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Presence of *Oenococcus oeni* and other lactic acid bacteria in grapes and wines from Priorat (Catalonia, Spain)

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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 study, a large survey of autochthonous LAB from the Catalan wine region of Priorat was made. A total of 761 LAB isolates, 254 from Grenache and Carignan grape berries and 507 from wines, were identified and typed. Around 70% of the isolates were *O. oeni*, mostly from wines, but remarkably, 53 of them were isolated from grapes. A minimum spanning tree of *O. oeni* strains constructed from the multilocus variable number tandem repeat analysis showed considerable phylogenetic diversity. Other non-*Oenococcus* species were also identified and typed, *Lactobacillus plantarum* being predominant in grapes. Other LAB isolates were *Pediococcus pentosaceus*, *Fructobacillus tropaeoli*, *L. mali*, *L. lindneri* and *L. sanfranciscensis*. High-throughput sequencing (HTS) analysis was also carried out with grape samples, and *Oenococcus* and *Lactobacillus* were detected in significant quantities, which corroborates the culturing results from the same samples.

1. Introduction

The occurrence of lactic acid bacteria (LAB) as *Pediococcus*, *Lactobacillus* and *Leuconostoc* in musts from freshly crushed grapes has been reported previously (Godálová et al., 2016; Pardo & Zúñiga, 1992). However, few studies have described the detection of *Oenococcus oeni* directly from grape berries (Garijo et al., 2011; Renouf, Claisse, & Lonvaud-Funel, 2007).

O. oeni is the species that is best adapted to wine conditions and it is usually found in wines during malolactic fermentation (MLF) (Bordas et al., 2013; González-Arenzana, Santamaría, López, & López-Alfaro, 2013; Henick-Kling, 1993; Wibowo, Eschenbruch, Davis, Fleet, & Lee, 1985) or it is commercially used for MLF induction. MLF is the bacterial-driven decarboxylation of L-malic acid to L-lactic acid and CO_2, resulting in deacidification, flavour modifications and the microbial stability of wine (Bartowsky, 2005; Davis, Wibowo, Lee, & Fleet, 1988; Liu, 2002; Lonvaud-Funel, 1999).

The use of native *O. oeni* strains for MLF has considerable potential as a more environmentally friendly wine production strategy in areas such as Priorat (southern Catalonia, Spain), a standout wine region. Most of the vineyards in the area minimize pesticide treatment is given, so most of the wines produced are ecologic, and LAB are rarely inoculated there.

The main objective of this study was to isolate and identify autochthonous LAB strains in healthy grapes and wines from Priorat. This collection of LAB isolates could be used in the future to select the strains that are most representative of the *terroir* so that they can be used as specific starter cultures by the region's cellars.

As well as isolating and cultivating microorganisms, high-throughput sequencing (HTS) makes it possible to analyse complex microbial communities via short amplicons, usually hypervariable domains of prokaryotic 16S rDNA (Caporaso et al., 2012). The HTS technique has recently been used in samples of botrytized wines (Bokulich, Joseph, Allen, Benson, & Mills, 2012), winery equipment and surfaces (Bokulich, Ohta, Richardson, & Mills, 2013), grapes from California (Bokulich, Thorngate, Richardson, & Mills, 2014), Merlot grapevines (Zarraonaindia et al., 2015) and fermenting Riesling grapes (Piao et al., 2015). These studies revealed the presence of various LAB, and *Oenococcus* DNA was detected only during the alcoholic fermentation of a Riesling (Piao et al., 2015).

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Table 1
Properties of Priorat region where samples of grapes and wines were taken.

Code	Property	Location	Appellation of origin [a]
A	Ferrer Bobet	Porrera	DOQ Priorat
B	Mas Sinén	Poboleda	DOQ Priorat
C	Roca de les Dotze	La Morera	DOQ Priorat
D	Scala Dei	Escaladei	DOQ Priorat
E	Mas Martinet	Gratallops	DOQ Priorat
F	Jaume Sabaté	Vilella Baixa	DOQ Priorat
G	Genium	Poboleda	DOQ Priorat
H	Mas del Botó	Alforja	DO Tarragona
I [b]	Laurona	Falset	DO Montsant

[a] DOQ: qualified appellation of origin; DO: appellation of origin.
[b] Only wine samples.

Recently, we applied HTS to analyse the microbial communities present in grape berries from Priorat (Portillo & Mas, 2016; Portillo, Franquès, Araque, Reguant, & Bordons, 2016) and, in this study, we make a deeper analysis of the LAB population, and particularly of *Oenococcus*.

2. Materials and methods

2.1. Sampling

Samples were collected in nine different properties from the 2012 and 2013 vintages (Table 1). Thirty samples of two bunches of healthy Grenache and Carignan grape berries (Table 2) were aseptically collected from eight vineyards on these properties a few days before harvesting. In addition, 44 samples of different wines from the nine wineries (Table 1) in the final phase of spontaneous MLF were collected using sterile plastic 50 mL tubes (Table 3). No malolactic starter cultures were used, the alcohol content of the wines was high (13.5–16%) and the pH was 3–3.7.

2.2. Isolation and growth conditions

All grape samples were processed in three ways: grape must, pulp and whole berries. The must (5 mL) and pulp (1 g) were obtained after homogenizing the grape samples (Stomacher-400: 2500 rpm, 2.5 min) and incubating at room temperature without light for 3 h. Three whole berries (equivalent to 3 g) were not homogenized and were treated separately. All these samples were cultured in 10 mL of liquid MRSm3 medium, which is MRS (De Man, Rogosa, & Sharpe, 1960) supplemented with L-malic acid (3 g/L), fructose (5 g/L), nystatin (100 mg/L), sodium azide (25 mg/L), L-cysteine (0.5 g/L) and tomato juice (100 mL/L), at pH 5. Then they were incubated for 15 days in 10% CO_2 atmosphere at 27 °C. When growth was observed by turbidity, an aliquot was cultured in solid MRSm1 (20 g/L agar), which is MRSm3 without nystatin and Na-azide, in the same conditions. After 15 days of growth, 30 colonies were picked randomly from each plate, and cultured in 1 mL of MRSm1 broth. Wine samples were cultured directly in solid MRSm3 at pH 5 and plates were incubated 15 days in 10% CO_2 atmosphere at 27 °C. All isolates confirmed to be LAB were kept at −20 °C with glycerol.

2.3. Identification and strain typing of Oenococcus oeni

The majority of LAB isolates with cocci morphology were confirmed to be *O. oeni* by the species-specific PCR according to Zapparoli, Torriani, Pesente, and Dellaglio (1998). The DNA extraction was performed according to Ruiz-Barba, Maldonado-Barragán, and Jiménez-Díaz (2005).

Isolates of *O. oeni* were typed by the multilocus variable number tandem repeat (VNTR) method, following Claisse and Lonvaud-Funel (2012). Briefly, the primers were mixed in two separate solutions – Multiplex-1 (M1: 0.025 pmol of TR1 primer pairs and 0.1 pmol of TR2 ones) and Multiplex-2 (M2: 0.025 pmol of each TR3, TR4 and TR5 primer pairs) – using the Qiagen PCR multiplex kit (Qiagen, Hilden, Germany) in a total volume of 10 μL, as described by the manufacturer. Samples were analysed using capillary electrophoresis by MWG-Eurofins-Operon (France). Then, 1 μL of the size standard (GenScan™ 1200 LIZ®, Applied Biosystems) was added to each of them. After a 5 min denaturalisation at 95 °C, samples migrated for 5 min in a 3130xl Genetic Analyser (Applied Biosystems). The results obtained were analysed using GeneMarker

Table 2
Numbers of identified isolates and typed strains for different LAB species (*O.*: *Oenococcus*, *L.*: *Lactobacillus*, *F.*: *Fructobacillus*, *P.*: *Pediococcus*) from 20 grape samples of eight properties. Coincident strains with those of wine samples (Table 3) are marked in bold type.

Sample	Vintage	Property [a]	Variety [b]	Isolates identified	*O. oeni*		*L. plantarum*	*L. sanfranciscensis*	*L. lindneri*	*L. mali*	*F. tropaeoli*	*P. pentosaceus*
					n. isolates	Main strains						
2GN	2012	B	GN	3	–		1	–	2	–	–	–
3GN	2012	A	GN	19	–		15	–	3	1	–	–
4CA	2012	D	CA	17	2		3	–	5	1	6	–
3GNesp	2012	A	GN	2	–		–	–	2	–	–	–
3GNinoL	2012	A	GN	1	–		–	–	–	1	–	–
3CAesp	2012	A	CA	19	–		19	–	–	–	–	–
3CAinoL	2012	A	CA	19	2	**1Pw16**	–	–	2	2	13	–
1G	2013	A	GN	1	–		–	–	–	–	–	1
3G	2013	E	GN	10	–		–	10	–	–	–	–
4G	2013	E	GN	19	–		–	19	–	–	–	–
5G	2013	E	CA	5	4	1Pw1, **1Pw2**	1	–	–	–	–	–
7G	2013	C	GN	11	–		11	–	–	–	–	–
8G	2013	G	CA	24	3		18	–	–	–	–	3
10G	2013	B	CA	10	10	1Pw1, 2Pw2, 2Pw3	–	–	–	–	–	–
13G	2013	D	GN	11	11	1Pw1, 2Pw3	–	–	–	–	–	–
14G	2013	A	CA	6	6	1Pw1, 2Pw3	–	–	–	–	–	–
15G	2013	C	CA	29	–		29	–	–	–	–	–
16G	2013	F	CA	12	3		4	–	–	–	–	5
17G	2013	H	GN	21	8	1Pw1, **1Pw2**, 2Pw2	12	–	–	–	–	1
18G	2013	H	CA	15	4	1Pw1	10	–	–	–	–	1
TOTAL	2	8	2	254	53		123	29	14	5	19	11
n strains					16		43	5	6	4	11	4

[a] See Table 1.
[b] GN, Grenache; CA, Carignan.