YEAST AND BACTERIA ANALYSIS OF GRAPE, WINE AND CELLAR EQUIPMENTS BY PCR-DGGE.

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Abstract

Aims: Microorganisms play an important role in winemaking. Most of their metabolisms are beneficial to the wine quality but several species have spoilage effects. Microbiological knowledge are crucial for improving species and strains identification and avoiding contamination by spoilage agents. Our objective was to follow wine microbial consortia at each stage of the process from grapes to bottles, and also on cellar equipment surface: barrels for the yeast analysis and tanks for the bacteria analysis.

Methods and results: For a global microbial monitoring, molecular tools are particularly efficient. We chose the PCR-DGGE for direct analyses without cultivation step. We used the *rpoB* gene as target for the bacteria, and the 26S rRNA gene for the yeast. Species inventories were made during the whole winemaking process, which had never been done before.

Conclusion: From the grape surface to storage in bottles, the microbial diversity decreased. Only the most resistant species were able to survive to the ethanol, SO_2, low oxygen and low pH constraints. Among them, *Pediococcus parvulus* for the bacteria and *Brettanomyces bruxellensis* for the yeast were the most resistant.

Significance and impact of study: The use of a molecular method independent of the culture stage of the microorganisms like the PCR-DGGE has allowed to have a more exhaustive vision of the diversity of the bacteria and yeasts species and to follow their evolution during the winemaking and in the cellar environment . These follow-ups could be extended to other estates in order to study the causes and the consequences of certain variations. The detection of these two prejudicial species was an argument for the PCR-DGGE approach in wine microbial studies.

Keys words: winemaking, yeast, bacteria, species, diversity, PCR-DGGE

Résumé

Objectif : Les microorganismes jouent un rôle déterminant dans l'élaboration des vins. La plupart de leurs métabolismes sont bénéfiques pour la qualité du vin (fermentations alcoolique et malolactique), mais certaines espèces ont une activité préjudiciable, comme la production des phénols volatils par la levure *Brettanomyces bruxellensis*. Une meilleure connaissance des populations microbiennes est cruciale pour améliorer l'identification des espèces et éviter la multiplication des espèces d'altération. Notre objectif était de suivre le consortium microbien du vin à chaque étape du procédé de vinification depuis la baie de raisin jusqu'à la bouteille, mais aussi au chai, à la surface des cuves pour l'analyse des bactéries, et, des barriques pour celle des levures.

Méthodes and résultats : Pour avoir une vision globale des suivis microbiologiques, les méthodes de biologie moléculaire sont particulièrement efficaces. Nous avons utilisé les analyses directes par PCR-DGGE sans étape de culture, en ciblant le gène *rpoB* pour l'étude des bactéries et le gène de l'ARN 26S pour l'identification levures. Des inventaires des espèces sont proposés à chaque étape du procédé de vinification, ce qui n'avait jamais été réalisé auparavant par des méthodes moléculaires.

Conclusion : Depuis la baie de raisin, la diversité microbienne ne cesse de diminuer. Seules les espèces les plus résistantes aux contraintes œnologiques (TAV, pH, SO_2…) survivent selon leurs avantages sélectifs lors des conditions rencontrées. Parmi ces espèces les plus résistantes, on note, chez les bactéries, l'espèce *Pediococcus parvulus* et, parmi les levures, l'espèce *Brettanomyces bruxellensis*.

Signification et mpact de l'étude : L'utilisation de méthodes moléculaires indépendantes de l'étape de culture des microorganismes, comme la méthode de PCR-DGGE directe, permet d'avoir une vision plus exhaustive de la diversité des espèces de bactéries et de levures présentes dans le vin et de suivre leur évolution au cours du processus de vinification. Ces suivis mettent en évidence des différences intéressantes, mais globalement les deux espèces les plus résistantes sont *B. bruxellensis* et *P. parvulus*. Ce sont deux espèces d'altération des vins. Cela est un argument supplémentaire pour les analyses directes par PCR-DGGE. Ces travaux devraient être élargis à d'autres domaines afin d'étudier les causes et les conséquences des variations de l'écosystème microbien.

Mots clefs : vinification, levure, bactérie, espèce, diversité, PCR-DGGE

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J. Int. Sci. Vigne Vin, 2007, **41**, n°1, 51-61

INTRODUCTION

Wine is the product of complex microbial interactions between yeast and bacteria species. After berries crushing, the yeasts, mainly *Saccharomyces cerevisiae* (*S. cerevisiae*) consume sugars and produce ethanol during the alcoholic fermentation (AF). Then, lactic acid bacteria (LAB), mainly *Oenococcus oeni* (*O. oeni*), convert the L-malic acid into L-lactic acid during the malolactic fermentation. In addition to alcohol production and acidity decrease, these metabolisms contribute to the wine chemical composition.

However, the microbial intervention is more extended and several post fermenting microbial metabolisms are prejudicial to wine sensorial qualities. This is particularly the case for volatile phenols synthesis by the yeast *Brettanomyces bruxellensis* (*B. bruxellensis*) conferring off-odours to wine (Chatonnet *et al.*, 1992), and exopolysaccharides (Lonvaud-Funel and Joyeux, 1988), biogenic amines (Coton *et al.*, 1998) and ethyl carbamate (Uthurry *et al.*, 2005) productions by some LAB strains. Therefore, microbial survey is of great importance to favour the development of beneficial species and to avoid contamination by spoilage agents (Loureiro and Malfeito-Ferreira, 2003; Porret *et al.* 2004).

In the past, wine microbial species had been monitored by plate isolation on selective nutritive medium and microscope observation and they were identified by physiological tests (Lafon-Lafourcade and Joyeux, 1979). These methods are time consuming and tedious. Moreover the preliminary cultivation step can lead to biased results since their strong dependence on the physiological state (Masco *et al.*, 2005). Development of molecular biology has provided alternative to this limitation and culture independent techniques are emerged. Among them, the denaturing gradient gel electrophoresis (DGGE) is a tool of species identification present in complex microbial mixtures (Ercolini, 2004). That combines the species-specific sequence amplification and their separation according to species-genotype characteristics (Muyser, 1999). DGGE targets DNA fragments with identical sizes. It bases their separation according their nucleotide sequence.

For bacteria species, primers targeting a region of the beta subunit gene of the RNA-polymerase, *rpoB*, have been designed (Renouf *et al.*, 2006a). For the yeast, the primers currently used target the D1/D2 domain of the 26S subunit of the ribosomal DNA (Cocolin *et al.*, 2000). After direct DNA extraction, the combination of bacterial and yeast PCR-DGGE profiles represents the global diversity of the wine microbial community and its dynamics during winemaking. The aim of this work was to use the PCR-DGGE to make exhaustive inventory of bacteria and yeast to obtain a better understanding of the wine microbial consortium complexity and diversity at each stage of the winemaking process and also on cellar equipment.

MATERIALS AND METHODS

1. Sampling

Grape, fermenting musts, wines were collected in three vineyards localized in the Bordeaux area: Libournais (L), Graves (G), and Medoc (M) according Renouf *et al.* (2005, 2006b) recommendations. In each estate, the plot monitored during grape ripening is followed all along the winemaking process until bottling. The main characteristics of vineyards and wines sampled are listed in table 1.

Then, we studied old bottles of non-filtered red wines produced in these estates. For each estate, two vintages, an old and a more recent, were studied in triplicate, in 2006 (table 4).

The cellar equipment was also surveyed by analysis of the water used during cleaning operations at the devatting and the racking moments.

2. DNA extraction

From sample, similar protocol was used for bacteria and yeast. 100 mL of berries, vinery material washing solutions or 10 mL of wine were centrifuged (10,000 g, 30 min, 4°C). The pellet was washed in 1 mL of Tris 10 mM (GenApex) - EDTA 1 mM (GenApex) (TE) buffer. After a second centrifugation (10,000 g, 15 min, 4°C), the supernatant was discarded and the pellet resuspended in 300 µL of 5 mM EDTA pH8. 300 µL of glass beads were added (Ø 0.1 mm) and samples were mixed at very high speed for 15 min. Then, 300 µL of nuclei lysis (Promega) and 200 µL of protein precipitation solution (Promega) were added and mixed for 20 s. Precipitation of cellular fragments was made on ice for 15 min. After centrifugation (10,000 g, 5 min, 4°C), the supernatant was transferred in a new clean tube containing 100 µL of PolyVinylPyrrolidone (PVP) (Sigma-Aldrich) 10% solution was added and mixed to precipitate the polyphenols. After centrifugation (10,000g, 5 min, 4°C) the supernatant was transferred to a new clean tube containing 500 µL of isopropanol. The tube was gently mixed by inversion until a visible mass of DNA could be observable. After incubation at -20°C for 2 hours and centrifugation (10,000 g, 20 min, 4°C), 500 µL of ethanol 70% (vol/vol) were added to the pellet before an ultimate stage of centrifugation (10,000 g, 5 min, 4°C). Ethanol was sucked up and the tube dried. Then PPI (Pour Preparation Injectable) (Cooper) water with 1 µL of RNase (Promega) was used to rehydrate DNA to final

Table 1 - Main characteristics of vineyards and domains of samples.

		L	G	M
Localization		Libournais	Graves	Médoc
Grape variety		Merlot	Cabernet-Franc	Cabernet-Sauvignon
Initial cold maceration		Yes 5 days at 10°C	No	No
Alcoholic fermentation		Indigenous flora Wooden tank	Indigenous flora Stainless tank	Levain 522 Davis Stainless tank
Malolactic fermentation		Indigenous flora New Barrels	Indigenous flora Stainless tank	Indigenous flora New and old Barrels
Wines parameters after MLF	**Alcohol content (% v/v)**	13.9	12.6	12.4
	Residual sugars (g/L)	<0.2	<0.2	<0.2
	TPI at the end of MLF	88	78	66
	SO_2 free (mg/L of H_2SO_4) at the end of ageing	27	23	26
	pH	3.44	3.78	3.82

concentration of 50 µg/Ml. After spent overnight at 4°C, DNAs were stored at -20°C.

3. DGGE procedures

For yeast, the D1 region of the 26S rRNA gene was amplified by PCR using the NL1-GC forward primer (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GCG GGC CAT ATC AAT AAG CGG AGG AAA-3') and the LS2 reverse primer (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin *et al.* 2000). The GC clamp underlined is necessary to prevent the duplex DNA artefact formation (MYERS *et al.*, 1985). The PCR and the migration procedure were made according to the protocol developed by Renouf *et al.* (2006c).

For bacteria, then DNA extracted was amplified by using the primer set: rpoB1 (5 'ATTGACCACTTG GGTAACCGTCG-3'), rpoB1o (5'-TCGATCACT-TAGGCAATC-GTCG-3') and rpoB2 (5'-ACGATCACGGGTCA-AACCACC-3'). The GC clamp was added to primer rpoB2. The DGGE was performed according the Renouf *et al.*'s (2006a) protocol.

4. Sequences comparison and phylogenetic analysis

From gels, each band was excised with small blocks of acrylamide containing the DNA. They were placed in 2 mL of TE buffer overnight at 4°C in order to allow DNA diffusion out of the gel. The recuperated DNAs are used for reamplification with primers (without GC-clamp) and reaction conditions used for PCR-DGGE analyses. Then, amplicons were purified using Qiaquick® PCR purification kit (Qiagen) and sequenced. These sequences are compared with sequences available in databases (Genbank). Neighbour-joining phylogenetic trees (Saitou and Nei, 1987) using MEGA version 2.1 software were built (Kumar *et al.*, 2001) with the closest referenced sequence. The phylogenetic distance was calculated according to Kimura's method. 1,000 repetitions were made for bootstrap (Felsentein, 1985).

5. Species frequency of detection.

The PCR-DGGE does not provide directly quantitative information. But it is possible to determine the frequency of detection of a band on DGGE gel during a monitoring. For example, concerning the study of grape berries, ten samples were carried out in the estate G. On yeasts DGGE gels, the species *Aureobasidium pullulans* was detected in seven samples. Consequently, the detection frequency of *Aureobasidium pullulans* to the estate G is of 70%. Finally the frequency of detection of a species is the number of times where the band characteristic of this species appears on DGGE gel during a monitoring.

RESULTS AND DISCUSSION

1. PCR-DGGE analysis on the berry surface

The high number of sequences obtained during PCR-DGGE analyses revealed a very large and diverse

Table 2. Species identified by PCR-DGGE during analysis on the berry surface from the three vineyards and frequency of their detection.

		Micro-organism group	Vineyard / number of samples L / 8	G / 10	M /6	Frequency of the species detection
Yeast	*Aureobasidium pullulans*	I	38%	70%	67%	58%
	Candida cantarelli	II	25%	10%	17%	17%
	Candida stellata	II	38%	20%	33%	30%
	Cryptococcus sp.	I	88%	60%	67%	71%
	Lipomyces spencerartinsaie	I	13%	0%	0%	4%
	Hanseniaspora uvarum	II	13%	40%	0%	18%
	Pichia anomala	II	25%	50%	33%	36%
	Rhodotorula sp.	I	38%	40%	50%	43%
	Sporobolomyces sp.	I	13%	20%	0%	11%
	Sporidiobolus sp.	I	13%	30%	17%	20%
	S. cerevisiae	III	13%	10%	0%	8%
Bacteria	*Bacillus* sp.	I	25%	10%	17%	17%
	Burkloderia vietnamiensis	I	75%	60%	50%	62%
	Enterobacter sp.	I	0%	20%	17%	12%
	Enterococcus sp.	II	13%	0%	33%	15%
	G. oxydans	II	38%	20%	67%	41%
	Lactobacillus sp.	II	63%	40%	33%	45%
	Leifsonia sp.	I	13%	0%	17%	10%
	Leuconostoc mesenteroides	I	13%	0%	17%	10%
	O. oeni	III	25%	60%	33%	39%
	P. parvulus	II	13%	10%	50%	24%
	Pseudomonas sp.	I	0%	20%	17%	12%
	Serratia sp.	I	38%	60%	33%	44%

community on berries surface (Table 2). That included species unknown in wine: *Aurebasidium pullulans*, *Burkholderia vietnamiensis*… They were detected with high frequency. These species should play an important role within grape microbial ecosystem (Renouf *et al.*, 2005). The species commonly fermenting in wine (*S. cerevisiae*, *O. oeni*) were also present but in minor proportion, as well some wine spoilage species: *Pediococcus parvulus* (*P. parvulus*), *Gluconobacter oxydans* (*G. oxydans*)… For bacteria, *Lactobacillus* sp. were an important group of grape's species. They were also known to intervene during the first step of the winemaking (Renouf *et al.*, 2006a). It was also the case of the *Candida stellata*, *Candida cantarelli*, *Mestchnikowia fructicola* and *Pichia anomala* yeast species (Clemente-Jimenez, 2004; Renouf *et al.*, 2006e).

In fact, species detected on grapes were divided into three groups. The first group contained the species specific of the grape's flora without fermentation ability and unknown in wine (*Aureobasidium pullulans, Burkholderia vietnamiensis*). The second group contained the species with some fermentation ability (*Lactobacillus* sp., *Pichia* sp., *Candida* sp. *Metschnikowia* sp.). They could act during the first stages of winemaking. The third group is composed by the main fermenting species: *S. cerevisiae* and *O. oeni* (Table 3).

The proportion of these groups changed during the grape ripening (Figure 1). The first group was dominant at the beginning of the berry development. Then, the proportion of the two other groups increased. At the harvest, when the microbial populations have reached to their maxima (Renouf *et al.*, 2005) the second group achieved its maxima.

2. PCR-DGGE analysis during the winemaking

During AF and MLF, the microbial diversity decreased (Figure 2). Before fermentations, yeast and bacteria species

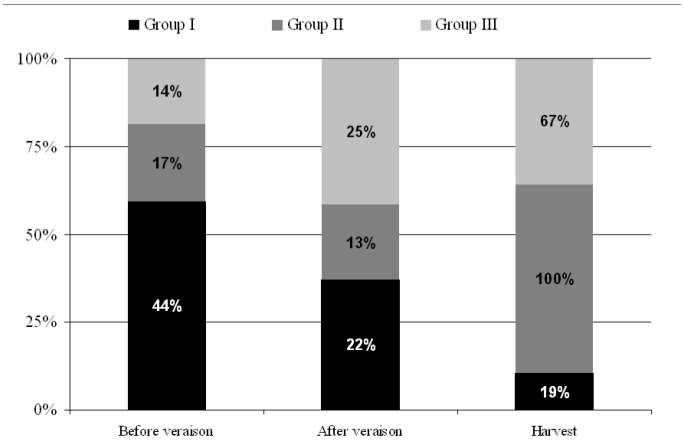


Figure 1. Evolution of the percentage of the three different groups of berry's micro-organisms during grape ripening.

Table 3. Species identified by PCR-DGGE from berries analysis and their frequency of detection according to the stage of sampling during the berry development.

			L			G			M			Average for the three vineyards		
			Before veraison	After veraison	Harvest	Before veraison	After veraison	Harvest	Before veraison	After veraison	Harvest	Before veraison	After veraison	Harvest
		Samples	3	4	1	4	5	1	2	3	1	9	12	3
	Yeasts	*Aureobasidium pullulans*	100%	0%	0%	100%	40%	100%	100%	67%	0%	100%	36%	33%
		Cryptococcus sp.	100%	50%	0%	50%	60%	100%	50%	100%	0%	67%	70%	33%
		Lipomyces spencerartinsaie	33%	0%	0%	0%	0%	0%	0%	0%	0%	11%	0%	0%
		Rhodotorula sp.	67%	0%	100%	100%	0%	0%	100%	33%	0%	89%	11%	33%
		Sporobolomyces sp.	33%	0%	0%	50%	0%	0%	0%	0%	0%	28%	0%	0%
		Sporidiobolus sp.	33%	0%	0%	50%	20%	0%	0%	33%	0%	28%	18%	0%
Group I		**Total for yeast**	**61%**	**8%**	**17%**	**58%**	**20%**	**33%**	**42%**	**39%**	**0%**	**54%**	**22%**	**17%**
	Bacteria	*Bacillus sp.*	67%	0%	0%	0%	20%	0%	50%	0%	0%	39%	7%	0%
		Burkloderia vietnamiensis	67%	75%	100%	75%	40%	100%	50%	67%	0%	64%	61%	67%
		Enterobacter sp.	0%	0%	0%	0%	40%	0%	0%	33%	0%	0%	24%	0%
		Leifsonia sp.	33%	0%	0%	0%	0%	0%	0%	0%	100%	11%	0%	33%
		Pseudomonas sp.	0%	25%	0%	0%	40%	0%	50%	0%	0%	17%	22%	0%
		Serratia sp.	67%	25%	0%	75%	40%	100%	100%	0%	0%	81%	22%	33%
		Total for bacteria	**39%**	**21%**	**17%**	**25%**	**30%**	**33%**	**42%**	**17%**	**17%**	**35%**	**23%**	**22%**
	TOTAL FOR GROUP I		**50%**	**15%**	**17%**	**42%**	**25%**	**33%**	**42%**	**28%**	**8%**	**44%**	**22%**	**19%**

J. Int. Sci. Vigne Vin, 2007, **41**, n°1, 51-61

Table 4. (suite)

Group II	Yeasts	*Candida cantarelli*	33%	0%	100%	0%	0%	100%	0%	0%	100%	11%	0%	100%
		Candida stellata	33%	25%	100%	0%	0%	200%	50%	0%	100%	28%	8%	133%
		Hanseniaspora uvarum	0%	0%	100%	0%	60%	100%	0%	0%	0%	0%	20%	67%
		Pichia anomala	33%	0%	100%	50%	40%	100%	0%	33%	100%	28%	24%	100%
		Total for yeast	**25%**	**6%**	**100%**	**13%**	**25%**	**125%**	**13%**	**8%**	**75%**	**17%**	**13%**	**100%**
	Bacteria	*Enterococcus* sp.	33%	0%	0%	0%	0%	0%	50%	67%	100%	28%	22%	33%
		G. oxydans	33%	50%	100%	0%	20%	100%	0%	33%	100%	11%	34%	100%
		Lactobacillus sp.	67%	50%	100%	25%	40%	100%	50%	0%	0%	47%	30%	67%
		Leuconostoc mesenteroides	0%	25%	0%	0%	0%	0%	0%	33%	100%	0%	19%	33%
		P .parvulus	0%	0%	100%	0%	20%	0%	0%	33%	100%	0%	18%	67%
		Total for bacteria	**27%**	**25%**	**60%**	**5%**	**16%**	**40%**	**20%**	**33%**	**80%**	**17%**	**25%**	**60%**
	TOTAL FOR GROUP II		**26%**	**17%**	**78%**	**8%**	**20%**	**78%**	**17%**	**22%**	**78%**	**17%**	**20%**	**78%**
Group III	Yeasts	*S. cerevisiae*	0%	25%	0%	0%	0%	100%	0%	0%	0%	0%	8%	33%
	Bacteria	*O. oeni*	33%	0%	100%	50%	60%	100%	0%	67%	100%	28%	42%	100%
	TOTAL FOR GROUP III		**17%**	**13%**	**50%**	**25%**	**30%**	**100%**	**0%**	**33%**	**50%**	**14%**	**25%**	**67%**

Table 4. Yeast and bacteria species identified during PCR-DGGE analyses of old and recent bottle of wine produced in the estate L, G and M.

The analyses are made in 2006. -* means that no band was visualized on the gel and the PCR amplification was negative

Estate	G		L		M	
Vintage	1974	2000	1981	1998	1974	2001
Yeast	*B. bruxellensis* *Zygosaccharomyces bailii* *Rhodotorula* sp.	*B. bruxellensis* *S. cerevisiae*	*B. bruxellensis*	*B. bruxellensis* *S. cerevisiae*	*B. bruxellensis*	*B. bruxellensis* *S. cerevisiae* *Pichia anomala*
Bacteria	-*	*O. oeni*	*O. oeni* *P. parvulus*	*O. oeni*	*O. oeni*	*O. oeni* *Lactobacillus plantarum*

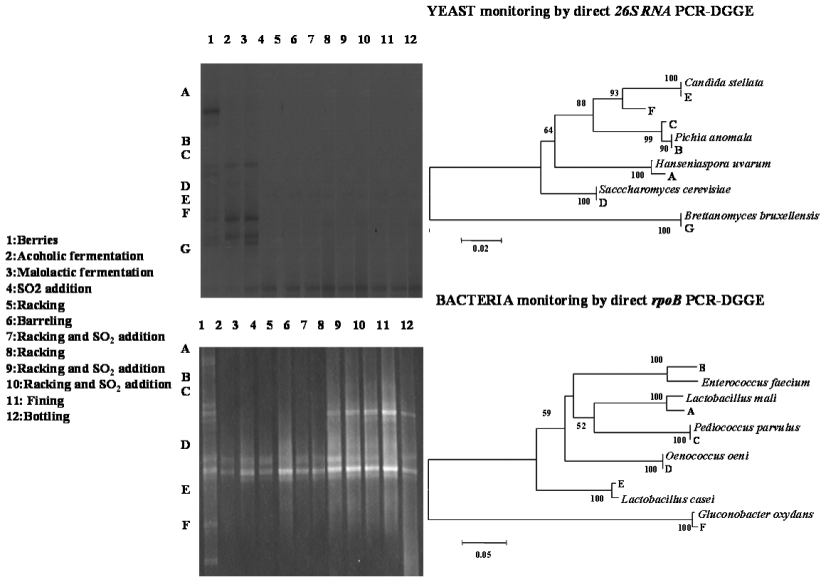
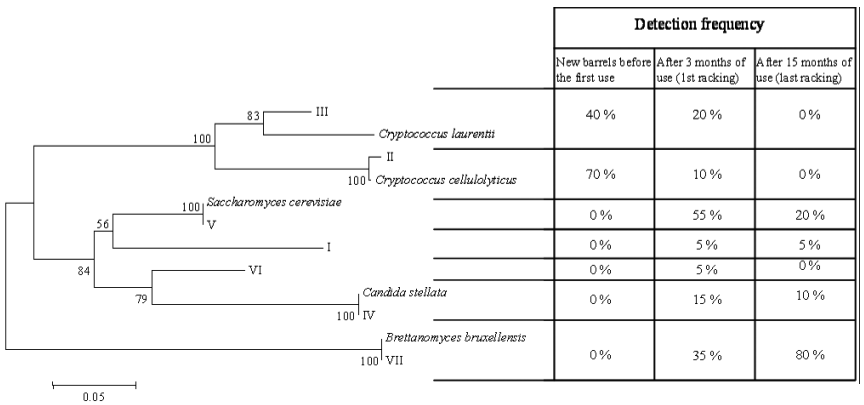


Figure 2. Yeast and bacteria DGGE monitoring all along the winemaking process since the berries crushing until the bottling for the wine of domain G.

The numbers given in the branches of the phylogenetics trees are the bootstrap values obtained after 1,000 repetitions; 0.03 and 0.05 represents the branch length scale for respectively the yeast and the bacteria analysis.



	Detection frequency		
	New barrels before the first use	After 3 months of use (1st racking)	After 15 months of use (last racking)
Cryptococcus laurentii	40 %	20 %	0 %
Cryptococcus cellulolyticus	70 %	10 %	0 %
Saccharomyces cerevisiae	0 %	55 %	20 %
	0 %	5 %	5 %
	0 %	5 %	0 %
Candida stellata	0 %	15 %	10 %
Brettanomyces bruxellensis	0 %	35 %	80 %

Figure 3. Neighbour-joining phylogenetic of the sequences extracted from DGGE gels during the analysis of the water used to wash the barrels and the frequency of their detection experiments performed in domain G.

The numbers given in the branches are the bootstrap values after 1,000 repetitions; 0.05 represents the scale for the phylogenetic branch length.

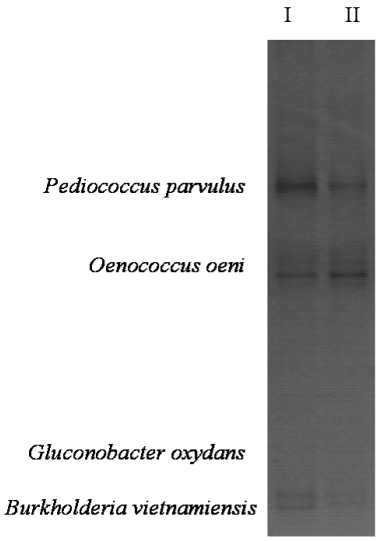


Figure 4. Analysis of the water used to wash a stainless tank which has contained wine during malolactic fermentation (I) and 6 months after (II).

detected by PCR-DGGE were respectively *Candida stellata*, *Candida cantarelli*, *Pichia anomala…* and *G. oxydans*, *Lactobacillus plantarum*, *Lactobacillus mali*, *P. parvulus*. They corresponded to the species of the second group. These species could actively grow in must. Other works reported that these species and their growth in must could be influenced by oenological practices such as the sulphite dioxide addition (Ciani et Pepe, 2002), the use of initial cold maceration (Renouf *et al.*, 2006e), the addition of commercial strain starter (Raspor *et al.*, 2002), and, also by the oxygenation (Strehaiano, 1990) and chemical parameters of must (Bely *et al.*, 1990; Alexandre and Charpentier, 1998).

After, the predominant species became *S. cerevisiae* during the AF and *O. oeni* during the MLF: the species of the third group. The second group species disappeared of the DGGE gels probably due to their low ethanol tolerance (Pina *et al.*, 2004). Finally, at the end of fermentations *B. bruxellensis*, *P. parvulus* only cohabited with residual fermentative species.

B. bruxellensis was detectable by direct PCR-DGGE analysis for the first time at the end of the alcoholic fermentation. After the post-fermentation sulphur dioxide addition it became the major yeast species whereas *S. cerevisiae* species was in decline (Renouf *et al.*, 2006c). This could be explained by the high ethanol tolerance (Medawar *et al.*, 2003) and the poor nutritive requirement (Usanga *et al.*, 2000) of *B. bruxellensis*. Concerning bacteria, *P. parvulus* was detected on berries, then it was not detectable probably due to the *O. oeni* predominance during fermentations, and it reappeared at the beginning of ageing.

These observations underlined the disadvantage of direct tools of identification. In fact, detection threshold was dependent on the diversity but also on the population levels, and ratio between dominant and minor species (Ercolini, 2004). Depending on stage of the winemaking, the best-adapted species constituted the overwhelming majority leading detection of minor species difficult. Prakitchaiwattana *et al.* (2004) reported that ratio of different species exceeding 1,000-fold made the detection of minor species difficult. It was probably the case for the yeast *B. bruxellensis* before the end of the alcoholic fermentation. Previous studies (Barbin, 2006; Renouf and Lonvaud-Funel, 2007) reported that the *B. bruxellensis* detection on grape was possible but only by an enrichment step. *B. bruxellensis* should be present all along the alcoholic fermentation but in minor proportion. At the end of the alcoholic, when *S. cerevisiae* declined and *B. bruxellensis* resisted (Renouf *et al.*, 2006c) the ratio

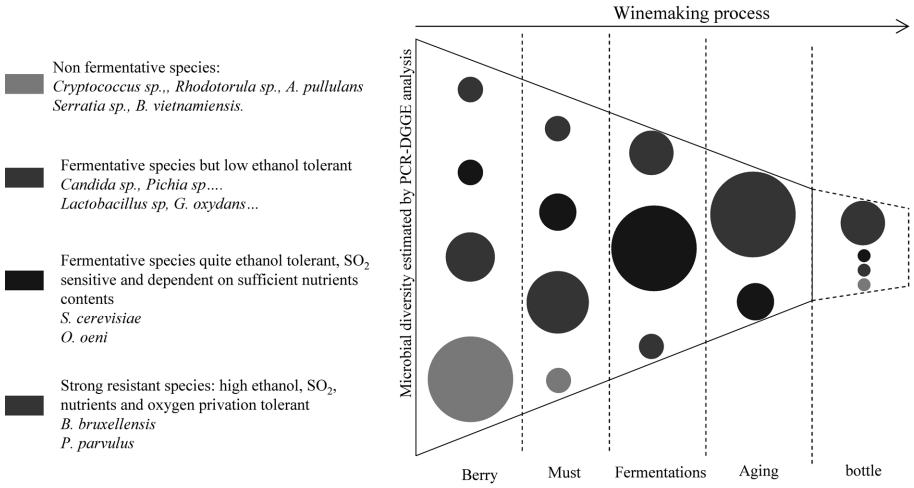


Figure 5. Illustration of the microbial diversity changes all along the winemaking process.
Circle size shows the proportion of the species group.

between these populations decreased. Then, *B. bruxellensis* could be revealed by direct PCR-DGGE analysis. Similar phenomena should be occurred between *O. oeni* and *P. parvulus*. The first dominated during MLF and the second was only detected at the end when *O. oeni* declined. Therefore, failure to detect some species by direct DGGE analysis did not mean that the species was absent, but only that some species were less numerous than others.

Detection of species present at low concentrations appeared to be difficult using direct PCR-DGGE when another species predominated but it was not really restricting because these cases were found during fermentations when *S. cerevisiae* and *O. oeni* are always dominating. Revelation of the spoilage species was more important during ageing when microbial population was lower and less diversified. At these moments, PCR-DGGE provided information necessary to act quickly against the growth of spoilage species.

3. PCR-DGGE analysis from surface of wood barrels

The winery equipment, notably the barrels, was also suspected to be a source of spoilage species (Chatonnet *et al.*, 1993; Laureano*et al.*, 2003). The control of the microorganisms present on their surface by PCR-DGGE revealed wine species: *O. oeni* and *S. cerevisiae*, but also specific wood yeast species belonging to the *Cryptococcus* genera (Figure 3). The latter were usually not present in wine and should be extracted from wood.

Species originated from the wine colonized the porous structure of wooden barrels since their detection increased with wine contact. On the contrary, wooden species disappeared.

On stainless tank surface, *O. oeni* was detected with also *P. parvulus* and, more surprising, species originated from berries like *Burkholderia vitenamiensis* (Figure 4). The detection of these species in water use for tank washing after the devatting was remarkable. At this moment *O. oeni* was the only bacteria species detected in wine. Therefore, species originated from the grape could survive on the tank surface despite they are unable to actively grow in the wine.

Retention of many yeast and bacteria species on cellar equipment surface can be considered as indigenous microflora reservoirs leading to some microbial cellar specificity (Renouf *et al.*, 2006d) but also as source of contaminants agents. Hence, it was recommended to proceed at efficient barrels and tanks cleaning before their use.

4. PCR-DGGE analysis of old bottled wine

Although large amount of information about microorganisms during the winemaking existed in the literature, the presence of remaining species in bottled wine was poorly documented. But the microorganisms could survive in bottle as revealed by the PCR-DGGE analyses (Table 4). In all bottles, *B. bruxellensis* was the predominant yeast species. That should leads to the production of 4-ethylphenol and 4-ethylguaiacol and wine sensorial alteration. That underlines the great interest of the microorganism removing by filtration (Ubeda *et al.*, 1999) or thermal treatment (Couto *et al.*, 2005) before bottling. The main species intervening during fermentations (*S. cerevisiae*, *O. oeni*) were also detected but less often. In fact, *S. cerevisiae* was only detected in recent vintages. Some species classified into the second group during berries analyses, *Lactobacillus plantarum* for the bacteria and *Pichia anomala* for the yeast, were also detected in some bottles. The detection of *R. mucilaginosa*, which was never found since berries studies, confirmed previous works (Ubeda *et al.*, 1999; Nisiotou and Gibson, 2005) reporting its ability to acquire progressively strong resistance. That may implicated the passage by a viable but nonculturable state at very low population during the winemaking process (Divol and Lonvaud-Funel, 2005). *Zygosaccha-romyces bailii* was also detected in oldest wines. This species is known to be able to referment sweet bottled wines, mainly due to its sulphite tolerance at high ethanol levels (Loureiro and Malfeito-Ferreira, 2003).

CONCLUSION

Previous tools used in wine microbial studies needed a preliminary cultivation step on selective media. That provided incomplete representation of the true diversity. The PCR-DGGE has offered an alternative free of cultivation steps and provided greater profiles representing a global view of wine microbial consortia. By this way, the main wine species: *S. cerevisiae* and *O. oeni* were easily distinguished from the other species, like the main spoilage species: *B. bruxellensis* and *P. parvulus*. That conferred strong argument for using the PCR-DGGE approach during winemaking monitoring at an industrial scale. With this method it was also possible to compare the yeast and bacteria species present in different vineyards and to connect the presence of *B. bruxellensis* or other spoilage species with particular wine defects.

Based on DGGE patterns, temporal changes of the microbial diversity all along the winemaking could be estimated (Figure 5). For yeast and bacteria, the species diversity was high on grape. After crushing, it became to fall. During the initial stages of the winemaking, the fermentative species (*Lactobacillus* sp., *Pichia* sp.) with

low ethanol tolerance were predominant. Then, *S.cerevisiae* and *O.oeni* predominated during fermentations. Biochemical changes during fermentations (ethanol, pH, etc.) and the SO_2 addition at their end were the main events acting on species diversity. The most resistant species were *B. bruxellensis* and *P. parvulus*. They were also the most dreaded species due to their abilities to spoil the wine. Moreover, these species survived in wine long time after the bottling. Therefore it was particularly important to prevent their growth by sensitive identification tool like direct PCR-DGGE analysis and efficient elimination before bottling.

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