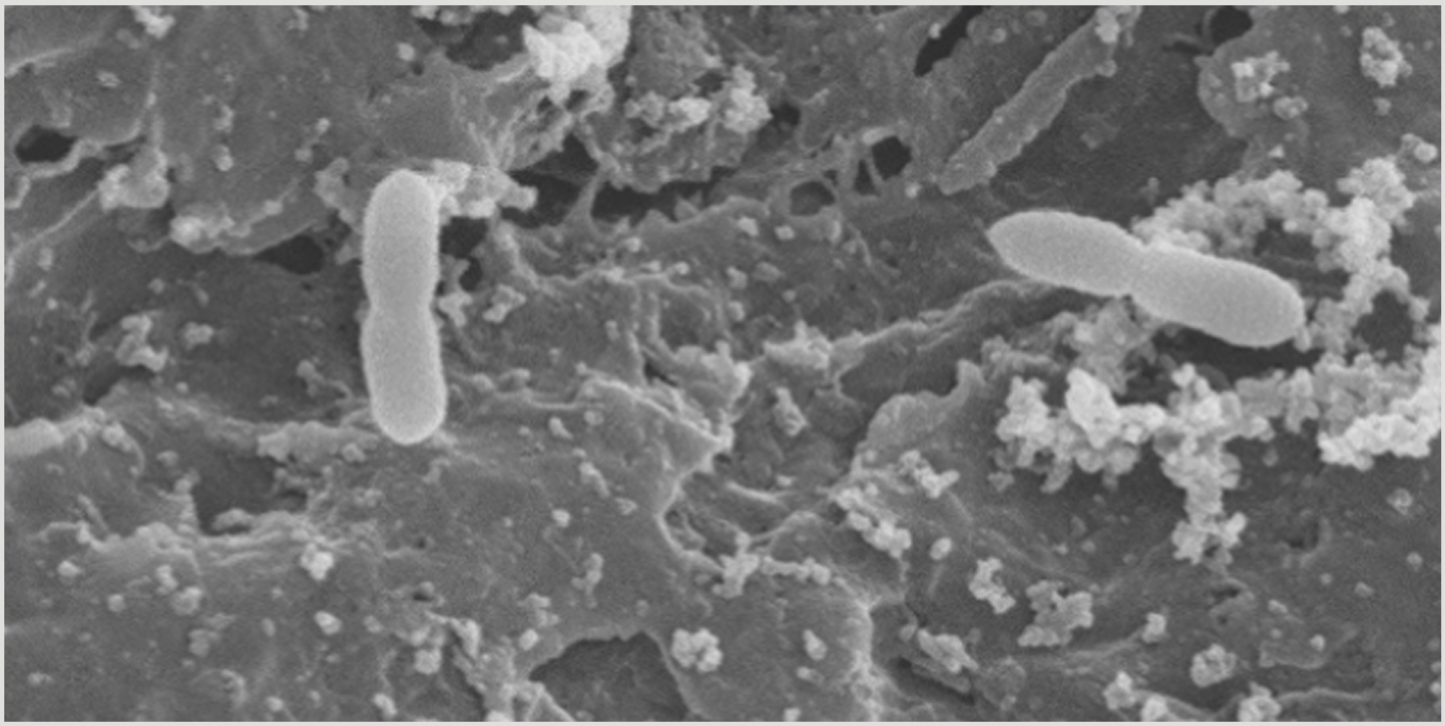
UNIVERSITAT ROVIRA i VIRGILI

Characterization of *Oenococcus oeni* and other lactic acid bacteria from the vine-wine ecosystem in Priorat

JUDITH FRANQUÈS MONTSERRAT



DOCTORAL THESIS
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Judith Franquès Montserrat

Characterization of *Oenococcus oeni* and other lactic acid bacteria from the vine-wine ecosystem in Priorat

Doctoral Thesis

Supervised by Dr. Isabel Araque, Dr. Albert Bordons and Dr. Cristina Reguant

Department of Biochemistry and Biotechnology - Universitat Rovira i Virgili

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This doctoral research was carried out between 2012-2018 and directed by the Lactic Acid Bacteria group of the Oenological Biotechnology research group (Department of Biochemistry and Biotechnology, Faculty of Oenology) at the Universitat Rovira i Virgili (URV). The doctoral thesis was supervised by Dr. Isabel Araque, Dr. Albert Bordons and Dr. Cristina Reguant. The research was financially supported by the European project FP7-SME-2012-Grant 315065 with the pre-doctoral grant 2012BPURV-28 from the Universitat Rovira i Virgili, and a mobility grant 2013CTP00024 from Generalitat de Catalunya. An international phase was completed at the Institute des Sciences de la Vigne et du Vin (Bordeaux, France) under the supervision of Dr. Patrick Lucas.

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WE STATE that the present study, entitled "*Characterization of* Oenococcus oeni *and other lactic acid bacteria from the vine-wine ecosystem in Priorat*", presented by Judith Franquès Montserrat for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university. This thesis is eligible to apply for the Degree of Doctor with International Mention.

Tarragona, 29th June 2018

Doctoral Thesis Supervisors

Dr. Isabel Araque Dr. Albert Bordons Dr. Cristina Reguant

"It is not so very important for a person to learn facts. For that he does not really need a college. He can learn them from books. The value of an education in a liberal arts college is not the learning of many facts, but the training of the mind to think something that cannot be learned from textbooks."

Albert Einstein, 1921.

Per a la meva família i l'Albert.

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CONTENTS

SUMMARY

Oenococcus oeni, the lactic acid bacterium (LAB) mainly responsible for malolactic fermentation (MLF), has been repeatedly isolated from wines, but hardly ever from grapes. In this work, the LAB biodiversity of the vine-wine ecosystem was established, and a large survey of autochthonous LAB from the Catalan wine region of Priorat was achieved, too. 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 presence of *Oenococcus* and *Lactobacillus* in grapes was also confirmed by high-throughput sequencing.

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 and its MLF was carried out successfully. 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.

RÉSUMÉ

Oenococcus oeni, l'espèce de bactéries de l'acide lactique (BAL) principalement responsable de la fermentation malolactique (FML), a été isolée à plusieurs reprises sur les vins, mais rarement sur les raisins. Dans ce travail, la biodiversité de BAL de l'écosystème vignoble-vin a été établie, et une vaste enquête sur BAL autochtone de la région viticole catalane du Priorat a été réalisée aussi. Au total, 1.904 isolats de BAL, issus de grenache et de carignan et de vins de différentes caves, ont été identifiés et typés. Environ 70% des isolats étaient des *O. oeni*, principalement des vins, mais remarquablement, 53 d'entre eux ont été isolés des raisins. D'autres espèces non-*Oenococcus* ont également été identifiées et typées, *Lactobacillus plantarum* étant prédominant dans les raisins. La présence d' *Oenococcus* et de *Lactobacillus* dans les raisins a également été confirmée par séquençage massif.

La possibilité d'utiliser certaines de ces souches autochtones a été étudiée. A partir d'eux, 45 souches d'*O. oeni* ont été sélectionnées et caractérisées en fonction de leur dégradation de l'acide L-malique, de la résistance à un pH bas et d'un éthanol élevé, et de l'absence de gènes d'amines biogènes. Les trois souches présentant les caractéristiques les plus souhaitables ont été inoculées dans de vrais vins et la FML a été réalisée avec succès. Les caractéristiques des vins obtenus suggèrent l'utilisation possible de l'une des souches comme un bon candidat pour la culture starter. Ainsi, les souches autochtones ont le potentiel d'être utilisées, après avoir été sélectionnées, comme inoculum de vrais vins, donc elles sont bien adaptées aux conditions de cette zone spécifique et peuvent garder les caractéristiques du terroir.

RESUM

Oenococcus oeni, l'espècie de bacteris làctics (BL) que són els principals responsables de la fermentació malolàctica (FML), s'han aïllat repetidament dels vins, però quasi mai des del raïm. En aquesta tesi, la biodiversitat de BL de l'ecosistema vinya-vi va ser establerta i també es va realitzar un ampli estudi sobre els BL autòctons de la regió vitivinícola catalana del Priorat. Es van identificar i tipificar un total de 1.904 aïllats de BL, de raïm de garnatxa i de raïm de carinyena, així com de vins de diferents cellers. Al voltant del 70% dels aïllats van ser *O. oeni*, principalment de vi, però sorprenentment, 53 d'ells es van aïllar de raïm. Es van identificar i tipificar també altres espècies no-*Oenococcus*, essent *Lactobacillus plantarum* la predominant en raïm. La presència d'*Oenococcus* i *Lactobacillus* en raïm també es va confirmar per seqüenciació massiva.

Es va estudiar la possibilitat d'utilitzar algunes d'aquestes soques autòctones. D'aquestes, es van seleccionar i caracteritzar 45 soques d'*O. oeni* en base a la degradació de l'àcid L-màlic, la resistència al pH baix i a les altes concentracions d'etanol, i l'absència de gens d'amines biògenes. Les tres soques amb les característiques més desitjables es van inocular en vins reals, dels quals la FML es va dur a terme amb èxit. Les característiques dels vins obtinguts van suggerir el possible ús d'una de les soques com a bona candidata per a un potencial cultiu iniciador. Per tant, les soques autòctones podrien ser utilitzades, després de ser seleccionades, com inòcul de vins reals, ja que estan ben adaptades a les condicions d'aquesta àrea específica i poden mantenir les característiques del terroir.

1. INTRODUCTION

1.1 Microbial diversity in winemaking

The first evidence of human production of fermented beverages is found in the Neolithic village of Jiahu in China, as early as the 7000 BC (McGovern et al., 2004). For winemaking, the earliest evidence comes from the site of Hajji Firuz Tepe in the northern Zagros Mountains in Mesopotamia (5400-5000 BC) (McGovern et al., 1996). Vineyards and grape wine production gradually spread to adjacent regions during the centuries and this fermentative process stimulated fortuitous domestication of several microbial species (Fay and Benavides, 2005).

Winemaking is a microbiological process carried out by a community of yeast and bacteria. After the end of the alcoholic fermentation (AF) where yeast, such as *S. cerevisiae* or non-*Saccharomyces,* carry out the conversion of sugars to ethanol, lactic acid bacteria (LAB), such as *Oenococcus oeni,* perform the malolactic conversion of malic acid to lactic acid in most wines, especially red wine.

Natural fermentations have been key to human development and are probably the oldest form of food preservation. In addition to preservation and providing variety to the diet, there are further important consequences of this process, such as the organoleptical changes of the final product.

The generalized use of prescribed starter cultures that obscure the native microbiota results in the resemblance of analytical and sensory properties of wines, depriving them of variability, complexity and personality. On the other hand, the use of indigenous *S. cerevisiae* or non-*Saccharomyces* (wild) yeasts, as of *O. oeni* or non-*Oenococcus* LAB species is a tool to create authenticity. Indigenous yeasts and LAB can assure the evolvement of the typical sensory properties of wine from a given region while the influence of wild yeasts marks the 'wild-ferment' character of wines.

In the first place, yeasts conduct the AF, and also have a prominent role in shaping wine quality. Different yeast species or strains impart diverse organoleptic profiles to wines, adding to the complexity and richness of wine aroma and flavour. Currently,

winemakers worldwide add commercial *Saccharomyces cerevisiae* as starter cultures, to ensure a predictable, reproducible and controlled fermentation.

Secondly, LAB, mainly strains of *Oenococcus oeni*, conduct the MLF (Henick-Kling, 1993; Wibowo et al*.,* 1985). During this fermentation L-malic acid is decarboxylated to L-lactic acid, resulting in wine deacidification. This is a crucial step in red winemaking as it provides enhanced organoleptic qualities, as the increase of soft mouth feel, flavour and microbiological stability of the final product (Bartowsky, 2005; Davis et al., 1988; Cappello et al*.*, 2017; Liu S.Q., 2002; Lonvaud-Funel, 1999). MLF may also have some undesirable effects on wine quality, due to the production of off-flavours, reduction in colour and formation of biogenic amines. The overall effect of MLF is principally dependent on the species and strains that perform the fermentation.

Traditionally, MLF was conducted spontaneously by the native wine bacteria. This practice is still applied in several wineries worldwide. However, spontaneous MLF is highly unpredictable, in that the onset, the rate, the completion and the production of off-flavours and biogenic amines cannot be controlled. To overcome these obstacles, the use of selected MLF starter cultures has been applied during the last four decades. Starter cultures can be selected on the basis of improving flavour and aroma, ensuring control of the time and the rate of MLF and reducing the potential for spoilage by other bacteria. *O. oeni* are preferably used thanks to their resistance to alcohol concentration, pH and SO_2 content of wine. Despite the clear benefits that selected MLF starters can deliver to the wine industry, only a small number of commercial starter cultures have been shown to successfully perform MLF, after the first *O. oeni* starter strain MLF was introduced in 1984. At the same time there is an increasing demand for new MLF starters with defined technological and flavouring properties, which will also meet proposed regulatory safety issues on biogenic amine production.

1.1.1 Unveiling the “wild” wine microbiota in Priorat region

In the prestigious winemaking region of Priorat in southern Catalonia (northeast Spain), mainly Carignan and/or Grenache cultivars are produced (Figure 1). 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. Priorat vineyards are characterized by high temperatures in summer (maximum temperature 35ºC), cold

winters (minimum temperature -4ºC) and low levels of rainfall (400-600mm/year), which yielded wines with high alcohol content. In this area, wines easily reach an ethanol content of 14% and sometimes higher (De Herralde et al., 2012). The low acidity of these wines together with the earlier fruit maturation patterns diminishes their L-malic acid content, thus restricting the growth of *O. oeni*.

Over the years, the emerging 'New World' wine-producing countries have led to fierce competition for wine market share. In addition, consumers' sophisticated preferences call for superior wines of distinct regional characteristics (terroir wines), as well as for wines made through natural and ecological procedures. There is also an increasing interest from consumers for biogenic amine-free wines. Those trends are mirrored by the raise in the production of ecological wine in the EU, the increased marketability of wines obtained without the addition of commercial yeast, the shift from table to superior wines and the introduction of limits on biogenic amine content in wine.

Figure 1: Grenache vineyard in Escaladei, close to Montsant mountains (Priorat, South Catalonia, Spain).

This raises an opportunity for 'Old World' wine-producing areas with peculiar terroirs and ancient tradition, like Priorat region, to make a dynamic comeback with the production of novel wines of ultra-premium quality that will be created according to natural operations. The use of indigenous *Saccharomyces* or non-*Saccharomyces* yeasts and LAB may offer a great potential in addressing the aforementioned critical issues in modern winemaking.

The preferences of consumers call for superior wines from a particular region to possess unique qualities and character (terroir wines) that differentiate them from wines of the same variety from other regions (Bisson et al., 2002). Wines perceived as being of high quality can be produced anywhere, even though, according to the concept of terroir, the local environment will influence the composition of the wine produced in a specific growing region (Gilbert et al., 2014; Zarraonaindia et al., 2015). Among other things, this involves the contribution of the indigenous microbiota in shaping the unique quality of the wine (Bartowsky et al., 2015). The trend of consumer preferences to the ecological wines represents an opportunity for traditional and peculiar terroirs.

This thesis is framed in the study of autochthonous microbiota in the Priorat region, carried out in the European Wildwine project FP7-SME-2012-Grant 315065. Four different countries took part in this project: Greece, Italy, France and Spain. From them, different universities and wineries worked together to perform this investigation. Château de Bellevue and Château Guiraud (France), Cavino (Greece), Araldica Vini Piemontesi s.c.a. (Italy) and Ferrer Bobet (Spain) were the wineries which participated, and the universities were the University of Turin (Italy), the University Victor Segalen Bordeaux II and the Entav-ITV (France) and finally, the University Rovira i Virgili (Spain). This whole European project objective was the achievement of multi-strain indigenous yeast and bacterial starters for 'Wild-ferment' wine production. In this sense, the use of native LAB as inoculum can offer great potential (Ruiz et al., 2010).

Today climate change poses a major additional problem for MLF. Over the last 10–30 years evidence of earlier fruit maturation patterns and consequently, modified vine development have been observed, both of which have been attributed to rising temperatures worldwide (Jones et al., 2005). The faster ripening of the grapes leads to a higher sugar content and therefore a higher ethanol content in the wines (Mira de Orduña, 2010; Webb et al., 2011).

1.2 Lactic acid bacteria biodiversity of the vine-wine ecosystem

1.2.1 From the grapes to the winery

Bacteria can readily colonize any surface including parts of the plant above the ground (Figure 2) affecting the health of their hosts in diverse ways. The surface of grape berries represents a complex natural reservoir of bacterial microbiota originating from the surrounding environment (Zarraonaindia et al., 2015). Grapevine bacteria play a key role not only in plant health, but also in crop quality and yields which can influence the winemaking process (Nisiotou et al., 2011; Verginer et al., 2010).

Many bacteria associated with grape surface cannot survive the extreme conditions of wine fermentation but their metabolic activity on the grape surface can have long-ranging consequences and they are undoubtedly included in the initial fermentation steps. Recently, Splivallo et al. (2014) demonstrated that bacteria associated with truffle-fruiting bodies contribute to truffle aroma. Thus, grape surface bacteria may play a significant role influencing the flavour, colour, and quality of the final product but this aspect still remains to be studied.

A study performed by Renouf et al. (2006) detected the most important species during all winemaking steps. It revealed the LAB population diversity on grape surface, in the fresh must and the predominance of *O. oeni* after the beginning of AF. During AF, production of ethanol and decrease of sugar concentrations is an additional selection pressure, illustrated by a LAB diversity decrease.

After AF and during MLF, analyses of dominant DGGE bands revealed that *O. oeni* was the most resistant bacteria detected in the wine, which is a well-known phenomenon (Kunkee, 1991). Cruz-Pio et al. (2017) demonstrated that the *O. oeni* isolates from grape must fermented more carbohydrates and were metabolically more diverse than the isolates isolated from wine. On the contrary, higher genomic diversity was found in the group of wine isolates, which demonstrates a high metabolic and genetic intraspecific diversity in *O. oeni*.

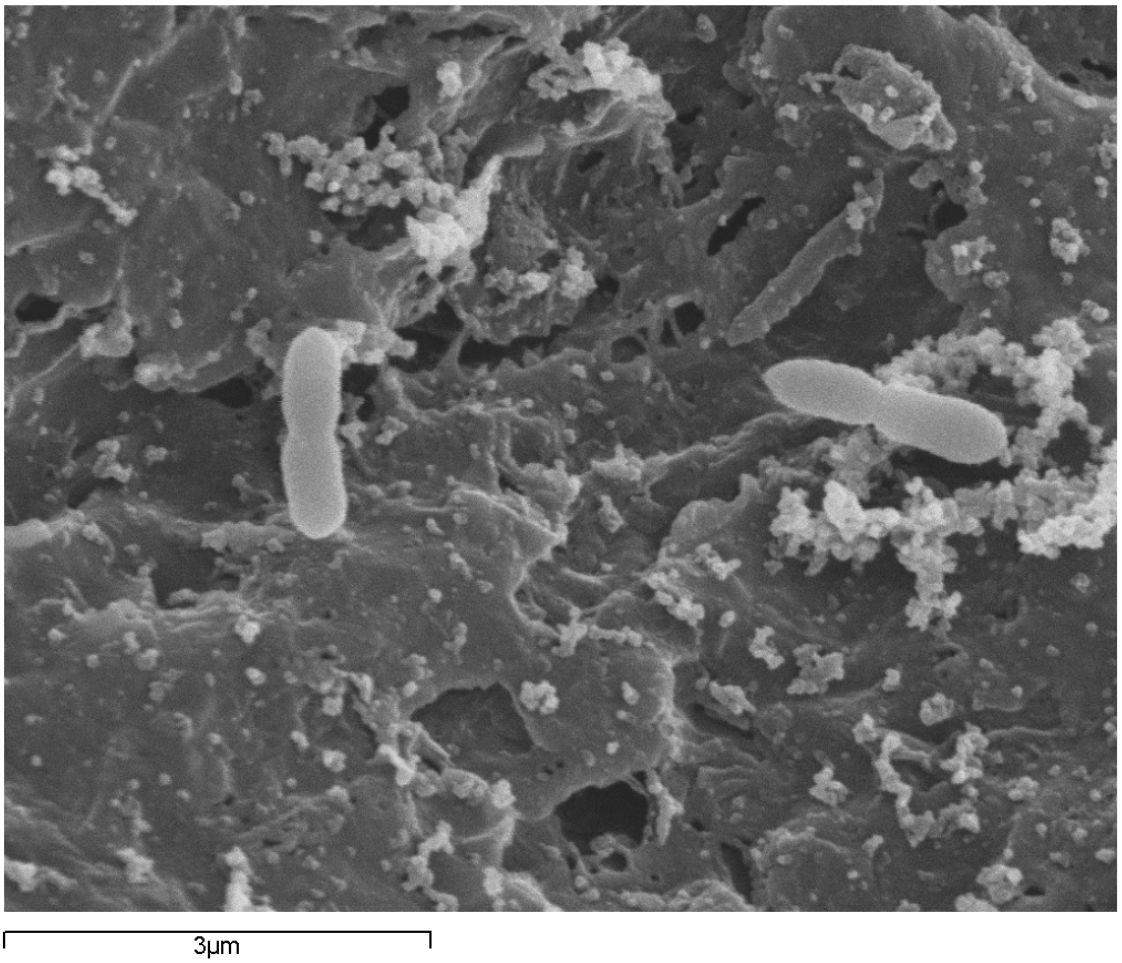

Figure 2: Bacilli in a sample of grape skin (SEM, Servei de Recursos Científics i Tècnics, URV).

1.2.2 The bacterial ecosystem found in must

Most research has concentrated on bacteria of oenological interest, like acetic acid and LAB present in the microbiota on grape berries (Bae et al., 2006; Nisiotou et al., 2011). *Oenococcus oeni* and some other LAB species are known to perform the MLF or to promote spoilage of wine depending on the species or strain.

Nevertheless, the role of other bacteria in wine fermentation has been mostly ignored (Barata et al., 2012). Furthermore, previous studies of grape-associated microbiota have been limited by methodological biases of culture-based techniques (Nisiotou and Nychas, 2007; Renouf et al., 2005, 2007) and low resolution of early molecular techniques (Martins et al., 2012). It is well reported that only a fraction of most environmental bacteria have been cultivated (Amann et al., 1995). Advances in massive

parallel short-amplicon (100-600bp) sequencing technologies have revealed a bacterial diversity of grape berries much more elevated than previously thought and important ecological questions on the grapevine microbiome are being answered (Bokulich et al., 2014; Leveau and Tech, 2011; Perazzolli et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Zarraonaindia et al., 2015). For example, from recent high-throughput studies we know that the bacterial community on leaves differed, both in size and structure, from that on berries (Leveau and Tech, 2011) and that soil serves as a key source of vine-associated bacteria with edaphic factors influencing the native grapevine microbiome, being the microbial community of soils from the same viticultural region quite heterogeneous (Zarraonaindia et al., 2015).

Furthermore, grape-associated microbial biogeography is nonrandomly associated with regional, varietal, and climatic factors across multiscale viticultural zones (Bokulich et al., 2014), fungi communities from the same vineyard can be highly variable (Setati et al., 2012) and the most abundant yeast at the beginning and the middle of the fermentation of grapes cultivated under different treatments were detected (David et al., 2014). However, so far no high-throughput study has investigated bacterial diversity of grape varietal communities within the same grapevine-growing region where climatic and regional factors are expected to be similar and produce wines of similar characteristics. Thus, it is ecologically relevant to know if bacterial communities of a single viticulture region are different and which factor influences the population changes.

The occurrence of various LAB as *Pediococcus*, *Lactobacillus* and *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 *Oenococcus oeni* directly from the grape berries (Garijo et al., 2011; Renouf et al., 2007), or from the grape juice (Saguir et al., 2009).

1.3 *Oenococcus oeni*

Louis Pasteur and Hermann Müller-Thurgau recognized the bacterial causes of MLF over a century ago (Müller-Thurgau, 1891; Müller-Thurgau and Osterwalder, 1913; Pasteur, 1873), but it was not until the mid 1960s that the organism responsible was

isolated, characterized and named *Leuconostoc oenos* by Ellen Garvie (Garvie, 1967). With the introduction of molecular techniques, however, a new genus, *Oenococcus*, was described, and *Leuconostoc oenos* was reclassified as *O. oeni*, originally the sole species within this genus (Dicks et al., 1995a), although later, another two species have been proposed: *O. kitaharae* (Endo and Okada, 2006), isolated from a composting distilled shochu residue, and *O. alcoholitolerans* (Badotti et al., 2014), isolated from cachaça fermentation and bioethanol plants. The three species are associated with different ethanol-containing environments, and they have different adaptive and metabolic capacities.

The genus *Oenococcus* belongs to the phylum *Firmicutes*. This Gram positive bacterium is catalase negative, microaerophilic, meaning that it grows best at low oxygen concentrations, obligately heterofermentative (glucose is fermented to D-(-)-lactic acid, CO_2, and ethanol or acetate) and acidophilic (Dicks et al., 1995a).

The pan-genome of *O. oeni* presents low GC% content, as with other Gram positive bacteria, and it has a small size (1.8 Mb) compared with other LAB species like *Lactobacillus plantarum* (3.3 Mb) or the main model of Gram positive bacteria *Bacillus subtilis* (4.1 Mb).

To date, June 2018, 219 different genomes of *O. oeni* are available in the database of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). PSU-1 strain was the first sequenced genome from this species (Mills et al., 2005) and nowadays it is the only complete annotated genome published.

1.3.1 What makes *O. oeni* the best MLF candidate ?

O. oeni is the species that is best adapted to wine conditions and it is usually found in wines during 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 are not always successful as they are limited under the harsh environmental conditions of wine (Coucheney et al., 2005; Spano and Massa, 2006; Ruiz et al., 2010), mainly the presence of ethanol.

Such as other wine LAB, *O. oeni* has fastidious nutritional requirements. Some studies (Peynaud et al., 1965; Terrade and Mira de Orduña, 2009) reflect the larger number of

amino acid requirements for oenococci than lactobacilli, and a slower growth rate, too. However, this species, due to selective pressure, has developed adaptive strategies that enable it to out-compete other potential MLF bacteria during the later stages of vinification and thus dominate in wine. It is, for example, well adapted to high ethanol concentrations (<15% v/v), low pH (as low as 2.9) and limited nutrient availability (Bartowsky, 2005). *O. oeni* is the most resistant to ethanol; however, at concentrations >12 % (v/v), ethanol can affect growth and malolactic activity (Capucho and San Romão, 1994, Zapparoli et al., 2009).

Moreover, other typical harsh conditions of wine like few nutrients, phenolics, low pH, restrict cell growth such that MLF is sluggish or even fails (Carreté et al., 2002). To survive and adapt to this harsh environment, *O. oeni* has various strategies, including the production of ATP by consuming organic acids (mainly L-malic, but also citric acid), the synthesis of stress proteins (Beltramo et al., 2004), and modifications in the composition of its membranes (Silveira et al., 2004).

1.3.1.1 Resistance to harsh conditions

Bacteria recognize different environmental changes and can trigger an appropriate response, which commonly is a simultaneous reaction to a wide variety of stresses. Also, the various cellular systems interact with each other by a complex global regulatory network leading the cell to equilibrium under different conditions.

The oldest and simplest method of identifying superior strains is to take advantage of natural diversity, isolating strains from nature and screening them for desired traits. Originally fermentations were typically optimized through inoculation via a small quantity of a previously performed, successful fermentation (*pied de cuve*). These successive inoculations have created populations of LAB that are suited specifically to the particular fermentation environment.

O. oeni is a prime example of a LAB evolved to occupy a very specific ecological niche, explaining its relative tolerance to the fluctuating environment of alcoholic fermentation and the harsh conditions of wine in which it must survive. Intraspecific diversity among different strains isolated from wineries worldwide has been

observed (Bridier et al., 2010) implying diversity in the tolerance to different stress conditions.

As mentioned, the multiple stresses contained in wine provoke a complex response of *O. oeni*. Among the stress response mechanisms of *O. oeni* (Figure 3), in this section it will be considered mainly the effects of two wine parameters, ethanol and low pH, due to their relevant inhibitory effect on *O. oeni*.

The ethanol toxicity is generally attributed to the partitioning of ethanol in the hydrophobic lipid bilayer, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes (Lonvaud-Funel et al., 1988). Moreover, the effects of ethanol in *O. oeni* cells and have shown that ethanol acts as a disordering agent of the *O. oeni* cytoplasmic membrane (Silveira et al., 2002, 2003) and negatively affects metabolic activity, as it promotes the low pH effect, boosting the proton entrance.

Low pH appears as a crucial parameter that limits bacterial growth in wine and consequently the desired MLF activity. Moreover, low pH is linked to two requirements for *O. oeni* to survive and function in wine: the activation of a proton-extruding ATPase and the proton motive force (PMF) generated by MLF. The ATPase membrane system is well known for its key role in the acid tolerance of bacteria. Induction of its activity and expression has been associated with increased resistance to low pH (Kobayashi, 1985) due to the maintenance of the intracellular environment and the control of the energetic status of the cell through the PMF. Another important requirement for *O. oeni* cell is the activation of several genes and their corresponding synthesis of stress proteins.

Cellular redox systems like glutathione and thioredoxin are activated during the acclimation of *O. oeni* to harsh wine conditions. It has been observed that both glutathione (Margalef-Català et al., 2016b, 2017c) and thioredoxins (Guzzo et al., 2000; Margalef-Català et al., 2017b; Renouf et al., 2008) are involved in the stress response and may have a protective role, too.

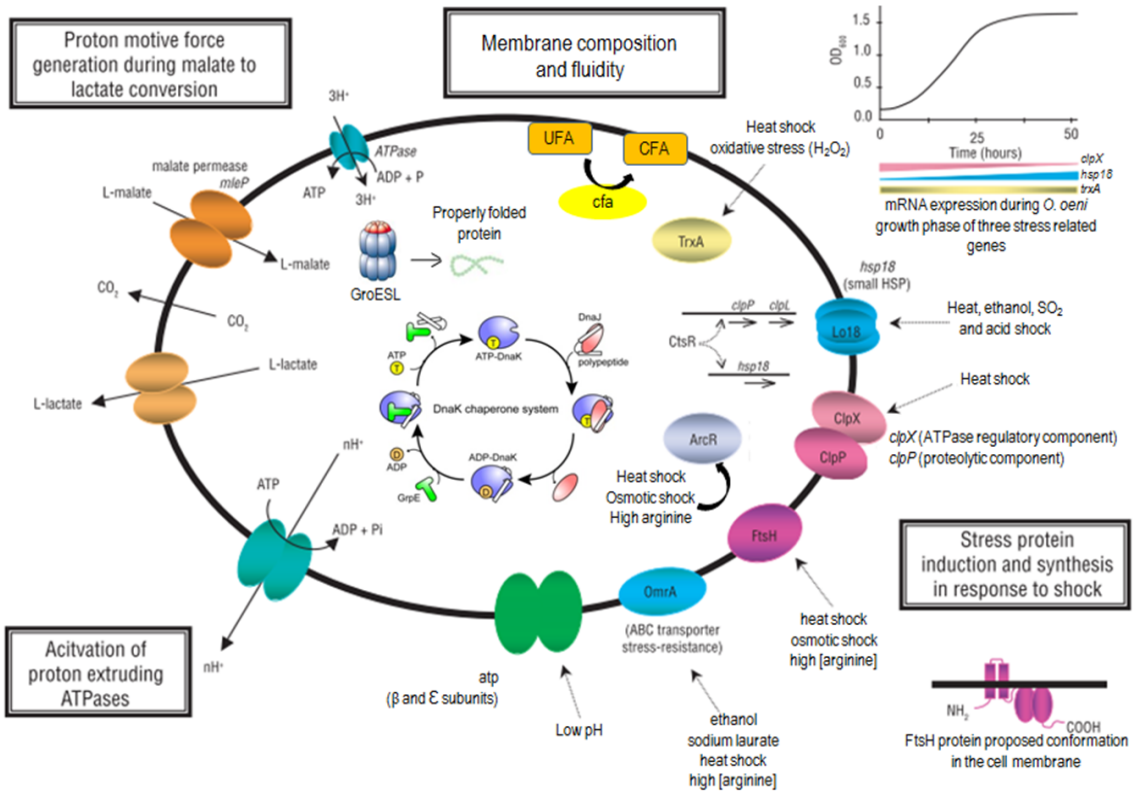

Figure 3. Mechanisms that are important in conferring, in *O. oeni*, the ability to survive in wine: the membrane composition, the proton motive force generated by malic acid metabolism, the activation of proton-extruding ATPase, and the stress protein induction and synthesis in response to shock. Adapted from Bartowsky (2005).

1.3.2 *O. oeni* diversity

Some studies described *O. oeni* is phylogenetically very homogeneous, as revealed by the homologous DNA fragments analysed, according to the ISR sequences (Zavaleta et al., 1996) and the global DNA homology reported (Dicks, et al., 1990, 1995b). The main difference in the genome appears to arise from the occurrence of two forms of DNA arrangement during the relatively short history of this species, as demonstrated by several analyses at the genomic organization level. Although data indicate that *O. oeni* follows a closely clonal model of evolution (Selander et al., 1994) genetic transfer may occur, as suggested by results of plasmid analysis (Zavaleta et al., 1997).

It has been proposed that *O. oeni* (formerly *Leuconostoc oenos*) represents a fast-evolving organism since, compared to all other members of the genus *Leuconostoc* and other LAB, considerable numbers of nucleotide positions in the 16S rRNA show an

unusually high rate of substitutions accompanied by an atypical phenotype and no significant DNA-DNA similarity (Yang et al., 1989). Results from Zavaleta et al. (1997) showed that homologous sequences, including noncoding DNA apparently not subjected to selection, are, unexpectedly, identical among strains that revealed considerable divergence of the genomic organization and a different plasmid content.

It is possible that selective pressure on *O. oeni* was strong over the period when adaptation to the wine-making environment was achieved. Nevertheless, the hypothesis of rapid evolution can be sustained only if we consider this microorganism, at the present stage, to be a true *Leuconostoc*. For example, a single microorganism belonging to a different genus at a relatively long phylogenetic distance from *Leuconostoc* (such as *Lactococcus* or *Streptococcus*) also shows a relatively unusual 16S rRNA sequence and different phenotype. Since this organism has been considered a member of a different genus, *Oenococcus*, the same considerations apply.

Marcobal et al. (2008) suggested that the genus *Oenococcus* is hypermutable due to the loss of the mismatch repair pathway (genes *mutS* and *mutL*), which occurred with the divergence away from the *Leuconostoc* branch. This would explain the observed high level of allelic polymorphism (de Las Rivas et al., 2004; Delaherche et al., 2006; Zé-Zé et al., 2008) among known *O. oeni* isolates and likely contributed to the high ecological competitiveness of this genus to acidic and alcoholic environments.

Several studies have generated controversial data related to the intraspecific taxonomic structure of *O. oeni*, too. Lactate dehydrogenases, carbohydrate fermentation, and cellular fatty acid patterns have shown considerable diversity among strains of *O. oeni* (Garvie, 1967; Garvie, 1969; Garvie, 1986; Garvie et al., 1980; Tracey and Britz, 1987, 1989). Pulsed-field gel electrophoresis (PFGE) has shown the existence of 20 genomic patterns in *O. oeni*, and the strains used fell in two major groups (Tenreiro et al., 1994).

Moreover, numerous studies based on molecular methods were performed during the past 30 years to evaluate the diversity of *O. oeni* strains in regional wines. They revealed that there is a huge diversity in each region, with up to 10 different strains simultaneously in the same vat during MLF (Bilhère et al., 2009; Bridier et al., 2010; Cappello et al., 2010; López et al., 2007; Sternes and Borneman, 2016). However, some strains persist in the cellar during several consecutive vintages (Gónzalez-Arenzana et al., 2015; Kelly et al., 1993; Larisika et al., 2008; Reguant and Bordons, 2003). Until

recently it was unclear whether strains were specific to a region or not. This is an important issue for determining whether they contribute to the unique properties of regional wines, and if they can be considered as a microbial component of the terroir. Recent surveys have shown that strains present in a region may belong to different genetic groups (phylogroups A and B, as described above) and that they ferment the local wines more or less efficiently (Bordas et al., 2013; Garofalo et al., 2015; Gónzalez-Arenzana et al., 2014).

A comparison of nearly 3,000 *O. oeni* isolates from different vineyards has confirmed that there is a considerable strain diversity in the regions and that each region holds a unique set of several hundreds of strains, which is in agreement with previous studies suggesting that vineyards represent different microbial terroirs (Bokulich et al., 2014; Knight et al., 2015). However, the strains present in a region belong to different genetic groups, some of which are also detected in distant locations, indicating that they are not genetically exclusive to any particular region (El Khoury et al., 2017). In contrast, there are clear cases of adaptation to different products (cider, wine) or different types of wines (white wines from champagne) (Campbell-Sills et al., 2015; El Khoury et al., 2017).

Like other industrial species of microorganisms, phenotypic variation in *O. oeni* will have direct economic consequences through impacts on product quality and production efficiencies. A thorough understanding of the basis of this variation therefore provides the means to improve the industrial performance of these strains or to easily screen for new strains with multiple, desirable traits. Borneman et al. (2012**)** provide a solid foundation for the investigation of phenotypic diversity in *O. oeni* by providing whole-genome sequences for a large cohort of strains from both commercial and environmental sources.

Significant variation across the strains that were investigated were identified in Borneman et al. (2012), including differences in cell wall synthesis and sugar utilization, that were largely due to differential insertion of large, multi-genic nucleotide fragments. These differences can be used to inform research on the industrial implications of this genetic variation while allowing for the identification of strains with combinations of desirable genetic, and therefore phenotypic, characteristics.

1.4 Malolactic fermentation

LAB are responsible for many fermented foods and they have been isolated from wine at various states of vinification (Du Plessis et al., 2004; Gindreau et al., 2001). These bacteria normally undertake the malolactic fermentation (MLF) spontaneously. However, LAB viability depends on the capacity of the cell to adapt to wine conditions.

Even though the existence of other wine species as *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo et al., 1985), and despite the fact that recent red wine MLF trials with strains of *Lactobacillus plantarum* have been performed (Bravo-Ferrada et al., 2013; Lerm et al., 2011), *Oenococcus oeni* is the principal bacterium responsible for MLF (Lonvaud-Funel, 1999). As its name implies, *Oenococcus oeni* holds major importance in the field of oenology due to the organoleptic and microbiological changes produced in wine.

The removal of L-malic acid, one of the major energy sources in wine, during MLF reduces the risk of the growth of spoilage microorganisms. Also, MLF ameliorates acidity and further contributes compounds that result in wine of increased aroma and flavour complexity. Most well described is diacetyl; however, the production of esters, alcohols, and other carbonyl compounds contribute to the buttery, spicy, vanilla, and smoky notes as well as a softer, fuller mouthfeel seen in wines post-MLF (Malherbe et al., 2012; Sumby et al., 2010).

As it can be seen in Figure 4, apart from its deacidification, other benefits have been attached to MLF as the removal of a potential substrate for spoilage bacteria, which imparts microbial stability to wine (Maicas et al., 1999) and the decrease in titratable acidity, which can influence the sensory properties of wine by decreasing sourness (de Revel et al., 1999). The responsibility for the conversion of L-malate to L-lactate is a single enzyme, malate decarboxylase (MleA), which is often referred to the malolactic enzyme (Kunkee, 1991). This conversion indeed produces CO_2, which escapes from wine by bubbling. While the active transport of L-malic acid into the cell is performed by malate permease (MleP), the lactate transport out of the cell is still unclear. Moreover, all of the MLF process is regulated by a regulatory protein, MleR (Betteridge et al., 2015).

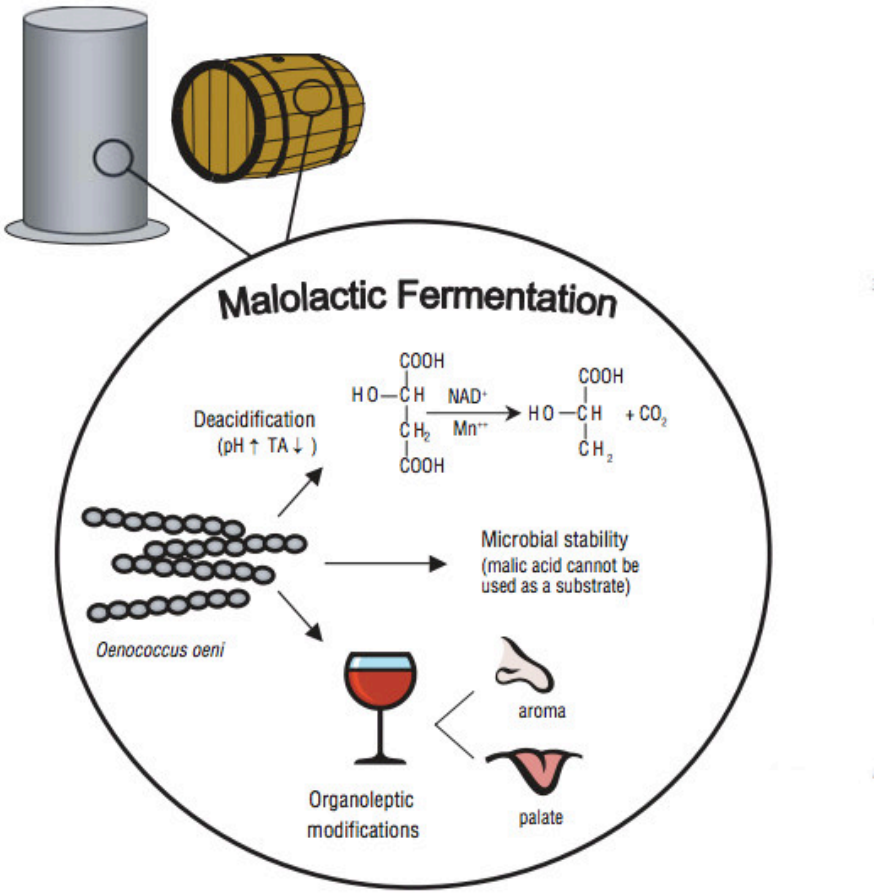

Figure 4. The three main consequences of MLF: microbial stability of the wine through the removal of a possible carbon source (L-malic acid) for other microorganisms, deacidification of wine (increase in pH [0.1–0.2 units] and decrease in titratable acidity [TA]), and bestows sensory changes (aroma and palate) in the wine (Bartowsky, 2005).

1.4.1 Biochemistry and genetics of the MLF

In general terms, MLF is the bacterial-driven decarboxylation of L-malic acid to L-lactic acid and CO_2, (Bartowsky, 2005; Davis et al., 1988; Liu S.Q., 2002; Lonvaud-Funel, 1999). More specifically, MLF is technically not a fermentation but the enzymatic decarboxylation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid by LAB (Figure 5). In a reaction requiring NAD^+ and Mn^{2+} as cofactors, the malolactic enzyme (EC 1.1.1.38) of the lactic acid bacteria enables direct conversion of L-malic acid into L-lactic acid and carbon dioxide (Battermann and Radler, 1991; Caspritz and Radler, 1983; Naouri et al., 1990).

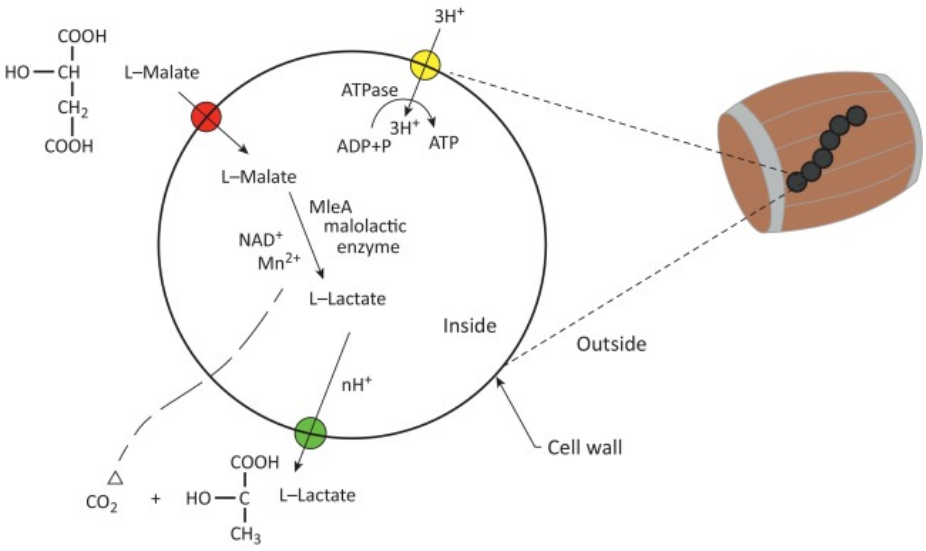

Figure 5. MLF involves the active transport of L-malic acid into the cell by malate permease (MleP, red). Decarboxylation of L-malic acid is facilitated by the malolactic enzyme (MleA) and requires NAD^+ and Mn^{2+} as cofactors before lactate is finally transported out of the cell (green). This process is controlled by a regulatory protein, MleR. The increase in the intracellular pH by MLF confers an energy advantage to the cell. The resulting increase in the proton motive force across the cell membrane combined with specific ATPases (yellow) facilitates the production of ATP (Betteridge et al., 2015).

Although MLF increases the pH of the wine, this increase does not stimulate the growth of *O. oeni*. The three genes responsible for this fermentation are present in a single cluster, with *mleA* (encoding malolactic enzyme) and *mleP* (encoding malate permease) on the same operon and *mleR* encoding the regulatory protein transcribed in the opposite direction. Maximal activity of MleA is seen at pH 5.0 and 37 °C and is noncompetitively inhibited by ethanol, underscoring the less-than-ideal nature of the wine environment (Betteridge et al., 2015). Moreover, as it is shown in Figure 5 the energy obtained through the use of malate produces a sufficient PMF for the synthesis of ATP (Cox and Henick-Kling, 1995).

The main substrates of the metabolism of LAB in wine are L-malic acid, citric acid, and pentose and hexose traces (Davis et al., 1985; Kunkee, 1974). Some research is related to the conditions of the synthesis of these compounds (de Revel et al., 1989) and to their contents in wines and their organoleptic consequences (Bertrand et al., 1984; de Revel

and Bertrand, 1993, 1994; Henick Kling, 1995). The reduction of dicarbonyl compounds into hydroxy ketones and diols has also been studied (de Revel, 1992).

The conversion of L-malic acid carried out by *O. oeni*, starts when its population reaches 10^6 CFU/ml after the alcoholic fermentation. *O. oeni* leads to the transformation of L-malic acid to L-lactic acid through an enzymatic decarboxylation (Peynaud and Domercq, 1968). Also, during its activity in wine, *O. oeni* cells can ferment residual sugars, hexoses and pentoses left by yeasts (Ribéreau-Gayon et al., 2006). Moreover, the production of secondary metabolites increases the sensory qualities of the final product (Malherbe et al., 2012; Sumby et al., 2010).

1.4.2 Factors influencing the MLF

Over centuries of selective pressure, *O. oeni* has adapted to high ethanol concentrations (<15% v/v), low pH (as low as 2.9) and limited nutrient availability; hostile conditions typical of wine. However, despite being *O. oeni* the species more adapted to wine, the induction of this fermentation remains problematic (Reguant et al., 2005a).

O. oeni starters deal with several stresses including low temperature, SO_2 concentration, short-chain fatty acid presence, phenolic compounds, low pH and ethanol content (Davis et al., 1988; Lonvaud-Funel, 1999; Spano and Massa, 2006). Depending on the wine production area, the composition of L-malic acid will also be different, from 0,5 to 10 g/L (Lonvaud-Funel, 1999; Bordas et al., 2013).

The action of all these factors on *O. oeni* has been studied in order to enhance the knowledge of the cellular adaptation this bacterium. Low temperatures affect growth rate and increase lag phase (Fugelsang, 1997). Sulphur dioxide reduces ATPase activity and decreases cell viability (Carreté et al., 2002; Reguant et al., 2005a). Phenolic compounds can produce breakdown of the LAB cell membrane (García-Ruiz et al., 2011) or can affect *O. oeni*'s growth and metabolism in other different ways depending on their type and concentration (Reguant et al., 2000). Generally, the most common phenolic compounds like phenol carboxylic acids, such as gallic (3,4,5-trihydroxybenzoic acid), caffeic (3,4-dihydroxy-cinnamic acid), ferulic (3-methoxy-4-hydroxy-cinnamic acid) and *p*-coumaric (4-hydroxy-cinnamic acid), and flavonoids, such as catechin (a proanthocyanidin: cyanidanol) and quercetin (a flavonol: 3,3′,4′,5,7-

pentahydroxyflavone), have no effects at low concentrations, but hydroxycinnamic acids are inhibitory at high concentrations, and the effects were greatest for coumaric and ferulic acids (Reguant et al., 2000; Stead, 1993). On the other hand, the presence of gallic acid seems to stimulate growth of *O. oeni* (Reguant et al., 2000; Vivas et al., 1995). Malolactic fermentation can be stimulated in the presence of catechin and quercetin, but increasingly delayed with increasing amounts of *p*-coumaric acid (Cornu et al., 1984; Reguant et al., 2000). Gallic acid appears to delay or inhibit the formation of acetic acid from citric acid (Reguant et al., 2000).

Yeast fatty acids such as decanoic and dodecanoic acid are powerful inhibitors of LAB growth because, like ethanol, they alter the bacterial membrane (Lonvaud-Funel et al., 1988). Finally, low pH reduces *O. oeni* growth and malolactic activity (Tourdot-Maréchal et al., 1999). Other difficulties in MLF have been ascribed to phage attack (Gindreau and Lonvaud-Funel, 1999; Poblet-Icart et al., 1998). However, as the phages readily disappear through inactivation by wine components, it seems that they are not responsible for influencing MLF (Lonvaud-Funel, 1999).

MLF and the growth of *O. oeni* are clearly inhibited by several of the physiochemical properties of wine. The four main wine parameters inducing stress and affecting MLF are ethanol (can exceed 15% v/v), low pH (typically less than 3.5), SO_2 (over 10 mg/L), and low temperature (can be below 12°C) (Table 1). These stressors have various cellular targets and mechanisms, which often work in combination to produce a more severe impact on growth or the enzymes involved in MLF. For example, in exploring the individual impacts of acid (pH 5.5 to pH 3.5), ethanol [0–10% (v/v)] or cold shock (30°C to 14°C) on membrane fluidity (Chu-Ky et al., 2005), near-total loss of cell viability could be demonstrated after only 30 min of exposure to a combined wine-like acid (pH 3.5) and ethanol [10% (v/v)] environment.

The interaction between yeasts, both *Saccharomyces* and no-*Saccharomyces*, and LAB can affect the MLF performance, depending also on the strain of each one (Su et al., 2014). Some of the effects of these inhibitory interactions could be explained as the result of nutrient competition, such as yeast assimilable nitrogen or amino acids (Costello et al., 2003). SO_2 and medium chain fatty acids are inhibitor compounds in the antagonism between yeast and *O. oeni* as well (Nehme et al., 2008). The release of

mannoproteins during the autolytic process of the yeasts can stimulate LAB growth (Diez et al., 2010)

Improved tolerance of such abiotic stress would appear to be beneficial in increasing the efficiency of MLF. Experimental evidence supports this, since *O. oeni* strains performing faster MLF also show increased relative expression of several stress response genes (Olguín et al., 2010). Similarly, the better-performing strains also showed an increased expression of *mleA*, if only its importance was greatest for determining the initial MLF velocity.

Table 1. Key inhibitors in wine of MLF and their mechanisms of inhibition. Adapted from Betteridge et al. (2015).

Inhibitor	Comment	Optimal condition	Typical wine conditions	Inhibitory mechanism	References
Ethanol	Produced during alcoholic fermentation	Up to 5% stimulates growth	12-15% (v/v)	Disrupts cell membrane structure and alters fluidity	Da Silveira and Abee, 2009
Low pH	Acidity from grape berries and winemaker intervention	4.8-5.5	2.5-3.5	Reduces growth and malolactic activity	Tourdot-Maréchal et al., 1999
Low temperature	Wineries often rely on ambient temperature for MLF	25°C	12-20°C	Affects growth rate and increases lag phase	Fugelsang, 1997
SO_2	Produced by yeasts and added to prevent spoilage during processing	0 mg/L	10-70 mg/L	Reduces ATPase activity, decreases cell viability	Carreté et al., 2002

1.4.3 Management of the MLF

The development of MLF is a difficult and time-consuming process that does not always proceed favourably under the natural conditions of wine (Maicas, 2001).

Most of the commercial starters for MLF contain strains of *O. oeni*, distributed by several companies: Lallemand and its related Danstar, Chr. Hansen, Laffort, Agrovin, Enartis, Oenobrands, Scott Lab., Bioprox, Wyeast, 2B-FermControl, and others. In order to increase the possibilities of success, the same companies also offer nutrients and preservatives that can help to activate the MLF. Until freeze-dried starter culture was available for direct inoculation (Nielsen et al., 1996), commercial preparations required reactivation before inoculation into wine (Nault et al., 1995).

Additional new strategies such as co-inoculation, using mixed LAB cultures, immobilized cells or immobilized enzymes have the potential to reduce the duration of MLF and risks associated with sequential MLF.

Starter cultures can be co-inoculated with yeast (at the beginning or toward the end of AF), or sequentially (after AF) (Bartowsky et al., 2015). Generally, it has been demonstrated that bacteria inoculated in must performed better than those inoculated after AF, especially when cell growth conditions are not favourable (Azzolini et al., 2010).

Some *Lactobacillus* species have also showed the ability to survive the harsh wine conditions; the species *Lactobacillus plantarum* has shown the most potential as a starter culture (Berbegal et al., 2016; Iorizzo et al., 2016; Lerm et al., 2011; Lucio et al., 2016, 2017). This versatile bacterium tolerates ethanol up to 14% v/v and has similar SO_2 tolerance of *O. oeni* (Cappello et al., 2017). The introduction of some *L. plantarum* strains to the fermenting musts could significantly modify the wine aroma profile due to a different enzymatic profile. Due to these characteristics, some of the malolactic starters contain strains belonging to other LAB species, mainly *Lactobacillus plantarum*, such as Viniflora® plantarum from Chr. Hansen, or ML PrimeTM from Lallemand.

Some years ago, the interest in using immobilized cells in the wine making process arose, as these strategies offer numerous technical and economic advantages, compared to the conventional free cell system (Nedović et al., 2011). Lactic acid bacteria have also been immobilized with positive results. Several immobilization systems have been studied for MLF or simultaneous AF and MLF, such as alginate beads (Bleve et al., 2016), delignified cellulosic materials (DCM) (Agouridis et al., 2005, 2008) and

DCM/starch gel composite biocatalyst (Servetas et al., 2013). Recently, the encapsulation of bacteria into a Si–Al gel has been achieved, and it is an exceptional application for the enhancement of MLF in red wines (Simó et al., 2017).

A membrane reactor using free *O. oeni* enzyme was developed by Formisyn et al. (1997). This process requires the availability of manganese (Mn^{2+}) and NAD^+, which act as cofactors (Davis et al., 1985; Kunkee, 1997; Schümann et al., 2013). Immobilized enzymes allow for continuous processing, which in turn can lead to lower production costs and energy consumption. A further benefit when compared with free enzymes in solution is that immobilized enzymes are more robust and are often more stable and resistant to environmental changes (Krajewska, 2004). Köhler et al. (2013) reported a method for efficient concurrent tandem catalysis that could potentially be used to enable utilization of Mle catalysis with regeneration of NAD^+.

Expression of malolactic enzyme can be performed in yeast. In this case, the malic enzyme predominately converts malic acid into pyruvic acid, which is further metabolized to ethanol and carbon dioxide under fermentative conditions via the maloethanolic pathway (Volschenk et al., 2003). Two tactics have been utilized to express malolactic enzyme in *S. cerevisiae*: co-expression of the malate permease gene and surface display of malolactic enzyme. The malate permease gene (*mae1*) of the fission yeast *Schizosaccharomyces pombe* has been co-expressed with either the *L. lactis* malolactic gene (*mleS*) or the *O. oeni* malolactic gene (*mleA*) in *S. cerevisiae* (Husnik et al., 2006, 2007; Volschenk et al., 1997). *Schizosaccharomyces pombe* species completely transform the malic acid of the must into ethanol, thanks to its particular metabolism of maloalcoholic fermentation (Loira et al., 2015). In this respect, *S. pombe* perform effective malic acid deacidification and significantly reduces the levels of biogenic amines and ethyl carbamate precursors without the need for any secondary bacterial MLF (Benito et al., 2014, 2016).

The application of similar commercial bacterial starters across different world regions may lead to certain product uniformity (Mas et al., 2016). Hence, the application of an autochthonous starter culture, well adapted to the conditions of a specific wine-producing area, has already been suggested (Nielsen et al., 1996; Ruiz et al., 2010). For this reason, several studies have been performed on the characterization of *O. oeni*

biodiversity with the aim of selecting putative autochthonous starter cultures (Bordas et al., 2013; Capozzi et al., 2010, 2014; El Khoury et al., 2017; González-Arenzana et al., 2014; Lamontanara et al., 2014; Mesas et al., 2011; Reguant and Bordons, 2003; Solieri et al., 2010; Wang P. et al., 2016), and this diversity is important within the same location (Cappello et al., 2010; López et al., 2007).

1.4.4 Selection criteria of *O. oeni* strains for wine inoculation

Nowadays, the trend in selecting *O. oeni* strains is the practice of isolating indigenous malolactic bacteria from the same cellar or its environment to develop starter cultures that can be used to enhance the regional identity of wines. There is evidence that regional branding is an effective means of a point of difference for marketing purposes, and especially because is mounting evidence that the local microbiota contributes to a wine's terroir (Bartowsky et al., 2015).

Selection of strains for wine inoculation is usually performed by classical tests based essentially on the survival in wine and monitoring the consumption of L-malic acid (Henick-Kling et al., 1989). Some of the criteria to be taken into account when developing bacterial cultures are listed in Table 2 (Torriani et al., 2011) and include resistance to ethanol and SO_2, resistance to bacteriophages (Poblet et al., 1998), ability to grow at low pH levels, no health hazard for the end consumer, and resistance to technological stress (freezing, freeze-drying, hydration, and inoculation into wine), among others (Henick-Kling, 1995).

Additional criteria are that strains have a high malolactic performance in different types of wine, the production of desirable flavours, a low acetic acid production, no production of ropy polysaccharides neither off-flavours, and a good compatibility with the yeast strains used for alcoholic fermentation (Torriani et al., 2011). Knowledge of *O. oeni* physiology in stress conditions can be used to generate tools based on molecular and physiological approaches allowing more precise characterization of strains. Among the metabolic and enzymatic systems that could be used to this end, L-malic acid metabolism and ATPase activity are of great interest (Coucheney et al., 2005).

Table 2. Guidelines for the selection of commercial malolactic starters for oenological applications (Torriani et al., 2011).

Categories	Property
Stress resistance	Resistance to high levels of ethanol (14% v/v)
	Tolerance to pH 3.0
	Resistance to high SO_2 concentrations
	Resistance to low temperatures
	Bacteriophages resistance, not lysogenic
Technological performances	High malolactic activity
	Ability to perform MLF in different types of wine
	Satisfactory growth in a synthetic medium
	Production of desirable flavours or enhancement of fruity aromas
	Low acetic acid production at the pH of grape juice and wine
	No production of ropy polysaccharides
	No production of off-flavours
	Compatibility with the yeast used for the alcoholic fermentation
	Can be freeze-dried
Safety	No production of biogenic amines
	No production of ethyl carbamate
	Inability to transmit antibiotic resistance genes

Furthermore, the use of a selected malolactic starter should be used to avoid the presence of biogenic amines (BA) in wines. BAs are organic bases, endowed with biological activity, that are commonly present in living organisms. Some of the symptoms that they may cause are headache, respiratory distress, heart palpitation, hyper- or hypotension, and several allergic reactions (Silla-Santos, 1996). MLF is the main mechanism of BA formation, especially of histamine, tyramine, and putrescine (Marcobal et al., 2006), being the LAB strains the main organisms responsible for BA

accumulation, especially for tyramine and histamine (Gardini et al., 2005; Landete et al., 2005; Lonvaud-Funel, 2001). Strategies based on PCR amplification of the corresponding genes have been designed to detect BA-producing LAB (de las Rivas et al., 2006; Landete et al., 2007, 2011), in order to apply early control measures to avoid the development of these bacteria.

1.5 Identification and characterization of the *O. oeni* and other non-*Oenococcus* strains in wine environment

1.5.1 Molecular methods for LAB species identification and strain typing

1.5.1.1 Culture-dependent techniques

Culture-dependent techniques need first to culture the cells and then identify or quantify them with the DNA extracted from isolated colonies, using a molecular method. These techniques are more reliable than phenotypic methods, because the identification and/or quantification is more accurate.

Nevertheless, they may often fail to characterize minor populations or microorganisms, for which selective enrichment is necessary, stressed or weakened cells often need specific culture conditions to recover and to become culturable, and all the microorganisms which are not culturable will not be detected. Moreover, the variability of isolated samples (in this case grape and wine) is higher. Furthermore, the identification of *O. oeni*, the species most common in wines, is especially difficult because it has a slow growth, there is a large variability in some tests, and this species has remarkable poor ability for sugar fermentation. Therefore, it is advisable to use molecular methods, which are reliable, accurate and fast.

Species identification

In order to identify whether an isolate is *O. oeni*, one of the most commonly used method is a species-specific PCR, although there are many molecular techniques available. This method is performed with specific primers (On1 and On2) for a gene

fragment of the malolactic enzyme of *O. oeni* (Zapparoli et al., 1998), and it gives a 1025 bp amplified DNA, easily detectable by electrophoresis.

One of the most practical methods for identifying different species of LAB is 16S-ARDRA, used also for a variety of microorganisms. In this method, 16S rDNA is amplified by PCR with specific primers and then digested by restriction enzymes *Mse*I, *Bfa*I, and *Alu*I (Rodas et al., 2003). In this way, these authors were able to discriminate 32 LAB species by their band profiles, including several *Lactobacillus* species, *L. mesenteroides*, *O. oeni*, *Pediococcus parvulus*, and *P. pentosaceus*. In case of doubtful profiles, it is useful to sequence the fragment 16S rDNA to confirm the species.

Lb. plantarum is one of the most frequent species found in grape and wine environments, but sometimes, it is difficult to differentiate it from the other two genotypically closely related species: *Lb. pentosus* and *Lb. paraplantarum*. For differentiating them, the best method is using the multiplex PCR assay with *recA* gene–derived primers, designed by Torriani et al. (2001). The RecA protein is implicated in DNA recombination and its gene is ubiquitous, having been proposed as a good phylogenetic marker for related species.

Strain typing

Since it is necessary to recognize which strain is the one isolated and identified, typing it is the next step (Carreté et al., 2006; Reguant et al., 2005a, b; Ruiz et al., 2010).

Several methods have been developed to type strains of *O. oeni*, among which two have to be highlighted. The first is the total DNA macrorestriction and separation of large fragments obtained by pulsed-field gel electrophoresis (PFGE) (López et al., 2008; Ruiz et al., 2008; Zapparoli et al., 2000), which provides specific profiles of bands very reproducible and easy to analyse for each strain, but it is quite a laborious technique.

The second one is the technique of random-amplified polymorphic DNA (RAPD)-PCR, which includes an arbitrary single short oligonucleotide (10–15 bp) as primer. Each strain presents amplification fragments that are different in size and number. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be characteristic of a particular strain. It is simple, quick and very discriminatory between strains. The only drawback is its low reproducibility. For increasing the reproducibility, this method was optimized with a RAPD multiplex

technique (Reguant and Bordons, 2003), where two primers are used simultaneously: one of the most discriminant primers already used in RAPD, called Coc (Cocconcelli et al., 1995), along with one of the two primers of the aforementioned species-specific PCR, called On2 (Zapparoli et al., 1998). This multiplex method yields a profile of discriminating bands for each strain that is reproducible in different trials. Another method related to this multiplex PCR has been developed that allows the simultaneous species identification and strain typing of *O. oeni* (Araque et al., 2009b), based on the combined use of the earlier-mentioned species-specific PCR primers with the other mentioned primer Coc used in RAPD-PCR analysis.

Another good and accurate method for typing *O. oeni* strains is using multilocus variable number of tandem repeats (VNTRs). This technique is based on the presence of VNTRs at a specific locus due to DNA polymerase slippage during replication. The complete TR is amplified and sized using a capillary electrophoresis system. It has been successfully used for typing an extensive collection of *O. oeni* strains (Claisse and Lonvaud-Funel, 2012), and recently improved by multiplexing amplifications in two separate PCR mixtures for five loci, taking advantage of the high performance of capillary electrophoresis (Claisse and Lonvaud-Funel, 2014).

The analysis of the diversity of *O. oeni* strains and the population structure of the species can be studied with multilocus sequence typing (MLST), too. It is a strategy based on the sequence polymorphism of a set of genes, usually 7–10, which generate data that can be used not only for strain differentiation but also for evolutionary and population studies. MLST has revealed a higher genetic diversity in *O. oeni* than indicated by ribotyping analysis (de las Rivas et al., 2004). Nevertheless, it seems that MLST is not able to distinguish among some strains, and it should not be so discriminant as RAPD-PCR or VNTR methods (Bordas et al., 2013).

Finally, typing strains of different wine *Lactobacillus* strains can be carried out with restriction fragment length polymorphism followed by PFGE, as shown by Rodas et al. (2005). Another good technique used both for identifying species and for typing strains of *Lactobacillus* is the rep-PCR finger-printing, using the GTG5 primer, that targets these bacterial DNA repetitive elements and that is suitable for a high throughput of strains (Gevers et al., 2001).

1.5.1.2 Culture-independent techniques

Culture-independent techniques use molecular techniques to identify and/or quantify wine microorganisms and do not require the microorganisms to be cultured previously (Rantsiou et al., 2005). These methods provide better information about the population, because they are not biased by the microorganisms that do not grow or do not grow well in a plate. The presence of viable but non-culturable microorganisms has been previously described in wine (Divol and Lonvaud-Funel, 2005; Millet and Lonvaud-Funel, 2000). Millet and Lonvaud-Funel (2000) studied the behaviour of various wine microorganisms and found a viable population between 104-105 cells/ml with the DEFT (direct epifluorescence technique) but lower than 1 CFU/ml with colony counts.

Some methods have been developed for detecting and quantifying *O. oeni* directly in wine. One of these is real-time quantitative PCR (RT-qPCR) method, which allows sensitive detection of the DNA product, ensures detection during the linear range of amplification, eliminates the need for post-PCR analysis, and incorporates specialized software to simplify data analysis. Real-time PCR detects and quantifies a fluorescent donor, the signal of which increases in direct proportion to the quantity of PCR product obtained. The most used probes are TaqMan, which are characterized by having a donor photochrome together with an acceptor photochrome (quencher). When they are both bound in the probe, the acceptor quenches the fluorescence emitted by the donor. When the *Taq* polymerase releases the acceptor photochrome the donor fluorescence is emitted. The information is represented as an amplification curve (Ct versus quantity of cell), which provides the cycle number for which the intensity of the donor emission increases compared with the background noise. The technique has high specificity and sensibility, and is quick. Nevertheless, all these parameters strictly depend on the primer design.

For instance, a RT-qPCR method has been developed with the specific primers for the malolactic enzyme and fluorogenic probes in order to quantify genomic DNA from wine samples without sample plating (Pinzani et al., 2004). Recently, Soares-Santos et al. (2017) have developed a novel quantitative PCR assay called Cells-qPCR. This method provides a highly sensitive and specific tool to detect and quantify yeasts, LAB and acetic acid bacteria species in wine-related matrices. This methodology does not require DNA extraction and overcomes the presence of reaction inhibitors like

polyphenols and ethanol. The Cells-qPCR technique can be applied directly from samples without having to dilute, and the presence of non-target microorganisms does not alter the method's specificity but allows proper target microorganism quantification.

The direct detection of cells of *O. oeni* and other species present in wine samples using fluorescent *in situ* hybridization (FISH) with 16S rDNA-specific probes labelled with fluorophores (Blasco et al., 2003; Sohier and Lonvaud-Funel, 1998). It allows the direct identification and quantification of bacterial species at microscopic level without previous cultivation. Generally, these probes are 15 to 20 nucleotides in length and are covalently labelled at the 5' end with a fluorescent dye. The technique combines the simplicity of microscopy and the specificity of DNA/RNA hybridization. In theory FISH could detect single cells, but in practice the detection limit is often 104 cells/ml. In general, it is less sensitive than PCR-based techniques (Hogardt et al., 2000; Moreno et al., 2003; Poppert et al., 2005). Another limitation is insufficient automation for high sample size throughput (Amann et al., 2001). Similar FISH protocols for fast identification of *O. oeni* based on the 5S rDNA and the ITS-2 region (23S-5S internal transcribed spacer) have also been developed (Hirschhäuser et al., 2005).

Flow cytometry simultaneously measures and then analyses multiple physical characteristics of single particles, such as cells. These cells flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. Cell viability can also be directly assessed by using fluorescent dyes to view the metabolic state of yeast and bacteria in wine (Boyd et al., 2003; Chaney et al., 2006; Herrero et al., 2006; Malacrino et al., 2001). Flow cytometry can be combined with FISH to selectively enumerate mixed microbial populations and carry out a high resolution automated analysis (Amann et al., 1990). The main advantage of this technique is its sensitivity (it can detect one cell in a million).

Recently developed high-throughput sequencing (HTS) technologies, such as the 454 pyrosequencing of amplicons, can be used to characterize the microbial diversity of environmental ecosystems more precisely (Ercolini, 2013; Galimberti et al., 2015; Solieri et al., 2013). Amplicon pyrosequencing is an automated high-throughput sequencing technique involving the synthesis of single-stranded deoxyribonucleic acids and detection of the light generated by pyrophosphate release in a luciferase-coupled

reaction (Margulies et al., 2005). This technique can be used for the rapid and accurate sequencing of nucleotide sequences from all species present in the sample, making it possible to study population structure.

Short-amplicon sequencing techniques have been used to monitor seasonal changes in winery-resident microbiota and to determine the bacterial diversity of botrytized wines (Bokulich et al., 2012a, 2012b) and Chinese traditional sourdough (Liu T. et al., 2016), to monitor seasonal changes in winery-resident microbiota (Bokulich et al., 2013), to analyse the microbial biogeography of grapes from a Californian region (Bokulich et al., 2014) or to study the diversity and dynamics during wine fermentation of Grenache grape variety (Portillo and Mas, 2016).

Advances in massive parallel short-amplicon (100-600bp) sequencing technologies have revealed a bacterial diversity of grape berries much more elevated than previously thought and important ecological questions on the grapevine microbiome are being answered (Bokulich et al., 2014; Perazzolli et al., 2014; Pinto et al., 2014; Taylor et al., 2014). For example, from high-throughput studies we know that the bacterial community on leaves differed, both in size and structure, from that on berries (Leveau and Tech, 2011) and that soil serves as a key source of vine-associated bacteria with edaphic factors influencing the native grapevine microbiome, being the microbial community of soils from the same viticultural region quite heterogeneous (Zarraonaindia et al., 2015). Moreover, the high-throughput sequencing of amplicons has recently been used to evaluate bacterial diversity and monitor microbial quality in fermented sausages (Wang X. et al., 2018) and to explore the diversity and biotechnological potential of lactic acid bacteria from traditional Peruvian fermented potatos (Jiménez et al., 2018).

Polymerase chain reaction amplification with different primers, in combination with denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient electrophoresis (Ampe et al., 2001; Ercolini, 2004; Giannino et al., 2009; Meroth et al., 2003; Miambi et al., 2003). These techniques consist of amplifying of a conserved region of the genome, generally ribosomal genes and to run the amplicons in a denaturing gradient polyacrylamide gel (PCR-DGGE). This method also has the theoretical potential to detect differences of as little as a few base pairs between species, although they also present some limitations (Prakitchaiwattana et al., 2004).

A species-specific multiplex PCR system for a rapid and simultaneous detection of LAB species frequently occurring in wine have been proposed (Petri et al., 2013). The species-specific markers for each species, called sequence-characterized amplified regions (SCARs), have been developed from RAPD markers, applying the DNA fingerprint method of nested specifically amplified polymorphic DNA (nSAPD)-PCR. This method has been shown to be efficient in wine samples, detecting populations higher than 10^3 CFU/mL for each species.

2. HYPOTHESIS AND OBJECTIVES

The main target of study has been the isolation and the study of *Oenococcus oeni* and other lactic acid bacteria (LAB) strains from the vine-wine ecosystem in Priorat. After the alcoholic fermentation, in order to promote *O. oeni* development and malolactic fermentation (MLF), selected starters can be inoculated. Unfortunately, due to the wine harsh conditions as low pH and high ethanol content, the loss of *O. oeni* viability is a fact (Reguant et al., 2005a). The recent research on *O. oeni* has been focused on the isolation and characterization of promising MLF starters (Bordas et al., 2013; Capozzi et al., 2010; Mesas et al., 2011; Solieri et al., 2010). This thesis 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 where minimized pesticide treatment is given, so the wines produced are ecologic, during two consecutive vintages. Thus, we compared the LAB composition of grapes and wines across the different vineyards and vintages providing useful information about the LAB biodiversity within this particular oenological area.

Within this framework, the **hypothesis** of this thesis was that **the use of autochthonous *O. oeni* and other LAB can preserve better terroir characteristics in ecological wines of Priorat region.**

Thus, the main **objective** of this thesis was the characterization of LAB biodiversity and the selection of the most representative strains with terroir characteristics for their use as starter cultures in the Priorat region.

In order to assess the established assumption the following **specific objectives** were attained to:

1. Establish the heterogeneity of the bacterial community associated with different varietals at Priorat grapes and characterize the identified communities. (CHAPTER I)
2. Assess the indigenous diversity of LAB in healthy grapes and wines from Priorat, involving the isolation and identification of the local LAB biodiversity associated with its terroir. (CHAPTER II)
3. Characterize the LAB strains isolated from Priorat wine samples in order to select those with oenological potential as MLF starter cultures in pilot scale wine production. (CHAPTER III)

The results of this thesis could provide an oenological LAB culture collection for the Priorat wine region. 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. Also, this knowledge will allow wine industry to apply induced wild fermentations in the development of novel products that are reproducible, premium, attractive to consumers and in accordance with the demands of the global market for natural wines.

3. RESULTS

CHAPTER I

Bacterial Diversity of Grenache and Carignan Grapes Surface from Different Vineyards at Priorat Wine Region (Catalonia, Spain)

CHAPTER II

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

CHAPTER III

Selection and characterization of autochthonous strains of *Oenococcus oeni* for vinification in Priorat (Catalonia, Spain)

CHAPTER I

Bacterial Diversity of Grenache and Carignan Grapes Surface from Different Vineyards at Priorat Wine Region (Catalonia, Spain)

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Abstract

Epiphytic bacteria on grape berries play a critical role in grape health and quality, which decisively influence the winemaking process. Despite their importance, the bacteria related with grape berries surface remains understudied and most previous work has been based on culture-dependent methods, which offer a limited view of the actual diversity. Herein, we used high-throughput sequencing to investigate the bacterial diversity on the surface from two grape varieties, Grenache and Carignan, and compared them across five vineyards included within Priorat region (Spain). We could detect up to 14 bacterial phyla with *Firmicutes* (37.6% *Bacillales* and 14% *Lactobacillales*), *Proteobacteria* (16.8% *Pseumonodales* and 11.6% *Enterobacteriales*) and *Actinobacteria* (3.4% *Actinomycetales*) being the most abundant. Bacterial community was different at each vineyard being grape varietal, geographical situation and orientation related with changes in bacterial populations. The most abundant bacterial taxa and those driving differences between the vineyards and grape varietals were identified. This study indicates that bacterial community heterogeneities can be influenced by geographic factors like orientation.

Keywords

Next generation sequencing - Bacterial diversity - Grape surface - Vineyard orientation

Introduction

Bacteria can readily colonize any surface including parts of the plant above the ground affecting the health of their hosts in diverse ways. The surface of grape berries represents a complex natural reservoir of bacterial microbiota originating from the surrounding environment (Zarraonaindia et al., 2015). Grapevine bacteria play a key role not only in plant health, but also in crop quality and yields which can influence the winemaking process (Nisiotou et al., 2011; Verginer et al., 2010). Many bacteria associated with grape surface cannot survive the extreme conditions of wine fermentation but their metabolic activity on the grape surface can have long-ranging consequences and they are undoubtedly included in the initial fermentation steps. Recently, Splivallo et al. (2014) demonstrated that bacteria associated with truffle-fruiting bodies contribute to truffle aroma. Thus, grape surface bacteria may play a significant role influencing the flavour, colour, and quality of the final product but this aspect still remains to be studied.

Despite their importance, the diversity of epiphytic bacteria on grape berries remains poorly described. Most research has concentrated on bacteria of oenological interest, like acetic acid and lactic acid bacteria (LAB) present in the microbiota on grape berries (Bae et al., 2006; Nisiotou et al., 2011). Acetic acid bacteria are usually related with spoilage of wine and *Oenococcus oeni* and some other LAB species are known to perform the malolactic fermentation or to promote spoilage of wine depending on the species or strain.

Nevertheless, the role of other bacteria in wine fermentation has been mostly ignored (reviewed in Barata et al., 2012). Furthermore, previous studies of grape-associated microbiota have been limited by methodological biases of culture-based techniques (Nisiotou and Nychas, 2007; Renouf et al., 2005, 2007) and low resolution of early molecular techniques (Martins et al., 2012). It is well reported that only a fraction of most environmental bacteria have been cultivated (Amann et al., 1995). Advances in massive parallel short-amplicon (100-600bp) sequencing technologies have revealed a bacterial diversity of grape berries much more elevated than previously thought and important ecological questions on the grapevine microbiome are being answered (Bokulich et al., 2014; Leveau and Tech, 2011; Perazzolli et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Zarraonaindia et al., 2015). For example, from recent high-

throughput studies we know that the bacterial community on leaves differed, both in size and structure, from that on berries (Leveau and Tech, 2011) and that soil serves as a key source of vine-associated bacteria with edaphic factors influencing the native grapevine microbiome, being the microbial community of soils from the same viticultural region quite heterogeneous (Zarraonaindia et al., 2015). Furthermore, grape-associated microbial biogeography is nonrandomly associated with regional, varietal, and climatic factors across multiscale viticultural zones (Bokulich et al., 2014), fungi communities from the same vineyard can be highly variable (Setati et al., 2012) and the most abundant yeast at the beginning and the middle of the fermentation of grapes cultivated under different treatments were detected (David et al., 2014). However, so far no high-throughput study has investigated bacterial diversity of grape varietal communities within the same grapevine growing region where climatic and regional factors are expected to be similar and produce wines of similar characteristics. Thus, it is ecologically relevant to know if bacterial communities of a single viticulture region are different and which factor influences the population changes.

Our goal was to test for heterogeneity of the bacterial community associated with different varietal at Priorat grapes and characterize the observed communities. For that reason, the present study characterizes the bacterial communities of Grenache and Carignan grape varieties across seven vineyards within the same viticultural zone, the Priorat region, by using 16S rRNA amplicon sequencing.

Materials and Methods

Sample collection

Grape samples were collected at 7 vineyards on Priorat region near Tarragona (Catalonia, Spain) located within 15 km^2 (Table 1, Supplementary Fig.1). These vineyards were denominated Ferrer Bobet (FB), Mas Martinet (MM), Jaume Sabaté (JS), Roca de les Dotze (RD), and Mas del Botó (MB) and produced mainly Carignan and/or Grenache cultivars with a unique rootstock of each varietal and managed under similar ecologic conditions. Priorat vineyards are characterized by high temperatures in summer (maximum temperature 35ºC), cold winters (minimum temperature -4ºC) and low levels of rainfall (400-600mm/year), which yielded wines with high alcohol content

(13-16%). The vineyards are planted on the slopes on terraces at altitudes of between 100 m and 700 m above sea level with a marked contrast between the valleys and the higher areas and there are both freezing winds from the North-North West and also warm and humid one from the East-South East. Five replicate grape clusters from different plants from equidistant intervals were collected from each cultivar and vineyard in order to capture the heterogeneity present in each vineyard lot (Setati et al., 2012). Vineyards were sampled one, two or three times depending on their size. The date of sampling was at maturation of grapes at each vineyard, just before the harvest of 2013 and preserved at 4ºC on sterile plastic bags, resulting in 19 samples (Table 1; 10 from Grenache and 9 from Carignan). The grape clusters of each variety and vineyard were destemmed and crushed to obtain grape must upon arrival to the laboratory within the next hour of recollection. The grape must (including seeds and skin) of each sample was centrifuged at 4ºC and 4,000 x g during 10 minutes and the pellet immediately frozen at -80ºC until DNA extraction. In addition, four 50ml samples of grape must fermented at final malolactic fermentation were collected. These wine samples consisted of mixed Carignan and Grenache grapes must harvested and fermented at "Mas Martinet" cellar.

Table 1
Description of the collected samples and their location.

Sample ID	Vineyard	Variety	Nº Samples	Coordinates	Elevation (m)	Orientation[a]
FB-Gx	Ferrer Bobet	Grenache	2	N41.1767, E0.8607	464.8	E
FB-Cy	Ferrer Bobet	Carignan	2	N41.1797, E0.8604	439.8	F
MM-Gx	Mas Martinet	Grenache	2	N41.1781, E0.7937	199.8	S
MM-Cy	Mas Martinet	Carignan	3	N41.177403, E0.794808	205.1	S
JS-Gx	Jaume Sabaté	Grenache	2	N41.218875, E0.749972	233.5	E
JS-Cy	Jaume Sabaté	Carignan	1	N41.2662, E0.8784	229.6	F
RD-Gx	Roca de les Dotze	Grenache	1	N41.265672, E0.879789	625.7	E
RD-Cy	Roca de les Dotze	Carignan	2	N41.218564, E0.749825	636.1	F
MB-Gx	Mas del Botó	Grenache	3	N41.196468, F0.919531	553.4	S
MB-Cy	Mas del Botó	Carignan	1	N41.196944, E0.920636	570.6	E
Wine	Ferrer Bobet	Mix	4	-	-	-

[a] E: East, S: South, F: Flat

DNA extraction and sequencing

Genomic DNA was extracted from grape must and wine samples (19 grape must and 4 wine) using the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. Extracted DNA concentration was measured by nanodrop, adjusted with molecular grade water to a concentration of 50ng/µL and 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/799R with adapters for the sequencing by the equipment PMG from Ion Torrent with chips 318. The universal primer 515F (GTGCCAGCMGCCGCGGTAA) included a 10-bp barcode unique to each amplified sample. The reverse primer was a modification of the universal primer 799R (CVGGGTATCTAATCCBGTT, Chelius and Triplett, 2001). We used this primer pair because it has been found to be particularly suited for short read sequencing studies (Ghyselinck et al., 2013) and because the primer 799R had been previously used to amplify bacteria from plants avoiding the amplification of host chloroplast sequences (Chelius and Triplett, 2001). Primers sequences used in this study with their adapters and barcodes are listed in Supplementary Table 1.

PCR reactions contained 5-100 ng DNA template, 1× GoTaq Green Master Mix (Promega), 1 mM MgCl2, and 2 pmol of each primer. Reaction conditions consisted of an initial 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and a final extension of 72 °C for 10 min. PCR products were pooled by sample and cleaned using a GeneRead Size Selection lit (Qiagen, Hilden, Germany). Cleaned PCR products were submitted to Centre for Omic Sciences (Reus, Spain) where their quality was checked by a Bionalyzer and their quantity adjusted for sequencing.

Data analysis

Raw sequences were demultiplexed and quality filtered using QIIME v1.8.0 (Caporaso et al., 2010a). Reads were discarded if the average quality score of the read was <25, if the length of the read was <200 or >400 and any read containing one or more ambiguous base calls. 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. OTU sequences were aligned using PYNAST (Caporaso et al., 2010b) against a template alignment of the Greengenes core set filtered at 97% similarity. OTU taxonomy was determined using the RDP classifier retrained toward the GreenGenes bacterial 16S rRNA database (13_8 release) (DeSantis et al., 2006) at 97% similarity (Wang et al., 2007). Chimeric sequences were identified and removed using ChimeraSlayer (Haas et al., 2011) and a phylogenic tree was generated from the filtered alignment using FastTree (Price et al., 2009). 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 rarified to an even depth of 850 sequences per sample in comparisons of all sample types in this study. Samples represented by less than 850 sequences following all quality filtering steps were discarded.

Statistical analysis

Alpha diversity (within-sample species richness) estimates were calculated by analysing the observed species, Shannon, Simpson and Chao1 indexes and Good´s coverage. Comparisons of alpha diversity between categories or sample clusters were run on QIIME.

Beta-diversity (between-sample microbial community dissimilarity) estimates were calculated within QIIME using weighted UniFrac distance (Lozupone and Knight, 2005) between samples. Ordination by non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) were used to summarize and visualize patterns in species composition. ANOSIM (an analogue of univariate ANOVA which tests for differences between groups of samples) were run in PRIMER v6 (Clarke and Gorley, 2006) to determine significant differences in phylogenetic or species diversity among experimental factors (vineyard origin, vineyard orientation and variety). The identification of the taxa explaining the similarity between samples type and the strongest variation of bacterial communities in each vineyard, geographical orientation and grape varietal was done with SIMPER (similarity percentage) run in PRIMER v6 (Clarke and Gorley, 2006). Comparative Mantel test on distance matrices were run in PRIMER v6 (Clarke and Gorley, 2006) with the tool RELATE to examine the correlations among geographic coordinates (latitude, longitude and altitude) and microbial community structures (based on UniFrac distances). Wine fermented samples

were considered apart for statistical analysis as they were non representative from must samples.

Results and Discussion

Sequence analysis

The bacterial community of the most commonly grown grape varieties in Priorat, Grenache and Carignan, were analysed independently at 5 different vineyards from the same region (Supplementary Fig. 1). Must samples consisting of destemmed, crushed grapes were analysed with a short-amplicon sequencing approach to characterize bacterial community composition. After removal of low quality sequences, those failing alignment or annotated as host sequences, and singleton sequences, 405,668 16S rRNA V4 amplicon sequences were generated from 19 must and 4 fermented wine samples, with an average of 17,637 sequences per sample. These sequences had an average of 299bp (ranging from 200 to 400bp) and clustered into 6,556 operational taxonomic units (OTUs; 97% nucleotide identity).

Bacterial diversity of Grenache and Carignan grapes from Priorat

The ecological diversity of grape must bacterial communities was estimated using various diversity indexes (Table 2). The observed OTUs of Grenache and Carignan musts samples ranged from 94 to 172. Alpha diversity of Grenache and Carignan must samples was not significantly different between the selected vineyards, their grape variety or geographical location. Wine fermented samples harbored a significantly higher diversity of OTUs (195) than Grenache or Carignan must samples (R=0468, P=0.001). However, the elevated diversity of sequences from wine samples was found within the same genera, as most of the different OTUs of these samples could be assigned to just 21 genera while grape must samples ranged from 30 to 137 genera (Table 2). In fact, 99% of the wine fermented sequences were just related to the genus *Oenococcus*. This fact is justified as the wine samples were taken at the end of the malolactic fermentation where *Oenococcus oeni* has been described as the most abundant genera (Lonvaud-Funel, 1999) and previous studies have described an elevated microdiversity of autochthonous *Oenococcus oeni* in wines conducting malolactic fermentation (Garofalo et al., 2015). It is difficult to ascertain to what extent

the observed microdiversity of *Oenococcus* represents ecologically differentiated populations.

Table 2
Alpha diversity obtained by the average of the different samples replicates using 850 sequences by sample.

	Chao1	Simpson	Shannon	Observed OTUs	Assigned genera	Good´s coverage
FB-Gx[a]	328.5	0.93	5.40	172	119	0.88
FB-Cy	232.1	0.38	2.08	98	41	0.92
MM-Gx	303.4	0.59	3.13	119	58	0.90
MM-Cy	374.5	0.40	2.19	112	117	0.90
JS-Gx	367.7	0.94	5.35	142	137	0.90
Js-Cy	272.8	0.36	1.96	94	32	0.92
MB-Gx	215.5	0.44	2.28	96	67	0.92
MB-Cy	341.0	0.71	3.52	132	30	0.89
RD-Gx	320.6	0.97	6.02	166	61	0.90
RD-Cy	324.3	0.80	4.11	128	72	0.90
Wine	703.4	0.62	3.70	195	21	0.82

[a] Gx: Grenache; Cy: Carignan; FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; MB: Mas Botó; RD: Roca de les Dotze

Rarefaction plots for observed OTUs at 850 sequences deep were close to reach a plateau for most samples except wine fermented ones (Supplementary Fig.2). Good's coverage for the samples, which provides an estimate of sampling completeness using a probability calculation with randomly selected sequences, was an average of 90% (±2.8) when calculated with 97% species level phylotypes (Table 2). These results suggest that the level of selected sequences (850 sequences per sample) would identify the majority of bacterial phylotypes present in the Priorat must samples and could be used to compare sample type.

Phylogenetic composition of the bacterial community of Grenache and Carignan grapes

Altogether, 14 bacterial phyla were detected in the Grenache and Carignan grape must samples, of which 2 phyla had no cultured representatives (Fig. 1, Supplementary Table 2). The 6556 different OTUs from this study were included in 19 predominant genera and more than 100 genera at abundance of less than 0.5% on average (Fig. 2) revealing an elevated bacterial diversity unprecedented from culture-dependent studies that barely

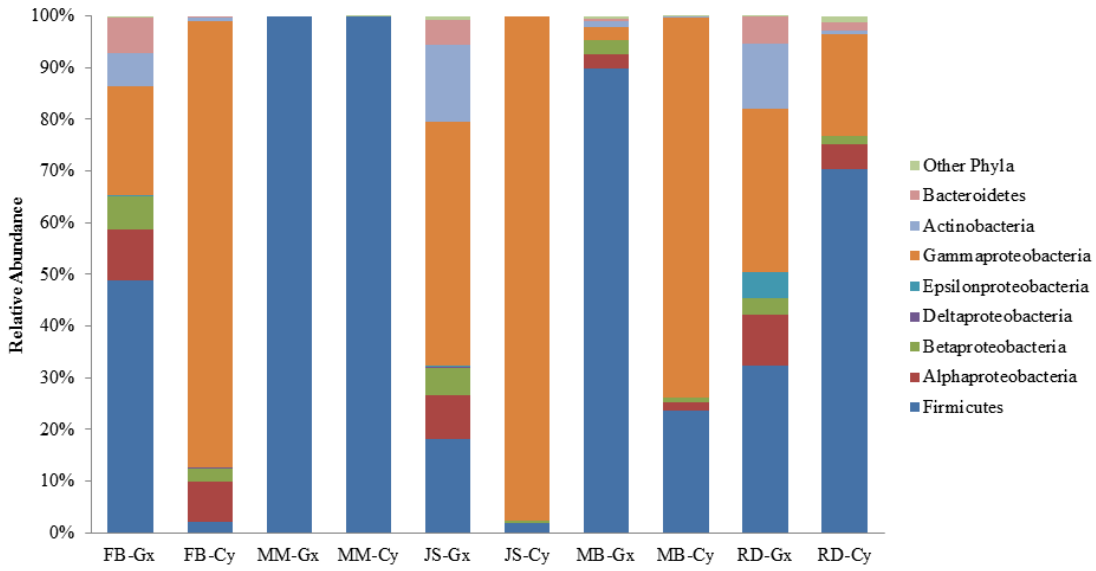

Fig. 1. Average relative abundance of dominant bacterial phyla at each sample type. "Other Phyla" include Fusobacteria, Acidobacteria, Deinococcus-Thermus, Chloroflexi, Verrucomicrobia, Planctomycetes, Armatimonadetes, FBP, SR1 and Spirochaetes.

detected a dozen genera within *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla (Barata et al., 2012; Martins et al., 2012). This is not surprising as it is well reported that only a fraction of most environmental bacteria have been cultivated (Amann et al., 1995), at least on standard culture media. Taxonomic composition of the bacterial communities of grape must varied greatly across the selected vineyards (Fig. 1) but predominantly consisted of the orders *Bacillales* (37.6%), *Pseumonodales* (16.8%), *Lactobacillales* (14%), *Enterobacteriales* (11.6%) and *Actinomycetales* (3.4%). Previous culture based studies on grape bacteria mainly detected *Pseudomonadales* (31-51%) and *Micrococcales* (14-21%) (Martins et al., 2012). Our results are in agreement with the dominant taxonomic groups found by HTS techniques on Chardonnay, Cabernet and Zinfandel grapes at California (Bokulich et al., 2014) thought proportions differed from the results obtained for Merlot grapes at New York were *Proteobacteria*, mainly belonging to *Sphingomonadales* and *Pseudomonadales*, represented up to 80.7% of community at the grape samples (Zarraonaindia et al., 2015). Organisms from the detected families are found in a wide range of environments, including soil and air, that have been previously been proposed as sources and reservoir for potential plant-associated bacteria (Bowers et al., 2011) included grapevine microbiota (Gilbert et al.,

2014; Zarraonaindia et al., 2015). Bacillus, Enterobacter and Acinetobacter were abundant genera in the studied must samples (Fig. 2) and have been frequently isolated from grapes but were usually considered innocuous contaminants as they do not have the ability to grow during the wine fermentation process (Barata et al., 2012). Other genera like *Streptococcus* and *Erwinia* also represented an important fraction of the grape must in this study (Fig. 2) and have been occasionally detected by culture in vineyard environments (Barata et al., 2012). Recently, Perazzolli et al. (2014) detected *Pseudomonas*, *Erwinia* and *Acetobacter* in all grapevine plants they surveyed by pyrosequencing which mean these phyllosphere genera could be transferred or shared with grape berries easily. *Pseudomonas* and *Bacillus* sp., can act as biological disease suppression agents, stimulating plant growth and health (Bulgari et al., 2009; Compant et al., 2011; West et al., 2010).

Acetic bacteria, related to spoilage of the wine, represented a minimal proportion of the sequences of this study; and the LAB, related with malolactic fermentation of wine, are usually considered as minor partners of grape microbiota according to microbiological culture methods. Next generation techniques are recently changing that view with percentages of LAB ranging from 15 to 30% of total bacterial communities in grapes (This study; Bokulich et al., 2014; Pinto et al., 2014).

Within LAB, the genus *Oenococcus*, and specifically the species *O. oeni,* is the main agent of malolactic fermentation of wines conferring unique organoleptic properties (Bartowsky, 2005; Davis et al., 1988; Liu, 2002; Lonvaud-Funel, 1999). This genus has been seldom isolated from grapes in the vineyard (Garijo et al., 2009) and its DNA has been detected just once, in a sample of grapes from Bordeaux (Renouf et al., 2005, 2007). Anyway, to our knowledge, *Oenococcus* has never been detected through massive sequencing from grapes berries, must or grape vine thought other genera from the same family like *Leuconostoc* have been previously detected. This study found members of *Oenococcus* in most samples, accounting for 5.5% (on average) of the bacterial communities in grape musts from Priorat region. These results were contrasted by the isolation from the same grape samples on LAB specific medium (MRS medium) of 174 LAB isolates identified as *Oenoccocus oeni* (28%) *Lactobacillus* (66%) and *Pediococcus* (6%) (Franquès et al., unpublished data).

Order, genus	FB-Gx	FB-Cy	MM-Gx	MM-Cy	JS-Gx	JS-Cy	MB-Gx	MB-Cy	RD-Gx	RD-Cy	Wine
Bacillales, *Bacillus*	0.5	0.1	99.7	99.8	1.8	3.6	88.3	21.7	3.6	20.0	0.0
Lactobacillales, *Oenococcus*	16.5	1.1	0.1	0.0	7.5	13.2	0.3	0.1	13.2	2.8	99.0
Pseudomonadales, *Acinetobacter*	11.9	0.6	0.0	0.0	14.9	15.2	1.5	63.6	15.2	11.3	0.1
Enterobacteriales, *Erwinia*	1.8	82.8	0.0	0.0	2.7	4.6	0.1	8.7	4.6	3.5	0.1
Lactobacillales, *Streptococcus*	15.7	0.0	0.0	0.0	3.2	6.2	0.3	1.2	6.2	33.8	0.1
Pseudomonadales, *Pseudomonas*	1.9	0.3	0.0	0.0	17.0	1.8	0.7	0.2	1.8	1.4	0.0
Pasteurellales, *Haemophilus*	1.3	0.1	0.0	0.0	0.5	0.4	0.0	0.4	0.4	0.1	0.0
Pseudomonadales, *Enhydrobacter*	2.2	0.1	0.0	0.0	8.2	6.5	0.0	0.0	6.5	0.9	0.1
Rhizobiales, *Methylobacterium*	7.4	0.0	0.0	0.0	1.2	3.9	0.4	0.0	3.9	0.7	0.0
Clostridiales, *Veillonella*	3.0	0.0	0.0	0.0	0.7	1.0	0.0	0.1	1.0	5.1	0.0
Actinomycetales, *Corynebacterium*	0.7	0.1	0.0	0.0	4.8	9.9	0.2	0.0	9.9	0.2	0.0
Lactobacillales, *Lactobacillus*	9.5	0.0	0.0	0.0	0.1	3.0	0.0	0.0	3.0	0.2	0.0
Neisseriales, *Neisseria*	1.8	0.0	0.0	0.0	0.1	1.5	0.0	0.6	1.5	0.4	0.0
Rhodospirillales, *Gluconobacter*	0.1	7.1	0.0	0.0	0.3	0.0	0.0	1.1	0.0	0.0	0.0
Sphingomonadales, *Sphingomonas*	0.5	0.5	0.0	0.0	1.6	0.8	2.0	0.2	0.8	0.1	0.0
Burkholderiales, Other	0.6	1.5	0.0	0.0	2.2	1.0	1.5	0.2	1.0	0.0	0.0
Bacillales, *Staphylococcus*	1.5	0.0	0.0	0.0	1.8	1.5	0.3	0.1	1.5	0.8	0.0
Lactobacillales, Other	0.5	0.1	0.0	0.0	0.3	0.8	0.0	0.4	0.8	3.1	0.0
Actinomycetales, *Micrococcus*	1.3	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.1	0.0
Other genera	21.3	5.8	0.4	0.1	27.6	25.1	4.2	1.2	25.1	15.6	0.5

Fig. 2. Heatmap displaying relative abundances of the most abundant genera detected in grape musts from Priorat averaged by sample type. "Other genera" is represented by phylogenetic groups detected by less than 0.5% on average of all sample types. The colour scale is from intense red for the most abundant taxonomic groups to intense blue for less abundant. ("Other genera" include members of the genera *Deinococcus, Micrococcus, Rothia, Mycobacterium, Rhodococcus, Friedmanniella, Nocardioides, Propionibacterium, Actinomycetospora, Pseudonocardia, Saccharomonospora, Bifidobacterium, Atopobium, Collinsella, Rubrobacter, Fimbriimonas, Flavisolibacter, Sediminibacterium, Prevotella, Bacteroides, Dysgonomonas, Porphyromonas, Adhaeribacter, Hymenobacter, Pontibacter, Spirosoma, Chryseobacterium, Cloacibacterium, Wautersiella, Capnocytophaga, Flavobacterium, Pedobacter, Alicyclobacillus, Geobacillus, Aneurinibacillus, Paenibacillus, Lysinibacillus, Sporosarcina, Gemella, Granulicatella, Enterococcus, Pediococcus, Leuconostoc, Lactococcus, Streptococcus, Turicibacter, Jan-68, Anaerococcus, Finegoldia, Peptoniphilus, WAL, Clostridium, Ruminococcus, Blautia, Moryella, Oribacterium;* and other unidentified genera belonging to orders Ellin 6075, iii1-15, Actinomycetales, Solirubrobacterales, Sphingobacteriales, JG30-KF-CM45, BSA2B-08, Clostridiales, Rhizobiales and Rhodospirillales; and to families Geodermatophilaceae, Intrasporangiaceae, Kineosporiaceae, Microbacteriaceae, Moraxellaceae, Halomonadaceae, Methylocystaceae, Micromonosporaceae, Gaiellaceae, Nocardioidaceae, Chitinophagaceae, Porphyromonadaceae, Cytophagaceae, Sphingobacteriaceae, Planococcaceae, Thermoactinomycetaceae, Aerococcaceae, Leuconostocaceae, Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae, Caulobacteraceae, Bradyrhizobiaceae, Methylobacteriaceae, Idiomarinaceae, Rhizobiaceae, Hyphomonadaceae, Rhodobacteraceae, Acetobacteraceae, Sphingomonadaceae, Alcaligenaceae, Comamonadaceae, Oxalobacteraceae, Rhodocyclaceae, Aeromonadaceae.)

The bacterial community of wine samples at final malolactic fermentation from Mas Martinet cellar were mostly composed by the genus *Oenococcus* (99%) but genera like *Acinetobacter*, *Pseudomonas*, *Methylobacterium*, *Paracoccus* and others were detected at minor proportions (Fig. 2). It has been previously described that *Sphingomonas* and *Methylobacterium* can survive the wine fermentation process (Bokulich et al., 2012) but the role and impact of these and other detected genera on quality and organoleptic properties of wine remain unknown. The general percentage of *Oenococcus* in the grapes from MM vineyard used for the making of wine fermented samples of this study

was low (0.1% of total bacterial community). However, the most abundant OTU related to *Oenococcus* in wine fermented samples (OTU ID 32722) was also present at MM grape must samples (specifically at Grenache grapes) and represented the most abundant OTU related to *Oenococcus* in grape must samples from the rest of vineyards. This result indicates that OTU 32722 is the best adapted for the conditions of wine fermented at MM cellar and was highly selected during malolactic fermentation.

Vineyard origin, grape varietal and geographical situation defines grape must bacterial communities

Based on results from previous studies describing differences between different varietals and viticultural regions (Bokulich et al., 2014), we hypothesized that bacterial communities would cluster according to the analysed grape varieties (Grenache and Carignan) and that vineyards sampled at shorter spatial distance should have a bacterial community more similar in composition than those vineyards sampled at higher distances.

Table 3
ANOSIM of category effects on microbial diversity pattern based on UniFrac distance matrix excluding fermentation wine samples and calculated in PRIMER v6.

Group	Factor	R	P
All	Vineyard	0.367	0.001
Grenache	Vineyard	0.677	0.006
Carignan	Vineyard	1	0.002
All	Variety	0.191	0.005
All	Orientation	0.84	0.001
Grenache	Orientation	0.8	0.01
Carignan	Orientation	0.897	0.01

Community structure varied widely across different selected vineyards within Priorat region (Table 3, Fig. 3), exerting the origin of the samples a significant impact on bacterial genetic diversity (weighted UniFrac $R_{ANOSIM} = 0.367$ $P < 0.001$). However, other factors as geographical orientation and grape variety also influenced deeply on the bacterial composition of Grenache and Carignan grapes (Table 3, Fig. 3). Previous studies have shown intra-vineyard heterogeneity to be high in aboveground tissues (Setati et al., 2012, Zarraonaindia et al., 2015). In our study, bacterial communities on grapes surface from the same vineyard were, on average, more similar than

communities from different vineyards as replicates of samples belonging to the same grape variety and same vineyard cluster closer at the NMDS (non-metric multidimensional scaling) than samples from different vineyards, which means that intra-vineyard community differences were smaller than the inter-vineyard ones (Fig. 3A). We consider that the sampling procedure was adequate to capture heterogeneity of each vineyard as various plants evenly distributed were sampled along each plot and some plots were sampled two or three times, depending on the size of the plot.

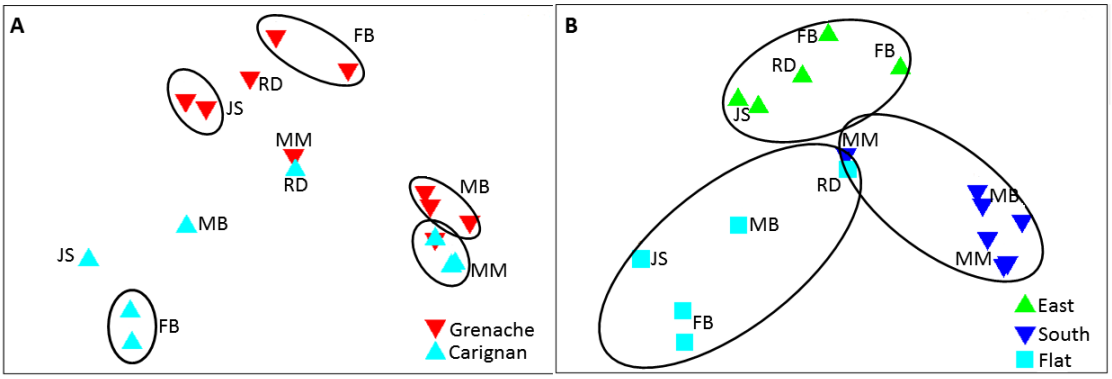

Fig. 3. Non-metric multidimensional scaling (NMDS) plots of the weighted pairwise UniFrac distances between Grenache and Carignan musts samples from five Priorat vineyards, FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó. (A) Clustering of bacterial communities by grape varietal with replicates of samples circled by a black loop (R_{ANOSIM}= 0.191; P=0.005); (B) Clustering of Grenache and Carignan musts samples by geographical orientation (R_{ANOSIM}= 0.84; P=0.001).

Bokulich et al. (2014) found that similarities in microbial communities from different Californian regions separated by more than 500 km followed the coastline indicating an environmental trend. The vineyards from the present study followed a similar, ecologic management of the grapevines and they were relatively closely located (15 km maximum distance) so that changes in bacterial populations due to differences on climatological parameters could be ruled out. However, it is possible that the irregular topography generates differences in altitude and geographical orientation of the vineyards could also influence locally in environmental parameters such as insolation-shading or humidity. Some of the sampled vineyards had weak or no orientation because they were in a fairly flat area but some vineyards were sampled on a hillside facing either East or South. These three categories (East, South and Flat) were used to group samples and we found that musts bacterial community similarities were significantly influenced by vineyard geographical orientation (R_{ANOSIM} = 0.84, P =

0.001) suggesting that environmental differences between hillsides probably underlie the observed community changes (Table 3).

Table 4
SIMPER analysis results for those OTUs contributing more than 5% to the similarity between samples (A and C) or similarity within samples (B and D) according to vineyard orientation (A and B) or grape varietal (C and D).

A.

	Dissimilarity %	**Genus**	**Contribution %**
East & Flat	89.6	*Erwinia*	24.2
		Acinetobacter	18.8
		Bacillus	6
		Oenococcus	5.7
East & South	85.6	*Bacillus*	45.6
		Acinetobacter	7.8
		Oenococcus	5.3
South & Flat	93.34	*Bacillus*	45.6
		Erwinia	22

B.

	Similarity %	**Genus**	**Contribution %**
East	35	*Acinetobacter*	30
		Oenococcus	11
		Streptococcus	9.5
South	21.3	*Bacillus*	88
Flat	12.3	*Erwinia*	65.1
		Acinetobacter	5.1

C.

	Dissimilarity %	**Genus**	**Contribution %**
Grenache & Carignan	89.5	*Bacillus*	41.2
		Acinetobacter	12
		Erwinia	12

D.

	Similarity %	**Genus**	**Contribution %**
Grenache	21.54	*Bacillus*	27
		Acinetobacter	26
		Oenococcus	6.6
		Streptococcus	6.3
Carignan	11.16	*Bacillus*	46.4
		Erwinia	24.4
		Acinetobacter	17.7

Bacterial communities from Grenache and Carignan grapes across selected vineyards from Priorat region resulted globally different (R_{ANOSIM}= 0.191; P=0.005) (Fig. 3A). This result has been previously observed for other grapes varieties (Bokulich et al., 2014; Zarraonaindia et al., 2015). Also, must samples within each grape variety harbored significantly different communities at each vineyard and clustered by vineyard orientation (Table 3, Fig 3). We found that Carignan must bacterial community was significantly related with geographical coordinates (latitude, longitude, altitude) (R_{RELATE}= 0.682, P= 0.001). Thus, bacterial communities associated to Carignan grapes from closer vineyards were more similar than those from distant vineyards. However, we could not observe a clear relationship between Grenache bacterial communities and location (R_{RELATE}= 0.037, P= 0.32) indicating that others factors (i.e. vineyard origin or orientation) showed more influence on bacterial community composition than the geographical situation.

Bacterial community of wine fermented samples was very stable within replicates and resulted clearly different from Grenache or Carignan grape must (Supplementary Fig. 3) thought on the PCoA representation wine samples migrated closer to MB and MM vineyard (the grapes used for the wine making come from MM vineyard).

Taxonomic groups driving differences between bacterial communities of grape from Priorat vineyards

SIMPER analysis revealed which taxa contributed the most to dissimilarity of samples and similarity between samples according to their orientation or grape varietal (Table 4). According to geographical orientation, the differential relative abundances of *Bacillus*, *Erwinia, Acinetobacter* and *Oenococcus* contributed the most to dissimilarities between grapes from East-, South-oriented and Flat (no orientation considered) vineyards (Table 4A). *Oenococcus, Acinetobacter* and *Streptococcus* were the genera contributing the most to similarities within East-oriented vineyard musts bacterial communities; *Bacillus* in South-oriented vineyards; *Erwinia* and *Acinetobacter* in Flat or not-oriented vineyards (Table 4B). According to grape varietal, *Bacillus*, *Acinetobacter* and *Erwinia* accounted for the higher contribution to dissimilarities between Grenache and Carignan grape must bacterial communities, being also the proportion of these genera together with *Oenococcus* and *Streptococcus* the mayor responsible of the similarities within Grenache or Carignan samples (Table 4C, D).

Table 5
List of OTUs shared by the samples within each factor or group category.

Factor	Group	Shared Percentage[a]	OTU ID	Taxonomic Affiliation
Vineyard	All	100%	40910	*Bacillus* (Bacillales)
Orientation	East	100%	1114	*Oxalobacteraceae* (Burkholderiales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			11667	*Haemophilus* (Pasteurellales)
			12900	*Methylobacterium* (Rhizobiales)
			18038	*Sphingomonas* (Sphingomonadales)
			28121	*Erwinia* (Enterobacteriales)
			32722	*Oenococcus* (Lactobacillales)
			40910	*Bacillus* (Bacillales)
			43218	*Pseudomonas* (Pseudomonadales)
	South	100%	40910	*Bacillus* (Bacillales)
	Flat	100%	830, 40910	*Bacillus* (Bacillales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			4511	*Aeromonadaceae* (Aeromonadales)
			12900	*Methylobacterium* (Rhizobiales)
			11173, 18425	Micrococcaceae (Actinomyctales)
			16121	*Streptococcus* (Lactobacillales)
			32722	*Oenococcus* (Lactobacillales)
			28121	*Erwinia* (Enterobacteriales)
			26940	*Staphylococcus* (Bacillales)
			6565	*Enhydrobacter* (Pseudomonadales)
Varietal	Grenache	100%	1114	*Oxalobacteraceae* (Burkholderiales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			12900	*Methylobacterium* (Rhizobiales)
			40910	*Bacillus (Bacillales)*
	Carignan	100%	40910	*Bacillus* (Bacillales)

[a] Percentage of samples within each group sharing a specific OTU.

These results show that the vineyard origin, its orientation and grape varietal determine the presence and proportion of these bacterial genera and could influence ultimately the composition of fermentative populations, which could determine regional wine characteristics. It may be interesting to relate the observed changes in bacterial communities with differences in wine physicochemical properties and the potential use of this knowledge to enhance organoleptic and chemosensory perception of wines.

Core bacterial community of Grenache and Carignan grapes at Priorat

We found just one OTU shared by all must samples that was related to *Bacillus* (Table 5, Fig. 4) and various OTUs were shared within pairs of vineyards (Fig. 4). Besides, we examined the bacterial community grouped according to each orientation category and resulted in several OTUs shared by samples clustered as East-oriented and Flat or not oriented vineyards, while 1 OTU related to *Bacillus* was present at 100% of South-oriented samples (Table 5). Five and one OTUs were shared by all Carignan and Grenache must samples, respectively. Most of the genera comprising the core OTUs of the samples were related to plant and soil environments with the exception of *Oenococcus* that was related to wine fermented and could be found at every East and South oriented samples (Table 5). This *Oenococcus* related OTU (ID 32722) was the most abundant both in grape must samples and wine fermented samples.

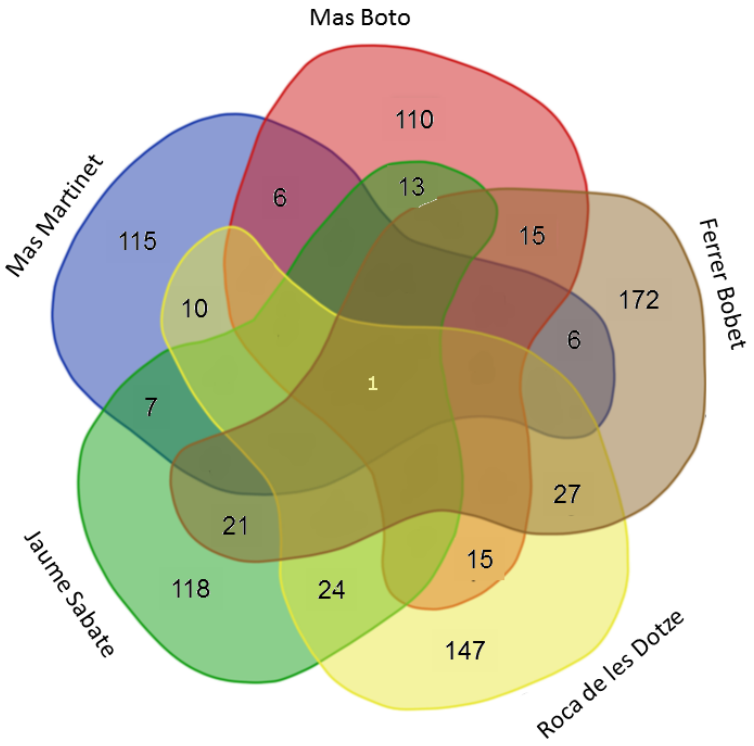

Fig. 4. Venn diagram showing the OTUs shared by the five vineyards. Overlapping coloured areas indicated the number of shared OTUs between pairs of vineyards.

These results confirm the nonrandom distribution of bacterial taxa present in different grape musts across selected vineyards and point to the possible consequences of the

metabolic activity of these specific taxonomic groups over wine quality and its characterization, even within vineyards from the same growing region, which had not been previously described. Whether these populations' patterns actively produce discriminative chemosensory characteristics of wines within Priorat vineyards must be experimentally demonstrated.

Conclusions

The most abundant bacterial taxa of Grenache and Carignan grapes were investigated for the first time by NGS. Regional patterns in grape berries bacterial communities suggest that local environmental conditions and grape varietal are responsible for driving bacterial diversity within a single viticultural zone. We were able to relate bacterial communities with vineyard geographical orientation, vineyard origin and grape variety within the same growing region. This study showed for the first time nonrandom distribution of grape bacteria across differently oriented vineyards, specifically within the Priorat region, which allows to propose that these characteristic bacterial communities could be used to drive specific wine properties and naturally enforce distinctive terroir characteristics in local wine blends

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary data

Fig. S1. Map of the sampled vineyards at the Priorat. FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó.

Table S1
Relative abundance of bacterial phyla averaged by sample type. "Other phyla" include Thermi, Gemmatimonadetes, Planctomycetes, Cyanobacteria, TM7 and SR1.

	FB-Gx	FB-Cy	MM-Gx	MM-Cy	JS-Gx	JS-Cy	RD-Gx	RD-Cy	G-Gx	G-Cy	MB-Gx	MB-Cy	MS-Gx	SD-Gx	MM-W
Firmicutes	40.54	1.62	73.04	99.91	21.89	70.81	27.16	4.46	11.89	22.70	90.27	12.97	52.43	29.19	99.12
Proteobacteria	41.01	98.38	22.50	0.09	53.11	25.27	52.70	89.86	69.46	62.16	7.39	85.95	28.92	45.14	0.74
Alphaproteobacteria	10.68	0.27	2.36	0.09	8.92	4.19	9.32	26.35	3.24	2.43	2.52	9.46	0.27	11.89	0.34
Betaproteobacteria	4.66	0.00	1.15	0.00	4.05	1.62	3.38	1.35	4.86	2.43	2.52	1.76	14.32	3.78	0.07
Deltaproteobacteria	0.27	0.00	0.00	0.00	0.00	0.00	0.14	0.14	0.27	0.00	0.00	0.00	0.27	0.00	0.00
Epsilonproteobacteria	0.00	0.00	0.14	0.00	0.27	0.14	3.38	0.14	0.54	0.00	0.00	0.00	1.08	0.00	0.00
Gammaproteobacteria	25.41	98.11	18.85	0.00	39.86	19.32	36.49	61.89	60.54	57.30	2.34	74.73	12.97	29.46	0.34
Actinobacteria	8.18	0.00	2.30	0.00	18.92	0.68	14.86	4.86	10.81	9.46	0.99	0.95	1.89	14.86	0.00
Bacteroidetes	9.80	0.00	1.89	0.00	5.68	2.16	4.05	0.81	2.97	4.86	0.99	0.14	11.89	9.19	0.14
Fusobacteria	0.27	0.00	0.00	0.00	0.00	1.08	0.14	0.00	2.70	0.27	0.00	0.00	4.05	0.27	0.00
Chloroflexi	0.00	0.00	0.20	0.00	0.41	0.00	0.27	0.00	0.27	0.00	0.00	0.00	0.00	0.54	0.00
Acidobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.81	0.00	0.27	0.27	0.00	0.00	0.27	0.00	0.00
Other Phyla	0.20	0.00	0.07	0.00	0.00	0.00	0.00	0.00	1.62	0.27	0.36	0.00	0.54	0.81	0.00

Gx: Grenache; Cy: Carignan; W: Wine; FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; G: Genium; MB: Mas Botó; MS: Mas Sinén

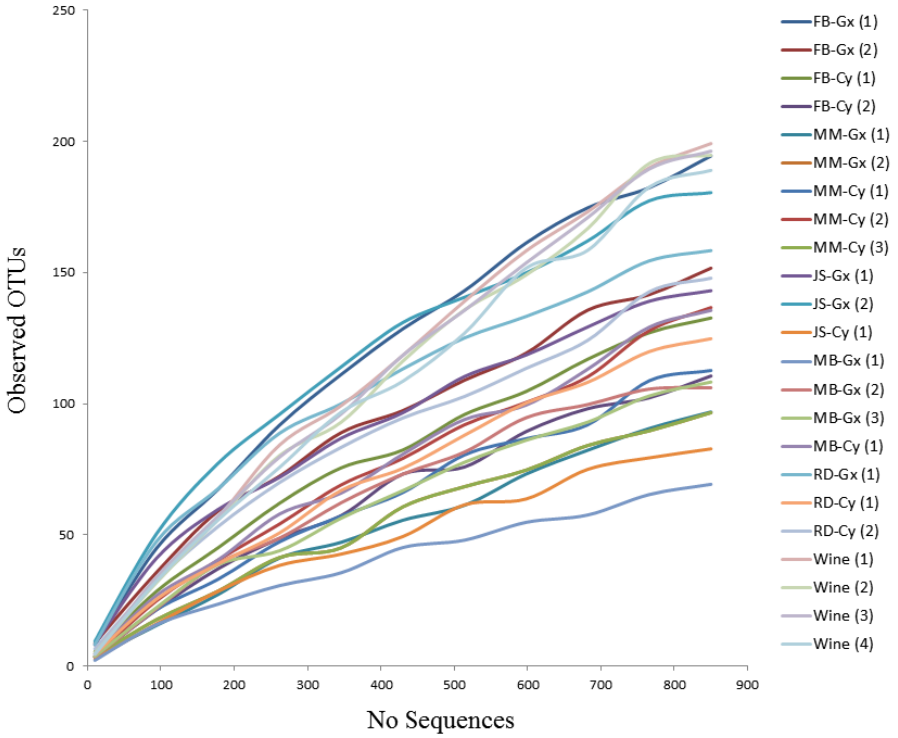

Fig. S2. Rarefaction plot for observed OTUs calculated at 850 sequences per sample.

Table S2
V4 primers and barcodes used in this study.

REVERSE PRIMER

ADAPTER	*KEY*	*BARCODE*	*LINKER*	*TARGET 16S PRIMER*
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CTAAGGTAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TAAGGAGAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AAGAGGATTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TACCAAGATC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CAGAAGGAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CTGCAAGTTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTCGTGATTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTCCGATAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGAGCGGAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CTGACCGAAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCCTCGAATC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TAGGTGGTTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTAACGGAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTGGAGTGTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTAGAGGTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTGGATGAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTATTCGTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	AGGCAATTGC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTAGTCGGAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CAGATCCATC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCGCAATTAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTCGAGACGC	GAT	GTGCCAGCMGCCGCGGTAA

FORWARD PRIMER

ADAPTER	*LINKER*	*TARGET 16S PRIMER*
CCTCTCTATGGGCAGTCGGTGAT	CC	CVGGGTATCTAATCCBGTT

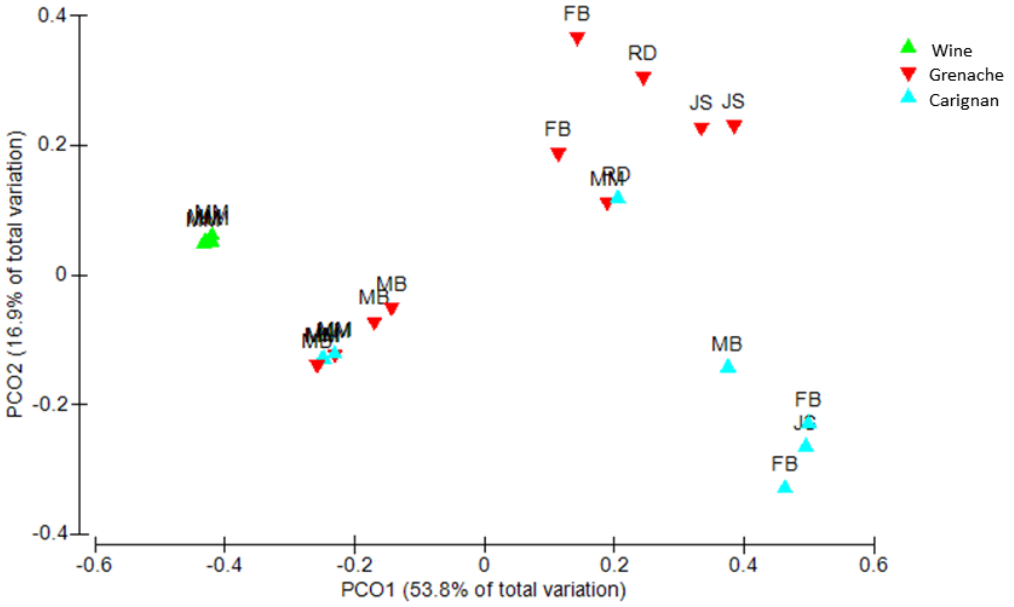

Fig. S3. Weighted UniFrac distance PCoA of bacterial communities at Priorat Vineyards including Grenache and Carignan must samples and wine fermentation samples. FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó.

CHAPTER II

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.

Keywords

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

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*.

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

Materials and Methods

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.

Isolation and growth conditions

All grape samples were processed in three ways: grape must, pulp and whole berries.

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.

(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.

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 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 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.

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.

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).

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.

Results

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.

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.

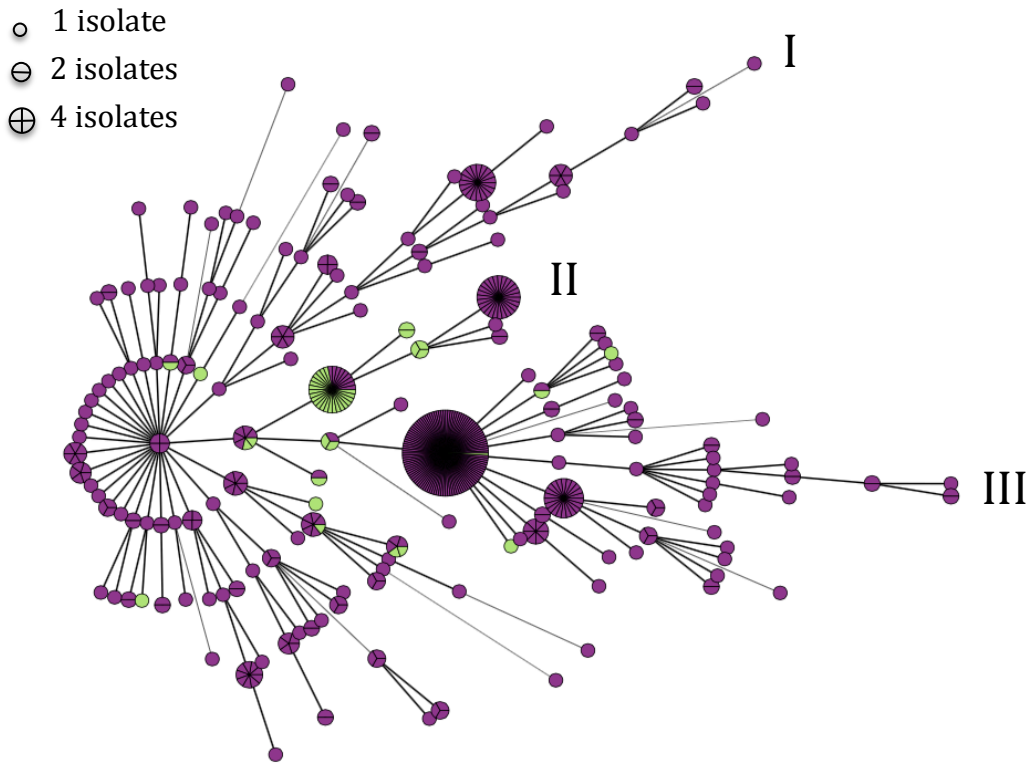

Fig. 1. Minimum spanning tree (MST) of 164 *O. oeni* strains typified by VNTR, according their isolation from grapes (green) or wines (violet). Each circle represents a strain and their diameters are proportional to the number of isolates for every strain. The biggest circle of cluster III is strain 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.

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).

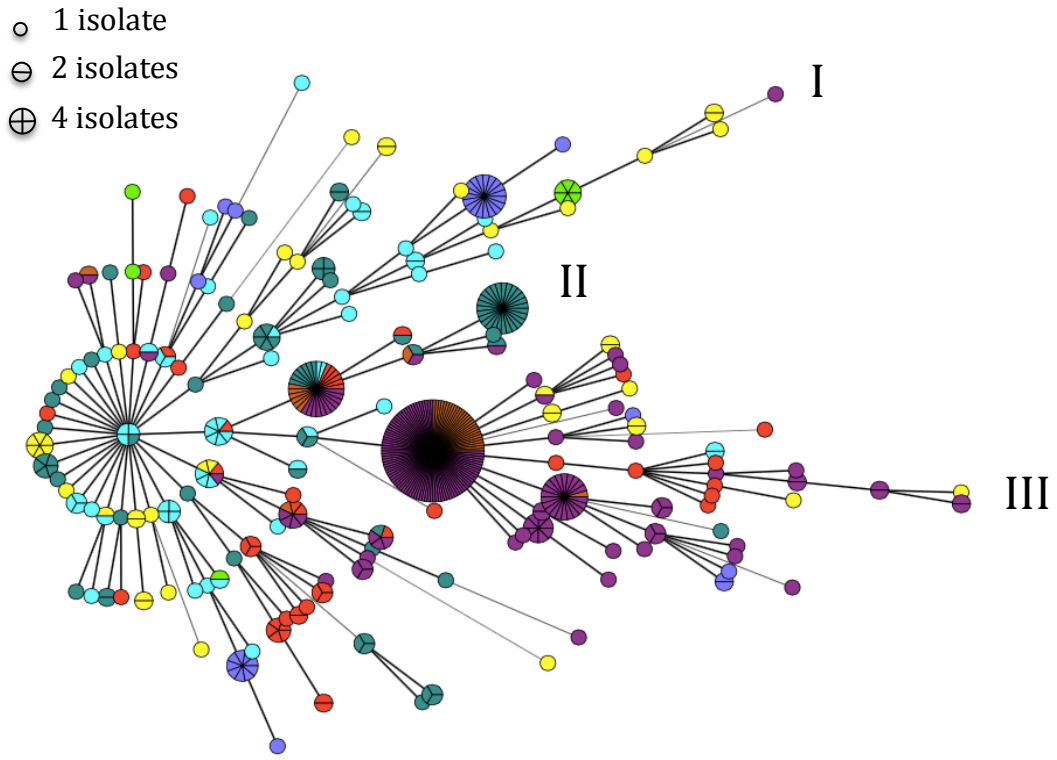

Fig. 2. Minimum spanning tree of 164 *O. oeni* strains typified by VNTR, according the different properties from which they were isolated: A (violet), B (brown), D (turquoise), E (blue cyan), F (yellow), G (blue purple), H (red) and I (green). See Fig. 1 for more details.

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

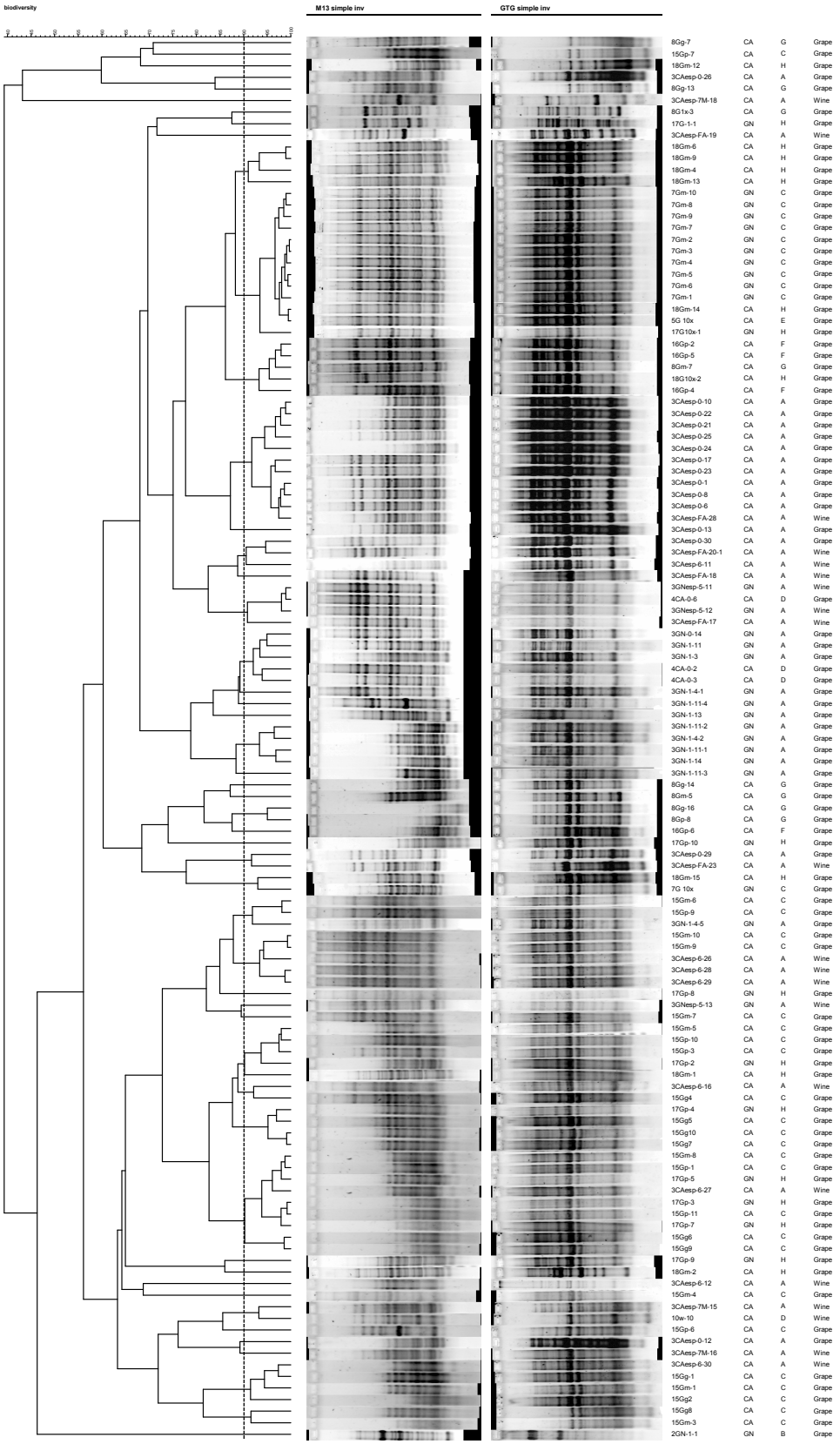

Fig. 3. Phylogenetic dendrogram obtained from RAPD-PCR(M13) and GTG_5-PCR fingerprints for 121 *Lactobacillus plantarum* isolates of this work. Similarities (%) are shown at left. Isolate names and source (variety, property and grape/wine: see Table 1) are at right.

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.

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.

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

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

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.

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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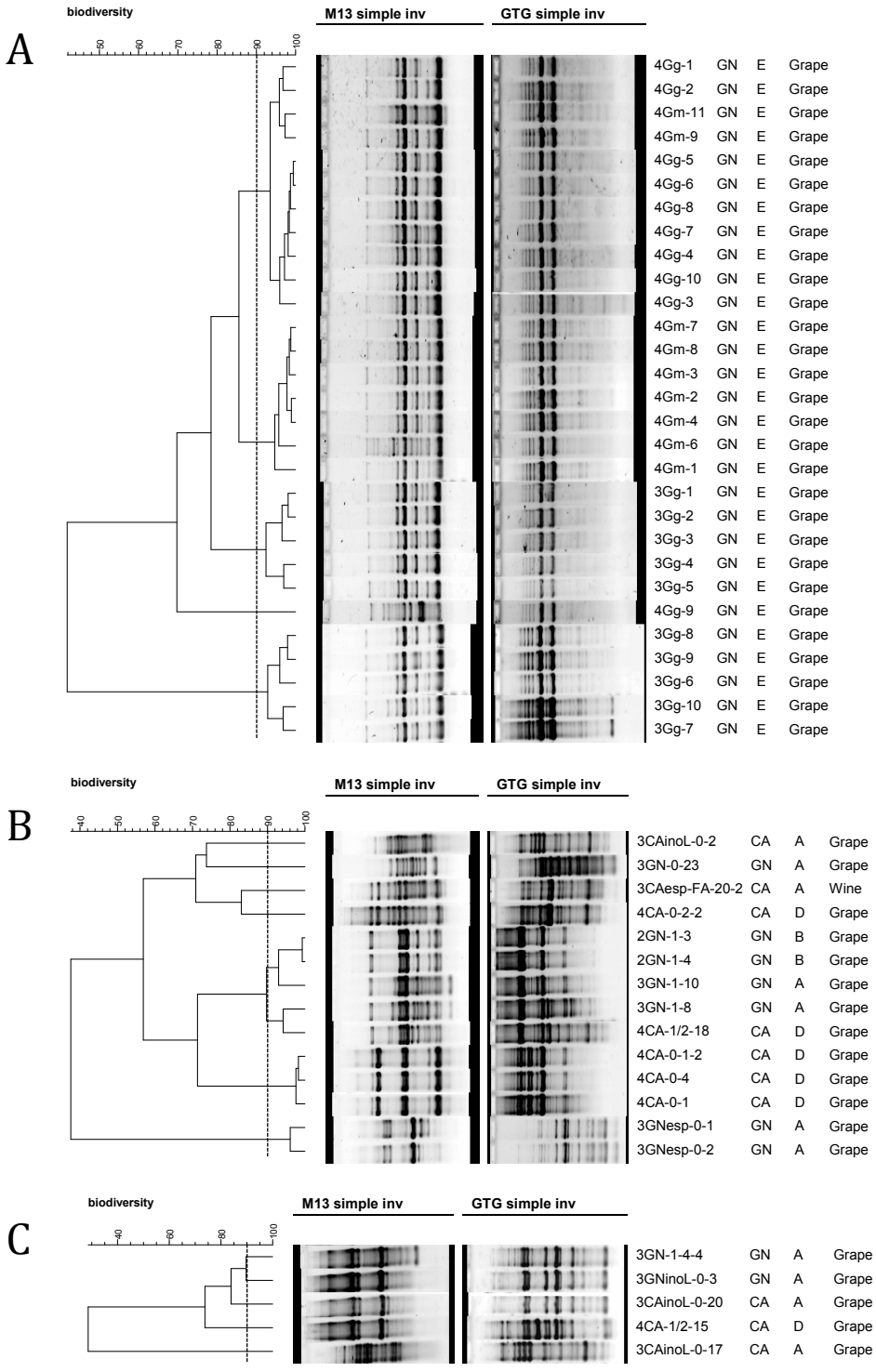
Supplementary data



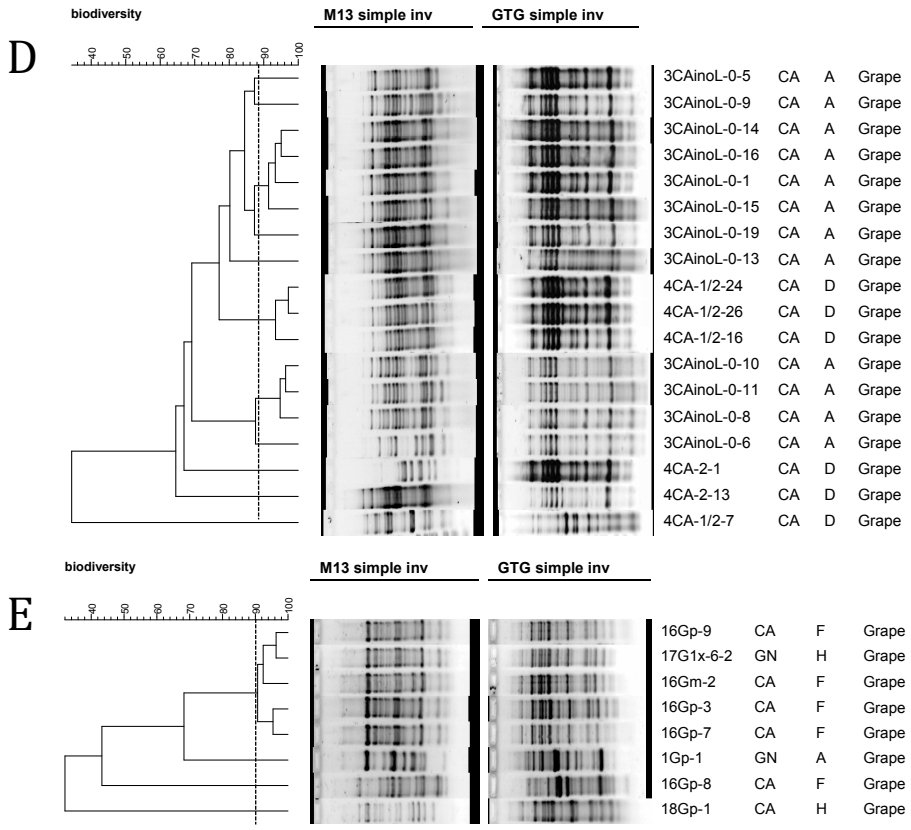


Fig. S1. Phylogenetic dendrograms obtained from RAPD-PCR(M13) and GTG_5-PCR fingerprints for 29 *Lactobacillus sanfranciscensis* isolates (A), 14 *L. lindneri* (B), five *L. mali* (C), 18 *Fructobacillus tropaeoli* (D) and eight *Pediococcus pentosaceus* (E), all isolated in this work. Similarities (%)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

CHAPTER III

Selection and characterization of autochthonous strains of *Oenococcus oeni* for vinification in Priorat (Catalonia, Spain)

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Abstract

The use of autochthonous strains of *Oenococcus oeni* for inducing malolactic fermentation (MLF) in wines is increasing because they are well adapted to the conditions of a specific area. The main aim of this work was to select *O. oeni* strains from Priorat (Catalonia, Spain) wines that would be able to carry out the MLF while maintaining the characteristics of the wine. Forty-five autochthonous strains were selected based on their degradation of L-malic acid, resistance to low pH and high ethanol, and the absence of biogenic amine genes. The three strains with the best characteristics were inoculated and MLF was carried out successfully with the final wines showing good characteristics. One of the inoculated *O. oeni* strains would be a good candidate to study as possible starter culture. It shows that autochthonous *O. oeni* strains, once selected, have the potential to be used as an inoculum for wines.

Keywords

Autochthonous strains - Malolactic fermentation - *Oenococcus oeni* - Priorat - Wine

Introduction

Oenococcus oeni is the major species among lactic acid bacteria (LAB) involved in the malolactic fermentation (MLF) of wine (Wibowo et al., 1985; Henick-Kling, 1993). During MLF, L-malic acid is decarboxylated to L-lactic acid, resulting in wine deacidification. This is a crucial step in red winemaking as it provides enhanced organoleptic qualities and microbial stabilization of the wine (Davis et al., 1988; Lonvaud-Funel, 1999; Liu, 2002; Bartowsky, 2005; Cappello et al., 2017).

MLF can be induced by inoculating commercial starters of *O. oeni*. However, this is not always successful because wine is a very harsh environment for bacterial growth (Coucheney et al., 2005; Spano and Massa, 2006; Ruiz et al., 2010), mainly due to the presence of ethanol (Capucho and San Romão, 1994; Zapparoli et al., 2009). The other limiting conditions of wine (few nutrients, phenolic compounds, low pH) may restrict cell viability in such a way as to make MLF sluggish or even fail (Carreté et al., 2002). In addition, the application of similar commercial bacterial starters across different world regions may lead to a certain product uniformity (Mas et al., 2016). Hence, the application of an autochthonous starter culture, well adapted to the conditions of a specific wine-producing area, has already been suggested (Nielsen et al., 1996; Ruiz et al., 2010). For this reason, several studies have been performed on the characterization of *O. oeni* biodiversity with the aim of selecting putative autochthonous starter cultures (Capozzi et al., 2010; Capozzi et al., 2014; González-Arenzana et al., 2014; Lamontanara et al., 2014).

Climate change poses a major additional problem for MLF. Over the last 10-30 years, evidences of earlier fruit maturation patterns, and consequently modified vine development, have been observed, both of which have been attributed to rising temperatures worldwide (Jones et al., 2005). The faster ripening of the grapes leads to a higher sugar content and therefore a higher ethanol content in the wines (Mira de Orduña, 2010; Webb et al., 2011). In the prestigious qualified appellation of Priorat in southern Catalonia (northeast Spain), wines easily reach an ethanol content of 14%, and sometimes more (De Herralde et al., 2012). The low acidity of these wines together with the earlier fruit maturation patterns diminishes their L-malic acid content, thus restricting the growth of *O. oeni*. Resistance to these harsh conditions (high ethanol and low pH) was the main criterion for strain selection in this work.

The preferences of consumers call for superior wines from a particular region to possess unique qualities and character (terroir wines) that differentiate them from wines of the same variety from other regions (Bisson et al., 2002). Wines perceived as being of high quality can be produced anywhere, even though, according to the concept of terroir, the local environment will influence the composition of the wine produced in a specific growing region (Gilbert et al., 2014; Zarraonaindia et al., 2015). Among other things, this involves the contribution of the indigenous microbiota in shaping the unique quality of the wine (Bartowsky et al., 2015).

A huge diversity of autochthonous *O. oeni* strains performing MLF has been found in wines (Reguant and Bordons, 2003; El Khoury et al., 2017), and this diversity is important within the same location (López et al., 2007; Cappello et al., 2010). Population structure analyses of strains in wines from diverse geographic origins have shown that there are two major genetic groups of *O. oeni* strains, known as A and B (Bilhère et al., 2009; Bridier et al., 2010). For this reason, a minimal genetic characterization of the selected strains using the single nucleotide polymorphism (SNP) method was included as an aim in this work.

Another decisive characteristic for our strain selection was the absence of the ability to produce biogenic amines (BA), which are compounds that are undesirable in wines because they may induce headaches, respiratory distress, hyper-hypotension and various allergenic disorders (Silla-Santos, 1996). Different results have been reported for BA production by *O. oeni* and other LAB and it is of utmost importance to avoid formation of these amines during MLF (Costantini et al., 2006; Landete et al., 2007a).

The main aim of this study was therefore to characterize LAB strains isolated from Priorat wine samples in order to select those with the best characteristics for application as oenological starter cultures. Hence, the selected strains needed to be evaluated by inoculating them into a real Priorat wine in the cellar, checking their imposition and analysing the final wines.

Materials and Methods

Strains

A total of 45 autochthonous LAB strains (Table 1) isolated from Priorat wines from vintages 2012 and 2013 and described in a previous study (Franquès et al., 2017) were chosen following the criterion of having been isolated at least twice in two different wines. They consisted of 41 *O. oeni* strains, two *Lactobacillus plantarum* strains, one *Fructobacillus tropaeoli* strain and one *L. mali* strain. Six of the *O. oeni* strains were found in both 2012 and 2013 vintages.

L-malic acid degradation test in a wine-like medium

The strains were grown in MRSm1 (Franquès et al., 2017), which is MRS (De Man et al., 1960) supplemented with L-malic acid (3 g/L), fructose (5 g/L), L-cysteine (0.5 g/L) and tomato juice (100 mL/L) at pH 5, until A_{600nm} = 1.6. The pellet obtained was inoculated (2%) into 50 mL of wine-like medium (WLM) (Bordas et al., 2013), which contained ethanol (12 or 14% v/v) added aseptically to the following sterilized base medium: 2 g/L fructose, 2 g/L tartaric acid, 0.5 g/L citric acid, 2 g/L L-malic acid, 5 g/L yeast extract, 0.1 g/L acetic acid, and 5 g/L glycerol, adjusted to pH 3.4 with 1 N NaOH. Then, it was incubated at 20ºC, 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.

Stress resistance test

The strains were precultured in a grape juice medium (GJM) similar to that used for El Khoury et al. (2017), which contained per liter 250 mL white grape juice (final sugar content of 50 g/L), 5 g yeast extract, 1 mL Tween 80, and 6% (v/v) ethanol, at pH 4. When the population reached approximately 10^8 CFU/mL, they were inoculated (0.2%) into 10 mL tubes of the same GJM so that a stress resistance test could be carried out in eight conditions combining different pH (2.8, 3, 3.3, 3.6 and 4) and ethanol concentrations (6, 12, 14 and 16%, v/v). The growth of the strains at 20ºC was followed for 3 weeks, checking the OD_{600} every 48 hours with a POLARstar Omega spectrophotometer (BMG Labtech).

Table 1
LAB strains used and characterized in this study.

Strains	Species	Efficiency group [a]	Resistance group [b]	Phylogroup [c]	Gene *odc*	Gene *hdc*
1Pw1 †	*Oenococcus oeni*	E3	R1	B	—	—
1Pw3	,,	E2	—	—	—	+
1Pw4	,,	E3	R1	B	—	—
1Pw5	,,	E1	—	—	—	+
1Pw6	,,	E3	—	—	—	—
1Pw7	,,	E3	—	—	—	+
1Pw8	,,	E3	—	—	—	—
1Pw9	,,	E3	R4	A	—	—
1Pw10	,,	E2	—	—	—	+
1Pw11	,,	E3	—	—	—	+
1Pw12	,,	E3	—	—	—	+
1Pw13	,,	E3	R4	A	—	—
1Pw14	,,	E3	—	—	—	+
1Pw15	,,	E3	—	—	—	—
1Pw16 †	,,	E3	—	—	—	—
1Pw17 †	,,	E1	R3	A	—	—
1Pw18	,,	E3	—	—	—	+
1Pw19	,,	E1	—	—	+	—
1Pw20	,,	E3	R1	B	—	—
1Pw2 †	,,	E3	R1	B	—	—
2Pw2	,,	E3	R1	B	—	—
2Pw3	,,	E3	R1	B	—	—
2Pw5	,,	E3	—	—	—	—
2Pw6	,,	E3	—	—	—	—
2Pw7 †	,,	E3	—	—	—	—
2Pw8	,,	E3	—	—	—	—
2Pw9	,,	E1	—	—	—	—
2Pw10	,,	E2	R2	A	—	—
2Pw11	,,	E3	R1	B	—	—
2Pw12	,,	E3	R1	B	—	—
2Pw13	,,	E3	R2	B	—	—
2Pw14	,,	E3	R2	A	—	—
2Pw15	,,	E3	R4	A	—	—
2Pw16	,,	E3	R3	A	—	—
2Pw17	,,	E3	R3	A	—	—
2Pw19 †	,,	E3	—	—	—	—
2Pw20	,,	E3	R3	A	—	—
2Pw21	,,	E3	R2	B	—	—
2Pw22	,,	E3	R4	A	—	—
2Pw23	,,	E3	R4	A	—	—
2Pw24	,,	E3	R4	A	—	—
1Ptr11	*Fructobacillus tropaeoli*	E1	R1	—	—	—
1Pma1	*Lactobacillus mali*	E1	—	—	—	—
1Ppl21	*L. plantarum*	E1	—	—	—	—
1Ppl24	,,	E1	R1	—	—	—

[a] Efficiency groups of strains according to Table 3, with E1 being the least efficient and E3 the most efficient.
[b] R1 is the group with the least resistant strains and R4 the most resistant strains. Strains with an empty field belong to group R0, since they were unable to grow with 6% ethanol at pH 4.
[c] Phylogroup of *O. oeni* strains. † These strains were found in both the 2012 and 2013 vintages.

Biogenic amine gene detection

The detection of the histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tdc*) and ornithine decarboxylase (*odc*) genes was performed by specific PCRs. The DNA extraction was performed according to Ruiz-Barba et al. (2005). The *hdc* gene was detected using HDC3 and HDC4 primers (Coton and Coton, 2005), the *tdc* gene using P1-rev and p0303 primers (Landete et al., 2007b), and the *odc* gene using primers 3 and 16 (Marcobal et al., 2005). LAB strains having the BA genes were used as positive controls: *Lactobacillus* sp. 30a (ATCC 33222) for *hdc*, *L. brevis* Enolab 4415 (kindly provided by Sergi Ferrer, University of Valencia) for *tdc*, and *O. oeni* Enolab 4783 (also provided by S. Ferrer) for *odc*.

The *hdc* PCR products were analysed using MultiNA equipment (Microchip Electrophoresis System for DNA/RNA Analysis, Shimadzu) and the MultiNA kit (DNA 100-1500 bp, Shimadzu). SYBR Gold buffer (Invitrogen) diluted 100 times in TE (pH 8) and the molecular marker phiX174-HaeIII digest (Promega) were used. The *tdc* and *odc* PCR products were checked by electrophoresis in 1.2% (w/v) agarose gels with Tris-borate-EDTA buffer (TBE) 0.5x (80V, 1h30) and dyed with ethidium bromide. DNA molecular weight markers 1KB Plus Invitrogen (REF 10787-018) were used for reference purposes.

Classification of strains in phylogroups using SNP genotyping

The simple nucleotide polymorphism (SNP) technique was used to analyse *O. oeni* strains and include them in phylogroups A and B. Two other *O. oeni* strains with previously characterized SNPs (Campbell-Sills et al., 2015) were also included in the study: PSU-1 (ATCC-BAA-331) and ATCC-BAA-1163, which belong to groups A and B, respectively. Before genotyping, a real time PCR was performed to compare the DNA samples with each other and check the DNA concentration of each.

SNP methodology was applied following Campbell-Sills et al. (2015) and El Khoury et al. (2017). Manual curation and selection were performed to select 39 SNPs, which were amplified and sequenced using the Sequenom strategy. The genotyping results of these SNPs for each strain were concatenated into a single sequence of 39 bp. The sequence alignments and phylogroup analysis were performed using MEGA software

6.0.5 (Tamura et al., 2013) with 1000 bootstrap replications on neighbour-joining distance calculation using Kimura 2-parameter.

Performance of MLF in industrial wines inoculated with the selected strains

The selected strains (WW strains, from the Wildwine project) were used as starter culture for the inoculation of two industrial wines (one from Grenache and the other from Carignan) at the Ferrer-Bobet winery, located on road T-740, between Falset and Porrera, in Priorat. The alcoholic fermentations (AF) were carried out with autochthonous *S. cerevisiae* strains (CECT13132, CECT13133 and CECT13134) isolated in the same Priorat area (Mas et al., 2015; Padilla et al., 2017). The main analytical characteristics at the end of AF are shown in Table 2. For each wine, the strains were grown separately in 1.5 L of MRSm1 medium until OD_{600} = 1.6, corresponding to 10^9 CFU/mL. The total pellet obtained was washed with saline solution and used to inoculate (2%) oak barrels containing 225 L of wine. Other barrels were inoculated with the commercial *O. oeni* CH11 strain (Viniflora® CH11, from Chr. Hansen A/S, Hørsholm, Denmark) as a control, following the manufacturer's indications.

All barrels were kept in the Ferrer-Bobet winery cellar and set aside for MLF at 20ºC. L-malic acid consumption was followed by enzymatic analysis in the laboratory of the same winery. The final MLF samples were collected and cultured in solid MRSm3 (Franquès et al., 2017), which is MRSm1 supplemented with nystatin (100 mg/L) and sodium azide (25 mg/L). In order to confirm the presence of the bacteria inoculated, 30 colonies of each sample were selected and their DNA extracted, then they were typed using the VNTR technique (Claisse and Lonvaud-Funel, 2012) including the modifications made by Franquès et al. (2017).

Wine chemical analysis

The main chemical characteristics (sugars, ethanol, glycerol, pH, total and volatile acidity, organic acids, nitrogen, sulphur dioxide and phenolics; see details in Table 2) of the final real wines after MLF by the selected strains were analysed following OIV methods (OIV, 2009) by the Catalan Institute of Vineyard and Wine (INCAVI, Vilafranca del Penedès, Catalonia, Spain).

Table 2
Main analytical characteristics of the wines made at the Ferrer-Bobet winery after MLF with inoculated strains of *O. oeni*. WW: mix of selected strains (1Pw13, 2Pw15 and 2Pw22) from the Wildwine project. CO: commercial *O. oeni* strain CH11. AF: alcoholic fermentation.

Inoculated strains		**WW**	**CO**	**WW**	**CO**
Wine variety		Grenache	Grenache	Carignan	Carignan
Contents at end of AF	Glucose + fructose (g/L)	0.25		0.45	
	pH	3.20		3.18	
	Ethanol (%)	14.95±0.11		13.95±0.10	
	Acetic acid (g/L)	0.31		0.44	
	L-malic acid (g/L)	0.4		1.0	
Glucose + fructose	g/L	0.16	0.23	0.13	0.21
Glycerol	g/L	6.80	6.80	8.66	8.43
pH		3.32	3.32	3.34	3.33
Tartaric acid	g/L	2.41	2.49	2.51	2.47
Total acidity	g/L	5.7±0.2	5.9±0.2	6.5±0.2	6.5±0.2
Volatile acidity	g/L	0.45±0.07	0.41±0.08	0.55±0.09	0.54± 0.08
L-lactic acid	g/L	0.41	0.21	0.72	0.7
L-malic acid	g/L	< 0.1	0.4	< 0.1	< 0.1
Citric acid	g/L	< 0.05	< 0.05	< 0.05	< 0.05
Alpha-amino nitrogen	mg/L	13.2	55.9	47.7	48.2
Ammoniacal nitrogen	mg/L	7.8	7.9	5.9	6.5
Folin-Ciocalteu Index		62.96	67.71	55.32	53.89
Anthocyanins	mg/L	466	484	555	546
Tannins	g/L	3.71	3.76	2.53	2.50

Results and discussion

In a previous study (Franquès et al., 2017), a survey of autochthonous LAB was carried out in the Catalan wine region of Priorat, with 166 strains being identified and typed. Of these, the 45 that were isolated at least twice were chosen to select strains with the greatest potential as malolactic cultures.

L-malic acid degradation test in wine-like medium

The 45 strains (41 *O. oeni* strains and four non-*Oenococcus* strains) were characterized by their L-malic acid degradation efficiency and their fermentation speed in WLM at 12% and 14% ethanol (Table 3). A clear difference could be seen between most strains of *O. oeni* and the few strains of other species. Regarding the *O. oeni* strains, three different behaviour groups were observed, with 75% (group E3) being accounted for by strains that consumed 100% of the L-malic acid (2 g/L) in both 12% and 14% (v/v) ethanol fermentations and were the quickest to do so. Some of the most rapid strains reached L-malic acid consumptions of 42 mg/L/h and 28 mg/L/h in the presence of 12% and 14% ethanol, respectively. This speed is equivalent to a consumption of 2 g/L L-malic acid in just four days.

Table 3
L-malic acid consumption (2 g/L) in wine-like medium (WLM) and MLF speed of the predominant strains from different species. Strains are grouped by their efficiency according to both degradation and MLF speed. Assays were done in duplicate with both 12% and 14% (v/v) ethanol.

Species	Efficiency group	No. of strains	% no. strains / total	% L-malic acid consumed		MLF speed (mg/L L-malic acid/h)	
				12% Ethanol	14% Ethanol	12% Ethanol	14% Ethanol
O. oeni	E3	34	75	100	100	11-42	14-28
	E2	3	7	100	80-100	11-14	3-7
	E1	4	9	50-100	50-100	4-21	4-21
L. mali	E1	1	2	100	73	15	5
F. tropaeoli	E1	1	2	100	66	15	5
L. plantarum	E1	2	4	36-100	< 50	4-15	4-5

The four non-*Oenococcus* isolates were considered as belonging to group E1 because none of them consumed more than 80% of the L-malic acid in 14% (v/v) ethanol, despite the fact that most of them consumed all the L-malic acid in 12% ethanol. *L. mali* and *F. tropaeoli* were slower than most *O. oeni* strains but quicker than the *L. plantarum* strains in both the 12% and 14% ethanol fermentations. The better performance of *O. oeni* over other species confirms once again its known characteristic of being the predominant LAB of MLF in wine (Wibowo et al., 1985; González-Arenzana et al., 2013).

Stress resistance test

Using both the viable culture results and the OD_{600} measurements over three weeks, the studied isolates were classified into five different groups depending on their degree of resistance, with R0 being the least resistant and R4 the most resistant, as shown in Table 1.

Twenty-one strains were unable to grow in the preculture step (pH 4 and 6% ethanol) in GJM. These were tagged as R0 and discarded from the experiment, as they could not grow in the least stressful condition. These 21 strains included 14 *O. oeni* that had been considered part of the more efficient E3 group in the previous experiment. Regarding these differences, it must be borne in mind that the efficiency assay was done in WLM, which is not as restrictive as the GJM medium used in the stress resistance test. Moreover, the inocula for WLM were grown in the rich MRS medium at 2%, whereas the inocula for GJM were grown in GJM at 0.2%. Therefore, the initial population in WLM was higher than in GJM. Finally, it must be taken into account that the goal of the efficiency assay was to measure the degradation of L-malic acid, while that of the resistance test was to see the possible growth under the different stress conditions.

The 12 isolates in group R1 showed good growth at pH 4, 3.6 and 3.3 with 6% ethanol. The growth kinetics of strain 1Pw4, representative of this R1 group, can be seen in Figure 1. In all other conditions, there was no growth even after three weeks of tracking. The four isolates in group R2 showed good growth in 6% ethanol and pH 4, 3.6 and 3.3 conditions. During the third week, the R2 isolates showed little growth in the medium with 12% ethanol and pH 4. The five isolates in group R3 showed active growth in 6% ethanol and pH 4, 3.6 and 3.3 conditions and in 12% ethanol and pH 4. Little growth was also noticed during the third week in the medium with 14% ethanol and pH 4.

Finally, there were six isolates in group R4 that showed good growth in 6% ethanol and pH 4, 3.6 and 3.3 conditions and in 12% and 14% ethanol and pH 4 conditions. Little growth was also noticed during the third week in the medium with 6% ethanol and pH 3, as shown in Figure 2 for strain 2Pw22, which is representative of this R4 group.

Of the few non-*Oenococcus* isolates, only the *F. tropaeoli* and one of the *L. plantarum* strains were classified in the R1 resistance group (Table 1). Nevertheless, all these isolates presented very low efficiency when degrading L-malic acid (Table 3).

Biogenic amine gene detection

MLF is generally considered to be a crucial factor for BA production, and studies have shown that the main BA generated in this phase are putrescine, histamine and tyramine (Lonvaud-Funel, 2001; Marcobal et al., 2006). Consequently, the isolates were tested for the presence of the corresponding genes.

None of the strains tested contained the *tdc* gene fragment, only one (1Pw19) contained the *odc* gene fragment, and eight (18%) contained the *hdc* gene fragment (Table 1). The strains that contained any of the three BA genes were discarded in the selection. This ensured that those selected would have no risk of producing these amines. Incidentally, all the strains harbouring BA genes were also discarded in the stress resistance test (see above).

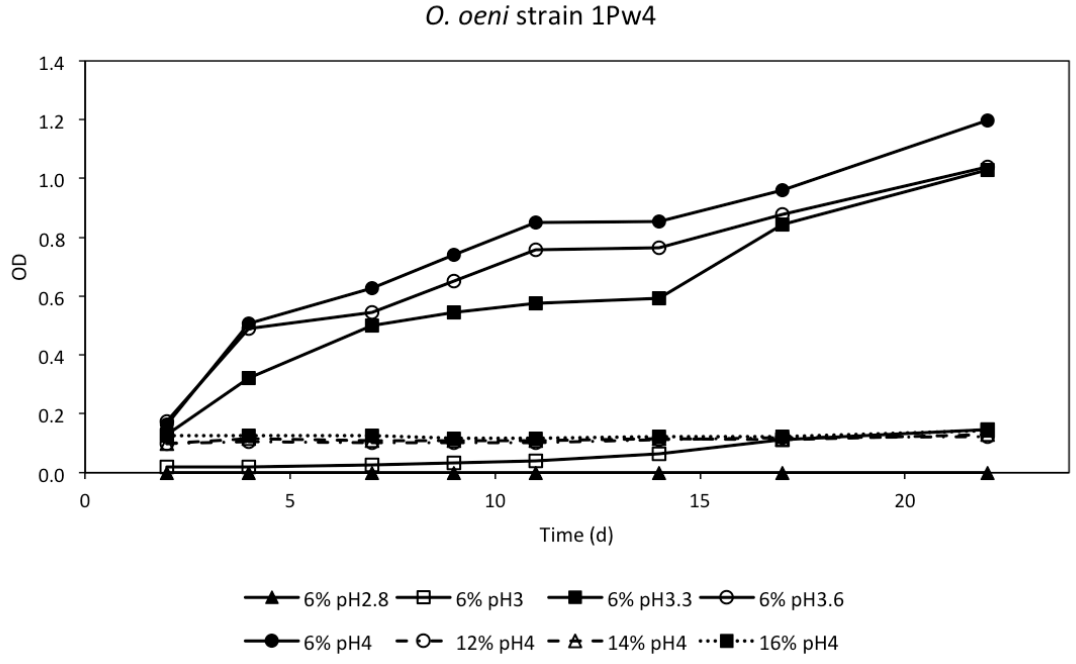

Fig. 1. Growth kinetics of *O. oeni* 1Pw4, a representative strain of the R1 resistance group.

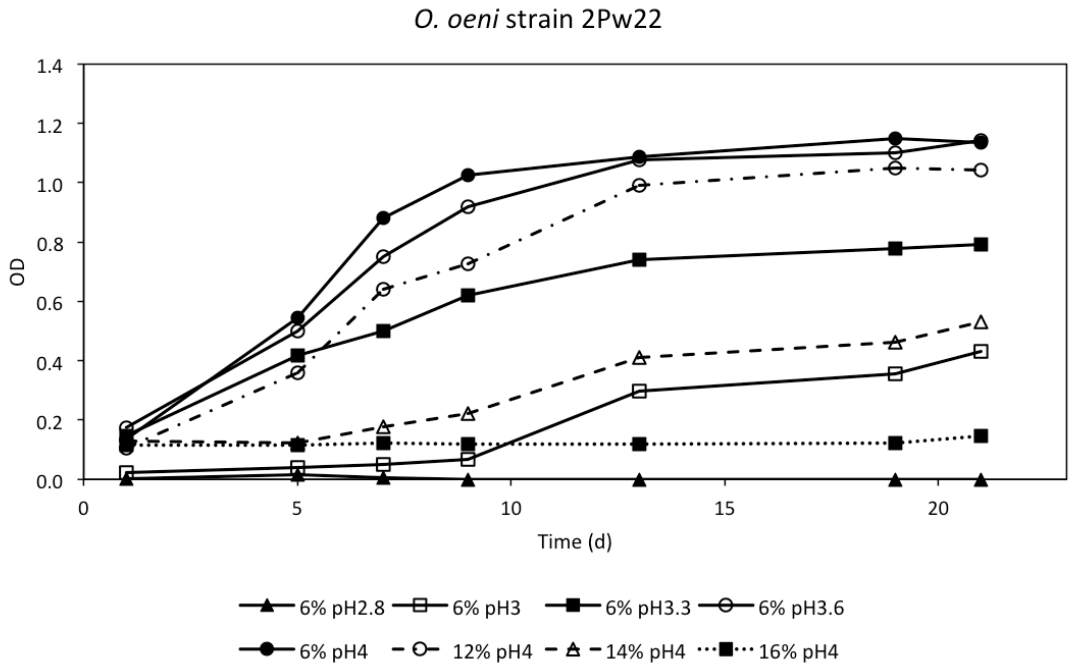

Fig. 2. Growth kinetics of *O. oeni* 2Pw22, a representative strain of the R4 resistance group.

Classification of O. oeni strains in phylogroups using SNP genotyping

SNP genotyping was carried out on 22 *O. oeni* preselected strains, in line with the above results and discarding the least resistant strains (group R0) and those containing BA genes. The 22 strains are those shown in Table 1 with their resistance group (R1, R2, R3 or R4). They could be assigned to the A and B subpopulations described earlier (Bilhère et al., 2009; Borneman et al., 2012; Campbell-Sills et al., 2015; El Khoury et al., 2017). In order to verify this, the known PSU-1 and ATCC-BAA-1163 strains representative of the A and B subpopulations, respectively, were included in the SNP analysis.

A total of 39 SNPs were manually selected following El Khoury et al. (2017) and checked for each of the 22 selected strains and for the two "control" strains characterized previously (Bridier et al., 2010). SNP data analysis revealed that all 24 strains possessed SNP combinations corresponding to seven of the predefined sequence types (ST) (El Khoury et al., 2017). Using these data, an unrooted tree was reconstructed by the neighbour-joining method (Figure 3). The result confirmed the assignment of all strains to groups A (upper branch) and B (lower branch) (Figure 3).

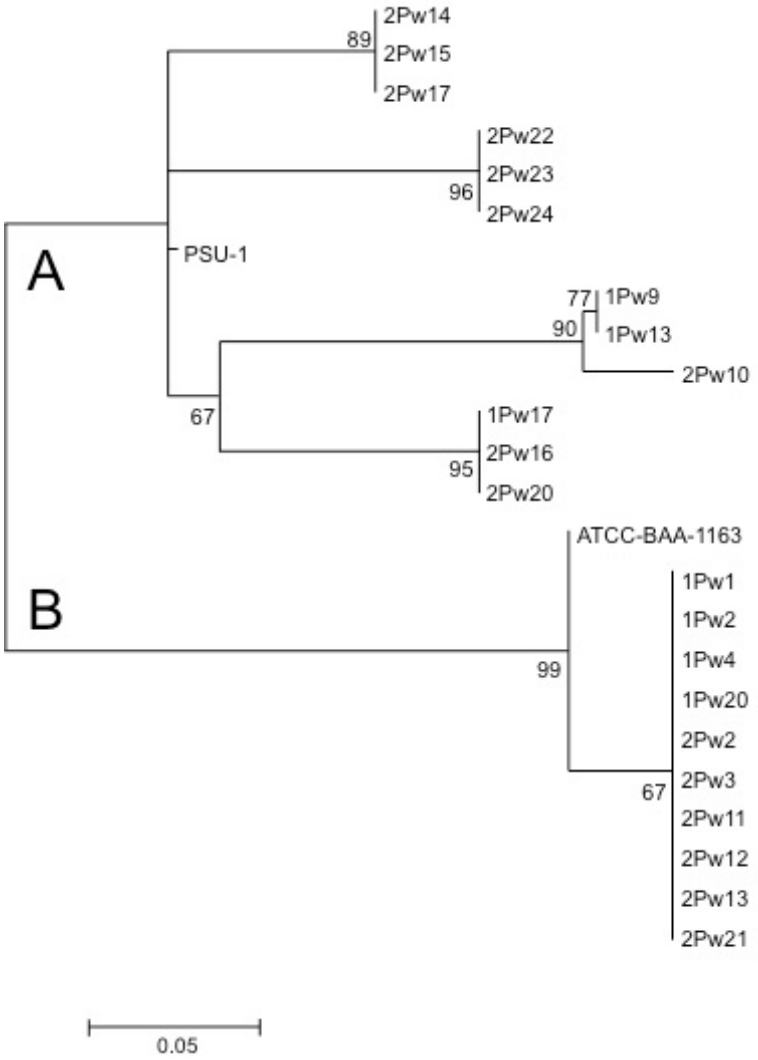

Fig. 3. Distribution of preselected *O. oeni* strains in phylogroups. The neighbour-joining tree was constructed using the 39 concatenated sequences of single nucleotide polymorphism (SNP) identified by analysing 24 strains. The number of nodes indicates the bootstrap values (%). The scale bar represents the number of substitutions per site.

As can be seen in Figure 3, 12 of the 22 strains were assigned to phylogroup A and the other 10 to group B. The strains with the best characteristics, i.e. the six in group R4 (Table 1), are located in phylogroup A. This agrees with the hypothesis that it is usually the A strains that are best adapted to wine conditions (Campbell-Sills et al., 2015), but we must not forget that some B strains with good malolactic behaviour in MLF were also isolated by our group from other wines of the same region (Bordas et al., 2013).

Performance of MLF in industrial wines inoculated with three selected strains of O. oeni

Of the initial 45 LAB strains, the best were selected according to the results shown above, i.e. the L-malic acid degradation test, the absence of BA genes and the stress resistance test. The best strains (Table 1) were the six in the resistance group R4 (1Pw9, 1Pw13, 2Pw15, 2Pw22, 2Pw23 and 2Pw24), which were assigned to the best efficiency group E3 and lacked the three BA genes. Bearing in mind that some of these strains presented the same SNP profile (see Figure 3: 1Pw9-1Pw13 and 2Pw22-2Pw23-2Pw24), three were selected, one from each SNP profile: 1Pw13, 2Pw15 and 2Pw22. All were *O. oeni* and had been isolated from different cellars (Franquès et al., 2017). As expected, the strains with the best inoculum characteristics were from *O. oeni*, the species most used for MLF induction (Kunkee, 1984; Solieri et al., 2010).

Barrels from the Ferrer-Bobet cellar with 225 L of Grenache and Carignan wines were inoculated with a mixed pellet of these three selected WW strains (1:1:1), previously grown in MRSm1 medium. As a control, other barrels of the same wines were inoculated with a commercial strain (CH11) of *O. oeni*.

The inoculum prepared with the three WW strains completed MLF in one Grenache (10 d) and one Carignan (77 d) wine. Final viable cell numbers of LAB (in MRSm3) were 10^4 and 10^5 CFU/mL in these Grenache and Carignan wines, respectively. In spite of these low numbers, all colonies were verified to be *O. oeni*. The imposition of two inoculated *O. oeni* strains (1Pw13 and 2Pw22) in those MLFs was confirmed by typing 30 colonies using the VNTR technique (Table 4). Another strain (profile "I"), which was not inoculated, was found at a low concentration (4%) in the Carignan wine. This "I" strain had been isolated previously in the same cellar (Franquès et al., 2017). The 2Pw15 strain was not recovered from any wine. The 1Pw13 strain was recovered from every wine and was the predominant strain in every case. The profile of the commercial strain *O. oeni* CH11 was found exclusively in the wines inoculated with this strain.

MLF was successfully carried out in the Grenache and Carignan wines in the cellar. This was despite the high ethanol content, especially for the Grenache wine (14.95%). The duration of MLF with WW strains was shorter in the Grenache than in the Carignan wine. Initial L-malic acid was low in the wines (0.4 and 1 g/L in Grenache and Carignan, respectively), but these concentrations are the ones currently measured in

Priorat wines. The better MLF performance in the Grenache than in the Carignan wine despite the harsher conditions (more ethanol and less L-malic acid) must surely be due to the different wine matrix of the two wine varieties, and in the Carignan there is probably some growth-limiting nutrients or other inhibiting substances (Gockowiak and Henschke, 2003).

Table 4. Proportions of *O. oeni* strains found at the end of MLF carried out in the cellar and inoculated with the three selected strains (1Pw13, 2Pw15 and 2Pw22). Proportions were obtained by typing 30 colonies of each sample by the VNTR technique. "I" is a wild (not an inoculated) strain that was previously found in the same cellar.

Wine	**Grenache**	**Carignan**
Duration of MLF (d)	10	77
***O. oeni* strain**	**Proportions (%)**	
1Pw13	73	88
2Pw22	27	8
"I"	—	4

MLF in the Grenache wines was performed by autochthonous strains and not by the commercial strain. Thus, the WW strains selected and used as inoculum were efficient. It is worth noting that the 1Pw13 strain, which was detected as the predominant strain in both the Grenache and Carignan wines (Table 4), performed the MLF in relatively short times. Moreover, this strain managed MLF without special previous adaptation, since the WW strains were grown in rich MRS medium and then harvested and inoculated directly into the wine. Therefore, it can be suggested that 1Pw13 is a good candidate for a starter culture and also for studying the molecular mechanisms of stress response to wine adaptation.

Wine chemical analysis

The results of the main analytical characteristics of the final wines after MLF using the three selected strains are shown in Table 2. It can be seen that all the wines had a high ethanol content, nearly 15% in the Grenache.

The initial L-malic acid was low, especially in this Grenache, with only 0.4 g/L. MLF was carried out quickly by WW strains in this wine and after 10 days the L-malic acid was exhausted. However, in the Grenache wine inoculated with the commercial *O. oeni* strain, the L-malic acid content did not decrease after more than two months. Consequently, the L-lactic acid content rose to 0.41 g/L in the Grenache wine with WW strains and only to 0.21 g/L in the one inoculated with the commercial strain. MLF was slower in the Carignan wines, but after 77 days L-malic acid was exhausted in the Carignan wine with WW strains, and two days earlier in the one inoculated with the commercial strain. Since the initial content of L-malic acid was 1 g/L in the Carignan wines, the L-lactic acid content (around 0.7 g/L) was higher than in the Grenache, as expected.

Meanwhile, citric acid was not detected (< 0.05 g/L) in any of the final wines (Table 2). It suggests that it was completely consumed by the same LAB strains, probably in connection with the lower initial concentration of L-malic acid in these wines. *O. oeni* usually degrades citric acid slightly after L-malic acid consumption (Bartowsky and Henschke, 2004), and here the low levels of L-malic acid would have facilitated the rapid consumption of citric acid. Despite this, volatile acidity remained at a reasonable level.

Other differences between the wines inoculated with WW strains and the commercial strain include the final residual sugar contents, which were slightly lower in those inoculated with WW strains (0.16 and 0.13 g/L glucose+fructose in Grenache and Carignan, respectively) than in those inoculated with the commercial strain (0.23 and 0.21 g/L in Grenache and Carignan, respectively). Another difference was that the alpha-amino nitrogen content was much lower in the Grenache wine with WW strains (13.2 g/L) than in the one inoculated with the commercial strain (55.9 g/L). This lower residual sugar content and alpha-amino nitrogen content in the wines fermented with WW strains can be seen as another positive characteristic of these strains. It means that they have an active metabolism under these harsh conditions and that these wines would run less risk of being contaminated by other bacteria.

Conclusions

In this study, three *O. oeni* strains were selected from 45 autochthonous LAB strains from Priorat wines based on their efficiency in degrading L-malic acid and most especially their resistance to high ethanol and low pH. The absence of biogenic amine genes in these strains was verified, and the SNP analysis placed them in phylogroup A, the same group in which other good wine-adapted strains have been found. After being inoculated into industrial wines, one of the strains showed good performance when carrying out the MLF. The characteristics of these wines suggest that this strain would be a good candidate for starter culture.

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4. GENERAL DISCUSSION AND PERSPECTIVES

The malolactic fermentation (MLF), which usually occurs after alcoholic fermentation (AF), and especially in red wines, 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 *Oenococcus oeni* (Henick-Kling, 1993). The trend of consumer preferences to the ecological wines represents an opportunity for traditional and peculiar terroirs such as Priorat. In this sense, the use of native LAB as inoculum can offer great potential (Ruiz et al., 2010). The occurrence of various LAB as *Pediococcus*, *Lactobacillus* and *Leuconostoc* species in musts from freshly crushed grapes has been reported previously (Godálová et al., 2016; Pardo and Zúñiga, 1992). However, very few studies have described the detection or isolation of *Oenococcus oeni* directly from the grape berries (Garijo et al., 2011; Renouf et al., 2007) or from the grape juice (Saguir et al., 2009).

Since 1990, the URV LAB research team is working in different aspects of the MLF. Factors like high ethanol content, low pH and phenolic compounds presence have been linked to a delay in the start of MLF (Reguant et al., 2000, 2005a; Rozès et al., 2003). The development of a reproducible method for molecular typing strains of *O. oeni* (Reguant and Bordons, 2003; Zapparoli et al., 2000) and a multiplex PCR technique that allows the simultaneous species identification and strain typing of *O. oeni* (Araque et al., 2009b) provided the study of population dynamics of these strains during wine MLF. Also, a better knowledge of the molecular processes involved in *O. oeni* tolerance to wine was required. Therefore the URV LAB research team has studied on *O. oeni* the role of specific metabolisms like citrate (Olguín et al., 2009) and arginine degradation (Araque et al., 2009a, 2011, 2013, 2016), the ATPase activity (Carreté et al., 2002), the relative expression of certain genes under wine stress conditions (Bordas et al., 2015; Olguín et al., 2010) and the characterization of the ethanol effect using microarrays and proteomic techniques (Olguín et al., 2015). In parallel, the isolation and selection of

autochthonous strains of *O. oeni* isolated from nearby vineyards have enlarged the collection of highly ethanol-tolerant strains as promising starters (Bordas et al., 2013). Moreover, the evaluation of the *O. oeni* acclimation to wine conditions in order to have a better understanding of which mechanisms are activated, being the attention focused on two cellular redox systems, glutathione and thioredoxin, not studied yet in this bacterium has been performed (Margalef-Català et al., 2016a, 2016b, 2017a, 2017b, 2017c). Finally, the interactions between oenological yeasts and LAB, especially *Oenococcus oeni*, have been investigated placing the emphasis on non-*Saccharomyces* effects due to their recent increased interest in winemaking (Balmaseda et al., 2018). Among the different works carried out, one of the main objectives of the group has been to unveil the adaptation of *O. oeni* into wine after the alcoholic fermentation in order to prevent a possible stuck of MLF process. Nowadays, using autochthonous isolates from the Priorat region and taking advantage of the selection techniques available, the study of the acclimation to wine seems more feasible.

In the light of these previous results, the objective of this thesis was to establish the LAB biodiversity of the vine-wine ecosystem in the Priorat region, as well as to isolate and characterize these LAB. Also, the attention was focused on the selection of the most representative strains with terroir characteristics for their use as starter cultures in this region.

In a first instance, the heterogeneity of the bacterial community associated with different varietals at Priorat grapes was established and the identified communities were characterized (**Chapter I**). The study of the biodiversity of the Priorat grapes is important in order to know which bacteria are present there, to investigate how this diversity changes from vine to wine, to look for the possible presence of *O. oeni* in grapes, and also to select *O. oeni* or other non-*Oenococcus* LAB strains to overcome the harsh wine conditions and preserve the terroir characteristics.

Secondly, it was important to assess the indigenous diversity of LAB in healthy grapes and wines from Priorat. To achieve this goal, in **Chapter II** the isolation and identification of the local LAB in healthy grapes and wines from Priorat biodiversity associated with its terroir was performed. Moreover, the establishment of the

heterogeneity of the bacterial community associated with different varietals, as well as its autochthonous diversity were comprehensively isolated, identified and typed.

For the final chapter of the thesis (**Chapter III**), the most predominant strains isolated in **Chapter II** were characterized. The three LAB strains that showed the best characteristics were used as a MLF starter culture and successfully performed MLF in pilot scale wine production.

Bacterial diversity in grape samples from Priorat region was analysed both via HTS (**Chapter I**) and culturing (**Chapter II**). Grenache and Carignan must samples were not significantly different between the selected vineyards, as well as their grape variety or geographical location, using the short-amplicon sequencing approach (**Chapter I**). In relation to grapes sampling, three different cultures were carried out in **Chapter II**: 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%). Grape pulp was the method that permitted to isolate more *O. oeni*. Although there were no statistical differences, the total number of LAB isolates of the different species was greater in samples of Carignan than in Grenache (**Chapter II**), 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.

The HTS study found strains of *Oenococcus* in most samples, accounting for 5.5% (on average) of the bacterial communities in grape musts from Priorat region (**Chapter I**). This is an important fact as, to our knowledge, *Oenococcus* had never been detected before through massive sequencing from grapes berries, must or grape vine thought other genera from the same family like *Leuconostoc* have been previously detected. This detection of *Oenococcus* by HTS has been confirmed recently (Lleixà et al., 2018), with a higher proportion at the end of alcoholic fermentation, and related to the sane state of the grapes.

Oenococcus DNA was detected (**Chapter I**) even in samples in which no isolates of *O. oeni* were obtained (**Chapter II**), 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 more 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.

A total of 761 LAB isolates were identified and typed, being the majority *O. oeni* strains. As expected, most of the 538 isolates of *O. oeni* came from samples of wine performing MLF (Bordas et al., 2013; González-Arenzana et al., 2013; Reguant and Bordons, 2003; Reguant et al., 2005a; Wibowo, 1985). One of the most considerable aspects of this thesis was finding 53 *O. oeni* strains directly isolated from grapes (**Chapter II**). In some studies, such as Bae et al. (2006), Sieiro et al. (1990), and recently Godálová et al. (2016), some LAB were isolated from grapes, but not *O. oeni*. Only Garijo et al. (2011) were able to isolate a colony of *O. oeni* from grapes. 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; Reguant and Bordons, 2003; Wang P. et al., 2016). Moreover, the DNA of *O. oeni* was detected in grapes (Renouf et al., 2005, 2007) by PCR-DGGE of the *rpoB* gene, although no *Oenococcus* has been isolated.

Lactobacillus, the most abundant genus isolated from grapes (**Chapter II**), was also detected by HTS (1%; **Chapter I**), 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. The most abundant species of LAB found in grape samples was *L. plantarum* (48% of isolates; **Chapter II**), present in both vintages and on all the properties. This species were 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). *L. sanfranciscensis* was never previously

isolated from grape must, but was detected during winemaking via PCR-DGGE (Renouf et al., 2007).

In many regions from Spain (Cruz-Pio et al., 2017; González-Arenzana et al., 2013; Sieiro et al., 1990), Portugal (Cruz-Pio et al., 2017), France (El Khoury et al., 2017), central Europe (Godálová et al., 2016), Italy (Garofalo et al., 2015) and Argentina (Saguir et al., 2009), LAB autochthonous starters were studied, but in Priorat there were only a few preliminary studies about it (Reguant and Bordons, 2003; Bordas et al., 2013). Thus, **Chapter III** performs the characterization of the LAB strains isolated in **Chapter II** from Priorat grape and wine samples.

Both *O. oeni* and non-*Oenococcus* isolates were found in grapes and wine samples (**Chapter II**). In the case of grapes, *L. plantarum* was the predominant species found. In the other hand, in the case of wine samples *O. oeni* was the predominant species found. A great quantity of both *O. oeni* and non-*Oenococcus* strains were isolated, but finally, the characterization showed that the best characteristics to become a MLF starter belonged to *O. oeni* (**Chapter III**), is the species that is best adapted to wine conditions (Bartowsky, 2005; Bordas et al., 2013; González-Arenzana et al., 2013; Henick-Kling, 1993; Wibowo et al., 1985).

A clear difference was seen between the characterization results from most strains of *O. oeni* and the few strains of non-*Oenococcus* species (**Chapter III**). From the total LAB strains isolated (**Chapter II**), the 45 strains (41 *O. oeni* strains and four non-*Oenococcus* strains) were characterized. The best were selected according to the results obtained, i.e. the L-malic acid degradation test, the absence of BA genes and the stress resistance test. The best strains were the six in the resistance group R4, which were assigned to the best efficiency group E3 and lacked the three BA genes. Bearing in mind that some of these strains presented the same SNP profile, three were selected, one from each SNP profile: 1Pw13, 2Pw15 and 2Pw22. As expected, the strains with the best inoculum characteristics were from *O. oeni* (**Chapter II**), the species most used for MLF induction (Kunkee, 1984; Solieri et al., 2010).

As it has been already remarked, one of the important achievements of this thesis was the isolation of 53 *O. oeni* from grape samples (**Chapter II**). However, the results obtained from the characterization of the grape *O. oeni* isolates showed weak

characteristics for being good candidates (**Chapter III**). The efficiency of L-malic degradation in wine-like medium and fermentative speed were evaluated from the predominant strains from different species. The four *O. oeni* strains isolated from grapes consumed the 100 % of L-malic, as the three *O. oeni* strains selected as a MLF starter did, but they performed the MLF slower. Moreover, the strains isolated from grapes belonged to R1 group, which is the less resistance grade to ethanol and low pH, and the three *O. oeni* strains selected as MLF starter belonged to R4, the most resistance one. Finally, the four *O. oeni* strains isolated from grapes were located in phylogroup B and the three *O. oeni* strains selected as MLF starter are located in phylogroup A. This agrees with the hypothesis that usually the strains located at phylogroup A are the best ones adapted to wine conditions (Campbell-Sills et al., 2015), but we must not forget some strains located at phylogroup B with good malolactic behaviour in MLF were also isolated by our group from other wines of the same region (Bordas et al., 2013).

In summary, this work has been focused on *O. oeni* isolation, identification and characterization from both grapes and wines from the Priorat region. 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 to carry out MLF successfully and final wines showed good chemical characteristics. The imposition of two of the inoculated strains in those MLF was confirmed, being 1Pw13 the predominant strain in all wines. In conclusion, a complete description of the biodiversity of the Priorat region has been obtained, and the characteristics of the obtained wines suggested the possible use of one of the strains as good candidate for starter culture.

The results obtained have opened new doors and will be useful for future studies of *O. oeni* and non-*Oenococcus* autochthonous MLF starters for Priorat wines. These data should be considered for further analysis using these and/or other *O. oeni* strains and different conditions. Also, the increase in popularity of autochthonous isolates starters can lead to focus the attention on the impact of the genomic features of the nearby *O. oeni* strains as some of them harboured specific genomic regions that may increase the natural genetic diversity of species for technological purposes. Moreover, special attention should be taken on the interactions between oenological yeasts, both

Saccharomyces and no-*Saccharomyces*, and LAB, and the compatibility of the different strains used, as it can affect the MLF (Diez et al., 2010; Costello et al., 2003; Nehme et al., 2008; Su et al., 2014). The identification of genes and proteins induced or repressed under wine-like media conditions can be useful to go deep into some specific pathways and their regulation, like 4'-0-(B-D-glucopyranosyl)-D-pantothenic acid, sugar or nitrogen metabolism.

The beneficial effect of using autochthonous MLF starters could be finally correlated with the activities of the enzymes involved, thus, finishing the characterisation of specific strains since their genome sequenced can be compared between those which are better MLF starters. Results obtained should be also complemented by other studies as metagenomics and proteomics. It could be interesting to perform the genome sequencing of the autochthonous MLF starters and other non-MLF-efficient strains obtained in order to compare them and to select strains for each of the genes to unveil their role and essentiality in this species. (Campbell-Sills et al., 2015, 2017; Margalef-Català et al., 2017c; González-Arenzana et al., 2015).

5. CONCLUSIONS

The main conclusions obtained from this PhD Thesis are:

1. The heterogeneity of the bacterial community associated with different varietals at Priorat grapes has been established and its identified species have been characterized.
2. The most abundant bacterial taxa of Grenache and Carignan grapes were investigated for the first time by high-throughput sequencing.
3. Regional patterns in grape berries bacterial communities suggest that local environmental conditions and grape varietal are responsible for driving bacterial diversity within a single viticultural zone.
4. Bacterial communities of grapes are related with vineyard, its geographical orientation and grape variety within the same growing region.
5. The indigenous diversity of lactic acid bacteria in healthy grapes and wines from Priorat has been assessed, involving their isolation and the identification of their biodiversity associated with its terroir.
6. High-throughput sequencing analysis confirmed a considerable presence of *Oenococcus* and *Lactobacillus* in the grape samples.
7. A large survey, consisting of 30 samples of healthy Grenache and Carignan grape berries and 44 wine samples, was carried out on autochthonous lactic acid bacteria from vineyards and wines in Priorat.
8. A total of 53 strains of *O. oeni* have been isolated from Grenache and Carignan grapes.
9. Some of these *O. oeni* strains from grapes have been isolated from the wines made with those same grapes, showing a good adaptation to cellar conditions.
10. A considerable diversity of typed *O. oeni* strains was determined by the phylogenetic distribution, which has been confirmed by the Simpson's index of diversity.

11. Several strains of *Lactobacillus* and other non-*Oenococcus* lactic acid bacteria were isolated from grapes and typed, from which 60% were *L. plantarum,* 13% *F. tropaeoli,* 9% *L. lindneri,* 8% *L. sanfranciscensis,* 5% *P. pentosaceus* and 5% *L. mali.*
12. Most of the non-*Oenococcus* isolates found are *L. plantarum*, from which eight strains were coincident in both grapes and wine, and showed a high Simpson's index of diversity (0.967).
13. The lactic acid bacteria strains isolated from Priorat wine samples have been characterized in order to select those with oenological potential as malolactic starter cultures in pilot scale wine production.
14. Three *O. oeni* strains were selected from 45 autochthonous LAB strains from Priorat wines based on their efficiency in degrading L-malic acid and most especially their resistance to high ethanol and low pH.
15. The absence of biogenic amine genes in these strains was verified.
16. SNP analysis placed these strains in phylogroup A, the same group of other good wine-adapted strains.
17. *O. oeni* 1Pw13 strain is a good candidate for starter culture for carrying out the MLF in wines because it has performed successfully the MLF in the industrial wines produced, as their characteristics suggest.

CONCLUSIONS

Les principals conclusions obtingudes d'aquesta tesi doctoral són:

1. La heterogeneïtat de la comunitat bacteriana associada amb diferents varietals de raïms del Priorat ha estat establida i les espècies que se n'han identificat han estat caracteritzades.
2. La taxa bacteriana més abundant de raïm de les varietats de garnatxa i carinyena ha estat estudiada per primera vegada mitjançant seqüenciació d'alt rendiment.
3. Els patrons regionals de comunitats bacterianes en raïm suggereixen que les condicions mediambientals locals i la varietat de raïm són responsables d'aportar la diversitat bacteriana en una mateixa zona viticultural.
4. La vinya d'origen, la seva orientació geogràfica i la varietat de raïm influencien sobre la composició de les comunitats bacterianes presents al raïm dins la mateixa regió.
5. La diversitat autòctona dels bacteris làctics presents en raïm sa i en vi del Priorat ha estat avaluada, i se n'ha aïllat i identificat la seva biodiversitat associada amb el seu terroir.
6. L'anàlisi mitjançant seqüenciació d'alt rendiment ha confirmat una considerable presència d' *Oenococcus* i *Lactobacillus* en les mostres de raïm.
7. Es va dur a terme un exhaustiu estudi dels bacteris làctics autòctons provinents de vinyes i vins del Priorat, que va consistir en 30 mostres de raïm sa de les varietats de garnatxa i carinyena i 44 mostres de vi.
8. Un total de 53 soques de *O. oeni* han estat aïllades des de raïm de les varietats de garnatxa i carinyena.
9. Algunes d'aquestes soques d'*O. oeni* provinents de raïm també han estat aïllades en el vi que es va fer amb aquell raïm, cosa que mostra que tenen una bona adaptació a les condicions del celler.

10. Es va determinar una considerable diversitat de *O. oeni* tipificats mitjançant la distribució filogenètica, que ha estat confirmada per l'índex de diversitat Simpson.
11. Diverses soques de *Lactobacillus* i altres bacteris làctics no-*Oenococcus* es van aïllar des de raïm i es van tipificar, dels quals el 60% eren *L. plantarum,* el 13% *F. tropaeoli,* el 9% *L. lindneri,* el 8% *L. sanfranciscensis,* el 5% *P. pentosaceus* i el 5% *L. mali.*
12. La majoria dels aïllats no-*Oenococcus* trobats són *L. plantarum,* dels quals vuit soques es van trobar tant en raïm com en vi, i mostraven un alt índex de diversitat de Simpson (0.967).
13. Les soques de bacteris làctics trobades en mostres de vi del Priorat han estat caracteritzades per tal de seleccionar aquelles amb el potencial enològic com a cultius iniciadors de la fermentació malolàctica en producció de vi a escala pilot.
14. Tres soques d'*O. oeni* van ser seleccionades de 45 soques de bacteris làctics autòctons del Priorat procedents de vi en base a la seva eficiència en la degradació de l'àcid L-màlic i sobretot especialment a la seva resistència a alta concentració d'etanol i baix pH.
15. L'absència de gens d'amines biògenes en aquestes soques es va verificar.
16. L'anàlisi de SNP va situar aquestes soques en el filogrup A, el mateix grup que altres soques ben adaptades al vi.
17. La soca d'*O. oeni* 1Pw13 és una bona candidata com a cultiu iniciador per a dur a terme la fermentació malolàctica en vins perquè ha dut a terme amb èxit la fermentació malolàctica en els vins industrials produïts, tal i com les seves característiques suggereixen.

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ANNEX

CURRICULUM VITAE

Judith Franquès Montserrat was born on 1989 in Tarragona, Spain. In 2007 she took up studies in Biotechnology at the Universitat Rovira and Virgili (URV) in Tarragona. She obtained her degree in 2011. During her degree project research at the Hospital Universitari Joan XXIII (Tarragona) she learned how the clinical laboratories of Microbiology, Molecular Biology and the Biobank of the hospital work.

She carried out the master's Applied Microbiology thesis working on a research project on the frequency of congenital infection in our environment. During this period, she did part of her thesis in the Hospital de Sant Pau i de la Santa Creu (Barcelona), department of Microbiology, under the supervision of Dr. Núria Rabella.

After master's graduation in 2012, Judith started working as a PhD student in the lactic acid bacteria team from the Oenological Biotecnological group of URV. Her thesis has been supervised by Dr. Isabel Araque, Dr. Cristina Reguant and Dr. Albert Bordons from the same university. Thesis research has been mainly focused on the isolation of *Oenococcus oeni* and other non-*Oenococcus* strains and their typification and characterization. At the third year she has gone to the Institut des Sciences de la Vigne et du Vin (Bordeaux, France) to perform, during a three-months internship, the genetic and technological characterization of the pre-selected LAB strains, under the supervision of Dr. Patrick Lucas.

Work experience

June - September 2010	**Hospital Universitari Joan XXIII (Tarragona)**
Occupation:	Degree's training stage.
Main activities:	Learning how the clinical laboratories of Microbiology, Molecular biology and the Biobank of the hospital work.

October 2011- July 2012	**Hospital de Sant Pau i la Santa Creu (Barcelona)**
Charge:	Master's degree training stage.
Function:	Learning how to work as a researcher in the microbiological environment. Conducting a research project on the frequency of congenital infection in our environment.
September 2012 - Present	**Universitat Rovira i Virgili (Tarragona)**
Charge:	PhD student.
Function:	Researcher in the European Project Wildwine. Conducting a research project on lactic acid bacteria from the vine-wine ecosystem in Priorat.
April - July 2014	**Institute des Sciences de la Vigne et du Vin (Bordeaux)**
Occupation:	PhD stage.
Main activities:	Genetic and technological characterization of lactic acid bacteria.
May 2015 - June 2016	**Enolab – University of Valencia (Valencia)**
Occupation:	PhD training stages.
Main activities:	Learning how to work with Bionumerics program. Genetic study of the absence of biogenic amine genes in lactic acid bacteria.

Education and training

2012-2018	**PhD in Enology and Biotechnology** Universitat Rovira i Virgili.
2011-2012	**Master degree of Applied Microbiology** Universitat Autònoma de Barcelona.
2007-2011	**Biotechnology degree.** Universitat Rovira i Virgili.
2015-2016	**Advanced French (level 5 EOI, B2)** EOI (Official school of languages) of Tarragona.
2013-2014	**French: level 3 EOI, B1** EOI (Official school of languages) of Tarragona.
2007	**Advanced English (level 5 EOI, B2)** EOI (Official school of languages) of Tarragona.

2006 **FCE (*First Certificate of English of Cambridge*)**
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Publications

Franquès, J., Araque, I., El Khoury, M., Lucas, P., Reguant, C., and Bordons, A. 2018. Selection and characterization of autochthonous strains of *Oenococcus oeni* for vinification in Priorat (Catalonia, Spain). OENO One. 52, 45-56. https://doi.org/10.20870/oeno-one.2018.52.1.1908

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List of oral conferences

Franquès, J., Araque, I., El Khoury, M., Lucas, P., Reguant, C., and Bordons, A. Characterization of autochthonous strains of *Oenococcus oeni* for vinification in Priorat. XX Congreso Nacional de Microbiología de los Alimentos (SEM). León, Spain. September 2016. **Conference in Spanish.**

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