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Review

Molecular approaches for the identification and characterisation of oenological lactic acid bacteria

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Together with yeast, lactic acid bacteria are the main microorganisms involved in winemaking. Lactic acid bacteria conduct malolactic fermentation, which is important in wines, since it changes the composition of wine, improving its organoleptic quality. In recent years, a great number of different molecular techniques for the identification and characterisation of lactic acid bacteria of oenological interest has been developed. In addition to species identification, using these techniques it is possible to know the genetic diversity of specific species and the evolution of bacteria population during winemaking. Moreover, an additional advantage is that they allow the study of wine microbiota without preliminary isolation and culture steps. In this paper, the main molecular techniques described in the literature for the detection, identification, typification and quantification of bacteria species and strains are reviewed.

Key words: Wine, lactic acid bacteria, molecular identification and characterisation.

INTRODUCTION

Winemaking is a complex microbial process involving yeasts and bacteria. Although they are both naturally present on grape skins (Renouf et al., 2007), they can also be found in barrels, tanks and the equipment used during vinification. Yeasts are predominant in wine and carry out the alcoholic fermentation, while lactic acid bacteria are responsible for malolactic fermentation (MLF). During winemaking, MLF reduces the acidity of wine (by conversion of L-malic acid into L-lactic acid) positively contributing to the microbial stability and organoleptic quality of the final product (Moreno-Arribas and Polo, 2005).

A great amount of research has been focused on the description and ecology of lactic acid bacteria in wine. In recent years, their involvement in winemaking, distribution and succession in musts, wines and during fermentation have been extensively studied (Lonvaud-Funel, 1999; Versari et al., 1999; Moreno-Arribas and Lonvaud-Funel, 2001; Liu et al., 2002; Matthews et al., 2006).

Wine lactic acid bacteria has a complex ecology and identically to many other fermented food products they experience a steady growth state during vinification. Because of the low pH, the scarce content of nutrients and the relatively high ethanol content (in the case of wine), only a limited number of bacteria species can grow and develop in grape must and wine. Table 1 shows the main lactic acid bacteria species found in grape musts and wines. Lactic acid bacteria from grape, must or wine belongs to 2 families representing 3 genera. *Lactobacillaceae* are represented by the genus *Lactobacillus* and *Streptococcaceae* are represented by *Oenococcus* and *Pediococcus. O. oeni* is the bacterial species predominating at the end of the alcoholic fermentation. This is the best adapted species to grow in difficult conditions, such as those imposed by the medium (low pH and high ethanol concentration) (Davis et al., 1986; van Vuuren and Dicks, 1993), therefore, for most wines, it is the main species responsible for malolactic fermentation.

ISOLATION AND IDENTIFICATION OF WINE LACTIC ACID BACTERIA

Most of the bacteria grown in wine can be isolated by traditional microbiological techniques, such as plating them in favourable nourishing mediums. This technique

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Table 1. Main lactic acid bacteria species isolated from musts and wines.

Genus	Species	Winemaking step employed for bacteria isolation	Some references
Pediococcus	*P. damnosus*	Must, alcoholic fermentation, wine, spoiled wine ('ropiness')	Garvie, 1986; Davis et al., 1986; Edwards and Jensen, 1992 ; Gindreau et al., 2001
	P. parvulus	Must, alcoholic fermentation, wine	
	P. pentosaceus	Must, alcoholic fermentation, wine	
Leuconostoc	*L. mesenteroides*	Grape, must, wine	Martinez-Murcia et al., 1993
Oenococcus	*O. oeni*	Grape, must, alcoholic fermentation, malolactic fermentation, oak barrel ageing	Dicks et al., 1995
Lactobacillus	*L. plantarum*	Grape,must, wine, base wine for brandy manufacture	
	L. brevis	Must, alcoholic fermentation, wine	
	L. hilgardii	Wine, alcoholic fermentation	Fornachon, 1957; Costello et al., 1983; Lafon- Lafourcade et al., 1983; Davis et al., 1986; Sieiro et al., 1990; Edwards et al., 2000; Du Plessis et al., 2004; Beneduce et al., 2004; Moreno-Arribas and Polo, 2008
	L. casei	Must, wine	
	L. paracasei	Must, wine	
	L. zeae	Biologically aged wine	
	L. vini	Wine	
	L. mali	Grape, must, wine	
	L. kunkeei	Grape, alcoholic fermentation, alcoholic fermentation in spoiled wines	
	L. lindneri	Grape	
	L. kefiri	Grape	
	L. vermiforme	Wine	
	L. trichodes	Spoiled wine	
	L. fermentum	Alcoholic fermentation	
	L. cellobiosus	Alcoholic fermentation	
	L. nageli	Alcoholic fermentation in spoiled wines	

involves a serial dilution of the wine sample in sterile physiological water (0.9% NaCl) and a further plating of the solution in specific media. Usually, anaerobic Gram-positive bacteria, which comprise most lactic acid bacteria are grown on MRS agar (de Man Rogosa and Sharpe) medium at pH 4.8. Cycloheximide 0.1% is then added to inhibit yeast growth. Plates are incubated at 30°C for 10 - 15 days. Wibowo et al. (1985) showed that the addition of tomato juice, grape juice, malic acid or different sugars to MRS medium increases bacterial growth. MRS supplemented with 10% tomato juice is the most common medium used to isolate and cultivate wine lactic acid bacteria. In order to obtain pure cultures, each colony is inoculated in liquid MRS medium and incubated at 30°C. The bacterial population obtained can then be identified through the use of traditional or molecular methods. However, plating methods can yield ambiguous results, since many bacteria have similar nutritional requirements and can grow under similar conditions.

Conventionally, wine lactic acid bacteria are identified by their morphological and biochemical characteristics. However, results can be imprecise and the methodology involved used to be very time consuming. Traditional methods for the identification of lactic acid bacteria are based on phenotypic analysis. These methods are based on the study of morphological characteristics of the cells, the nature of their metabolic products and their ability to assimilate certain substrates.

Identically, as accounting for other wine micro-organisms, in the last decade, the use of molecular biology techniques has allowed important progresses in the classification and identification of wine lactic acid bacteria. The design of DNA probes and specific primers to be used for sensitive PCR procedures have been successfully applied in oenology for the detection of desirable and undesirable strains. In the following sections the main molecular techniques described in the literature for detection, identification, typification and quantification of lactic acid bacteria species and strains from oenological origin are described. Special focus is paid on their usefulness, advantages and disadvantages. In addition, their potential application in research and/or oenological laboratories, wine cellars, service companies and administrative centres are also discussed.

MOLECULAR METHODS FOR IDENTIFICATION OF LACTIC ACID BACTERIA

Study of the genes encoding the small subunit or 16S ribosomal RNA

The 16S rRNA gene sequence has been widely used as

a molecular method to estimate phylogenetic relationships among bacteria. More recently its use has also been proposed for the identification of unknown bacteria. The 16S rRNA gene is highly conserved among bacterial species, but it has some variable zones that can be used for identification purposes. These zones can be amplified by specific primers and the sequence can be introduced in available on-line data bases. The identification is based on the similarity with other sequences within the data base. Although it is very useful and simple method for the identification of genus and species of bacteria, it does not allow differentiation of subspecies (Sato et al., 2001; du Plessis et al., 2004; Moreno-Arribas and Polo, 2008).

Nucleic acid hybridation: DNA-DNA AND DNA-RRNA

Acid nucleic hybridization techniques are important for the detection and identification of microorganisms allowing higher resolution than those based on the 16S rRNA gene sequence. From a taxonomic point of view the 16S-23S rRNA region from the bacteria genome has been proposed as a useful tool for species identification (Rodas et al., 2003, 2005). The common principle of all the techniques based on "nucleic primers" is to reveal the presence of a DNA or RNA fragments complementary to those of the primer by hybridisation. Therefore, the choice of the primer sequence is an outstanding decision, since it will determine the taxonomic level of the study. Depending on the application, there are 2 hybridization procedures, (i) methods based on DNA extraction from the strain to be identified and (ii) methods applied on the whole cells. In the first one, the restriction fragments obtained after total DNA digestion are separated by electrophoresis on agarose gels and transferred to nylon or nitrocellulose membranes for their following hybridisation with the selected DNA primers. The probes are previously marked. The use of ribosomal or other genes allows us to verify whether a specific strain belongs to species or subspecies categories by comparison of the observed polymorphism regarding to the size of the fragments from a particular region in the genome. When there is a large number of samples, a common variation of this technique, is to drop the strain DNA directly on a solid support and hybridise it with the selected probe ("*dot*"). Different authors have applied this method to identify wine lactic acid bacteria (Dicks et al., 1995; Lonvaud-Funel et al., 1989; Lonvaud-Funel et al., 1991a; Sato et al, 2001).

In the methods based on using the whole yeast, after the lyses of bacteria cells in a petri plate, the nucleic acids are fixed on a solid support and directly hybridised with DNA or RNA from a specific strain. Using specific DNA probes, this technique allows the identification of several lactic acid bacteria species present in grape must or wine simultaneously (Lonvaud-Funel et al., 1991b; Sohier and Lonvaud-Funel, 1998).

Lonvaud-Funel et al. (1989, 1991a) described a method for the identification of lactic acid bacteria during vinification and wine storage by DNA-DNA hybridisation. Genomic DNA of the strain to be identified was hybridised with total genomic DNA probes extracted from reference strains. They found that this method was particularly efficient when used for colony hybridisation to study mixed populations. In this case, at least 5 different species could be detected (Lonvaud-Funel et al., 1991b). DNA-DNA hybridisation is a method that provides greater resolution than 16S rDNA sequencing. It has been successfully employed in the identification of several wine lactic acid bacteria species in complex mixtures, however because of amount of work necessary it is not often used. Another drawback of this technique is that data on DNA homology obtained from different laboratories are often discordant because of the use of different technical approaches or because the experiments are not performed in standard conditions. Different probes based on the 16S-23S rRNA spacer region from the bacterial genome have been synthesised, allowing us to obtain useful taxonomic information, even for the identification at the species level.

A useful technique for detection and simultaneous counting of lactic acid bacterial species in wine is the Fluorescence Hybridization *in situ* technique (FISH), which is based on the design of specific species oligonucleotides marked with a fluorescence label. The Ribosomal RNA is the target for these probes. In the first step, bacterial cells are permeabilised, which allows the probe to penetrate through the bacteria wall and reach the ribosome. Once there, it will find its complementary 16s RNA sequence and bind to it. Therefore, during this step the fluorochrome will be fixed to the ribosome of the target species. Then, using a fluorescence microscope it will be possible to simultaneously detect, count and identify various types of microorganisms in a sample (Blasco et al., 2003). Figure 1 shows an example of a microscopic observation of a wine analysed by FISH. One of the main advantages of this method, is that it is very fast, because it does not require the cultivation of the sample and secondly, it can be applied to wine cellar conditions.

PCR-based methods

The fastest molecular techniques are based on FISH and PCR techniques which are mainly used to discriminate among wine lactic acid bacteria species/strains. The most commonly used techniques for the differentiation of lactic acid bacteria to species level are described later afterwards and mainly include RAPD (Random Amplified polymorphic DNA), specific PCR, ARDRA (Restriction analysis of amplified rDNA) and DGGE and TGGE (Denaturing and temperature gradient gel electrophoresis respectively)

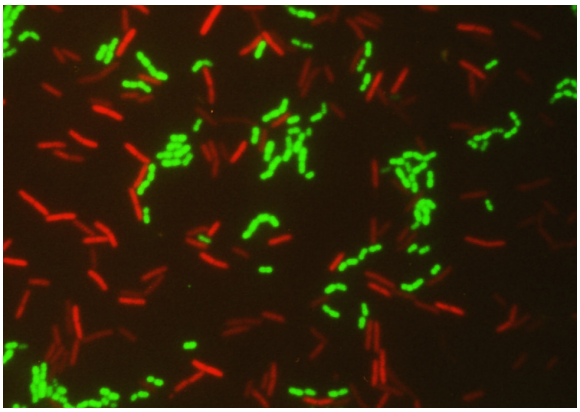

Figure 1. Microphotography of a wine analyzed by FISH, containing *Lactobacillus plantarum* (in red) detected with a rhodamine probe and *Leuconostoc mesenteroides* (in green) detected with a fluorescein probe. With permission from Moreno-Arribas et al. (2008).

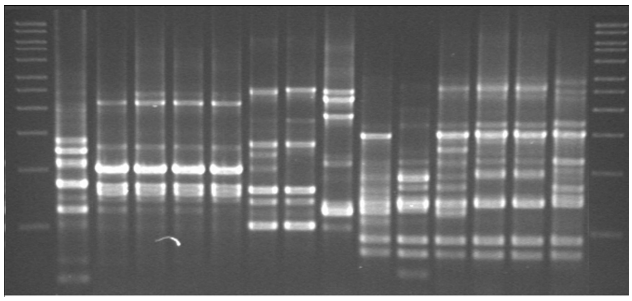

Figure 2. RAPD-PCR fingerprinting of different wine lactic acid bacteria species. With permission from Constantini et al. (2009).

RAPD: This technique has been described as a useful technique for both identification and typing (Du Plessis and Dicks, 1995; Zavaleta et al., 1997; Sohier et al., 1999; Bartowsky et al., 2003; Rodas et al., 2005). Although in RAPD fingerprints, it has been observed certain variability, a better reproducibility can be achieved under carefully controlled conditions. The main advantage of the proposed system lies in the fact that once a high reproducibility is reached, the method is fast, practical, easy to perform and inexpensive.

Molecular typing systems using RAPD and multiplex PCR have been developed for the characterization of different *O. oeni* strains that develop during wine manufacture and/or to monitor the successful implantation of malolactic starter cultures. Zavaleta et al. (1997) and Reguant and Bordons (2003) applied RAPD analysis, using different conditions, to evaluate intraspecific gene-tic diversity of *O. oeni*, and found that most strains showed unique RAPD patterns, they proposed this method as a good tool to study the population dynamics of bacteria during malolactic fermentation (Figure 2).

Species-specific primer: Bartowsky and Henschke (1999) designed specific primers to detect *O. oeni* in grape juice and wine samples. Recently, it has been developed specific primers and fluorogenic probes able to target the gene encoding the malolactic enzyme of *O. oeni* that have been used in real time PCR assays (Pinzani et al., 2004). Real time PCR is an emerging technique that allows rapid quantification of micro-organisms avoiding the plating step, this is a suitable method for monitoring fermentation and allows early and prompt corrective measures to regulate bacterial growth.

ARDRA: Restriction analysis of amplified rDNA (ARDRA) has been used to differentiate a variety of micoroganisms (Ventura et al., 2000; Rodas et al., 2003). This technique is useful as it can simplify and clarify the identification of lactobacilli. 16S-ARDRA has advantages over RAPD, for example, firstly it is less dependent on reaction conditions and the interpretation of results is easier. 16S-ARDRA generates species-specific patterns in the majority of the species studied, but it is not useful for typing purposes because the 16S rRNA gene sequence is highly conserved at the species level (Rodas et al., 2005).

DGGE: Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) have been developed to rapidly analyse microbial communities by sequence-specific separation of PCR-amplified fragments. This technique has been recently applied to evaluate the microbial diversity of several environments (Cocolin et al, 2000; Ercolini, 2004). It was also used to test the purity of bacterial strains, to monitor bacteria from environmental samples and to study the dynamics of specific populations according to environmental variations. This technique enables the separation of polymerase chain reaction amplicons of the same size but of different sequences. The amplicons in the gels are subjected to an increased denaturing environment and the migration stops when DNA fragments are completely denatured. DGGE has recently been applied to study wine microbial ecology giving an exhaustive profile of the species present in wine (Bae et al., 2006; Renouf et al., 2006, 2007, Spano et al., 2007). Some of the results reported in the literature have shown that this technique, based on the rpoB gene as a molecular marker, is a reproducible and suitable tool and may be of great value for winemakers in monitoring microorganism spoilage during wine fermentation (Spano et al., 2007).

Real-time PCR: Specific primers and fluorescent probes which detect the gene that codifies the malolactic enzyme of *O. oeni* have recently been developed and they have also been used in real time PCR trials (Pinzani et al., 2004). It is an emerging technique that allows the fast quantification of microorganisms avoiding growth step in plates. In addition, it is an adequate method to monitor

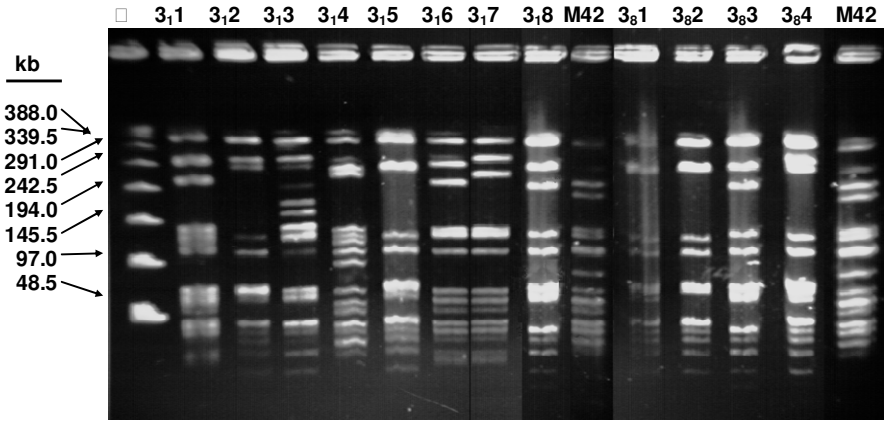

Figure 3. PFGE profiles of genomic DNA from indigenous *O. oeni* strains isolated from a wine after 3 days of inoculation with *O. oeni* M42. The genomic profile of strain M42 is shown in line 15 as a reference. Molecular weight standard: phage λ concatemers. With permission from Moreno-Arribas et al. (2008).

the progress of malolactic fermentation, allowing to undertake fast correction measurements to regulate the bacterial growth, since it is as useful for the detection as for the counting.

STRAIN IDENTIFICATION METHODS

The main objective of any microbial classification system is to identify the species level, which is the basic unit of the taxonomic grouping. Nevertheless, from an industrial point of view, the discrimination or classification of different strains or genotypes of a same species are of increasing interest. This is mainly due to the differences in transformation products (metabolites) and technological properties the strains of the same species could exhibit. One of the advantages of the use of molecular techniques is that this can permit intraspecific classification. In the oenological industry, the possibility of characterising different *O. oeni* strains has been of great importance for the selection of malolactic fermentation starter cultures.

Pulsed field gel electrophoresis (PFGE) employs restriction enzymes that digest microbial DNA, which is then subjected to electrophoretic separation. The restriction with endonuclease ApaI has been shown to be an efficient method to reveal polymorphism between *O. oeni* strains (Zapparoli et al., 2000; Pardo et al., 1998). Also, the enzymes SfiI, NotI and SmaI have been used to differentiate the intraspecific level in diverse wine *Lactobacillus* species (Rodas et al., 2005). After separation, DNA fragments are compared in order to evaluate the variability among strains belonging to the same species. This is a very useful technique, when used to study the diversity of populations during inoculated malolactic fermentations with the objective of carrying out the monitoring of the establishment of different populations and to differentiate among native and inoculated *O. oeni* strains (Gindreau et al., 1997; Pozo-Bayón et al., 2005; Hernández et al., 2007). The main disadvantage is that this technique is quite laborious and time-consuming and requires special and expensive equipment. Figure 3 shows an example of PFGE profiles of *O. oeni* wild strains.

Multilocus sequence typing (MLST) has emerged as a powerful new DNA-typing tool for the evaluation of intraspecies genetic relatedness. In MLST methods, several bacterial “housekeeping” genes are compared on the basis of the partial nucleotide sequence, all sequence types are represented by a single strain and all the strains can be distinguished from each other, because of a unique allele combination. This method has shown a high degree of intraspecies discriminatory power for bacterial and fungal pathogens. De la Rivas et al. (2003) applied this technique to discriminate *O. oeni* at the strain level, they determined the degree of allelic variation in 5 genes of *O. oeni* and showed that the % of variable sites was high, indicating a considerably high degree of genetic diversity. Therefore, MLST was demonstrated to be a powerful method to discriminate *O. oeni* at the strain level and the data obtained could be applied to study the population structure and its evolutionary mechanism.

Multiple PCR systems that use different pairs of primers to simultaneously detect lactic acid bacteria producers of histamine, tyramine and putrescine, the major wine biogenic amines have recently been published (Marcobal et al., 2005; Constantini et al., 2006). The detection of these biogenic amines producing bacteria is important in fermented foods, since these compounds can cause adverse effects in sensitive humans, but there are also economical concerns (Marcobal et al., 2006; Moreno-Arribas and Polo, 2005, 2008). Real-time PCR allows the detection of the bacteria cell number potentially producers of biogenic amines in wine and it also

constitutes a tool of interest to evaluate the risk of production of these compounds from a determined bacterial population. For example it seems that in the case of the production of histamine, the risk appears when the population of producer bacteria strains is over 10^3 cel/ml (Lucas et al., 2008).

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