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

Judit Franquès, Isabel Araque, Elisabet Palahí, María del Carmen Portillo, Cristina Reguant, Albert Bordons *

Grup de Biotecnologia Enològica, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, c/ Marcel·lí Domingo 1, Campus Sescelades N4, 43005 Tarragona, Catalonia, Spain.

*Corresponding author: Albert Bordons

Tel: +34 977 558043; Fax: +34 977 558232; E-mail address: albert.bordons@urv.cat

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.

Keywords:

Oenococcus oeni - Grape - Wine - Lactic acid bacteria - High-throughput sequencing

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 and Zúñiga, 1992). However, few studies have described the detection of *Oenococcus oeni* directly from grape berries (Garijo et al., 2011; Renouf et al., 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 et al., 2013; Henick-Kling, 1993; Wibowo et al., 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 et al., 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 et al., 2012), winery equipment and surfaces (Bokulich et al., 2013), grapes from California (Bokulich et al., 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). Recently, we applied HTS to analyse the microbial communities present in grape berries from Priorat (Portillo et al., 2016; Portillo and Mas, 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 et al., 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 27ºC. 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 27ºC. All isolates confirmed to be LAB were kept at -20ºC 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 et al. (1998). The DNA extraction was performed according to Ruiz-Barba et al. (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 L, as described by the manufacturer. Samples were analysed using capillary electrophoresis by MWG-Eurofins-Operon (France). Then, 1 L of the size standard (GenScan^{TM} 1200 LIZ®, Applied Biosystems) was added to each of them. After a 5 min denaturalisation at 95ºC, samples migrated for 5 min in a 3130xl Genetic Analyser (Applied Biosystems). The results obtained were analysed using GeneMarker V2.4.0 (SoftGenetics LLC., State College, PA, USA) and the script used by El Khoury et al. (2016). The discriminatory power of VNTR (i.e. the ability to distinguish between unrelated strains of *O. oeni*) was calculated using Simpson's index of diversity, DI (Hunter and Gaston, 1988). A minimum spanning tree (MST) was constructed with BioNumerics software (Applied Maths, version 6.6) in order to distribute the strains according to their VNTR profiles.

*2.4. Species identification and strain typing of lactobacilli and other non-*Oenococcus *isolates*

Non-*Oenococcus* isolates were identified with the 16S-ARDRA method and *MseI* digestion according to Rodas et al. (2003). DNA was extracted and PCR products were separated as described in *2.3*. The profiles obtained were confirmed by 16S fragment sequencing by Macrogen (Rodas et al., 2005). All isolates identified as *Lactobacillus plantarum* were confirmed by *recA* gene multiplex PCR using the same primers and conditions as Torriani et al. (2001).

All non-*Oenococcus* were typed using rep-PCR with GTG_5 (Hurtado et al., 2010) and RAPD-PCR with M13 primer (Zapparoli et al., 2000) techniques. The results obtained from both techniques were analysed together with BioNumerics software (Applied Maths). The similarities between digitized profiles were calculated using the Pearson correlation with 1% optimization and 2.5% curve smoothing, and an unweighted pair group algorithm with arithmetic averages (UPGMA) dendrogram was derived from the profiles. The dendrograms were analysed bearing in mind that the strains were defined at a minimum similarity level of 90%. The strain diversity of *L. plantarum*, the main species found in grape samples, was calculated using Simpson's DI (Hunter and Gaston, 1988).

2.5. Analysis of LAB on the grapes surface by high-throughput DNA sequencing (HTS)

DNA extraction and sequencing. The same grape samples (18) used for LAB isolation from the 2013 vintage were analysed by HTS. Genomic DNA was extracted from grape must samples in duplicate as described in Portillo et al. (2016). Extracted DNA was stored at -20°C until further processing. The V4 region of the 16S rRNA gene was amplified in triplicate for each sample replicate using the primer pair 515F/799Rm with adapters for the sequencing by the equipment PMG from Ion Torrent with chips 318. Amplification and sequencing were performed as described elsewhere for the analysis of bacterial communities (Portillo et al. 2016). Briefly, the universal primer 515F (GTGCCAGCMGCCGCGGTAA) included a 10-bp barcode unique to each amplified sample. The reverse primer 799Rm (CVGGGTATCTAATCCBGTT) was a modification of the universal primer 799R (CKGGGTATCTAATCCMGTT) (Chelius and Triplett, 2001) in which the two nucleotides K and M were substituted by the nucleotides V and B. This modification makes it possible to include the amplification of the genus *Oenococcus*, which was otherwise discriminated by the 799R primer, as we tested *in silico*. All primer sequences used in this study with their adapters and barcodes and PCR reactions were the same as in Portillo et al. (2016). PCR products were pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen). Cleaned PCR products were submitted to the Centre for Omic Sciences (Reus, Spain) where their quality was checked by a bioanalyser and their quantity adjusted for sequencing.

Data analysis. Raw sequences were analysed using QIIME v1.8.0 (Caporaso et al., 2010) and treated as described previously in Portillo et al. (2016). Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based (Edgar, 2010) open-reference OTU-picking workflow, with a threshold of 97% pairwise identity. A final OTU table was created, excluding unaligned sequences, singletons (sequences observed just once), and sequences matching plant mitochondria. To avoid biases generated by differences in sequencing depth, the OTU table was rarefied to an even depth of 370 sequences per sample in comparisons of all sample types in this study. Samples represented by less than 370 sequences after all the quality filtering steps were discarded.

Estimates of alpha diversity (within-sample species richness) were calculated by analysing the Chao1 and Observed-OTU indexes. The Chao1 index considers the total observed species and the number of species observed just once and twice (singletons and doubletons). The observed OTU index considers the total number of observed species.

3. Results

3.1. Isolation of LAB from grapes and wines and identification of species

Grape samples showed growth in MRSm3 plates in 20 out of the 30 cases, from which 565 LAB isolates were obtained. LAB isolates were obtained from grapes by the three ways of processing the samples: 23% of the isolates came from whole berries, 40% from must and 36% from pulp. For *L. plantarum* the proportions were 15%, 48% and 36% respectively, and for *O. oeni* they were 8%, 38% and 54%.

The samples of wines on which MLF was performed showed growth in MRSm3 plates in 40 out of the 49 cases, from which 1,339 LAB isolates were obtained. The number of isolates was considerable so we decided to identify and typify only 254 isolates (45%) from grapes (Table 2), and 507 isolates (38%) from wine (Table 3). They were chosen randomly, about 10 colonies from each plate (which had 20-30 colonies each), so the number of colonies was proportional to the different morphologies.

The predominant species in grape samples was *L. plantarum* (48.4%), followed by *O. oeni* (21%). Other species found are also shown in Table 2. A total of 123 *L. plantarum* isolates came from 11 grape samples from all eight vineyards. A total of 53 *O. oeni* isolates were obtained from only 10 of the 20 grape samples (Table 2). Both *L. plantarum* and *O oeni* were isolated more frequently in Carignan (68% and 64%, respectively) than in Grenache grapes (32% and 36% respectively), but these differences were not statistically significant, because of the considerable variability between samples. The predominant species in wine samples (Table 3) was *O. oeni* (95.7%), and a few *L. plantarum* (21 isolates) and one *L. lindneri* were also isolated from them.

3.2. Typing and phylogenetic distribution of O. oeni *isolates*

The 538 *O. oeni* isolates from grapes and wines were typed by the multilocus VNTR method, resulting 16 different profiles from grapes and 150 from wines (Tables 2 and 3). Two of these VNTR profiles – *id est*, strains – (1Pw2 and 1Pw16) were present in grapes and wine samples, and 11 strains were present in different wines. Therefore, 164 *O. oeni* strains were obtained.

The number of alleles in the VNTR-PCR products of these *O. oeni* isolates were 43, 5, 5, 4 and 4 for TR1, TR2, TR3, TR4 and TR5, respectively, and the number of repeats varied from 2 to 51 for TR1, 1 to 5 for TR2 and for TR3, 2 to 5 for TR4, and 1 to 4 for TR5. Some of the dominant alleles were found in more than 70% of the strains, such as allele 3 for TR2, allele 4 for TR3 and allele 3 for TR4. When Simpson's index of diversity was analysed, TR1 showed the highest discriminatory power (DI = 0.883), followed by TR5 (DI = 0.701). The other TR showed low DIs: 0.450 (TR4), 0.440 (TR3) and 0.168 (TR2). When all five TR regions were combined together, the 164 strains mentioned above were obtained, with a Simpson DI = 0.935. These DIs were also calculated separately for the isolates from grapes (DI = 0.725) and for those from wines (DI = 0.928).

The most abundant *O. oeni* strain was 1Pw16 (Tables 2 and 3). Six *O. oeni* strains were found in both vintages: 1Pw1, 1Pw2, 1Pw16, 1Pw17, 2Pw7 and 2Pw19. Some of the VNTR profiles found in low proportions, exclusively in cellars A and E, were coincident with profiles of two *O. oeni* commercial strains.

As shown in the MST (Figs. 1 and 2), the *O. oeni* population found was quite diverse, but there were three phylogenetic clusters that contained more than half of the strains: cluster I (11% isolates), II (14% isolates) and III (41% isolates). Clusters II and III have a common tree root (that is to say, strain 1Pw2) found in grape and wine samples.

Two strains were predominant in cluster II (Fig. 1): 1Pw1, which was isolated from both grapes and wines, and 1Pw13, found exclusively in wine samples. On the other hand, strains in cluster I and most of those in cluster III were isolated exclusively from wines. The strain 1Pw16 stands out from the others as being the predominant strain in wine but it was also found in grapes (small green sector inside the circle) (Fig. 1).

3.3. Typing and phylogenetic distribution of non-Oenococcus *isolates*

The 223 isolates of non-*Oenococcus* LAB species (Tables 2 and 3) were typed using rep-PCR with GTG_5 and RAPD-PCR with M13 primer. Some of the isolates (23 *L. plantarum*, one *F. tropaeoli* and three *P. pentosaceus*) were not typed due to difficulties in growing them and obtaining enough DNA. Eight strains of *L. plantarum* were coincident in both grapes and wine.

For each species, a dendrogram was obtained with typed isolates (Fig. 3 for *L. plantarum* and Fig. S1 in the supplementary data for the other five species). Considering that strains were regarded as different if they showed a similarity below 90%, a wide variety of strains was obtained for each species: 50 *L. plantarum,* 11 *F. tropaeoli*, seven *L. lindneri*, six *L. sanfranciscensis*, four *P. pentosaceus* and four *L. mali*.

Simpson's index of diversity was calculated for *L. plantarum*, the dominant non-*Oenococcus* species found, from the numbers of the 121 isolates distributed in each of the 50 strains (Fig. 3). The obtained DI was high (0.967). As can be seen, the dendrogram of *L. plantarum* showed two clusters with a similarity of 40%. The number of *L. plantarum* strains from Carignan (31) was greater than from Grenache (11 strains), as was the case for isolates.

3.4. Analysis of LAB on the grapes surface by high-throughput DNA sequencing (HTS)

Grape samples from the 2013 vintage (Table 4) were analysed in duplicate by the HTS method and 418,000 sequences were obtained (257 bp on average). Moreover, 6,558 different OTUs were obtained with a 97% of identity. In order to compare the taxonomic composition and diversity, 370 sequences were randomly chosen in each sample. Samples 10G and 12G were discarded for having fewer than 370 sequences. Grenache samples showed a higher diversity of OTUs (eight samples, with an average of 94 OTUs) than Carignan samples (six samples, with an average of 72 OTUs). Results were similar between sampling and DNA extraction replicates. Thus, according to the observed OTUs (Table 4) and the Chao1 index, 1G, 2G, 5G, 7G, 13G and 18G seem to be the samples that showed most species richness.

Firmicutes was the main phyla detected in half of the samples (Table 4). *Oenococcus* was detected in 15 of the 16 samples and it was the most abundant genus in the Grenache samples 1G and 2G (17.3%). *Lactobacillus* was detected in 9 of the 16 samples, mostly in samples 1G and 2G, with 5.6%. The average proportion of *Oenococcus* and *Lactobacillus* detected in all the samples was 5.8% and 1%, respectively.

4. Discussion

In this study, the autochthonous LAB from vineyards and wines in the Priorat region were comprehensively isolated, identified and typed. A total of 761 LAB isolates were identified and typed (Tables 2 and 3). As expected, most of the 538 isolates of *O. oeni* came from samples of wine performing MLF. However, the most considerable aspect was finding 53 *O. oeni* isolates from grapes (Table 2). 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. Moreover, the DNA of *O. oeni* has been detected in grapes (Renouf et al., 2005; Renouf et al., 2007) by PCR-DGGE of the *rpoB* gene, although no *Oenococcus* has been isolated. Alternatively, *O. oeni* has been isolated from grape juice samples already that had entered in the cellar (Cruz-Pio et al., 2017; Saguir et al., 2009).

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. The three methods gave similar proportions for isolates of *L. plantarum*, and the proportions found in whole berries were even higher for all the LAB isolates (23%).

The total number of LAB isolates of the different species (Table 2) 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 and Morrison, 1989) may allow a better microbial adherence to Carignan.

The most abundant species found in grape samples was *L. 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 and Zúñiga, 1992; Rodas et al., 2005).

Some other LAB species previously reported in grapes and wine were also found: for example, *P. pentosaceus* (Lonvaud-Funel et al., 1991*)*, *L. mali* (Rodas et al., 2005), *L. lindneri* (Bae et al., 2006) and *F. tropaeoli* (González-Arenzana et al., 2012). On the other hand, *L. sanfranciscensis* has never been previously isolated from grape must. In fact, it is exclusive to sourdough because of its preference for fermenting maltose and for its requirement of yeast extractives (Gobbetti and Corsetti, 1997). This suggests a contamination, or, more probably, a misidentification because the 16S sequence of *L. sanfranciscensis* is very similar to that of other *Lactobacillus*, such as *L. florum* (Endo et al., 2010) or *L. fructivorans* (Valcheva et al., 2007)*,* which are more related to flowers and fruits.

The VNTR profiles of *O. oeni* isolates gave a total of 164 different strains. The PCR products of the five TRs gave numbers of alleles and their repeats similar to those found by

Claisse and Lonvaud (2012), who developed this method for *O. oeni*. Simpson's indexes of discrimination showed good differentiation between strains, particularly for TR1 (DI = 0.883) and the combination of the five TR together (DI = 0.935). All this gives us confidence in these VNTR results. The diversity index was much higher in wine isolates (DI = 0.928) than in those from grape berries (DI = 0.725). Somehow similarly, very recently Cruz-Pio et al. (2017) have found a higher genotypic diversity index in wine isolates than in grape must ones.

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: 1Pw2 and 1Pw16. They were the most abundant strains isolated in this study (Figs. 1 and 2). Further work is required with these autochthonous *O. oeni* strains in order to understand their technological abilities in the performance of MLF. Moreover, sequencing their genomes and comparing them with those of other known strains in order to perform phylogenomic analyses, as in Campbell-Sills et al. (2015), would reveal the specific genetic features related with their abilities. Additionally, two *O. oeni* commercial strains were found in low proportions in cellars A and E. Notably, cellar A was the only one using commercial strains for other wines, but not the wines used here.

Minimum spanning trees (MST) were constructed with the VNTR data of *O. oeni* strains (Figs. 1 and 2). Our analysis has found 164 *O. oeni* strains isolated from 10 grape samples and 44 Priorat wines, which shows a picture of the biodiversity of this species in this small but prestigious wine region. It also shows the genetic relationships between these strains, as previously described (Claisse and Lonvaud-Funel, 2014). Most of the 16 *O. oeni* strains isolated from grapes (Fig. 1, green circles) were relatively close, located in clusters II and III. The results show that the common tree root of clusters II and III (strain 1Pw2) was isolated from grapes and wines, and that these clusters include most strains in grapes, from several properties (Fig. 2). Therefore, this strain could be the phylogenetic origin of most of the strains now found in this wine region.

The few previously published studies describing *O. oeni* strains with MST of VNTR patterns (Claisse and Lonvaud-Funel, 2012; El Khoury et al., 2016) also included many strains and suggested that there was a considerable genotypic diversity of *O. oeni* and that several phylogenetic subgroups were somehow related with the geographical origin. Nevertheless, they studied strains from very different wine producing areas, while in the current study all the strains were isolated in the small region of Priorat. Recently, Garofalo et al. (2015) have also found a huge diversity of strains in isolates from wines from the North-Apulian region. Our results suggest that the strain diversity of *O. oeni* may be higher than previously thought, even within vineyards and properties, at least in a region such as Priorat, which is geographically very diverse and where malolactic starters have scarcely been used.

This study is the first survey of non-*Oenococcus* LAB strains in grapes and wines, and seeks to determine the phylogenetic distribution of strains belonging to six different LAB species. Simpson's index of discrimination calculated for the typed isolates of *L. plantarum* showed good differentiation between strains, with a very high diversity (DI = 0.967). We did not find a clear relationship between phylogenetically closer strains and the varieties or properties (Fig. 3). Nevertheless, most isolates of the same strain were from the same property and variety. This confirms the goodness of the dendrogram.

The grape samples of 2013 vintage used for LAB isolation were also analysed by HTS. The complete analysis of the bacterial communities in these samples was recently reported (Portillo et al., 2016). *Firmicutes* was one of the main phyla detected besides *Proteobacteria* (Table 4). Previous HTS studies of grape microbiota had found *Proteobacteria* to be the main phylum (Bokulich et al., 2012). Nevertheless, it has recently been shown that bacterial communities are strongly influenced by geographical region or variety, and the *Firmicutes* phylum has been found to be predominant in some areas (Bokulich et al., 2014).

To our knowledge, HTS has never detected *Oenococcus* in grape berries, although it has detected other related genera such as *Leuconostoc* (Bokulich et al., 2012). An average of almost 6% of *Oenococcus* means that it has a not negligible presence in grapes. A key factor in finding the DNA of *Oenococcus* was the use of the primer 799Rm, slightly modified from 799R, so that the amplification of this genus, otherwise discriminated, could be included.

Most of the samples that showed greater bacterial diversity according to Chao1 and Observed OTU indexes (Table 4) were the ones that also presented the highest proportion of *Oenococcus* (for example 1G, 2G, 7G and 13G). As expected, the samples with most *Oenococcus* (1G and 2G) were the ones with highest proportion of family *Leuconostocaceae*. *Lactobacillus,* the most abundant genus isolated from grapes, was also detected by HTS (1%), but to a lesser extent than *Oenococcus*. This may be because faster-growing LAB such as *Lactobacillus* would be isolated more abundantly than other species, while HTS analyses the bacterial DNA and so it is proportional to the real bacterial numbers.

Oenococcus DNA was detected even in samples in which no isolates of *O. oeni* were obtained, such as 1G or 7G (Table 2), which suggests either that their population is very low, or that their cells were VBNC (viable but not culturable) (Millet and Lonvaud-Funel, 2000). Unlike *Lactobacillus*, *Oenococcus* is always difficult to culture. Anyway, HTS and culture methods were complementary and, most importantly, the HTS results confirmed the presence of *Oenococcus*, which had also been isolated from the grape berry samples.

5. Conclusions

A large survey was carried out on autochthonous LAB from vineyards and wines in Priorat. The most important contribution of the study was that several strains of *O. oeni* were isolated from grapes. The phylogenetic distribution of typed *O. oeni* strains showed considerable diversity, which was confirmed by the Simpson's index of diversity. Besides *O.*

oeni, several strains of *Lactobacillus* and other LAB species were isolated and typed, mainly from grapes. Most of them were *L. plantarum*, which also showed considerable diversity. Furthermore, the HTS analysis confirmed a considerable presence of *Oenococcus* and *Lactobacillus* in the grape samples.

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FIGURE LEGENDS

Fig. 1. Minimum spanning tree (MST) of 164 *O. oeni* strains typified by VNTR, according their isolation from grapes (green) or wines (violet). Each circle represents a strain and their diameters are proportional to the number of isolates for every strain. The biggest circle of cluster III is strain 1Pw16. The biggest ones of cluster II are strains 1Pw1 (left) and 1Pw13 (right). C_1 and C_2 are isolates coincident with profiles of two commercial strains.

Fig. 2. Minimum spanning tree of 164 *O. oeni* strains typified by VNTR, according the different properties from which they were isolated: A (violet), B (brown), D (turquoise), E (blue cyan), F (yellow), G (blue purple), H (red) and I (green). See Fig. 1 for more details.

Fig. 3. Phylogenetic dendrogram obtained from RAPD-PCR(M13) and GTG_5-PCR fingerprints for 121 *Lactobacillus plantarum* isolates of this work. Similarities (%) are shown at left. Isolate names and source (variety, property and grape/wine: see Table 1) are at right.

Supplementary Figures

Fig. S1. Phylogenetic dendrograms obtained from RAPD-PCR(M13) and GTG_5-PCR fingerprints for 29 *Lactobacillus sanfranciscensis* isolates (A), 14 *L. lindneri* (B), five *L. mali* (C), 18 *Fructobacillus tropaeoli* (D) and eight *Pediococcus pentosaceus* (E), all isolated in this work. Similarities (%) are shown at left. Isolate names and source (variety, property and grape/wine: see Table 1) are at right.

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

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.

Table 3

Numbers of identified isolates and typed strains for different LAB species (*O.*: *Oenococcus*, *L.*: *Lactobacillus*) from 44 wine samples of nine wineries. Coincident strains with those of grape samples (Table 2) are marked in bold type.

Sample	Vintage	Property [a]	Varieties of wine [b]	Isolates identified	*O. oeni*		*L. plantarum*	*L. lindneri*
					n. isolates	Main strains		
12-1w	2012	E	GN+S+CA	11	11	**1Pw2**, 1Pw7	-	-
12-2w	2012	E	GN+CS+M	10	10	1Pw3, 1Pw7	-	-
12-3w	2012	E	GN	11	11	1Pw2, 1Pw4, 1Pw9	-	-
12-4w	2012	E	M	10	10	**1Pw2**, 1Pw3, 1Pw5, 1Pw7	-	-
12-5w	2012	F	GN	10	10	1Pw6, 1Pw7	-	-
12-6w	2012	F	GN	10	10	1Pw7	-	-
12-7w	2012	D	GN+S	10	10	1Pw13	-	-
12-8w	2012	D	GN	10	10	1Pw13	-	-
12-9w	2012	D	GN	10	10	1Pw8, 1Pw9, 1Pw10	-	-
12-10w	2012	D	CS	11	10	1Pw9, 1Pw11	1	-
12-11w	2012	D	CA+GN	11	11	1Pw10, 1Pw11, 1Pw12, 1P13	-	-
12-12w	2012	D	S	10	10	1Pw5, 1Pw9, 1Pw10, 1Pw12, 1Pw13	-	-
12-13w	2012	D	CA	10	10	1Pw13	-	-
12-14w	2012	I	GN	10	10	1Pw14	-	-
12-15w	2012	A	GN	10	10	1Pw16, 1Pw17	-	-
12-16w	2012	A	Rejected grapes	10	10	1Pw15, **1Pw16**	-	-
12-17w	2012	B	GN+CA	10	10	**1Pw16**	-	-
12-18w	2012	B	GN	10	10	**1Pw16**	-	-
12-19w	2012	B	GN+CS+S+CA	10	10	**1Pw16**, 1Pw17	-	-
12-20w	2012	G	GN	10	10	1Pw18	-	-
12-21w	2012	G	M	10	10	1Pw19	-	-
12-22w	2012	G	M	10	10	1Pw19	-	-
12-23w	2012	A	CA	10	10	1Pw15, **1Pw16**	-	-
12-24w	2012	A	CA	10	10	**1Pw16**, 1Pw17	-	-
12-3GNesp	2012	A	GN	35	32	1Pw15, **1Pw16**, 1Pw17	3	-
12-3CAesp	2012	A	CA	38	20	1Pw1, 1Pw15	17	1
12-3CAinoL	2012	A	CA	36	36	1Pw1	-	-
12-3GNinoL	2012	A	GN	10	10	**1Pw16**, 1Pw17	-	-
13-1w	2013	E	GN+CA+S	9	9	**1Pw2**, 2Pw6	-	-
13-2w	2013	E	GN+CA+S	10	10	2Pw7, 2Pw8	-	-
13-3w	2013	F	CA+CS	10	10	2Pw9		-
13-4w	2013	F	GN	10	10	2Pw10	-	
13-5w	2013	F	GN	9	9	2Pw11, 2Pw12	-	-
13-6w	2013	G	CA+GN	10	10	2Pw13	-	-
13-10w	2013	D	CA	3	3		-	-
13-11w	2013	D	GN	3	3		-	-
13-12w	2013	H	GN	10	10	2Pw14, 2Pw17		-
13-13w	2013	H	GN	10	10	2Pw14, 2Pw15, 2Pw16, 2Pw17	-	-
13-14w	2013	H	CA	10	10	2Pw16		-
13-15w	2013	H	CS	10	10	2Pw2, 2Pw16, 2Pw17	-	-
13-17w	2013	A	GN	10	10	1Pw17, 2Pw2, 2Pw5,2Pw19, 2Pw21	-	
13-18w	2013	A	GN	10	10	1Pw17, 2Pw5,2Pw20	-	-
13-19w	2013	A	CA	10	10	1Pw17, 2Pw2, 2Pw5, 2Pw21	-	
13-Vi2FB	2013	A	GN	10	10	2Pw22, 2Pw23, 2Pw24	-	-
TOTAL		9		507	485		21	1
n strains					150		15	1

[a] See Table 1

[b] GN, Grenache; S, Syrah; CA, Carignan; CS, Cabernet Sauvignon; M, Merlot.

Table 4

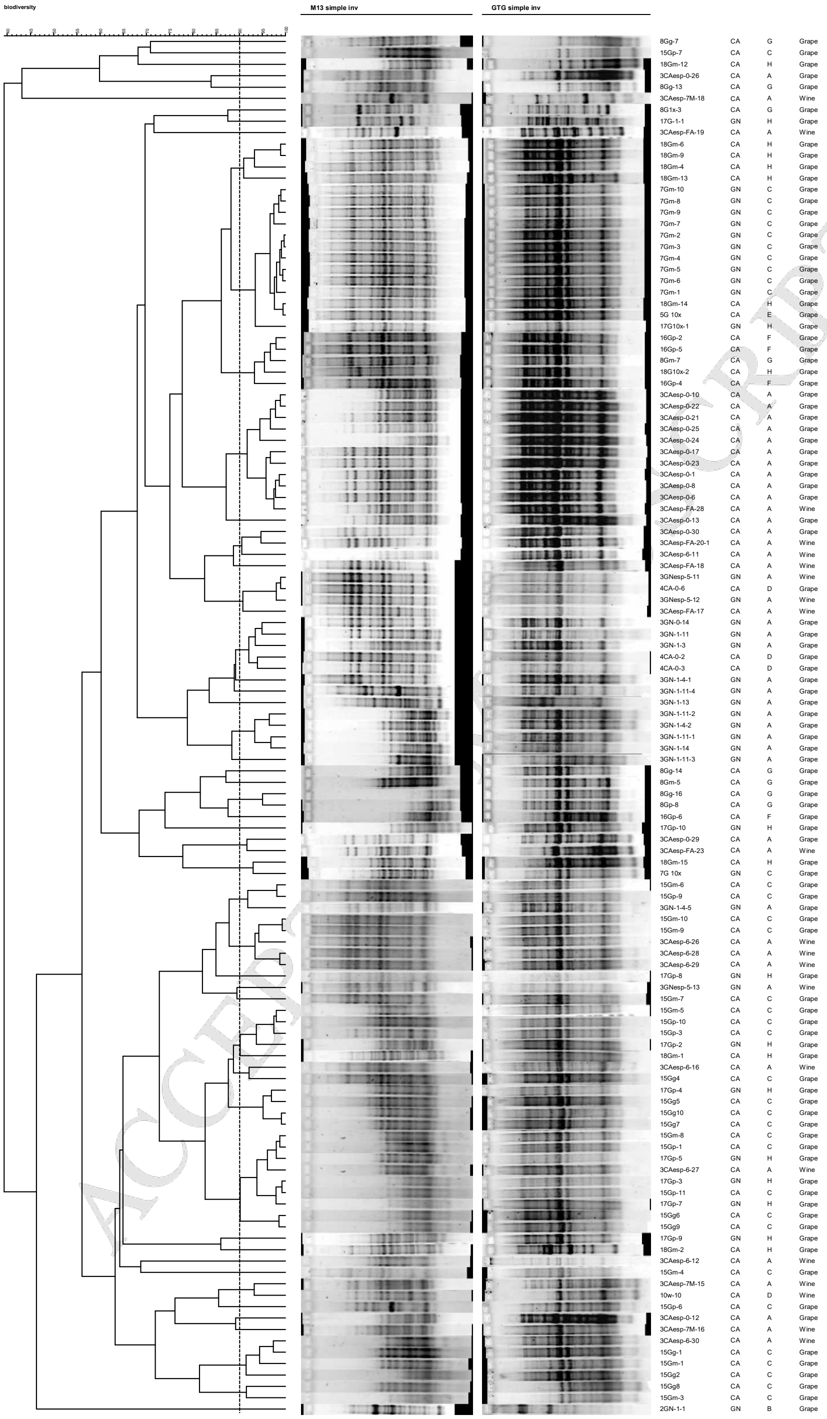
Main bacterial phyla, family and genera found (especially *Oenococcus* and *Lactobacillus*) in grapes surface analysed by high-throughput sequencing. All samples were from 2013 vintage. Data are the average of duplicates for each sample.

Sample	Property [a]	Variety [b]	% *Oenococcus* from total	% *Lactobacillus* from total	% Main family, genus	% Main phyla	Alpha diversity index	
							Chao1	Observed OTUs
1G	A	GN	17.3	5.6	17.3% *Leuconostocaceae*, *Oenococcus*	41% Firmicutes	246	111
2G	A	GN	17.3	5.6	17.3% *Leuconostocaceae*, *Oenococcus*	41% Firmicutes	246	111
3G	E	GN	3	0.3	66.8% *Bacillaceae, Bacillus*	73% Firmicutes	205	77
4G	E	GN	3	0.3	66.8% *Bacillaceae, Bacillus*	73% Firmicutes	205	77
5G	E	CA	0	0	98.6% *Bacillaceae, Bacillus*	100% Firmicutes	229	61
6G	F	GN	11.3	0.9	14.6% *Pseudomonadaceae, Pseudomonas*	40% Gammaproteobacteria	178	95
7G	C	GN	12.1	1.8	16.5% *Moraxellaceae, Acinetobacter*	36% Gammaproteobacteria	296	113
8G	G	CA	2.9	0	27.8% *Enterobacteriaceae, Enterobacter*	57% Gammaproteobacteria	217	106
9G	G	GN	6.7	0	27.8% *Enterobacteriaceae,* other genera	61% Gammaproteobacteria	140	89
11G	B	GN	0.3	0	38.6% *Streptococcaceae, Streptococcus*	52% Firmicutes	222	96
13G	D	GN	14.5	0.8	17.6% *Moraxellaceae, Acinetobacter*	29% Gammaproteobacteria	222.	116
14G	A	CA	1.3	0	94.6 % *Enterobacteriaceae, Erwinia*	98% Gammaproteobacteria	126	52
15G	C	CA	0.5	1.0	54.7% *Moraxellaceae, Acinetobacter*	62% Gammaproteobacteria	138	64
16G	F	CA	2.7	0.1	35.4% *Streptococcaceae, Streptococcus*	71% Firmicutes	196	71
17G	H	GN	0.4	0	88.7% *Bacillaceae, Bacillus*	90% Firmicutes	165	52
18G	H	CA	0.5	0	36.6% *Enterobacteriaceae, Erwinia*	75% Gammaproteobacteria	262	75

[a] See Table 1

[b] GN, Grenache; CA, Carignan

Fig. 3.


biodiversity
40
45
50
55
60
65
70
75
80
85
90
95
100
M13 simple inv
GTG simple inv
8Gg-7 CA G Grape
15Gp-7 CA C Grape
18Gm-12 CA H Grape
3CAesp-0-26 CA A Grape
8Gg-13 CA G Grape
3CAesp-7M-18 CA A Wine
8G1x-3 CA G Grape
17G-1-1 GN H Grape
3CAesp-FA-19 CA A Wine
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18Gm-9 CA H Grape
18Gm-4 CA H Grape
18Gm-13 CA H Grape
7Gm-10 GN C Grape
7Gm-8 GN C Grape
7Gm-9 GN C Grape
7Gm-7 GN C Grape
7Gm-2 GN C Grape
7Gm-3 GN C Grape
7Gm-4 GN C Grape
7Gm-5 GN C Grape
7Gm-6 GN C Grape
7Gm-1 GN C Grape
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5G 10x CA E Grape
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16Gp-5 CA F Grape
8Gm-7 CA G Grape
18G10x-2 CA H Grape
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3CAesp-0-6 CA A Grape
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3CAesp-FA-18 CA A Wine
3GNesp-5-11 GN A Wine
4CA-0-6 CA D Grape
3GNesp-5-12 GN A Wine
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3GN-0-14 GN A Grape
3GN-1-11 GN A Grape
3GN-1-3 GN A Grape
4CA-0-2 CA D Grape
4CA-0-3 CA D Grape
3GN-1-4-1 GN A Grape
3GN-1-11-4 GN A Grape
3GN-1-13 GN A Grape
3GN-1-11-2 GN A Grape
3GN-1-4-2 GN A Grape
3GN-1-11-1 GN A Grape
3GN-1-14 GN A Grape
3GN-1-11-3 GN A Grape
8Gg-14 CA G Grape
8Gm-5 CA G Grape
8Gg-16 CA G Grape
8Gp-8 CA G Grape
16Gp-6 CA F Grape
17Gp-10 GN H Grape
3CAesp-0-29 CA A Grape
3CAesp-FA-23 CA A Wine
18Gm-15 CA H Grape
7G 10x GN C Grape
15Gm-6 CA C Grape
15Gp-9 CA C Grape
3GN-1-4-5 GN A Grape
15Gm-10 CA C Grape
15Gm-9 CA C Grape
3CAesp-6-26 CA A Wine
3CAesp-6-28 CA A Wine
3CAesp-6-29 CA A Wine
17Gp-8 GN H Grape
3GNesp-5-13 GN A Wine
15Gm-7 CA C Grape
15Gm-5 CA C Grape
15Gp-10 CA C Grape
15Gp-3 CA C Grape
17Gp-2 GN H Grape
18Gm-1 CA H Grape
3CAesp-6-16 CA A Wine
15Gg4 CA C Grape
17Gp-4 GN H Grape
15Gg5 CA C Grape
15Gg10 CA C Grape
15Gg7 CA C Grape
15Gm-8 CA C Grape
15Gp-1 CA C Grape
17Gp-5 GN H Grape
3CAesp-6-27 CA A Wine
17Gp-3 GN H Grape
15Gp-11 CA C Grape
17Gp-7 GN H Grape
15Gg6 CA C Grape
15Gg9 CA C Grape
17Gp-9 GN H Grape
18Gm-2 CA H Grape
3CAesp-6-12 CA A Wine
15Gm-4 CA C Grape
3CAesp-7M-15 CA A Wine
10w-10 CA D Wine
15Gp-6 CA C Grape
3CAesp-0-12 CA A Grape
3CAesp-7M-16 CA A Wine
3CAesp-6-30 CA A Wine
15Gg-1 CA C Grape
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2GN-1-1 GN B Grape

Fig. 1.

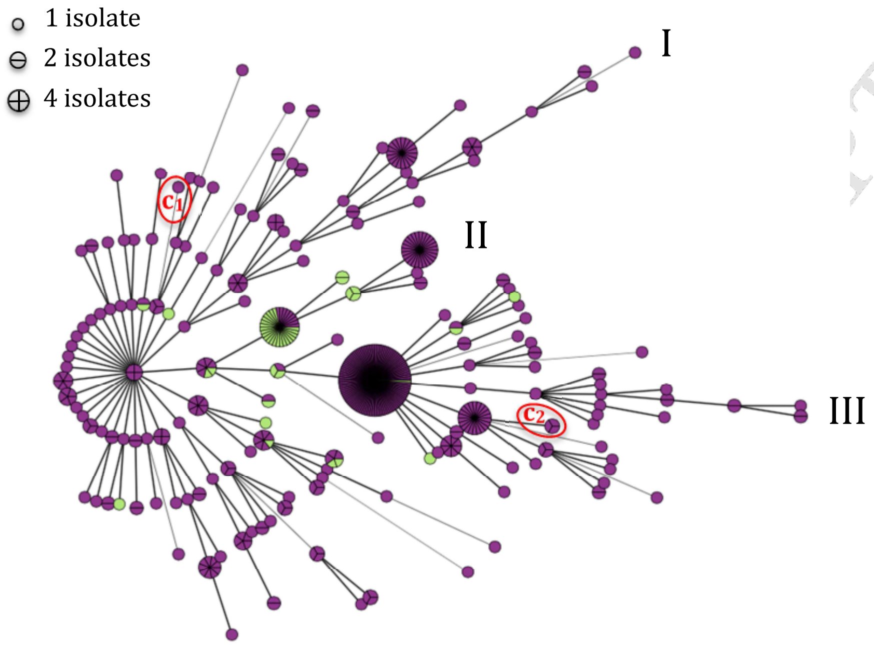
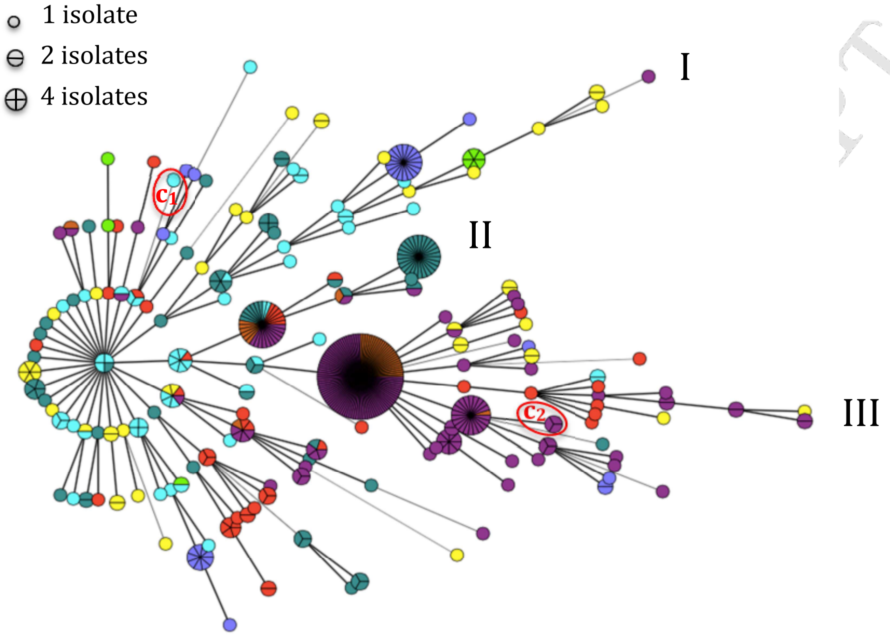


Fig. 2.



Highlights

- Autochthonous lactic acid bacteria from vineyards and wines of Priorat region were analysed.
- *Oenococcus oeni* was isolated from several samples of grape berries.
- A great diversity of *O. oeni* strains from Priorat wines was observed.
- High-throughput sequencing confirmed the presence of *Oenococcus* in grapes.