Techniques for dealing with awkward malolactic fermentations

Vincent Renouf[1], Stéphane La Guerche[2], Virginie Moine[1], Marie-Laure Murat[2]

[1] LAFFORT - BP17 - 33072 BORDEAUX - vincent.renouf@laffort.com; virginie.moine@laffort.com.

[2] Laboratoire SARCO – filiale de recherche du groupe LAFFORT– BP 40, 33072 Bordeaux – slaguerche@sarco.fr, marielaure.murat@sarco.fr

INTRODUCTION

Alcoholic fermentation (AF) is generally followed by a second process, malolactic fermentation (MLF), caused by lactic bacteria, mainly *Oenococcus oeni* species. This reduces acidity in the wine and develops its aromas, as well as ensuring microbiological stabilization.

Like yeasts, indigenous lactic bacteria in wine originate from the vineyard environment, where they are already present on the surface of the grapes. A large number of species are present on grapes, but changes in the environment (addition of SO_2, ethanol production, etc.) gradually reduce this diversity. After AF, there is generally only one species left: *O. oeni* (Renouf et al. 2006). The total population of lactic bacteria also decreases from approximately $10^3/10^4$ CFU/mL to $10/10^2$ CFU/mL O. oeni by the end of AF. Then the yeasts involved in AF die off and leave the ecosystem to the bacteria. *O. oeni* have not only room to develop, but also plenty of their preferred substrate: L-malic acid. From $10/10^2$ CFU/mL, the *O. oeni* population increases to $10^6/10^7$ CFU/mL, enabling the malolactic transformation to start (Figure 1).

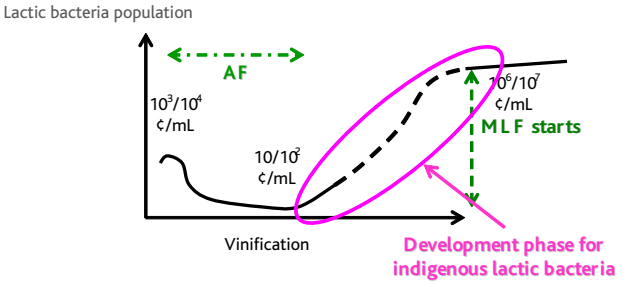

Figure 1: Variation in lactic bacteria population during fermentation

This growth phase lasts a variable amount of time, depending partly on the characteristics of the wine (Table I), but mainly on the growth capacity of the indigenous *O. oeni* strains. These develop at varying rates according to their physiological and genetic characteristics. As a result, the latency phase before MLF starts is unpredictable with indigenous flora, as it is impossible to assess the growth capacity of the highly diverse strains present. Ideally, the latency phase should not exceed two to three weeks. If conditions are too harsh and the indigenous bacteria are deficient, it may take much longer and, in extreme cases, winemakers have been obliged to wait until spring for MLF to be completed. If MLF has not started before cold weather sets in, it is no longer feasible to maintain a suitable temperature for the bacteria to develop in the wine (18°C – 20°C).

Table I: Impact of physicochemical parameters on malolactic fermentability

	pH	A.C.V (% vol.)	Total SO_2 (mg/L)	L-Malic acid (g/L)	Temperature (°C)
Favorable conditions	≥ 3,3	≤ 13	≤ 40 mg/L	≤ 3 g/L	18-25
Difficult conditions	< 3,2	> 15% vol	> 70 mg/L	> 4 g/L	14-18

NB: The parameters interact.

The use of a malolactic starter is recommended to prevent these problems (Figure 2). After AF, adding a large population of selected *O. oeni* reduces the latency phase. Besides having the «finished» wine ready for aging sooner, quicker MLF also makes fermentation safer, as it can be protected by sulfiting sooner as well. It is well known that the longer MLF takes to start, the greater the risk that *Brettanomyces* will develop (Renouf and Murat 2008). Finally, reducing the time required to complete MLF minimizes the energy costs necessary to maintain the wine at a favorable temperature for lactic bacteria growth (18-20°C). Protocols have been developed for inoculating bacteria during AF to optimize the timing of MLF. Early co-inoculation consists of adding malolactic starter 24 h after AF starts and late co-inoculation means adding bacteria around the end of AF, when 0°Brix (Murat et al. 2007, Renouf et al. 2008). This optimizes the timing of MLF and maintaining controlled microbiological activity «blocks» the proliferation of spoilage organisms (*Brettanomyces*). Furthermore, it also eliminates the need to heat the wine, as it is still warm enough at the end of AF for the bacteria to develop properly.

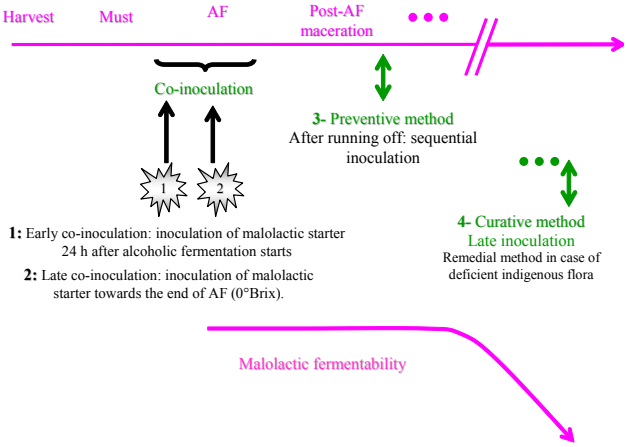

Figure 2: Diagram showing the effect of adding malolactic starters at different stages in the winemaking process.

However, a preventative utilization of malolactic starter has not yet been universally adopted. As a result, MLF may start excessively late in certain wines deficient in indigenous flora. This situation is problematic for several reasons. It obviously delays the presentation of the finished wine to potential buyers, but, much more importantly, there is a major risk of microbiological spoilage. Furthermore, the later MLF starts, the more hostile conditions become to the growth of lactic bacteria, as inhibiting factors accumulate and cold winter temperatures put additional stress on the bacteria cells. There is also greater competition with other, more resistant microorganisms, like *Brettanomyces*. Restarting MLF in spring is, therefore, by no means an easy, risk-free operation. The objective of this article is to outline effective solutions to this problem.

1- Analyzing the microbiological status of the wine

Increasing temperatures in spring promote the growth of all the microorganisms present in the wine. Consequently, it is essential to monitor any residual microflora, particularly *Brettanomyces*. Among the many analysis methods now available, quantitative PCR is the most effective for detecting *Brettanomyces*. Its molecular technology ensures reliable results and its speed facilitates rapid treatment if contamination is detected. Epifluorescence microscopy is useful for further analysis of *Brettanomyces*, as it provides a comprehensive observation of all the viable microbial cells in a wine, making it possible to count yeasts as well as active bacteria. Observation under a microscope reveals the shape of each cell: elongated *Brettanomyces* and small, shell-shaped *Pediococci*, generally in groups of four (tetrads) are thus easily identifiable. Similarly, it is possible to count the *O. oeni* (long chains of shells) necessary to trigger MLF.

2- Analyzing the physicochemical status of the wine.

The following characteristics are important in determining the malolactic fermentability of a wine: % Alcohol, glucose-fructose, pH, L-malic acid, free SO_2, and total SO_2. However, other inhibiting metabolites, such as short- and medium-chain fatty acids, may accumulate in wine when MLF is delayed. Produced by yeasts during AF, as well as *Brettanomyces* (Romano et al. 2008), C_8 (octanoic acid), C_{10} (decanoic acid) and C_{12} (dodecanoic acid) fatty acids strongly inhibit MLF bacterial growth and enzyme activity, particularly by disrupting membrane function (Lonvaud-Funel et al. 1988). An assay of the fatty acids gives a good indication of the wine's toxicity for lactic bacteria. Ethanol exacerbates their toxic effect. These parameters also interact with pH and total SO_2 content (Table III).

Table III: Examples of octanoic (C8) and decanoic (C10) acid concentrations measured in wines made from various grape varieties at the end of AF.

	Wines with good malolactic fermentabilitya[a]			Wines with poor malolactic fermentability[b]		
Grape variety	Cabernet - Sauvignon	Syrah	Sémillon	Merlot	Tannat	Sauvignon
C8 (mg/L)	15,5	22,2	14,2	43,7	18,8	20,7
C10 (mg/L)	2,9	3,8	8,8	13,6	11,5	7,5
ACV (% Vol.)	13,8	13,9	12,4	13,5	14,8	14,4
pH	3,36	3,35	3,08	3,68	3,12	3,10
total SO_2 (mg/L)	17	12	16	36	44	22

[a]: MLF completed in under 30 days.

[b]: MLF completed in over 30 days.

Finally, in view of the competition between lactic bacteria and *Brettanomyces*, an assay of volatile phenols provides a useful indication of the extent of any contamination. If the sum of the ethyl-4-phenol and ethyl-4-guaiacol concentrations before MLF exceeds a few micrograms per milliliter, microbiological analysis is essential to confirm the presence of *Brettanomyces*.

3- Adapting the MLF protocol

The analyses described above should make it possible to establish a suitable protocol to ensure the successful completion of late MLF.

First case: A large, indigenous population of O. oeni (>10^5 cells/mL) and no Brettanomyces (<10 cells/mL).

Ideally, for MLF to start spontaneously, even late in the season, analysis should reveal an *O. oeni* population of at least 10^5 cells/mL, but no *Brettanomyces* (<10 cells). In this case, the objective is to promote development of the bacteria. The temperature of the wine is raised (> 18°C) to bring the bacteria out of their latent state and nutrients are provided (vitamins, nitrogen compounds, etc., e.g. by adding 20 - 30 g/hL **MALOSTART®, LAFFORT**) to correct probable deficiencies in the medium. Analyses are also required to check that the indigenous lactic bacteria do not have a tendency to produce biogenic amines (Lucas et al. 2008).

Second case: Low indigenous *O. oeni* population (<10^5 cells/mL) and/ or presence of *Brettanomyces* (>10 cells/mL).

When the indigenous lactic bacteria population is below 10^5 cells/mL and/ or *Brettanomyces* has been detected, even at very low levels (< 10 cell/mL), it is absolutely essential to use a malolactic starter. A massive inoculation of selected *O. oeni* (10^6/ 10^7 cells/mL) is required to ensure that the bacteria take over the ecosystem and trigger MLF.

However, if the *Brettanomyces* population is excessively high (> 10^4/10^5 cells/mL), the malolactic starter / *Brettanomyces* ratio is less favorable. In this case, the first step must be to reduce the *Brettanomyces* population by racking under inert gas or filtering (1 µm filter) before the malolactic starter is inoculated. Once potential competition from *Brettanomyces* has been eliminated, the ecosystem will become more favorable for the development of lactic bacteria. To ensure the success of inoculation at this stage, described as «late» or «curative» (Figure 2), the malolactic starter must be adapted to the conditions in the medium, which, as described above, are harsher than those prevailing at the end of AF.

It is, therefore, advisable to adapt the inoculation protocol by adding an acclimatization stage. The objective is to enable the commercial bacteria to adapt to the conditions in the wine, thus optimizing their survival rate after inoculation. We, therefore, recommend adding the recommended dose of bacteria for the entire vat to a sample representing 1 - 10 % of the total volume. Then progress in MLF is monitored (twice-weekly assays of L-malic acid and volatile acidity) to determine the best time for transferring the sample back into the vat, ensuring that the wine is inoculated with fully active *O. oeni*. The vat sample is ready for use before MLF is completed. Indeed, by the time the L-malic acid content approaches zero, the lactic bacteria population is already on the decline. The ideal time for transferring the sample to the full vat is two-thirds of the way through MLF, when the lactic bacteria are most active and population levels are at their peak. Of course, the bacteria require proper nutrition, so the wine used to activate the lactic bacteria should be supplemented (**MALOSTART®, LAFFORT**), to provide the nutrients essential for their development and proliferation.

The vat-sample phase also offers an ideal opportunity to «adjust» certain parameters in the wine, in order to optimize adaptation of the bacteria. For example, if the wine's pH is very low (< 3.2), adding potassium bicarbonate to the vat sample (e.g. to obtain pH 3.4) provides a more favorable environment for the bacteria to proliferate. Similarly, if the free residual SO_2 content of the vat sample is too high (> 10 mg/L), it should be reduced (e.g. by controlled aeration).

To illustrate the effectiveness of this strategy, figure 3 shows the results obtained with a batch of Merlot (250 hL) harvested in October 2007 that had still not undergone MLF by March 2008 (L-malic acid content on 03/03/08: 2.9 g/L). Having established that there was no *Brettanomyces* contamination, the MLF problem was solved by inoculating **LACTOENOS SB3®** bacteria (**LAFFORT**) (1g/hL) into a 12.5 hL wine vat, supplemented with 20 g/hL **MALOSTART®** when the lees were being stirred with nitrogen (temperature maintained at 22°C). MLF had started in the small vat 8 days after seeding. **MALOSTART®** (20 g/hL) was added again when the wine was pumped over under nitrogen, to ensure that malolactic activity started strongly. After 14 days, the L-malic acid content had dropped to 0.8 g/L and the 12.5 hL sample was used to inoculate 250 hL wine in a temperature-controlled stainless-steel vat at 20°C. A final Malostart (20 g/hL) supplement was added during pumping over under nitrogen to homogenize the wine. MLF was completed 32 days after the start of the operation (18 days after the transfer to the 250 hL vat), whereas the indigenous flora had not even started after five months!

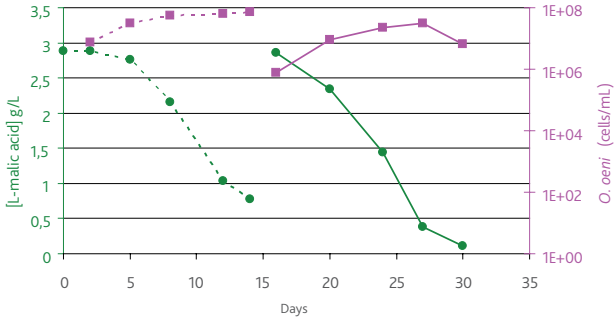

Figure 3: Variations in L-malic acid content and lactic bacteria population in the vat sample (dotted line) and the full vat (solid line). Before seeding, the indigenous O. oeni population was below 10^3 cells/mL).

4- Detoxifying the medium

In addition to providing nutrients for the bacteria, it is also essential to detoxify the medium. The toxic effect of short- and medium-chain fatty acids, described above, must be eliminated to enhance the wine's malolactic fermentability. The most effective method is to treat the wine with yeast hulls. These «hulls» consist of yeast cell walls purified from lysed cells (yeast extract), which have a tremendous capacity to adsorb fatty acids. Several yeast hull preparations are available (e.g. **BIOCELL®**). This treatment (30 / 40 g/hL), combined with thorough homogenization, 48 h before the malolactic starter is added, enhances the survival rate of the bacteria following inoculation. Certain lactic bacteria activators also contain yeast hulls, thus combining nutrient and detoxifying properties. This is the case of **MALOSTART®**, which may be added during inoculation, but also afterwards, if the bacteria fail to proliferate properly. **MALOSTART®** stimulates bacterial activity and triggers MLF (Figure 4).

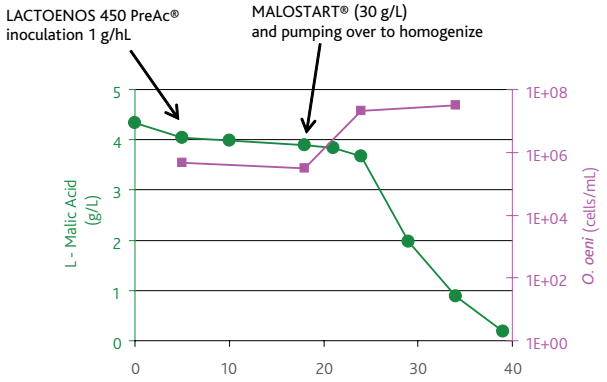

Figure 4: Examples of utilizing MALOSTART® to facilitate the activation of inoculated malolactic starter.

CONCLUSION

When a malolactic starter has not been inoculated during (co-inoculated) or immediately after AF (sequential, preventive inoculation) and the indigenous lactic bacteria have not been capable of triggering spontaneous MLF, this process may not even have started when temperatures drop in winter. The bacteria remain inactive due to the cold and it becomes urgent to complete MLF quickly as soon as spring comes. During this latency phase, conditions become even tougher for the indigenous *O. oeni*, due to accumulation of inhibiting compounds, nutrient depletion of the medium, competition with other microorganisms, etc. Consequently, as the indigenous bacteria were incapable of triggering MLF before the winter, they will probably never be able to do so. The utilization of a malolactic starter then becomes inevitable. The first issue to be resolved is competition from *Brettanomyces*, which continue to develop during the bacteria's latency period, as they are more resistant to inhibiting factors. Finally, it is vital to adapt the inoculation protocol to the specific parameters. Irrespective of the malolactic starter chosen, we recommend using selected bacteria with an acclimatization stage in a vat sample before inoculation into the full vat, to ensure that the bacteria reach optimum population and activity levels. Special care must be taken to provide proper nutrition, by supplementing the wine with an activator to correct any deficiencies. Finally, it is also recommended to assess the toxicity of the medium by measuring concentrations of short- and medium-chain fatty acids, mainly octanoic and decanoic acid. If necessary, the wine may be detoxified by treating it with a yeast-hull preparation. This is a very effective tool for use in combination with malolactic starter.

References

Lonvaud-Funel, A., Joyeux, A., Dessens. C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolism. J. Sc. Food Agric. 44, 183-191.

Lucas P., Claisse O., and Lonvaud-Funel A. 2008. High Frequency of Histamine-Producing Bacteria in the Enological Environment and Instability of the Histidine Decarboxylase Production Phenotype. Appl. Envir. Microbiol. 2008 74: 811-817.

Murat, M.L. Gindreau, E ; Augustin, C., Fuster, Hantz, E., 2007. De la bonne gestion de la FML, interactions levures / bactéries, applications à l'inoculation séquentielle et à la co-inoculation, 125, 49-53.

Renouf, V., Murat, M.L., 2008. L'utilisation de levains malolactiques pour une meilleure maîtrise du risque *Brettanomyces*. Rev. Œnol. 126, 11-15.

Renouf, V., Gourraud, C., Murat, M.L., 2008. Les différentes alternatives d'utilisation des levains malolactiques. Rev. Œnol. 128, 39-43

Romano et al. 2008. Growth and volatile compound production by *Brettanomyces/ Dekkera bruxellensis* in red wine. J. Appl. Microbiol. 104, 1577-1585

LAFFORT
l'œnologie par nature

BP 17 – 33072 BORDEAUX CEDEX – Tél.: +33 (0)5 56 86 53 04 – Fax: +33 (0)5 56 86 30 50 – www.laffort.com