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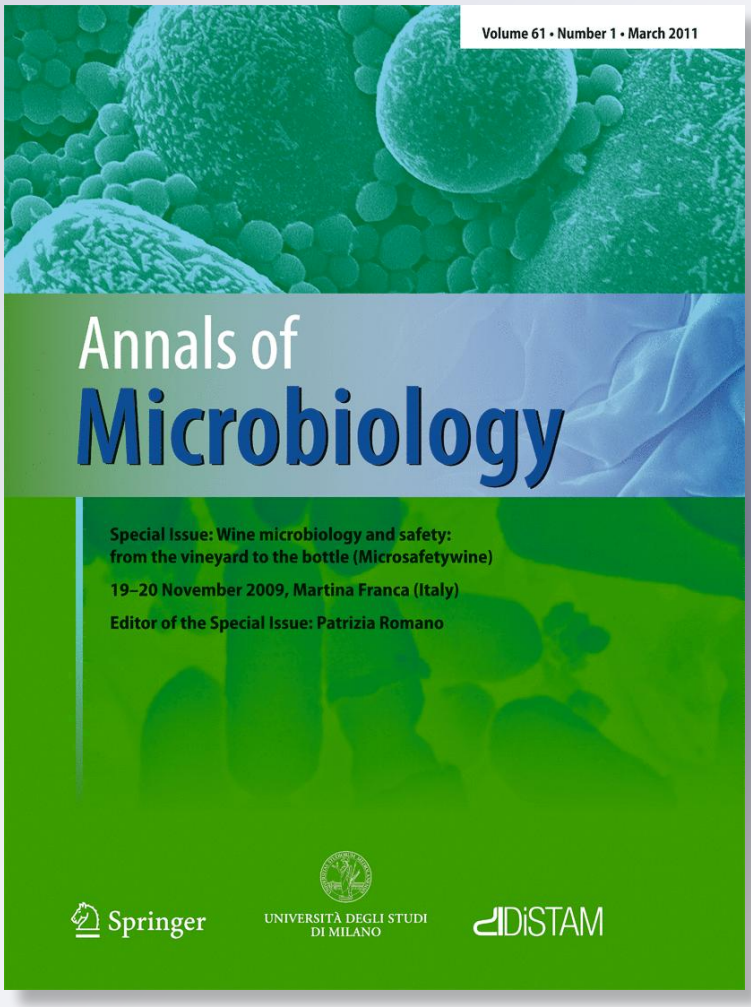

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ORIGINAL PAPERS

Ethyl carbamate content in wines with malolactic fermentation induced at different points in the vinification process

M. C. Masqué · M. Soler · B. Zaplana · R. Franquet · S. Rico · X. Elorduy · A. Puig · E. Bertran · F. Capdevila · A. T. Palacios · S. V. Romero · J. M. Heras · S. Krieger-Weber

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Abstract Ethyl carbamate (EC) is a carcinogenic compound found in fermented food and beverages such as wine. Although its carcinogenic potential in animals is known, information regarding its effects in humans remains insufficient, thus there is increasing interest in its research. EC content is higher in products with high alcohol content and in aged products. The main precursor involved in EC production in wine is urea, which is produced by metabolism of arginine by yeast, but there is also evidence that EC levels can increase after malolactic fermentation (MLF). Some lactic acid bacteria (LAB) can degrade the arginine present in must and wine via the arginine deiminase pathway, producing citrulline and carbamyl phosphate. Both compounds can react with ethanol in acidic conditions and produce EC. Our research group is studying the influence of MLF induced at different points of wine-making on the quality of the resulting wine. Among other parameters, the content of toxic compounds such as EC was evaluated. Results so far indicate that EC levels at the end of MLF were quite low (less than 3 μg/l) in all cases, i.e. below the existing legal limit (e.g. 30 μg/l in Canada). In almost all wines, EC concentrations increased after 8 months of storage as has been described by other authors. In some of the wines in which MLF was carried out by selected LAB, the increase in EC concentration was lower.

Keywords Ethyl carbamate · Malolactic fermentation · Co-inoculation · Lactic acid bacteria · Wine

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Introduction

Ethyl carbamate (EC), also known as urethane, is a known animal carcinogen (Mirvish 1968) that is detected in some wines and other alcoholic beverages in significant amounts. It increases with alcohol degree and aging of wine. The main precursor involved in EC production in wine is urea produced by metabolism of arginine by yeast (Ought et al. 1988; Monteiro et al. 1989) but there is also evidence that EC levels can increase after malolactic fermentation (MLF; Uthurry et al. 2004, 2006). Some lactic acid bacteria (LAB) can degrade the arginine present in must and wine via the arginine deiminase (ADI) pathway and produce citrulline and carbamyl phosphate (Liu et al. 1995, 1996; Arena and Manca de Nadra 2002). Both compounds can react with ethanol and produce EC. This reaction is favoured by high temperatures and acidic conditions (Ough et al. 1988).

Most *Oenococcus oeni* strains (Mira de Orduña et al. 2000), heterofermentative lactobacilli (Tonon and Lonvaud-Funel 2002) and some homofermentative LAB (Spano et al. 2004) have been described as being able to degrade arginine (Araque et al. 2009). Certain factors in the process of wine production can also help increase the amount of EC precursors, i.e. some culture practices, fermentation processes, conditions during maceration and wine storage, etc. pH is a very important factor in LAB development and metabolism and, as a result, in MLF development. In wine, a pH value lower than 3.3 limits LAB development, whereas LAB growth is favoured at pH > 3.7.

The induction of MLF by inoculation of selected LAB is already an extended practice in wineries; however, the best point at which to add LAB during the winemaking process is still a topic of discussion. Usually, LAB inoculation is carried out once alcoholic fermentation (AF) has been completed, but many researchers have been studying simultaneous inoculation of yeast and LAB. It has been demonstrated that LAB are better acclimatised to must, MLF takes place more efficiently (unless AF was faster than expected), and the wine achieves microbiological stability earlier (Bellman and Kunkee 1985; King and Beelman 1986; Krieger 1989; Franco 1992; Masqué et al.

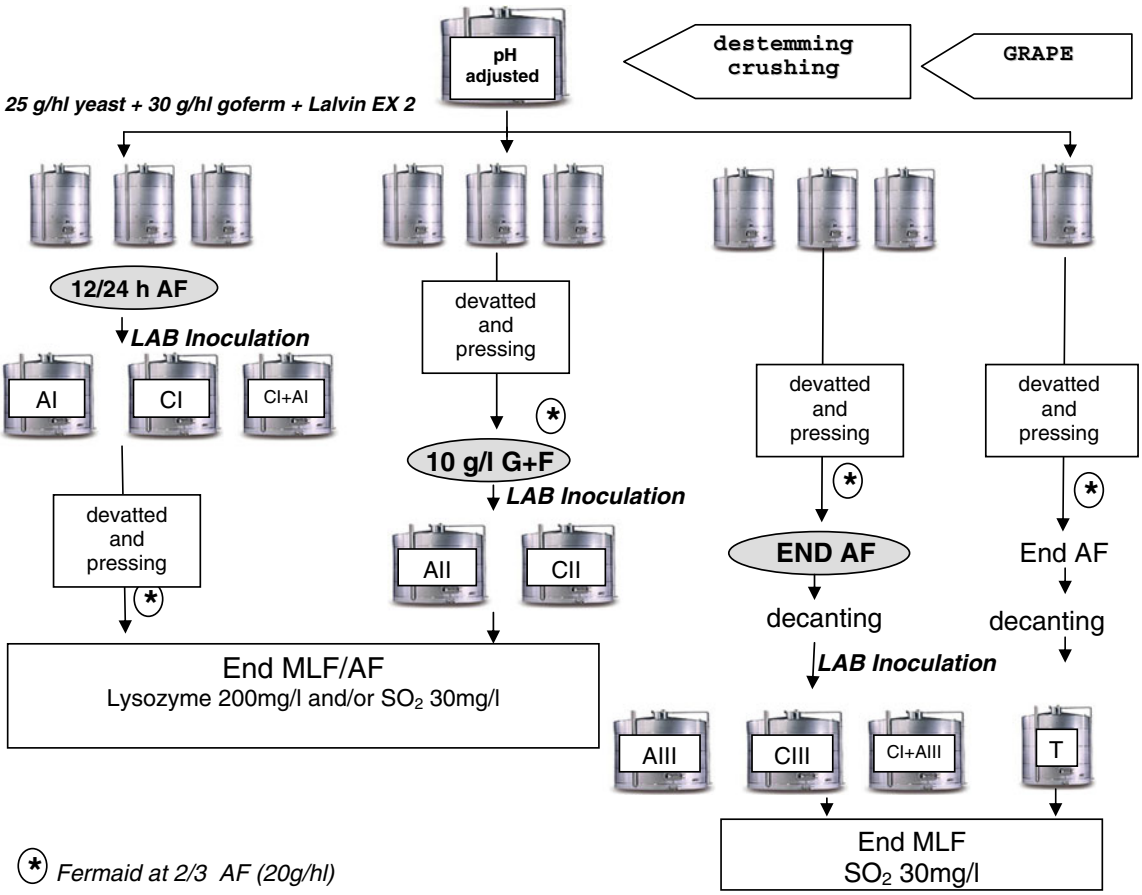

Fig. 1 Wine-making process for red wine: *AF* alcoholic fermentation, *MLF* malolactic fermentation, *LAB* lactic acid bacteria, *A* strain of *Oenococcus oeni* R1098, *C* strain *Lactobacillus plantarum* CECT 5671, *T* non-inoculated, *G+F* glucose + fructose

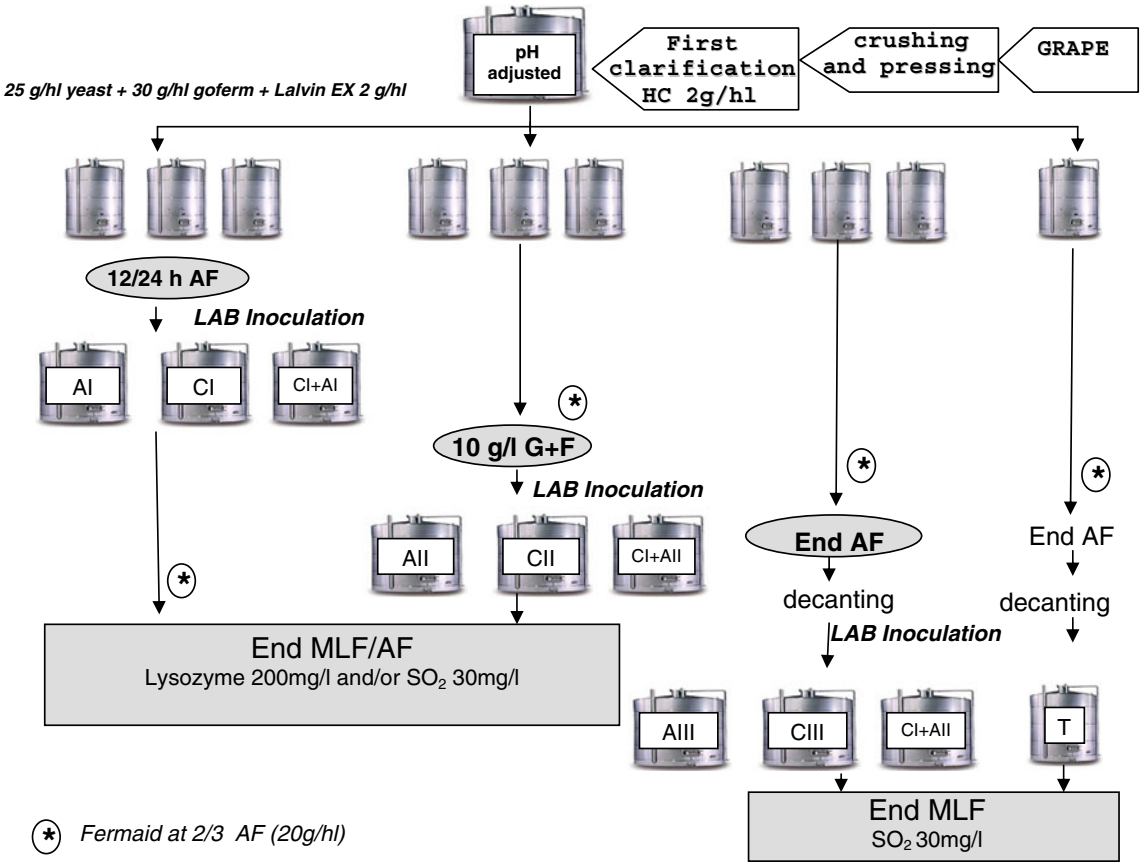

Fig. 2 Wine-making process for white wine. Definitions as in Fig. 1

1994). Recent works have observed that wines elaborated with simultaneous yeast-LAB inoculation are fruitier and keep more varietal character than wines elaborated by sequential AF and MLF (Krieger 2006; Palacios et al. 2007a, b). Our research group at INCAVI (Institut Català de la Vinya i el Vi, Reus, Spain) carried out a project aimed at studying the influence on wine quality of MLF induced at different points during the wine-making process. Among other parameters, the EC content was quantified and related to the strain inoculated and time of inoculation.

Materials and methods

Experiments were carried out with two grape varieties, one white (Chardonnay) and one red (Tempranillo). As pH is an important factor for both LAB metabolism and EC production, must pH was adjusted to two values for each variety: higher (3.7) and lower (3.3 for Chardonnay and 3.5 for Tempranillo).

We used two LAB strains: (A) *O. oeni* R1098 (Lallemand, Montreal, Canada) and (C) *Lactobacillus plantarum* CECT 5671 (Ru11) isolated from Tempranillo grape must from Tarragona (Masqué and Bordons 1996).

The winemaking process used was the traditional one used for red (Fig. 1) and white (Fig. 2) wines. Microvinifications were carried out in volumes of 100 L. MLF was induced at three different points during the wine-

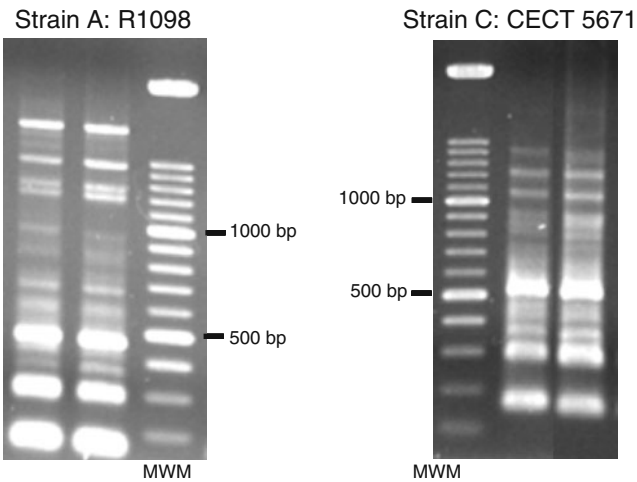

Fig. 3 Random amplified polymorphic DNA (RAPD) patterns of the inoculated LAB starters: strain A (*O. oeni* R1098) and strain C (*L. plantarum* CECT 5671) used as pattern. *MWM* Molecular weight marker 100 bp ladder

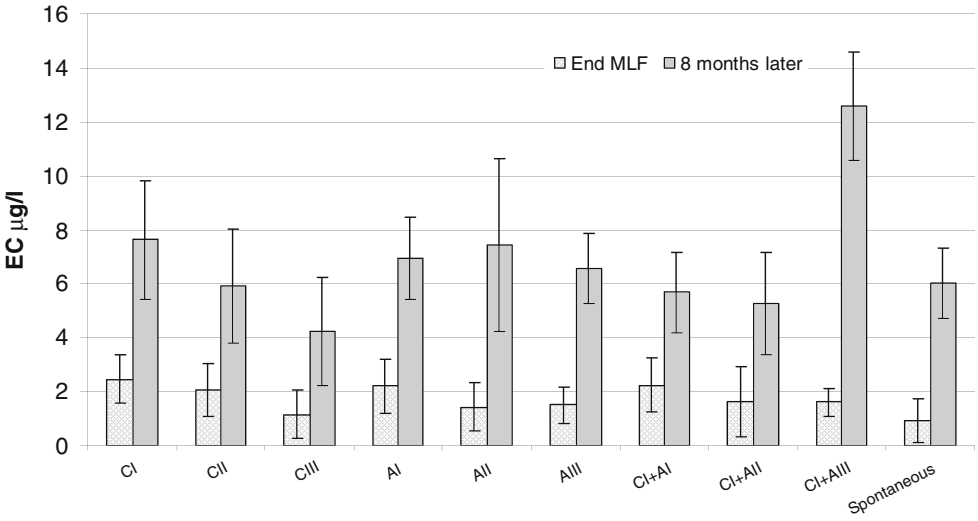

Fig. 4 Ethyl carbamate (EC) concentration in wines at the end of MLF and after 8 months of aging for Chardonnay variety at pH 3.3. Strains: C (*L. plantarum* CECT 5671), A (*O. oeni* R1098). Inoculation point: I (12/24 h beginning of AF), II (at ~10 g/l glucose+fructose), and III (after AF). Data shown are the mean of two replicates and standard deviations

making process: (I) at 12/24 h from the beginning of AF, (II) when the sugar concentration was around 10 g/l, and (III) after AF. Each strain was inoculated at three points for each variety and pH (CI, CII, CIII for strain C and AI, AII, AIII for strain A). There was a third line with simultaneous or sequential inoculation, where strain C was always inoculated at time I and strain A was inoculated at time I (CI+AI), II (CI+AII) and III (CI+AIII).

For each wine, EC was quantified at the end of MLF and after 8 months of storage. EC was analysed using gas chromatography (GC) with a selective mass spectrometer (MS). Previously, the sample was extracted by solid-phase in an Extrelut NT 20 column (Merck, Darmstadt, Germany) as described by Romero et al. 2009. Each sample was analysed in duplicate.

Implantation percentages of inoculated strains were calculated by typing using random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) with the primer M13 (5′-GAGGGTGGCGGTTCT-3′; Stenlid et al. 1994) as follows: a representative volume sample of each tank was taken when two-thirds of the initial concentration of L-malic acid had been degraded (2/3 MLF). An adequate dilution of the wine was plated on MRS medium (De Man et al. 1960) modified with the addition of 4 g/l DL-malic acid, 10 g/l fructose and supplemented with 3% tomato juice and 50 mg/l nystatin. The incubation time was 7 days at 28°C. DNA was amplified from some randomly chosen colonies that appeared on the medium without a previous step of DNA extraction. For this, a cell suspension of each colony (at least 28 colonies) was performed in 10 µl distilled water.

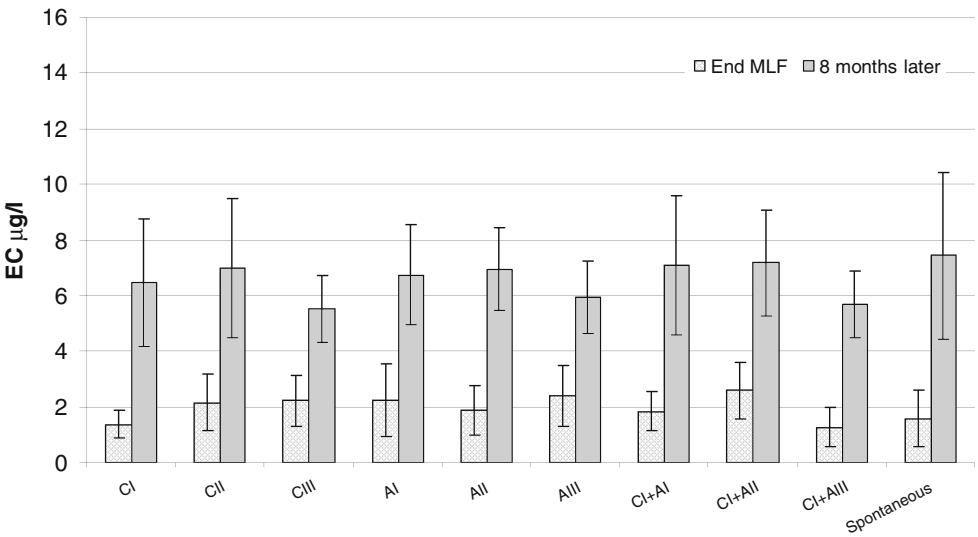

Fig. 5 EC concentration in wines at the end of MLF and after 8 months of aging for Chardonnay variety at pH 3.7. Strains: C (*L. plantarum* CECT 5671), A (*O. oeni* R1098). Inoculation point: I (12/24 h beginning of AF), II (at ~10 g/l glucose+fructose) and III (after AF). Data shown are the mean of two replicates and standard deviations

Table 1 Imposed percentages of strains at two-thirds of malolactic fermentation (2/3 MLF) in Chardonnay microvinifications at pH 3.3. Strains: *C* (*L. plantarum* CECT 5671); *A* (*O. oeni* R1098); *a*, *b*, *B*, *D*, *F*, *G* (indigenous strains). Inoculation point: *I* [12/24 h beginning of alcoholic fermentation (AF)], *II* (~10 g/l glucose+fructose), and *III* (after AF)

Microvinification	Inoculated strain	Other profiles
CI	0%	91% D+8% F + 1% (A+B)
CII	0%	1% A+99% G
CIII	4%	96% a
AI	100%	0%
AII	87%	9% a+4% b
AIII	90%	3% a+7% b
CI+AI	100% A+0% C	0%
CI+AII	100% A+0% C	0%
CI+AIII	63% A+0% C	37% G
Spontaneous		99.96% F+0,.2% G+0.02% A

Table 2 Imposed percentages of strains at 2/3 MLF in Chardonnay microvinifications at pH 3.7. Strains: *C* (*L. plantarum* CECT 5671); *A* (*O. oeni* R1098); *a*, *b*, *c* (indigenous strains). Inoculation point: *I* (12/24 h beginning of AF), *II* (~10 g/l glucose+fructose), and *III* (after AF)

Microvinification	Inoculated strain	Other profiles
CI	4%	96% A
CII	6%	88% A+6% b
CIII	5%	82% A+9% b+5% c
AI	100%	0%
AII	96%	4% a
AIII	90%	10% a
CI+AI	94% A+6 % C	0%
CI+AII	96% A+4% C	0%
CI+AIII	96% A+4% C	0%
Spontaneous		92% A+8% (a,b,c)

PCR amplification were performed in a 20 μl mixture containing 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 1×NH_4 reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8 at 25°C] and 1.5 U of *Taq* DNA polymerase (Biotaq™ DNA Polymerase, Bioline, London, UK), along with 1 μM primer M13. A preincubation step at 94°C for 5 min was carried out with a mixture of distilled water, primer M13 and 1 μl cell suspension in order to achieve denaturing of the DNA and penetration of the primer into the cells. The rest of reaction mixture was then added and the following amplification conditions were applied in an Eppendorf Mastercycler Gradient apparatus: cycles 1–15: DNA denaturation at 94°C for 30 s, annealing at 35°C for 30 s, and extension at 72°C for 1 min; cycles 16–40: denaturation at 94°C for 30 s, annealing at 35°C for 30 s, and extension at 72°C for 1 min increasing 25 s in each cycle (Tompkins et al. 1996). A final extension at 72°C for 10 min was performed. Amplification products were visualized on 1% agarose gel (GellyPhor®LE) made with Tris/boric acid/EDTA buffer and stained with ethidium bromide. The same procedure was applied to obtain RAPD patterns of the inoculated LAB starters (see Fig. 3).

Results and discussion

Chardonnay

EC concentrations are presented in Figs. 4 and 5, and Tables 1 and 2 list the percentage of strain imposition. Looking at Figs. 4 and 5, we can see that EC content at the end of MLF was lower than 3 μg/l in all wines. EC concentration is lower at pH 3.3 (Fig. 4) than at pH 3.7 (Fig. 5), probably because the growth of yeasts and bacteria

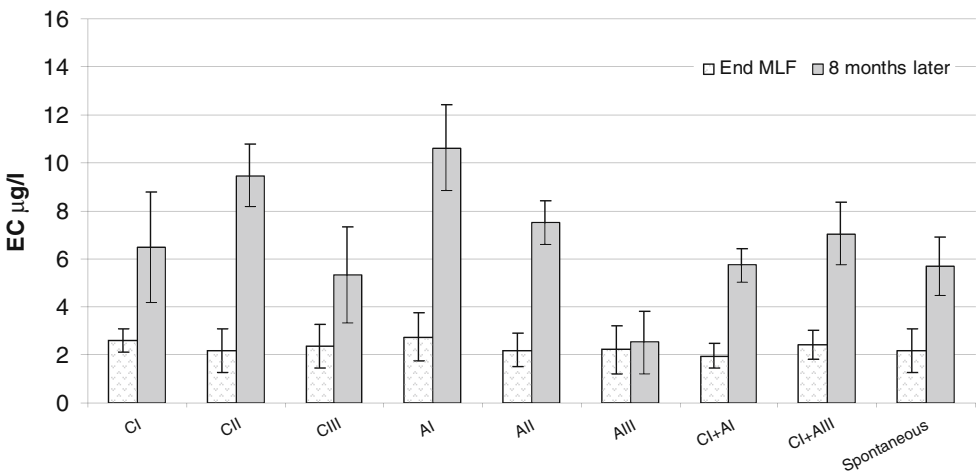

Fig. 6 EC concentration in wines at the end of MLF and after 8 months of aging for Tempranillo variety at pH 3.5. Strains: *C* (*L. plantarum* CECT 5671), *A* (*O. oeni* R1098). Inoculation point: *I* (12/24 beginning of AF), *II* (~10 g/l glucose+fructose) and **III** (after AF). Data shown are the mean of two replicates and standard deviations

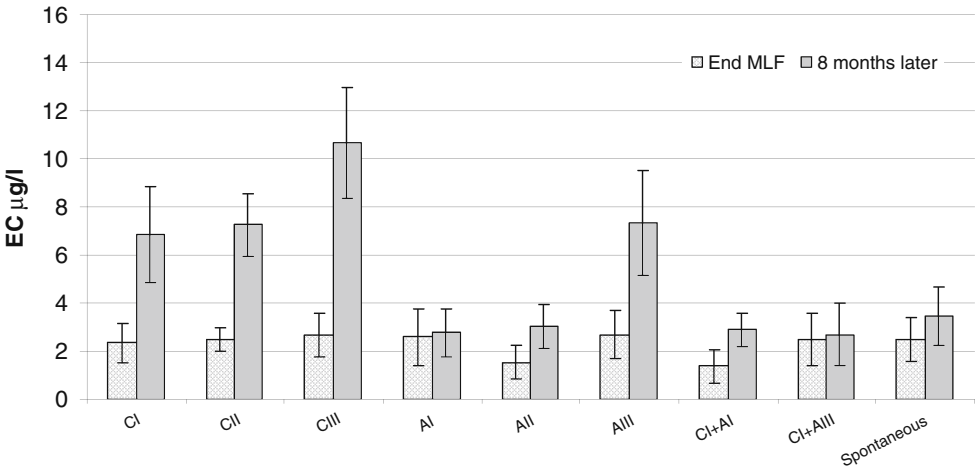

Fig. 7 EC concentration in wines at the end of MLF and after 8 months of aging for Tempranillo variety at pH 3.7. Strains: *C* (*L. plantarum* CECT 5671), *A* (*O. oeni* R1098). Inoculation point: *I* (12/24 beginning of AF), *II* (~10 g/l glucose+fructose) and *III* (after AF). Data shown are the mean of two replicates and standard deviations

is more favoured and there is more metabolic activity at pH 3.7 than at pH 3.3 and, as a result, generation of EC precursors is greater at pH 3.7 (Romero et al. 2009). For CI, AI and CI+AI microvinifications inoculated at 12/24 h from the beginning of AF at pH 3.3, EC content is a little bit higher. This effect is not observed at higher pH, where some wines inoculated in this first phase have less EC content than others (CI, CI+AI). Thus, there is no clear influence of the point of LAB inoculation on EC content.

After 8 months of storage, increased EC concentration was evident in all wines, as described by other authors (Stevens and Ough 1993; Uthurry et al. 2004). The production of EC is a slow process at normal wine storage temperatures and, as a result, EC concentration increases with time. As it occurs at the end of MLF, for wines after 8 months, there are greater differences in EC content between wines at pH 3.3 than at pH 3.7, at which all wines had similar EC concentrations. As can be seen in Table 2 at pH 3.7, strain A was implanted in almost all microvinifications, including those in which strain A had not been inoculated. Probably, during sampling, a contamination with this strain had taken place from microvinifications in which strain A had been inoculated to those in which strain A had not been inoculated, and this would have resulted in all microvinifications having similar development.

For Chardonnay microvinifications, we found no evidence of any influence on EC content in the wine of the strain carrying out MLF.

Tempranillo

Figures 6 and 7 show results of EC content and Tables 3 and 4 show percentages of strain implantation for Tempranillo microvinifications. At the end of MLF, the results obtained were similar to those with Chardonnay. EC

Table 3 Imposed percentages of strains at 2/3 MLF in Tempranillo microvinifications at pH 3.5. Strains: *C* (*L. plantarum* CECT 5671); *A* (*O. oeni* R1098); *a*, *b*, *c*, *d*, *e*, *D*, *E*, *F* (indigenous strains). Inoculation point: *I* (12/24 beginning of AF), *II* (~10 g/l glucose+fructose) and *III* (after AF)

Microvinification	Inoculated strain	Other profiles
CI	0%	32% a+36% b+14% c+18% d
CII	0%	26% a+4% b+70% c
CIII	0%	100% A
AI	100%	0%
AII	89%	11%a
AIII	97%	3% F
CI+AI	100% A+0% C	0%
CI+AIII	95% A+0% C	4% D+1% E
Spontaneous		12% a+27% b+23% c+13% d+23%e

Table 4 Imposed percentages of strains at 2/3 MLF in Tempranillo microvinifications at pH 3.7. Strains: C (*L. plantarum* CECT 5671); A (*O. oeni* R1098); *a*, *b*, *c*, *B*, *D*, *G*, *H* (indigenous strains). Inoculation point: *I* (12/24 beginning of AF), *II* (~10 g/l glucose+fructose) and *III* (after AF)

Microvinification	Inoculated strain	Other profiles
CI	0%	37% a+63% b
CII	0%	18% a+32% b+50% c
CIII	4%	96% A
AI	100%	0%
AII	96%	4% a
AIII	4%	33% D+4% B+58% H
CI+AI	88% A+4% C	4% a+4%b
CI+AIII	100% A+0% C	0%
Spontaneous		100% (A+G)

concentration was less than 3μg/l at both pHs in all wines. There were minor differences between wines, but no correlation with LAB inoculation point was found.

At pH 3.5 (Fig. 6), an increase of EC was detected after 8 months of storage in all wines. However, it is important to emphasise that in some wines obtained from microvinifications where strain A was imposed (Table 3), the increase was smaller (CIII, AIII). At pH 3.7 (Fig. 7), an increase of EC content was also detected. But, in this case, in almost all microvinifications (except CIII) in which strain A was imposed (Table 4) and where this strain carried out MLF, the increase of EC was very low (AI, AII, CI+AI, CI+AIII). In the case of spontaneous (no strain inoculated) microvinifications, the profile of strain A was detected as one of the prevailing two (Table 4), which was also one of the microvinifications with a smaller increase in EC after 8 months of storage.

Therefore, in contrast to the results we saw in Chardonnay microvinifications, some of the results obtained in Tempranillo showed a possible influence of strain imposed on EC generation, but no evidence was found of any influence on EC content of the point at which LAB inoculation takes place.

Conclusions

For the wines obtained in this work, EC levels at the end of MLF were around 3 μg/l for both varieties and pHs. In almost all wines these levels increased after 8 months of storage, but there was no wine with more than 15 μg/l. No influence of the point at which LAB inoculation takes place in EC content was found. For Tempranillo, in some wines where MLF was carried out by strain R1098 (A), the increase in EC concentration was lower. These results could indicate an influence of imposed strain on EC formation, probably because of the higher or lower activity of the ADI pathway of this strain. Therefore, in order to complete this study, quantification of citrulline and carbamyl phosphate is needed.

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