Mastering malolactic fermentation – how to manage the nutrition of wine bacteria and minimise the effect of inhibitors

Keywords: Malolactic fermentation, nutrition, inhibitors.

1. Introduction
Wine bacteria are characterised as having complex nutritional needs. In this review, we will detail the wine bacteria’s requirements for carbon, and for nutrients containing nitrogen, vitamins and minerals. When a must or wine lacks certain nutritional elements, it can have a major impact on malolactic fermentation (MLF). Therefore, it is important to understand the nutritional needs of wine bacteria and to learn about the tools that are available to the winemaker for successful MLF.

2. Wine bacteria have complex nutritional requirements

2.1 Carbohydrate metabolism
In wine, sugars are the primary source of energy for lactic bacteria, playing an essential role in their growth. The main sugars in wine (hexoses) are glucose and fructose. Lactic bacteria are capable of utilising both sources, although Oenococcus oeni prefers fructose, and with Oenococcus oeni, the co-metabolism of glucose and fructose is advantageous in terms of energy. At the end of alcoholic fermentation (AF), the glucose-fructose concentration is low, but still meets the bacteria’s needs, because the concentration of bacteria is also low. Indeed, most lactic bacteria are capable of utilising other monosaccharides present in the wine (e.g. arabinose, mannose, galactose, xylose, etc.), as well as the polysaccharides and glycosylate compounds. O. oeni has extracellular glycosidase activity (Guilloux-Benatier et al., 1993 & Guilloux-Benatier et al., 2000). Several other studies have identified this glycosidase activity, including Grimaldi et al. (2000), MacMahon et al. (1999) and Mansfield et al. (2002), and we now know that numerous aroma precursors are conjugated with glucose residues or glucose disaccharides. These aroma compounds are released through the action of wine bacteria and the sugars are consumed.

2.2 Organic acid metabolism
The main organic acids present in grape must and wine at the end of AF and transformed by Oenococcus oeni are malic and citric acids.

2.2.1 Malic acid
The concentration of malic acid in the must depends on the degree of maturity of the grape and varies from 0.7 to 8.6 g/L (Cabanas & Cabanis, 1998). The main reaction of MLF is the decarboxylation of diacid L-malic acid in wine into monoacid L-lactic acid. In wine, the malolactic system is a mechanism that allows O. oeni to recover energy in the form of adenosine triphosphate (ATP) synthesis and to maintain an intracellular pH level favourable for enzyme activities and cell growth.

Malic acid penetrates into the cell in its anionic form to be decarboxylated into lactic acid in the cell’s cytoplasm. The decarboxylation allows the consumption of an intracellular proton and the expulsion of protons by lactate/H+ symporters. A proton gradient – or “proton-motive force” – from the wine medium towards the cell interior maintains the intracellular pH of the bacteria (about 6.0) and leads to forming energy as ATP.

2.2.2 Citric acid
Citric acid, an important component of grape must and wine, has a concentration that varies from 0.1 to 0.7 g/L. The degradation pathway of citric acid by lactic bacteria leads to the formation of three types of compounds: acetic acid, lipids and acetoinic derivatives (acetoin, butanediol and diacetyl). The metabolism of citric acid is also a source of energy for O. oeni.

In general, the consumption of citric acid begins later than malic acid. With the O. oeni species, the effect of the strain is important in terms of the moment of attack of the citrate and the speed of consumption of the citric acid. While certain strains that produce high levels of diacetyl begin to consume citric acid from the mid-point of MLF, other strains – of greater interest to winemakers in order to avoid buttery notes – do not start metabolising citric acid until there is no more malic acid left to consume (Krieger, 2012).

3. Metabolism of nitrogen sources
The free amino acids and those that come from the hydrolysis of peptides are the main sources of nitrogen used by wine bacteria. Contrary to what happens in yeast, the amino acids necessary for wine bacteria cannot be synthesised from ammonia nitrogen. Therefore, the amino acids must either come from the medium or be synthesised by the bacteria, via the carboxylic precursor amino acids.

3.1 Amino acids
The bacteria’s need for amino acids depends not only on the species but on the strain as well. The identification of the essential amino acids for O. oeni has been the subject of numerous studies. The preferred technique consists of comparing the growth of the bacteria in a medium containing all the necessary amino acids (a complete medium) with the bacteria in a medium from which one of these amino acids is missing.

The research of Garvie (1967), Fourcassié et al. (1992), Remize et al. (2006) and Terrade et al. (2009) has explored the needs of, respectively, nine, six, five and two strains of O. oeni. The differences in the methodologies utilised by the researchers (e.g. growth medium, strains utilised, washing the biomass stage, successive transplanting, etc.) explain the differences among the results obtained with this research.
The following table, adapted from the book “Les bactéries lactiques en oenologie” by Alexandre et al., summarises the results of this research.

Depending on each case, the amino acid missing from the culture medium is said to be:

- “Essential” when the biomass formed represents less than 20% of the biomass in the control culture.
- “Necessary” when the biomass formed represents from 20% to 80% of the biomass in the control culture.
- “Indifferent” when the biomass formed represents more than 80% of the biomass in the control culture.

Arginine is an essential amino acid for the 22 strains studied. Amino acids such as glutamic acid, isoleucine, tryptophan, methionine, valine, cysteine, tyrosine, histidine and phenylalanine are essential for the growth of the majority of strains.

The absence of proline or alanine in the medium does not affect the growth of most strains. Consequently, they are considered indifferent amino acids.

The results obtained by Remize et al. (2006) for two selected *O. oeni* strains are presented in Figure 1 and 2.

Researchers are also interested in the requirements for *Lactobacillus* strains. In Terrade et al. (2009), the authors conclude that the number of amino acids essential to a strain of *L. buchneri* and a strain of *L. hilgardii* is fewer than the number required by two strains of *O. oeni*: five and eight amino acids are essential to *L. buchneri* and *L. hilgardii* respectively, compared to 13 and 16 amino acids essential to the two strains of *O. oeni*.

In practice, the number of amino acids essential to a strain is important, indicating that in a medium lacking amino acids the *Lactobacillus* strains, which are usually undesirable, will develop more easily than the *O. oeni* strains.

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**TABLE 1. Comparison of the amino acid needs of 22 strains of *Oenococcus oeni* (adapted from “Les bactéries lactiques en oenologie” by Alexandre et al.).**

<table>
<thead>
<tr>
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<td>Essential</td>
<td>Essential</td>
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<td>Isoleucine</td>
<td>Essential</td>
<td>Essential</td>
<td>Essential (3/5) Necessary (2/5)</td>
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<td>Valine</td>
<td>Essential</td>
<td>Essential (3/6) Necessary (3/6)</td>
<td>Essential (4/5) Necessary (1/5)</td>
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<td>Essential (4/6) Indifferent (2/6)</td>
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<td>Essential (1/6) Indifferent (5/6)</td>
<td>Essential (1/5) Necessary (4/5)</td>
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3.2 Peptides

Besides free amino acids, peptides can constitute a distinct source of nitrogen. Wine bacteria have both proteolytic activities (protein degradation) and peptidolytic activities (peptide degradation), and they can obtain the amino acids necessary or essential to their growth via peptides.

Research on the nature of the nitrogen metabolised by *O. oeni* indicates that the presence of yeast peptides (0.5 to 10 kDa fractions) in the medium contributed particularly to the growth of *O. oeni* compared to a medium containing only free amino acids (Remize *et al.*, 2005). It was also shown that peptide metabolism was followed by the release of free amino acids into the medium.

Peptides appear to be the key nitrogen source for the development of *O. oeni*. From an energy perspective, the bacteria cells take advantage of peptide consumption. Indeed, it is quite probable that certain peptides are more likely than others to be hydrolysed and transported by the bacteria. These peptides are particularly stimulating because they allow energy to be generated with greater productivity (Alexandre *et al.*, 2008).

In practice, we know that in some varietals, such as Chardonnay, it is often more difficult to carry on MLF, even in the absence of inhibitors (e.g. low pH and high molecular SO₂). The reason is suspected to be a lack of essential nutrients. Trials have been carried out in the Lallemand R & D laboratory to assess the impact of adding various peptides to a Chardonnay wine. The results were obtained after adding a peptide considered to be particularly stimulating.

Experiment protocol:
- Medium: Chardonnay white wine (pH 3.2, ethanol 12.9%, total SO₂ <25 mg/L and free SO₂ <5 mg/L).
- Preparation of three mediums per strain:
  - Control: wine with no addition.
  - Wine + peptide to 5 mg/L.
  - Wine + peptide to 20 mg/L.
- Inoculation of each medium after rehydrating the MBR® bacteria A, B, C and D in non-chlorinated water for 15 minutes at 20°C.
- Incubation of trials in glass containers at 20°C.
- Follow-up of the bacteria population by counting on a modified MRS gel medium.

Figure 3 presents the bacteria population seven days after inoculation. Adding this peptide at a rate of 5 mg/L to the Chardonnay white wine has a net stimulating effect on the growth of the four selected strains utilised in the trial. Studies of *O. oeni* have shown that bound amino acids transported inside the cell are released in their free form back into the medium. Although they were transported, the peptides are not necessarily consumed (Ritt *et al.*, 2008). The hypothesis suggested was that this transport was carried out with the aim of saving energy. Indeed, it appears that this internalisation of bound amino acids requires less energy than free amino acids.
TABLE 2. The essential nutrients for four strains of oenological bacteria (Terrade et al., 2009).

<table>
<thead>
<tr>
<th></th>
<th>O. oeni S1</th>
<th>O. oeni S2</th>
<th>L. buchneri</th>
<th>L. hilgardii MHP</th>
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<td>D-Ribose</td>
<td>D-Ribose</td>
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<tr>
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<td>K$_2$HPO$_4$</td>
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<td>L-Histidine</td>
<td>L-Proline</td>
<td>L-Phenylalanine</td>
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<td>L-Proline</td>
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<tr>
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<td>L-Valine</td>
<td>L-Leucine</td>
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<td>L-Isoleucine</td>
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<td>Riboflavin</td>
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<td>Ca-D-pantothenate</td>
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FIGURE 3. Impact of adding a peptide in two different concentrations (5 mg/L and 20 mg/L) on the growth of four strains of Oenococcus oeni bacteria in a Chardonnay white wine (Lallemand R & D).

(Konings, 2002 & Kunji et al., 1993), which can be transported by different pathways. In addition, the excretion of free amino acids by proton symporters generates the proton-motive force necessary for ATP synthesis. This peptide could be shown to be advantageous from an energy point of view by improving the resistance of the bacteria in an acid medium (pH 3.2).

4. Vitamin requirements

Lactic bacteria need several vitamins from the B family, especially pantothenic acid (vitamin B5), biotin (B8), thiamine (B1) and niacin (B3). Although pyridoxine (B6) and riboflavin (B2) are not essential, they may contribute nonetheless to the optimal growth of strains. Pantothenic acid, present in great quantity in tomato juice, as well as grape and apple juice, was long considered a special growth factor for O. oeni, and is in numerous culture mediums for the species.

In a study by Terrade et al. (2009), it was shown that the presence of niacin (B3) and pantothenic acid is essential for the growth of four wine bacteria (two O. oeni and two Lactobacillus sp.), and riboflavin was necessary for two Lactobacillus sp. only. Pyridoxine (B6) had a stimulating effect on the growth of all four strains.
5. **Influence of minerals**

Certain minerals, including magnesium, manganese, potassium and sodium, are important as they are enzyme cofactors or they intervene in the transport mechanisms. Few studies have taken an interest in the need for minerals of these strains.

Wine bacteria have nutritional requirements as complex as they are varied. Satisfying these requirements contributes to mastering MLF. Consequently, any lack of these activating elements for MLF must be remedied with specific activators, obtained from specific inactivated yeasts, which are natural sources of amino acids, peptides, vitamins and minerals, and are vital to the growth and survival of *O. oeni*.

6. **Strategies to ensure the completion of MLF**

6.1 **Wine is a medium not conducive to the development of bacteria**

Wine is a stressful environment for bacteria. Acidity, alcohol, sulphites and temperature (below 18°C) are factors that inhibit the growth of bacteria. In addition, the yeast produces inhibiting factors during AF, such as SO₂, medium-chain fatty acids and peptide inhibitors. The lack of nutrients in the medium is also inhibiting. Certain oenological practices, like clarification, eliminate the nutrients and suspended particles favourable to the growth of bacteria. As explained above, certain amino acids are indispensable to the growth of *O. oeni*. Towards the end of AF, the level of organic nitrogen varies considerably from one wine to another. Renouf (2013) has published the results of his analysis of the level of essential amino acids at the end of AF and before MLF in wines from diverse varietals (Merlot, Cabernet Sauvignon, Malbec, Chardonnay, Syrah, Tannat and Pinot Noir) and various appellations. He reports on the variations in factors, classified from 1 to 10, on the total level of amino acids vital to the growth of *O. oeni*. The completion of MLF was particularly difficult in the wine with the greatest deficiencies.

6.2 **Utilising malolactic fermentation activators**

It is generally accepted that smooth AF is based on sufficient nutrients for the yeasts. MLF also requires sufficient nutrients for the bacteria, and there are now solutions for smooth and complete MLF.

Figure 4 presents the results of a trial carried out on a Cabernet Sauvignon wine, whose analysis (ethanol 14%, pH 3.56, total SO₂ <25 mg/L and free SO₂ 5 mg/L) was within the parameters recommended for the Alpha MBR® bacteria.

Three MLF protocols were compared:
- Direct inoculation into wine with Alpha MBR® bacteria.
- Direct inoculation into wine with the Alpha MBR® bacteria, plus...
FIGURE 6. Malolactic fermentation kinetics (malic acid concentration) in a Chardonnay wine (control) and in the same wine after adding extracts of Cabernet Sauvignon (CS1, CS2 and CS3) and Tannat (T) (Lonvaud-Funel, 2013).

FIGURE 7. Bacteria growth in a Chardonnay wine to which extracts of Cabernet Sauvignon (CS1, CS2 and CS3) were added and the same wine enriched with inactivated yeasts Y1 and Y2 (Lonvaud-Funel, 2013).

FIGURE 8. Bacteria growth in a Chardonnay wine to which extracts of Tannat (T) were added and the same wine enriched with inactivated yeasts Y1 and Y2 (Lonvaud-Funel, 2013).
FIGURE 9. Bacteria populations at 8 days and 14 days after inoculation with three different bacteria strains, with and without the addition of ML Red Boost™ inactivated yeast preparation.

FIGURE 10. Duration of malolactic fermentation with three different bacteria strains, with and without the addition of ML Red Boost™ inactivated yeast preparation.

the addition of the Opti’Malo Plus® fermentation activator at a rate of 20 g/L.
- Control wine not inoculated with selected bacteria.

The trial temperature was 18°C.

In the control wine, which was not inoculated with the selected bacteria, the bacteria population remained very low for the 21 days of the trial, never going above 1 x 10^4 CFU/mL. With such a low bacteria population, the malic acid concentration remained stable.

In the wine inoculated with the selected bacteria and no added bacteria nutrients, the bacteria population maintained itself for the first seven days after inoculation, then began a slow decline. Ten days after inoculation, the death of the bacteria led to the end of malic acid degradation.

However, in the wine with the added MLF activator, the rapid and sustained growth of bacteria was observed after inoculation. Malic acid was completely metabolised after 11 days. This wine was probably lacking at least one element essential to the development of Alpha bacteria.

6.2.1 The case of white wines

As explained above, in the case of white wines, especially those from the Chardonnay varietal, MLF is more difficult to launch.

The effects of inoculating the wine with diverse bacteria strains and adding diverse MLF activators were studied in Chardonnay wines. The study was interested notably in inactivated yeast preparations containing the activator peptide whose beneficial effect is shown in Figure 1. The results presented in Figure 5 outline the study’s observations.

As part of this research into Chardonnay wine, the following protocols were compared:
- Inoculated with the Alpha, Beta or PN4 bacteria strain.
- MLF activator added at a rate of 20 g/L.
- Types of activator: the A nutrient (control activator) and a new activator high in peptides (Opti’ML Blanc®).

According to the strain utilised, the length of MLF with no added nutrients will be 28 to 30 days. In this medium, very likely lacking elements essential to the growth of bacteria, adding a complex
nutrient high in amino acids, peptides and vitamins will considerably shorten the duration of MLF, whatever the strain. For the Alpha and Beta wine bacteria, the new activator is much more effective than the control nutrient.

6.2.2 The case of concentrated red wines

In practice, successful MLF is also difficult to attain in concentrated red wines high in polyphenols, which is generally the case with Merlot and Tannat wines, in the southwest of France, and on Graciano in Spain.

Much research has been carried out with the aim of exploring the impact of polyphenols on the growth and viability of lactic bacteria, and on the metabolism of malic acid degradation, with sometimes contradictory results.

Apparently, polyphenols can have an effect that is sometimes stimulating and sometimes inhibiting on bacteria growth and activity, depending on the strain, as well as the nature and the concentration of the polyphenols tested. For the moment, data on the molecular mechanisms in play is very rare.

Several studies have confirmed the stimulating effect of gallic acid on the growth of O. oeni strains and the rate of malic acid degradation (Lombardi et al., 2012, Reguant et al., 2000 & Vivas et al., 1997).

As for hydroxycinnamic acids (Garcia-Ruiz et al., 2009), it was shown by Campos et al. (2003) that the L. hilgardii and Pediococcus pentosaceus bacteria are strongly inhibited by p-coumaric and caffeic acids. The inhibiting effects of the three hydroxycinnamic acids (caffeic, ferulic and p-coumaric) also affect O. oeni bacteria (Reguant et al., 2000).

Opinions diverge as to the effect of flavonols, which some say are inhibiting (Cushnie & Lambert, 2005) and others say are stimulating (Reguant et al., 2000) on the growth and the rate of malic acid degradation.

It seems the presence of catechin and epicatechin, in concentrations usually observed in wine, does not have an inhibiting effect on the growth of O. oeni (10 to 200 mg/L) (Lombardi et al., 2012). Catechin would even stimulate the growth of O. oeni, with this effect increasing the higher the concentration in the wine (Reguant et al., 2000 & Alberto et al., 2001).

The condensed tannins would be very toxic (Vivas et al., 2000), even at very low concentrations, under the levels usually found in wine (0.5 g/L) (Figueiredo et al., 2007).

The simultaneous presence of molecules that activate and inhibit growth, viability and MLF create a balance that usually facilitates the growth of lactic bacteria. In addition, numerous tannins are polymerised with other molecules, which can reduce the toxic effects. Launching MLF will be even more difficult in a wine containing essentially weakly polymerised tannins.

Recently, trials were conducted with the goal of exploring the impact of polyphenolic extracts on MLF (Lonvaud-Funel, 2013). These extracts were taken from three grape varieties: Merlot, Cabernet Sauvignon and Tannat. Each extraction utilised 200 mL of wine. After evaporation at a temperature of 30°C, the resulting residue was resuspended in acidified water. The polyphenolic fraction was measured after eliminating the sugars and acids by column purification. The extracts were added to a Chardonnay wine (ethanol 12.0%, pH 3.5 and total SO2 <20 mg/L) in concentrations equal to the initial polyphenol concentrations in the wine.

The trials carried out with different extracts showed that, for the two O. oeni strains, bacteria growth is inhibited by adding extracts from Cabernet Sauvignon and from Tannat (the data obtained for one of these strains are presented in Figure 6). The result is a significant slowing of malic acid degradation. Although MLF was completed in 10 days in the Chardonnay wine to which no polyphenolic extract was added, there was 1 to 1.5 g/L of malic acid in the wine enriched with the extract.

In these mediums, adding the two inactivated yeast preparations – Y1 and Y2 – was studied with the aim of lessening the inhibiting effects on MLF.

As can be seen in Figure 7 and 8, MLF was stimulated in the Chardonnay wine to which the inactivated yeast preparations were added, as compared to the Chardonnay wine control. We can conclude that these preparations contributed to eliminating the inhibiting effect associated with the adding of polyphenolic extracts. The inactivated yeast preparations could have an impact on several levels, notably via the liaison of yeast derivatives with tannins, which lowers their toxicity to bacteria. Measuring the redox potential also indicates that this will decrease with the addition of two nutrients, which also contributes to stimulating bacteria growth. These activators obviously contribute to enriching the wine’s nutritional profile, and by the same token, stimulate bacteria growth.

Concentrated red wines, produced during the 2012 and 2013 harvests, were submitted to additional trials with the Y1 inactivated yeast (ML Red Boost™).

Figure 9 and 10 show the results for a Tannat wine presenting the following characteristics: ethanol 14.6%, pH 3.6, total SO2 <25 mg/L, free SO2 <5 mg/L and total polyphenol index [TPI] 90. For the three strains studied, ML Red Boost™ was added 24 hours before adding the bacteria in order to make the medium more conducive to the growth of O. oeni (by decreasing the inhibiting action of the tannins). This had a pronounced impact on the rate of development for the lactic bacteria. In fact, eight days after inoculation, the bacteria population of strains A and B was 60% higher in the mediums treated with the ML Red Boost™ activator than in the non-treated mediums.

For strain 7, the gap was astounding: the size of the bacteria population in the wine treated with the activator was 700 times higher than in the wine inoculated with the bacteria but not treated with the activator. Two weeks after inoculation, the differences in the populations sizes were greater still, which means much shorter MLF times in all the mediums that were supplemented with nutrients (see Figure 10).

7. Conclusion

Although the overall mechanism of malolactic fermentation (MLF) is fairly well known, the specific exploration of the metabolism of Oenococcus oeni began only recently and is now the subject of numerous studies. The belief that L-malic acid is sufficient to meet the energy needs associated with the development of O. oeni is still widespread. However, the truth is otherwise. In fact, lactic bacteria are particularly demanding micro-organisms with complex nutritional needs. The absence of certain nutrients essential to the implantation, growth and metabolism of O. oeni can cause delayed, even failed MLF. Fortunately, there are solutions for this problem. Nutritional deficiencies and bacteria inhibitors vary from one medium to another. Their negative impact can be limited by adding fermentation activators, the choice of which differs for white and for red wines.

We have described several specific nutrients for which different formulations have been optimised to particular applications and requirements. The Opti’ML Blanc® activator, for instance, was designed to stimulate the growth of selected bacteria and thereby shorten the duration of MLF in white wines associated with difficult MLF, which is the case for certain Chardonnay wines. Designed for red wines rich in polyphenols, the ML Red Boost™ activator, which
must be added prior to inoculation with selected bacteria, helps encourage the implantation of the selected bacteria.

References