MICROBIAL CHANGES DURING MALOLACTIC FERMENTATION IN RED WINE ELABORATION

MODIFICATION DE L'ÉCOSYSTÈME MICROBIEN PENDANT LA FERMENTATION MALOLACTIQUE LORS DE L'ÉLABORATION DES VINS ROUGES

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Abstract : Winemaking is based on complex microbial interactions. They result in alcoholic and malolactic fermentation. In some cases undesirable micro-organisms pass beyond a limit and become prejudicial to wine quality. It is particularly the case of *Brettanomyces bruxellensis* which produces volatile phenols.
Most of wine microbial studies have been focused on only one species and that can lead to incomplete and biased results by neglecting possible interactions between the populations. The aim of this study was to obtain a global survey of wine microflora and its quantitative and qualitative changes during the malolactic fermentation, the last microbial intervention before sulphur dioxide addition. The results were obtained by chemical wine analysis, conventional microbiological methods and molecular tools for microbial identification (PCR-ITS-RFLP, PCR-DGGE). In this study, conducted under cellar scale conditions, several oenological parameters were considered: two different cellars, three grape varieties, MLF in tank or in barrels, use of malolactic starters or indigenous flora.
Interactions appeared, mainly between *Oenococcus oeni* and *B. bruxellensis*, but also between *O. oeni* strains. Some explanations are suggested and further investigations are proposed.

Résumé : L'élaboration du vin rouge nécessite l'intervention de différentes populations microbiennes. Les levures, principalement *Saccharomyces cerevisiae* transforment les sucres en éthanol durant la fermentation alcoolique. Puis les bactéries lactiques décarboxylent l'acide malique en acide lactique durant la fermentation malolactique. Mais les interventions microbiennes ne se limitent pas aux activités fermentaires et c'est l'ensemble des métabolismes microbiens qui participent aux qualités aromatiques du vin. Certains sont préjudiciables et altèrent les qualités du vin. C'est particulièrement le cas de la levure *Brettanomyces bruxellensis* qui produit des phénols volatils et confère des odeurs désagréables. La majorité des études microbiennes en œnologie sont ciblées sur une seule espèce microbienne, la plupart du temps sur *Saccharomyces cerevisiae* ou *Oenococcus oeni*. Mais l'écosystème microbien du vin est très divers et complexe et s'affranchir de cette diversité peut conduire à des résultats incomplets et des interprétations erronées négligeant les interactions possibles entre les espèces. Pour pouvoir intégrer ces interactions, il est fondamental de considérer l'écosystème microbien dans sa globalité et d'analyser toutes les populations microbiennes présentes : levures totales, levures non-*Sacharomyces*, bactéries lactiques et bactéries acétiques. Cela permet d'avoir une vision systémique et exhaustive des relations entre les populations microbiennes. L'objectif de ces travaux est d'évaluer l'impact de la fermentation malolactique sur l'ensemble de l'écosystème microbien. Ces travaux ont été menés à l'échelle du chai sur deux châteaux du vignoble bordelais (Graves et Médoc) et plusieurs paramètres œno-techniques ont été considérés : trois cépages différents (Merlot, Cabernet-Sauvignon, Cabernet-Franc), fermentation conduite en cuves inox ou en barriques, utilisation de levains malolactique ou recours à la flore indigène. L'isolement et le dénombrement des populations microbiennes ont été réalisés à l'aide de milieux nutritifs sélectifs et les espèces ont été identifiées par des méthodes moléculaires (PCR-RFLP-ITS pour les levures et PCR-DGGE pour les bactéries). Ces données microbiologiques ont été confrontées aux paramètres physico-chimiques des vins étudiés (pH, TAV…).
La fermentation malolactique, dernière intervention microbienne favorisée par les vinificateurs avant l'ajout de dioxyde de soufre et la stabilisation microbienne provoque des changements des paramètres chimiques du vin (baisse de l'acidité, modification de la couleur…) ; elle est apparue comme une étape particulièrement favorable aux interactions entre les populations microbiennes. Parmi les bactéries lactiques, l'espèce *O. oeni* devient majoritaire et réalise la conversion de l'acide malique en acide lactique. Les bactéries acétiques absentes lors de la fermentation alcoolique augmentent également à la fin de la fermentation malolactique. Durant cette fermentation, les levures du genre *Saccharomyces* disparaissent tandis que les levures *B. bruxellensis* se multiplient et deviennent majoritaire à la fin de la fermentation. Le vin est plus favorable au développement de *B. bruxellensis* lors du développement d'*O. oeni* et la consommation effective d'acide malique. Cela peut s'expliciter soit par des interactions directes entre les cellules des deux espèces soit par la modification des paramètres physicochimiques du vin (augmentation du pH, baisse de l'acide malique…). Les fermentations malolactiques languissantes sont apparues particulièrement risquées car plus le temps mis pour consommer l'acide malique est long plus la quantité de *B. bruxellensis* présente est importante. Cela souligne la nécessité des contrôles microbiologiques lors de la fermentation malolactique et l'intérêt du développement de levains malolactiques de plus en plus efficaces afin de réduire le temps de la fermentation et d'éviter le développement des *Brettanomyces* et l'altération des vins.

Key words : MLF, LAB, *Brettanomyces bruxellensis*, Interactions.

Mots clés : FML, bactéries lactiques, *Brettanomyces bruxellensis*, interactions

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J. Int. Sci. Vigne Vin, 2005, **39**, n°4, 179-190

INTRODUCTION

Winemaking is based on complex microbial collaboration. After crushing, yeasts, mainly *Saccharomyces cerevisiae* species, ferment sugars, which are naturally present in must, to produce ethanol during alcoholic fermentation (AF). Then lactic acid bacteria (LAB), mainly *Oenococcus oeni*, convert malic acid into lactic acid by decarboxylation during the malolactic fermentation (MLF). In addition to alcohol production and acidity decrease, other metabolisms produce aroma responsible for sensorial wine properties.

After MLF, winemakers try to reduce microbial population by sulphating, racking, fining, filtration and thermal treatment in order to avoid any microbial growth. Indeed, *Brettanomyces bruxellensis* yeast species can spoil wines by developing off-odours which have been described as mousy, wet wool, medicinal, smoky, spicy (FUGELSANG and ZOECKLEIN 2003; LOUREIRO and MALFAITO-FERREIRA 2003). *Pedioccocus* sp. bacteria can modify wine viscosity by producing exopolysaccharides (WALLING *et al.*, 2005). Other microbial metabolisms can have a toxicological potential effect by producing biogenic amines (COTON *et al.*, 1998) and ethyl carbamate (UTHURRY *et al.*, 2005). Microbiological alterations are the highest winemaker preoccupation during the aging period.

After it was established that the primary origin of *B. bruxellensis* and *Pediococcus parvulus* was the grape berries themselves, conditions of their growth and detection in wine have been the focus of several studies (DELAHERCHE *et al.*, 2004).

Species and strains diversity is very important in wine. Thus studies which focused on only one of them within such complex mixture should lead to incomplete and biased results. It is fundamental to have a systemic approach of the wine microflora in order to integrate the possible interactions between each species.

MLF is a key step of red wine elaboration. It allows a deacidification (LONVAUD-FUNEL, 1999) and sensorial modifications (DE REVEL *et al.*, 1999) and winemakers try hard to do it. They can favour the development of the indigenous flora or use commercial malolactic starters (GINDREAU *et al.*, 1997). Moreover MLF is the last desirable microbial intervention in winemaking before the addition of sulphur dioxide in order to discard microbial population.

The goal of this work was to investigate the whole wine microflora and to characterize each population: total yeasts (TY), non-*Saccharomyces* yeast (NS), lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during the MLF. Different conditions were considered: grape variety, physicochemical parameters of the wines (alcohol content, pH, colour intensity…), and oenological practices such as MLF in barrels or tank, addition of malolactic starters or indigenous flora. This study revealed significant interactions between LAB and yeast populations. It gave a global survey of wine microflora during and after MLF leading to a better understanding of microbial interactions.

MATERIALS AND METHODS

I - SAMPLES

Wine samples from different cellars in various areas of the Bordeaux region were collected at several winemaking stages: harvesting, tank filling and homogenization, maceration before fermentation, alcoholic fermentation, post-fermentation maceration, running off, malolactic fermentation, racking, and sulphur dioxide addition. Samples were collected with sterile material and conserved in an isotherm package until their treatment at the laboratory. In the first cellar, named G, we followed the winemaking of three grape variety plots: Merlot, Cabernet-Sauvignon and Cabernet-Franc. For these wines, AF and MLF were conduced by the indigenous microflora. In the second cellar, named M, we followed two tanks of Merlot wines (A and B). Alcoholic fermentation was conducted with commercial active dried yeasts. After fermentation, the wine was transferred into new barrels which were from a homogenous pool. They were inoculated with an *O. oeni* starter for malolactic fermentation following a direct inoculation protocol. These

Table I - Chemical analysis of wine before MLF for cellar M experiment

(CI: Color Intensity, TPI: Total Polyphenol Index).

Analyses chimiques des vins avant fermentation malolactique pour les expérimentations menées au chai M.

(CI: Intensité Colorante, TPI: Index des Polyphénols Totaux)

	A	B
Alcohol % vol	13.9	13.95
Residual sugars g/L	0.7	0.8
Total acidity g/L H_2SO_4	3.85	3.87
Volatile acidity g/L H_2SO_4	0.08	0.08
pH	3.63	3.59
SO_2 free mg/L	7	10
SO_2 total mg/L	41	50
Malic acid g/L	1.85	1.86
CI	1.81	1.84
OD 420	0.53	0.55
OD 520	1.08	1.1
TPI	63.4	69.8

inoculations were made 34 days after the harvest. Six different starters were used: lots I-a, I-b, II, III, IV and V, in addition to the indigenous flora (0). It makes 12 different conditions (A-0, A-I-a, A-I-b, A-II, A-III, A-IV, A-V, B-0, B-I-a, B-I-b, B-IV and B-V) for the two tanks A and B. I-a and I-b were inoculated by the same *O. oeni* strain that differed by their preparation method. Some of these strains are still under a selection procedure (I-a, I-b, II and III), and others are commercial starters (IV and V). The starters were suspended in room temperature water and they were added into wine. After the inoculation, the wine was homogenized by sticking. Each condition was made in duplicate barrels. Wine analyses performed, before bacterial inoculation, are reported in table I.

II - ISOLATION OF MICROBIAL POPULATION AND CELL COUNTS

Serial dilutions of each sample were used to inoculate in triplicate plates of four different nutritive medium. The yeast were cultivated on YPG medium containing glucose 20 g/L, bactotryptone 10 g/L, yeast extract 10 g/L and agar 25 g/L , pH adjusted to 5.0 using orthophosphoric acid. To count total yeast population (TY), after sterilization, the medium was supplemented with biphenyl (Fluka) (0.015 %w/v) and chloramphenicol (0.01 %w/v) (Sigma Aldrich) to respectively inhibit mould development and bacterial growth. The addition of 0.1 % (w/v) cycloheximide (Sigma Aldrich) eliminated the *Saccharomyces* sp. and allowed for the numeration of non-*Saccharomyces* (NS) yeast population. At 25 °C, incubation lasted 5 days to count the TY and 10 days for the NS. The *Saccharomyces* population was estimated by subtracting the NS from the TY population. LAB were isolated on MRS plates: *Lactobacilli* MRS broth (Difco) 55 g, D-L malic acid (Prolabo) 10 g, agar 20 g, pH 4.8 with NaOH 10N. Growth of yeast was inhibited by adding 50 mg/L of pimaricine (Delvocid, DSM Food Specialties) and growth of AAB was inhibited by incubation under anaerobic conditions using an anaerobic system with palladium catalyst (BBL). LAB plates were incubated at 25 °C for 10 days. AAB were grown on MRS plates containing 100 mg/L of pimaricine and 20 mg/L of penicillin (Sigma Aldrich) to inhibit the growth of yeast and gram positive bacteria, respectively. AAB plates were incubated in aerobic conditions at 25 °C for 5 days.

In order to estimate the total different microbial populations present in the wines, we calculated the integral of the curve representing the dynamic evolution of the microbial population. These sums were made by weighting of the surface delimited by the population curve plotted on a linear graphic and the time axis for the studied interval.

III - YEAST IDENTIFICATION

1) DNA extraction

Two different protocols for DNA extraction were used according to the material considered: isolated colony or whole complex biomass.

2) DNA extraction from whole biomass on plates

After 5 days of incubation, the biomass collected from TY plates were suspended in 2 mL of Tris 10 mM -EDTA 1 mM (TE) and were centrifuged at 10 000 g at 4 °C for 20 min. Then, the supernatant was discarded. DNA extraction from the pelleted cells was made according to classic phenol/ chloroform method as described by AUSUBEL *et al.* (1995). After precipitation, DNAs were rehydrated with 100 µL of PPI (Pour Préparation Injectable, Cooper) water containing 2 µL of RNAse solution (Qiagen) at 4 °C overnight. The DNAs were conserved at -20 °C until PCR analysis.

3) Analysis of isolated yeast colony

The following identification experiments were made on Petri dishes carrying between 30 and 300 colonies. For each plate, 20 % of the colonies were tested and the results were given in species percentage (table III).

The PCRs were performed directly on the colonies isolated from the plates. Colony biomass was collected with a sterile tip and suspended in 20 µL of PPI water. 5 µL of the cell suspension were deposited on a FTA® card (Whatman). The cards were impregnated with a solution which allowed cell lysis and protein denaturation, enabling a direct DNA extraction (HANSEN and BLAKESLEY, 1998). DNAs were stabilized and immobilized at room temperature. Punches from FTA card containing the DNA were placed in clean PCR microtubes and washed by two successive solutions. Firstly, with a 100 µL Reagent buffer (Whatman) for 5 min, thereafter with 200 µL of TE buffer for 5 min. The TE buffer was carefully removed by pipetting and the microtube containing the FTA patch was dried at 50 °C for 10 min in the microtube.

4) PCR-ITS-RFLP

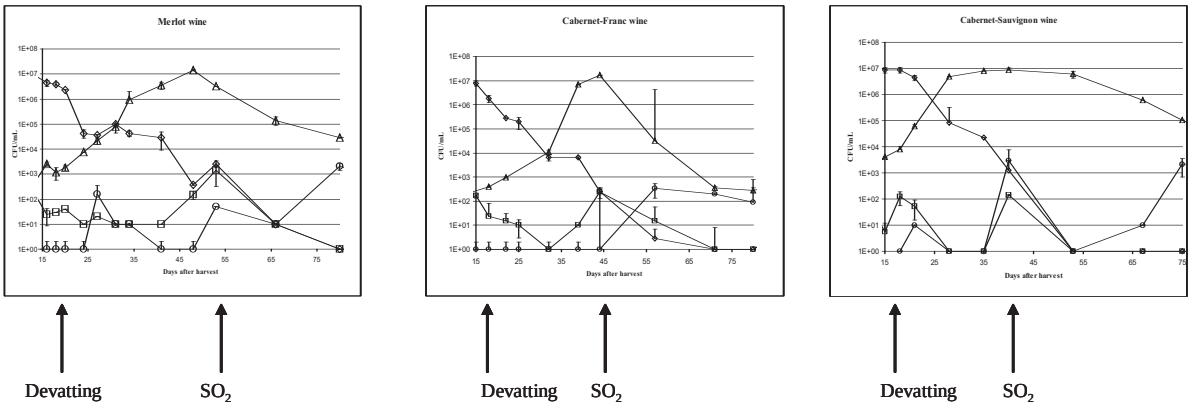
Yeast identification was done by RFLP analysis of the *5.8S rRNA* gene and the two ribosomal internal transcribed spacers (ITS1 and ITS2) (GUILLAMON *et al.* 1998). The PCR was performed with the forward primer ITS1 (5'- TCCGTAGGTGAACCTGCGG- 3') and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplification was done in a 50 µL reaction mixture containing 4 µL of commercial PCR mix (QBiogene), each primer at a concentration of 0.5 µM and a FTA patch containing the DNA template or 2 µL of DNA prepara-

tion solution. PCR conditions were as follows: initial denaturation at 95 °C for 10 min; 40 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The reaction was conducted in a Bio-Rad thermocycler. 10 µL of PCR products were deposited on a 2 % agarose (Eurobio) gel. When whole biomass was analyzed, different bands were shown on electrophoresis gel. Then, the interesting bands were carefully excised with a sterilized razor blade, and the block of agarose containing the DNA was put in sterile 1.5 mL microcentrifuge tube. 100 µL TE buffer was added and the DNA was allowed to diffuse out of the gel overnight at 4 °C. 2 µL of TE buffer containing the DNA were used for the re-amplification with the same primers. 5 µL of the re-amplified DNA were analyzed in a 1.5 % agarose gel to confirm the re-amplification. 45 µL of this product were purified (Qiaquick, Qiagen) and used to enzymatic digestions. 10 µL of PCR product were digested separately with CfoI, HaeIII and HinfI, respectively. The digestion mixture contained 10 µL of the purified PCR product, 1 µL of commercial enzyme solution, 2 µL of buffer provided by Boehringer, for CfoI, also 2 µL of 10X BSA and up to a total volume of 20 µL with distilled water. The digestion occurred at 37 °C for 5 h. The restriction fragments were separated on a 3 % agarose gel. After migration, the PCR products and the restriction fragments on their gel were visualized under UV light after ethidium bromide staining (Invitrogen). Sizes of DNA fragments were estimated by comparison with a DNA length standard (100 bp ladder, Invitrogen). The length of PCR products and restriction fragments allowed for yeast identification (table II). For each ITS pattern, DNA, from FTA patch for the isolated colonies or recuperated after band excision from agarose gel for the whole biomasses, was re-amplified, purified (Qiaquick PCR puirification Kit, Qiagen) and sent for sequencing (Millegen, France).

Table II - Size in bp of PCR products and restriction fragments of identified yeast species

Tailles en pb des produits PCR et des fragments de restriction des espèces de levures identifiées.

	Length of the PCR products (pb)	Length of the restriction fragments		
		CfoI	HaeIII	HinfI
Saccharomyces cerevisiae	850	390+380	320+230+170+130	380+160
Brettanomyces bruxellensis	490	250+150+90	390+90	270+220
Candida cantarelli	700	310+310+80	440+180+80	360+170+170



Time between harvest and devatting = 20 d
Time between devatting and the end of MLF= 35 d
pH= 3.79
Alcohol (%vol) =13.74
Free SO_2 10 days after the post-fermenting sulphur dioxide addition = 25 mg/L
Total SO_2 10 days after the post-fermenting sulphur dioxide addition = 36 mg/L

Time between harvest and devatting = 16 d
Time between devatting and the end of MLF= 28 d
pH= 3.80
Alcohol (%vol) =12.61
Free SO_2 10 days after the post-fermenting sulphur dioxide addition = 25 mg/L
Total SO_2 10 days after the post-fermenting sulphur dioxide addition = 67 mg/L

Time between harvest and devatting = 17 d
Time between devatting and the end of MLF= 20 d
pH= 3.79
Alcohol (%vol) = 12.41
Free SO_2 10 days after the post-fermenting sulphur dioxide addition = 25 mg/L
Total SO_2 10 days after the post-fermenting sulphur dioxide addition = 60 mg/L

Figure 1 - Time course of microbial population since the devatting operation and until several days after sulphur dioxide addition and chemical analysis just after the end of MLF for the three studied wines in cellar G

(◊ = TY population, ❑ = NS population, Δ = LAB population and ❍ = AAB population).

Evolution des populations microbiennes

(◊ = population de levures totales, ❑ = NS population de levures non-*Saccharomyces*, Δ = population de bactéries lactiques et ❍ = population de bactéries acétiques) depuis l'écoulage et jusqu'à plusieurs jours après le sulfitage post-fermentaire et analyses chimiques des trois vins du chai G réalisées juste après la fin de la fermentation malolactique

IV - LAB POPULATION ANALYSIS

1) LAB species identification

Analyses were made on the whole biomass from the LAB plates. After 10 days of incubation, biomasses from LAB plates were collected with 2 mL of TE buffer. After 15 min centrifugation (15 °C, 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.* (2006a) protocol.

2) Implantation control

Implantation controls were made as previously described by GINDREAU *et al.* (1997), using pulse-field gel electrophoresis method to separate NotI-restricted bacterial DNA fragments.

V - CHEMICAL ANALYSIS

Conventional analysis: total acidity, volatile acidity, alcohol content, free and total SO_2, reducing sugars, colour intensity (CI), 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). Analyses were carried out after the MLF was completed, just after sulphur addition. Malic acid concentration was measured by the enzymatic method (Boehringer-Mannheim).

RESULTS

I - EVOLUTION OF YEAST AND BACTERIA AFTER THE AF IN THREE DIFFERENT VARIETIES OF WINES

In the three cases, LAB population increased progressively after devatting but at different rate to reach a maximum concentration of 10^7 CFU/mL (figure 1). The time necessary to complete MLF differed significantly (figure 2). After devatting, the malic acid concentration was close for the three wines however in the Cabernet-Sauvignon wine MLF was faster. *O. oeni* was the only LAB species detected by PCR/DGGE-*rpoB* after devatting, during MLF and also after sulphur dioxide addition (data not shown). During the growth of LAB population and MLF, the AAB population remained at low level. It grew in Merlot and Cabernet-Sauvignon wines at the end of MLF even while LAB population was maxima. Then sulphur addition stopped the AAB increase. For Cabernet-Franc, the AAB population started to grow after sulphur dioxide addition. Finally, at the end of the monitoring, AAB population was between 10^2 and 10^3 CFU/mL according to the wine. Regarding yeasts, after

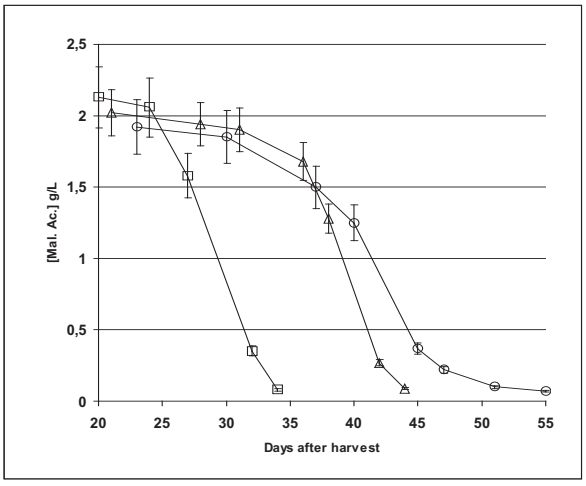


Figure 2 - L-malic acid consumption for the three wines of cellar G (❍ = Merlot, Δ= Cabernet-Franc and ❑ = Cabernet-Sauvignon).

Cinétique de consommation de l'acide L-malique pour les trois vins du chai G (❍ = Merlot, Δ = Cabernet-Franc and ❑ = Cabernet-Sauvignon).

Table III - Yeast species between the devatting and the end of malolactic fermentation identified by PCR-ITS-RFLP analysis on isolated colonies randomly picked on TY plates.

Espèces de levures identifiées par PCR-ITS-RFLP réalisée sur un échantillon de colonies isolées sur les milieux de cultures de levures totales entre l'écoulage et la fin de la fermentation malolactique.

		Merlot	Cabernet-Franc	Cabernet-Sauvignon
Devatting	*S. cerevisiae*	100%	90%	100%
	B. bruxellensis	-	5%	-
	C. cantarelli	-	5%	-
Beginning of MLF	*S. cerevisiae*	100%	100%	100%
	B. bruxellensis	-	0%	-
[Malic acid] = 1 g/L	*S. cerevisiae*	60%	85%	100%
	B. bruxellensis	40%	15%	-
End of MLF	*S. cerevisiae*	30%	5%	65%
	B. bruxellensis	70%	95%	35%

devatting, TY population decreased regularly during MLF. The NS population which previously stayed at a low level since the beginning of the alcoholic fermentation, reached the same number as TY population at the end of MLF, just before sulphur addition. Thus TY was actually NS and this was confirmed by molecular identification of isolated yeasts (table III). After devatting *S. cerevisiae* was the predominant species present in wine. For Cabernet-Franc, two non-*Saccharomyces* species were detected at a minor level: *B. bruxellensis* and *Candida cantarelli*. Then, *B. bruxellensis* was the only non-*Saccharomyces* species detected. Therefore the NS population could be assimilated to *B. bruxellensis* population. At mid-MLF, *B. bruxellensis* was detected in the three wines. When MLF was completed, *B. bruxellensis* has become the major species in Merlot and Cabernet-Franc wines. After the sulphur dioxide addition, *B. bruxellensis* was the only yeast species detected by PCR-ITS-RFLP in the three wines (figure 3).

Table IV shows the time between the devatting and the end of MLF, and the NS population and LAB population integrals during this period for the three wines. Significant differences of the time necessary to consume all malic acid were found. On the contrary there was no significant difference between LAB population integrals between the devatting and the sulphur dioxide addition. In the three cases, a sum of 10^8 CFU.Δt/mL LAB was necessary to consume all malic acid. In addition a relationship appeared between the NS population integral and the time required for MLF completion. Indeed, the more the MLF was long, the more the NS population integral was high: in Merlot, MLF was the longest and NS population was the highest as was the proportion of *B. bruxellensis* species at the end of MLF (table IV)

II - EVOLUTION OF POPULATIONS IN WINES INOCULATED BY MALOLACTIC STARTERS

First, the efficiency of inoculation was checked. Thirty minutes after inoculation, the LAB population was numbered. Indigenous microflora was at very low level, indicating a probably long lag phase before the beginning of spontaneous MLF. For starters, LAB populations were in range of 10^5-10^6 C FU/mL which are normal values for direct-inoculation starters, except for A-III which was found at only 10 CFU/mL, a non acceptable value for a correct inoculation. In this case, the bacteria present in the preparation were unable to survive after direct wine inoculation. Then, three different cases could be distinguished (an example of each case is given in figure 4). First, for the indigenous microflora and the previously mentioned starter failed experiment, LAB populations increased gradually to reach an optimum of 10^7 CFU/mL after more than 70 days. In a second case, cell concentrations at the inoculation were correct, but the LAB population decreased until to 10^3 CFU/mL, then it followed the same evolution as the indigenous microflora with an increasing step. The viable bacteria present in the

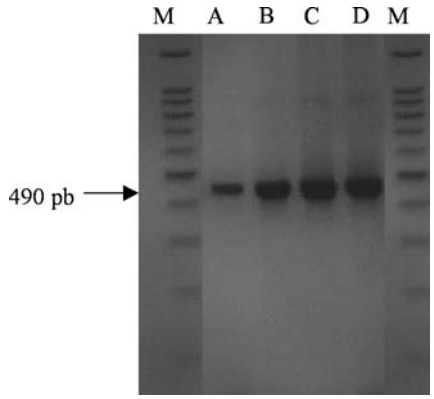


Figure 3 - PCR-ITS on DNA extracted from whole biomass from TY plates and samples taken 7 days after sulphur dioxide addition.
Lane A = *B. bruxellensis* species control FOEBL 0417 strain, lane B = Merlot wine, lane C = Cabernet-Franc wine, lane D = Cabernet-Sauvignon wine, Lanes M = 100 bp ladder (Promega).

PCR-ITS réalisées sur l'ADN extrait des biomasses totales collectées sur les boites de Pétri de levures totales 7 jours après le sulfitage post-fermentaire.
Puit A = *B. bruxellensis* contrôle positif souche FOEBL 0417, puit B = vin de Merlot, puit C= vin de Cabernet-Franc et puit D = vin de Cabernet-Sauvignon, puits M = marqueur de 100 pb (Promega).

Table IV - Integral of NS yeast and LAB population and the time between the devatting and the end of the FML in cellar G. Integral represents the total CFU/mL during the time Δt.

Intégrale de la population de levures non-*Saccharomyces* et de bactéries lactiques entre l'écoulage et la fin de la fermentation malolactique au chai G. Ces intégrales représentent la totalité des cellules (en UFC/mL) qui se sont développées dans le vin durant l'intervalle de temps Δt considéré.

Wines	Days between devatting and the end of FML	Integral of NS population (CFU.Δt/mL)	Integral of LAB population (CFU.Δt/mL)
Merlot	**35**	**$7.8 \pm 0.1 \times 10^3$**	**$1.1 \pm 0.2 \times 10^8$**
Cabernet-Franc	**28**	**$1.27 \pm 0.04 \times 10^3$**	**$1.03 \pm 0.02 \times 10^8$**
Cabernet-Sauvignon	**20**	**$8.2 \pm 0.2 \times 10^2$**	**$1.05 \pm 0.05 \times 10^8$**

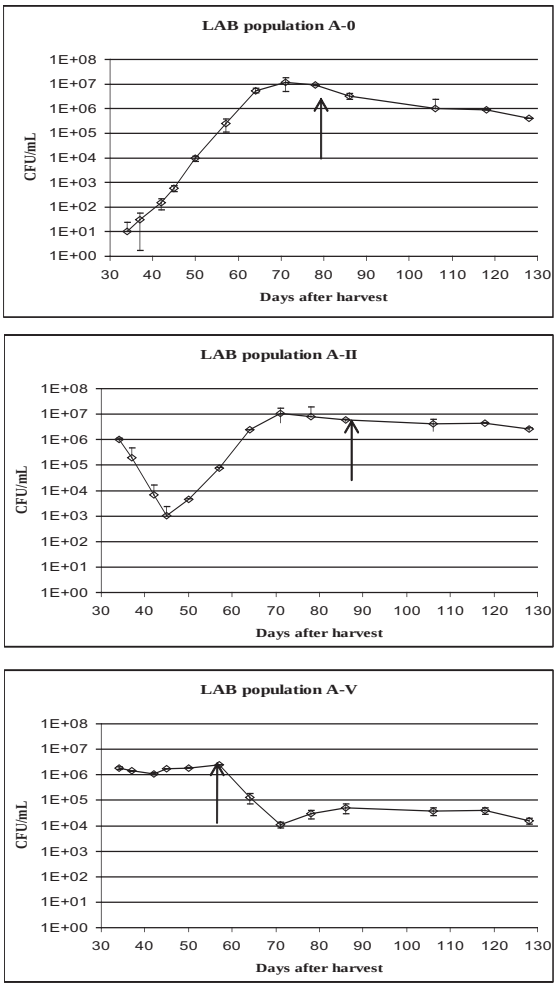


Figure 4 - Evolution of LAB populations in cellar M experiment.
Arrows show the end of MLF and sulphur dioxide addition.

Evolution de la population de bactéries lactiques durant les suivis réalisés au château M.
Les flèches représentent la fin de la fermentation malolactique et le sulfitage post-fermentaire.

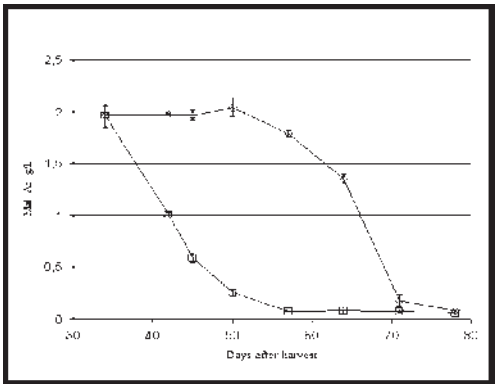


Figure 5 - Examples of concave (❒) and convex (◊) acid-L-malic consumption.

Exemples de cinétique concave (❒) et convexe (◊) de consommation de l'acide-L-malique.

freeze-dried preparation were unable to survive and to grow after being stressed by the inoculation into the wine. Finally in the other experiments, the LAB population stayed at high level during all the MLF process (above 10^6 CFU/mL). Concerning malic acid consumption, two kinds of kinetics were observed (figure 5). Wines inoculated with the strain V presented a concave evolution and the time after inoculation necessary to complete MLF was respectively 25 and 37 days. The other experiments presented a convex evolution and the end of MLF was delayed. Implantations were positive for strains IV and V in all wines. Concerning I-a and I-b starters preparations, result was positive for wine A and negative for B. In the other wines, where the LAB populations and malic acid consumption were similar to the indigenous assay the result was negative. In addition in the cases where the starters could not be detected the time needed for MLF completion was even longer than with the indigenous microflora. Concerning AAB population all experiments showed the same results. An example is given in figure 6. During MLF, AAB populations were low and they increased just after the end of MLF and sulphur dioxide addition. At the end of the experiment, they reached a level of 10^4 CFU/mL.

At the beginning of the MLF, TY population progressively decreased and NS population remained low. Then, when malic acid consumption became significant, the NS population increased to the same level as the TY population. At the end of MLF, *Saccharomyces* species were negligible and all numbered yeast were NS species (figure 7). The figure 8 shows PCR-ITS-RFLP results obtained during MLF. The same evolution was observed in all cases. At the beginning of MLF, only *S. cerevisiae* was detected. Then, at the middle of MLF, *S. cerevisiae* and *B. bruxellensis* species were both detected and finally only *B. bruxellensis* band could be seen on the gel. The

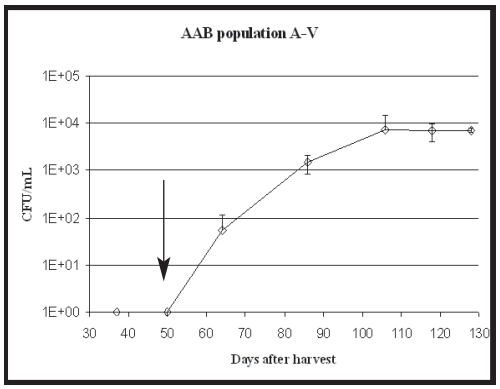


Figure 6 - AAB population in a malolactic starter experiment. The arrow shows the end of MLF.

Évolution de la population en bactéries acétiques observée dans le cas d'utilisation d'un levain malolactique. La flèche représente la fin de la fermentation malolactique.

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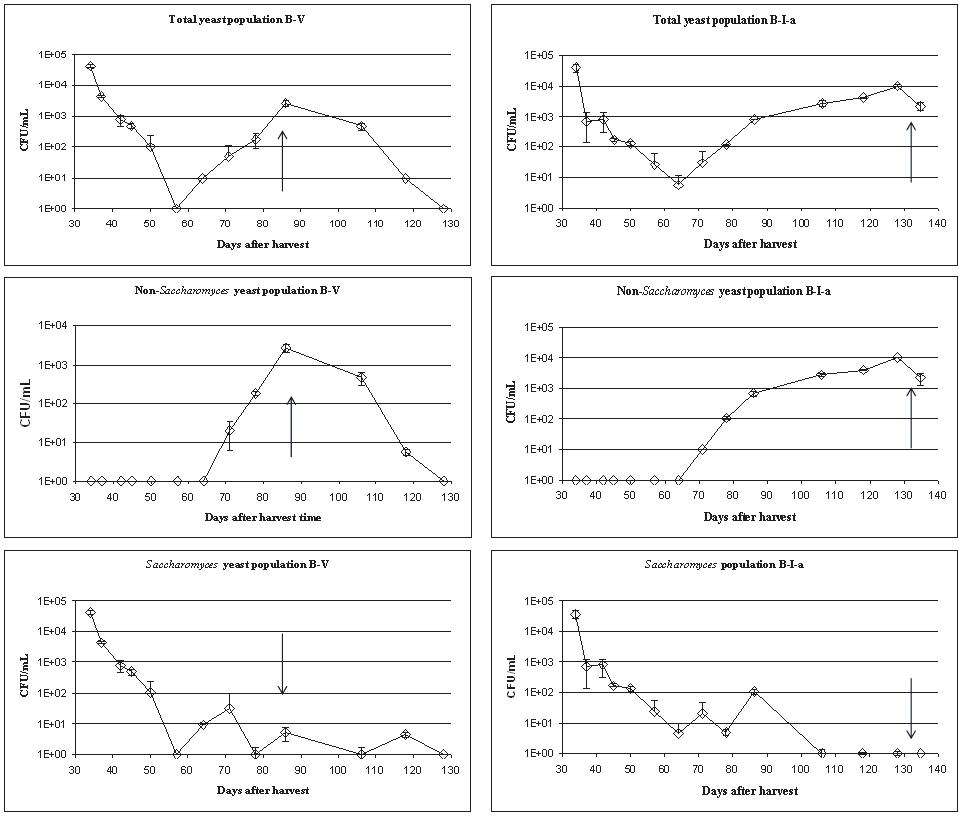

Figure 7 - Yeast population evolution during MLF in two experiments of cellar M. *Saccharomyces* population is estimated by subtracting NS population from TY population.

End of MLF is indicated by an arrow. Ten days after the sulphur dioxide addition, the wine B-V contained 22 mg/L of free SO_2 and 66 mg/L of total SO_2, and the wine B-I-a contained 23 mg/L of free SO_2 and 88 mg/L of total SO_2.

Évolution des populations de levures durant une fermentation rapide et une fermentation languissante au chai M. La population de levures du genre Saccharomyces est estimée par soustraction de la population de levures non-*Saccharomyces* à la population de levures totales.

La fin de la fermentation malolactique est indiquée par une flèche. 10 jours après le sulfitage postfermentaire, le vin B-V contenait 22 mg/L de SO_2 libre et 66 mg/L de SO_2 total, et le vin B-I-a contenait 23 mg/L de SO_2 libre et 88 mg/L de SO_2 total.

figure 9 shows the relationship between the integral of *B. bruxellensis* population and the duration of MLF. The longer MLF was, the higher was the sum of *B. bruxellensis.* Therefore an exponential correlation between the *B. bruxellensis* population integral and the time between inoculation and the end of MLF was evidenced.

DISCUSSION

When MLF was conduced by indigenous LAB in tank, the LAB population, composed by *O. oeni* (WIBOWO *et al.*, 1985; RENOUF *et al.*, 2006a), increased progressively in all grape varieties wines after devatting. Despite the same initial level (10^3 CFU/mL), the delay necessary to achieve MLF differed according the wine probably because the alcohol content was different. Indeed, MLF was more difficult in Merlot (13.74 % v/v) than in Cabernet-Sauvignon (12.41 % v/v). Ethanol is considered as one of the main inhibitor factor for *O. oeni* growth (RIBÉREAU-GAYON *et al.*, 1998). The peak of LAB population was obtained 8 days after devatting for Cabernet-Sauvignon, whereas it took 30 days for the merlot wine. Also when malolactic starters were used, the wine which had the highest alcohol content presented also the longest MLF. An important observation made from this study is that despite the variability of the time necessary to achieve MLF in the different wines, maximal populations were similar, and interestingly, calculated integrals of population, from devatting to the end of MLF, were also the same (table IV). That means that the total *O. oeni* biomass involved in a complete MLF process should be constant. Some of our results strongly suggest interactions between *O. oeni* strains, probably involved in MLF duration. Indeed, in some starters experiment *O. oeni* population was high just after inoculation but after a stationary phase, it decreased and the evolution was similar to that of indigenous flora. Moreover, controls made during MLF showed implantation failure. It seems that the *O. oeni* inoculated strains were able to survive in wine after direct inoculation, but they were

unable neither to grow nor to perform MLF. Therefore, after probable interactions between indigenous and starter strains, the indigenous microflora got the upper hand but MLF was delayed by comparison with indigenous flora experiment. Similar observations were made in other experiments where the delay necessary to complete MLF with some malolactic starter was longer than for indigenous flora (data not shown). Interactions between the *O. oeni* strains should have occurred. Interactions between *O. oeni* and other common LAB species such as *Lactobacillus* sp. and *Pediococcus* sp. had been previously studied (LONVAUD-FUNEL and JOYEUX, 1993; EDWARDS *et al.*, 1994), but study of interactions between strains of the same species are more complicated. It needs heavy methods to describe the growth of each strain. To resolve this problem ALBASI *et al.* (2002) developed a bioreactor in order to perform mixed cultures by keeping microbial populations separated by a membrane which allowed substrates and products to flow freely. Further investigations using similar process should be made to understand *O. oeni* strains interactions.

After the end of MLF, in the same time of the fall of LAB population, the AAB population increased. That confirms previously studies according them the AAB became dominant during the later stages of fermentation and in wine (JOYEUX *et al.*, 1984; DRYSDALE AND FLEET, 1985). At this stage of the wine elaboration the AAB species should be *Acetobacter* species which prefer ethanol as carbon source (DE LEY *et al.*, 1984) whereas the other main oenological AAB species, *Gluconobacter oxydans*, prefers a sugary rich environment (DU TOIT and LAMBRECHTS, 2002). The increases of the AAB population after the sulphur dioxide addition may be explained by the relative tolerance of *Acetobacter aceti* species to SO_2 (DU TOIT *et al.*, 2005). The incomplete elimination of *Acetobacter* species by sulphur dioxide addition is problematic for the winemakers because these species may affect the sensory properties of the end product. Winemakers should combine the SO_2 with other winemaking procedures: racking (RENOUF and LONVAUD-FUNEL, 2004), high cellar hygiene and good barrels management (LONVAUD-FUNEL, 2001), low temperature and oxygen levels (MILLET, 2001).

Concerning yeast population, important changes were observed. After devatting, TY population, mainly composed by *S. cerevisiae*, decreased. That should be explained by low residual fermentable sugars and the limited ethanol tolerance of *Saccharomyces* species (ALEXANDRE *et al.*, 1993). When MLF started, *B. bruxellensis* was detected and became the major yeast species at the end of MLF. No detection at earlier stage does not mean that *B. bruxellensis* was absent. Indeed results of molecular methods for specific species detection within a complex microbial mixture depend in some extent on the ratio species. *B. bruxellensis* should be present at very low level but they were masked by predominant *Saccharomyces* species. After post-fermentation sulphur dioxide addition, *B. bruxellensis* was the only yeast species detected in all studied wines, even where *S. cerevisiae* was still present at the end of MLF. That confirmed other cellars observation which suggested that *B. bruxellensis* is more tolerant to low sugar concentration, ethanol stress, and SO_2 than *S. cerevisiae* (RENOUF *et al.*, 2006b) In the slowest MLF, *B. bruxellensis* population overcame the threshold of 10^3 CFU/mL which is considered by several authors as a critical population for volatile phenols production (RENOUF and LONVAUD, 2005).

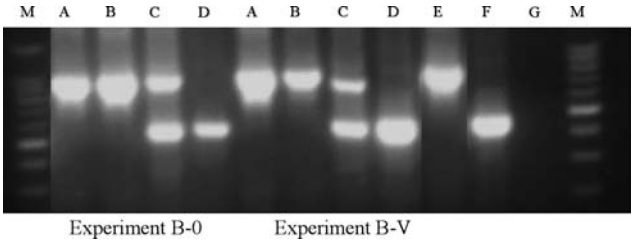

Figure 8 - PCR-ITS gel on DNAs extracted on whole biomass from TY plates for two cellar M experiments.
Lanes M = 100 bp ladder (Promega), Lanes A = Beginning of the MLF follow, Lanes B = Beginning of malic acid degradation, Lanes C = Middle of the MLF (acid malic = 1 g/L), Lanes D = end of MLF before sulphur dioxide addition, Lanes E = *S. cerevisiae* species positive control, Lane F = *B. bruxellensis* species positive control and Lane G = negative control.

Gel de PCR-ITS réalisées sur les ADN extraits des biomasses totales collectées sur les boîtes de levures totales lors de expérimentations menées au chai
M. M = marqueur de 100 pb, A= début du suivi, B= début de la consommation de l'acide malique, C= milieu de la fermentation malolactique (acide malique = 1 g/L), D= fin de la fermentation malolactique avant le sulfitage post-fermentaire, E= *S. cerevisiae* contrôle positif, F= *B. bruxellensis* contrôle positif et G= contrôle négatif de PCR.

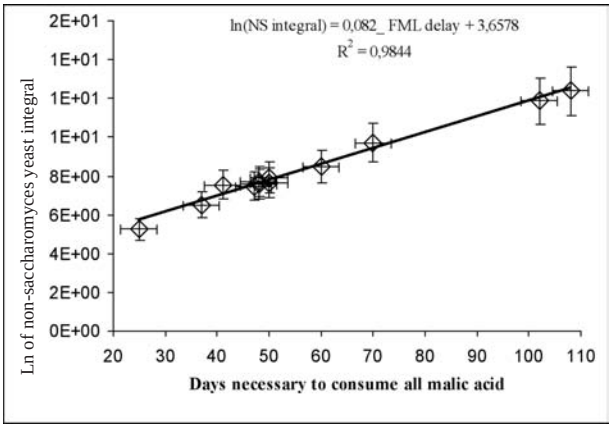

Figure 9 - Correlation between the integral of the NS population and the delay necessary to complete MLF.

Corrélation entre l'intégrale de la population de levures non-*Saccharomyces* et le temps nécessaire à l'achèvement de la fermentation malolactique.

The calculation of the integral of *B. bruxellensis* population during the time necessary to achieve MLF showed an opposite relation between the sum of *B. bruxellensis* and the MLF rate. Sluggish MLF promoted the *B. bruxellensis* development. This phenomenon was always observed in these experiments. Then MLF should modify wine properties favouring *B. bruxellensis* growth when wines are not yet protected by SO_2. Therefore, we suggest that any factor that inhibits LAB growth or survival and delays the MLF, as a consequence may favor *B. bruxellensis* growth. Among these factors, the alcohol content is important and *B. bruxellensis* ethanol tolerance is particularly remarkable. MEDAWAR *et al*. (2003) show that *B. bruxellensis* can grow in synthetic medium containing up to 12 % (v/v) of ethanol, whereas for CAPUCHO and SAN ROMANO (1994), 4 % (v/v) has been reported to reduce the growth rate of LAB. The higher the alcohol content was, the higher the risk of MLF starter culture failure and unsuccessful MLF was. Another factor which may explain difference in MLF duration is the acetaldehyde-bound SO_2 degradation by LAB species. Acetaldehyde is mainly formed during alcoholic fermentation by the *Saccharomyces* species metabolism (LIU and PILONE, 2000). In wine, acetaldehyde bounds SO_2 and decreases free SO_2 and its antimicrobial role. During MLF the sum of free SO_2 released by *O. oeni* depends of the strain ability to degrade acetaldehyde-bound SO_2. When free SO_2 released is high, it may cause stuck and sluggish MLF, due to the very sensitive SO_2 effect on *O. oeni* (HENICK-KLING and PARK 1994; CARRETE *et al*., 2002; REGUANT *et al*., 2005), whereas *B. bruxellensis* which is more resistant to SO_2, should be less affected in its growth. The pH is also an important wine parameter that can influence *O. oeni* growth (BRITZ *et al.*, 1990). Progressive deacidification of wine during MLF may explicate the easier *B. bruxellensis* development at the end of MLF. In this study, the highest *B. bruxellensis* level was found in the wines with the highest pH. The pH effect on the ratio of active molecular sulphur H_2SO_4 and inactive sulphate ion (MACRIS and MARKAKIS, 1974) is well known (RIBÉREAU-GAYON *et al*., 2000) and the maintenance of a lower pH is important to have a higher percentage of the SO_2 free molecular form.

In fact, *B. bruxellensis* seemed be characterized by poor homeostatic requirement. It supports high degree of alcohol (MEDAWAR *et al*., 2003). Its nutrition requirement is low (ROSE and HARRISON, 1971; AGUILAR-USCANDA *et al.*, 2000). The total use up of glucose and fructose at the end of alcoholic fermentation is limiting (RENOUF *et al.*, 2006b). Whereas the phenolic acids inhibit the most part of wine micro-organism (WAUTERS *et al.*, 2001; CAMPOS *et al.*, 2003), *B. bruxellensis* can consume them. Its growth is stimulated as soon as they wine is aerated, but it can also perfectly survive in an anaerobic environment as it is the case in the lees (RENOUF and LONVAUD-FUNEL, 2004). All those make *B. bruxellensis* particularly resistant species against the environmental stresses whereas the other wine yeast and bacteria species are more sensitive. That should lead to the selection of *B. bruxellensis* all along the winemaking procedure.

CONCLUSION

In order to understand microbial phenomena during winemaking it is crucial to have a global approach of the system. For that, the combined use of conventional microbiological methods such as numbering and isolating on selective nutritive media, and molecular methods of species identification and physicochemical analysis allow to get a global view. These followings during winemaking revealed quantitative and qualitative dynamic microbial changes. Interactions between each population may occur and should influence further wine taste. In addition to the classical interpretation of population curve, we have suggested to calculate the integral of the population curves to estimate the sum of cells present during a considered delay.

The malolactic fermentation appeared like a key stage of the winemaking. During this step, crucial modifications appeared concerning acetic acid bacteria and yeast population more particularly concerning the growth of the spoilage yeast *B. bruxellensis*. In fact, *B. bruxellensis* growth in wine was easier after the growth of *O. oeni*, when malic acid consumption was effective. This phenomenon was observed for different cellars, grape varieties, and different oenological practices and may be explained by direct cell interactions or by changes of wine properties resulting from *O. oeni* growth and MLF. Sluggish MLF promoted *B. bruxellensis* multiplication and the population could overcome the threshold of 10^3 CFU/mL, which is sufficient for volatile phenols production. That underlines the necessity of MLF management and the interest of efficient *O. oeni* starters to prevent the growth *B. bruxellensis* and wine alteration.

Acknowledgements : The authors wish to thank F. Ardouin, J.C Berrouet, C. Chevalier, J.P. Masclef, V. Millet, E. Tourbier, A. Vauthier and K. Van Leeuwen for their winemaking assistance and for supplying wine samples?

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Manuscrit reçu le 12 juillet 2005 ; accepté pour publication, après modifications le 15 septembre 2005

