THE WINE MICROBIAL CONSORTIUM: A REAL TERROIR CHARACTERISTIC

LE CONSORTIUM MICROBIEN DU VIN : UNE RÉELLE CARACTÉRISTIQUE DU TERROIR

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Abstract: Yeast, bacteria, species and strains play a key role in the winemaking process by producing metabolites, which determine wine sensorial qualities. Therefore microbial population enumeration, species identification and strain discrimination from berry surface at harvest to storage in bottle are fundamental. The microbial diversity and significance of its variation according to the estate localization have not really been thoroughly considered in literature. This is the focus of this work. That should be of great interest because the spontaneous microbial population dynamics associated with a wine producing estate provide information on what might be considered as the method to obtain specific terroir typed wine. The both use of conventional microbiological methods like microbial population enumeration on nutritive selective media and efficient molecular tools of species identification like PCR-RFLP for yeasts and PCR-DGGE for bacteria and strains discrimination have demonstrated significant microbial differences between different estates localized in the Bordeaux area. Theses results appeared very interesting since certain microbial species are clearly specific of certain estates, in particular the bacterium Pediococcus parvulus, and also some strains of Saccharomyces cerevisiae, of Oenococcus oeni and Brettanomyces bruxellensis. Within an estate this specificity persists from one year to another. These differences observed suggest that the indigenous winemaking processes can contribute to the specificity of the wines produced on the various estates.. That concludes to the hypothesis of a microbial part in wines speficities.

Résumé : Les espèces et les souches de levures jouent un rôle majeur lors de la vinification car les métabolites qu'ils produisent influencent les qualités aromatiques du vin. Par conséquent, les suivis microbiologiques des espèces et des souches impliquées depuis le raisin au vignoble jusqu'au conditionnement du vin sont fondamentaux. Les variations de la diversité microbienne selon la localisation du vignoble et les différences observées entre les chais n'ont pas fait, jusqu'à présent, l'objet de nombreux travaux. La comparaison de la diversité des souches et des espèces entre différents domaines est l'objectif de ce travail. Les techniques classiques de microbiologie comme l'isolement et le dénombrement des populations sur des milieux de cultures sélectifs associées aux méthodes de biologie moléculaire d'identification des espèces par PCR-RFLP pour les levures et par PCR-DGGE pour les bactéries et de discrimination des souches ont révélé des différences entre les domaines étudiés. Ces observations se sont révélées très intéressantes puisque certaines espèces microbiennes sont clairement apparues spécifiques de certains domaines, notamment la bactérie *Pediococcus parvulus*, ainsi que certaines souches de *Saccharomyces cerevisiae*, d'*Oenococcus oeni* et de *Brettanomyces bruxellensis*. Au sein d'un domaine, cette typicité persiste également d'un millésime à l'autre. Ces différences observées suggèrent que les vinifications indigènes peuvent concourir à la typicité de vins produits dans différents domaines. Ces différences d'espèces et de souches peuvent réellement aboutir à l'existence d'une composante microbiologique à la notion de spécificité des vins.

Key words: microbial ecology, species, strains, diversity, specificity

Mots-clés : écologie microbienne, espèces, souches, diversité, spécificité

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INTRODUCTION

Winemaking is a complex microbial process. Yeast and bacteria play key roles. Alcoholic fermentation performed by yeast and malolactic fermentation performed by lactic acid bacteria (LAB) are beneficial metabolisms but other metabolisms are prejudicial (LONVAUD-FUNEL, 1999; LOUREIRO and MALFEITO-FERREIRA, 2003). It is notably the case of volatile phenols by the yeast *Brettanomyces bruxellensis* (CHATONNET *et al.*, 1992), biogenic amines (LONVAUD-FUNEL, 2001) and exopolysaccharides (LONVAUD-FUNEL and JOYEUX, 1988) by certain LAB species. Sensorial qualities of the wine integrate all the microbial growths, which intervene during its elaboration. The production of sensorial compounds is strongly dependent on the species. For instance, *Pichia* and *Candida* produce large amounts of ethyl acetate, whereas *Saccharomyces* and *Torulaspora* generate small amounts of this ester (PLATA *et al.*, 2003). Concerning bacteria, it was reported that *Pediococcus* and *Lactobacillus* genera degrade higher amounts of glycerol than the other LAB species (CLAISSE and LONVAUD-FUNEL, 2001; PASTERIS *et al.*, 2005). Other metabolism characteristics are strains dependent. It is notably the case of glycerol production by *Saccharomyces cerevisiae* (NIKOLAOU *et al.*, 2006) and carbamate ethyl production by *Oenococcus oeni* (UTHURRY *et al.*, 2005). The quantities of volatile phenols suitable to be produced by *B. bruxellensis* are also suspected be function of the strains (RENOUF *et al.*, 2006e).

Monitoring of microbial populations, species identification and distinction of the strains among a species, at each stage of the winemaking process are therefore of great importance. That is possible by both use of conventional microbiological methods to evaluate population levels and efficient molecular tools, which allow efficient microbial species identification and strain discrimination.

Performing experiments in Bordeaux region in four different vineyards on berries since the berry set and at each stage of the wine elaboration, we evaluated (i) the impact of estate localization on the population level at the harvest period, (ii) species detected and detection frequency according to the vineyard, (iii) strains of *S. cerevisiae*, *B. bruxellensis* and *O. oeni* diversity in each cellar, in order to evaluate if the microbial consortium can be considered such as a real terroir characteristic.

MATERIALS AND METHODS

I - GRAPE AND WINE SAMPLES

Grape and wine samples were collected, according to RENOUF *et al.* recommendations (2005, 2006b), from several domains in the Bordeaux area: Graves (A), Libournais (B, C) and Médoc (D).

II - ISOLATION OF MICROBIAL POPULATION AND CELL COUNTS

Yeasts were plated on YPG medium containing glucose 20 g/L, bactotryptone 10 g/L, yeast extract 10 g/L and agar 20 g/L, pH adjusted to 5.0 using orthophosphoric acid. For counting total yeast population (TY), after sterilization, the medium was supplemented with biphenyl (0.015 %w/v) and chloramphenicol (0.01 %w/v) to respectively inhibit mould development and bacterial growth. Addition of 0.1% (w/v) cycloheximide eliminated the *Saccharomyces* sp. and allowed isolation of non-*Saccharomyces* (NS) yeast. At 25°C, the incubation lasted 5 days for TY and 10 days for NS. Lactic acid bacteria were isolated on MRS plates: Lactobacilli MRS broth 55 g/L, D-L malic acid 10 g/L, agar 20 g/L, pH 4.8 with NaOH 10N. Growth of yeasts was inhibited by adding 50 mg/L of pimaricine and growth of acetic acid bacteria was inhibited by incubation under anaerobic conditions. LAB plates were incubated at 25 °C for 10 days. In each case, colonies were counted on plates with 30 to 300 colonies.

II - MICROBIAL IDENTIFICATION

1) Direct DNA extraction

An universal protocol for bacteria and yeast studies and for berry washing solutions, fermenting musts, wine during ageing, and bottled wine samples was used. Microbial biomass was collected from 10 mL of fermenting must, 100 mL of wine during aging and in bottle, 100 mL of berry washing solution and winery equipment cleaning solution by centrifugation (30 min, 10,000 g, 4 °C), and the pellet was washed in 2 mL of Tris 10mM (GenApex)-EDTA 1 mM (GenApex) (TE) buffer. After a second centrifugation (10,000 g for 15 min at 4°C), the supernatant was discarded and the pellet re-suspended in 300 µL of 0.5mM EDTA pH 8. Three hundred µL of glass beads were added (Ø 0.1 mm) and samples were mixed at maximum speed for 15 min. Then, 500 µL of nuclei lysis (Promega) and 300 µL of protein precipitation solution (Promega) were added and mixed for 30 s. Precipitation of cellular fragments was made on ice for 5 min. After another centrifugation (10,000 g for 5 min at 4 °C), the supernatant containing the DNA was transferred to a new microcentrifuge tube. Residual polyphenols were precipitated after addition of 100 µL of a 10 % PolyVinyl-Pyrrolidone (PVP) (Sigma-Aldrich) solution and vortexing at high speed for 10s. For highly tannic wines, this step can be repeated. After centrifugation (10,000 g for 5 min at 4 °C), the supernatant was transferred to a 1.5 mL microcentrifuge tube containing 500 µL of isopropanol. The tube was gently mixed by inversion until a visible mass of DNA could be seen, and left at -20 °C for three hours. After centrifugation (10,000 g for 20 min at 4 °C), 300 µL of a room temperature 70 % ethanol solution were added to the pellet before a final cen-

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trifugation (10,000 g for 5 min at 4 °C). Ethanol was carefully removed and the tube dried. Fifty µL of PPI (Pour Preparation Injectable) water with 1 µL of RNase (Promega) were used to rehydrate DNA overnight at 4 °C. After rehydratation, this DNA was stored at -20 °C. This extraction can also be used to process biomasses collected on Petri dishes. The biomass was collected in TE buffer before the first step of centrifugation. DNA concentrations were standardized (50 ng/µL) by measuring optical density at 260 nm with a SmartSpec (+) Bio-Rad spectrophotometer.

2) Yeast species identification

Yeast species identification was performed by molecular tools. DNA directly extracted from samples (RENOUF *et al.*, 2006d) was analyzed by PCR-DGGE targeting the D1/D2 domains of the 26S RNA gene (COCOLIN *et al.*, 2000). We also used the RFLP analysis of the 5.8S rRNA gene and its two ribosomal internal transcribed spacers (ITS1 and ITS2) (GUILLAMON *et al.*, 1998) on isolated colonies from TY plates in order to add quantitative data to the qualitative information provided by the PCR-DGGE. Percentage of each species was estimated. The frequency of detection is also provided. For a species, it is the relationship between the number of times where it was detected and the total number of samples carried out. The sum of the frequencies of detections of the whole of the species can obviously exceed 100%, which is naturally not the case of the sum of the proportions of each species. But, it is important not to assimilate the frequency of detection of a species during a continuous monitoring and the percentage of the species within the population during a single analysis. The frequency of detection is more relevant since it integrates the variations usually observed during grape maturation into the vineyard (RENOUF *et al.*, 2005) or during the winemaking into the cellar (RENOUF *et al.*, 2006d). Consequently the differences in frequency observed are more significant than specific variations of the proportion of each species. Based on NS colonies, *B. bruxellensis* species was identified by using specific-species nested-PCR method developed by IBEAS *et al.* (1996).

3) Yeast strains discrimination

Identification of *S. cerevisiae* at strain level was done by PCR according to method and δ12/δ21 primers developed by LEGRAS and KARST (2003). *S. cerevisiae* strain discrimination experiments were made from samples of cellars A, C, and D. No starters were ever used in the winery C, two known strains were employed only in white must vat in the cellar A, and one strain was employed in D where only red wine was elaborated.

The typication of the *B. bruxellensis* strains was made by REA-PFGE according to the MIOT-SERTIER and LONVAUD-FUNEL (2006) protocol.

4) Bacteria species identification

DNA directly extracted from samples was analyzed by PCR-DGGE targeting the *rpoB* gene according to RENOUF *et al.* (2006a) protocol. Colonies isolated on LAB plates were also tested by using a species-specific PCR method for *O. oeni* (DIVOL *et al.*, 2003). That gave the percentage of the *O. oeni* species in the LAB population. Percentages of other species were estimated by their detection of their specific band on DGGE gel.

5) *Oenococcus oeni* strains discrimination

The typication of isolated *O. oeni* was based on multiplex RAPD-PCR method using two primers: On2 and Coc determined by ZAPPAROLI *et al.* (1998) and COCCONCELLI *et al.* (1995) respectively and both used by REGUANT and BORDONS (2003).

IV - STATISTICAL ANALYSIS

The effects of domain factors were analysed by using Sigmastat® software. When the probability (p) was less than 0.05, it was accepted that the variable under consideration had a significant effect on the population number.

RESULTS AND DISCUSSION

First, we focused on the frequency of detection for five yeast species from berries surface (table I), which is the ratio between positive samples for the considered species detection and the total number of samples perfor-

Table I - Detection frequency of five yeast species on four vineyards.

Fréquence de détection de cinq espèces de levures sur les quatre domaines

	Vineyard A	Vineyard B	Vineyard C	Vineyard D
Candida cantarelli	0 %	60 %	5 %	0 %
Pichia anomala	50 %	30 %	35 %	40 %
Metschnikowia fructicola	30 %	0 %	0 %	0 %
Rhodotorula graminis	20 %	15 %	20 %	25 %
Bulleromyces albus	0 %	10 %	0 %	40 %

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med in an estate. Among yeast species, *Candida cantarelli*, *Pichia anomala* and *Metschnikowia fructicola* are well known to ferment sugars during early stages of fermentation, when the ethanol concentration is still low. They should contribute to wine complexity. *Rhodotorula graminis* and *Bulleromyces albus* are significant in the berry microbial ecosystem (RENOUF *et al.*, 2005) but not known to play a role after crushing.

R. graminis was detected with no significant difference in the four vineyards but *B. albus* was only detected in B and D. *P. anomala* was detected with similar frequencies in the four vineyards whereas *M. fructicola* was detected only in the vineyard C. *C. cantarelli* was a predominant species in the vineyard B but it was never detected in A and D.

Similar observations were made about bacteria (table II). *Gluconobacter oxydans* was detected without significant difference in vineyards, like *Burkholderia vietnamiensis*, which is a crucial species in the bacteria community on berries (RENOUF *et al.*, 2005). *Pediococcus parvulus* was only detected in vineyard B and D with significant difference of their detection frequency.

These results underlined the importance of geographical location on the presence of certain yeast and bacteria species, which could be considered as particular residents of each vineyard.

These factors may also influence the level of yeast and bacteria population from A, B, C and D vineyard obtained during three successive vintages (2003, 2004 and 2005) (figure 1). Differences in the mean total yeast populations among the different vineyard were statistically significant ($p<0.001$). Population in vineyard D was higher than in vineyard A, in vineyard B, and in vineyard C. Moreover, total yeast populations statistically differed according to the vintage ($p<0.001$). Total yeast populations in 2005 were higher than in 2004 and 2003. However, effect of vineyard was independent on the vintage ($p<0.001$). Concerning total bacteria population, the population was lower in vineyard C during all vintages.

Consequently, the level of yeast and bacteria populations, and species detected seemed to be function of the vineyard. The presence of certain species on berries (*M. fructicola* and *P. parvulus*), able to persist in wine (RENOUF *et al.* 2006a, 2006c), may have a real impact on wine quality. Other species (*B. albus*) unable to resist in wine may only affect the maintenance of the microbial ecosystem on grape berries and also on the preservation of high population levels of microorganisms (RENOUF *et al.*, 2005). Yeast and bacteria populations

Table II - Frequency of the detection of three bacteria species on four vineyards.

Fréquence de détection de trois espèces de bactéries sur les quatre domaines.

	Vineyard A	Vineyard B	Vineyard C	Vineyard D
Gluconobacter oxydans	40 %	40 %	50 %	35 %
Pediococcus parvulus	0 %	10 %	0 %	90 %
Burkholderia vietnamiensis	60 %	50 %	30 %	40 %

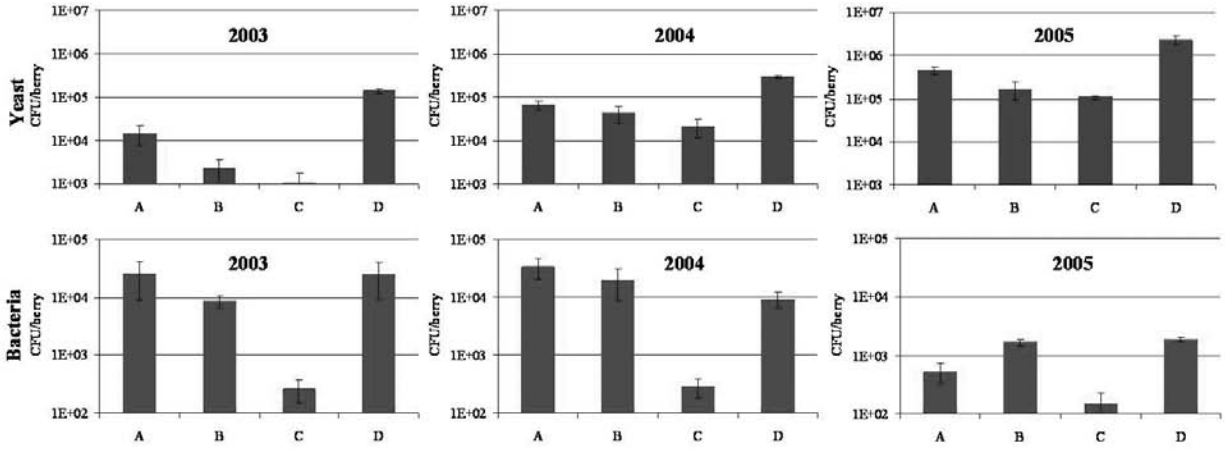


Figure 1 - Total yeast and total bacteria populations on berries at harvest in the four vineyards (A, B, C and D) during three vintages (2003, 2004 and 2005).

Populations de levures totales et de bactéries totales sur les baies au moment des vendanges sur les quatre domaines étudiés durant trois millésimes (2003, 2004 and 2005)

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on berries surface at harvest moment have a significant effect at the beginning of the winemaking (RENOUF *et al.*, 2006c). Therefore, differences observed between each vineyard according to their geographical localization underline the part of the indigenous microflora, which may be considered as an estate specificity.

Then, we evaluated the *S. cerevisiae*, *B. bruxellensis* and *O. oeni* diversity at a strain level in the different cellars at different stages of the winemaking process. For

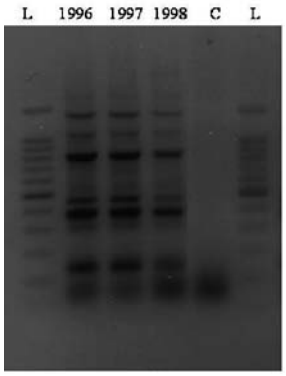


Figure 2 - Comparison of *S. cerevisiae* strains isolated from bottled wines produced in the cellar C for three different vintages (L 100 pb ladder, C: negative control).

Comparaison des souches de *S. cerevisiae* isolées dans des bouteilles de trois millésimes différents produites au chai C (L marqueur 100 pb, C : control négatif)

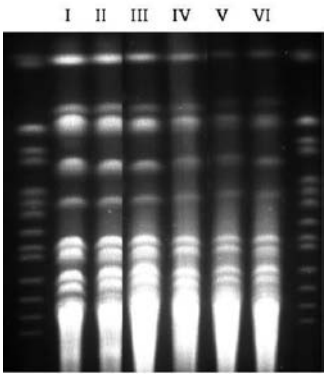


Figure 3 - Comparison of strain profile of *B. bruxellensis* isolated at different stages of the winemaking process in the estate A.

I: berries at berry set, II: berries at the beginning at the veraison, III: at the end of the veraison, IV: berries at harvest, V: must in fermentation and VI: Wine at the aging.

Comparaison des profils des souches de *B. bruxellensis* isolées au domaine A à différentes étapes du processus de vinification.

I : sur les baies à la nouaison, II : sur les baies au début de la véraison, III : sur les baies à la fin de la véraison, IV : sur les baies au moment des vendanges, V : dans le moût en fermentation et VI : dans le vin durant l'élevage.

S. cerevisiae and *O. oeni*, survival in wine and fermentative ability were clearly strain dependent characters which result form genomic differences between strains (DELAHERCHE *et al.*, 2006). It is probable that it is the same for the production ability of volatile phenols by *B. bruxellensis* strains. The number of *S. cerevisiae* strains detected during different vintages and also their frequency varied between the four cellars. Many *S. cerevisiae* strains were detected during fermentations in cellar C. For a total of 25 colonies analyzed each year, the average number of different strains was 8 in the cellar C. Among them, two were detected each year. Concerning cellars A and D, it was respectively 5 and 4. In cellar A, the starter employed in white must was not always detected in red must. In cellar D, the starter strain cohabited with two indigenous strains mainly at the beginning of alcoholic fermentation where the strain diversity was maximal (RENOUF *et al.*, 2006c). These strains were detected during different vintages. Concerning the indigenous strains, there was no strain common in the different cellars. Each cellar owned its specific pools of indigenous *S. cerevisiae* strains suitable to act during alcoholic fermentation. Even if the number of indigenous strains could be influenced by the use of starters, the winery and oenological environment has a significant effect. In some cases, residual *S. cerevisiae* strains could be remained in the bottled wine. Finally, the comparison of several wines produced in the same cellar showed that the residual strain had a similar profile. This strain should be a specific resident in the final wines of the cellar (figure 2).

The *B. bruxellensis* strain discrimination is also a great interest because it was the most prejudicial yeast species in wine due to its production of volatile phenols. In cellar A, we monitored *B. bruxellensis* at different stages of the winemaking process. A similar REA-PFGE profile was observed from grape berries and wine (figure 3). In cellar B, comparison of the *B. bruxellensis* strains was made during malolactic fermentation, which seemed to be a critical stage (RENOUF *et al.*, 2006c) for their growth in wine, for three successive vintages. A same strain was isolated and identified for three successive vintages (figure 3). Therefore, each estate had its specific *B. bruxellensis* strain, which should be considered as constant resident in the winery.

Concerning bacteria, we focused on the *O. oeni* strains isolated during MLF. The main strain was specific to each cellar (figure 5). In a same cellar, a major RAPD profile was similar for different vintages. In bottles, the bacteria diversity is lower than during winemaking and ageing. Table III lists the bacteria population and the species identified in old wines produced in cellars A, C and D. The levels of residual LAB population were statistically ($p=0.03$) different according to the cellar. Survival of high LAB population was strongly dependent on the

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Table IIII - Lactic acid bacteria populations and species isolated and identified from bottled wines produced in the cellars A, C and D (- means that no residual LAB was counted).

Population de bactéries lactiques et espèces isolées et identifiées dans les bouteilles produites dans les chais A, C et D (- signifie qu'aucune population résiduelle de bactéries lactiques est dénombrée).

	Vintages	1949	1981	1993	1996	1998	2003
A	LAB population (CFU/mL)	-	-	-	1.1×10^2	-	1.7×10^2
	Species identified	-	-	-	*O. oeni*	-	*O. oeni*
C	LAB population (CFU/mL)	-	1.7×10^3	4.7×10^2	2.7×10^2	1.1×10^3	5.4×10^2
	Species identified	-		*O. oeni*	*O. oeni*	*O. oeni*	*O. oeni*
D	LAB population (CFU/mL)	4.1×10^6	3.5×10^5	1.5×10^4	8.5×10^5	7.3×10^5	6.5×10^6
	Species identified	*P. parvulus*	*O. oeni* *P. parvulus*	*O. oeni* *P. parvulus*	*O. oeni* *P. parvulus*	*O. oeni* *P. parvulus*	*O. oeni* *P. parvulus*

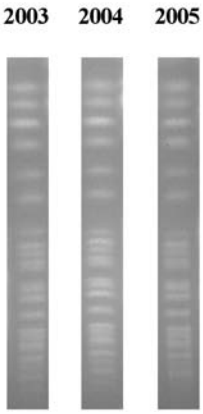


Figure 4 - Comparison of *B. bruxellensis* strains isolated during malolactic fermentation in the cellar B for three successive vintages.

Comparaison de souches de *B. bruxellensis* isolées durant la fermentation malolactique au chai B pendant trois millésimes successifs.

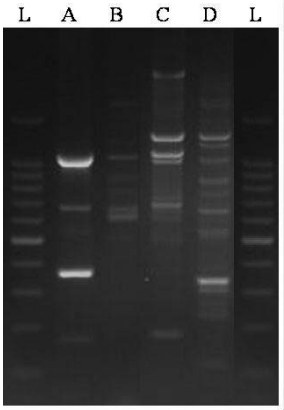


Figure 5 - Comparison of the profile of the *O. oeni* strain involved in the MLF in the four cellars (L: 100 pb ladder).

Comparaison du profil des souches d'*O. oeni* isolées au moment de la FML dans les quatre chais. (L : marqueur 100 pb).

oenological practices, notably the level of sulphating, fining and filtration performed before bottling. RAPD profiles have previously revealed that the residual *O. oeni* strains were different according to the cellars, and since the resistance in wine was strongly strains dependent, it was possible to attribute the survival of high level of LAB population in certain cellar by the result of certain strain specificity. For D, LAB population was always the highest and the detection of *O. oeni* was always associated to *P. parvulus*. In very old vintage (1949), *P. parvulus* was alone. This phenomenon may also be attributed to a cellar characteristic. Moreover, in this cellar the detection frequency of *P. parvulus* was also the highest during the vineyard monitoring like previously mentioned. That demonstrated the part played by the grape microbial consortium on species acting in wine (RENOUF and LONVAUD-FUNEL, 2006). Combination of vineyard indigenous flora associated with oenological practices currently used in the cellar may be determinant part in specific terroir characteristics.

CONCLUSION

Wine sensorial qualities are affected by components mainly formed during fermentation and also by microbial growth after the post-fermentation SO_2 addition, notably the spoilage by certain species like *B. bruxellensis*, which confers off-odours to wine. In fact, wine composition depends on a number of variables including grape variety, soil type, winemaking practices, but the micro-

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bial species and strain diversity has also a crucial implication.

Results obtained in this study show that microbial populations, species and strain diversity differ according to the vineyard, which could be a result of viticulture practices selecting the most resistant species but also of certain terroir specificities. This is also significant during the first stage of the winemaking process when the berries enter in the cellar. Among species dominating at early stage of winemaking certain were detected only in one estate. These species may contribute to wine quality if they proliferate extensively enough. They may be involved in the maintenance of the typical profile of the wine. Concerning *S. cerevisiae* and *O. oeni*, the main ferment agents, strain comparison carried out between the different cellars and at each stage of the process suggested that strains should be considered as constant and specific of a single cellar. Similar results were obtained for the main spoilage yeast species, *B. bruxellensis*. The volatile phenol amounts are probably strain dependent and some certain cellar should be more sensitive to the « Brett » problem. *P. parvulus* seemed to be a specific resident in certain vineyard and corresponding cellar. Its growth may also be prejudicial due to the high frequency of ropy strains. These indigenous microbial species and strains associated with a wine producing area should be directly involved in the elaboration of wine with specific terroir characteristics by acting on fermentation kinetics, metabolite profiles and alteration risks during ageing.

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