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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.

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