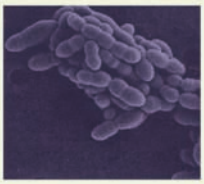
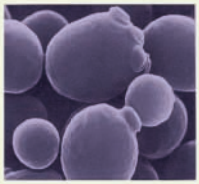
101 QUESTIONS about MLF

Everything you always wanted to know about malolactic fermentation but were afraid to ask!





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LAFFORT
l'œnologie par nature

101 QUESTIONS ABOUT MLF

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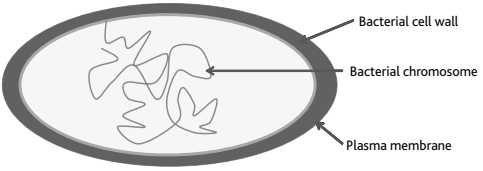
SUMMARY

ŒNOLOGICAL PRACTICES AND FERMENTATION MANAGEMENT

GENERAL MICROBIOLOGY

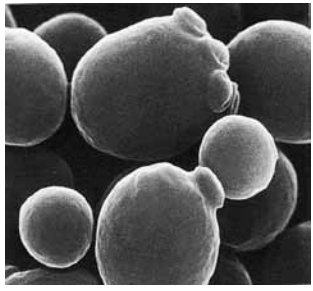
1. What is a bacterium?



Bacteria are unicellular, prokaryotic (with no nucleus) living organisms. They are characterised by a single cell devoid of organelles with the genetic material contained in a circular chain of DNA. Bacteria have peptidoglycan cell wall. The constituent differences of the cell walls are used to differentiate bacteria. Bacteria measure a few micrometers (µm) and are found in different forms: spherical (coccus), elongated or rod (bacillus) forms. Aside from these morphological differences, bacteria are also differentiated in terms of metabolism. In wine, the principal bacteria found are lactic bacteria (*Œnococcus œni*, lactobacilles, *Pediococcus*) and acetic acid bacteria.

2. What is the difference between a bacterium and a yeast?

Yeasts and bacteria are unicellular living organisms. While yeasts possess a nucleus containing DNA and organelles that compartmentalise the intracellular medium, bacteria are characterised by the absence of nucleus and organelles. In a bacterium the DNA is free in the cytoplasm. Bacteria are prokaryotes (without nucleus), yeasts are eukaryotes (with nucleus). The size of a yeast cell is approximately 10 µm. Bacteria are significantly smaller. *Œnococcus œni* , the MLF bacterium is a small sphere measuring between 0,5 and 0,7 µm. Despite its relatively small size, a bacterium can accomplish significantly more sophisticated molecular mechanisms than a yeast can.



Photograph of a Saccharomyces cerevisiae culture, the principal yeast in wine.

3. What is the genome of a bacterium?

DNA is the molecule that stores the genetic information of all living beings (except for a few viruses that use RNA). DNA sequences are made up of genes, and the total number of genes of an organism represents its genome. In bacteria, the genome is essentially made up of a single circular chromosome that can potentially be complemented by extra-chromosomal structures: plasmids. The chromosome of the bacterium *Œnococcus œni*, principal lactic bacterium in MLF, has 1.9 million base pairs, which represents approximately 1700 genes, while the number can vary according to the strain. By way of comparison, yeasts possess approximately 4000 genes and Man has 25,000. At present, just one single genome of an *Œnococcus œni* strain has been entirely sequenced and its principal genes annotated. But a number of other studies are currently in progress, notably at the ISVV in Bordeaux. Complete sequencing of a genome provides a vast amount of information about the genes and the way the bacterium functions.

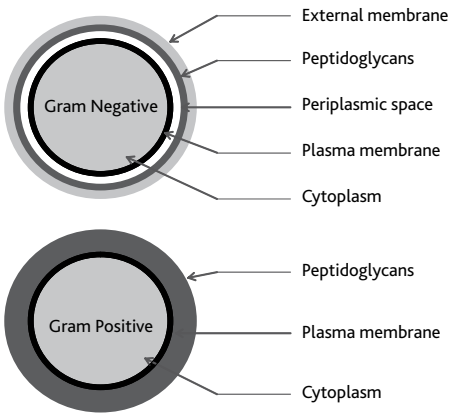
4. What are the principal differences between lactic bacteria and acetic acid bacteria in wine?

Lactic bacteria and acetic acid bacteria are the two main bacterial groups in wine. But they are very different, both in terms of morphology and metabolism.

Lactic bacteria belong to the group of Gram positive bacteria. Acetic acid bacteria are Gram-negative bacteria. Gram staining is a method for distinguishing bacteria according to their cell wall structure. Gram staining today remains a benchmark method of analysis in bacteriology. Gram positive bacteria retain the stain used for the analysis, thus responding positively to the staining.

Apart from this difference, it is important to underline that acetic acid bacteria are aerobic bacteria, that is, they require oxygen for development, while lactic bacteria are facultative anaerobic (aerobic tolerant). They develop best in a medium without oxygen but tolerate its presence.

Finally, from an oenological point of view, the major difference is the fact that acetic acid bacteria lead to the production of acetic acid while lactic bacteria produce principally lactic acid.



Organizational differences in the cell walls in Gram-positive bacteria (lactic bacteria in wine) and Gram-negative bacteria (acetic acid bacteria in wine)

5. What are the characteristics of lactic bacteria?

Lactic bacteria are bacteria that transform sugars and acids. They are found everywhere in nature. In Man, lactic bacteria are normally present in the skin and the digestive system, where they carry out numerous functions. Notably they create a hostile environment for pathogenic bacteria (an acidic medium thanks to the production of lactic acid). In the food industry they are used to ferment dairy products, rendering them more digestible while also increasing bioavailability of vitamins and minerals.

6. Lactic bacteria and wine: what is malolactic fermentation?

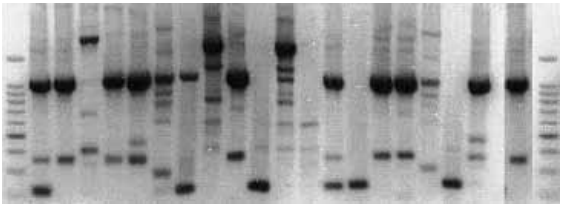
MLF is the transformation of L-malic acid into L-lactic acid with the release of carbon dioxide (CO_2). This reaction is catalysed by the malolactic enzyme. This enzyme is located inside the lactic bacteria cell in the wine. To function, it requires essential co-factors: magnesium (Mg^{2+}) and manganese (Mn^{2+}) ions, and also a pH of 5.8, which requires the lactic bacteria in wine (a more acidic medium) to constantly regulate their intracellular pH.

7. Several types of lactic bacteria; homo or heterofermentative?

The glucose consumption pathway taken by lactic bacteria is also a criterion for classifying lactic bacteria. Some only produce lactic acid; others also produce acetic acid, CO_2 and ethanol. The former are called homofermentative, the latter heterofermentative. Among lactic bacteria in wine, the species of the *Pediococcus* genus are homofermentative, the species of the *Œnococcus*, *Leuconostoc* and *Lactobacillus* genera are heterofermentative.

8. How are bacteria classified (Families, species, strains)?

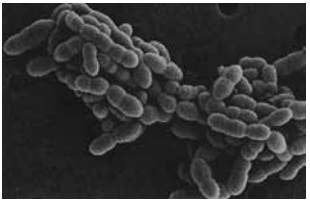
From the very first observations carried out in the microbial world, Man has tried to classify micro-organisms according to their similarities and differences. To begin with, distinction criteria were simply morphological criteria (coccus or bacillus, Gram-positive or Gram-negative…), with time, tests became increasingly specific. Understanding bacteria physiology enabled the development of phenotypic, and later, genetic tests (each bacterium having specific DNA sequences). Bacteria are classified in families, species and finally strains. The official definition of the bacterial species is that two bacteria belong to the same species if the percentage of DNA homology is more than 70%. No equally precise definition exists for distinguishing strains. One mutation on the entire bacterial genome can be sufficient for distinguishing between two strains of the same species.



Observation of Œnococcus œni strain diversity in a wine using molecular biology. Each profile corresponds to a particular strain.

9. Which lactic acid bacteria species is dominant in wine?

While the number of lactic bacteria species present on the grape and in the must is elevated, as vinification progresses, the species diversity declines. Oenological constraints: pH, SO_2, reduced quantity of available carbon substrates and especially the production of alcohol, all render the medium hostile to a large number of species. The species that is most apt at withstanding the oenological constraints is *Œnococcus œni*. It tolerates alcohol reasonably well, adapts to the wine's pH and L-malic acid is an adequate source of carbon for its growth.



Photograph of a Œnococcus œni culture

Œnococcus œni is the main species responsible for malolactic fermentation in wine. Malolactic yeast starters (LACTOENOS 350 PreAc®…) available on the market are all strains of *Œnococcus œni*.

10. Why was Leuconostoc œnos renamed *Œnococcus œni*?

Leuconostoc œnos, the principal lactic bacteria in wine was renamed *Œnococcus œni* following a compilation of fermentation properties and phylogenetic analyses that revealed the necessity to define an *Œnococcus* genus distinct from the *Leuconostoc* genus. For a long time, *Œnococcus œni* was the only species representative of the *Œnococcus* genus, clear proof of its particular properties. Nevertheless, recent studies have led to believe that within the *Œnococcus œni* species, it could be possible to separate several sub-species. Until recently, and with the identification of a new *Œnococcus* species; *Œnococcus* kitaharae, *Œnococcus œni* was the only species within the *Œnococcus* genus; an atypical situation in bacterial systematics, proving the significant diversity between *Œnococcus œni* strains.

11. What is a bacteriophage?

A bacteriophage is a virus that only affects bacteria. It is an organism incapable of reproduction by its own means. It has to inject its genetic material into the bacterium. It diverts its enzymatic machinery and when it has replicated, it kills the bacterium, leading to inactivity and a drop in the bacterial population. Bacteriophages can provide an explanation for certain stubborn MLFs. However, it should be emphasised that a bacteriophage is specific to a target bacterium, affecting the indigenous population but not the cells of a malolactic yeast starter, which are insensitive to the bacteriophage.

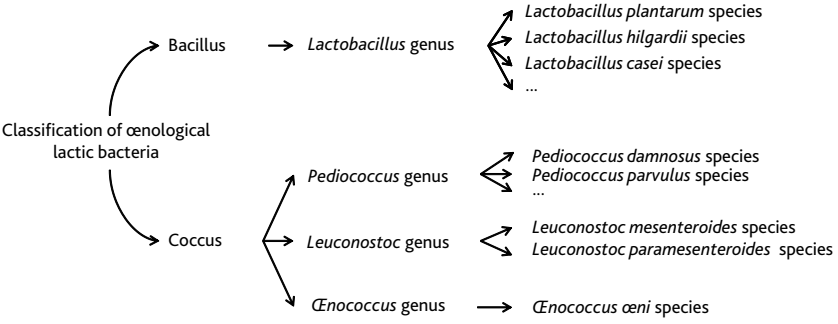
12. What is a plasmid?

A plasmid is a DNA molecule that is distinct from chromosomal DNA. It is capable of autonomous replication. Plasmids are generally circular. Their size varies from a few hundred nitrogenous bases to several thousand. Their replication method is autonomous and independent of the bacterial chromosome. They can carry genes that are essential to the qualities of the bacterium. Plasmids can be spontaneously lost during cell division, and notably during extraction in laboratory conditions. In *Œnococcus œni*, the particular properties of certain strains are associated with the presence of certain plasmids. It can be a case of advantageous plasmids, which contain genes that are resistant to certain stress, or detrimental plasmids, such as the plasmid discovered by Patrick Lucas at the ISW in Bordeaux which contains genes implicated in histamine synthesis, the principal biogenic amine found in wine. Plasmids (genetic partners of lactic bacteria) maintain the integrity and identity of the bacteria for expressing their metabolic characteristics. LAFFORT, in collaboration with Patrick Lucas from the University of Bordeaux oenological faculty, is working on this issue in order to obtain selection tools that take these characteristics into account and that ensure the stability of the strains selected for yeast starters (during industrial manufacturing process, the advantageous plasmids must not be lost).

BACTERIA AND ŒNOLOGY

13. Which lactic bacteria are found in wine?

Several dozen species of lactic bacteria intervene in oenology. They belong to the *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Œnococcus* species. The main species involved in MLF is *Œnococcus œni*, being the best equipped to resist the particular conditions found in wine and having the best capacity for degrading L-lactic acid.



Certain strains of *Œnococcus œni* have remarkable oenological aptitudes, the best being utilized for producing malolactic bacteria starters. Others strains can be detrimental to wine quality, such as indigenous strains that produce biogenic amines. The other most frequently encountered species in oenology are *Lactobacillus* plantarum in the must and *Pediococcus* damnosus in certain wines at the end of maturation.

14. Where do wine bacteria originate from?

Indigenous lactic bacteria in wine originate naturally from the vineyard, where they cover the surface of the grape berry along with yeasts and molds. However the microflora of the grape is extremely variable both in terms of quantity (population levels present on a berry vary from one to one million cells) and diversity. Vineyard practices and Mother Nature play a major role in the development of berry microflora populations.

15. Is it possible to utilized the bacteria population present on the grape grape in the winemaking process?

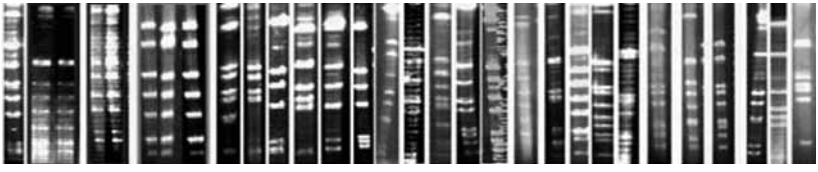
Studies that have been carried out on grape micro flora are ongoing. The microflora that resides on the grape is diverse and highly complex. The principal oenological micro-organisms, including *Œnococcus œni*, have been detected on the surface of the grape. However, a large number of factors play a role (climate, viticultural practices, phytosanitary treatments) and given the current state of our knowledge, it is impossible to act on grape microflora. It is only in the cellar that these populations can be controlled. The microflora brought to the cellar by the grape can be estimated with microbiological analyses and controlled by using selected yeast and bacteria strains in order to avoid spoilage. The massive addition of a selected bacteria strain (malolactic bacteria starter) significantly reduces the detrimental implications of indigenous flora: biogenic amines, volatile acidity, volatile phenols…

16. How can lactic bacteria in wine be identified?

Using a wine sample, it is possible to study the lactic bacteria present. Simple microscopic observation reveals useful initial information. Bacteria, being smaller than yeasts, are easily detected. Using the Gram staining system, lactic bacteria (Gram-positive) can be distinguished from acetic acid bacteria (Gram-negative). By using a cellular viability marker, dead cells can be distinguished from live cells with epifluorescence microscopy: live cells appear fluorescent green.

Molecular biology techniques are used more and more frequently to establish species identity. This can be by hybridization, PCR or sequencing. To distinguish between strains within one species, pulsed field electrophoresis is carried out for *Œnococcus œni*, a type of karyotype of the bacterial chromosome.

Below is an *Œnococcus œni* strain differentiation carried out by Emmanuel Gindreau in 2003 in the laboratory of Professor Aline Lonvaud-Funel using pulsed field electrophoresis. A profile corresponds to each distinct strain. These profiles can also be exploited to establish phylogenetic trees that group together the strains presenting the most resemblances. These studies have defined the strain groups with an oenological advantage.



Example of pulsed field gel electrophoresis

17. Are all lactic bacteria beneficial for wine quality?

No, certain metabolic pathways are detrimental to wine quality: glycerol degradation (bitterness disease), production of exopolysaccharides (graisse disease) and biogenic amines… These pathways are characteristic of certain species: glycerol degradation is specific to specific species of the *Lactobacillus* genus and graisse disease is generally caused by the *Pediococcus* genus. But this can also be characteristic of certain strains within a species. For example, in *Œnococcus œni* certain strains produce biogenic amines, contrary to strains isolated for producing malolactic bacteria starters.
In 2005, Patrick Lucas demonstrated on over 250 wines in the Bordeaux region that 70% of the wines presented a population of biogenic amine producing indigenous bacteria in excess of 10^3 cell/mL of wine. It was a majority of *Œnococcus œni* strains that had carried out MLF. The preventive usage of a selected strain (non-producing) would have significantly decreased the risk of negative alteration.
Finally, defects of indigenous flora are unpredictable, and the best way to control this phenomenon is prevention and the use of a malolactic bacteria starter.

18. What are the principal defects caused by lactic bacteria in wine?

The principal defects caused by lactic bacteria are (in order of importance and frequency):

- Increase in volatile acidity.
- Degradation of hexoses and pentoses (sugars) into a combination of L-lactic acid and D-lactic acid (a dosage of over 0.3 g/L of D-lactic acid is a sign of lactic disease).
- Biogenic amine production.
- High production of exopolysaccharides: graisse disease.
- Glycerol degradation and production of acrolein: bitterness disease.
- Tartaric acid degradation and production of acetic acid: tourne disease (loss of flavor).
- Mannitol production: mannitic disease (sour taste).

Bitterness, tourne and mannitic diseases are very rare nowadays. On the other hand, a high biogenic amine content is increasingly frequent, and poses a genuine health problem.

19. What is the advantage of distinguishing between strains within the *Œnococcus œni* species?

Identification criteria for a bacterium allow us to classify it successively by family, then by species and finally to characterize it as a unique and specific entity, i.e. a strain. A family contains several species and each species has several thousand strains.
Numerous differences exist between the strains of a same species. For example, LACTOENOS 350 PreAc® is a strain initially selected for its remarkable capacity

to resist against low pH; LACTOENOS 450 PreAc® is a strain that is particularly tolerant to high degrees of alcohol. Certain defect producing metabolic pathways are also strain-dependent. This is notably the case for biogenic amine production by *Œnococcus œni* strains. Not all O. œni strains possess the genes implicated in biogenic amine production. Some indigenous strains can produce several dozen milligrams of histamine while other strains do not produce any at all. The same applies to graisse disease and *Pediococcus*: some are known as 'ropy' as they produce high quantities of exopolysaccharides, while others produce only a small amount or none at all. Finally, each strain must be considered as a specific individual with its own qualities and defects. The use of malolactic bacteria starters permits the bacterium with maximum superior qualities and limited defect producing metabolism to be selected. During indigenous MLFs it is impossible to control the defects of the strains that are in action. The use of malolactic bacteria starters, selected for their absence of detrimental metabolism is the best weapon against indigenous defect production.

20. Is the malolactic enzyme unique to lactic bacteria in wine?

It was during the 1970's that the first malolactic enzyme was extracted from a bacterium isolated in wine. Since then, its presence has been revealed in a number of other lactic bacteria, including those in the dairy industry, where malic acid does not exist.
This enzyme is a complex of two identical protein sub-units. Its activity requires the presence of a cofactor, NAD+, and divalent ions Mg^{2+} or Mn^{2+}. The other dicarboxylic acids found in wine; lactic acid (product of the reaction), tartaric acid, citric acid and succinic acid are all reaction inhibitors. L-malic acid, despite being a substrate of the reaction, also has a repressive effect when present at levels above 5 g/L. This can be explained by the fact that L-malic acid, on entering the bacterium, releases its protons which acidify the cell's interior. The bacteria are consequently forced to provide a supplementary effort to control its intracellular pH (which must remain stable at around 5.9). Without taking other parameters into account (alcohol, pH, temperature, SO_2…) malolactic fermentability is at its highest at between 1 and 5 g/L of L-malic acid.

21. Why does malolactic fermentation modify wine acidity?

During MLF, multiple biological transformations occur, the most noteworthy being a drop in total acidity (approximately 0.4 g of H_2SO_4 per gram of consumed L-malic acid) and an increase in pH (a 5 to 10% rise in pH can be observed during MLF). This is due to the transformation of a di-acid, L-malic acid, into a mono-acid, L-lactic acid, and also due to other organic acids: citric acid, pyruvic acid… that are used by the lactic bacteria during MLF. For these reasons, besides microbiological stability, there is an increasing demand for MLF on dry white wines or extremely acidic rosé wines. It should be noted that if a must is acidified with malic acid as authorized by European regulations since 2009 (see effective regulation conditions); the preparation used contains a racemic combination of both isomeric forms of malic acid (L and D). In this case, lactic bacteria

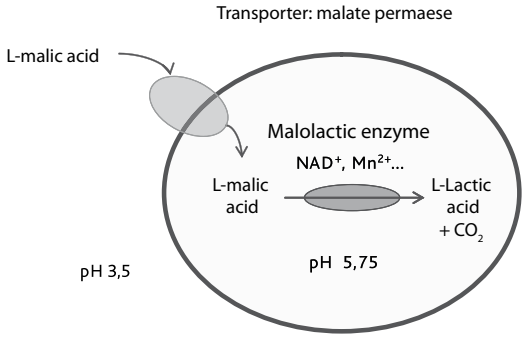
development will lead to degradation of L form of the isomer, leaving only d-malic acid to remain in the wine.

22. Why does malolactic fermentation contribute towards microbiological stability in wine?

Besides its effect on acidity, MLF is also implemented for microbiological stability, especially in red wines and certain effervescent white wines for two main reasons. First, by degrading the L-malic acid and other nutrients (vitamins, nitrogenous compounds) during MLF, the bacteria reduce the available quantities of these compounds for other microbial usage, which could be sources of defects. Secondly, good MLF management (preventive use of a malolactic bacteria starter) helps maintain an active and selected microflora following yeast decline at the end of AF. This prevents spoilage bacteria from entering, especially *Brettanomyces* or biogenic amine producing lactic bacteria that cannot develop during this period where the microbial ecosystem is dominated.

23. Why is cellular viability essential for malolactic activity?

Enzymatic activity cannot be dissociated with cellular viability. For the malolactic enzyme to act, L-malic acid must first penetrate the cell. This active transport function is conducted by malate permease, which is coded by a gene situated on the bacterial chromosome adjacent to the one for the malolactic enzyme. Consequently, during enzyme synthesis the cell is also ensured a sufficient number of available transporters.



It is equally important to recall that one of the important characteristics of the malolactic enzyme is its functioning pH, which is 5.8. As wine is markedly more acidic, maintaining this pH requires a significant effort from the cell to export the protons. This is only possible when the cell demonstrates excellent viability.

24. What does malolactic fermentation provide the cells with?

From the very first studies on MLF, it appeared evident that the reaction was beneficial to bacteria growth, but due to the fact that the reaction supplies neither ATP nor NADH++ H+, which are the two principal cellular energy sources, the reaction hypothesis needed to be pursued further. The supplied energy is, in fact, generated at the membrane level via translocation of malate molecules (entry of the reactant according to its concentration gradient) and lactate molecules (product exit also according to its concentration gradient). Decarboxylation of malic acid also provokes alkalization of the cytoplasm. This pH difference between the interior and exterior of the cell generates a proton pump that is used as a motor for ATP synthesis.

25. What role does citric acid play for lactic bacteria?

L-malic acid is not the only substrate for lactic bacteria in wine. While present at a much lower concentration than malic acid, citric acid is also an important substrate for wine bacteria. Citric acid is systematically consumed during MLF but at a much slower speed than malic acid, to such an extent that when MLF is completed, wines still contain up to 100 - 200 mg/L, i.e. 30 to 60% of the initial citric acid concentration. Nevertheless citric acid is an important substrate for lactic bacteria due to the fact that at the start of their growth phase, its degradation is essential for synthesizing membrane lipids. While L-malic acid degradation is favorable for wine quality, that of citric acid is questionable. Citric acid degradation leads either to the production of volatile acidity, acetoinic compounds (diacetyl, acetoin, butanediol) or lipids. In favorable growth conditions, bacteria develop rapidly and they have high lipid requirements. Consequently the degradation pathway of citric acid is mainly used to provide lipids. Conversely, in limiting growth conditions, the bacteria will use the citric acid mainly to produce acetoinic compounds. The most well-known one, diacetyl, is responsible for buttery odors.
The more sluggish the MLF, the more diacetyl is formed. After malolactic fermentation diacetyl concentration is quite variable; from 2 to 10 mg/L and sometimes higher. It is preferable that the diacetyl content should not exceed 5 to 6 mg/L in wine. Within this range we can consider that the diacetyl contributes to the wine's bouquet; above this it is detrimental.
The use of a malolactic bacteria starter is the most efficient tool for limiting diacetyl production: on the one hand the selected strains intrinsically produce little diacetyl, on the other, carrying out a straightforward and regular MLF limits its production.

26. Are all strains of the *Œnococcus œni* species beneficial to wine?

No, a number of metabolic pathways/end-products that are specific to certain indigenous strains are detrimental. Certain indigenous strains can produce biogenic amines and/or contribute to seriously increasing volatile acidity. Strains selected as

malolactic bacteria starters have passed selection tests that verify the absence of these detrimental characteristics.
Besides poor metabolic attributes, not all *Œnococcus* strains present the same fermentation aptitudes. Underactive indigenous strains can be the origin of sluggish MLF while efficient and correctly used malolactic bacteria starters can considerably reduce the time required for MLF to complete. Sluggish MLFs are an open door to diverse spoilage, notably the production of volatile phenols by *Brettanomyces* yeasts.

27. What are the methods for monitoring MLF?

Although the impressions perceived during wine tastings (slight effervescence due to CO_2 production, appearance of milky or buttery aromas…) can provide information about the possible triggering of an MLF, fermentation must be monitored out by regularly measuring the L-malic acid. This can be done with paper chromatography or by using enzyme kits. The latter should be preferred as they are faster and more precise.
Besides monitoring the L-malic acid content, it is also useful to evaluate the level of the lactic bacteria population, either indigenous bacteria or selected strains. Lactic bacteria monitoring can be carried out by cell/colony count or by epifluorescence observation. The latter is particularly pertinent as it provides information rapidly (no culture waiting time) about the number of active *Œnococcus* cells.
When using malolactic bacteria starters, it is a good idea to check inoculation efficiency 24 to 48 hours following bacteria inoculation, by enumerating bacterial flora using epifluorescence. This is the survival rate measurement.
After inoculation, the lactic bacteria population must have a minimum rate of 5.10^5 cells/mL. Additionally, one can ensure that the fermenting strain is definitely the one that has been inoculated by carrying out the genetic analysis (PCR) on the strains present in the wine during MLF.

28. How can one be certain that MLF has started?

MLF is considered to have started when L-malic acid degradation is detectable through analysis.
To evaluate whether MLF has started, all that is required is to measure the L-malic acid concentration in wine. The best method is enzymatic measurement, which offers extremely precise measurements. As soon as a drop of over 0.2 g/L is observed, it can be considered that MLF has definitely started.
For the decrease in L-malic acid to be effective, a minimal population of 10^6 active *Œnococcus œni* cells/mL is considered to be necessary.

29. How can one confirm that MLF has completed?

The initial L-malic acid content of a must or wine at the end of AF can be between 1 and 6 g/L (or even much higher for certain acidic white wines, up to 14 g/L). The aim of MLF is to convert it entirely into L-lactic acid.
For many years, estimating MLF triggering and completion was limited by the precision of the analytical methods used. Paper chromatography only offered a qualitative vision of the transformation phenomenon of L-malic acid into L-lactic acid. Currently, the use of enzymatic kits has improved measurement accuracy.
Today, regulations set final and maximum L-malic contents. For red wines, this threshold is generally 0.3 g/L. Certain enologists consider that MLF completion has been attained when the L-malic acid content is less than 0.2 g/L. it is important to take into consideration that L-malic acid is a carbon substrate that can also be used by other microbial species (it enters into the Krebs cycle used by yeast). For the purpose of microbial stabilization objectives, it is always beneficial to reduce its content as much as possible, hence sluggish MLF finishes should be avoided. The use of malolactic bacteria starters ensures complete depletion of the L-malic acid reserve. Consequently this facilitates microbial stabilization.

30. How do malolactic bacteria starters fight against spoilage micro-organisms?

The use of malolactic bacteria starters intervenes at a key moment in vinification, where the AF yeasts decline and free up an ecosystem that is favorable to the development of spoilage flora, such as *Brettanomyces* or indigenous biogenic amine-producing lactic bacteria. Consequently, the malolactic bacteria starter can monopolize the ecological niche and prevent the spoilage bacteria from developing after AF. By promoting a faster malolactic fermentation, the wines can be sulfured earlier, thereby closing the window of opportunity for spoilage.

31. How are malolactic bacteria starter strains selected?

Selection of the best *Œnococcus œni* strains is divided into three phases:

- Selection of wines that are likely to provide the most pertinent strains.
  These are wines with remarkably rapid MLF despite difficult conditions: high alcohol (alcoholic strength by volume), low pH, low temperature…
- In the laboratory the bacteria are isolated from the wine by successive cultures on selective media.
- The physiological and genetic qualities of the collected strains are studied in order to retain only those which perform the best. During these tests, we select for the absence of detrimental characteristics (metabolic pathways). Our selection process

ensures the malolactic bacteria starters are free of microbial-origin deviations. Laffort Malolactic bacteria starters do not produce biogenic amines and their activities result in very low levels of volatile acidity and diacetyl.

Recently, breakthroughs in knowledge concerning genetic diversity in O. œni strains has led to the development of genetic tests which are utilized now in ML starter selection work. The genetic tests make it easy to ensure the ML strains have intrinsic aptitudes for withstanding constraints particular to the wine industry. LACTOENOS 350 PreAc® was thus the first bacterium to be selected using the genetic tools that revealed its high resistance to the lowest pHs.
Now, genetics provides markers for the intrinsic aptitudes of strains for withstanding specific constraints (conditions). This consists of verifying the presence of specific pertinent genes of the bacterial chromosome by using PCR. Research projects currently led by LAFFORT should allow the study spectrum to be broadened to include important genes also localized on plasmids.
Finally, let us not forget that all marketed bacteria are 'natural' bacteria isolated from wine. None are derived from genetic modifications or from controlled cross-breeding, as bacteria only reproduce by simple cell division (asexual reproduction).

Selection of wines with physical-chemical parameters
that are restricting for the bacteria during spontaneous MLF
↓
Isolation of the lactic bacteria on selective nutritive
culture media
↓
Verification that they belong to the *Œnococcus œni* species
↓
Verification of the isolated strain identity
↓
Physiological tests (resistance to alcohol, pH…) in the laboratory
↓
Genetic tests to confirm the strain's oenological aptitudes
↓
Verification of the absence of detrimental metabolisms
↓
Vinification tests in real cellar conditions
↓
Commercial productivity tests (strain's aptitude to be produced
commercially in the form of a freeze-dried bacterium)
↓
Availability of the commercial ML starter

32. Are all malolactic bacteria starters equivalent?

No, all bacteria starter strains do not have the same characteristics. For example, some are adapted for direct inoculation while others require a phase of re-acclimatization. As each bacteria starter is derived from a particular strain, capacities to resist against a particular parameter vary from one bacteria starter to another. For example, LACTOENOS 350 PreAc® easily withstands acidic pHs.
While in yeasts, the production of aromas is also a strain-dependant characteristic that varies according to the ADY offered, this is less manifest in bacteria except for a few exceptions: for example, LACTOENOS SB3® is recognized for its good revelation of woody notes during MLF in barrels or in contact with wood chips, probably due to particular enzymes that interact with the wood compounds.

33. In a packet of malolactic bacteria starter, are all the cells identical?

Bacteria are asexual organisms, production taking place via cell division: a cell, named the mother cell, will produce two identical cells known as daughter cells, which in turn become mother cells, etc… This binary fission mode of reproduction means that each daughter cell is identical to the initial mother cell. In the production of ML starters, the selected strain is produced in optimal control conditions to exclude contamination. In this way, all the cells present in the malolactic bacteria starter possess the same identity and the same qualities as the strain that was initially selected in the laboratory.

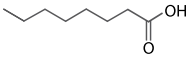
34. What pH increase is generally observed during a MLF?

Depending on the initial L-malic acid concentration, the relative pH increase consecutive to MLF is generally between 5 and 10% of the initial value. That is, a wine with a pH of 3.5 could see its pH increase up to 3.85 after MLF. However the increase in pH cannot be genuinely correlated with the quantity of L-malic acid consumed because other organic acids in the grape are equally important: tartaric acid, citric acid, succinic acid and pyruvic acid. Besides L-malic acid, MLF bacteria also degrade small quantities of citric and pyruvic acid for example.
It should be noted here that for L-malic acid only the L isomer is transformed by the MLF bacteria.

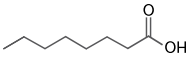
35. What is the total decrease in acidity generally observed during a MLF?

In general it is considered that the decrease in total acidity is approximately 0.4 g/L of H_2SO_4 per gram of L-malic acid consumed. This appears to be the most important impact the MLF bacteria have on the organoleptic qualities of the wine.

36. Why are Octanoic acid and decanoic acid enemies of lactic bacteria?



Octanoic acid

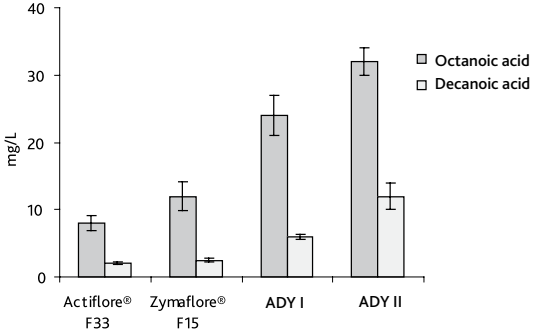


Decanoic acid

Medium chain fatty acids: octanoic acid (C8) and decanoic (C10) acid are two important inhibitors of lactic bacteria in wine.

These fatty acids take advantage of their short aliphatic chains and insert themselves between the membrane phospholipids in the bacteria, affecting membrane fluidity; an essential parameter for cellular viability. The antibacterial effect of these compounds is such that they are sometimes used in high concentrations as antibiotics to treat infectious diseases of bacterial origin.

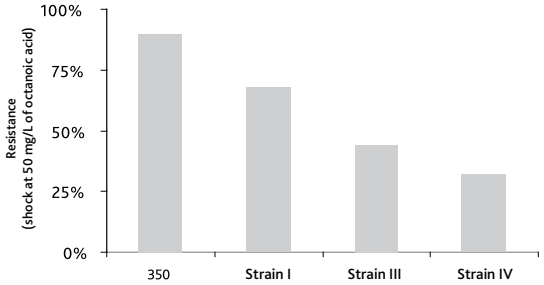
Medium chain fatty acids are principally produced by AF yeasts. The phenomenon is strain-dependent, meaning that certain strains produce less than others. For example, ACTIFLORE F33® or Zymaflore F15® are among the ADYs that produce the least. They consequently favor rapid MLF start-up.



Brettanomyces bruxellensis yeasts produce a large quantity of octanoic and decanoic acid (these compounds interact with other molecules produced by *Brettanomyces* conferring the negative characteristic "brett" notes). The production of octanoic and decanoic acids by *Brettanomyces bruxellensis* also partially explains why it is generally difficult to trigger a malolactic fermentation following the development of a *Brettanomyces bruxellensis* infection. To remedy high octanoic and decanoic acid contents, the most effective treatment is to add specialized yeast cell walls such as BIOCELL® adsorb these toxic compounds. It is also important to use a bacteria strain recognized for its high tolerance to fatty acids, such as LACTOENOS 350 PreAc®. In wine, it is considered that concentrations of octanoic acid greater than 25 mg/L and/or 5 mg/L of decanoic acid affect malolactic fermentability. These values are observed in almost all wines that, during the spring following the harvest, have not yet undergone MLF because these compounds have accumulated since the end of AF.

37. What influences octanoic and decanoic acid contents in wines?

The production of octanoic and decanoic acids depends principally on the yeast strains that carry out AF. Certain yeast strains produce very few medium chain fatty acids, rendering MLF particularly easy, this being the case for ZYMAFLORE F15® or ACTIFLORE F33®, while others produce more, and can delay MLF triggering. In reviewing a wide range of analyses, it has been observed that average concentrations at the end of AF are distinctly higher in white wines than in red wines. Moreover, in white wines, the lower the pH, the higher the octanoic and decanoic acid content tends to be. In red wines, average contents are approximately 15 mg/L for octanoic acid and 2.5 mg/L for decanoic acid, while in white wines these values increase to 30 mg/L for octanoic acid and 5 mg/L for decanoic acid, and sometimes even as high as in excess of 50 mg/L for octanoic acid and 25 mg/L for decanoic acids in more acidic white wines. This difference could explain why, besides acidity, MLFs are more difficult to carry out on white wines than on reds.
Finally, it has been demonstrated that *Brettanomyces* produce high quantities of medium chain fatty acids which could explain, in addition to the problem of ecological niche occupation, why *Brettanomyces* development slows down MLF triggering.
Not all bacteria strains tolerate octanoic and decanoic acids in the same way, some being distinctly more sensitive than others. Resistance to high levels of octanoic and decanoic acids was first taken into consideration during LACTOENOS 350 PreAc® bacterium selection. On average, LACTOENOS 350 PreAc® tolerates octanoic and decanoic acid concentrations twice has high as other malolactic bacteria starters available on the market.



38. *Brettanomyces bruxellensis* / *Œnococcus œni*, friend or foe?

Brettanomyces bruxellensis and *Œnococcus œni* both are resistant to alcohol and are ready to populate wine after AF is complete. Between the end of the alcoholic fermentation and the beginning of the malolactic the wine is particularly vulnerable to the *Brettanomyces* growth. It is the competition between *Brettanomyces* and *Œnococcus*, not a synergistic relationship. The longer it takes MLF to begin, the more time the *Brettanomyces bruxellensis* has to develop. In order to shift the system in

favor of MLF triggering, the preventive usage of a malolactic bacteria starter is the most effective tool. Inoculation of a massive *Œnococcus œni* population will block the *Brettanomyces bruxellensis* before they can develop. In optimal fermentation conditions, the addition of a ML starter can be implemented before the end of AF, which gives the ML bacteria a head start over *Brettanomyces*.

39. What are the interactions between *Saccharomyces cerevisiae* and *Œnococcus œni*?

While traditionally (that is to say except in co-inoculation) AF yeasts (*Saccharomyces cerevisiae*), and MLF bacteria (*Œnococcus œni*) are not active simultaneously, bacteria development is considerably dependent on yeast activity. AF yeasts are for the most part responsible for the conditions of the medium in which the bacteria evolve, therefore indirect interactions exist between AF yeasts and MLF bacteria. Besides ethanol, certain yeast products are detrimental to the bacteria. This is the case for SO_2 and medium chain fatty acids. Most of the time, the yeasts are beneficial to the bacteria. By self autolysis (cell destruction) after AF, they release nutrients required for bacteria growth, notably nitrogenous compounds that are essential to the bacteria. The ML bacteria are incapable of assimilating inorganic nitrogen. They are consequently highly dependent on the nitrogenous compounds released by the yeasts. This underlines the importance of controlling the assimilable nitrogen in the must and using organic nitrogen (NUTRISTART ORGANIQ®) for good AF development and also to ensure good malolactic fermentability. Yeast autolysates are also beneficial as cell wall fragments adsorb compounds on their surfaces that are toxic to the bacteria, such as medium chain fatty acids.

40. What are biogenic amines?

Biogenic amines are the second cause of food poisoning in Europe, despite the fact that these incidents are considered to be only moderately dangerous. They are mainly found in fish, cooked meats, sauerkraut, ripened cheeses and also in fermented beverages: beer, cider and wine.

Biogenic amines are molecules produced by decarboxylation of certain amino acids. The most frequently found in wine are histamine, tyramine and putrescine. In sensitive subjects, they provoke headaches, respiratory complications, heart palpitations, allergic reactions and blood pressure problems. In our liver, there are enzymes that are specifically intended to eliminate biogenic amines, but these enzymes are inhibited by ethanol. This explains why, despite the fact that biogenic amine contents in wine are generally lower than in other products, they cause genuine health problems for the consumer, to the extent that their content in wine is rigorously regulated for export to certain countries (10 mg/L in Switzerland). Other biogenic amines: cadaverine, putrescine… when present in high concentrations, can play a genuinely detrimental role on a sensorial level.

The production of biogenic amines varies according to the substrates available, to wine

pH, but especially to lactic bacteria species and strain.
In particular, in *Œnococcus œni* and lactic bacteria in general, biogenic amines are produced by the action of specific enzymes: tyrosine-decarboxylase, histidine decarboxylase. However, not all strains possess the genes that code for these enzymes. Patrick Lucas has demonstrated that the genes implicated in histamine production are not present in the *Œnococcus œni* bacterial chromosome, but on a plasmid. The absence of these genes and consequently the absence of the biogenic amine production metabolism are systematically checked during malolactic bacteria starter selection. The LACTOENOS® bacteria are thus "incapable" of producing biogenic amines. When they are inoculated, they reduce indigenous flora activity, including that of biogenic amines. Inoculated wines thus present biogenic amine contents that are significantly lower than those in spontaneous MLF wines. Inoculation using selected bacteria is thus the best means of prevention against biogenic amines in wine.

41. Is there a higher risk of biogenic amines in difficult conditions?

The metabolic pathways involved in the production of biogenic amines constitute an advantage for the strains that possess them. The exchange between the amino acid and the corresponding amine contributes towards reinforcing the electrochemical gradient and the propelling proton force across the membrane, and consequently the cells' energy reserve. When conditions become restrictive (high ethanol content, temperature decrease during the winter period…), the biogenic amine-producing indigenous strains are more likely to survive. In this case, only the use of a malolactic bacteria starter is likely to prevent biogenic amine production. Laffort selected strains do not produce biogenic amines, but will still tolerate the restrictive conditions as they possesses other biochemical pathways to ensure its survival and optimal activity.

42. Ethyl carbamate, are lactic bacteria the guilty party?

Ethyl carbamate is a cancer causing molecule that despite being present in weak concentrations in wine is sometimes the object of severe controls for wine importation to certain countries such as Canada or the United States.
In theory, lactic bacteria in general and *Œnococcus œni* in particular, are capable of producing ethyl carbamate. The lactic bacteria species generally implicated is *Lactobacillus* plantarum, which transforms Arginine into citrulline and is then used to produce ethyl carbamate. In reality, the principal source of ethyl carbamate in wine comes from urea produced by the indigenous yeasts during alcoholic fermentation. In most cases, ethyl carbamate levels are very low in wine, even when produced by indigenous yeast.

43. What is the significance of D or L-lactic acid?

Lactic acid is a carboxylic acid, its chemical formula is $C_3H_6O_3$, with an asymmetric carbon. Two lactic acid enantiomers exist: L-lactic acid and D-lactic acid. L-lactic acid is the direct product of L-malic acid degradation that is naturally present in grapes. When sugars remain at the end of AF, the bacteria can metabolize the sugars instead of malic acid. The products of this metabolism are L and D-lactic acids, ethanol and acetic acid. The quantity of ethanol formed is in all cases negligible in relation to the wine's alcohol concentration. Likewise, only a small quantity of L-lactic acid is formed from the sugars in comparison to the MLF activity. On the other hand, D-lactic acid is a genuine indicator that the sugars are being used by the lactic bacteria.
Ultimately this leads to an increase in the acetic acid content. A concentration of D-lactic acid greater than 0.5 g/L confirms lactic disease. The presence of D-lactic acid in wine is not the consequence of D-malic acid degradation by MLF bacteria but the degradation of sugars by the bacteria. Lactobacilles in the must can be the origin of this problem.

44. Where do the buttery/milky aromas in MLF originate from?

Citric acid is another acid in the must that is not metabolized by the yeasts and can be used by the bacteria. Besides lipids and acetic acid, diacetyl is one of the products from degradation of citric acid by the lactic bacteria. It confers buttery or milky notes to the wine. It is considered that at below 5 mg/L, diacetyl intervenes favorably in the wine's aromatic complexity. Above this, it can be perceived as an organoleptic defect, notably in white, rosé or red wines intended for fast consumption.

45. How does maturing on lees reduce the buttery perception from MLF?

Once it is produced by the lactic bacteria, diacetyl is reduced into acetoin, a less-odorous molecule. This reductase diacetyl activity is always present in lees and consequently the diacetyl concentration drops, reducing the buttery aroma. Sulphiting also plays an important role as diacetyl can combine with SO_2 leading to a drop in the content of free, aromatic diacetyl. Nevertheless, the combination is a phenomenon of balance, and when the SO_2 level decreases, the aromatic impact can increase.

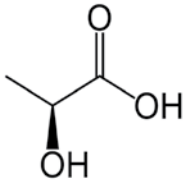
46. Diacetyl and amino acids, what is the double effect of diacetyl?

Owing to its ketone functions, diacetyl is a highly reactive molecule. It reacts notably with amino acids, and particularly with sulfur containing amino acids to form odorant molecules with sulfurous, floral or toasted notes.

47. Why do lactic bacteria increase volatile acidity?

Before answering this question, it is important to emphasize that volatile acidity production is inherent to all microbial developments in wine, whether it is indigenous yeasts, *Brettanomyces* yeast, AF yeasts, lactic or acetic bacteria. Depending on the metabolic mechanisms used that differ from one species to another, and also from one strain to another, the contribution towards increasing volatile acidity can be minimal or excessive (acetic bacteria, *Brettanomyces*…).
Controlling the volatile acidity increase is one of the main objectives when selecting yeast and bacteria starters. An ADY or a malolactic bacteria starter intrinsically produces less volatile acidity than an indigenous strain. Besides these qualities that are linked to cell physiology, yeast starters also shorten the phase during which the micro-organisms can produce volatile acidity. A fast, controlled MLF using a malolactic bacteria starter also helps reduce the microbial period of activity and shorten the phase of volatile acidity production.

48. Oxygen, friend or foe for lactic bacteria?



Bacteria are anaerobic micro-organisms that tolerate oxygen to a certain extent (facultative anaerobic or aerobic tolerant). Without being particularly adapted to aerobic life, lactic bacteria can take advantage of the oxygen present in a micro-aerated environment in the presence of a small quantity of carbon substrates. Operations that supply wine with moderate quantities of oxygen including racking or lees stirring; can accelerate the development of lactic bacteria. In late co-inoculation, when the ML starter is added towards the end of alcoholic fermentation (at a density of 1010 or 2.5 Brix°), it is generally observed that the bacteria take advantage of the end of AF and post-fermentative maceration to adapt to the wine and monopolize the medium. However, MLF triggering does not generally occur until racking. This allows the two fermentation phases to be clearly separated (firstly alcoholic fermentation, then malolactic fermentation). Finally, it is possible that aerating during racking contributes to reducing the potential concentration of free SO_2that is not recommended for bacteria.

ŒNOLOGICAL PRACTICES AND FERMENTATION MANAGEMENT

49. Why use a malolactic bacteria starter?

Despite its importance in wine production, MLF often remains a random phase of vinification. In order to control and optimize MLF, the preventive usage of a malolactic bacteria Starter is the most effective tool. Launched in the early 1990's, ML starters are used more and more frequently by winemakers concerned with monitoring their fermentation procedure and controlling risks of microbial spoilage (*Brettanomyces bruxellensis*). The economic and environmental advantages to using high-performance ML starters are significant. A malolactic bacteria starter costs less than a tank that must be maintained at a particular temperature during sluggish indigenous MLFs and the carbon footprint for producing and using an ML starter is six times lower than the carbon footprint for heating a tank in the cellar. Also, The lag time of the indigenous flora development is avoided by the addition of a massive O. oeni population (106 cells/mL) in the ML starter (Table below).

Modality	Number of trials monitored in 2008	Number of days between AF end and MLF end
Late coinoculation	5	12-32
Sequential inoculation	7	20-42
Indigenous MLF	12	34-78

The time saved in MLF development reduces the total heating time of the tanks. One way to eliminate tank heating completely is to use the co-inoculation method. Statistically, when an ML starter is used early (co-inoculation), the purchasing investment of the bacteria starter is paid off by the energy saved. (Table below).

Modality	Number of trials monitored in 2008	Average number of days heating	Cost of malolactic east starter	Average heating cost
Late coinoculation + 450 PreAc	5	0	1,2 €/hL	0 €/hL
Sequential inoculation + 450 PreAc	7	12	1,2 €/hL	0,42 €/hL
Indigenous MLF	12	45	0 €/hL	1,68 €/hL

Using a malolactic bacteria starter also helps ensure that the microbial niche is monopolized in the wine, which helps inhibit the growth of deleterious bacteria. As an example, the risks of Brettanomyces development during vinification are entirely controlled when a high-performance malolactic bacteria Starter is used.

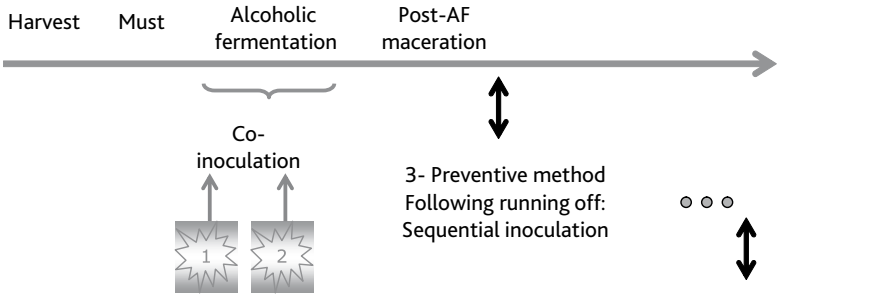
50. What are the different stages for using a malolactic starter?

Traditionally the ML bacteria starter is introduced into the wine after racking; this is sequential inoculation. The ML starter can also be introduced earlier during fermentation. This is referred to as co-inoculation. Two distinctions can be made:

- Early co-inoculation where the ML starter is introduced 24 hours after AF fermentation start-up. In this case, MLF begins before AF is completed.
- Late co-inoculation which consists of adding the malolactic bacteria starter at around a density of 1010 or 2.5 Brix°. In this case MLF is triggered once AF has completed.

Early co-inoculation optimises the effectiveness of the ML starter. The time saved is optimal. MLF is generally completed in just a few days after AF.

A large majority of quick-to-market wines are now vinified using this procedure. Late co-inoculation does not reduce the amount of time required for MLF start-up as much. On the other hand, it does allow excellent prevention of spoilage as the bacterium is in position to dominate at the moment when the AF yeast begins to decline. The ML bacteria will take over from the dying yeast population and dominate the ecosystem at the expense of indigenous spoilage flora. These operative models are the most efficient in terms of time saving and alteration prevention. The malolactic bacteria Starter can also be used later, in urgent situations, when indigenous flora is insufficient. In this case, conditions are more problematic: accumulation of toxic compounds, nutritive deficiencies, development of competing micro organisms… An adapted protocol must be implemented (the use of a robust strain such as LACTOENOS 350 PreAc®, detoxification using yeast cell walls (BIOCELL®)…)



1: Inoculation of the malolactic bacteria starter: 24h after fermentation start-up
Early co-inoculation
MLF starts before the end of AF

2: Inoculation of the malolactic bacteria starter towards the end of AF (approx 1010 density/ 2.5 Brix)
Late co-inoculation
MLF starts once AF has completed

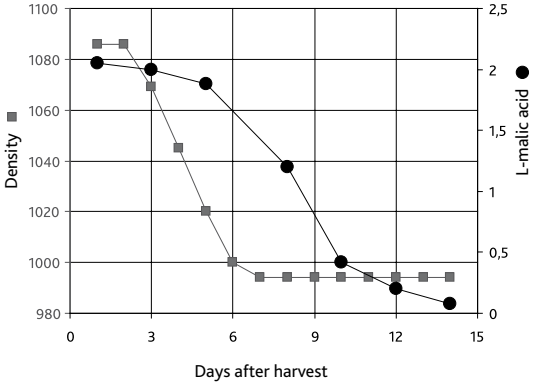
51. What is the reason for industry wide increase in ML bacteria usage?

When they were first marketed, malolactic bacteria starters were seen as relief measures in cases where indigenous MLFs had not begun after several weeks or months of waiting. Obviously, the longer MLF takes to start, the worse the conditions become for the bacteria: nutritive deficiency or competition with other micro-organisms. For this reason, late inoculations do not always offer the best success rates. Now it is recommended to use the selected bacteria preventively. Ideally, their usage should not be considered as a curative method, but rather as a tool for controlled MLF management.
By opting for an addition right after vinification, the malolactic bacteria Starter's effectiveness is optimized. The amount of time saved is significant and the medium is protected against spoilage bacteria. The earliest inoculation techniques (co-inoculation) offer the best success rates and allow recurrent cases of problematic MLF on certain batches to be remedied.

52. What is co-inoculation?

Yeast/bacteria co-inoculation is an alternative to the more familiar or traditional sequential fermentation system. It consists of inoculating bacteria into the must at the beginning of AF. As soon as the yeast activity slows down, the bacteria can take over and MLF is promptly triggered. These techniques have been developed over the last

ten years and their benefits are immensely popular: time saving, spoilage prevention, and reduced energy costs… Co-inoculation ensures clean fermentations and prevents the infection of spoilage bacteria. It preserves the qualities of the wine obtained from alcoholic fermentation. As the wine is stabilized more rapidly by the addition of SO_2, the fruity and fermentative aromas in red wines are retained. In white wines, improved glutathione protection is observed. For example, in early co-inoculation, the glutathione content at the end of MLF is equivalent to that measured at the end of AF. Whereas during late indigenous MLF, the glutathione content can drop considerably due to the fact that the wine is exposed to oxygen prior to post-fermentative SO_2 addition.



53. Early or late co-inoculation?

Early co-inoculation consists of adding the bacteria 24 hours following the start of fermentation, while in the case of late co-inoculation, the bacteria are inoculated towards the end of AF (from a density of 1010 or 2.5 brix).

Early co-inoculation saves time as MLF begins before AF is completed. In late co-inoculation, the bacteria adapt to the medium while AF is finishing and MLF only begins when the AF is completed, once the yeast population starts to decline.

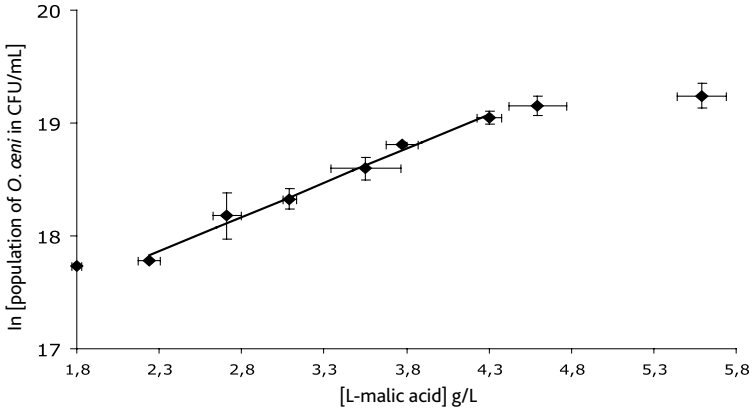
Early co-inoculation should be utilized in healthy fermentation conditions: moderate final alcohol, clean fruit, and vineyard blocks which are known to finish AF strong. With questionable conditions: high potential alcohol, mold, and blocks with historical stuck fermentations, it is best to avoid early co-inoculation. Once you are confident that the AF curve looks good and the wine will finish AF, add the ML starter (this option being the late-co-inoculation method).

54. Co-inoculation or sequential inoculation in the event of heat treated harvests?

In thermovinification, the temperature increase considerably reduces indigenous microbial flora derived from the grape. However, heating does not sterilize the must and the most resistant flora can survive. With the elimination of the most sensitive species, those that are resistant have command of an ecosystem that is favorable to their growth. Among these species, *Brettanomyces* is one of the most resistant. Consequently, a thermal treatment should be followed up with rigorous control of the microbial flora: yeasting for AF and ML bacteria inoculation for MLF.
Here, early co-inoculation has a particular relevance: colonizing the must as quickly as possible at the beginning of fermentation, avoiding the occurrence of a microbiological void and thus contaminations. Also, the temperature rise contributes to reducing the availability of heat-sensitive nutrients such as vitamins. As a consequence it is also recommended to closely monitor yeast nutrition (NUTRISTART®, THIAZOTE®…) and bacteria nutrition (MALOSTART®) in thermovinification.

55. What is the minimal bacteria population required for significant degradation of L-malic acid?

The quantity of degraded L-malic acid is directly correlated with the number of active *Œnococcus œni* cells. At a given moment, a cell degrades a certain number of L-malic acid molecules, and thus 100 cells degrade 100 times this number. For degradation to be significant (perceptible in analysis) a minimum population of 10^6 active *Œnococcus œni* cells is required per milliliter of wine.
Malolactic bacteria Starters are prepared in such a way that a 1 g/hL inoculation ensures this population with 10^6 cells per milliliter of wine. After a period of adaptation to the wine conditions (generally between 3 and 10 days), the selected bacteria trigger MLF.
It is important to emphasize that the speed of L-malic acid degradation is generally greater with a selected strain than with the indigenous flora as fermentation kinetics is a selection criterion for an industrial strain.
Of course the duration of MLF depends on the bacterium used, but also on the quantity of L-malic acid. The higher the quantity, the more bacteria cells are required to consume it. The graph below illustrated the relation between the L-malic acid concentration and the quantity of *Œnococcus œni* required to degrade it. It should be noted that above 5 g/L of L-malic acid, an inhibiting effect exists on the MLF bacteria. Wines with a high L-malic acid content are consequently not the easiest to ferment.



56. Why, occasionally, do analyses count $10^6/10^7$ *Œnoccocus œni* cells in wine when MLF has not been triggered or is stuck?

The correlation previously mentioned between the number of *Œnococcus œni* cells and MLF is only valid if the cells are active. In certain cases (competition with other micro-organisms, with high alcohol, low pH, excessively low temperature, presence of inhibiting compounds such as medium chain fatty acids) it is possible for the cells to be present in the wine in high population levels, without the malic acid degradation being necessarily effective. In this case, to promote malolactic activity start-up, a good understanding of the reasons for this latency is required: the presence of a significant yeast population, nutritional deficiency, excessively restrictive development conditions (alcohol, pH, temperature), then, an attempt to find a solution must be made. Occasionally the bacteria present have a weak malolactic activity; they persist in the medium using substrates other than the L-malic acid: amino acids, citric acid. They must be eliminated (lysozyme, filtration…) because they can bring about spoilage (biogenic amines, diacetyl, an increase in volatile acidity).

It is not a good idea to add the selected bacteria to the wine if there is already a large population present in the wine (the population level can be determined rapidly by epifluorescence microscopy or by quantitative PCR), as competition phenomena can occur and increase inhibition of MLF start-up. Measurement of these bacteria-inhibiting compounds such as medium chain fatty acids (octanoic and decanoic acid) provides information about malolactic fermentability. If the content is high, a curative treatment using yeast cell walls (BIOCELL®) allows the inhibitory compounds to be removed.

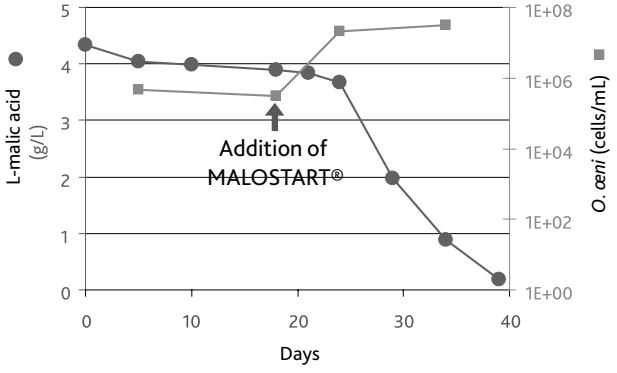
57. What is an MLF activator?

An MLF activator, such as MALOSTART® is a mixture that is rich in elements essential to the malolactic activity of the bacteria present in the medium. MALOSTART® provides vitamins, amino acids (except for those implicated in the production of biogenic amines) and the co-factors that are essential to the malolactic enzyme: Mg^{2+} et Mn^{2+} ions. In the event of a sufficient lactic bacteria population without malolactic activity, MALOSTART® promotes MLF triggering by activating the bacteria that are present. MALOSTART® also contributes nutrient for deficiency problems during late MLF.

Beware of confusing ENERGIZER®, an acclimatization bacteria preparation and MALOSTART®.

ENERGIZER® (PreAc® bacteria) provides bacteria with the elements that will help them survive in the must or the wine during inoculation. MALOSTART® supplies the compounds required for activating the bacteria in the event of deficiency in the medium (late inoculation).

MALOSTART® is used for rapid activation of the selected bacteria. In late co-inoculation, when MLF triggering is desired as soon as AF has finished, a 30 g/hL addition of MALOSTART® promotes triggering (see graph below). MALOSTART® is not specific to selected bacteria strains. It can thus also be used when indigenous bacterial flora is present at a sufficient level. It is recommended to ensure that there is an absence of spoilage bacteria such as *Brettanomyces*, so that the latter cannot benefit from the addition of the activator for their own growth.



58. What are the factors that influence MLF start-up?

Factors influencing MLF start up are: pH. Alcohol, temperature, SO_2, and medium chain fatty acids. Each factor must be considered when choosing the ML Starter for your wine. In general, malolactic bacteria Starter s are selected for their remarkable capacity for growth and activity. They are always more capable of developing where the indigenous

flora would be inadequate in the face of too many inhibiting elements.

59. Why is it more risky to implement MLF than Alcoholic Fermentation (AF)?

Bacteria are added into a in a more hostile and less standardized medium than must. Yeasts always find ideal conditions (sugars, nutrients, temperature, oxygen…) for their development, to such an extent that it is always more delicate to trigger a MLF than an AF. After AF, the bacteria must first adapt to the medium before beginning MLF.
A lag phase of a few days should always be anticipated between bacteria inoculation and the beginning of L-malic acid degradation.
Only the co-inoculation technique allows this latency period to be averted. As in this case, the bacteria are added into the must under favorable conditions (low alcohol and nutrient-rich medium).
It is essential to take particular care of lactic bacteria and the conditions of their growth in wine. The water used to rehydrate the bacterial preparation (non-chlorinated water), the temperature of this water and the wine, nutritional deficiencies, control of indigenous populations such as *Brettanomyces*… these are all key factors for ensuring successful inoculation.

60. Can difficulties for carrying out MLF be anticipated?

The conditions under which MLF triggering can be difficult are listed in the table below:

Difficult conditions MLF	
Alcohol (% vol.)	>13
pH	<3,3
Total SO_2 (mg/L)	>40
L-malic acid (g/L)	< 1 and > 5
Temperature (°C)	< 16 (and > 30 in co-inoculation)
Medium chain fatty acids (mg/L)	Octanoic acid > 25 Decanoic acid > 5

These conditions are cumulative. As an example, when the wine's alcohol is 13% vol, MLF is more delicate at a pH of 3.3 than at a pH of 3.6. Likewise, at a pH of 3.6, MLF will be easier with an alcohol of 12% vol than at 15% vol.
The presence of a competing population (*Brettanomyces* for example) is also a parameter that can complicate MLF start. It is considered that with as low as 10^3 cells/mL of *Brettanomyces*, bacterial inoculation becomes more risky.
It is important to underline that the choice of an adapted ML starter resolves certain

problematic cases. For example, the LACTOENOS 450 PreAc® can be active up to an alcohol of 17 % vol, and the LACTOENOS 350 PreAc® bacterium will be efficient in very acidic wines (down to a pH of 2.9) or in wines with a high concentration of medium chain fatty acids.
Finally, the L-malic acid content is also a parameter to be taken into consideration. Below 1 g/L of L-malic acid, it is difficult to trigger MLF as the quantity is not sufficient for the bacteria to choose this metabolic pathway. MLF is also complicated when the content is greater than 5 g/L because the L-malic acid, on entering the cell, releases its protons and acidifies the intracellular medium, which must be counterbalanced by the bacterium at the expense of its fermentative activity. Lastly, fermentability is optimal with an L-malic acid content of between 1 and 5 g/L.

61. PH, ethanol, temperature… how do these parameters have a cumulative restricting effect on bacteria?

Environmental constraints first act on the bacteria wall and membrane, which are key elements for cellular viability (capacity to persist in the wine) and vitality (aptitude for activity in the wine) The role of the cell's membrane structure is to detect environmental pressures and activate the appropriate protection mechanisms. But it is also the critical structure for essential enzymatic reactions (transport, synthesis…).

- Ethanol acts on the membrane by inserting itself between the membranous lipids and by modifying fluidity, consequently affecting the effectiveness of these reactions.
- Temperature also acts on membrane fluidity: an excessively high temperature will liquefy the cell membrane, too low it becomes rigid.
- Medium chain fatty acids (octanoic and decanoic acids) also penetrate between phospholipids, whose aliphatic chains are longer, disrupting the structure's cohesion and fluidity.

Membrane fluidity, like pH, controls the activity of the membranous enzymes that are essential to the bacteria, such as nutritive element transporters, and ATP synthesis enzymes… Even though the cell has genetic and biochemical tools to help it to adapt, the more these difficult conditions accumulate, the more the bacteria will struggle to survive.

62. What is the optimal temperature for MLF?

Lactic bacteria are, like all micro-organisms, temperature sensitive. Optimal growth and activity are obtained at temperatures of between 18°C and 25°C. But in wine, other previously mentioned parameters (pH, alcohol…) also intervene: a wine with a pH of 3.6 can ferment at 18°C while a wine with a pH of 3.0 would need a temperature closer to 22°C. In all cases it is important to take the temperature into consideration and monitor its consistency once bacterial activity has begun. Temperature variations are indeed as detrimental as excessively low temperatures. To this effect, it is important to emphasise

the importance of controlling the temperature of the water or wine used to rehydrate the bacteria. Ideally, it should be between 20°C and 25°C and not have a differential of more than 5°C with the temperature of the wine to be inoculated.
In co-inoculation, the superior temperature limit can be increased to 28°C as the bacteria are introduced into a medium that is less rich in alcohol. After AF, if the alcohol is elevated (>14% vol), it is advisable not to exceed 25°C during post-fermentative maceration in order to allow the bacteria added in late co-inoculation to develop.

63. Why is good AF management the first step towards successful MLF?

As seen previously, yeast metabolism has a direct influence on bacteria development. The use of ADYs ensures the implementation of yeast strains that are compatible with the lactic bacteria, while indigenous strains can produce metabolites that are likely to hinder bacteria development. Moreover, good management of yeast nutrition (SUPERSTART®, NUTRISTART®, or NUTRISTART® ORGANIQ) presents an obvious advantage for the lactic bacteria. The bacteria will draw a large part of the nutrients required for their growth from yeast cell autolysis. The more growth factors that are available to the yeasts, the more of these compounds the bacteria will in turn be able to use for optimal development. Finally, good AF management also helps control development of *Brettanomyces* and thus reduces the risk of competition between *Brettanomyces* and the lactic bacteria at the start of MLF. In co-inoculation, interactions between AF yeasts and MLF bacteria are numerous, due to their cohabitation. It is possible to choose yeasts that promote malolactic fermentability (low production of medium chain fatty acids, good autolysis speed…) such as ZYMAFLORE F15® or RB2® or ACTIFLORE® F33 and BO213, but also to opt for a bacterium that has a good resistance to medium chain fatty acids such as LACTOENOS 350 PreAc®.

64. Why, in certain cases, does wine deacidification help the bacteria?

The pH difference between the wine and the intracellular medium of a bacterium (pH=5.8/6) is such that acidity is the principal constraint for lactic bacteria that must develop in wine. Consequently, to promote bacteria growth, it is possible to play on pH by carrying out a deacidification. Generally, this is carried out by adding calcium carbonate or potassium bicarbonate. In the case of a restrictive pH, it is best to deacidify a portion of the wine from the tank to be treated, then add the malolactic bacteria Starter to it, thus optimizing its activity. Once MLF has been started, this yeast starter can be integrated into the rest of the wine.

65. Should SO_2 be added to wine only when the malic acid content has reached 0 g/L?

In truth, entirely eliminating all L-malic acid from the wine by MLF is practically impossible. When the concentration becomes very weak, the bacteria find themselves in limited substrate conditions and their L-malic acid degradation activity thus declines. The bacteria will then turn to another substrate, in this case, citric acid, which engenders the production of diacetyl and buttery aroma at the end of MLF. For this reason it is preferable to sulphite the wine as soon as the 0.2 g/L threshold is reached. Residual enzymatic activity consumes a few more molecules of L-malic acid, hence ensuring the wine's microbiological stability (earlier sulphiting) and in addition avoiding the production of buttery aromas at the end of MLF.

66. How do lactic bacteria influence the level of bound SO_2 in a wine?

Acetaldehyde, pyruvic acid and diacetyl all have a strong ability to bind with SO_2. Certain lactic bacteria are capable of degrading acetaldehyde and pyruvic acid, resulting in lower concentrations of these compounds at the end of MLF. An MLF that is controlled using a selected strain generally permits the reduction of SO_2 dosages required for stabilizing the wine, as final acetaldehyde and pyruvic acid contents will be lower. It should also be specified that acetaldehyde is mainly produced by yeasts during AF. In this sense, co-inoculation is the best way to force the bacteria into immediately consuming the acetaldehyde produced by the yeasts.
Controlling diacetyl production at the end of MLF also allows the SO_2 combination rate to be reduced. In conclusion, a wine fermented by a bacterium that is both capable of degrading acetaldehyde and pyruvic acid and forming little diacetyl such as LACTOENOS 350 PreAc® will have a significantly lower level of bound SO_2.

67. Why are selected bacteria eliminated more easily after post-fermentation sulphiting than indigenous bacteria?

Selected bacteria are added to wine in order to carry out MLF; they do not belong to the wine's microbiological ecology. They are inoculated at a rate of 10^6 cells/mL so as to prevail over the indigenous flora and to trigger MLF rapidly, but ultimately their adaptation time is relatively short. At the end of MLF, when the wine is sulphited, they are not prepared for survival and are thus easily eliminated.
During indigenous MLFs, a different phenomenon occurs as the *Œnococcus œni* that are present come from the grape and have already resisted all the environmental pressures since the harvest: initial SO_2 addition, alcoholic fermentation and the resulting changes in medium. They are thus particularly well-adapted to these environmental variations. The addition of SO_2 at the end of MLF will simply be a new environmental modification to which the majority of the cells that have survived will be able to resist.

Finally, recent genetic studies have shown that two sub-groups can be distinguished amongst the *Œnococcus œni* strains. The first sub-group contains strains with remarkable fermentative aptitudes but that are SO2 sensitive, while the second sub-group corresponds to the most SO2 resistant strains (the diagram below illustrates this dynamic). Malolactic bacteria Starter s belong to the first sub-group. After indigenous MLFs, the lactic flora tends to persist in the wine during maturing (*Œnococcus* and/or Pediococcus) and can produce biogenic amines or worse, Graisse Disease.

68. Why is it dangerous to add a second bacterium following the failure of an initial inoculation?

When the ecosystem is occupied by a population that is present at a relatively high level, even if the cells are inactive in terms of fermentation, re-inoculating the medium is a delicate process as competitions are established between populations. When large populations of viable *Œnococcus œni* are observed (epifluorescence analysis) without there necessarily being any perceivable malolactic activity, it is always recommended to try and promote the activity of these cells rather than adding new bacteria. For this, heating the tank (20°C) and the addition of specific nutrients for the bacteria such as MALOSTART® (on the condition that the absence of *Brettanomyces* has been verified beforehand) can be beneficial.

69. Is the use of a nutrient hazardous if other undesirable micro organisms are present?

When MLF takes a long time to start and lactic bacteria are observed in the wine, the initial reflex is to control the temperature of the wine (18°C/20°C) and to use a specific nutrient for the bacteria (MALOSTART®) in order to promote MLF triggering. Nevertheless, the action of this activator can also be used by other undesirable microbial populations. If spoilage species are also present in the wine, such as *Brettanomyces*, the risk of these practices is to unintentionally promote their development. Consequently, MALOSTART® must only be used once an *Œnococcus œni* flora control has been carried out (there is no point supplying an activator if the bacteria are not present) and an alteration flora control, principally for *Brettanomyces*. It is thus only recommended to use MALOSTART® if the following conditions are brought together:

- *Œnococcus œni* population > 10^5 cells/mL (as is the case after the addition of a malolactic bacteria Starter)
- A difference of 10^3 cells between the *Brettanomyces* population and that of *Œnococcus*, in favour of the latter.

70. Can a malolactic bacteria starter be maintained in the cellar?

Œnococcus œni is a species that demonstrates considerable intraspecies diversity. Countless strains of *Œnococcus œni* exist and their metabolism is specific and different from one strain to another.
Geneticists consider *Œnococcus œni* as a fast-evolving species. Contrary to other bacteria, *Œnococcus œni* strains lack genetic elements that are involved in DNA reparation, hence they mutate more easily). Finally, the recombination phenomenon, i.e. the modification of the genetic heritage during replication, is a frequent phenomenon in O. œni. This can easily be observed in the laboratory. By successively transplanting the bacteria onto several different media, their phenotypic traits are modified. When trying to maintain a malolactic bacteria Starter in the cellar, these phenomena can occur. They can lead to physiological characteristic modifications of the cells that can be detrimental if they manage to integrate defect producing metabolic pathways. Finally, "maintaining" bacteria in the cellar can also increase strain sensitivity to bacteriophages.

71. What is the cost of using a malolactic bacteria starter?

The average cost of malolactic bacteria Starter s currently available generally ranges from $1.5/hL for acclimatization bacteria and $4/hL for direct inoculation bacteria, which represents between $0.001 and $0.029 per bottle. This investment must be considered in comparison with fermentation safety and the reduction in risks offered by the ML starter.
Furthermore, using a malolactic starter helps reduce the amount of time the containers are heated. There is a genuine energy savings: in early co-inoculation for example, heating costs are nonexistent because MLF is triggered at the mild temperature of the must right in the middle of alcoholic fermentation. For barrel MLFs, wine loss related to evaporation is reduced. Finally, it should be noted that late sluggish MLFs incur labor and analysis costs that can be avoided by using early, controlled inoculation with selected bacteria.

72. What is the cost of inoculation compared to the cost of heating a tank?

Lactic bacteria selected for ML starters are more efficient than indigenous bacteria. The massive addition of a high-performance strain enables MLF to be established more rapidly. With a ML starter MLF start-up is no longer a random phenomenon but a controlled operation. Consequently, bacterial inoculation reduces MLF duration and thus the time required to heat the wine. The longer it takes MLF to begin, the higher the energy costs needed to maintain the wine at the correct temperature. When indigenous MLFs are really stubborn, the additional energy costs generated are for the most part higher than the price of the malolactic bacteria Starter .

Savings are all the more important when the addition of the bacteria starter is carried out early: in co-inoculation for example, the bacteria benefit from the mild temperature of the fermenting must to develop and generally it is not necessary to heat the wine for MLF to take place. Conversely, in cases of extremely delayed MLF (after the winter for example), the heating costs required to bring the wine temperature up to 18°C / 20 °C are considerable. These costs can be avoided by early and controlled usage of a malolactic bacteria Starter .

Modality	Number of trials monitored in 2008	Cost of malolactic east starter	Average heating cost
Late co-inoculation	5	1,2 €/hL	0 €/hL
Indigenous MLF	5	0 €/hL	1,98 €/hL

73. Why is it more cost-effective to use a malolactic bacteria Starter than heating a tank that must be maintained at a suitable temperature for indigenous flora?

Numerous comparisons have been made between the cost of spontaneous MLF and the cost of using malolactic bacteria Starter s. These studies demonstrate that the use of commercial bacteria starters is considerably less expensive than the heating costs of wines inherent to indigenous MLF. By compiling these trials, the cost of a malolactic bacteria Starter used in late co-inoculation (LACTOENOS 350 PreAc® or 450 PreAc®) is estimated at $1.56hl while heating costs for carrying out an indigenous MLF is on average $2.57/hL.
It is important to emphasize that by co-inoculating the ML starter at the beginning of alcoholic fermentation (early co-inoculation) or at the end (late co-inoculation), it is not necessary to heat the wine. Indeed, the lag phase takes place at the mild temperature at the end of AF.

74. In wine, aside from L-malic acid, which nutrients are essential to bacteria for carrying out MLF?

Like all organisms, except for carbon substrates (L-malic acid for *Œnococcus* during MLF) that provide energy, bacterial cells need other nutrients to ensure their multiplication. A large number of studies have demonstrated the protective action of certain fatty acids and sterols. They act by consolidating the membranous structure of the bacteria cells, which reduces the inhibiting effect of ethanol and accentuates bacteria resistance. For

these reasons, certain strains such as LACTOENOS 350 PreAc® or 450 PreAc® are offered with a reacclimatization phase in the presence of a preparation. The latter combines all the survival factors that improve the effectiveness of the inoculation and cell survival in the wine. For the most delicate wines (high alcohol, low pH), nutrient supply to the bacteria can be boosted by using MLF activators such as MALOSTART®.

75. Why do the bacteria also have high nitrogen requirements?

Lactic bacteria require amino acids for protein synthesis. Certain amino acids are "essential" because the cells are incapable of synthesizing them. In O. œni, glutamic acid, valine, leucine, isoleucine, methionine, phenylalanine, serine, arginine, tyrosine and tryptophan are essential. Generally at the end of alcoholic fermentation, amino acid contents are sufficiently high in the wine as the yeasts, after having used them momentarily, release them by autolysis. These nutrients are then available for the bacteria. Nevertheless, in the case of late MLF such as spring MLF, deficiencies occur frequently and it can be necessary to complement the medium with specific nutrients (MALOSTART®), in order to provide the bacteria with the required dosage of amino acids.

76. Why not use tap water (chlorinated) for rehydrating malolactic bacteria starters?

To preserve the bacteriological sanitation of tap water, an average of 0.1 – 0.2 mg/L of chlorine is added. If the malolactic bacteria starter is rehydrated in chlorinated water, its survival capacity will be severely reduced and its efficiency affected. It is thus recommended to use mineral water, or to allow the water to sit 24 hours before incorporating the bacteria.

77. Can an opened packet of bacteria be stored?

Storage of a freeze-dried malolactic bacteria Starter (such as LACTOENOS bacteria) in its sealed packaging ensures the absence of water available to the cells. Storing an opened packet promotes bacteria moistening and significantly alters their storage. In the presence of even a minute quantity of water, the bacteria will not be able to develop effectively, will break dormancy and lose their efficiency for future usage. Once a packet has been opened, maximum recommended storage time is around 3 to 4 days in the refrigerator. To reduce these losses, different packagings are available. The LACTOENOS SB3® bacterium exists in 2.5hL packets, which corresponds to the volume required for a Bordeaux style barrel (225 l).

78. Why are storage conditions more flexible for freeze-dried bacteria than for frozen bacteria?

Freeze drying is a process of quick freezing then vacuumed evaporation of free water that can be divided into three cycles:

- The first stage: freezing, where the products are refrigerated at temperatures of around -80°C, the water is transformed into ice.
- The second stage: primary drying which takes place in vacuum (100 µbar) and consists of sublimating the free ice, i.e. transforming the water directly from the solid to vapor state without passing through the liquid state.
- The third stage: secondary drying (5 µbar) allows the water molecules trapped at the surface of the dried products to be extracted by desorption.

The objective of freeze drying is to eliminate the water in a product while retaining its volume, its aspect and its properties. An entirely dehydrated product is obtained.
It can be stored because the free water required for all biochemical activity has been removed.

In comparison, frozen bacteria have been halted at the first stage, that is, that the free water has been trapped by the freezing process but has not been eliminated. The product must always be stored frozen because an increase in temperature can cause liquid water release, resulting in biochemical reactions that are likely to alter the quality of the product.

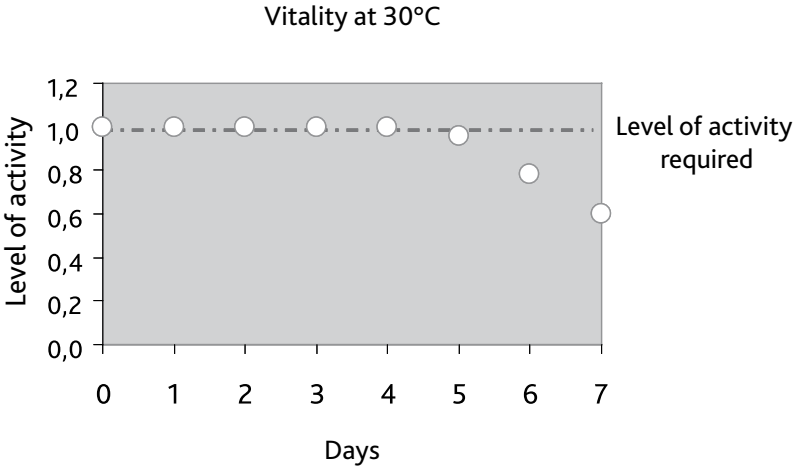
79. Why recommend storing freeze-dried bacteria in a cold environment?

In reality, water exists in the biological universe in two distinct forms:

- In the first form, it is incorporated into the molecular structures within the cells. This is known as bound water; intramolecular and intracellular water.
- In the second form, it circulates outside of these structures to provide the nutritive elements required for their functioning and ensures waste elimination; this is free water.

Freeze drying excludes free water from the bacteria but not bound water. For this reason it is appropriate to maintain the freeze-dried bacteria in a cold environment in order to safeguard their storage. However, interactions between the bound water and the matter are much stronger than interactions with free water, to such an extent that when the temperature of a product that has been refrigerated increases, the bound water remains bound and the free water is released first. In humans, when serious dehydration occurs, the rate of bound water does not vary but the rate of free water drops… Bound water only diminishes in humans with age, this is the aging process… The same applies to bacteria. Frozen bacteria that possess free water can "age" and alter very quickly if the product defrosts, while freeze-dried bacteria can survive a few days at room temperature with no loss of quality. Studies have shown that freeze-dried bacteria could tolerate a

temperature of 25°C for 7 days then returned to a cold environment without losing their efficiency (at 30°C/5 days). Storage conditions for freeze-dried bacteria are consequently a good deal more permissive than storage conditions for frozen bacteria. The illustration below demonstrates optimal viability storage for bacterium LACTOENOS 450 PreAc®, stored at 30°C for 5 days.

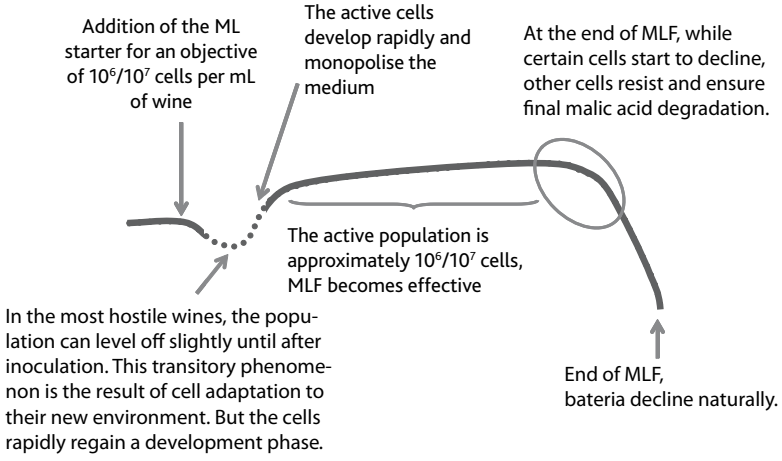


80. What is a "fermenting starter"?

For wines that are particularly hostile to the development of micro-organisms: high alcohol, low pH… it is difficult for the malolactic bacteria Starter added directly into the wine to survive, however high-performance it may be.
To improve the chances of a successful operation, it is possible to implement a fermenting starter. This consists of placing a reduced volume of wine (between 2 and 5% of the tank's final volume) in conditions that are favorable to bacteria development (nutrition using MALOSTART®, temperature 22°C – 25°C). The bacteria are then "excessively" inoculated into this volume of wine. For example, if the objective is to obtain 10^7 cells/mL in the tank, by making a 1% fermenting starter, 10^9 cells/mL are put into the fermenting starter. As the conditions are more favorable, they develop more easily. When there is significant L-malic acid degradation in the fermenting starter, it is introduced into the tank. The active bacteria are hence implanted into the wine mass. Within the LACTOENOS range, B16® Standard is the bacterium to use as a fermenting starter. It is recommended in difficult cases and when there are no time constraints (fermenting starters are long and fastidious operations). But in general, the "fermenting starter" principal can be applied to all commercial bacteria to ensure their success in the most delicate conditions and when there are no time limitations.

81. Is it better to wait until all the L-malic acid has been consumed in the fermenting starter before introducing it into the final volume?

When a malolactic bacteria starter is used, the bacteria undergo different phases of development. Optimal degradation of L-malic acid during MLF is ensured by a maximum-sized active population. However, at the end of MLF, when the L-malic acid is almost entirely consumed, the bacteria begin to "run out of steam". Fermentation end is neither the peak of fermentation activity nor the peak of cell viability. To optimize the fermenting starter, the addition must be carried out in the final tank when the content has dropped by 75% and not wait until all the malic acid has been completely degraded. The bacteria population and activity are thus at their height. The graph below represents bacterial dynamics during an inoculation.



82. Is it beneficial to use the lees of a batch that has carried out MLF to inoculate a batch that has not yet started MLF?

At first sight this method can appear judicious but in reality remains risky for several reasons:

- There is a decline in activity of lactic bacteria at the end of fermentation. Adding inactive bacteria is of little interest and needlessly monopolizes the ecosystem.
- Each batch presents analytical particularities: alcohol, pH of the original batch and the batch to be fermented are not necessarily identical, and the bacteria are forced to adapt to their new environment. This adaptation is all the more delicate because the lees bacteria are at the end of their lifecycle and find themselves in an atypical

environment that is very different to that of the wine mass. MLF will be even more difficult to trigger.

• Lees constitute a highly favorable environment for microbial development, especially for *Brettanomyces* yeasts that sediment and here find ideal conditions for multiplication. Before any usage of lees, it is essential to control the absence of *Brettanomyces* at the risk of contaminating the wine.

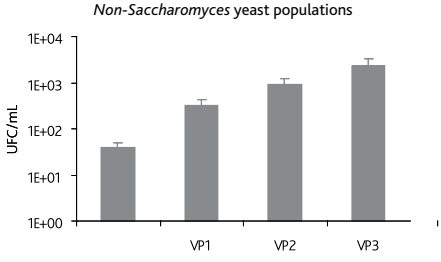
83. Why is MLF generally more rapid in press wines than in the corresponding free-run wines?

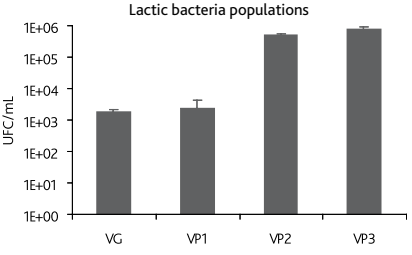
Generally MLF is triggered more quickly in press wine than in free-run wine for two main reasons:

i) In a certain manner, pressing will ensure a higher population in the press wine. During comparative counts, the microbial population rate is indeed systematically higher in press wines than in free-run wines. Also, the higher the pressure, the larger the populations (P2 generally have a larger quantity of micro-organisms, *Brettanomyces* yeasts and lactic bacteria than P1). In indigenous MLF, the press wine, that has "recuperated" more bacteria than the free-run wine, will begin its MLF more rapidly. To compensate for the loss of indigenous bacteria in the free-run wine, the best solution is to add a malolactic bacteria Starter , all the more so because it contains less micro-organisms and has an ecological niche that is easier to colonize.

ii) Besides microbial cells, press wine is generally richer in nutrients that can be used by the bacteria. PH is also higher. The lactic bacteria consequently find a more favorable environment in the press wine. To remedy the nutrient deficiency in the free-run wine, a nutritional complement can be used, such as MALOSTART®.

First press wine: VP1: 0<p<300 mbar
Second press wine: VP2: 300<p<900 mbar
Third press wine: VP1: 900<p<1400 mbar





84. Why are yeast cell walls used to improve malolactic fermentability?

Besides ethanol, medium chain fatty acids released by the yeast metabolism are one of the inhibitors of lactic bacteria. Their toxic effect can be efficiently eliminated by treating the wine with yeast cell walls (BIOCELL®). This procedure, primarily developed to limit AF arrests, presents the same advantage for promoting MLF. Yeast cell walls, made up of lipid-free yeast cell hulls, are the solid residue obtained once the cell content has been collected (referred to as yeast extract). Adding cell walls to wine uses their capacity for adsorbing fatty acids and other inhibiting metabolites.
It is recommended to add BIOCELL® cell walls in a dosage of approximately 20 to 40 g/hL. During the 48 hours following the addition, several homogenations (closed circuit) should be carried out, in order to promote the phenomenon of adsorption and inhibition removal. Then bacteria must be inoculated rapidly or the bacteria that are present must be activated by adding MALOSTART® so that the inhibition removal does not benefit the spoilage micro-organisms.

85. Should the wine be homogenized after adding a malolactic bacteria starter?

In co-inoculation, the malolactic bacteria Starter must be incorporated under the cap. It is not necessary to carry out a homogenizing pump-over, as the convection inherent to the yeasts' fermentative activity is sufficient to activate bacteria diffusion into the wine volume. At this stage, not supplying oxygen also helps avoid a deviation of the bacteria metabolism towards the usage of the sugars.
The phenomenon is different for a late inoculation, after racking-off for example. Homogenizing by conducting an unaerated pump-over in the tank, or stirring for wines held in barrels is a good method for facilitating bacteria adaptation to the wine (nutrients put back into suspension) and for ensuring homogenous dispersion. It is however recommended to correctly control the cleanness of the lees as they can host a large *Brettanomyces* population without this being noticeable in the wine.

86. Will the initial sulphiting at the crusher affect the ML starter growth?

Generally when the grapes exit the destemmer/crusher, an initial SO_2 addition is made.. The dosage traditionally ranges from 3 to 8 g/hL. The importance of this is mainly its anti-oxidation, anti-oxidase and dissolving action. The SO_2 helps preserve and maintain the color of the freshly extracted must. But this initial SO_2 addition is not without significance for the micro-organisms either: it can slow down the natural development of the indigenous species present initially. While it does not have a notable effect on the beneficial yeasts for alcoholic fermentation, it can have a significant impact on future bacteria development. When the must has become the ethanol-rich and nutrient-poor wine, the presence of SO_2 will constitute a supplementary factor that renders the medium even more hostile to the lactic bacteria. This phenomenon will be accentuated

in musts where the pH is relatively low.
SO_2 resistance is not a pertinent criterion during strain selection for malolactic bacteria Starters, because the bacteria must not resist post-fermentation sulphiting.
The yeasts that are implemented during AF can produce SO_2 and amplify the inhibiting phenomenon of residual SO_2 for the bacteria. The selected yeast strains naturally produce less SO_2 than indigenous strains. Their implementation is thus compatible with a satisfactory MLF triggering. This is not always the case for indigenous AF where SO_2 contents produced by the yeasts can exceed several thousand milligrams per liter.

87. Why can the tartaric acid concentration decrease in wine during MLF?

The tartaric acid content sometimes decreases during MLF. Besides variations in tartaric acid solubility that are independent of microbiological activity, it is important to note that certain indigenous bacteria can also degrade tartaric acid. In this case, the degradation is always associated with a phenomenon that is detrimental to the quality of the wine. Total acidity decreases while volatile acidity and the concentration of succinic acid increase, to such an extent that the wine becomes weaker and disagreeable odors appear (when succinic acid exceeds 0.5g/L).

88. Do lactic bacteria produce ethyl acetate?

Contrary to acetic bacteria which possess enzymes that catalyse ethanol and acetic acid esterification, lactic bacteria do not directly produce ethyl acetate. In wine, the principal fermentation ester produced by the lactic bacteria is ethyl lactate. In this case, the proportion of R and S-enantiomers varies depending on fermentation activity. If MLF has not taken place in the wine, the ethyl lactate content is approximately ten milligrams and it is the R-entantiomer that predominates. In a wine in which MLF has occurred, the ethyl lactate content can be up to ten times higher with a predominance of the S-enantiomer.

89. Will Lactic bacteria produce fruity notes in wine?

MLF lactic bacteria are capable of metabolizing sulphur containing amino acids: methionine and cysteine. It is now recognized that sulfanyl-3-methyl propionic acid, a compound derived from this metabolism, positively contributes to red fruit aromas in wine. It is, in our current state of knowledge, the only compound clearly identified as being implicated in the aromatic impact of lactic bacteria during MLF.
Inversely, it is recognized that sluggish MLFs, that delay the wine's microbiological stability, are detrimental to the fruity aromas derived from alcoholic fermentation. With the indigenous flora, it should be specified that certain compounds such as biogenic amines can mask aromas.

Research, led by Professor Gilles de Revel at the University of Bordeaux Oenology Faculty (ISVV), is currently in progress, studying the effect of bacteria and fruitiness in wine. While the initial results show that it is difficult to establish the existence of a bacteria strain effect used during MLF and fruity notes, it would appear that certain vinification pathways protect fruity aromas more than others. This would be the case in co-inoculation.

90. How does bacteria inoculation influence wine color?

For a long time, certain enologist considered that a MLF that was slow to start could be beneficial for color stabilisation in wine. This was especially evoked for Pinot noir vinification in Burgundy. This does not result from direct bacteria activity, but is the consequence of late sulphiting. It should be noted that delaying sulphiting is a hazardous practice in sluggish MLFs given the risks of alteration during this period.
For Bordeaux grape varieties, recent studies have shown that even the earliest inoculation of selected bacteria (early co-inoculation) with early wine SO_2 addition leads to no damage whatsoever in terms of color stability and the organoleptic qualities of the wine.

91. Is the lag phase between the end of AF and MLF start-up favorable for color increase and stabilization?

The lower the MLF temperature, the more the color will be stabilized. On the temperature scale to which the bacteria can adapt (16 to 25°C), it is preferable to be within the lower limit.
Color stability will also be encouraged by moderate sulphiting at the end of MLF.
Finally, the usage of a malolactic bacteria starter is not detrimental to color stability, quite the contrary:

- After AF, if the wine lacks spoilage bacteria, the use of a ML starter enables the moment that MLF starts to be controlled.
- The strains selected for the bacteria starters present advantages in comparison to indigenous strains and are more sensitive to environmental constraints. With a malolactic bacteria starter, if the wine does not present restricting analytical parameters (alcohol, pH…) it is possible to effectuate MLF at 16 - 18°C, whereas to boost the triggering of indigenous flora, it is often necessary to heat the wine or the cellar.
- Limiting the dosage of SO_2 at the end of MLF is only possible if the ecosystem already lacks spoilage bacteria. For this, the use of a ML starter is a highly advantageous tool.

92. Barrel or tank: which container should be used for MLF?

MLF can be carried out in tanks or in barrels. Both these containers have advantages and disadvantages that should be rationalized in terms of objectives and available means.

	Advantages	Disadvantages
Tank	• More homogenous wine. • Temperature control. • Safer microbiological conditions. • Reduced loss of wine. • Limited labor.	• Slow CO_2 elimination. • Aromatic neutrality.
Barrel	• Interaction between the lactic bacteria and wood compounds. • Lees stirring to encourage MLF triggering builds mouthfeel. • Progressive CO_2 elimination.	• Each barrel constitutes a sample of the initial wine, the microflora can be heterogeneous and bring about significant differences from one barrel to another. • Difficult to control temperature. • Loss of wine. • Labor requirements higher.

*Lactic bacteria interaction with the wood can occur in tank with wood staves or in barrel. MLF done in the presence of wood will allow for better integration of oak character in the wine.

93. Will lactic bacteria influence the oak character in wines?

For a long time, winemakers wondered whether the woody aromas that sometimes appeared in wines during barrel MLF were solely related to the early contact of the wine with the wood or whether lactic bacteria played a role in this phenomenon. Recent studies have shown that lactic bacteria, mainly due to their glycosidase activity, promote the release of oak wood volatile compounds, such as vanillin. To promote these woody aromas in wine, the use of a malolactic bacteria Starter in synergy with the wood is thus evident. This can be done during a barrel MLF, but also by using wood chips / staves in the tank. The latter also provide roundness and sweetness.

94. Wood; a substrate for lactic bacteria?

It has been brought to light that lactic bacteria contribute to triggering five types of glycosidase activities: ß-glucosidase, ß-apiosidase, ß-xylosidase, ß-rhamnosidase and ß-arabinosidase. It is probable that these activities play a role in bacteria nutrition by providing them with a sugar source in the depleted wine medium that is housed in barrels following vinifications and during maturation.

95. Can a wine be heat treated before triggering MLF?

When MLF is not triggered naturally, *Brettanomyces* yeasts can work their way in during the lag phase between the two fermentations and contaminate the wine. Resorting to a flash-pasteurization can be considered in order to eliminate the *Brettanomyces*. It is important to consider that pasteurization is not a specific treatment. It will not only act on the *Brettanomyces* but also on all the indigenous bacteria.
Under the action of the heat, these bacteria will undergo intense cellular stress before dying. During this stress, the cells utilise a defence metabolism which is accompanied by toxic compounds synthesis. Even if the cells disappear, these compounds persist in the wine following treatment, to such an extent that starting MLF after a flash pasteurization is always risky.
It is thus more beneficial to manage the first part of vinification well in order to avoid early contamination by *Brettanomyces*. Inoculating the wine after AF with malolactic bacteria Starters is the most efficient solution.
However, if *Brettanomyces* development is observed before MLF, it is preferable to filter the wine rather than heat it. The *Brettanomyces* will be eliminated by the filtration and the wine will be more favorable to inoculation with a malolactic bacteria Starter.

96. How does lysozyme inhibit lactic bacteria?

Lysozyme is a hydrolytic enzyme discovered by Alexander Fleming in 1922. It is found naturally in a certain number of secretions (tears, saliva…). It is also extracted from egg white (which characterizes its usage in winemaking). Lysozyme degrades lactic bacteria cell walls, by hydrolyzing the bacteria cell wall bonds. It can be used at all stages of vinification (must, fermenting must, wine).
Lysozyme contributes towards microbiological stabilization in wine after MLF to avoid the development of lactic bacteria during maturing. It can also be used to control untimely development of indigenous lactic bacteria before the use of a malolactic bacteria Starter . In this case, a stabilization period of 5 to 7 days is required between the addition of lysozyme and bacteria inoculation. This time period can be reduced by bentonite treatment.

97. For what length of time does lysozyme inhibit bacteria in red wine?

The principal factors that contribute to lysozyme activity loss in wine are polyphenols and tannins ; to such an extent that lysozyme will see its activity reduced more rapidly in a red wine than in a white wine. It is consequently considered that lysozyme will have no more effect after AF when added to a red grape must.

98. Can the addition of lysozyme during a stuck fermentation (to impede the increase in volatile acidity) hinder the use of future malolactic bacteria starters?

No, because lysozyme added to the juice will react with different compounds that will progressively decrease its inhibiting activity. If in doubt, in order to ensure MLF triggering following a lysozyme treatment, a bentonite treatment can be carried out prior to bacteria inoculation.

99. What are the advantages and disadvantages of lysozyme for controlling lactic flora?

Lysozyme has no effect on yeast. This specificity can be considered as an advantage if the aim is to selectively fight against lactic bacteria, or as a disadvantage when the aim is to stabilize the wine's overall microflora. In the latter case, the lysozyme can be complementary to SO_2. For example, "ropy" *Pediococcus* can present good resistance to SO_2 but are eliminated by the lytic activity of the lysozyme. Moreover, contrary to SO_2, its activity increases when the pH rises. A certain advantage of lysozyme is the absence of influence on the organoleptic qualities of the wine as well as its innocuousness for the consumer, contrary to excessive additions of SO_2.

100. Is lysozyme sufficient for stabilising the wine following MLF?

At the end of MLF, lysozyme can be used to reduce the population of lactic bacteria. However it should not be forgotten that lysozyme is only active on lactic bacteria (not at all on *Brettanomyces* yeasts for example). Its usage cannot therefore be justified for replacing SO_2after vinification to reduce the wine's microbial load at the beginning of maturing and to ensure microbiological stability.

101. Are lactic bacteria sensitive to the residue of fungicide/pesticide treatments in the vineyard?

It is effectively worth asking the question, as risky cases of MLF are sometimes difficult to understand. However, while the inhibiting effect of certain fungicides used on the grape is clearly demonstrated for yeasts in the wine, very few studies have been carried out on lactic bacteria up until now. Work should be undertaken in order to gain better understanding of this phenomenon.

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Malolactic fermentation remains an essential stage of vinification. It has a major influence on the analytical and gustatory characteristics of wine, and also plays a necessary role in microbiological stability.

Controlling MLF is still a delicate operation and numerous problems can be encountered. Consequences are always detrimental, not only from an œnological point of view, with wine alterations, but also in economic terms, with marketing delays that are always detrimental.

For over 10 years, LAFFORT has been actively participating in research on MLF through the Sarco laboratory, in collaboration with the Bordeaux Faculty of Oenology and Professor Aline Lonvaud Funel's laboratory. Three strains of latest-generation bacteria have emerged from this research: Lactoenos® SB3, Lactoenos 350 PreAc® and Lactoenos 450 PreAc®, offering a comprehensive range of bacteria for optimal MLF control for any situation (low pH, high TA...).

The research led by LAFFORT has also provided a better understanding of certain issues inherent to MLF (short or medium chain fatty acids, bacteria and Brettanomyces interactions...) and practical, efficient solutions can be specified (early or late co-inoculation, MLF restart protocol...).

This publication is a compilation of this work in the form of a questionnaire. The most frequently asked questions have been identified and listed, and simple and practical answers given, aiming to meet practitioners' demands to the best of our ability.

Livrets techniques, notes d'information consultables sur le site internet : www.laffort.com:

- Du bon usage des activateurs de fermentation
- De la bonne gestion de la FML
- Cas particulier de la gestion des fermentations : la co-inoculation levures/ bactéries.