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Received: 15 March 2019 | Accepted: 10 January 2020 | Published: 4 February 2020
DOI:10.20870/oeno-one.2020.54.1.2435

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ABSTRACT

Grape berries host a complex microbial community that plays a predominant role in grape quality prior to harvest and in the winemaking process. Like other carpospheric habitats, the epiphytic microbial community of grape berries is influenced by several factors. Climate plays an essential role in grape composition and quality, and its impact on berry microbiota was recently demonstrated. However, there is still a lack of information about how environmental conditions influence the system in grape berries.

In this study, the microbial communities of grape berries from two wine appellations characterized by specific climatological conditions were analyzed. The results revealed that the size of cultivable communities (bacteria, yeast and filamentous fungi) and the diversity and richness of the microbial community profiles were higher in vineyards found to have lower temperatures. The metabolic activity (*Community-Level Physiological Profiles*) of the epiphytic microbial community was lower in warm climates. Water activity (aw) had a positive effect on the cultivable population, on metabolic activity, and on microbial diversity.

The results showed that the microclimate affects the microbial population associated with grape berries through the aw of grape skins.

KEYWORDS

grape berries, metabolic activity, microbial communities, water activity

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/2435

INTRODUCTION

Grape berries sustain a complex microbial ecosystem that harbors a diverse array of bacteria, yeast and filamentous fungi, as reviewed by (Barata *et al.*, 2012). The epiphytic microbial community of wine grapes influences the rape quality prior to the harvest, the winemaking process, and the final wine quality (Pretorius, 2000; Fleet *et al.*, 2002; Nisiotou *et al.*, 2011). As with other carpospheric habitats, it is affected by several factors, including maturity stage (Rementeria *et al.*, 2003; Martins, 2012), phytosanitary products (Comitini and Ciani, 2008; Cordero-Bueso *et al.*, 2011; Martins *et al.*, 2012; Setati *et al.*, 2012), geographical location (Li *et al.*, 2010; Taylor *et al.*, 2014), grape variety (Sabate *et al.*, 2002; Raspor *et al.*, 2006), grape berry composition (Martins *et al.*, 2014), and biotic factors such as Botrytis *cinerea* (Barbe *et al.*, 2001; Nisiotou *et al.*, 2011).

The impact of the climate on bacterial and fungal species associated with grape berysurfaces has been previously established (Querol *et al.*, 1990; Combina *et al.*, 2005; Brilli *et al.*, 2014; Paterson *et al.*, 2018). It is a key component of environmental conditions involved in the viticulture concept called *terroir* (Leeuwen and Seguin, 2006). The microclimate (local atmospheric zone where the climatic conditions differ from the surrounding areas) is a key element that discriminates dramatically the quality of grapes within the same wine region (Pereira *et al.*, 2006; Lecourieux *et al.*, 2017). The grape berry maturation process is highly linked to climate conditions, impacting the concentration of sugars and acids, the polyphenol content, and other quality determinants such as the fruit's phytosanitary status (Martin *et al.*, 2016).

Previous work has showed that rainfall in the days before the harvest influences the diversity of yeast and leads to an increase in population size (Combina *et al.*, 2005). The authors suggest that the grape volume increases as a result of rain, and allows the release of nutrients from the pulp by exosmosis phenomena to the berry surface. Čadež *et al.* (2010) showed that in rainy and cold harvests the yeast population increases. The opposite result was described by Comitini and Ciani (2008), who found lower levels of microbial populations in rainy and fresh vintages, and by Rementeria *et al.* (2003) who noted higher population counts in dry and hot vintages.

The biogeographic distribution of grape microbiome was the topic of a recent study by Gayevskiy and Goddard (2012) concerning yeasts in three different wine producing regions, characterized by specific climates. The results showed that fruit-associated fungal communities differ in space. The microbial biogeography of grape berries is conditioned by climate, and populations are correlated to specific climatic conditions, suggesting a link between vineyard environmental conditions and microbial inhabitation (Bokulich *et al.*, 2014; Grangeteau *et al.*, 2016). Vintage variations substantially affect microbial communities within small geographic scales (vineyards) but not at a larger scale (macroregions). However, little is known about how microclimate heterogeneity and environmental microclimate parameters impact microbial community. Climate is linked with an important parameter for the development of microorganisms on grape berry surfaces: the water activity (aw) (Fermaud *et al.*, 2011).

The term 'water activity' refers to freely available water, unbound to nutrient molecules, which allows microbial growth and supports biochemical reactions involved in the spoilage process (Rockland and Nishi, 1980). Water activity is defined as the ratio of the vapor pressure of water in a material to the vapor pressure of pure water at the same temperature. Water activity depends on temperature due to changes in water binding, dissociation of water, the solubility of solutes in water, or the state of the matrix (Wrolstad *et al.*, 2005). The influence of climate on the aw of the grape skin is still to be studied, but Fermaud *et al.* (2011) showed significant interaction between the relative humidity (RH) and the aw.

Aw is as an important parameter in grape microbial growth and metabolism (Rousseau and Doneche, 2001). Aw determination has been used to provide information about the susceptibility of grapes to the microbial detrimental effects of pathogenic fungus such as B. *cinerea*, and mycotoxin producing fungi such as *Aspergillus Niger* (Esteban *et al.*, 2006; Fermaud *et al.*, 2011).

Our hypothesis is that climatic conditions can impact the grape skin aw, and consequently affect microbial communities. The aim of this study was to test the relationship between aw and the grape berry microbial communities.

Grape berry samples were collected from vineyards in the same area but in two different wine appellations characterized by contrasting

climatological conditions (one appellation was warmer and drier than the other). The aw from the grape skin and the structure and activity of the microbial community were determined, using both culture-dependent and independent approaches and a community-level cultural approach called community-level physiological profiles (CLPP) to characterize the microbial activity.

MATERIALS AND METHODS

1. Site description and sampling design

This study was conducted in the Libourne wine area (south-west France), in two different wine appellations: Pomerol (44° 55' 52'' N, 0° 12' 16'' W, 34 m altitude) and Puisseguin St Émilion (44° 55' 26'' N, 0°04' 38'' W, 91 m altitude) characterized by specific climatological conditions (Bois, 2007). Different climatic data from the beginning of veraison until the over-ripe stage (corresponding to stages 34, and 39 of the modified E-L system for identifying major and intermediate grape vine growth stages, respectively; Coombe, 1995) were recorded in both appellations. Weather stations were located at equal distances to or less than km from the plots. In Pomerol, for that period, the average temperature was 19.36ºC, the RH was 69.55 % and the precipitation was 74.50 mm. In Puisseguin, the corresponding values were 18.03ºC, 74.47 %, and 76.00 mm, respectively. In Pomerol, veraison began on 28 July 2010 and the over-ripe stage began on 6 October 2010. In Puisseguin, veraison began on 5 August 2010 and the over-ripe stage began on 14 October 2010. The sum of grow degree days from 1 May 2010 to 31 October 2010 was 1517.30 and 1352.70 (°C) for Pomerol and Puisseguin, respectively.

Two plots (plot I and plot II), approximately 400 meters apart, were selected in each appellation for very similar characteristics: grape variety (Merlot), age, pruning system, canopy management, and sun exposure. Plot I in Pomerol and Puisseguin was managed with an organic farming system, plot II with conventional farming.

In the trial year, both organic plots were treated with dusting sulfur and with different copper formulations of metallic copper. The conventional plots were both treated with Freeland (Glyphosate 36 %, SL. Dow Agrosciences) as herbicide, Cascade (Flufénoxuron 10, BASF) and Explicit (Indoxacarbe 15, DuPont) as insecticide and miticide, and Eperon (métalaxyl-M 3.88 + mancozèbe 64, EC. Syngenta) and Roxam Combi (Zoxamide 6,15 % + Mancozèbe 68,9 %, Philagro) as fungicides.

In each plot, three sampling plots were chosen, consisting of five vines. Samples were collected at harvest ripe. At each sampling date and location, approximately 1 kg of undamaged grapes with their pedicels were aseptically removed from several bunches and put in sterile bags. Grape berries were collected from the top, middle and bottom of the clusters as well as from the sun-exposed and shaded sides. Grapes were transported to the laboratory in refrigerated boxes and analyzed within 12 h after collection.

2. Measurement of the water activity in grape skins

For each sample of 250 undamaged berries, 14 were randomly chosen and used to measure water activity individually according to the method of Detieux-Belleau et al, 2009. Beforehand, the pedicel was surrounded with paraffin to avoid exchanges from this zone and to consider only those from the skin surface. The berries were placed individually in the chamber of an Aw-Sprint (Novasina) aw-meter, thermo-regulated at 30 °C. The stability factor was adjusted to 6 min and the plot final aw value was calculated by using Novalog software (Deytieux-Belleau *et al.*, 2009).

3. Microbial biomass recovery

The cell suspension was filtered in order to obtain enough biomass for DNA extraction. Each sample consisted of 250 berries, in sterilized flasks with 500 mL isotonic solution containing 0.1 peptone and 0.01 % Tween 80, and was subjected to orbital shaking at 150 rpm for 1 h (Prakitchaiwattana *et al.*, 2004). Cell suspensions were separated from the berries and an aliquot of 1 mL of the suspension was used to inoculate the culture medium. The rest was filtered through a 0.2 µm pore size, 47 mm diameter cellulose acetate filter (Sartorius AG, Göttingen, Germany).

4. Colony isolation and counting

From the cell suspensions previously described, yeast and yeast-like populations 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), respectively, to inhibit mold and bacterial growth. Samples were spread at ten-fold serial

dilution in triplicate and in plates for aerobiosis at 26ºC for 5 days. Around 30 colonies were randomly selected and plated onto fresh LT plates purified by subcloning and stored at -80 °C with 33 % glycerol for further genetic identification.

Bacterial communities of cell suspensions were cultured by spreading at ten-fold serial dilutions in triplicate on 1/10 diluted LB (1 g L^{-1} bactotryptone, 1 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl, and 20 g L^{-1} agar) and 150 mg L^{-1} biphenyl (Acros) to inhibit yeast and mold growth. Total aerobic and aero-tolerant bacteria were grown on 1/10 diluted LB medium under aerobic conditions at 25 °C for 5 days. For each sample, 20–24 colonies were picked from the samples on 1/10 diluted LB medium randomly and plated onto fresh 1/10 diluted LB plates purified by subcloning and stored at -80 °C with 33 % of glycerol for further genetic identification.

5. Isolate identification based on rRNA gene sequence.

DNA was extracted and stored from the isolates using the FTA® CloneSaver™ card (Whatman® BioScience, USA), as described by Zott *et al.* (2008). Yeast and yeast-like DNA was used as a template and 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 72ºC, with a final elongation at 72ºC 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.

For bacterial isolates, PCR targeted 16S rRNA with primers 8F and 1063R (Wang and Qian, 2009) was run in a final volume of 50 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 5 % glycerol, 0.08 % NP-40, 0.05 % Tween-20, 25 units mL^{-1} Taq DNA polymerase, and 200 nM of each primer. Amplification was carried out with initial denaturation at 95 °C for 5 min, 30 cycles at 94 °C and 58 °C for 1 min each, 72 °C for 1.5 min, and 72 °C for 7 min.

The PCR products obtained from the yeast, yeast-like and bacterial isolates 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). The yeast and yeast-like 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 HE802466 and HE802507 to HE802544. For bacterial isolates, identification was considered valid when the identity of a contiguous sequence of 343 pb-989 pb was at least 98 %. The 16S rDNA sequences obtained were deposited in the EMBL Nucleotide Sequence Database under the webin ID number Hx2000041988.

6. Community analysis by CE-SSCP (capillary electrophoresis-single strand conformation polymorphism)

DNA was directly extracted 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, with 5'-GAGACCGATAGCGAACAAG-3' and NL4:5'-GGTCCGTGTTTCAAGACGG-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 × 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. PCR products were checked by 1 % Tris-borate-EDTA agarose gel electrophoresis prior to SSCP analysis. 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. Then, 1 µL 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). DNA 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 ×), 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).

CE-SSCP profiles were analyzed by calculating the Shannon diversity indices as follows:

$$H' = -\sum \left[\frac{n_i}{N} log\left(\frac{n_i}{N}\right)\right]$$

where H' is the Shannon diversity index, N 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). Phylotype richness (S) was estimated by counting the number of peaks detected in each CE-SSCP profile) (Michelland *et al.*, 2010).

7. T-RFLP analysis of DNA samples extracted from cell suspensions

DNA samples extracted from cell suspensions were homogenized with the FastPrep®-24 cell disruptor (MP Biomedicals, Irvine, CA), as previously described, and incubated twice at 70 °C for 15 min, with homogenization between incubations. Samples were centrifuged at 13,000 g and 20 °C for 10 min. The supernatants were collected, incubated with 1/10 volume of 3 M potassium acetate pH 5.2 at 4 °C for 10 min, and centrifuged at 14,000 g for 5 min. DNA was precipitated with one volume of ice-cold isopropanol, washed with 70 % ethanol, dried, and diluted in water. Quality was checked by electrophoresis on 1 % agarose gels. DNA was then quantified using a NanoVue (GE Healthcare).

DNA was amplified using a nested PCR. The first PCR amplified a 1398 bp region of the 16S rRNA gene, using primers 8F (5′AGAGTTTGATCCTGGCTCAG3′) and 1406R (5′ACGGGCGGTGTGTRC3′) (Schute *et al.*, 2008), and the second one a 1055 bp region, using primers 8F and 1063R (5′CTCACGRCACGAGCTGACG3′) (Wang and Qian, 2009). For the second PCR, the 8F primer was fluorescently labeled on the 5′ end with 6-FAM (6-carboxyfluorescein). The PCR reaction mix was the same as that used for CE-SSCP analysis. PCR conditions for the first amplification were: 95 °C for 5 min, 20 cycles at 94 °C and 58 °C for 1 min each, 72 °C for 1.5 min, and 72 °C for 7 min. The second program was the same but for only 15 cycles. PCR products (100 ng) were purified with the Geneclean Turbo Kit (Qbiogene) and digested with 3 U Hae III or Hinf I enzymes (New England Biolabs). Fluorescently labeled terminal-restriction fragments (T-RFs) were separated by capillary electrophoresis on an ABI prism 310 (Applied Biosystems). Approximately 10 ng digested DNA was mixed with 9 µL de-ionized formamide and 0.5 µL 5-carboxytetramethylrhodamine size standard (TAMRA 500), denatured at 95 °C for 5 min, and immediately chilled on ice prior to electrophoresis. After a 10 s injection step, electrophoresis was performed at 15 kV for 30 min. T-RFLP peaks were analysed using GeneScan software (ABI), profiles were compiled to produce data matrices, the background noise was reduced and T-RF heights were normalized (Dunbar *et al.*, 2001). Only T-RFs between 50 pb and 500 pb were analyzed. The profiles were aligned using T-align software, with a confidence interval of 1.0 (Smith *et al.*, 2005).

For the T-RFLP analysis, Shannon–Weaver diversity indices (H') were calculated using peak heights as a metric of abundance for each T-RFLP profile, as described previously (Hill *et al.*, 2003). The phylotype richness (S) was calculated as the total number of distinct T-RF peaks.

8. Analysis of community-level physiological profiles

A Biolog EcoPlates (Biolog Inc., Hayward, CA, USA) system was used to analyze CLPP. This method proved to be very useful to characterize catabolic diversity from various environments (Garland, 1996). This technique provides a rapid and highly reproducible method to measure functional diversity of bacteria and fungi (Classen *et al.*, 2003).

The 96-well Biolog microtiter plates containing three replicate wells for each of 31 carbon sources and a redox dye (Insam and Rangger, 1997) were inoculated with 100 µl of cell suspensions collected from the grape berries. The EcoPlates were incubated at 25 °C and optical density (OD) recorded after 48 and 120 hours with a

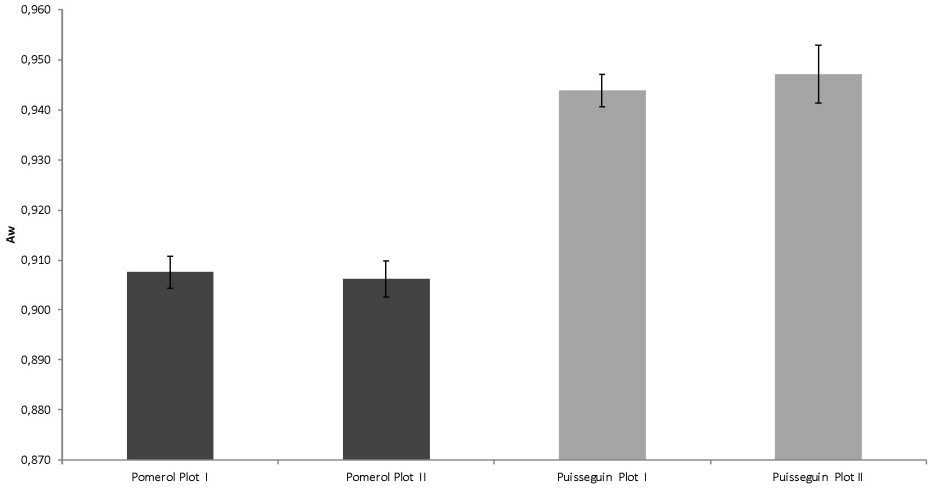


FIGURE 1. Water activity (aw) on grape skins from Polerol and Puisseguin.
Results show significantly higher values of aw on grapes from Puisseguin ($p<0.00001$). Bars represent standard deviation of the mean.

spectrophotometer (Synergy TM HT, Biotek) using a 590-nm filter. The average value for the optical density for all the carbon sources resulted in the average well color development (AWCD). The average catabolic activity was calculated as the sum of absorbance for all 31 carbon sources and, if divided by 31, is equivalent to the AWCD calculated by Garland (1996). Only data collected after 48 h were considered, and analyses using 120 h incubation times did not provide more information.

9. Statistical data analyses

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

The data were treated with different statistical analysis:

i) One- and two-way ANOVA was used to access the statistical significance of the differences in all four vineyards for aw, microbial counts, diversity and richness of CE-SSCP and T-RFLP profiles, and microbial metabolic activity. Data with non-normal residuals were subjected to logarithmic transformation prior to parametric analysis.

ii) Pearson's correlation test and regression plots were used in order to determine the relations between the following: aw and microbial counts (LT and 1/10 diluted LB media); aw and the microbial metabolic activity (AWCD); aw and metabolic diversity indices (H Met).

iii) Because the grape samples were collected in real field conditions, and each farming system has its specificities that may influence the microbial population of grape berries, we decided to create four linear models: metabolic diversity (H Met); microbial metabolic activity (AWCD); the LT population or 10 diluted LB population as dependent variable; the farming system as a categorical predictor; aw as a continuous predictor.

To study the metabolic diversity of the microbial community, Shannon diversity indices were calculated as follows:

$$H' = \sum_{i=1}^{i=s} pi \ \ln(pi)$$

where pi is the proportion of a microbial activity on substrate i in total microbial activity, and s is the number of substrates (Stephan *et al.*, 2000).

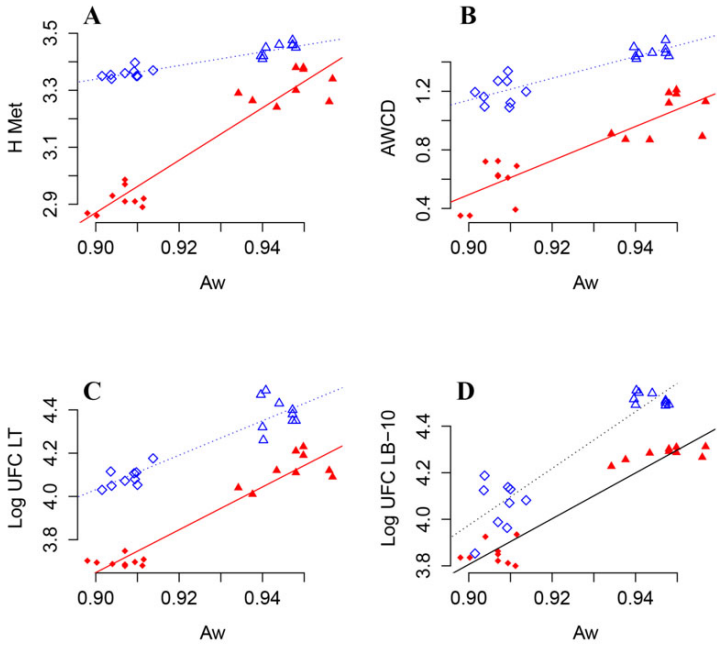
RESULTS

1. Water activity in grape skins

Aw data obtained from the four different plots were submitted to statistical analyses (two-way ANOVA). Results show significant differences between samples from different wine regions, revealing an effect of plot localization on the aw of the grape skins (Figure 1).

Aw was significantly higher in the Puisseguin plots. The farming system has no significant effect. Correlation analyses between water activity (aw), metabolic diversity (H Met), microbial metabolic activity (AWCD), LT population and 10 diluted LB population were carried out for both appellations (Figure 2).

To evaluate the effect of aw on these variables, four linear models were created (supplementary data, Table 2). Due to the possible influence



	Regression summary for dependent variables		R^2	p
A	aw × H Met	Farming system I	0.902	< 2.304E-09
		Farming system II	0.908	< 2.976 E-09
B	aw × AWCD	Farming system I	0.826	< 1.747E-07
		Farming system II	0.896	< 2.014E-07
C	aw × log cfu LT	Farming system I	0.863	< 2.578 E-07
		Farming system II	0.891	< 2.218 E-07
D	aw × log cfu LB-10	Farming system I	0.886	< 5.812 E-09
		Farming system II	0.877	< 3.749 E-08

FIGURE 2. Correlation between the water activity (aw) in grape skin and (A) the metabolic diversity (H Met) of the microbial community, (B) the microbial metabolic activity (AWCD), (C) the LT population, (D) the 1/10 diluted LB population and the respective regression summary.
Blue diamonds and blue triangles represent Pomerol and Puisseguin samples from Farming system I (organic) and red diamonds and red triangles represent Pomerol and Puisseguin samples from Farming system II (conventional), respectively.

TABLE 2 (A). Number of yeast and filamentous fungi isolates within different samples.

A. Yeast and filamentous fungi		Source of isolation			
		Pomerol		Puisseguin	
		Vineyard I	Vineyard II	Vineyard I	Vineyard II
Aureobasidium sp.	*A. proteae*	10	4	4	5
	A. pullulans	8	1	1	1
Cladosporium sp.	*C. cladosporioides*		2	4	1
	C. macrocarpum			2	
	C. silenes		1		
Cryptococcus sp	*C. carnescens*	1			
Metschnikowia sp.	*M. fructicola*			1	1
Penicillium sp.	*P. glabrum*			9	7
Phoma sp.	*P. negriana*			2	
	P. pedeiae	1			
	P. aliena	1			
Rhodotorula sp.	*R. glutinis*		2		1
Sporidiobolus sp.	*S. pararoseus*		12	1	7
Total		21	22	24	23

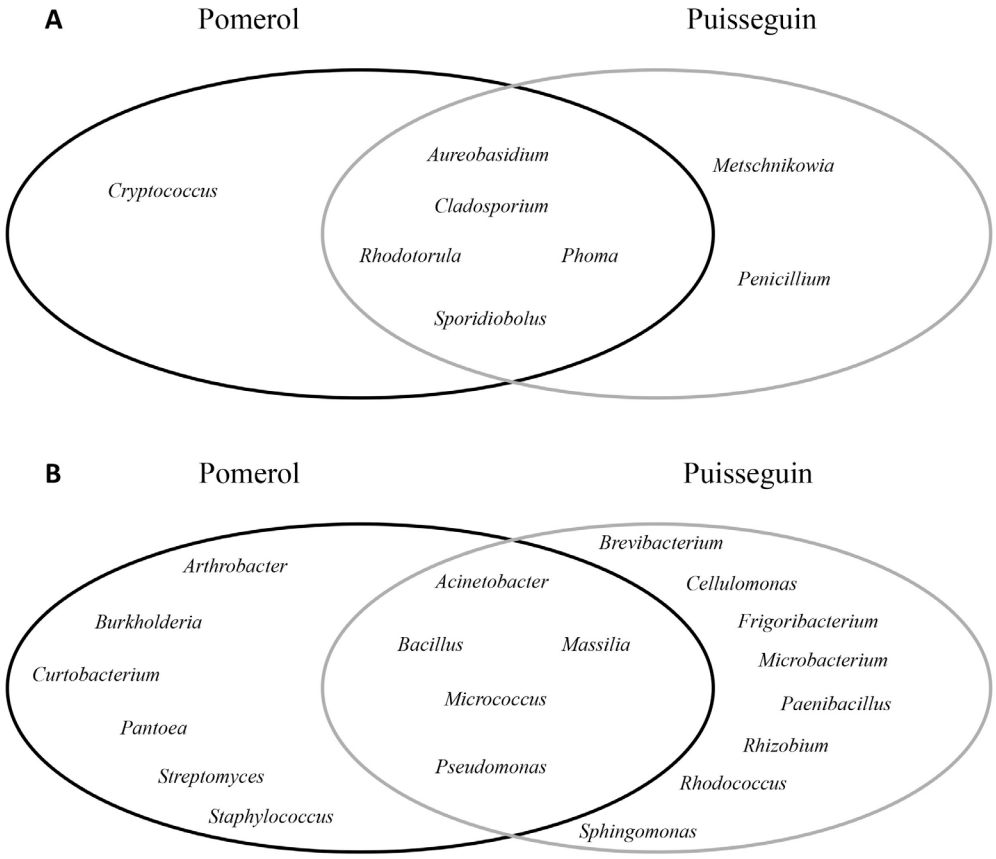


FIGURE 3. Two-way Venn diagram depicting the shared and unshared genera of (A) fungi and (B) bacteria of samples from Pomerol and Puisseguin vineyards.

of farming system on the dependent variables (H Met, AWCD, LT population and 10 diluted LB population), we used it as a categorical predictor.

The univariate tests of significance showed a significant effect of aw over each of the dependent variables (i.e. H Met, AWCD, LT population and 10 diluted LB population).

2. Size of cultivable communities

Populations grown on LT and 1/10 diluted LB media are shown in Table 1. The highest population counts in both media were detected in the Puisseguin samples. The differences were significant for LT and LB populations on the one-way ANOVA analysis with p-values of 1.69 E-08 and of 1.80 E-11, respectively.

A positive correlation was shown between the cultivable population and the aw for each vineyard, (Figure 2 C,D). The regression summary for LT and 1/10 diluted LB in the different vineyards indicates that aw had a positive effect on the cultivable population.

3. Diversity of cultivable population

From the randomly picked colonies, a total of 90 isolates in LT medium and 90 isolates in 1/10 diluted LB were assigned to species level with a classification threshold above 98. In LT medium, 13 different species belonging to 8 genera were distinguished (Table 2 A).

The most abundant Ascomycota species included *Aureobasidium proteae* (23 isolates), *A. pullulans* species (11), *Cladosporium cladosporioides* (7), and *Penicillium glabrum* (16), whereas the most abundant *Basidimycota phylum* included 20 *Sporidiobolus* isolates that have been all assigned to *Sporidiobolus pararoseus* species.

The most abundant genus *Aureobasidium* was present in all samples, although with varying abundance. Some of the genera were specific to a wine appellation: *Penicillium* and *Metschnikowia* in Puisseguin and *Cryptococcus* in Pomerol (Table 2 A, Figure.3).

The identification of isolates from 1/10 diluted LB medium showed the presence of 28 different

TABLE 3. (A) Shannon diversity index (H') and phylotype richness (S) obtained from CE-SSCP and T-RFLP profiles. (B) Shannon diversity index based on community-level physiological profiles (H') and average well color development (AWCD).

		Pomerol		Puisseguin	
		Plot I	Plot II	Plot I	Plot II
A	CE – SSCP (H')	3.71 (±0.032)	3.48 (±0.04)	4.10 *** (±0.031)	3.92 ** (±0.081)
	CE – SSCP (S)	8.33 (±0.577)	6.33 (±0.577)	10.67 (±1.528)	7.67 * (±0.577)
	T-RFLP (H')	1.29 (±0.084)	1.16 (±0.045)	1.83 ** (±0.070)	1.42 * (±0.012)
	T-RFLP (S)	13.67 (±0.577)	12.33 (±0.577)	16.00 * (±1.0)	14.33 * (±0.577)
B	Metabolic diversity (H')	3.36 (±0.012)	2.92 (±0.031)	3.45 *** (±0.019)	3.31 *** (±0.049)
	AWCD	1.19 (±0.067)	0.56 (±0.133)	1.47*** (±0.029)	.,04** (±0.138)

(A) Values in brackets represent standard deviation (n=3). (B) Values in brackets represent standard deviation (n=18). Significant differences (one-way ANOVA) between Pomerol and Puisseguin samples are represented as *p < 0.05, **p < 0.005, ***p < 0.001.

species belonging to 19 genera (Table 2B). The most abundant genera were *Micrococcus* (8 isolates of *M. luteus* and 10 of *M. endophyticus*) followed by *Pseudomonas* (15 isolates).

Some of the genera were specific to a wine appellation: B*revibacterium, Cellulomonas, Frigoribacterium, Microbacterium, Paenibacillus, Rhizobium, Rhodococcus* and *Sphingomonas* at Puisseguin, and *Arthrobacter, Burkholderia, Curtobacterium, Pantoea, Streptomyces* and *Staphylococcus* at Pomerol (Table 2B and Figure 3).

4 Community analysis by CE-SSCP and T-RFLP

The fungal and bacterial communities were analyzed by CE-SSCP and T-RFLP, respectively, from DNA extracted directly from microbial biomass retained on the filtration membranes. The diversity and richness indices were calculated and mean values of Pomerol and Puisseguin samples were compared separately according to the farming system by one-way ANOVA. There was a significant difference between samples of Puisseguin and Pomerol, in almost all indices, with exception of the phylotype richness (S) of the CE-SSCP profiles (Table 3).

The diversity and richness of the microbial community profiles were, in all samples, significantly higher in Puisseguin vineyards.

DISCUSSION

Epiphytic microbial communities of grape berries have been the subject of increasing interest from researchers, winegrowers and winemakers. This interest is in part due to the important role that epiphytic microbial communities have in pest management, crop protection and wine quality.

In the present work, we analyzed the epiphytic microbial community of wine appellations characterized by specific micro-climatic conditions. Despite the small distance of around 12 km between Pomerol and Puisseguin, Pomerol vineyard is warm and dry whereas Puisseguin is more humid and cold (Bois, 2007).

At harvest stage, aw was significantly higher for samples originated from humid and cold climate appellation compared to those from warm and dry climate appellation. There was a significant effect of the vineyard location on the aw but no effect of the farming system. We found that aw had a positive effect on the cultivable population as well as on metabolic activity and diversity.

Cultivable communities (bacteria, yeast and filamentous fungi) and metabolic activity were higher in the Puisseguin samples. Some of the fungal genera were specific to a wine appellation: *Penicillium* and *Metschnikowia* in *Puisseguin* and *Cryptococcus* in Pomerol.

In a previous study, Rousseau and Doneche (2001) showed that *Metschnikowia* species were negatively influenced by decreasing the aw and that its growth was higher at aw values close to 0.945 (the average aw value of Puisseguin samples) than 0.907 (the average aw value of Pomerol samples). Moreover Abellana *et al.* (2001) concluded that at the same temperature the growth rates of *Penicillium* increase at higher aw.

As for bacteria, we also observed that some genera were specific to a wine appellation: *Brevibacterium, Cellulomonas, Microbacterium, Paenibacillus,*

Rhizobium, Rhodococcus and *Sphingomonas* in Puisseguin, and *Arthrobacter, Burkholderia, Curtobacterium, Pantoea, Streptomyces* and *Staphylococcus* in Pomerol.

Results of the bacterial and fungal community structure analysis by T-RFLP and CE-SSCP, respectively, showed that the diversity and richness of the microbial community profiles were, in all samples, higher at Puisseguin vineyards compared to Pomerol. In relation to the climate characteristics of each area, these results confirm previous works that shows microorganism growth rate was consistently decreased at low aw (Plaza *et al.*, 2003). A reduction in aw has a dramatic effect on fungal and bacterial growth, with an increase in the lag phase and decreases in the growth rate and cell yield (Scott, 1957.). Many bacteria have their maximum growth rates between aw 0.990 and 0.995 and, from the maximum point the growth rate diminishes until the minimum aw for growth is reached. For example, the growth rate of *S. aureus* at aw 0.90 is only 10 % of its maximum growth rate (Scott, 1957). At aw values lower than the minimum for growth, cells either remain dormant or die. Another important point for microorganism growth is the composition in solute media growth. Generally, NaCl, KCI, glucose and sucrose show similar patterns while glycerol permits growth at lower aw values. Several examples presented by Sperber (1983) show similar minimum aw values for growth when NaCI or glucose are the solutes, but markedly lower values when glycerol is the solute.

Conversely, it has been known that reduction of water activity has a variable effect on different genera of bacteria and yeast. As aw is reduced, some bacteria stop growing at high values, while others are able to grow at much lower values. Indeed, the intracellular aw of cells is slightly lower than that of the external medium so that cells are able to maintain turgor pressure. When the aw of the external medium is reduced, cells are subjected to osmotic shock and rapidly lose water. Koujima *et al.* (1978) have shown that *S. aureus* loses about 50 % of its intracellular water when switched from a medium of aw 0.995 to one of aw 0.950. Under similar conditions, Gibson (1973) has shown that the cell volume of *Salmonella typhimurium* decreases 44 %. More recently, Torres *et al.* (2003) showed that a modification of lower aw-levels (0.721–0.901) with glycerol did not maintain the viability of the *Candida sake* cells while at higher aw-levels (0.93–0.95) with either glycerol or PEG improved the viability. But *Candida sake* cells maintained viabilities > 60 % when sugars such as trehalose and polyols such as glycerol and PEG were used as protectants in liquid formulations.

Altogether, our results suggest that microclimate through aw of grapes skins impact the microbial population of grape berries. Aw appears to be a key component to explain how microclimate can affect grape berry microbiota and its geographic distribution, thus resulting in *terroir*: specific microbial community.

Moreover, on the berries there are some epiphytic genera identified in this work. that have been described in the literature as biological control agents (BCAs) used against phytopathogenic infections of the grapevine. Thus, the use of these BCAs and their antagonistic activity could be disrupted by the differences in aw. BCA is regarded by many wine growers as promising in crop protection, and studies on this matter have multiplied substantially in the last years (Compant and Mathieu, 2016). In viticulture some of the most common BCAs are fungi genera such as *Aureobasidium*, *Sporidiobolus, Ampelomyces, Streptomyces* and *Trichoderma*, (Martins, 2012; Schmid *et al.*, 2011) bacteria like *Bacillus spp*. or *Pseudomonas spp*. (Compant *et al.*, 2011), and yeasts like *Candida, Metschnikowia* or *Pichia* (Santos *et al.*, 2004; Sipiczki, 2006; Tasin *et al.*, 2009). A key point for successful BCA colonization is the climatic condition (Whipps, 2001). Under field conditions, the plant or fruit surface is subjected to fluctuations of temperature, vapor pressure deficit, surface wetness, etc. (Burrage, 1971), and this variation will influence the implementation and activity of the BCA and can reduce its efficiency (Elad and Stewart, 2007). For this reason BCA needs to be applied when climatic conditions are favorable to vine colonization (Whipps, 2001).

The effectiveness of BCAs depends largely on the environmental condition of the ecosystem where they are inoculated. Knowledge about the ecological requirements of the epiphytic community is important in order to predict their behavior in natural situations.

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