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Changes in sour rotten grape berry microbiota during ripening and wine fermentation

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ABSTRACT

This study investigated the microbiota of sour rotten wine grapes and its impact on wine fermentations. Yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) were enumerated and identified on sound and sour rot grapes during the ripening stage. The alteration of the ecological balance induced by sour rot was particularly evidenced 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 than those found in sound grapes. LAB populations were low detected from both healthy and sour rotten grapes. The yeast species *Issatchenkia occidentalis*, *Zygoascus hellenicus* and *Zygosaccharomyces bailii* and the AAB species *Gluconacetobacter hansenii*, *Gluconacetobacter intermedius* and *Acetobacter malorum*, were recovered from damaged grapes and resulting grape juices in the winery. *Acetobacter orleaniensis* and *Acetobacter syzygii* were only recovered from sour rotten grapes.

Dekkera bruxellensis and *Oenococcus oeni* were only recovered after wine fermentation induced by starter inoculation, irrespective of grape health, probably originating from cellar environment. After malolactic fermentation, racking and sulphur dioxide addition the only remaining species were the yeast *Trigonopsis cantarellii* and *Saccharomyces cerevisiae*, independently of the grape health status.

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 et al., 1986; Wolf et al., 1990; Zoecklein et al., 1992). Rotten bunches have a strong and pungent odour 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 et al., 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,b). 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,b; Bisiach et al., 1982; 1986; Guerzoni and Marchetti, 1987; Guerzoni and Marchetti, 1982; Marchetti et al., 1984). 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* (grey 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 grey 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.

Previous work from our laboratory, based on accurate berry sampling, has shown that grape damage by sour rot induced dramatic

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increases on yeast counts and number of species since the beginning of grape ripening (Barata et al., 2008a,b). However, the knowledge of the complex microbial changes 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 (i) to investigate the changes 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 (ii) 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 38° 42′31.57″ N and longitude 9° 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 3 different ripening phases: i) after veraison (10 August 2007); ii) middle of ripening (22 August 2007); and iii) harvest time (7 September 2007). A total of 3 bunches from each vine set were aseptically collected and transported to the laboratory in sterile bags at 5 °C. 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.

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 38° 42′26.36″ N and longitude 9° 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 sulphur 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 h) was performed at cellar temperature (18 ± 2 °C), 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 20 L 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; and iii) after MLF and addition of sulphur dioxide.

2.3. Microbiological analyses

2.3.1. 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 and ZDM for the isolation of *Dekkera*/*Brettanomyces* and *Zygosaccharomyces* species, respectively, or other non-*Saccharomyces* species as previously reported by (Barata et al., 2008a,b). Total yeast enumeration was obtained from the GYP plates, recording the number of colony forming unit (CFU) counts.

In case of *Dekkera*/*Brettanomyces* species, a secondary procedure was performed using the enrichment medium EBB (Renouf and Lonvaud-Funel, 2007). 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 °C in an 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 °C 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 two to five representative isolates from each morphology were selected for identification. When colonies from the same type were identified as different species, the respective proportion was changed accordingly. 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 °C and subcultured every month.

2.3.2. 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 °C 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 microscopical observation.

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 °C up to 8 days. The different colony/cell morphologies found in each positive tubes of each dilution were registered and representative isolates, as mentioned before for yeasts, 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 °C 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.

2.3.3. 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 were 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. The usual species contaminating fermentations are not inhibited by 10 mg/L cycloheximide (Rodrigues et al., 2001). Plates were incubated at 25 °C 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.

2.4. 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 re-suspended 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) thermocycler using the primers (ITS1 and ITS4) and conditions described in Esteve-Zarzoso et al. (1999). Lengths 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 (Kurtzman and Robnett, 1998). 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 *H. uvarum*/*H. guilliermondii*/*H. opuntiae* was differentiated by growth on GYP agar plates incubated at 37 °C (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 *Lachancea* clade former species (*L. fermentati*, *L. cidri*, *L. thermotolerans* and *L. 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 (Kurtzman, 2003; Kurtzman and Fell, 1998). *Pichia deserticola* was differentiated from *Candida pseudolambica* by growth on GYP agar plates incubated at 37 °C 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).

2.5. 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 re-suspended in 50 μL of lysis solution (0.25% sodium dodecyl sulphate, 0.05 N NaOH) and incubated at 100 °C for 15 min (boiling water bath). The suspension was cooled at room temperature, centrifuged (5000 *g*, 4 °C, 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 region 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 10 μL 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 cerevisiae*/*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.

2.6. 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 °C 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 *Fsp*BI 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 establish 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.

2.7. 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 (Table S1).

3. Results and discussion

3.1. Isolation and identification of yeasts, AAB and LAB

A total of 203 yeasts isolates, recovered from sound and rotten grape samples during the 3 ripening phases, and 97 yeast isolates obtained from grape musts and wine samples throughout the 3 winemaking phases, were selected for identification. A total of 15 species were successfully identified by comparison of the *Cfo*I, *Hae*III and *Hinf*I restriction profiles contained in the Yeast-id database (see Table S1 in supplementary material). The *Dra*I endonuclease enabled the identification of *C. zemplinina* while *H. uvarum* and *H. guilliermondii* were distinguished by *Dde*I and *Dra*I restriction profiles. *P. deserticola* and *Lachancea thermotolerans* were identified by additional biochemical tests. The remaining 10 species were identified by sequencing of D1/D2 domains of the large subunit 26S rRNA gene and respective accession numbers deposited in GenBank (see Table S1).

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). 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) 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) (see Table S2 in supplementary material). These identifications, except for *A. orleaniensis*; 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. These molecular approaches allowed the obtention of 6 different restriction patterns (III, IV, V, VIII, IX and X) associated to the species *A. cerevisiae* (III), *A. malorum* (IV), *A. orleaniensis* (V), *Ga. europaeus* (VIII), *Ga. intermedius* (IX) and *Ga. saccharivorans* (X) (see Table S2).

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 *Fsp*BI and *Mse*I endonucleases. Two of them (XIV and XV) matched with the characteristic patterns of *Lactobacillus brevis* and *O. oeni*, 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* sp. for the pattern XIII (see Table S2).

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 *Kocuria kristinae* (see Table S2). Table S3 in supplementary material, shows the patterns obtained with each restriction endonucleases 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.

3.2. Sour rot effect on microbial populations during grape ripening

The population changes in 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 3 different bunches collected from 3 different vines, and analysed in duplicate at each ripening phase. The standard deviation of the duplicates was less than 15% of the mean (results not shown).

3.2.1. Changes in yeast populations 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 1). In healthy grapes, 7 ascomycetous and 3 basidiomycetous yeasts species were recovered during the 3 maturation phases. Regarding basidiomycetes, the red to pinkish ballistoconidia-forming *Sporobolomyces roseus* species was isolated since veraison until harvest, while the non pigmented

Table 1
Changes of yeast 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).

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). Seven ascomycetous yeast species were also recovered from sound grape samples during maturation. *Aureobasidium pullulans* was the single species isolated in every ripening phase and in all vine sets. This ascomycetous yeast-like fungus is a common inhabitant 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 refer to *A. pullulans* as the main species isolated from immature, mature, and both damaged and undamaged grapes. *C. 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 homogeneous distribution on grape surfaces, since they were not detected in some vine sets. 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 burtonii*, detected in immature sound grapes and *S. vini*, in both immature and mature grapes (Table 1).

In the case of sour rot damaged grapes, total yeast counts and yeast diversity species in rotten grape samples were much higher than those found in sound grapes. Total yeast 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). 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. A total of 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 (Table 1).

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 applied. Indeed, *Z. hellenicus* and *Z. meyerae*, were only recovered in DBDM and ZDM media, while the minority species *C. stellimalicola*, *H. guilliermondii* and the 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. We have already isolated it from sound, sour rotten and honeydew damaged grapes in the vineyard (Barata et al., 2008a,b), 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.

Z. hellenicus and *Issatchenkia* spp. have been consistently isolated only from sour rotten grapes in a 4-year 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. Interestingly, Nisiotou and Nychas (2007) in a survey of yeast populations residing on healthy and *Botrytis*-infected grapes, found that the grey rotten grape samples had higher yeast populations and were also associated to the presence of *I. occidentalis*, *I. terricola* and *Z. bailii*.

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). In addition, *D. bruxellensis* was not isolated from grapes despite the use of EEB selective enrichment medium.

3.2.2. AAB and LAB changes during ripening

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

Table 2 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 during a vintage characterized 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 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 (Table 2). 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). .

The recently new proposed *Ga. saccharivorans* (Lisdiyanti et al., 2006) and *A. malorum* have been detected in grapes by Valera et al. (2011). *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).

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 2). 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

Table 2
Changes in bacterial 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.

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 (*O. oeni*, *Lactobacillus* spp., *Pediococcus* spp.) were not detected on grape berries. 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).

3.3. 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.

3.3.1. Changes in yeast populations during wine fermentation

The overall results of this survey are listed in Table 3. 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 1), 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 3). This difference should be due either to the contamination of injured berries hidden in apparently sound bunches or to equipment contamination.

A total of 9 ascomycetous yeast species were recovered from the sound must sample (VAT) (Table 3). 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 (see Table 1). 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 3).

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. 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,b). 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.

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

Table 3
Evolution of yeasts, acid bacteria (AAB) and lactic acid bacteria (LAB) populations during wine fermentations containing sound and 30% rotten grapes. Results reported as log CFU/ml (yeasts) and log MPN/mL (bacteria).

Organism	Species	Medium	100% sound grapes vinification[a]					30% Sour rot vinification[a]				
			VAT	AF		MLF		VAT	AF		MLF	
				30[b]	100[b]	30[b]	100[b]		30[b]	100[b]	30[b]	100[b]
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 yeast counts (CFU/ml)	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[c]	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.
[b] Initial SO_2 concentration (mg/kg) added on initial vatting phase.
[c] nd — not detected in 1 mL of wine sample.

typing strain characterization was performed, we cannot 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 3). 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).

3.3.2. AAB and LAB changes 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 3). However, low counts of *Ga. hansenii* and *A. malorum* were also obtained. 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. This species was already mentioned by du Toit and Lambrechts (2002) and González et al. (2004) in wines. *Ga. saccharivorans* was isolated by Kato et al. (2011) from white wines. 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 3 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* while *A. aceti* was reduced by about 2 log counts.

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. *O. oeni* was not isolated from freshly grape musts where only *Enterococcus* spp. were present (Table 3) 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. 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, including *O. oeni*, were detected in 1 mL of all analysed wine samples (Table 3).

4. Conclusions

The results obtained in this work support our view that grape damage is the main driving force altering berry microbiota. Grape damage increases sugar accessibility and creates 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ijfoodmicro.2011.12.029.

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