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In Vivo PCR-DGGE Analysis of *Lactobacillus plantarum* and *Oenococcus oeni* Populations in Red Wine

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Abstract. In order to monitor *Lactobacillus plantarum* and *Oenococcus oeni* in red wine produced with Italian grape (variety ''Primitivo di Puglia''), a polymerase chain reaction– denaturing gradient gel electrophoresis (PCR-DGGE) approach using the *rpoB* as gene target was established. Wine was treated or not with potassium metabisulphite and supplemented with a commercial bacterial starter of *O. oeni* to encourage malolactic fermentation. Samples were taken from the vinification tanks at 4, 10, 16, 22, and 28 days after the start of alcoholic fermentation. Genomic DNA was directly isolated from wine and identification of lactic acid bacteria was performed using primers *rpoB1*, *rpoB1O*, and *rpoB2* able to amplify a region of 336 bp corresponding to the *rpoB* gene. Amplified fragments were separated in a 30–60% DGGE gradient, and the ability of the PCR-DGGE analysis to distinguish *L. plantarum* and *O. oeni* was assessed. The results reported suggest that the PCR-DGGE method, based on the *rpoB* gene as molecular marker, is a reproducible and suitable tool and may be of great value for wine makers in order to monitor spoilage microorganisms during wine fermentation.

Lactobacillus plantarum is a flexible species that is encountered in a variety of environmental niches, including fermented beverage, meat, and many vegetable or plant fermentations [1, 2, 3, 12, 26]. The ecological flexibility of *L. plantarum* is reflected by the observation that this species has one of the largest genomes known among lactic acid bacteria [13, 16]. The large size of its genome is thought to be related to the diversity of environmental niches in which *L. plantarum* is encountered. *L. plantarum* is frequently isolated from red wine undergoing malolactic fermentation (MFL) and sterilized with sulphite [2, 22, 23]. Although *L. plantarum* is capable of MFL, it usually contributes to production of undesirable products such as biogenic amine and precursors of ethyl carbamate [14, 15, 15, 24] and it is therefore of general concern because of its spoilage nature. Sensitive and reliable methods for its detection in fermented beverages are therefore of great importance in order to monitor population changes during fermentation.

The improvement of molecular tools, usually based on polymerase chain reaction (PCR) techniques, has allowed a fast and sensitive characterization of the majority of wine Lactic Acid Bacteria (LAB). Community analysis of bacteria using molecular methods such as PCR amplification of the 16S rRNA gene (*rDNA*) in combination with denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) is commonly performed in microbial ecology [4, 17, 11, 25]. Recently, the *rpoB* gene codifying for the RNA polymerase beta subunit has been used as an alternative to the 16S RNA gene [8, 9, 18, 19, 20] although the database of the sequence is less documented than that of the *16S rRNA* gene and therefore, each DGGE band cannot easily be attributed to a species.

The primary focus of this work was to use a PCR-DGGE *rpoB*-based methodology on DNA directly isolated from wine samples, and to monitor the predominance of *L. plantarum* and *O. oeni* species in red

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wine produced with or without sulphur dioxide and supplemented with selected malolactic starter.

Materials and Methods

Wine and Samples Collection. Samples of red wine made with Italian grape (variety "Primitivo di Puglia") were collected from winemakers located in Foggia (Italy). "Primitivo" grapes were harvested at 22°C, destemmed and crushed, and the must was then divided in two different samples (**A** and **B**). Sample **A** was treated with 80 mg L^{-1} of potassium metabisulfite ($K_2S_2O_5$) and supplemented with a commercial *Oenococcus oeni* starter (named Lalvin 31) 12 days after the start of alcoholic fermentation (residual sugars < 2 g L^{-1}). Sample **B** was only supplemented with the bacterial starter without adding $K_2S_2O_5$.

Inoculation with malolactic bacteria (10^6 cells mL^{-1}) was carried out after rehydration of cells in warm sterile water at 30°C for 30 min. Samples were taken directly from the vinification tanks at 4, 10, 16, 22, and 28 days after the start of alcoholic fermentation. The pH value of each sample was measured in duplicate, and samples were stored in cold ice prior to total DNA extraction (between 1 h and 1.30 h since samples were collected).

Bacterial Strains, Plasmids, and Growth Conditions. The *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) used for cloning procedures were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg mL^{-1}) when required. Plasmid DNA was purified with Wizard *Plus* SV Minipreps (Promega) and DNA sequencing was performed on both strands with universal primers (T7 and SP6) by a commercial facility (MWG Biotech, Germany). The plasmid pGEM-T easy vector (Promega) was used as a general vector for cloning and sequencing.

L. plantarum strain Lp90 [24] and *O. oeni* DSM 20252 were grown without shaking at 28°C in an MRS broth (Oxoid, Basingstoke, UK) adjusted to pH 6.8 or in a modified MRS adjusted to pH 5, respectively [3]. Thereafter, a single colony was obtained by spreading 100 μL of MRS broth in MRS plus 15 g L^{-1} of Agar (Oxoid, Milan, Italy). Plates were then incubated for 48 h at 28°C.

Microbial Analysis. In order to count lactic acid bacteria, serial dilutions of each wine sample were used to inoculate plates of MRS agar adjusted to pH 5 [3, 19]. The MRS agar plates were then incubated at 25°C for 5 days in anaerobic conditions using an anaerobic system envelope with palladium catalyst (BBL).

Amplification of the *rpoB* Gene from *Lactobacillus plantarum* and *Oenococcus oeni*. Genomic DNA of *L. plantarum* and *O. oeni* was isolated from a single colony, with the Microbial DNA extraction kit (CABRU, Milan, Italy) according to manufacturer's procedure. For the PCR experiment, about 100 ng of genomic DNA was added to a 50-(L PCR mixture containing 1.25 U of Taq polymerase (Qiagen, Milan, Italy) 0.2 mM of each dATP, dTTP, dGTP, dCTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.4 mM of primers *rpoB1* (ATTGACCACTTGGGTAACCGTCG), *rpoB1O* (ATCGATCACTT AGGCAATCGTCG), and *rpoB2* (CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGGCACGATCACGGGTCAAACCA CC) [19, 20]. Primer *rpoB2* has a GC-rich clamped DNA sequence in order to avoid duplex DNA problems and to improve detection of single-base changes [21]. The reaction mix was cycled through the following temperature profile: 94°C 5 min; 94°C 1 min; 58°C 1 min; 72°C 1 min for the first 15 cycles, then 15 cycles at 52°C as annealing temperature. The PCR reaction was terminated at 72°C for 10 min and thereafter cooled to 4°C. PCR fragments were analyzed on gel electrophoresis carried out by applying 5 μL of sample to 1.5% agarose gels. Gels were run for about 45 min at 110 V in TAE 1X buffer (0.04 M Tris/acetate, EDTA 1 mM) to check the quality and size of PCR products before DGGE or sequencing.

After electrophoresis, the gel was stained with ethidium bromide 1 μg mL^{-1} and washed for 10 min. Subsequently the gel image was acquired with a Gel Doc 2000 (Biorad). The amplified products (with a size of approximately 336 bp) were cloned, sequenced, and sequence comparison was performed. Hence, a reference ladder was made by adding an equal amount (about 200 ng of PCR fragments) of the *rpoB* amplicons and used in the DGGE analysis.

Extraction of DNA from Fermented Wine Samples and Identification of *Lactobacillus plantarum* and *Oenococcus oeni* by PCR-DGGE. At each step of wine fermentation, duplicate 10-g samples were homogenized in a stomacher bag with 10 mL of saline-peptone water for 1 min. After each preparation had settled for 1 min, two 1.8-ml subsamples were placed in 2-mL screw-cap tubes and total DNA was extracted using a Powersoil DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's procedure. After DNA was isolated, 500 μL of phenol–chloroform–isoamyl alcohol (25:24:1; pH 6.7; Sigma) was added to each tube and the tubes were then centrifuged at 12,000 *g* at 4°C for 10 min, the aqueous phases was collected, and DNA was precipitated with 2.5 (vol/vol) ice-cold absolute ethanol and 1/10 (v/v) NaAcetate 3M pH 3.5. The DNA was collected by centrifugation at 14,000 *g* at 4°C for 10 min, and the pellet was dried under vacuum at room temperature. Fifty microliters of sterile water was added and the preparation was incubated for 30 min at 45°C to facilitate nucleic acid solubilization. One microliter of DNase-free RNase (Invitrogen) was added to digest RNA, during incubation at 37°C for 1 h. PCR conditions employed were as reported above. In order to test the absence of Taq polymerase inhibitors, primers pA and pH were used to amplify a region of approximately 1 kb of eubacterial 16S-rDNA [10].

The Decode Universal Mutation Detection System™ (BioRad, Hercules, CA) was used for the sequence-specific separation of the PCR products. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [wt/vol] acrylamide:bisacrylamide 37.5:1) and a 30–60% urea-formamide denaturing gradient (100% corresponds to 7 M urea and 40% [w/v] formamide). The gel was subjected to a constant voltage of 60 V for 12 h at 60°C. After electrophoresis, the gel was stained for 20 min in a SYBR Green solution (Cambrex, Bio Science Rockland, Inc.) and analyzed under UV illumination (Versa-Doc 4000 Imaging System, BioRad).

When PCR products were destined for sequencing analysis, bands were excised and an unclamped forward primer was used for the generation of the amplicons. The amplification mixture and conditions employed were reported above. PCR products were checked by agarose gel electrophoresis and sent for sequencing to a commercial facility (MWG Biotech, Germany).

Analyses of DNA sequences were carried out using a set of programs accessible at the sites DEAMBULUM (www.infobiogen.fr/services/deambulum/fr) and NCBI (www.ncbi.nlm.nih.gov).

Results and Discussion

The main problem to deal with in an in vivo molecular approach is the recovery of nucleic acids (DNA and RNA) directly from the food or the environmental samples. In this paper, a method to extract DNA from wine samples, mainly based on a commercial DNA extraction kit usually used for soil samples, was

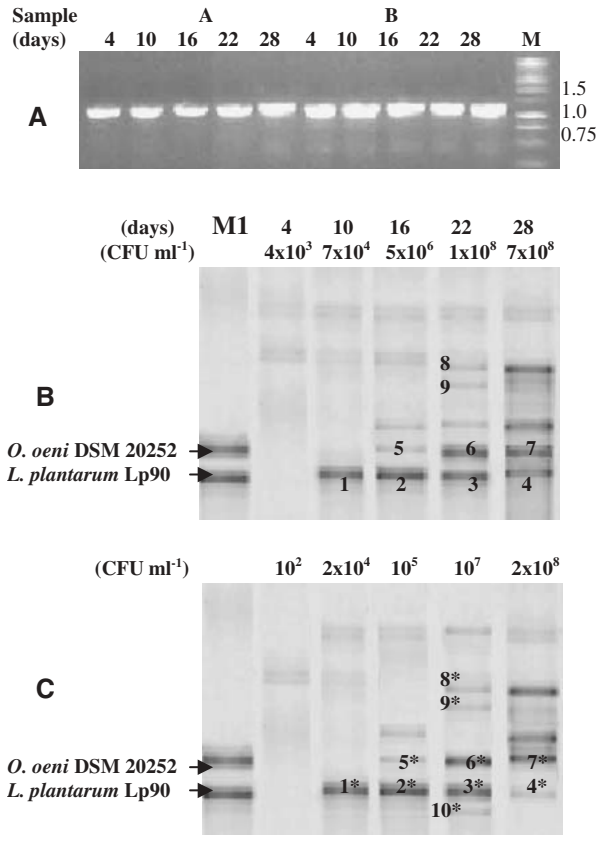

Fig. 1. **A** DNA isolated from samples A and B and amplified with primers specific for the eubacterial 16S rDNA. **B** Polymerase chain reaction– denaturing gradient gel electrophoresis (PCR-DGGE) analysis performed on DNA extracted from red wine supplemented with the *Oenococcus oeni* starter. **C** PCR-DGGE analysis performed on DNA extracted from red wine treated with 80 mg L^{-1} of potassium metabisulfite ($K_2S_2O_5$) and supplemented with a commercial *Oenococcus oeni* starter (named Lalvin 31) 12 days after the start of alcoholic fermentation. **M:** 1-kb molecular ladder (Promega). Molecular masses (kb) are reported on the left. **M1**: molecular reference ladder made by adding an equal amount of the *rpoB* amplicons isolated from *Lactobacillus plantarum* strain Lp 90 and *Oenococcus oeni* DSM 20252.

developed. The purity of the isolated DNA was tested in a PCR experiment using primers specific for the eubacterial 16S rDNA (Fig. 1A). In all of the samples analyzed, the isolated DNA give a fragment of about 1.2 kb corresponding to the internal portion of the 16S rDNA gene. Therefore, the DNA extraction employed in this study is able to recover DNA suitable for PCR experiments. Furthermore, total DNA was amplified with the oligonucleotides set *rpoB1*, *rpoB1O*, and *rpoB2* and analyzed by DGGE experiments. A molecular ladder constitute with the PCR-*rpoB* amplicons obtained from *L. plantarum* strain Lp 90 and *O. oeni* DSM 20252 was used as reference marker.

After separation of the *rpoB* amplicons in a denaturant gradient of 30–60%, several PCR fragments were differentiated from must samples (Fig. 1B and C). The number of PCR fragments increased from 4 to 28 days after the start of alcoholic fermentation in both samples. Wine samples collected after 22 and 28 days presented the largest number of bands of which bands 8, 9 (Fig. 1B) and 8*, 9*, and 10* (Fig. 1C) were absent from the DGGE profiles of the wine samples collected after 10 and 16 days. A similar number of DGGE fragments was observed for DNA isolated from wine samples treated or not with $K_2S_2O_5$. PCR-*rpoB* fragments (Fig. 1B and 1C bands 1, 2, 3, 4, 5, 6, 7, and bands 1*, 2*, 3*, 4*, 5*, 6*, and 7*, respectively) migrated at the same size with the *O. oeni* and *L. plantarum* reference ladder, suggesting that they belong to *O. oeni* and *L. plantarum* species. To establish whether the *rpoB* amplicons were clearly homologues to *L. plantarum* and *O. oeni* species, all of the 14 DGGE fragments were excised and a confirmatory DGGE run was performed to check for the presence of a single band. Excised bands were then re-amplified and sent for sequencing to a commercial facility (MWG Biotech, Germany). Comparison of the sequenced fragments with the *rpoB* genes previously identified from *L. plantarum* strain ATCC8014 and *O. oeni* strain ATCC23277 (EMBL accession numbers AY875849 and AY875845 respectively) [19] confirmed that the amplicons correspond to the internal portion of the *rpoB* gene**.** DGGE-*rpoB* amplicons homologues to *L. plantarum* and *O. oeni* populations were already observed 10 days (*L. plantarum*) and 16 days (*O. oeni*) after the start of alcoholic fermentation, and were represented also at 22 and 28 days in wine supplemented with bacterial starter and treated with $K_2S_2O_5$ (Fig. 1C) or supplemented only with bacterial starter (Fig. 1B). In particular, *L. plantarum* species was identified at 10, 16, 22, and 28 days after the start of alcoholic fermentation, whereas *O. oeni* was detected only after 16 days, suggesting that, initially, the predominant LAB population in wine samples was mainly *L. plantarum* species. The DGGE profiles were in accordance with plate count analysis. Colony-forming units (CFU) mL^{-1} increased from 4 to 28 days in both samples A and B. However, the CFU mL^{-1} recovered was always lower in sample treated with $K_2S_2O_5$, and a decrease in DGGE fragment intensities, mainly corresponding to *L. plantarum* species, was observed 28 days after the start of alcoholic fermentation in sample supplemented with bacterial starter and treated with $K_2S_2O_5$ (Fig. 1C). Nevertheless, *L. plantarum* was still detected by PCR-*rpoB*/DGGE.

Discussion

The use of the PCR-DGGE approach has been recently developed to study several food ecosystems [5, 7, 18]. In some of these studies, the PCR target is usually a region of the *16S rRNA* gene. However, due to interspecies heterogeneity of the *16S rRNA* gene sequence, targeting a region of this gene can lead to the detection of several bands when only one species is present [6].

In this study, an alternative approach to 16S rDNA-target identification of *L. plantarum* and *O. oeni* populations in red wine undergoing MFL was assessed, involving sequence variation in the *rpoB* gene encoding the beta subunit of RNA polymerase. The PCR-DGGE methodology developed was applied in vivo to determine the dynamics of species directly involved in MFL such as *O. oeni* or frequently isolated from red wine and considered as spoilage microorganisms such as *L. plantarum*. PCR-DGGE analysis clearly suggests that *L. plantarum* was the predominant population at the beginning of MFL in our samples. *L. plantarum* species was already observed 10 days after the start of alcoholic fermentation in sample treated with or without $K_2S_2O_5$ and was still detectable at 16 and 22 days. It did not disappear at 28 days even in wine supplemented with $K_2S_2O_5$. *RpoB* amplicons related to *O. oeni* species were observed only 16 days after the start of alcoholic fermentation in samples supplemented with $K_2S_2O_5$ or not. Furthermore, the *O. oeni* population was apparently unaffected by sulphite treatment also 28 days after the start of alcoholic fermentation, suggesting that *O. oeni* is somehow tolerant to sulphite. In modern winemaking, sulphite is used extensively for its suppression of yeasts and bacterial action and its antioxidant properties. Tolerance to sulphite is a valuable trait in selecting wine strains used as starter [15]. *Lactobacillus* is frequently predominant as species and can induce spoilage in wines treated with low doses of sulphite [15]. However, in our samples, a survival of *L. plantarum* in wine must supplemented with 80 mg L^{-1} of $K_2S_2O_5$ has been observed. This result suggests that also spoilage microorganisms such as *L. plantarum* have developed mechanisms able to escape or tolerate high doses of sulphite.

PCR-DGGE fragments unrelated to *L. plantarum* and *O. oeni* species were also observed in both samples A and B. However, our aim was focused on the detection and survival of *O. oeni* and *L. plantarum*, and the discrimination of each species and their evolution are more important than giving a name to each band. PCR-DGGE analysis using the *rpoB* as gene target may be considered a reliable technique in order to monitor bacterial starter extensively used in fermented beverages. Moreover, it could be a useful tool to investigate the dynamic changes of spoilage microorganisms and to assess their viability in a stressful environmental such as wine.

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