THE ROLE OF OCTANOIC AND DECANOIC ACIDS IN INHIBITING MALOLACTIC FERMENTATION IN WINES: PROBLEMS AND SOLUTIONS

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ABSTRACT

Malo Lactic Fermentation (MLF) is not always an operation perfectly managed and some failures are still observed. Classical wine parameters such as pH, ethanol content, SO_2 and temperature influence the development of bacteria. When they are too restricting they delay MLF completion. Cases of failure however are still observed in wines with favourable values of these parameters. These unexplained cases of failure raise a general question about of the malolactic fermentability: the factors that make a wine more or less favourable to MLF achievement.

Among parameters influencing the progress of MLF, medium chain fatty acids (octanoic (C8) and decanoic (C10) acids) play a very important role that is often overlooked.

Our work on hundreds of wines of different types and origins clearly demonstrates the toxicity of these compounds to lactic acid bacteria. Their production by yeasts at the end of alcoholic fermentation strongly influences malolactic fermentability.

The first part of our work is devoted to highlighting the phenomenon of inhibition by C8 and C10 acids on MLF bacteria and the definition of the thresholds of inhibition. After a study on the origin of fatty acids in the wines, we then propose practical solutions to address this issue.

INTRODUCTION: BASICS OF MALOLACTIC FERMENTABILITY

Malolactic fermentation (MLF) is an essential stage in winemaking. It reduces the wine's acidity, contributes to its aromatic development, and is partly responsible for ensuring its microbiological stability. Many winemakers have become aware of the unpredictable, risky nature of indigenous MLF including spoilage, unwanted organoleptic characteristics, delayed market release, high cost of heating, and others and now use commercial malolactic starter cultures to control the start of MLF. This practice is not yet in systematic use and wineries may still experience problems with MLF. In most cases, the causes are easily identifiable: excessive SO_2, high alcohol content, low pH, uncontrolled temperatures, and unreliable indigenous flora (Table I). Other factors may also be responsible for these MLF problems, particularly parameters in the medium that may inhibit lactic acid bacteria.

	Total SO_2 (mg/L)	pH	Ethanol content (% Vol.)	Temperature (°C/°F)	Indigenous flora of non-Saccharomyces yeasts (Brettanomyces ...) and / or of indigenous bacteria, before using a malolactic bacteria (cell/mL).
Optimun conditions	≤ 60	≥ 3.3	≤ 14.	≥ 16 / 61	≤ 1.10^3
Difficult conditions	≥ 70	≤ 3.2	≥ 15	≤ 15 / 60	≥ 1.10^4

Table 1. Evolution of the citric acid in a Chardonnay wine inoculated after the end of AF with Lactoenos 350 PreAc®. Wine parameters before the bacterial addition: Ethanol = 13.2% vol., pH = 3.3, L-malic acid = 2.4g/L, citric acid = 0.68g/L.

Like all microorganisms, *Œnococcus œni* bacteria have specific requirements: temperature, pH, etc. *O. œni* also have nutritional requirements, particularly in terms of nitrogen as, unlike yeasts, they are incapable of assimilating inorganic nitrogen. They absorb their nitrogen supply exclusively from organic nitrogen compounds present, requiring specific amino acids be present in the medium. These include glutamic acid, arginine, isoleucine, leucine, methionine, phenylalanine, serine, tryptophan, tyrosine, and valine. The development of lactic bacteria also requires certain vitamins, including thiamine (vitamin B1), riboflavin (vitamin B2), pantothenic acid (vitamin B5), nicotinic acid (vitamin B3 or PP), and folic acid (vitamin B9) (Lonvaud-Funel et al. 2010). Certain minerals are also essential, particularly magnesium and manganese ions, which are indispensable co-factors for the malolactic enzyme. A deficiency of any one of these compounds may inhibit MLF.

These deficiencies may be caused by delaying the start of MLF, as the medium gradually and irreversibly loses its nutrients. This situation may be remedied by the utilization of starters, designed to provide non-limiting quantities of the substrates required by the bacteria (Renouf et al. 2009).

TOXICITY OF THE MEDIUM: A KEY FACTOR

Deficiencies do not provide an adequate explanation for all the difficulties observed in practice. Laboratory tests have shown that it is very difficult, or even impossible, to put the same wine through MLF several times, even if L-malic acid and nutrients are added at the end of each MLF (Lonvaud-Funel et al. 2010). This observation indicates that nutrition is only one key factor. The toxicity of certain compounds in wine for lactic bacteria must also play a critical role. The main compounds responsible for toxicity in the medium are produced by yeasts. Another factor is SO_2. Depending on the yeast strain responsible for AF, the quantity of total SO_2 (around 10 mg/L) may vary but these variations are generally small compared to the total SO_2 obtained by sulfuring the must before fermentation (Renouf et al. 2008).

Short- and medium-chain fatty acids (C4 - C10) produced by yeasts are certainly more powerful inhibitors. Early research by Lonvaud-Funel et al. (1988) demonstrated that octanoic (C8) and decanoic (C10) acids were among the most powerful inhibitors. These amphiphilic compounds, polar due to their COOH functions and hydrophobic due to their short aliphatic chains, may interfere between membrane phospholipids and disrupt membrane fluidity. This is the key component in many biochemical reactions, enzymes, and transporters, which are essential for absorbing nutrients, excreting waste, and synthesizing energy, thus making them key factors for bacterial viability and vitality. This disruption phenomenon is similar in every way to the effect of ethanol on bacteria. Ethanol and C8 and C10 fatty acids thus have a cumulative inhibiting effect on bacterial activity (Capucho and San Romano 1993). The inhibiting role of C8 and C10 is not only due to a disruption in the cell membrane organization, however. These compounds also affect the membrane endoderm, dissociating and releasing a proton into the bacteria cytosol. Consequently, they contribute to acidifying the intracellular medium of the bacteria, which is usually much more alkaline than wine. The pH inside an *O. œni* cell is in the range of 5.8, while the value for wine is generally between 3.0 (for the most acidic white wines) and 4.0 (for the least acidic red wines). This difference in pH is essential to maintain the electrochemical gradient required by the bacteria for energy production and is also required by many intracellular enzymes including, in particular, malolactic enzymes which are incapable of functioning correctly if the pH of their environment is not maintained around 5.8. Consequently, when large quantities of C8 and C10 are present, the MLF bacteria have to make an additional effort to regulate their intracellular pH at the expense of the activities that interest winemakers:

bacteria development and malolactic conversion.

INHIBITION OF MLF BY OCTANOIC (C8) AND DECANOIC (C10) ACIDS IN WINE.

The graph below shows the difference in MLF kinetics after the bacteria were inoculated in a wine enriched with varying concentrations of octanoic and decanoic acids at the end of alcoholic fermentation (AF). At concentrations of 20 mg/L C8 and 4 mg/L C10, MLF required twice as long as it did when the values were half (C8= 5 mg/L and C10 = 2 mg/L). In the sample with the highest fatty acid content (C8 = 50 mg/L and C10 = 20 mg/L), MLF had not even started 3 months after inoculation.

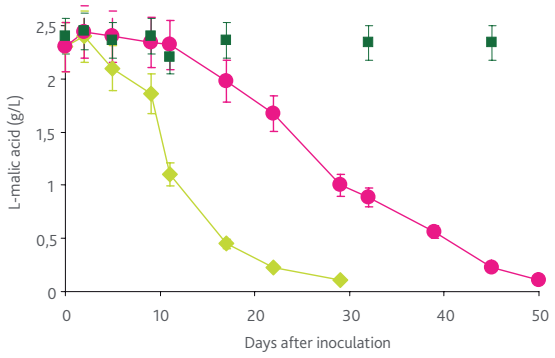

Figure 1. MLF monitor in a wine with C8 =5 mg/L and C10=2 mg/L (◆), in the same wine with C8 =20 mg/L and C10=4 mg/L (●) and in the same wine with C8 =50 mg/L and C10=20 mg/L (■). The wine is obtained by microvinification with a commercial red grape juice (Ethanol=11.2 % vol., pH=3.1, Total SO2=24 mg/L, temperature=20°C/68°F). Trials are made in triplicate. Malolactic starter is inoculated at the initial population of 106 cell/mL at day 0.*

**NB : The AF had been conducted with the yeast Actiflore F33 (Laffort), one of the strain producing the least important levels of C8 and C10 which has permitted to obtain a post-AF with naturally low in C8 and C10 for complementation with appropriate doses.*

In order to explain this phenomenon, the C8 and C10 in a total of 282 wines of different types (red, white, etc.) and origins (northern and southern hemisphere) were assayed at the end of AF for 3 successive vintages. The mean concentrations obtained were 14.9 mg/L for C8 and 3.8 mg/L for C10. The wines were ranked in different groups. Those where MLF was completed normally with indigenous or inoculated bacteria (group I, MLF started less than one month after running off in the case of indigenous MLF, or less than two weeks after the selected bacteria were inoculated) and those where MLF was difficult (group II). Once again, the impact of C8 and C10 concentrations was clearly illustrated (Figure 2): the wines that had MLF problems contained significantly higher concentrations of C8 and C10 than those where MLF went smoothly.

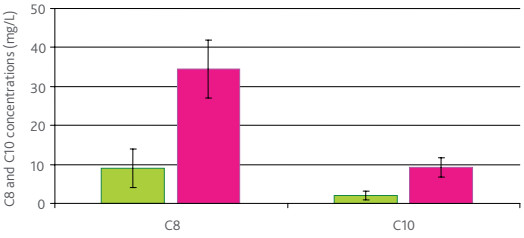

Figure 2: Of the 282 wines studied: 156 were not presented problems of MFL (group I in green) and showed average levels of C8 (9.0 ± 5.0) and C10 (2.1 ± 1.1) relatively low. The 126 wines in which MLF was problematic (group II in pink) showed significantly higher average levels of C8 (34.4 ± 7.4) and C10 (9.3 ± 2.5).

Besides the usual categories (grape varieties, color, etc.), an additional factor proved to be relevant in classifying these wines: their geographical origin. Wines from countries in the Southern hemisphere, especially Australia, South Africa, and New Zealand, contained much lower concentrations of C8 and C10 than wines from the Northern hemisphere (Table II).

Countries	C8 (mg/L)	C10 (mg/L)
England	61.5±27.4	14.5±6.0
Portugal	34.8±18.6	8.7±5.6
Switzerland	24.8±18.7	7.8±1.8
Italy	24.4±14.2	7.5±3.5
Germany	23.9±8.5	6.4±2.7
France	21.9±12.9	4.5±1.0
China	18.0±8.7	1.1±0.2
USA	12.2±7.0	2.8±1.4
Spain	11.4±2.3	2.5±0.5
Chile	8.1±5.8	1.9±0.8
South Africa	3.9±1.2	1.9±0.5
New Zealand	3.7±0.4	0.6±0.3
Australia	1.9±0.2	1.0±0.4

Table II: Average levels of fatty acids measured at the end of AF according to the country of origin.

The average concentrations in French wines were: 21.9 mg/L C8 and 4.5 mg/L C10. These values are similar to those found in wines from neighboring countries: Switzerland, Italy and Germany. Portuguese wines had much higher concentrations, which probably explain why 10 out of 12 Portuguese wines analyzed had problems with MLF. On the contrary, Spanish wines contained relatively low concentrations compared to other European countries and, indeed, the majority of wines analyzed had no problems with MLF (24 out of 28 wines analyzed). The proportion of French, Italian, Australian, and South African wines analyzed that had MLF problems was more homogeneous, making it possible to analyze the results in greater detail without bias and draw truly relevant conclusions (Table III). The variation in values for wines from these countries confirmed both the difference in C8 and C10 concentrations between wines that did or did not experience difficulties during MLF and the difference between the mean concentrations in wines from the Northern and Southern hemispheres.

Country	Wine without MLF issues			Wines with MLF issues		
	Number of wines analyzed	C8 (mg/L)	C10 (mg/L)	Number of wines analyzed	C8 (mg/L)	C10 (mg/L)
France	48	12.9±2.1	2.7±0.6	44	37.3±9.7	10.6±5.7
Italy	22	11.2±1.2	3.4±0.4	22	38.4±8.4	12.6±6.6
South Africa	17	3.5±1.6	1.7±0.5	15	11.1±5.4	2.8±0.3
Australia	16	2.9±1.5	1.0±0.4	12	12.3±3.2	3.6±2.2

Table III: Differences in fatty acid content in wines with or without MLF issues in France, Italy, South Africa and Australia.

Therefore in a majority of the cases studied, C8 and C10 concentrations provided excellent indicators of malolactic fermentability, as the values at the end of AF were significantly correlated with successful or problematic MLF. Furthermore, the Northern hemisphere wines considered (French and Italian) had higher mean concentrations than the South African and Australian wines. However, the Australian and South-African wines that had issues during MLF had mean C8 and C10 values equivalent to those of French and Italian wines that had no difficulty with MLF. This indicated that MLF problems due to fatty acids were mainly a European issue.

CRITICAL THRESHOLD VALUES OF OCTANOIC (C8) AND DECANOIC (C10) ACIDS FOR MALOLACTIC FERMENTABILITY

Regardless of the country concerned, it is interesting to note that the maximum values for wines that had no problems with MLF were 23.8 mg/L C8 and 4.8 mg/L C10. C8 concentrations in the wines that had difficulties during MLF were mainly between 26 and 35 mg/L. It is important to emphasize that the wines that still had problems with MLF although their C8 content was under 10 mg/L had other atypical parameters, particularly low pH (below 3.1) and/or very high total SO_2 (>60 mg/L) and/or ethanol content. These factors already made the medium sufficiently hostile to bacteria, without further inhibition due to C8 and C10.

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The analytical parameters of wines that had problems with MLF and C8 values over 25 mg/L were much less atypical: pH always between 3.55 and 3.9, Alcohol always below 15 % vol., and total SO_2 below 60 mg/L. In these cases, difficult MLF may therefore be directly attributed to the inhibiting effect of the fatty acids. In tests carried out in triplicate under laboratory conditions, using a Merlot wine (pH =3.5, Alcohol=13% and total SO_2 =55 mg/L, C10=2.7 mg/L, 20°C/68°F) and 5 different strains of bacteria. MLF went smoothly provided the amount of C8 added did not exceed 25 mg/L. Between 25 mg/L and 40 mg/L, the latency phase before MLF started was be multiplied by 2 or 3, depending on the specific strain of bacteria. Above 30 mg/L, 3 out of 5 bacteria strains tested never managed to complete MLF.

Considering all these observations, the mean threshold value of C8 for inhibiting MLF bacteria in wine was established at 25 mg/L. Below that value, the bacteria apparently resisted the C8 and completed MLF without difficulty in most cases, provided that the other parameters did not reach limiting values. Above 25 mg/L C8, the inhibiting effect was extremely severe and likely to cause difficulties with completing MLF. Similar experiments established a threshold inhibition value of 5 mg/L for C10.

It is important to note that octanoic and decanoic acids are present together in wine with a relatively consistent ratio of 5 C8 for 1 C10 (C8/C10 = 5.15 ±1.56 in the 282 assays carried out during this study). The threshold values proposed above reflect this ratio. This ratio indicates that C10 does not, in fact, have a stronger inhibiting effect on bacteria than C8, but is simply present in naturally lower concentrations in wine.

TOOLS FOR SOLVING THE PROBLEM

The observations presented above raised a number of issues concerning the variations in C8 and C10 concentrations in the wines analyzed. Several hypotheses were considered.

The first was the strain of *Saccharomyces cerevisiae*, which is mainly active during AF. Values produced by two *Saccharomyces cerevisiae* yeast strains used for AF varied by a factor of 1 to 5. Under similar conditions, one strain may produce very small quantities of fatty acids, while another produces quantities considerably over the threshold mentioned above. These observations provide a partial explanation of the interactions between the ***Saccharomyces cerevisiae*** yeast strains responsible for AF and *Œnococcus œni* bacteria, particularly why certain activated dried yeast (ADY) strains are more favorable to MLF than others. The conditions surrounding the yeasts during AF also play a fundamental role. The initial parameters of the must including pH, sugar content, available nitrogen levels, the amount of SO_2 added to the must, and fermentation temperatures, are key factors that impact yeast proliferation and survival. They have a direct effect on the yeast metabolism and, therefore, considerable impact on C8 and C10 production. In general, any conditions that make AF difficult cause stress for the yeast, resulting in larger quantities of secondary metabolites, such as C8 and C10.

Among these parameters, initial available nitrogen content plays a key role. Significantly, the available nitrogen content of wines from the Southern hemisphere is generally particularly high, compared to values obtained in Europe, where nitrogen deficiency is relatively common. This hypothesis was confirmed by analyzing several wines where the initial available nitrogen content was known, revealing a correlation between this value and the C8 content at the end of AF (all other conditions being equal in musts fermented with the same yeast strain) (Figure 3).

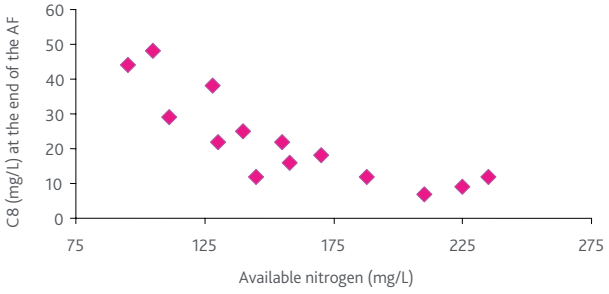

Figure 3: Test of correlation between the initial value of the available nitrogen concentration in must and C8 at the end of the FA for musts of different origins fermented with the same strain of yeast (Zymaflore FX10. Laffort. France).

The C8 and C10 production kinetics revealed that these compounds were produced by the ***Saccharomyces*** yeasts at the end of AF, at a density of approximately 1,000 (Renouf et al. 2008). As a result, the values were higher when the yeast had difficulty completing the breakdown of the sugars. This confirmed that stress certainly contributed to increased production of C8 and C10 at the end of AF. Research focusing on the ***Saccharomyces*** yeast metabolism for producing C8 and C10 will be conducted in the near future to elucidate these phenomena and, in particular, the interactions between the yeast's nitrogen supply and its production of fatty acids (Torija et al. 2003), especially C8 and C10.

Other research has demonstrated that non-***Saccharomyces*** yeasts, particularly ***Brettanomyces bruxellensis***, also produce large quantities of C8 and C10 (Romano et al. 2008). Consequently, the presence of indigenous yeasts alongside ***Saccharomyces cerevisiae*** may also cause an increase in C8 and C10 concentrations at the end of AF. In particular, this explains why MLF generally has difficulty starting following certain cases of sluggish AF, where ***Brettanomyces bruxellensis*** yeasts have developed while the ***Saccharomyce***s were having trouble maintaining their population in the medium.

Practically, effective tools are already available to deal with this key issue of fatty acids and ensure a successful MLF, even in the most awkward cases. Three different preventive or curative approaches may be considered.

The first consists of starting MLF before the fatty acids are produced by the yeasts. As previously mentioned, fatty acid production generally starts at a density of 1,000, so co-inoculation of yeast / bacteria offers interesting possibilities (Murat et al. 2007). The early co-inoculation technique consists of adding bacteria 24 hours after AF has started, thus giving the bacteria the chance to adapt to a medium with very low concentrations of C8 and C10, this generally resulting in a very short latency phase before MLF starts. When the AF yeasts start to produce fatty acids, bacteria are already active and are thus able to adapt to increasing concentrations of these compounds, starting when the fermenting must reaches critical density (Figure 4). The bacteria can also adapt to the increasing ethanol content as AF progresses. To summarize, co-inoculated bacteria develop physiological tools that enable them to acclimatize to an increasingly hostile medium. This is a general principle of microbiology: it is always easier for any type of microorganism to adapt to a changing medium, with increasingly hostile selection constraints, than to be inoculated directly into an extremely hostile medium. Bacteria added after C8 and C10 production must resist the inhibitor immediately and have no possibility of acclimatizing, as peak toxicity has already been reached (final concentrations of C8 and C10) prior to inoculation.

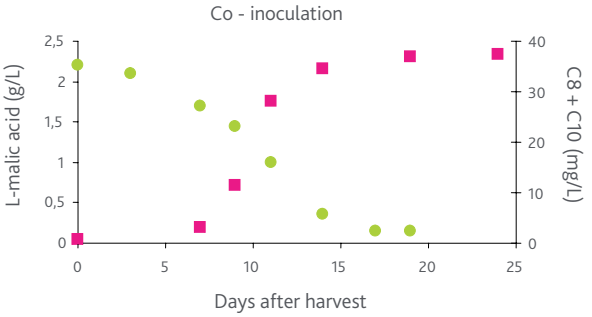

Figure 4: Kinetics of degradation of L-malic acid (●) and the production of the C8 and C10 (■) on the co-inoculation modality (Lactoenos 350 PreAc (Laffort. France) at 10ppm 24 hrs after AF has started (D +3)). The MLF has begun on day 9 and was completed on day 17.

Two other approaches may be considered at later stages in the process, if analysis at the end of AF reveals excessively high concentrations of C8 and C10. The first is to use a strain of lactic bacteria that is particularly resistant to fatty acids. Recent research (Renouf and Favier 2010) has reported that different *Œnococcus œni* strains exhibit varying resistance to C8 and C10. In laboratory experiments using a model medium, certain

strains were able to resist much higher concentrations than others. Among these, **LACTOENOS 350 PreAc®**, initially selected for its resistance to low pH (Renouf et al. 2009), is the most tolerant to C8 and C10 (Table IV). Indeed, it is probably the most resistant strain currently available to winemakers. This may be due to its tolerance to low pH, which enables it to offset the release of protons with the acid functions of C8 and C10 when they penetrate inside the cell.

Strain	Inhibitory [C8 + C10](mg/L)
Starter I	35
Lactoenos 350®	65
Starter II	50
Starter III	15
Starter IV	50
Starter V	35
Starter VI	25
Starter VII	30
Starter VIII	30
Starter IX	35

Table IV: Comparison of inhibition threshold of C8 and C10 for different malolactic starter in wine (pH = 3.4, Ethanol = 14% Vol., Total SO2 = 54 mg / L).

The third option consists of acting directly on the toxicity of the medium. Yeast hull preparations are well known to play a role in adsorbing C8 and C10. However, once again, this adsorption capacity is highly dependent on the specific products and the way they are used. To optimize adsorption, it is recommended to add a pure yeast hull preparation 48 hours before seeding with selected bacteria, to detoxify the wine prior to inoculation. During this 48-hour period, it is recommended to pump the wine over in a closed circuit (without adding O_2) every 6 hours until the bacteria are added, to maximize adsorption of C8 and C10 by the yeast-hull surface. It is recommended to assay C8 and C10 and analyze the wine's microbiological status prior to using this technique. This is to check that the yeasts responsible for the production of C8 and C10 are no longer present in the medium and ensure that it does not contain any spoilage microorganisms (*Brettanomyces*, lactic bacteria that produce biogenic amines. etc.) that would take advantage of the disinhibiting effect of the yeast hull treatment to proliferate. If any of these undesirable microorganisms are present, they must be eliminated by racking or filtration prior to starting the yeast-hull treatment.

CONCLUSIONS

During our extensive research into MLF, this assay of octanoic and decanoic acids in 282 wines of different types and origins confirmed the major inhibiting capacity of these compounds. MLF has difficulty starting when octanoic acid content is over 25 mg/L and/ or decanoic acid exceeds 5 mg/L.

These compounds are produced toward the end of AF, due to yeast activity, in quantities that depend partly on the yeast strain, but mainly on the conditions in the medium. Although further studies are required to elucidate this phenomenon, our results highlight the need for careful AF management to facilitate MLF. One key parameter is correcting available nitrogen deficiencies. The development of new, organic, nitrogen-based activators (**NUTRISTART OrganiQ®**, Laffort, France) opens up interesting possibilities.

In all cases, to ensure the success of MLF it is necessary to take octanoic and decanoic acids into account and adopt the following options: early yeast / bacteria co-inoculation so that the bacteria develop in a completely favorable medium, utilizing a bacteria strain that tolerates high concentrations of C8 and C10, and a curative detoxification treatment with yeasts hulls before the bacteria are inoculated for late MLF.

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