Chapter 2
Biochemical Transformations Produced by Malolactic Fermentation

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2.1 Introduction

Malolactic fermentation (MLF) in wine is by definition the enzymatic conversion of L-malic acid to L-lactic acid, a secondary process which usually follows primary (alcoholic) fermentation of wine but may also occur concurrently. This reduction of malic acid to lactic acid is not a true fermentation, but rather an enzymatic reaction performed by lactic acid bacteria (LAB) after their exponential growth phase. MLF is mainly performed by *Oenococcus oeni*, a species that can withstand the low pH (<3.5), high ethanol (>10 vol.%) and high SO_2 levels (50 mg/L) found in wine. More resistant strains of *Lactobacillus*, *Leuconostoc* and *Pediococcus* can also grow in wine and contribute to MLF; especially if the wine pH exceeds 3.5 (Davis et al. 1986; Wibowo et al. 1985). The most important benefits of MLF are the deacidification of high acid wines mainly produced in cool climates, LAB contribute to wine flavour and aroma complexity and improve microbial stability (Lonvaud-Funel 1999; Moreno-Arribas and Polo 2005).

Unfortunately, uncontrolled MLF also presents a risk of wine spoilage by compounds that can produce off-flavours (including acetic acid, volatile phenols and mousiness) or that may be hazardous to human health (such as ethyl carbamate and biogenic amines). The most important aspects of the development of LAB and MLF in wines are dealt with in this chapter.

2.2 Ecology and Development of Lactic Acid Bacteria During Vinification

2.2.1 Lactic Acid Bacteria in Wine

Winemaking is a complex microbial process involving yeasts and bacteria. They are both naturally present on grape skins (Renouf et al. 2005), but are also found in barrels, tanks and the equipment used during vinification. A large amount of research has focused on the description and ecology of LAB in wine; their involvement in winemaking, their distribution and their succession in musts, in wine and during fermentation have been extensively studied.

The LAB from grape, musts or wine belong to two families representing three genera. *Lactobacillaceae* are represented by the genus *Lactobacillus*, and *Streptococcaceae* are represented by *Oenococcus* and *Pediococcus.*

2.2.1.1 *Lactobacillus*

Lactobacillus represents a highly diverse group of Gram-positive, microaerophilic bacteria; its cells are non-mobile and they have long rod-like forms or short rods (Kandler and Weiss 1986) and can appear as single cells, in pairs or in chains of different sizes. Bacteria belonging to this genus are facultative anaerobes and require a rich medium containing fermentable sugar.

They are divided into two groups in relation to their hexose metabolism:

– Strict heterofermenters (*L. brevis, L. hilgardii*)
– Facultative heterofermenters (*L. casei, L. plantarum*)

In the heterofermentative metabolism, glucose is transformed into lactic acid and other compounds such as acetic acid, ethanol and carbon dioxide, as shown in Fig. 2.1.

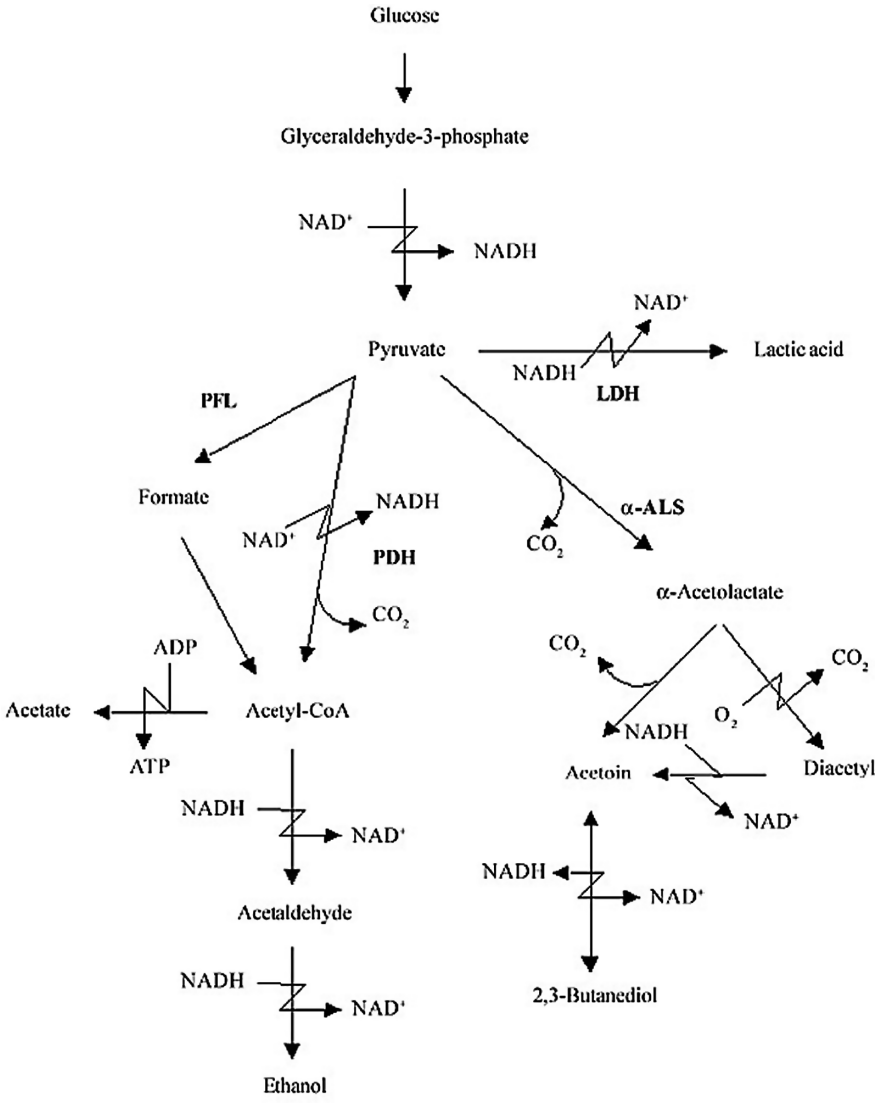

Fig. 2.1 Schematic pathway of heterofermentative metabolism. Intermediate and final glucose metabolism products are indicated by *arrows*. Catalytic enzymes are abbreviated in *bold* (LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PFL: pyruvate-formate lyase; α-ALS: acetolactate synthase) (Miyoshi et al. 2003)

There is also a third group comprised of strict homofermenters that has never been found in wine.

Several species of *Lactobacillus* have been isolated from grapes and wines worldwide, including *L. brevis*, *L. buchneri*, *L. casei*, *L. paracasei*, *L. cellobiosus*, *L. curvatus*, *L. delbrueckii*, *L. diolivorans*, *L. fructivorans*, *L. heterohiochii*, *L. hilgardii*, *L. jensenii, L. kunkeei*, *L. leichmanni*, *L. lindneri, L. mali*, *L. nagelli*, *L. paracasei, L. plantarum*, *L. trichodes, L. vermiforme*, *L. vini*, *L. yamanashiensis* and *L. zeae* (Douglas and Cruess 1936; 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).

2.2.1.2 *Pediococcus*

Cells are non-mobile and have a spherical shape; these are the only LAB that separate into two planes, which results in the formation of pairs, tetrads or large clumps of spherical cells.

Bacteria belonging to this genera are facultative anaerobes and require a rich medium containing growth factor and fermentable sugar for their development. Their optimum temperature is 25–30 °C with a pH value of 6. They are homofermentative, which means that all the glucose is metabolized into lactic acid and they do not ferment pentose.

Among the approved species of *Pediococcus* (Garvie 1986), only four have been isolated from wines: *P. damnosus*, *P. parvulus*, *P. inopinatus* and *P. pentosaceus* (Davis et al. 1986; Edwards and Jensen 1992); *P. pentosaceus* and *P. parvulus* are the most common species in this medium.

2.2.1.3 *Oenococcus*

Oenococcus oeni is described as a Gram-positive non-mobile coccus and frequently occurs in pairs and chains of different sizes (Fig. 2.2).

Oenococcus is a facultative acidophilic anaerobe and grows at pH 4.8 with temperatures between 18 °C and 30 °C. It requires a rich medium supplemented with tomato juice or grape juice, and its growth is not inhibited in the presence of 10% ethanol. Glucose is fermented in lactic acid, carbon dioxide, acetic acid and ethanol (it is a heterofermenter). It converts malate into lactate and CO_2 in the presence of fermentable carbohydrate.

Wine bacteria belonging to the genus *Oenococcus* were previously classified as *Leuconostoc oenos* by Garvie (1967) and were the only acidophilic members of the genus *Leuconostoc*. Later, phylogenetic studies revealed that *L. oenos* represented a distinct subline separate from other *Leuconostoc* spp. (Martínez-Murcia et al. 1993), and this bacterium was, finally, assigned to a new genus: *Oenococcus* (Dicks et al. 1995).

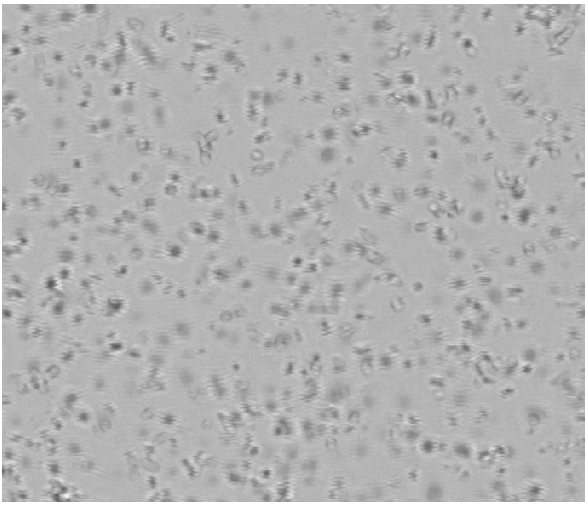

Fig. 2.2 *Oenococcus oeni* cells observed under optical microscope (CRA - Centro di ricerca per l'Enologia)

2.2.2 Development During Vinification

The growth of different microorganisms in wine tends to follow a specific order. During the harvest period, bacteria and yeasts colonize the winery. LAB are present on the grape surface and in must at very low levels, they are represented by *L. plantarum*, *L. casei, L. mesenteroides* and *O. oeni*. In the first few days of fermentation they multiply but their population is limited to levels of about 10^4 cells/mL. As alcoholic fermentation advances, these values decrease to 10^2 cells/mL: sensitivity to ethanol and low pH may explain the decline in cell population. After a lag phase, the surviving cells start multiplying and can reach populations of 10^6–10^8 cells/mL, during which stage MLF occurs (Fleet et al. 1984). This is completed when the bacteria reach a stationary phase.

O. oeni is the main species of LAB identified after primary fermentation and during MLF. Its development occurs naturally but it can be increased by raising the wine temperature to 20–25 °C and under conditions of low SO_2 (less than 15–20 mg/L "free"). After completion of MLF, other bacteria, such as *Lactobacillus* and *Pediococcus,* can take over. These stages overlap, giving rise to interactions between different types of bacteria, as well as between bacteria and yeasts.

Under standard conditions, LAB remain viable in wine during storage, exhibiting no tendency for further growth and showing only a slow progressive decline in viability over a long storage period. Carre (1982) observed a small decrease from 10^7 to 10^5cell/ml after 6 months of storage. Even if these cells do not multiply, they can metabolize some substances and produce unwanted compounds that can impair wine quality, especially due to the action of *Pediococcus* and *Lactobacillus* strains.

Wine is often a poor source of nutrients and these unfavourable conditions can make MLF very difficult. Temperature, pH, alcohol, SO_2 and nutrient availability all affect bacterial growth and activity. High and low temperatures will inhibit malolactic bacteria; high levels of alcohol and SO_2 can even kill them. Stuck or sluggish MLF may be caused by difficult conditions in the wine or by the malolactic bacteria not being able to multiply and reach the minimum population required for this process. In some cases, several weeks or months are required to obtain an appropriate number of cells able to degrade the malic acid present in red wines. Nowadays, it is becoming a common practice to directly inoculate a concentrated starter culture containing a selected malolactically-active bacterial strain in wine.

2.2.3 Microbial Interactions

2.2.3.1 Yeasts-Bacteria Interactions

The interrelationships between LAB and yeasts play an essential role during fermentation and in the final product. In complex ecosystems, the microorganisms may compete for the same substrates (Fleet 1990) or synergistically promote growth and wine is the product of these complex interactions between yeasts and bacteria. Results, however, are controversial. While some authors retain that these interactions are inhibitory, others consider them to be stimulatory.

Patynowski et al. (2002) showed that yeasts produce an unidentified inhibitory factor (maybe a toxic metabolite) that could be responsible for the inhibition of bacterial growth. These results could explain the antagonism between yeasts and malolactic bacteria, since yeasts are known to produce compounds during alcoholic fermentation such as ethanol, SO_2, medium-chain fatty acids and antibacterial proteins/peptides (Weeks et al. 1969; De Oliva et al. 2004; Comitini et al. 2005; Osborne and Edwards 2007). The nature and quantity of peptides and other molecules released by yeasts are different depending on winemaking techniques and the yeast strain.

In contrast to inhibition, in other studies these relationships have been shown to be positive for bacteria because yeasts may promote their growth and stimulate MLF. Challinor and Rose (1954) observed 13 interrelationships between yeasts and *Lactobacillus* spp. and in each of them the yeast appeared to be the active microorganism, synthesising the missing substances like vitamins, aminoacids or purine, essential for growth of the *Lactobacillus*. Kennes et al. (1991) showed that when *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were grown in co-culture in a glucose-citrate medium under acid conditions, *S. cerevisiae* reduced the lactic acid produced by *lactobacillus* and thereby stabilized pH, encouraging the fermentation of citrate by *Lactobacillus.*

2.2.3.2 Bacteria-Bacteria Interactions

LAB can synthesise compounds with metabolic activity such as H_2O_2, organic acids and bacteriocins. Several studies have been conducted on bacteriocin production;

Lonvaud-Funel and Joyeux (1993) and Strasser de Saad and Manca de Nadra (1993) tackled this problem for wine LAB and two bacteriocins were discovered:

- Brevicin, produced by *Lactobacillus brevis,* has a broad range of action and can also inhibit *O. oeni, P. damnosus* and *L. brevis*; it is a small thermostable protein of 3 KDa and can act in a wide pH range.
- Caseicin, produced by *L. casei*, has a higher molecular weight, but is less stable.

Antibacterial activity has also been observed in *P. pentosaceus* and in one strain of *L. plantarum* that strongly inhibits the growth of *O. oeni, L. mesenteroides* and *L. hilgardii.* The discovery of these molecules gives only an indication of the true situation in wine. These could be species or strain-specific, so further studies are required to understand these relationships better. Fernandez and Manca de Nadra (2006) recently studied the interaction between a proteolitic strain of *O. oeni* and a non-proteolitic strain of *P. pentosaceus* and found a mutualism in the mixed culture, providing new knowledge about the metabolic interaction between LAB.

2.3 Isolation and Identification of Wine Lactic Acid Bacteria

Most bacteria growing in wine could be isolated by traditional microbiological techniques, such as plating them on a favourable nutritious medium. This involves serially diluting the wine sample in sterile physiological water (0.9% NaCl), then each solution is plated onto a specific medium. Usually, anaerobic Gram-positive bacteria, which comprise most LAB, are grown on MRS agar (de Man Rogosa and Sharpe) medium pH 4.8; and cyclohexamide 0.1% is 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. Usually, MRS supplemented with 10% tomato juice is the medium used to isolate and cultivate wine lactic acid bacteria. In order to obtain pure cultures, each colony is inoculated in liquid medium MRS and incubated at 30°C and the bacterial population obtained can be identified with traditional or molecular methods. Plating methods can yield ambiguous results, since many bacteria have similar nutritional needs and can grow under similar conditions.

2.3.1 Traditional Methods

Traditional methods used to identify LAB are based on phenotypic analysis: these methods study the morphological characteristics of the cells, the nature of their metabolic products and their ability to assimilate certain substrates.

Morphologic characteristic can be identified using microscopy, and depending on the shape of the cells it may be possible to establish which genus they belong to; this

observation can be coupled with the Gram coloration test which verifies whether the cells studied are Gram-positive or not.

In the second step, the unidentified strain is grown in a medium containing only glucose as carbon source, after which the metabolic products are analysed:

- If the strain is a homofermenter, lactic acid will be the only metabolic product (*Pediococcus*)
- Of gas production is observed, the strain is a heterofermenter and this can be confirmed by analysing the presence of ethanol and acetic acid (*Oenococcus*, *Lactobacillus*)

The latest method that has been used to classify bacteria at species level, makes use of a system called API 50 CH (bio-Mérieux). This kit enables the genus *Lactobacillus* and related organisms to be identified. It is a ready-to-use medium which shows the fermentation profile of 49 carbohydrates (hexose, pentoses and others) on the API 50 CH strip of the microorganism to be studied. The bacterial suspension (made in a medium containing all the ingredients necessary for growth) is inoculated in each microtube of the strip. To assure anaerobiosis, the tubes are sealed with paraffin. During incubation, carbohydrates are fermented to acids, causing the pH to drop, detected by the colour change of the indicator: yellow indicating a positive character. The results make up the biochemical profile of the strain and are used in its identification or typing.

The fermentation profile is not well adapted to characterize LAB isolated from wine: bacteria are in optimal growth conditions and this does not give a true indication of the real metabolism in wine, which is influenced by environmental conditions. In general, the discriminating power is not high and several subcultures are required to obtain a stable profile. Therefore, a clear within-species identification by simple phenotypic tests may, sometimes, be difficult, and these tests are also labour-intensive and time-consuming.

2.3.2 Molecular Methods

The development of molecular techniques has opened up new perspectives for characterizing microorganisms from fermented foods and beverages. They provide outstanding tools for typing, taxonomy and evolution of bacteria in food processes (Giraffa and Neviani 2000; Germond et al. 2003).

2.3.2.1 16S rRNA Sequencing

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but recently it has also become important as a means to identify an unknown bacterium to genus or species level. This gene is highly conserved, it is amplified with specific primers and the resulting

sequence is inserted into the databases available online, and by the similarity obtained with other sequences it is possible to identify the unknown bacterium.

The sequence analysis method is very good to identify the organisms at genus and species level but it does not differentiate at the subspecies level.

2.3.2.2 G+C Content and DNA Hybridization

Estimation of the DNA nitrous base ratio (or G+C molar percent) is a classical genotypic method, constituting an integral part of a standard description of a bacterial taxon (Botina et al. 2006). These values vary from 24% to 76% among various bacteria (Schleifer and Kilpper-Balz 1987). It has been demonstrated, with high statistical significance, that among strains of a single species, the variation in the G+C ratio does not exceed 3%, compared with 10% in congeneric bacteria. LAB have a low (less than 50%) content of G+C pairs. In particular, *Oenococcus* has 38–44%; *Pediococcus* has 34–42% and *Lactobacillus* has 36–47%. This method does not allow the discrimination of species with similar GC values.

DNA-DNA hybridization is a method that provides more resolution than 16S rDNA sequencing, and has been used to describe bacterial species (Wayne et al. 1987). The 16S-23S rRNA spacer region has been suggested as a suitable region of the bacterial genome from which to derive useful taxonomic information, particularly with regard to identification at the species level (Whiley et al. 1995) and probes have been synthesized on its sequences to characterize bacterial species.

Lonvaud-Funel et al. (1989, 1991a) described the identification of LAB during vinification and wine storage by DNA-DNA hybridization. Genomic DNA of the strain to identify was hybridized with total genomic DNA probes extracted from reference strains. They found that this method was particularly efficient when used in colony hybridization to study mixed populations: at least five different species can be detected in a mixture with this system (Lonvaud-Funel et al. 1991b).

In spite of these values, the method is not popular. Major disadvantages include the laborious nature of pairwise cross-hybridizations and the impossibility of establishing a central database. Another disadvantage of the method is its high sensitivity to physiological parameters. Moreover, the data on DNA homology obtained in different laboratories are often discordant because of using different technical approaches or not complying with standard experimental conditions.

2.3.2.3 PCR-Based Methods

RAPD: This technique has been described as a useful technique for both identification and typing (Cocconcelli et al. 1995; Nigatu et al. 2001; Du Plessis and Dicks 1995; Sohier et al. 1999). Although variability has been observed in RAPD fingerprints, 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 (Rossetti and Giraffa 2005). Figure 2.3 shows an example of RAPD analysis of different LAB with primer M13 (Rossetti and Giraffa 2005).

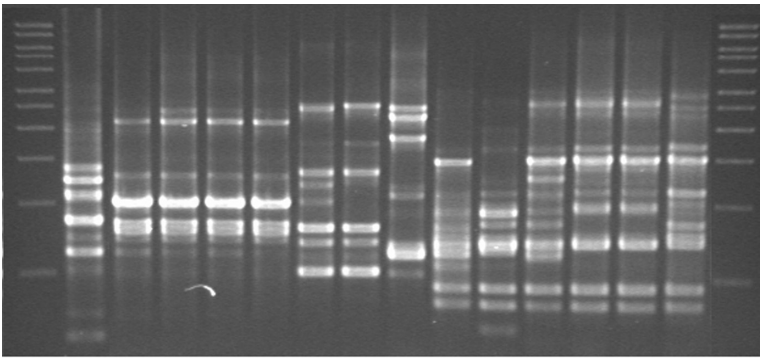

Fig. 2.3 RAPD-PCR fingerprinting of different wine lactic acid bacteria species (CRA-Istituto Sperimentale per l'Enologia)

Species-specific primer: Bartkowsky and Henschke (1999) designed specific primers to detect *O. oeni* in grape juice and wine samples. Recently, specific primers and fluorogenic probes, targeting the gene encoding malolactic enzyme of *O. oeni,* were developed and used in real time PCR assays (Pinzani et al. 2004). Real time PCR is an emerging technique that allows rapid quantification of microorganisms avoiding the plating step; this is a suitable method for monitoring fermentations 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 microorganism (Ventura et al. 2000; Rodas et al. 2003; Collado and Hernandez 2007). This technique is useful to simplify and clarify the identification of lactobacilli. 16S-ARDRA has advantages over RAPD: it is less dependent on reaction conditions and the interpretation of results is easier. 16S-ARDRA generates species-specific patterns in the majority of species studied, but 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 analyze microbial communities rapidly by sequence-specific separation of PCR-amplified fragments (Fleske et al. 1998). This technique has been recently applied to evaluate the microbial diversity of several environments (Ampe et al. 1999; Gelsomino et al. 1999; Cocolin et al. 2000; Ercolini 2004) and to "profile" complex microbial communities (Heuer et al. 1997). 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 (Tenske et al. 1996). This technique enables the separation of polymerase chain reaction amplicons of the same size but of different sequence; the amplicons in the gels are subjected to an increasingly denaturing environment; the migration is stopped when DNA fragments are completely denatured. Recently, DGGE has been applied to study wine microbial ecology giving an exhaustive profile of the species present in wine (Renouf et al. 2006, 2007). The results reported that this technique,

based on *rpoB* gene as a molecular marker, is a reproducible and suitable tool and may be of great value for winemakers to monitor spoilage microorganism during wine fermentation (Spano et al. 2007).

2.3.2.4 Strain Identification

Pulsed field gel electrophoresis (PFGE) uses restriction enzymes to digest microbial DNA, which is then subjected to electrophoretic separation (Arbeit et al. 1990; Maslow et al. 1993). The restriction with endonuclease ApaI was shown to be an efficient method to reveal polymorphism between *O. oeni* strains (Zapparoli et al. 2000). DNA fragments after separation are then compared to evaluate the variability among strains belonging to the same species. The disadvantage is that this technique is laborious and time-consuming and requires special equipment. Figure 2.4 shows an example of PFGE profiles of *O. oeni*.

Zavaleta et al. (1997) and Reguant (2003) applied RAPD analysis, using different conditions, to evaluate intraspecific genetic diversity of *O. oen*i, 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 MLF.

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 las Rivas et al. (2003) applied this technique to discriminate *O. oeni* at the strain level; they determined the degree of allelic variation in

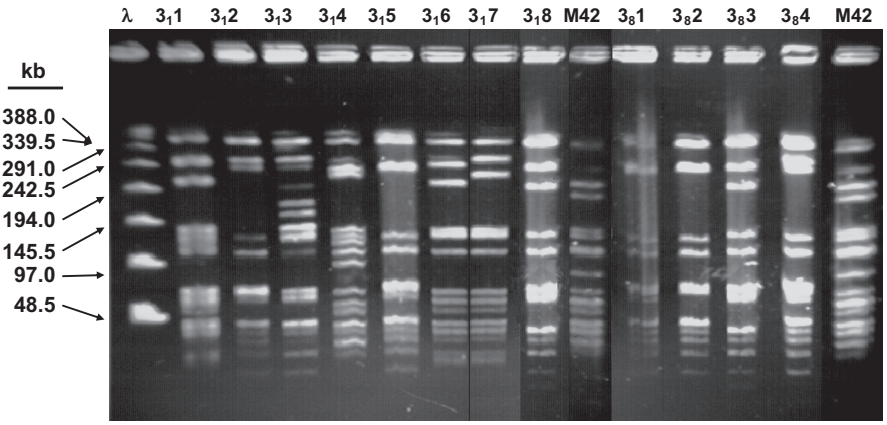

Fig. 2.4 PFGE profiles genomic DNA from indigenous *O. oeni* strains isolated from a wine after 3 days of inoculation with *O. oeni* M42. The genomic profile of strain M42 is shown in line 15 as a reference. Molecular weight standard: phage λ concatemers (From Moreno-Arribas et al. 2008b, with permission)

five genes of *O. oeni* and showed that the percentage 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.

2.4 Relevant Aspects of Lactic Acid Bacteria Metabolisms in Wines

Of all the metabolic activities that lactic acid bacteria can carry out in wine, the most important, or desirable, in winemaking is the breakdown of malic acid, but only when it is intended for this to be removed completely from the wine by malolactic fermentation. Although the breakdown of malic and citric acids has considerable consequences from a winemaking perspective, it is also evident that lactic acid bacteria metabolise other wine substrates to ensure their multiplication, including sugars, tartaric acid, glycerine and also some amino acids. We will now describe some of the metabolic transformations that have received most attention in the literature, or which have important repercussions in winemaking.

2.4.1 Carbohydrate Metabolism

Sugars are the main energy sources for bacterial growth, which tend to prefer to use glucose and trehalose more than others. However, the metabolic routes of sugars have not yet been completely elucidated for enological lactic acid bacteria, especially for *O. oeni*. Depending on the species of lactobacilli and cocci, these ferment either by glycolysis (homofermentation) or by the pentose route (heterofermentation). However, only the latter process generates acetic acid that increases the wine's volatile acidity. Nonetheless, in normal vinification, without incidences, when the LAB multiply in the medium, only sugars not fermented by yeasts remain in the medium. In general, this corresponds to hundreds of mg/L of glucose and fructose and grape must pentoses (xylose and arabinose). The residual sugars are sufficient to supply the energy required for bacterial growth and to permit the formation of biomasses that will later carry out the MLF.

Wine lactic acid bacteria can degrade polysaccharides and *O. oeni* has been shown to have an extracellular β (1→3) glucanase activity (Guilloux-Benatier et al. 2000).

2.4.2 Organic Acid Metabolism

The ability to metabolise malic and citric acids is widespread among lactic acid bacteria strains that develop after alcoholic fermentation, and can lead to a great

number and diversity of organoleptic changes. The one most studied is the breakdown of malic acid, in the phase known as "malolactic fermentation" and, more recently, citric acid breakdown, and its association with improved wine sensorial characteristics has also received attention.

2.4.2.1 Transformation of Malic Acid

This is the main reaction of MLF. Chemically it consists of a simple decarboxylation of the L-malic acid in wine into L-lactic acid. Biochemically, it is the result of activity of the malolactic enzyme, characteristic of lactic acid bacteria. This transformation has a dual effect. On the one hand, it deacidifies the wine, in other words, it raises the pH, an effect that is greater at higher initial quantities of malic acid. It also gives the wine a smoother taste, replacing the acidic and astringent flavour of the malic acid, by the smoother flavour of the lactic acid.

This is the main reaction by which MLF causes discrete changes in the organoleptic characteristics of a wine, and is why the second fermentation is especially recommendable for most red and many white wines. The duration of this transformation of malic acid depends on the initial amount of this acid present and the total population of bacteria that have multiplied in the wine. However, for the same biomass formed, this process can be slowed down as a consequence of certain inhibitors in the wine, which have not yet been identified.

The malolactic enzyme is dimeric and is comprised of two identical subunits of 60 kDa using NAD^+ and Mn^+ as cofactors. There are numerous studies into the biochemical characteristics of this enzyme in many bacteria species, such as *L. casei* (Battermann and Radler 1991) *L. plantarum* (Schüzt and Radler 1974), *L. mesenteroides* (Lonvaud-Funel and Strasser de Saad 1982) and *O. oeni* (Naouri et al. 1990). These studies have shown that this enzyme functions via a sequentially arranged mechanism, in which the cofactors of the reaction, Mn^{2+} and NAD^+, become fixed before l-malate. Moreover, the activity is induced by the reaction of the malic acid substrate. Also, the malolactic enzyme of *O. oeni* has been genetically characterised. In the *mle* locus of *O. oeni* the malolactic operon can be found, composed of three genes, gene *mleA* that encodes the malolactic enzyme, gene *mleP* that encodes the malate-permease and the *mleR* gene, which encodes the regulator that activates transcription of the malolactic operon (Labarre et al. 1996).

2.4.2.2 Breakdown of Citric Acid

While the wine contains several g/L of L-malic acid before MLF, it usually only contains between 200 mg/L and 300 mg/L of citric acid. Although the citric acid is only present in low concentrations, it is of considerable importance. On the one hand, its metabolic pathway leads to production of acetic acid, in other words, it increases the volatile acidity of the wine. However, the most important enological significance associated with fermentation of citrate is the production of diacetyl and other acetonic compounds, which affect the wine aroma.

At low levels (5 mg/L), diacetyl is considered to add complexity to wine aroma since it can impart positive *nutty* or *caramel* characteristics, although at levels above 5 mg/L it can result in spoilage, creating an intense *buttery* or *butterscotch* flavour, and is perceived as a flaw. Microbial formation of diacetyl is a dynamic process and its concentration in wine depends on several factors: bacterial strain, pH, wine contact with lees, SO_2 content (Martineau and Henick-Kling 1995; Nielsen and Richelieu 1999). The sensory threshold for the compound can vary depending on the levels of certain wine components, such as sulfur dioxide. It can also be produced as a metabolite of citric acid when all the malic acid has been used up. However, diacetyl rarely taints wine to levels where it becomes undrinkable.

2.4.3 Metabolism of Phenolic Compounds

To date, most studies on the interactions between phenolic compounds and LAB in wines refer to the metabolism of hydroxycinnamic acids (ferulic and coumaric acids), by different bacteria species, resulting in the formation of volatile phenols (4-ethylguaiacol and 4-ethylphenol) (Cavin et al. 1993; Gury et al. 2004). These derivatives can have a significant influence on wine aroma since they are regarded as sources of phenolic off-flavors in wine, due to their characteristic aroma and low detection threshold (Cavin et al. 1993). In wines, the amounts of these compounds are generally low and are, usually, limited by the concentrations of their precursors. Hernández et al. (2006) showed that *trans*-caftaric and *trans*-coutaric acids are substrates of LAB that can exhibit cinnamoyl esterase activities during MLF, increasing the concentration of hydroxycinnamic acids. An additional source of caffeic and *p*-coumaric acids may come from the hydrolysis of cinnamoyl-glucoside anthocyanins (Moreno-Arribas et al. 2008a), as well as from other hydroxycinnamic derivatives by LAB enzymatic activity. Furthermore, according to Hernández et al. (2007), it seems that among wine LAB, this activity could be strain-dependent and could also depend on the isomeric form of the above-mentioned esters, since only the *trans*-isomers were involved in the reaction. Besides wine LAB, free phenolic acids can also be metabolized by other wine microorganisms, mainly *Brettanomyces*/*Dekkera* (Chatonnet et al. 1995) to form 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives in wine. Thus, on the basis of these observations, it can be deduced that LAB could contribute to the differences in vinylphenol levels found in wines.

2.4.4 Hydrolysis of Glycosides

The release of glycosidically-bound aroma compounds, such as monoterpenes, C_{13} norisoprenoids, and aliphatic alcohols, can be achieved by the action of glycosidase enzymes. β-Glycosidase activity has not been much studied in wine LAB. McMahon et al. (1999) detected a low β-glycosidase activity in *O. oeni* OSU and a

low activity of α-L-ramnopyranosidase in *O. oeni* Viniflora oenos. However, other authors reported significant β-glycosidase activities in different *O. oeni* strains in model systems (Grimaldi et al. 2000; Ugliano et al. 2003; D'Inceddo et al. 2004) and during red wine production (Ugliano and Moio 2006). These results suggest that the LAB of wine have the potential to hydrolyse glycoconjugates that affect wine aroma.

2.4.5 Metabolism of Amino Acids

Cysteine and methionine are metabolised by bacteria that form diverse sulphated compounds, including hydrogen sulphide and methanethiol. The metabolism of methionine has been the most studied. The LAB isolated from wine are able to degrade methionine to form methanethiol, dimethyl disulphide, 3-(methyldisulphanyl) propan-1-ol and 3-(methyldisulphanyl) propionic acid. These compounds are formed in greater quantities by *O. oeni* than *Lactobacillus*. Methanethiol and 3-(methyldisulphanyl) propan-1-ol are characterized by putrid faecal like aromas and cooked cabbage descriptors, respectively (Pripis-Nicolau et al. 2004). A reaction in wine can occur between α- dicarbonyl compounds and aminoacids, in particular cysteine; several aromas can arise, including sulphury, floral, toasted and roasted aromas. Many of the compounds produced in this way have been identified in wine, and because of their low olfactory threshold could play an important role in wine aroma and flavour.

2.4.6 Breakdown of Proteins and Peptides

The peptide fraction of an industrially manufactured red wine has been studied during MLF, and it was found that wine LAB have the potential to hydrolyze wine proteins (Alcaide-Hidalgo et al. 2008), although some authors have consider that this activity is not widespread among oenococci strains (Leitao et al. 2000). However, the ability of *O. oeni* to exhibit extracellular protease activity able to release peptides and free amino acids during MLF in white (Manca de Nadra et al. 1997) and red wines has also been demonstrated (Manca de Nadra et al. 1999). The oligopeptide utilization of *O. oeni* was characterized only recently (Ritt et al. 2008) and *O. oeni* was found to be able to transport oligopeptides with two to five-amino acid residues and then to hydrolyse them further.

2.5 Contribution of Malolactic Fermentation to the Organoleptic Characteristics of Wine

Different studies have focused on the biosynthesis of aroma compounds during MLF and the concomitant organoleptic consequences (Laurent et al. 1994). Maicas et al. (1999) demonstrated that MLF noticeably changes major and minor volatile

compounds which are beneficial to wine flavour. Pozo-Bayón et al. (2005) investigated the changes in volatile compounds before and after MLF, carried out by four different starter cultures of the species *Oenococcus oeni* and *Lactobacillus plantarum,* and found significant metabolic differences between both species. Aroma/ flavour attributes also seemed to vary according to the strain used for inducing MLF.

According to Henick-Kling (1993), MLF increases the fruity and buttery aromas but reduces vegetable or grassy aromas. Formation and hydrolysis of esters during MLF may also lead to an increase in the fruity aroma and it is, probably, due to the action of LAB esterases responsible for the synthesis and degradation of these compounds. However, to date there are no studies that demonstrate these changes. The reduction in vegetable or grassy aromas could be due to the catabolism of aldehydes by lactic acid bacteria. *O. oeni* can catabolise acetaldehyde, converting it into ethanol and acetate (Osborne et al. 2000).

As well as fruity and buttery aromas, MLF has also been associated with other characteristic aromas such as floral, roasted, vanilla, sweet, woody, smoked, bitter, honey, etc. (Henick-Kling 1993; Sauvageot and Vivier 1997). However, further studies are required to be able to relate the wine characteristics that are modified during malolactic fermentation with the production and/or degradation of a specific chemical compound by wine lactic acid bacteria. With this information, the winemaker can choose the best strain of lactic acid bacteria to obtain wine with a specific aroma or flavour.

In general, the change in colour of red wines after MLF corresponds to a reduced intensity with less blue tones, mainly due to the possible adsorption of anthocyanins, especially the methoxylated ones, by the bacterial cell walls, aided by the rise in pH which produces the transformation from malic to lactic acid and the decreased levels of free sulphurous anhydride (Suárez-Lepe and Íñigo-Leal 2003). Recently, new data were provided about the effect of MLF on the concentration of the phenolic compounds of red wines. The changes in four different groups of anthocyanins (simple glucosides, acetyl glucosides, cinnamoyl glucosides and pyroanthocyanins) were studied by HPLC-PAD-MS during MLF in barrel or in tank of an industrial red wine (Moreno-Arribas et al. 2008a). It was shown that the effect of the container used seems to be more important than the metabolic activity of the bacteria responsible for the process. Hydroxycinnamic acids (*trans*-caffeic and *trans-p*-coumaric) and their derivatives (*trans*-caftaric and *trans p*-coutaric acids) were the main compounds modified by MLF, independently of the use of stainless steel or barrel (Hernández et al. 2006). Taking into account that phenolic acids can act as anthocyanin copigments, stabilizing the colour of wine, higher contents of these compounds will have a positive effect on the colour.

The lactic acid bacteria may cause polysaccharides to be released in a wine (Dols-Lafalgue et al. 2007). These compounds can increase the sensation of volume or body of wines, and can also be polymerized with the grape or wood tannins, reducing sensations of roughness or astringency, and producing more complex flavours.

2.6 New Trends in the Performance of Malolactic Fermentation in Wineries

2.6.1 Use of Malolactic Starter Cultures

The induction of MLF through the use of selected starters gives some advantages: a better control of the start of fermentation, of its progress and of the strain that completes this process. In fact, the inoculum of selected bacteria, generally constituted by only one strain or mixtures of 2–3 strains of *O. Oeni,* prevents the development of bacteria belonging to the genera *Lactobacillus* and *Pediococcus*. These contaminating species can produce high concentrations of acetic acid that can impair the organoleptic quality of the wine and also substances undesirable from a health perspective such as the biogenic amines (Straub et al. 1995; Moreno-Arribas et al. 2003; Costantini et al. 2006).

In synthesis, the inoculum of selected bacteria permits:

- The beginning of MLF to be controlled: if the bacterial population has been correctly controlled at the end of alcoholic fermentation, the wine will contain few bacterial cells and spontaneous MLF could occur after weeks or even months
- Wine quality from being impaired by the development of contaminating bacteria
- The organoleptic characteristics of the wine to be selected; in fact, MLF not only represents a process of deacidification of the wine but, depending on the strain employed, it can also influence the organoleptic characteristics, preventing the production of negative secondary metabolites

Currently, different starters for MLF are commercialized as lyophilized preparations. Nevertheless, the vitality of these starters can decrease after they are inoculated in wine (Krieger et al. 1993). From the perspective of cell vitality it would, therefore, be preferable to use fresh or frozen preparations. However, these solutions are not feasible on a large scale: the fresh preparation must be produced in situ, and the frozen ones are difficult to keep, especially when they must be transported for long distances. The rehydration phase of the lyophilised cells is a delicate and important phase since it allows the cells to recover the viability required to survive in the wine (Nielsen et al. 1996). The use of a starter culture with a dilute microbial population renders the inoculum almost useless. On the other hand, although a low cell viability negatively influences the result of the inoculum, a high vitality does not always guarantee the success of MLF. A variable behaviour in wine has been shown for the different bacteria strains, reflecting their different ability to adapt and variable malolactic activity in wine (Malacrinò et al. 2003). One bacterial strain may be unable to adapt in wines with limiting chemical-physical characteristics, while another strain in the same conditions is able to adapt and to multiply.

Another important practical factor to consider in the use of starters for MLF is the possible sensitivity to bacteriophages. The sensitivity of bacteria to phages is

very different, depending on the strain. Some authors (Sozzi et al. 1982) claimed that MLF may be interrupted, delayed, or completely inhibited by the action of phage active against lactic bacteria. Other authors (Poblet-Icart et al. 1998) studied the induction bacteriophage of *O. oeni* and showed that 45% of the strains studied proved to be lysogenic, suggesting that lysogeny is widespread among bacteria isolated from wines during MLF. Since the phages of *O. oeni* are lysogenic, they concluded that they would not represent a critical problem for MLF, even if they agree with Sozzi et al. (1982) on the need to inoculate a mixture of strains with different sensitivities to phages to avoid starter culture failure and problems during MLF.

2.6.2 Time of Inoculation/Co-inoculation

The most common decision is to inoculate selected bacteria at the end of alcoholic fermentation, to avoid an excess development of LAB, which can give high quantities of acetic acid. In recent years a co-inoculum of selected yeasts and bacteria has been proposed to induce simultaneous alcoholic fermentation and MLF to increase the adaptation of LAB to wine, particularly as this concerns adaptation to a high ethanol contents. Co-inoculation at different times has been studied by some authors (Henick-Kling and Park 1994; Rosi et al. 2003; Jussier et al. 2006). In the case of co-inoculum, but also when the selected bacteria are inoculated at the end of alcoholic fermentation, the phenomenon of yeast-bacteria interaction should be considered (King and Beelman 1986; Lemaresquier 1987; Delaquis et al. 2000; Larsen et al. 2003). In recent work, Alexandre et al. (2004) studied the interactions between *Saccharomyces cerevisiae* and *Oenococcus oeni* in wine and showed that yeasts can oppose or stimulate MLF. Recently, Osborne and Edwards (2007) found that a strain of *Saccharomyces cerevisiae* could produce a peptide responsible for inhibiting MLF. A successful co-inoculum will strongly depend on the selection of suitable yeast-bacterium combinations (Alexandre et al. 2004; Jussier et al. 2006).

2.6.3 Malolactic fermentation in Barrels/Microoxygenation

Microoxygenation is a technique that involves the addition of small and controlled quantities of oxygen to the wine. This technique is mainly used to stabilize the colour of red wines, since oxygen in small quantities favours polymerization reactions among anthocyanins and tannins (Atanasova et al. 2002). Globally, total anthocyanins decrease, but what is formed, combined with tannins, leads to a product which is more intensely coloured and more stable over time than the initial compounds.

An important reaction is that of acetaldehyde polymerization (Saucier et al. 1997; Romero and Baker 2000). This reaction not only increases the colouring intensity, but also intensifies the blue coloration (od 620 nm) that is responsible for the mauve tones in wine.

Because microoxygenation delays the beginning of MLF, this should be completed before inoculating the selected bacteria. Another reason to induce MLF after microoxygenation is because the lactic bacteria consume acetaldehyde, an essential intermediate in the polymerization reactions among phenolic compounds, as seen above.

Slow and controlled oxidation occurs in wooden containers. The use of wood in the refinement of wines furnishes volatile and non-volatile compounds, including polysaccharides and polyphenols that, together with a slow oxygenation process, help to stabilize the wine colour.

2.7 Wine Spoilage by Lactic Acid Bacteria

2.7.1 Aspects Related to the Organoleptic Quality of Wines

Besides carrying out MLF, under certain conditions LAB can also cause undesirable changes in wine flavour which render the wine undrinkable. Many species of LAB do not conduct MLF and their growth in wine can cause some serious wine spoilage. The nature and extent of this spoilage depend on several factors such as the type of bacteria, composition of the wine and vinification practices; specific types of spoilage are associated with a restricted number of lactobacilli.

These bacteria can partially metabolize more complex wine components such as phenolic compounds, aromatic compounds or aroma precursors present in small quantities and the resulting products can have important organoleptic repercussions on wine quality. Excess volatile acidity, mannitol taint, ropiness, mousiness, acrolein formation and bitterness, tartaric acid degradation, diacetyl overproduction and rancidness, as well as the very unpleasant geranium off-flavour are often the consequence of uncontrolled growth of some species of *Lactabacillus* (*e.g.*, *L. brevis*, *L. hilgardii*, *L. plantarum*), *Leuconostoc* (*e.g.*, *L. mesenteroides*), *Streptococcus* (*S. mucilaginosus*) and *Pediococcus* (*e.g.*, *P. cerevisiae*) (Pretorius, 2001).

When alcoholic fermentation is too slow or when it stops, conditions are favourable for bacterial development. LAB ferment different quantities of sugars that have not been totally fermented by yeasts and produce acetic acid and D-lactic acid. This alteration is called "*Lactic disease*" (piqûre lactique) and is characterised by a high volatile acidity that depreciates the wine. If this volatile acidity exceeds the limit of 1 g/L, the wine is unmarketable (Lonvaud-Funel 1999). This spoilage also occurs in fortified wine where *O. oeni, L. hilgardii, L. fructivorans* and L. *plantarum* are active in spite of very high ethanol contents.

The microbiological breakdown of glycerol forms acrolein, a product which causes *bitterness* in wine by binding with phenolic components (Singleton 1995). Ethanol increases the intensity of the bitter taste, as well as the duration of the bitter sensation (Noble 1994). An increased alcohol concentration resulted in an increase in the bitter sensation (Fischer and Noble 1994). *Lactobacillus brevis* and *L. buchneri*, isolated from spoiled wine, can metabolize glycerol in the presence of

glucose or fructose, resulting in the formation of 3-hydroxy propanal (also known as 3-hydroxy propionaldehyde, 3-HPA), which is subsequently reduced to 1,3-propanediol (Schutz and Radler 1984a,b). 3-Hydroxy propionaldehyde is a precursor of acrolein. The conversion of glycerol to 3-HPA in co-metabolism with glucose or fructose is not restricted to wine lactobacilli. *L. collinoides,* isolated from spoiled cider and fermented apple juice, can also do this (Claisse and Lonvaud-Funel 2000). Physiologically, the co-metabolism of sugar and glycerol is important to these lactobacilli, since additional ATP is generated from acetyl phosphate (Veiga-da-Cunha and Foster 1992).

Some strains of *L. brevis* cause "*mannitol taint*" by enzymatic reduction of fructose to mannitol. Mannitol is a polyol produced in heterofermentative metabolism. Its perception is often complicated as it generally exists in wine alongside other defects, but it is usually described as viscous and ester-like, combined with a sweet and irritating finish (Du Toit and Pretorius 2000). Mannitol is usually produced in wines that undergo MLF with a high level of residual sugars still present.

Tartaric acid is relatively stable to bacterial activity and can only be metabolized by some *Lactobacillus* species with the production of acetic acid, lactic acid and succinic acid (Kandler 1983). When tartaric acid is metabolised, the volatile acidity increases and the wine acquires an acetic aroma and a disagreeable taste; this degradation can be total or partial depending on the bacteria population, but it always decreases wine quality. The tartaric acid degrading capacity is restricted to only a few species: Radler And Yannissis (1972) found it in four strains of *L. plantarum* and one strain of *L. brevis*.

Several strains of LAB isolated from wine were tested for their abilities to metabolize ferulic and *p*-coumaric acids. Cavin et al. (1993) showed that these acids were strongly decarboxylated by growing cultures of *Lactobacillus brevis, Lactobacillus plantarum*, and *Pediococcus;* when decarboxylation was observed, volatile phenols (4-ethylguaiacol and 4-ethylphenol) were detected, indicating the possibility of reduction of the side chain before or after decarboxylation. Couto et al. (2006) reported *L. collinoides* as a producer of volatile phenols, although strain specificity concerning this capacity was observed. *L. mali, L. sake, L. viridescens*, and *P. acidilactici* were also found to be able to produce volatile compounds but they only perform the decarboxylation step. Volatile phenols cause animal taints such as horse sweat, wet animal and urine that are usually attributed to *Brettanomyces* spoilage.

Wine with an increased viscosity and a slimy appearance is called "*ropy*". This aspect is due to the production of dextrane or glucane produced by *Leuconostoc* and *Pediococcus* (Fugelsang 1997; Lonvaud-Funel 1999). These polysaccharides are mainly produced by *P. damnosus* and their production is linked to a plasmid of approximately 5500 bp; the ropy phenotype disappears when the plasmid is lost. These ropy strains are much more tolerant to ethanol than others. Concentrations of glucane around 100 mg/L are high enough to give the wine this abnormal viscosity.

Mousiness is a wine fault most often attributed to *Brettanomyces* but can also originate from *L. brevis*, *L. fermentum*, and *L. hilgardii* (Du Toit and Pretorius 2000). The metabolism of ornithine and lysine is associated with the formation of N-heterocycles, 2-acetyl-1-pyrrolene, 2-acetyltetrahydropyridine and

2-ethyltetrahydropyridine. These compounds are not volatile at wine pH, but in the mouth where pH is neutral they become very apparent, giving a nauseating aroma of *mouse urine*.

2.7.2 Aspects Related to the Hygienic Quality of Wines

The metabolism of amino acids does not affect the taste, but is problematic at a toxicological level, because it increases the concentrations of biogenic amines and ethyl carbamate precursors in wine.

Biogenic amines are natural compounds present in different types of foods and beverages, such as cheese, fish, beer, and wine. Histamine and tyramine, when ingested, can have adverse reactions that affect the nervous and vascular systems (Silla 1996; Bover-Cid and Holzapfel 1999). Putrescine is also potentially dangerous, because it can react with nitrites to form carcinogenic nitrosamine (Halasz et al. 1994). Biogenic amines are mainly produced by decarboxylation of the precursor amino acid through the substrate-specific enzymes of microorganisms that can be present in food. The enzymes on which most research has focused are histidine decarboxylase (HDC), which catalyzes the formation of histamine (Coton et al. 1998); tyrosine decarboxylase (TDC), which is specific for tyramine formation (Moreno-Arribas et al. 2000); and ornithine decarboxylase (ODC), which catalyzes the formation of putrescine (Marcobal et al. 2004). The production of biogenic amines in wine should be considered an important criterion in the selection of starter cultures and in the study of the characteristics of the autochthonous microflora present in the wine environment. Several papers have reported conflicting results but, in general, the presence of biogenic amines in wine is correlated with wine spoilage and, especially due to the action of different *Lactobacillus* strains (Straub et al. 1995; Moreno-Arribas et al. 2003; Costantini et al. 2006). More information about the chemical and biochemical features of the production of biogenic amines in wines is found in Chapter 6A.

Ethyl Carbamate besides malic acid, some heterofermentative wine LAB are capable of forming small amounts of citrulline from degradation of the amino acid arginine. The excretion of citrulline is of toxicological concern, since it is a precursor in the formation of carcinogenic EC (ethyl carbamate) in wine (Zimmerli And Schlatter 1991). From the results obtained, Mira de Orduña et al. (2001) concluded that the risk of citrulline formation by malolactic bacteria in wines with high residual arginine concentrations can be reduced by carrying out MLF with pure oenococcal cultures and by precisely establishing complete malolactic conversion, which must be followed by inhibition of bacterial activity.

Also, in this case, research results indicate the need for caution in the selection of starter cultures for MLF in wine, since citrulline formation from arginine degradation could result in ethyl carbamate production, even at normal temperatures, during prolonged storage. In addition, spontaneous MLF by undefined strains should be avoided, as this may lead to formation of ethyl carbamate precursors (Liu et al. 1994).

2.8 Methods for Managing Lactic Acid Bacteria Growth

In winemaking it is especially important to control MLF effectively to avoid possible bacterial alterations. On the other hand, although MLF is sometimes difficult to induce in wineries, prevention or inhibition of the growth and development of LAB in wine is also a difficult task. In practise during vinification, by adding sulphur dioxide (SO_2) LAB are eliminated after all the wine's malic acid has been degraded. SO_2 has numerous properties as a preservative in wines; these include its antioxidant, antioxidasic and selective antimicrobial effect, especially against LAB. Today this is therefore considered to be an essential treatment in winemaking and preservative technology. However, the use of this additive is strictly controlled, since high doses can cause organoleptic alterations in the final product (undesirable aromas of the sulphurous gas, or when this is reduced to hydrosulphate and mercaptanes) and, especially, owing to the risks to human health of consuming this substance. In addition, a first move to increase food safety has been taken by the EU through a legislation that regulates the use of sulphites as preservatives. Henceforth directives 2000/13/EC, 2003/89/EC and 2007/68/EC request the systematic labelling of allergens or similar incorporated in food products, including wine. Since the 25th of November 2005, the mandatory and particular mention of the presence of sulphites in foodstuffs is also required as soon as the concentration exceeds 10 mg/L or 10 mg/kg. Because of these effects, in recent years there has been a growing tendency to reduce the maximum limits permitted in musts and wines. Although as yet there is no known compound that can replace SO_2 with all its enological properties, there is great interest in the search for other preservatives, harmless to health, that can replace or at least complement the action of SO_2, making it possible to reduce its levels in wines.

With regard to products with antimicrobial activity complementary to SO_2, recently dimethyldicarbonate (DMDC) has been described as being able to inhibit the development of yeasts and LAB, permitting the dose of SO_2 to be reduced in some types of wines (Renouf et al. 2008). Other alternatives have been introduced based on "natural antimicrobial agents", of which the use of lysozyme is especially important and some antimicrobial peptides or bacteriocins (Navarro et al. 2000; Du Toit et al. 2002). Since lysozyme can cause IgE-mediated immune reactions in some individuals (Mine and Zhang 2002), its presence in food products, including wine, can cause some concern. To date, nisin is the only bacteriocin that can be obtained commercially, and although this has been shown to be effective at inhibiting the growth of spoilage bacteria in wines (Radler et al. 1990a,b; Rojo-Bezares et al. 2007), it has not been authorized for use in enology. Other bacteriocins have been described to control the growth of LAB in wine (Bauer et al. 2005).

Recently, special attention has been paid to the effect of phenolic compounds on the growth and metabolism of LAB in wine in order to establish the extent to which these compounds are involved in malolactic fermentation during winemaking (García-Ruiz et al. 2008a). It has been suggested that phenolic compounds can behave as activators or inhibitors of bacterial growth depending on their chemical structure (substitutions in the phenolic ring) and concentration (Reguant et al. 2000;

Vivas et al. 1997). Recently, the evaluation of the dual antioxidant and antibacterial activity of 21 phenolic compounds mainly present in *Vitis Vinifera L.* belonging to different groups was examined (García-Ruiz et al. 2008b). Structure-activity relationships were probed for both antimicrobial and antioxidant properties of wine phenolics, confirming the potential of these compounds as an alternative to sulphites in winemaking.

2.9 Conclusions

In the last few years, the interest of the scientists in wine LAB has increased. Currently, the enologist has more and better ways to control the activity of lactic acid bacteria and to counter their effect on the quality of the wine through a multidisciplinary and more extensive vision. Of special importance, work on the natural diversity of the species *O. oeni*, the major control of its development during the MLF, and the contribution of precise aromatic notes depending on the type of wine is likely to continue. On the other hand, another line of prominent investigation will continue focusing on greater knowledge and control of the organoleptic impact and the security of the wine, and of the development and metabolism of LAB; the new tools involving advanced analytical techniques, as well as those of molecular biology, will enable continuous progress in this field.

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