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Research Article

Autochthonous *Oenococcus oeni* Strain to Avoid Histamine Formation in Red Wines: A Study in Real Winemaking Conditions

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Abstract: The production of wines with low biogenic amine (BA) concentrations is one of the current concerns in the wine sector and strategies to avoid their formation during winemaking are of especial interest. The aim of this work was to determine the influence of selected autochthonous *Oenococcus oeni* lactic acid bacteria (LAB) on the BA content in red wines and their prevalence against the indigenous microbiota to avoid BA formation. Sixty-seven red wines were elaborated at industrial scale in real winemaking conditions in three consecutive vintages. LAB implantation and BA concentrations in every wine obtained were determined at different stages of winemaking process. The results clearly indicated that the use of selected *O. oeni* strains unable to produce BA in combination with an adapted biomass production is a good strategy to control histamine production in wines. These practices, carried out over three consecutive years, were also observed

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to ensure the persistence of this selected autochthonous *O. oeni* strain CECT 9749 against other indigenous microbiota, in the whole winery. Furthermore, the analysis of BA content during wine aging in barrels indicated that low BA content was maintained, resulting in healthier wines for the consumer.

Keywords: autochthonous strains, biogenic amines, histamine, lactic acid bacteria, malolactic fermentation, *Oenococcus oeni*

Introduction

Biogenic amines (BA) are low molecular weight organic nitrogenous compounds naturally occurring in fermented foods and wines (Gardini et al. 2016). Some BA are present in grapes but they are mainly formed by amino acids decarboxylation through the enzymatic activity of microbial decarboxylases. In normal physiological conditions, BA are metabolized by gastrointestinal enzymes (Gardini et al., 2016, Ancín-Azpilicueta et al. 2019), however, the consumption of elevated BA doses could have adverse effects on consumer's health from a toxicological point of view (EFSA 2011). Histamine and tyramine are considered the most toxic amines, causing negative effects such as headaches, nausea, hypo- and hypertension, respiratory disorders, tachycardia and various allergic disorders among others (Landete et al. 2005, Ladero et al. 2010, Moreno-Arribas et al. 2010, Gardini et al. 2016).

Other BA, such as putrescine and cadaverine, are non-toxic polyamines but they can potentiate the toxic effects of BA through the inhibition of enzymes that detoxify histamine, tyramine and phenylethylamine (Straub et al. 1995, Özogul and Özogul 2019). In addition, these polyamines adversely affect wine sensory quality (Tomera 1999, García-Villar et al. 2007).

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Therefore, the presence of high BA concentrations is related to wine safety (EFSA 2011, Martuscelli et al. 2013), but they may also be indicators of wine quality and hygienic conditions (Del Prete et al. 2009, Gardini et al. 2016).

Currently, the European Union has not established a regulatory limit for histamine content or any other BA in wines. However, different countries in Europe have set different recommendation limits for histamine in wine ranging from 2 to 10 mg/L (Smit et al. 2008). The International Organisation of Vine and Wine (OIV) recommends not to exceed the limit of 12 mg/L of histamine.

Estimating safe levels of BA in wine is difficult since it depends on several factors such type of amine, concentration and the physiological conditions of consumers (individuals that do not have degrading mechanisms of these compounds) and the consumption of other BA-containing foods, which could increase their toxicity (Ancín-Azpilicueta et al. 2019). In addition, in humans, it has been demonstrated that ethanol and acetaldehyde may enhance the toxicity of amines through the inhibition of amino oxidase enzymes, responsible for amine degradation (Zimatkin and Anichtchik 1999).

Histamine, putrescine, cadaverine, tyramine, phenylethylamine and spermidine are the main BA present in wine (Moreno-Arribas and Polo 2009, EFSA 2011). Some BA, such as putrescine and spermidine, can be present in grapes (Landete et al. 2005, Izquierdo-Cañas et al. 2008, Del Prete et al. 2009), but most of them are produced during winemaking by microorganisms. The formation of BA requires the presence of amino acids, microorganisms with decarboxylase activity and the favorable conditions for their growth (Smit et al. 2008, Costantini et al. 2009, Moreno-Arribas et al. 2010). Some authors reported that yeasts can produce BA during

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alcoholic fermentation (AF) (Ancín-Azpilicueta et al. 2008, Smit et al. 2008) or during wine storage (Jiménez-Moreno et al. 2003, Hernández-Orte et al. 2008). Although reported results are contradictory, it is assumed that the greatest amount of BA, especially histamine, is produced during malolactic fermentation (MLF) through the decarboxylation activity of lactic acid bacteria (LAB) that transform amino acids into BA (Soufleros et al. 1998, Lonvaud-Funel 1999, Landete et al. 2005, Marcobal et al. 2006).

Oenococcus oeni is the most habitual species of LAB found after AF in both spontaneous MLF, due to the growth of indigenous strains, and inoculated MLF with selected strains (Moreno-Arribas et al. 2003, Nehme et al. 2010). This is due to the fact that *O. oeni* was best adapted to the harsh wine conditions (high ethanol, low pH, low nutrients and SO_2) (Lonvaud-Funel 1999). Other LAB species of different genera such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* can also grow in wine, especially if pH values are higher than 3.5 (Lonvaud-Funel 2001, Costantini et al. 2009). The ability of these bacterial species to produce BA seems to be strain-dependent (Berbegal et al. 2017), therefore, it is important to select LAB strains non-BA producers to minimize the BA content. Some authors reported that selected LAB without decarboxylase activity can be used to prevent BA formation in wines (Marcobal et al. 2006). Moreover, it was demonstrated that simultaneous yeast/ LAB co-inoculation was more effective to avoid BA production and for obtaining wines with better sensory characteristics (Massera et al 2009, Izquierdo-Cañas et al. 2014).

The occurrence of BA in wines has been studied in the last years and winemakers are looking for strategies to avoid their formation during winemaking to obtain wines with low BA concentrations (Benito 2019a). Different strategies to control the production of BA or to degrade

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BA have been suggested. The use of non-*Saccharomyces* strains that decrease malic acid content, or the usage of different retention systems (not used for quality wines) have been studied in depth (Benito 2019b, Rodríguez-Bencomo and Mehdi 2019). Nowadays, one of the main control strategies to prevent the formation of BA in wines at industrial scale is the inoculation of selected non-BA producers *O. oeni* strains (Moreno-Arribas et al. 2003, Izquierdo-Cañas et al. 2009). However, the induction of MLF by these commercial LAB was not always successful in a whole winery, due to different factors such as wine is a very harsh medium for LAB growth (Ruiz et al. 2010) or the competitive advantage of autochthonous microbiota to winery conditions. In this sense, some authors suggested the use of autochthonous selected LAB of a specific wine-producing area, which could improve the MLF development (Ruiz et al. 2010, Berbegal et al. 2017).

Therefore, the aim of this work was to study the influence of selected autochthonous *O. oeni* on the BA content of red wines from Ribera del Duero during three consecutive vintages (2016, 2017 and 2018) and study their prevalence against the indigenous microbiota to avoid BA formation in these wines. The importance of managing the adaptation of the LAB culture and how to ensure its presence in winery tanks was also studied in order to avoid histamine formation in wines. The effect of LAB inoculation and wine aging in oak barrels on BA production was also evaluated.

Materials and Methods

Winemaking conditions. Red wines were elaborated following the usual winemaking process in Pago de Carraovejas State winery, located in Peñafiel (Valladolid, Spain) in Ribera del Duero Geographical Indication in Spain. Vineyards, belonged to the winery, are disposed in the

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same valley between 850- and 950-meters elevation and are cultivated under organic farming conditions. Grape variety is 'Tinto Fino' ('Tempranillo') Carraovejas clone with 110 R rootstock.

Maturity control was checked using not only technical parameters (sugar, pH, Total acidity, weight/100 grape berries) but also quality parameters such “Glories method” (Cromoenos ®) that evaluates the phenolic maturity of grapes. It provides information on both, the quantity (total potential in anthocyanins and tannins) and the quality (anthocyanin extractability, seed maturity) of the polyphenols.

This work, performed during three consecutive harvests, was done with the real winemaking conditions and practices used at cellar facilities. Taking into consideration the differences in characteristics and grape yield accounted in each vintage, the experimental design was slightly different each year. However, in order to establish a suitable point of comparison, each vintage, half of the harvest was inoculated with the selected *O. oeni* CECT 9749 strain and compared with the other half.

In this study, three consecutive years were compared: 2016 (harvest start/end, 4 Oct/29 Oct), 2017 (harvest start/end, 19 Sept/30 Sept) and 2018 (harvest start/end, 29 Sept/12 Oct). Grapes were harvested manually and immediately transported to the winery in 12-kg boxes. Then, grapes went through double selection to avoid any rot or not mature cluster. After that, clusters were destemmed and lightly crushed.

For all vintages, the alcoholic fermentations were carried out in stainless steel tanks of 25,000 L and were filled in with 18,000 kg of red grapes. It was collected 396,000 kg, 324,000 kg and 486,000 kg, respectively in the three vintages, and were filled 22, 18 and 27 fermentation tanks (Table 1).

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Alcoholic fermentation (AF) was carried out by using the strain *Saccharomyces cerevisiae* CECT 12008 (Spanish Type Culture Collection) that was previously isolated and selected from the vineyards of Pago de Carraovejas State winery. Yeast strain was inoculated in the filling of each tank ensuring 10^6 cells/mL of must or mg of grape. Also, some fermentations were spontaneously developed by the indigenous yeasts in each harvest, to see the influence of spontaneous alcoholic fermentation in the development of the inoculated bacterial culture and/or in the production of biogenic amines.

Alcoholic fermentation kinetics were conducted at 24ºC ± 1ºC. Temperature (cap and liquid) and sugar content was measured daily during fermentation. Addition of SO_2 was done at 30 mg/L during the filling of the fermentation tanks. It was supplemented using organic nitrogen without diammonium phosphate (Actimax Natura, Agrovin) at 0.50 g/L doses, at the beginning of the fermentation. For color extraction, maceration was conducted along fermentation and pumping with aeration twice a day for color extraction. When fermentation and maceration finished, the wines were devatted and only the first pressings (55% yield) were taken.

Lactic acid bacteria strain and inocula preparation. An autochthonous *O. oeni* strain CECT 9749 was used to inoculate the experimental wines of this study. This strain was isolated and selected from red wines of the same winery in a previous study (Berbegal et al. 2017). This non-histaminogenic strain was selected because of its prevalence in wines, high alcohol resistance and high quality properties that not affects wine fruitiness. In addition, this strain was found to be well-adapted to the conditions of these wines, competing effectively with other indigenous LAB strains (Berbegal et al. 2017).

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Inoculum production was performed following a strict scale-up procedure to reach $>10^9$ CFU/mL. The selected *O. oeni* CECT 9749 strain was initially grown in MLO broth (Zúñiga et al. 1993) to reach an early stationary phase. Cells were then centrifuged (8,000 rpm, 10 min), washed with Ringer's solution and transferred to 10 mL culture media at a final concentration of 1×10^6 CFU/mL. Then, cellular concentration was scaled up through a three-stage (0.5 L, 10 L and finally, 80 L) procedure using a liquid production medium described in Berbegal et al. (2015), with several modifications that allowed the culture to adapt to the harsh conditions of red wines: use of red wine with more than 70 TPI (Total Polyphenol Index), higher alcohol degree, fermentation activator enhancers as Actimax NATURA (Agrovin S.A., Alcázar de San Juan, Ciudad Real, Spain) and cofactors (Mg, Mn, folic acid, ascorbic acid and retinol).

The industrial biomass production was performed in a fermenter with 80 L of production medium under sterile conditions (Bioprocess technology Bio-pro 100 L), and it was used to inoculate each tank of 25,000 L with the selected *O. oeni* strain (2×10^6 CFU/mL). A microbiological analysis of the three scale-up steps was carried out to certify the correct sterilization of the culture medium (Berbegal et al. 2015). This scale-up was done in Agrovin S.A. facilities.

Inoculation of lactic acid cultures and malolactic fermentation. The autochthonous selected *O. oeni* CECT 9749 strain was inoculated at the beginning of the AF, ensuring that free SO_2 was zero. The first tanks of each harvest (2016, 2017 and 2018) were inoculated, trying to maintain the same number of inoculated and non-inoculated tanks. The percentage of inoculated tanks in the three consecutive harvests were 40.9%, 55.6% and 59.3%, respectively (Table 1).

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Once AF was finished (glucose + fructose content < 1 g/L), MLF was conducted at 22ºC ± 1ºC, until malic acid was < 0.2 g/L. Then, it was considered that MLF was over.

Aging in cellar. After MLF, the wines were racked, corrected with SO_2 (approximately 0.5 of molecular SO_2), and later aged in French oak barrels (225 L) for 12 months in a cellar at 15°C and with a controlled relative humidity of about 75%–85%.

Chemicals. Chromatographic grade reagents were provided by Carlo Erba Reagents (Sabadell, Spain). 2-aminoheptanoic acid (internal standard) and diethylethoxymethylenemalonate (DEEMM) were from Fluka (Sigma-Aldrich, Germany), and the remaining reagents were supplied by Panreac (Madrid, Spain). Milli-Q grade water was obtained using a Millipore system (Bedford, MA). The biogenic amine standards were purchased from Sigma-Aldrich (Steinheim, Germany).

LAB and yeast counts and implantation. RAPD-based genotyping was used to determine the implantation of *O. oeni* CECT 9749 strain as described below. Before inoculation with *O. oeni* CECT 9749, grape musts were checked in order to determine the amount of wild LABs from vineyards (Figure 1A, red points). Then, wine samples were collected at three different moments for LAB determination (*O. oeni* inoculation, after 48h of inoculation and in the second third of the MLF) (Figure 1A black points). Samples were serially diluted and seeded onto MLO plates (Zúñiga et al. 1993). Plates were counted and ten single colonies were randomly picked from plates with 30-300 colonies and suspended in 10 µL of sterile milliU water (Millipore, Bedford, MA). These suspensions were used in RAPD (Random Amplification of Polymorphic DNA)-PCR amplification with M13 primers as described by Berbegal et al. (2017). In each electrophoresis gel, comparison between the electrophoretic band profiles obtained from LAB isolates and *O. oeni*

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CECT 9749 allowed to determine the percentage of implantation of the inoculated strain in each tank (Figure 1C, 1D).

A similar procedure was developed in order to check the cellular densities of yeasts and the implantation success of the inoculated yeast strain *S. cerevisiae* CECT 12008 (Figure 1B). Samples, taken at three stages of alcoholic fermentation (corresponding to densities (1.100, 1.040 and 0.990 g/cm^3), were seeded onto Malt Extract Agar and incubated. Yeasts colonies were counted and ten of them were randomly picked from plates for genotyping. Colonies were suspended in water and subjected to PCR-amplification of the interdelta regions (de Celis et al. 2019). The electrophoretic band pattern obtained for *S. cerevisiae* CECT 12008 was used for reliable comparisons into the same electrophoresis gel.

Analysis of biogenic amines. Nine biogenic amines (histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermidine, agmatine, tryptamine, and isoamylamine) were analyzed using the method described by Gómez-Alonso et al. (2007) with slight modifications (Ortega-Heras et al. 2014). Aminoenone derivatives of amines were obtained by reaction with DEEMM and after that, they were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) in an Agilent Technologies LC series 1200 with a diode array detection system (DAD) (Agilent, Stuttgart, Germany).

Samples were taken after MLF and after 12 months of aging in barrels after their respective racking. BA analyses were carried out in duplicate.

Analysis of oenological parameters. Standard oenological parameters in wines were determined according to the official analysis methods of OIV: pH, titratable acidity (as g/L tartaric

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224 acid), and alcohol degree (% vol: mL ethanol/100 mL wine). Malic, lactic and acetic acids were
225 analyzed using enzymatic kits in an Y15 Analyser (Biosystems, Barcelona, Spain).

226 SO_2 was determined by the official analysis methods of OIV (OIV-MA-AS323-04A).

227 These oenological parameters were analyzed in samples after AF and when MLF was
228 finalized. Analyses were performed immediately after sample collection.

229 **Statistical analyses.** A one-way analysis of variance and a Fisher Least Significant
230 Difference test (LSD) at a significant level of $p < 0.05$ was performed to determine the effects of
231 inoculation and of aging in oak barrels on the BA content, using Statgraphics Centurion XVII.

232 ## Results

233 **Oenococcus oeni implantation.** The selected *O. oeni* strain was implanted in all the
234 inoculated wines and in almost all the non-inoculated ones. Only in eight non-inoculated wines
235 from 2016 vintage (tanks 15-S to 22-S), the selected *O. oeni* strain was not found or it was found
236 under 50% (Table 2). In 2017 and 2018 vintage, the inoculated strain was present in the whole
237 winery. The LAB implantation percentage is shown in Tables 2 to 4.

238 Figure 1 (Figure 1C and 1D) shows electrophoretic patters (EP) of bands for ten randomly
239 picked colonies from MLO plates seeded with wines of two tanks of the 2016 vintage in order to
240 determine the percentage of *O. oeni* implantation. Overall, a strain is considered to be implanted
241 when it is clearly dominant, being the percentage of implantation superior to 80% (ie. eight of ten
242 EP are equal to the EP of the inoculated strain). In tank 16 1-I, the EP of all samples (lanes 2 to
243 11) were identical to the known EP (lane 12) of the inoculated *O. oeni* CECT 9749 strain, and
244 therefore the implantation was considered to be complete. However, in tank 16 3-I, 1 do not

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matched with the EP of the inoculated *O. oeni* strain, and the implantation was considered to be around 90%.

Fermentation kinetics. Kinetics of malic acid consumption and lactic acid production followed the same pattern in all fermentations performed in the three harvests of the study. As an example, Figure 1 (1C and 1D) shows malic acid/lactic acid kinetics during the progression of the fermentation in two selected tanks (Tank 16 1-I and 16 3-I). In both cases, one with a good (100%) implantation of the *S. cerevisiae* strain CECT 12008 (Tank 16 1-I, Figure 1B) and the other (Tank 16 3-I, Figure 1B), without a good implantation (0%) -similar to spontaneous-, the global kinetics of malic acid consumption and lactic acid production were similar. Furthermore, this fact, jointly considered with the results of Table 2, indicates that the influence of the yeast in the progression malolactic fermentation was little.

Biogenic amine analyses. Tables 2 to 4 show the BA concentrations found in all analyzed wines from the three vintages just after MLF and after 12 months aging in barrels. Spermidine and phenylethylamine were found in low concentrations and no statistically significant differences were observed between wines (data not shown). Agmatine, tryptamine and isoamylamine were not detected in any wine. Therefore, the study was focused on the content of the main BA (histamine, tyramine, cadaverine and putrescine) found in wines. Putrescine was the most abundant amine in all the wines, with concentrations ranging between 2.9-12.0 mg/L.

A clear difference in histamine and putrescine contents between the inoculated and the non-inoculated wines from 2016 vintage was observed in both stages, after MLF and after 12 months of aging in barrels (Table 2). Non-inoculated wines presented statistically significant higher histamine and putrescine concentrations than the inoculated ones. The wines with the *O. oeni* strain

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implanted showed a reduction of 93.6 % in histamine and 30.2 % in putrescine. Furthermore, it should be pointed out that those non-inoculated wines in which the selected *O. oeni* strain was also implanted (tanks 10-S to 14-S), showed the lowest histamine content.

On the contrary, the same was not observed in wines from 2017 and 2018 vintages (Tables 3 and 4). The inoculation did not produce statistically significant differences in histamine and putrescine concentrations. However, it should be taken into account that in these two vintages, the content of these BA was very low and the presence of the *O. oeni* CECT 9749 strain was detected in all the wines, not only in the inoculated ones but also in those non-inoculated.

No statistically significant differences were found in the tyramine and cadaverine concentrations of the wines due to *O. oeni* inoculation, with the exception of the wines from 2017 vintage. However, the differences found in these wines were low. After MLF, the inoculated wines had 1.30 mg/L of tyramine and 0.36 mg/L of cadaverine while the non-inoculated wines had 1.70 mg/L of tyramine and 0.49 mg/L of cadaverine (Table 3).

After 12 months of aging in barrels, significant increases were recorded for putrescine in the non-inoculated wines from 2016 vintage. Slight increments in BA of 2017 wines were observed, although, in general, they were very low.

General oenological characteristics. Mean values of the oenological parameters and the standard deviation of wines from the three consecutive vintages (2016, 2017 and 2018) at the end of AF and MLF are shown in Table 5. Wines from 2017 and 2018 showed a slightly higher alcohol content (15.4%vol.) than 2016 wines (14.8%vol.). In addition, 2017 wines also showed slight lower titratable acidity (5.2 g/L) and malic acid content (1.45 g/L) than wines obtained from 2016 and 2018 vintages (mean values of 5.9 g/L of tartaric acid and 1.9 g/L of malic acid). In spite of

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these slight differences, it can also be highlighted that wines from the three vintages showed similar oenological characteristics, with pH ranges around 3.6-3.8; titratable acidities between 5.2-6.1 g/L of tartaric acid; and alcoholic degrees between 14.7%vol.-15.6%vol.

Concerning the effect of the *O. oeni* inoculation, no statistically significant differences were found in most of the oenological parameters between the inoculated and the non-inoculated wines in each vintage, with the exception of the lactic and acetic acid concentrations in 2018 wines.

All wines completed the MLF since malic acid concentrations were below 0.2 g/L. Before aging, wines were corrected with SO_2 and molecular SO_2 was up of 0.5 (Table 5), allowing to maintain its antibacterial activity.

Discussion

Oenococcus oeni implantation. To ensure *O. oeni* implantation to avoid wild histaminogenic LAB development, an efficient production of the selected *O. oeni* strain was achieved. Highly active cultures exerting a good malic acid enzymatic activity prevent sugar consumption, avoiding increments in acetic acid and lactic acids.

Several studies have reported that the inoculation of bacterial starters before or during AF (simultaneous inoculation of yeast and LAB) allows better control of MLF in winemaking (Massera et al. 2009, Azzolini et al. 2010, Smit and DuToit 2013, Izquierdo-Cañas et al. 2014). In this sense, some preliminary experiences (data not shown) were conducted during 2013, 2014 and 2015 harvests. We observed that, when wines were inoculated with *O. oeni* CECT 9749 at the end of AF, wild LAB counts coming from grapes were high, making it impossible to ensure the implantation of the inoculated strain, consequently, histamine production was not reduced.

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Therefore, in this study, it was decided to inoculate a selected non-histaminogenic *O. oeni* strain at the beginning of AF.

The development of this strain was successful in all the inoculated wines of the three vintages. In 2016 vintage, the selected *O. oeni* strain was found in the 38% of the non-inoculated wines and in the following vintages, 2017 and 2018, it was found in all the wines, independently of the inoculation. This is a very interesting result since it has been observed that over the years, the autochthonous selected *O. oeni* strain is predominant during the elaboration of the wines of the whole winery, even in those wines that were not previously inoculated. Therefore, the addition of the well-adapted culture of *O. oeni* CECT 9749 strain to the different tanks during AF seems to generate a high prevalence of the strain during the three consecutive vintages studied. Similar results have been obtained using selected *Saccharomyces cerevisiae* strains as starters for wine fermentation, indicating that these practices could have an important incidence on microbial diversity in surrounding vineyards (de Celis et al. 2019).

In winery real conditions, yeast and bacteria does not work separately. Inoculated and non-inoculated tanks are processed using the same material (pumps, tubes, etc) and as the inoculated tanks are routinely the first to be used for fermentation, the dissemination of the bacterial cultures to the non-inoculated tanks is a feasible situation.

Biogenic amine analyses. The implantation results are according to the BA data of these wines. The non-inoculated and non-LAB implanted wines from 2016 presented the highest histamine contents (Table 3). Histamine formation occurs mainly during MLF, and no significant increases were observed during aging in barrels, with some exceptions. The increase of histamine was showed in the wines with very low initial concentrations, but this increase is not considered

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significant since the histamine values were always less than 1 mg/L. The results found in bibliography are contradictory respect to the BA formation mainly during MLF or during aging (Soufleros et al. 1998, Lonvaud-Funel 1999, Jiménez-Moreno et al. 2003, Landete et al. 2005, Marcobal et al. 2006, Hernández-Orte et al. 2008), and also are dependent on whether the MLF was carried out spontaneously or by inoculating LAB (Marcobal et al. 2006, Hernández-Orte et al. 2008). Hernández-Orte et al. (2008) showed an increase of histamine concentration after 6 months of oak aging that was higher in wines that conducted MLF with indigenous bacteria than in inoculated wines. Other authors indicated that LAB inoculation did not give rise to an increase of histamine during oak aging (Marcobal et al. 2006). The contradictory results found by different authors could be due to the prevalence or not of the non-histaminogenic strain, since some works evaluated the BA content but not the LAB strain that carried out the MLF. Taking into account our results, the presence of the *O. oeni* strain non-histamine producer during MLF avoids the increase of histamine values during the aging time.

Mean values of histamine in the inoculated and the non-inoculated wines from the three vintages studied after MLF and after 12 months of aging in barrels are shown in Figure 2. The use of the selected autochthonous *O. oeni* strain from the red wines of the winery has drastically reduced the content of histamine in its wines. A progressive reduction in histamine content in wines has been observed over the years. Histamine mean values have been reduced from 6 mg/L in inoculated wines and 18 mg/L in non-inoculated ones in 2011 (Berbegal et al. 2017) to values < 1 mg/L in wines of 2017 and 2018 vintages. These low histamine values were also found in the non-inoculated wines, but in those where the selected *O. oeni* strain has been implanted. This might be due to the prevalence of the selected *O. oeni* strain against other indigenous LAB that manage

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354 to avoid the growth of other populations' histamine producers. In addition, this also leads the
355 maintenance of low histamine levels during aging in barrels. Therefore, it seems that the
356 implantation of the selected *O. oeni* strain is determinant to reduce the risk of histamine formation
357 and to obtain wines with low BA concentrations. The influence of the use of selected *O. oeni* strain
358 was more significant in the histamine and putrescine content than in the cadaverine and tyramine.
359 Putrescine concentrations were also significantly higher in the non-inoculated wines than
360 in the inoculated ones but only in wines from 2016. Therefore, the indigenous microbiota that
361 conducted the MLF in these wines were responsible for producing these BA. These results are in
362 agreement with those found by other researchers who indicated that spontaneous MLF has more
363 risk to produce wines with high BA contents (Izquierdo-Cañas et al. 2008, Berbegal et al. 2017).
364 Significant increases were recorded for putrescine in the non-inoculated wines during the
365 aging process, mainly in 2016 vintage. Putrescine can be formed by the decarboxylation of
366 ornithine from the action of bacterial decarboxylase enzymes. In addition, Mangani et al. (2005)
367 have reported that *O. oeni* strains can produce putrescine, not only from ornithine, but also from
368 arginine, if they have the enzyme necessary to degrade arginine to ornithine. Arginine is one of
369 the major amino acids found in grape and wine, and may be the main responsible for the formation
370 of putrescine. The increase observed in the mean putrescine values from 5.13 to 12.0 mg/L was
371 mainly due to the great increase found in four wines (non-inoculated tanks 10-S, 12-S, 19-S and
372 21-S from 2016 vintage). These wines presented a high decrease in arginine (between 40-77%)
373 and ornithine (between 36-94%) during the aging time (data not shown). Therefore, although it
374 was not found a linear correlation between the degradation of the precursor amino acids and the
375 formation of putrescine, the decrease in the concentrations of these amino acids can lead to the

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376 formation of this BA by indigenous bacteria that have survived after MLF. Putrescine can reduce
377 sensorial quality at 20-30 mg/L in red wines (Barbieri et al. 2019), but only the non-inoculated
378 wine "tank 10-S" from 2016 vintage exceeded these values.

379 In general, the inoculation did not influence the content of tyramine and cadaverine. The
380 non-inoculated wines from 2017 showed a slightly higher content of tyramine and cadaverine than
381 the inoculated ones. However, these differences are not considered important since the values are
382 low, similar to those found in 2016 vintage and lower than those found in bibliography (Marcobal
383 et al. 2006, Hernández-Orte et al. 2008, Izquierdo-Cañas et al. 2008, EFSA 2011). These BA can
384 be found in grapes, before AF and MLF and their amount is related to grape maturation degree
385 and grape variety.

386 After 12 months of aging in barrels, no statistically significant differences were found in
387 tyramine and cadaverine concentration in both the inoculated and the non-inoculated wines. Only
388 it was observed a slight increase in 2017 wines, although they were very low, being the mean final
389 concentrations of tyramine lower than 2.5 mg/L and of cadaverine lower than 0.61 mg/L. These
390 results are in accordance with those found by Marcobal et al. (2006) and Hernández-Orte et al.
391 (2008) who neither found an increase of these BA after 6 or 12 months of aging in barrels. The
392 mean values of these BA found in our wines were lower than those reported by other authors
393 (Marcobal et al. 2006, Hernández-Orte et al. 2008, Izquierdo-Cañas et al. 2008, EFSA 2011), and
394 were similar to the concentrations reported by the EFSA (2011).

395 Although the toxicological role of BA in wines is still not well-known, it is desirable to
396 avoid their formation and to obtain wines with low BA concentrations, allowing the production of
397 healthier wines and with less allergic reactions (Ancín-Azpilicueta et al. 2019). The values of

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histamine found in the wines of this study were lower than those reported by the EFSA (2011), with the exception of the wines in which the selected *O. oeni* strain was not implanted. In addition, the concentrations of tyramine, putrescine and cadaverine found in all the studied wines were in the range of the data reported by the EFSA (2011).

General oenological characteristics. AF was generally conducted by the inoculation of a selected *S. cerevisiae* CECT 12008 strain (Tables 2 to 4), with no influence of the yeast strain on the compounds and parameters analyzed.

The LAB inoculation neither produced changes in the oenological parameters of the wines (Table 5). It was only found statistically significant differences between the inoculated wines and the non-inoculated wines from 2018 in lactic and acetic acid concentrations. However, the differences found in acetic acid were very low and the mean values ranged from 0.42 to 0.50 g/L.

On the other hand, Mendoza et al. (2011) showed an increase in volatile acidity in co-inoculated musts due to bacterial sugar catabolism. This reaction is more favorable in wines with high pH values. In this study, only the inoculated wines from 2018 vintage showed a slightly higher acetic acid concentration than the non-inoculated ones. However, the acetic acid values of 0.5 g/L do not suppose any negative effect in wines. Acclimatisation of the culture in a medium rich in malic acid, actives malolactic metabolism of the culture and avoids bacterial sugar catabolism during AF. Therefore, it could consider that the selected *O. oeni* strain did not produce an increase in volatile acidity, even when it is inoculated during AF, which agrees with the results obtained by other authors (Massera et al. 2009, Azzolini et al. 2010, Izquierdo-Cañas et al. 2014).

Alexandre et al. (2004) and Muñoz et al. (2014) observed that the use of simultaneous inoculation of yeasts and LAB resulted in sluggish AF. However, in this study, the inoculation of

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the selected *O. oeni* strain did not interfere in the yeast growth and the AF showed adequate development in all the wines. Both, the yeast and the LAB used in this study, were selected from the autochthonous microbiota of the surrounding vineyards, and did not give rise to the negative effects found by other authors, such as the increase in volatile acidity or sluggish AF, since these effects can be strain-dependent.

In spite of the high alcohol content of the wines (around 15-15.5 % vol.), the MLF was developed adequately and it was finalized in all wines. So, the selected *O. oeni* strain was adapted gradually to the ethanol content and wine characteristics (Jussier et al. 2006, Zapparoli et al. 2009, Azzolini et al. 2010), being resistant to high alcohol content (> 14.5 % vol) and able to compete with other bacterial species as *Pediococcus* or *Lactobacillus* that could reach the wines from vineyards (Lonvaud-Funel 2001, López et al. 2008, Berbegal et al. 2017).

Most of the researches that study the influence of the inoculation of LAB starters have been carried out at laboratory or pilot scale (Zapparoli et al. 2009, Azzolini et al. 2010, Mendoza et al. 2011, Izquierdo-Cañas et al. 2014, Muñoz et al. 2014, Ortega-Heras et al. 2014). Therefore, these results are important and significant since this study has been conducted in real winemaking conditions (18,000 kg of grapes in 25,000 L tanks) with sixty-seven wines in three consecutive vintages.

Conclusions

The selection of a non-histaminogenic *O. oeni* strain together with an adequate adaptation of the culture to wine conditions at the beginning of each harvest, is a good strategy to avoid the formation of BA in red wines, mainly histamine.

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This practice ensures the prevalence, against the indigenous microbiota, of the selected *O. oeni* CECT 9749 strain inoculated that can also be present even in the non-inoculated wines, and allowing reductions in the histamine content of all the wines of the winery.

The success of the selected *O. oeni* strain in the wines to avoid the production of BA, mainly histamine, was maintained after 12 months of aging in barrels.

This procedure prevents microbial alterations during the moments of fermentation in which wines are not protected with sulfur dioxide. For the same reason, sulfide addition for wine protection is lower, and even can be a tool for making wines with very low or without sulfides.

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Table 1 Experimental design of the experiences conducted in the present work during three consecutive vintages. The *O. oeni* CECT 9749 strain was used in the inoculated tanks.

Vintage	Feature	Inoculated tanks	Non-inoculated tanks	Total
2016	Number of tanks	9	13	22
	Volume (kg)	162,000	234,000	396,000
	Percentage (%)	40.9	59.1	100
2017	Number of tanks	10	8	18
	Volume (kg)	180,000	144,000	324,000
	Percentage (%)	55.6	44.4	100
2018	Number of tanks	16	11	27
	Volume (kg)	288,000	198,000	486,000
	Percentage (%)	59.3	40.7	100

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Table 2 Biogenic amines of the wines from 2016 vintage analyzed after malolactic fermentation (MLF) and after 12 months of aging in barrels[a].

Sample	Yeast inoculation	LAB inoculation	LAB implantation (%)	Histamine (mg/L)		Tyramine (mg/L)		Putrescine (mg/L)		Cadaverine (mg/L)	
				After MLF	12 months	After MLF	12 months	After MLF	12 months	After MLF	12 months
16-Tank 1-I [b]	yes	yes	100	nd [c]	2.00	1.85	1.38	3.10	4.03	0.35	0.43
16-Tank 2-I	yes	yes	100	nd	1.39	1.54	1.36	3.42	2.99	0.41	0.33
16-Tank 3-I	yes	yes	90	nd	2.40	1.60	1.45	2.75	2.92	0.38	0.37
16-Tank 4-I	yes	yes	80	1.14	1.62	1.42	1.20	4.31	3.79	0.37	0.33
16-Tank 5-I	yes	yes	100	nd	1.18	1.29	1.35	2.36	2.43	0.34	0.32
16-Tank 6-I	yes	yes	100	nd	3.80	1.55	1.54	4.64	4.64	0.41	0.39
16-Tank 7-I	yes	yes	100	nd	1.00	1.51	1.31	3.72	3.43	0.37	0.36
16-Tank 8-I	yes	yes	100	1.11	1.76	1.54	1.33	3.42	3.24	0.35	0.34
16-Tank 9-I	yes	yes	100	1.16	1.41	2.20	1.69	2.99	2.91	0.42	0.38
MEAN				0.38 a, y	1.84 a, z	1.61 a, y	1.40 a, y	3.41 a, y	3.38 a, y	0.38 a, y	0.36 a, y
Standard deviation				0.57	0.85	0.27	0.14	0.73	0.68	0.03	0.04
16-Tank 10- S [b]	yes	no	100	2.64	6.88	2.22	1.91	4.10	42.5	0.50	0.44
16-Tank 11- S	yes	no	80	4.32	5.95	1.32	1.13	7.69	7.15	0.39	0.33
16-Tank 12- S	yes	no	100	nd	2.63	2.74	2.15	5.91	19.0	0.45	0.41
16-Tank 13- S	no	no	100	nd	3.54	1.54	1.45	3.58	3.31	0.39	0.35
16-Tank 14- S	yes	no	100	1.50	1.70	1.00	1.05	5.46	4.39	0.29	0.28
16-Tank 15- S	yes	no	0	**16.4**	**16.3**	1.87	1.84	10.1	12.0	0.53	0.47
16-Tank 16- S	yes	no	0	**18.7**	**17.3**	1.57	1.36	6.42	11.2	0.37	0.36
16-Tank 17- S	yes	no	0	**12.8**	**15.8**	1.73	1.45	4.40	4.55	0.35	0.32
16-Tank 18- S	yes	no	40	**14.3**	**12.8**	1.28	0.98	3.12	2.93	0.36	0.32
16-Tank 19- S	yes	no	0	**15.7**	**17.4**	1.66	1.42	3.87	17.6	0.36	0.41
16-Tank 20- S	yes	no	0	**17.1**	**17.4**	1.89	1.66	3.01	3.30	0.39	0.39
16-Tank 21- S	yes	no	0	8.45	**12.6**	1.61	5.94	4.78	24.8	0.28	0.81
16-Tank 22- S	yes	no	0	**10.7**	**12.5**	1.06	1.24	4.23	3.84	0.32	0.31
MEAN				9.42 b, y	11.0 b, y	1.65 a, y	1.81 a, y	5.13 b, y	12.0 b, z	0.38 a, y	0.40 a, y
Standard deviation				6.96	6.02	0.47	1.29	2.01	11.59	0.07	0.14

[a]Mean values with different letters in each compound indicate statistically significant differences at $p < 0.05$. Letters (a, b) indicate differences between the inoculated and the non-inoculated wines in the same aging stage and letters (y, z) indicate differences between aging stages. Histamine values > 10 mg/L (up OIV recommendation) are marked in bold.
[b]I: inoculated wines: S: spontaneous MLF or non-inoculated wines. [c] nd: no detected.

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Table 3 Biogenic amines of the wines from 2017 vintage analyzed after malolactic fermentation (MLF) and after 12 months of aging in barrels[a].

Sample	Yeast inoculation	LAB inoculation	LAB implantation (%)	Histamine (mg/L)		Tyramine (mg/L)		Putrescine (mg/L)		Cadaverine (mg/L)	
				After MLF	12 months	After MLF	12 months	After MLF	12 months	After MLF	12 months
17-Tank 1-I[b]	yes	yes	100	< LQ[c]	0.28	1.15	1.42	2.96	3.46	0.31	0.35
17-Tank 2-I	yes	yes	100	0.32	1.42	0.68	1.08	2.57	3.31	0.35	0.40
17-Tank 3-I	yes	yes	100	< LQ	0.41	1.60	1.98	2.87	3.54	0.37	0.41
17-Tank 4-I	yes	yes	100	< LQ	0.35	1.22	1.57	2.60	3.11	0.36	0.41
17-Tank 5-I	yes	yes	100	nd[c]	< LQ	1.23	1.78	2.77	3.25	0.26	0.31
17-Tank 6-I	yes	yes	100	nd	0.58	2.03	2.87	2.95	3.58	0.39	0.47
17-Tank 7-I	yes	yes	100	< LQ	0.61	1.70	1.91	2.68	3.46	0.46	0.53
17-Tank 8-I	yes	yes	80	nd	nd	1.19	1.53	2.91	3.52	0.39	0.45
17-Tank 9-I	yes	yes	100	nd	0.27	0.85	1.14	2.86	3.43	0.36	0.40
17-Tank 10-I	yes	yes	100	nd	0.39	1.31	1.62	3.70	4.45	0.34	0.39
MEAN				< LQ a, y	0.43 a, z	1.30 a, y	1.69 a, z	2.89 a, y	3.51 a, z	0.36 a, y	0.41 a, z
Standard deviation					0.40	0.39	0.51	0.32	0.36	0.05	0.06
17-Tank 11-S[b]	yes	no	100	< LQ	0.51	1.62	1.87	2.48	3.24	0.47	0.53
17-Tank 12-S	yes	no	100	nd	0.28	1.91	2.49	2.95	4.80	0.48	0.58
17-Tank 13-S	yes	no	100	< LQ	< LQ	2.00	2.17	2.80	3.74	0.54	0.56
17-Tank 14-S	yes	no	100	nd	0.36	1.91	2.18	3.02	3.81	0.47	0.53
17-Tank 15-S	yes	no	100	< LQ	0.43	1.59	1.78	2.34	3.31	0.44	0.50
17-Tank 16-S	yes	no	100	0.44	0.45	1.63	1.95	3.19	3.82	0.59	0.61
17-Tank 17-S	no	no	80	0.38	0.35	1.22	1.55	3.09	3.74	0.45	0.50
17-Tank 18-S	no	no	100	nd	< LQ	1.75	2.26	3.59	5.13	0.46	0.52
MEAN				< LQ a, y	0.80 a, z	1.70 b, y	2.03 b, z	2.93 a, y	3.95 b, z	0.49 b, y	0.54 b, z
Standard deviation					0.43	0.25	0.30	0.40	0.67	0.05	0.04

[a]Mean values with different letters in each compound indicate statistically significant differences at $p < 0.05$. Letters (a, b) indicate differences between the inoculated and the non-inoculated wines in the same aging stage and letters (y, z) indicate differences between aging stages.
[b]I: inoculated wines: S: spontaneous MLF or non-inoculated wines.
[c]LQ: limit of quantification (0.20 mg/L); nd: no detected.

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Table 4 Biogenic amines of the wines from 2018 vintage analyzed after malolactic fermentation (MLF) and after 12 months of aging in barrels[a].

Sample	Yeast inoculation	LAB inoculation	LAB implantation (%)	Histamine (mg/L)		Tyramine (mg/L)		Putrescine (mg/L)		Cadaverine (mg/L)	
				After MLF	12 months	After MLF	12 months	After MLF	12 months	After MLF	12 months
18-Tank 1-I [b]	yes	yes	100	<LQ [c]	<LQ	1.53	1.50	3.61	3.47	0.45	0.42
18-Tank 2-I	yes	yes	100	0.39	0.34	1.23	1.53	2.10	2.33	0.44	0.42
18-Tank 3-I	yes	yes	100	0.42	0.40	2.31	2.32	4.49	4.26	0.56	0.52
18-Tank 4-I	yes	yes	100	0.41	0.38	1.88	1.65	5.04	4.65	0.55	0.48
18-Tank 5-I	yes	yes	100	0.32	0.30	2.13	2.30	3.03	2.98	0.52	0.49
18-Tank 6-I	yes	yes	100	0.62	0.59	2.49	2.27	3.35	3.13	0.59	0.53
18-Tank 7-I	yes	yes	100	0.62	0.57	2.58	2.35	3.31	3.10	0.59	0.53
18-Tank 8-I	yes	yes	100	0.45	0.42	1.54	1.57	3.21	3.02	0.45	0.41
18-Tank 9-I	yes	yes	100	0.50	0.51	1.56	1.61	3.21	3.18	0.44	0.42
18-Tank 10-I	yes	yes	100	0.41	0.51	2.58	1.85	4.63	3.22	0.47	0.57
18-Tank 11-I	yes	yes	100	0.38	0.40	2.31	2.26	4.54	4.31	0.57	0.51
18-Tank 12-I	yes	yes	100	0.42	0.41	2.15	2.17	3.74	3.62	0.56	0.51
18-Tank 13-I	yes	yes	100	0.46	0.45	2.49	2.41	3.93	3.76	0.58	0.54
18-Tank 14-I	yes	yes	100	0.31	0.30	2.93	2.92	4.35	4.65	0.56	0.64
18-Tank 15-I	yes	yes	100	0.58	0.60	2.65	2.44	3.46	3.29	0.59	0.50
18-Tank 16-I	yes	yes	100	0.26	0.28	3.30	3.15	4.04	3.87	0.67	0.61
MEAN				0.42 a, y	0.42 a, y	2.23 a, y	2.14 a, y	3.75 a, y	3.55 a, y	0.54 a, y	0.51 a, y
Standard deviation				0.12	0.12	0.56	0.49	0.75	0.66	0.07	0.07
18-Tank 17-S [b]	yes	no	100	0.32	0.40	1.52	1.54	3.01	3.12	0.45	0.47
18-Tank 18-S	no	no	100	0.28	0.88	1.79	2.44	4.55	5.03	0.41	0.46
18-Tank 19-S	yes	no	100	0.50	0.53	1.63	1.59	3.25	3.25	0.51	0.46
18-Tank 20-S	no	no	100	0.46	0.74	1.83	1.94	4.60	4.27	0.50	0.45
18-Tank 21-S	no	no	100	0.45	0.72	2.05	2.00	4.39	4.30	0.48	0.47
18-Tank 22-S	no	no	100	0.56	0.94	2.19	2.16	4.52	4.12	0.49	0.45
18-Tank 23-S	yes	no	100	0.41	0.75	2.58	2.55	4.63	4.50	0.47	0.45
18-Tank 24-S	no	no	100	0.50	0.61	2.70	2.74	4.71	4.37	0.40	0.42
18-Tank 25-S	yes	no	100	0.52	0.55	3.02	3.03	4.60	4.09	0.52	0.45
18-Tank 26-S	no	no	100	0.42	0.49	2.43	2.38	3.48	3.62	0.61	0.55
18-Tank 27-S	yes	no	100	0.26	0.44	2.11	1.86	4.30	3.96	0.56	0.48
MEAN				0.42 a, y	0.64 b, z	2.17 a, y	2.20 a, y	4.19 a, y	4.06 b, y	0.49 a, y	0.47 a, y
Standard deviation				0.10	0.18	0.47	0.47	0.62	0.56	0.06	0.03

[a]Mean values with different letters in each compound indicate statistically significant differences at $p < 0.05$. Letters (a, b) indicate differences between the inoculated and the non-inoculated wines in the same aging stage and letters (y, z) indicate differences between aging stages.
[b]I: inoculated wines: S: spontaneous MLF or non-inoculated wines.
[c]LQ: limit of quantification (0.20 mg/L).

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Table 5 Mean values and standard deviation of oenological parameters of the inoculated and the non-inoculated wines from the three vintages [a].

		After alcoholic fermentation				After malolactic fermentation			Before aging	
		pH	Titratable acidity[b]	Malic acid (g/L)	Lactic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Alcohol[b]	Free SO_2 (mg/L)	Molecular SO_2
2016 vintage										
inoculated	Mean	3.71	6.02	1.92	0.28	1.26	0.40	14.8	52	0.80
	SD [c]	0.10	0.40	0.16	0.09	0.14	0.04	0.5	3	0.11
non-inoculated	Mean	3.72	5.65	1.87	0.25	1.17	0.40	14.7	51	0.71
	SD	0.07	0.47	0.19	0.15	0.11	0.04	0.4	4	0.10
2017 vintage										
inoculated	Mean	3.78	5.20	1.59	0.56	1.21	0.43	15.4	49	0.66
	SD	0.07	0.42	0.59	0.16	0.10	0.06	0.4	4	0.09
non-inoculated	Mean	3.82	5.25	1.27	0.66	1.24	0.47	15.6	51	0.64
	SD	0.11	0.31	0.46	0.34	0.13	0.09	0.2	4	0.11
2018 vintage										
inoculated	Mean	3.65	6.08	1.91	0.57 b	1.60 b	0.50 b	15.4	49	0.56
	SD	0.10	0.59	0.64	0.39	0.23	0.10	0.3	8	0.10
non-inoculated	Mean	3.59	5.99	2.05	0.24 a	1.31 a	0.42 a	15.4	47	0.57
	SD	0.10	0.48	0.66	0.07	0.18	0.07	0.3	9	0.12

[a]Mean values with different letters in each compound and vintage indicate statistically significant differences at $p < 0.05$. Values without letters do not show statistically significant differences.
[b]Titratable acidity (as g/L tartaric acid); alcohol (% vol: mL ethanol/100 mL wine).
[c]SD: standard deviation.

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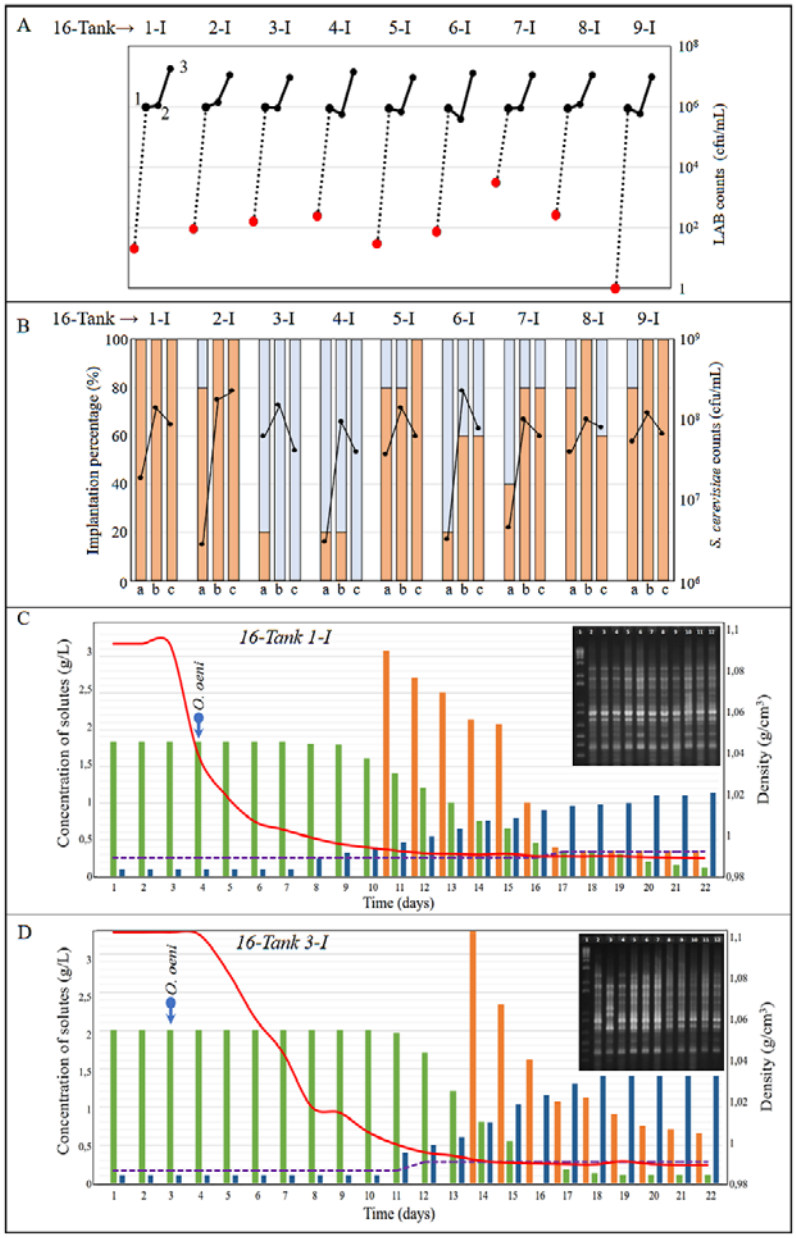

Figure 1 Microbiological control in 9 tanks from the 2016 vintage (Table 2) to exemplify the evolution of the inoculated tanks with the strains *O. oeni* CECT 9749 and *S. cerevisiae* CECT 12008. (**A**) LAB cell counts: three sampling times were used to follow *O. oeni* CECT 9749 development: 1, Inoculation of *O. oeni*; 2, LAB counts at 48h after inoculation; and 3, LAB counts at 2/3 of MLF. Additionally, LAB counts before inoculation were determined (red dots). (**B**) Progress of AF: *S cerevisiae* cell counts and implantation of the selected yeast strain: three samples, at different stages of the AF, were taken to follow *S. cerevisiae* CECT 12008 development. Orange bars indicate the implantation percentage of the strain CECT 12008, whereas light blue bars indicate the implantation percentage of other wild *S. cerevisiae* strains. (**C**, **D**) Fermentation kinetics during AF and MLF in two tanks (16-Tank 1-I and 16-Tank 3-I) inoculated with *O. oeni* CECT 9749. Arrows indicate the moment of each inocula addition. Malic acid (green), lactic acid (blue), glucose + fructose (orange), density (red) and acetic acid (violet). G+F concentrations above 3 g/L are not presented in order to facilitate the representation. RAPD-fingerprinting of LAB colonies isolated from each wine tank have been embedded into C and D. Lane 1: molecular weight markers; Lanes 2-11: RAPD band patterns of ten randomly picked colonies; Lane 12: RAPD band pattern of *O. oeni* CECT 9749.

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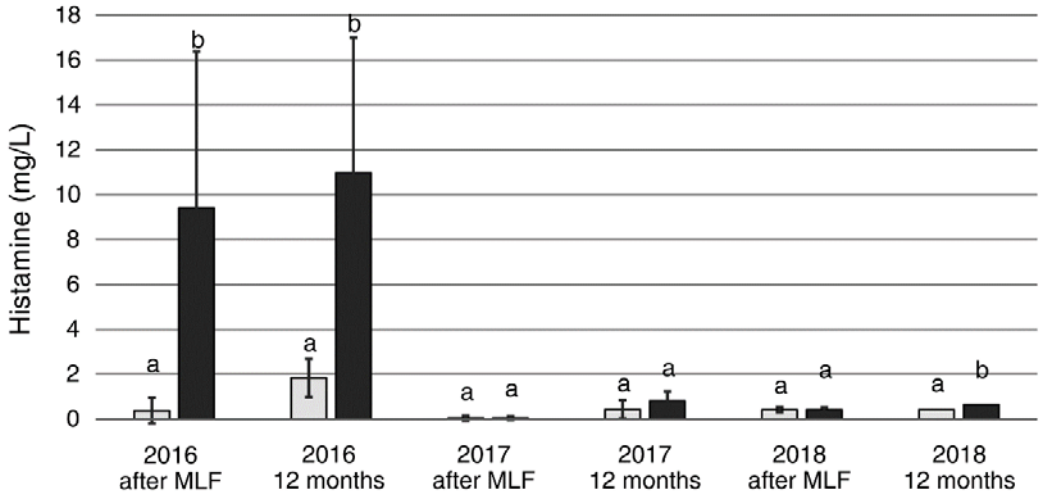

Figure 2 Mean values of histamine (mg/L) in the LAB inoculated (light grey) and the non-inoculated wines (dark grey) from the three vintages studied after MLF and after 12 months of aging in barrels. Mean values with different letters indicate statistically significant differences at $p < 0.05$.