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ORIGINAL ARTICLE

Ascomycetous yeast species recovered from grapes damaged by honeydew and sour rot

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Abstract

Aims: To identify ascomycetous yeasts recovered from sound and damaged grapes by the presence of honeydew or sour rot.

Methods and Results: In sound grapes, the mean yeast counts ranged from 3·20 ± 1·04 log CFU g^{-1} to 5·87 ± 0·64 log CFU g^{-1}. In honeydew grapes, the mean counts ranged from 3·88 ± 0·80 log CFU g^{-1} to 6·64 ± 0·77 log CFU g^{-1}. In sour rot grapes counts varied between 6·34 ± 1·03 and 7·68 ± 0·38 log CFU g^{-1}. *Hanseniaspora uvarum* was the most frequent species from sound samples. In both types of damage, the most frequent species were *Candida vanderwaltii*, *H. uvarum* and *Zygoascus hellenicus*. The latter species was recovered in high frequency because of the utilization of the selective medium DBDM (*Dekkera/Brettanomyces* differential medium). The scarce isolation frequency of the wine spoilage species *Zygosaccharomyces bailii* (in sour rotten grapes) and *Zygosaccharomyces bisporus* (in honeydew affected grapes) could only be demonstrated by the use of the selective medium ZDM (*Zygosaccharomyces* differential medium).

Conclusions: The isolation of several species only from damaged grapes indicates that damage constituted the main factor determining yeast diversity. The utilization of selective media is required for eliciting the recovery of potentially wine spoilage species.

Significance and Impact of the Study: The impact of damaged grapes in the yeast ecology of grapes has been underestimated.

Introduction

The study of the microbial communities of grapes is usually addressed to sound berries, being well established that mature sound grapes harbour microbial populations at levels of 10^4–10^6 CFU g^{-1} consisting mostly of yeasts and various species of lactic and acetic bacteria (Fleet 2003). Regarding yeasts, oxidative basidiomycetous yeasts, without any enological interest, like *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 the ascomycetes, apiculate 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). However, the microbial ecology of damaged grapes has been poorly studied and it is not clear if damaged grapes are significant vehicles of dangerous spoilage micro-organisms (Loureiro and Malfeito-Ferreira 2003). Several factors affect the dissemination of yeasts on the berry surface and berry rupture is associated with a sudden increase in microbial load to more than 10^6 CFU g^{-1} (Fleet 2003). In addition, the occurrence of fermentative species, like *Saccharomyces cerevisiae*, is higher when grape skin is damaged and juice escapes onto the grape surface (Mortimer and Polsinelli 1999).

Bearing in mind that wine spoilage species are also fermentative, Loureiro and Malfeito-Ferreira (2003) hypothetized that the knowledge of their dissemination could greatly improve if more attention was given to the microbiology of damaged grapes.

Damaged grapes include those attacked by several types of rot, by insects or hail and heavy rain (as reviewed by Loureiro and Malfeito-Ferreira 2003). Sour rot is characterized by the main role of yeasts in the rotting process and has already been the object of other studies (Bisiach *et al.* 1986; Guerzoni and Marchetti 1987). Another type of damaged grapes is that caused by mealybugs (mostly *Pseudococcus* and *Planococcus* species) excreting honeydew that may not damage grape skin, but a high concentration of sugar is accumulated on the surface. In Portugal, this disease is regarded as the key pest in 15% of the cases by vineyard extensionists (Godinho and Franco 2001). In California and South Africa, the invasive vine mealybug, *Planococcus ficus,* has also emerged as a serious pest (Walton and Pringle 2004; Daane *et al.* 2004). As far as we are aware, the microbial ecology of grapes with honeydew has never been investigated, although the typical black colour because of the growth of filamentous fungi on the grape surface (sooty mold), and the abundant presence of ants is well known. Bearing in mind that mealybugs are phloem feeders and that honeydew is essentially a sugary excretion (Daane *et al.* 2004), it is conceivable that damaged grapes are a favourable habitat for yeast growth, especially osmophilic and osmotolerant species, such as those belonging to the genus *Zygosaccharomyces*.

Spoilage species usually grow slower than other microorganisms in general purpose culture media and, therefore, they tend to be underestimated in samples heavily contaminated (Loureiro *et al.* 2004). Therefore, the purpose of this work is to characterize the yeast flora of damaged grapes using culture media favouring the recovery of wine spoilage species.

Materials and methods

Sample collection and yeast recovery

Vineyards were located in different Portuguese wine regions. Sound and honeydew affected berries or grape bunches were aseptically picked with ethanol-sterilized scissors from different vines randomly selected in the core of the vineyard and transported to the laboratory and stored at 5°C. Vineyard plots were not subjected to fungicide treatments within 2 weaks before sample collection. During the 3-year study, different procedures of sample obtention were performed to ensure the recovery of potentially wine spoilage yeast species.

In the 2002 harvest, about 10 g of sound and damaged berries were aseptically removed from the respective bunches and diluted twofold with peptone water (1 g l^{-1} peptone with 0·1 g l^{-1} Tween 80) in 250-ml Erlenmeyer flasks and vigourously vortexed for 2 min. Decimal dilutions were obtained with peptone water and spreaded on plates (duplicates). Exactly 0·1 ml from each dilution was inoculated on MEP [48 g l^{-1} malt extract agar (Merck, Darmstadt, Germany), 0·5 g l^{-1} biphenyl (Fluka, Buchs, Switzerland) and 0·1 g l^{-1} oxytetracycline (Oxoid, Basingstoke, UK)] and YGCO [40 g l^{-1} yeast extract glucose chloramphenicol agar (Merck), 0·01 g l^{-1} bromophenol blue (Merck), 0·1 g l^{-1} oligomycin (Sigma, St Louis, MO)]. Incubation was carried out at 25°C for 10 days. Colonies were differentiated according to their morphology and representative types were counted. Strains were purified by streaking thrice onto plates of GYP (20 g l^{-1} glucose, 5 g l^{-1} yeast extract, 5 g l^{-1} peptone 20 g l^{-1} agar) and incubated at 25°C for 3 days. Pure cultures were maintained on GYP slants at 4°C.

During the 2003 vintage bunches with different degrees of damage were picked and transported to the laboratory. Four different types of berries were obtained: (i) sound berries from sound clusters; (ii) sound berries from partially damaged clusters; (iii) damaged berries from partially damaged clusters; and (iv) damaged berries from fully damaged clusters. A total of 15 berries of each type were cut from single clusters bearing those types of grapes. Each berry was suspended in single test tubes with different culture media: GYP10 [100 g l^{-1} glucose, 5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5% (v/v) ethanol and 0·1 g l^{-1} chloramphenicol], GYP50 (500 g l^{-1} glucose, 5 g l^{-1} yeast extract and 5 g l^{-1} peptone), DBDM (Rodrigues *et al.* 2001) and ZDM (Schuller *et al.* 2000). After 7 days (GYP10 and GYP50) and 20 days (DBDM and ZDM) of incubation at 25°C, the number of growth-positive tubes was recorded and a loopful was streaked onto the respective solid culture media to purify the grown yeast strains. In parallel, total yeast countings were obtained by collecting single berries, weighing and serially diluting with peptone water. Colonies were counted after incubation on GYP plates after 7 days at 25°C. Walking plates (DBDM agar) of individual mealybugs were prepared in the vineyard and incubated as described before. Isolate purification was performed as in 2002.

In the 2004 harvest two protocols were followed. As in 2003 vintage, a total of 15 berries of the sound or the damaged type were cut from single clusters bearing those types of grapes. Each berry (weighed) was suspended and put in single test tubes with the previous culture media (GYP10, DBDM and ZDM). In the second protocol, total yeast countings and isolates purification were performed from 10 sound or damaged berries of one or two clusters

from the same vine. After weighing, vortexing for 2 min and serially diluting, 0·1 ml was spread (duplicates) onto plates of several culture media: GYP, GYP50, de Mann Rogosa Sharpe (MRS; 52 g l^{-1}; Oxoid), MRSTJ [800 ml l^{-1} of MRS mixed with 200 ml l^{-1} of tomato juice (Oxoid)], DBDM and ZDM. Total countings were obtained after incubation at 25°C for 7 days (GYP, GYP50, MRS and MRSTJ) and 20 days (DBDM and ZDM). Colonies were selected according to their morphology and purified by restreaking on GYP plates.

Biochemical characterization and identification

Purified strains were first characterized regarding the urease reaction. Only the urease-negative strains (*Ascomycetes*) were identified by restriction analysis of the 5·8S-intervening sequence (ITS) rDNA. This region was amplified using the primers ITS1 and ITS4 (Esteve-Zarzoso *et al.* 1999). Cells were collected from a fresh colony and resuspended in the polymerase chain reaction (PCR) mixture. The suspension was heated in a Mastercycler personal (Eppendorf, Hamburg, Germany) thermocycler at 95°C for 15 min. Then, two units of DNA polymerase (Biotools, Madrid, Spain) was added to each tube. PCR conditions were: 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 followed by an extension cycle at 72°C for 10 min. The amplified DNA (10 μl or 0·5–10 μg of DNA) was digested with three restriction endonucleases, *Cfo*I, *Hae*III and *Hin*fI (Roche Molecular Biochemicals, Mannheim, Germany), according to the supplier's instructions. The amplified PCR product and their corresponding restriction fragments were separated on 1·4% and 3% agarose gels, respectively. 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 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 and NL-4 (O'Donnell 1993). PCR reactions were performed in a PROGENE thermocycler (Techne, UK) as follows: a first denaturation step at 95°C for 5 min, followed by 40 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 30 s, with a final extension of 10 min at 72°C. The PCR products were cleaned with the Perfectprep Gel Cleanup (Eppendorf) and then directly sequenced using the BigDye terminator v3·1 cycle sequencing kit (Applied Biosystems, Warrington, UK), following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer, model 310. 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 MEGA version 3·1 software (Kumar *et al.* 2004), 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 performed using biochemical reactions (Kurtzman and 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°C and growth on DBDM plates. *Zygosaccharomyces fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii*, were differentiated by growth on GYP agar plates incubated at 37°C and 40°C, and growth with D-galactose and 0·01% cycloheximide.

Results

Yeast quantification in sound and damaged grapes

In the vintage of 2002, sampling of about 10 g of sound- and honeydew-damaged grapes was performed in several vineyards during the ripening period from the 24th of July to the 11th of September (2 or 3 weeks before harvesting and just before harvesting). We did not find a clear tendency to higher yeast counts with grape maturation (results not shown), therefore, average yeast countings were determined as a function of grape health and culture media used (Table 1). Sound grapes harboured 3·72 ± 0·91 log CFU g^{-1} (MEP medium) or 3·20 ± 1·04 log CFU g^{-1} (YGCO medium), against 3·96 ± 1·34 log CFU g^{-1} (MEP) or 3·88 ± 0·80 log CFU g^{-1} (YGCO) in honeydew-damaged grapes. The total log mean yeast counts were lower on sound grapes than in honeydew grapes, but the mean values were not statistically different between both types of grapes and between each culture media (one-way ANOVA, $P < 0·05$). By the end of the harvest season till the postmaturation period (26 September–30 November) grapes damaged by sour rot appeared in other vineyards and we had the opportunity to pick only damaged samples. The mean log CFU g^{-1} were 7·68 ± 0·38 (MEP) and 6·34 ± 1·03 (YGCO) which were statistically higher than the average of sound- or honeydew-affected grapes.

In 2003, grape sampling was different from 2002. A single vineyard, where both types of damages were present, was chosen and countings corresponded to the analysis of single berries using one culture medium (GYP). In the first sampling date (3 September) only honeydew-affected grapes were observed and the results were statistically equivalent, yielding 3·69 ± 0·22 log CFU g^{-1} for sound grapes and 3·32 ± 1·40 log CFU g^{-1} for damaged grapes.

Table 1 Mean yeast counts and standard deviation (log CFU g^{-1}) of sound and damaged grapes obtained during the 3-year study (number of samples are shown in brackets)

Harvest	Grape varieties	Date	Medium*	Sound grapes	Honeydew grapes	Sour rot grapes
2002	Several	Several	MEP	3·72 ± 0·91 (9)	3·96 ± 1·34 (18)	7·68 ± 0·38 (4)
			YGCO	3·20 ± 1·04 (8)	3·88 ± 0·80 (15)	6·34 ± 1·03 (13)
2003	Red (Periquita)	3 September	GYP	3·69 ± 0·22 (2)	3·32 ± 1·40 (2)	– †
		16 September		4·48 ± 0·35 (2)	4·77 ± 1·28 (2)	–
				5·93 ±1·69 (2)	–	5·71 ± 2·50 (2)
2004	White (Bical)	7 August and 1 September	GYP	5·87 ± 0·64 (4)	6·45 ± 0·56 (4)	–
			GYP50	5·78 ± 0·91 (2)	6·09 ± 1·44 (2)	–
			MRS	5·75 ± 1·06 (2)	6·51 ± 0·82 (2)	–
			MRSTJ	5·80 ± 0·88 (2)	6·64 ± 0·77 (2)	–
			DBDM	4·11 ± 0·74 (2)	4·49 ± 1·41 (2)	–
		7 August	ZDM*	<1 (1)	<1 (1)	–
		1 September	ZDM†	<1 (1)	1·93 (1)	–

*MEP, malt extract agar; YGCO, yeast extract glucose chloramphenicol agar; GYP, glucose, yeast extract and peptone agar; MRS, de Mann Rogosa Sharpe medium; MRST J, 800 ml l^{-1} of MRS mixed with 200 ml l^{-1} of tomato juice.
†Damage not present.

On 16th September (before harvest) also statistically equal average values of 4·48 ± 0·35 and 4·77 ± 1·28 log CFU g^{-1} were obtained for sound and honeydew grapes, respectively. In this date a group of vine plants was observed to harbour grapes affected only by sour rot. In Table 1 the results are presented as the average of each health status which do not show the different microbial loads observed. In fact, sound berries collected from sound bunches yielded 4·73 log CFU g^{-1} while sound berries from partially damaged bunches yielded 7·12 log CFU g^{-1}. Damaged berries from partially damaged bunches harboured 7·48 log CFU g^{-1} while berries from fully damaged bunches contained 3·94 log CFU g^{-1}.

In 2004, samples of about 10 g of berries were obtained in one vineyard where only honeydew damage was observed. Within each type of grapes, the media GYP, GYP50, MRS and MRSTJ yielded statistically equivalent mean log counts (one-way ANOVA, $P < 0·05$) (Table 1). The average results of DBDM, although low, were not statistically different from these because of the high variability of the results. Significant differences were produced by the selective medium ZDM, which only enabled yeast growth in one sample obtained before harvest (Table 1). Considering only the former four culture media, the mean log counts of sound grapes ranged from 5·75 ± 1·06 (MRS medium), to 5·87 ± 0·64 (GYP medium), while damaged grapes yielded mean log counts of 6·09 ± 1·44 (GYP50 medium) to 6·64 ± 0·77 (MRSTJ medium).

Yeast identification

The strains recovered from grape samples were first tested for the urease reaction to estimate the proportion of *Ascomycetes* (urease-negative) and *Basidiomycetes* (urease-positive). This test gives positive results for two ascomycetous species and one genera (Kurtzman and Fell 1998). One species is *Schizosaccharomyces pombe* which is easily recognized by its morphology and was not detected in this survey. The other yeasts, *Yarrowia lipolytica* and *Lipomyces* spp. are not regarded as contaminants of grapes (Fleet *et al.* 2002) and do not have technological significance in winemaking (Loureiro and Malfeito-Ferreira 2003). Table 2 shows the results according to the culture media and the type of grapes. The media MEP, YGCO, GYP, GYP10, GYP50, MRS and MRSTJ yielded 36%, 27% and 55% of ascomycetous yeasts in sound, honeydew and sour rot samples, respectively. The selective medium DBDM yielded higher proportions of ascomycetes (52%, 37% and 100%, respectively). The rather high selective ability of ZDM was shown by the low number of recovered strains, without allowing the detection of basidiomycetes. Overall, concerning the grape's health, the proportion of ascomycetes was slightly lower in honeydew grapes (32%) than in sound grapes (38%), while sour rot damage resulted in 58% of the ascomycetes. Basidiomycetous yeasts are not regarded as relevant to winemaking and were not further investigated.

Identifications of ascomycetous species was first performed by restriction fragment length polymorphism (RFLP) of 5·8S-ITS rDNA and results are shown in Table 3. A total of 21 different restriction patterns were found, from which it was possible to assign 17 species. This typing method was not able to identify four different patterns, leading to further characterization by sequencing or biochemical tests.

Table 2 Distribution of ascomycetous strains (urease-negative) according to the isolation culture media and grape health, during the 3-year study

Medium	Number of strains isolated from sound samples	*Ascomycetes*	Number of strains isolated from honeydew samples	*Ascomycetes*	Number of strains isolated from sour rotten samples	*Ascomycetes*
MEP, YGCO, GYP, GYP10, GYP50, MRS and MRSTJ	154	36%	158	27%	64	55%
DBDM	29	52%	62	37%	3	100%
ZDM	0	0%	8	100%	2	100%
Total *Ascomycetes*	183	38%	228	32%	69	58%

MEP, malt extract agar; YGCO, yeast extract glucose chloramphenicol agar; GYP, glucose, yeast extract and peptone agar; MRS, de Mann Rogosa Sharpe medium; MRST J, 800 ml l^{-1} of MRS mixed with 200 ml l^{-1} of tomato juice.

Table 3 Strain identification by restriction fragment length polymorphism of the 5·8S-intervening sequence region obtained by using the restriction endonucleases *Cfo*I, *Hae*III and *Hin*fI, and accession number of the 26S sequences of the GenBank (percentage of similarity between brackets)

Species	Amplified product (bp)	*Cfo* I	*Hae* III	*Hinf* I	GenBank access number
Candida amapae	700	580 + 80	650	320 + 320	–
Candida apicola	510	220 + 190 + 100	400 + 90	230 + 130 + 130	–
Candida diversa	410	140 + 140 + 90	380	190 + 180	U71064 (100%)
Candida methanosorbosa	700	320 + 290 + 80	550 + 190	370 + 180 + 120	U76345 (100%)
Candida stellata	470	200 + 100 + 100	460	230 + 230	–
Candida vanderwaltii	490	210 + 120	480	240 + 240	–
Candida viswamathii	550	290 + 260	450 + 90	270 + 190 + 90	–
Hanseniaspora uvarum/Hanseniaspora guilliermondi/Dekkera anomala	800	340 + 340 + 120	790	380 + 210 + 180	–
Hanseniaspora osmophila	800	270 + 150 + 150	460 + 120 + 100	390 + 370	–
Issatchenkia terricola	450	130 + 100 + 90 + 85	290 + 125	240 + 105 + 105	–
Metschnikowia pulcherrima	400	210 + 190 + 90	280 + 100	200 + 190	
Pichia caribbica	625	300 + 265	400 + 115 + 90	320 + 300	AY187283 (100%)
Pichia fermentans	450	170 + 100 + 100 + 80	340 + 80	250 + 200	–
Pichia guilliermondi	650	300 + 270	400 + 120 + 80	320 + 300	–
Pichia kluyveri	420	180 + 110 + 80 + 50	390	250 + 210	–
Saccharomycopsis vini	690	550	380 + 150 + 80	290 + 180 + 130	–
Torulaspora pretoriensis	880	380 + 330 + 120	880	380 + 210 + 150	–
Zygoascus hellenicus	650	320 + 320	630	340 + 170 + 120	–
Zygosaccharomyces bailii	790	320 + 270 + 95 + 95	690 + 90	340 + 225 + 160 + 55	–
Zygosaccharomyces bisporus	790	300 + 280 + 100 + 90	690 + 90	390 + 230 + 160	–
Zygosaccharomyces fermentati/Zygosaccharomyces cidri/Kluyveromyces thermotolerans/Kluyveromyces waltii	680	315 + 285 + 95	310 + 215 + 90 + 90	355 + 345	EF463105·1 (99% with *Lachancea* spp.) AB087397·1 (98% with *Zygosaccharomyces* spp.)

One restriction pattern corresponded to *H. uvarum, H. guilliermondii* and *D. anomala*. These strains grew at 37°C and did not grow on plates of DBDM, indicating the presence of *Hanseniaspora uvarum* or its anamorph *Kloeckera apiculata*. Another group of strains showing similar restriction profiles included the species *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii*. Their 26S rRNA gene sequencing results yielded *Lachancea* spp.

(99% similarity) and *Zygosaccharomyces* spp. (98% similarity) as the closest relatives (Table 3). The genus *Lachancea* includes the aforementioned four species with similar restriction profiles. These strains displayed positive growth with D-galactose and at 37°C, and did not grow under 40°C and 0·01% cycloheximide. These physiological results are consistent with the old epithet *K. thermotolerans* and not with *Z. cidri, Z. fermentati* or *K. waltii*. Therefore, we assigned those strains to *Lachancea thermotolerans*.

Finally, the species *Candida diversa*, *Candida methanosorbosa* and *Pichia caribbica* were identified by 26S sequencing because of the absence of species assignment for their restriction profiles in the Yeast-id database (http://www.Yeast-id.com) (Table 3).

Yeast species dissemination in sound and damaged grapes

The different sampling approaches used in the three vintages determined the yeast diversity. Dissemination studies should include a large number of samples, in several years and, in ecological terms, the isolation of a species is more meaningful than its quantification (Lachance 2003). This reasoning also holds true in our case, where we are more interested in establishing the presence of wine spoilage species in grapes than to quantify its isolates. Therefore, we present in Table 4 the species identified in grape samples, consisting of one sample of about 10 berries in 2002 and 2004 or of one berry in 2003, according to the health status and the culture media.

In sound grapes a total of 10 species were identified, *H. uvarum* being the most frequent (11 samples in the three vintages). *Metschnikowia pulcherrima* was present in three samples (2003 and 2004). *Candida* spp. was represented by six species in six samples, during the three vintages. *Pichia guilliermondii* and *Torulaspora pretoriensis* were recovered from one sample each, in 2004.

In honeydew-damaged grapes a total of 14 species were present, where *Candida vanderwaltii*, *H. uvarum* and *Zygoascus hellenicus* (teleomorph of *Candida steatolytica*) were the most frequent. Other *Candida* spp., *Issatchenkia terricola*, *M. pulcherrima* and *Saccharomycopsis vini* were sporadicly isolated. Also infrequent was the recovery of *L. thermotolerans*, *T. pretoriensis* and *Z. bisporus*.

In sour rot-damaged grapes a total of eight species were recovered, being *C. vanderwaltii*, *H. uvarum* and *Zygoascus hellenicus* the most frequent. Other *Candida* spp., *Hanseniaspora osmophila*, *I. terricola* and *M. pulcherrima* were

Table 4 Number of positive samples for each species in the 3-year study according to the type of damage and the culture medium

		General purpose medium*			DBDM			ZDM		
Species	Vintage year	Sound	Honeydew	Sour rot	Sound	Honeydew	Sour rot	Sound	Honeydew	Sour rot
Candida amapae	2004				1					
Candida apicola	2004				1					
Candida diversa	2003	1								
Candida methanosorbosa	2004		1							
Candida stellata	2002, 2003	1		2						
Candida vanderwaltii	2002, 2003, 2004	1	5	4						
Candida viswamathii	2004				1					
Hanseniaspora uvarum	2002, 2003, 2004	11	6	4						
Hanseniaspora osmophila	2002			1						
Issatchenkia terricola	2003			1						
Lachancea thermotolerans	2004		2							
Metschnikowia pulcherrima	2003, 2004	3	1	1						
Pichia caribbica	2003		2							
Pichia fermentans	2003		1							
Pichia guilliermondii	2004				1	1				
Pichia kluyveri	2003		1							
Saccharomycopsis vini	2004					1				
Torulaspora pretoriensis	2004	1	2							
Zygoascus hellenicus	2003, 2004			1		9	4		2	1
Zygosaccharomyces bailii	2003									1
Zygosaccharomyces bisporus	2004								1	

*Culture media: MEP, malt extract agar; YGCO, yeast extract glucose chloramphenicol agar; GYP, glucose, yeast extract and peptone agar; GYP10; GYP50; MRS, de Mann Rogosa Sharpe medium; MRST J, 800 ml l^{-1} of MRS mixed with 200 ml l^{-1} of tomato juice.

seldom isolated. Also isolated from only one sample was *Z. bailii*.

Concerning the selective culture media DBDM and ZDM, the results presented in Table 4 showed that they were essential to reveal the high frequence of *Zygoascus hellenicus* in damaged grapes, and the presence of *Z. bisporus* in honeydew grapes and of *Z. bailii* in sour rotten grapes. In addition, two DBDM walking plates of mealybugs collected in one vineyard in 2003, demonstrated the unique presence of *Zygoascus hellenicus* (results not shown).

Discussion

The total yeast counts of sour rotten grapes were significantly higher than those of the healthy grapes, as already observed by Guerzoni and Marchetti (1987). This result may be explained by the release of juice after skin rupture supporting the growth of higher yeast numbers and increasing the proportion of *Ascomycetes*. Our 2003 results showing the influence of bunch health on berry yeast counts emphasizes the need for careful berry sampling. On one hand sound berries must be obtained from fully sound bunches, otherwise countings may be overestimated for sound berries. On the other hand, the lower counts of damaged berries picked from fully rotten bunches suggests that, as rotting proceeds, conditions become more stressful for yeast proliferation.

As far as we are aware, this study provides the first yeast quantification for honeydew grapes, showing higher, but not statistically different average values from sound grapes. This result is surprising because honeydew is rich in sugar and aminoacids that could stimulate yeast multiplication. Our hypothesis is that honeydew also bears antimicrobial compounds, similar to those present in honey (Bogdanov 2006), although inhibiting yeast growth.

Honeydew and sour rot grapes also differ in the proportion of ascomycetous yeasts present. While honeydew grapes bear proportions of *Ascomycetes* similar to those of the sound grapes, sour rot grapes yielded higher percentages of this group of yeasts.

The influence of grape soundness on yeast ecology was mostly observed by the increase in yeast diversity, specially related to the high recovery rate of *Zygoascus hellenicus* and with the rare presence of *Z. bailii* and *Z. bisporus*. Taking into consideration the species isolated during the three vintages, 10 ascomycetous species were isolated from sound grapes while 17 species were present in both types of the damaged samples.

In healthy and damaged grapes, the most frequent species was *H. uvarum* (teleomorph of *K. apiculata*). Less frequent, but yet common, isolates belong to the genus *Candida* spp. and *Pichia* spp. and to the species *M. pulcherrima*. These genus or species are common contaminants of grapes (Fleet 2003) and their isolation was not unexpected. The effect of damage on the proportion of these most common genus or species appears to be the decrease in the predominance of *H. uvarum* and *M. pulcherrima* and the higher proportion of *C. vanderwaltii*. These genera are not regarded as dangerous wine spoilers (Loureiro and Malfeito-Ferreira 2003).

The species *Zygoascus hellenicus*, or its anamorph, *C. steatolytica* (Smith *et al.* 2005) has been seldom 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 but not from sound grapes. Our results showed its isolation from several locations, in two different vintages, only from damaged grapes and from mealybug walking plates. Its frequent recovery was mostly because of the utilization of the selective DBDM media. We are not aware of wine spoilage because of this species but it would be interesting to understand its isolation from damaged grapes in proportions higher or similar to the most common *H. uvarum*, *M. pulcherrima* or *C. vanderwaltii*.

The selective medium DBDM was used having in mind the possible recovery of the 4-ethylphenol-producing species *Dekkera bruxellensis*. We have not found this species but another 4-ethylphenol-producing species, *P. guilliermondii*. When reported, this species (or its anamorph *C. guilliermondii*) is regarded as a rare contaminant of grapes and musts (Jolly *et al.* 2003). However, we have already isolated it from grapes in the vineyard, grape juices, grape stems, wines and insects using DBDM (Dias *et al.* 2003; Martorell *et al.* 2006). Therefore, although with low frequency, it seems to be a common inhabitant of wine-making environments. It has the ability to produce 4-ethylphenol in grape juices before fermentation but does not seem to spoil wines by volatile phenol production (Barata *et al.* 2006).

Torulaspora pretoriensis was isolated only in 2004 from sound and damaged grapes and has not been mentioned as a grape contaminant. It belongs to a genus where the species *Torulaspora delbrueckii* is known as a wine spoiler (Minarik 1983) but we are not aware of spoilage outbreaks caused by *T. pretoriensis*.

The species *L. thermotolerans* was only isolated in 2004 from damaged grapes. This new epithet resulted from the gathering of several species showing closely related DNA sequences (*Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii*) (Kurtzman 2003). Xufre *et al.* (2006) reported the isolation of *K. thermotolerans* from white grape juices. *Zygosaccharomyces fermentati* (or *Z. cidri*) was reported in

the initial step of sherry fermentation at the winery level (Esteve-Zarzoso *et al.* 2001) and has the ability to produce volatile compounds in synthetic sherry-like medium (Freeman *et al.* 1977). Romano and Suzzi (1993) isolated *Z. fermentati* at the end of laboratorial grape juice fermentation obtained by the winery crushing of grapes. To the best of our knowledge, there are no reported wine spoilage outbreaks because of these four sibling species. However, the afinity with *Zygosaccharomyces* spp. justifies the future investigation of any possible spoilage ability.

In technological terms, the most significant result of our work was related to the isolation of the osmotolerant and acidophilic species, *Z. bailii* and *Z. bisporus*. This was achieved by the use of the selective media ZDM. This medium was directed to *Z. baili*, which is regarded as one of the most dangerous wine spoilage species (Loureiro and Malfeito-Ferreira 2003). The frequency of *Z. bisporus* isolation from foods is much lower than that of *Z. bailii*, but it has a similar ability to cause food spoilage and it is also preservative-resistant (Pitt and Hocking 1999). Jolly *et al.* (2003) reported the rare isolation of *Z. bailii* and *Zygosaccharomyces* spp. from sound Chardonnay grapes. We are not aware of reports mentioning the recovery of *Z. bisporus* from grapes but Romano and Suzzi (1993) isolated three strains from grape juices, among 29 strains of *Z. bailli* or *Z. fermentati*. In spite of the ability of *Z. bisporus*, isolated from sherry film, to resist to sorbic acid and sulfur dioxide (Splittstoesser *et al.* 1978) and to produce odorous acyloins in sherry wines (Neuser *et al.* 2000), it is not usually regarded as a dangerous wine spoilage yeast (Loureiro and Malfeito-Ferreira 2003). As with *L. thermotolerans*, future studies might elucidate its role in wine spoilage.

The sampling protocol determined that berries with different health levels were collected from the same bunch or from the same vine and so environmental factors (e.g. grape varieties, vineyards, regions, fungicides and climatic conditions) should exert the same effect on the species diversity of both types of grapes. This fact strongly indicates that grape damage constituted 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 *Zygoascus hellenicus* and by the scarce, but technologically relevant, isolation of *Z. bailii* and *Z. bisporus*. This change may be explained by the release of grape juice in sour rot or by the composition of honeydew excreted by mealybugs on vines. Although the latter is not known (Daane, personnal communication), it should be similar to others consisting of aqueous solutions of various sugars (e.g. glucose, fructose, trehalose and melezitose) and amino acids (Fischer and Shingleton 2001; Fischer *et al.* 2002; Wäckers 2005). The chemical composition of honeydews varies with insect species (mainly aphids) and host plants, being reported sugar contents higher than 1 mol l^{-1} (Karley *et al.* 2005) or as high as 140 g l^{-1} together with amino acids concentrations up to 22·6 mmol l^{-1} (Fischer *et al.* 2002). The mealybug *Saccharococcus sacchari* in sugarcane stems produces acidic honeydew with pH values of about 3 (Ashbolt and Inkerman 1990). These authors showed that the epiphytic microbiota was dominated by acetic bacteria and acidophilic yeasts. In addition, ants are commonly associated with vine mealybugs (Daane *et al.* 2004) and prefer melezitose (Fischer and Shingleton 2001). The conjunction of these factors, if valid in honeydew excreted by mealybugs on grape surface, may explain the colonization by *Zygosaccharomyces* spp. which are characterized by osmotolerant, fructophilic and acidophilic species. Although present in low numbers, these species may be veiculated into the winery by the grapes during harvesting, and further colonize wines and equipments.

Our results concur with the statement of Fleet (2003) that the impact of damaged grapes in the yeast ecology of wine-related environments has been underestimated so far. We showed that the use of selective media is crucial to characterize yeast ecology but this observation is not new. Early ecological works on wine environments had already reflected the need for selective media to recover minority and/or slow-growing species (van der Walt and van der Kerken 1961; Davenport 1974; Florenzano *et al.* 1977). This awareness was not profited by recent ecological works which show a strong emphasis on yeast identification by molecular biological methods. As these methods depend on cell growth, if only general purpose media are used, there is no deeper insight on species diversity. Even the recent direct molecular techniques are not sensitive enough to recover minority species (Prakitchaiwattana *et al.* 2004) and so are not able to detect minor, but technologically significant, differences in the ecology of damaged grapes. Therefore, to ascertain the extent of the impact of damage on yeast diversity is required to use selective media directed to the recovery of minority species, among which stand the potentially spoiling species, as hypothetized by Loureiro and Malfeito-Ferreira (2003). Then, reliable identification molecular techniques are indispensable for the study of a particular yeast community (Lachance 2003). In addition, our conclusions were based on a high number of analysed samples, in different vintages and different vineyards, to minimize the effect of the intrinsic variability of yeast populations on grapes. In future works we will check if some yeast species can be used as indicators of grape damage, irrespective of the vineyard, grape variety or climatic conditions.

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