J. Microbiol. Biotechnol. (2009), **19**(9), 1005–1012
doi: 10.4014/jmb.0811.602
First published online 26 March 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Evaluation of Lysozyme to Control Vinification Process and Histamine Production in Rioja Wines

López, Isabel[1]*, Pilar Santamaría[1], Carmen Tenorio[1], Patrocinio Garijo[1], Ana Rosa Gutiérrez[2], and Rosa López[1]

ICVV, Instituto de Ciencias de la Vid y el Vino ([1]Servicio de Investigación y Desarrollo Tecnológico Agroalimentario del Gobierno de La Rioja, [2]Universidad de La Rioja and CSIC). C/ Madre de Dios 51, 26006 Logroño (La Rioja), Spain

Received: November 3, 2008 / Revised: February 18, 2009 / Accepted: February 28, 2009

Lysozyme and the reduction of metabisulfite addition to red wine were evaluated during a winemaking process and after malolactic fermentation (MLF). Treatment, with lysozyme, of the must from Tempranillo grapes and at the end of alcoholic fermentation (AF) caused the 100% implantation of the inoculated bacterial strain and shortened the duration of MLF by 7 days. At the end of the MLF, wines treated with lysozyme showed lower volatile acidity, color intensity, and biogenic amine content. The differences in color intensity disappeared during wine stabilization. The lysozyme addition after MLF led to lower histamine concentrations in wines. These phenomena occurred irrespective of the lactic acid bacteria (LAB) proliferation control and of the *Oenococcus oeni* dominant strain identified at this period. The results of this study show the significance of preventive use of lysozyme in vinification of red wine to maintain low histamine levels and ensure a successful implantation of inoculated *O. oeni* starters.

Keywords: Histamine, lactic acid bacteria, lysozyme, malolactic fermentation, wine

Sulfur dioxide is commonly used to control spoilage bacteria in wines, but may cause some unwanted effects, such as negative sensory characteristics, and allergic reactions in sensitive people, or can be a source of hydrogen sulfite formation. The antimicrobial activity of SO_2 decreases as wine pH becomes higher, making it more difficult to microbiologically stabilize wines with low acidity. Other antimicrobials such as sorbic acid and dimethyl carbonate are active against yeasts but have limited activity against bacteria [9, 10]. Nisin is a bacteriocin that may be an alternative [5, 45], but at present its addition to wine is not approved.

Lysozyme is an enzyme isolated from hen egg albumen [26] and its lytic activity against most lactic acid bacteria (LAB) associated with wine has recently been found to be useful in controlling bacterial activity in wines [8, 16, 38]. However, it does not substitute the antioxidant properties of sulfur dioxide [11], and although it is highly active against Gram-positive bacteria, it has little activity against Gram-negative bacteria and is inactive against the eukaryotic cell wall [36]. Moreover, the bacteriolytic action of lysozyme in wines is affected by the polyphenolic components, which may bind to lysozyme [12]. It has been observed that lysozyme is more active in white wines than in red ones, which is attributed to the difference in polyphenolic content [2].

Lysozyme has several applications, including delaying malolactic fermentation (MLF) in red wines, allowing longer macerations and micro-oxygenation to improve color stabilization and polyphenolic extraction [20–22, 27, 40]. The lysozyme addition can also prevent the increase of volatile acidity during stuck or sluggish alcoholic fermentation (AF) and delay or inhibit MLF in white wines [16, 17, 19].

In recent years, wine industries have moved towards using pure starter cultures of selected LAB to promote a reliable and rapid malic acid bioconversion, and thus ensuring a better control and predictability of the reaction [47]. Commercial strains directly inoculated into wine improved significantly the control of MLF [37], but in many cases, the implantation of the cultures did not always occur and the indigenous microbiota carried out MLF [4, 31, 33] with the risk of volatile acidity, biogenic amine production, and unnecessary costs. In this context, addition of lysozyme can decrease endogenous LAB populations and favor LAB inoculated implantation by avoiding LAB competition.

*Corresponding author
Phone: +34-941-291383; Fax: +34-941-291392;
E-mail: isabel.lopez@larioja.org

*Servicio de Investigación y Desarrollo Tecnológico Agroalimentario. Ctra. de Mendavia-Logroño (NA 134, km. 88), 26071 Logroño (La Rioja), Spain

Moreover, stabilization after winemaking is one of the problems that worry winemakers the most. Once the wine is ready to age or to bottle, the enologist has to find a way to maintain the quality of the final product. Wine stabilization after AF or MLF is usually carried out by metabisulfite addition. The minimum free SO_2 concentration is adjusted to 30 mg/l in order to protect wine from microbial development. However, this operation can be a problem, because sometimes it is easy to exceed the total concentration of 140 mg/l SO_2 allowed on red wines by the regulation of the Rioja Appelation, which could be dangerous to health, especially in allergic people. Moreover, this addition dose not always prevent the development of LAB or the enzymatic reactions that lead to the formation of biogenic amines [6].

Studies conducted so far have been made with white grape varieties such as Chardonnay, Sauvignon Blanc, Gewürztraminer, and Riesling [2, 16, 18], and red varieties such as Pinot Noir, Syrah, and Cabernet Sauvignon [2, 16, 18, 20, 25], but not with Tempranillo; most of them have been conducted at laboratory scale and analyzed vinification or aging separately without tipification of the LAB involved in the processes. Tempranillo is the classic red grape variety of Spain and native to Rioja Appelation, which has great economic significance for this region because it represents over 75% of cultivated area. Therefore, the goal of this research was to study the use of lysozyme in vinification and during the stabilization processes of red wine elaborated from Tempranillo grapes, determining proper usage of this compound in a semi-industrial scale to reduce SO_2 concentration, improve LAB implantation, and keep histamine levels low.

Materials and Methods

Wine Elaboration

Vinifications were performed at the experimental winery of CIDA Research Centre of the Spanish northern region of La Rioja, from a vintage characterized by a special climatology (low precipitations and high average temperatures) throughout the region. Wines were elaborated from 1,500 kg c.v. Tempranillo local grapes, and after destemming and crushing operations they were placed homogeneously in six fermentation vats for AF. Three of them were sulfited with 60 mg/l SO_2 and the others were treated with 30 mg/l SO_2 and after 1 h with 150 mg/l lysozyme, following manufacturer instructions (Biostar S.A. Toledo, Spain). Table 1 shows the different treatments made during the vinification process. AF was induced with the commercial *Saccharomyces cerevisiae* strain Uvaferm VRB (Lallemand S.A.S. St. Simon, France) and musts were acidified with 1.5 g/l tartaric acid. At the end of AF, a new addition of 150 mg/l lysozyme was made in the wine previously treated with lysozyme. After 48 h, wines were racked, and 2 days later the wine without lysozyme was placed into six 50-l vats for MLF: three were not inoculated and performed MLF with the indigenous microbiota (referred to as "1A") and three were inoculated with the commercial *O. oeni* strain Uvaferm Alpha "U" (Lallemand S.A.S.) (referred to as "1B"). The wine treated with lysozyme was placed into another three 50-l vats ("2C") and inoculated with Uvaferm Alpha 96 h after the last lysozyme addition. The spontaneous MLF was not made in the wine treated with lysozyme because the fermentation under these conditions was not conducted successfully in previous experiences (unpublished data). Temperature was maintained around 22°C and MLF was followed by measuring of wine L-malic acid content using the L-malic acid Enzymatic BioAnalysis (Boehringer-93 Mannheim/R-Biopharm, Darmstadt. Germany).

Wine samples were taken from fermentation tanks for further analysis at different times: before SO_2 or/and lysozyme addition to musts, 24 h later, vigorous or tumultuous AF (density around 1,025), final AF (<2 g/l glucose+fructose), after racking, before LAB inoculation, 48 h after inoculation, and at consumption of 10%, 30%, 60%, and 80% of the initial malic acid.

Wine Stabilization

After MLF, wines were acidified with 1 g/l tartaric acid to achieve pHs around 3.60, and four conservation experiments (a, b, c, and d) were carried out in duplicate (12 l vats) from each one: a, 30 mg/l SO_2 addition; b, 15 mg/l SO_2 and 200 mg/l lysozyme; c, 15 mg/l SO_2 and 300 mg/l lysozyme; and d, 300 mg/l lysozyme addition (Table 1). Temperature was maintained at 16°C. Wine samples were taken from stabilization tanks 1 and 2 months after conservation treatments.

Bacterial Enumeration and Isolation

Ten ml wine samples were processed as described before by López *et al.* [32] with some modifications. Samples were spun at 100 ×*g* for 3 min at 4°C (Sorwall RC-5 B Refrigerated 103 Superspeed Centrifuge). Pellets containing fermentation debris were discarded

Table 1. Treatments during the vinification process.

Treatment in must	Tank	Treatment after AF	Tank	Treatment after MLF			
				30 mg/l SO_2	15 mg/l SO_2 +200 mg/l lysozyme	15 mg/l SO_2 +300 mg/l lysozyme	300 mg/l lysozyme
60 mg/l SO_2	1	No inoculation	1A	a*	b	c	d
		Bacterial inoculation	1B	a	b	c	d
30 mg/l SO_2 +150 mg/l lysozyme	2	Bacterial inoculation 150 mg/l lysozyme	2C	a	b	c	d

*For example, 1Aa: vinification without lysozyme, not inoculated, and 30 mg/l SO_2 addition after MLF.

and supernatants were spun at 1,000 ×*g* for 10 min. Pellets were collected, and after appropriate dilutions in sterile saline solution (0.9% NaCl) they were seeded in duplicates onto MRS agar (Scharlau Chemie S.A., Barcelona. Spain) plates with 200 μg of cycloheximide per ml (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.). Samples were incubated at 30°C under strict anaerobic conditions (GasPak, Oxoid Ltd., Basingstoke, England) for at least 10 days and viable counts were obtained as the number of CFU/ml. Ten colonies of each sample were taken for re-isolation. They were grown on MLO medium (Scharlau Chemie S.A.) with 3% of bacto agar (Difco, Madrid, Spain) plates, at 30°C under 98% humidity and 10% CO_2 atmosphere. Strains were stored in 20% sterile skim milk (Difco, Madrid, Spain) at −20°C.

Species Identification

Species identification was carried out by previously recommended methods [24] and by the API 50 CHL kit and APILAB Plus software using the API 50 CHL version 4.0 database (BioMérieux S.A., Marcy l'Etoile, France). *L. plantarum* and *O. oeni* species were confirmed by the species-specific PCR method described by Quere *et al.* [43] and Zapparoli *et al.* [46].

Typification of Strains

Pulsed-field gel electrophoresis (PFGE) was carried out according to the method described by Birren and Lai [3], with some modifications [32] for agarose block preparation. *O. oeni* cells from a fresh overnight culture on MLO agar were suspended in 3 ml of saline solution to a turbidity equivalent of McFarland standard NO. 1. This solution was harvested, washed with 3 ml of 50 mM EDTA (pH 8), and submitted to macrorestriction analysis with SfiI endonuclease by PFGE following the method reported by López *et al.* [32].

Analysis of Histamine

The method used to determine histamine in wines was the method reported by Crespo and Lasa [7]. A Perkin Elmer 410 chromatographic system, with a LS-4 fluorometric detector and a PE LCI-100 integrator, was used. Chromatographic separations were carried out in a Spherisorb ODS 2 column (15×0.46 cm, 3 μm particle size). *o*-Phthaldialdehyde was used in the derivatization reaction. The mobile phase composition was as follows: methanol and sodium acetate buffer (pH 6, 0.05 M) and tetrahydrofurane (99:1). The excitation and emission wavelengths were 340 nm and 420 nm, respectively. The amount of sample injected was 20 μl and a constant temperature of 45°C was maintained.

Other Analyses

Analytical parameters of wines were determined as described by the EC official methods [13]. Color intensity was calculated as the sum of OD 420 nm, 520 nm, and 620 nm; tonality as the OD 420/OD 520; and total phenols as OD 280 nm.

An informal sensory analysis was conducted, after a 2-month storage period, by seven experienced wine sensory panellists in the sensory laboratory at the CIDA Research Centre.

Statistical Analysis

All the statistical analyses were performed using software (version 11.0) from SYSTAT Inc., 2004. Significance of the differences was evaluated by the one-way ANOVA with Tukey's post hoc test. Different letters indicated significance at the $p \leq 0.05$ level.

Results and Discussion

Alcoholic Fermentation

The chemical composition of the initial must was as follows (before acidification): probable alcohol content 14.6% v/v; pH 3.68; total acidity 6.92 g/l (as tartaric acid); malic acid 2.49 g/l; ammonium 134 mg/l; assimilable nitrogen 263 mg/l nitrogen. The AF development was not affected by the lysozyme addition to the must. AF completion lasted for 10 days in

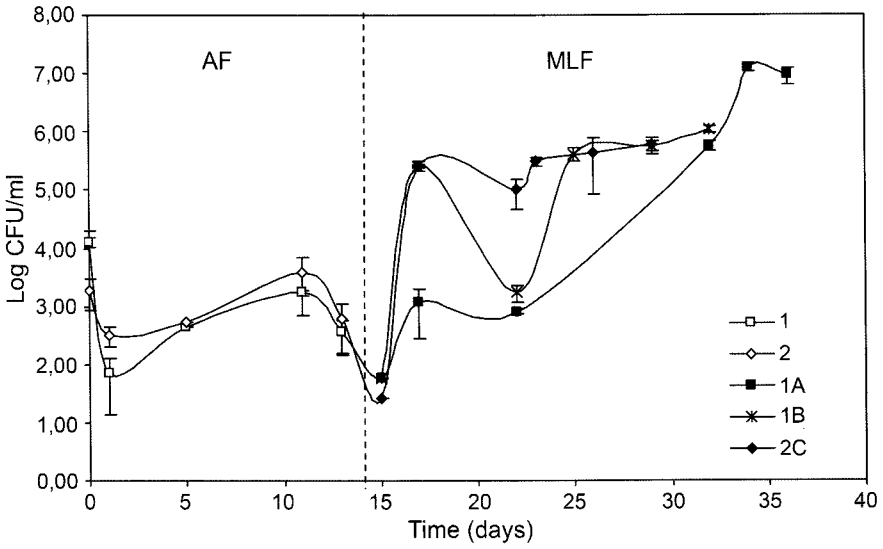

Fig. 1. Viable LAB counts during AF and MLF.
□ 1, must treated with 60 mg/l SO_2; ◇ 2, must treated with 30 mg/l SO_2+150 mg/l lysozyme. MLF: ■ 1A, spontaneous MLF; * 1B, wine inoculated with commercial *O. oeni* and not treated previously with lysozyme; ◆ 2C, wine treated with lysozyme and inoculated with commercial *O. oeni*. Vertical bars represent the standard deviation (SD) of measurements.

both 60 mg/l SO_2 and 30 mg/l SO_2+150 mg/l lysozyme treatments (data not shown). Fig. 1 shows the viable bacterial counts during AF. The initial bacterial populations decreased with the treatments applied (1, SO_2; or 2, SO_2+lysozyme), and the addition of sulfur dioxide initially appeared to be more efficient, although at the end of AF (final AF) bacterial populations became very similar. Of the total isolates obtained at final AF in the SO_2-treated fermentation tanks (1), 77.7% of them were *L. plantarum* and 22.3 % were *O. oeni* (Fig. 2). The treatment with lysozyme (2) gave 95.5% *L. plantarum* and 4.5% *O. oeni*. In this context, Delfini and Formica [8] described that strains of *L. plantarum* were more resistant to the action of lysozyme than strains of *O. oeni.*

The analytical compositions of wines before MLF are shown in Table 2. Wines treated with lysozyme presented lower levels of total and volatile acidity, color intensity, and total phenols, whereas anthocyans were higher. There were no significant differences in the histamine composition.

Similar results have been reported by other authors. Gao *et al.* [16] described that the addition of lysozyme may be a useful tool to control the growth of spoilage LAB and to reduce the production of volatile acidity. The increase in titratable acidity may be related to the rise in volatile acidity, as suggested by Aiken and Noble [1] and Pomar and Gonzalez- [42]. In our study, this increase was not very important and could be caused by the different clonal distribution of *O. oeni*. It has also been observed that the addition of lysozyme decreases the red wine color [25] and phenolic content in association with the formation of a light precipitate [2].

Malolactic Fermentation

The development of the MLF was related to the viable populations of LAB and there was a correlation between bacterial population and the decrease in malic acid. MLF of the inoculated wines lasted for 14 days (wine treated with lysozyme) to 17 days (wine untreated with lysozyme), and the spontaneous MLF lasted for 21 days (data not shown).

Fig. 1 shows the viable bacterial counts during MLF. Forty-eight hours after inoculation, bacterial populations were near 10^6 CFU/ml for both the lysozyme-treated (2C) and untreated (1B) tanks, enough population to start the degradation of malic acid [14, 44], and 10^3 CFU/ml for wines with spontaneous MLF (1A). From this moment, viable bacterial counts showed different behaviors. They were approximately constant until the end of MLF for the lysozyme-treated wines (2C); decreased at the next point and reached again the same level, for the inoculated and lysozyme untreated wines (1B); and increased until 10^7 CFU/ml for the spontaneous MLF (1A). The inoculation with *O. oeni* commercial strain "U" and treatment with lysozyme was the most efficient and the duration of MLF was shortened by 7 days when compared with control wines with spontaneous MLF.

Species identification at half-way through the MLF or tumultuous MLF (60% malic acid consumption) showed 3.3% of isolates identified as *L. plantarum* in the wine with spontaneous MLF (1A), whereas all the other isolates were identified as *O. oeni* (Fig. 2). *O. oeni* is usually the only organism isolated in tumultuous MLF and therefore the main organism responsible for MLF development [32]. In

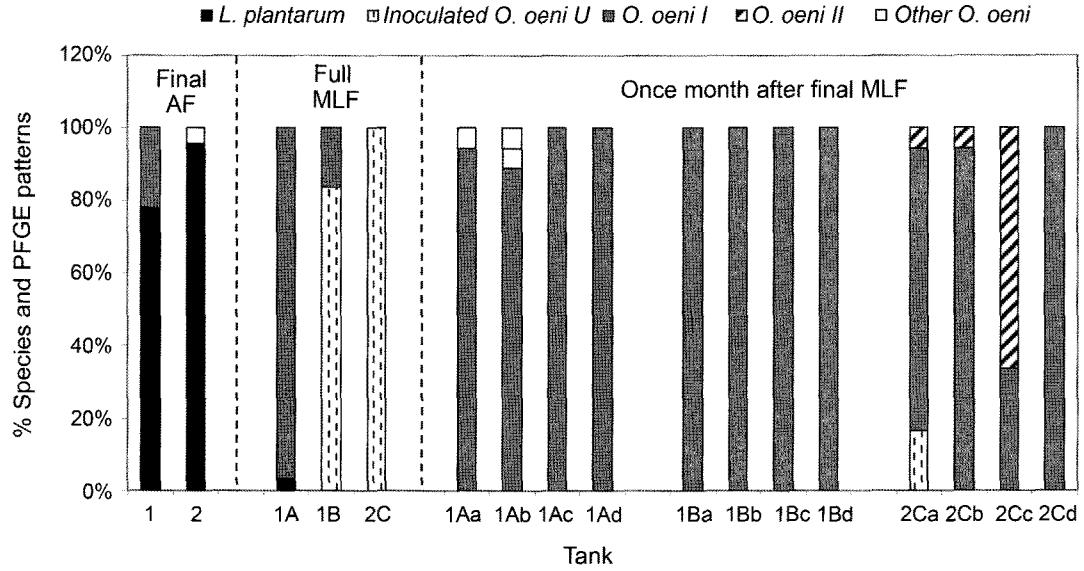

Fig. 2. Species identification and percentage of *O. oeni* PFGE patterns during winemaking.
Lanes: 1, must treated with 60 mg/l SO_2; 2, must treated with 30 mg/l SO_2+150 mg/l lysozyme; 1A, spontaneous MLF and not treated previously with lysozyme; 1B, inoculated MLF and not treated previously with lysozyme; 2C, inoculated MLF and treated with lysozyme. Additions after final MLF: a, 30 mg/l SO_2; b, 15 mg/l SO_2 and 200 mg/l lysozyme; c, 15 mg/l SO_2 and 300 mg/l lysozyme; and d, 300 mg/l lysozyme.

Table 2. Analytical composition of wines before and after MLF.

Analytical parameter	Before MLF		After MLF		
	1*	2	1A	1B	2C
Alcohol (% v/v)	14.4	14.0	-‡	-	-
pH	3.71	3.74	3.84 b	3.79 a	3.83 b
Total acidity (g/l tartaric acid)	6.97b**	6.72a	5.75	5.79	5.62
Tartaric acid (g/l)	2.05	2.09	2.11	2.21	2.16
Malic acid (g/l)	2.18	2.17	nd	nd	nd
Lactic acid (g/l)	nd†	nd	1.54 a	1.68 b	1.54 a
Volatile acidity (g/l acetic acid)	0.46b	0.40a	0.57 b	0.55 b	0.47 a
Citric acid (mg/l)	523	514	381 b	298 a	318 a
Glycerol (g/l)	10.7	10.6	11.0	10.8	10.5
Glucose+Fructose (g/l)	1.10b	0.94a	-	-	-
Color intensity (OD 420+520+620 nm)	6.84b	6.45a	7.06 c	6.69 b	6.00 a
Tonality (OD420/OD520 nm)	0.88	0.90	0.97	0.96	0.97
Anthocyans (mg/l)	204a	220b	191 a	212 b	198 ab
Total phenols (OD 280 nm)	48.6b	45.9a	48.0 b	48.2 b	42.7 a
Histamine (mg/l)	0.01	0.01	9.27c	1.57b	0.01a

*1: must treated with 60 mg/l SO_2; 2: must treated with 30 mg/l SO_2+150 mg/l lysozyme; 1A: spontaneous MLF and not treated previously with lysozyme; 1B: inoculated MLF and not treated previously with lysozyme; 2C: inoculated MLF and treated with lysozyme.
**Different letters indicate significance at the $p \leq 0.05$ level to the same vinification time. No letters means no significant differences at the $p \leq 0.05$ level.
†Not detected.
‡Not analyzed.

this study, *L. plantarum* was isolated together with *O. oeni* and survived in a spontaneous MLF of red wine.

Typification analysis of the *O. oeni* isolates showed a low clonal diversity. The isolates from tank 1A (spontaneous MLF) rendered one PFGE pattern, referred to as "I" and indistinguible to the one identified at the fermentation tank treated with SO_2 at final AF (Fig. 2). Two PFGE patterns corresponding to two clearly distinct strains appeared at tank 1B (inoculated MLF without lysozyme): pattern "I" appeared in 16.6% of the isolates and coexisted with the dominant and inoculated strain "U", suggesting that a competition between bacteria inoculated and endogenous strain occurred. In this case, without lysozyme addition, the implantation of inoculated bacterial strain was not 100%.

The inoculation with the commercial strain "U" at tank 2C (lysozyme addition) was successful and the percentage of implantation was 100% because all the PFGE patterns were identical to that of the inoculated strain (Fig. 2).

The lysozyme-treated and inoculated wine (2C) showed the lower volatile acidity, colour intensity and total phenols after MLF (Table 2). The lower histamine production was also from the wine of tank 2C (Table 2, Fig. 3), despite the fact that the pH (pH=3.83) was higher than the pH (pH=3.50) proposed by Landete *et al.* [29] as critical to the production of high quantities of biogenic amines. This result may be related to the total implantation of the inoculated bacteria. Other authors showed similar results, finding a minor biogenic amine production and volatile acidity when the implantation of the inoculated strain was 100% [16, 35, 39]. Histamine was much higher in tank 1A (spontaneous MLF) than in the other tanks, almost reaching the legal regulation in Switzerland (10 mg/l for histamine) [23]. There was high histamine production and it was not possible to detect other species different to *O. oeni* (only a low percentage of *L. plantarum*, Fig. 2) like *L. hilgardii* or *P. parvulus* whose metabolism was reported as responsible for high quantities of amines [28].

Wine Stabilization

In general, the bacterial counts remained at high levels (around 10^5 CFU/ml) 1 or 2 months after final MLF (data not shown), and they were independent of the different treatments applied. Other authors [18] have also reported high LAB populations in wines after MLF.

The lysozyme effectiveness to microbial stabilization could have been reduced by reaction with phenolic compounds present in the wine composition, as has been previously reported [2]. The dosage of the antiseptic products assayed was not enough to successfully stabilize bacterial development after MLF.

Fig. 2 shows 100% of isolates identified as *O. oeni*, 1 month after final MLF. PFGE patterns analyzed at this moment revealed that the inoculated strain "U" was not isolated, irrespective of the strain inoculation and the implantation level, except for a 16.7% in tank 2Ca. Different endogenous strains appeared as distinguible to the ones isolated at previous stages, but the initial pattern "I" was the majority in most of the analyzed wines. The clonal diversity was again very low, being this pattern the one that best adapted to the red wine characteristics as well as to the winemaking conditions. The next important strain was referred to as "II" and was isolated at three of the studied tanks (Fig. 2).

The lysozyme treatment or/and its combination with SO_2 at the stabilization process led to lower histamine

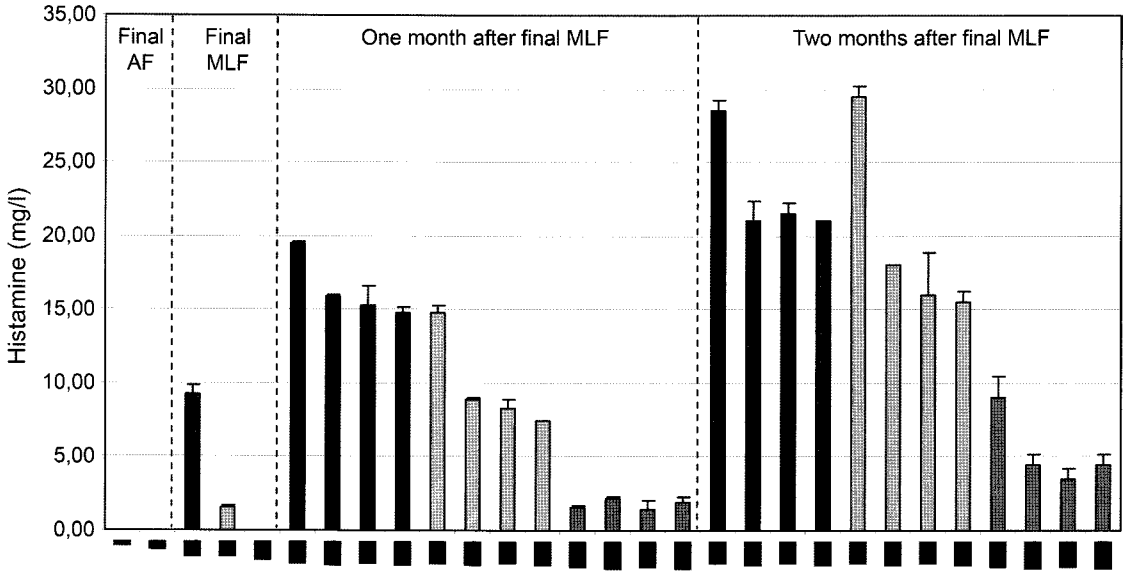

Fig. 3. Histamine content in wines during elaboration and stabilization processes.
Lanes: 1, must treated with 60 mg/l SO_2; 2, must treated with 30 mg/l SO_2+150 mg/l lysozyme; ■ 1A, spontaneous MLF and not treated previously with lysozyme; ▩ 1B, inoculated MLF and not treated previously with lysozyme; ■ 2C, inoculated MLF and treated with lysozyme. Additions after final MLF: a, 30 mg/l SO_2; b, 15 mg/l SO_2 and 200 mg/l lysozyme; c, 15 mg/l SO_2 and 300 mg/l lysozyme; and d, 300 mg/l lysozyme. Vertical bars represent the standard deviation (SD) of the measurements.

levels. Wines treated with lysozyme during the vinification step (2Ca, 2Cb, 2Cc, and 2Cd tanks) (Fig. 3) showed lower histamine concentrations 1 and 2 months after the end of MLF. However, in the case of musts treated only with SO_2 (1A and 1B tanks), the combination or treatment with lysozyme during conservation was less efficient, leading to intermediate histamine levels in wines. Tanks treated only with metabisulfite throughout vinification (1Aa and 1Ba) were the ones that showed higher histamine levels at subsequent stages. Conversely to the results reported by Marcobal *et al.* [34] and on the basis of our results, we can conclude that metabisulfite addition was not enough to prevent amine formation after MLF and during wine aging.

No correlation was found between viable LAB population and histamine production. Those tanks where we expected to find a lower bacterial growth because of their low histamine content, showed similar or higher LAB counts than the wines with a lower histamine level. The treatment with either 200 mg/l or 300 mg/l of lysozyme did not make any difference. The major "I" and "II" patterns were found isolated both in wines with high and low histamine content. Therefore, there was no correlation between the strain distribution and the production of this amine.

Gafner *et al.* [15] did not find correlations between cellular counts and biogenic amine concentration, although they estimated that 10^3 CFU/ml was enough for their production. On the contrary, Polo *et al.* [41] were able to relate the biogenic amine content with the viable LAB populations after aging for 8 months.

The results of this study suggest that lysozyme is much more efficient than metabisulfite to maintain low histamine levels in red wine, with endogenous microbiota very well adapted to develop in the pH, acidity, alcohol, and total phenol conditions of this wine. Landete *et al.* [30] studied the regulation of *hdc* expression and HDC activity by different enological factors such as pH, temperature, ethanol, sugars, malic and citric acids, SO_2, histamine, histidine, and cellular growth phase. In our study, none of these factors nor the identified strain justified the final histamine production in wines, and only lysozyme seemed to have an effect, which was not by means of decreasing the bacterial growth. The focus of further studies will be knowing if lysozyme could be playing a role in the regulation of the *hdc* gene expression or the enzyme activity.

The wine analytical composition 1 month after MLF showed significant differences in color intensity, anthocyans, and total phenol content, being the lowest in wines treated with lysozyme (data not shown). There were not significant differences in color intensity passed 2 months after MLF.

An informal assessment of the wines was carried out to determine if there was a detectable difference between the different treatments (data not shown). Variations in red wine color were not noticed by the sensory panel. Treatment with lysozyme did not cause changes either in the aroma or the palate of the red wines tested. Therefore, there was no sensorial impact of lysozyme in red wine over a 2-month storage period.

In summary, the bacteriolytic activity of lysozyme has mainly been used to control MLF, but the results of this study suggest the convenience of using lysozyme during different stages of vinification (a preventive use in musts before settling and the beginning of AF and also during the

stabilization and conservation processes, especially when the health status of the grapes favors the endogenous bacterial development) to maintain low histamine levels in wine. Its use also was very beneficial to achieve complete implantation of the commercial *O. oeni* inoculated in this study. The histamine production in these wines could not be easily explained and warrants further investigations.

Acknowledgment

This work was supported by the INIA grant VIN03-047 and FEDER of the European Community.

REFERENCES

1. Aiken, J. W. and A. C. Noble. 1984. Composition and sensory properties of Cabernet Sauvignon wine aged in French versus American oak barrels. *Vitis* **23:** 27–36.
2. Bartowsky, E. J., P. J. Costello, A. Villa, and P. A. Henschke. 2004. The chemical and sensorial effects of lysozyme addition to red and white wines over six months cellar storage. *Aust. J. Grape Wine Res.* **10:** 143–150.
3. Birren, B. and E. Lai. 1993. Preparation of DNA for pulsed field analysis, pp. 25–74. *In* B. J. Hartcourt (ed.), *Pulsed Field Gel Electrophoresis. A Practical Guide.* Academic Press, San Diego.
4. Blateyron, L., C. Bonnefond, and D. Granes. 2007. Maîtrise des fermentations malolactiques des vins rouges vinifiés en maceration traditionnelle: Etude experimentale de facteurs technologiques jouant un rôle sur la fermentescibilité des vins. Proceedings of the XXXth World Congress of Vine and Wine, Budapest, Hungary. Available at: http://www.oiv2007.hu/documents/viniculture/159_poiv07 _actes_fml.pdf. Accessed October 2008.
5. Chung, W. and R. E. W. Hancock. 2000. Action of lysozyme and nisin mixtures against lactic acid bacteria. *Int. J. Food Microbiol.* **60:** 25–32.
6. Coton, E., G. Rollan, A. Bertrand, and A. Lonvaud-Funel. 1998. Histamine producing lactic acid bacteria in wines: Early detection, frequency and distribution. *Am. J. Enol. Viticult.* **49:** 199–204.
7. Crespo, M. I. and B. V. Lasa. 1994. Determination of biogenic-amines and other amines in wine by an optimized HPLC method with polarity gradient elution. *Am. J. Enol. Viticult.* **45:** 460–463.
8. Delfini, C. and J. V. Formica. 2001. *Wine Microbiology. Science and Technology,* pp. 67, 149. Marcel Dekker, New York.
9. Delfini, C., M. Cersosimo, V. Del Prete, M. Strano, G. Gaetano, A. Pagliara, and S. Ambro. 2004. Resistance screening assay of wine lactic acid bacteria on lysozyme: Efficacy of lysozyme in unclarified grape musts. *J. Agr. Food Chem.* **52:** 1861–1866.
10. Delfini, C., P. Gaia, R. Schellino, M. Strano, A. Pagliara, and S. Ambrò. 2002. Fermentability of grape must alter inhibition with dimethyl dicarbonate (DMDC). *J. Agr. Food Chem.* **50:** 5605–5611.
11. Desert, S. and L. Hubert. 2002. Le dioxyde de soufre est-il irremplacable?. *Rev. Oenologues* **102:** 35–36.
12. Eggert, K., H. M. Rawel, M. S. P. Nikfardjam, and J. Kroll. 2006. Interactions between lysozyme and wine components. *Deut. Lebensm. Rundsch.* **102:** 472–478.
13. European Community. 1990. Community methods for the analysis of wine. Commission Regulation (ECC) No. 26/76/90 of 17 September 1990. *Off. J. Eur. Commun.* **33** (L272)**:** 1–191.
14. Feuillat, M., M. Guilloux-Benatier, and V. Gerbaux. 1985. Acceleration of the malolactic fermentation in wine. *Rev. Française d'Oenologie* **25:** 45–54.
15. Gafner, J., A. Bachmann, A. Frei, F. Hesford, N. Porret, and K. Schneider. 2006. Efectos de los contaminantes microbiológicos inducidos por la fermentación malólactica descontrolada en vino, pp. 69–74. In III Encuentro enológico. Fermentación maloláctica Fundación para la cultura del vino, Madrid.
16. Gao, Y. C., G. P. Zhang, S. Krentz, S. Darius, J. Power, and G. Lagarde. 2002. Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. *Aust. J. Grape Wine Res.* **8:** 76–83.
17. Gerbaux, V. and C. Monamy. 2000. Les amines biogènes dans les vins de Bourgogne. 1er partie: Teneurs, origine et maîtrise dans les vins. *Rev. Française d'Oenologie* **183:** 25–28.
18. Gerbaux, V., A. Villa, C. Monamy, and A. Bertrand. 1997. Use of lysozyme to inhibit malolactic fermentation and to stabilize wine after malolactic fermentation. *Am. J. Enol. Viticult.* **48:** 49–54.
19. Gerbaux, V., E. Meistermann, Ph. Cottereau, C. Barriere, C. Cuinier, J. L. Berger, and A. Villa. 1999. Use of lysozyme in enology. *Bulletin de l'OIV* **72:** 348–343.
20. Gerland, C. 2006. Optimisation de la micro-oxygénation grâce au lysozyme. *Rev. Oenologues* **120:** 45–47.
21. Gerland, C. and N. Sieczkowski. 2004. Lysozyme: Nouvelles acquisitions pour une utilization mieux maîtrisée. *Rev. Oenologues* **113:** 14–16.
22. Gerland, C., V. Gerbaux, and A. Villa. 1999. Le lysozyme: Nouvel outil biotechnologique pour maitriser les bactéries lactiques. *Rev. Oenologues* **93:** 44–46.
23. Grossmann, M., I. Smit, O. Loehnertz, and A. Ansorge. 2007. Biogenic amines and grapes: Effect of microbes and fining agents. *In* Proceedings of the International Symposium Microbiology and Food Safety of Wine "Microsafetywine", Vilafranca del Penedès, Spain.
24. Holt, J., N. Krieg, P. Sneath, J. Staley, and S. Williams. 1994. *In* W. R. Hensyl (ed.), *Bergey's Manual of Deteriminative Bacteriology.* Williams and Wilkins, Baltimore.
25. Jilbert, R., J. A. Kennedy, and M. A. Daeschel. 2005. Lysozyme interaction with anthocyanin and pigmented polymer fractions from Pinot Noir wine. *Am. J. Enol. Viticult.* **56:** 303A–303A.
26. Kim, W. K. and B. H. Chung. 1999. Direct purification of lysozyme from hen egg white using high density mixed mode adsorbent. *J. Microbiol. Biotechnol.* **9:** 292–296.
27. Lagarde, G., J. Power, Y. C. Gao, E. Pilatte, M. Nygaard, and S. Krentz. 2000. Etude de l' effet du lysozyme sur différentes souches d' *Oenococcus oeni*: Applications dans la gestion de la fermentation malolactique. *Rev. Française d'Oenologie* **185:** 26–29.
28. Landete, J. M., I. Pardo, and S. Ferrer. 2006. Histamine, histidine, and growth-phase mediated regulation of the histidine decarboxylase gene in lactic acid bacteria isolated from wine. *FEMS Microbiol. Lett.* **260:** 84–90.

29. Landete, J. M., S. Ferrer, and I. Pardo. 2005. Which lactic acid bacteria are responsible for histamine production in wine? *J. Appl. Microbiol.* **99:** 580–586.
30. Landete, J. M., S. Ferrer, and I. Pardo. 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control* **18:** 1569–1574.
31. López, I., C. Tenorio, M. Zarazaga, M. Dizy, C. Torres, and F. Ruiz-Larrea. 2007. Evidence of mixed wild populations of *Oenococcus oeni* strains during wine spontaneous malolactic fermentations. *Eur. Food Res. Technol.* **226:** 215–223.
32. López, I., R. López, P. Santamaría, C. Torres, and F. Ruiz-Larrea. 2008. Performance of malolactic fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains isolated from Rioja red wines. *Vitis* **47:** 123–129.
33. López, R., P. Santamaría, P. Garijo, S. Epifanio, A. R. Gutiérrez, I. López, C. Tenorio, and F. Ruiz-Larrea. 2001. Induccion de la fermentacion malolactica en vinos tintos de Rioja con cultivos comerciales de bacterias lácticas. *In* Proceedings of the National Symposium of Winemakers, Pamplona, Spain.
34. Marcobal, A., P. J. Martin-Alvarez, M. C. Polo, R. Munoz, and M. V. Moreno-Arribas. 2006. Formation of biogenic amines throughout the industrial manufacture of red wine. *J. Food Protect.* **69:** 397–404.
35. Martin-Alvarez, P. J., A. Marcobal, C. Polo, and M. V. Moreno-Arribas. 2006. Influence of technological practices on biogenic amine contents in red wines. *Eur. Food Res. Technol.* **222:** 420–424.
36. McKenzie, H. A. and F. H. White. 1991. Lysozyme and alpha-lactalbumin: Structure, function, and interrelationships. *Adv. Protein Chem.* **41:** 173–315.
37. Nielsen, J., C. Prahl, and A. Lonvaud-Funel. 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *Am. J. Enol. Viticult.* **47:** 42–48.
38. Nygaard, M., L. Petersen, E. Pilatte, and G. Lagarde. 2002. Prophylactic use of lysozyme to control indigenous lactic acid bacteria during alcoholic fermentation. *In* Proceedings of the ASEV 53rd Annual Meeting, Portland, Oregon.
39. Olmos, P., A. Puig, E. Bartra, F. Capdevila, M. Vilavella, and S. Míguez. 2007. Control de la implantación de bacterias lácticas y formación de aminas biógenas en fermentaciones malolácticas en vinos tintos. *In* Proceedings of the International Symposium on Microbiology and Food Safety of Wine "Microsafetywine", Vilafranca del Penedès, Spain.
40. Pilatte, E., B. Cauchy-Alvin, J. C. Crachereau, and J. P. Gervais. 2005. Gestion de la micro-oxygénation et de la fermentation malolactique des vins rouges. *Rev. Oenologues* **116:** 4–8.
41. Polo, L., S. Ferrer, and I. Pardo. 2007. Efecto de la lisozima, SO_2, microoxigenación, tipo de recipiente y adición de cultivos malolácticos sobre la generación de aminas en vinos de Tempranillo, pp. 143–145. *In* M. Ramirez *et al.* (eds.), *Avances en ciencias y técnicas enológicas Transferencia de tecnología de la red GIENOL al sector vitivinícola.* Badajoz.
42. Pomar, M. and L. A. Gonzalez-Mendoza. 2001. Changes in composition and sensory quality of red wine aged in American and French oak barrels. *J. Int. Sci. Vigne Vin.* **35:** 41–48.
43. Quere, F., A. Deschamps, and M. C. Urdaci. 1997. DNA probe and PCR-specific reaction for *Lactobacillus plantarum*. *J. Appl. Microbiol.* **82:** 783–790.
44. Reguant, C. 2006. Avances y perspectivas en la selección de cultivos iniciadores para la fermentación maloláctica, pp. 9–16. In Sobre la conducción de la calidad de los vinos en la transición de la fermentación tumultuosa a la maloláctica. Cursos Rioja XXI, Gráficas Quintana, Haro.
45. Rojo-Bezares, B., Y. Sáenz, M. Zarazaga, C. Torres, and F. Ruiz-Larrea. 2007. Antimicrobial activity of nisin against *Oenococcus oeni* and other wine bacteria. *Int. J. Food Microbiol.* **116:** 32–36.
46. Zapparoli, G., S. Torriani, P. Pesente, and F. Dellaglio. 1998. Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Lett. Appl. Microbiol.* **27:** 243–246.
47. Zhang, D. and R. Lovitt. 2006. Performance assessment of malolactic fermenting bacteria *Oenococcus oeni* and *Lactobacillus brevis* in continuous culture. *Appl. Microbiol. Biotechnol.* **69:** 658–664.