ARTICLE

CITRIC ACID METABOLISM IN LACTIC BACTERIA AND CONTROLLING THE DIACETYL CONTENT IN WINE

LAFFORT info, Vincent RENOUF[1] & Marie-Laure MURAT[2]

[1] LAFFORT® - BP17 - 33072 BORDEAUX - vincent.renouf@laffort.com
[2] EXCELL® Laboratory - Research subsidiary of the LAFFORT® group Bordeaux, France

INTRODUCTION

Diacetyl (2,3-butanedione: $C_4H_6O_2$, Figure 1) is an acetoinic molecule responsible for the 'buttery' character perceived in wine during malolactic fermentation (MLF), which is by no means universally appreciated by wine tasters.

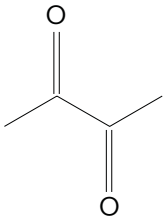

Figure 1. The chemical formula of diacetyl.

In wine, diacetyl is mainly produced by lactic bacteria, particularly the *Œnococcus œni* species, which is responsible for MLF (Renouf et al 2006). Diacetyl and other acetoinic molecules produced by lactic bacteria (acetoin and butanediol) are the degradation by-products of citric acid (Figure 2), one of the organic acids naturally present in grape juice.

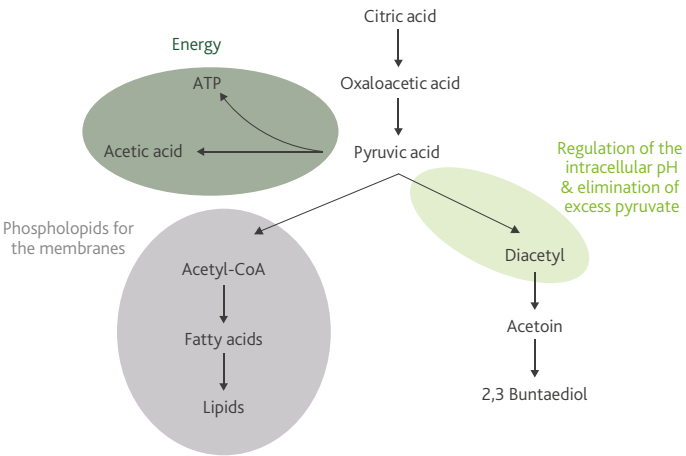

Figure 2. Pathways of citric acid degradation by lactic acid bacteria.

Pyruvate is the key metabolite at the crossroads of these metabolic pathways. It may be either used to synthesise lipids that are used in turn to build membrane phospholipids, which are essential components of the cell membrane, or consumed to produce acetoinic molecules.

The formation of these molecules is therefore considered a detoxification process for cells, which must eliminate excess pyruvate once the phospholipid demand is satisfied. The formation pathway for acetoinic compounds is also essential as it contributes to regulating intracellular pH. Citric acid is also used as an energy source by lactic bacteria. Firstly, the acetyl phosphate molecules produced from pyruvate are converted to acetic acid, releasing phosphate used for adenosine triphosphate (ATP) synthesis (Wagner et al. 2005), and secondly, the decarboxylation and translocation of the citrate molecule (which exists in the ionised form H_2citrate- at wine pH) are the two components of the proton driving force that generates energy (Seiz et al. 1963, Ramos et al. 1995), which is also the case during the malolactic transformation reaction.

Under conditions favourable for bacteria, the metabolic pathway of the pyruvate molecule is oriented towards lipids or diacetyl, depending on the cell's lipid and energy requirements. Conversely, under limiting growth conditions, the bacteria mainly use citric acid to produce acetoinic compounds.

In wine, the organoleptic impact of diacetyl has been debated for many years (Peynaud 1947, Rankine et al. 1995). At the end of MLF, concentrations vary between 1 and 10 mg/L, or sometimes even higher. Wine tasters generally agree that the diacetyl content must not exceed 5-6 mg/L (Davis et al. 1986), although it depends on the characteristics of each wine (Martineau et al. 1995). Below that level it is considered to contribute to the wine's bouquet, while higher concentrations have a negative impact. Chardonnay wines generally have the highest concentrations and, unsurprisingly, the impact of diacetyl is mainly a concern in white wines that undergo MLF (acidic wines and/or those intended for ageing, or base wines for sparkling wine).

MLF is intended to reduce acidity and ensure microbiological stabilisation, but must not leave the wine overwhelmed by heavy buttery aromas.

This article presents a brief summary of the essential knowledge on this subject and the results of recent experiments. It proposes practical solutions for controlling the diacetyl content of wine while ensuring successful completion of MLF.

CITRIC ACID IS AN INDISPENSABLE SUBSTRATE FOR BACTERIA

As previously mentioned, the citric acid degradation pathway provides *O. œni* key elements for its cell viability (population) and vitality (activity). The degradation of citric acid leads to energy production, regeneration of reducing capacity, regulation of intracellular pH, and membrane phospholipid synthesis. The vast majority of indigenous strains and those selected for malolactic starters use this pathway during their development in wine.

Citric acid is present in grapes, and even if concentrations may be modified by yeast activity, they generally range from 0.2-1 g/L at the end of alcoholic fermentation (AF), rarely more. This is not proportionate to the total acidity (TA) of the wine; for example, citric acid concentrations in wines with high TA (12 g/L H_2SO_4) may be lower than those in wines with a TA of 6 g/L H_2SO_4. Furthermore, not all citric acid is consumed during MLF, generally resulting in 20 - 50% consumption of the initial concentration. Figure 3 illustrates the usual variation in citric acid content during MLF.

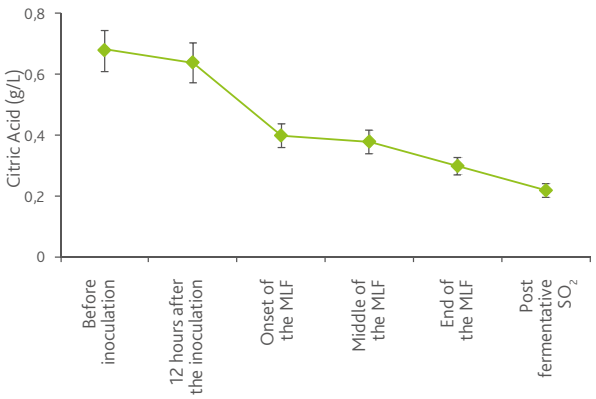

Figure 3. Evolution of the citric acid in a Chardonnay wine inoculated after the end of AF with **LACTOENOS® 350 PREAC**. Wine parameters before the bacterial addition: ETHANOL = 13.2% vol., pH = 3.3, L-MALIC ACID = 2.4 g/L, CITRIC ACID = 0.68 g/L.

As shown in Figure 3, citric acid consumption occurs mainly between inoculation with bacteria and the start of MLF. Apparently the cells need citric acid to survive inoculation in their new environment. This is due to the fact that the bacteria not only need energy at that time but they also have to regulate their intracellular pH (5.5 - 6) to a new acid environment, i.e. wine. Once they have adapted to the medium, the bacteria proliferate and need to produce new membranes, which requires an increase in phospholipid synthesis and therefore a supply of citric acid. Once the population has reached a sufficient level, the bacteria mainly consume malic acid, probably as it is naturally present in much larger quantities in wine than citric acid. It is important to emphasise that the phenomenon is identical when bacteria are co-inoculated with yeast into must with high sugar content. Among the available substrates, lactic bacteria initially degrade a small quantity of citric acid and then turn to malic acid, only using minute quantities of glucose and fructose, which remain the key target of the yeasts that are also active in the must at that time. Careful monitoring of the co-inoculation process demonstrated the kinetics of substrate use (data not shown).

Citric acid is not consumed by the bacteria during the remainder of MLF, with the exception of small amounts degraded right at the end of the process.

Figure 3 shows that a total of 0.46 g/L citric acid was used by the bacteria in that particular wine sample during MLF. This resulted in a final diacetyl content of 2.2 mg/L. This is well below the theoretical yield of the reaction (according to Bartowsky and Henschke (2004), 1 mol citric acid produces 0.5 mol acetoinic compounds). These results therefore indicate that the citric acid degradation products were used by the bacteria for other essential purposes (energy, phospholipids), rather than simple diacetyl production.

The quantity of diacetyl produced also depends on the aptitude of the bacteria and any environmental stresses to which they are subjected. Indeed, as previously described, diversion from the citrate to the pyruvate pathways depends exclusively on physiological requirements. In general, factors such as pH, high temperatures and the general composition of the wine medium, which affect bacterial growth, modify diacetyl production levels.

In view of the negative aromatic impact of diacetyl, microbiologists attempted to identify strains of *O. oeni* that were incapable of degrading citric acid under any environmental conditions. These strains did not possess the genes involved in synthesis of the enzymes responsible for citrate degradation. However, in view of previous observations of the adaptive advantages conferred by the citric acid degradation pathway, it was unsurprising that these strains had difficulty developing in wine under difficult conditions, and that they performed far poorer than other malolactic starters. As illustrated by the comparative test in Figure 4, the latency phase of the malolactic starter incapable of degrading citric acid was twice as long as that of the conventional starter, even under favourable conditions (% alc., pH and temperature). On completion of MLF, the conventional starter had only degraded 0.17 g/L citric acid

and produced less than 2 mg/L diacetyl (1.8 mg/L). Furthermore, although the non-citrate metabolising starter had not degraded any citric acid, volatile acidity was significantly higher than with the conventional malolactic starter (0.36 g/L H_2SO_4 compared with 0.25 g/L H_2SO_4). The longer latency phase certainly contributed to this increase in volatile acidity by giving other microorganisms a chance to develop.

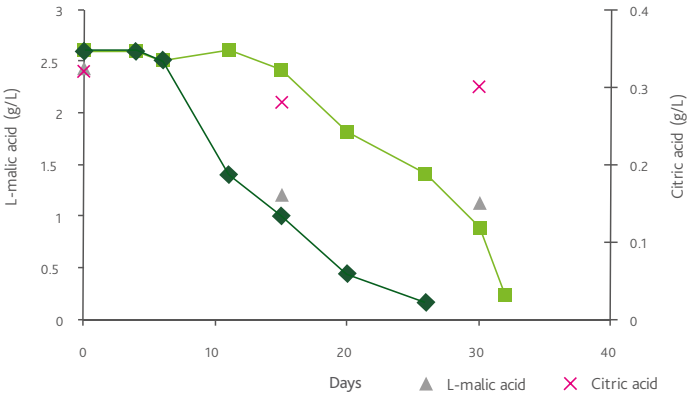

Figure 4. Comparison between a conventional malolactic starter (in green: **LACTOENOS SB3®**, **LAFFORT®**) and a malolactic starter incapable of degrading citric acid (in pink) in a Merlot wine (ACV = 13.4% vol, pH = 3.68, L-MALIC ACID = 2.6 g/L, CITRIC ACID = 0.32 g/L (test carried out in the laboratory at 25°C / 77°F)).

Depriving bacteria of the possibility of using citric acid and its associated benefits for cell development was therefore not the optimum solution as it was detrimental to the starter's efficiency in adapting to the medium, delaying the triggering of malolactic activity and leading to higher acetic acid production. Fortunately, there are other solutions to diacetyl management.

CONDITIONS FOR DIACETYL PRODUCTION BY BACTERIA

As previously mentioned, bacteria require citric acid when phospholipid and energy demands are high and intracellular pH regulation is necessary. Phospholipid demand depends on bacteria development. This factor is unavoidable as it is now well known that a minimum population of 10^6-10^7 cells/mL is required to trigger MLF. On the contrary, energy demand and pH regulation depend directly on the parameters of the bacterial environment. In general, these requirements increase whenever the bacteria are growing under difficult conditions. For example, a 30% increase in diacetyl production was measured in a Chardonnay wine during MLF when pH = 3.1 rather than pH = 3.5, all other conditions being equal. In the same wine at pH = 3.5 diacetyl production increased by 12% when the total sulfur dioxide (SO_2) content was reduced (25/45 mg/L).

It is, however, difficult to draw general conclusions on the basis of these observations as many parameters must be taken into consideration. For example, considering the impact of temperature, diacetyl production is much higher at 25°C (77°F) than 16°C (60°F), which is only significant in wines with a high alcohol content (Table 1). High temperatures probably accentuate the impact of ethanol, obliging the bacteria to metabolise citric acid. It is also known that high temperature and ethanol have a direct impact on cell membranes, probably resulting in a higher phospholipid demand. The situation was reverse in wine with a lower alcohol content – diacetyl production was slightly higher at 16°C (60°F) than 25°C (77°F). Consequently, it would certainly be preferable to maintain the temperature in the vicinity of 20°C (68°F), i.e. the optimum temperature for bacterial growth in wine. Finally, note that the relatively high diacetyl values obtained during this test were probably due to the fact that the wine was supplemented with citric acid, which also confirmed that the risk of diacetyl production increases concurrently with wine citric acid content.

ACV (% VOL)	15		12	
Temperature	16°C (60°F)	25°C (77°F)	16°C (60°F)	25°C (77°F)
Diacetyl mg/L	2.3 ± 0.7	33.5 ± 1.1	7.2 ± 0.9	5.4 ± 0.9

Table 1. Effect of temperature and alcohol content by volume (ACV) on diacetyl production at the end of MLF in a wine with a high initial citric acid content (2.5 g/L) (laboratory test).

It should also be emphasised that the duration of MLF plays a decisive role in diacetyl production. Irrespective of circumstances, the longer it takes to complete MLF, the larger the quantity of citric acid degraded and therefore the greater the risk of diacetyl production. If MLF has started but completion is sluggish, then this is a sign that the bacteria are having problems and will probably start to degrade citric acid.
Finally, note that the final concentration in wine is also moderated by the rate of diacetyl reduction. Indeed, like all ketones, diacetyl is an unstable compound which can be rapidly reduced to the corresponding alcohol – acetoin and then butanediol in this case. These molecules are much less odoriferous than diacetyl, therefore the diacetyl formation rate depends not only on the citric acid degradation rate, but also the acetoin conversion rate. This conversion is carried out by *Saccharomyces* yeasts, in particular when they are in decline at the end of AF, as well as *O. oeni*, also during their decline but at the end of MLF (Martineau and Henick-Kling 1995).

HOW CAN THE DIACETYL CONTENT IN WINE BE CONTROLLED AT THE END OF MLF?

In view of the previous observations, the first essential point is to ensure that the medium is as favourable as possible for the bacteria and that conditions are optimal for MLF, i.e. moderate sulphiting of the grapes to restrict the amount of residual SO_2 when the bacteria are added, and maintaining stable temperatures as close as possible

to 20°C (68°F) during MLF. The choice of yeast strains is also a major factor as, firstly, the quantity of pyruvate varies at the end of AF depending on the yeast strain, and secondly, the yeasts interact with the bacteria in several ways (Alexandre et al. 2004). Different yeasts make the medium more or less favourable to bacterial growth, depending on their production of SO_2 and medium-chain fatty acids (Murat et al. 2007). The yeast autolysis rate at the end of AF also plays a key role as, firstly, it adds nutrients required by the bacteria to the medium, and secondly, as previously described, the yeasts have a strong diacetyl reductase activity during their decline phase.

It is also essential to use a bacterial strain that is suited to the conditions in the wine. Specially selected bacteria are required for wines with a high alcohol content or low pH. The inoculation stage is also a key factor. While in the past, selected bacteria were added once AF had been completed, co-inoculation techniques – where the bacteria are inoculated into fermenting must – are now increasingly widely used. The main objectives are to complete MLF more rapidly and to maintain effective control of the microbial ecosystem in the wine during fermentation by imposing selected strains of yeast and bacteria. This prevents contamination due to microfloral spoilage (Renouf et al. 2008a), while ensuring more economical and ecological control of MLF. For example, when the bacteria are active at the high temperatures of AF, it is unnecessary to heat the wine, as can be required to complete MLF (Renouf et al. 2008b, Laurent et al. 2009). Adding the bacteria to the must at the beginning of AF also provides them with a more favourable medium for their development. Must is a nutrient-rich, warm and low alcohol environment, which facilitates the rapid adaptation of the bacteria, so they need less citric acid after inoculation. More importantly, the diacetyl produced during MLF may be immediately reduced via the diacetyl reductase activity of the yeast in the medium. As a result, the final diacetyl content of the wine may be halved by co-inoculation, as compared with late MLF.
The timing of post-fermentation sulphiting also has a major impact. Close monitoring of the L-malic acid degradation kinetics during MLF (assays on a twice-weekly basis) is recommended when diacetyl content is a major concern for the winemaker. This enables the winemaker to sulphite the wine rapidly as soon as MLF is complete to avoid the second phase of citric acid consumption, which occurs at that time. When the L-malic acid content is equivalent to that of citric acid (about 0.3 g/L, on average), the bacteria are once again just as likely to use either of these substrates. In many cases, they consume a little citric acid and produce diacetyl at that time; therefore, although the bacteria consume a much higher proportion of L-malic acid during the first part of MLF, when there is no more L-malic acid available, or equal substrate availability, they start degrading citric acid again. This is why it is recommended to sulphite the wine as soon as the L-malic acid content drops below the threshold of 0.2 - 0.3 g/L. In this case, the last few milligrams of L-malic acid are degraded by the residual enzyme activity of the bacterial cells inhibited by sulphiting, which then do not survive long enough to start consuming citric acid.

Finally, the way the wine is stored after MLF (i.e. whether it remains in prolonged contact with the lees or is run-off rapidly) has a major impact on the final diacetyl concentration (Nielsen et al. 1999). The yeast lees reduce diacetyl to acetoin and then butanediol, which have perception thresholds over 100 times higher than diacetyl. Sulphiting also has a significant impact, as diacetyl combination is reversible. When SO_2 is present, the concentration of free diacetyl drops and, on the contrary, its aromatic impact increases when SO_2 levels are inadequate. Of course, good management of the diacetyl content upstream in the process also contributes to reducing the amount of SO_2 required.

CONCLUSION

The degradation of citric acid by lactic bacteria during MLF should not be considered a real problem. First of all, the metabolic pathways for degrading citric acid are necessary for lactic bacteria to perform efficiently in the medium, even under stressful conditions. Secondly, it is possible to prevent excessive production of diacetyl by applying simple rules: using a malolactic starter suited to the conditions, developing early co-inoculation in the most sensitive wines, compliance with proper sulphiting and temperature conditions, regular monitoring throughout MLF to ensure that post-fermentation sulphiting is carried out before the shortage of L-malic acid causes the lactic bacteria to consume citrate, and finally, regular monitoring of free SO_2 following post-fermentation sulphiting. These simple measures are much more effective than using lactic bacteria that are incapable of degrading citric acid, as these bacteria are less efficient at malolactic conversion, which raises several other significant quality control issues.

Finally, it should be noted that its ketone functions make diacetyl a highly reactive compound capable of combining with S-based amino acids to produce odoriferous molecules with desirable floral or toasty aromas. A great deal of further work is required to clarify the role of MLF in developing wine aroma and flavour (De Revel et al. 1999, Malherbe et al. 2009), but it is quite clear that preventing the controlled production of diacetyl probably means depriving the wine of certain compounds that contribute to its aromatic complexity at the end of MLF.

REFERENCES

Alexandre, H; Costello, PJ; Remize, F; Guzzo, J and Guilloux-Benatier, M, 2004. *Saccharomyces cerevisiae – Œnococcus œni* interactions in wine: current knowledge and perspectives. Int. J. Food Microbiol. 92, 141-154.

Bartowsky, E and Henschke, PA, 2004. The 'buttery' attribute of wine-diacetyl-desirability, spoilage and beyond. Int. J. Food Microbiol. 96, 235-252.

Davis, CR; Wibowo, D; Lee, TH and Fleet, GH, 1986. Growth and metabolism of lactic acid bacteria during fermentation and conservation of some Australian wines. Food. Technol. Aust. 38, 35-40.

De Revel, G; Martin, N; Pripis-Nicolau, L; Lonvaud-Funel, A and Bertrand, A, 1999. Contribution to the knowledge of malolactic fermentation influence in wine aroma. J. Agric. Food Chem. 47, 4003-4008.

Laurent, M; Valade, M; Andrieux, A and Monocomble, D, 2009. Faire la fermentation malolactique sans chauffage, Vignerons Champenois, 6, 51-64.

Malherbe, S; Tredoux, AGJ; du Toit, M and Nieuwoudt, HH, 2009. MLF definitely makes a difference: the aroma facts. SASEV congress. Cape Town, South Africa, July 28-30.

Martineau, B; Acree, TE and Henick-Kling, T, 1995. Effect of wine type on the detection thereshold for diacétyle. Food Res. Int. 28, 139-143.

Martineau, B and Henick-Kling, T, 1995. Formation and degradation of diacétyle in wine during alcoholic fermentation with *Saccharomyces cerevisiae* strain EC 1118 and malolactic fermentation with *Leuconostoc œnos* strain MCW. Am. J. Enol. Vitic. 46, 442-448.

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

Nielsen, JC and Richelieu, M, 1999. Control of flavour development in wine during and after malolactic fermentation by *Œnococcus œni*. Appl. Environ. Microbiol. 53, 533-541.

Peynaud, E, 1947. Le 2,3-butylèneglycol, l'acétylméthylcarbimol et le diacétyle dans les vins. Bull. OIV 20, 16-19.

Ramos, A; Lolkema, JS; Konings, WN and Santos, H, 1995. Enzyme basis for pH regulation of citrate and pyruvate metabolism by *Leuconostoc œnos*. J. Bacteriol. 176, 4899-4905.

Rankine, BC; Fornachon, JCM and Bridson, DA, 1969. Diacetyl in Australian dry red and its significance in wine quality Vitis. 8, 129-134.

Renouf, V; Claisse, O; Miot-Sertier, C and Lonvaud-Funel, A, 2006. LAB evolution during winemaking: use of rpoB gene as a target for PCR-DGGE analysis. Food Microbiol. 23, 136-145.

Renouf, V; Gourraud, C and Murat, ML, 2008a. Les différentes alternatives d'utilisation des levains malolactiques, Rev Oenol. 128, 39-43.

Renouf, V; Barona de la Fuente, F and Murat, ML, 2008b Ce que coûtent réellement les levains malolactiques. Rev. Fr. Œnol. 232, cahier technique.

Seitz, EW; Sandine, WE; Elliker, PR and Day, EA, 1963. Studies on diacétyle biosynthesis by *Streptococcus diacetilactis*. Can. J. Microbiol. 9, 431-441.

Wagner, N; Hon Tran, Q; Richter, H; Selzer, PM and Unden, G, 2005. Pyruvate fermentation by *Œnococcus œni* and *Leuconostoc mesenteroides* and role of pyruvate dehydrogenase in anaerobic fermentation. Appl. Environ. Microbiol. 71, 4966-4971.

MALOLACTIC NUTRITION

PRODUCT	PURPOSE	PREPARATION	SPECIAL CONSIDERATIONS	ADDITION RATE RANGE
MALOBOOST®	Promote MLF activity and kinetics.	10 x weight in water or wine.	Use during MLF. Can be added 24 hours after beginning of MLF.	200 - 400 ppm (20 - 40 g/hL)
ENERGIZER®	**LACTOENOS® 450 PREAC** preparation only.	For 50 hL. Add with bacteria to 1L of wine + 1L of water at 68°F (20°C). Follow protocol for inoculation.	**LACTOENOS® 450 PREAC** only. Use as described in protocol.	50 ppm (5 g/hL)
REACTIVATER	**LACTOENOS® B16 STANDARD** preparation only.	For 50 hL Add with bacteria to 5L of wine + 5L of water at 68°F (20°C). Follow protocol for build up.	**LACTOENOS® B16 STANDARD** only. Use as described in protocol.	60 ppm (6 g/hL)