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SHORT COMMUNICATIONS

Biogenic amine synthesis in high quality Tempranillo wines. Relationship with lactic acid bacteria and vinification conditions

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Abstract Due to toxicological and economical concerns, there is considerable interest in establishing which enological practices promote biogenic amine accumulation in wines. Effects of SO_2 and lysozyme, malolactic fermentation (MLF) management and ageing have been studied. The type of bacteria performing MLF and ageing proved to be the main factors influencing biogenic amine content of wine, specifically Tempranillo from Somontano appellation (Huesca, Spain), produced at an industrial scale. Sulphur dioxide and lysozyme, at the doses used, were not sufficient to reduce lactic acid bacteria populations. Treatments to inhibit MLF were not able to prevent histamine production. No relationship was found between the type of vessel in which MLF took place and subsequent biogenic amine content.

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Introduction

Biogenic amines (BA) are low molecular weight organic bases, frequently formed in food and beverages (Silla Santos 1996; ten Brink et al. 1990). These compounds can cause severe human health problems when present in high concentrations (ten Brink et al. 1990). Twenty-four amines have been identified in wines, with putrescine being the most abundant (Vázquez-Lasa et al. 1998; Lehtonen 1996), followed by isoamylamine, histamine, tyramine and phenylethylamine in decreasing concentrations (Lehtonen 1996). Histamine, tyramine, phenylethylamine, cadaverine, tryptamine and putrescine are responsible for toxic effects, although the most dangerous is histamine (Anli and Bayram 2009). Upper limits for histamine in wine have been recommended in Germany (2 mg l^{-1}), Belgium (5–6 mg l^{-1}), and France (8 mg l^{-1}), whereas Switzerland has established a limit of 10 mg l^{-1} as a tolerable value (Landete et al. 2005b). Such limits imply severe barriers to wine-exporting countries.

Some amines such as putrescine, ethanolamine, and ethylamine are common constituents of grapes (Del Prete et al. 2009), although these and others could be produced by microorganisms from amino acids present in must or wine (Ancín-Azpilicueta et al. 2008). Only microorganisms displaying decarboxylase activity are able to produce BA from amino acids in wine.

Factors affecting amine concentration in wine include the type of grape and must characteristics, vintage, geographical location and soil composition, wine style and type, enological practices such as skin maceration

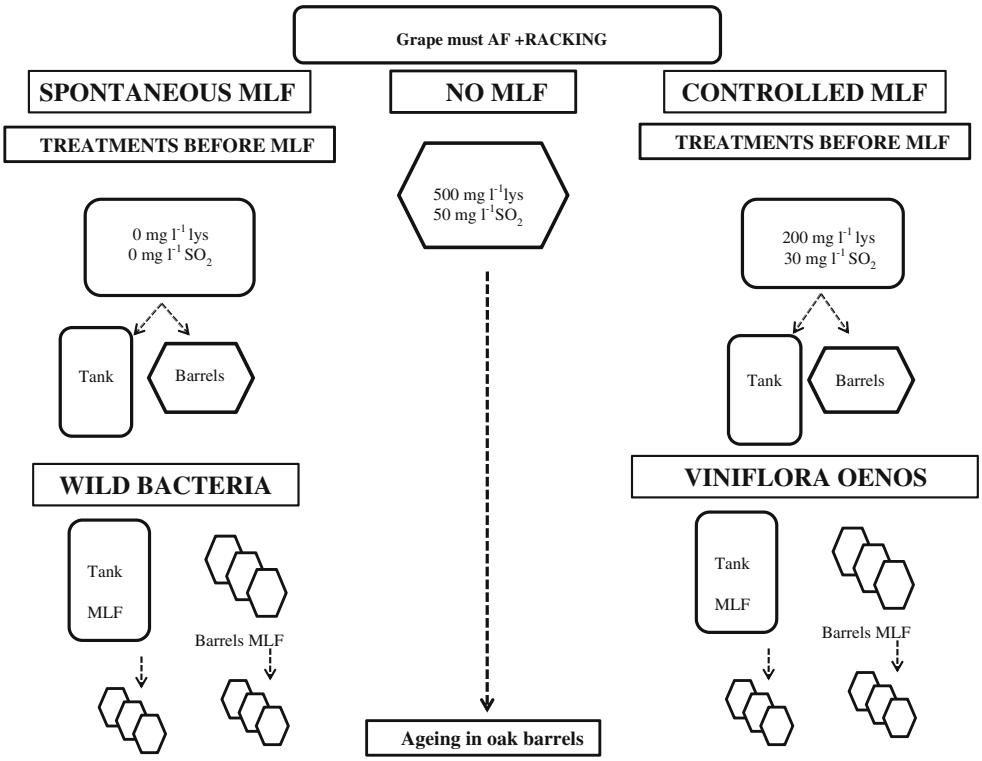

Fig. 1 Flow chart of vinification conditions. *AF* Alcoholic fermentation, *lys* lysozyme, *MLF* malolactic fermentation

duration, SO_2 doses, pectolytic enzyme addition, length of contact with lees, alcoholic and malolactic fermentation (MLF), use of commercial malolactic starters, and ageing (Anli and Bayram 2009; Ancín-Azpilicueta et al. 2008; Smit et al. 2008). Recently, Costantini et al. (2009) have pointed out that BA in wine could be caused by bacterial contamination of commercial yeast starters. They found that *Lactobacillus brevis* and a presumptive *L. rossiae* were able to produce tyramine and histamine in a synthetic medium (Costantini et al. 2009).

Our objective was to study the influence of certain winemaking conditions on lactic acid bacteria (LAB) populations and biogenic amine (BA) accumulation during industrial production of Tempranillo wines from the Somontano region in Spain. Effects of SO_2 and lysozyme doses, MLF management and ageing, have all been studied.

Materials and methods

Industrial vinifications

The flow chart of treatments applied during vinification is given in Fig. 1.

Tempranillo must (80,000 l) was added with 50 mg l^{-1} of SO_2, and inoculated at a concentration of 20 mg l^{-1} with a commercial yeast strain (UCLMS 377; Bio Springer Maisons-Alfort, France). Fermentation took place for a week. When the wine reached 13º (alcoholic degrees; 13% vol/vol) it was racked and divided and subdivided into different vessels where it was subjected to different treatments, as described in Fig. 1. Some batches of wine were placed in new 225-l French oak barrels (Seguin Moreau) and others in stainless steel tanks. Wines to be inoculated with a malolactic starter were treated with 30 mg l^{-1} SO_2 and 200 mg l^{-1} lysozyme; to avoid MLF, wines were treated with 50 mg l^{-1} SO_2 and 500 mg l^{-1} lysozyme, while wines in which spontaneous MLF was desired were not treated. Some wines underwent controlled MLF, having been inoculated with *Viniflora oenos* (Christian Hansen, Hoersholm, DK) at 5.8 mg l^{-1}, whereas in other wines, MLF was spontaneous. Once MLF had taken place, wines were treated with tartaric acid and SO_2 in order to reach a total acidity of 3.7 g l^{-1} (expressed as tartaric acid) and SO_2-free concentration of 32 mg l^{-1}. Wines that underwent MLF in stainless steel tanks were aged in oak barrels for 6 months. The same ageing time was applied to wines that underwent MLF in barrels (see Fig. 1).

Samples were taken at the end of alcoholic fermentation, 48 h after SO_2 addition, 48 h after lysozyme treatment, at the end of MLF, and 20 days, 2, 4 and 6 months after MLF.

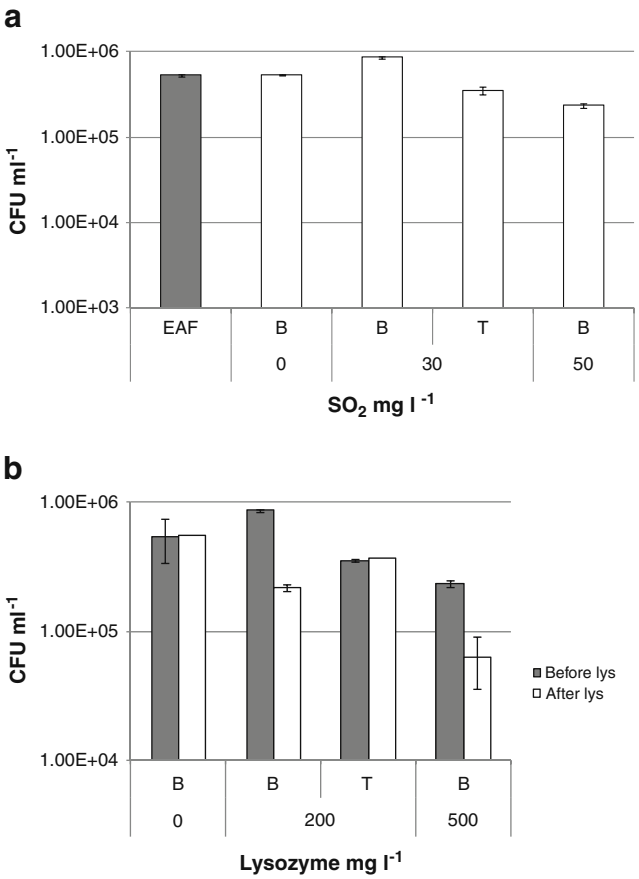

Fig. 2 Effects of SO_2, (**a**) and lysozyme additions (**b**) on total LAB populations. Values correspond to samples taken 48 h after treatment. *B* Oak barrels, *EAF* bacterial counts at the end of alcoholic fermentation, *T* stainless steel tank, *lys* lysozyme

Quantification of BA by HPLC

Cadaverine, histamine, putrescine and tyramine were quantified by HPLC, using the same equipment and chromatographic conditions described previously (Hernández-Orte et al. 2006).

LAB quantification, identification and typing

LAB populations were determined by plating samples on MRS (Scharlab, Barcelona) for lactobacilli and pediococci and MLO plates for *Oenococcus oeni* (Maicas et al. 2000) added with 0.15 mg l^{-1} of Actistab (Gist-Brocades) to avoid yeast and mould growth. Plates were incubated at 28° C for up to 7 days in anaerobic jars. The colony-forming units were counted and isolated on the same medium. Identification of isolates was performed by 16S-ARDRA analysis (Rodas et al. 2003). Molecular characterization at strain level was achieved by RAPD, using primer and amplification conditions described previously (Zapparoli et al. 2000). Similarity of RAPD profiles was analysed using the BioNumerics software version 2.5 (Applied Maths, Kortrijk, Belgium) using the Pearson's product moment correlation coefficient and the UPGMA as clustering method (Rodas et al. 2005).

Statistical analysis

Data on bacterial counts and BA during the vinifications were processed by analysis of variance (ANOVA) to test the effect of SO_2 and lysozyme on LAB populations, and the type of vat, MLF bacteria and ageing on LAB counts and BA concentrations after MLF and ageing oak (Hernández-Orte et al. 2009) The analyses were carried out using StatView (SAS Institute, Cary, NC, USA) for Windows, version 5.0.

Results and discussion

The only LAB species recovered from samples throughout the vinification process was *O. oeni*.

Influence of SO_2 and lysozyme on LAB populations

An SO_2 concentration of 30 mg l^{-1} added, after alcoholic fermentation, to wines destined for inoculation did not decrease the *O. oeni* population, but gave rise to a slight increase (Fig. 2a). There was only a slight reduction in bacterial viability when a dose of 50 mg l^{-1} SO_2 was used. This low antibacterial effect of SO_2 was not surprising: Reguant et al. (2005) found that 100 mg l^{-1} of SO_2 was not enough to kill LAB a few days after application, nor to prevent MLF in two of the three experiments they performed. These observations would mean that, in our case, SO_2 prevented cells from multiplying but did not kill them, i.e. exhibited a bacteriostatic effect. Differences found between SO_2 doses and *O. oeni* counts were statistically non-significant with $p=0.2491$.

Lysozyme addition did not exert an important effect on *O. oeni* populations, as can be deduced from Fig. 2b. One can see that 200 mg l^{-1} did not have a clear effect: a 4% increase in the *O. oeni* population was observed in tanks while there was a 75% decrease in barrels. A similar decrease (71%) occurred when 500 mg l^{-1} were added to barrels. Response to lysozyme did not seem to be directly related to either SO_2 concentration or vessel type. Statistically significant differences in counts, at 95% probability, were found between 0 and 500 mg l^{-1} lysozyme ($p=0.013$) but not between 0 and 200 mg l^{-1} ($p=0.4297$). Previous works showed contradictory results on populations and biogenic amine content after lysozyme treatment. Thus, some authors report that lysozyme

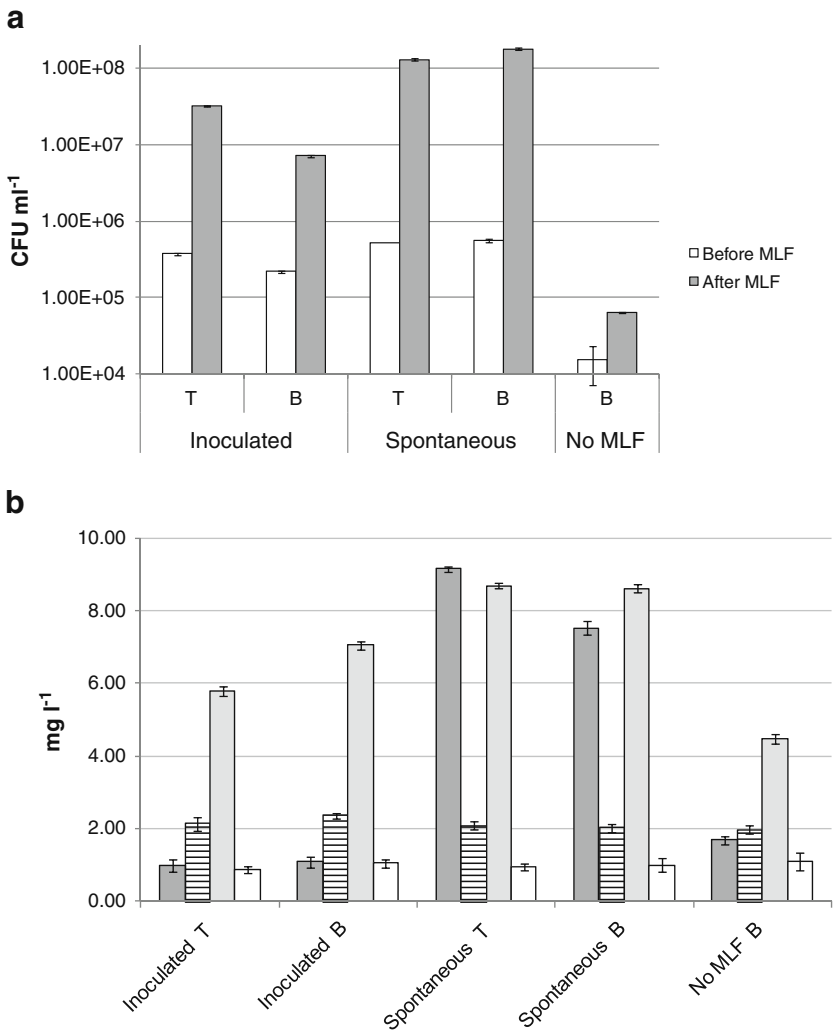

Fig. 3 Effect of management of malolactic fermentation (vessel and malolactic bacteria used) on *Oenococcus oeni* populations (**a**) and cadaverine, histamine, putrescine and tyramine at the end of MLF (**b**). *T* stainless steel tank, *B* oak barrels, *MLF* malolactic fermentation

guaranteed the prevalence of commercial malolactic bacteria over wild LAB and, consequently, low biogenic amine concentration (López et al. 2009; Gao et al. 2002). However, other authors found that the extent of the lysozyme effect depended on the sensitivity of the strains present in the wine (Pilatte et al. 2000). An interesting study by Tirelli and De Noni (2007) showed that SO_2 and polyphenolic compounds depressed lysozyme solubility and effectiveness, probably by sulphonation of enzyme disulphide bonds or protein binding, respectively. Sulphur dioxide and lysozyme interacted and formed mono-thiosulphonated lysozyme, which is inactive. This reaction was favoured by increasing pH values and sulphur dioxide concentration.

A dose of 50 mg l^{-1} SO_2 added to wine before the addition of 500 mg l^{-1} lysozyme, in order to prevent MLF, seemed to diminish the lytic activity of the enzyme, as deduced by the low effect on *O. oeni* viability when compared with to that of the 200 mg l^{-1} concentration.

Effect of MLF management on LAB populations and BA content

The concentration of *O. oeni* at the end of MLF was higher in wines which underwent this process spontaneously. As expected, the lowest counts were recorded in wines in which MLF was prevented (in these cases, samples were taken at a time in which others had completed this fermentation), see Fig. 3a. Differences between inoculated or non-inoculated wines were significant (p=0.036) for counts obtained after MLF. The type of vessel in which MLF took place did not influence (p=0.7275) bacterial populations: in MLF controlled wines, lower counts were obtained in barrels, whereas in spontaneous MLF wine, this was the case for the tank. Different RAPD profiles corresponding to wild strains were obtained from spontaneously fermented wines, and also those inoculated in barrels (Fig. 4).

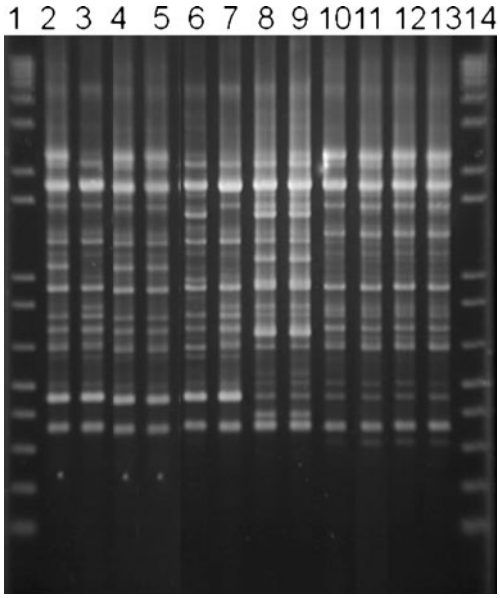

Fig. 4 RAPD patterns obtained by PCR with M13. *Lanes 1 and 14* 1-kb ladder Plus, Invitrogen, *lanes 2–7* wild strains isolated from spontaneous MLF, *lane 8 Vinoflora oenos*, *lane 9* strain isolated after MLF from stainless steel tanks and barrels inoculated with *Viniflora oenos*, *lane 10* wild strain isolated after MLF from inoculated barrels (putrescine producer), *lanes 11–13* wild strains isolated during ageing in all wines, except in those which performed MLF in stainless steel tanks

Total amine concentration was observed to be clearly higher in wines performing spontaneous MLF (average 20.08 mg l^{-1}) than in controlled MLF wines (average 10.68 mg l^{-1}); the lowest amounts were recorded in wines in which MLF was prevented (average 9.23 mg l^{-1}). The main difference between controlled or spontaneous conditions, after spontaneous MLF, was histamine concentration: 8.34 mg l^{-1} average in non-inoculated wines, and 1.04 average in inoculated wines. Putrescine concentrations in the same conditions averaged 8.65 and 6.41, respectively. Tyramine and cadaverine were similar in both types of MLF. We found significant differences between inoculated or spontaneously fermented wines for histamine, putrescine ($p<0.001$ for both), and tyramine ($p=0.0356$). Smit et al. (2008) found that the use of commercial strains considerably reduced the risk of histamine formation; however, this is not always true as the highest putrescine concentration was found in barrels inoculated with *Viniflora oenos*. This unexpected result could be explained by the isolation of a wild strain besides *Viniflora oenos* at the end of MLF (Fig. 4). Both strains developed at the same time, with *Viniflora oenos* being surpassed by the wild one, as can be deduced from the higher counts obtained in the barrels. The presence of the wild putrescine producer strain only in barrels could be due or to a residual population present in the barrels, or to better development, due to the more oxidative conditions found in this type of recipient. The presence of *O. oeni* strains able to produce histamine and putrescine, tyramine and cadaverine has previously been reported, although it must be specified that these traits are strain-dependent (Guerrini et al. 2002; Rosi et al. 2009; Landete et al. 2005a). High levels of both histamine and putrescine were found in wines after spontaneous MLF. We were able to detect putrescine but not histamine *O. oeni* producers at this stage (data not shown). RAPD profiles of putrescine producers were similar to those observed in inoculated wines performing MLF in the barrels (see Fig. 4). The origin of histamine is probably due to other LAB species, such as *L. hilgardii*, able to develop in wines undergoing MLF and to produce high levels of this amine (Landete et al. 2005a; Pardo and Zúñiga 1992). This species was not recovered on the plates, probably due to a lower relative concentration compared to *O. oeni*. The relationship between BA and MLF is controversial; thus some authors have demonstrated an important increase in amines after this process (Izquierdo Cañas et al. 2008; Marcobal et al. 2006; Marques et al. 2008), whereas others have found that amines decrease once malic acid degradation has been accomplished (Buteau et al. 1984). Differences between results can be explained by the fact that LAB developing in each case have different abilities to produce BA (Landete et al. 2005a). The first reports about LAB species responsible for BA synthesis pointed to spoilage bacteria, such as *Pediococcus damnosus*, but later it was demonstrated that other current wine species were also able to produce BA, such as *Lactobacillus brevis*, *L. hilgardii*, *L. mali* and indeed *O. oeni* (Landete et al. 2005a).

The type of vessel in which MLF took place seemed unrelated to amine concentration. Non-significant differences were found for this effect on bacterial and BA concentrations ($p>0.05$). Comparison of total amines in wines that underwent spontaneous MLF showed similar results in the tank and the barrels, and the same was found for each individual amine. These results are in agreement with those obtained by Alcaide-Hidalgo et al. (2007).

Effect of barrel ageing on LAB populations and BA content

The evolution of *O. oeni* populations and BA were monitored during a 6-month ageing period.

O. oeni populations decreased parallel to ageing in all wines, although at different rates. Twenty days after MLF completion, LAB populations were in the range of 1.2–7.6 x 10^6 CFU ml^{-1} in three of the five conditions tested. In the case of wines in which spontaneous MLF took place, higher viable populations remained longer: 40–3,000 CFU ml^{-1} after 6 months ageing, whereas no bacteria were detected after 2 months ageing in the rest of the wines

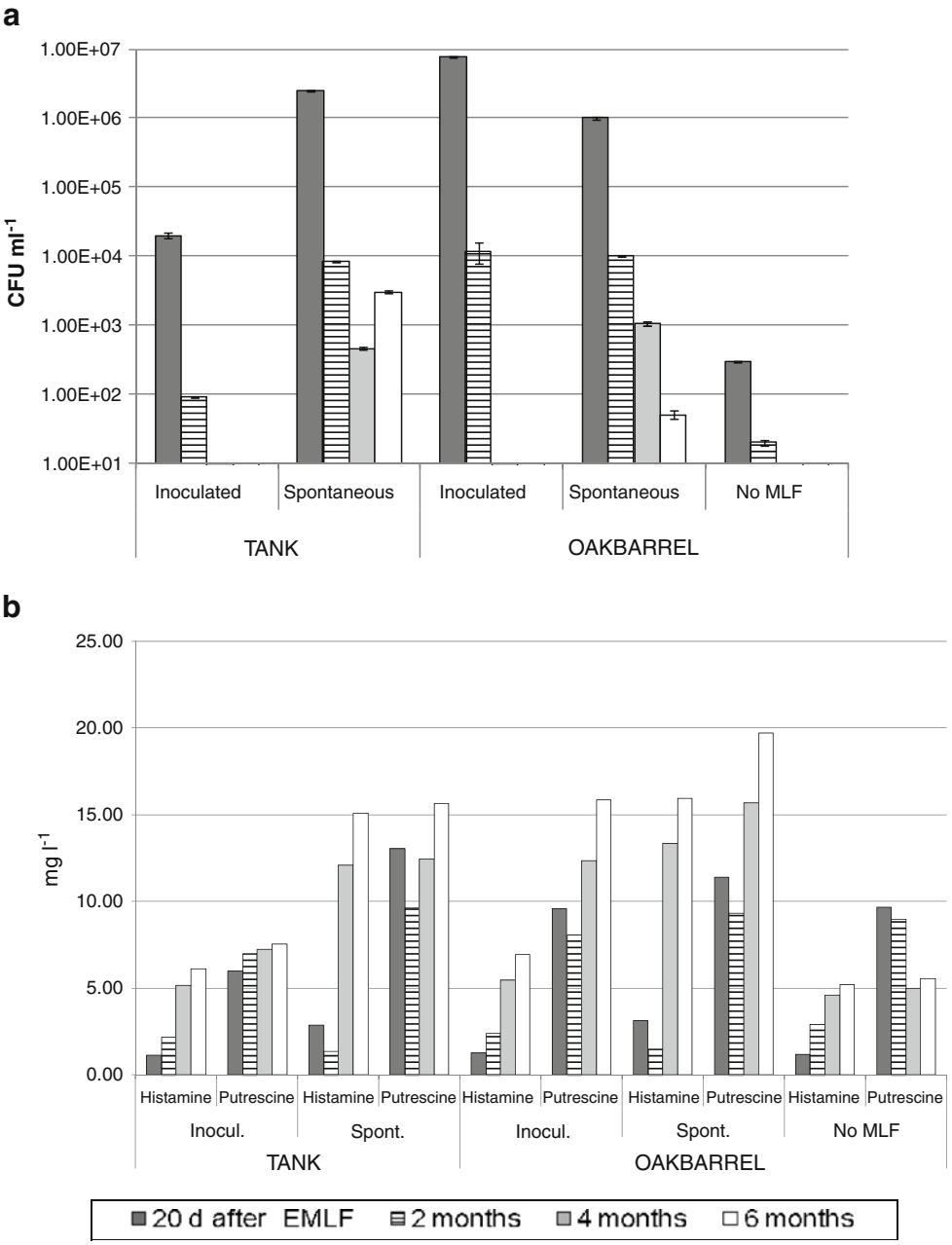

Fig. 5 **a** Dynamics of *Oenococcus oeni* populations, and **b** histamine and putrescine evolution, during ageing periods of 20 days, 2, 4 and 6 months after malolactic fermentation. *d* Days, *Inocul.* inoculated with *Viniflora Oenos*, *Spont.* spontaneous MLF, *EMLF* end of malolactic fermentation

(Fig. 5a). It could be deduced that population dynamics were not related to the type of vessel in which MLF took place, but to whether it had been controlled or spontaneous (Fig. 5a). Differences regarding the MLF vessel effect, and MLF strain on *O. oeni* counts during ageing were not significant ($p>0.1$), but they were between ageing time and counts ($p=0.0031$). Strains showing the RAPD profile corresponding to the putrescine producer were found in all the barrels, except in those filled with wines in which MLF was performed in tank with *Viniflora oenos*.

During ageing, a general increase in total BA was recorded (Fig. 5b). This increase was due mainly to histamine and putrescine, whereas tyramine and cadaverine diminished slightly or remained unchanged at the end of ageing. Significant differences were found for ageing time regarding histamine ($p<0.001$) and putrescine ($p<0.0961$) content. A decrease in certain amines has been reported by some authors after alcoholic fermentation and ageing. The reasons given for this fact are, in the first case, co-precipitation with fine lees (Jiménez Moreno et al. 2003) and, in the second, degradation by amine oxidases (Marques et al. 2008). At the end of ageing, the highest total BA concentration was found in wines that had performed spontaneous MLF (36.55 mg l^{-1}), intermediate concentrations in wines with controlled MLF (21.18 mg l^{-1}), and the lowest ones in those in which this fermentation was

prevented (13.45 mg l^{-1}). Although less important increases in BA were recorded in wines that performed MLF by *Viniflora oenos*, histamine and putrescine increased during ageing mainly in wines that underwent MLF in the barrels. Statistically significant differences were found between time and MLF strain ($p<0.001$), and between this last variable and histamine and putrescine concentrations at the end of ageing ($p=0.0108$ and <0.001, respectively), but not between MLF vessel and BA ($p=0.7836$ for histamine and 0.3874 for putrescine). It was clear that wild LAB strains that developed after controlled MLF were responsible for histamine and putrescine production. Lack of correlation between histamine and putrescine production in some wines pointed to the presence of different strains or species with different amine-forming abilities. This observation is supported by the fact that an increase in histamine is accompanied by a decrease in putrescine in wines in which MLF was prevented.

In general, the longer the LAB population remained during the ageing process, the higher the BA amounts found. Ancín-Azpilicueta et al. (2008) suggested that biogenic amines during wine ageing in oak barrels could be due to residual populations of LAB in the wine, which obtained energy through decarboxylation of amino acids. This explanation fits in with our results: amine-producing wild strains were able to survive longer than *Viniflora oenos*, as can be deduced from RAPD profiles of the strains isolated during ageing (Fig. 4).

In conclusion, the main factors influencing BA content of wine at an industrial production level were the type of bacteria performing MLF and ageing. The use of safe commercial strains could prevent BA from increasing during MLF, but such strains were not always able to prevail over wild populations. Furthermore, a later colonisation by wild strains with decarboxylating activities could occur, which could in turn promote BA synthesis in both inoculated or non-inoculated wines, or even in those receiving treatment to prevent MLF. Thus, proper microbiological stabilization is necessary to avoid the risk of increased BA during ageing.

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