GLOBAL SURVEY OF THE MICROBIAL ECOSYSTEM DURING ALCOHOLIC FERMENTATION IN WINEMAKING

ÉVOLUTION DE L'ÉCOSYSTÈME MICROBIEN PENDANT LA VINIFICATION

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Abstract: The alcoholic fermentation is a crucial winemaking step. Its failure is problematic. In spite of several studies to understand and elucidate these problems wine global microbial ecology has never been considered. Using conventional microbiological methods and sensitive molecular tools we monitored the alcoholic fermentations of different red grape varieties in several cellars located in Bordeaux area. These observations were made during three successive vintages in different oenological conditions. The effect of the addition of commercial active dried yeast and of initial cold maceration was studied. All these factors were compiled and their effects on microbial changes were investigated. Some of them acted directly on the microbial population of berries surface at the harvest time and should have impact on alcoholic fermentation. They could modify the microbial changes which in some cases could lead to sluggish fermentation. In these cases, we focused on the *Brettanomyces bruxellensis* spoilage problem. The risk of further contamination was discussed according to the alcoholic fermentation development.

Résumé: La fermentation alcoolique est une étape déterminante de la vinification. Son échec ainsi que ses fins difficiles et languissantes sont problématiques. Pour comprendre et élucider ces problèmes, de nombreuses études ont été réalisées. Mais jamais l'écologie microbienne du raisin et du moût n'avait réellement été prise en compte. L'objectif de ce travail est de démontrer l'importance de la diversité microbienne, et, notamment du nombre et des espèces de levures présentes sur la baie de raisins et dans le moût, sur le déroulement de la fermentation alcoolique. En utilisant des méthodes classiques de microbiologie : dénombrement des différentes populations microbiennes sur des milieux de culture sélectifs et des méthodes moléculaires d'identification des espèces et des souches microbiennes, nous avons suivi les fermentations alcooliques de plusieurs lots de différents cépages et dans différents domaines de la région bordelaise. Ces suivis ont été réalisés durant trois millésimes successifs. Ils ont permis d'étudier plusieurs pratiques œnotechniques comme la dose de SO_2 ajoutée dans le moût lors de la cuvaison, l'utilisation de souches de levures commerciales ou le recours à la flore indigène et la mise en œuvre de macérations initiales à froid. Tous ces facteurs ont été comparés et leurs effets sur le consortium microbien étudiés. Certains agissent directement sur les populations microbiennes présentes sur la baie de raisin au moment des vendanges et se répercutent sur le déroulement de la fermentation alcoolique. Certains paramètres de la diversité microbienne peuvent conduire à des cas de fermentations alcooliques languissantes. Dans ce cas, nous nous sommes concentrés sur l'espèce d'altération : *Brettanomyces bruxellensis*. Les risques de contamination par cette espèce ont été soulevés et discutés selon le déroulement de la fermentation alcoolique.

Key words: winemaking, alcoholic fermentation, microbial ecosystem, *Brettanomyces bruxellensis*

Mots clés : vinification, fermentation alcoolique, écosystème microbien, *Brettanomyces bruxellensis*

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INTRODUCTION

During the winemaking, the first microbial intervention is the glucose and fructose conversion into ethanol and carbon dioxide during the alcoholic fermentation (AF) by yeasts. The main species responsible for the AF is *Saccharomyces cerevisiae* (*S. cerevisiae*). This yeast is naturally present on the grape berry surface however in minority compared to the other micro-organisms: bacteria, fungi, non-*Saccharomyces* yeasts (MARTINI *et al.*, 1996, RENOUF *et al.*, 2005). Nevertheless *S. cerevisiae* is by far the most dominant yeast species colonizing surfaces in wineries (PRETORIUS 2000) on tank (PETRUCCIOLI *et al.*, 2002) and barrels surface (RENOUF *et al.*, 2006). This pool of *S. cerevisiae* strains constitutes the indigenous microflora of each cellar (SANTAMARIÁ *et al.*, 2005).

After the berries crushing, the beginning of the AF can be favoured by addition of commercial active dried strains of *S. cerevisiae* which are selected according to their fermenting abilities and their sensorial contribution (NIKOLAOU *et al.*, 2006). It can be preceded by an initial cold maceration which favours the polyphenols extraction. The winemaker can also let acting the indigenous microflora. In all cases, the AF must be fast and complete: the *S. cerevisiae* growth must be efficient enough to consume all sugars and to monopolize the microbial ecosystem in order to limit the growth of other microorganism, notably the spoilage agents like the yeast *Brettanomyces bruxellensis* (*B. bruxellensis*) and the lactic acid bacteria (LAB).

However, the use of commercial active dried strains is not always satisfactory. It is quite common to encounter inoculation failure. Nevertheless, even if the inoculation is positive, the end of the fermentation can be sluggish and difficult. In some other cases, residual sugar remains in wine (BISSON, 1999). Stuck and sluggish AF are a great oenological problem. In addition to the extended processing times, it often leads to off-taste and off flavors of the final product due to the microbial instability. The understanding of these phenomena is poor and it is difficult to have a rapid and correct diagnosis in order to provide efficient treatment to the winemaker.

Several factors of stuck and sluggish fermentation have already been revealed. First, some parameters of the must are suspected such as nitrogen (BELY *et al.*, 1990) and vitamins deficiency (ALFENORE *et al.*, 2002) or the presence of residual pesticides (DOIGNON and ROZES, 1992). Then, even if fermentation is an anaerobic microbial process, the yeast required oxygen in order to favour the synthesis of sterols and unsaturated fatty acids which are crucial for plasma membrane fluidity (SALMON *et al.*, 1998). This factor is involved in preserving the activity and viability when ethanol concentration has become high (JIMENEZ and BENITEZ, 1987). However, in practical fermentation it is difficult to precisely control the amount of oxygen transferred to the must and the lack of oxygen (STREHAIANO, 1990; BLATEYRON and SABLAYROLLES, 2001) can also be a common cause of sluggish fermentation. The fermentation by-products are also considered as AF inhibitor, particularly decanoic, acetic acid (EDWARDS *et al.*, 1990, 1999) and the final fermentation product, ethanol, which affects the cell viability (INGRAM and BUTTKE, 1984; D'AMORE and GRAHAM, 1987). Statistical studies of numerous musts had led to kinetic model and inhibitory effect estimation (BOVEE *et al.*, 1990: DUBOIS *et al.*, 1996). Theses studies have been focused only on *S. cerevisiae* single growth and the other microbial species which in reality are present in the must, were never considered. Experiments were performed on the laboratory scale and never in the real cellar condition. That probably biased the results.

During three successive vintages (2003, 2004, 2005), we investigated different cases of winemaking at real cellar scale. We choose to have a global survey of the microflora by the use of both conventional microbiological methods and molecular tools for species and strains identification. The objective of this work was to have a microbial systemic approach integrating the possible interactions between species on the grape berries surface at the harvest time, in the must and during the winemaking. Different oenological practices were monitored. These observations have given some coherent explanations about stuck and sluggish fermentations which are often described but not yet understood. We also studied the impact on the AF on the growth of the most dreaded spoilage agent, *B. bruxellensis*.

MATERIALS AND METHODS

I - GRAPE AND WINE SAMPLES

We collected grape and wine samples from several chateaux localized in different areas of Bordeaux appellation: Libournais (A), Graves (B) and Médoc (C). The sampling was done according to RENOUF *et al.* (2005) recommendations for the grape berry analyses and RENOUF *et al.* (2006) for the wine.

Concerning the grape berry microbial analyses, we studied several plots of different red grape varieties: Merlot, Cabernet-Sauvignon, Cabernet Franc and Petit Verdot at the harvest. These analyses were made during the three successive vintages: 2003, 2004 and 2005. In 2005, winemaking included different practices listed in table I.

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Table I - List of the most important oenological practices and must parameters in the wines in the four château where samples were collected during 2005 winemaking.

Liste des importantes pratiques œnotechniques et des paramètres des moûts des vins des quatre châteaux où les échantillons ont été collectés en 2005.

Cellar	Grape varieties	Wine	SO_2 addition of the must ($g.hL^{-1}$)	Glucose and fructose cencentrations of the must ($g.L^{-1}$)	pH of the must	Pre-fermenting cold-maceration	Assimilable nitrogen ($mg.L^{-1}$)	Total Acidity of the must ($g.L^{-1}$) (H_2SO_4)	Addition of commercial active dried yeast strain	Nature of the tank	Average of temperature during the fermentation
Chateau A	Merlot	A-I	8	251	3.44	5 days at 10°C	224	4.46	No	Wood	28°C
		A-II	8	247	3.42	5 days at 10°C	214	4.36	No	Wood	28°C
		A-III	8	248	3.46	5 days at 10°C	222	4.27	No	Wood	28°C
Chateau B	Merlot	B-I	5	249	3.55	No	240	3.3	522 Davis strains	Stainless	27°C
	Cabernet-Sauvignon	B-II	5	235	3.50	No	237	3.8	522 Davis strains	Stainless	26°C
Chateau C	Cabernet-Sauvignon	C-I	7	211	3.74	3 days at 10°C	204	2.1	No	Stainless	27°C
		C-II	7	211	3.74	3 days at 10°C	219	2.1	522 Davis strains	Stainless	27°C
		C-III	7	210	3.74	3 days at 10°C	216	2.1	Strain selected in the cellar during previous winemaking	Stainless	27°C

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II - MICROBIOLOGICAL ANALYSES

1) Colony isolation and count

The grape berry washing solution, the must and the wine were subjected to conventional microbial analysis to determine the Total Yeast (TY), non-*Saccharomyces* (NS), Lactic Acid Bacteria (LAB) and acetic acid bacteria (AAB) populations. We also studied the anaerobic Gram negative bacteria (AGN) population. For each analysis, 100 µL of undiluted sample and serial dilutions (10^{-1} to 10^{-4}) were plated out by spreading using sterile micro-marble on four selective nutritive media. For yeast, the medium contained yeast extract 10 $g.L^{-1}$, bactotryptone 10 $g.L^{-1}$, glucose 20 $g.L^{-1}$ and agar 20 $g.L^{-1}$. The pH was adjusted to 5.0 with orthophosphoric acid. For TY cultivation, this medium was supplemented with biphenyl (0.015 % w/v) (Fluka) and chloramphenicol (0.01 % w/v) (Sigma Aldrich) to inhibit mould development and bacterial growth. The addition of 0.05 % (w/v in acetone) of cycloheximide (Sigma-Aldrich) prevented the *Saccharomyces* spp. growth and allowed the numeration of the NS yeast population. At 25 °C, incubation lasted 5 days for the TY and 10 days for the NS. For bacteria, the medium consisted of commercial red grape juice 250 $mL.L^{-1}$, yeast extract 5 $g.L^{-1}$, Tween 80 1 $mL.L^{-1}$ and agar 20 $g.L^{-1}$. The pH was adjusted to 5.0 with KOH 10N. The yeast and mould development was inhibited by adding 50 $mg.L^{-1}$ of pimaricine (Delvocid, DSM Food specialities). For numbering LAB population, the AAB growth was inhibited by anaerobic incubation using an anaerobic system envelope with palladium catalyst (BBL) during 7 days at 25 °C. For the AAB population numbering, the similar medium was supplemented with 30 $mg.L^{-1}$ of penicillin (Sigma Aldrich) to prevent the growth of Gram positive bacteria. The incubation lasted 3 days at 25°C. Anaerobic Gram negative bacteria grew for 5 days at 25 °C on Zymomonas pimaricine penicillin (ZPP) medium : glucose 20 $g.L^{-1}$, peptone 5 $g.L^{-1}$, yeast extract 3 $g.L^{-1}$, malt extract 3 $g.L^{-1}$, agar 20 $g.L^{-1}$ and pH adjusted to 5.0 with orthophosphoric acid (COTON and COTON, 2003). The growth of yeast and Gram positive bacteria were, respectively, inhibited by adding 100 $mg.L^{-1}$ of pimaricine and 50 $mg.L^{-1}$ of penicillin. The ZPP plates were incubated in anaerobic condition using similar equipment as for LAB plates. Each counting was done in triplicate.

III - MICRO-ORGANISM IDENTIFICATION

1) Yeast study

The yeast species identification was performed by molecular tools. The yeast DNA was directly extracted from the biomass collected from the TY or NS plates according to RENOUF *et al.* (2006) and DNA extraction protocol of AUSUBEL (1995). Then, the identification was based on sequence analysis of D1/D2 domains of the rRNA 26S gene by PCR-DGGE (COCOLIN *et al.*, 2000). From the acrylamide gel each interesting band was excised in order to proceed to sequencing. The identification of unknown sequence was made by alignment and phylogenetic comparison (SAITOU and NEI, 1987) with the sequences available in the data bank.

In order to add quantitative data to the qualitative information provided by the PCR-DGGE, we also used the RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS1 and ITS2) (ESTEVE-ZARZOSO *et al.*, 1999) on isolated colonies. This method allowed estimating the percentage of each species (CLEMENTE-JIMENEZ *et al.*, 2004)

2) Bacteria analysis

Analyses were made on the whole biomass from the LAB plates. After 7 days of incubation, biomass from

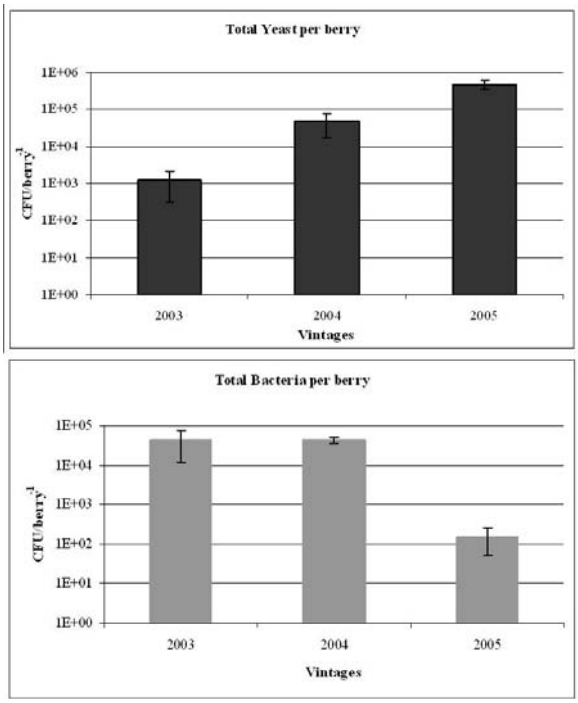


Figure 1 - Average of the Total Yeast (TY) population counted on the TY medium, and of the Total Bacteria (TB) population counted on the LAB, AAB and ZPP medium for three successive vintages for about twenty plots and four red varieties: Merlot, Cabernet-Sauvignon, Cabernet-Franc and Petit-Verdot.
The errors bars are the standard deviation.

Moyenne des populations de levures totales (TY) dénombrées sur le milieu TY, et bactéries totales (TB) dénombrées sur les milieux LAB, AAB et ZPP pour les trois millésimes successifs pour vingt parcelles et quatre cépages de raisins rouges : Merlot, Cabernet-Sauvignon, Cabernet Franc et Petit Verdot.
Les barres d'erreurs représentent les écart-types.

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LAB plates were collected with 2 mL of deionised sterile water. After centrifugation (15 min, 10,000 g, 4 °C) the supernatant was discarded. Then DNA was extracted and analyzed by PCR-DGGE targeting the rpoB gene according to RENOUF *et al.*, (2006) protocol. Then, we focused on *O. oeni* species. Colonies were isolated on LAB plates and 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. The percentages of the other species were estimated by the frequency of the detection of their specific band on DGGE gel.

IV - IDENTIFICATION OF *SACCHAROMYCES CEREVISIAE* STRAINS

The identification of *S. cerevisiae* at the strain level was done by PCR according to the method developed by LEGRAS and KARST (2003) by using the primers δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ21 (5'-CATCATTAACACCGTATATGA-3'). The PCR δ12/δ21 can be performed directly on DNA extracted from the total biomass collected on TY plates, but also on isolated colonies in order to evaluate the proportion of each strain.

V - IDENTIFICATION OF *OENOCOCCUS OENI* STRAINS

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 used by REGUANT *et al.* (2005).

VI - IDENTIFICATION OF *BRETTANOMYCES BRUXELLENSIS* STRAINS

Based on colonies isolated from NS plates, *B. bruxellensis* species was identified by using specific-species nested-PCR method developed by IBEAS *et al.* (1996). 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.

VI - CHEMICAL ANALYSIS

Conventional analysis: pH, total acidity, volatile acidity, alcohol content, free and total SO_2, and total polyphenol index (TPI), were carried out by the official methods or the usual methods recommended by the International Organization of the Vine and Wine (OIV) (1990). Malic acid, glucose and fructose concentrations were measured by the enzymatic method (Boehringer-Mannheim). Volatile phenols were extracted by dichloromethane from a 50 mL sample and they were separated by collecting the organic phase of the mixture. The quantification was achieved by gas chromatography (CHATONNET and BOIDRON, 1988).

VII - STATISTICAL ANALYSIS

The effect of different factors: vintage, grape variety, pH and SO_2 added after the press on total yeast population on the grape berry surface and in the corresponding fresh must were analysed by using Sigmastat software (two way ANOVA test). 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

I - MICROBIAL POPULATION ON THE GRAPE BERRIES SURFACE AT THE HARVEST AND IN MUST BEFORE THE BEGINNING OF THE ALCOHOLIC FERMENTATION

On the figure 1, the total yeast population per berry at harvest has been counted on TY plates during the three successive vintages: 2003, 2004 and 2005. TY population changed according to the year. In 2005, it was significantly greater than 2003. This effect was statically confirmed by the analysis of data in table III. There was a statistically significant difference of the median values between the vintage and the yeast population on the berries ($p<0.001$).The total bacteria population per berry was also function of the year and varied at the opposite. In 2005 bacteria were less important than in 2004 and 2003. The diversity of yeast species also depended on the year. Indeed, in 2003 and 2004, the large major yeast species detected on berries surface belonged to the non-fermentative (BARNETT *et al.*, 1983) yeast with a clear domination of *Cryptococcus generum* (table II). In 2005, there was equilibrium between these non-fermentative yeasts and fermentative yeasts. The *Pichia* and *Metschnikowia genera* were more represented on berries at harvest in 2005.

After crushing the sulphite was currently added to the must. The quantities of SO_2 generally used are comprised between 3 $g.hL^{-1}$ and 8 $g.hL^{-1}$ (table I). But after statistical test, the difference in the median values between the quantities of SO_2 and the ratio of yeast must populations and berries populations is not great enough and there was no statistically significant difference ($p=0.365$). These quantities of SO_2 did not affect also the species diversity. Indeed, despite the addition of 8 $g.hL^{-1}$ of SO_2 into the fresh must similar D1/D2 DGGE profiles could be seen on grape berries surface, in must just after the crushing, just after the sulphiting and three days after, during initial cold maceration (figure 2).

In the case of the cellar B wines, where no maceration occurred, yeast increased after crushing, it was similar for LAB and AAB contrary to AGN bacteria population which significantly fell down. The main spe-

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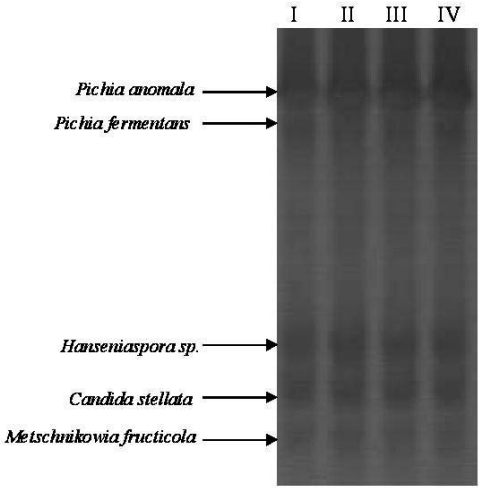


Figure 2 - DGGE profiles of yeast biomass collected from TY plate at four initial stages of the winemaking process.

I: on grape berries surface at the harvest, II: in must just after the crushing, III: in must just after the addition of SO_2 at the tank homogenization and IV: at the middle of initial cold maceration. The quantity of SO_2 added was 8 $g.hL^{-1}$ and the pH of the must was 3.38.

Profil de DGGE levure réalisée sur les biomasses collectées sur les boîtes TY durant quatre premières étapes de la vinification.

I : sur les baies de raisins au moment des vendanges, II : dans le moût après le foulage, III : dans le moût après l'addition de SO_2 et le premier remontage d'homogénéisation et IV : au milieu de la macération initiale à froid. La quantité de sulfitage vendange est de 8 $g.hL^{-1}$ et le pH du moût est de 3.38.

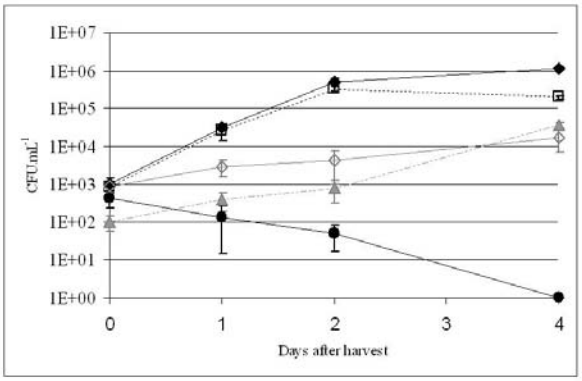


Figure 3 - Example of the routine microbial populations changes during the first days of the winemaking.

◆: total yeast population, ❑: non-*Saccharomyces*, ◊: Acetic Acid Bacteria population, ▲: Lactic Acid Bacteria, and ● : Gram Negative Anaerobic population. This example was a Merlot plot, its main characteristics were: pH=3.74, Total acidity 2.1 $g.L^{-1}$ H_2SO_4, [glucose + fructose] =232 $mg.L^{-1}$.

Exemple des changements microbiens observés classiquement durant les premiers jours de la vinification.

◆ : levures totales, ❑ : levures non-*Saccharomyces*, ◊: bactéries acétiques, ▲ : bactéries lactiques, ● : bactéries à Gram négatif anaérobiques. L'exemple est un lot de merlot dont les principales caractéristiques étaient : pH=3.74, acidité totale=2.1 $g.L^{-1}$ deH_2SO_4, [glucose + fructose] =232 $mg.L^{-1}$.

Table II - Yeast species identified by PCR-RFLP-ITS and changes in the percentages of the different species on berries surface at harvest during the three vintages from all samples in the different vineyards.

Espèces de levures identifiées par PCR-RFLP-ITS et les changements de pourcentage des espèces détectées sur les baies de raisins au moment des vendanges au cours des trois millésimes et pour l'ensemble des échantillons prélevés sur les différents vignobles.

	Family	Genera	2003		2004		2005	
Non-fermentative yeasts	Sporiodiobolaceae	*Rhodosporidium sp.*	12%	80%	8%	62%	4%	52%
		Rhodotorula sp.	6%		10%		6%	
		Sporobolomyces sp.	-		6%		-	
	Tremellaceae	*Cryptococcus sp.*	44%		34%		38%	
		Bulleromyces sp.	10%		4%		4%	
	Dothioracae	*Aureobasidium pullulans*	8%		-		-	
Fermentative yeasts	Candidacae	*Candida sp.*	6%	20%	22%	38%	2%	48%
	Dipodascaceae	*Yarrowia sp.*	2%		2%		-	
	Lipomycetacae	*Lipomyces sp.*	-		2%		-	
	Saccharomycetaceae	*Debaryomyces sp.*	6%		2%		4%	
		Issatchenkia sp.	2%		-		-	
		Pichia sp.	-		6%		16%	
		Saccharomyces sp.	-		2%		2%	
		Kluyveromyces sp.	-		-		-	
	Saccharomycodaceae	*Hansenispora sp.*	4%		2%		6%	
		Metschnikowia sp.	-		-		18%	

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cies of this bacterial population, identified by PCR-DGGE-rpoB analysis were *Burkholderia vietnamiensis* and species closed to the Leifsonia and Enterobacter genera. These bacteria which represent approximately 10 % of the total bacteria population on berries surface gradually disappeared in must, while the LAB and AAB population overcame 10^4 CFU.mL^{-1} (figure 3).

II - MICROBIAL CHANGES DURING ALCOHOLIC FERMENTATION

1) Microbial dynamics during classical alcoholic fermentation

The figure 4 shows the microbial changes observed during a routine alcoholic fermentation performed after addition of commercial active dried yeast for the wine B-I in the cellar B. Similar changes were also observed for the Cabernet-Sauvignon wine (B-II) in this cellar. The commercial strain has been added just after the tank filling and the homogenization on the second day after the harvest. The TY population increased to reach a maximum concentration of 3.107 CFU.mL^{-1}. At this moment, the density has begun to fall. During the growth of TY population, the other microbial population decreased. At the end of the fermentation, the LAB and the NS were approximately at the same level (10^2 CFU.mL^{-1}). There was also an evolution of the proportion of the species among these populations (table IV). The species *O. oeni* and *B. bruxellensis* which were in minor proportion in the must became the predominant species among, respectively, the LAB and the NS yeast population. Nevertheless, despite the fall of the LAB and NS populations during the AF, the individual levels of *O. oeni* and *B. bruxellensis*, estimated by considering the proportion of these species among their respective population and the total level of them, were quite constant during the AF.

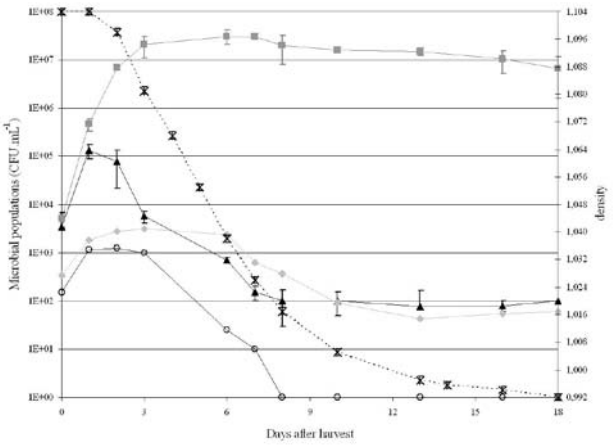


Figure 4 - Changes of the microbial populations
(■: Total Yeast population, ▲ : non-Saccharomyces population, ◆: Lactic Acid Bacteria population and ❍: Acetic Acid Bacteria population, the errors bars show the standard deviations) during the alcoholic fermentation (x: density of the must) of a Merlot wine of a cellar in the Graves appellation. The chemical data of the wine after alcoholic fermentation were: pH=3.59, Total Acidity=4.3 g.L-1 of H_2SO_4, Volatile Acidity=0.21 g.L^{-1} H_2SO_4, Alcohol content= 14.89 % (v/v), malic acid concentration= 1.25 g.L^{-1} and TPI=64.

Changements des populations microbiennes
(■: levures totales, ▲: levures non-*Saccharomyces* population, ◆: bactéries lactiques and bactéries acétiques, les barres d'erreurs représentent les écart-types) durant la fermentation alcoolique (x: densité du moût) d'un vin de Merlot dans un chai de l'appellation des Graves. Les données chimiques du vin à la fin de la fermentation alcoolique étaient : pH=3.59, acidité totale=4.3 g.L^{-1} of H_2SO_4, acidité volatile=0.21 g.L^{-1} of H_2SO_4, T.A.V.= 14.89 % (v/v), concentration en acide malique= 1.25 g.L^{-1} et TPI=64.

Within, the species there was also an evolution of the strains. In fresh must, ten RAPD profiles could been seen on a sample of twenty colonies of *O. oeni*, but only two major RAPD profiles at the end of AF (figure 5). Concerning the *B. bruxellensis* strains only two REA-PFGE profiles could be seen in fresh must before the AF (figure 6). At the end, only profile III was observable. Concerning S. *cerevisiae*, the PCR revealed a positive implantation of the commercial strains all along the AF. This strain was the only one detected.

2) Atypical alcoholic fermentation

a) Inoculation problem

In chateau C, an initial maceration for three days at 10 °C occurred. Then, the must was inoculated in tank C-II with strain 522D and tank C-III with a strain previously isolated in the cellar. Tank C-I was not inoculated (table I)

The dominant strain during AF was checked by PCR δ12/δ21 profile. In wine C-III, the implantation

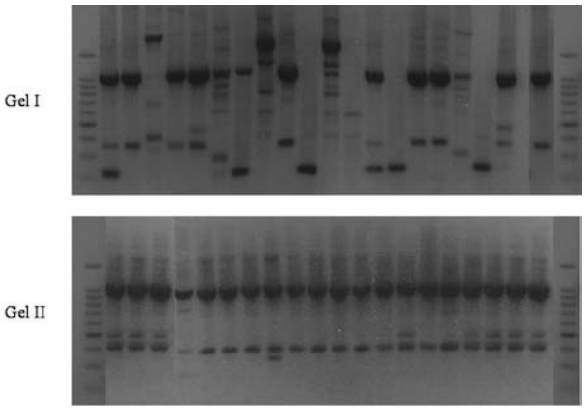


Figure 5 - Changes of RAPD profiles of *O. oeni* strains before and after the alcoholic fermentation.
Gel I: *O. oeni* colonies isolated from must, Gel II: *O. oeni* colonies isolated at the end of AF.

Changement des profiles RAPD des souches d'*O. oeni* avant et après la fermentation alcoolique.
Gel I : colonies d'*O. oeni* isolées dans le moût, Gel II : colonies d'*O. oeni* isolées à la fin de la fermentation alcoolique.

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Figure 6 - REA-PFGE of *B. bruxellensis* strains isolated from B-I wine.

I= S. cerevisiae profile used as ladder, II: 1st profile of *B. bruxellensis*, III: 2nd profile of *B. bruxellensis*.

Discrimination des souches de *B. bruxellensis* isolées du vin B-I par REA-PFGE.

I= profile de *S. cerevisiae* utilisées comme marqueur, II : 1er profile de *B. bruxellensis* et III : 2nd profile de *B. bruxellensis*.

of the selected strain was satisfactory during the first step of the AF: the population of TY reached more than 10^8 CFU.mL^{-1} (figure 7) and only a δ12/δ21 PCR profile corresponding to the commercial strain was noticed. Since day 10 of fermentation the TY population began to fall and the rate of fermentation decreased. At this stage, the δ12/δ21 PCR profile changed. Other profiles appeared. At this moment, the density was about 1, the alcohol content closed to 12 % vol/vol and the TY population was closed to the TY population numbered in the tank C-I. By comparison in wine C-II the implantation was positive all along the AF and the density rapidly decreased. For the wine C-III the end of fermentation was sluggish and at this time the NS population grew and reached to 2.10^3 CFU.mL^{-1} at the end of AF. This population was even higher than for the wine C-I. At this moment, the species detected, in the wine C-III, were *Candida stellata*, *Torulaspora delbrueckii* and *B. bruxellensis*. The latter represented more than 75 % of NS species. The chemical data of the three wines at the end of AF are listed in table IV. The ethanol content was lower in the non-inoculated wine, as well as the pH. At, contrary the volatile phenols concentrations were slightly higher than in inoculated wines. But all these differences were not really significant.

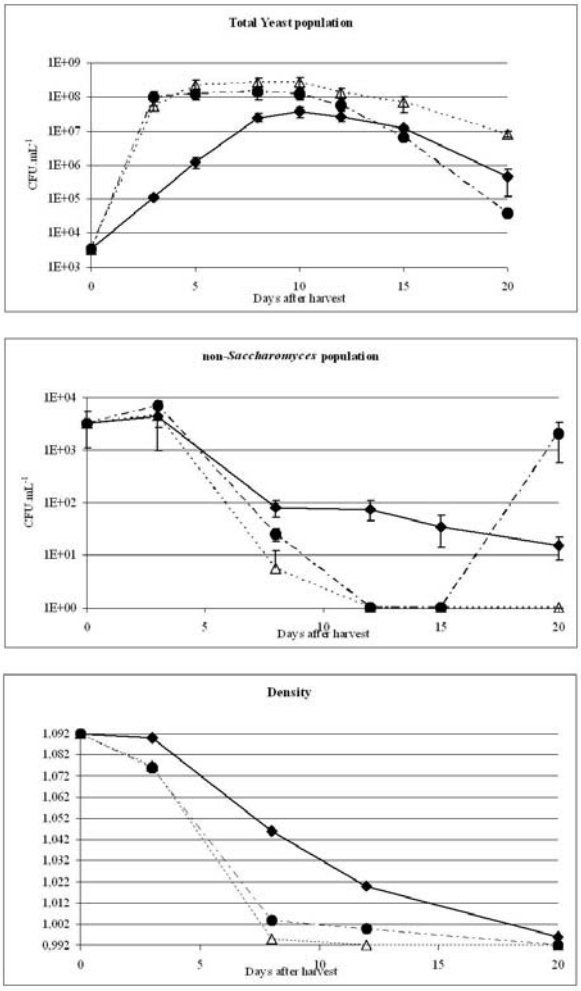


Figure 7 - Evolution of Total Yeast and non-*Saccharomyces* yeast population and the density in the three wines studied at the cellar C.

◆: non inoculated wine (C-I), Δ: inoculated wine with positive implantation all along the fermentation (C-II), ●: inoculated wine with a shift of *S. cerevisiae* strains (C-III).

Évolution de la densité, des populations de levures totales et des levures non-*Saccharomyces* dans les trois vins étudiées au château C.

◆: vin non-inoculé (C-I), Δ: vin inoculé dont l'implantation est positive durant toute la fermentation alcoolique (C-II), ●: vin inoculé dont l'implantation n'a été positive que durant les premières étapes de la fermentation (C-III).

3) Fermentation with indigenous yeast

In the cellar A, after the berries crushing, the beginning of the AF was preceded by an initial cold maceration for five days. During this period, the tank cooling was operated by addition of dry ice and circulation of cold water. For the three wines studied, the yeast population slightly decreased in tank I while despite in tank II and III, the TY population was multiplied by 100 until reached 10^6 CFU.mL^{-1}. Only NS composed the population. Figure 8 shows a phylogenetic tree of D1/D2 domain of

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Table III - Effects on grape varieties, pH, vintage, and SO_2 added after the press on total yeast population on berries and in the must before addition of selected strain.

Effets du cépage, du millésime, et du SO_2 ajouté après le foulage des baies sur la population de levures totales sur les baies de raisins et dans le moût avant la levurage.

Grape varieties	Vintages	pH of the must	SO_2 added at the press exit g.hL^{-1}	Total yeast population on berries surface at harvest (CFU.berry^{-1})	Total yeast population in must after the sulphiting (CFU.mL^{-1})	Ratio of total yeast population in must after the sulphiting and before the addition of active dried yeast (CFU.mL^{-1}) to the yeast population on berries surface at harvest (CFU.berry^{-1})
Merlot	2004	3.65	3	3.75×10^{4}	2.50×10^{6}	66.7
Merlot	2005	3.72	4	4.00×10^{4}	8.50×10^{5}	21.3
Cabernet-Sauvignon	2003	3.88	4	2.20×10^{3}	5.26×10^{5}	239.1
Merlot	2003	3.44	5	2.90×10^{3}	2.00×10^{4}	6.9
Merlot	2004	3.52	5	1.55×10^{4}	2.25×10^{4}	1.5
Merlot	2005	3.44	6	5.50×10^{4}	7.50×10^{5}	13.6
Petit-Verdot	2005	3.84	6	2.50×10^{5}	1.70×10^{7}	68.0
Cabernet-Sauvignon	2003	3.46	6	9.00×10^{3}	3.10×10^{4}	3.4
Cabernet-Sauvignon	2004	3.55	6	1.00×10^{4}	2.40×10^{6}	240.0
Cabernet-Sauvignon	2005	3.66	6	1.00×10^{5}	2.10×10^{5}	2.1
Merlot	2003	3.53	7	2.00×10^{2}	3.20×10^{4}	160.0
Cabernet-Franc	2003	3.78	7	1.50×10^{5}	3.96×10^{6}	26.4
Petit-Verdot	2003	3.92	7	3.25×10^{3}	7.96×10^{5}	244.9
Cabernet-Sauvignon	2004	3.67	7	1.60×10^{3}	1.00×10^{4}	6.3
Merlot	2005	3.41	7	3.40×10^{4}	1.20×10^{5}	3.5
Cabernet-Franc	2005	3.77	7	7.40×10^{6}	2.50×10^{7}	3.4
Cabernet-Sauvignon	2004	3.63	8	6.00×10^{3}	2.00×10^{4}	3.3
Merlot	2005	3.39	8	1.10×10^{4}	2.20×10^{4}	2.0
Merlot	2005	3.55	8	1.00×10^{5}	7.50×10^{6}	75.0
Cabernet-Sauvignon	2005	3.74	8	5.70×10^{4}	3.85×10^{5}	6.8

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Table IV - NS yeast and LAB species and changes in percentages before and after the alcoholic fermentation.

Espèces de levures non-*Saccharomyces* et de bactéries lactiques et variations de leur pourcentage lors d,e la fermentation alcoolique

Non-*Saccharomyces* Yeast species identified by PCR-RFLP-ITS	Beginning of the AF : density =1.08		End of the AF: density =0.99	
	% of each species	Concentrations of each species (CFU.mL^{-1})	% of each species	Concentrations of each species (CFU.mL^{-1})
Brettanomyces bruxellensis	02	1.1×10^2	80	0.8×10^2
Candida cantarelli	12	6.7×10^2	8	0.08×10^2
Candida stellata	12	6.7×10^2	4	0.04×10^2
Debaryomyces hansenii	14	7.7×10^2	-	-
Hanseniaspora uvarum	22	12.2×10^2	-	-
Metschnikowia pulcherrima	12	6.7×10^2	-	-
Pichia anomala	18	10.0×10^2	8	0.08×10^2
Pichia fermentans	08	4.4×10^2	-	-
TOTAL	100	5.55×10^3	100	1×10^2

Lactic acid bacteria species identified by PCR- *rpoB*	Beginning of the AF : density =1.08		End of the AF: density =0.99	
	% of each species	Concentrations of each species (CFU.mL^{-1})	% of each species	Concentrations of each species (CFU.mL^{-1})
Bacillus sp.	8	2.5×10^2	-	-
Lactobacillus caseï	4	1.2×10^2	-	-
Lactobacillus plantarum	40	12.4×10^2	12	0.8×10^2
Lactobacillus sp.	4	1.2×10^2	-	-
Leuconostoc mesenteroides	12	3.7×10^2	-	-
Oenococcus oeni	20	6.2×10^2	80	5.6×10^2
Pediococcus damnosus	4	1.2×10^2	-	-
Pediococcus parvulus	4	1.2×10^2	8	0.6×10^2
Pediococcus pentosaceus	4	1.2×10^2	-	-
TOTAL	100	3.1×10^3	100	7×10^2

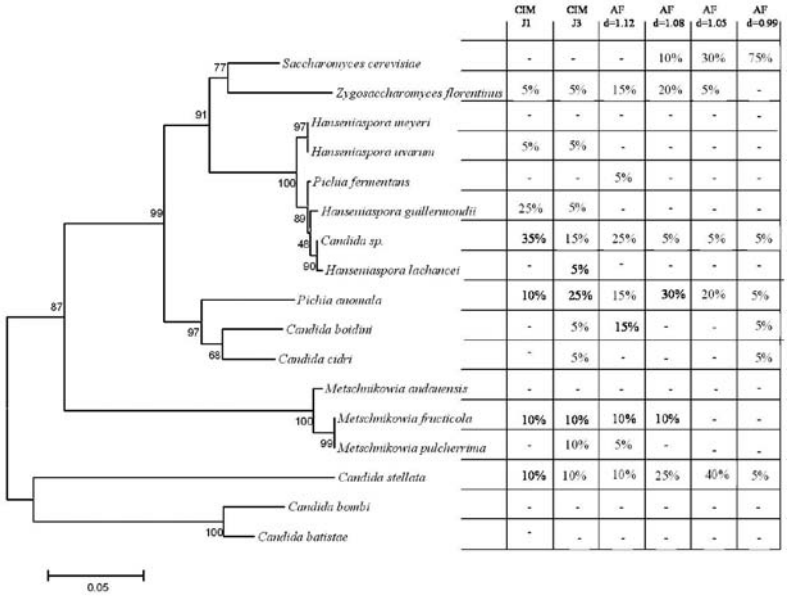


Figure 8 - Neighbour-joining tree of all yeast sequences obtained by PCR-DGGE NL1/LS2 analysis and their percentage estimated by PCR-ITS at different time of the fermentation process.

The numbers given in the branches are the bootstrap values after 1000 repetitions; only significant values higher than 50% are shown. 0.05 represents the scale for the phylogenetic branches length. CIM= Cold Initial Maceration, AF= Alcoholic Fermentation., d= density.

Arbre phylogénétique construit selon la méthode des plus proches voisins pour toutes les séquences de levures obtenues par la PCR-DGGE NL1/LS2 et leur poucentage estimé par la PCR- ITS à différents temps de la fermentation.

Les nombres donnés sur les branches sont les valeurs de boostrap obtenues après 1000 répétitions, seules les valeurs supérieures à 50 % sont retenues. 0,05 représente l'échelle de la longueur phylogénétique.

Table V - Chemical analysis of wines I, II and III at the end of AF for cellar C experiment (TPI: Total Polyphenol Index).

Analyses chimiques des vins I, II et III à la fin de la fermentation alcoolique pour les expériences du château C. (TPI : Index des polyphénols totaux).

Wine	C-I	C-II	C-III
Ethanol content (% vol/vol)	12.34	12.55	12.47
Glucose + frcutose ($g.L^{-1}$)	2	<1.7	<1.7
pH	3.55	3.58	3.61
Total acidity ($g.L^{-1}$ d'H_2SO_4)	4.75	4.45	4.15
Volatile acidity ($g.L^{-1}$ d'H_2SO_4)	<0.1	<0.1	0.14
Malic acid ($g.L^{-1}$)	2.5	2.4	2.1
T.P.I.	64	62	60
4-ethylphenol ($\mu g.L^{-1}$)	6±1	2±1	9±1
4-ethylguaiacol ($\mu g.L^{-1}$)	0	0	0
4-vinylphenol ($\mu g.L^{-1}$)	103±2	96±7	78±4
4-vinylguaiacol ($\mu g.L^{-1}$)	9±1	7±2	6±2

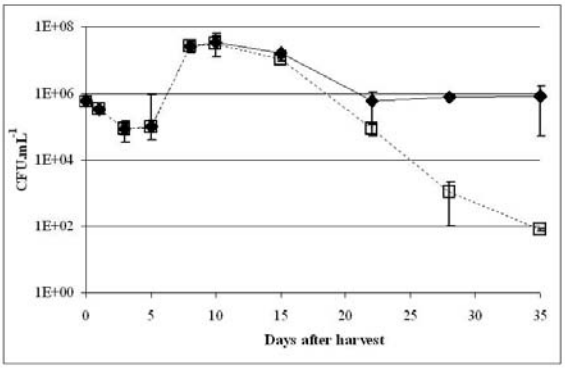


Figure 9 - Evolution of Total Yeast (◆) and non-*Saccharomyces* (❑) yeast populations during the fermentation of the wine A-I.

Évolution des populations de levures totales (◆) et de levures non-*Saccharomyces* (❑) durant la fermentation du vin A-I.

rRNA 26S gene of the species and their percentage. The main species at the end of the maceration was *Pichia anomala*. The TY and the NS populations were similar even after the increase of temperature (28.5 °C) and the beginning of the sugar consumption. Then, at the middle of the AF, NS species still represented more than two third of the TY population. The high percentage of *Candida stellata* (40%) and *Pichia anomala* (20%) was remarkable by comparison with *S. cerevisiae* (30%) at this stage of the fermentation. In previous vintages (2003 and 2004), in the same cellar and the must of the similar parcel, the NS decreased from the beginning of sugar consumption and at the middle of AF (density = 1.05) the NS population represented less than 1 % of the TY population. In 2005, the level of NS and TY populations was clearly different only after the 20th day of fermentation (figure 9) with the *S. cerevisiae* domination. The analysis of *S. cerevisiae* strains by PCR δ12/δ21 revealed a change of the strains all along the fermentation. The profile I was the only detectable during the first step of AF. From the middle of AF (density=1.05) four other profiles (II, III, IV and IV) were appeared (figure 10).

DISCUSSION

According to the year, the quantity and the quality of the yeasts on berries surface change. These changes should be due to the interactions regulating the microbial community on berries surface (RENOUF *et al.*, 2005). They include environmental factors such as agrochemical applications and physical and chemical properties of the berry surface liable for the microbial adhesion. The climatic conditions should also play a crucial role on microbial population (LONGO *et al.*, 1991). All these parameters can explain the differences observed in the successive vintages mainly when the climatic conditions during the grape ripening were so different as for the studied vintages. In 2005 climatic conditions led to overripening of fruit that has favoured water fruit loss trough transpiration (HAMILTON and COOMBE, 1992) and modified the stem and pellicle of the berries (ROGERS *et al.*, 2004). That should directly affect the microbial populations by modifying the adhesion and growth condition. During the ripening the concentration of sugars, of malic acid, of tartaric acid and available nitrogen are the main components influenced by climatic conditions. In 2005, the malic acid and tartaric acid concentrations

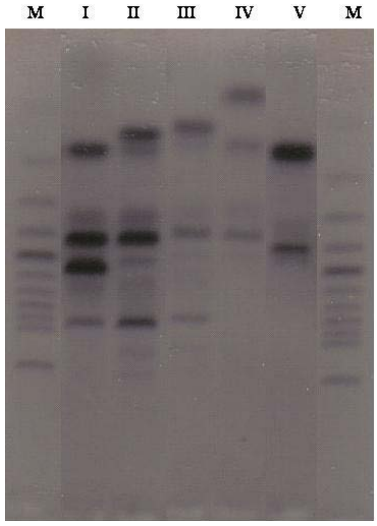


Figure 10 - The fives (I, II, III, IV, and V) PCR δ12/δ21 profiles of the different *S. cerevisiae* isolated during A-I wine fermentation. M:100 pb ladder.

Les cinq (I, II, III, IV, et V) profiles PCR δ12/δ21 des différentes souches de *S. cerevisiae* isolées durant la fermentation du vin A-I. M : 100 pb marqueur.

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were slightly lower than in the previous vintages, while the nitrogen content was closed to that of 2004 and notably higher than in 2003. There was a significant effect of the vintage and pH on the yeast population in fresh must ($p<0.001$) and all these parameters were strongly dependent on the plot, the grape variety and also on the terroir. However it is not possible to establish any correlation between these data and the microbial population. Further investigations at larger scale and for several vintages should be necessary.

After crushing, the microbial populations find a more favourable medium. The fermentative yeast, particularly the *S. cerevisiae* species are the best adapted to fresh must. The addition of commercial active dried strain of *S. cerevisiae* has to favour the *S. cerevisiae* predominance. In a routine alcoholic fermentation, the *S. cerevisiae* population reached 10^7 CFU.mL^{-1} and stayed at this high level during the fermentation whereas the nutrients were not limiting. The arrest of growth for *S. cerevisiae* as well as its limitation for the other yeast species could in part be explained by the high cell density and cell-cell contact inhibitory mechanism (NISSON *et al.*, 2003). The *S. cerevisiae* monopolized the microbial ecosystem.

The increase of ethanol leads to several cellular and molecular disorders, mainly at the level of the membrane integrity (JONES, 1989). A selection of the most resistant species took place among the bacteria and the yeast populations. The AAB and AGN bacteria populations were innumerable at the end of the AF. Before that, PCR-DGGE-rpoB analyses revealed that the major species of these groups were respectively *Gluconobacter oxydans* and *Bukholderia vietnamiensis*. The first is able to use sugar as organic carbon source but did not resist to ethanol (DU TOIT and LAMBRECHTS, 2002). The second is one the major species detected on berries surface during the grape development (RENOUF *et al.*, 2005). It should play a significant role in the microbial community on berries by producing protective exopolysaccharide film. Moreover its ability to grow on berries surface on dried and poor environment could be higher than survival at the hyper-osmolarity of the must.

Concerning the NS yeast and the LAB populations after a first stage of growth they decreased during the AF. That coincided with a fall of the species diversity. At the end of the fermentation these populations were constituted by two major species, which was respectively *B. bruxellensis* and *O. oeni*. Their resistance to the ethanol stress are well known and these phenomena were previously described (RENOUF *et al.*, 2006). In addition the strain diversity within these species also decreased showing a selection phenomenon. The tolerance to the AF conditions should be based on strain dependent characters.

Nevertheless the predominance of the *S. cerevisiae* since the beginning of the AF was not always observed in all cases. During the 2005 vintage, the high level population of NS yeast on berries could be a possible cause of failures in yeast starter implantation. The manufacturers recommend adding the commercial active dried yeast in order to obtain a population of 10^6 CFU.mL^{-1}. This is enough to impose a ratio of 10^3-10^4 between the commercial strains and the indigenous yeast in classical vintage. However that was not the case in 2005, since some NS species were higher than 10^6 CFU.mL^{-1} in fresh must. Competitions between the commercial strains and the indigenous species should have occurred. These competitions should be influenced by the quality of the commercial strains and also by the nature of the indigenous species and the proportion of the fermentative non-*Saccharomyces* species. It could be the case of *Metschnikowia fructicola* and *Zygosaccharomyces florentinus*, which have been suspected to act in re-fermentation phenomena in white winemaking (DIVOL 2004). In addition, most of the identified species in 2005, are known to be well adapted to environmental stress such as sugars and ethanol concentration, low pH and low temperature (FREDLUND *et al.*, 2002). Then, the more the composition of grape must such as sugar concentrations (LAFON-LAFOURCADE *et al.*, 1979), phenolics compounds content and the pH (LAFON-LAFOURCADE and RIBÉREAU-GAYON 1983) is atypical, the more these indigenous species should be favoured. The increase of SO_2 added after crushing cannot be considered as an alternative for reducing the indigenous population. Its effect was insignificant. Moreover it is important not to use excessively SO_2. Indeed, some *S. cerevisiae* strains are able to produce significant quantities of SO_2 and that can lead to problem for the further step of the winemaking notably for the implantation of *O. oeni* starter (HENICK-KLING and PARK, 1994).

The initial cold maceration should also favour the indigenous non-*Saccharomyces* yeast adaptation to the must by cross tolerance mechanism. Low temperature should boost a metabolism of defence and synthesis of preservative agents as trehalose (THEVELEIN, 1984), glycogen (FRANÇOIS *et al.*, 1997) and chaperones Hsp proteins (SALES *et al.*, 2000). Low temperature and ethanol lead to similar cell disorders: membrane fluidity, decrease of water activity. Then, cross tolerance should be effective. After cold maceration, when the temperature increased, the yeast could grow and began the AF. They had a reserve of protective molecules previously used against the cold conditions which may allow a greater ethanol tolerance. Moreover some previous studies have shown that certain NS species have a more intrinsic robustness to low temperature than *S. cerevisiae* species (HEARD and FLEET, 1988). Hence, it was observed

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that NS species were more likely to make stronger contribution to fermentation conducted at low temperature (NOVO *et al.*, 2003). Cross tolerance phenomena could also been effective when the must was highly concentrated in sugars. Indeed, the high sugar content of the must and the ethanol produced during the fermentation led to an osmotic stress (QUEROL *et al.*, 2003) and the protective response of the cells are similar.

The preservation of high non-*Saccharomyces* population levels at the beginning of the AF should affect the duration of the alcoholic fermentation. Indeed, the AF rate is directly dependent on two main parameters: the total viable biomass and the specific rate of sugar consumption of the individual cells. According to several authors, the second parameter is the most important, because a sluggish fermentation occurs when the specific rate of fermentation decreases even though viable biomass remains high (ALEXANDRE and CHARPENTIER 1998). The fermentative capacities of the NS species are lower than that of *S. cerevisiae* species. In non-inoculated wine, a slowing down of the fermentation rate and a stagnation of the NS population was observable when the density reached 1.0 and the kinetic of sugar consumption has decreased. At this moment, *S. cerevisiae* was predominant. But its population, estimated by subtracting NS to TY population, and the fermentation rate were still lower than for usual fermentation. The implantation of *S. cerevisiae* was difficult when the AF has started by NS yeast. The toxin production by NS yeast could explain these phenomena (COMINTINI *et al.*, 2004). *Pichia anomala*, which is one of the major species identified during the first steps of the AF, is known for its significant killer activity on *S. cerevisiae* (YAP *et al.*, 2000). But interactions between the declining NS and the active *S. cerevisiae* should also act. When the NS species was dominant, only one strain of *S. cerevisiae* was identified. Afterwards, at the end of the fermentation the *S. cerevisiae* strain diversity was higher. They overcame the NS population whereas the initial strain was unable to impose upon NS yeast.

At the end of the sluggish fermentation the evolution of the NS population seemed to depend on the use of commercial starter strain or of the indigenous strain requirement. When a commercial strain was used, the NS population decreased just after the inoculation. The starter should have inhibited the indigenous species (VAN VUUREN and JACOBS, 1992). When the fermentation was sluggish, the TY population decreased, the shift of PCR δ12/δ21 profile was effective and showed that the NS population has started to growth. The major species responsible for this growth was *B. bruxellensis*. *B. bruxellensis* is not very demanding from nutritional point of view by comparison with *S. cerevisiae* (USCANGA *et al.*, 2000) and its resistance against high ethanol concentration and the conditions at the end of AF are remarkable (HOLLOWAY *et al.*, 1992; RENOUF *et al.*, 2006). This species was neither previously detected on the berries surface nor in the grape must by the molecular tools used. But that did not mean that *B. bruxellensis* was totally absent. It should be present but in great minority. Indeed, the molecular methods used to detect yeast species in wine are not enough efficient to identify the great minor yeast species. If the minor species of yeast are more than 100 times lower than the major species, as it is the case on the surface of grape berries, they are not detected (PRAKITCHAIWATTANA *et al.*, 2004).

For the non-inoculated wines, the NS species were not inhibited at the beginning of AF by the massive supply of exogenous S. *cerevisiae*. Their population was stabilized until the ethanol content was too high. At the end of AF the residual NS species: *Candida* sp., *Pichia* sp. remained close to 10^3 UFC.mL^{-1} but without *B. bruxellensis* appearance. *B. bruxellensis* should be present but at a very low level and the preservation of high total active yeast population all along the fermentation did not allow its growth. That could be explained by the occupation of the microbial ecosystem and the killer toxins production, for instance by the *Pichia* sp. genera which could have killer effect on *S. cerevisiae* as it was previously mentioned and which had also a remarkable inhibitory on *B. bruxellensis* growth (YAP *et al.*, 2000; COMITINI *et al.*, 2004). In the inoculated wine, the starter should inhibit the major NS species present in the fresh must. The NS population remained at a very low level all along the fermentation. However when the inoculated strain failed to achieve the fermentation, the *S. cerevisiae* population declined, the microbial ecosystem became vacant. The *B. bruxellensis* which should be previously present at undetectable level and was probably the more resistant species to the conditions of the fermentation's end, took advantage of this situation to grow. It reached the critical level for spoilage at 10^3 CFU.mL^{-1} (RENOUF and LONVAUD-FUNEL, 2005). Even if, the quantity of volatile phenols produced was not yet important, the spoilage could be suspected during the further steps of the winemaking notably after the malolactic fermentation when the wine was more favourable to *B. bruxellensis* growth (RENOUF *et al.*, 2005). However, the vinylphenol and vinylguiacol measured may have been produced by the *S. cerevisiae* (CHATONNET *et al.*, 1989; SHINOHARA *et al.*, 2000). In order to avoid the growth of *B. bruxellensis* at the end of sluggish fermentation it is crucial to increase the robustness of the selected strain to the conditions of the end of the fermentation in the selection starter process. Further investigations should be made to favour those inhibiting which would significantly inhibit *B. bruxellensis* strains by the production of killer toxins.

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CONCLUSION

The alcoholic fermentation is a crucial step of the winemaking. Its success depends on oenological practices but also on properties of the must and the qualities of the microbial species. During certain vintages must are more difficult to convert into wine. Trials of stuck and sluggish explanations and predictions were not satisfactory mainly because the previous works were focused on biochemical must parameters and the single culture of the S. *cerevisiae*. Our work has given a new view of these phenomena by considering the global microbial consortium. The most common wild yeasts on grapes were non-*Saccharomyces* species. After crushing, microbial consortium was modified by several environmental stresses: high sugar, low pH, addition of SO_2, low temperature. The survival, adaptation and the fermentation performance depends on the severity of the stresses which is function of the must (sugars, nitrogen) and the oenological practices (oxygen, temperature), and the level of the population and its quality. According to the level of population and the intrinsic robustness and genetic potential of the species, the non-*Saccharomyces* species could grow during the early stages of fermentation. The most part of must bacteria were inhibited, only certain *O. oeni* strains were able to survive. When yeast starters were used, they took advantage to the indigenous yeast species in the most cases. When the end of fermentation was sluggish, the *S. cerevisiae* strains were more diversified and different than at the beginning. These strains should resist to all the inhibitory compounds by all the yeasts which have grown previously. This change of *S. cerevisiae* dominant strains has coincided with a fall of total yeast population and slowing down of fermentation rate. In inoculated wines, this phenomenon was also associated with a significant growth of non-*Saccharomyces* growth, mainly the *B. bruxellensis* specie. *B. bruxellensis* is well adapted to the condition of end of fermentation. It was able to take advantage of the decline of S. *cerevisiae*. In non-inoculated wines, equilibrium between indigenous *S. cerevisiae* strains and the initial NS species (*Pichia* sp., *Candida* sp.) was established. This ecosystem was never vacant and *B. bruxellensis* could not grow even at the end of the fermentation. Even the sum of volatile phenols was not yet high, the risk of *B. bruxellensis* contamination should be more important in sluggish inoculated fermentation than in indigenous fermentation.

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