OPTIMIZATION OF THE MANAGEMENT CONDITIONS OF MALOLACTIC FERMENTATION IN RED WINES OF THE NEMEA REGION

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Abstract

Malolactic fermentation (MLF) and its impact on wine quality are often not taken into consideration in the modern Greek winemaking reality. Relatively low initial L-malic acid concentration, rarely exceeding 2.0 g/l, in conjunction with rather high pH values and warm temperatures during the winemaking period result, in most cases, in spontaneously triggered malolactic fermentations. Malic acid will be depleted by indigenous LAB and winemakers will consider "the job completed" neglecting the fact that quality and health issues have been raised by this "wild" non-controlled process. Quality issues would be mainly undesired products such as volatile acidity, diacetyl, acetoin, volatile phenols and others, where health issues would be related with biogenic amine production.
Inoculation with selected Lactic Acid Bacteria (LAB) strains provides a good tool to overcome these issues provided that it is conducted in optimum conditions. Time of inoculation of LAB seems to be the most crucial factor. We have demonstrated that inoculation after the completion of the alcoholic fermentation as well as early inoculation at the beginning of fermentation improve performances but not erase problems. LAB inoculation when 50% of reducing sugars are fermented seems to be the most adapted MLF modality in the AOC Nemea region, leading to better MLF kinetic that yields higher quality end products.

Key words: Malolactic fermentation, lactic acid bacteria, lysozyme, volatile acidity.

Introduction

Malolactic fermentation (MLF) is very important particularly for skin fermented red wines as it improves their acidity balance and flavor complexity. Its beneficial impact has been shown in Greek wines as well (Soufleros *et al*., 1996). MLF typically follows alcoholic (yeast) fermentation (AF) and is carried out by indigenous lactic acid bacteria (LAB), which convert the dicarboxylic L-malic acid into the monocarboxylic L-lactic acid. This results in a reduced titratable acidity and in an increase of the pH. However, MLF not only represents a biological deacidification process, it also exerts a significant impact on the organoleptic aspects of wine (Henick-Kling, 1993). These sensory effects can be positive or negative, depending on the bacterial species, and, more specifically, the strain of LAB employed to conduct the MLF (Liu, 2002). Strains of *Oenococcus oeni* generally produce desired flavor change and

different strains of *O. oeni* can have a different flavor impact. *O. oeni*, formerly known as *Leuconostoc oenos* (Dicks *et al*., 1995), is a facultative anaerobe, can be propagated in a variety of low pH media (pH 4.2-4.8) and its cells are spherical and occur in chains when grown on solid media. Growth is generally slow and can take from 5 to 7 days to form visible colonies at incubation temperatures between 20°-30°C (Van Vuuren and Dicks, 1991).

Winemakers typically rely on the indigenous bacterial microflora to complete a timely and desirable malolactic fermentation. This may take several months, may occur in some barrels and tanks but not in others and may be responsible for the occurrence of problems related to indigenous LAB species currying out the MLF (several *Lactobacillus* spp. or *Pediococcus* spp.) (Lonvaud-Funel, 2001) and which may cause a range of undesirable changes to wine sensory properties, altered wine colour, and may even lead to the generation of biogenic amines (BA) (Davis *et al*., 1985). Histamine, derived from the decarboxylation of histidine, is believed to cause a reaction in sensitive individuals if the wine contains more than 0.1 mg/L. Biogenic amine synthesis usually occurs in wines exhibiting a high pH and Greek wines could be considered as high risk on this matter. Although recent studies on Greek wines revealed acceptable levels on BA content (Pramateftaki *et al*., 2006; Soufleros *et al*., 2007) vigilance and controls are required.

Another problem is associated with the risk of the development of *Brettanomyces bruxellensis* and its deleterious effect on wine aromas due to volatile phenol production (Renouf and Murat, 2008). For the above reasons, induction of the MLF by the use of selected bacterial starter cultures has become the preferred option in USA recently, in Europe it is becoming more and more popular and its beneficial impact is recognized in Greece (Soufleros et al., 2007). Furthermore in order to facilitate the absolute domination of the selected LAB starter culture in the media, the use of Lysozyme enzyme has been proposed (Dell'Acqua, 1996; Gerbaux *et al*., 1997) as an efficient way to destroy all indigenous LAB strains prior to inoculation with the selected starter culture.

In the past 15 years, the quality of malolactic starter cultures has been drastically improved. The starter cultures available for direct inoculation into wine are easy to handle and allow for better control over the MLF. Using this new generation of ML starter cultures permits the early onset as well as the rapid completion of MLF. The most desirable time for inoculation depends on many vinification factors, the most important of which are juice chemistry, the yeast strain used to produce the wine, and winemaking techniques.

Given the importance of yeast and bacterial interactions in dictating the success of malolactic fermentation, the precise timing of the addition of MLF starters to the wine has been the subject of much debate. Those in favour (Beelman *et al*., 1982; 1985) of inoculating the must with MLF bacteria (MLB) at the same time as the fermentation yeast believe that the bacteria have an increased chance of growing because they have better access to nutrients. They also feel this practice allows the MLF to complete before the termination of the alcoholic fermentation. Although favourable results have been reported using this regime, poor growth of bacteria and limited malic acid degradation have been observed by others (Gallander, 1979; Lafon-Lafourcade *et al*., 1983). If the alcoholic fermentation is delayed, there is a distinct possibility that the malolactic bacteria will also metabolize sugars in the

grape must. This can result in poor alcohol production by the fermentation yeast, as well as the production of elevated levels of acetic acid (Lafon-Lafourcade *et al*., 1983) in the final product.

Inoculating with malolactic bacteria at the end of alcoholic fermentation creates a different set of problems. The wine at this stage is often severely depleted in nutrients and the concentration of ethanol is generally high. Both conditions can cause a significant delay in the completion of MLF (Beelman *et al*., 1982; Lafon-Lafourcade *et al*., 1983) depending on the characteristics of the MLB strain employed. It should be noted that, although ethanol generally inhibits the growth of bacteria, MLF can still occur even when cells are not actively dividing, or dividing at a slower rate. It has been argued that MLB inoculated into the wine after completion of the alcoholic fermentation benefit from the presence of dead yeast cells. There is significant evidence to suggest that the practice of maintaining the wine in contact with yeast lees can enhance MLF by furnishing the bacteria with nutrients through the process of yeast autolysis (Beelman *et al*., 1982). The autolytic activity of wine yeast during aging on lees can greatly affect the concentrations of nitrogenous compounds available to MLB, including amino acids, peptides and proteins (Fornairon-Bonnefond *et al*., 2001). Other macromolecules, such as glucans and mannoproteins, are also released during yeast autolysis and have been observed to stimulate bacterial growth (Guilloux-Benatier *et al*., 1995). In addition, small amounts of CO_2 produced by yeast during fermentative metabolism provide an environment favourable for the growth of LAB. It has been suggested that leaving wine on yeast lees specifically to maintain a higher level of CO_2 may further encourage MLF (Gallander, 1979). Although protease activities, macromolecule production, autolytic capacity and CO_2 production can stimulate LAB growth and MLF, it should be noted that such a dynamic environment is difficult to control and any effects are likely to be dependent upon both the yeast and the MLB employed.

The optimum time for the addition of MLF starters to the wine or to the fermenting must, in relation with the temperature regime, and their consequences in MLF kinetics and organoleptic characteristics in the Greek red variety Agiorgitiko in the AOC of Nemea was the subject of this study.

Materials and methods

Yeast Strains. An alcohol-resistant *Saccharomyces cerevisiae* dry yeast strain (UV NEM, UVAFERM) isolated from the agricultural area of Nemea, Greece, was used for winemaking. The inoculation rate was 25 g of dry yeast / hl of must. Prior to inoculation the dry yeast was re-hydrated in 35^{o}C tap water (1:10 w/v) additioned with yeast activator GoFerm – Lallemand (25 g activator / hl water) for 20 min. The temperature of the inoculum, before incorporating it to the must, was adjusted at the temperature of the must plus 10^{o}C.

Lactic bacteria strains. For MLF experiments a relatively alcohol-resistant *O.oeni* lyophilized strain was used (Lalvin VP41, Lallemand). The inoculation rate was 0.5 g lyophilized bacteria / hl wine (or fermenting must). Prior to inoculation the lyophilized bacteria were re-hydrated in 30^{o}C mineral table water for 15 min.

Lysozyme enzyme. Lysozyme enzyme (Lallzyme Lyso, Lallemand) was added at 30 g/hl of must in order to destroy all Gram^{+} indigenous flora.

Winemaking process. Some 2,000 kg of healthy Agiorgitiko grapes were hand-picked, destemed and crashed. A total of 50 mg/l of SO_2 was added to the grape pomace just after crashing. The temperature of the pomace was adjusted to 8^{o}C and the skins were left to macerate in the must for 48 hours. After this maceration period some 1,000 l. of must were separated from the solids and distributed in 10 stainless steal temperature-controlled vats, of a capacity of 100 l. each.

The fresh must at this point had a potential alcohol strength of 13.0 % vol., a 5.1 g/l. titrable acidity (in tartaric acid), a 1.89 g/l. L-malic acid and a pH of 3.53. All 10 vats were inoculated with UV NEM *Saccharomyces cerevisiae* strain, as described above, and temperature was adjusted at 28^{o}C throughout alcoholic fermentation.

The kinetic of alcoholic fermentations in all vats was monitored by following the evolution of the density as well as the reducing sugar concentration of the fermenting must.

The 10 vats were tagged as following : Ia, Ib, IIa, IIb, IIIa, IIIb, IVa, IVb, Va & Vb. Conditions IIIa & IIIb were also additioned with lysozyme enzyme just after the yeast inoculation.

Malolactic fermentation conditions. According to the time of lactic bacteria inoculation in relation with the alcoholic fermentation stage 5 MLF patterns were created: Vats Ia & Ib didn't receive any MLB inoculum and they were left to undergo MLF with the indigenous bacterial flora. Vats IIa, IIb, IIIa & IIIb were inoculated with *O. oeni* lyophilized strain (VP41) just after completion of alcoholic fermentation. The deference between conditions II & III lies in the fact that IIIa & IIIb had already received the lysozyme enzyme addition. Vats IVa & IVb were inoculated with VP41 when the 50 % of the reducing sugars were fermented by yeast. Finally vats Va & Vb were inoculated with VP41 in the 24 h that followed the yeast inoculation.

Once alcoholic fermentation was completed, temperature of vats Ia, IIa, IIIa, IVa & Va was adjusted at 25 – 27^{o}C while temperature of the "b" series was adjusted at 30 – 32^{o}C.

The kinetic of MLF in all vats was monitored by following the evolution of L-malic acid concentration.

Analytical methods.

Determination of L-malic acid concentrations is carried out through enzymatic analysis (Boehringer Mannheim kit), reducing sugars concentration is determined with the Lüff method and volatile acidity (VA), on the end products, is determined with the distillation reference method. The comparison of the VA levels between the different MLF conditions served as the main quality criteria.

Furthermore the influence of the lysozyme addition upon the color of the wine was determined by measuring the colour intensity (C.I.) on the end product by spectrophotometry on 420, 520 & 620 nm.

Finally a quality assessment of all samples was carried out through a panel of 12 wine-judges and their quality ranking was statistically analyzed by means of the Kramer model (Fribourg et al., 1989).

Results and discussion.

As shown in Fig.1, spontaneous MLF occurred in the wine that had not receive any LAB addition. This spontaneous MLF started 8 days after the end of the alcoholic fermentation (AF) and was completed 14

days later (thus a total of 22 days after the end of the AF). The relatively rapid MLF in this case is mainly due to the favorable pH of the Agiorgitiko wine that was 3.68 at the end of the AF.

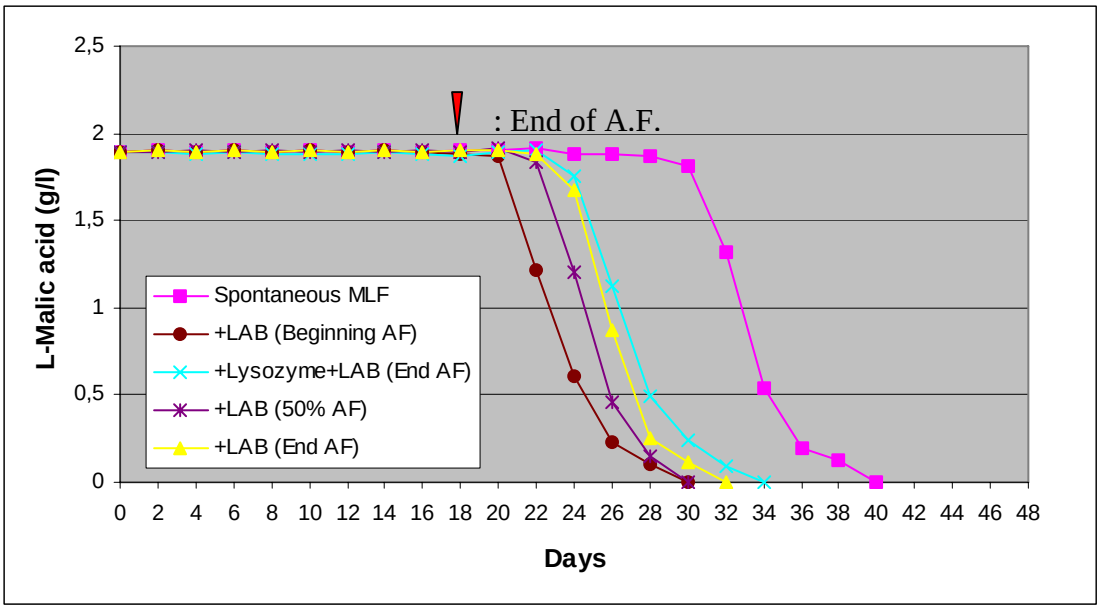

Figure 1 : MLF kinetic at 25-27°C.

Nevertheless the spontaneous condition was the slowest in comparison with all other four MLF conditions that had received LAB addition. This is due to the lower levels of the bacterial population.

The condition that was treated with lysozyme showed the second slower kinetic of L-malic acid depletion (a total of 16 days after the end of the AF).

The most rapid depletion of the L-malic acid is observed when selected LAB where added either 24 h after the yeast inoculation or when the 50 % of reducing sugars where fermented.

A similar result pattern is seen when the MLF is carried-out at a higher temperature of 30 – 32°C (Fig. 2) instead of 25 – 27°C as previously.

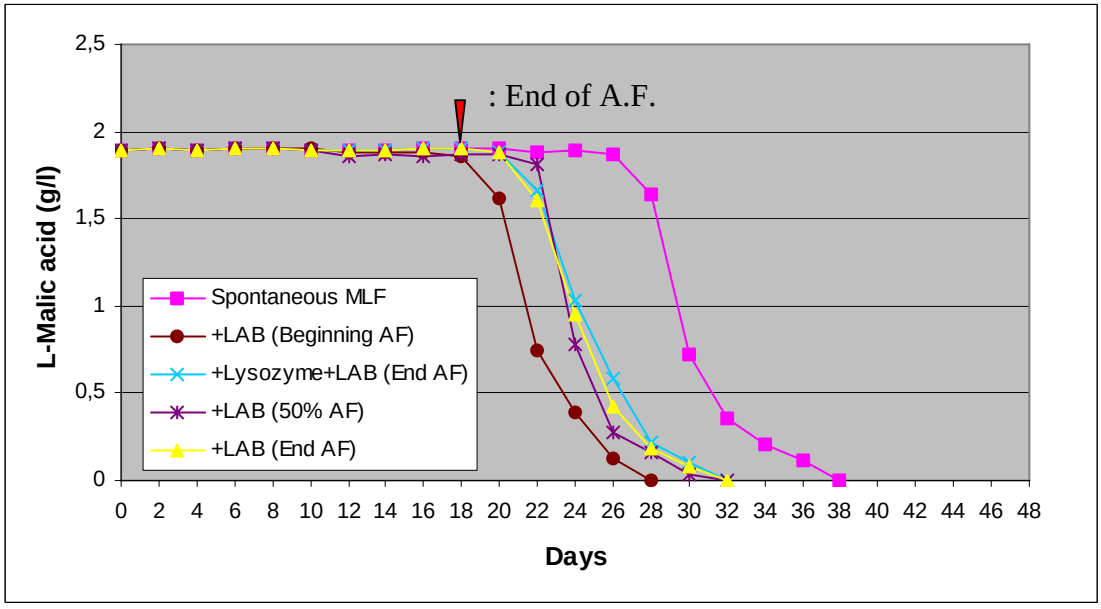

Figure 2 : MLF kinetic at 30-32°C.

The spontaneously occurred MLF, although faster by 2 days in comparison to the 25-27°C experiment, remains the slowest of 5 conditions. The condition that was inoculated with LAB just 24h after the yeast inoculations appears to be the most rapid one, where all other 3 conditions don't seem to present any significant kinetic differences what so ever.

In a general way inoculation with LAB boosts-up MLF, if compared against the spontaneously occurring MLF, and this statement is valid regardless the modality of inoculation or the temperature of the media.

From a different perspective the gain of these few days, registered in both experiments (25-27°C and 30-32°C) when selected LAB where used, would have been of no significant impact if it wasn't coupled with an obvious quality improvement for some of the modalities.
Quality of the end products has been evaluated through the indirect parameter of the volatile acidity (VA) produced during the MLF and also through a blind tasting of the end products by a panel of wine-assessment specialists.
As it can be seen on Fig. 3, final products do have significant differences as it concerns the VA registered at the day of completion of the MLF. Higher VA is a strong indication of an "unhealthy" process that leads to wines of a lesser quality.

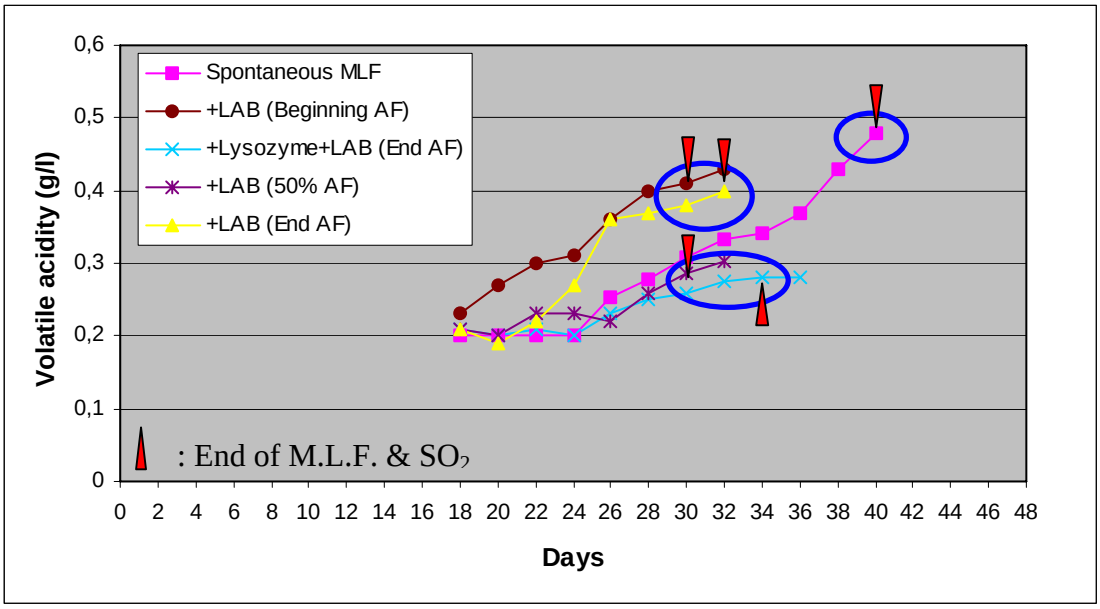

Figure 3 : Volatile acidity production during MLF at 25-27°C.

At the 25-27°C experiment we can clearly group the results in three distinct groups. The worst performance is the one that corresponds to the spontaneously triggered MLF by the indigenous flora, where the VA reached a value of 0.48 g/l.

At the antipode of this result, both wines produced after LAB inoculation, either at 50% of the alcoholic fermentation or when the sample was previously treated with lysozyme, present significantly lower VA levels (0.285 & 0.28 g/l respectively).
The two remaining LAB inoculation modalities (at the beginning and at the end of the alcoholic fermentation) led to intermediate VA production levels of 0.41 and 0.4 g/l respectively.

A rather similar VA pattern is also observed when MLF was carried-out at 30-32°C (Fig. 4). In this case two distinct VA groups are formed. As in the 25-27°C condition, the two wines produced after LAB inoculation, either at 50% of the alcoholic fermentation or when the sample was previously treated with lysozyme, present again significantly lower VA levels (0.324 & 0.3 g/l respectively).

The second VA group includes the remaining 3 MLF modalities and presents higher VA levels on the final products. More specifically the wine that resulted from the spontaneously triggered MLF had the higher VA at 0.486 g/l, the wine the resulted from LAB inoculation at the beginning of the AF was also at a very high level of 0.473 g/l and, finally, the wine that resulted from LAB inoculation at the end of the AF presented a VA of 0.44 g/l.

As previously mentioned VA production is a strong indication of quality of the final product but nevertheless it can't reflect the actual level of appreciation that the wines would benefit once tasted.

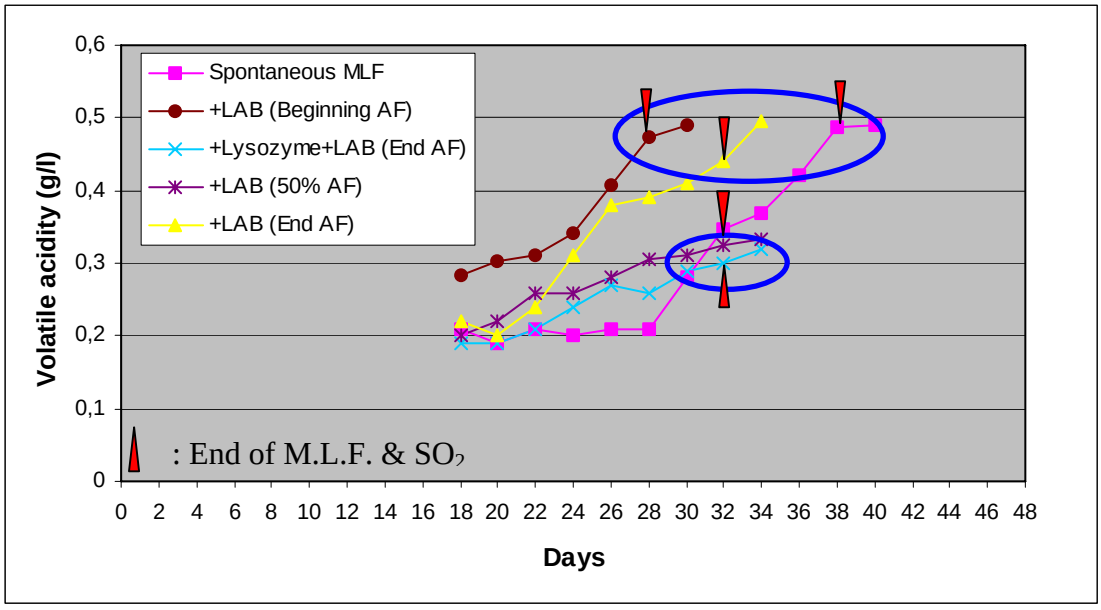

Figure 4 : Volatile acidity production during MLF at 30-32°C.

To over come this difficulty we lead a blind tasting of the wines produced with MLF at 25-27°C. A panel of 10 wine-appreciation specialists tasted the 5 wines and ranked them in order of preference (1st the considered as "the best" to 5th the considered as "the worst"). On Table 1 we give the deliberation of the 10 panelists.

Table 1 : Ranking by order of preference the 5 wines issued after MLF at 25-27°C.

	Order of preference					n
MLF condition	1st	2nd	3rd	4th	5th	
Spontaneous MLF			1	3	6	10
+ LAB (Beginning AF)		1	2	4	3	10
+ Lysozyme + LAB (End AF)	7	1	1	1		10
+ LAB (50% AF)	3	6	1			10
+ LAB (End AF)		2	5	2	1	10
n of judgments	10	10	10	10	10	

Once the ranking completed we proceeded to a statistical analysis by Kramer. The score of each wine was calculated by adding up the products of rank by the number of times that this rank has been attributed. Table 2 gives these products as well as the score for each wine.

According to Kramer's analysis for 5 samples of wine ranked by 10 panelists and for a threshold of significance of 1% the wines that score between 18 and 42 don't present any significantly important differences. The wines scoring 42 and above are judged as significantly worst were the ones that score 18 or below are judged as significantly superiors.

As seen in Table 2, the wine issued from the spontaneously triggered MLF by the indigenous bacterial flora scored 45, thus judged as the significantly worst of the lot. The wines that resulted after inoculation with LAB either at the beginning or at the end of the alcoholic fermentation scored respectively 39 & 32, thus they don't have any significant differences. Finally the two wines produced after LAB inoculation, either at 50% of the alcoholic fermentation or when the sample was previously treated with lysozyme, scored 18 & 16 respectively. It's therefore these two samples the ones judged as the significantly better wines, with a small preference towards the wine produced with the lysozyme treatment.

Table 2 : By Kramer statistical analysis of 5 wines issued after MLF at 25-27°C.

	Products (rank x nb of times)					**Score**
Spontaneous MLF	0	0	3	12	30	**45**
+ LAB (Beginning AF)	0	2	6	16	15	**39**
+ Lysozyme + LAB (End AF)	7	2	3	4	0	**16**
+ LAB (50% AF)	3	12	3	0	0	**18**
+ LAB (End AF)	0	4	15	8	5	**32**

This hedonic appreciation analysis was carried out only for the wines that derived from the MLFs that were conducted at 25-27°C. It was considered not necessary to run the same procedure for the 30-32°C scenario since it's widely accepted that it represent a "non quality" practice during wine making, a fact that is indirectly shown in this paper through the VA results.

The “sanitation” of the grape must by destroying all indigenous lactic flora through the lysozyme addition in conjunction with the inoculation of the new wine with LAB just at the end of the alcoholic fermentation would so far be the MLF modality that led to better results regardless the slowest kinetic of the phenomenon.

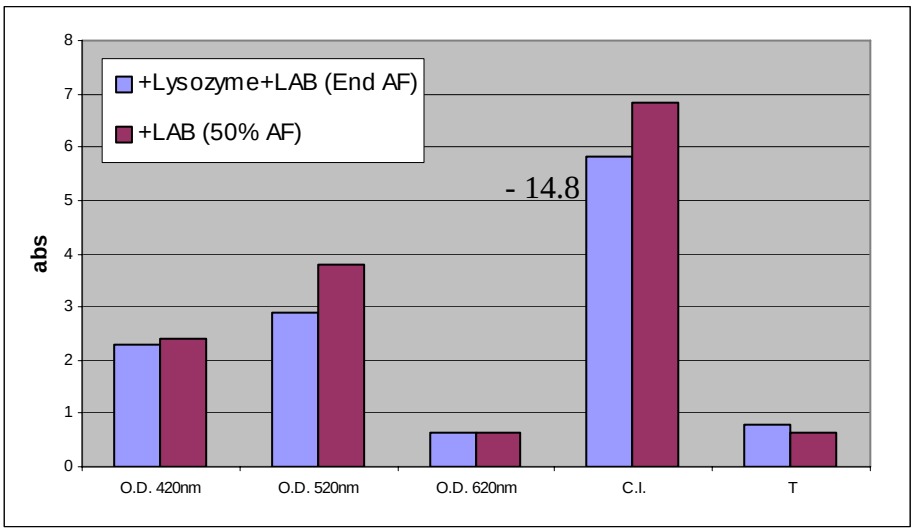

Figure 5 : Colour measurements for the two best judged wine samples.

However colour measurements (Fig. 5) reveled an alarming – 14.8 % of the colour intensity (C.I.) between the wine issued from the lysozyme treatment and the one that was produced when LAB were added at the 50% of the alcoholic fermentation.

Conclusions.

Taking into consideration the results referring to the kinetic of the MLF as well as the quality of the resulting wines, it seems clearly that for a grape variety that has a potential alcohol strength of 13.0 % vol., some 1.7 – 2.0 g/l. L-malic acid and a pH between to 3.5 – 3.6, such as the Agiorgitiko of Nemea, the optimum MLF pattern is the inoculation of the fermenting must with a selected LAB strain when the reducing sugars are fermented down to the 50% of their initial concentration. The temperature of the media after the end of the alcoholic fermentation and during the MLF should be monitored between 25 and 27^{o}C.

By doing so, the LAB added in the fermenting must have at their disposition enough time to adapt in a media that still has favourable conditions such as relatively low alcohol concentration, practically inexistent free SO_2, abundance of nutrients, convenient temperature and CO_2 saturated atmosphere.

Inoculating just after the completion of the alcoholic fermentation has been – and still remains so without any obvious reason for many winemakers – the preferred approach. We have demonstrated that this is a poor practice with relatively slow kinetics and relatively high concentration of undesirable by-products. The fact that selected Lactic Acid Bacteria are added to an unfavourable and hostile environment (poor in nutrients and rich growth inhibitors such as ethanol, fatty acids & their esters)

prolongs their time of adaptation and reduces their viability. Meanwhile the indigenous bacterial flora, an unknown mixture of "good" & "bad" strains, has had at its disposition all the time necessary to adapt in the media. If this technique is finally selected, winemakers should be aware that – at best - FML would be finally carried-out both from the selected added LAB as well as the indigenous bacterial flora, with the expected poor results on kinetic and quality.

Very early LAB inoculation, or even co-inoculation with yeast, remains conspicuous in the Nemea / Agiorgitiko conditions since there is a high risk of a rapid L-malic depletion before the end of the alcoholic fermentation, an undesirable condition that would permit to LAB to metabolise unfermented sugars, resulting in even greater quality issues.

Finally, the spontaneously triggered MLF by the indigenous bacterial flora has been shown in all scenarios as the worst choice leading to slow kinetics and undesirable by-products such as higher volatile acidity.

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