IDENTIFICATION OF AUTOCHTHONOUS \textit{OENOCCUS OENI} FROM GRAPES AND WINES OF PRIORAT (CATALONIA, SPAIN) AND THEIR SELECTION FOR VINIFICATION

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Introduction

The malolactic fermentation (MLF) usually occurs after alcoholic fermentation (AF), especially in red wines, which contributes to an organoleptic improvement of wine and to its microbiological stability (Bartowsky, 2005). This process is carried out by lactic acid bacteria (LAB), particularly by \textit{Oenococcus oeni} (Henick-Kling, 1993). The trend of consumer preferences to the ecological wines represents an opportunity for traditional and peculiar terroirs. In this sense, the use of native LAB as inoculum can offer great potential (Ruiz et al., 2010).

The occurrence of various lactic acid bacteria (LAB) as \textit{Pediococcus}, \textit{Lactobacillus} and \textit{Leuconostoc} species in musts from freshly crushed grapes has been reported previously (Godálová et al., 2016; Pardo and Zúñiga, 1992). However, few studies have described the detection or isolation of \textit{Oenococcus oeni} directly from the grape berries (Garijo et al., 2011; Renouf et al., 2007), or from the grape juice (Saguir et al., 2009).

The main objective of this study was to isolate, identify and characterize autochthonous LAB from vineyards and wines of Priorat. Our aim was to expand the knowledge of the LAB species and strains present in healthy grapes and wine from the Priorat region, by studying nine different vineyards and wineries during two consecutive vintages. Thus we compared the LAB composition of grapes and wines across the different vineyards and vintages providing useful information about specific strains within an oenological area. This information together with the collection of LAB isolates could be used in the future to select the most representative strains with terroir characteristics for their use as starter cultures.

Materials and methods

2.1. Sampling

Samples were collected in nine different properties of Priorat wine region. Thirty samples of Grenache and Carignan healthy grape berries, consisting of two bunches each, were aseptically collected few days before harvesting, using both gloves and ethanol-sterilized scissors. In addition, 44 samples of wines made with the same grapes were collected using sterile plastic tubes of 50 mL volume. Samples corresponded from two consecutive vintages (2012 and 2013). The wines had high alcohol content (13.5-16%) and a pH of 3-3.7. Wine samples were taken at the final phase of spontaneous MLF.

2.2. Isolation and growth conditions

Grape samples were homogenized (Stomacher-400 Circulator Seward: 2500 rpm, 2.5 min) and the homogenized was incubated at room temperature without light during 3 h. Then the grape must (5...
mL) and pulp (1 g) obtained, and three whole berries (equivalent to 3 g) without homogenizing, were treated separately. These samples were cultured according to Franquès et al. (2017). All isolates confirmed to be LAB (morphology, Gram positive, catalase negative) were kept at -20°C with glycerol.

2.3. Identification and strain typing of *Oenococcus oeni*
Species-specific PCR targeting the malolactic enzyme gene was used to identify *O. oeni* isolates according to Zapparoli et al. (1998). Identified isolates were typed by the multilocus variable number tandem repeat (VNTR) method, following Claisse and Lonvaud-Funel (2012).

2.4. Species identification and strain typing of lactobacilli and other non-*Oenococcus*
All non-*Oenococcus* isolates were identified with 16S-ARDRA method and MseI digestion according to Rodas et al. (2003). The different profiles obtained were confirmed by 16S fragment sequencing by Macrogen (Rodas et al., 2005). All isolates identified as *Lactobacillus plan tarum* were confirmed by recA gene multiplex PCR following Torriani et al. (2001). Subsequently, lactobacilli and other non-*Oenococcus* species were typed using rep-PCR with GTG$_5$ (Hurtado et al., 2010) and RAPD-PCR with M13 primer (Zapparoli et al., 2000) techniques.

2.5. Technological characterization of strains
L-malic acid degradation test was performed in wine-like medium (WLM) (Bordas et al. 2013) with L-malic acid (2 g/L) and ethanol (12 and 14% v/v) at pH 3.4, and incubated at 20°C, in duplicate for each strain. The L-malic acid was measured enzymatically (Miura One, TDI S.A.) and both the L-malic acid consumption and fermentation speed were calculated.

The detection of genes responsible for biogenic amine production was performed by specific PCRs according the following methods: histidine decarboxylase gene (*hdc*), Coton & Coton (2005); tyrosine decarboxylase gene (*tdc*), Landete et al. (2007); and ornithine decarboxylase gene (*odc*), Marcobal et al. (2005).

**Results and discussion**
The total samples analyzed provided us of 1,904 LAB isolates. Most of them were confirmed to be *Oenococcus oeni* by specific PCR technique, and remarkably, 53 of them were isolated from grapes. In some studies, such as Bae et al. (2006), Sieiro et al. (1990), and recently Godálová et al. (2016), some LAB have been isolated from grapes, but not *O. oeni*. Only Garijo et al. (2011) have been able to isolate a colony of *O. oeni* from grapes. *O. oeni* was isolated from grape samples in this study thanks to the exhaustive sampling and the use of media that were richer than usual. Moreover, a sample pre-enrichment was used, and three different cultures were carried out: must, pulp and whole berries. Using the three different cultures was useful and complementary, since *O. oeni* was isolated mostly from must and pulp (38 and 54% respectively), but there was also a non-negligible 8% from whole berries.

Some grapes were processed for their observation with the Scanning Electronic Microscope (SEM) and probable LAB were observed (Figure 1).
The total number of LAB isolates of the different species was greater in samples of Carignan than in Grenache, although there were no statistical differences, despite the fact that similar numbers of samples from both varieties had been analysed. One possible explanation could be the differences in skin composition, since the thicker skin of Carignan than Grenache grapes (Rosenquist & Morrison, 1989) may allow a better microbial adherence to Carignan. The most abundant species found in grape samples was *Lactobacillus plantarum* (48% of isolates), present in both vintages and on all the properties. This species has been reported several times in grape juice or must (Fleet et al. 1984; Pardo & Zúñiga, 1992; Rodas et al., 2005). Some other LAB species previously reported in grapes and wine were also found: for example, *Lactobacillus mali* (Rodas et al., 2005) and *Fructobacillus tropaeoli* (González-Arenzana et al. 2013).

The *O. oeni* identified isolates were typified using VNTR, obtaining 164 different profiles. Some *O. oeni* strains were found in both vintages, and some were found on different properties. Significantly, two strains were coincident in grapes and in wines made with these grapes. They were the most abundant strains isolated in this study. Other non-*Oenococcus* species were typified with rep-PCR with GTG₅ technique, obtaining a wide variety of profiles (Franquès et al, 2017).
The 45 predominant strains, from *O. oeni* and other LAB, were characterized by their degradation of L-malic acid (Table 1), the resistance to low pH and high ethanol, and the absence of biogenic amine genes.

<table>
<thead>
<tr>
<th>Species</th>
<th>N. of strains</th>
<th>% L-malic consumed</th>
<th>MLF speed (mg/L L-malic acid/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12 % Ethanol</td>
<td>14 % Ethanol</td>
</tr>
<tr>
<td><em>O. oeni</em></td>
<td>34</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
<td>80-100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>50-100</td>
<td>50-100</td>
</tr>
<tr>
<td><em>F. tropaeoli</em></td>
<td>1</td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td><em>L. mali</em></td>
<td>1</td>
<td>100</td>
<td>73</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>2</td>
<td>36-100</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

*Table 1:* Efficiency of L-malic degradation (2 g/L) in wine-like medium (WLM) from studied strains (by duplicate). Abbreviations: *O., Oenococcus; F., Fructobacillus; L, Lactobacillus.*

A clear difference could be seen between most strains of *O. oeni* and the few strains of other species. A proportion of 75% of *O. oeni* strains were able to consume all 2 g/L of L-malic acid at both ethanol concentrations and at the considerable speed of more than 15 mg/L L-malic acid per h. In some strains the remarkable speed of 40 mg/L/h was reached, which is equivalent to a consumption of 2 g/L L-malic acid in just four days. The better performance of *O. oeni* over other species confirms once again its known characteristic of being the predominant LAB of MLF in wine (González-Arenzana et al. 2013, Wibowo et al. 1985).

MLF is generally considered to be a crucial factor for BA production, and studies have shown that in this phase the main BA generated are putrescine, histamine and tyramine (Lonvaud-Funel 2001, Marcobal et al. 2006), so the detection of their producing genes was performed on our isolates. The few strains in which these genes were detected were discarded from the selection, ensuring that those selected would have no risk of producing these amines.

The three *O. oeni* strains with the most desirable characteristics were selected regarding the obtained results: the L-malic acid degradation test, the absence of BA’s genes and the results of the stress resistance test. These were used in winery Ferrer-Bobet to inoculate 225 L oak barrels of Grenache and Carignan wines, MLF was carried out successfully and final wines showed good
chemical and sensorial characteristics. The imposition of two of the inoculated strains in those MLF was confirmed, being 1Pw13 the predominant strain in both Grenache and Carignan wines. The characteristics of the obtained wines suggested the possible use of one of the strains as good candidate for starter culture.

Conclusions

A large survey of autochthonous LAB from the Catalan wine region of Priorat was achieved. By the first time, remarkably several strains of *O. oeni* were isolated directly from grapes. The 45 preselected strains of *O. oeni* from Priorat were characterized regarding the efficiency of MLF and the growth at harsh specific conditions of these specific wines, keeping the terroir characteristics. From these, three strains were selected and they carried out successfully the MLF in Grenache and Carignan wines in cellar.

Acknowledgements

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References


Abstract

Oenococcus oeni, the lactic acid bacterium (LAB) mainly responsible for malolactic fermentation, has been repeatedly isolated from wines, but hardly ever from grapes. In this work, a large survey of autochthonous LAB from the Catalan wine region of Priorat was achieved. A total of 1,904 LAB isolates, from Grenache and Carignan grape berries and from wines of different cellars, were identified and typed. Around 70% of isolates were O. oeni, mostly from wines, but remarkably, 53 of them were isolated from grapes. Other non-Oenococcus species were also identified and typed, being Lactobacillus plantarum the predominant one in grapes.

The possibility of using some of these autochthonous strains was studied. From them, 45 O. oeni strains were selected and characterized in base of their degradation of L-malic acid, the resistance to low pH and high ethanol, and the absence of biogenic amine genes. The three strains with the most desirable characteristics were inoculated in real wines, MLF was carried out successfully and final wines showed good chemical and sensorial characteristics. The characteristics of the obtained wines suggested the possible use of one of the strains as good candidate for starter culture. Thereby, autochthonous strains have the potential to be used, after being selected, as inoculum of real wines, they are well adapted to the conditions of this specific area and can keep the terroir characteristics.