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Influence of the farming system on the epiphytic yeasts and yeast-like fungi colonizing grape berries during the ripening process

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ABSTRACT

Grape berries are colonized by a wide array of epiphytic microorganisms such as yeast and filamentous fungi. This microbiota plays a major role in crop health and also interferes with the winemaking process. In this study, culture-dependent and -independent methods were used to investigate the dynamics and diversity of the yeast and yeast-like microorganisms on the grape berry surface during maturation and the influence of cropping systems in this microflora. The results showed a significant impact of both the farming system and the maturity stage on the epiphytic yeast and yeast-like community. A quantitative approach based on counting cultivable populations indicated an increase in the yeast and yeast-like population during the grape ripening process, reaching a maximum when the berries became overripe. The cultivable yeast and yeast-like population also varied significantly depending on the farming system. Microorganism counts were significantly higher for organically- than conventionally-farmed grapes. The yeast and yeast-like community structures were analysed by culture independent methods, using CE-SSCP. The results revealed changes in the genetic structure of the yeast and yeast-like community throughout the ripening process, as well as the impact of the farming system. Copper-based fungicide treatments were revealed as the main factor responsible for the differences in microbial population densities between samples of different farming systems. The results showed a negative correlation between copper levels and yeast and yeast-like populations, providing evidence that copper inhibited this epiphytic community. Taken together, our results showed that shifts in the microbial community were related to changes in the composition of the grape–berry surface, particularly sugar exudation and the occurrence of copper residues from pesticide treatments.

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1. Introduction

Grape berries are colonized by a complex, dynamic microbial ecosystem, which encompasses a wide array of epiphytic microorganisms, such as bacteria, yeast, and filamentous fungi (review by Barata et al., 2012). This microbiota plays a major role in crop health and also interferes with the winemaking process, potentially having major repercussions on wine quality, as reported by Barbe et al. (2001), Nisiotou et al. (2011), and Verginer et al. (2010).

The ecology of filamentous fungi and yeast colonizing grapes has been widely studied due to their impact on wine quality (review by Pretorius, 2000). Research has also focused on a number of pathogenic fungi that affect grapes, including *Erysiphe necator* (the causal agent of grapevine powdery mildew), *Botrytis cinerea* (gray rot), and the peronosporomycete, *Plamospara viticola* (downy mildew). However, saprophytic molds, like *Aspergillus* spp., *Cladosporium* spp., and *Penicillium* spp. are also responsible for grape rots and, indirectly, food spoilage due to their mycotoxin production.

Grape berries are the primary source of yeast, which play a prominent role in the grape quality prior to harvesting, as well as throughout the winemaking process (Fleet et al., 2002). Previous studies have indicated that the genera *Aureobasidium* (yeast-like fungus), *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Issatchenkia Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Sporidiobolus*, *Torulaspora*, and *Zygosaccharomyces* are the most frequently isolated on grape berries (Sabate et al., 2002; Fleet et al., 2002; Prakitchaiwattana et al., 2004; Raspor et al., 2006; Nisiotou and Nychas, 2007; Chavan et al., 2009). However, the main agent of alcoholic fermentation, *Saccharomyces cerevisiae*, is rarely isolated from grape berry samples (Mortimer and Polsinelli, 1999).

Other "Non-*Saccharomyces*" species such as *Candida zemplinina*, *Hanseniaspora* spp, *Pichia kudriavzevii*, *Metschikowia pulcherrina*, and *Torulaspora delbrueckii* add to the diversity of this community and

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have been detected during fermentation, particularly during the pre-fermentation stage (Zott et al., 2008). From a winemaking perspective, these yeasts make a useful contribution to the aromatic complexity of wines (Renault et al., 2009; Zott et al., 2010). While some grape berry yeasts are potentially beneficial in the winemaking process, other species are detrimental to wine quality. This is the case of the genus *Brettanomyces*, known as the main spoilage yeast in red wines. Its ability to produce volatile phenols from hydroxycinnamic acids results in off-flavors that deteriorate the overall quality of the wine (Loureiro and Malfeito-Ferreira, 2003).

Another important point that has received less attention is that epiphytic yeasts on grape berries like *Aureobasidium pullulans*, may have antagonist effects on other microorganisms and are even starting to be used to control deleterious microorganisms, such as *Aspergillus carbonarius*, *A. niger* (Prakitchaiwattana et al., 2004; Bleve et al., 2006; de Felice et al., 2008), and *B. cinerea* (Duhail, 1999).

As is the case in other carpospheric habitats, the grape microbial community is influenced by several factors, such as the maturity stage (Rementeria et al., 2003; Renouf et al., 2005; Martins et al., 2012) and the use of phytosanitary products (Comitini and Ciani, 2008; Martins et al., 2012). Previous research into the impact of these phytosanitary treatments on grape–berry yeast communities (Comitini and Ciani, 2008; Čadež et al., 2010; Cordero-Bueso et al., 2011; Grube et al., 2011; Schmid et al., 2011) revealed that fungicide treatments caused a decrease in yeast populations. These studies were only carried out during the harvest-ripe stage and berry-surface microbiota was analysed after crushing the grape berries.

Drastic reductions in fungicide applications to treat vineyard pathogens will have a considerable impact on future plant protection strategies, leading to the testing and implementation of new phytosanitary practices. For instance, one of the aims of organic viticulture is to protect vines without using synthetic chemical pesticides, replacing them with copper-based molecules. However, previous studies have already shown that copper-based fungicides cause significant changes in the size and structure of microbial communities (Stirling et al., 1999; Tom-Petersen et al., 2003; Berg et al., 2005; Ranjard et al., 2006; Verginer et al., 2010; Martins et al., 2012).

The Single Strand Conformation Polymorphism (SSCP) relies on electrophoretic separation based on differences in DNA sequences: single-stranded DNAs of equal sizes are separated on a non-denaturing gel based on differences in mobility caused by their folded secondary structure (Kirk et al., 2004). One of the main advantages of SSCP is that it can be used to detect rapid changes in microbial communities in the absence of prior knowledge about their composition (Garbeva et al., 2004). This method also avoids the biases introduced by culture-based methods. Additionally, these techniques have been recently used to study the diversity and dynamics of microbial communities in different environments (Vallance et al., 2009, 2012).

In this work, culture-dependent and SSCP method were used to study the dynamics and structure of the epiphytic yeast and yeast-like community colonizing grape berries during the ripening process. The quantitative and qualitative influence of organic versus conventional systems on the microbial community was also investigated. In view of the frequent use of copper-based products as alternatives to synthetic fungicides, especially in organically-farmed vineyards, this study focused on the impact of copper on epiphytic yeasts on grape berries.

2. Materials and methods

2.1. Site description and sampling design

This study was performed in the Libourne wine area (southwest France), in two different wine appellations: Pomerol (44°55′ 52″ N, 0°12′ 16″W, 34 m altitude) and Lussac St Emilion (44°57′15″N 0°06′12″ W, 77 m altitude), in 2010.

Each vineyard is characterized by specific climatological conditions (Bois, 2007). The following data were obtained from the vineyard weather stations during the growing season from the beginning of the ripening process to the overripe stage: mean air temperatures were 19.36 °C and 18.70 º C and rainfall was 74.5 mm and 65.5 mm in Pomerol and Lussac-St-Emilion, respectively.

Two vineyards, approximately 400 meters apart, were selected in each appellation according to the farming system, i.e. organic and conventional. Both organic and conventional vineyards had very similar characteristics: grape variety (Merlot), age, pruning system, canopy management, and sun exposure. During the experiment, the organic vineyards were treated with Heliosoufre (Helioterpern; sulfur SC) and various copper formulations, such as Heliocuivre (Helioterpern; copper hydroxide SC), Nordox 50 (Nordox; cuprous oxide WP), and Champ flo (Nufarm; copper hydroxide SC). The conventional vineyards were treated with several agricultural chemicals: Freeland herbicide (Dow Agrosciences; glyphosate acid SL); Cascade insecticide (BASF; flufenoxuronm DC); and Explicit miticide (DuPont; indoxacarb SC); and Nordox 75 (Nordox; cuprous oxide WG); Eperon (Syngenta, metalaxyl-M mancozeb WG.); Roxam Combi (Philagro; zoxamide and mancozèbe WG); Valiant Flash (Bayer CropScience; cymoxanil, folpet, fosétyl WG), and Mikal flash (Bayer CropScience; folpet, fosetyl WG) fungicides.

Three sampling points, each corresponding to five vines, were selected in each vineyard. To evaluate changes in the microbial ecosystem throughout grape maturation, samples were collected at five different growth stages: the beginning of the berry ripening (BRB) process, veraison (BV), berries not quite ripe (BQR), harvest ripe (HR), and overripe (OR), corresponding to stages 34, 35, 37, 38, and 39, respectively, in the modified E-L system for identifying major and intermediate grapevine growth stages (Coombe, 1995). At each sampling date and location, approximately 1 kg of undamaged grapes with their pedicels attached were aseptically removed from several bunches and put in sterile bags. Grapes were transported to the laboratory in refrigerated boxes and analyzed within 12 h after collection.

2.2. Microbial biomass recovery

Each sample consisted of 250 undamaged berries, randomly and aseptically removed from the bunches, were placed in sterilized flasks with 500 ml isotonic solution containing 0.1 % peptone and 0.01% Tween 80 and subjected to orbital shaking at 150 rpm for 1 h (Prakitchaiwattana et al., 2004). The cell suspensions obtained were separated from the berries for downstream analysis. An aliquot of 1 ml of the suspension was used to inoculate culture medium and the rest was filtered through a 0.2 μm pore size, 47-mm diameter cellulose acetate filter (Sartorius AG, Göttingen, Germany).

2.3. Copper content of the cell suspensions

The copper concentration of the cell suspensions was assayed using a Perkin–Elmer (Norwalk, CT, USA) AAnalyst 100 atomic absorption spectrometer, equipped with a deuterium-arc lamp background corrector and air-acetylene burner (2.1 L/min flow rate), with absorbance measurements at a wavelength of 324.8 nm and a 15 mA lamp operating current. Absorbance measurements were transformed into concentration data using calibration curves constructed using 1.0 mg/L copper atomic absorption standard in nitric acid (VWR BDH Prolabo), diluted to concentrations ranging from 0.1 to 5 mg/L in an isotonic solution.

2.4. Sugar content in grape berry exudates

The sugar content of grape berry exudates was assessed by quantifying D-Glucose and D-Fructose in the cell suspensions obtained from the grape berry washes, using a UV enzymatic kit Cat No. 139106 (Boehringer Mannheim, Germany), according to the manufacturer's

instructions, with a SmartSpec™ 3000 Spectrophotometer (BioRad, Oakland, USA).

2.5. Colony isolation and counting

Yeast and yeast-like populations in the cell suspensions collected from the grape berries were assessed using a specific YPD-based medium named LT (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, and 30 g/L agar, pH adjusted to 4.8 with orthophosphoric acid), supplemented with 0.15 g/L biphenyl (Fluka, France) and 0.1 g/L chloramphenicol (Sigma Aldrich, France) to inhibit mold development and bacterial growth, respectively. Samples were spread at tenfold serial dilution in triplicate and incubated under aerobic conditions at 26 ºC for 5 days. Plates containing between 30 and 300 colonies were counted and colony-forming units (CFU) per mL were recorded. For samples at harvest-ripe stage, around 30 colonies were randomly picked and plated onto fresh LT plates. The purity of the colonies was verified by subcloning and they were stored at −80 °C on 33% glycerol stocks for further genetic identification.

2.6. Isolate identification based on rRNA LSU sequencing

DNA was extracted and stored from the isolates using the FTA® CloneSaver™ card (Whatman® BioScience, USA), as described by Zott et al. (2008). DNA was used as a template to be amplified by PCR targeting the D1/D2 variable domains of the ribosomal DNA large-subunit, using NL1 and NL4 primers (Kurtzman and Robnett, 1997). PCR conditions consisted of a preliminary denaturation step at 95 °C for 5 min, followed by 30 denaturation cycles at 95 ºC, annealing for 2 min at 52 ºC, and elongation for 2 min at 72 ºC,with a final elongation at 72 ºC for 10 min. The PCR was run in a final volume of 25 μL, containing 4 mL Taq & Go™ commercial PCR mix (MP Biomedicals, Carlsbad, USA), 200 nM of each primer, and one FTA patch containing the DNA template. The PCR products were then sequenced in double-strand form using the Sanger dideoxynucleotide method by GATC Biotech Inc. (Konstanz, Germany). The sequences were compared with other rDNA sequences in the GenBank, using the NCBI BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). Identification was considered valid when the identity of a contiguous sequence of around 550 bp was at least 98%. The rDNA sequences obtained were deposited in the EMBL Nucleotide Sequence Database under accession numbers HE802423 to HE802506.

2.7. Community analysis by CE-SSCP (Capillary Electrophoresis-Single Strand Conformation Polymorphism)

Several filamentous fungus and yeast species previously described (Barata et al., 2012) as associated with vine and wine environments, as well as *Vitis vinifera*, var. Merlot DNA were used to test the specificity of the selected primers and PCR conditions (see the supplementary data S1). Strains were obtained from the CRB Œnologie (www.crboeno.univ-bordeauxsegalen.fr) (Université Bordeaux Segalen, Bordeaux, France) and the CLIB collections (Collection de Levure d'Intérêt Biotechnologique, Thiverval-Grignon, France, http://www.inra.fr/internet/produits/cirmlevures). One *Penicillium expansum* and one *B. cinerea* strain were from our laboratory's collection and the *Saccharomyces cerevisiae* strain used was commercially produced (Actiflore F33, Biolaffort, Bordeaux, France). All strains were grown on LT medium at 26 ºC for 5 days, except *Penicillium expansum* and *B. cinerea*, which were propagated on potato dextrose agar.

DNA was extracted from the reference strains, as described in 2.6. In the case of environmental samples, DNA was extracted directly from the microbial biomass retained on the filtration membranes after rinsing the grape-berries, as described by Martins et al. (2013).

The divergent D1/D2 domain of the LSU rRNA gene was amplified using NL3A: 5′-GAGACCGATAGCGAACAAG-3′ and NL4:5′-GGTCCGTG TTTCAAGACGG-3′ primers (O'Donnell, 1993). Both primers were fluorescently labeled with 6-Carboxyfluorescein (6-FAM). DNA was amplified by PCR in a reaction mixture (25 μL final volume) consisting of 1 μL DNA template, 0.2 mM of each deoxynucleoside triphosphate, 2 ng/μL final sample volume of each primer, 2.5 μL 10 x *Pfu* Turbo buffer, and 0.05 units *Pfu* Turbo DNA polymerase (Agilent Technologies). The cycling conditions were as follows: enzyme activation at 95 °C for 2 min; 35 denaturation cycles at 95 °C for 30 s, hybridization for 30 s, extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The PCR products were checked by 1% Tris-borate-EDTA agarose gel electrophoresis prior to SSCP analysis. The lengths of the DNA amplification fragments from all fungi tested were around 200 bp. CE-SSCP analyses were performed on an ABI Prism 3130 XL genetic analyzer (Applied Biosystems, Courtaboeuf, France), equipped with four 36-cm capillaries. One microlitre of the PCR product was mixed with 18.8 μL Hi-Di formamide (Applied Biosystems) and 0.2 μL Genescan 400 HD ROX standard internal DNA molecular size marker (Applied Biosystems). The sample mixture was denatured at 95 °C for 5 min, immediately cooled on ice, and then loaded onto the instrument. The non-denaturing polymer consisted of 5% POP conformational analysis polymer (Applied Biosystems), 10% glycerol, EDTA buffer (10 x), and ultrapure water (Applied Biosystems). The migration voltage was set to 12 kV at 32 °C. Samples were allowed to comigrate with the fluorescent size standard (GeneScan 400 ROX) to facilitate comparison of sample migration profiles. CE-SSCP profiles were aligned and normalized with the StatFingerprints library package (Micheland et al., 2009), from R version 2.9.2 (RDC Team, 2009), using a standard procedure (Fromin et al., 2002).

2.8. Statistical data analyses

Statistical data were analysed using Statistica V.7 software (Statsoft Inc., Tulsa, OK, USA).

Statistical differences between copper and sugar concentrations in the samples were evaluated by one-way ANOVA, followed by post-hoc testing (Tukey's HSD multiple-comparison test, $p < 0.05$).

Two-way ANOVA was used to assess the impact of farming systems and berry-growth stage on the microbial community (microbial counts, diversity and richness of CE-SSCP profiles) and Tukey's test for comparisons between regions. Data with non-normal distributions were subjected to logarithmic transformation prior to parametric analysis.

Pearson's correlation test was used to determine the relations between copper and sugar concentrations and cultivable population. The CE-SSCP profiles were explored using a centered, scaled PCA, with samples as statistical observations. The Shannon diversity indices were calculated as follows:

$$H' = -\Sigma[(ni/N)\ log(ni/N)]$$

where (H′) is the Shannon diversity index, (ni) is the intensity or height of the individual CE-SSCP peaks, and (N) is the sum of the intensity or height of all the bands or peaks (Hong et al., 2007). Richness index (S) was estimated by counting the number of peaks detected in each CE-SSCP profile (Michelland et al., 2010).

Similarities between CE-SSCP profiles were evaluated by hierarchical clustering analysis. Similarities between data sets were quantified by calculating Euclidian distance and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used as the linkage criterion.

3. Results

3.1. Copper and sugar concentrations in the cell suspensions

Copper concentrations in the cell suspensions varied according to the vineyard (Table 1) and farming system, with higher concentrations

Table 1
Copper and sugar concentrations (D-glucose + D-fructose) in the wash solutions at the various grape-growth stages in two regions (Lussac and Pomerol) and with different farming systems (organic and conventional). Values in brackets represent standard deviation (n = 3). * indicates significant differences between organic and conventional samples—one way ANOVA ($p < 0.05$).

Vineyards	Copper (μg /g fresh weight of grape berries)					D-Glu + D-Frut (mg /g fresh weight of grape berries)				
	Berry ripening beginning (BRB)	Veraison (BV)	Berries not quite ripe (BQR)	Berries harvest ripe (HR)	Berries overripe (OR)	Berry ripening beginning (BRB)	Veraison (BV)	Berries not quite ripe (BQR)	Berries harvest ripe (HR)	Berries overripe (OR)
Lussac — Organic	1.40 (±0.49)	0.49 (±0.09)	0.58 (±0.14)	0.60 (±0.18)	0.43 (±0.02)	0.32 (±0.07)	0.33 (±0.05)	0.39 (±0.05)	0.56 (±0.02)	1.69 (±0.02)
Lussac — Conventional	1.38 (±0.56)	0.88* (±0.12)	1.74* (±0.55)	1.05* (±0.25)	0.41 (±0.06)	0.34 (±0.02)	0.36 (±0.04)	0.41 (±0.03)	0.58 (±0.02)	1.72 (±0.11)
Pomerol — Organic	0.86 (±0.33)	0.32 (±0.07)	0.41 (±0.17)	0.21 (±0.06)	0.05 (±0.003)	0.41 (±0.04)	0.45 (±0.07)	0.42 (±0.03)	0.6 (±0.03)	1.82 (±0.06)
Pomerol — Conventional	1.51* (±0.19)	1.97* (±0.30)	1.89* (±0.66)	1.39* (±0.62)	0.69* (±0.77)	0.33 (±0.02)	0.39 (±0.02)	0.4 (±0.04)	0.51 (±0.04)	1.74 (±0.03)

in conventionally farmed grapes at almost all stages (one way ANOVA ($p < 0.05$).

There were no statistically significant differences between the two farming systems in the mean values for sugar concentrations (data not shown); a continuous increase in sugars was observed throughout the ripening process (Table 1).

One-way ANOVA analysis revealed statistically significant differences between some of the berry-growth (BRB) and -ripening stages (HR and OR). Post-hoc comparisons using the Tukey HSD test indicated that the mean sugar concentration in the overripe stage was significantly different from all other stages (data not shown).

3.2. Size of cultivable communities

Populations grown on LT media are reported in Table 2. Two-way ANOVA revealed a significant effect of the farming system and berry-growth stage on the cultivable populations in both Lussac and Pomerol vineyards (Table 2). In Lussac the partition sum of squares indicated that berry-growth stage was the preponderant factor, explaining 59.56% of the variation, with 30.11% for the farming system factor and 7.77% for the interaction between these factors. Similar results were obtained in Pomerol: berry growth stage explained 58.38% of the variation, with 26.99% for the farming system and 9.41% for the interaction between these factors.

In both Pomerol and Lussac, the population was larger in organic than conventional vineyards. The highest population counts were in the overripe stage, irrespective of the wine region and farming system.

Post-hoc tests revealed several homogeneous groups (Tukey's HSD, $P < 0.05$). Regarding the farming system factor, samples from conventional and organic vineyards fell into two homogeneous groups in both Lussac and Pomerol. Concerning the berry-growth stage, in Lussac, the earliest (BRB) and latest (OR) stages each formed homogeneous groups distinct from all the other samples. In Pomerol, three homogeneous groups were formed, consisting of the OR stage, the BV stage, and the samples from the BQR, HR and BRB stages together (Table 2). For details on the variance analyses see the supplementary data (S2).

An evaluation of the relationship between copper levels and population density over the veraison period revealed a negative correlation for both Lussac ($r = -0.822$, $p < 0.001$) and Pomerol ($r = -0.799$, $p < 0.001$), suggesting that copper had an inhibiting effect on the cultivable population (Fig. 1A) (r = Pearson correlation coefficient). A comparison of population size and sugar concentrations in cell suspension revealed a positive correlation (Fig. 1B) for both Lussac ($r = 0.765$, $p < 0.001$) and Pomerol ($r = 0.775$, $p < 0.001$) vineyards, thus explaining higher population counts detected in the overripe stage.

3.3. Cultivable population diversity at harvest stage

From 96 randomly-picked colonies, 7% did not survive subculturing. The DNA of the remaining isolates was extracted and sequenced using the D1/D2 domain of the rDNA large-subunit. All the strains were assigned to a specific genus with a classification threshold above 98%. Overall, 7 genera were distinguished (Fig. 2). Representatives of the Ascomycota phylum included: *Aureobasidium proteae* (14 isolates), *A. pullulans* species (26), *Cladosporium silenes* (1), *Phoma pedeiae* (1), *Phoma negriana* (1), *Phoma aliena* (1), and *Epicoccum nigrum* (1). The Basidimycota species represented included: *Sporidiobolus* (21 isolates which have been all assigned to *Sporidiobolus pararoseus* species), *Rhodotorula* (7 isolates all assigned to *Rhodotorula glutinis* specie), and *Cryptococcus*: *C. carnescens* (1isolate), *C. tephrensis* (1), *C. terrestris* (2), *C. victoriae* (1). Among the genera identified, *Aureobasidium was* more frequently associated with organic (76 and 85% of all isolates in Lussac and Pomerol, respectively) than conventional samples (5 and 22% of all isolates) (one-way ANOVA analysis, $p = 0.021$). On the contrary, the *Sporidiobolus* genus was more frequently associated with

Table 2
Cultivable populations on berries from organic and conventional vineyards in two wine regions (Lussac and Pomerol), at five different stages, determined in the wash solution by plate counts on LT medium and expressed as CFU/g fresh weight of grape berries).

Vineyards	log10 CFU/g fresh weight of grape berry				
	Berry ripening beginning (BRB)	Veraison (BV)	Berries not quite ripe (BQR)	Berries harvest ripe (HR)	Berries overripe (OR)
Lussac — Organic	4.14 (±0.03)	4.2 (±0.03)	4.4 (±0.02)	4.41 (±0.06)	4.99 (±0.04)
Lussac — Conventional	3.42 (±0.03)	4.12 (±0.09)	3.69 (±0.18)	4.03 (±0.05)	4.57 (±0.02)
Pomerol — Organic	3.98 (±0.07)	4.07 (±0.13)	4.01 (±0.12)	4.09 (±0.04)	4.67 (±0.11)
Pomerol — Conventional	3.85 (±0.09)	3.21 (±0.02)	3.65 (±0.03)	3.7 (±0.02)	4.4 (±0.17)

conventional (56 and 30% of all isolates) than organic samples (0 and 5% of all isolates) (one-way ANOVA analysis, $p = 0.024$).

3.4. Effect of the ripening process and farming system on fungal community structure

The impact of the farming system on these fungal communities was analyzed by CE-SSCP fingerprint patterns of DNA extracted directly from berry wash solutions. The diversity and richness indices were calculated and mean values compared by two-way ANOVA, revealing statistically significant effects of the farming system and ripening process on the diversity and richness of the CE-SSCP profiles (Table 3A and B).

The diversity and richness of the profiles increased throughout the ripening process, reaching a maximum at the overripe stage.

In all samples, the partition sum of squares indicated that the growth-stage factor was preponderant, explaining 50 to 59 % of the

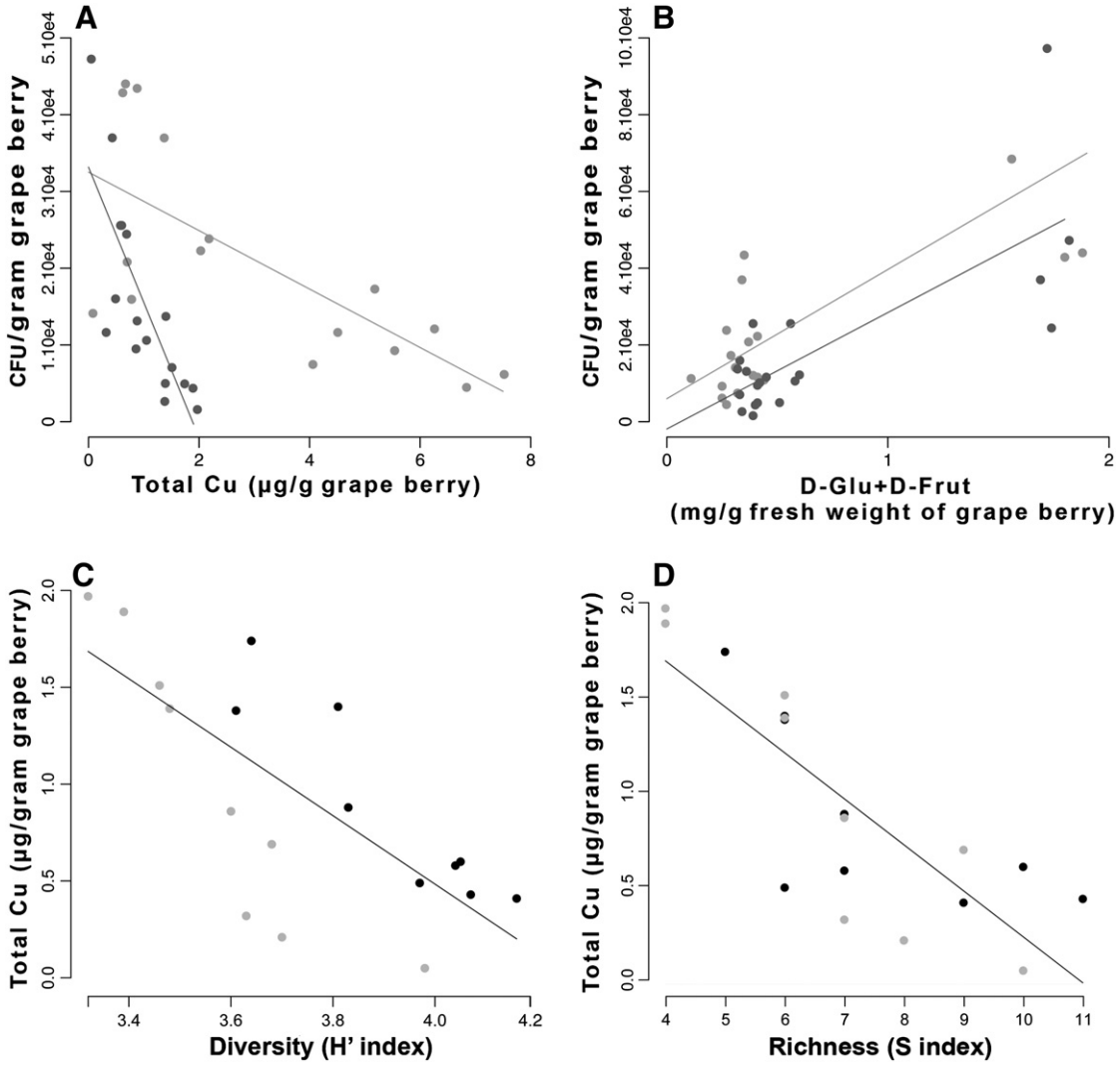

Fig. 1. (A) Correlation between copper and cultivable populations over the veraison period: Lussac (black circles) ($R^2 = 0.775$; Pearson correlation coefficient $= -0.822$, $p < 0001$, $n = 16$) and Pomerol (gray circles) ($R^2 = 0.670$; Pearson correlation coefficient $t = -0.799$, $p < 0.001$, $n = 16$). (B) Correlation between sugars and cultivable population density over the veraison period: Lussac (black circles) ($R^2 = 0.759$; Pearson correlation coefficient $= 0.765$, $p < 0.001$, $n = 20$) and Pomerol (gray circles) ($R^2 = 0.760$; Pearson correlation coefficient $= 0.775$, $p < 0.000$, $n = 18$). (C) Correlation between copper and diversity indices calculated from CE-SSCP profiles for samples in the harvest-ripe and overripe stages: Lussac (black circles) ($R^2 = 0.801$; Pearson correlation coefficient $= -0.862$, $p < 0001$, $n = 9$) and Pomerol (gray circles) ($R^2 = 0.770$; Pearson correlation coefficient $= -0.869$, $p < 0.001$, $n = 9$). (D) Correlation between copper and richness indices calculated from CE-SSCP profiles for samples in the harvest-ripe and overripe stages: Lussac (black circles) ($R^2 = 0.812$; Pearson correlation coefficient $= -0.871$, $p < 0001$, $n = 9$) and Pomerol (gray circles) ($R^2 = 0.790$; Pearson correlation coefficient $= -0.872$, $p < 0.001$, $n = 9$).

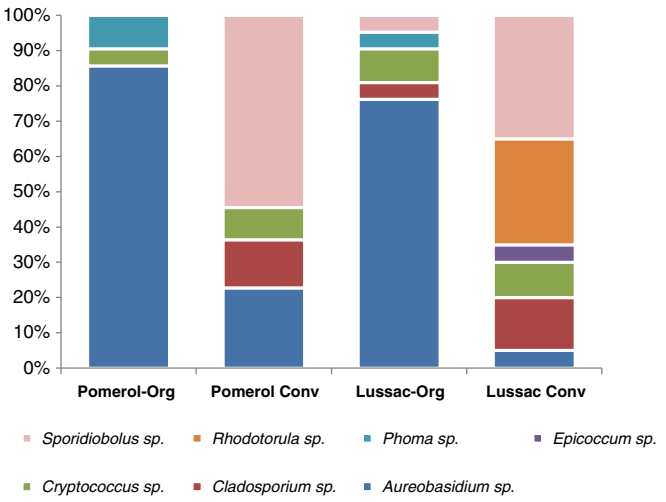

Fig. 2. Abundance of cultivable genera in grape samples from organic and conventional farming systems in Lussac and Pomerol regions at harvest-ripe stage (2010 vintage). Bar plots shows relative percentages by genus of isolates in each sample.

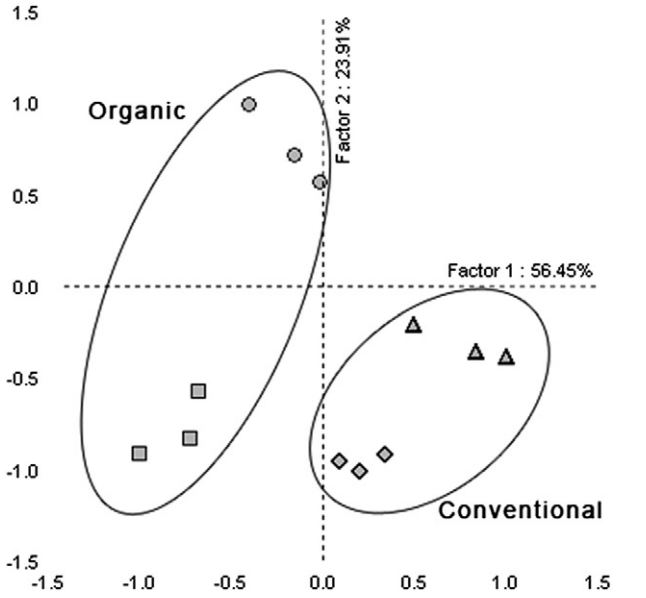

Fig. 3. Principal-component analysis based on fungal community structure assessed by rRNA LSU gene CE-SSCP, for samples at harvest-ripe stage from 2010 vintage. Squares (□) and circles (○) indicate organic samples from Lussac and Pomerol vineyards, respectively.

variation, followed, in a majority of cases, by the farming system. Post-hoc tests revealed several homogeneous groups. The latest stages, OR and HR, were frequently distinct from the earlier stages (Table 3A and B). For details on the variance analyses concerning the effects of the farming system and ripening process on the diversity and richness of the CE-SSCP profiles see the supplementary data (S3).

Concerning the farming system effect, diversity (H′) and richness (S) values in both Lussac and Pomerol were higher for organic than conventional vineyards. In addition, a negative correlation indicated that copper had a negative impact on the diversity and richness of the fungal population (Fig. 1C and D).

As harvest-ripe is the key stage from an enological standpoint, the profiles from samples at this stage were analyzed in detail. A comparison of HR samples profiles by cluster analysis revealed a clear difference according to the farming system (data not shown). The samples from conventional vineyards were all gathered in one group, distinct from the organic samples. These two groups were divided into two subgroups — Lussac versus Pomerol.

The PCA analysis results of the CE-SSCP profiles are shown in Fig. 3. The first two principal components explained 80.36 % of total variance. The profile distribution over the score plot indicated different trends according to the farming system. Conventional samples were all on the right-hand lower segment of the first factorial plane, while the organic samples were all on the left-hand side of the first factorial plane, with the Lussac samples in the lower segment and the Pomerol samples in the upper segment).

4. Discussion

Previous research investigated the impact of abiotic factors, such as phytosanitary treatments and grape maturity stages on the epiphytic yeasts colonizing grape berries (Rementeria et al., 2003; Comitini and Ciani, 2008; Čadež et al., 2010; Cordero-Bueso et al., 2011; Grube et al., 2011; Schmid et al., 2011; Milanovic et al., 2013). However, the majority of these studies only focused on the harvest-ripe stage and the berry-surface microbiota was analyzed after crushing the grape berries with a stomacher machine. This subsequent enrichment steps inevitably induced microbial selection. In order to avoid this artifact, in this study, the epiphytic community was collected directly from the grape berry surface by washing it with an isotonic solution.

Our results showed a significant impact of both the farming system and the maturity stage on the epiphytic yeast and yeast-like community. Shifts in the microbial community were related to changes in the composition of the grape–berry surface, particularly sugar exudation and the occurrence of copper residues from pesticide treatments.

Irrespective of the vineyard considered, cultivable yeast and yeast-like populations increased during the grape ripening process, reaching

Table 3
Diversity (H′ index) and (B) richness (S index) obtained from CE-SSCP profiles at different grape-growth stages.

A	H′ (Shannon diversity index)				
Vineyards	Berry ripening beginning (BRB)	Veraison (BV)	Berries not quite ripe (BQR)	Berries harvest ripe (HR)	Berries overripe (OR)
Lussac — Organic	3.81 (0.12)	3.97 (±0.01)	4.04 (±0.03)	4.05 (±0.04)	4.07 (±0.05)
Lussac — Conventional	3.61 (±0.02)	3.83 (±0.03)	3.64 (±0.05)	3.97 (±0.02)	4.16 (±0.05)
Pomerol — Organic	3.6 (±0.02)	3.63 (±0.02)	3.61 (±0.07)	3.7 (±0.03)	3.98 (±0.01)
Pomerol — Conventional	3.46 (±0.03)	3.32 (±0.08)	3.39 (±0.10)	3.48 (±0.05)	3.68 (±0.04)
B	S (Species richness)				
Vineyards	Berry ripening beginning (BRB)	Veraison (BV)	Berries not quite ripe (BQR)	Berries harvest ripe (HR)	Berries overripe (OR)
Lussac — Organic	6 (±1,15)	6 (±0,58)	7 (±1,00)	10 (±1,00)	11 (±1,15)
Lussac — Conventional	6 (±0.58)	7 (±1.15)	5 (±1.73)	6 (±0.58)	9 (±1.00)
Pomerol — Organic	7 (±1.53)	7 (±0.58)	7 (±1.73)	8 (±1.15)	10 (±0.58)
Pomerol — Conventional	6 (±0.58)	4 (±1.00)	4 (±1.15)	6 (±1.00)	9 (±0.58)

a maximum when the berries became overripe. Similar observations were made in the same vineyards in 2009 vintage (Martins, 2012) and same results concerning grape–berry bacterial microbiota was reported by Martins et al. (2012), but the reasons for these increases remain to be determined. One hypothesis is that changes in microbial population density and sugar content on the grape skin are directly related to modifications in the nutrient composition of grape–berry exudates.

The cultivable fungal population also varied significantly depending on the farming system. In both Lussac and Pomerol vineyards, the microorganism counts were significantly higher for organically- than conventionally-farmed grapes, while copper levels were higher in conventional vineyards.

A comparison of organic versus conventional vineyards in the two wine producing areas, considering various spatial and temporal parameters, such as temperature, humidity, water activity, and grape-skin exudates, produced similar results for all the grape samples (data not shown). Consequently, copper-based fungicide treatments were revealed as the main factor responsible for the differences in microbial population densities. Ecotoxicity of copper on several microbial populations colonizing various ecosystems, including grape-berries (Martins et al., 2012), has been reported by Ellis et al. (2002), Brandt et al. (2006), and Ranjard et al. (2006), but its impact on epiphytic grape yeasts had not previously been evaluated. The potential negative impact of copper on grape-colonizing microbiota was raised by Verginer et al. (2010), but was never evaluated. The present results showed a negative correlation between copper levels and yeast and yeast-like populations, providing evidence that copper inhibited this epiphytic community.

These culture-independent data revealed changes in the genetic structure of the yeast and yeast-like community throughout the ripening process, as well as the impact of the farming system. The increasing diversity and richness of the CE-SSCP profiles throughout the ripening process may be partly due to the increase in sugar exudation on the grape skin.

As regards the two abiotic factors investigated, the farming system induced limited changes in comparison with the maturity stage, but the two were statistically significantly different. H′ and S values were higher for organic than conventional grapes, possibly due to the higher copper concentrations detected in conventional vineyards.

The CE-SSCP profile distribution over the score plot and in the cluster analysis showed different trends depending on the farming system, making it possible to distinguish between samples from conventional and organic farming systems.

Aureobasidium was the predominant genus in organic grapes, while *Sporidiobolus* was more frequently associated with conventional samples. Similar observations were made by Grube et al. (2011), who hypothesized that the predominance of *Aureobasidium* in organic grapes was related to copper- and sulfur-tolerance in strains from this genus. In our study, the organic grapes where this genus was the most abundant had lower quantities of copper than those from conventional vineyards. However, in previous vintages, copper concentrations in grapes from organic plots were often higher than in conventional ones, so this phytosanitary history, included the non-use of synthetic chemical pesticides, probably acted as a selection factor over the years, favoring the colonization of *Aureobasidium* in the organic vineyards (Martins, 2012).

The epiphytic species *A. pullulans* is well adapted to the phyllosphere and carposphere, and is widely distributed throughout vine environments (Martins, 2012). This yeast-like fungus is of great biotechnological importance thanks to its potential for controlling grape-berry spoilage microorganisms (Dik et al., 1999; Schena et al., 2003; Dimakopoulou et al., 2008). A biopesticide was developed and commercialized to take advantage of its capacities as a biocontrol agent against pathogens (Chi et al., 2009).

The species *Epicoccum nigrum* has also been reported to be effective biological agent for grape berry pathogen *B. cinerea* and *P. viticola* (Fowler et al., 1999; Kortekamp, 1997), and the yeast *R. glutinis* showed an inhibitory activity against postharvest fungal pathogens (Lima et al., 1999). The presence of those species are particular important, as they influence the grape berry sanitary status, and consequently impact the fermentation process and the wine quality.

The prevalence of *Sporidiobolus* in conventional vineyards may also be related to phytosanitary practices, reflecting its specific resistance to some active fungicide components frequently used in the conventional farming system.

In conclusion, these results contribute to the understanding of the influence of abiotic factors on the dynamics and structure of epiphytic fungal communities on grape berries. This field is of particular importance in view of the role epiphytic yeast and yeast-like in plant health and the fact that grape berries are the primary source of the microbial communities that play a prominent role in the winemaking process. Further research is required to explore other factors besides copper treatment and maturity stage that impact the size and composition of the microbial communities on the grape–berry surface. The use of other culture-independent approaches, including next-generation sequencing methods, is likely to provide a comprehensive survey of the epiphytic yeast and yeast-like communities on grape berries and the factors affecting them.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.002.

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