USE OF DIRECT INOCULATION MALOLACTIC STARTERS: SETTLING, EFFICIENCY AND SENSORIAL IMPACT

UTILISATION DE LEVAINS MALOLACTIQUES A ENSEMENCEMENT DIRECT : IMPLANTATION, EFFICACITÉ ET IMPACT SENSORIEL

E. GINDREAU[1, 2], H. KEIM[1], G. de REVEL[1], A. BERTRAND[1]
and Aline LONVAUD-FUNEL[1]*
1 : Faculté d'Œnologie, Unité associée INRA. Université Victor Segalen Bordeaux 2,
351, cours de la Libération, 33405 Talence, France.
2 : SARCO, ZA la Jacquotte, 11 rue Aristide Bergès, 33270 Floirac, France.

Abstract : Four direct inoculation malolactic starters were used in this study. They conducted malolactic fermentation in a merlot wine in barrels. We compared the behaviour of these strains at multiple levels. Inoculation efficiency was evaluated, and great differences were observed among the starters. Implantation controls were also carried out. They were all clearly positive. The rates of malate degradation were compared. They were all different, but MLF in the inoculated barrels always finished before the non-inoculated control. Physicochemical and sensory analysis were done. No major differences were seen concerning chemical data. However, some significant differences were observed at the sensory level. Positive implantation controls allowed to conclude that these differences originated from the starter strains used in this study.

Résumé : Quatre levains malolactiques à ensemencement direct ont été utilisés pour cette étude. Ils ont réalisé la fermentation malolactique dans un vin de merlot en barriques. Nous avons comparé le comportement de ces différentes souches à plusieurs niveaux. L'efficacité de l'inoculation a été évaluée et de fortes variations ont été observées. Certains levains ont paru moins résistants à l'inoculation que d'autres. Les barriques correspondantes ont dû être ensemencées de nouveau pour que la fermentation malolactique se déclenche. Nous avons réalisé des contrôles d'implantation en utilisant la technique d'électrophorèse en champ pulsé. Ils ont été clairement positifs dans tous les cas. Les cinétiques de dégradation de l'acide malique ont également été comparées. Elles étaient très différentes selon les levains. Dans tous les cas, pour les vins inoculés par les différents levains, la fermentation malolactique s'est terminée plus rapidement que dans la barrique de contrôle non ensemencée. Enfin, des analyses physicochimiques et sensorielles ont été conduites. Aucune différence majeure concernant les données chimiques n'a été observée entre les vins obtenus. Seule l'acidité volatile est retrouvée en concentration un peu plus faible dans les lots inoculés par rapport au témoin non ensemencé. Les teneurs en amines biogènes ont été mesurées. Leur teneur est moindre dans tous les essais inoculés, comparé au témoin non ensemencé. Au niveau sensoriel, des différences significatives ont été mises en évidences. Elles portent sur les descripteurs "fruits rouges, café et noix de coco". Les contrôles d'implantation positifs suggèrent une implication directe de la souche bactérienne dans les qualités sensorielles des vins.

Key words: malolactic starter, implantation control, aromas, biogenic amines

Mots clés: levain malolactique, contrôle d'implantation, arômes, amines biogènes

INTRODUCTION

The benefit of malolactic fermentation (MLF) for wine quality is now well established. *Œnococcus œni* is generally responsible for MLF, as it is the best adapted bacteria to the harsh conditions encountered in wine (low pH, presence of alcohol and sulfite...).

Wine-makers are more and more attentive to a good development of MLF, and the number of different commercial malolactic starters is increasing. Emergence of freeze-dried starters used for direct inoculation was a considerable progress for MLF control (NIELSEN *et al.*, 1996). Specific starters have been developed for different kinds of wine: some strains seem more adapted to conduce MLF in white wines, and others in red wines. The multiplication of different starters makes it necessary to develop sensitive methods to identify these strains, in order to compare their properties. Molecular

*Correspondance : aline.lonvaud@œnologie.u-bordeaux2.fr

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methods can be used, such as randomly amplified polymorphic DNA (RAPD) (ZAVALETA *et al.*, 1997; ZAPPAROLI *et al.*, 2000), or pulse field gel electrophoresis (PFGE) (KELLY *et al.*, 1993; TENREIRO *et al.*, 1994). The use of the latter discriminates strains used as starters from indigenous flora, and therefore can be used for implantation controls (GINDREAU *et al.*, 1997).

Besides the use of malolactic starters to manage MLF, sensory aspect may also be considered. Numerous studies pointed out organoleptic changes consecutively to MLF (for reviews, see LONVAUD-FUNEL, 1999; VERSARI *et al.*, 1999). Some studies also deal with changes according to the strain used as starter (McDANIEL *et al.*, 1987; RODRIGUEZ *et al.*, 1990; de REVEL *et al.*, 1999). Contradictory results are obtained. Significant differences can be seen between wines obtained with different starters, but other results show that these differences are never very significant, nor reproducible (LONVAUD-FUNEL, 1995). Thus when data concerning the correct implantation of the different starters are missing, this kind of results should be taken cautiously. Our objective in this work was to conduce MLF in a red wine with different strains and check the correct implantation. The behaviour of the different strains and putative sensory changes, in relation with the presence of a strain in wine, were evaluated.

MATERIALS AND METHODS

I. STARTERS

Four different starters have been used : BL1, BL2, BL3 and BL4. BL1 and BL2 are two strains undergoing a selection procedure. BL3 and BL4 are two commercial starters. All the strains are freeze-dried and used as starters for direct inoculation. According to the manufacturer instructions, depending of the strains, they were suspended in warm water (25 °C) during 15 min, and added to the wine; or they were added directly into the wine. After the inoculation, the wine was homogenised.

Table I - Physicochemical analysis of the wine before MLF

Analyse physicochimique du vin avant FML

Alcohol (vol) %	13.40
Reducing sugars g/L	1.3
Total acidity (tartaric acid) g/L	6.96
Volatile acidity (acetic acid) g/L	0.25
pH	3.59
Free SO_2 mg/L	2
Total SO_2 mg/L	18
Malic acid g/L	2.56

II. WINE-MAKING CONDITIONS

We used a Merlot wine, in the region of Lalande de Pomerol (Néac, France). Alcoholic fermentation was conduced by indigenous yeast. After post-fermentary maceration, the wine was transferred into one-year-old barrels (used once, for a red wine vinification) and inoculated. The barrels (225 L) were from a homogenous pool. For each starter, three barrels were used. One barrel was used to fill in the two others, which were analyzed. Wine analyses performed before bacterial inoculation are reported table I.

III. MICROBIOLOGICAL AND PHYSICOCHEMICAL ANALYSES

For bacterial count, wine samples were collected after homogenisation. Adequate dilutions were plated onto MRS (de MAN *et al.*, 1960) plates solidified with agar 30 g/l. Yeast growth was inhibited by addition of 100 mg/l pimaricin. Plates were incubated for 10 days in anaerobiosis.

Implantation controls were done as previously described (GINDREAU *et al.*, 1997), using pulse-field gel electrophoresis method to separate *Not*I-restricted bacterial chromosome fragments.

Traditional analysis (total acidity, volatile acidity, alcohol content, free and total SO_2, reducing sugars, color intensity, total polyphenol index) were carried out by the official methods or the usual methods recommended by the International Organization of the Vine and Wine (OIV). These analyses were carried out after MLF was completed, just after sulfite addition. Malic acid concentration was measured by the enzymatic method (Roche, Mannheim, Germany).

IV. BIOGENIC AMINE DETERMINATION

The wine was centrifuged at 10 000 g for 10 min. The supernatant fluids were filtered through a 0.45 µm Millipore HAWP membrane and analysed in duplicate for the presence of biogenic amines by reversed phase HPLC of the o-phthaldialdehyde (OPA) derivatives according to the method described by PEREIRA MONTEIRO and BERTRAND (1994). A Waters 501 liquid chromatograph (Milford, MA, USA) was used and controlled by a HP Chemstation (Hewlett-Packard, Avondable, PA, USA). All separations were performed on a Lichrochart cartridge containing a Lichrospher RP18 column (length 10 cm, diameter 5 µm; Merck, Darmstad, Germany) and a pre-column of the same type. Detection was by fluorescence using a Jasco-821-FP (Jasco, Tokyo, Japan) with a 350 nm excitation filter and a 445 nm emission filter.

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V. SENSORY EVALUATION

A panel of 21 experts (teachers and students of the Faculté d'Œnologie) participated to the sensory evaluation. Triangle tests (AFNOR NF V 09-013) were conduced in order to compare inoculated wines versus non-inoculated control. With the wines perceived as different from the control, a quantitative descriptive analysis (AFNOR NF V 09-016) was carried out, with a card of 7 descriptors. For data acquisition, the FIZZ software (Biosystems, Couternon, France) was used. The notes were interpreted using the results of a variance analysis (ANOVA) to two factors (wines, judges) carried out on each of the seven descriptors. The results of the triangle tests were analyzed by the probability theory that the number of right answers follows a binomial distribution B (n,1/3) where n is the size of the panel (n = 21). The products were regarded as differently perceived for a probability lower than 5 %.

RESULTS

Table II - Inoculation rate for the different starters (cfu/ml).

IF is the indigenous flora control (non-inoculated).

Taux d'inoculation pour les différents levains (ufc/ml).

IF représente le témoin flore indigène (non ensemencé).

IF	20
BL1	10^3
BL2	10^3
BL3	2.3×10^5
BL4	10^6

I. INOCULATION EFFICIENCY

Thirty minutes after the inoculation, viable lactic acid bacteria population was numbered. The results are shown in table II. Indigenous flora was at a very low level (20 cfu/ml), indicating a probably long lag phase before the beginning of spontaneous MLF. BL3 and BL4 were in the range of 10^5-10^6, which is a normal value for direct-inoculation starters. BL1 and BL2 were found at only 10^3 cfu/ml, a non acceptable value for a correct inoculation. Total viable bacteria in the package were controlled and they were normal : 4.10^{11} cfu/g and 3.10^{11} cfu/g were counted for BL1 and BL2 respectively. This indicates that the viable bacteria present in the preparation were unable to survive after being stressed by the inoculation into the wine. Thus we had to inoculate for a second time the barrels containing BL1 and BL2. The new counts were 10^5 and 10^6 cfu/ml for BL1 and BL2 respectively. At this moment, indigenous flora was composed of 3.10^4 cfu/ml. This concentration was not much higher than that of BL1, suggesting a possible difficult implantation for this starter.

II. IMPLANTATION CONTROLS

At the beginning of MLF (i.e. when malic acid started to be degraded), implantation controls were done. For each barrel, we compared the pattern obtained from restriction of genomic DNA extracted from total lactic flora with that of the pure starter. The different patterns are shown in figure 1. There was a perfect identity between the 2 kinds of pattern, for each starter. The lactic microflora present in the barrels was predominantly composed of the inoculated starter in each case. Moreover, the patterns obtained in inoculated barrels were different than the pattern obtained for indigenous flora, from the non-inoculated barrel. These results indicate a perfect implantation for each starter, even

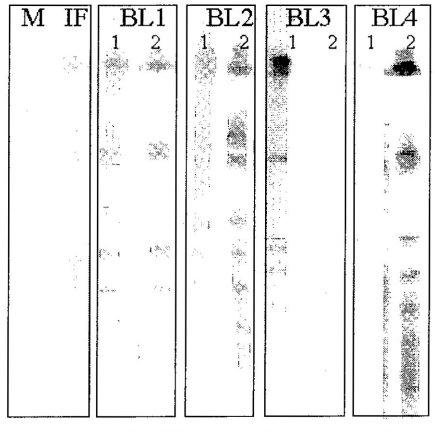


Fig. 1 - Implantation controls : PFGE restriction patterns for pure starters (lanes 1) and for flora from inoculated barrels (lanes 2).

IF: indigenous flora control. M: molecular weight marker MidRange II PFGE Biolabs.

Contrôles d'implantation : profils de restriction obtenus par PFGE des levains purs (pistes 1) et de la flore issue des barriques inoculées (pistes 2).

IF : témoin flore indigène. M : marqueur de taille MidRange II PFGE Biolabs.

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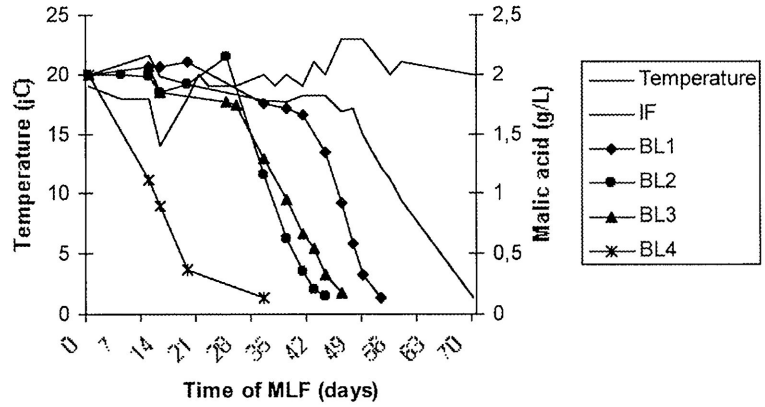


Fig. 2 - Malic acid degradation kinetics by the different starters.

IF is the non-inoculated control.

Cinétiques de dégradation de l'acide malique par les différents levains.

IF représente le témoin non-inoculé.

Table III - Wine physicochemical analysis after MLF.

CI: color intensity, TPI: total polyphenol index, IF: indigenous flora control

Analyse physicochimique des vins après FML.

CI : intensité colorante, TPI : indice des polyphénols totaux, IF : témoin flore indigène

	Alcohol % vol	Total acidity (tartaric acid g/L)	Volatile acidity (acetic acid g/L)	pH	CI	TPI
IF	13.40	4.65	0.45	3.82	1.31	63.3
BL1	13.40	4.73	0.42	3.85	1.22	63.0
BL2	13.40	4.65	0.39	3.81	1.26	63.1
BL3	13.40	4.80	0.35	3.80	1.25	63.2
BL4	13.40	4.65	0.39	3.82	1.37	64.3

III. MALOLACTIC EFFICIENCY

Malic acid degradation was measured all along the experiment (figure 2). A strong heterogeneity among the strains is observed. The most efficient strain was BL4, which completed MLF in 33 days. It ended 11 days before BL2, 13 days before BL3 and 21 days before BL1. Indigenous flora necessitated 71 days to degrade all malic acid. It should be noted that even if some strains are less efficient than others, all starters completed MLF before the non-inoculated control, confirming that a malolactic inoculation is of major interest. Temperature was also controlled all along the experiment. The corresponding curve in figure 2 shows that a quick decrease (from 18 to 14°C) occurred between days 11 and 13. At this date, MLF had started only with BL4, and this temperature change did not affect MLF conducted by this starter. However, it is possible that this breakdown could have delayed MLF conducted by other strains, particularly for BL3, which was at a cell concentration sufficient to initiate the MLF process at this moment. BL1 and BL2 were at an insufficient concentration at this moment, and a re-inoculation was necessary to undergo MLF.

IV. INFLUENCE ON WINE QUALITY

Chemical analysis were done after MLF ended with the different starters. Some results are given in table III. No significant difference was observed. Volatile acidity was a little lower in trials using malolatic starters compared to the indigenous flora. This difference may be explained by the time needed to complete MLF. In the non-inoculated barrels, bacterial population stayed longer in the wine, whereas the wine was stabilised earlier in the inoculated barrels.

At the end of MLF, biogenic amines concentration was measured. We also measured this concentration in a barrel in which MLF had been inhibited by addition of SO_2. Histamine and tyramine were detected at a low concentration, and no difference could be found according the trials ((table IV). Only the concentration of putrescine (diaminobutane) showed differences. It was higher in the wines which underwent MLF than in the wine that did not. Moreover, all inoculated wines contained less putrescine than the indigenous flora control. The concentration increased twofold in the indigenous flora trial compared to the absence of MLF. The concentration found in the BL1 trial was the highest

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Table IV - Biogenic amines concentration in wines

No MLF : control without MLF, IF : indigenous flora control.

Concentration des amines biogènes dans les vins

No MLF : témoin sans FML, IF : témoin flore indigène.

	Histamine (mg/L)	Tyramine (mg/L)	Putrescine (mg/L)
No MLF	0.1	0.2	7.1
IF	0.2	0.2	15.5
BL1	0.1	0.2	11.5
BL2	0.1	0.2	8
BL3	0.1	0.2	8.4
BL4	0.1	0.2	6.5

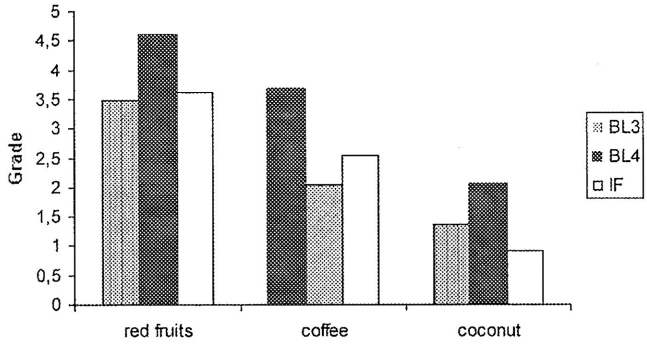


Fig. 3- Intensity of descriptors that significantly distinguish the wines

Intensité des descripteurs permettant une distinction significative des vins

Table V - Triangle tests results

IF : indigenous flora control. The difference is significant when probability is less than 0.05.

Résultats des tests triangulaires

IF : témoin flore indigène. La différence est significative si la probabilité est inférieure à 0.05.

Tests	Total responses	Correct responses	Probability level
IF / BL1	21	8	0.3992
IF / BL2	21	8	0.3992
IF / BL3	21	12	0.0212
IF / BL4	21	11	0.0557

for inoculated barrels, but lower than for spontaneous MLF. This increase in biogenic amine concentration can be correlated with the kinetics of malate degradation : BL1 was the less efficient strain. In these conditions, we can not exclude that indigenous flora could have emerged in the final step of MLF, and participated in biogenic amines formation.

Sensorial impact of the different strains was evaluated. A panel of 21 tasters underwent triangular tests in order to differentiate each wine from non-inoculated wine. Results are shown in table V. Only the wine inoculated by BL3 strain was significantly perceived as different from the non-inoculated wine. Eleven tasters felt a difference between wine inoculated by BL4 and non-inoculated wine. Signification threshold is 0.0557, just higher than the threshold of 5 %, thus this wine was also retained for subsequent analysis. Only 8 tasters perceived a difference between BL1 and BL2-inoculated wines from the non-inoculated. So, the wines perceived as the most different were those which underwent MLF in a efficient way, with no re-inoculation necessary.

For the three retained wines (BL3, BL4 and non-inoculated), sensorial patterns were established defined during pre-tastings and triangular tests. The descriptors are red fruits, vegetal, coffee, spiced, wood, coconut and butter. Among them, only red fruits, coffee and coconut allowed to significantly distinguish the wines (fig. 3). BL4 starter gave the highest ranked wine for red fruits and coconut descriptors, but for which the coffee descriptor was not strongly perceived. This descriptor was the most strongly felt in the wine inoculated by BL3. A Duncan test showed the red fruits and coffee descriptors significantly characterized wines obtained with BL4 and BL3 respectively. However the coconut descriptor only allowed to make a distinction between wines obtained with the indigenous flora and BL4.

DISCUSSION

Studies concerning strain involvement in wine modifications during MLF are of major interest. Numerous results concerning this aspect are presented in professional journals. Starters are compared on kinetics and sensorial aspects. Data concerning implantation are often lacking, even if they are a prerequisite for all subsequent analysis. Differences brought by the different strains are sometimes expected as weak, and can be easily influenced by the participation of indigenous flora to the process of MLF. Thus, rigorously conducted implantation controls allow to minimize the possible artefacts. In our study, implantation controls are unambiguous, and we considered that all differences seen between starters were strain dependent. Restriction patterns revealed during implantation controls were all different, indicating that different strains composed the starters used in this experiment. Thus, difference between trials were not totally unexpected.

Concerning inoculation efficiency, different behaviours were observed. Two strains (BL3 and BL4) were resistant to the inoculation and were numbered in a range compatible with a rapid start of MLF, whereas two other strains did not survive after the inoculation stress. They had to be inoculated a second time. This failure was not due to a low viable cell concentration

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in the preparation: it was measured in the order of 10^{11} cfu/g which is a correct concentration for a direct inoculation. Whether the strains were unable to grow in wine or were incorrectly prepared (default in pre-adaptation during the manufactory process) remains to be shown. The wine used in this experiment was not particularly harsh, as other starters succeeded to complete MLF.

The delay necessary to complete MLF differed significantly between starters. However, inoculated wines always completed MLF before the non-inoculated control, confirming the benefit of malolactic starters to manage MLF. Acidity and colour of wines obtained with the different starters were not significantly different. Results show that the quicker the MLF was, the lower the volatile acidity was. This is understandable as the wines which underwent MLF rapidly were stabilized (sulfite added) earlier, thus inhibiting other bacterial metabolisms responsible for volatile acidity production. However, the citric acid degradation measurement did not support this observation, as we found that the strain that the most degraded citric acid was BL2 and the strain BL1 left the highest concentration of citrate in the wine. Thus, in this experiment, whether other metabolisms were involved in the volatile acidity is possible.

On a sensorial aspect, observed differences were weak. Using a 21 tasters panel, only one wine (BL3 inoculated) was significantly perceived as different from the non-inoculated control. These slight differences confirm that unambiguous implantation controls are obligatory to propose an involvement of a given bacterial strain in sensorial modifications. Using sensorial patterns, red fruits, coconut and coffee descriptors were retained as differently perceived. This result shows that different sensorial characters can be brought by distinct bacterial strains. Whether the same character is specific for one particular strain remains to be shown. We have to use different kinds of wine (grape variety, label...) in order to confirm this.

CONCLUSION

This study was conducted in order to compare different malolactic starters on fermentation efficiency and on sensorial impact levels. Results showed that strains possess different behaviours when inoculated : some are more sensitive to the inoculation stress than others. The more resistant strains were also the more efficient to conduce a rapid MLF. These wines contained less volatile acidity than the wines in which MLF was slower. Moreover, the most different wines on a sensorial aspect were those inoculated by the most efficient strains.

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REFERENCES

AFNOR, 1995. *Contrôle de la qualité des produits alimentaires: analyse sensorielle.* 5e éd. Ed. AFNOR, Paris.

DE MAN J.C., ROGOSA M and SHARPE M.E., 1960. A medium for the cultivation of *Lactobacilli. J. Appl. Bacteriol.*, **23**, 130-135.

DE REVEL G., MARTIN N., PRIPIS-NICOLAU L., LONVAUD-FUNEL A., and BERTRAND A., 1999. Contribution to the knowledge of Malolactic fermentation influence on wine aroma. *J. Agric. Food. Chem.*, **47**, 4003-4008.

GINDREAU E., JOYEUX A., DE REVEL G., CLAISSE O. and LONVAUD-FUNEL A., 1997. Évaluation de l'établissement des levains malolactiques au sein de la microflore bactérienne indigène. *J. Int. Sci. Vigne Vin*, **31**, 197-202.

KELLY W.J., HUANG C.M. and ASMUNDSON R.V., 1993. Comparison of *Leuconostoc œnos* strains by pulse-field gel electrophoresis. *Appl. Environ. Microbiol.*, **59**, 3969-3972.

LONVAUD-FUNEL A., 1995. Microbiology of the malolactic fermentation: Molecular aspects. *FEMS Microbiol. Lett.*, **126**, 209-214.

LONVAUD-FUNEL A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie Van Leeuwenhoek*, **76**, 317-331.

MCDANIEL M., HENDERSON L.A., WATSON B.T. and HEATHERBELL D., 1987. Sensory panel training and screening for descriptive analysis of the aroma of pinot noir wine fermented by several strains of malolactic bacteria. *J. Sens. Studies.*, **2**, 149-167.

NIELSEN J.C., PRAHL C. and LONVAUD-FUNEL A., 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc œnos* cultures. *Am. J. Enol. Vitic.*, **47**, 42-48.

PEREIRA MONTEIRO M.J. and BERTRAND A., 1994. Validation d'une méthode de dosage. Application à l'analyse des amines biogènes du vin. *Bull. OIV*, **765-766**, 917-962.

RODRIGUEZ S.B., AMBERG E., THORNTON R.J. and MCLELLAN M.R., 1990. Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of *Leuconostoc œnos*. *J. Appl. Bacteriol.*, **68**, 139-144.

TENREIRO R., SANTOS M.A., PAVEIA H. and VIEIRA G., 1994. Inter-strain relationship among wine *leuconostocs* and their divergence from other *Leuconostoc* species, as revealed by low frequency restriction frag-

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ment analysis of genomic DNA. *J. Appl. Bacteriol.*, **77**, 271-280.

VERSARI A., PARPINELLO G.P. and CATTANEO M., 1999. *Leuconostoc œnos* and malolactic fermentation in wine: a review. *J. Indust. Microbiol. Biotechnol.*, **23**, 447-455.

ZAPAROLI G., REGUANT C., BORDONS A., TORRIANI S. and DELLAGLIO F., 2000. Genomic DNA fingerprinting of *Œnococcus œni* strains by pulse-field gel electrophoresis and randomly amplified polymorphic DNA-PCR. *Curr. Microbiol.*, **40**, 351-355.

ZAVALETA A.I., MARTINEZ-MURCIA A.J. and RODRIGUEZ-VALERA F., 1997. Intraspecific genetic diversity of *Œnococcus œni* as derived from DNA fingerprinting and sequence analysis. *Appl. Environ. Microbiol.*, **63**, 1261-1267.

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