Prevalence of *Lactobacillus plantarum* and *Oenococcus oeni* during spontaneous malolactic fermentation in Patagonian red wines revealed by polymerase chain reaction-denaturing gradient gel electrophoresis with two targeted genes

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Abstract

Background and Aims: Information regarding the composition and dynamics of microbial communities throughout winemaking is always valuable to control the process and contributes to improving the quality of wine. The aims of this work were to analyse the diversity of lactic acid bacteria (LAB) species present during the spontaneous malolactic fermentation (MLF) of Patagonian red wines, and to isolate and identify native LAB species.

Methods and Results: Samples of Merlot and Pinot Noir wines were collected during three vintages at one commercial cellar. Through polymerase chain reaction (PCR)-*rpo*B/denaturing gradient gel electrophoresis (DGGE), it was possible to identify 15 LAB species and through PCR-*16S rRNA* V3 region/DGGE, 11 LAB species, during spontaneous MLF of these wines, mostly, *Oenococcus oeni* and *Lactobacillus plantarum*. These two species were also largely recovered by culture and analysed by the random amplified polymorphic DNA–PCR technique. Isolates of these two LAB species exhibited considerable genotypic heterogeneity.

Conclusions: The prevalence of *O. oeni* and *Lb. plantarum* in all samples suggests that both species are involved in leading the spontaneous MLF of these Patagonian wines. The analysis of the LAB microbiota in wine by PCR-DGGE using two gene regions enabled addition to the number of species that can usually be detected during MLF.

Significance of the Study: The study offered a more complete knowledge of the LAB community present in wines in a cellar in the Argentine Patagonia during the spontaneous MLF. Our findings are particularly relevant, because they contribute to the possibility of designing an MLF starter composed of native strains of *Lb. plantarum* and *O. oeni*.

Keywords: Lactobacillus plantarum, *malolactic fermentation*, Oenococcus oeni, *Patagonian red wine*, *PCR-DGGE*

Introduction

Winemaking is a complex process in which yeasts and lactic acid bacteria (LAB) play a significant role. Yeasts consume sugars to produce ethanol and lead the alcoholic fermentation (AF). The malolactic fermentation (MLF) is responsible for the conversion of L-malic acid to L-lactic acid and CO_2, causing a reduction in the total acidity of wine. It occurs during or after AF and is carried out by LAB species, mainly *Oenococcus oeni* (Lafon-Lafourcade et al. 1983, Lonvaud-Funel 1999, Pozo-Bayón et al. 2005). This biological deacidification is always accompanied by the generation of additional flavours and by microbiological stability for wines (Lonvaud-Funel 1999, Maicas et al. 1999, Moreno-Arribas and Polo 2005). Spontaneous MLF can be unpredictable and involves several risks, such as a considerable increase in volatile acidity, consumption of residual sugars and formation of undesirable metabolites (López et al. 2008). An excess of acetic acid, the synthesis of glucan, biogenic amines and precursors of ethyl carbamate are undesirable (Lonvaud-Funel 1999).

The use of starter cultures of selected LAB from each oenological region takes advantage of the natural adaptation of strains to wine characteristics, and maintains regional characteristics (Izquierdo et al. 2004, López et al. 2008). To be successful, however, information on the composition and dynamics of microbial communities throughout the winemaking process is necessary to identify the indigenous microorganisms with which the starter strain has to compete.

Culture-dependent techniques fail to reveal the diversity of wine LAB populations (Millet and Lonvaud-Funel 2000), because those populations, numerically less important, under stress or in a viable but non-cultivable (VBNC) state, are hardly recovered, leading to errors of evaluation of the ecology of complex microbial ecosystems (Cocolin et al. 2011). Thus, culture-independent fingerprinting techniques, based on polymerase chain reaction (PCR) amplification of total DNA obtained from the sample, and the analysis of community amplicons by denaturing gradient gel electrophoresis (DGGE), have been successfully employed to assess the biodiversity and

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population dynamics occurring in wine (Renouf et al. 2007, Ruiz et al. 2010, González-Arenzana et al. 2012, 2013a,b, Bokulich et al. 2013), and on the grape berry surface (Renouf et al. 2005). The main limitation of this technique is the difficulty of distinguishing every genotype from the community, and the overestimation of particular populations because of the detection of VBNC organisms. Extraction of DNA is decisive in order to obtain a reliable and reproducible quantification of the microorganisms present (Schramm and Amann 1999).

The diversity of the LAB population during spontaneous MLF of Tempranillo wines (Ruiz et al. 2010) has been analysed using the V3 region of the *16S rRNA* gene. Nevertheless, to overcome multiple banding patterns caused by the heterogeneity of the *16S rRNA* gene, the encoding gene for the β-subunit of RNA polymerase (*rpo*B gene) was proposed as an alternative gene for PCR-DGGE analysis (Dahllof et al. 2000, Renouf et al. 2006). Ruiz et al. (2010), however, pointed out that the discriminatory capacity obtained with PCR-*rpo*B/DGGE was insufficient to monitor the bacterial composition during the winemaking process, because some species usually present during MLF were not adequately separated. Recently, the use of PCR-*rpo*B/DGGE in addition with PCR-*16S rRNA*/DGGE of V4 and V5 regions was valuable to evaluate the dynamics of LAB populations in Rioja wines (González-Arenzana et al. 2012). Therefore, we propose that PCR-DGGE analysis with two targeted genes, *rpo*B and the V3 region of *16S rRNA*, could provide an improved knowledge of the diversity of LAB species during spontaneous MLF of Patagonian wines.

In Argentina, Saguir et al. (2009) have provided the only report on the diversity of LAB species predominant in grape juice at the beginning of MLF in a Malbec wine. The biological diversity of LAB, however, associated with spontaneous MLF of Patagonian wines has not yet been examined. Consequently, the aim of this work was: (i) to study the dynamics of LAB populations during spontaneous MLF in wines produced in Northern Patagonia by the PCR-DGGE method using two gene regions; and (ii) to isolate and identify indigenous LAB in these wines.

Materials and methods

Wine samples

Samples of Merlot wines were collected during the 2008 and 2012 vintages and Pinot Noir wines from the 2010 and 2012 vintages, at the oldest commercial cellar (105 years old) in Northern Patagonia, Argentina. The AF of one of the Merlot wines from the 2008 vintage was promoted by must inoculation with *Saccharomyces cerevisiae* F10 Laffort (Bordeaux, France) [guided fermentation (GAF)]. In the remaining vinifications, the AF was spontaneous [natural fermentation (NAF)]. Malolactic fermentation was spontaneous in all vinifications analysed; it is important to note that the cellar sampled had never used commercial malolactic starters.

The chemical composition of the wines was evaluated at the end of AF: 2010 Pinot Noir ethanol 14.5%, total SO_2 96 mg/L, pH 3.82; 2012 Pinot Noir ethanol 14.1%, total SO_2 92 mg/L, pH 3.78; 2008 Merlot ethanol 12.2%, total SO_2 83.3 mg/L, pH 3.52; 2012 Merlot ethanol 12.2 %, total SO_2 83.3 mg/L, pH 3.45; and 2008 Merlot with GAF ethanol 12.6%, total SO_2 83 mg/L, pH 3.35.

Samples were collected aseptically according to the following schedule: at the end of AF (MLF_1), at day 14 (MLF_2), and at day 35 (MLF_3). At the stage MLF_1, the L (+) malic acid concentration ranged between 4.6 and 3.9 g/L. The last sample (MLF_3) was taken when the L (+) malic acid concentration ranged between 0.8 and 0.5 g/L.

Diversity analysis by polymerase chain reaction-denaturing gradient gel electrophoresis

Direct DNA extraction from wine samples for culture-independent methods. Biomass was collected from 100 mL of wine, and washed with 2 mL TE buffer [Tris 10 mmol/L, ethylenediaminetetraacetic acid (EDTA) 1 mmol/L, pH 8]. The pellet was resuspended in 300 µL 0.5 mmol/L EDTA, pH 8, and 300 µL of glass beads (Ø = 0.1 mm) were added. Samples were mixed at maximum speed for 10 min (Mini Beadbeater, Biospec Products, Bartlesville, OK, USA). Then, DNA samples were obtained according to Spano et al. (2007).

Polymerase chain reaction amplification for rpoB/ polymerase chain reaction-denaturing gradient gel electrophoresis. The DNA of wine samples was amplified with primers rpoB1 (5′-ATTGACCACTTGGGTAACCGTCG-3′), rpoB1o (5′-ATCGATCACTTAGGCAATCGTCG-3′) and rpoB2 (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG GGGCACGATCACGGGTCAAACCACC-3) according to Spano et al. (2007) in order to obtain a 336 bp fragment of *rpo*B gene. The rpoB2 primer has a GC-rich clamp DNA sequence that improves PCR-DGGE separation (Scheffield et al. 1989).

Polymerase chain reaction amplification for 16S rRNA V3 region/polymerase chain reaction-denaturing gradient gel electrophoresis. The DNA of wine samples was amplified by two successive PCR reactions according to Ogier et al. (2002). To amplify a first 700 bp fragment of the *16S rRNA* gene that included the V3 region, W01 [5′-GAGTTTGATC (AC) TGGCTC-3′] and W012 [5′-TACGCATTTCACC (GT) CTACA-3′] primers (Genbiotech, Buenos Aires, Argentina) were used. The *16S rRNA* gene fragment including the V3 region was amplified using HDA1-GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGC GGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3′) and HDA2 (5′-GTATTACCGCGGCTGCTGGCA-3′) primers (Ogier et al. 2002, Ruiz et al. 2010).

Conditions of denaturing gradient gel electrophoresis. The DNA amplification products from the different stages of MLF and from reference strains were subjected to DGGE analysis on equipment manufactured by Cleaver Scientific Ltd (Rugby, England) using 16 × 16 × 1 mm gels. Electrophoresis was undertaken at 60°C in 1 × TAE buffer (40 mmol/L Tris-acetate, 2 mmol/L EDTA, pH 8.0) with 8% polyacrylamide gels containing 30–60% urea–formamide linear denaturing gradient [100% solution of 7 mol/L urea and 40% (v/v) formamide], increasing in the direction of electrophoresis, for 2 h at 180 V for amplicons of the V3–16S *rRNA* gene (Ruiz et al. 2010), and for 24 h at 60 V for amplicons of the *rpo*B gene. Following electrophoresis, gels were stained for 30 min with a Red gel solution (Genbiotech).

Sequencing of DNA. The discriminatory capacity of the PCR-DGGE technique was evaluated under the conditions employed with several reference strains: *O. oeni* (ATCC 27310), *Lb. plantarum* (ATCC 14917), *Lb. casei* (ATCC 393) and *Lb. rhamnosus* (ATCC 7469). In order to identify the bacterial species, the PCR-DGGE bands were cut from the gels immediately after staining, eluted and re-amplified according to Bae et al. (2006). Amplified products were purified with QIAgen

PCR Purification Kit (Promega Corp., Madison, WI, USA) and sequenced by Macrogen Inc. (Seoul, South Korea). The sequences of the *16S rRNA* V3 gene region and the *rpo*B gene fragment were compared with the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). All sequences obtained were submitted to the GenBank (www.ncbi.nlm.nih.gov/genbank) (see the results and discussion section for accession numbers).

Isolation and identification of lactic acid bacteria by culture-dependent methods

Isolation of lactic acid bacteria. Samples of Merlot and Pinot Noir wines were initially plated directly onto MRS agar (De Man et al. 1960) (Biokar, Beauvais Cedex, France) supplemented with tomato juice 15% v/v (MRST) and cycloheximide 100 mg/L (Sigma-Aldrich de Argentina, Buenos Aires, Argentina) to prevent growth of yeasts and other fungi. These samples were also subjected to a previous enrichment culture on MRS broth pH 4.8 supplemented with tomato juice and cycloheximide. Samples from enrichment cultures were transferred at 0, 5 and 10 days to MRS agar (Pan et al. 1982, Bae et al. 2006). In contrast, samples were plated directly onto specific medium for *Leuconostoc oenos* (MLO) medium (Caspritz and Radler 1983) to isolate *O. oeni*. Microaerophilic incubation was carried out at 28°C for 10 days using the candle jar method. All isolates were considered to be LAB based on their positive Gram reaction, non-motility and absence of catalase activity. Isolates were preserved in MRS or MLO broth with 30% glycerol at −80°C.

DNA extraction from lactic acid bacteria isolates. Genomic DNA was obtained from LAB isolates and reference strains according to the previously described method (Bravo-Ferrada et al. 2011). Samples of DNA were quantified with a Nanodrop spectrophotometer (Thermo Scientific, 1000, Instrumentación Científica SA, Buenos Aires, Argentina) and visualised on a 1.0% agarose gel.

Identification of LAB isolates. The reference strains used for LAB identification by PCR-restriction fragment length polymorphism (RFLP) were: *O. oeni* ATCC 27310, *Lb. plantarum* ATCC 14917, *Lb. brevis* (ATCC 8287), *P. pentosaceus* (ATCC 10791), *Lb. collinoides* (ATCC 27611), *Lb. fermentum* (ATCC 9338), *Lb. casei* (ATCC 393), *Lb. paracasei* (ATCC 27216), *Lb. mali* (ATCC 27304) and *L. mesenteroides* (ATCC 23386). These strains were selected according to LAB species previously found in Patagonian Pinot Noir wines (Bravo-Ferrada et al. 2013).

A 294 bp region of the *rpo*B gene from LAB isolates was amplified with the primers *rpo*B1, *rpo*B1o and *rpo*B2. Amplifications were performed by touchdown PCR according to Claisse et al. (2007). Amplicons were subjected to restriction analysis with *Aci*I and *Hinf*I enzymes (Fermentas International Inc., Burlington, ON, Canada). Restriction products were detected by electrophoresis on 3% agarose gel using a 50 bp ladder marker as standard size (Productos Bio-Lógicos, Bernal, Argentina).

Cloning and sequencing of rpoB fragments. Amplicons of the *rpo*B gene from LAB isolates that were difficult to identify by the PCR-RFLP method were cloned in pGEM -T Easy Vector System (Promega Corp.) and sequenced. Sequencing of DNA was performed with universal primers (T7 and SP6) (Macrogen Korea, Geumcheon-gu, Seoul, South Korea) and partial *rpo*B gene sequences were deposited in the GenBank.

Lactobacillus plantarum and *O. oeni* isolates were analysed by random amplified polymorphic DNA (RAPD)-PCR with the single primer Coc (5′-AGCAGCGTGG 3′) (Cocconcelli et al. 1995). The PCR reactions were amplified as previously described (Delfederico et al. 2006, Bravo-Ferrada et al. 2011) and PCR products were resolved by electrophoresis in a 2% agarose gel. Fragment lengths were estimated by comparison to a 100 bp ladder marker as a size standard (Productos Bio-Lógicos). High-resolution photographs of RAPD-PCR gels were obtained by Kodak Electrophoresis Documentation and Analysis System 120, and subsequently analysed using Kodak Digital Science V.3.0.2 (Eastman Kodak Company, Rochester, NY, USA). Patterns were evaluated by calculating the genetic similarity index using a simple matching coefficient (Apostol et al. 1993). The unweighed pair group method using arithmetic averages (UPGMA) cluster analysis was carried out using PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA, USA).

Results and discussion

Diversity of lactic acid bacteria species in Patagonian Merlot wines detected by polymerase chain reaction-denaturing gradient gel electrophoresis

Many authors have focused on the study of LAB of oenological origin (Lafon-Lafourcade et al. 1983, Bartowsky et al. 2003, Bae et al. 2006, Renouf et al. 2007, Barata et al. 2012, González-Arenzana et al. 2012, 2013a,b) from different winegrowing regions of the world. The description of LAB species diversity is highly dependent of the methodology used, environmental conditions and grape cultivar. Saguir et al. (2009) employed culture-dependent methods to analyse the LAB predominant in grape juice and at the beginning of MLF in Malbec wines from the Northern wine-producing region of Argentina. Patagonia is the southernmost wine-producing region of Argentina and one of the southernmost of the world. This area has optimal agro-ecological conditions that favour high-quality viticulture and conservation of natural microbiota associated with grapes, because agrochemicals are not used. For this reason, we examined the LAB microbiota associated with spontaneous MLF in a Patagonian cellar, as the first stage in a long-term study with the ultimate goal of designing autochthonous MLF starter cultures.

The culture-independent PCR-DGGE technique was applied using two genome regions, a fragment of the housekeeping *rpo*B gene and the V3 variable region of the *16S rRNA* gene, in order to achieve a more complete description of the LAB diversity through the combined use of both gene regions.

The LAB species identified by PCR-*rpo*B/DGGE during NAF-MLF in the 2008 Merlot were (Figure 1a): *Lb. plantarum* (Accession Number: JX406827), *Lb. reuteri* (JX406830), *Lb. buchneri* (KF147809), *Lb. collinoides* (KF147810), *Lb. rhamnosus* (KF147816), *Lb. paracasei* (KF147813) and *Lb. fermentum* (KF147808). We also detected three cocci species, *L. mesenteroides* (JX406832), *P. pentosaceus* (JX406829) and *O. oeni* (JX406828).

The number of electrophoresis bands obtained from the 2008 Merlot (GAF-MLF) decreased as MLF progressed, exhibiting only two bands at the final stage, which corresponded to *O. oeni* and *Lb. plantarum* LAB species. In NAF-MLF from 2008, Merlot the number of bands obtained was the same, but with different electrophoretic mobility according to the species detected (Figure 1a). The LAB species *Lb. rhamnosus* and *L. mesenteroides* were detected in the early and mid-stages of NAF-MLF, whereas in GAF-MLF *Lb. rhamnosus* remained until

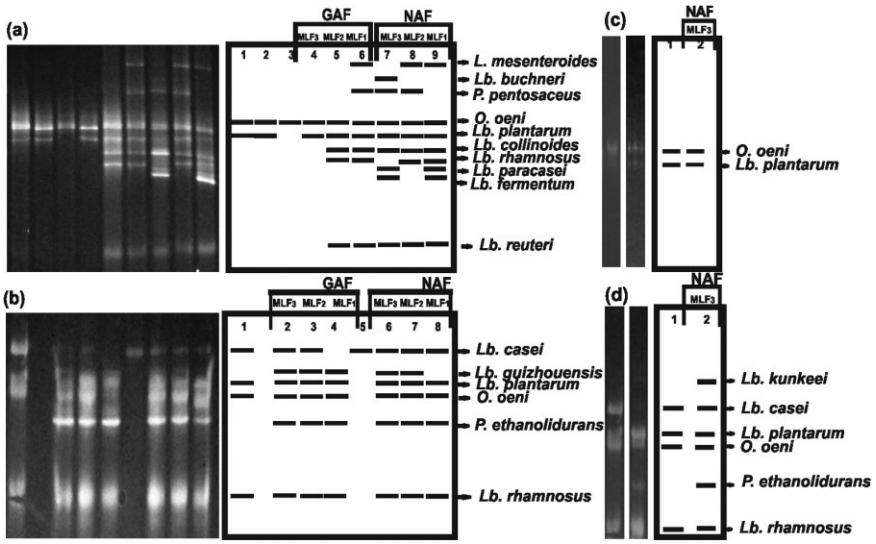

Figure 1. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles of lactic acid bacteria species associated with the 2008 and 2012 Merlot wine samples, from different stages of natural alcoholic fermentation-malolactic fermentation (NAF-MLF) and guided alcoholic fermentation (GAF-MLF). (a) PCR-*rpo*B/DGGE of the 2008 Merlot: 1, *Lb. plantarum* and *O. oeni* control strains; 2, mix of DNA amplification products from *O. oeni* and *Lb. plantarum*; 3, *O. oeni* control strain; 4 to 6, three MLF stages of Merlot with GAF; and 7 to 9, three MLF stages of Merlot with NAF. (b) PCR-*16S rRNA* V3/DGGE of the 2008 Merlot : 1, *Lb. plantarum*, *Lb. casei*, *Lb. rhamnosus* and *O. oeni* control strains; 2 to 4, three MLF stages of Merlot with GAF; 5, *Lb. casei* control strain; and 6 to 8, three MLF stages of Merlot with NAF. (c) PCR-*rpo*B/DGGE of the 2012 Merlot : 1, *Lb. plantarum* and *O. oeni* control strains;and 2, MLF_3. (d) PCR-*16S rRNA* V3/DGGE of the 2012 Merlot: 1, *Lb. plantarum*, *Lb. casei*, *Lb. rhamnosus* and *O. oeni* control strains; and 2, MLF_3.

MLF2, and *L. mesenteroides* was only detected at MLF1 (Figure 1a). *Lactobacillus paracasei* and *Lb. fermentum* were detected only at stages 1 and 3 of NAF-MLF from the 2008 Merlot. This result was not obtained from any of the other wines analysed and, more interestingly, this was consistent with the results obtained using culture-dependent methods (see below). In MLF from the 2012 Merlot (Figure 1c), *Lb. plantarum* (KF514143) and *O. oeni* (KF514142) were the only LAB species detected at MLF_3.

The diversity analysis performed with the *rpo*B gene revealed the predominance of *O. oeni* and *Lb. plantarum* as major bands in the 2008 and 2012 Merlot wines (Figure 1a,c). In addition to *O. oeni* and *Lb. plantarum*, however, other LAB species, such as *Lb. collinoides* and *Lb. reuteri,* were detected at almost all stages of NAF-MLF and GAF-MLF from the 2008 Merlot (Figure 1a).

The analysis of LAB diversity by PCR-*16S rRNA* V3/DGGE, undertaken to complement the information obtained with the PCR-*rpo*B/DGGE gene, yielded the following results. Samples from the Merlot wines, taken at three MLF stages, showed profiles with a variable number of bands, ranging from five to six. No differences were observed on the number and type of LAB species identified from the 2008 Merlot NAF or GAF vinifications (Figure 1b). We detected four *Lactobacillus* species, *Lb. plantarum* (JX128253), *Lb. rhamnosus* (JX128255), *Lb. casei* (JX128251) and *Lb. guizhouensis* (JX128252), and the presence of two cocci species, *O. oeni* (JX128256) and *P. ethanolidurans* (JX128254). *Lactobacillus plantarum*, *Lb. rhamnosus*, *O. oeni* and *P. ethanolidurans* were found at each MLF stage. Furthermore, *Lb. casei* was detected at each stage of NAF-MLF and at MLF_2 and MLF_3 of GAF-MLF. In contrast, *Lb. guizhouensis* (JX128252) was revealed at each stage of GAF-MLF and at stages 2 and 3 of NAF-MLF (Figure 1b). To our knowledge, this is the first time that this LAB species has been found in wine. It has been previously reported in traditional rice fermented sour dough in China (Zhang et al. 2011).

The *Lactobacillus* sp. species identified from the 2012 Merlot were *Lb. plantarum* (KF058557), *Lb. rhamnosus* (KF058550) and *Lb. kunkeei* (KF551239). Two cocci species were identified as well, *O. oeni* (KF058539) and *P. ethanolidurans* (KF058542) (Figure 1d). All species of *Lactobacillus* sp. detected have been previously described as oenological species with favourable sensory properties, except for *Lb. kunkeei*, which has been reported in association with damaged grapes (Bae et al. 2006) and wines with retarded fermentations (Edwards et al. 1998a,b). Fourteen LAB species were identified using the two targeted genes and only three, *Lb. plantarum*, *Lb. rhamnosus* and *O. oeni,* were detected in all samples.

Patagonian Pinot Noir wines

Samples from a 2010 Pinot Noir at three stages of spontaneous MLF were analysed with both gene regions, whereas only the MLF_3 of spontaneous MLF from 2012 Pinot noir was analysed. Figure 2a,c shows the PCR-*rpo*B/DGGE profiles obtained. The number of bands detected in MLF_1 and MLF_2 was higher than that detected in MLF_3. The species *O. oeni* (KF147822), *Lb. plantarum* (KF147819) and *Lactococcus lactis* (KF147815) were detected at each stage of MLF. Interestingly, *L. lactis* has seldom been detected in wines and grapes (Bae et al. 2006, Mesas et al. 2011, González-Arenzana et al. 2013b). In MLF_1 and MLF_2, other LAB species were also detected, such as *Lb. sakei* (KF147811), *Lb. brevis* (KF147817), *Lb. delbrueckii* (KF147825) and *P. acidilactici* (KF147822) (Figure 2a). *Pediococcus pentosaceus* (KF147826) was also detected at MLF_3. In contrast, *Lb. plantarum* (KF147823) and *O. oeni* (KF058540) were the only LAB species detected in the 2012 Pinot Noir by PCR-*rpo*B/DGGE (Figure 2c).

Figure 2b,d show the PCR-*16S rRNA* V3/ DGGE profiles obtained from the 2010 and 2012 Pinot Noir, respectively. From the 2010 Pinot Noir, MLF_1, MLF_2 and MLF_3 exhibit the same number of bands but with different electrophoretic mobility,

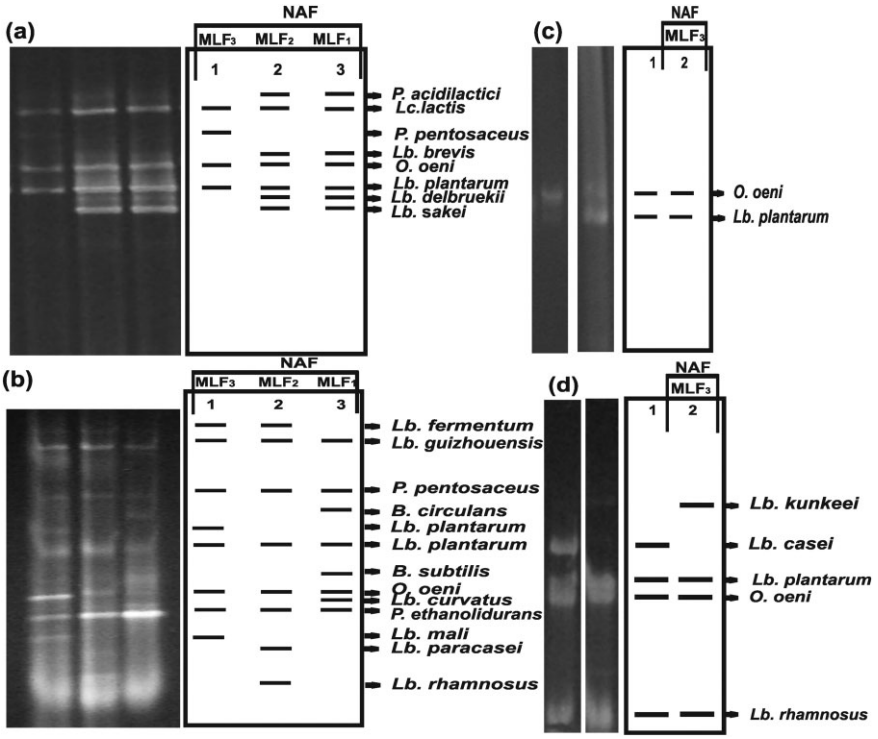

Figure 2. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles of lactic acid bacteria species associated to Pinot Noir wine samples from the 2010 and 2012 vintages at different stages of natural alcoholic fermentation-malolactic fermentation (NAF-MLF). (a) PCR-*rpo*B/DGGE of 2010 Pinot noir: 1 to 3, three MLF stages. (b) PCR-*16S rRNA* V3/DGGE of 2010 Pinot noir: 1 to 3, three MLF stages. (c) PCR-*rpo*B/DGGE of 2012 Pinot Noir: 1, *Lb. plantarum* and *O. oeni* control strains; and 2, final MLF stage. (d) PCR-*16S rRNA* V3/DGGE of the 2012 Pinot Noir: 1, *Lb. plantarum*, *Lb. casei*, *Lb. rhamnosus* and *O. oeni* control strains; and 2, final MLF stage.

according to the species detected at each stage. The species *O. oeni* (KF058541) and *Lb. plantarum* (KF058554) were detected at all MLF stages from this wine sample, as well as the species *Lb. guizhouensis* (KF058552), *P. pentosaceus* (KF058558) and *P. ethanolidurans* (KF058553). *Lactobacillus curvatus* (KF058560) was detected only in MLF_1, while *Lb. paracasei* (KF514140) was found in MLF_2 and *Lb. mali* in MLF_3. *Lactobacillus fermentum* (KF058547) was present in MLF_2 and MLF_3. We observed two bands of different size belonging to the species *Lb. plantarum* (KF058556) in MLF_3, because of the existence of repeated copies of the *16S rRNA* gene (Coenye and Vandamme 2003). Two species belonging to *Bacillus* were detected in MLF_1, *B. circulans* (KF058546) and *B. subtilis* (KF058559), which could be the result of a soil contamination (Figure 2b). Furthermore, the use of the *16S rRNA* V3 gene region revealed the presence of *O. oeni*, *Lb. plantarum*, *Lb. kunkeei* (KF551240) and *Lb. rhamnosus* (KF058548) from the 2012 Pinot Noir (Figure 2d). The combined use of two targeted gene regions in Pinot Noir wines led to the identification of 11 species of *Lactobacillus* sp., three species of *Pediococcus*, one *Lactococcus* and one *Oenococcus*, a total of 16 LAB species associated with these wines.

The LAB species *Lb. casei*, *Lb. guizhouensis*, *Lb. plantarum*, *Lb. fermentum* and *O. oeni* were detected in both Pinot Noir and Merlot wines. Comparing the results obtained by PCR-*rpo*B/DGGE and PCR-*16S rRNA*/DGGE, it appears that the *rpo*B gene shows a greater ability for discriminating among the main LAB cocci species found in wines. These results were somewhat expected, because the primers chosen were based on the *rpo*B gene sequence that is usually selected to discriminate cocci species (Renouf et al. 2006). *Pediococcus ethanolidurans*, however, was not detected by PCR-*rpo*B/DGGE gene, suggesting that the primers used have some limitations for the detection of cocci; this limitation was solved by using primers of the *16S rRNA* gene. The presence of *O. oeni* and *Lb. plantarum* was easily detected by both genes from Merlot as well as from Pinot Noir wines.

The results obtained by PCR-DGGE of the *16S rRNA* and *rpo*B genes were similar to those reported by Renouf et al. (2006). Nisiotou et al. (2011), using PCR-*16S rRNA* of the V1–V3 region/ DGGE, identified 13 bacterial species, with *Lb. plantarum* being the only LAB species. Similar to our work, the PCR-*16S rRNA* of V3 region/DGGE used by Ruiz et al. (2010) detected *O. oeni* and *Lb. casei*, but the remaining species detected were not LAB species. Results from other studies (Meroth et al. 2003, Pérez-Pulido et al. 2005, Bokulich et al. 2013) also revealed differences in the microbial composition of fermented foods depending on whether culture-dependent or culture-independent methods were used. In agreement with our results, González-Arenzana et al. (2013b) detected 13 LAB species (nine by PCR-*16S rRNA*/DGGE of V4–V5 region, and four by PCR-*rpo*B/DGGE); many of these LAB species were also detected in our work by the combination of *rpo*B and *16S rRNA* V3 gene regions. Therefore, our results reinforce the idea that the use of the *16S rRNA* V3 gene region in combination with the *rpo*B gene enhances the diversity analysis of the LAB community, given that different species present in varying proportion could be detected and amplified by the two pairs of primers used.

Isolation and identification of lactic acid bacteria

Lactic acid bacteria isolates were obtained from three stages of spontaneous MLF (GAF and NAF) from the 2008 Merlot wines, and from the 2010 and 2012 Pinot Noir wines, under enrichment conditions, but were not recovered by direct plating on MRST agar, suggesting either a low density of LAB populations or the presence of bacteria in the VBNC state. Similar results were reported by Bae et al. (2006), who showed that enriched conditions substantially increased the frequency of isolation of LAB from grapes. In our laboratory, the MRST medium has been successfully used to isolate *O. oeni*, but in small numbers (Bravo-Ferrada et al. 2011), as reported by other authors (Sico et al. 2008).

We recovered 43 isolates of *O. oeni* from NAF-MLF and 22 from GAF-MLF from the Merlot wines, using the MLO medium.

Isolates of LAB recovered from GAF-MLF wine samples were fewer than those obtained from NAF-MLF wine samples (42 and 83 isolates, respectively). This result might indicate an inhibiting effect on LAB growth in this wine caused by the *S. cerevisiae* commercial starter culture used in the AF. It has been pointed out that the relationship between yeast and bacteria is strain dependent (Fornachon 1968, Fleet 2003), and some strains of *S. cerevisiae* could inhibit the subsequent growth of *O. oeni* and MLF (Markides 1993, Costello et al. 2008). The use of indigenous AF starter strains, naturally adapted to each winery region, could reduce this competition problem (Curilén et al. 2009).

The PCR-RFLP analysis using *Aci*I and *Hinf*I showed that the species most frequently isolated in both NAF-MLF and GAF-MLF from the Merlot wines were *O. oeni* and *Lb. plantarum*, followed by *Lb. paracasei*. Isolates of *Lb. paracasei* were found at MLF_1 and MLF_3 of NAF-MLF. This unusual result is consistent with those found by culture-independent methods (Figure 1a), and may be due to some issue during the vinification process that could have affected the detection and isolation of *Lb. paracasei* from the middle stage of the NAF-MLF, considering that it was detected only in the 2008 Merlot wine. Isolates of *Lb. fermentum* and *L. mesenteroides* were recovered only at the middle stage of NAF-MLF. Isolates belonging to the Bacillus genus, however, were recovered at each MLF stage (data not shown) and also detected by DGGE. Bae et al. (2004) reported that the presence of some *Bacillus* species can inhibit growth and decrease the likelihood of recovering LAB from grapes. The NAF-MLF samples showed a slightly greater diversity of LAB isolates than that of the GAF-MLF samples, and according to the results obtained by PCR-*rpo*B/DGGE gene (Figure 1a), the isolation of LAB species appears to decline during the GAF-MLF. From the 2010 and 2012 Pinot Noir wines, in which AF was a spontaneous process, *Lb. plantarum* and *O. oeni* were largely recovered. Other *Lactobacillus* species isolated from these wines were *Lb. brevis* and *Lb. rhamnosus*.

Some isolates, unidentifiable by restriction analysis, were selected to obtain the sequence of *rpo*B gene fragments. Comparison of sequences with those available in the GenBank database revealed that they were ≥ 97% similar to *Lb. plantarum* (JX406833 and JX406834).

The use of culture-dependent methods allowed the isolation of six LAB species from the Merlot wines, and four from the Pinot Noir wines, all of them also detected by culture-independent methods. Those LAB detected by PCR-DGGE but not isolated may either be present at low concentration in wine, have strict nutritional requirements or be largely dependent on the microbial consortium of wine to grow.

Oenococcus oeni and *Lb. plantarum* were the bacterial species most frequently isolated throughout MLF, and were also revealed as the main species by PCR-DGGE analysis in all wine samples. Despite *O. oeni* having been previously described as the main LAB species involved in MLF (Davis et al. 1985, Lonvaud-Funel 1999), other LAB species may also play a role in this process. The abundance of *Lb. plantarum* detected in Patagonian red wines suggests a positive intervention during the MLF process. Furthermore, *Lb. plantarum* has been reported as a flexible species that can grow in wines (Navarro et al. 2000, Du Plessis et al. 2004, Rojo-Bezares et al. 2007) and possesses resistance mechanisms to tolerate high ethanol concentration and low pH in the growth medium (G-Alegría et al. 2004, Rojo-Bezares et al. 2007). The presence of *Lb. plantarum* in wine fermentations has been well described (Beneduce et al. 2004, Spano et al. 2007, Nisiotou et al. 2011). It has been associated with the deterioration of wine quality, through the production

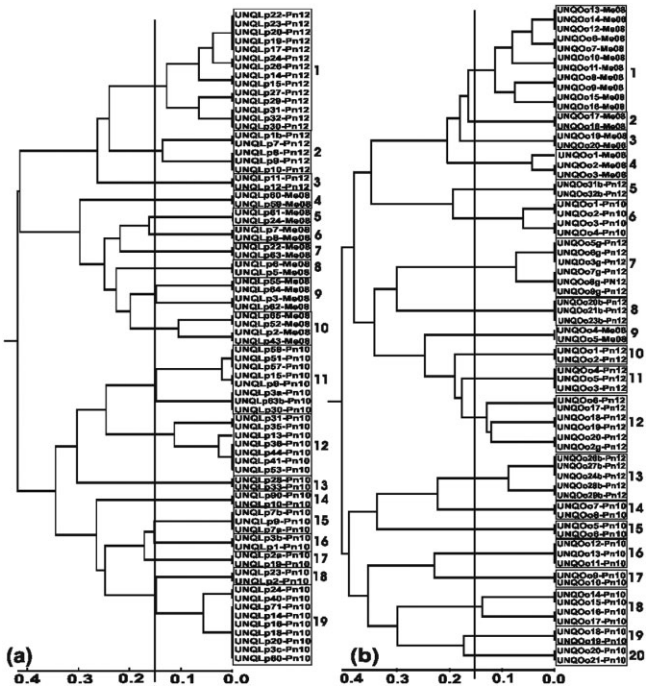

Figure 3. Clustering of lactic acid bacteria isolates from wine samples [2008 Merlot – NAF (Me08), 2010 Pinot noir (Pn10), and 2012 Pinot Noir (Pn12)]. Dendrograms were obtained by unweighted pair group method using arithmetic averages analysis of Coc RAPD-PCR. (a) *Lb. plantarum* isolates, (b) *O. oeni* isolates.

of biogenic amines and precursors of ethyl carbamate (Lonvaud-Funel 1999, Liu 2002). Nonetheless, previous results obtained in our laboratory showed that several *Lb. plantarum* strains isolated from spontaneous MLF of Patagonian Pinot Noir wines have good oenological and technological properties that would enable their use as starter cultures (Bravo-Ferrada et al. 2013).

Typing of isolates

Because *Lb. plantarum* and *O. oeni* were the most prevalent species recovered from the wine samples, all isolates belonging to these species were subjected to further study. The genetic heterogeneity of these isolates was determined by numerical analysis of DNA profiles obtained by RAPD–PCR using the Coc primer, which allowed isolates from each wine to be grouped into clusters defined at a minimum similarity level of 85.5%. The UPGMA dendrogram generated from electrophoretic profiles illustrates the genomic variability and the possible clonal relationships between each single genomic fingerprinting (Figure 3a,b). The number of different genotypes was 19 out of 76 isolates for *Lb. plantarum* (type variation of 25%) and 20 out of 68 isolates for *O. oeni* (type variation of 29.4%). Clusters 1 and 19 from *Lb. plantarum* include the largest number of members (14 and nine isolates, respectively), with cluster 1 being the most abundant for the 2012 Pinot Noir and cluster 19 the predominant one for the 2010 Pinot Noir. In contrast, cluster 1 from *O. oeni* consisted of 11 isolates, while clusters 7 and 12 included six members. Among the *O. oeni* isolates, cluster 1 was the predominant one for the Merlot wine, and clusters 7 and 12 were the main genotypes for the 2012 Pinot Noir.

Lactobacillus plantarum strains isolated from different wines were grouped into different clusters; distribution of RAPD genotypes showed nine clusters out of 37 isolates for the 2010 Pinot Noir, three clusters out of 21 isolates for the 2012 Pinot

Noir and seven clusters out of 18 isolates for the 2008 Merlot. Strains of *O. oeni* isolated from different wines were grouped into different clusters as well. Distribution of RAPD genotypes showed eight clusters for 21 *O. oeni* isolates from 2010 Pinot Noir, seven clusters for 27 isolates from the 2012 Pinot Noir and five clusters for 20 isolates from the 2008 Merlot. Interestingly, cluster 9 from *O. oeni* from the 2008 Merlot is closely related to strains from the 2012 Pinot Noir. All clusters from both, *Lb. plantarum* and *O. oeni* included members which showed a 100% similarity among them, meaning that members of the same strain were repeatedly isolated. Moreover, the genetic diversity data of 53 *Lb. plantarum* isolates obtained from a Patagonian 2008 Pinot Noir wine from the same cellar we sampled yielded 12 different genotypic clusters (Bravo-Ferrada et al. 2013), in agreement with the results now reported. Furthermore, different RAPD patterns for isolates of *O. oeni* and *Lb. plantarum* were often recovered in most of the wine samples analysed by Solieri et al. (2009). Bartowsky et al. (2003) investigated the genetic variation of *O. oeni* present in wine fermentations with fruit from a single source (Cabernet Sauvignon, Padthaway, SA, Australia, vintage 2002) using RAPD techniques, and found a wide genetic variation among the isolates. Similar results were obtained by other authors using pulsed-field gel electrophoresis which confirms that several strains can occur in a single spontaneous MLF (Ruiz et al. 2010, González-Arenzana et al. 2012).

There were some interesting differences between the wines analysed here: the 2008 Merlot showed the most complex polymorphism for *Lb. plantarum* species, but the least complex for *O. oeni*. For the latter, the highest strain diversity was observed in the 2010 Pinot Noir. We found a good correlation between RAPD profiles and wine samples, with only two cases where isolates coming from different samples showed similar RAPD patterns.

Conclusions

Our research represents the first study of LAB microbiota from spontaneous MLF of wines produced in the Argentine Patagonia in one commercial cellar. The prevalence of *O. oeni* and *Lb. plantarum* in all wine samples suggests that both species are involved in leading spontaneous MLF of the Pinot Noir, as well as in the Merlot wines tested. The variations observed between the results obtained from culture-dependent and culture-independent methods suggest that a combined approach is needed to detect dominant and rare bacterial species. This study has provided a more complete view of the composition of the bacterial community present during the spontaneous MLF of Patagonian red wines. Our research showed the greatest amount of LAB described so far during spontaneous MLF in red wines, and reported for the first time the presence of *Lb. guizhouensis* in fermenting wine samples. The application of PCR/DGGE increased the number of LAB species usually detected during MLF in a wine. The results from PCR/DGGE with two targeted genes were complementary and should be further employed to obtain a better knowledge of LAB ecology in wine.

The commercial cellar from which the wine samples were obtained has been active for more than 100 years. It produces young red wines and has never made use of starter bacterial cultures. The genetic diversity data from both *Lb. plantarum* and *O. oeni* isolates demonstrate a considerable genotypic heterogeneity through the MLF in all vinifications studied. This suggests that there are several strains, none of them implanted in the cellar, that come from grapes and lead these spontaneous MLFs, highlighting the need to use an MLF starter to exert a better control of the process. It would be interesting to determine in future studies the viability of the best adapted indigenous strains during MLF, as well as their role in the process and their effect on the sensory properties of wines.

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