# 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 the sensorial qualities of wine. Therefore microbial population numeration, species identification and strains discrimination from berry surface at harvest to storage in bottle are fundamental. The microbial diversity and significance of its variation according to vineyard and cellar have not really been thoroughly considered in literature, and 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 numbering the wine microbial populations and efficient molecular tools of species identification and strains discrimination has demonstrated the microbial differences according to the estate revealing the microbial part in specific terroir characteristic.

**Key words:** microbial ecology, species, strains

## Introduction

Winemaking is a complex microbial process. Yeast and bacteria play a key role. Alcoholic fermentation performed by yeast and malolactic fermentation performed by lactic acid bacteria (LAB) are beneficial metabolisms (ROMANO *et al*., 2003) but other metabolisms are prejudicial (SPONHOLTZ, 1993; LOUREIRO and MALFEITO-FERREIRA, 2003). It is notably the case of volatile phenols by the yeast *Brettanomyces bruxellensis*, biogenic amines and exopolysaccharides by certain LAB species. Sensorial qualities of the wine integrate all the microbial growths which have intervened 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 certain metabolism characteristics are strains dependent. It is notably the case of glycerol production by *Saccharomyces cerevisiae* (NIKOLAOU *et al*., 2006) and the 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. 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 and efficient molecular tools.
Performing experiments in Bordeaux region in height different vineyards on berries since the berry set and at each stage of the wine elaboration, we evaluated (i) the impact of terroir 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 to which extent the microbial consortium can be considered such as a real terroir characteristic

## Materials

### Grape and wines samples

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

**Isolation of microbial population and cell counts**

Yeast 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, 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 yeast 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.

**Yeast population analysis**

- Species identification

Yeast species identification was performed by molecular tools. We 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 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. Based on NS colonies, *B. bruxellensis* species was identified by using specific-species nested-PCR method developed by IBEAS *et al.* (1996).

- 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 F. 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 F where only red wine was elaborated.
The typication of the *B. bruxellensis* at the same level was made by REA-PFGE according to the MIOT-SERTIER and LONVAUD-FUNEL (2006) protocol.

**Bacteria population analysis**

- 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 the detection frequency of their specific band on DGGE gel.

- *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.

**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 detection frequency for five yeast species from berries surface (table 1). Among them *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 contribute to wine complexity. *Rhodotorula graminis* and *Bulleromyces albus* are significant in the berries microbial ecosystem (RENOUF *et al.*, 2005) but not known to play a role after crushing.

**Table 1 - Detection frequency of five yeast species on four vineyards.**

| | 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 % |

*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 also made about bacteria (table II).

**Table 2 - Frequency of the detection of three bacteria species on three vineyards.**

| | 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 % |

*Gluconobacter oxydans* was detected without significant different frequencies in the different vineyard, such as *Burkholderia vietnamiensis* a crucial species in the bacteria community on berries (RENOUF *et al*. 2005). *Pediococcus parvulus* was only detected in vineyard B and D. These results underlined the importance of geographical location on the presence of certain yeast and bacteria 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).

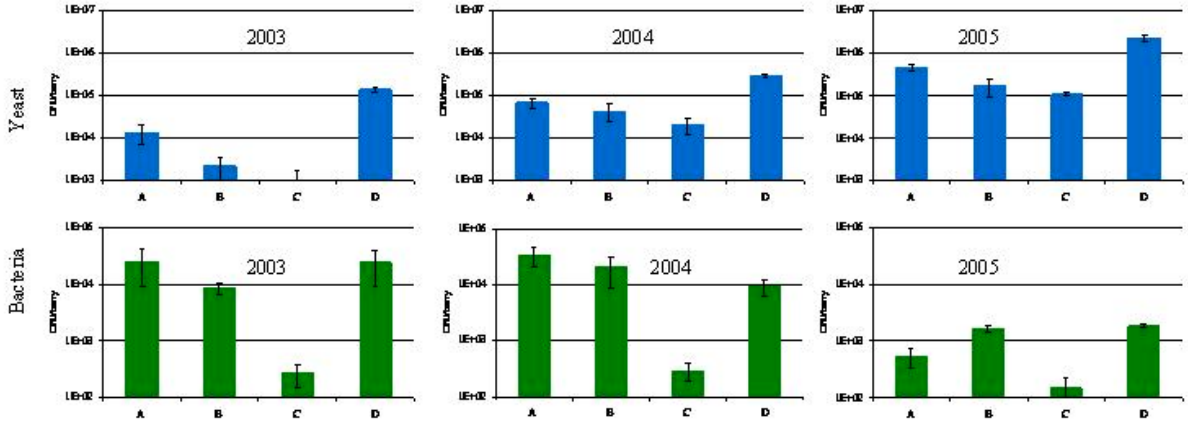


**Figure 1 - Total yeast and bacteria populations on berries at harvest in four vineyards (A, B, C and D) during three vintages (2003, 2004 and 2005).**

Differences in the mean total yeast populations values 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 population values 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, 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 level populations of microorganisms (RENOUF *et al.*, 2005). The levels of yeast and bacteria populations on berries surface at harvest moment have a significant effect at the beginning of the winemaking (RENOUF *et al.*, 2006c). Therefore, difference according to the vineyard localization underlines the role played by the vineyard indigenous microflora which may be considered as a terroir characteristic.

Then, we evaluated strain repartition of *S. cerevisiae*, *B. bruxellensis* and *O. oeni* in the different cellars at different stages of the winemaking process. The number of *S. cerevisiae* strains detected at each vintage and their frequency varied between the three cellars. Many *S. cerevisiae* strains were detected during fermentations in cellar C: for 25 colonies analyzed each year the average number of different strains was 8. Among them, two predominant yeast strains were detected each year. Concerning cellar C and F it was respectively 5 and 4. In C, the starter employed in white must was not always detected in red must. In F, the starter strain cohabited with two indigenous strains mainly at the beginning of alcoholic fermentation. These strains were detected in different vintages. Concerning the indigenous strains, there was no strain common in the different cellars. 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 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).


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

The *B. bruxellensis* strains discrimination is also a great interest. In cellar A, we monitored *B. bruxellensis* at each time of the winemaking process. A Similar profile was observed from grape berries and wine (figure 3).

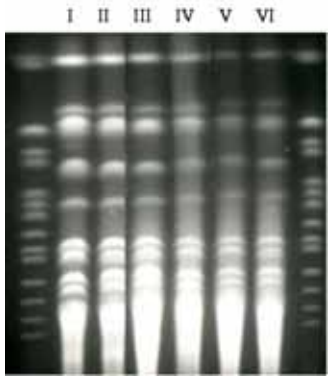


**Figure 3 - Comparison of strain profile of *B. bruxellensis* isolated at different stage of the winemaking process. I: Berries at berry set, II: Berries at the beginning at the veraison, III at the end of the veraison, IV Berries at harvest, V: Must in fermentation and VI: Wine at the aging.**

That confirmed the importance of the vineyard indigenous microflora. In cellar B, comparison of the *B. bruxellensis* strain at malolactic fermentation which seemed to be a critical stage for their growth in wine (RENOUF *et al.*, 2006c) showed that it was the same strain for three successive vintages (figure 3). For cellar (G) a strain isolated from old bottled wines had the same profile. Therefore, the *B. bruxellensis* strains should be considered as constant residents in the winery.

Concerning *O. oeni*, we focused on the strains isolated during MLF. The main strain involved in MLF in the five cellars was specific to the cellar.

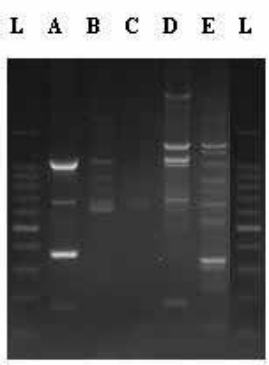


**Figure 4 - Comparison of the profile of the *O. oeni* strain involved in five different cellars (A, B, C, D and E) (L:100 pb ladder).**

In the same cellar, one of the major RAPD profile during the winemaking was similar for different vintages. In bottled wine, the bacteria diversity is lower than during winemaking and aging. Table 3, lists the bacteria population and species identified in old wines produced in three cellars.

**Table 3 - Lactic acid bacteria population and species in wines produced in three cellars.**

| | Vintages | 1949 | 1981 | 1993 | 1996 | 1998 | 2003 |
|---|---|---|---|---|---|---|---|
| **A** | LAB population (CFU/mL) | - | <1 CFU/mL | <1 CFU/mL | $1.1\times10^2$ | <1 CFU/mL | $1.7\times10^2$ |
| | Species identified | - | - | - | *O. oeni* | - | *O. oeni* |
| **C** | LAB population (CFU/mL) | - | $1.7\times10^3$ | $4.7\times10^2$ | $2.7\times10^2$ | $1.1\times10^3$ | $5.4\times10^2$ |
| | Species identified | - | | *O. oeni* | *O. oeni* | *O. oeni* | *O. oeni* |
| **D** | LAB population (CFU/mL) | $4.1\times10^6$ | $3.5\times10^5$ | $1.5\times10^4$ | $8.5\times10^5$ | $7.3\times10^5$ | $6.5\times10^6$ |
| | Species identified | *P. parvulus* | *O. oeni* *P. parvulus* | *O. oeni* *P. parvulus* | *O. oeni* *P. parvulus* | *O. oeni* *P. parvulus* | *O. oeni* *P. parvulus* |

The levels of residual population were statistically significant ($p$=0.03) different according to the cellar. Survival of high LAB population was strongly dependent on the oenological practices, notably the level of sulphating, fining and filtration performed before bottling. RAPD profiles showed that the residual *O. oeni* strains were different according to the cellars. Since the resistance in wine was strongly dependent on the strains, it was possible to attribute the survival of high level of LAB population in certain cellar by the strain specificity impact. 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 in the vineyard monitoring. Combination of vineyard indigenous flora associated with oenological practices currently used in the cellar may be determinant part in specific terroir characteristics.

# Conclusion

Sensorial quality of wine is affected by components mainly formed during fermentation and microbial growth even after the post-fermentation $SO_2$ addition. Wine composition depends on a number of variables including grape variety, soil type, winemaking practices and the microbial species play also a crucial role. Spontaneous fermentations, notably, involve numerous microbial species and strains which may influence the sensorial properties of the resulting wine. Results obtained in this study show that microbial populations and diversity in the vineyard differ according to the vineyard resulting of viticulture practices but also of terroir specificities. This is 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 be prejudicial due to the high frequency of ropy strains.

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