days at 25 ºC, in the case of GYP and DBDM media, respectively, and 28 ºC up to 5 days in the case of GY media. After enrichment, yeast and bacterial isolates were obtained by streaking 10 µL (triplicates) of each media with microbial development onto

GYP and GY plates, respectively. Representative isolates of each type of colony/cell morphology found on plates were isolated, purified and selected for identification.

2.5.4 Acetic acid and gluconic acid determinations

The two main chemical markers of sour rot were quantified for those grape sets which showed sour rot symptoms at the final of *in-vitro* tests. Acetic acid was determined in the final grape musts samples using a Reflectoquant RQflex10® (Merck, Darmstadt, Germany) device and the Reflectoquant® Acetic Acid test strips (Merck), according to the manufacturer's instructions. Gluconic acid was quantified using the D-Gluconic Acid/D-Glucono-δ-lactone enzymatic assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland), according to the manufacturer's instructions.

2.6 Identification of yeasts isolates

Yeast isolates recovered from grapes and *Drosophila* spp. flies (2008 vintage) and 2009 *in-vitro* trials were identified by restriction fragment length polymorphism (RFLP) analysis of the 5.8S-ITS rDNA region (Esteve-Zarzoso *et al.*, 1999). PCR amplifications were carried out from fresh colonies (48-h) resuspended directly in the PCR mixture, or from extracted DNA obtained after a previous enzymatic treatment with liticase (5 mg/mL), following the procedure described in Cryer *et al.* (1975). PCR amplification conditions, restriction reactions with *Cfo*I, *Hae*III and *Hin*fI (Bioron, GmbH, Ludwigshafen, Germany) restriction endonucleases and electrophoretic conditions were performed as described in Barata *et al.* (2008a). Restriction patterns were determined by comparison with 100-bp DNA ladder (Bioron) using the Quantity One 1-D analysis software (BioRad) and compared with those contained in the Yeast-id database at www.yeast-id.com (Valencia University and CSIC, Spain).

Yeast isolates sharing similar restriction patterns or with misidentified patterns, were grouped and representative isolates of each case were identified by sequencing the D1/D2 domains of the 26S rRNA gene as described in Barata *et al.* (2008a). Sequences were edited and assembled using BioEdit Sequence Alignment Editor version 7.0.1 software (Hall, 1999), and then subjected to the GenBank BLASTn search tool of the NCBI database (http://ncbi.nlm.nih.gov/blast) to retrieve sequences of closely related taxa.

The cluster *Hanseniaspora uvarum/H. guilliermondii/H. opuntiae* was differentiated by growth on GYP agar plates incubated at 37 ºC (Smith, 1998) and by restriction of 5.8S-ITS region with *Dde*I and *Dra*I (Bioron) endonuclease in accordance with Cadez *et al.*(2002) and Nisiotou and Nychas (2007), respectively. The *Lachance*a clade former species (*L. fermentati*, *L. cidri*, *L. thermotolerans* and *L. waltii)* were differentiated by growth on GYP agar plates incubated at 37 ºC and 40 ºC, and growth with D-galactose and 0.01% cycloheximide (Kurtzman, 2003; Kurtzman and Fell, 1998). *Candida stellata* was distinguished from *C. zemplinina* by digestion of the 5.8S-ITS region with *Dra*I (Bioron) endonuclease in accordance with Sipiczki (2004).

2.7 Identification of AAB isolates

Gram staining and catalase tests were initially carried out for all bacterial isolates. Gram-negative, catalase positive isolates with ellipsoidal to rod-shaped cells were considered as putative acetic acid bacteria. These isolates were identified by RFLP of PCR-amplified 16S rRNA gene and RFLP of PCR-amplified 16S-23S ITS region according to González *et al.* (2006). DNA extraction was carried out as follows: fresh cultures were grown on GY plates and 3 to 5 bacterial colonies were picked and ressuspended in 50 μL of lysis solution (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and incubated at 100 ºC for 15 min (boiling water bath). The suspension was cooled at room temperature, centrifugated (5000 *g*, 4 ºC, 5 min) and the supernatant containing the released DNA was diluted 100-fold with sterile miliQ water and used as template for PCR amplification. The 16S rRNA gene and 16S-23S ITS regions were amplified using the primers and thermocycling conditions designed by Ruiz *et al.* (2000). PCR amplifications were carried out in 50 μL reaction volume, consisting of 2 μL bacterial DNA lysate and 48 μL amplification mixture, containing 10μL of 5× amplification buffer Green Go Taq Flexi (Promega), 200 μM of dNTPs mixture, 2 mM $MgCl_2$, 0.3 μM of each primer and 2.5 U Go Taq® (Promega). For the restriction reactions of the 16S rRNA gene-amplified products, 5 μL of each PCR product was digested with 10 U of *Alu*I (Fermentas, Thermo Fisher Scientific Inc., Leicestershire, UK), *Cfo*I, *Hinf*I, *Tru9*I (Bioron) and *Taq*I (Metabion, Martinsried, Germany) restriction endonucleases, as recommended by the manufacturers.

Isolates inconclusively identified by RFLP of 16S rRNA gene *Acetobacter cerevisae*/*A. orleaniensis*/*A. malorum*, were resolved by RFLP of 16S-23S ITS region, digesting the

amplified 16S-23S ITS product (5 μL) with *Alu*I and *Cfo*I endonucleases (González *et al.*, 2006).

The PCR products and restriction fragments were detected and separated by 1.0% (w/v) and 3.5% (w/v) agarose gel electrophoresis in 1× TBE buffer, respectively. Restriction patterns were obtained after estimation of fragment sizes by comparing their mobility against a 100-bp DNA Ladder (Bioron), as described above.

Results of RFLP analysis were confirmed by sequencing the amplified 16S rRNA gene of representative isolates of each restriction pattern. For those isolates, the amplified 16S rDNA products were purified, sequenced and the final corrected sequences were submitted to the BLASTn network service, as previously described.

2.8 Nucleotide sequences accession numbers

Nucleotide sequences were deposited in the NCBI GenBank database library under the accession numbers JN004183 to JN004201.

3. Results and Discussion

3.1 Effect of the grape cluster coverage on the development of sour rot

The sanitary state of all selected bunches maintained free or protected inside the plastic structures covered with nylon cloth during ripening was visually analyzed at the harvest time. Table 2 shows that the integrity of grape surfaces since the early ripening phases and the absence of vectors are critical for the final sanitary state of bunches and for the sour rot development. For each modality, the visual observation was carried out for the three bunches used in both independent sampling (vine set A and B). As expected, healthy bunches with unblemished berries maintained their perfect sanitary state until harvest, independently of the presence of vectors. However, when sound bunches were wounded and left on the vineyard without protection of nylon nets (Sw_F), two different results were observed: for 50% of the cases the wounded berries rotted, while the other 50% showed wound's healing. On the contrary, when the same type of bunches were maintained protected inside the nylon nets (Sw_P), it was observed the

healing of wounds in all the tested bunches, which did not shown any sign of rotting at the harvest time.

Concerning grape samples with sour rot symptoms protected with nylon nets (SR_P), results showed that nets avoided, for the most studied bunches, the spread of sour rot for the remaining parts of the clusters. Nevertheless, one bunch in each vine set rotted completely. This last result could be explained by the observation at the harvest time of adult *Drosophila* spp. flies and larvae inside the closed structure involving these two rotten bunches, which possibly hatched from eggs or larvae already present on the initially affected berries.

Finally, the six grape bunches (vine set A and B) with initial signs of sour rot and left freely during ripening (SR_F), were totally rotten at the harvest time, emphasizing the role of *Drosophila* spp. for the development and disease extension. Moreover, the constant presence of *Drosophila* flies surrounding the rotten clusters and a large number of larvae inside the rotten berries were observed.

Table 2. Effect of grape bunches protection with nylon nets during ripening in the final sanitary state at the harvest type. Results reported as the number of bunches associated to each sanitary state.

Assay	Sanitary state at *veráison*	Bunches	Sanitary state at harvest[a]							
			Sound		Wound's healing		Partially rotten		Totally rotten	
			(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)
S_F	Sound	Free	3	3						
S_P		Protected	3	3						
Sw_F	Sound with wounds	Free			2	1	1	2		
Sw_P		Protected			3	3				
SR_F	Sour rot symptoms	Free							3	3
SR_P		Protected					2	2	1	1

[a] (A) and (B) represent the two independent sampling (vine sets).

3.2 Effect of *Drosophila* spp. on yeast populations isolated from grapes

A similar isolation strategy to that successfully applied in our previous works (Barata *et al.*, 2008a, 2008b) was carried out by using either general purpose media (GYP) or specific and differential media (DBDM and ZDM), for the isolation and enumeration of dominant and minority yeast species present on grapes surface. A total of 159 yeasts isolates representative of the diversity found in all analyzed grape samples were selected for identification. The RFLP of 5.8S-ITS analysis performed for these isolates

generated 26 different restriction patterns obtained with *Cfo*I, *Hae*III, *Hinf, Dde*I and *Dra*I restriction endonucleases, of which 11 were successfully identified by comparison with the restriction profiles contained in the Yeast-id database, while 15 were unidentified by this method. Representative isolates of each pattern included in the latter group were subsequently identified by sequencing the D1/D2 domains of the 26S rRNA gene. Detailed restriction fragments data of each identified yeast species by PCR-RFLP of the 5.8S-ITS region are available as supplementary material (Table S1).

Table 3 shows the diversity and enumeration of yeasts species recovered from the six grapes modalities analyzed at harvest time after being left protected or unprotected from vectors action during ripening. In order to evaluate the effect of bunch protection, the yeast species patterns recovered at harvest time on the different modalities were compared with those found after *veráison* on sound and initially sour rotten bunches.

Just after *veráison*, as previously observed by Barata *et al.* (2008a), the amount and diversity of yeasts species was deeply changed on bunches with initial sour rot symptoms. Sour rotten bunches had in average more 4.25 log CFU g^{-1} of total yeast counts than the healthy grapes samples, and showed a species pattern composed by 13 different ascomycetous yeast species dominated by *C. zemplinina*, *C. apicola* and *H. uvarum* and characterized by the absence of basidiomycetes. Moreover, sour rotten samples showed the presence of the sour rot zymological markers *Z. hellenicus*, *P. terricola* and *P. kudriavzevii* (the two latter species were formerly named as *Issatchenkia* spp.), in accordance with the previous works of Barata *et al.* (Barata *et al.*, 2008a, 2008b). On the contrary, sound grapes had lower yeasts species diversity characterized by only 3 ascomycetous (*C. diversa, C. zemplinina* and *H. uvarum*) and 3 basidiomycetous yeast species (*Cryptococcus magnus, Cr. carnescens* and *Rhodotorula* spp.) and by the dominance of the ascomycetous yeast-like fungus *Aureobasidium pullulans*.

Since all the covered and free sound bunches (S_P and S_F) remained healthy until the harvest time (Table 2), such a significant change in their yeast microbial flora would not be expected. Indeed, in spite of a slight increase on total yeast populations, similar diversity patterns were found on both sound bunches modalities in comparison with the initial ripening phase. As observed just after *veráison*, yeast flora was characterized by several basidiomycetous species (*Cr. flavescens*, *Cr. laurentii*, *Sporobolomyces roseus* and *Rhodotorula* spp.) and by the dominance of the ascomycetous species *C. zemplinina* and *A. pullulans* (Table 3).

Table 3. Microbial yeast populations isolated from sound bunches and with initial sour rot symptoms after the veráison period and effect of bunches coverage during ripening on yeast dynamic populations[a]

Yeast species	After *veráison* [b]				Harvest time[b]												GenBank Acession no.[c]
					Protected bunches with net						Free bunches						
	S		SR		S_P		Sw_P		SR_P		S_F		Sw_F		SR_F		
	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	
Basidiomycetes																	
Cryptococcus aureus							3.40										JN004192
Cryptococcus carnescens		1.18															JN004185
Cryptococcus flavescens							0.70					1.78					JN004195
Cryptococcus laurentii						2.74											
Cryptococcus magnus	3.18																JN004184
Cryptococcus spp.								3.00			2.70	3.00					JN004190
Sporobolomyces roseus						0.70	3.70	3.70			2.48	2.88					
Rhodotorula spp.	2.70	1.00			1.00	2.70											JN004191
Ascomycetes																	
Aureobasidium pullulans	3.30	2.08	4.07	3.89	3.30	3.54	4.90	4.48	4.40	4.18	3.54	3.54	4.18	5.98	5.00	4.70	JN004193
Aureobasidium spp.		1.70															JN004186
Candida amapae					2.18						1.18	1.65	1.48				
Candida apicola			6.29	6.28					6.39	6.93					7.73	7.11	JN004197
Candida azyma									4.24	2.95					4.13	5.23	JN004198
Candida diversa	3.00																
Candida zemplinina	2.48		7.08	7.23	4.06	2.60	3.08	3.40	5.78	6.49	4.04	3.81	4.48	6.39	6.00	7.11	JN004183
Hanseniaspora guilliermondii			5.42	5.28	3.48				5.65	5.44	2.95	3.18	3.30		6.09	6.07	
Hanseniaspora uvarum	1.60		6.12	5.98			3.81	2.93	5.81	5.00			2.74	3.30	6.48	6.60	JN004196
Lachancea thermotolerans			5.65	4.70						4.18				4.70		5.00	
Metchnikowia pulcherrima				4.18					5.22	4.74			3.12	4.25	4.18	3.40	
Pichia kluyveri			3.00	5.13													JN004187
Pichia kudriavzevii			5.40	6.00													JN004188
Pichia sporocuriosa			3.52	2.18						2.26						2.30	JN004199
Pichia terricola			5.78	5.40					5.30	4.70			3.87	5.35	5.48	5.48	
Saccharomycopsis vini			5.00	4.93	1.90					3.20					3.74	5.00	
Zygoascus hellenicus			4.90	4.14					5.24	4.12			4.05	4.92	5.73	5.23	
Zygosaccharomyces bisporus									4.70	5.88				4.63	6.00	6.30	
Total counts	3.72	2.29	7.18	7.31	4.22	3.69	5.01	4.59	6.62	7.10	4.18	4.12	4.75	6.57	7.77	7.51	

[a] Results reported as log CFU/g. [b] Type of bunches: S, sound;SR, Sour rot symptoms; Sw , sound with wounds; (A) and (B) represent the two independent sampling (vine sets). [c] GenBank accession number of the sequences deposited in NCBI GenBank database library.

These results show that unblemished berries show constant numbers and species diversity through ripening or have similar variability to that found on the surface of other parts of the phylloplane (Fonseca and Inácio, 2006).

Noteworthy changes on grapes yeast flora were detected at the moment from which sound berries were wounded and maintained free or protected inside the nets. In case of protected bunches (Sw_P), a typical yeast species profile of mature healthy bunches was observed (Table 3), which is in accordance with the final sanitary state observed at harvest, characterized by the total healing of their wounds and absence of any type of sour rot symptoms (Table 2). On the contrary, wounded bunches visited by *Drosophila* flies (Sw_F), were characterized by higher total yeast populations (5.66 CFU g^{-1} in average of both sampling sets), absence of basidiomycetes, and the presence of the typical sour rot colonizing yeasts *P. terricola*, *Z. hellenicus* and *Zygosaccharomyces bisporus* (Table 3).

Finally, identical yeast species profiles were recovered from both covered (SR_P) and free (SR_F) bunch sets with initial sour rot symptoms. Thirteen ascomycetous yeast species were isolated from these samples, among which high populations of *Z. bisporus* occurred. This similarity at yeast flora level does not reflect, however, the final grape sanitary state of bunches at harvest time. In fact, the complete sour rot dissemination throughout the entire cluster was observed in all insect-visited bunches, while for the most of the protected bunches sour rot symptoms stayed confined only to the early infected berries (Table 2).

3.3 Isolation of yeasts from wild *Drosophila* spp. flies

The enrichment strategy used for the isolation of yeasts from wild *Drosophila* spp. flies captured in the vineyard, allowed the recovery of 20 yeast isolates from the bodies surface (10) and digestive tract of flies (10). Three different RFLP patterns were obtained in each group of isolates. These patterns matched with the yeast species *C. zemplinina*, *H. uvarum* and *Z. hellenicus*, for the group of bodies surface isolates and *H. uvarum, P. terricola* and *Z. hellenicus*, in case of isolates obtained from the *Drosophila* macerated sets (Table 4).

These results are in line with the studies of Fermaud *et al.* (2000, 2002) which observed by electron microscopy the presence *Kloeckera apiculata* (anamorph of *H. uvarum*) yeasts cells on the bodies surface (thorax and legs) of adult *Drosophila* flies.

These authors also analyzed the microflora of the surface and gut of adult flies captured in vineyards of three different regions in France and isolated *K. apiculata, C. stellata* and *M. pulcherrima* either from the flies' surface or gut, and also *P. membranifacies* and *C. krusei* only from the gut.

It should be highlighted from our results the isolation of *P. terricola* and *Z. hellenicus*, which have been consistently isolated only from sour rotten grapes in previous works and considered as zymological markers of this disease (Barata *et al.*, 2008a, 2008b). Therefore, these results seem to indicate clearly that *Drosophila* spp. flies are vectors of the main yeast flora that typically colonize the surface of sour rotten grapes and support the empirical hypothesis which holds that fruit flies are involved in the spread of grapevine sour rot.

Table 4. Yeast species recovered from the surface and macerate of wild *Drosophila* spp. flies captured in the vineyard.

Isolate	RFLP Pattern	Yeast species	Captured wild *Drosophila* spp.			
			Intact		Macerated	
			GYP	DBDM	GYP	DBDM
WD1, WD2, WD3	Y15	*C. zemplinina*	+	–	–	–
WD4, WD5,WD11, WD12, WD13	Y18	*H. uvarum*	+	–	+	–
WD14, WD15	Y25	*P. terricola*	–	–	+	–
WD6, WD7, WD8, WD9, WD10, WD16, WD17,WD18, WD19, WD20	Y28	*Z. hellenicus*	+	+	+	+

3.4 Influence of bunch protection on chemical composition of grape musts

Table 5 illustrates the effect of bunch protection on the changing of the oenological parameters of grape musts. The results obtained by FTIR analysis showed that several chemical parameters were significantly affected when wounded sound (Sw) or with initial symptoms of sour rot (SR) bunches were kept protected from the influence of insects. In order to evaluate the significance of these changes, a set of unpaired *t*- tests was performed between must samples obtained from protected and free bunches. Table 6 summarizes the oenological parameters with significant differences between samples. The samples of grape musts obtained from wounded sound bunches left under *Drosophila* influence, showed significantly higher values of total acidity, volatile acidity, accompanied with a significant drop of pH, when compared with those obtained from the covered bunches. Moreover the results of the sour rot and yeast activity

sanitary indexes were significantly higher for the free bunch samples, which were classified as having a “slight attack” contrasting with the classification of “good grapes” for the covered bunch.

Table 5. Chemical composition of musts obtained from the six grape bunches modalities, determined at harvest time by FTIR analysis.

Sanitary state at *veráison*	Bunches	Enological parameters[a]						Sanitary state indexes			
		Glu-Fru (g/L)	Total acidity (g/L)	pH	Tartaric acid (g/L)	Malic acid (g/L)	Volatile acidity (g/L)	Gray Rot (0-60)	Sour Rot (0-30)	Yeast Activity (0-30)	Lactic Bacteria (0-2)
Sound (S)	Free (F)	197	3.68	3.34	3.55	2.80	0.02	33	3	6	1
		(6)	(0.06)	(0.03)	(0.07)	(0.14)	(0.01)	(4)	(0)	(0)	(0)
	Protected (P)	190	3.72	3.31	3.35	2.75	0.01	31	3	6	1
		(12)	(0.17)	(0.06)	(0.07)	(0.07)	(0.01)	(2)	(0)	(0)	(0)
Sound with wounds (Sw)	Free (F)	252	4.95	3.14	3.55	3.00	0.18	37	12	11	1
		(18)	(0.21)	(0.02)	(0.49)	(0.14)	(0.01)	(3)	(1)	(1)	(0)
	Protected (P)	230	3.27	3.45	3.75	2.90	0.06	31	3	6	1
		(13)	(0.05)	(0.04)	(0.07)	(0.14)	(0.01)	(4)	(0)	(0)	(0)
Sour rot symptoms (SR)	Free (F)	321	8.09	3.07	3.75	2.55	0.89	51	25	16	2
		(9)	(0.23)	(0.04)	(0.07)	(0.07)	(0.13)	(2)	(4)	(1)	(0)
	Protected (P)	261	4.85	3.17	3.80	2.45	0.19	42	13	13	1
		(13)	(0.78)	(0.02)	(0.14)	(0.35)	(0.01)	(3)	(1)	(1)	(0)

[a] Average values of independent duplicate samples; standard deviation between brackets; Glu-Fru, glucose-fructose content.

Indeed, these results are in agreement with the previously described microbial observations of the final sanitary characteristics of bunches at harvest (Table 2).

Interestingly, the chemical profile of the grape must samples obtained from the covered wounded bunches (Sw_P), was similar to those registered for both sets of sound bunches (S_F and S_P). Bearing in mind that the totality of wounded bunches which were not visited by *Drosophila* flies, or other insects, healed their wounds, we speculate that the vine plant activated a defense mechanism leading to the healing of berry skin lesions.

A similar trend was observed for the free and protected bunches with initial sour rot symptoms. Table 6 shows that the musts from free bunches were characterized by significant higher values of glucose-fructose, total acidity, volatile acidity, sour rot index and a slight lower of pH values, than those kept in the absence of the vectors. These chemical changes were recently defined by Barata *et al.* (2011b) as the typical changing pattern arising from the sour rotting process. The vectors role becomes clear

since significant lower levels of volatile acidity and sour rot index were observed in the covered bunches, which were classified as “slight attack” in contrast to the “serious to severe attack” associated to free bunches. Therefore these results clearly suggest that *Drosophila* flies could be the main sources of AAB which typically colonize the surface of sour rotten grapes, as stated by Fermaud *et al.* (2000).

No significant differences were found between both protected and free sound bunches for all the parameters tested, which shows that *Drosophila* flies induced chemical changes in musts only when the physical barrier posed by the berry skin was broken.

Table 6. Significant effects of grape bunches protection in the chemical composition of grape musts[a].

Sanitary state	Parameters	Av. Diff[b] (P-F)	*t*	*p*(t)[c]	
Sound with wounds (Sw)	Total acidity (g/L)	-1.69	10.94	0.008	***
	pH	0.32	-9.39	0.011	**
	Volatile acidity (g/L)	-0.13	11.18	0.008	***
	Sour rot index	-8.50	17.00	0.003	***
	Yeast activity index	-4.50	9.00	0.012	**
Sour rot symptoms (SR)	Glucose-Fructose (g/L)	-60	5.36	0.033	**
	Total acidity (g/L)	-3.24	5.63	0.030	**
	pH	0.10	-3.43	0.075	*
	Volatile acidity (g/L)	-0.70	7.28	0.018	**
	Sour rot index	-12.00	4.71	0.042	**

[a] Results of an unpaired *t*-test between must samples obtained from protected bunches (P) and their corresponding controls from free bunches (F); [b] Average difference protected bunches (P) free bunches (F); [c] Notations of *, ** and ***, indicate significance at $p < 0.1$, $p < 0.05$ and $p < 0.01$, respectively.

3.5 *In-vitro* sour rot development tests

The developed method involved the use of commercial table grapes inoculated with several strains of yeasts and bacteria species belonging to the typical microbiota of sour rotten berries, after sequential steps of rinsing, surface disinfection and washing of the berries. After microbial inoculation, healthy and wounded berries were incubated with the presence and absence of adult *Drosophila* spp. flies, which were reared in laboratory from wild capture *Drosophila* flies. Thus, it was possible to create in the laboratory, an *in-vitro* method able to induce the typical symptoms of sour rot and find the biological agent without which sour rot does not occur. Table 7 summarizes the global data obtained during the *in-vitro* sour rotting assays.

Table 7. *In-vitro* sour rotting assays. Effects of the inoculated strain and *Drosophila* spp.

Inoculated strain	Assay[a]	Initial counts[b] (log CFU/g)		Berries characterization[c]					Final counts[b] (log CFU/g)		Metabolites[d]	
		TY	TB	1.	2.	3.	4.	Sour rot (%)	TY	TB	ACET (g/L)	GLUC (g/L)
C. zemplinina	C	5.56	<1[e]	–	–	–	NA[f]	0	ND[g]	ND	ND	ND
ISA 2339	D	5.35	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.47	<1	+	–	–	–	0	7.93	<1	0.24	0.07
	WD	5.29	<1	++	++	++	–	86	7.55	6.81	7.00	10.79
H.uvarum	C	5.06	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2294	D	5.08	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.15	<1	+	–	–	+	0	7.25	<1	0.11	0.01
	WD	5.19	<1	+	++	++	–	71	6.70	7.38	3.37	0.98
I. occidentalis	C	4.60	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2384	D	4.68	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.06	<1	+	–	–	–	0	8.11	<1	0.10	0.09
	WD	5.13	<1	++	++	++	–	71	8.29	6.78	1.73	1.22
I. orientalis	C	5.75	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2381	D	5.53	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.53	<1	+	–	–	+	0	6.92	<1	0.08	0.07
	WD	5.38	<1	++	++	+	–	100	7.72	5.48	0.75	0.09
I. terricola	C	4.53	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2322	D	4.41	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	4.70	<1	+	–	–	+	0	6.38	<1	0.09	0.06
	WD	4.66	<1	+	+	+	–	86	6.64	4.78	0.58	0.17
L. thermotolerans	C	5.38	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2380	D	5.60	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.48	<1	+	–	–	–	0	7.70	<1	0.14	0.01
	WD	5.53	<1	+	++	++	–	86	6.87	7.26	4.50	0.51
S. cerevisiae	C	4.89	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 1000	D	4.56	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.41	<1	+	–	–	+	0	7.34	<1	<0.04	0.01
	WD	5.45	<1	++	+++	+++	–	100	7.19	>8.60	3.67	8.73
Z. hellenicus	C	5.34	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2324	D	5.02	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	4.53	<1	+	–	–	–	0	8.44	<1	0.25	0.04
	WD	5.35	<1	++	++	++	–	71	7.58	5.56	1.19	7.07
Z.bailii	C	5.16	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA 2295	D	5.18	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.51	<1	+	–	–	–	0	7.51	<1	0.04	0.08
	WD	5.34	<1	++	+++	+++	–	100	6.53	5.00	9.20	2.49
Z. bisporus	C	5.30	<1	–	–	–	NA	0	ND	ND	ND	ND
ISA2336	D	5.26	<1	–	–	–	NA	0	ND	ND	ND	ND
	W	5.56	<1	+	–	–	–	0	8.00	<1	0.10	0.10
	WD	5.68	<1	++	++	+	–	100	7.97	<1	0.49	0.17
Ga. saccharivorans	C	<1	5.73	–	–	–	NA	0	ND	ND	ND	ND
CBISA 4244	D	<1	5.62	+	+	++	NA	28	6.87	6.00	2.05	7.15
	W	<1	5.58	+	++	++	–	71	<1	6.98	1.43	8.02
	WD	<1	5.79	+++	+++	+++	–	100	7.64	6.21	12.80	29.20
E. durans	C	<1	4.72	–	–	–	NA	0	ND	ND	<0.04	ND
CBISA 4315	D	<1	5.06	–	–	–	NA	0	ND	ND	<0.04	ND
	W	<1	5.15	+	–	–	+	0	ND	ND	<0.04	ND
	WD	<1	4.72	+	+++	+	–	100	8.39	7.83	0.41	5.56
Control berries	Blank	2.64	1.90	ND	ND	ND	NA	ND	ND	ND	ND	ND
(NI)[h]	C	<1	<1	–	–	–	NA	0	ND	ND	<0.04	0.03
	D	<1	<1	–	–	–	NA	0	ND	ND	0.16	0.16
	W	<1	<1	–	–	–	+	0	ND	ND	0.20	0.08
	WD	<1	<1	+	+	+	–	43	4.08	5.51	0.47	6.06

[a] C, sound berries (control set); D, sound berries with *Drosophila* flies; W, sound wounded berries; WD, sound wounded berries with *Drosophila* flies; Blank, unsterilized sound berries. [b] TY, total yeasts (GYP plates); TB, total bacteria (TSA plates for yeasts and control berries trials, MRS plates for LAB trial). [c] 1. Browning, 2. Disaggregation of the internal tissues, 3. Acetic acid odor, 4. Wound's healing. [d] ACET,acetic acid, GLUC, gluconic acid. [e] <1 CFU/g. [f] NA, not applicable. [g] ND, not determined. [h] NI, not inoculated.

The results of the initial microbiological analyses which were carried out just after berry inoculation with each individual tested yeast or bacteria strain, showed that the berries were efficiently disinfected, since only the inoculated strain were recovered. The immersion of berries in a cellular suspension containing 10^7 metabolically active cells/mL of each selected strain allowed the production of berries with an average initial microbial charge of 5.19 log CFU g^{-1} and 5.30 log CFU g^{-1}, in case of yeasts and bacterial strains, respectively.

Concerning yeast inoculation trials, Table 7 shows that independently of the inoculated strain, healthy and unblemished berries either in the absence (C) or presence (D) of *Drosophila* flies did not develop any symptoms of sour rot. In all of these trials, none of the sour rot symptoms were observed, whereby no further microbial and chemical analyzes were performed.

Interestingly, the induction of wounds in sound berries followed by the inoculation with the yeasts strains, did not contribute by itself for the developing of sour rot. Indeed, none of the tested yeast strain induced the disease. Moreover, wounds healed for 40% of the tested yeast strains (*H. uvarum, P. kudriavzevii, P. terricola* and *S. cerevisiae*). This phenomenon was previously observed *in-vivo* during the experimental survey performed on the vineyard, in all the tested wounded sound bunches which were not visited by the vectors and also in 50% of the unprotected wounded bunches (Table 2). The final microbiological analyzes carried out after the *in-vitro* incubation period, showed that all yeasts strains increased their populations at the surface of wounded berries (2.31 log CFU g^{-1} in average), and no viable bacterial cells were found (<1 CFU g^{-1}). In addition, low concentrations of acetic acid and gluconic acid were detected, independently of the yeast strains inoculated (Table 7). These results seem to demonstrate that none of the yeast species had the ability to induce sour rot by itself and should not be regarded as primary agents responsible for this disease.

A completely different scenario was observed when sound wounded berries were inoculated with the AAB strain of *Gluconacetobacter saccharivorans*. After incubation, 71% of the inoculated grape berries developed clearly the typical symptoms of sour rot and high concentration of the acetic (1.43 g L^{-1}) and gluconic (8.02 g L^{-1}) acids were found in the respective final grape must. These modifications were only due the action of this AAB species, since no viable counts of total yeasts were found after incubation (Table 7).

Contrarily to the observed with all yeasts strains, 28% of the sound berries set inoculated with the AAB strain and maintained with *Drosophila* adult flies, developed sour rot. However we believe that this result may have been due to the presence of small wounds which were not visual detected during the selection of the sound berries.

The results obtained with the wounded sound berries sets maintained in contact with *Drosophila* spp. (WD), clearly evidenced that *Drosophila* flies are a key factor without which no sour rot development occurs. Independently of the yeast or bacterial inoculated strains, all the WD assays culminated in the development of sour rot and very high concentrations of acetic and gluconic acids were detected in the respective grape musts. Regarding yeast inoculation sets, with the exception for the trial inoculated with *Z. bisporus*, very high bacterial counts were recovered after incubation of all remaining berries sets, demonstrating that *Drosophila* flies acts as inoculation sources of bacterial populations responsible for sour rot onset. On the other hand, very high yeasts populations were found when only *Ga. saccharivorans* (7.64 log CFU g^{-1}) and *E. durans* (8.39 CFU g^{-1}) were inoculated in wounded berries in the presence of flies. Moreover, high counts of total yeasts (4.08 CFU g^{-1}) and bacteria (5.51 CFU g^{-1}) were recovered from the wounded control berries (not inoculated) incubated with *Drosophila*. All these results clearly indicate that *Drosophila* flies played a role as inoculation and dispersion vectors of the sour rot causal agents.

3.6 Identification of isolates recovered from *in-vitro* sour rotten berries and reared *Drosophila* flies

Twenty six yeast and sixteen bacterial isolates representative of the microbial diversity found in all analyzed berries sets after incubation were selected for identification. In case of the wounded berries sets (W) inoculated with the ten different yeasts strains, only a single type of colony morphology was found for each individual set, after the incubation period. The analysis of the RFLP patterns of the isolates representative of each morphology confirmed that only the inoculated species were present on the berries surface (data not shown). Table 8 lists the yeasts and bacterial species isolated from the berries sets for which sour rot development was observed.

Results showed that beyond the inoculated yeast strain, the yeast species *H. uvarum* and the AAB species *G. oxydans* and *A. malorum* were recovered after incubation with *Drosophila* flies (WD).

Table 8. Microbial flora recovered from berries sets which developed sour rot during the *in-vitro* tests.

Inoculated species (Strain)	Assay[a]	Isolates after in-vitro rotting development					
		Isolate[b]	Colony[c]		RFLP Pattern	Species	GenBank Acession no.[d]
C. zemplinina	WD	Lf1	Y	+	Y15	*C. zemplinina*	
(ISA 2339)		Lf2	Y	–	Y18	*H. uvarum*	
		Bf1	B	NA	B3	*G. oxydans*	
H.uvarum	WD	Lf5	Y	+	Y18	*H. uvarum*	
(ISA 2294)		Bf6	B	NA	B2	*A. malorum*	
I. occidentalis[e]	WD	Lf7	Y	+	Y23	*P. occidentalis*	
(ISA 2384)		Bf3	B	NA	B3	*G. oxydans*	
I. orientalis[f]	WD	Lf9	Y	+	Y22	*P. kudriavzevii*	
(ISA 2381)		Bf4	B	NA	B2	*A. malorum*	
I. terricola[g]	WD	Lf11	Y	+	Y25	*P. terricola*	
(ISA 2322)		Bf5	B	NA	B2	*A. malorum*	
L. thermotolerans	WD	Lf25	Y	+	Y19	*L. thermotolerans*	
(ISA 2380)		Bf17	B	NA	B2	*A. malorum*	
S. cerevisiae	WD	Lf13	Y	+	Y26	*S. cerevisiae*	
(ISA 1000)		Bf7	B	NA	B3	*G. oxydans*	
Z. hellenicus	WD	Lf3	Y	+	Y28	*Z. hellenicus*	
(ISA 2324)		Bf2	B	NA	B3	*G. oxydans*	JN004204
Z.bailii	WD	Lf20	Y	+	Y29	*Z. bailii*	
(ISA 2295)		Bf13	B	NA	B3	*G. oxydans*	
Z. bisporus	WD	Lf18	Y	+	Y30	*Z. bisporus*	
(ISA2336)							
Ga. saccharivorans	D	Lf16	Y	NA	Y18	*H. uvarum*	
(CBISA 4244)		Bf10	B	+	B4	*Ga. saccharivorans*	
		Bf11	B	–	B2	*A. malorum*	
	W	Bf12	B	+	B4	*Ga. saccharivorans*	
	WD	Lf14; Lf15	Y	NA	Y18	*H. uvarum*	
		Bf8	B	+	B4	*Ga. saccharivorans*	
		Bf9	B	NA	B2	*A. malorum*	JN004205
E. durans	WD	Bf16	B	–	B1	*A. cibinongensis*	JN004206
(CBISA 4315)		Lf23	Y	NA	Y16	*C. oleophila*	JN004207
Control berries	WD	Lf21	Y	NA	Y15	*C. zemplinina*	
(NI)[h]		Lf22	Y	NA	Y20	*M. pulcherrima*	
		Bf14	B	NA	B3	*G. oxydans*	
		Bf15	B	NA	B2	*A. malorum*	

[a] D, sound berries with *Drosophila* flies; W, wounded berries; WD, wounded berries with *Drosophila* flies. [b] Y,yeast; B, bacteria. [c] +, colony and cells morphology similar to the inoculated species; –, different colony morphology; NA, not applicable. [d] GenBank accession number of the sequences deposited in NCBI GenBank database library. [e] renamed as *Pichia occidentalis*; [f] renamed as *Pichia kudriavzevii*; [g] renamed *as Pichia terricola*. [h] NI, not inoculated.

In particular, *G. oxydans* and *A. malorum* were isolated in 50 and 40% of the yeasts inoculation trials, respectively. Furthermore, these two AAB species were also isolated from the WD control berries set together with *C. zemplinina* and *M. pulcherrima* yeast species. *Hanseniaspora uvarum* and *A. malorum* were also detected in both D and WD berries set inoculated with *Ga. saccharivorans*, while *Candida oleophila* and *A. cibinongensis* were found in the WD berries set inoculated with *E. durans*.

The microbial flora analysis of the reared-*Drosophila* flies used in this survey established the origin of most of the species found on the in-vitro sour rotten berries.

Six yeast and ten AAB isolates were recovered from a macerate sample of 50 adult reared-*Drosophila* spp. flies after the enrichment step in GYP and GY broths respectively. Among yeast isolates, two restriction patterns matched with the ascomycetous yeasts *C. zemplinina* and *H. uvarum*, while for the AAB isolates, *G. oxydans*, *A. malorum* and *A. cibinongensis* were identified (Table 9). As previously observed in the analysis of wild captured *Drosophila* spp. flies, *C. zemplinina* and *H. uvarum* were also recovered from the bodies of laboratory-reared flies, which suggest that these two yeast species could be part of the natural floral of the surface or digestive tract of *Drosophila* flies.

Table 9. Microbial flora isolated from the laboratory-reared *Drosophila* flies.

Reared *Drosophila* flies macerate				**GenBank Acession no[a]**
Isolate	**RFLP Pattern**	**Enrichment media**	**Species**	
L1, L2, L3	Y15	GYP	*C. zemplinina*	
L4, L5, L6	Y18	GYP	*H. uvarum*	
DR1, DR2, DR3,	B3	GY	*G. oxydans*	JN004201
DR5, DR8, DR9, DR10	B2	GY	*A. malorum*	JN004202
				JN004203
DR6	B1	GY	*A. cibinongensis*	JF7188429

[a] GenBank accession number of the sequence deposited in NCBI GenBank database library.

4. Conclusions

The results of this work together with previous research (Barata *et al.* 2011, submitted for publication) enabled us to tentatively explain the ecological interactions between vine plant, microorganisms and insects underlying the development of sour rot. In this vineplant-microorganism-*Drosophila* model system, the first event should be the damage of berry skin by any biotic (e.g. grape moths, phytopathogenic moulds, birds) or abiotic (e.g. rain, berry abrasion in tight bunches) factors. The injury triggers a plant defense response mechanism leading to skin healing if *Drosophila* spp. are not present. These insects transport yeasts and acetic acid bacteria that initiate a spontaneous fermentative process on the berry surface. First, fermentative yeasts proliferate and produce ethanol but are not able to induce sour rot alone. Simultaneously, *Gluconobacter* and *Gluconoacetobacter* spp. also attain high cell numbers, producing gluconic acid and acetic acid. Finally, *Acetobacter* oxidizes ethanol

leading to higher concentrations of acetic acid. Therefore, the specific ability to produce acetic and gluconic acid in high levels explain why acetic acid bacteria should be regarded as the etiological agents of sour rot. The volatiles produced attract larger *Drosophila* spp. populations spreading sour rot to neighbour injured berries which physiological defensive response is not fast enough to heal the skin wounds. If there is no injury, except for *D. suzukii* (Calabria *et al.*, 2010), the common *Drosophila* spp., mainly *melanogaster*, have not the ability to perforate the berry skin and sour rot is not triggered, being berry microbiota dominated by basidiomycetous yeasts and *A. pullulans*, similarly to the aerial parts of other plants (Fonseca and Inácio, 2006).

Two stages remain to be elucidated. The first is related with the initial insect attraction signal. Volatile chemicals, such as ethanol, acetic acid, ethyl acetate and acetaldehyde (Hoffmann, 1985; West, 1961), ethyl phenylacetate (Zhu *et al.*, 2003) and acetoin (Stensmyr *et al.*, 2003) are known to attract drosophilids. In particular, ethyl phenylacetate and acetoin were found in significantly higher concentrations in wines affected by sour rot (Barata *et al.*, 2011a). These latter authors speculated that the high concentration of phenylacetic acid, precursor of ethyl phenylacetate, found in sour rotten berries was the result of a plant defense mechanism, because it is a plant auxin (Wightman and Lighty, 1982). Then, it is conceivable that the first event attracting fruit flies is the production of phenylacetic acid which acts also as a pheromone (Cossé *et al.*, 2002; Wightman and Lighty, 1982) but we are not aware of specific research done with *Drosophila* spp.. The second stage is related with the mechanisms underlying the prevention of skin healing in the presence of insects. It is conceivable that microbial enzymes produced during growth or the fermentative metabolites prevent the curative action mediated by phenylacetic acid, but further research is necessary to clarify this issue.

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Supplementary material

Figure S1. Representative samples of free (I) and protected (II) grape bunches used in the vineyard experimental trial. Free bunches under influence of insects: sound (S_F); sound with manually induced wounds (Sw_F) and with initial sour rot symptoms (SR_F). Bunches protected from vectors influence: sound (S_P); sound with manually induced wounds (Sw_P) and initial sour rot symptoms (SR_P).

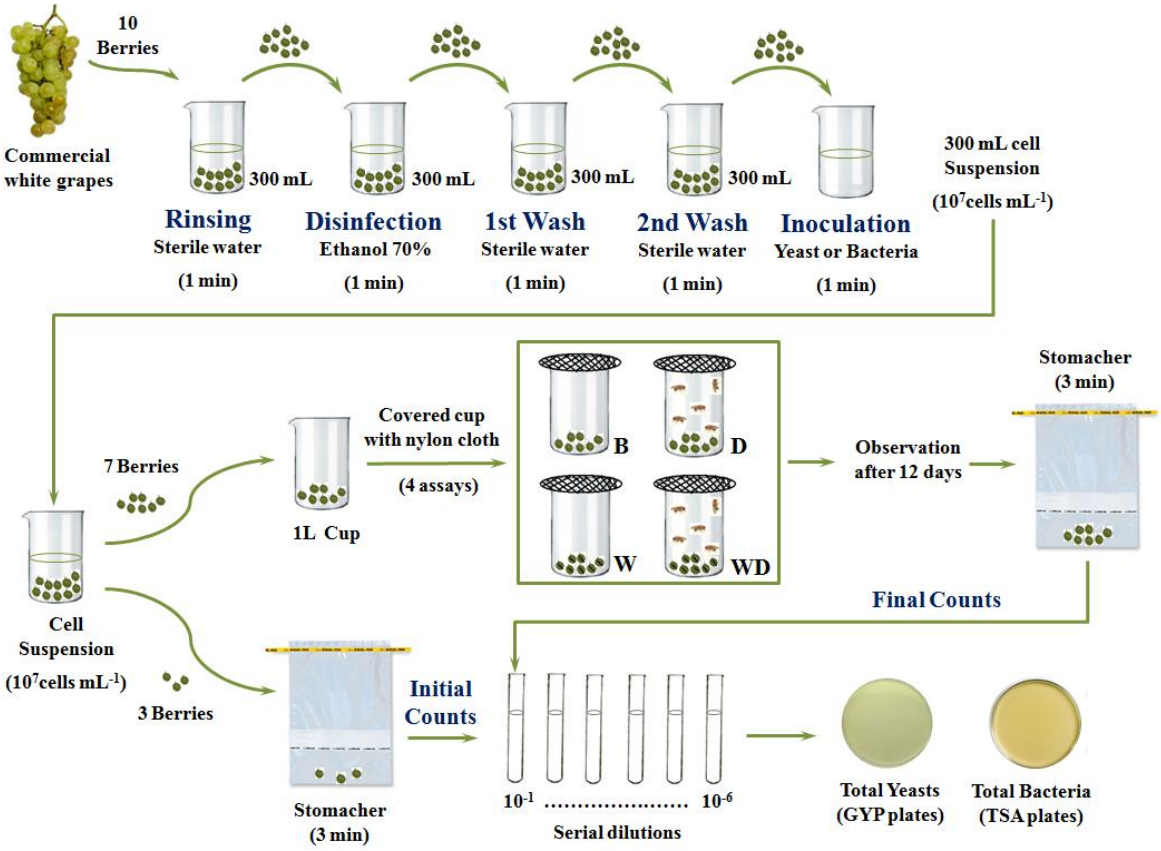

Figure S2. Experimental procedure carried out in sour rot development *in vitro* tests.

Figure S3. Example of the four grape modalities used in the in-vitro sour rot development assay. Inoculated strain: *S. cerevisiae* yeast strain ISA 1000. Modalities: C (control set) – sound berries, D - sound berries in the presence of *Drosophila* spp. flies; W – wounded sound berries; WD - wounded berries in the presence of *Drosophila* spp. flies. Stages: I – just after strain inoculation, II – incubation in sterile 1L cup during 12 days, III – final berries sanitary state after incubation.

Table S1 Electroforetic restriction fragments sizes (bp) of the amplified 5.8S-ITS rDNA region of the yeast isolates and tested yeast strains and of the amplified 16S and 16S-23S ITS rDNA of the AAB isolates.

RFLP Pattern	Yeast species	PCR-RFLP of 5.8S-ITS region					
		AP	*Cfo*I	*Hae*III	*Hin*fI	*Dde*I	*Dra*I
Y1	*Cryptococcus aureus*	530	270-230-80-50	530	295-180-70		
Y2	*Cryptococcus carnescens*	580	290-260	360-100-50	270-270		
Y3	*Cryptococcus flavescens*	580	265-120-70	540	310-180-60		
Y4	*Cryptococcus laurentii*	520	285-250	360-130-70	270-270		
Y5	*Cryptococcus magnus*	630	360-285	500-90-50	265-240-150		
Y6	*Cryptococcus* spp.	580	285-250	360-130-60	270-180-80		
Y7	*Sporobolomyces roseus*	630	300-230-90	600	280-130-110-110		
Y8	*Rhodotorula* spp.	650	310-225-100	600	220-130-90		
Y9	*Aureobasidium pullulans*	600	190-170-90-90	450-155	280-180-135		
Y10	*Aureobasidium* spp.	620	195-150-150-110	480-90	295-295		
Y11	*Candida amapae*	650	580-70	650	320-320		
Y12	*Candida apicola*	480	220-180	400-50	240-120-120		
Y13	*Candida azyma*	460	210-195-90	460	230-200		
Y14	*Candida diversa*	420	160-100-100	420	220-200		
Y15	*Candida zemplinina*	480	210-110-110-60	480	240-240		310-130
Y16	*Candida oleophila*	620	290-290-40	420-140-80	320-310		
Y17	*Hanseniaspora guilliermondii*	775	320-310-120	775	350-200-170	380-180-95-80	
Y18	*Hanseniaspora uvarum*	775	320-310-120	775	350-200-170	300-170-90-90	
Y19	*Lachancea thermotolerans*	700	320-280-60	290-210-80-80	340-340		
Y20	*Metschnikowia pulcherrima*	400	210-100-95	290-100	210-190		
Y21	*Pichia kluyveri*	480	170-110-80-50	380-90	250-220		
Y22	*Pichia kudriavzevii*	520	210-190-70-50	400-90	220-150-130		
Y23	*Pichia occidentalis*	480	220-105-70-70	300-90-70	250-110-100		
Y24	*Pichia sporocuriosa*	450	130-90-90-80-70	280-135	230-190		
Y25	*Pichia terricola*	450	130-95-95-80	290-130	230-110-110		
Y26	*Saccharomyces cerevisiae*	850	380-330-140	310-230-170-130	380-370-120		
Y27	*Saccharomycopsis vini*	600	550	380-170-90	290-195 -140		
Y28	*Zygoascus hellenicus*	650	320-320	630	340-170-130		
Y29	*Zygosaccharomyces bailii*	780	320-270-95-95	690-90	340-220-160		
Y30	*Zygosaccharomyces bisporus*	780	300-280-110-90	680-100	380-230-160		
	AAB species		PCR-RFLP of 16S region				
			*Alu*I	*Cfo*I	*Hin*fI	*Tru*9I	*Taq*I
B1	*Acetobacter cibinongensis*	1450	450-310-270-190-120-50	520-440-210-150-140	980-200-200-80	520-350-250-140-120-90	720-390-230
B2	*Acetobacter malorum*	1450	320-280-240-210-190-120-50	520-350-210-150-140-70	980-200-200-80	530-340-320-150-120	900-370-230
B3	*Gluconobacter oxydans*	1450	550-280-200-190-120-70	430-340-180-160-140-110	950-270-190		
B4	*Gluconobacter saccharivorans*	1450	790-490-120	430-220-190-170-150-140	980-270-190		
			PCR-RFLP of 16S-23S ITS rDNA				
			*Alu*I	*Cfo*I	*Pvu*II		
B2	*Acetobacter malorum*	870	480-210-120	400-210-170-70			
B4	*Gluconobacter saccharivorans*	830			460-340		

CHAPTER 7 Final Conclusions and Future Perspectives

Final Conclusions and Future Perspectives

1. Microbial ecology of grape berry

The striking effect of sour rot on the microbial ecology of grapes has been clearly demonstrated by the results obtained during the course of this work. Available knowledge on this subject describes a series of the factors affecting grape ecology but without weighing the different importance of each one. Our results enabled us to explain the succession of different microbial species as a function of berry damage. Therefore, future studies on the effect of biotic or abiotic factors can only be adequately studied under equal berry injury.

This new insight was only possible because of an accurate berry sampling. Many studies use grape bunches as sampling object but this approach is not valid because damaged berries hidden in a visually intact bunches affect significantly the numbers and diversity of microbial colonisers.

2. Processing of grapes affected by sour rot

Sour rotten grapes can be processed in relatively high proportions without affecting wine quality, although production yields are reduced. The main issue is to process the grapes adequately and direct the wines for quick consumption. This result was somewhat familiar to winemakers but now we can explain the phenomenon in scientific terms.

This output has an important practical implication concerning the evaluation of grapes at winery reception. Contrarily to other types of rot, it is acceptable to ferment grapes affected by sour rot up to 30% without grape depreciation.

3. Effect of damage on wine flavour

The detrimental influence of damaged grapes on quality is long known by enologists. Surprisingly, the mechanisms explaining the detrimental effect of grape damage are not well understood. Apparently grape juice composition is affected negatively giving

rise to unbalanced wines, but the origin of molecules responsible for the disorder is not fully understood.

The effect of sour rot on wine flavour was mainly influenced by the increasing levels of phenylacetic acid on grape juices that are esterified to ethyl phenylacetate during wine storage. This explanation was given by us for the first time and opens the way to explain other wine defects as a consequence of grape damage. It is interesting to note that ethyl phenylacetate as also been associated with the “sweet-like” off-odor of Italian wines (Tat *et al.*, 2007) and that the precursor of another molecule responsible for the so-called “premature red wine aging” is a furan fatty acid (FFA) (Pons *et al.*, 2008). This molecule is present in cutin (Velíšek and Cejpek, 2006) and is related with plant defense mechanisms (Batna and Spiteller, 1994). These defects have not been demonstrated as associated to plant defense mechanisms, but it is conceivable that FFA and phenylacetic acid are produced as a response to berry injury. These aroma precursors in grape juices originate higher levels of aromatic compounds that affect wine flavor during storage. It remains to be seen if this “honey” attribute in wine can be regarded as a taint, indicating the use of grapes with poor health status.

The deleterious effects mediated by plant defense mechanisms seem to be different from the effects of damage provoked by other fungal contaminants. Present knowledge is mainly related with the defects provoked by vine diseases like powdery mildew (*Uncinula necator*) or gray rot (*Botrytis cinerea*) (Calonnec *et al.*, 2004, Stummer *et al.*, 2005, Darriet *et al.*, 2002). The reported defects are due to either deterioration of phenolic compounds (anthocyanins, hydroxycinnamic acids and flavanols), diminishing of desirable aroma molecules or production of highly odorous compounds. As a consequence, these diseases are deleterious under rather low percentages of damage, contrarily to sour rot.

Therefore, we hypothesize that grape damage affects wine quality by three different ways, according to the type of vine plant aggression:

a) Production of deleterious volatile molecules and modification of berry chemical composition by phytopathogenic fungi. It occurs until véraison and the plant defense responses are mediated by the production of compounds dependent on the plant metabolic pathways active until this ripening stage (Ferreira *et al.*, 2006) and on the fungal metabolism.

b) The second occurs when berries become resistant to host specific parasites but become sensitive to fungal saprohytes, like *Botrytis cinerea*. This mould has strong

oxidasic (lacase and tirosinase) and esterasic activities, changing juice chemical composition and producing highly odorous molecules.

c) The third is related with the production of plant defense molecules which act as precursors of unwanted aromatic volatiles. The attack is dependent on berry skin injury and the vine response is to produce molecules responsible for tissue healing. The injury opens the way to opportunist microorganisms, like yeasts, bacteria or moulds which may affect wine flavour during fermentation and wine storage.

4. Interactions between different organisms in sour rot

Sour rot emerged as an example of the interactions between several organisms in nature. The trilogy plant-insects-microorganisms was clearly illustrated by this "case study".

The disease occurs after fruit injury, making nutrients accessible to opportunistic microorganisms like ascomycetous yeasts and acetic acid bacteria. These have the ability to ferment sugars and produce volatile compounds that attract drosophilids which disseminate the microorganisms through other wounded fruits. It remains to be seen if insects are first attracted by the auxin phenylacetic acid produced by the plant as a defense mechanism. If injured berries are not visited by insects, the microbial residents of oxidative nature do not ferment the sugar enabling skin healing by the plant. The rotting process may begin during véraison with rapid increase in yeast numbers and acetic acid bacteria. The yeast species seem to be the same along the ripening period but *Acetobacter* spp. become the dominant acetic acid bacteria producing high levels of acetic acid by oxidation of the ethanol produced by the yeasts. At harvest there seems to be a reduction in yeast diversity probably due to the stress imposed by berry dehydration and acidification.

Therefore, the etiological agents of sour rot are acetic acid bacteria but their activity is stimulated by the consortium with fermenting yeasts and the dissemination of the disease depends on the presence of large drosophilid populations attracted by volatile organic compounds released by the plant.

The methodologies used in this work could be further employed in the study of other vine rots appearing after berry véraison, which seem to be increasingly more prevalent when compared to gray rot, probably due to climate changes (Steel *et al.*, 2011).

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APPENDIX I

Scientific Outputs

I.1 Accepted and submitted papers in peer reviewed international journals

Barata, A., González, S., Malfeito-Ferreira, M., Querol, A. and Loureiro V. (2008). Sour rot damaged grapes are sources of wine spoilage yeasts. *FEMS Yeast Research,* **8**: 1008–1017.

Barata, A., Pais, A., Malfeito-Ferreira, M., Loureiro, V. (2011). Influence of sour rotten grapes on the chemical composition and quality of grape must and wine. *European Food Research and Technology*, DOI: 10.1007/s00217-00011-01505-x.

Barata, A., Campo, E., Malfeito-Ferreira, M., Loureiro, V., Cacho, J., Ferreira, V. (2011). Analytical and Sensorial Characterization of the Aroma of Wines Produced with Sour Rotten Grapes Using GC-O and GC-MS: Identification of Key Aroma Compounds. *Journal of Agricultural and Food Chemistry*, 59, 2543-2553.

Barata, A., Malfeito-Ferreira, M., Loureiro, V. (2011). Dynamics of sour rotten grape berry microbiota during ripening and wine fermentation. *International Journal of Food Microbiology* (submitted for publication).

Barata, A., Santos, S., Malfeito-Ferreira, M., Loureiro, V. (2011). The role of the insect *Drosophila* spp. and of the grape berry microbiota in the development of sour rot disease. *Microbial Ecology* (accepted for publication).

Barata, A., Malfeito-Ferreira, M. and Loureiro, V. (2011). The microbial ecology of wine grape berry. *International Journal of Food Microbiology* (Review article submitted for publication).

I.2 Papers in conference proceedings

Malfeito-Ferreira, M., **Barata, A**., Loureiro, V. (2011). Construction of a wine out of grapes affected by sour rot. Proceedings of the XXXIV World Congress of Vine and Wine “The Wine Construction” Portugal, 20-27th Junho 2011. Um Porto para o Mundo, Associação para o Congresso OIV 2011 (eds.), Porto, Portugal. ISBN: 978-989-20-2449-3.

Barata, A., Campo, E., Malfeito-Ferreira, M., Loureiro, V., Cacho, J., Ferreira, V. (2011). Analytical and sensorial characterization of the aroma of wines produced with sour rotten grapes using GC-O and GC-MS: Identification of key aroma compounds. Wine active compounds 2011. Proceedings of the 2nd Edition of the International Conference Series on Wine Active Compounds. Chassagne, D. and Régis, D. (eds), Gougeon Université de Bourgogne, OenoPluriMedia, pp. 191-193.

Malfeito-Ferreira, M., **Barata, A.**, Loureiro, V. (2011). Influenza del marciume acido sulla vinificazione, la composizione chimica e l'aroma del vino, Workshop Qualitá, Innovazione e Territorio: La Ricerca Microbiologica per i Vini del Futuro. P.R.C. Repubbliche srl, Palermo, Castello Utveggio. 17-19.

Barata, A., Seborro, F., Malfeito-Ferreira, M., and Loureiro, V. (2007). Damaged grapes are vectors of wine spoilage yeast species. In: Œno 2007: 8th International Symposium of Oenology, Bordeaux, France. Vigne et Vin Publications Internationales (Eds), Villenave d’Ornon, France, Vol. 2, pp. 159-162.

I.3 Oral presentations in international conferences and meetings

Malfeito-Ferreira, M., **Barata, A.**, Pais, A. e Loureiro, V. (2011). Como minimizar os efeitos da podridão ácida nos vinhos. I Seminário de Vitivinicultura Atlântica, Construir o Futuro, Vila da madalena, Pico, Açores, Portugal, June 2011.

Malfeito-Ferreira, M., **Barata, A.**, Loureiro, V. (2011). Influenza del marciume acido sulla vinificazione, la composizione chimica e l'aroma del vino. Workshop Qualitá, Innovazione e Territorio: La ricerca microbiológica per i vini del futuro, Palermo, Castello Utveggio, Italy, February 2011.

Santos, S., **Barata, A.**, M Malfeito-Ferreira, M. e Loureiro, V. (2010). Desenvolvimento *in-vitro* de podridão ácida em uvas na presença de *Drosophila* spp. XVIII Jornadas de Biologia de Leveduras "Professor Nicolau van Uden", Lisboa, Portugal, June 2010.

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Barata, A., Malfeito-Ferreira, M., Loureiro, V. (2008). Vinificação de uvas com podridão ácida. 1as Conferências da Tapada – "A qualidade das uvas", Lisboa, Portugal, October 2008.

Barata, A. (2008). Isolation of wine spoilage yeasts from sour rot damaged grapes. Seminar "Spoilage wine yeasts: between science and technology", Facoltà di Agraria, Università di Pisa, Italy, May 2008.

Barata, A., González S., Malfeito-Ferreira, M., Querol, A. and Loureiro, V. (2007). Sour rot damaged grapes are vectors of wine spoilage yeast species. ISSY26: International Specialized Symposium on Yeasts, Sorrento, Italy, June 2007.

Barata, A., Seborro, F., Malfeito-Ferreira, M., and Loureiro, V. (2007). Identificação de leveduras isoladas de uvas afectadas por melada de cochonilha. 7º Simpósio de Viticultura do Alentejo, Évora, Portugal, May 2007.

I.4 Poster presentations in international conferences

Malfeito-Ferreira, M., **Barata, A.**, Loureiro, V. (2011). Construction of a wine out of grapes affected by sour rot. XXXIV World Congress of Vine and Wine (OIV), "The Wine Construction", Porto, Portugal, June 2011.

Malfeito-Ferreira, M., **Barata, A.** e Loureiro, V. (2011). Podridão ácida: uma doença emergente a ter em conta na viticultura atlântica. I Seminário de Vitivinicultura Atlântica, Construir o Futuro, Vila da madalena, Pico, Açores, Portugal, June 2011.

Barata, A., Campo, E., Malfeito-Ferreira, M., Loureiro, V., Cacho, J., Ferreira, V. (2011). Analytical and Sensorial Characterization of the Aroma of Wines Produced with Sour Rotten Grapes Using GC-O and GC-MS: Identification of Key Aroma Compounds. WAC 2011 – "Wine Active Compounds - International Conference", Beaune, France, March 2011.

Barata, A., Santos, S., M Malfeito-Ferreira, M. e Loureiro, V. (2009). Fruit flies (*Drosophila* spp.) are essential to the development of sour rot in grapes. II International Symposium "Microsafetywine", Wine Microbiology and Safety: from the Vineyard to the bottle, Martina Franca, Italy, November 2009.

Barata, A., Seborro, F., Malfeito-Ferreira, M., and Loureiro, V. (2007). Damaged grapes are vectors of wine spoilage yeast species. Œno 2007: 8th International Symposium of Oenology, Bordeaux, France, June 2007.