# Suitability of Different PCR-DGGE Primer Sets for the Monitoring of Lactic Acid Bacteria in Wine

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**Lactic acid bacteria (LAB) play a dual role in winemaking as they are the main effectors of malolactic fermentation, but some members can also cause wine spoilage. PCR-DGGE has proved to be a quick tool to study the LAB community and their fluctuation in wine. For detecting wine-associated LAB by PCR-DGGE, the primer sets *WLAB1/WLAB2$^{GC}$*, *WBAC1/WBAC2$^{GC}$*, *Lac1/Lac1o/Lac2$^{GC}$*, *341f$^{GC}$/518r* and *rpoB1/rpoB1o/rpoB2$^{GC}$* were tested and evaluated in this study. The primer systems were assessed by the separation of LAB reference strains on DGGE gels and by attributing the resulting amplicons to defined species. Subsequently, the detection of LAB in wine samples and enrichments thereof was compared. While the primer systems *WBAC1/WBAC2$^{GC}$* and *341f$^{GC}$/518r* were not appropriate, the *Lac1/Lac1o/Lac2$^{GC}$* primer set performed well. However, multiple bands complicated the evaluation. The *rpoB1/rpoB1o/rpoB2$^{GC}$* set seemed to be promising for the detection of LAB in wine, although further improvements in terms of the detection limit need to be done. Due to the pronounced sensitivity and the sufficient discrimination of LAB at species level, the *WLAB1/WLAB2$^{GC}$* primer system was found to be most suitable for studying the occurrence of LAB in wine.**

## INTRODUCTION

Winemaking is a complex microbial process in which primarily yeasts, but also lactic acid bacteria (LAB), play pivotal roles (Lonvaud-Funel, 1999). Malolactic fermentation (MLF) can occur at the end of the alcoholic fermentation conducted by yeasts (Lonvaud-Funel, 1999). This fermentation is usually desirable in most of the red wines, some white cultivars, including Chardonnay, some sparkling wines and also in cool-climate Riesling wines (Lerm *et al*., 2010; Knoll *et al*., 2012). MLF is the bacterial conversion of L-malic acid to L-lactic acid and $CO_2$ (Bousbouras & Kunkee, 1971) and exerts significant influences on wine acidity, flavour and microbiological stability (Moreno-Arribas & Polo, 2005). The main effectors of MLF are LAB. Of these, *Oenococcus oeni* is the species mainly responsible for MLF (Davis *et al*., 1986), as it is the species that has accommodated the best to the difficult fermentation conditions, such as low pH values and high ethanol concentrations (Wibowo *et al*., 1988). Due to its particular role, this species is commonly used as starter culture to promote MLF (Mills *et al*., 2005). Furthermore, it is not very frequently reported to be associated with off flavours like volatile acidity and mousiness, spoilage like ropiness, or the formation of undesirable metabolites such as ethyl carbamate and biogenic amines, which can be caused by other wine-related LAB (Mills *et al*., 2005).

Owing to these observations there is a need to control MLF to enhance the positive attributes or to reduce potential negative impacts on the particular wine (Mills *et al*., 2005). Traditional culture-based techniques are often used to detect LAB in wine samples (Cho *et al*., 2011) but, especially in case of the main effector *O. oeni*, up to 14 days are required to yield results. Such long cultivation periods, however, do not allow the carrying out of possible oenological prevention or operation in wine production (Pinzani *et al*., 2004). Thus, several culture-independent methods (e.g. PCR-DGGE, qPCR) have been developed because they overcome the problems described above.

PCR-denaturing gradient gel electrophoresis (DGGE) is a commonly used culture-independent fingerprinting technique for the rapid analysis of microbial communities and has been used to analyse LAB in food (Cocolin *et al*., 2001). This technique is applied to separate a mixture of PCR amplicons of the same size but of different sequences (Ercolini, 2004). Double-stranded PCR amplicons in the gel are subjected to an increasingly denaturing environment. The migration is stopped when the DNA fragments are completely denatured (Renouf *et al*., 2007), yielding patterns that visualise the genetic diversity of the investigated microbial community (Ercolini, 2004).

Implementing a new method in the own laboratory always requires intensive literature research. In terms of PCR-DGGE applied to study wine LAB microbiota, several

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different primer sets and PCR conditions have already been introduced by researchers. Of these, five primer sets were shortlisted, as they seemed to be appropriate (Lopez *et al*., 2003; Rantsiou *et al*., 2004; Endo & Okada, 2005; Bae *et al*., 2006; Renouf *et al*., 2006a; Spano *et al*., 2007). The objective of this study was to partly modify and extensively test and evaluate these primer systems regarding their suitability to monitor LAB in wine. The results of this study can be consulted to investigate the presence of LAB in wine by PCR-DGGE.

## MATERIALS AND METHODS

### Strains and growth conditions

Bacterial reference strains and their corresponding growth conditions (medium and temperature) used in this study are listed in Table 1. The strains were grown anaerobically (85% $N_2$, 10% $CO_2$, 5% $H_2$) using a MACS VA 500 micro-aerophilic workstation (Don Whitley Scientific, Shipley, U.K.).

In addition, further LAB (*Enterococcus faecalis* LMG 7937$^T$, *Enterococcus faecium* LMG 11423$^T$; *Streptococcus thermophilus* LMG 6897$^T$ and *Tetragenococcus muriaticus* LMG 18498$^T$) were included in the tests in order to evaluate the specificity of primer *Lac1o*. Except for *Tetragenococcus muriaticus*, all strains were grown on MRS medium (deMan, Rogosa, Sharpe; Merck, Darmstadt, Germany): *Streptococcus thermophilus* anaerobically at 37°C and *Enterococcus faecalis* as well as *Enterococcus faecium*

TABLE 1
LAB reference strains and growth conditions.

| Genus | Species | Subspecies | Source | Growth conditions |
|---|---|---|---|---|
| *Lactobacillus* | *brevis* | - | LMG[a] 6906$^T$ | MRS, 30°C |
| *Lactobacillus* | *buchneri* | - | LMG 6892$^T$ | MRS, 37°C |
| *Lactobacillus* | *casei* | - | LMG 6904$^T$ | MRS, 30°C |
| *Lactobacillus* | *collinoides* | - | LMG 9194$^T$ | MRS, 30°C |
| *Lactobacillus* | *coryniformis* | *torquens* | LMG 9197$^T$ | MRS, 30°C |
| *Lactobacillus* | *curvatus* | *curvatus* | LMG 9198$^T$ | MRS, 30°C |
| *Lactobacillus* | *delbrueckii* | *delbrueckii* | LMG 6412$^T$ | MRS, 37°C |
| *Lactobacillus* | *farciminis* | - | LMG 9200$^T$ | MRS, 30°C |
| *Lactobacillus* | *fermentum* | - | LMG 6902$^T$ | MRS, 37°C |
| *Lactobacillus* | *fructivorans* | - | LMG 9201$^T$ | MRS, 30°C |
| *Lactobacillus* | *hilgardii* | - | LMG 6895$^T$ | MRS, 30°C |
| *Lactobacillus* | *lindneri* | - | LMG 14528$^T$ | MRS, 30°C |
| *Lactobacillus* | *mali* | - | LMG 6899$^T$ | MRS, 30°C |
| *Lactobacillus* | *nageli* | - | LMG 21593$^T$ | MRS, 37°C |
| *Lactobacillus* | *paracasei* | *paracasei* | LMG 13087$^T$ | MRS, 30°C |
| *Lactobacillus* | *pentosus* | - | LMG 10755$^T$ | MRS, 30°C |
| *Lactobacillus* | *plantarum* | - | LMG 6907$^T$ | MRS, 30°C |
| *Lactobacillus* | *rhamnosus* | - | LMG 6400$^T$ | MRS, 37°C |
| *Lactobacillus* | *zeae* | - | LMG 17315$^T$ | MRS, 37°C |
| *Lactococcus* | *lactis* | *lactis* | LMG 6890$^T$ | MRS, 30°C |
| *Leuconostoc* | *mesenteroides* | *mesenteroides* | LMG 6893$^T$ | MRS, 30°C |
| *Oenococcus* | *oeni* | - | LMG 9851$^T$ | MLO, 30°C |
| *Pediococcus* | *acidilactici* | - | LMG 11384$^T$ | MRS, 30°C |
| *Pediococcus* | *damnosus* | - | LMG 11484$^T$ | MRS, 30°C |
| *Pediococcus* | *inopinatus* | - | LMG 11409$^T$ | MRS, 30°C |
| *Pediococcus* | *parvulus* | - | LMG 11486$^T$ | MRS, 30°C |
| *Pediococcus* | *pentosaceus* | - | LMG 11488$^T$ | MRS, 30°C |
| *Weissella* | *confusa* | - | LMG 9497$^T$ | MRS, 30°C |
| *Weissella* | *paramesenteroides* | - | LMG 9852$^T$ | MRS, 30°C |

[a]LMG: BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium

aerobically at 37°C. *Tetragenococcus muriaticus* was cultivated on GYP sodium acetate mineral salts medium with 5% sodium chloride (BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium; medium 244) under aerobic conditions at 30°C.

Isolates of the accompanying bacterial flora (*Bacillus coagulans* MSB 29W, *Gluconobacter japonicus* MSB 32W, *Gluconobacter oxydans* MSB 107W and *Acetobacter aceti* MSB 109W) grown anaerobically on MLO medium (medium for *Leuconostoc oeni*; German Collection of Microorganisms and Cell Cultures (DSMZ); medium 59) at 30°C were also included.

**Wine samples and corresponding microbial enrichment cultures**

In addition to the reference strains described above, wine samples containing an unknown variety of microorganisms were investigated. However, as the detection limit of PCR-DGGE is around $10^4$ cells/mL or even higher (Andorrà *et al*., 2008), enrichment cultures of wine samples were also used for this investigation. For this purpose, 1 mL of wine sample was inoculated in 9 mL MRS and MLO medium and incubated for seven days at 30°C under anaerobic conditions to enhance naturally occurring wine LAB.

Initially, the DNA of 16 wine samples was isolated for the investigation. As the first PCR-DGGE results of these samples displayed no or only faint bands, their enrichments were used mainly to compare the performance of the different primer systems.

**DNA extraction**

DNA extraction from 2 mL of pure or enriched cultures was performed with the Archive Pure DNA Yeast & Gram+ Kit (5 Prime, Hamburg, Germany), according to the manufacturer's instructions.

The DNA from the wine sample was extracted directly using a protocol described by Renouf *et al*. (2009), with minor modifications. In brief, microbial cells were collected from 50 mL of wine by centrifugation (4 500 x *g*, 15 min, 4°C) and the pellet was washed in 600 µL TE buffer (10 mM Tris, 1 mM EDTA). After the next centrifugation step (10 000 x *g*, 7 min, 4°C), the supernatant was discarded and the pellet resuspended in 300 µL TE buffer. Furthermore, 300 µL of sterile glass beads were added and the samples were vortexed for 10 min at 4°C. The supernatant was mixed with 300 µL cell lysis solution (5 Prime). Subsequently, 200 µL of protein precipitation solution (5 Prime) were added and mixed. Precipitation of cellular fragments was done on ice for 5 min, followed by a centrifugation step at 10 000 x *g* for 3 min at 4°C. The supernatant was then transferred to a new 1.5 mL micro-centrifuge tube already containing 100 µL of 10% polyvinyl-pyrrolidone solution (PVP, Sigma-Aldrich, St. Louis, Missouri, USA) for the elimination of tannins. After vortexing and further centrifugation (10 000 x *g,* 10 min, 4°C), the supernatant was once more transferred to a new micro-centrifuge tube containing 300 µL of isopropanol. The tube was gently mixed by inversion and centrifuged at 10 000 x *g* for 3 min at 4°C. Subsequently, the supernatant was discarded, and 300 µL of 70% ethanol were added to the pellet and mixed by inversion. A final centrifugation step (10 000 x *g*, 3 min, 4°C) followed, and the ethanol was removed carefully. The tube was dried for 15 min. To rehydrate the DNA, 25 µL TE buffer and 0.5 µL RNase (4 mg/mL) were added to the sample overnight at 4°C. The DNA of the wine samples was stored at -20°C until use.

**DNA amplification and primers**

The primer systems *WLAB1/WLAB2*$^{GC}$ (Lopez *et al*., 2003), *WBAC1/WBAC2*$^{GC}$ (Lopez *et al*., 2003), *Lac1/Lac2*$^{GC}$*/Lac3* (Walter *et al*., 2001; Endo & Okada; 2005), *Lac1/Lac1o/Lac2*$^{GC}$ (Walter *et al*., 2001; this study) and *341f*$^{GC}$*/518r* (Bae *et al*., 2006; Muyzer *et al*., 1993) were used for the amplification of fragments of the bacterial 16S ribosomal RNA (rRNA) gene (Table 2). In addition, the primer system *rpoB1/rpoB1o/rpoB2*$^{GC}$ was applied for the duplication of fragments of the RNA polymerase beta subunit, *rpoB* (Renouf *et al*., 2006b; Spano *et al*., 2007).

PCR amplification was performed at a final volume of 25 µL with a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) containing a combination of the corresponding primers and template DNA, as indicated by the authors (Muyzer *et al.*, 1993; Walter *et al*., 2001; Lopez *et al*., 2003; Spano *et al*., 2007), and 2.5 µL 10 x PCR-buffer (Finnzymes, Vantaa, Finland), 0.5 µL dNTP-Mix (10 mM), and 0.5 µL DNA polymerase (2U/µL, Dynazyme II; Finnzymes). The remaining volume was filled up with sterile distilled water.

Amplicons were run on 2% agarose gels, stained with ethidium bromide and photographed under UV trans-illumination.

**DGGE**

The Dcode universal mutation detection system™ (Bio-Rad, Hercules, California, USA) was used for the sequence-specific separation of PCR products. These were run on 8% (w/v) polyacrylamide gels in TAE-buffer (40 mM Tris-acetate; 2 mM $Na_2$-EDTA x $H_2O$, pH 8.5) and a denaturing gradient as described originally or modified according to Table 2. The electrophoresis was performed at 85 V for 16 h in 1 x TAE-buffer at a constant temperature of 60°C.

**Band-matching analysis**

Using the BioNumerics software, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium), wine-associated LAB species were identified by performing band matching. Accordingly, bands were automatically assigned to band classes defined by the program. Uncertain bands were ignored. The assignments were corrected manually, leading to an optimisation of 0% (*WBAC*, *Lac*, *rpoB* primer set) or 0.5% (*WLAB*, *341f*$^{GC}$*/518r* primer set) and a position tolerance of 0.5% (*WLAB* primer set) or 1% (*WBAC*, *Lac*, *341f*$^{GC}$*/518r*, *rpoB* primer set).

**Sequence analysis**

After staining the DGGE gel, bands of interest were excised directly from the gels with a scalpel, mixed with 100 µL of 1x PCR buffer, and incubated overnight at 4°C. Two microlitres of this solution were used to re-amplify the PCR product. The PCR products were purified with the PCRExtract Mini Kit (5 Prime) and subjected to commercial sequencing (Eurofins

MWG Operon, Ebersberg, Germany). Sequence compilation and comparison were performed with the BLASTn program.

## RESULTS AND DISCUSSION

PCR-DGGE is a fast method for bacterial analysis, enabling the survey of LAB during winemaking (Renouf *et al*., 2006b). Therefore, a selection of primer systems proposed in the literature were checked against each other by testing a set of reference strains as well as wine samples and their enrichments.

Lopez *et al*. (2003) have already shown that a number of primers are not suited, as they also amplify nonbacterial DNA, resulting in a masking of bacterial populations in DGGE profiles. They therefore developed two new primer sets specifically for the amplification of bacterial 16S rRNA gene in wine fermentation samples. One primer set, termed *WLAB1/WLAB2$^{GC}$*, amplifies LAB, while the other one, termed *WBAC1/WBAC2$^{GC}$*, amplifies LAB and acetic acid bacteria (AAB). The primer set *WLAB1/WLAB2$^{GC}$* targets the V4 and V5 regions of the 16S rRNA gene and produces a fragment of approximately 400 bp (Lopez *et al*., 2003). Pure reference cultures were examined using this primer system. It was found that several LAB species exhibited similar electrophoretic mobilities, because all amplicons were only

TABLE 2
Primers tested for PCR-DGGE.

| Primer | Sequence (5' → 3') | Target region | Reference | Modified PCR-DGGE conditions |
|---|---|---|---|---|
| *WLAB1* | TCCGGATTTATTGGGCG-TAAAGCGA | 16S rRNA gene (V4 – V5) | Lopez *et al*., 2003 | PCR – amplification: Lopez et al., 2003<br>DGGE – denaturing gradient: 30 to 55% of urea and formamide |
| | WLAB2$^{GC}$CGCCCGCCGC-GCCCCGCGCCCGGCCC-GCCGCCCCCGCCCCTC-GAATTAAACCACAT-GCTCCA | | | |
| *WBAC1* | GTCGTCAGCTCGTGTC-GTGAGA | 16S rRNA gene (V7 – V8) | Lopez *et al*., 2003 | PCR – amplification: Lopez *et al*., 2003<br>DGGE – denaturing gradient: Lopez *et al*., 2003 |
| | WBAC2$^{GC}$CGCCCGCCGC-GCCCCGCGCCCGGCCC-GCCGCCCCCGCCCCCCC-GGGAACGTATTCACCGCG | | | |
| *Lac1* | AGCAGTAGGGAATCTTC-CA | 16S rRNA gene (V3) | Walter *et al*., 2001 | PCR – amplification: Walter *et al*., 2001<br>DGGE – denaturing gradient: 35 to 55% of urea and formamide |
| | Lac2$^{GC}$CGCCCGGGGC-GCGCCCCGGGCGGCCC-GGGGGCACCGGGGGAT-TYCACCGCTACACATG | | | |
| | Lac3AGCAG-TAGGGAATCTTCGG | | Endo & Okada, 2005 | |
| | Lac1o***T***GCAG-TAGGGAAT***T***TTC***CG***[a] | | this study | |
| *341f$^{GC}$* | CGCCCGCCGC-GCGCGGCGGGC-GGGGCGGGGGCAC-GGGGGGCCTACGGGAG-GCAGCAG | 16S rRNA gene (V3) | Muyzer *et al*., 1993 | PCR – amplification: Bae *et al*., 1993, except touchdown: 0,5°C/cycle<br>DGGE – denaturing gradient: 35 to 60% of urea and formamide |
| *518r* | ATTACCGCGGCTGCTGG | | | |
| *rpoB1* | ATTGACCACTTGGGTA-ACCGTCG | *rpo* gene | Renouf *et al*., 2006b; Spano *et al*., 2007 | PCR – amplification: Spano *et al*., 2007<br>DGGE – denaturing gradient: Renouf *et al*., 2006b |
| *rpoB1o* | ATCGATCACTTAG-GCAATCGTCG | (beta-subunit) | | |
| *rpoB2$^{GC}$* | CGCCCGCCGC-GCGCGGCGGGC-GGGGCGGGGGCAC-GGGGGGGCACGATCAC-GGGTCAAACCACC | | | |

[a]Modified nucleobases (bold and italic letters)

displayed in a small range of the denaturant concentration. Varying the concentration of denaturant of the electrophoresis gel did not improve the separation of the tested reference strains. Due to several copies of the targeted gene, some species even resulted in multiple bands, complicating the allocation of bands to certain LAB species. However, all of the tested LAB could be detected and differentiated, except for *Lb. casei* and *Lb. paracasei* (Fig. 1).

Primer set *WBAC1/WBAC2*$^{GC}$ targets the V7 to V8 regions of the 16S rRNA gene and produced an approximately 320 bp amplicon with all tested reference strains. With reference to Lopez *et al*. (2003), this primer system works particularly well to resolve AAB strains on DGGE. Anyhow, according to our results, this primer pair was not capable for analysing the LAB diversity in wine, as the separation of the tested reference strains was not sufficient and many species migrated to the same position (Fig. 1). Nevertheless, it was possible to discriminate between *Lb. casei* and *Lb. paracasei*. Compared to the *WLAB* primer system, more multiple and stronger bands were obtained with the reference strains, except for *Lb. buchneri* and *Lb. fructiovorans*, which resulted in weak bands. Along with all the LAB reference strains, the *Bacillus* sp. isolate was also detected. This isolate, as well as the AAB, produced amplicons at the same gel positions as LAB.

The primer pair *Lac1/Lac2*$^{GC}$ was designed for analysing the diversity of faecal or vaginal LAB and is specific for the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc*. The primer pair forms a 340 bp fragment of the V3 region of the 16S rRNA gene (Walter *et al*., 2001). An additional primer was constructed by Endo and Okada (2005) to extend the range of detectable LAB for the investigation of fermented foods. This *Lac3* primer attaches at the same position as *Lac1* and amplifies the 16S rRNA gene of *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Vagococcus* spp. and *Tetragenococcus* spp. Testing different primer combinations, Endo and Okada (2005) observed that the use of all three primers in a PCR at the same time was useful to analyse LAB diversity. Applying the primer mixture *Lac1/Lac2*$^{GC}$*/Lac3*, the most relevant wine LAB, *O. oeni*, was not amplified (data not shown). To overcome this problem, the primer *Lac1* or *Lac3* was modified in this work (*Lac1o*, Table 2). The specificity of the new primer set *Lac1*/*Lac1o*/*Lac2*$^{GC}$ was analysed using BLASTn and evaluated by performing PCR-DGGE with reference strains and isolates of the unwanted, accompanying bacterial wine micro-flora (for details see Materials and Methods). DGGE bands were obtained for all strains of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella* and *O. oeni*. In contrast, no bands were achieved for the genera *Enterococcus, Streptococcus*, *Tetragenococcus* (data not shown) and *Lactococcus*, for which the *Lac3* primer was designed by Endo and Okada (2005). However, these genera generally are not relevant for the fermentation processes of wines. Due to the generation of multiple bands for many reference strains, the evaluation of the results was difficult. Except for *Lb. paracasei*, the identification of species of the *Lactobacillus casei* and *Lb. plantarum* group, as well as the *Pediococcus* genus, was often only possible at genus or species group level (Fig. 1), although a good separation was achieved for all other reference strains. No bands on DGGE gel were obtained for the non-LAB bacteria *Bacillus coagulans, Acetobacter aceti* and *Gluconobacter* spp. (Fig. 1).

The universal primer set *341f*$^{GC}$*/518r*, designed by Muyzer *et al*. (1993), was applied successfully by Bae *et al*. (2006) to detect LAB associated with wine grapes. It amplifies a fragment of the V3 region of the 16S rRNA gene, forming a 233 bp product. The PCR product from the *Lb. fructivorans* reference strain was weak when using this primer set without GC-clamp, and resulted in no visible band on the DGGE gel. However, the distribution of the bands of all other LAB reference strains was good, although multiple bands per strain appeared (Fig. 1). In addition, different LAB of one genus or species group (e.g. the *Pd. damnosus*, *Pd. parvulus, Pd. inopinatus, Lactobacillus casei* and *Lb. plantarum* group) showed identical results on the DGGE gel. As this primer set is universal, faint bands of AAB and the *Bacillus* isolate were displayed, but not in the concentration range of LAB.

As ribosomal genes are present in several copies with different sequences (Rantsiou *et al*., 2004), all primer systems considered generated diverse amplicons, resulting in multiple bands on the gel. Thus, another primer set targeting the RNA polymerase beta subunit gene *rpoB*, which is only present as a single copy (Rantsiou *et al*., 2004), was also included in the tests. This primer set, originally developed by Renouf *et al*. (2006a), had already been used to study the effect of different oenological practices on LAB populations and their evolution during winemaking. Based on the *rpoB1/rpoB1o/rpoB2*$^{GC}$ primer system, the bands of the reference strains were well separated within this study (Fig. 2). However, the optimally expected single band per strain appeared as a main band with weak "double bands" in its neighbourhood (Renouf *et al*., 2006a). Amongst others, these bands may be due to an enzymatic process involving the TAQ polymerase (Janse *et al*., 2004). Nevertheless, the main bands were clearly separated and visible. In the case of wine samples, the unambiguous detection of main bands could even be improved with a mixture of various species. Otherwise, reference strains of some LAB species already found in wine could not be detected beside the accompanying bacterial flora (e.g. *Lc. lactis*, *Lb. brevis*, *Lb. casei*, *Lb. coryniformis*, *Lb. curvatus*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. fructivorans*, *Lb. hilgardii*, *Lb. lindneri*, *Lb. nageli, Lb. zeae* and *Pd. inopinatus*), or generated only faint bands (e.g. *Lb. paracasei* and *Pd. parvulus*). Although Renouf *et al*. (2006a) could determine the wine-relevant species *Lb. brevis* and *Lb. hilgardii* by PCR-DGGE, we could not produce amplicons for the used reference strains of these two species in our study when applying this primer system. However, *Lb. brevis* could be identified in one of the subsequently tested samples.

As PCR-DGGE patterns obtained with the reference strains should allow the tentative identification of DNA fragments in each sample, wine-associated LAB species were identified by matching their band distances to those of the reference strains using the BioNumerics software. *O. oeni*, *Lb. brevis*, members of the *Pediococcus* genus, the *Lb. buchneri*, *Lb. casei* and *Lb. plantarum* group were

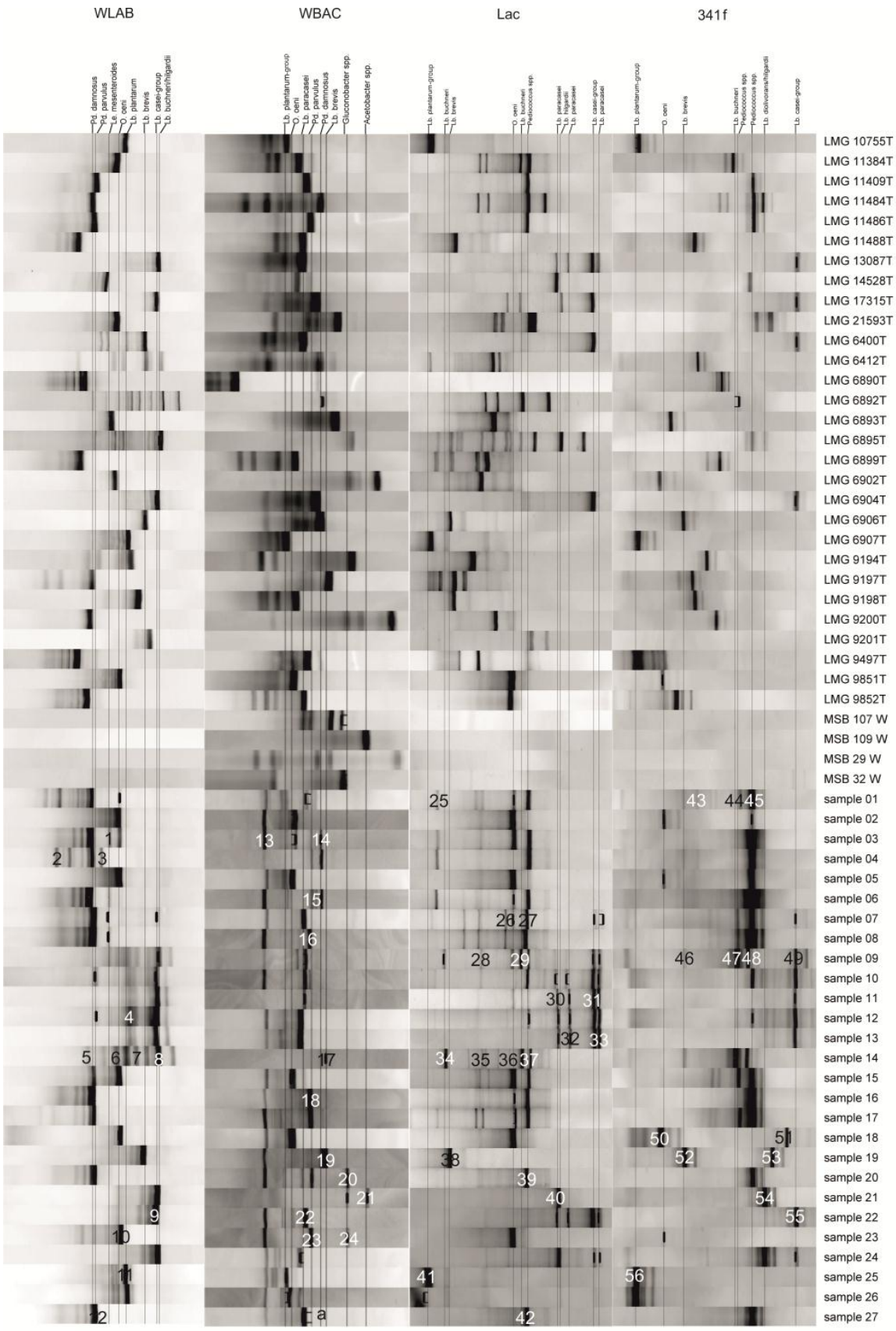


FIGURE 1

Digitised PCR-DGGE patterns of active ranges from 29 reference strains, four accompanying flora isolates, one wine sample and 26 wine enrichments with primer sets *WLAB1/WLAB2*$^{GC}$ (30% to 80%), *WBAC1/WBAC2*$^{GC}$ (45% to 75%), *Lac1/Lac1o/Lac2*$^{GC}$ (25% to 85%) and *341f*$^{GC}$*/518r* (10% to 80%). Vertical lines indicate the specified band classes. The bands labelled 1 to 56 are described in Table 4.

[a] band class assignment

TABLE 3
Detection of LAB in wine samples and/or their enrichment cultures based on band position, band pattern of species, sequencing results and comparison between different primer systems.

| Sample | Primer set | | | | |
|---|---|---|---|---|---|
| | ***WLAB1/WLAB2$^{GC}$*** | [a] ***WBAC1/WBAC2$^{GC}$*** | ***Lac1/Lac1o/Lac2$^{GC}$*** | ***341f$^{GC}$/518r*** | ***rpoB1/rpoB1o/rpoB2$^{GC}$*** |
| 1[b] | *O. oeni*, *Pd. damnosus* | *Pd. parvulus* | *O. oeni*, *Pediococcus* spp. | *Pediococcus* spp. | *O. oeni*, *Pd. damnosus* |
| 2[c] | *O. oeni* | *O. oeni* | *O. oeni* | *O. oeni*, *Pediococcus* spp. | *O. oeni* |
| 3[c] | *O. oeni, Pd. damnosus* | *O. oeni, Pd. damnosus* | *O. oeni*, *Pediococcus* spp. | *Pediococcus* spp. | *O. oeni*, *Pd. damnosus* |
| 4[c] | *Pd. damnosus* | *Pd. damnosus* | *Pediococcus* spp. | *Pediococcus* spp. | *Pd. damnosus* |
| 5[c] | *O. oeni* | *O. oeni* | *O. oeni* | *O. oeni*, *Pediococcus* spp. | *O. oeni* |
| 6[c] | *Le. mesenteroides*, *Pd. damnosus* | *Pd. damnosus* | *O. oeni, Pediococcus* spp. | *Pediococcus* spp. | *O. oeni*, *Pd. damnosus* |
| 7[c] | *Le. mesenteroides*, *Pd. parvulus, Lb. casei*-group | *Lb. paracasei* | *O. oeni, Pd. parvulus*, *Lb. paracasei* | *Pediococcus* spp., *Lb. casei*-group | *O. oeni*, *Pd. parvulus* |
| 8[c] | *Le. mesenteroides*, *Pd. parvulus* | *Pd. parvulus* | *Pediococcus* spp. | *Pediococcus* spp. | *Pd. parvulus* |
| 9[c] | *Lb. buchneri/hilgardii* | *Lb. paracasei* | *Lb. buchneri, Lb. paracasei* | *Lb. buchneri, Lb. casei*-group | *O. oeni, Lb. buchneri, Lb. rhamnosus* |
| 10[c] | *Lb. casei*-group, *Pd. parvulus* | *Lb. paracasei* | *Lb. paracasei*, *Pediococcus* spp. | *Lb. casei*-group, *Pediococcus* spp. | *O. oeni, Pd. parvulus* |
| 11[c] | *Lb. casei*-group | *Lb. paracasei* | *Lb. paracasei* | *Lb. casei*-group | *Lb. rhamnosus* |
| 12[c] | *Lb. casei*-group, *Pd. parvulus* | *Lb. paracasei* | *Lb. paracasei*, *Pediococcus* spp. | *Lb. casei*-group, *Pediococcus* spp. | *Lb. buchneri, Lb. rhamnosus* |
| 13[c] | *Lb. casei*-group | *Lb. paracasei* | *Lb. paracasei* | *Lb. casei*-group | *Lb. rhamnosus* |
| 14[c] | *Lb. buchneri* | *Lb. buchneri* | *Lb. buchneri* | *Lb.buchneri* | *Lb. buchneri* |
| 15[c] | *O. oeni*, *Pd. parvulus* | *O. oeni* | *O. oeni*, *Pediococcus* spp. | *Pediococcus* spp. | *O. oeni*, *Pd. parvulus* |
| 16[c] | *Pd. parvulus* | *Pd. parvulus* | *O. oeni*, *Pediococcus* spp. | *Pediococcus* spp. | *O. oeni*, *Pd. parvulus* |
| 17[c] | *Pd. parvulus* | *Pd. parvulus* | *O. oeni*, *Pediococcus* spp. | *Pediococcus* spp. | *Pd. parvulus, Pd. damnosus* |
| 18[c] | *O. oeni* | *O. oeni* | *O. oeni* | *O. oeni, Geminicoccus* spp. | *O. oeni* |
| 19[c] | *Lb. brevis* | *Lb. brevis* | *Lb. brevis* | *Lb. brevis, Enterobacter* spp. | *Lb. brevis* |
| 20[c] | *Pd. parvulus* | *Pd. parvulus, Gluconobacter* spp. | *Pd. parvulus* | *Pediococcus* spp. | *Pd. parvulus* |
| 21[c] | *Lb. buchneri/hilgardii* | *Acetobacter* spp., *Gluconobacter* spp. | *Lb. diolivorans* | *Lb. diolivorans/hilgardii* | *Lb. buchneri* |

TABLE 3 (CONTINUED)

| Sample | Primer set | | | | |
|---|---|---|---|---|---|
| | ***WLAB1/WLAB2$^{GC}$*** | [a] ***WBAC1/WBAC2$^{GC}$*** | ***Lac1/Lac1o/Lac2$^{GC}$*** | ***341f$^{GC}$/518r*** | ***rpoB1/rpoB1o/rpoB2$^{GC}$*** |
| 22 [c] | *Lb. casei*-group | *Lb. paracasei* | *Lb. paracasei* | *Lb. casei*-group | *Lb. paracasei* |
| 23 [c] | *O. oeni* | *O. oeni*, *Gluconobacter* spp. | *O. oeni* | *O. oeni* | - |
| 24 [c] | *Lb. buchneri/hilgardii* | *Lb. paracasei* | *Lb. diolivorans/hilgardii*, *Lb. paracasei* | *Lb. diolivorans/hilgardii*, *Lb. casei*-group | *Lb. paracasei* |
| 25 [c] | *Lb. plantarum* | *Lb. plantarum*-group | *Lb. plantarum*-group | *Lb. plantarum*-group | *Lb. plantarum* |
| 26 [c] | *Lb. plantarum* | *Lb. plantarum*-group | *Lb. plantarum*-group | *Lb. plantarum*-group | *Lb. plantarum* |
| 27 [c] | *Pd. parvulus* | *Pd. parvulus* | *Pd. parvulus* | *Pediococcus* spp. | *Pd. parvulus* |

[a] The highest band appeared in all strain mixtures (used as marker, data not shown) and samples with strong band signals. It therefore was not used for differentiation.
[b] wine sample, [c] enrichment sample




FIGURE 2
Digitised PCR-DGGE patterns of the active range (10% to 75%) from 29 reference strains, four accompanying flora isolates, one wine sample and 26 wine enrichments with primer set *rpoB1/rpoB1o/rpoB2$^{GC}$*. Vertical lines indicate the specified band classes. The bands labelled 57 to 67 are described in Table 4.
[a] band class assignment

TABLE 4
Similarities of sequenced bands.

| Primer system | Band | Closest relatives | GenBank accession no. | % sequence similarity |
|---|---|---|---|---|
| WLAB | 1, 10 | *O. oeni* | NR_075030.1 | 99%[a] |
| | 2, 3 | *Pd. damnosus* | NR_042087.1 | 99%[a] |
| | 4, 9 | *Lb. casei*, *Lb. paracasei*, *Lb. zeae* | NR_075032.1, NR_041054.1, NR_037122.1 | 99%[a] |
| | 5 - 8 | *Lb. buchneri* | NR_102772.1 | 99%[a] |
| | 11 | *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus* | NR_075041.1, NR_025447.1, NR_029133.1 | 99% |
| | 12 | *Pd. parvulus* | NR_029136.1 | 99% |
| WBAC | 13-15 | *Pd. damnosus* | NR_042087.1 | 99%[a] |
| | 16, 18 | *Pd. parvulus*, *Pd. ethanolidurans* | NR_029136.1, NR_043291.1 | 99%[a] |
| | 17 | *Lb. parabuchneri*, *Lb. buchneri* | NR_041293.1, NR_041294.1 | 99% |
| | 19 | *Lb. brevis* | NR_044704.1 | 99% |
| | 20, 24 | *Gluconobacter* spp. | NR_041047.1, NR_041050.1, NR_041049.1, NR_026118.1 | 99%[a] |
| | 21 | *Acetobacter* spp. | NR_028614.1, NR_025513.1, NR_025512.1 | 99% |
| | 22 | *Lb. paracasei*, *Lb. casei* | NR_041054.1, NR_043408.1 | 99% |
| | 23 | *O. oeni* | NR_040810.1 | 99% |
| Lac | 25 | *Pd. damnosus* | NR_042087.1 | 99% |
| | 26, 27, 39, 42 | *Pd. parvulus* | NR_029136.1 | 99%[a] |
| | 28, 29, 34-37 | *Lb. buchneri* | NR_041293.1 | 99%[a] |
| | 30 - 33 | *Lb. paracasei*, *Lb. casei*, *Lb. zeae* | NR_041054.1, NR_041893.1, NR_037122.1 | 99%[a] |
| | 38 | *Lb. brevis* | NR_044704.1 | 99% |
| | 40 | *Lb. diolivorans* | NR_037004.1 | 99% |
| | 41 | *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus* | NR_042394.1, NR_025447.1, NR_029133.1 | 99% |
| 341f | 43-45 | *Pd. parvulus*, *Pd. damnosus*, *Pd. inopinatus* | NR_029136.1, NR_042087.1, NR_025388.1 | 99%[a] |
| | 46-48 | *Lb. buchneri* | NR_041293.1 | 99%[a] |
| | 49, 55 | *Lb. rhamnosus*, *Lb. casei*, *Lb. paracasei*, *Lb. zeae* | NR_102778.1, NR_075032.1, NR_041054.1, NR_037122.1 | 97%, 99% |
| | 50 | *O. oeni* | NR_075030.1 | 100% |
| | 51 | *Geminicoccus* sp. | NR_042567.1 | 82% |
| | 52 | *Lb. brevis* | NR_075024.1 | 99% |
| | 53 | *Klebsiella* sp., *Erwinia* sp., *Enterobacter* sp. | NR_102982.1, NR_102820.1, NR_024640.1 | 100% |
| | 54 | *Lb. diolivorans*, *Lb. hilgardii* | NR_044708.2, NR_037004.1 | 99% |
| | 56 | *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus* | NR_075041.1, NR_025447.1, NR_029133.1 | 99% |
| rpoB | 57, 58 | *Pd. damnosus* | DQ176043.1 | 99%, 100% |
| | 59, 60, 64 | *O. oeni* | CP000411.1 | 98%[a] |
| | 61-63 | *Pd. parvulus* | AY875850.1 | 99%-100%[a] |
| | 65 | *Lb. brevis* | AP012167.1 | 98% |
| | 66 | *Lb. paracasei*, *Lb. casei* | CP007122.1, HE970764.1 | 99% |
| | 67 | *Lb. plantarum* | CP006033.1 | 99% |

[a] same similarity for all sequenced bands

detectable when investigating the samples using each selected primer system (Table 3). In addition to these LAB, weak bands corresponding to the species *Le. mesenteroides* were displayed by the *WLAB* set. This species, as well as the species detected by all primer sets, belong to the main LAB isolated from must and wines (Pozo-Bayón *et al*., 2009). Compared to the other primer systems used in our experiment, *O. oeni* was rarely detected by the *341f*$^{GC}$*/518r* primer set, whereas this species was frequently identified by the *Lac* and *rpoB* primer systems with specific primers for *O. oeni*. Furthermore, species of the *Lb. buchneri* group and the *Pediococcus* genus were rarely found by the *WBAC* primer set.

The detection of the diversity of species by various primer sets may be influenced by their differing affinity to different species (Bae *et al*., 2006). According to our observations, the primer pair used itself also affects the detection limit of PCR-DGGE. Thus, *O. oeni* could not or hardly be identified in sample 23 by the *rpoB* and *341f*$^{GC}$*/518r* primer sets respectively, whereas its presence was clearly detected by all other primer systems (Table 3). This detection limit even increases when competitive template DNAs are present (Andorrà *et al*., 2008). Furthermore, Bae *et al*. (2006), as well as Renouf *et al*. (2006b), concluded independently that their applied primer sets were only able to reveal the predominant species.

When applying the primer sets described above to investigate 27 wine samples and enrichment cultures, the same species were mostly detected by the *Lac1*/*Lac1o*/*Lac2*$^{GC}$ and WLAB1/WLAB2$^{GC}$ primer sets, followed by the *rpoB1/rpoB1o/rpoB2*$^{GC}$ primer systems. The biggest diversity of LAB species was also verified by these primer sets. The *WBAC1/WBAC2*$^{GC}$ and *341f*$^{GC}$*/518r* primer systems showed the poorest compliance.

Based on the sequence analysis performed for selected bands (Table 4), it turned out that the similarity of the sequences of LAB bands generated by primers targeting the 16S rRNA gene (*WLAB*, *WBAC*, *Lac* and *341f*$^{GC}$*/518r* systems) with those available in the database was ≥ 97%, whereas all tested *rpoB* sequences corresponded to database sequences with a similarity of ≥ 98%. Furthermore, sequences generated by the *rpoB* primer set were more discriminative for the identification of related LAB species than those produced by 16S rRNA gene primer systems, which is in accordance with the literature (Renouf *et al*., 2006a; Lv *et al*., 2012).

## CONCLUSIONS

Due to poor compliance with the other primer sets, the *WBAC1/WBAC2*$^{GC}$ and *341f*$^{GC}$*/518r* primer systems are not suitable to investigate the diversity of LAB involved in winemaking. In addition, multiple bands were frequently produced for the reference strains tested, complicating the allocation of a particular band to a defined species. Compared to these primer systems, the *Lac* set with the modified primer *Lac1o* exhibited slightly better performance, although multiple bands also were obtained. Due to the proper separation of different species on the gel, as well as their verification by a distinctive sequence analysis, the *rpoB1/rpoB1o/rpoB2*$^{GC}$ primer system seems to be a promising tool for monitoring the evolution of wine LAB. However, improvements should be made, as the detection limit of this set seems to be higher than that of the other primer sets. Owing to its pronounced sensitivity and its capability of discriminating to species level, the *WLAB1/WLAB2*$^{GC}$ primer set turned out to be advantageous for LAB detection purposes in wine.

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