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WINE QUALITY AND MALOLACTIC FERMENTATION

PROCEEDINGS
OF

LES XVI^e ENTRETIENS SCIENTIFIQUES LALLEMAND

LALLEMAND

FOREWORD

Wine producers must have increasingly technical oenological knowledge and must stay informed about the latest innovative practices. At the *XVI*^{es} *Entretiens Scientifiques Lallemand* in Porto, the experts in malolactic bacteria presented the most recent advances in the field.

This meeting, which brought together key people from more than 15 countries on five continents, took a more practical turn this year, welcoming a dozen oenologists and winemakers to share their perspectives on the use of selected malolactic bacteria. Representatives of the wine trade also talked about how they select wines, what they look for regarding quality and stability, and how their own choices were dictated by the expectations of consumers.

Today, the principal concerns of winemakers gravitate around two main poles – how to maintain the typical characteristics of the wine while offering consumers the utmost quality. Tim Atkin, Master of Wine and wine correspondent for The Observer in the U.K., the moderator for the round table, summarized the discussions on the use of selected malolactic bacteria thus: "No one wants wine to be standardized. And even if selected bacteria have a positive influence on the taste and aroma, malolactic fermentation represents only a part of the wine producer's work, which starts much sooner, in the vineyard itself."

Having obtained a number of different points of view during the debates and wine tastings, the technical team at Lallemand can direct the company's research towards offering oenological tools that are ever more adapted to the needs of the market.

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INDIGENOUS LACTIC ACID BACTERIA AND SELECTED LACTIC ACID BACTERIA

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Grape must and wine host a very diverse microflora comprising yeast and bacteria. Due to their specific ability to grow and to survive in the medium, the different microorganisms dominate, survive or die along the winemaking process. Yeast invade the medium first as they are well adapted to multiply in grape must, and they induce alcoholic fermentation (AF). In the meantime, lactic acid bacteria (LAB) resist and adapt, more or less efficiently, to the increasing adversity of the environment. A spontaneous selection is achieved. Malolactic starters prepared from indigenous *Oenococcus oeni* strains are used for better management of malolactic fermentation (MLF).

Indigenous LAB and their evolution during winemaking

At the very early stage of AF, the LAB microflora comprises seven or eight, perhaps more, different species (Table 1). The list will probably be longer when the investigations will use the efficient new molecular methods for identification.

Table 1 List of LAB species isolated from grape must and wines.

Genera	Species
Lactobacillus	*L. casei, L. plantarum, L. hilgardii, L. brevis, L. nagelii, L. kunkei, L. diolivorans, L. fructivorans*
Pediococcus	*P. parvulus, P. damnosus, P. pentosaceus*
Leuconostoc	*L. mesenteroides*
Oenococcus	*O. oeni*

However, whatever the species, the strain level is most important for winemaking. The variability inside a species may be related to a phenotype of oenological interest (production of volatile compounds or undesirable compounds).

LAB come from grapes and the level of the initial population varies according to the environment during the last days of maturation. Humidity, temperature and UV exposure probably influence directly and/or indirectly their viability inside the complex epiphytic microflora, including yeasts and fungi. Usually a population around 10^2 to 10^4 cfu/mL is present in the grape must before AF starts. Sulphiting, which is generally done to prevent oxidation, also limits the early growth of LAB. Yeasts, which are more adapted to growth in grape must, multiply very actively and reach a high population in a few hours or one or two days, starting the AF. At the same time, LAB also transitorily increase in population, but very soon the maximum is around 10^3-10^4 cfu/mL. Figure 1 shows the evolution of both types of microorganisms inoculated at the same time in a sterile grape must. The rapid growth of yeasts

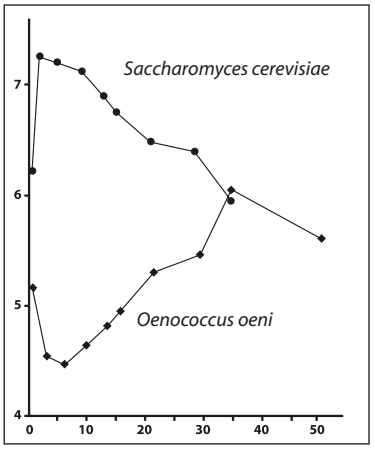


Figure 1 Evolution of *Saccharomyces cerevisiae* and *Oenococcus oeni* inoculated in a grape must.

coincides with the regression of bacteria that will grow further only during the decline phase of yeast.

Identification to the species level shows a continuous selection of the bacterial population. From up to seven or eight species identified in the grape must, the exclusive *O. oeni* species dominates at the end of AF (Table 2).

Table 2 Evolution of LAB species during alcoholic fermentation.

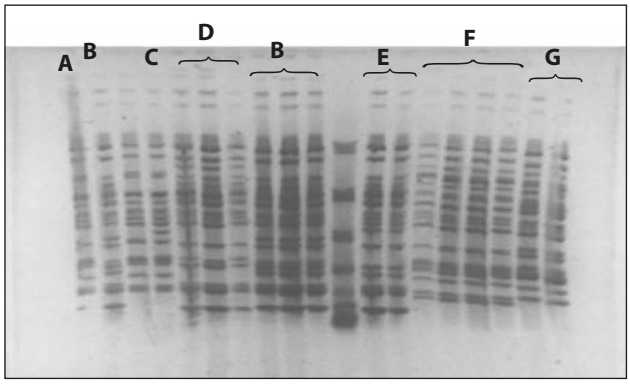
SPECIES	Days				
	0	3	6	10	18
Oenococcus oeni	**nd**	**nd**	**nd**	**4.3×10^3**	**3.4×10^6**
Leuconostoc mesenteroides	2.9×10^2	1.7×10^4	9.6×10^4	3.2×10^3	nd
Pedicoccus damnosus	6.0×10^2	3.8×10^4	3.7×10^4	4.9×10^3	nd
Lactobacillus hilgardii	1.1×10^3	8.0×10^4	4.0×10^4	4.4×10^3	nd
Lactobacillus brevis	nd	2.0×10^4	4.5×10^3	nd	nd
Lactobacillus plantarum	7.5×10^1	2.0×10^4	nd	nd	nd
Lactobacillus casei	7.7×10^1	2.0×10^4	nd	nd	nd
Total	**2.5×10^3**	**1.7×10^5**	**1.5×10^5**	**1.8×10^4**	**3.4×10^6**

nd: not detected

Selection is the result of numerous interactions between yeasts and LAB. It is also controlled by the tolerance of LAB to changing environmental conditions. As soon as the grapes are in the tank, the microflora must cope with new conditions. They are immersed in an acidic medium, very highly concentrated in sugars, and more and more deprived of oxygen. While yeasts multiply, they actively use sugars, but also amino acids and vitamins, and they accumulate in the medium products of their metabolism, e.g., ethanol, fatty acids, etc. In such conditions, LAB strains that first survived the acidity and perhaps the osmotic conditions of the grape must face other constraints due to nutritional limitation in essential amino acids and to the toxicity of yeast metabolites. The medium becomes more and more selective such that only the best strains survive. These strains will grow after yeast decline. They are predominantly *O. oeni*. At lower populations, other LAB species remain viable, but do not multiply during the winemaking; they may induce spoilage later during storage. The competition between strains of the two main wine microorganisms, *Saccharomyces cerevisiae* and *O. oeni*, results in the successive inhibition of bacteria and the acceleration of the yeast decline phase by the growing bacteria. In some conditions, especially if pH is too high, the competition does not exist, or is lessened. A higher pH favours the bacteria, but does not really influence yeasts. As a result, LAB can multiply earlier during the process, inducing or not a decrease of the AF rate or even stuck AF. Sulphiting grapes prevents this problem. Increasing toxicity and transitory nutritional limitation are the main factors of LAB inhibition by yeast, while yeast autolysis is crucial for growth of the surviving LAB. In a controlled laboratory medium, using a pure culture of *Saccharomyces cerevisiae* and *O. oeni*, it is evident that the interactions vary according to the strains. In practice, it also depends on the composition of the medium, so that laboratory findings cannot always be verified. The study of interactions between wine microorganisms is a very difficult problem to address.

When MLF starts spontaneously, the LAB population has reached 10^6 cfu/mL. *O. oeni* dominates and several strains are present. During MLF, several strains can be identified (Figure 2). This diversity results from the initial *O. oeni* microflora on grapes, from the environment and its evolution during AF, and finally from specific interactions with the dominant yeast strains. In addition, diversity is probably the best explanation for the cohabitation of *O. oeni* strains and bacteriophages without any disturbance of the MLF process.

Figure 2 Genome patterns of 18 isolates from a wine during malolactic fermentation (restriction by NotI).



Main and minor metabolisms of indigenous LAB

Malic acid degradation is always considered as the main metabolism, certainly from the winemaker's point of view. However, LAB can use numerous wine components as substrates. While a component is transformed, products accumulate in the wine and participate in the final wine composition, and therefore in the quality of the wine. Wine LAB metabolisms can be shared as: i) metabolisms common to all bacterial species and strains; ii) frequently encountered metabolisms; and iii) occasional and rare metabolisms.

L-malic acid decarboxylation seems to be a general property of all strains and species that have been isolated from wine. An exception should be strains (perhaps all species) of *Lactobacillus casei*, which in our collection did not degrade malic acid. It is catalyzed by the malolactic enzyme, distinct from the malic enzyme. The malolactic activity of a strain depends on the specific activity of the enzyme, which can be different from one strain to another. However, the strains' effectiveness in carrying out MLF differs due to their variable aptitude for growing in wine and forming a high bacterial biomass. The activity is also closely linked to the membrane integrity, which guarantees the optimum reaction conditions inside the cell. Thus only viable bacteria have the activity. Indirectly, the malolactic reaction provides energy to the bacteria through the exchange of substrate/product and protons at the membrane level.

General to LAB cells is the fermentation of sugars left by yeasts. This is the main source of energy, especially for heterofermentative bacteria which ferment glucose, fructose and pentoses. Even if they are present only in relatively low concentrations, at a total of 1 g/L or less, sugars are a significant source of energy and carbon compounds. Besides sugar and L-malic acid, the other common metabolisms of wine LAB have not yet been studied.

Citric acid and arginine metabolisms are frequently encountered. According to current knowledge, it seems that citric acid is degraded by all the heterofermentative cocci and homofermentative lactobacilli. *O. oeni* strains in general use citric acid in a complex pathway that provides energy to the cell and a carbon source for lipid synthesis. Carbon from the citric acid molecule is incorporated in the fatty acids necessary for phospholipids and membrane. Part of the pathway is funnelled to acetoinic compounds. Even if the citric acid amount is low (200-300 mg/L), it participates, with the other substrates, in the nutrition and growth of the bacteria. During MLF, its degradation is slower than L-malic acid, but it continues, even after sulphiting. Arginine, which is one of the most important amino acids in grape must, is first actively used by yeast, and then eventually released at the end of AF and during yeast autolysis. Arginine degradation is a phenotypical character used for LAB classification. For a long time, it has been used for differentiation of heterofermentative *lactobacilli*, and *O. oeni* was usually described as arginine negative. Recently, the gene system that determines the ADI pathway in *O. oeni* was identified. It involves a 10 kb region of the chromosome that is totally absent in the arginine negative strains. Recent studies show that in a collection of several dozens of *O. oeni* isolated from wines, about 74% of the strains can degrade arginine by the arginine deiminase pathway. This metabolism is involved in providing energy, as ATP is produced. Moreover, at least in the very closed environment of the cell, the increase of pH due to deamination helps deal with the acidic environment. The products of the ADI pathway are ammonium, citrulline and carbamylphosphate. This metabolism increases the concentration of ethylcarbamate precursors in wine. However, their involvement is much lower and negligible compared to urea released by yeasts.

Amino acid decarboxylation, glycerol degradation and production of exopolysaccharide, which increases wine viscosity, are much more occasional than the metabolisms mentioned above. So far, it has been shown they are not attached to a given species; they can appear in any species. Amino acid decarboxylation has been studied for histidine and tyrosine; it produces the undesirable histamine and tyramine. By a mechanism analogous to the malolactic reaction, decarboxylation provides energy at the membrane level. The genes encoding all the system for the amino acid/biogenic amine transport, and decarboxylation have been identified and sequenced for strains of *O. oeni* and *L. hilgardii*. Molecular tests show that they are present in other species. These decarboxylation pathways can be considered as a very positive option for the strains, providing additional energy sources. It is possible that such strains can remain in wine longer, when all preferential nutriments have been totally used. Like bacteria of other microbiological niches, wine LAB can degrade glycerol. There are two pathways. Glycerol can be oxidized after phosphorylation by the glycerokinase to dihydroxyacetone phosphate, which connects the glycolysis and produces pyruvate, then the usual products from the latter. The other way uses the glycerol dehydratase, which produces 1,3 propanediol. In this pathway, the 3 hydroxyproprionaldehyde is an intermediary, of which a small amount leads to acrolein by dehydratation. The genes encoding the glycerol dehydratase and other related proteins have been identified. They are used specifically to detect such bacteria in wine. Several strains belonging to *L. hilgardii* and *L. diolivorans* were isolated from wines. Strains of the first species could degrade glycerol both ways while *L. diolivorans* could degrade it only to 1,3 propanediol. Both can be involved in release of acrolein in wine, which, after combing with phenoilic compounds, induces bitterness.

Impact on sensorial quality

As a result from the metabolisms described above and all the others that are still unknown, the composition of the final wine is changed. Obviously, the main incidence of

LAB growth in wine is deacidification by the malolactic reaction. Its impact depends on the initial malic acid concentration, which can vary from 1 g/L to more than 6-7 g/L for the most acidic wines.

The quantity of citric acid degraded is much lower than malic acid. However, its incidence is significant as acetic acid and diacetyl are produced. Diacetyl, with its buttery aroma, plays a great role in the changes induced by MLF. It increases the aroma and taste complexity of wine. Its threshold level depends on the wine structure. At higher concentration, the butter flavour and aroma are not always accepted by consumers. The level of diacetyl is supposed to depend on the strain, but it is probably more dependent on the conditions of MLF, which determine the growth rate and the total population. From citric acid and other substrates, LAB release other ketonic compounds in the medium. These very reactive molecules are involved in chemical reactions with free amino acids and quickly produce very odorant compounds at acidic pH and low temperature (Table 3). Although it has not been fully established yet, it is reasonable to assume their contribution to wine aroma. Some of these molecules have a very low threshold of sensorial impact.

TABLE 3 List of components produced by chemical reactions between ketones and cystein.

Heterocycles: pyrazines, alkylpyrazines, thiazoles, etc. *sulphurous, popcorn, almond, toasted, roasted, ripe fruit*
Oxidized heterocycles: 2-furane methanethiol, thiophene-2-thiol *coffee, rubber*
Most important: formation of 2-acetylthiazol, 2-acetyl-2-thiazoline *heavy odour of popcorn, almond*

Among the numerous biochemical pathways that produce odorous compounds, the methionine metabolism is significant. The final products are low boiling point sulphurous compounds, such as methanethiol and dimethylsulphide, as well as methional, 3-methylsulphanyl propanol and 3-methylsulphanyl propionic acid, which are characterized by chocolate and roasted odours. During MLF, *O. oeni* increases their concentration, which finally exceeds their sensorial threshold. The present results do not reveal significant differences according to strains. It is necessary to evaluate the possible pertinence of this character in strain selection. However, much more work is needed to identify the biochemical pathways that change the composition of wine during MLF.

Use and selection of malolactic starters

Malolactic starters are the concentrated biomass of *O. oeni* strains selected among collections of isolates. The first and only reason for using malolactic starters in the 1980s was to force malic acid degradation when it did not occur spontaneously. The deficiency of the indigenous microflora is offset by the massive addition of a highly concentrated suspension of *O. oeni*. However, today the attitude has changed and malolactic starters are added at the time the winemaker wishes. Most often, they are still used after AF, when sugars have been fermented by yeasts. In this condition there is no risk of competition with the active yeast biomass, and therefore less risk of volatile acidity production from heterolactic fermentation. Usually added when the delay between AF and MLF becomes too long, they can also been added just after racking. If the starter is correctly implanted, the MLF is carried on by the selected strain, which will dominate the indigenous LAB. The medium is invaded by the starter and, if present, undesirable indigenous bacteria are outnumbered. Although they are not really discarded, spoilage strains cannot grow, which limits their negative impact. Moreover, like ADY (active dry yeast) for AF, it is proven that the final impact on the sensorial quality depends on the *O. oeni* strain chosen for the starter. Therefore, once knowledge on aromas produced by LAB has improved, it is expected that the winemaker will choose the starter according to the type of wine.

However, unlike with ADY, the first problem with malolactic starters is their reliability. LAB must be viable to degrade malic acid. The stress when the starter is added to wine is so great that only part of the population can survive, so sometimes the inoculation fails. The efficiency and reliability of malolactic starters has greatly improved within the past 10 years, but they are still unpredictable in the most difficult wines where they are needed.

So far, selection of *O. oeni* strains for starters has been very time-consuming work based on the isolation of bacterial collections, then several steps that focus on their adaptability to wine environments, then the sensorial impact. Tolerance to acidity and high ethanol are the most important criteria. Obviously, these selection factors cannot fully represent the very complex constraints of wine. This explains why, even when the basic conditions for temperature, pH and sulphur dioxide are optimal, starters lose their viability and activity.

Molecular biology applied to indigenous LAB and malolactic starters

The usual methods of molecular biology, such as DNA/DNA hybridization, PCR and fingerprinting, have brought new tools for wine microbiology and especially for wine LAB. It is now possible to identify at the species and the strain level the LAB isolated from grape must or wines.

These methods are routinely used in the detection of spoilage bacteria (and yeasts) during winemaking and aging, and for the final microbiological analysis before wine bottling. They are also helpful in the malolactic starter industry.

In the selection procedure for malolactic starters, the first step is the identification of isolates after cultivation on nutrient plates. *O. oeni* strains can be identified by DNA/DNA hybridization using a specific probe in colony hybridization, or by PCR after clone isolation.

Once the collection of *O. oeni* is done, each strain must be tested for its possible biogenic amine producing activity. The test is done either by DNA/DNA hybridization using DNA sequences of the amino acid decarboxylases (histidine decarboxylase or tyrosine decarboxylase), or by PCR using the corresponding primers. They can also be tested for the presence of a prophage with the same methods.

When undesirable strains have been eliminated, those that are retained for the final collection are typed. The best method for O. *oeni* is the restriction pattern obtained after digestion by the restriction enzyme NotI and pulse field gel electrophoresis. So far, it is recognized that each strain is characterized by its profile. This property is also used for controlling the efficiency of the starter during MLF. The restriction pattern of the biomass harvested in the fermenting wine is compared to the profile of the starter. It is then possible to conclude whether the strain is implanted or not.

For the moment, knowledge of the genetics of wine LAB is exploited exclusively in these applications. However, current research in the genomics of *O. oeni* should result in the future with very new approaches to strain selection. Most of the research currently done on the *O. oeni* genome is focused on the adaptation of the bacteria to the harsh conditions of wine. Of course, several mechanisms are involved in the survival and adaptation of the bacteria after inoculation. The objectives of the research are to find the genetic determinants of the cell response and to understand the cell strategy. This will lead to pertinent genetic markers which will be used for screening the collection of *O. oeni* candidates. This approach will be much more rapid and precise than the cascade of laboratory tests based on tolerance with the best known inhibitors, acidity, ethanol and sulphur dioxide. It is hypothesized that several genetic markers should be used to eliminate the less interesting strains and continue selection with a limited number, all recognized as the best for survival to the stress of inoculation.

Eventually, research on the metabolisms of wine substrates that have a significant influence on the composition should be performed, also to the molecular level. In consequence, genetic markers attached to the related biochemical pathways would be added to the others, to increase not only the reliability of starters, but also their practical interest in the elaboration of the wine quality (Table 4).

Table 4 Future research needed on *Oenococcus oeni*.

INDIGENOUS STRAINS	SELECTED STRAINS
Ecology: Evaluation of diversity in the *O. oeni* species. Relationship between diversity and technology of vine and wine.	**Improvement of adaptability:** Selection for wine tolerance – genetic markers. Optimization of industrial processes.
Physiology and metabolism: Transformation of wine components (aromas, macromolecules, polyphenols) down to the molecular level. Genetics of the main mechanisms of adaptation to wine conditions.	**Effect on sensorial quality:** Selection for production of aroma compounds or precursors – metabolisms and key genetic factors associated.

THE BUTTERY ATTRIBUTE OF WINE – DIACETYL. DESIRABILITY, SPOILAGE AND BEYOND. BUTTER OR NO BUTTER.

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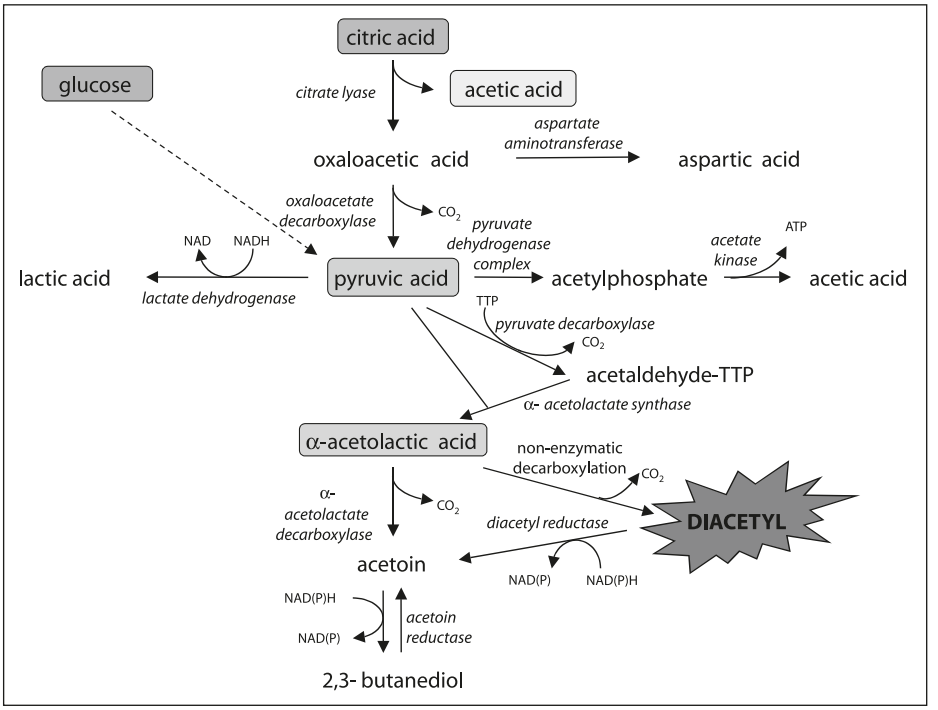
Abstract

Diacetyl is a major flavour metabolite produced by lactic acid bacteria (LAB), including the wine-associated LAB, *Oenococcus oeni*, the preferred species for inducing malolactic fermentation (MLF). Diacetyl imparts a buttery aroma and flavour to many fermented foods and beverages. In wine, diacetyl has important stylistic implications. The biosynthesis of diacetyl is dependent upon citric acid metabolism and diacetyl is an intermediate metabolite, that can be further reduced to acetoin and the polyol, 2,3-butanediol. Factors that can affect the formation and concentration of diacetyl in wine include the LAB and ML strain(s) present, wine chemical and physical parameters (pH, temperature, citric acid, sulphur dioxide, aeration) and the presence of yeast lees. By manipulating various winemaking conditions, it is possible to manage the diacetyl concentration of a wine.

Metabolism of diacetyl

The diketone, diacetyl (2,3-butanedione), is associated with the buttery character of wine and is formed as an intermediate metabolite in the reductive decarboxylation of pyruvic acid to 2,3-butanediol (Figure 1). Pyruvic acid, which when it is derived from the co-metabolism of sugar and citric acid, stimulates the formation of 2,3-butanediol. This series of reactions contributes to the redox balance of cellular metabolism. Yeasts also contribute to the diacetyl content of wine, but due to the highly reductive conditions that exist at the end of fermentation,

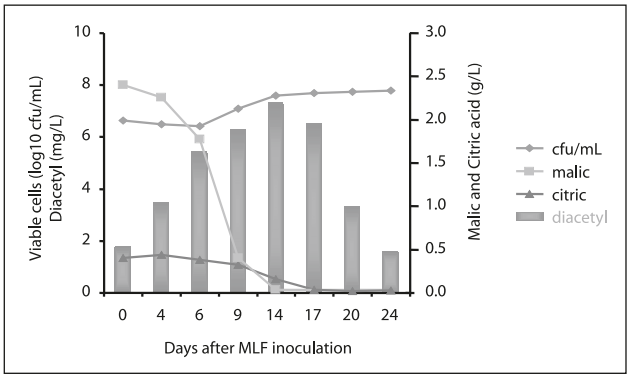
Figure 1 Pathway for citric acid metabolism by *Oenococcus oeni*.



the concentration of diacetyl is usually below its detection threshold (Martineau et al., 1995a). The amount of diacetyl, and hence the buttery attribute in wine, can therefore be regarded to be relatively unstable when microorganisms are present.

The formation and degradation of diacetyl is closely linked to the growth of ML bacteria and the metabolism of sugar, malic acid and citric acid. The relationship between some of these parameters in a laboratory scale fermentation using a 1998 Barossa Valley Cabernet Sauvignon wine inoculated with the commercial *O. oeni* strain Lalvin EQ54 is shown in Figure 2 (Bartowsky and Henschke, 2000). Due to the high inoculum of ML bacteria (~5x10^6 cfu/mL) produced by this preparation, malic acid degradation commenced immediately and the maximum rate of degradation coincided with the period of rapid bacterial growth. Citric acid metabolism commenced after approximately 25% of the malic acid had been degraded. The diacetyl content of the wine increased to a maximum when approximately 95% of the malic acid had been converted to lactic acid and 75% of the citric acid had been consumed. When all of the citric acid had been metabolized, the concentration of diacetyl had decreased from its maximum value to about twice the initial concentration. With continued incubation, the final diacetyl concentration approximated the initial concentration.

Figure 2 Malolactic fermentation in Cabernet Sauvignon wine by commercial *Oenococcus oeni* strain Lalvin EQ54. Wine composition: residual sugar < 0.1 g/L, 2.6 g/L malic acid, 0.5 g/L citric acid, 12.5% v/v ethanol, 1.9 mg/L diacetyl, pH of 3.5. The bacterial starter culture was prepared and used according to the manufacturer's recommendations.

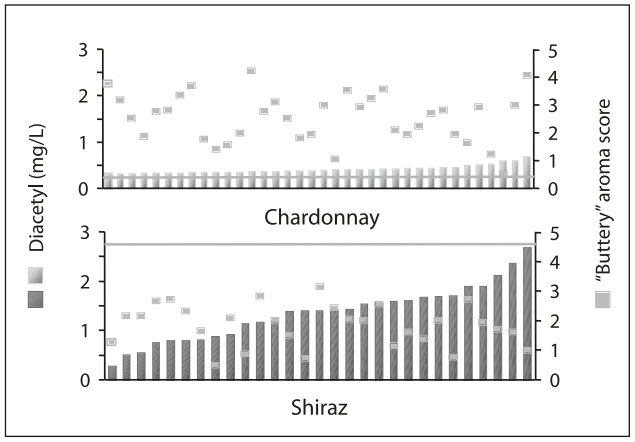


Sensory aspects of diacetyl

When present at a high concentration (exceeding 5-7 mg/L), diacetyl is regarded by many to be undesirable in the wine, whereas, at around 1-4 mg/L and depending on the style and type of wine, it is considered to contribute a desirable buttery or butterscotch flavour character (Rankine et al., 1969; Davis et al., 1985). The aroma threshold for diacetyl is dependent on the wine type and has been determined to vary from 0.2 mg/L for Chardonnay wine, 0.9 mg/L for Pinot Noir to 2.8 mg/L for Cabernet Sauvignon wine (Martineau et al., 1995a). The sensory perception of diacetyl in wine is also highly dependent upon the presence of other compounds present in the wine (Bartowsky et al., 2002a, b).

The sensory perception of a flavour compound in wine may be influenced by the chemical constituents of the wine in which it is presented. This can be demonstrated by the results of a survey of commercially finished Australian wines produced between 1992 and 1999 (36 Chardonnay and 29 Shiraz wines) (Bartowsky et al., 1997; Bartowsky et al., 2002b; Bartowsky and Henschke, 2004), in which the sensory perception of diacetyl was related to the actual concentration in wine (Hayasaka and Bartowsky, 1999). For most of the Chardonnay wines, the diacetyl content was near the sensory threshold, whereas in all of the Shiraz wines the diacetyl content was below the sensory threshold (Figure 3). From the sensory panel results, we can see that there is a range of buttery scores for wines with similar diacetyl content. The buttery aroma, while significantly correlated with diacetyl concentration in the Chardonnay wines, was not predicted very well by the regression equation, but the prediction was improved when the free sulphur dioxide concentration of the wines was taken into account. There was a weak, although statistically significant, correlation between diacetyl concentration and buttery aroma scores for the red wine data set

Figure 3 The diacetyl content and "buttery" sensory perception of Australian Chardonnay (36) and Shiraz (29) wines. The concentration of diacetyl in individual wines is ranked in order of those with the lowest to those with the highest. A trained sensory panel of 20 participants rated the "buttery" aroma score for the wines on a scale of 0 to 9 (0 indicated that the buttery attribute could not be perceived, while 9 was defined as high intensity). The line at diacetyl of 0.2 mg/L (Chardonnay) and 2.8 mg/L (Shiraz) indicates the reported sensory thresholds for diacetyl in these wines.



when the sulphur dioxide concentration was taken into account. This weak correlation may have been due, in part, to the generally low concentration of diacetyl in relation to the published aroma threshold in red wine. These wines were of varying ages, styles and from different viticultural regions, so the presence of other wine flavour compounds is likely to have contributed to or modified the sensory impact of diacetyl.

Winemaking factors that can affect the diacetyl content

A variety of factors, including some which are amenable to control by the winemaker, can greatly affect the concentration of diacetyl in wine, and are summarized in Table 1.

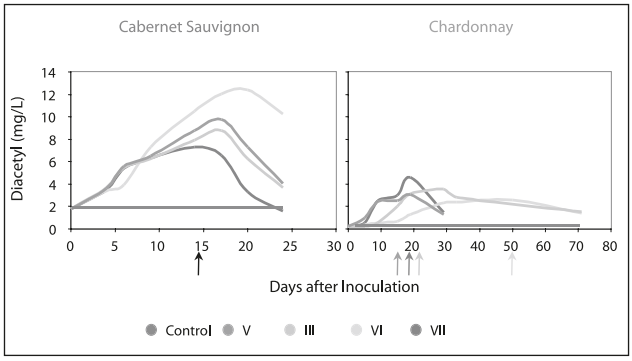
Table 1 Factors that can affect the diacetyl content of wine (adapted from Martineau et al., 1995b).

Winemaking factors	Effect on diacetyl concentration and /or sensory perception
Malolactic bacterial strain	*O. oeni* strains vary in production of diacetyl
Wine type	Red wine versus white wine favours diacetyl
Inoculation rate of malolactic bacteria	Lower inoculation rate (10^4 vs. 10^6 cfu/mL) favours diacetyl production
Contact with active yeast culture (lees)	Yeast contact reduces diacetyl content of wine
Contact of wine with air during MLF	Oxygen favours oxidation of a-acetolactate to diacetyl
Sulphur dioxide content	• SO_2 binds diacetyl, which renders it sensorily inactive • SO_2 addition inhibits yeast/bacteria activity and stabilizes diacetyl content at time of addition
Citric acid concentration	Favours diacetyl production, however, acetic acid is also produced
Temperature at which MLF is conducted	18°C vs. 25°C may favour diacetyl production
pH of wine at which MLF is conducted	Lower pH may favour diacetyl production
Fermentable sugar concentration	Conflicting information; residual sugar may reduce diacetyl production

Malolactic bacterial strain

The performance, growth and metabolism by an *O. oeni* strain are very much dependent upon the chemical composition of the wine. Nevertheless, based on research conducted under various conditions in different winegrowing regions, certain malolactic (ML) bacterial strains have been observed to produce a higher residual concentration of diacetyl in wines than other strains. In the example shown in Figure 4, the metabolism of diacetyl has been followed during and after MLF in a Cabernet Sauvignon and Chardonnay wine of similar chemical composition with four commercial *O. oeni* strains. The overall total diacetyl synthesized by the *O. oeni* strains is higher in the red wine, which has also been previously noted in the diacetyl sensory study (Figure 3). There is variation in the diacetyl peak concentration with each of the *O. oeni* strains.

Figure 4 Diacetyl metabolism by four commercial *Oenococcus oeni* strains during malolactic fermentation in Cabernet Sauvignon and Chardonnay wine with similar chemical composition (12.5-13% v/v alcohol, pH 3.5, 2.5 g/L malic acid, 0.2 g/L citric acid).The arrows indicate the time at which all the malic acid was completely metabolized.



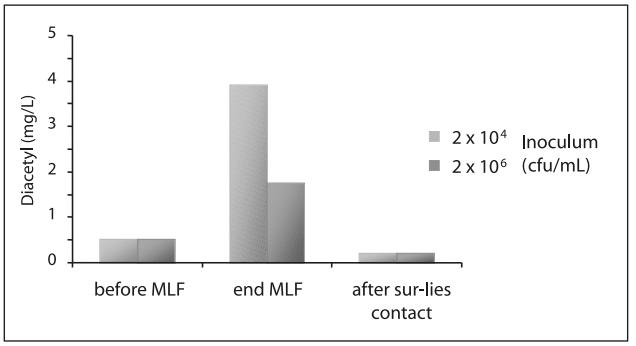
Inoculation rate of malolactic bacteria

The inoculation dosage of bacteria markedly affects the time for induction and completion of MLF. Contemporary commercial cultures typically deliver 0.5 - 5 x 10^6 cfu/mL bacteria when prepared and inoculated as recommended. However, a transient decline in the viable population often occurs upon inoculation due to delayed adaptation to the chemically harsh conditions (for example, low pH and high sulphite and ethanol concentration) of the wine. Bacterial growth may approach 10^8 cfu/mL under favourable conditions.

Significant malate degradation is not observed until the viable population reaches 0.5 - 1 x 10^6 cfu/mL, and a high rate of degradation is associated with a rapidly growing culture (Gockowiak and Henschke, 2003). It has been observed by several researchers (Lonvaud-Funel et al., 1984;

Krieger et al., 2000) that a lower inoculation rate, such as in 10^4-10^5 cfu/mL, can result in higher diacetyl accumulation in wine. Observations made from a winery trial illustrate this point with *O. oeni* strain V and a Pinot Noir wine (Figure 5). After the completion of MLF, the wine inoculated with 2 x 10^4 cfu/mL bacteria had an eight-fold increase in diacetyl, whereas the wine inoculated at 2 x 10^6 cfu/mL diacetyl only increased three-fold. The increased final diacetyl content of the wine inoculated at the lower dosage is likely to be due to the extended growth and fermentation period.

Figure 5 Diacetyl content of Pinot Noir wine with malolactic fermentation conducted by commercial *Oenococcus oeni* strain V with two different inoculation rates (adapted from Krieger et al., 2000).



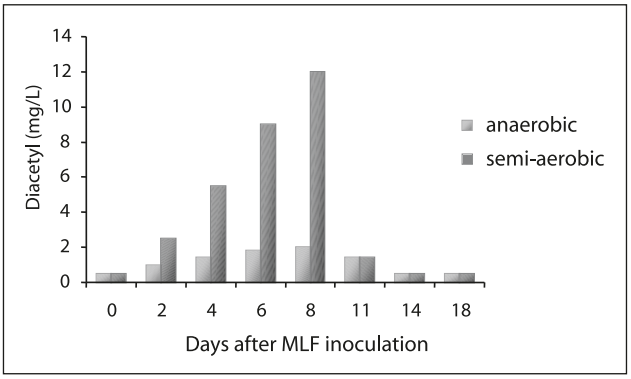
Also illustrated in this example is the impact of yeast lees and extended wine contact on diacetyl concentration. As *Saccharomyces cerevisiae* wine yeasts are capable of synthesizing diacetyl as well as degrading it, the presence of yeast lees, especially when stirred, can further reduce the wine diacetyl content.

Impact of air (oxygen) on diacetyl formation

Malolactic fermentation in wine is essentially an anaerobic process whereby wine contact with air (oxygen) is kept to a minimum, mainly to prevent oxidation of the wine and the growth of spoilage bacteria and film-forming yeasts. The conversion of α-acetolactate to diacetyl is a non-enzymatic decarboxylation (Figure 1) that is enhanced by the presence of oxygen.

The enhancing effect of a limited exposure of wine to air during MLF with *O. oeni* strain VII in a Chardonnay wine is shown in Figure 6 (Nielsen and Richelieu, 1999). Although the degradation of malic and citric acids and cell growth were not greatly affected by limited exposure to air, the amount of diacetyl that accumulated in the wine varied greatly, with the formation of 2 mg/L under anaerobic conditions and 12 mg/L under semi-aerobic conditions.

Figure 6 Effect of anaerobic and semi-aerobic conditions on diacetyl metabolism during MLF in Chardonnay wine. MLF *Oenococcus oeni* strain VII (adapted from Nielsen and Richelieu, 1999).

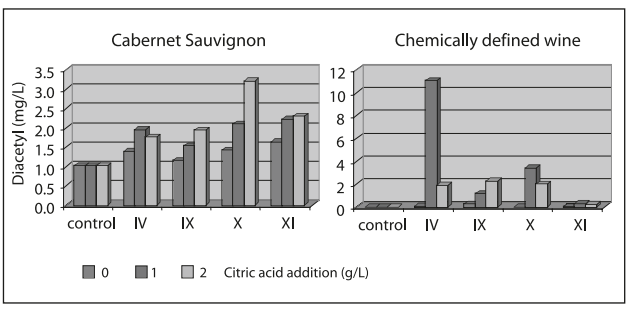


Citric acid content of wine

Citric acid, a grape-derived organic acid, is commonly present in wine in the range of 0.1-0.7 g/L and most strains of *O. oeni* are able to metabolize this acid during MLF (pathway shown in Figure 1). The metabolism of citric acid is normally delayed relative to that of malic acid, and consequently its depletion from wine may not occur until after malic acid depletion (Figure 2).

Higher peak concentrations of diacetyl generally correlate with an elevated concentration of citric acid (Figure 7). However, the magnitude of the relationship depends on many other factors. The addition of citric acid to grape must or wine for the purpose of evaluating diacetyl accumulation should be approached with caution. Even though an addition of citric acid to wine can be accompanied by an increase of diacetyl, the formation of other flavour metabolites, particularly acetic acid, can also result (Henick-Kling and Park, 1994). Citric acid addition will affect the titratable acidity (TA), and as some yeasts are also capable of metabolizing this acid unexpected changes in the concentration of diacetyl and TA could result.

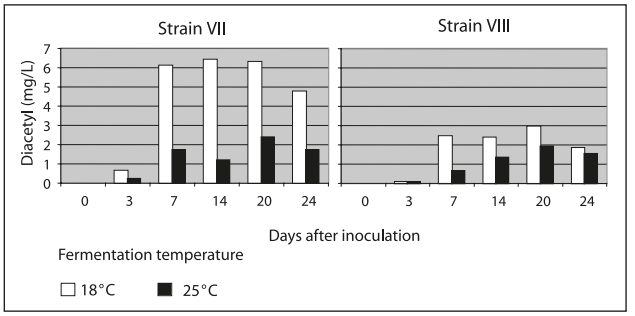
Figure 7 Effect of different concentrations of citric acid on the diacetyl content of Cabernet Sauvignon wine and chemically defined wine (10% v/v ethanol, pH 3.5) after MLF with four *O. oeni* strains (Burvill, 1996).



Temperature at which MLF is conducted

Although ML bacteria have a growth optimum temperature in laboratory media of approximately 27ºC, growth in wine is restricted to the range 15–25ºC with an optimum for most *O. oeni* cultures around 20–22ºC. MLF conducted at lower temperatures, such as 18ºC rather than 25ºC, tends to be slower, but wines accumulate a higher concentration of diacetyl (Figure 8).

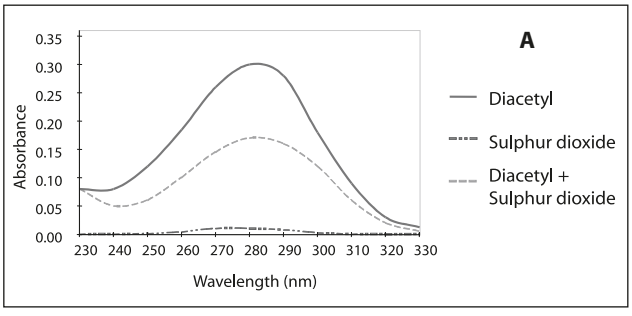
Figure 8 Diacetyl concentration in chemically defined wine (11% v/v ethanol, pH 3.5) during MLF conducted by *O. oeni* strains VII and VIII (Hart, 1997).

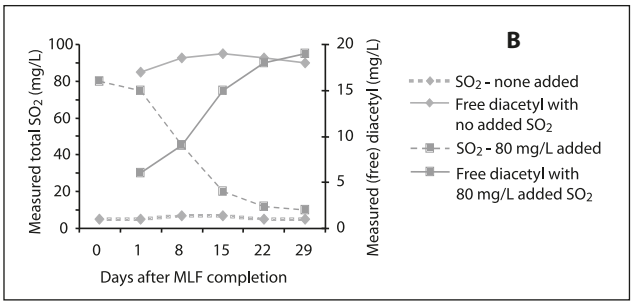


Sulphur dioxide and diacetyl

Sulphur dioxide (SO_2) has complex roles in the wine-making process, including antioxidant and antimicrobial properties. It is able to interact with carbonyl compounds, including acetaldehyde and diacetyl in a reversible manner (Figure 9a). In the presence of SO_2, the concentration of free diacetyl in wine is lowered, however as the SO_2 content decreases, as for example during aging, the ratio of free diacetyl will increase again, thus increasing its sensory impact (Nielsen and Richelieu, 1999) (Figure 9b).

Figure 9 Interactions between sulphur dioxide (SO_2) and diacetyl. (A) Absorbance of 10% aqueous ethanol solution with the addition of either 1 mg/L diacetyl, 5 mg/L SO_2 or 1 mg/L diacetyl + 5 mg/L SO_2 (Hart, 1997). (B) Effect of storage time on diacetyl and total SO_2 concentrations in Chardonnay wine with an initial 20 mg/L of diacetyl and addition of 80 mg/L SO_2 (adapted from Nielsen and Richelieu, 1999).

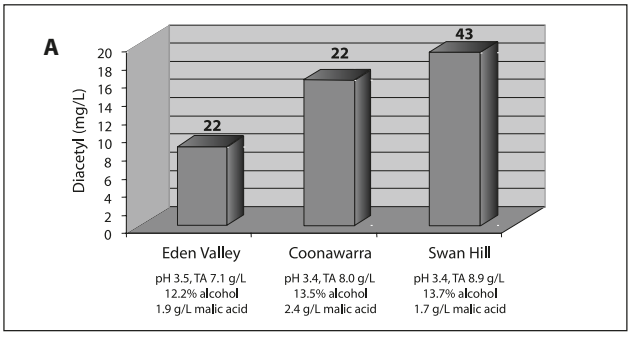


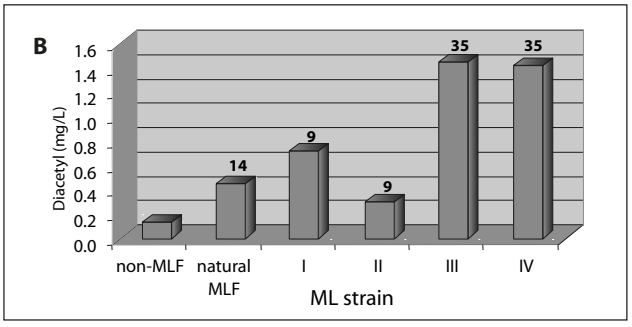


Duration of MLF

The duration of MLF is dependent upon numerous factors, including bacterial strain, chemical composition of the wine and wine temperature. Wines which undergo a prolonged MLF, for whatever reason, tend to have a higher diacetyl content (Figure 10). Three Cabernet Sauvignon wines sourced from different Australian viticultural regions were inoculated for MLF with commercial *O. oeni* strain VII and the diacetyl content was determined at the end of MLF (Figure 10a). A Chardonnay wine that underwent MLF with five different ML strains showed varying lengths of MLF, as well as final concentrations of diacetyl (Figure 10b) (Bartowsky et al, 2002a). In both examples, the highest diacetyl concentration was observed in the wines that had the longest MLF duration.

Figure 10 Duration of malolactic fermentation (days) and its effect on final diacetyl concentration. (A) In three Cabernet Sauvignon (2000) wines sourced from different viticultural regions and inoculated with *O. oeni* strain VII (McCarthy, 2000). (B) In a Chardonnay wine (Langhorne Creek, 2001; pH 3.3, TA 5.8 g/L, 12.5% v/v alcohol, 2.1 g/L malic acid, 0.2 g/L citric acid) with five MLF treatments.





Summary

There are numerous factors that can influence the diacetyl content and, hence, the buttery character of wine. Therefore, in order to achieve a particular buttery level, it is necessary to combine a number of factors that are either favourable or not to diacetyl content. The starting point is to choose the most appropriate ML bacterial strain for conducting the MLF, and then consider the wine composition and MLF conditions.

Increasing the buttery diacetyl impact of a wine can be achieved by using a lower than usual inoculum of a high diacetyl-producing strain in the absence of active yeast, such as after racking wine off yeast lees. The diacetyl content should then be stabilized by filtration to remove bacteria (and yeast if present) to prevent remetabolism, and the addition of sufficient SO_2 to prevent further microbial activity. Use of citric acid to augment diacetyl content entails the risk of acetic acid production, and limited air contact also needs great care. As it might be difficult to achieve the desired level of impact through the MLF process, blending a portion of wine made with a high diacetyl content with the remainder would facilitate achievement of the required level. A low diacetyl content can be achieved by using an appropriate strain inoculated during the late stage of the alcoholic fermentation, and, if necessary, maintaining the wine on stirred lees until the diacetyl becomes undetectable.

Acknowledgments

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APTITUDE OF SELECTED AND WILD STRAINS OF *OENOCOCCUS OENI* TO INDUCE MLF IN HARSH WINES

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Introduction

Malolactic fermentation (MLF) has important consequences on the quality of wine and thus oenologists' efforts are directed at having greater control of how and when the fermentation takes place. The introduction of *Oenococcus oeni* cultures for direct use in wine has greatly simplified the management of this fermentation [1, 2]. However, the inoculated bacterial strain is not always able to adapt to the difficult physico-chemical and nutritional conditions present in the wine. Selection of bacterial strains able to better tolerate the harsh physico-chemical properties of wine continues.

In our study, the capacity of 10 wild strains of *O. oeni* to carry out MLF in harsh conditions was evaluated. These strains belong to our collection of lactic acid bacteria, isolated over the course of nearly 20 years from wines of different varietals and production areas. For comparison purposes, a commercial culture of *O. oeni* was also included in the study. Since the wines employed were obtained from the same grape juice fermented by three different strains of *Saccharomyces cerevisiae*, information on the compatibility between malolactic bacteria and wine yeast could also be obtained. Yeast can deplete the nutrients and growth factors required by ML bacteria and may release bioactive metabolites (SO_2, fatty acids and macromolecules) that can, overall, stimulate, inhibit or have a negligible effect on the metabolism of ML bacteria [3-8].

Experimental plan

Alcoholic fermentation

Grape juice obtained from Sangiovese grapes from the 2003 vintage was divided in aliquots and inoculated with three different *S. cerevisiae* strains: 25 g/hL of BM45 and SLO rehydrated yeast and 2% (v/v) of 24-hour-old culture of the RP strain (from our wine yeast collection). After 10 days of skin contact (maceration) time, each wine was drained and then pressed. The free-run wine derived from duplicate fermentations was first reunited then centrifuged (approximately 3000 rpm x 15 min), sterile filtered (0.45 μm) and an aliquot (80 mL) was dispensed into 100 mL sterile glass bottles.

Malolactic fermentation

Wild strains of *O. oeni* were grown for five days at 28°C in liquid grape juice medium, pH 4.5; 2% (v/v) of culture was used to inoculate wines. The malolactic bacterium *O. oeni* Alfa strain (Uvaferm) was used as the control. The freeze-dried bacterial culture (containing $2x10^{11}$ cfu/g) was rehydrated according to the manufacturer's instructions and used at a level of 1 g/hL. Wines were incubated at 20°C.

Results

Table 1 summarizes the chemical composition of the three wines at the time of bacterial culture inoculation. Depending on the yeast strain, the wines are characterized by dif-

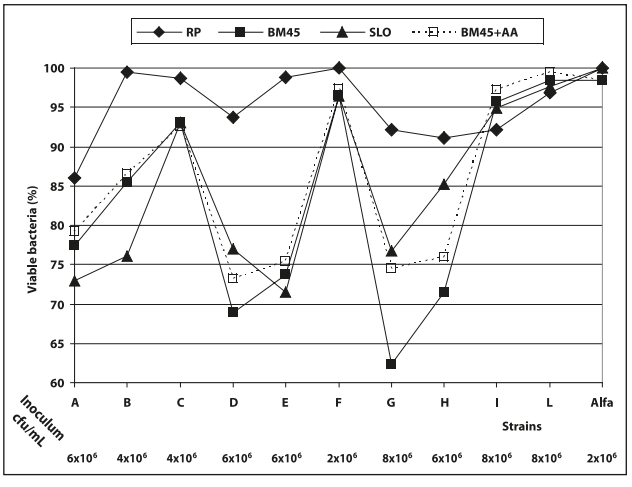
Table 1 Chemical analysis of Sangiovese wines at the moment of malolactic bacteria inoculation.

WINES	Glucose (g/L)	Fructose (g/L)	Ethanol (%)	pH	Total SO_2 (mg/L)	Free SO_2 (mg/L)	a-amino nitrogen (mg/L)	Malic acid (g/L)	Citric acid (g/L)	Succinic acid (g/L)
RP	0.25	0.47	14.00	3.50	27.50	6.80	96.00	1.20	0.47	1.51
SLO	0.07	0.55	15.40	3.50	24.30	5.00	69.00	1.00	0.30	1.56
BM45	0.13	0.89	15.20	3.50	35.80	7.70	55.00	1.24	0.40	1.52

ferent physico-chemical and nutritional conditions, which are more or less inhibiting for the ML bacteria. In fact, the wines are essentially different due to ethanol (ranging from 14 to 15.4%), total SO_2 and α-amino nitrogen content. In particular, BM45 wine shows an inhibiting level of total SO_2 (36 mg/L) and the poorest amino-nitrogen composition. Thus, the bacterial cultures will encounter a different composition in the medium which will influence their development and metabolic activity.

The performance of the wild and commercial strains was verified by evaluating the evolution of the bacterial population and the rate of L-malic acid degradation. Figure 1 shows the viable cell count performed 30 minutes after inoculation, and also includes the number of viable cells inoculated in the three wines, ranging from 2 to 8 x 10^6 cfu/mL. Depending on the bacterial strain and the wine in which it was inoculated, it is possible to observe an immediate drop in the bacterial population. However, bacterial survival after inoculum was independent of the number of viable cells added with the inoculum.

Figure 1 Viable cells (%) of different *O. oeni* strains after 30 minutes of wine inoculation.



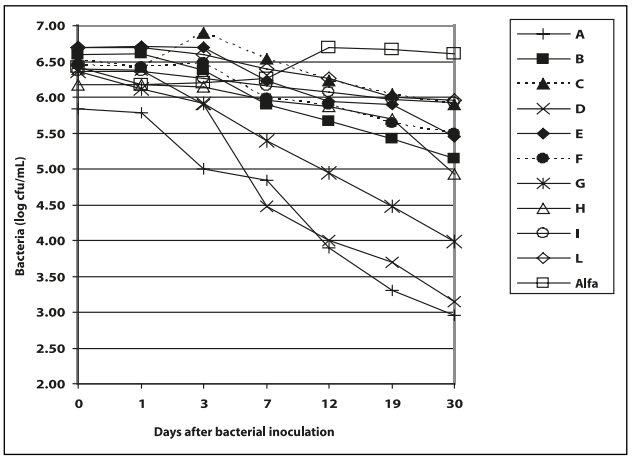
In the wine obtained with the RP yeast strain, 10 out of 11 bacterial strains maintained a high level of viability (90-100%); only strain A showed a lower survival (86%). On the other hand, in the wines obtained with BM45 and SLO yeasts, characterized by a more inhibiting composition, cell viability of the bacterial strains showed a wider range of variation: six strains (A, B, D, E, G, H) out of 11 exhibited an immediate and drastic decrease in cell viability. The addition of amino acids to BM45 wine seemed to stimulate the survival of those strains that had been more greatly inhibited by their contact with the wine. For example, strain G went from a cell viability percentage of 62 to 75. We can speculate the wild strains that show higher viability (five out of 11 strains) are naturally able to synthesize resistance factors to the harsh properties of wines. Indeed, they behave like the commercial culture of *O. oeni*, whose production foresees induction of the synthesis of stress proteins, to confer greater resistance to the cells directly inoculated in the wine [9].

The evolution of viable cells and L-malic acid degradation in wines is reported in Figure 2 and Figure 3 respectively. In RP wine (Figure 2a and 3a), strains A, D and G showed a progressive decrease in viable population, while L-malic acid was not metabolized. Strains B, E and H maintained their viable populations at a high level, however the degradation of L-malic acid was rather low (only 0.3-0.4 g/L of malic acid consumed). Strains C, F, I, L and Alfa maintained their viable population at about 10^6 cfu/mL. In strains C and F, malic acid degradation began immediately and terminated after five days; after seven days citric acid was also completely degraded. The remaining three strains concluded their MLF in 12 days, but after 30 days citric acid was only partially degraded. Wines obtained with SLO (Figure 2b and 3b) and BM45 yeasts (Figure 2c and 3c) were considerably more inhibiting for most of the inoculated strains. This inhibition is noted as a progressive and important loss of cell viability. Only strains C, F, I, L and Alfa maintained a viability level of 10^6-10^5 cfu/mL. However, rapid and complete MLF was carried out only by strain F in SLO wine, while in BM45 wine this strain left 200 mg/L of malic acid.

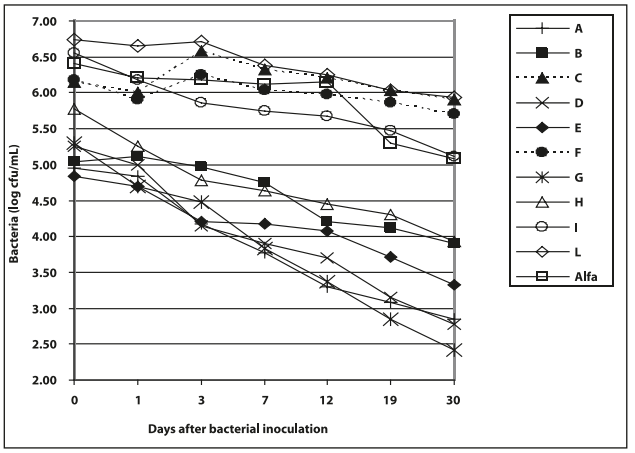
According to previous work [6], these results confirm that ethanol content is the discriminating factor for cell viability soon after inoculation, growing ability and malolactic activity of the strains investigated. This trend can be better verified by observing the chemical composition of wine at the time of inoculation. In SLO wine, with a total SO_2 content similar to that of RP wine, but with a higher alcohol content (15.4%), only one strain was able to complete MLF. On the contrary, in RP wine, with a lower alcohol content (about 14%), five strains rapidly completed MLF.

Figure 2 Evolution of viable cells in RP (A), SLO (B) and BM45 (C) wines.

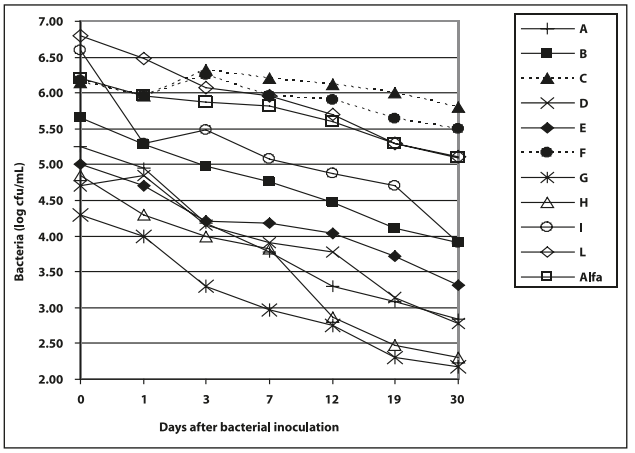
A RP wine (EtOH 14 %, total SO_2 27.5 mg/L, pH 3.5, α-amino nitrogen 96 mg/L)



B SLO wine (EtOH 15.4%, total SO_2 24.3 mg/L, pH 3.5, α-amino nitrogen 69 mg/L)



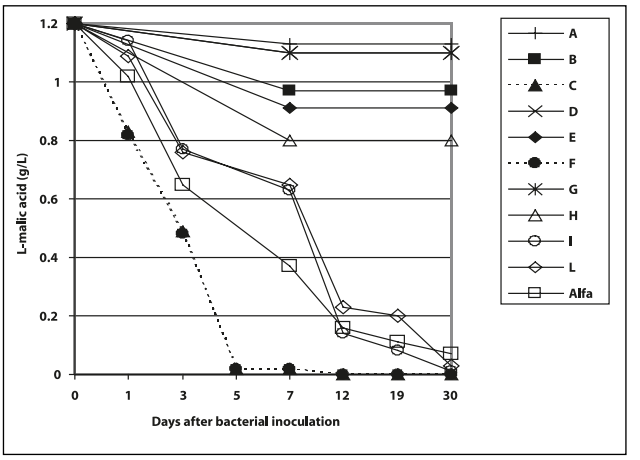
C BM45 wine (EtOH 15.2%, total SO_2 36 mg/L, pH 3.5, α-amino nitrogen 55 mg/L)



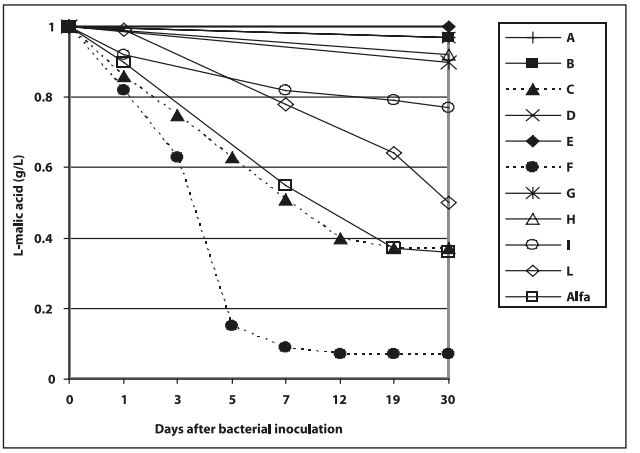
In BM45 wine however, having a high total SO_2 content as well as high alcohol content, MLF was not completed, not even by strain F.

Figure 3 L-malic acid degradation in RP (A), SLO (B) and BM45 (B) wines.

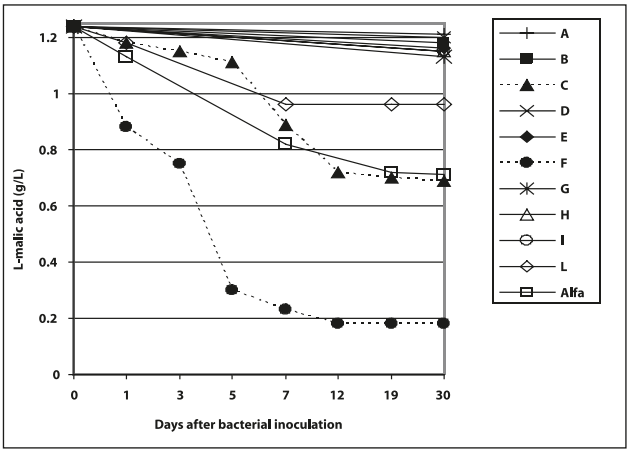
A RP wine



B SLO wine

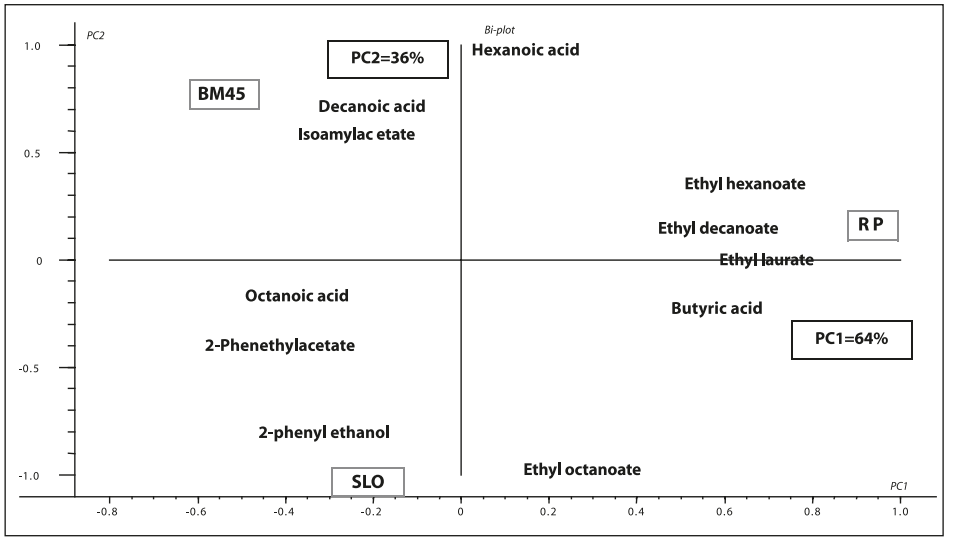


C BM45 wine



In order to understand if, along with the inhibition caused by ethanol and total SO_2, the bacterial strains also underwent the negative effect of other metabolites produced

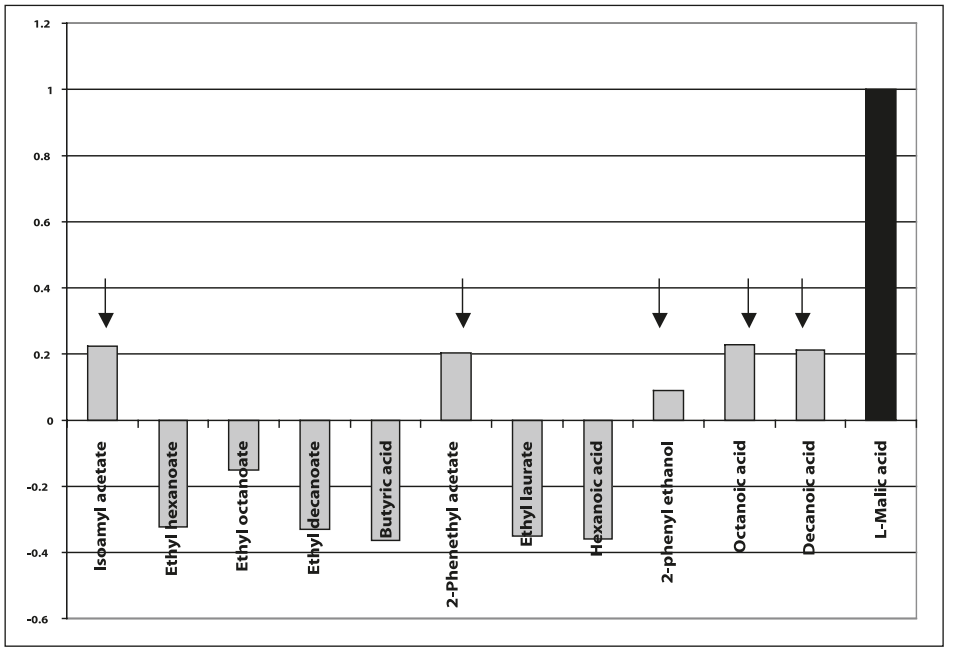
Figure 4 PCA of Sangiovese wine volatile compound data: projection of the volatile compound variables and wines on the first and second components.



by the yeast cells during alcoholic fermentation, we determined the contents of some volatile compounds in the wines. To compare the volatile compound profile of wines, a Principal Component Analysis (PCA) of data was performed (Figure 4). Wines show some differences in the content of volatile compounds produced by yeast strain. Medium-chain fatty acids, known to be toxic for lactic acid bacteria [6], were relatively similar among the three wines. Wines containing high concentrations of octanoic and decanoid acid and 2-phenyl ethanol have been reported as being more resistant to MLF success [4, 10]. RP wine has a higher content of ethyl esters, BM45 wine has a higher content of decanoic and isoamyl acetate, SLO wine has a higher content of 2-phenyl ethanol, 2-phenethyl acetate and octanoic acid. The volatile compound composition of wines was related to L-malic acid degradation by ML bacteria, by means of a PLS regression analysis (Figure 5). The results, expressed as loadings of the PLS model, show that a higher content in isoamyl acetate, 2-phenethyl acetate, octanoic and decanoic acids and 2-phenyl ethanol in wines resulted in a reduction of the ability of ML bacteria to transform L-malic acid.

Another aspect that may be involved in the interaction phenomena between yeasts and bacteria is related to the nitrogen composition of the wines examined (Figure 6). Amino acids are important for the growth of *O. oeni* strains, as a source of both s nitrogen and carbon [11]. In particular, isoleucine, glutamic acid, tryptophan and arginine are reported to be essential for correct malolactic activity and development of most *O. oeni* strains [12]. The different α-amino nitrogen contents of the three wines at the end of alcoholic fermentation point out that the three yeast strains have different needs with regard to nitrogen nutrition. Comparing the amino acid profile of the wines before inoculum of ML bacteria, BM45 wine, in agreement with its lower amino nitrogen content, has the poorest amino acid content.

Figure 5 Relationship between volatile compounds in wine before MLF and residual L-malic acid after MLF: PLS loadings.



Taking into account the nutritional requirements of *O. oeni* strains, we added an amino acid solution to BM45 wine, in order to increase its α-amino nitrogen level up to 100 mg/L (as in RP wine), to verify whether the viability and malolactic activity of the bacteria could be stimulated. In Figure 7, a comparison between the evolution of viable bacterial population and L-malic acid degradation in the control wine and the amino acid wine is reported. A slight stimulation of bacteria growth can be noted for all strains. At the same time, degradation of malic acid was also stimulated. In particular it is worth noting that strain F completed MLF.

Figure 6 HPLC aminoacidic profiles of wines.

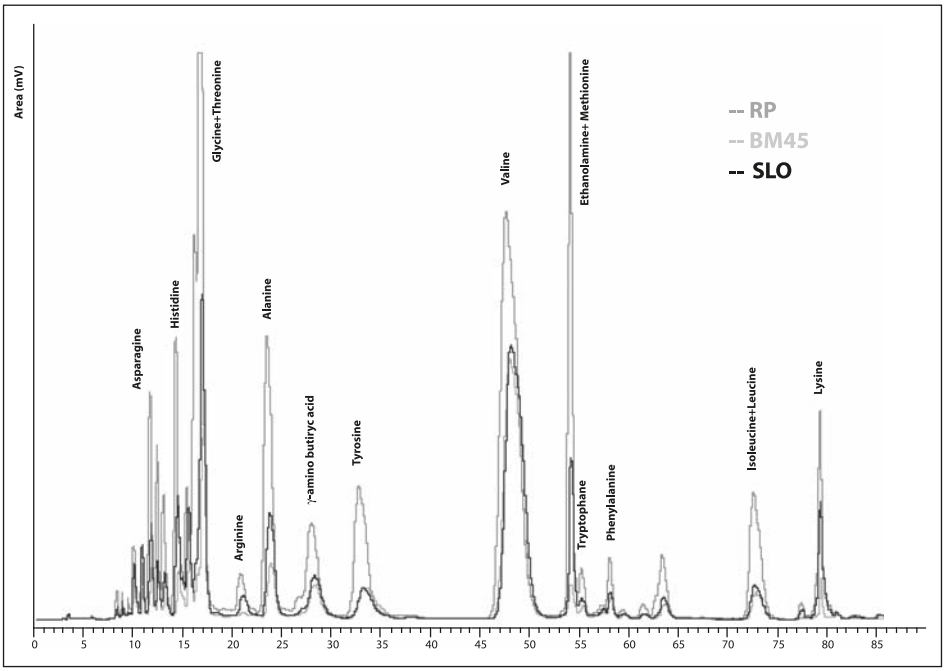
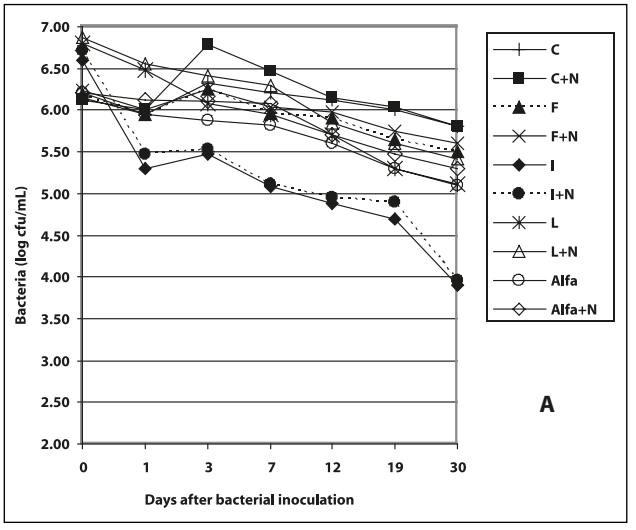
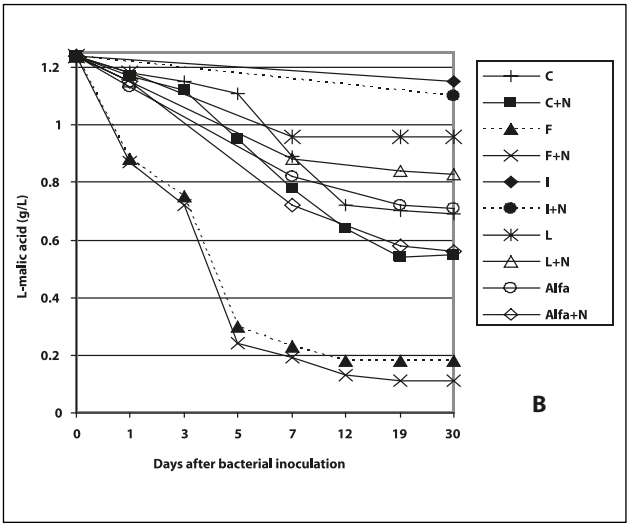


Figure 7 Evolution of viable cells
(A) and L-malic acid degradation (B) in BM45 control wine (α-N 55 mg/L) and BM45+N (α-N 100 mg/L).





Conclusion

This study has verified that the aptitude to carry out MLF in harsh conditions is closely tied to the physiological properties of the *O. oeni* strain inoculated. Among the 11 strains studied, only one was able to remain viable and active even in more difficult physico-chemical and nutritional conditions.

The other strains in this study were greatly influenced by the production of bioactive yeast metabolites and by a deficiency in nitrogen content induced by the yeast strain. Moreover, some strains performed MLF without growth in wine, but only in a maintenance mode, as previously observed by other authors [13]. Further work is in progress to search for new strains of malolactic bacteria and to study their compatibility with yeast strains used to conduct alcoholic fermentation, with the aim of improving the outcome of MLF even in harsh wines.

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YEAST-BACTERIA INTERACTION – POSSIBLE NUTRIENT STRATEGIES

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Yeast-bacteria interactions

The following are the biochemical routes in which wine yeast and bacteria interact.

Yeast produce:

- Sulphur dioxide – inhibits bacteria and other yeasts by inhibiting enzymes
- Alcohol – inhibits bacteria and other yeasts by disrupting lipid membranes, making them leaky
- Short chain fatty acids – inhibit bacteria by disrupting lipid membranes, making them leaky
- Antimicrobial peptides – inhibit bacteria and yeasts by disrupting lipid membranes

Yeast compete very aggressively for nutrients – by removing essential nutrients, other microorganisms are no longer able to grow.

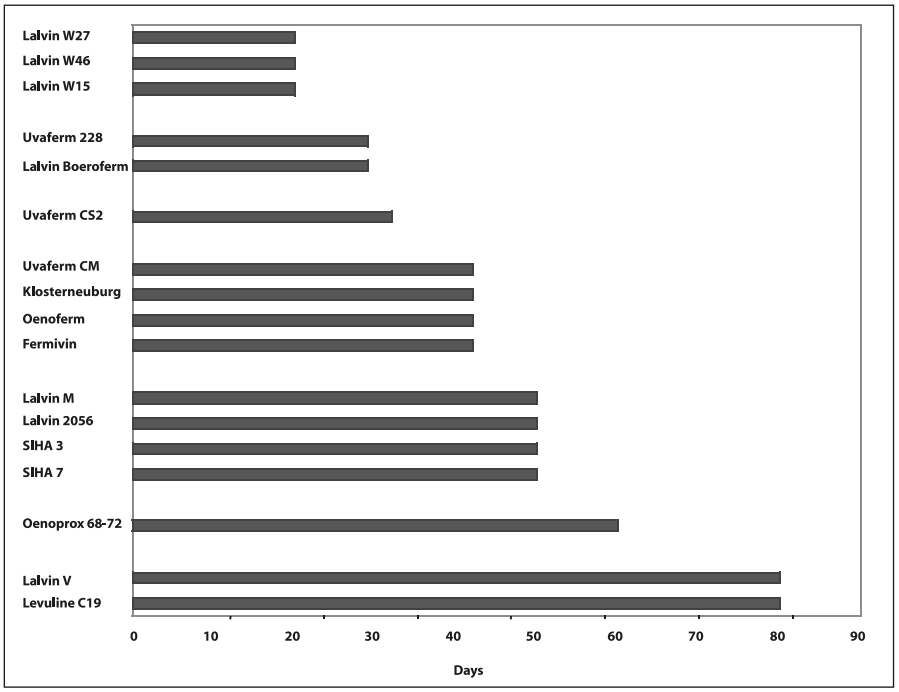
Lactic acid bacteria:

- Compete for nutrients – removing some essential nutrients can inhibit yeast growth
- Produce antimicrobial peptides – those produced by bacteria can disrupt yeast cell membranes, inhibiting their growth and killing them

Effect of yeast strain on malolactic fermentation

An example of how the interaction for nutrients and the production of antimicrobial compounds affect the onset and completion of malolactic fermentation (MLF) was given by Dr. Jürg Gafner (personal communication). Figure 1 shows the average length of time required for completion of spontaneous MLF in wines fermented with various yeast cultures. In this comparison, the grape must was divided

Figure 1 Time required to complete spontaneous malolactic fermentation in wines fermented with various yeast starter cultures. Length of malolactic fermentation was measured as days from completion of alcoholic fermentation to completion of malolactic fermentation (J. Gafner, personal communication).



into separate fermenters and each was inoculated with a different yeast starter culture. After completion of alcoholic fermentation, the wines were monitored for the onset and completion of spontaneous MLF. The total length of MLF in this comparison reflects the length of time required for the bacteria to grow and the rate of MLF. In this comparison, the length of MLF varied from 20 to about 80 days, and was affected by several factors. Since all fermentations started with the same must, each wine contained the same lactic acid bacteria population before the start of MLF. Once the yeasts started to grow and ferment they competed for nutrients and produced antimicrobial compounds, such as those listed above. During, towards the end and after completion of alcoholic fermentation, individual yeasts die and release their cell content into the wine. Through this process (autolysis), more nutrients are made available to the lactic acid bacteria. These wines were left in contact with the yeast lees (sediment) providing the bacteria with nutrients released from the autolyzing yeasts. The observed length of MLF is the sum of all these interactions. This comparison shows simply that yeasts affect the length of MLF. In the research conducted in the past several years in our laboratory and in other laboratories, researchers have been setting up experiments to study the individual effects of yeast metabolism on growth and malolactic activity of lactic acid bacteria. In order to manage successful MLF, we need to know how individual yeast starter cultures affect the growth and the malolactic activity of lactic acid bacteria.

Figure 2 shows how growth of *Oenococcus oeni* starter cultures was suppressed during alcoholic fermentation with four different yeasts. The analysis of free and total SO_2 in the wines during and after alcoholic fermentation (Figure 3) shows the viability of the bacteria cultures was suppressed by peak SO_2 concentrations during alcoholic fermentation. The bacteria grew again after the end of alcoholic fermentation when the SO_2 concentration in the wines had declined. The rate of bacteria recovery after alcoholic fermentation was generally, but not always, related to the amount of SO_2 produced by the different yeasts. This study shows the importance of SO_2 production by yeasts. *O. oeni* bacteria are very sensitive to inhibition by SO_2. The recovery of the bacteria was not always related to the concentration of SO_2 in the wine. This indicates that other factors, such as nutrient release from autolyzing yeast and possibly antimicrobial peptides, are playing a (lesser) role in this interaction.

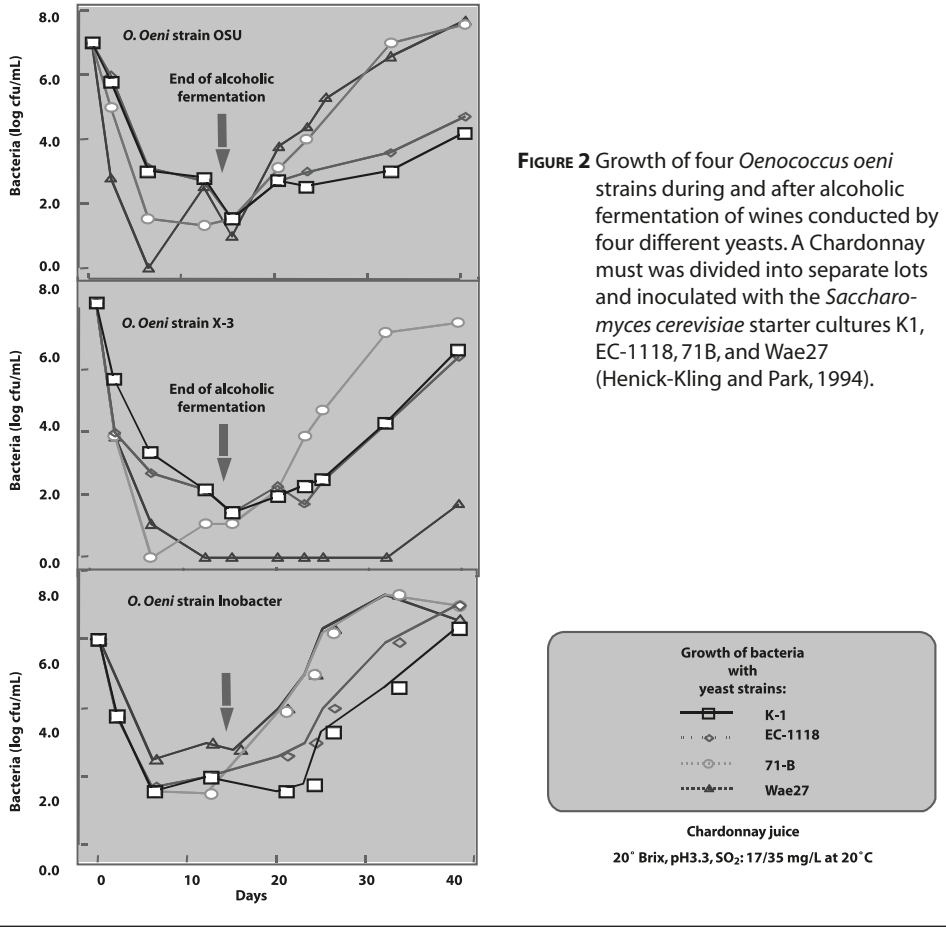
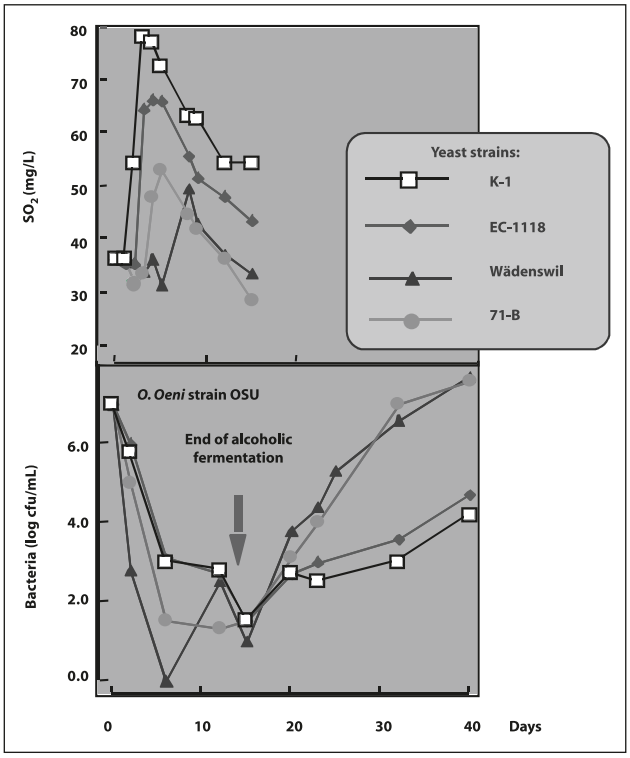


FIGURE 2 Growth of four *Oenococcus oeni* strains during and after alcoholic fermentation of wines conducted by four different yeasts. A Chardonnay must was divided into separate lots and inoculated with the *Saccharomyces cerevisiae* starter cultures K1, EC-1118, 71B, and Wae27 (Henick-Kling and Park, 1994).

Nutrient additions to stimulate malolactic fermentation

Nutrient additions to the grape must and to the wine can be used to lessen the competition for nutrients between yeasts and bacteria. Yeasts very effectively compete for available nutrients (sugars, amino nitrogen, vitamins, essential minerals, fatty acids) in the grape must and wine. During the early phase of growth, *Saccharomyces* yeasts very quickly take up available nutrients and store them inside the cell, thus making them very quickly unavailable to other, competing microorganisms. *O. oeni* bacteria also need essential nutrients (primarily amino acids and vitamins) preformed in the growth medium.

Figure 3 SO_2 production by 4 *Saccharomyces cerevisiae* starter culture yeasts in a Chardonnay juice (20 Brix, pH 3.3, SO_2 at inoculation: 17/35 mg/L).



SO_2 production by the yeast is also affected by the supply of nutrients. A lack of nitrogen nutrients causes an imbalance in the sulphur and nitrogen metabolism in the yeast and causes the yeast to excrete excess SO_2. Thus, providing a grape must with sufficient yeast available nitrogen can lower SO_2 production by *Saccharomyces* and lessen the inhibition of *O. oeni* by the SO_2. Also, supplementing the grape must to provide sufficient nitrogen and other nutrients for *Saccharomyces* should leave more nutrients in the wine during and after alcoholic fermentation. Theoretically, this will make the wine more conducive to growth by *Oenococcus* and help to achieve a successful MLF. We have started experiments to investigate this possibility.

Yeast nitrogen requirement for successful wine fermentation

Yeast assimilable nitrogen (YAN) is amino nitrogen and free ammonia. Fleet and Heard (1992) determined that a minimum 120-140 mg/L of YAN is required to avoid stuck fermentations.

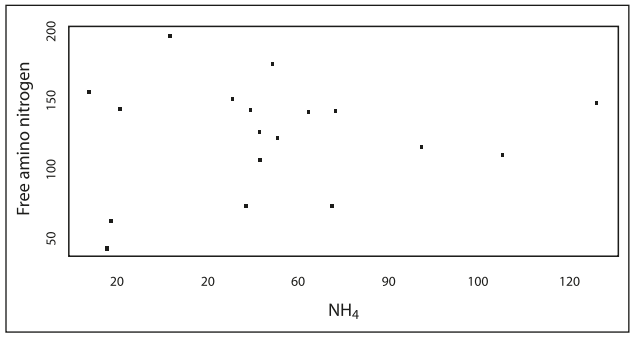
Ferreira-Monteiro and Bisson (1992) found that 392-473 mg/L of YAN is necessary to avoid stuck and sluggish fermentations and avoid sulphur off-flavours. Jiranek et al. (1995) came to a very similar conclusion, recommending 328-467 mg/L of YAN.

Yeast nitrogen demand varies with the extent of yeast growth, and with yeast strain, growth temperature, availability of oxygen, sugar concentration and competition with other microorganisms.

We found in work with the NY wine industry that 120-140 mg/L of YAN is not sufficient to ensure complete fermentation and to avoid fermentation off-odours (reduced sulphur and fatty acids). With nitrogen additions to the grape must we try to achieve a minimum of 300 mg/L of YAN.

To ensure sufficient nitrogen supply, it is, of course, necessary to have a reliable measurement. We found the free amino nitrogen method as described by Dukes et al. (1998) to be the most precise and reliable method. Together with the analysis of free ammonia with enzymatic analysis (Boehringer Mannheim kits) the results obtained with this method do estimate the YAN of grape must very well. We (Shively and Henick-Kling, 2001) have also found that the results obtained with the o-phthaldialdehyde/N-acetyl-L-cysteine method (Dukes et al., 1998) correlate very well with those obtained with a carefully executed formol titration. For free ammonia analysis, we prefer the enzymatic analysis. We found the analysis by ammonia electrode was not at all reliable. We also found no correlation between the amount of free ammonia and free amino nitrogen (Figure 4). Thus, a simple measurement of ammonia cannot be used to estimate the amount of YAN in a grape must.

Figure 4 Correlation between free ammonia concentration and free amino nitrogen concentration in grape must. There is no correlation!



New York State grape musts contain very low YAN (Edinger and Henick-Kling, 1994; Henick-Kling et al., 1996; Shively and Henick-Kling, 2001). Surveys of several grape varieties over several years (1993, 1994 and 1997) and several grape-growing regions in New York State showed a range of 101 to 232 mg/L of YAN for Cabernet Sauvignon, Cayuga White, Chardonnay, Pinot Noir, Riesling and Seyval Blanc (Table 1).

Table 1 Total assimilable nitrogen averages for six cultivars from three vintages (mg/L). The numbers in parentheses indicate the number of each cultivar assayed for ammonia and FAN.

Cultivar	Ammonia			FAN			Total Yeast Assimilable Nitrogen		
	1993	1994	1997	1993	1994	1997	1993	1994	1997
Cab. Sauv.	49(5)	69(5)	18(7)	74	142	43	123	211	61
Cayuga	68(2)	32(2)	21(9)	74	197	144	142	229	165
Chardonnay	46(13)	55(13)	50(18)	151	177	143	197	232	193
Pinot Noir	52(7)	88(21)	127(21)	135	116	148	187	204	275
Riesling	52(9)	56(9)	106(22)	102	123	110	154	179	216
Seyval Blanc	19(6)	14(6)	63(6)	82	156	142	101	170	205

Grape samples from New York vineyards collected in 1993 contained an average of 165 mg/L of YAN. The average YAN in 1994 was 204 mg/L, and in 1997 it was 181 mg/L (N = 120). Of 120 juice samples tested in 1997, the lowest YAN content was 61 mg/L and the highest was 275 mg/L. These surveys show that NY grape musts contain a minimum and even less than the minimum amount of nitrogen necessary for successful wine fermentation. Experience with yeast nitrogen supplementation of grape musts over the past 10 years has proven that grape must supplementation is very successful in avoiding stuck fermentations and fermentation off-flavours. In our New York State Wine Analytical Laboratory we have found that the incidence of stuck fermentations and wines with reduced sulphur off-odours in New York State has been reduced. We also have seen this benefit in the 200 and more wines we prepare each year in our experimental winemaking program. U.S. Federal law allows the addition of maximum 8 lb/1000 gal of diammonium hydrogen phosphate. Eight pounds of DAP per thousand gallons is approximately 960 mg/L of DAP and adds approximately 203 mg/L ammonia. Thus, the addition of the allowed maximum amount of DAP brings some grape musts to the recommended 300-500 mg/L of YAN (Monteiro and Bisson, 1992; Jiranek et al., 1995).

A study carried out over three years in a NY State vineyard showed that drought stress years (hot, dry summers, mainly August, September) irrigation increases the YAN content in grape must (Martinson et al., 2003) and foliar nitrogen applications (urea) can further increase YAN in grape must. The year 2001 was a hot, dry year in New York State. With a combination of irrigation and foliar nitrogen application, juice YAN reached the desired 300-400 mg/L.

Table 2 Yeast assimilable nitrogen content in hot/dry years and wet/cool year. Riesling vineyard in the Finger Lakes Region of New York State. Data from Martinson et al, 2003.

Treatments		Yeast Available Nitrogen (mg/L)		
Irrig.	Nitrogen	2001	2002	2003
No	0	181	153	405
	Foliar N	277	253	448
	Soil N	169	107	338
Yes	0	245	167	461
	Foliar N	329	235	438
	Soil N	245	169	399
Significance (P)				
Irrigation		0.001	ns	ns
N		0.001	0.001	ns

How to correct nutrient deficiencies

Yeast nutrient additions:

- Diammonium hydrogen phosphate, 1 g/L provides approximately 260 mg/L of ammonia
- Proprietary nutrient blends (some containing DAP), also contain vitamins, nucleic acids, and trace elements
- Yeast extract contains amino acids, fatty acids, nucleic acids, vitamins, minerals, etc.

Bacteria nutrient additions:

- Yeast extract contains amino acids, fatty acids, nucleic acids, vitamins, minerals
- Yeast hulls contain amino acids, fatty acids, nucleic acids, vitamins and minerals, and can help detoxify a wine by binding fungicides and antimicrobial peptides to the cell membrane and cell wall fragments
- Proprietary nutrient blends

The above nutrient supplements can be added at the rate recommended by the manufacturer.

Investigating nutrient competition and antimicrobial activity between *Saccharomyces* and *Oenococcus*

We carried out a range of experiments to separate the various possible interactions and to validate laboratory findings in industry practice.

Using interaction plating methods, we were able to study the nutrient competition and the production of antimicrobial substances such as bacteriocins and fatty acids. We used overlay methods and contact growth methods to determine whether antimicrobial substances are formed in a non-nutrient limiting situation. We also used filter discs, soaked in growth media in which various yeasts had been grown (the yeast were then removed), to investigate antimicrobial residues in the fermentation medium. The results of these studies were largely presented in a previous report (Lallemand Technical Meeting, Biarritz, France, 22-23 April 2002).

Figure 5 Growth and malolactic activity of selected *Oenococcus oeni* cultures in Chardonnay wine fermented with *Saccharomyces* EC-1118. The red and white colour code for each *O. oeni* strain number indicates whether it did or did not complete malolactic fermentation.

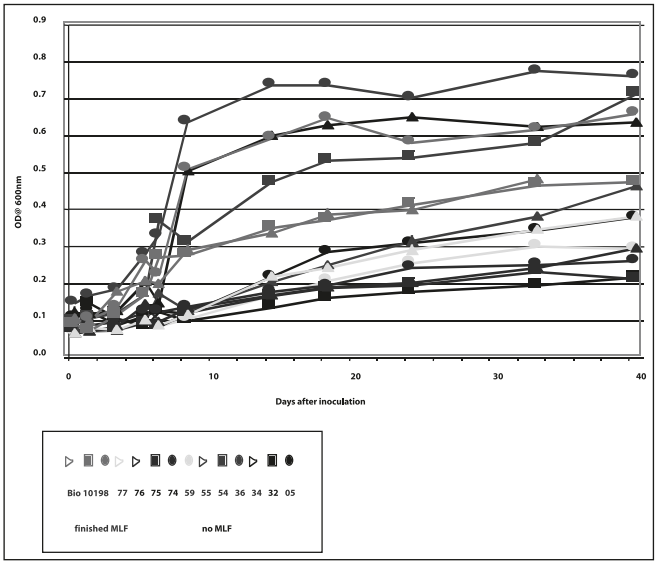
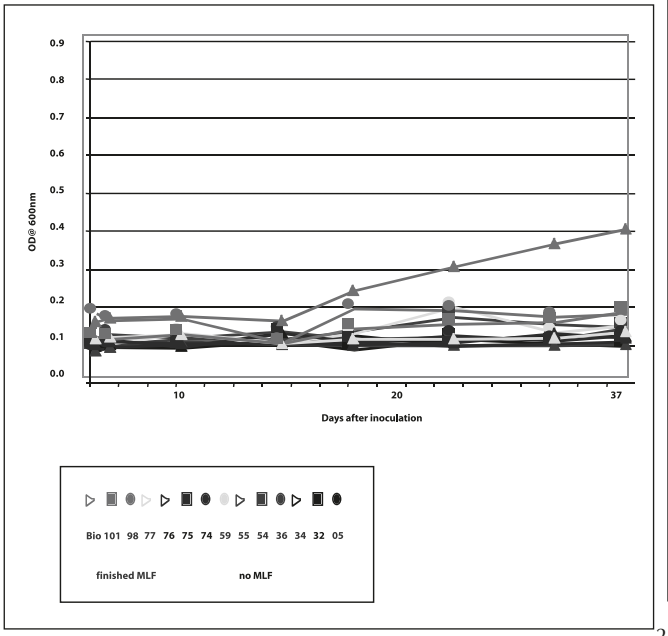


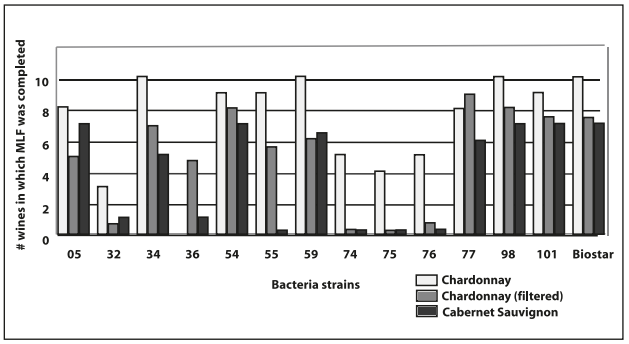
Figure 6 Growth and malolactic activity of selected *Oenococcus oeni* cultures in Chardonnay wine fermented with *Saccharomyces* BM45. The red and white colour code for each *O. oeni* strain number indicates whether it did or did not complete malolactic fermentation.



Laboratory scale fermentations were carried out in 10 mL tubes and in 25 to 500 mL Erlenmeyer flasks on orbital shakers in synthetic and in natural grape musts and wines. These experiments included simultaneous and successive inoculations with *Saccharomyces* and *Oenococcus* starter cultures. In liquid fermentation media we can only study growth requirements, but metabolic activity as well. As the results in Figures 5 and 6 show, in many wines *Oenococcus* starter cultures were able to grow, but MLF was partially or completely inhibited. The Chardonnay fermented by *Saccharomyces* EC-1118 allowed good growth for most of the 14 *Oenococcus* cultures tested. Only the weakest growing strains did not complete MLF (four out of 14 strains). The data in Figure 6 confirm this result. All *Oenococcus* strains inoculated in wine fermented with Saccharomyces BM45 showed very poor growth. Only one strain completed MLF. Growth of the bacteria seems to be linked to malolactic activity. The results with yeast AMH in the same must raise more questions. For most strains of *Oenococcus*, growth was quite good, 0.1 to 0.6 OD, though less than in the wine fermented by EC-1118, at 0.2 to 0.8 OD. Also success in completing MLF was lower in the wine fermented with AMH; only eight of 14 strains completed MLF. These results point to other interactions. Why did some of the strains grow yet not complete MLF? This does not appear to be a nutrient competition.

In laboratory scale fermentations of Chardonnay and Cabernet Sauvignon wines, we tested 15 cultures of *O. oeni* against 10 and seven yeast strains, respectively (Figure 7). In addition, half the Chardonnay wine was filtered through 0.45µ membranes to remove all yeasts. The other half of the Chardonnay was not filtered, neither was the Cabernet Sauvignon. The wines were pushed with a syringe through laboratory filters, causing some aeration of the wine when it left the filter. Malolactic fermentation was more successful in filtered Chardonnay than in non-filtered Chardonnay. We assume that some of the SO_2 produced during alcoholic fermentation was lost during filtration, thus making the wine more hospitable for the bacteria. Bacteria selections 32, 74, 75 and 76 performed reasonably well only in the filtered Chardonnay. This also indicates that some

Figure 7 Success of bacterial cultures completing malolactic fermentation in a Chardonnay wine fermented with 10 different yeasts and a Cabernet Sauvignon wine fermented with seven different yeasts. Part of the Chardonnay wine was filtered (0.45µm), the other part was not. The columns indicate the number of wines in which a particular bacteria culture completed MLF.



inhibitors were removed by filtration. All other bacteria performed well in the Cabernet Sauvignon. The best performing strains, 54, 98, 101 and Biostar, did well in all red wines and all filtered Chardonnays and in eight out of 10 non-filtered Chardonnays. These strains apparently were not inhibited by the yeasts tested here. The other bacteria did not complete MLF in wine fermented by at least one wine. Some yeasts (AMH, D47, EC-1118, W27) allowed MLF by all bacteria cultures tested here. Table 3 lists the residual primary amino nitrogen content and SO_2 content for each yeast used to ferment the Chardonnay. There is no correlation between the number of strains completing MLF in a particular wine and the YAN content of the wine. The SO_2 content is very similar in all wines, except in the wine fermented with *S. cerevisiae* BM45 which contained about three times more total SO_2. In another trial with a 2002 Chardonnay, the primary amino nitrogen content after yeast fermentation was very low (19-29 mg/L) in four of six of these wines, none of the five bacteria cultures completed MLF, in two of six wines only two bacteria cultures completed MLF. This result indicates that in wines with very low amino nitrogen content the nutrient supply for the bacteria might be limiting.

Table 3 Primary amino nitrogen and SO_2 content of Chardonnay wines (1999 NY Chardonnay) fermented with various yeast starter cultures. The same Chardonnay juice was used in each of the wines

		1° amino N mg/L	Free SO_2 mg/L	Total SO_2 mg/L	Adjusted pH
	Juice	119.38			3.50
Wine	D47	67.03	5.3	9.9	3.32
	W27	79.87	5.0	7.0	3.30
	SIHA3	76.17	5.1	15.4	3.32
	W15	63.62	4.9	8.5	3.29
	BM45	57.46	5.1	55.9	3.29
	EC-1118	70.08	5.6	15.3	3.29
	KN	55.39	5.8	12.7	3.30
	W46	52.92	5.6	12.5	3.29
	AMH	85.19	5.4	9.0	3.30
	CY3079	68.88	4.6	12.3	3.30

Amino nitrogen according to Dukes and Butzke, 1998

Strategies to increase amino nitrogen after completion of alcoholic fermentation

We wanted to test how much the amino nitrogen content after completion of alcoholic fermentation is increased by addition of ammonium nitrogen (DAP) to the grape must. We made 1 g/L and 2.5 g/L DAP additions to a Chardonnay, Cayuga White and Pinot Noir must, fermented each with *S. cerevisiae* DV10 and checked the amino nitrogen content after completion of alcoholic fermentation (Table 4).

Table 4 Amino nitrogen (mg/L) in must and wine fermented with EC-1118.

Diammonium hydrogen phosphate additions			
	No addition	**1.0 g/L added**	**2.5 g/L added**
Chardonnay			
Before ferment.	129	126	131
After ferment.	< 10	18	29
Cayuga White			
Before	177	181	184
After	<10	26	31
Pinot Noir			
Before	198	206	194
After	<10	29	34

Amino nitrogen according to Dukes and Butzke, 1998

All must had low amino nitrogen content. With the addition of 1.0 and 2.5 g/L of DAP the residual amino nitrogen after completion of yeast alcoholic fermentation was increased over no addition. Yet, even with a large addition of 2.5 g/L DAP, the remaining amino nitrogen content was still low. The yeast still preferred to use the amino nitrogen and left increasing amounts of ammonium in the wine (data not shown). Further trials with additions of more complex nutrient blends will show whether such low amino nitrogen content can indicate problems with MLF.

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IMPACT OF S-CONTAINING AMINO ACIDS AND GLUTATHIONE ON GROWTH OF *OENOCOCCUS OENI* AND MALOLACTIC FERMENTATION

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Introduction

Malolactic fermentation (MLF) by *Oenococcus oeni* is well known for transforming harsh malic acid in wine to smooth lactic acid and carbon dioxide. Recently it was shown that MLF improves organoleptic characteristics, impacts mouthfeel and structure and provides biological stability in the final wine (Henick-Kling, 1993; Krieger and Hammes, 1988; Lonvaud-Funel et al., 1998). Moreover, reduced sulphur off-flavours can be detected in some wines after MLF. Volatile sulphur (S)-containing compounds play a significant role in the flavour of wines. This is related to their high volatility, reactivity and impact at very low concentrations. Some of the S-substances are necessary for wine quality, while others are the cause of strong objectionable flavours (rotten eggs, cooked cabbage, cauliflower, burnt rubber, etc.), even at extremely low concentrations (e.g., H_2S, methanethiol [MeSH], ethanethiol [EtSH]). Certain thiols contribute to the typical sensory impression of grape varieties like Chenin blanc, Sauvignon blanc, Scheurebe, etc. It is well known that the formation of volatile S-compounds is affected by organic and inorganic S-containing substances and pesticides in grapes and musts. Other factors are the nutrient level in grapes and musts and the yeast metabolism during fermentation.

During recent years, a reoccurrence of off-flavours in wines during storage after treatment and bottling was noticeable. Those off-flavours can be related to a release of unpleasant volatile compounds from non-volatile or volatile precursors, like the hydrolysis of thioacetic acid esters to thiols and acetic acid. This depends on the chemical equilibrium. Therefore, an off-flavour can increase or reoccur. In comparison to the thioacetic acid esters, the mercaptanes have very low threshold values (>40 mg/L and <2 µg/L, respectively). Consequently, traces of mercaptanes are sufficient to induce a sulphur off-flavour.

The cause for an increase of reduced sulphur off-flavours after MLF or the storage of wine can be attributed to the increase of the pH-value which can change the chemical equilibrium or trigger further chemical reactions. On the other hand, the addition of SO_2 or ascorbic acid can reduce disulphides to the more odour-active thiols. Further-

more, the metabolism of lactic acid bacteria is discussed as a cause for the development of sulphur off-odours in wine. It is well known that lactic acid bacteria can metabolize S-containing amino acids like methionine and that several S-aroma compounds like methanethiol, dimethyl sulphide, dimethyldisulphide, methionol, methional and 3-(methylsulphanyl)propionic acid can be formed by degradation of this amino acid (Dias and Weimer, 1998; Bonnarme et al., 2000; Pripis-Nicolau et al., 2004). Most of these S-compounds are relevant for the cheese flavour in a number of cheeses. Cysteine can be the precursor of S-containing heterocycles, like certain thiazoles.

Therefore, research work was focused in the first stage on the influence of methionine, cysteine and glutathione (also in combination) on the growth of *Oenococcus oeni* and the duration of MLF (Schwarz, 2003; Kondzior, 2004).

Experimental procedure and results

Research trials were conducted in musts of various grape varieties to prove the impact of S-containing amino acids and glutathione on the growth of *Oenococcus oeni* strains. In the following two trials with Riesling and a German red wine variety, Dornfelder, are demonstrated. The MLF tests were carried out as microvinifications with a volume of 750 mL. All experiments were run in duplicates. The growth of lactic acid bacteria was controlled by agar platting and counting the colony forming units after incubation and by measuring the organic acids by HPLC and enzymatic determination.

Figure 1 shows the experimental design of the Dornfelder wine. The must was split into equal parts and the alcoholic fermentation was performed with two yeast strains, Lalvin Rhone 2056 and Lalvin BM 45. Controls without MLF of each of the fermented wines (without inoculation of lactic acid bacteria) were stored under the same conditions after adding SO_2 in a concentration of 70 mg/L to prevent a spontaneous MLF in those wines. The *O. oeni* strain OoA (Lallemand) was used to initiate MLF. The sulphur-containing amino acids and glutathione were added as single substances or in combinations with concentrations as demonstrated in Figure 1, except to the control wines. Both of the fermented wines had very low levels of assimilable nitrogen. This is indicated by the very low Ferm-N-value (<1) which is an indicator for free amino nitrogen.

Figure 1 Experimental design of a trial with the red wine variety Dornfelder.

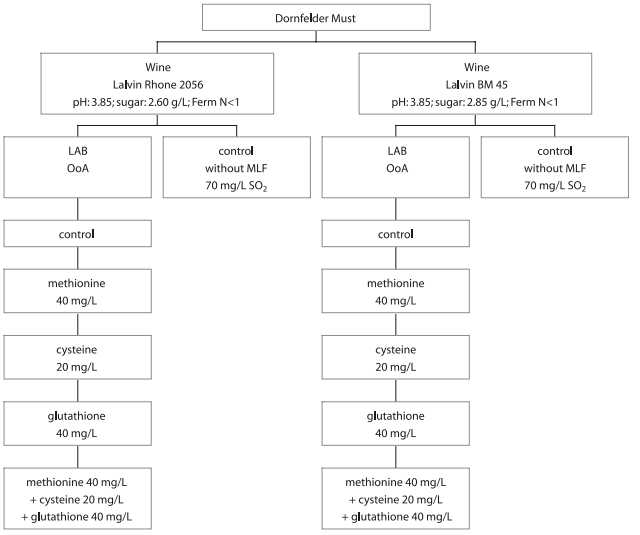


Figure 2 illustrates that the addition of cysteine (20 mg/L) increased the growth of lactic acid bacteria in both wines. The maximum cell count was measured in the wine, which was fermented with Lalvin Rhone 2056, eight days after bacteria inoculation. A similar effect was obtained in the samples with the addition of glutathione (40 mg/L). Glutathione contains cysteine as one moiety.

Figure 2 Growth of *Oenococcus oeni* strain OoA after addition of cysteine (20 mg/L) to two wines fermented with two different yeast strains (Lalvin Rhone 2056 [Rh] and Lalvin BM 45 [BM]) in comparison to the controls without addition.

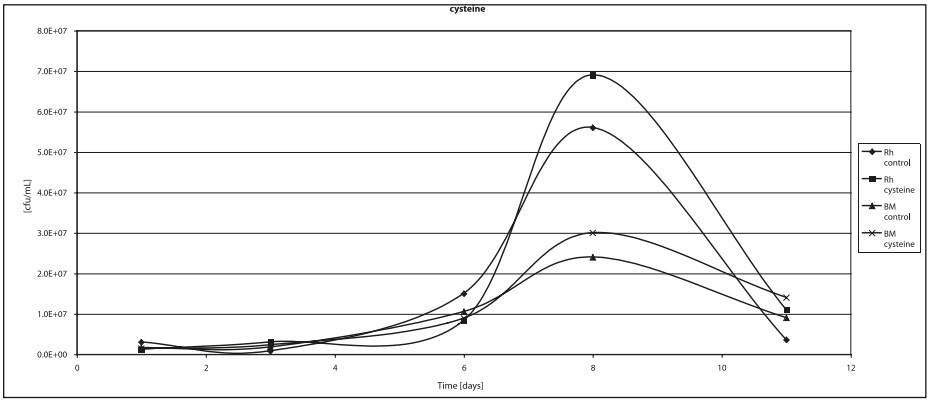


Figure 3 indicates that the highest numbers of cell-forming units were again detected in wines fermented with Lalvin Rhone 2056 to which glutathione was added.

Whereas the addition of methionine entailed rather a slight decrease of bacterial growth (Figure 4), especially in the variant fermented with Lalvin BM 45, a significantly lower number of cell-forming units was measured.

The influence on the development of lactic acid bacteria after the addition of cysteine, methionine and glutathione in combination is illustrated in Figure 5.

Figure 3 Growth of *Oenococcus oeni* strain OoA after addition of glutathione (40 mg/L) to two wines fermented with two different yeast strains (Lalvin Rhone 2056 [Rh] and Lalvin BM 45 [BM]) in comparison to the controls without addition.

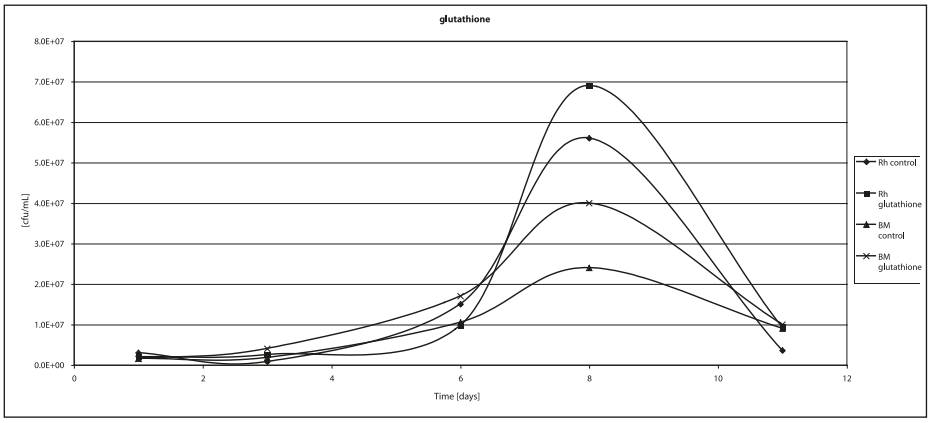


Figure 4 Growth of *Oenococcus oeni* strain OoA after addition of methionine (40 mg/L) to two wines fermented with two different yeast strains (Lalvin Rhone 2056 [Rh] and Lalvin BM 45 [BM]) in comparison to the controls without addition.

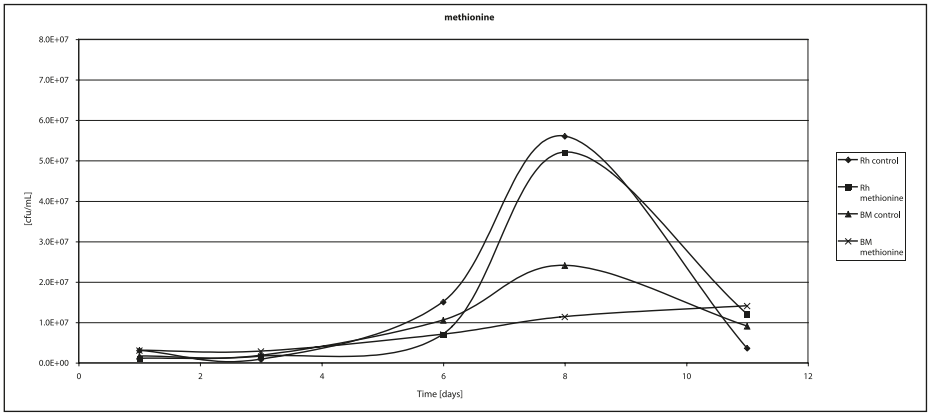
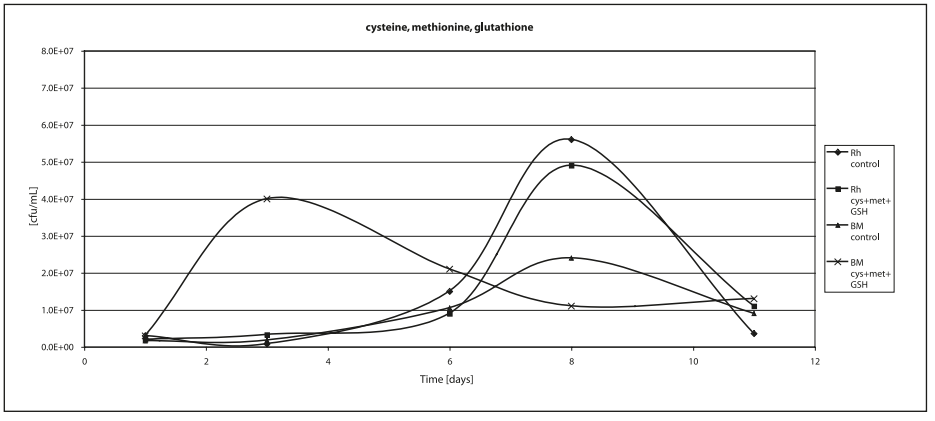


Figure 5 Growth of *Oenococcus oeni* strain OoA after addition of cysteine (20 mg/L), methionine (40 mg/L), glutathione (40 mg/L) to two wines fermented with two different yeast strains (Lalvin Rhone 2056 [Rh] and Lalvin BM 45 [BM]) in comparison to the controls without addition.



In the wine fermented with Lalvin Rhone 2056, the addition of methionine, cysteine and glutathione had a slight decrease in bacterial growth. The combination of all three compounds accelerated the development of lactic acid bacteria in the wine fermented with Lalvin BM 45. The maximum cell count was detected three days after bacteria inoculation.

Figures 6 and 7 point out that the addition of the two sulphur-containing amino acids and the tripeptide glutathione had no impact on MLF. The degradation of L-malate was not influenced by the supplements and there was also no influence of the yeast strains noticeable.

The experimental design of another trial with the variety Riesling is explained with the scheme presented in Figure 8. The must was divided into two equal parts and the alcoholic fermentation was carried out with the yeast strains Uvaferm CM and Lalvin EC-1118. Control wines without MLF were stored under the same conditions after the addition of SO_2 in a concentration of 70 mg/L to protect them from spontaneous MLF. Also in this trial *O. oeni* strain OoA (Lallemand) was used to perform MLF. The sulphur-containing amino acids and glutathione were added as single substances or in combinations with concentrations as was done in the trial with the Dornfelder must. The same concentrations of cysteine and methionine were used. Only the added amount of glutathione was reduced to 20 mg/L as shown in Figure 8.

Figure 6 Degradation of L-malate after addition of cysteine (20 mg/L), methionine (40 mg/L), glutathione (40 mg/L) and a combination of the two amino acids and the tripeptide to a wine fermented with yeast strain Lalvin Rhone 2056 in comparison to the control without addition.

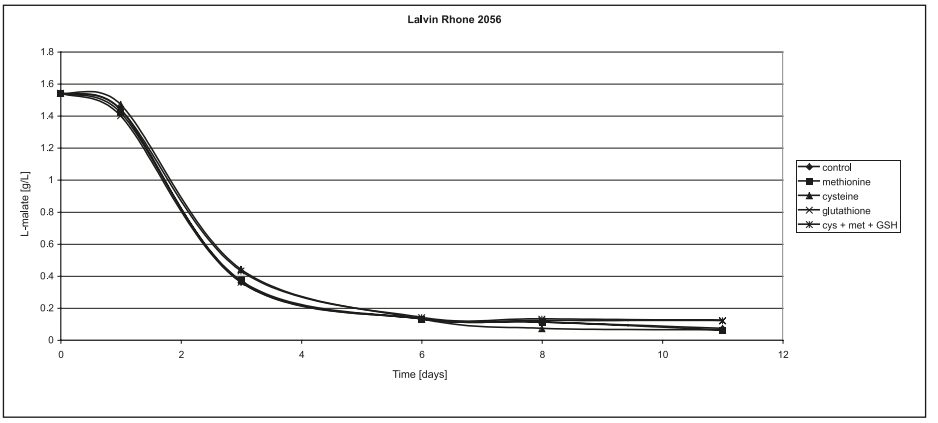


Figure 7 Degradation of L-malate after addition of cysteine (20 mg/L), methionine (40 mg/L), glutathione (40 mg/L) and a combination of the two amino acids and the tripeptide to a wine fermented with yeast strain Lalvin BM 45 in comparison to the control without addition.

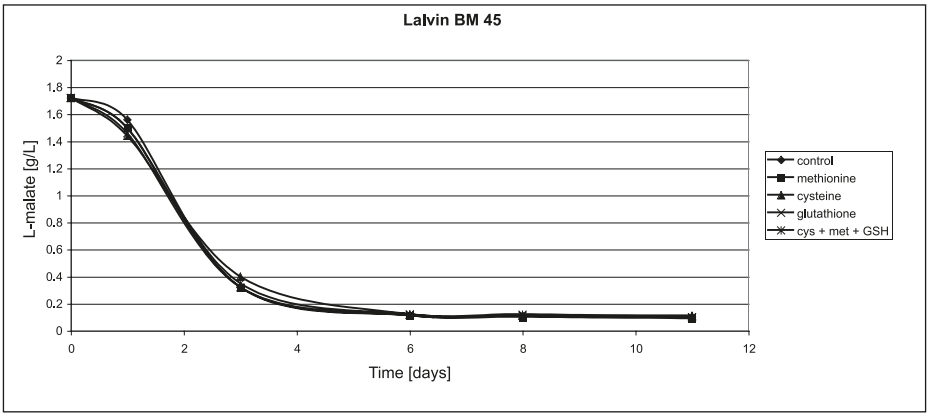


Figure 8 Experimental design of a trial with the variety Riesling.

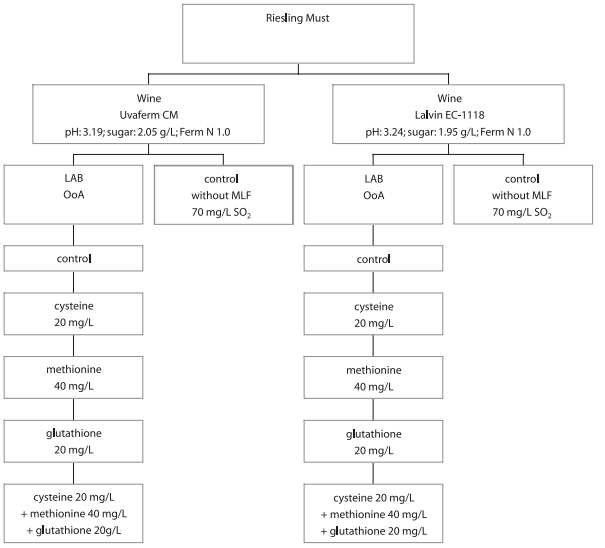


Figure 9 Degradation of L-malate after addition of cysteine (20 mg/L), methionine (40 mg/L), glutathione (40 mg/L) and a combination of the two amino acids and the tripeptide to a wine fermented with yeast strain Lalvin EC-1118 in comparison to the control without addition.

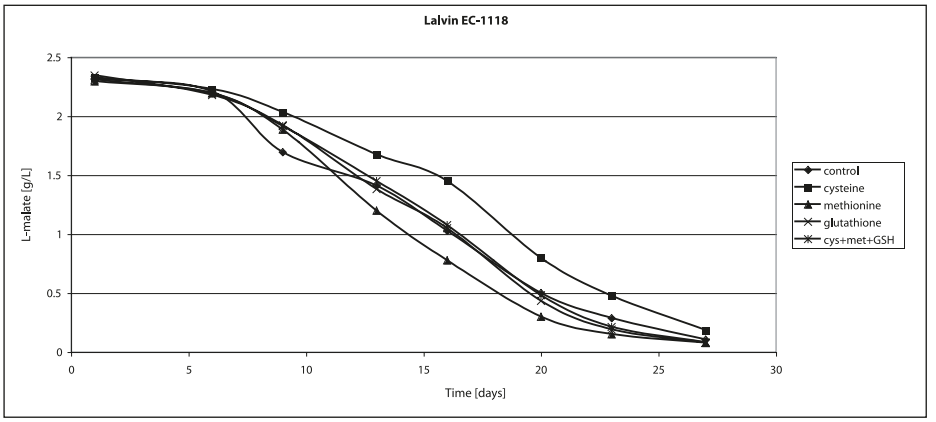
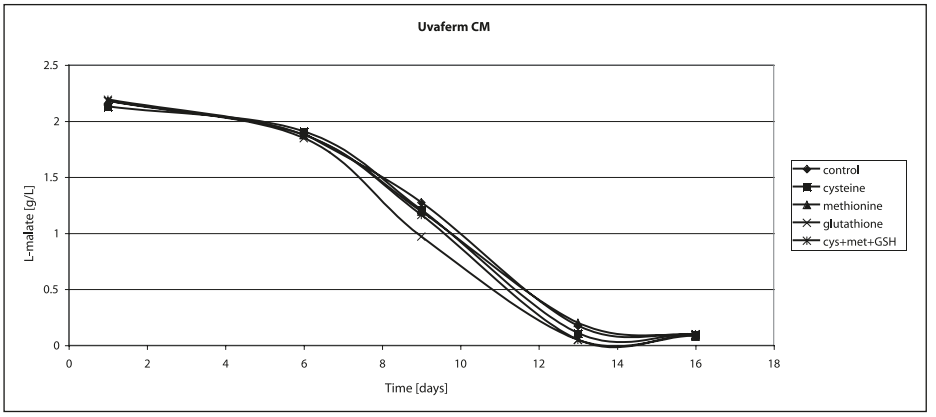


Figure 10 Degradation of L-malate after addition of cysteine (20 mg/L), methionine (40 mg/L), glutathione (40 mg/L) and a combination of the two amino acids and the tripeptide to a wine fermented with yeast strain Uvaferm CM in comparison to the control without addition.



Figures 9 and 10 illustrate that a slight effect on the degradation of L-malate clearly depended on the yeast strain used to ferment the wine and on the supplement added.

The addition of methionine accelerated MLF in the wine fermented with EC-1118. In contrast, the addition of cysteine seemed to slow down the degradation of L-malate, whereas the addition of glutathione promoted MLF a little in the wine fermented with Uvaferm CM (Figure 10).

Discussion and conclusions

Preliminary results showed that the addition of cysteine and glutathione to a fermented wine after alcoholic fermentation can promote the development of lactic acid bacteria and MLF. This effect seems to be influenced by the added concentration and by the nutrient composition of the wine, which is also affected by the nutrient requirement of the chosen yeast strain for alcoholic fermentation and by the applied strain of *Oenococcus oeni*. On the other hand, reactions of the SH-group in cysteine and glutathione with other ingredients of wine can be supposed, which probably vary the effect of their addition to the different wines.

Future research work is directed to organoleptic changes after the addition of S-containing amino acids and glutathione, as well as on the development of S-containing aroma compounds, because initial results indicate that the qualitative and quantitative composition of S-substances in wines can be changed during MLF.

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PRACTICAL IMPLICATIONS OF THE LACTIC ACID BACTERIA GENOME PROJECT: MALOLACTIC FERMENTATIONS

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Abstract

The Lactic Acid Bacteria Genome Consortium, in collaboration with the Joint Genome Institute, has generated draft genome sequences for several lactic acid bacteria. The species, sequenced in early 2002, are *Lactococcus lactis* ssp. *cremoris*, *Lactobacillus gasseri*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus* and *Oenococcus oeni*. Recently, the gaps in the draft sequences were closed and the finished genomes are currently being compiled for publication. What does this mean to the winemaker? First of all, five of these species are commonly isolated from the wine environment and several have prominent roles in the malolactic conversion. Indeed, *Oenococcus oeni* is the main species used commercially as a starter culture for the malolactic fermentation. The practical implications of this effort will come from the increased knowledge gained on the physiology (and ecology) of lactic acid bacteria in the wine environment. This will enable a more defined selection of malolactic starter cultures as well as a detailed view of how winemaking processes influence those cultures. An immediately applicable benefit emerging from the sequence data will be a new, and comprehensive, ability to discriminate malolactic strains, perhaps allowing for definition of "regional" genotypes that have arisen in various winemaking regions. An additional benefit will be the ability to identify genetic links to various spoilage characters produced by lactic acid bacteria. This will enhance our ability to identify spoilage-causing strains that emerge during wine production. In summary, public release of the genome sequence generated through the Lactic Acid Bacteria Genome Project has helped usher in a new era in the study of malolactic bacteria with both short-term and long-term benefits to the winemaker.

Genomics is a word not often used in conjunction with winemaking. Indeed, the layperson's (and many winemakers') view of winemaking is often at odds with modern technological advances such as genomics. Regardless, by providing a comprehensive view of the genetic landscape underlying various viticultural and winemaking events, genomics stands to forever change our understanding of the winemaking process. Genomics begins with the acquisition of a full genome sequence for the organism(s) in question. Other than *Saccharomyces cerevisiae*, few of the biological entities involved in winemaking have been fully sequenced. In this report I will describe a project recently undertaken to obtain full genome sequences for a number of lactic acid bacteria, important bacterial constituents in the winemaking process, and propose how this newly acquired sequence will benefit the average winemaker.

The lactic acid bacteria (LAB) play an essential role in various food and beverage fermentation processes. LAB are a group of Gram-positive bacteria that possess similar morphological, metabolic and physiological characteristics. A key metabolic distinction of this group is the rapid

production of lactic acid from sugars. Given their long history of safe human consumption, LAB are involved in the production of a range of value-added food and beverage products. The LAB perform the main bioconversion in fermented dairy products (cheese, yogurt, and fermented milk), fermented meats (sausage, fish) and fermented vegetables (cabbage, cucumbers, and olives). Most wineries use LAB to carry out the malolactic conversion, an important secondary fermentation in the production of wine [4]. In addition, LAB are involved in the production and processing of sourdough, soda crackers, silage, coffee, cocoa and tea [5, 6, 17]. These fermentations result in products of tremendous economic value and prominence in the world food supply. Moreover, all of these products are critically dependent on LAB for production.

LAB play a dual role in wine fermentations. LAB are prominent agents of wine spoilage through an impressive (and unfortunate!) panoply of mechanisms, most notably through heterofermentative production of acetic acid. A second, more amiable, role for LAB in winemaking is as the agent responsible for the malolactic fermentation. Numerous LAB can be isolated from musts or wines, including members of *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* [12]. *Oenococcus oeni*, the sole species in the genera *Oenococcus*, is currently the most favoured LAB species to carry out the malolactic conversion in wine. It possesses this status chiefly due to its superior ability to reproducibly grow in the harsh conditions of wine, to effectively carry out the malolactic conversion and to impart few, if any, off-flavours. This has led to the common use of oenococcal starter cultures to carry out the malolactic fermentations and prompted the ever-present search for new and different oenococcal isolates for certain oenological conditions and/or for specific flavour modifications.

While molecular research on LAB in general, and *Oenococcus* in particular, has advanced tremendously in recent years, relatively few genome sequences are available. Indeed at the time this project was initiated in the fall of 2000, not a single LAB genome sequence was publicly available, a significant hindrance to worldwide LAB research efforts. Public availability is a key aspect as unfettered access to whole genome sequences serves to uniformly foster research agendas and opportunities (across both corporate and national borders). To obtain genome sequence for fermentation-associated bacteria, we worked with the Joint Genome Institute (JGI), a high throughput sequencing facility run by the Department of Energy. Importantly, JGI has a policy of early public release of genome sequences as soon as the draft quality meets an appropriate internal standard, thereby ensuring rapid public access to high quality draft sequences.

We chose 11 different bacterial strains associated with fermented food, beverage and probiotic industries for genome sequencing (Table 1). The specific strains were chosen on the basis of both economic and scientific rationales. Each of the 11 strains is prominently involved with a commercial fermentation or, in the case of the probiotic strains, are added as a key component of a fermented product. The various strains also represent the breadth of the phylogenetic, morphological and metabolic diversity inherent in the LAB group. The strains are isolates from plant, dairy, fruit juice and human environments. Both thermophilic and mesophilic species are included, as well as species more tolerant to acid and alcohol environments. All three

Table 1 LABGC Project Microorganisms.

Microorganism	Strain	Genome Size	Predicted # Genes*	Investigator
Oenococcus oeni	ATCC BAA-331	1.8 Mb	1799	David Mills UC-Davis
Leuconostoc mesenteroides	ATCC 8293	2 Mb	1882	Fred Briedt USDA-ARS
Pediococcus pentosaceus	ATCC 25745	2 Mb	1673	LABGC
Lactobacillus casei	ATCC 334	2.5 Mb	2526	Jeff Broadbent Utah State U.
Lactobacillus brevis	ATCC 367	2 Mb	1573	Milton Saier UC-San Diego
Lactobacillus gasseri	ATCC 33323	1.8 Mb	1708	Todd Klaenhammer NCSU
Lactobacillus delbrueckii ssp. *bulgaricus*	BAA-365	2.3 Mb	1602	Jim Steele U. Wisconsin
Lactococcus lactis ssp. *cremoris*	SK11	2.3 Mb	2693	Bart Weimer Utah State U. Larry McKay U. Minnesota
Streptococcus thermophilus	ATCC BAA-491	1.8 Mb	1910	Robert Hutkins U. Nebraska
Bifidobacterium longum	DJ010A	2.1 Mb	2122	Dan O'Sullivan U. Minnesota
Brevibacterium linens	BL2	3 Mb	4231	Bart Weimer Utah State U.

*Computational gene prediction from the draft genome sequences

major fermentative pathways for production of lactic acid are represented (homofermentative, heterofermentative, bifidum pathway) [16]. Importantly, the collected strains span a significant phylogenetic distance, with most major phylogenetic groupings of the LAB represented (Figure 1) [1]. Genome sequences for the microorganisms will help resolve several current phylogenetic debates, including the origin of *Lactococcus lactis* ssp. *cremoris* [9], the genetic basis for the polyphasic taxonomy present in the *Lactobacillus casei-Pediococcus* group [5], and the debate on whether *Oenococcus oeni* is an example of a fast-evolving organism [18].

Figure 1 Phylogenetic tree of the food-grade LAB showing the strains sequenced as part of the LABGC project. The figure is based on similar tree in Axelsson, 1998.

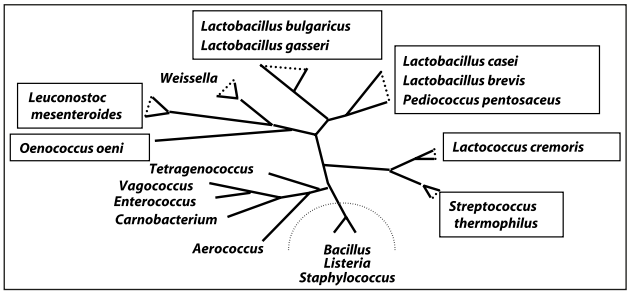
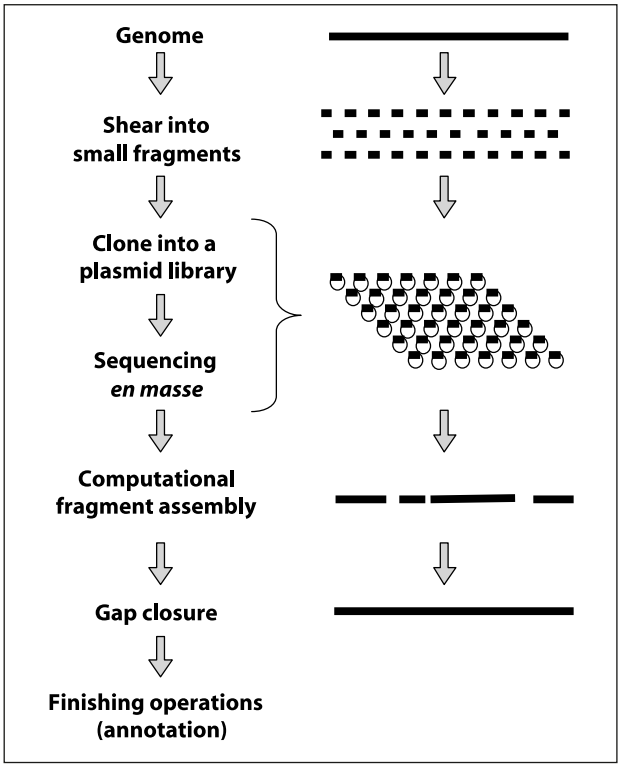


Figure 2 General steps in whole genome shotgun sequencing. For the LABGC project, the Joint Genome Institute produced a high quality draft sequence that contained some genome gaps. Gap closure is being carried out at Fidelity Systems Inc., through a specific collaboration with the LABGC.



To coordinate this effort, we assembled researchers throughout the United States into a working unit termed the Lactic Acid Bacteria Genome Consortium (LABGC). The mission of LABGC is to forward functional genomic studies on the LAB. Each LAB strain is linked to a designated investigator who was primarily responsible for gap closure and publishing of the genome sequence. During 2002 JGI generated draft sequences of the 11 genomes. This was accomplished by sequencing small-insert libraries (2-3 kb) of each microbe's DNA to achieve 10X coverage ("shotgun sequencing," Figure 2). Where possible, this coverage was supplemented with 5X coverage from large insert cosmid libraries (40 kb). The sequence data was incorporated into an assembly and then ordered into scaffolds. The draft sequence was then computationally annotated by Oak Ridge National Laboratories. This annotation can be viewed from the Joint Genome Institute Web site (http://www.jgi.doe.gov/). This Web site also allows researchers to scan through the draft annotation by metabolic pathway or functional gene categories. Moreover, specific DNA or protein searches are possible through a Basic Local Alignment Search Tool (BLAST) within the site. The LABGC group is currently working with Fidelity Systems Inc., a private company, to close the gaps of all 11 genomes, with completion expected in 2004.

What does this project mean to the winemaker?

In one sense, it could be argued that the wine industry has, and will, benefit the most from the LABGC project, as five different bacterial species sequenced can be isolated from the wine environment (*L. casei*, *L. brevis*, *P. pentosaceus*, *L. mesenteroides* and *O. oeni*). However, it will take time for the fruits of this accomplishment to directly benefit the winemaker. Perhaps the best analogy is the similar relationship between the recent completion of the human genome sequence and the average physician. During a medical check-up, your physician doesn't routinely go online and directly query the human genome sequence. However, access to the human genome sequence has fostered a number of new tests for various diseases. Thus through the creation of better diagnostic tools, the field of human genomics has greatly benefited the average physician by enhancing his ability to diagnose and, ideally, to correct disease. While this is but one simplistic example, the concept directly translates to how a winemaker may, in the future, use the fruits of the LABGC project; through the creation of better tools with which the winemaker may control malolactic fermentation.

Our ability to control and enhance malolactic fermentations requires an in-depth understanding of LAB physiology.

Consider that, to date, relatively few oenococcal genes, notably *mleA* and *mleT*, have been directly linked to the capacity of this organism to carry out malolactic fermentation #[8]. However, with access to the *O. oeni* genome sequence, researchers can use microarray technology to examine the expression of all genes in cells actively carrying out malolactic fermentation. Why is this important? Through these methods, additional genes linked to the malolactic conversion may be revealed, including specific control elements. One might predict that monitoring of key control elements will enhance our ability to diagnose both healthy and problem malolactic fermentations. In a similar vein, genome sequences from spoilage LAB (i.e., *Pediococci* and *Lactobacilli*) will advance efforts to understand taint production, as well as providing genetic markers to help us more rapidly diagnose specific taint-producing LAB. Thus, LAB genomics lays the groundwork for advances in fermentation control, both to encourage efficient malolactic conversion and to prevent LAB-based spoilage.

Another benefit will come from our enhanced ability to discriminate oenococcal strains. Strain differentiation is critical to ensure that starters are clonal prior to use in wine fermentations. In addition, precise methods for strain differentiation are necessary to better identify novel oenococcal strains with specific performance traits. Recently, several groups have examined the diversity of *O. oeni* strains isolated in and around wineries [10, 11, 14, 19, 21, 22]. Unfortunately, differentiation of strains has been difficult given the homogenous nature of the genus [20]. Regardless, studies have shown that multiple distinct strains can be isolated from the same wine undergoing spontaneous malolactic fermentation [7]. Most approaches to differentiate strains have relied on molecular survey techniques, such as RAPD or PFGE analysis. Unfortunately, these typing methods have revealed little information on actual differences in encoded gene content, particularly as it relates to strain performance.

Microarray-based strain differentiation, in which genomic DNA from a query strain is hybridized against a reference genome array made to the sequenced strain, is becoming a common method for identification of specific strain lineages [3, 13], as well as to uncover polymorphic regions within isolates [2]. Array-based differentiation is fundamentally more informative than survey methods like RAPD or PFGE, which only sample the genome at select sites. Using microarrays for strain differentiation, it will be possible to examine oenococcal diversity in great detail and gain an appreciation for the actual encoded differences in strains. For example, it is critical to determine what the common genes among most strains of oenococci are. This will help us define the core genome complement involved in growth in wine and the malolactic conversion. Do different genotypes emerge from different regional or environmental conditions? Arrays can be used to determine if oenococcal isolates obtained from different parts of the world truly possess significant genome differences and help reveal what these differences encode. In this fashion, array information on the exact gene-by-gene comparisons between strains will provide a more definitive measure of oenococcal diversity, and thereby enhance understanding of the relationship between a strain's performance and its underlying genome.

In summary, completion and publication of the genome sequences in the LABGC project will be a significant milestone in LAB research impacting researchers worldwide. In the context of modern biology, genome sequence provides the necessary template that advances research tremendously, revealing new insights into the physiology and ecology of the target organisms. By providing public access to a number of wine-related LAB genomes, the LABGC project will similarly advance wine research. This in turn will benefit the winemaker through the enhanced understanding of starter culture performance, as well as through identification of new robust starter strains.

Acknowledgements

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PERCEPTION BY WINE DRINKERS OF SENSORY DEFECTS CAUSED BY UNCONTROLLED MALOLACTIC FERMENTATION

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Summary

To carry out this study, additions of chemical compounds that are often generated by undesirable microbial metabolism during uncontrolled malolactic fermentation were made to a white wine made from Viura grapes and a red wine made from Tempranillo grapes. The additions ranged from a minimum concentration equivalent to the threshold of detection, up to the maximum concentration found in wine. The wines were then submitted in random order to a panel of tasters under blind tasting conditions. The panel was made up of wine drinkers, who had previously been informed about the risks of microbiological contamination during winemaking, in particular at the time of malolactic fermentation. The tasting also included control wines to which no additions had been made. In order to validate the defects found by the non-professional panel, the panel's findings were compared with those from an evaluation of the same samples carried out by an expert taster, who is totally blind.

Introduction

Wine is one of the oldest products in which microbiological processes make an important contribution to final product quality. The development of wine has been a well-known process for centuries, but new developments, in particular biological treatments, are more and more accepted and used in the cellar. The principal reason for this increased acceptance is that it is increasingly necessary to meet requirements specific to the market for wine, particularly in terms of quality. To meet this goal, certain conditions must be met:

- The contribution of certain chemical constituents to the sensory profile of wine must be managed. The amount of sulphur dioxide that is used must be optimized, particularly during malolactic fermentation (MLF).
- Limitation of components that may cause a risk for health: it is essential that the consumer sees wine as a safe, healthy beverage. Wine production processes must ensure that minimal quantities of biogenic amines (in particular histamine) and ethyl carbamate are present.
- Development and preservation of the general quality of the wine throughout its commercial life: this condition requires stabilization of aromatic and polyphenolic structure, as well those compounds responsible for wine colour.

Another interesting aspect of the market for wine is that product preferences can change very quickly. For example, R. Klein stated in 1997, "The taste of wine drinkers has changed over time, the majority of consumers prefer a fruity white wine with moderate acidity." This description applies quite well to currently preferred white wine styles. This is why the control of MLF is increasingly important in many types of wines.

Bacteria in wine - the dangers

One of the principal problems that bacteria can cause in wine is a rise in volatile acidity. An excess of diacetyl can also cause reduction of fruit character. Certain types of lactic acid bacteria are also able to produce undesirable flavours and tastes. Another risk of uncontrolled MLF is significant loss of colour, due to the enzymatic activity of the bacteria as well as an increase in pH. The production of histamine and ethyl carbamate, which are harmful to human health, is largely influenced by MLF. The organisms responsible for all these problems are some strains of *Oenococcus* and many strains of *Lactobacillus* and *Pediococcus*.

Wine defects that can result from lactic acid bacteria metabolism include the following:

- Lactic prickle: This fault appears under conditions favourable to the development of bacteria when sugar remains in the wine, such as slow or stopped fermentations. Sugars with six carbon atoms are transformed into ethanol, acid acetic and CO_2. The D isomer of lactic acid appears in the wine, whereas the MLF produces L-lactic acid. This phenomenon is caused by lactic acid bacteria.

- Bitterness: Decomposition of glycerol to acrolein. The combination of acrolein with tannins produces a very unpleasant bitter flavour on the finish of the wine.

- Production of volatile phenols: In red wines, 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol can form. These compounds contribute aromas similar to stables and horse sweat. The appearance of these compounds can be related to the action of certain strains of *Pediococcus* and *Lactobacillus*, although the microorganisms most frequently responsible for these defects are contaminant *Brettanomyces / Dekkera* yeasts.

- Mousiness: Some strains of the heterofermentative species of *Lactobacillus* and *Oenococcus oeni* can produce aromatic heterocyclic bases that cause a distinctive mousy flavour. This defect can also be generated by *Brettanomyces / Dekkera* yeast.

- Lastly, deterioration can occur that affects the public health status of the wine. The bacterial metabolism of arginine, for example, produces citruline and carbamylphosphate. If this latter material reacts with the urea that is produced by some yeasts during alcoholic fermentation, ethyl carbamate can be formed. This material is harmful to human health and is controlled by strict legal limits in many wine markets. The decarboxylation of specific amino acids can also lead to the presence in wine of various biogenic amines (histamine, putrescine, cadaverine, etc.), which, similar to ethyl carbamate, can be harmful to human health.

Bacteria in the wine, the positive contributions

The most obvious positive contribution of bacterial growth in wine is the reduction in acidity content, in particular of malic acid. The production of ethyl lactate, diacetyl and other substances by bacteria can also be favourable, as these compounds can add to aromatic complexity. In the same way, enhancement of varietal aroma, which has been demonstrated by certain authors (C. Gerland, 1999), the reduction of vegetal notes and the reduction of astringency and bitterness on the finish are other positive effects that bacterial growth can sometimes contribute. In certain cases, palate roundness and the expression of tannins are increased. Thanks to bacterial consumption of acetaldehyde, a phenomenon that is observed during the growth of certain strains (R. Mira de Orduña, 2001), bound SO_2 concentration is reduced, which makes it possible to decrease the amount of SO_2 that must be added. The causative organisms for these positive contributions are certain strains of lactic acid bacteria of the *Oenococcus* genus. This is why it is important to undertake inoculation of selected bacteria to obtain quality wines, which gives enhanced control of biological changes.

Organoleptic impact of MLF

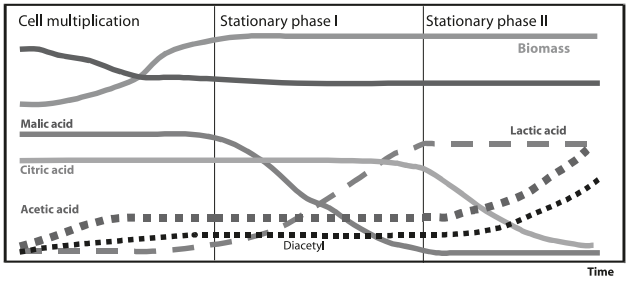
Wines that have completed MLF are generally evaluated similarly in tastings and given such positive descriptors as butter, nutty, yeast, honey, vanilla, leather, spices, earthy and toasty, with more body and roundness, silky tannins and greater length on the palate. On the other hand, uncontrolled malolactic fermentation can lead to the use of negative descriptors, like intense lactic aromas, acid yogurt, sweaty notes, acetic, intense bitterness on the finish and animal notes. The impact of diacetyl on the aromatic profile of wine is very variable. The aromas that it contributes can be very different, depending on its concentration. At levels of 5 to 14 mg/L, a buttery aroma is contributed, while at 2 to 4 mg/L, diacetyl confers nutty, caramel, yeast and wet peel notes. The detection threshold is higher in red wines than in white. This is why red wines can contain higher diacetyl concentrations without apparent negative impact. Bacterial consumption of citric acid contributes to diacetyl formation. Therefore, it is possible to manage the concentration of this acid to obtain appropriate levels of diacetyl for the intended wine style.

Metabolism of MLF

The evolution of bacteria in wine during MLF is characterized by three well differentiated metabolic phases (Figure 1).

1. Cell multiplication: Use of the sugar in the medium as a source of energy. No consumption of malic or citric acids is observed but production of acetic acid can be noted.
2. Stationary phase I: The bacteria do not use sugar. Malic acid is transformed into lactic acid, and there is neither degradation of citric acid nor production of acetic acid.
3. Stationary phase II: There is no degradation of sugar or catabolism of malate, but the bacteria consume citric acid to produce acetic acid and diacetyl in excess. This phase can be avoided in winemaking by employing SO_2 and lysozyme.

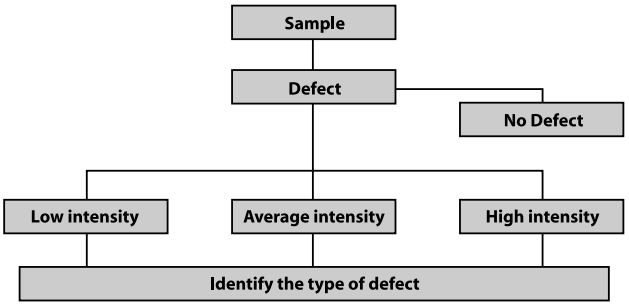
Figure 1 Evolution of different metabolites during MLF.



Materials and methods

Twenty-two wines were tasted during this study: two controls and 20 wines modified by the addition of various concentrations of diacetyl (white wine: 0.1 ppm, 5 ppm, 10 ppm; and red wine: 0.1 ppm, 10 ppm, 30 ppm), of volatile biogenic amines (putrescine and cadaverine in the red wine, in concentrations of 1 ppm, 10 ppm, 50 ppm and 100 ppm) and of ethyl phenols (2-ethyl-phenol and 2-ethyl-guaiacol in the red wine, concentrations of 425 mg/L, 800 mg/L and 1000 mg/L). The wines were arranged in six series according to these compounds (DB: diacetyl in white wine; DT: diacetyl in red wine; P: putrescine; C: cadaverine; EF: ethylphenol and EG: ethylguaiacol). The samples were presented to a tasting panel made up of 24 wine drinkers, who had previously been informed (by a short course of about 30 minutes) of the risks of microbial contamination during winemaking, and how it can generate the appearance of sensory defects. The results were collected by means of a questionnaire (Figure 2). These data were compared with the opinion of a professional taster, who is completely blind.

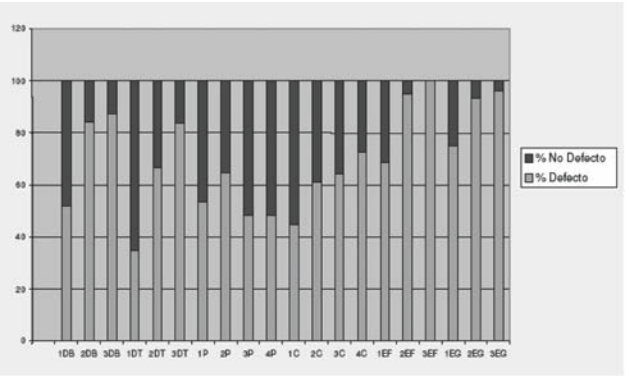
Figure 2 Questionnaire used during tasting.



Results obtained

The tasters perceived a high frequency of defects, which they identified using descriptors of their own choosing. In the case of the ethylphenols and diacetyl, the frequency of perception increased as the concentration of the added material increased, more for the white wine than for the red. This relationship was less obvious in the wines to which biogenic amines had been added (Figure 3). The defects that were easiest to identify were those caused by the additions of ethylphenols and diacetyl in the white wine.

Figure 3 Frequency of detection of the defects by the tasters.



No Defecto = No defect **Defecto** = Defect

The defects identified in the wines to which diacetyl had been added are different according to whether diacetyl was present in white or red wine. This also affected the frequency of detection of the defect. For the white wine, 31% of the tasters identified a defect but could not define it. The most frequently observed defects were described as buttery flavour, cheese-like notes and a tendency to appear oxidized, as if the wine was more advanced in age. For the reds, the frequency of non-identification reached 43%. Among the identified flavours, the most common were almond butter notes (Figures 4 and 5).

Figure 4 Descriptors for white wine with diacetyl.

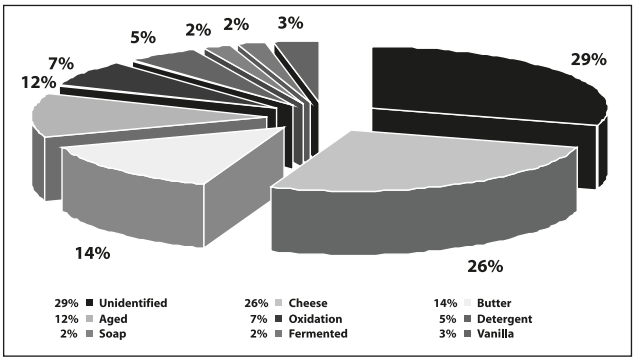
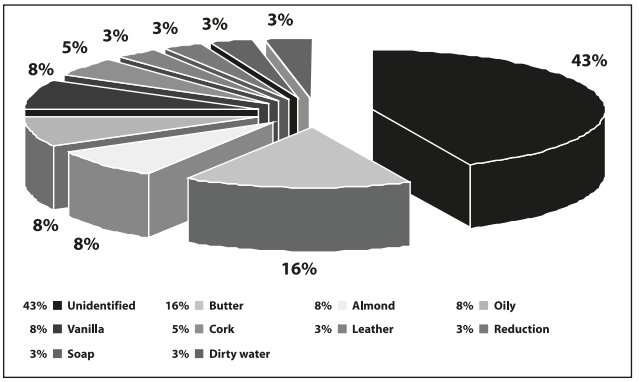
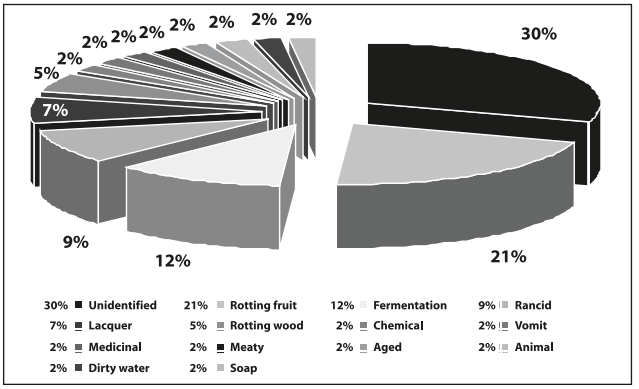


Figure 5 Descriptors for red wine with diacetyl.



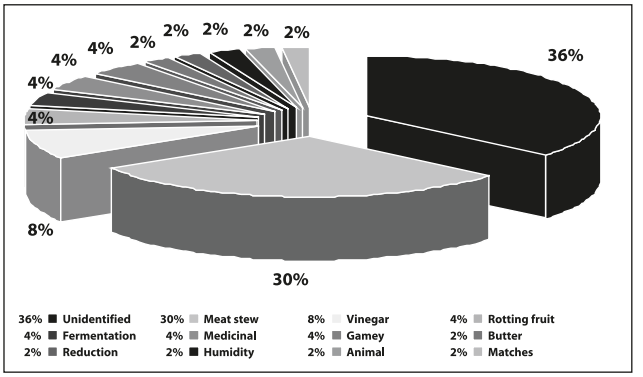
In the red wine modified by putrescine addition, no relationship was found between increasing concentration and better identification of defects; 30% of the tasters found a defect, but could not describe it. The words most used to describe the wines with added putrescine were rotten fruit, the sensation of fermentation, rancid and dirtiness (Figure 6).

Figure 6 Descriptors used for red wine with added putrescine.



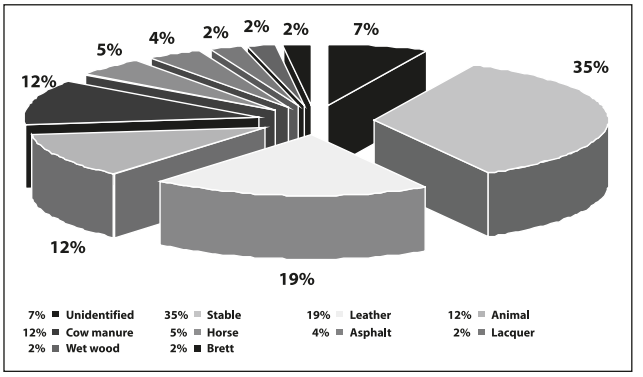
When the added biogenic amine was cadaverine, a tendency towards better identification of the defect with increasing concentration in the wine was noted; 36% of the tasters in the panel found a defect without managing to describe it. The most often used descriptors were meaty, vinegary and dirty (Figure 7).

Figure 7 Descriptors used for red wine with cadaverine.



The identification of problems by the tasters proved much easier in the wines to which ethylphenol had been added. A close correlation was apparent between increasing concentration and identification and description of the defect. In the case of 2-ethylphenol, only 7% of the tasters did not find descriptors to qualify the problem. The most often used descriptors were animal stable, leather, animal aromas, cow manure, horses and asphalt (Figure 8).

Figure 8 Descriptors for red wine with 2-ethylphenol.



For wines with added 2-ethylguaiacol, the frequency of perception of a defect by tasters who were unable to find a suitable term to describe the nature of the aroma or taste reached 31%. The recurring descriptors used to identify this problem were mould, medicinal and smoke (Figure 9).

Figure 9 Descriptors of red wine with 2-ethylguaiacol.

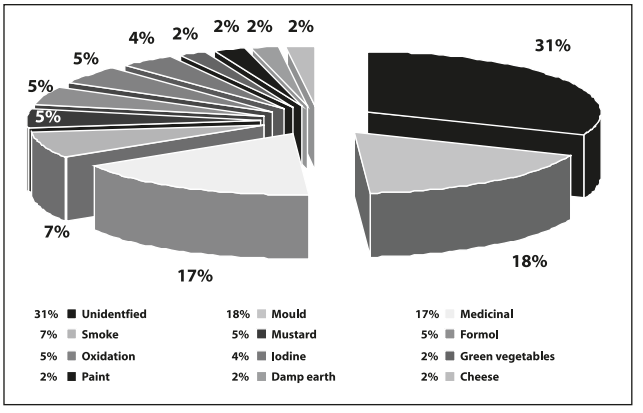


Table 1 Types of defects found and level of intensity allotted by the tasters.

Sample	Type of Defect	Intensity			No Defect	Defect	No Defect
		Low	Average	High			
1DB	Cheese III; Oxidized; Unidentified II; Soap; Butter	******	***	*	********* *****	13	14
2DB	Unidentified III; Oxidized I; Butter III; Aged; Yeast; Cheese; Detergent I	*****	****	********	*******	37	7
3DB	Lactic I, Unidentified IIIII; Gas; Aged III; Butter; Cheese III; Vanilla	********	******	*****	*****	35	5
1DT	Unidentified III; Bitter Almond; Soap	*****	**		********** ******	9	17
2DT	Unidentified IIIIII; Oil; Cork; Butter, Bitter Almond; Vanilla; Leather	*****	*******	*	**********	22	11
3DT	Unidentified IIII; Oil; Cork; Butter IIII; Reduced; Dirty water; Bitter Almond; Vanilla I	****	*******	******	*******	36	7
1P	Unidentified III; Chemical; Rot; Rotting fruit I; Vomit; Lacquer I	*****	****	*	********** ***	16	13
2P	Unidentified IIII; Rancid; Wet wood; Rot; Rotting fruit; Solvent; Fermentation I	*******	*****	*	**********	20	11
3P	Rancid I; Fermentation II; Medicinal; Meaty; Varnish; Unidentified	*****	***	*	********** ****	14	15
4P	Aged; Wet wood; Immature; Goat; Dirty water; Rot; Rotting fruit; Soap; Unidentified	*******	***		********** ***	13	14
1C	Unidentified II; Humidity III; Stew	***	*****		********** *****	13	16
2C	Unidentified I; Humidity III; Stew; Nail polish, Matches; Fermentation I; Butter	*******	***	**	**********	19	12
3C	Unidentified IIIII; Humidity III; Reduction; Overripe fruit; Vinegar	******** ***	**	*	**********	18	10
4C	Unidentified III; HumidityI; Game I; Vinegar II; Medicinal; Overripe fruit; Cheese	*******	*******	*	*********	24	9
1EF	Box; Unidentified III; Leather; Animal I; Stable II; Lacquer; Sweat	*****	*****	***	**********	24	11
2EF	Stable IIIIIII; Asphalt I; Leather III; Goat sweat; Animal II; Cow manure II	*	****	********** ******	***	57	3
3EF	Stable IIIIIIII; Leather IIIII; Goat sweat; Cow manure III; Animal I; Brett	*	*****	********** ********		65	0
1EG	Unidentified II; Oxidized; Medicinal II; Smoky; Cheese; Formol I; Iodine	***	******	*****	**********	30	10
2EG	Unidentified IIIIIII; Oxidized; Medicinal I; Smoky; Mould II; Green vegetables; Mustard II; Formol	*******	*******	*******	***	42	3
3EG	Unidentified IIII; Oxidized; Medicinal IIII; Smoky I; Mould IIII; Paint; Earthy, Formol	****	*********	*********	**	49	2

Table 1 lists the frequency of the detection of defects and the intensity that was allotted to them by each taster for each wine, and the descriptors that the panellists chose to describe the defect. Each defect is scored according to the frequency of detection and the intensity noted by tasters. For low intensity, the number of defects detected is multiplied by one. For average intensity, a factor of two is used, and for high intensity, the number of detected defects is multiplied by three.

To corroborate the tasting by the consumer panel, composed of people informed about the risks of microbial spoilage during winemaking, the same wines were tasted by an expert professional taster, a specialist in oenology who is totally blind. The taster was selected because of his particular ability to detect sensory problems in wines. The descriptions given to the wines by this taster are as follows.

- 1BD: Aromas of menthol, very pleasant, orange candy and strawberry. Syrupy aromas. Notes of milk and fresh cheese, soft butter and almond. A little fat on the nose, but pleasant due to its syrupy nuances. On the palate, sour, fruity with a buttery retronasal character.
- 2BD: Clean aroma, vanilla and cheese, with garlic and rancid butter characters, old shoe. These characters can be seen behind the thyme notes and the vinous character. The mouth is denser, with a sweeter sensation than the preceding wine. The lactic retronasal character is as obvious as it is on the nose, similar to boiling milk and goat's cheese, a little dirty.
- 3BD: Intense nose, dominated by butter and aromas of yeast, bread crumb, milk cake, bread crust and butter fat. Wheat notes and aromas of oxidation; like varnish. On the palate, buttery, with a hint of bacon and rillettes. Very lactic retronasal characters.
- 1TD: Aromas of sweat at first on the nose, fresh cheese, wet and dirty rags, hot milk, fresh cream. Dried fruit, but also spicy. Unctuous on the palate, with grilled notes and burnt bread. Retronasal characters of cream and liquor, cream and whisky.
- 2TD: Aromas of raspberry and vanilla yogurt on the nose, also burnt wood, it seems to have been matured in oak. Flavours of red licorice, boiling milk, cream Catalan, burnt sugar. Honey aromas become dominant when the wine is agitated. Palate is well developed, lactic retronasal characters, natural yogurt. The lactic notes dominate with time in the mouth.
- 3TD: Boiling milk aromas at first on the nose, with natural yogurt and honey. Lactic flavours are very dominant and cover everything else. Palate shows dominant lactic characters, which are reminiscent of curd. Finishes with notes of milk coffee, mocha and cocoa.
- 1P: Floral and strawberry aromas, spicy notes with a hint of humus. Meaty notes, reminiscent of black pudding, wet ground, earthworms. Salty flavour, saltpetre, crab and seafood notes, but also of freshly caught fish. Characters of rice, seafood paella and molluscs.
- 2P: Very mature fish dominates at first on the nose, with fruits in syrup, red licorice, tree bark. Notes of wool and cotton sweater. Aromas of salted and smoked cod, fish scales. On the palate, flavours of overcooked rice. Very unpleasant yeast notes, burnt rice. Rotting vegetation retronasal character.
- 3P: Clearly spicy at the start, the flavours of this wine become very unpleasant, of manure and rubbish. Very difficult to assess palate, vomit in mouth and a stinking retronasal character.
- 4P: Intense spice at first on nose. Geranium flower characters, wet plaster. Wet cork bark aromas, mould. Manure, rubbish truck, nappies. On the palate, animal manure, cattle shed.
- 1C: Meaty and spicy notes on the nose, human sweat, armpit, freshly cut hair. Slate mineral characters, pebbles. Aromas of dead rose petal, starting to rot. Reminiscent of cockroach. On the palate, very modified, very meaty, like veal stew with peas. Retronasal characters similar to insecticide.
- 2C: Aromas of plaster or wet cement freshly prepared in water. Aromas reminiscent of a hairdressing salon, with lacquer and acetone. Aromas of stagnant water, wet cave with mushrooms. Dirty cloth, sulphur notes similar to mercaptans. Very aggressive on the palate due to its meaty characters. Burnt retronasal characters, with very marked ash and mushroom.
- 3C: Dry plaster on the nose. Kitchen aromas, cooked chickpea. Old meat, strong humidity, broom dust, dirtiness. Salty water sensation on the palate, with earthy, aggressive tannins. The intensity of the problem increases with time in the mouth. Retronasal characters of lacquer and hair gel.
- 4C: Aroma dominated by human hair, wig, burnt hair. Dog-like notes and dirty brooms. Perception of volatile acidity. Anchovies marinated in vinegar. Very aggressive on the palate, with notes of dusty brooms and hairdressing products. Vinegar marinade retronasal characters, with rabbit or marinated partridge.

- 1EF: Strawberry candy sensations at first on the nose, syrupy soft sensation, mint and herb notes. Perception of decomposed yeast, rancid yeast and rancid fat. Metallic aromas similar to nails. Earthy, stale sewer water flavours, cattle shed. Palate has very aggressive tannins, metallic, reminiscent of wet iron, rusted nails and cleaning rags.
- 2EF: Aromas of plastic paint, varnish, tracing paper, wet paperboard, butane, synthetic plastic, bovine aroma, cow skin, cowhide, cattle shed and horse manure. On the palate, objectionable tannins, bitter, like rusted iron filings. Notes of tinplate and horse saddles, shoe leather and bitumen.
- 3EF: Horse sweat, dominance of animal and bitumen aromas, shoe. Human sweat, human armpit, very unpleasant. On the palate, reminiscent of horse manure and anthills. Very sour, formic acid, characters resembling ants.
- 1EG: Fruity aromas, very ripe banana, decomposing banana skin. Mould, mushroom, iodine aromas. Aromas of Mercurochrome, dentist's anaesthetic. Spicy on the palate, clove and bay tree. Very bitter, with bitter almond flavours.
- 2EG: Very spicy at first on the nose, clove and bay tree, banana liqueur. Intense aroma of liquid anaesthetic, pharmaceutical and medicinal notes, syrup, effervescent aspirin, ether. Mushroom and mould, wet straw and fresh hay. Iodine notes. Very medicinal on the palate, with syrup taste. Finishes with rubber, decomposed cork, rotten tree bark.
- 3EG: Very intense spiciness at first on the nose, accompanied by flavours of mycelial mushroom and green mould. Aromas of ash and wet earth. Smoky notes, hot tire, gum, car after braking. Musty taste very perceptible, which is reminiscent of yeast bitterness combined with a dominant iodine character.

Conclusions

- A consumer who is well informed of the risks of microbial problems in winemaking is able to detect sensory defects present in wine that are related to chemical compounds that can be generated by an uncontrolled malolactic fermentation. In spite of a lower sensory acuteness and frequency of detection, the descriptors chosen and used by the panel to define the aroma and flavour defects found in the wines were similar to and agreed with those used by a professional taster, who evaluated the same wine samples.
- Therefore, when they are involved in a tasting exercise that focuses on perceived sensory characteristics, regular wine drinkers are able to discriminate and distinguish between defective wines that have been affected by microbial problems and unaffected controls.
- These organoleptic defects can be avoided by controlling MLF through inoculating wine with selected bacteria and avoiding the presence of contaminants by maintaining hygienic conditions in the cellar during the winemaking process.

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MALOLACTIC FERMENTATION WITHOUT CONTROL… THE MASKS

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During the presentations at the *XVIes Entretiens Scientifiques Lallemand*, it was clearly seen that lactic acid bacteria also produce several other compounds besides lactic acid that are classed as secondary metabolites. These secondary metabolites can create complexity and influence the quality of the wine. They include ethyl lactate, diacetyl and other aromatic compounds. Wines that have undergone malolactic fermentation generally acquire characters described as lactic, smoky, spicy and reminiscent of dried fruits, with more body, enhanced tannins and better length on the palate.

However, instead of improving the wine, lactic acid bacteria can sometimes carry out undesirable changes. During wild malolactic fermentation carried out by indigenous *Lactobacillus* and *Pediococcus*, sensory deterioration can occur. In particular, excessive volatile acidity or diacetyl can be formed. These compounds are not only likely to mask the primary fruit flavours of the wine, they can contribute undesirable characters to the bouquet. For example, acetic acid is prickly on the nose and causes sourness. Diacetyl contributes an aroma giving complexity at low concentrations, resembling butter at medium level but at higher concentrations, diacetyl contributes nutty flavours or characters resembling caramel, yeast and even wet fur. The undesirable aromas and flavours generated by the growth of spoilage organisms can produce characters similar to acid yogurt, rancid butter and sweat. Often, odours resembling a horse stable in red wines and antiseptic in whites can be formed, due to the presence of volatile phenols. These characters can sometimes be accompanied by bitterness and metallic tannins on the palate. Uncontrolled growth of microorganisms is also likely to generate secondary compounds that are harmful, including such allergens as biogenic amines (histamine, putrescine, cadaverine) and such potentially carcinogenic compounds as ethyl carbamate and ochratoxin A.

A kit (Figure 1) was developed by Lallemand to evaluate the impact of those compounds in a specific wine selected by the winemaker. By adding to a selected wine different secondary compounds produced by wild bacteria that have been placed in test tubes, the winemaker can "smell" the consequence in wine. The kit consists of nine tubes containing aroma active compounds known to cause defects due to non-controlled MLF. The following compounds are included in the kit: putrescine, cadaverine, diacetyl, ethylphenol, ethylguaiacol, acetaldehyde, ethyl lactate, 4-hydro-pyridine, etoxy-hexan-dien. Table 1 describes the aroma impact of each compound.

Figure 1 Photo of the test tubes containing the aroma defects produced by non-controlled MLF.

Compounds	Sensory sensation	Concentration added to the test wine (final concentration in a 25 mL sample)	Concentration normally found in normal wine"
Putrescine	Putrefaction	100 mg/L	10 mg/L
Cadaverine	Rotten meat	100 mg/L	5 mg/L
Diacetyl	Buttery notes	30 mg/L	7-10 mg/L
Ethyl phenol	Horse sweat	1 mg/L	0.15 mg/L
Ethyl guiaicol	Burnt tire	1 mg/L	0.1 mg/L
Acetaldehyde	Green apple	50 mg/L	40 mg/L
Ethyl lactate	Milky	25 mg/L	15 mg/L
4-hydropyridine	Mousy taint	Not measurable[1]	0.05 mg/L
Etoxy-hexan-dien	Geranium	0.01 mg/L	0[2]

[1]The methology used to introduce this compound to the wine does not permit to know the real final concentration. This has to be considered as a indication.

[2]Etoxy–hexan-dien is formed from the metabolism of the sorbic acid by lactic acid bacteria. This compound could be used in wine for its antifungal properties (maximum dosage allowed=200 mg/L).

The goal of the kit is to educate winemakers and wine specialists on the potential risks associated with an uncontrolled MLF where wild lactic acid bacteria are producing the above compounds. The impact of those compounds on the final products represents a quality issue as the consumer (see article by Palacios et al.) can detect the aroma defect and, in some cases, identify it accurately. Consequently, it is important that winemakers be aware of these issues. It is better still to prevent any microbial contamination of the wine by controlling the malolactic fermentation. This is possible, by maintaining good hygienic conditions in the cellar, and by employing bacteria selected to carry out malolactic fermentation. These simple measures prevent the presence of microbial contaminants in the wine and, consequently, its deterioration.

DO MALOLACTIC BACTERIA INFLUENCE WINE QUALITY?

ROUND TABLE DISCUSSION

DO MALOLACTIC BACTERIA INFLUENCE WINE QUALITY? ROUND TABLE DISCUSSION

Tim ATKIN

Master of Wine, U.K.
Moderator
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Tim Atkin is one of Britain's leading wine writers and an internationally recognized expert on the subject. He is the wine correspondent of *The Observer*. He also writes for *Decanter*, *WINE International*, *Woman and Home*, and *Observer Food Monthly*. He is the winner of many awards, including the Glenfiddich Drink Writer Award in 1988, 1990 and 1993, and the Wine Guild of the United Kingdom's Wine Columnist of the Year in 1991, 1992, 1994 and 1996. In 1994, he was the first recipient of the Wines of France Award. The following year he was the co-winner of The Bunch Award, described by Auberon Waugh as the "Booker Prize of wine writing," and the winner of the Waterford Crystal Wine Correspondent of the Year Award. In 1999, 2002 and 2003 he was named Lanson Wine Writer of the Year.

Participants

Pedro Aibar
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Pedro Aibar is an agricultural engineer and an oenologist, and his responsibilities are mostly in the areas of winery design and wine development. He first worked in the old Somontano Cooperative, at the beginning of the Somontano area development. He then worked in the Viñas del Vero from its beginning, followed by the development of the Clarión and Gran Vos wines, as well as the most recent Secastilla and Blecua wines. Presently, he is working at the Viñas Del Vero winery, which produces 7 million bottles per year. They grow their own grapes and produce various styles of wines, using microoxygenation, spontaneous MLF in barrels and a variety of oenological processes.

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One of the most renowned professors at the Catholic University in Chile, Edmund Bordeu is also a famous winemaking consultant in South America. He was awarded the Viticulture and Winemaking merit by the Association of Engineers in Agronomy and Oenology in Chile, and more recently, the Recognition of Excellence by the Faculty of Agronomy and Forest Engineering at the Catholic University in Chile. Dr. Bordeu's current projects include the development of modern processing and managing techniques for better security and quality in winemaking.

Jeff Brinkman
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Jeff Brinkman is the chief winemaker at Husch Vineyards in Mendocino County, California. They very successfully use Alpha MLB in their wines. He will do all in-barrel malolactic fermentations that are inoculated at the end of alcoholic fermentation. They use selected malolactic bacteria cultures in reds, mostly to ensure they obtain a good quality clean wine.

Joao Correia
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Joao Correia is the technical director of viticulture and oenology at Companhia das Quintas in Portugal. They have wineries in different regions

of Portugal, including Douro, Beiras, Ribatejo, Bucelas, Alentejo and Setúbal, producing 2 million litres of wine, with 70% red and 30% white. Malolactic fermentation is not done in the whites to retain freshness and acidity. However, MLF is done in the reds with spontaneous fermentation.

Dominique Delteil
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As scientific director, Dominique Delteil does external and internal consultation in oenology in the south of France. He prepares training, does analysis and gathers scientific information for the ICV, as well as in wineries in Europe and abroad. He also coordinates the research and development aspects of ICV. He selected the malolactic bacteria called Elios 1, and has 20 years of experience in wine yeast selection. In addition of his research, Mr. Delteil consults for experimental and private wineries where he can do trials and applied fermentation management.

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A wine technologist, Sam Harrop holds a bachelor of Commerce degree from Auckland University and a diploma in Viticulture and Oenology from Lincoln University in New Zealand. He was a winemaker at Villa Maria in Esk Valley, New Zealand, and then a winemaker consultant in California. He was in Australia as a winemaker for Rosemount Estate in Hunter Valley. He has been a winemaker/technologist at Marks and Spencer in the United Kingdom for the past seven years. He obtained his Master of Wine in 2003. Now a private consultant, he also owns a winery in the south of France, Domaine Matassa.

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Glen James is senior winemaker/ winery manager at Penfolds, responsible for winemaking and production at Penfolds 40,000T Barossa Winery. They source their fruit from the Barossa Valley, Clare Valley, Eden Valley, Adelaide Hills, McLaren Vale and Langhorne Creek regions. They do malolactic fermentation in reds (Shiraz, Cabernet, Grenache, Mourvèdre, Sangiovese and Dolchetto, etc.). The majority of their wines are inoculated with yeast and bacteria.

Gernot Limbach
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Gernot Limbach is the oenologist at Henkell-Söhnlein in Wiesbaden, one of the largest sparkling wine producers in Germany, producing many millions of bottles of sparkling wines, and high quality still wines. *Oenococcus oeni* is used in the winemaking process to improve the quality of wines, as well as for safety.

Hélène Mingot
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Hélène Mingot obtained her National Diploma of Oenology in Toulouse. In 2001, she extended her experience, working alongside Professor Denis Dubourdieu in the winemaking process of white, red and sweet wines, such as Sauternes. In 2002, she joined the Gruppo Matura as assistant winemaker. Gruppo Matura is a consulting group based in Florence. They have a winemaking team of 12, working mostly in Tuscany, Umbria, Emilia and Romagna.

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David Molina is a sommelier from the Barcelona Sommelier School. He has worked in several famous restaurants in Barcelona. Part of the selection in Premier of Grand Cru Classé (Bordeaux), he was also a finalist in Gold Nose (Madrid) each year from 1997 to 2001. He worked at the Viniteca (Barcelona) as an adviser for clients and wine purchases. He is also a teacher at the Barcelona Sommelier School and gives courses at the University of Catalunya. He is now working for a new winery in Priorat area as manager of sales and marketing, and is a winemaking consultant.

François Naudé
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François Naudé is the chief winemaker at L'Avenir Winery in the Stellenbosch region of South Africa. They produce about 25,000 cases of wine per year, divided into 35% white and 65% red. Chardonnay, Chenin blanc and Sauvignon blanc are the principal white varieties, and the reds include Pinotage, Cabernet Sauvignon and Merlot. He likes to use selected bacteria in the reds and in Chardonnay. On the reds, he uses MLB in tanks and barrels, first for stability and then to use less SO_2 in his wines. On Chardonnay, he uses MLB only on some wines, to ensure a better acid balance because of the high acidity sometimes present in his juice.

Jacques Réjalot
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Jacques Réjalot is a winemaker and the former technical director for the Buzet cooperative. Buzet is a wine region in southwest France, independent from the Bordeaux area since 1911. They produce two types of wine: a young Merlot for local restaurants and another for aging. Mr. Réjalot was part of a CRAFT European project to select malolactic bacteria from different *terroirs*. He was able to adapt winemaking techniques to very tannic wines in the southwest of France. Now he is a consultant for several wineries. He is a fan of microoxygenation and prefers wines with lots of mouthfeel and fruit; he wants to adapt to the global market and bring wines to market earlier.

Rui Reguinga
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Rui Reguinga is a winemaking consultant with 14 years of experience in different regions of Portugal, including Dão, Estremadura, Ribatejo and Alentejo. He is responsible for the winemaking at 10 high quality producers. He has a strong focus on quality control and is equipped with a modern laboratory.

Oliver Schmidt
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Oliver Schmidt works at the Staatliche Lehr- und Versuchsanstalt fuer Obst- ind Weinbau Weinsberg in the Referat Kellerwirtschaft und Staatsweingut Weinsberg (Department of Oenology and State winery) as an extension microbiologist. He is in charge of teaching students, organizing and supervising oenological trials in the school, the state winery and the industry. He also consults in the wine industry in Wuerttemberg.

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Dr. Fernando Zamora teaches at the Rovira y Virgili University in Spain and is winemaker at Clos Mogador, Priorato, Spain, where they grow Grenache and Carignan, Cabernet Sauvignon, Syrah and Merlot.

A first for the *Entretiens Scientifiques Lallemand*!

Sixteen winemakers and oenologists from around the world came to share their experiences and philosophies regarding malolactic fermentation and how it influences wine quality in today's market, during the second half of the *XVI*^{es} *Entretiens Scientifiques Lallemand* in Porto, Portugal. New and Old World views were exchanged regarding practices particular to malolactic fermentation, and how these practices have evolved over time. How are selected malolactic bacteria used in different situations and for what reasons? From a consumer point of view, two wine retailers explained the purchase process of wine; including purchasing criteria and how this process impacts the winemakers they deal with. All those ideas and questions were discussed and an interesting and passionate debate followed, handled by moderator extraordinaire, Tim Atkin.

Master of Wine, and wine journalist at *The Observer* in the U.K., Mr. Atkin was the moderator of the round table discussion. His extensive knowledge of the wine world contributed positively to the discussions. With his quick sense of humour and vast experience tasting wines from around the world, he animated the debate by pulling out the key points from the scientific presentations of the previous day

(e.g., diacetyl management, sulphur aroma compounds, bacteria population) and stirring in winemaking practices from different wine regions, in relation to wine quality in today's market.

The German point of view

The German delegates, Gernot Limbach and Oliver Schmidt, are both advocates of the use of selected bacteria during malolactic fermentation. For Mr. Limbach, the use of malolactic bacteria has had important economic implications for sparkling wine production at Henkell; they have changed their winemaking practice from an inoculation of bacteria at mid-alcoholic fermentation to a co-inoculation (yeast and bacteria). With this method, they reduced malolactic fermentation (MLF) time from four to six months to three to four weeks. The gain of time is considerable, and a technical problem (length of MLF) was solved using this method. For Mr. Limbach, the completion of MLF is important as the wines are protected from instability and ready for the peak selling periods for sparkling wines, such as Christmas and New Year's.

In the past, Mr. Schmidt allowed spontaneous malolactic fermentation, but now prefers using selected bacteria. For him, it is a question of style, in order to be competitive and international. He wants to maintain the soft fruity characters with the help of selected bacteria in his Pinot Meunier and the Trollinger wines that are marketed for early consumption.

Even the French are doing it

The French winemakers are as varied as the wines in that country. However, both Jacques Réjalot, formerly from the Buzet cooperative and now a consultant, and Dominique Delteil, ICV scientific director and consultant, embrace the use of selected bacteria. Mr. Réjalot is a strong advocate of selected bacteria to obtain more aromatic wines and softer tannins, which is often difficult to obtain in the southwest of France for some red varietals. Following microoxygenation, he recommends a malolactic fermentation done in barrels for about three months, without SO_2.

"Malolactic fermentation in barrels is more and more popular," commented Sam Harrop, wine technologist at Marks and Spencer, "and it is all a question of layers to obtain complexity, and getting a balance during the assemblage of different wine styles."

Mr. Réjalot continued to explain that malolactic fermentation can be left to run longer at lower temperature, which has a strong flavour effect but does not appear to affect malic acid degradation. Dr. Thomas Henick-Kling gave a warning, however, on long malolactic fermentation and its contribution to the flavour of the wine, "The flavour contribution might not only be coming from *O. oeni*, but from other bacteria, as well as from *Brettanomyces*, especially in high pH wines."

As for Mr. Delteil, he explained that malolactic fermentation can be managed in different types of wines for different types of markets. For example, in Sauvignon blanc, MLF can be done on ripe grapes for a wine that has a certain quality level determined by the winemaker and the wine buyer. The importance of malolactic fermentation is obvious when you consider that malic acid, which is not degraded when there is no MLF, is dry and harsh, and it is even more perceptible with higher alcohol levels, such as 13-14%.

A word of caution from Mr. Delteil, "It's important to avoid practices that are 'dangerous' to the quality of the wine, such as malolactic fermentation during cap management in varietals like Carignan, because ill-timed malolactic fermentation can cause the loss of some aromas, like the nice juniper aroma associated with this varietal." Another point to consider is the interaction between yeast and bacteria, a situation that requires more and more attention to the proper combination. He ended by concluding that well managed malolactic fermentation will also help manage *Brettanomyces* problems, especially in high pH conditions.

Due south

"The mentality changes as you go south," said Pedro Aibar. "In Spain, spontaneous malolactic fermentation is easy. It can start easily under the cap during alcoholic fermentation. It is encouraged in most reds for biological security." In his winery, they do not use selected malolactic bacteria and carefully manage their malolactic fermentation, and rarely have problems starting malolactic fermentation. When they do have a problem with the onset of malolactic fermentation, they use selected bacteria. However, this creates other problems as the price of malolactic bacteria is sometimes a limiting factor, especially in some very price-sensitive wine regions in Spain.

Dr. Antonio Palacios, also from Spain, agreed that in some regions wineries sometimes cannot afford to buy malolactic bacteria. However, Dr. Fernando Zamora, professor of oenology and winemaker at Clos Mogador in Spain, was not convinced about the price issue, especially when the risk of losing the wine is high. He pointed out that the use of selected bacteria should not be curative but preventive.

Dr. Palacios continued by saying that they have no problem with the onset of spontaneous malolactic fermentation, but that the problems come later. When alcohol levels are up (even to 16%), it is often difficult to control MLF. High pH (over 3.7) further complicates the matter. If malolactic fermentation starts spontaneously, it will take place under the cap and can lead to problems with volatile acidity. Temperature and microoxygenation must be very carefully controlled. Like Mr. Aibar, Dr. Zamora does not always use selected malolactic fermentation because spontaneous fermentation starts very easily. However, it is not to maintain typicity, but rather a matter of habit. He does admit that spontaneous malolactic fermentation has to be carefully managed to avoid aromatic deviations.

Clean and stable wines in Portugal

In Portugal, the situation is similar to Spain, but the mentality is slowly changing, as Rui Reguinga, a wine consultant, commented. When he advises wineries in different regions in Portugal, he is primarily concerned with market demand issues. And modern markets favour ripe, fruity, soft tannin styles of wine. Malolactic fermentation is a concern in the sense that he wants it done as quickly as possible to keep the wines clean and fruity. Spontaneous malolactic fermentation is easy in the south of Portugal. Nevertheless, some winemakers will inoculate with selected malolactic bacteria, depending on the region and the style of the wine. When he has a high quality wine, he will inoculate with selected bacteria. This has been successful, especially with malolactic fermentation done in barrels, similar to the recommendations of Mr. Réjalot. One thing is certain; Mr. Reguinga does not want to take risks with high quality wines and in such cases he will definitely use selected bacteria.

Joao Correia from Portugal echoes similar thoughts. In Portugal, the use of selected bacteria is considered expensive. He will not be using them on less expensive wines, but won't take any risks for premium quality wine.

Reducing biogenic amines and selected bacteria

"The malolactic situation in Italy is like Spain," commented Hélène Mingot, from Gruppo Matura. In the case of white wines, Italian winemakers will prevent spontaneous malolactic fermentation to maintain the balance, crispness and fresh fruit in their wines. In some cases, malolactic fermentation is done to add complexity, especially to increase the diacetyl content, then followed by a blending of non malolactic and malolactic wines. Selected bacteria are not being used because spontaneous fermentation is easy and the cost of selected bacteria is considered high. On premium wines, however, oenologists recommend it to control the risk of off flavours.

"In difficult conditions, if spontaneous fermentation does not occur, the corrective use of selected bacteria does not always work," said Ms. Mingot, repeating comments made by some winemakers. "If the conditions for spontaneous fermentation are very harsh, then the selected bacteria will also have to survive in those difficult conditions, responded Dr. Sibylle Krieger. "Malolactic bacteria are very sensitive, and if the conditions are particularly difficult, then choosing the right bacteria strain with the proper nutrition is crucial, and in some cases, it might even be better to work with a *pied de cuve* for acclimatization."

Ms. Mingot was particularly concerned about the production of biogenic amines during spontaneous malolactic fermentation, as well as ethyl carbamate production. For the export market, it is becoming crucial to control the production of those compounds, as several importing countries severely control their concentrations. Selected bacteria, which are reputed to reduce the production of biogenic amines compared to spontaneous bacteria, could become more and more important, even in wineries where spontaneous fermentation is currently the rule.

The New World – How to handle risk

The New World philosophy is quite different. With most of his wines, Glen James, from Penfolds in Australia, will use selected bacteria. A clean and quick malolactic fermentation, which takes more or less a month, is necessary to avoid *Brettanomyces* problems. As Sam Harrop said earlier, it is often a question of style and balance, and that reflects well in the Penfolds' range of Chardonnays, where several wines are available on the market. Malolactic fermentation will be induced depending on climate considerations, as well as the price of the wine, where the most expensive wines are sure to go through malolactic fermentation. In other varietals, like Viognier, Riesling and Semillon, only partial malolactic fermentation will be done.

As chief winemaker at L'Avenir Winery, François Naudé, from South Africa, likes to have control over fermentation. They use selected bacteria because the risk of spontaneous fermentation with *Lactobacillus* and *Pediococcus* is very high. Malolactic fermentation is therefore done on reds for better stability. Selected bacteria will be used in tanks or barrels, especially with tannic wines. Another procedure is to re-inoculate with lees from an actively fermenting tank to a still tank; they have quite a success with

this method. Mr. Naudé is convinced of the results when using MLB since he obtains clean wines, and he does not want to take the risk of losing a tank or a barrel because of the production of undesirable aromas if spontaneous MLF goes wrong. Laughing, he said, "I don't want to lose my job, my wife and my house if the wine goes bad."

In whites, they do malolactic fermentation in Chardonnay only. They use selected bacteria for the reduction of malic acid and will do partial malolactic fermentation, depending on the vintage. They will not do malolactic fermentation on Chenin blanc as they do not want to lose the already restrained fruity character. Instead, they work with chemical de-acidification. For the same reason, they do not use malolactic fermentation in Sauvignon blanc. Instead, through viticultural practices, they manage to ripen the Sauvignon blanc to lower acidity, but they will correct high acidity eventually with chemical de-acidification.

Jeff Brinkman, the winemaker at Husch Vineyards in California, likes to have all his reds go through malolactic fermentation using selected bacteria. For Pinot noir, he will use barrels during the malolactic fermentation, while all the other reds go through the process in tanks. In his whites, he will do malolactic fermentation only in Chardonnay, and depending on balance and the style intended for the fruit and the brand, will do it in varying amounts. His preference goes to non-diacetyl types of wines, and the presentation by Dr. Eveline Bartowsky, listing all the conditions to enhance or reduce the buttery character, provided excellent insight on how to manage this character. "I'm using Enoferm Alpha, the selected bacteria, which helps me manage the buttery notes while maintaining the roundness and mouthfeel," Mr. Brinkman said. After using wild bacteria in several trials, he prefers the security and reliability of the selected bacteria. "I'm not willing to take the risk of losing my wine."

"The situation in Chile regarding the use of selected bacteria is quite similar to Spain and Italy," commented Dr. Edmund Bordeu. The problem is that the average wine price is low so that the price of selected bacteria becomes an issue. "Chilean winemakers usually think it's not a good idea to spend money on something that happens spontaneously." In reds, it is not a big concern, because they have high alcohol to control the bacteria population and then malolactic fermentation occurs quickly, but if they do have some problems with specific lots of wines, they will buy selected cultures. However, in cooler areas like Casablanca where Chardonnay is grown, they have some problems with spontaneous malolactic fermentation. There they use selected bacteria, but the price of the wines in this region can justify it.

Malolactic fermentation and wine quality: The market's point of view

Ultimately, the consumer – whether a connoisseur or not – will look for a good wine. If the wine of a winemaker finds a market, then the winemaker has done his job. Does the use of selected bacteria provide an assurance that the wine is "fitter" for the market? Both of the wine retailers have their opinions.

Mr. Harrop feels that from a retailer's perspective, it is critical to have biological stability. Therefore, it is crucial to do malolactic fermentation properly. Although he sees greater acceptance among his New World suppliers, he encourages all his wine producers, including those from Europe, to use selected bacteria for speed, stability and cleanness. "Style and quality issues are two factors that are critical," he said. Other important issues are the upcoming labelling legislation regarding sulphides, the presence of which can be reduced by using selected bacteria, and traceability. The control of raw materials has become stricter and the use of selected bacteria allows better records of the malolactic fermentation event. "I look for fast malolactic fermentation and complexity for the premium wines obtained with this fermentation. I truly believed that spontaneous fermentation would give me that complexity, but after tasting the wines fermented with and without selected bacteria, I'm changing my opinion and recommending selected bacteria for the premium wines, too," said Mr. Harrop.

David Molina, sommelier and wine buyer at Vila Viniteca in Barcelona, agrees with Mr. Harrop. The winery must consider to which consumer the wine is targeted. Once this is defined, the style of the wine is also defined. "I like a wine that is clean, and malolactic fermentation helps achieve this," said Mr. Molina. The winemaker has to know what the consumer wants, and consumers want to have quality every time they buy wine.

Last but not least: The debate on typicity

No true debate on wine could occur without a tasting. A German Riesling was presented, made with selected bacteria inoculated at different times, and the differences were surprising to all. The belief that malolactic fermentation will induce the loss of the fruit in some varietals was rapidly dismissed, as a well managed co-inoculation of yeast and selected bacteria clearly shows that the fruit character is maintained in Riesling without the production of volatile acidity. However, this cannot be done on all wines, and some conditions must be carefully controlled.

In the case of the French Merlot/Cabernet Sauvignon blend with six weeks of microoxygenation from Mr. Réjalot's trial, one wine was made with spontaneous malolactic fermentation and another with the selected bacteria VP41. The consensus was clear: everyone preferred the second wine fermented with VP41. Ms. Mingot found the inoculated wine to have more complexity on the nose, more fruit and better nose sensation. Mr. Aibar thought that the inoculated wine was softer, the tannin rounder, and the mouthfeel very distinctive. Mr. Atkin preferred it as well, because of its richer, better texture, more weight, and complexity.

Then Tempranillo wines from Rioja were presented. One had undergone spontaneous malolactic fermentation, and some winemakers thought it was too buttery, covering the fruit expression. The second wine, which was preferred over the spontaneous MLF wine, underwent malolactic fermentation with the help the selected bacteria Uvaferm Beta. The third wine, fermented with the selected bacteria VP41, was preferred overall. This wine had a nice mouthfeel, length and texture. Mr. James from Penfolds liked the good integration of the oak and the texture. It had the best finish and had soaked up the oak nicely. As for Mr. Harrop, he contributed that the sulphide in the wine helped the integration of fruit and oak. Dr. Doris Rauhut, who is an expert at sulphur compounds, confirmed his comment saying that good malolactic fermentation can bring positive sulphur compounds forward.

According to Mr. Molina, the Tempranillo that fermented spontaneously had the character typical of the varietal, whereas the other two represented more the modern way of producing Tempranillo. The question was finally raised regarding the typicity, the region and the grape. Ultimately, it becomes a winemaking decision, but he feels that winemakers should not lose the typicity. "How do you handle a changing market? The evolution of styles?" wondered Mr. Atkin.

In Mr. Reguinga's opinion, if the wine is clean, then you sell more of that wine, and the last two Tempranillo were a reflection of clean wines. Mr. Molina responded that the answer basically comes down to what kind of wine you want to make. "Wine has a culture, it is the sense of the wine, and you can lose this character if you use new technology". He echoed the traditional Old World reluctance to use new methods, while New World producers are usually more receptive to new ways of making better wines.

"We must not forget that even though selected bacteria are used, there is still an active indigenous population in the wine that will influence the sensory properties of the wine," commented Dr. Henick-Kling. All selected bacteria were also once indigenous. Complexity, typicity, those are two issues that are not easily defined. Several winemakers feel they prefer to make stylish wines and will achieve that through blending.

The loss of typicity and the fear of standardization are real concerns, and everyone agrees that the standardization of wine should be avoided. As Mr. Atkin commented, "I feel that the debate about standardization is not in the use of selected bacteria for malolactic fermentation. Malolactic fermentation is not the sole defining factor whether wine has typicity or not. When it is a matter of style, there is no right or wrong answer. If the wine sells, then there are no more questions."

Concluding remarks

Many ideas were exchanged during this debate, which has no doubt expanded the view of all present. Malolactic fermentation is an essential part of winemaking and careful control is essential, whether or not selected malolactic bacteria are used. Clean, stable wines, the reduction of such undesirable compounds as biogenic amines, risk management, bringing a wine to market that sells, and, ultimately, wine quality are the main concerns regarding malolactic fermentation and selected malolactic bacteria. It is easy to conclude that a clear trend is apparent: the Old World is more reticent than the New World about utilizing selected bacteria. Nevertheless, European winemakers, who are becoming more and more innovative and care about preserving the quality of their products, say they are open to using new tools for the winemaking process, in order to get the best out of the grapes.

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