

Dynamics of lactic acid bacteria populations in Rioja wines by PCR-DGGE, comparison with culture-dependent methods

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Abstract Lactic acid bacteria populations of red wine samples from industrial fermentations, including two different vinification methods were studied. For this investigation, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) analysis was employed to supplement previous results that were obtained by culture-dependent methods. PCR-DGGE was aimed to study two targeted genes, 16S ribosomal DNA (rDNA) and *rpoB*, and the results were useful to evaluate the microbial populations in wine samples. Moreover, an improvement of a detection limit determined so far for DGGE analysis was obtained with the method described in this study, what made possible to identify lactic acid bacteria populations below 10^1 colony-forming unit/mL. The species *Oenococcus oeni* was the most frequently detected bacterium, but identifications close to species *Oenococcus kitaharae* and *Lactococcus lactis* that are not often found in wine were firstly identified in samples of this research. PCR-DGGE allowed to detect 9 out of 11 lactic acid bacteria species identified in this study (nine by PCR-16S rDNA/DGGE and four by PCR-*rpoB*/DGGE), while five species were detected using the modified de Man, Rogosa and Sharpe agar. Therefore, the two methods were demonstrated to be complementary. This finding suggests that analysis of the lactic acid bacteria population structure in wine should be carried out using both culture-dependent and culture-independent techniques with more than one primer pair.

Keywords Lactic acid bacteria · PCR-DGGE · 16S rDNA · *rpoB* gene · Wine

Introduction

Winemaking is a complex process that involves numerous microorganisms from which the main microorganisms are yeast and bacteria. The yeasts lead the alcoholic fermentation (AF), wherein glucose is mainly converted to ethanol, and the lactic acid bacteria (LAB) perform the malolactic fermentation (MLF). MLF is a part of LAB metabolism and mainly consists on the transformation of L-malic acid into L-lactic acid and carbon dioxide, producing several intermediate and final compounds (Vanvuuren and Dicks 1993). This biological transformation is a recommended oenological practice for certain types of white wines and for almost all red wines because of its significant contribution to increasing the microbiological stability of wines (Alexandre et al. 2004; Lonvaud-Funel 1999, 2008) and to improving its sensorial characteristics (Andorrà et al. 2010; de Revel et al. 1999; Torriani et al. 2011).

The LAB ecology in wines represents a microbiological factor that should be monitored to achieve high-quality wines (Andorrà et al. 2010; Kim et al. 2011; Reguant et al. 2005). To understand the ecosystem structure of LAB in wines, many studies about LAB species in red wine have been conducted by using culture-dependent methods (González-Arenzana et al. 2012a, 2013), but results have always been limited by the ability of the microorganisms to develop their metabolisms in culture media (Cocolin et al. 2011a; Renouf et al. 2005a, b). Moreover, the presence of viable but non-culturable microorganisms in wine (Divol and Lonvaud-Funel 2005; Millet and Lonvaud-Funel 2000), which are not able to form colonies on microbiological media, has made necessary the use of different methods based on culture-independent techniques such as

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polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) to resolve this difficulty (Andorrà et al. 2008; Renouf et al. 2006a, b).

This qualitative technique makes possible separation of PCR amplicons of the same size but of different sequences in denaturing gradient gels (Cocolin and Mills 2003; Cocolin et al. 2011b). This technique is a common method employed to characterise microbial communities from specific environmental niches (Muyzer and Smalla 1998) because it enables detection of individual species as well as overall profiling of community structure changes with time. The 16S ribosomal DNA (rDNA) gene has been the most employed one among all the universal genes to detect LAB species by culture-independent techniques (López et al. 2003). However, several authors have proposed that PCR-DGGE technique should be focused on the study of *rpoB* gene in order to minimise the biases caused by intraspecies heterogeneity that 16S rDNA genes can produce (Renouf et al. 2006a, b).

In this research, LAB community structure and its evolution along the fermenting process of Rioja red wines was analysed with 16S rDNA and *rpoB*/PCR-DGGE. Rioja wine comes from the Rioja region of northern Spain. Rioja is a region with a long, glorious viniculture history and was the first Qualified Appellation of Origin region in Spain. Rioja wine, especially the red, is the most well-known Rioja style. Classic, bold, these wines taste mostly of their Tempranillo roots and have a bright, fresh flavour to them. The study described in this paper was conducted in these fermenting wines to expand on previous ecological research that used culture-dependent methods (González-Arenzana et al. 2012a).

Materials and methods

Wine fermentations and sampling

The samples of Tempranillo musts and red wines were taken from four wineries located in the Rioja Appellation in one vintage. The winemaking process involves manual harvesting of grapes followed by two different vinification methods that are representative of the most important types of red winemaking: wineries A, B and C, where AF was performed with the destemming and crushing method in stainless steel tanks, and winery D, where AF was conducted by the traditional semi-carbonic maceration method (Ribéreau-Gayon et al. 2007) in open cement tanks (Fig. 1). When AF was completed, wines were racked and placed in stainless steel tanks for the case of wineries A, B and C and in an open cement tank for winery D. The wines underwent spontaneous MLF with the endogenous microbiota (no starter inocula were used).

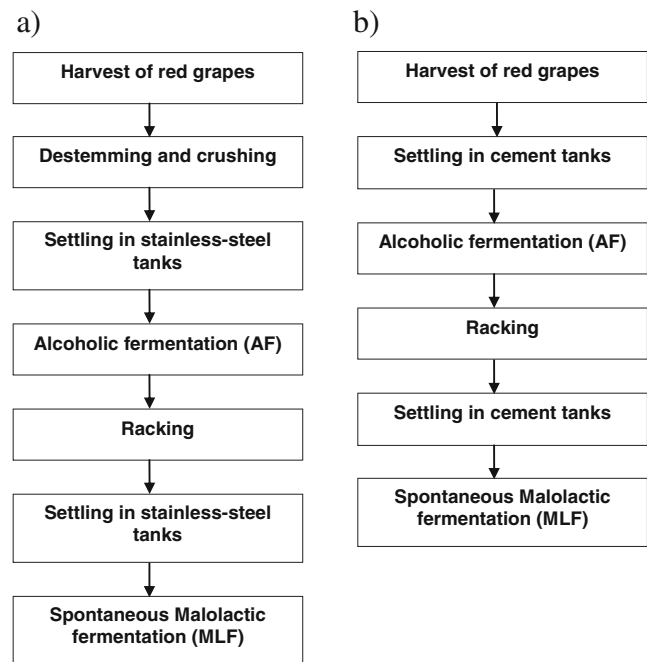


Fig. 1 Flow sheet for Tempranillo wine vinifications: **a** wineries A, B and C; **b** winery D

One fermentation tank was sampled in each winery. Must and wine samples were taken aseptically at different moments: must (stage 1), middle AF (density around 1,025; stage 2), at the end of AF (<2 g/L glucose + fructose; stage 3), initial MLF (consumption of 10 % of the initial malic acid; stage 4), middle MLF (consumption of 60 % of the initial malic acid; stage 5) and at the end of MLF (L-malic acid concentration of <0.5 g/L; stage 6). Wineries A and B were not sampled during AF.

Bacterial identification by culture-independent methods

Direct DNA extraction from wine samples for culture-independent methods

A previous study was performed to optimise direct DNA extraction from red wine (González-Arenzana et al. 2010). In this study, different commercial DNA extraction kits (Plant DNeasy kit from Qiagen, PowerSoil® and PowerFood® DNA isolation kit from MO BIO) were combined with the addition of zirconium hydroxide. The best result was obtained using the procedure described below. A volume of 10 mL of each sample was centrifuged (20 min, 4,000×g, 4 °C). The supernatant was discarded, and 1.2 mL of saline solution (NaCl 0.9 %) and 2.4 mL of zirconium hydroxide (7 g/L) were added to the pellet to facilitate pelleting of the bacteria in wine (Lucore et al. 2000). After 10 min of horizontal shaking at room temperature, the suspension was again centrifuged (10 min, 500×g, 7 °C).

The DNA was subsequently extracted and purified from the cell pellet using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA USA) and Fast Prep™ (FP120, BIO 101, Thermo Electron Corporation, USA) bead beater instrument (twice for 45 s at speed setting of 6) as per the manufacturer's instructions.

PCR conditions

PCR was performed using the Applied Biosystem, GeneAmp® PCR System 2700 thermocycler at a final volume of 50 µL. To amplify the region V4 to V5 of 16S rDNA gene, primers WLAB1 and WLAB2^{GC} were used as described by López et al. (2003). Moreover, the *rpoB*1, *rpoB*1o and *rpoB*2^{GC} primers were employed to amplify the region of the *rpoB* gene as described by Renouf et al. (2006a, b) with the following modifications: 0.5 µM of each primer, 1 mM dNTP mix and 0.5 µL of PfuUltra II Fusion HS DNA Polymerase (Stratagene).

DGGE analysis

The separation of the respective PCR products was performed with the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA). PCR products were run on 8 % (w/v) polyacrylamide gels in TAE buffer (2 M Tris, 1 M glacial acetic acid and 50 mM EDTA pH 8) at a constant temperature of 60 °C. WLAB1–WLAB2^{GC} amplicons were separated with gels containing 35 to 55 % urea–formamide gradient, and electrophoresis was performed for 10 min at 20 V and for 18 h at 80 V. *rpoB* amplicons were separated with gels containing 32 to 50 % urea–formamide gradient, and the electrophoresis was performed for 10 min at 20 V and for 15 h at 60 V. Gels were stained in ethidium bromide after the electrophoresis and then were visualised with UV transillumination (Gel Doc, Bio-Rad). Blocks of the polyacrylamide gels which contained the selected DGGE bands were excised and subsequently incubated overnight in 20 µL of sterile and pure water at 4 °C to make DNA bands diffuse to the liquid. One microlitre of this elution was reamplified using the PCR conditions described above to DNA sequencing.

DNA sequencing and phylogenetic analysis

PCR products were sequenced by Macrogen, Inc. (Seoul, South Korea). The quality and characteristic of the obtained sequences were analysed with the software InfoQuest™ 5.1, and only those products considered as appropriate were used for comparison to the GenBank nucleotide database with the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). The 16S rDNA and *rpoB* sequences were deposited in the GenBank nucleotide database under the accession

numbers JQ838834–JQ838875 and JQ838876–JQ838890, respectively. After this preliminary study, our sequences were assembled and submitted to phylogenetic and evolutionary analysis with the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.1 (Tamura et al. 2007). The neighbour-joining method (Saitou and Nei 1987) identified the relationships between the obtained sequences and the reliability of the identifications that were provided by the GenBank databases. The bootstrap test was based on 1,000 random revamping (Felsenstein 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2007) that made it possible to calculate the units that were equivalent to the base substitutions per site.

Bacterial identification by culture-dependent methods

The bacterial isolation was performed in de Man, Rogosa and Sharpe (MRS) agar (Scharlau Chemie S.A., Barcelona, Spain) plates supplemented with tomato juice (10 % v/v), fructose (6 g/L), cysteine HCl (0.5 g/L), DL-malic acid (5 g/L) and 50 mg/L of pyrimicin to avoid yeast growth (Acofarma, S. Coop., Spain), and species identification was carried out as described by González-Arenzana et al. (2012a).

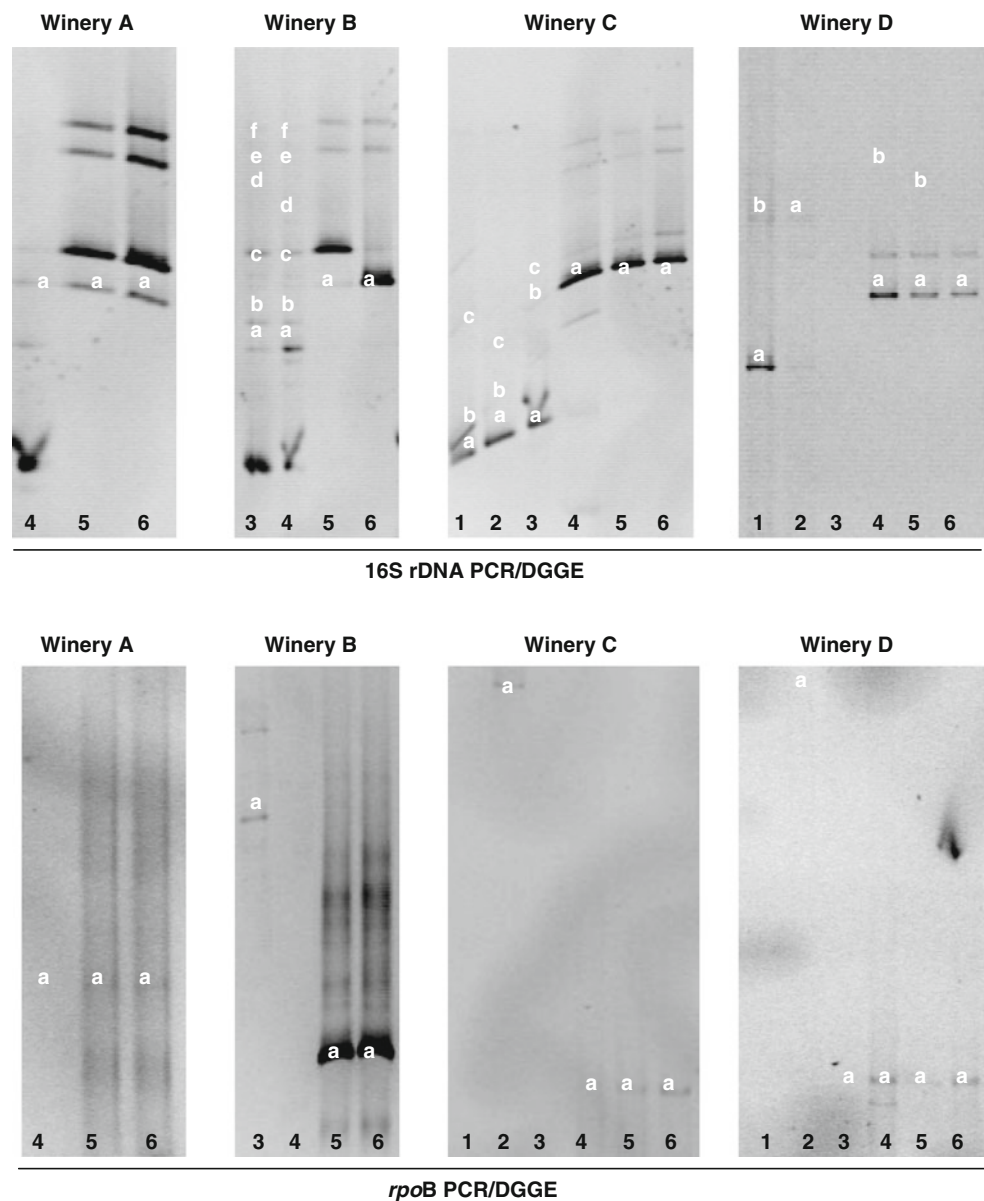
Results

LAB species detected by PCR-16S rDNA/DGGE

The DNA was extracted directly from must and wine samples and was submitted to PCR targeted to the 16S rDNA gene and later to DGGE. One hundred thirty-seven bands were retrieved from different DGGE gels (Fig. 2) and were sequenced by Macrogen, Inc.

The obtained sequences were carefully analysed with the bioinformatic software InfoQuest™ 5.1. Only those with reliable sequencing were included in the GenBank Data Library, referred each one to an accession number and then submitted to BLAST searches at the National Centre of Biotechnology Information GenBank. Some sequences were clearly identified, and they corresponded to sequences available in the databank, and others were compared to the most closely related species (Fig. 3). Finally, 38 sequences were processed with the MEGA software 4.0.2 and shaped the phylogenetic neighbour-joining tree displayed in Fig. 3. This tree was established by two main ramifications or branches. The “branch a” was composed by the LAB families *Leuconostocaceae* and *Lactobacillaceae*. The family *Leuconostocaceae* was formed by the genera *Oenococcus* and *Leuconostoc*. Eighteen bands were identified as *Oenococcus oeni*; three, close to *Oenococcus kitaharae*;

Fig. 2 PCR-DGGE profiles of the bacterial communities associated to the must or wine samples of the different wineries at each vinification stage (1 must, 2 middle AF, 3 final AF, 4 initial MLF, 5 middle MLF, 6 final MLF). Letters correspond with bands used in the phylogenetic analysis



four, as or similar to *Leuconostoc mesenteroides*; and finally, one, close to *Leuconostoc fallax*. The family *Lactobacillaceae* was integrated by the genera *Lactobacillus* and *Pediococcus*. Five sequences were identified as or close to *Lactobacillus plantarum*; two, near to *Lactobacillus buchneri*; one, as *Pediococcus pentosaceus*; and two, close to *Pediococcus parvulus*. Otherwise, the “branch b” included two sequences belonging to the family *Streptococcaceae* and the genus *Lactococcus*, which were identified as close to the species *Lactococcus lactis*.

LAB species detected by PCR-*rpoB*/DGGE

The LAB species detected in the fermenting wine samples with PCR-*rpoB*/DGGE are displayed in Fig. 4. In this case, 29 bands were sequenced, and 15 were considered qualified

enough to be submitted in the GenBank nucleotide database. These sequences were linked to a different accession number and were included in the phylogenetic study. Two branches could be observed in the tree (Fig. 4). The branch a was made up by sequences belonging to the families *Leuconostocaceae* and *Lactobacillaceae*. Eight sequences were identified as *O. oeni*; one, as *L. mesenteroides*; and two, similar to *L. buchneri*. The family *Lactobacillaceae* was also represented in the branch b where four sequences were confirmed near to *P. parvulus*.

Distribution of LAB in each winery and stage of vinification

The LAB species identified at each fermentative stage in every sampled winery are shown in Table 1. In this table, both results from culture-independent (PCR-16S rDNA/DGGE

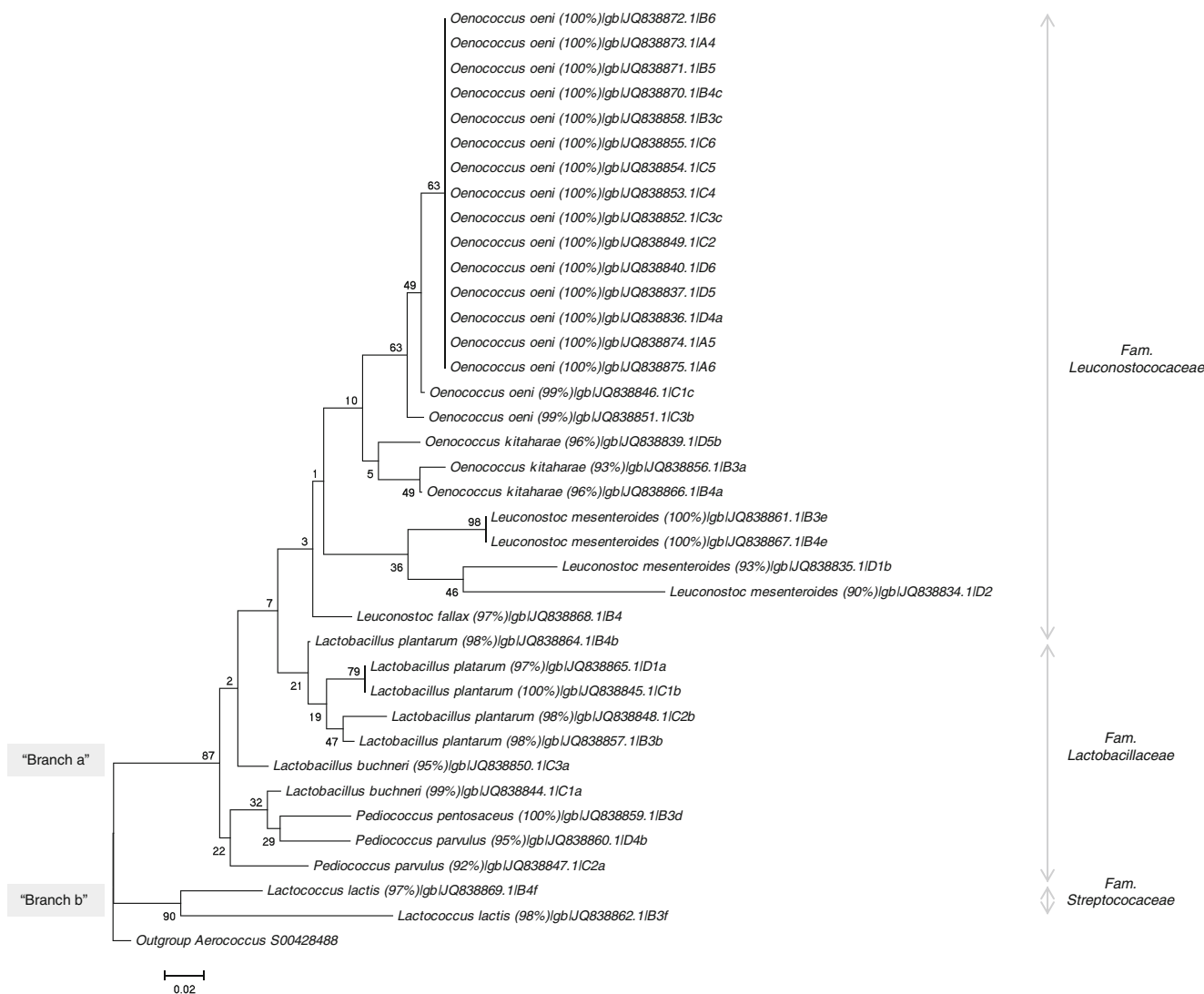


Fig. 3 Phylogenetic relationship of DNA sequences obtained from 16S rDNA PCR/DGGE. The phylogenetic tree was inferred using the neighbour-joining method (Saitou and Nei 1987) (1,000 bootstrap analysis). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2007) and are in the

units of the number of base substitutions per site. Each sequence is defined with the most accurate identification and the identity percentage (%), with the given accession number and with a *letter* and a *number* that mean the winery and isolation stage, respectively

and PCR-*rpoB*/DGGE) and culture-dependent techniques of a previous study (González-Arenzana et al. 2012a) of the same wine samples are included.

Cellars A and B were not sampled during AF, while C and D wineries were studied during AF and MLF. In relation to the LAB species distribution during the process of winemaking, it was observed by culture-independent methods that six species were detected at final AF (stage 3) in winery B and between one and three species in wineries C and D during the whole AF process. In relation to the LAB diversity during MLF, it was observed that *O. oeni* was the only species found at this period in fermenting wines from cellars A and C. In contrast, five different identifications besides *O. oeni* were found in the wines from winery B

at stage 4. In addition, identifications close to *P. parvulus* were also detected, apart from *O. oeni*, at all the sampled MLF stages in winery D, and in the middle period (stage 5), another LAB species was found and was later identified near to *O. kitaharae*.

Discussion

In this study, the LAB community structure and its evolution along the fermenting process of Rioja wines were analysed using 16S rDNA (Fig. 3) and *rpoB*/PCR-DGGE (Fig. 4).

The PCR-16S rDNA/DGGE enabled us to detect the highest number species of this study (nine) belonging to

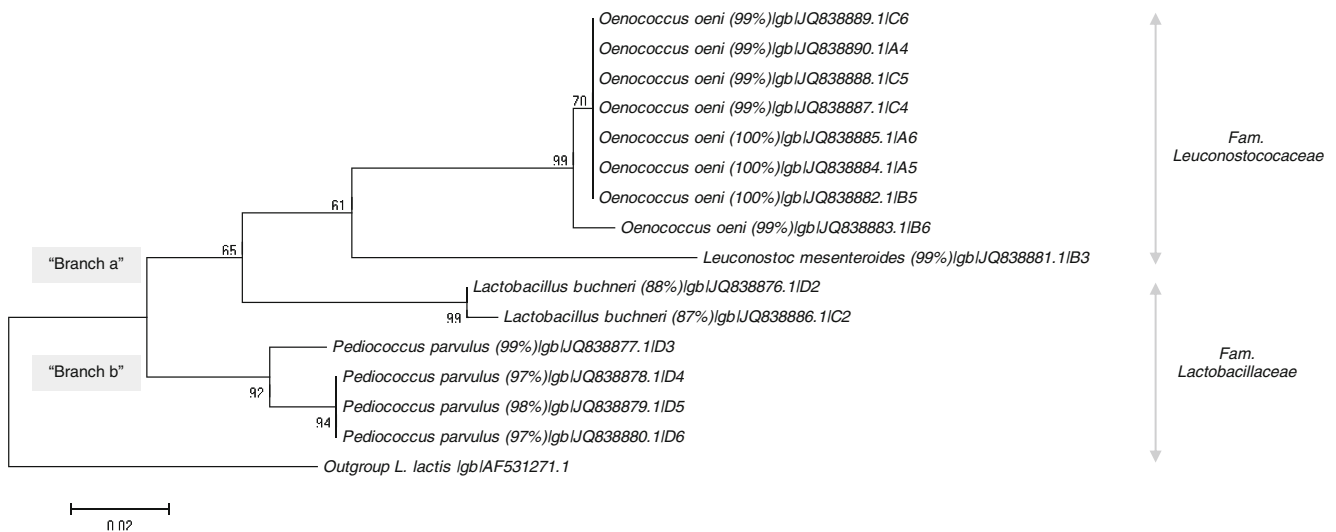


Fig. 4 Phylogenetic relationship of DNA sequences obtained from *rpoB* PCR/DGGE. The phylogenetic tree was inferred using the neighbour-joining method (Saitou and Nei 1987) (1,000 bootstrap analysis). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2007) and are in the units of

the number of base substitutions per site. Each sequence is defined with the most accurate identification and the identity percentage (%), with the given accession number and with a letter and a number that mean the winery and isolation stage, respectively

three families such as *Leuconostocaceae* and *Lactobacillaceae* that are widely found in winemaking according to the literature (Dicks and Endo 2009; Zhang et al. 2011) and *Streptococcaceae* that is rarely associated to wine. These three families were represented by five genera. The genus *Oenococcus* was the most representative inside the family *Leuconostocaceae*, and *O. oeni* was the most detected LAB species due to its great adaptation to the wine conditions (López et al. 2008; Renouf and Favier 2010). Surprisingly, in the genus *Oenococcus*, several sequences were identified with BLAST close to *O. kitaharae* (JQ838839, JQ838856 and JQ838866), despite having never been detected in wine because of its poor adaptation to the stressful wine environment (Endo and Okada 2006). *O. kitaharae* could have been present at the wine samples, but not necessarily with an active metabolism during fermentation, as the PCR-DGGE was based on DNA/PCR-DGGE (Cocolin et al. 2011a). To the best of our knowledge, the proximity of those sequences placed *O. kitaharae* in the group of the genus *Oenococcus* in the phylogenetic tree (Fig. 3), which meant that the identification in GenBank nucleotide database may have been adequate. However, the unusual detection of *O. kitaharae* in fermenting wines could be due to the Library of GenBank nucleotide database not being complete enough to achieve a more accurate identification. These sequences could be considered to a different group inside the genus *Oenococcus* as other authors have already reported in their studies (Lucena et al. 2010; Renouf et al. 2009). In relation to the genus *Leuconostoc*, species such as *L. mesenteroides* and *L. fallax* that were previously reported by many authors in wines have been also detected

in this research (Dicks and Endo 2009; Ogier et al. 2008). Relating to the genus *Lactobacillus* (Fig. 3), some sequences were identified as similar to *L. plantarum* and *L. buchneri*. The first one has been considered as habitual species in stages previous to the beginning of MLF because they have malolactic activities, but they are not usually adapted to the pH and ethanol of wine (Dicks and Endo 2009; du Toit et al. 2011). The species *L. buchneri* that was isolated from must and wines has been deeply analysed along with *O. oeni* in relation to arginine metabolism (Liu et al. 1994; Mira de Orduña et al. 2001). Inside this same family and very close to the genus *Lactobacillus*, two species of the genus *Pediococcus* were included. These species are sometimes related to spoilage wines or to difficult MLF situations (Dobson et al. 2002). *P. parvulus* is a species usually present in must and wine samples that can produce extracellular polysaccharides (Dols-Lafargue et al. 2008), and regarding to *P. pentosaceus*, it is known that under glucose limitation, it is able to use glycerol-producing pyruvate (Ribéreau-Gayon et al. 2007). Finally, the other genus found in this study with 16S rDNA/PCR-DGGE was *Lactococcus*, specifically the species *L. lactis* which has been usually attached to dairy products. This species is not very frequently reported in wine samples, although it has been recently detected in grapes samples from Australian vineyards (Bae et al. 2006), in musts from Ribera Sacra (Mesas et al. 2011) and from Rioja (López-Alfaro 2004).

Respect to the analysis of DGGE based on the PCR of the *rpoB* gene was rather a lack of results although allowed to detect four different species such as *O. oeni*, *L. mesenteroides*, *L. buchneri* and *P. parvulus*. The main reason to use PCR-

Table 1 Detected LAB species by PCR-DGGE of 16S rDNA and *rpoB* genes and by culture-dependent methods (González-Arenzana et al. 2012a) in each sampled moment (I must, 2 tumultuous AF, 3 final AF, 4 initial MLF, 5 tumultuous MLF, 6 final MLF) and at each winery

LAB species	A			B			C			D			
	4	5	6	3	4 ^a	5	6	1	2	3	4	5	6
<i>Lactobacillus buchneri</i>	-	-	-	-	-	-	-	a	b	a	-	-	-
<i>Lactobacillus mali</i>	-	-	-	-	c ⁵⁰	-	-	-	c ¹⁵	-	-	-	-
<i>Lactobacillus plantarum</i>	-	-	-	a	ac ³⁶	-	-	a	ac ⁸	-	-	-	c ⁵⁰
<i>Lactobacillus</i> sp.	-	-	-	-	c ⁷	-	-	-	-	-	-	-	c ⁶⁷
<i>Lactococcus lactis</i>	-	-	-	a	a	-	-	-	-	-	-	-	-
<i>Leuconostoc fallax</i>	-	-	-	-	a	-	-	-	-	-	-	-	-
<i>Leuconostoc mesenteroides</i>	-	-	-	ab	ac ⁷	-	-	-	-	-	-	-	a ac ⁵⁰
<i>Oenococcus kitaharae</i>	-	-	-	a	a	-	-	-	-	-	-	-	-
<i>Oenococcus oeni</i>	abc	abc	abc	a	a	abc	abc	ac	ac ⁷⁷	ac	abc	abc	c ³³
<i>Pediococcus parvulus</i>	-	-	-	-	-	-	-	-	a	-	-	-	b
<i>Pediococcus pentosaceus</i>	-	-	-	a	-	-	-	-	-	-	-	-	-
LAB species no. (PCR-16S rDNA/DGGE)	1	1	1	6	6	1	1	3	3	2	1	1	2
LAB species no. (PCR-rpoB/DGGE)	1	1	1	1	0	1	1	0	1	0	1	1	0
LAB species no. (culture-independent method)	1	1	1	6	6	1	1	3	4	2	1	1	2
LAB species no. (culture-dependent method)	1	1	1	ml ^b	4	1	1	1	3	1	1	1	2
CFU/mL	10 ⁵	10 ⁷	10 ⁷	10 ²	10 ²	10 ⁶	10 ⁷	10 ¹	10 ²	10 ³	10 ⁵	10 ⁶	10 ⁸
LAB species no./winery (culture-independent method)	1	1	1	7	7	1	1	4	4	6	6	6	6
LAB species no./winery (culture-dependent method)	1	1	1	5	5	1	1	3	3	4	4	4	4
Total LAB species no. at each winery	1	1	1	9	9	1	1	5	5	7	7	7	7

Lowercase letters indicate that species are detected by the following: a, 16S rDNA/PCR-DGGE; b, *rpoB*/PCR-DGGE; c, culture-dependent method; - not detected. Number indicates the percentages of each isolated species; no number means 100 % of appearance

^a MLF was not started

^b Colonies were not isolated at this fermentation stage

rpoB/DGGE was the deviation caused by the presence of several copies of the 16S rDNA gene in the genome of some microorganisms (Renouf et al. 2006a, b). However, in certain wine samples that were submitted to PCR-*rpoB*/DGGE, more than five bands proceeding from the same direct DNA extraction were later identified as the same species (data not shown). Thus, we were not able to avoid the intraspecies heterogeneity associated to 16S rDNA gene (Case et al. 2007). In addition to these considerations, it was important to take into account that GenBank nucleotide database includes few sequences of *rpoB* gene from different LAB species, and this lack of sequences made the identification rather limited, as other authors have already described (Ruiz et al. 2010). Nevertheless, the results that were obtained with PCR-*rpoB*/DGGE could be complementary to PCR-16S rDNA/DGGE results when the study was focused on each fermentation stage.

A comparison of the PCR-16S rDNA and *rpoB*/DGGE results with those obtained from the identification of the colonies isolated from modified MRS plates in a previous study (González-Arenzana et al. 2012a) revealed coincidental results in certain wine samples, but some differences in others. A total of 11 different LAB species were identified in the four studied wineries by traditional and culture-independent methods. PCR-DGGE of the 16S rDNA-targeted gene allowed identifying nine species in comparison with the four species that were detected by PCR-*rpoB*/DGGE and the five species detected by culture in plates of the samples. Thus, *L. buchneri*, *L. lactis*, *L. fallax*, *O. kitaharae*, *P. parvulus* and *P. pentosaceus* were not detected in the employed culture medium, while *Lactobacillus mali* was not detected by PCR-DGGE.

The LAB species distribution at each winery during the winemaking process revealed different situations. In winery A, only *O. oeni* was detected by PCR-DGGE during MLF, and this finding was concordant with the plating results in which the 100 % of the isolates were identified as *O. oeni*.

The winery B showed the highest LAB species richness, detecting 9 out of 11 LAB species that were identified in this study. At the end of AF (no colony isolation performed, stage 3), six LAB species were found; *L. mesenteroides* was the only species detected by DGGE with the two targeted genes and the only one detected by PCR-*rpoB*/DGGE. This coincidental result could be due to the presence of an important population of this species in the analysed samples (Cocolin et al. 2011a). A great LAB species diversity (six species, between them *L. lactis*, *L. fallax*, *L. mesenteroides*, *O. kitaharae*, *O. oeni* and *L. plantarum*) was also observed at the initial MLF (stage 4) and was in accordance with the results that were obtained from culture plates (four identified species) in terms of the highest detected species diversity, but not in terms of microbial composition. This observation has been also reported in other fermented foods (Merouh et al. 2003; Pérez-Pulido et al. 2006). Moreover, *L. mali* was

the 50 % of the species isolated in plate, but it was not detected by PCR-DGGE. This lack of concordance between techniques could be related first to the effect of the enrichment medium that would favour growth of some species over the others. In this sense, other authors have already reported that high populations on plating media were not found by PCR-DGGE (Rantsiou et al. 2008). Secondly, this disagreement could also be in relation to other technique biases such as co-migrations, primer competition and limit of detection (Cocolin et al. 2011a). In this winery B, *O. oeni* was not able to grow on plating media despite being detected by PCR-DGGE at stage 4, most likely due to the competition with other LAB species that were present in the media, which could suggest that MLF did not actually begin as was indicated by the viable LAB count and the malic acid consumption at this stage (González-Arenzana et al. 2012a). Moreover, the *O. oeni* detection with the two targeted genes during stages 5 and 6 was in agreement with the results derived from the previous plating study and supported viability DGGE in analysing large populations (10^6 – 10^7 colony-forming unit (CFU)/mL) from winemaking samples (Bester et al. 2010).

The whole process of winemaking (AF and MLF) was studied in wineries C and D. Curiously, in winery C, *O. oeni* was identified by PCR-DGGE and also in the MRS agar during every stage of AF and MLF, despite the fact that the LAB viable populations were low at some stages (10^1 CFU/mL at stage 1 and 10^2 CFU/mL at stage 2). This fact meant an increase in the detection limit for PCR-DGGE previously established by Cocolin et al. (2011b). During the early AF (stages 1 and 2), four LAB species were detected in addition to *O. oeni*, being *L. plantarum* noticed by PCR-16S rDNA/DGGE and by plating. *L. mali* was not detected by culture-independent techniques similar to the results from winery B. *L. buchneri* and *P. parvulus* were detected in AF by PCR-DGGE which was in agreement with previous studies (Renouf et al. 2005a, b). During MLF, *O. oeni* was the only species detected by all the employed techniques, supporting previous studies (González-Arenzana et al. 2012a, b), which highlighted the enormous adaptation of this species to the strict wine conditions.

Surprisingly, in winery D, *L. mesenteroides* and *L. plantarum* were detected at stage 1 by PCR-16S rDNA/DGGE, despite any LAB species that was able to grow in plating media at this moment. This result improved the detection limit for PCR-DGGE which is described above. The immobilisation with metal hydroxides to concentrate food-borne bacteria was developed by Lucore for bacterial detection by cultural and molecular methods in dairy products by Lucore et al. (2000) and was applied to bacterial DNA extraction for RT-PCR in wine by Phister et al. (2007). To our knowledge, this study details for the first time that direct DNA extraction with a soil kit has been

combined with the application of zirconium hydroxide, and the present study demonstrated that this method was a successful strategy to study the dynamics of LAB species in red wine samples by PCR-DGGE.

In this winery D, four LAB species that were different to *O. oeni* were detected using the only one of the methods at middle and final AF (LAB populations around 10^1 CFU/mL). *L. mesenteroides* was an exception because it was detected by PCR-16S DNA/DGGE and by plating. During MLF (LAB populations of $>10^6$ CFU/mL), identifying two different LAB species, in addition to *O. oeni*, was also surprising. This finding represents for the first time that *O. kitaharae* and *P. parvulus* were detected together with *O. oeni* in a correct (without quality deviation) wine fermentation that was conducted in open cement tanks with whole grapes. This peculiar result was in accordance with the suggestion that the employed winemaking method might create a special ecosystem with its own microbiota, as reported in a previous study (González-Arenzana et al. 2012a).

In this study, results obtained by culture-independent methods were in agreement with the ones in culture in plating media. Both methods were complementary during AF (with low LAB viable populations) and during MLF (with high populations), but in most of the studied fermentation stages, PCR-DGGE allowed to detect a greater number of species that were observed growing on MRS agar. The highest LAB species diversity was detected during AF, and an alternation of species was observed at a previous stage of the beginning of MLF as it has been reported in other wine fermentations (López-Alfaro 2004; Renouf et al. 2005a, b; Ruiz-Larrea et al. 2001).

In summary, this study contributed to increasing the knowledge of LAB diversity in red fermenting wines from La Rioja (Spain). The method developed in this study allowed to improve the detection limit that has been established to date for the T/DGGE direct analysis below 10^1 CFU/mL in some wine samples. It was firstly showed in the presence of the species *O. kitaharae*-like and *L. lactis* in fermenting wine samples. PCR/DGGE allowed us to increase the number of LAB species usually detected during MLF in a wine. Further research will be necessary to determine if this result was in relation to the different types of winemaking carried out in winery D (semi-carbonic maceration). The results from PCR/DGGE with two targeted genes and plating were complementary throughout the entire winemaking process; therefore, a parallel analysis based on culture-dependent and culture-independent methods should be developed to achieve a complete knowledge of LAB ecology in wine.

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