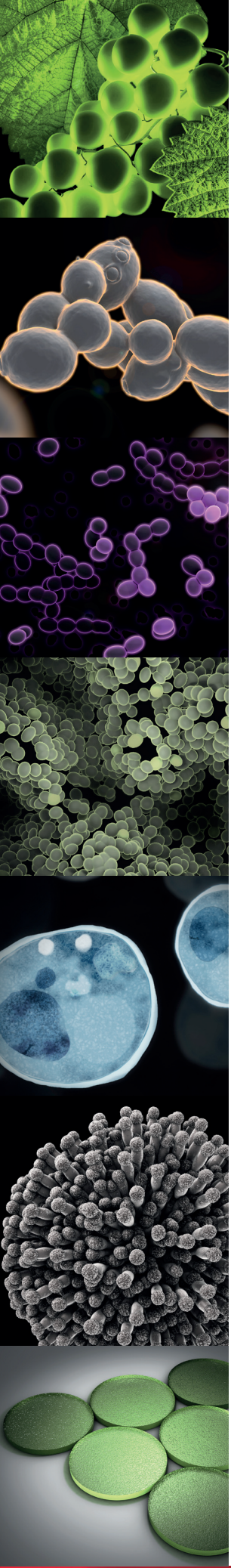

CO-INOCULATION OF WINE BACTERIA DURING WINE FERMENTATION: FROM NEW FAD TO A RECOGNIZED PRACTICE

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Introduction

Malolactic fermentation (MLF) occurs in wine as the result of the metabolic activity of wine lactic acid bacteria (LAB) with the enzymatic decarboxylation of L-malic acid into L-lactic acid and carbon dioxide. MLF reduces wine acidity, allows for a better wine stability, and influences wine sensory profiles, all of which are considered to be beneficial to wine quality. MLF can occur during or after alcoholic fermentation and is carried out by one or more species of LAB. Strains from four genera were identified as the principal organisms involved in MLF: *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (du Toit et al., 2011). Not all of the indigenous LAB are efficient and some of them can produce certain off-aromas negatively impacting wine quality. *Oenococcus oeni* is the predominant species, and the best adapted to achieve MLF notably under difficult wine conditions with a positive impact on the wine sensory profile.

The tendency to reduce or avoid SO_2 additions, and increasing grape must pH due to global warming augment the risk of excessive growth of wild spoilage bacteria, which can damage wine quality. Among various off-flavors that can appear with uncontrolled MLF, it is well known that some spontaneous wine lactic acid bacteria can produce biogenic amines (mainly histamine, putrescine, and cadaverine) which can be detrimental to human health, but also impact negatively on the wine sensory profile or mask varietal aromas. It is also known that some specific wine bacteria (*O. oeni* and *L. plantarum*) have also the capacity to degrade tartaric acid ester bound hydroxycinnamic acids, resulting in an increase of the corresponding free forms e.g. coumaric acid in the wine, which are precursors for volatile phenol production by *Brettanomyces*.

Selecting the approriate wine bacteria is thus crucial in the development and stability of the wine, from a quality point of view (reduction of faults), sensory point of view (wine style) as well as from an economic point of view (quality wine, earlier release date).

The importance of inoculation with a well-characterized wine bacteria

A full range of reliable *Oenococcus oeni* bacteria have been selected and characterized to ensure a fast and complete MLF in different wines conditions. The use of selected bacteria such as VP41™, O-MEGA™, ALPHA™, PN4™, or Silka™ results in a better control over L-malic acid degradation and guarantees that no biogenic amines nor ethyl phenols precursors are produced. Each wine bacteria is also well characterized for their sensory impact. Studies show that flavor compounds produced by wine LAB impart recognizable changes to the flavor characteristics of a wine and several studies show that different wine LAB will have distinct sensory effects in wines. For example, generally, VP41™ will contribute to produce fruity wines, O-MEGA™ will express fresh wines and ALPHA™ will develop roundness into wines. In parallel, the timing of the bacterial inoculation and the cell population in the wine after inoculation will also influence the sensory profile (Abrahamse and Bartowsky, 2012).

More recently, a new generation of *Lactobacillus plantarum* were selected in order to offer a solution to the consequences of global warming by being able to conduct a very fast and safe MLF, when inoculated one day after yeast (co-inoculation) in high pH red wine vinification. *L. plantarum* uses a homofermentative pathway for sugar metabolism and thus does not produce acetic acid when growing in grape juice. *L. plantarum* ML Prime™ has been well characterised for MLF performance, sensory impact and screened to ensure that it does not produce biogenic amines nor volatile phenols precursors.

Different timing of inoculation

Traditionally, when selected wine bacteria are used, inoculation is performed at the completion of alcoholic fermentation (AF). Since 1980, researchers have explored the possibility of inoculating wine LAB into the grape must together with the yeast or shortly after the yeast at the beginning of the alcoholic fermentation.

Today, we have identified 2 different timing throughout the winemaking process for inoculating wine LAB into the wine. (Figure 1).

Co-inoculation with yeast

- Selected wine lactic acid bacteria added 24 to 48 hours after yeast addition (48 to 72 hours if 80 to 100 ppm of SO_2 is added at crush). This practice is now getting very popular, because of its various benefits.

Post-alcoholic fermentation

- Selected wine lactic acid bacteria are added at the end of, or just after the completion of AF

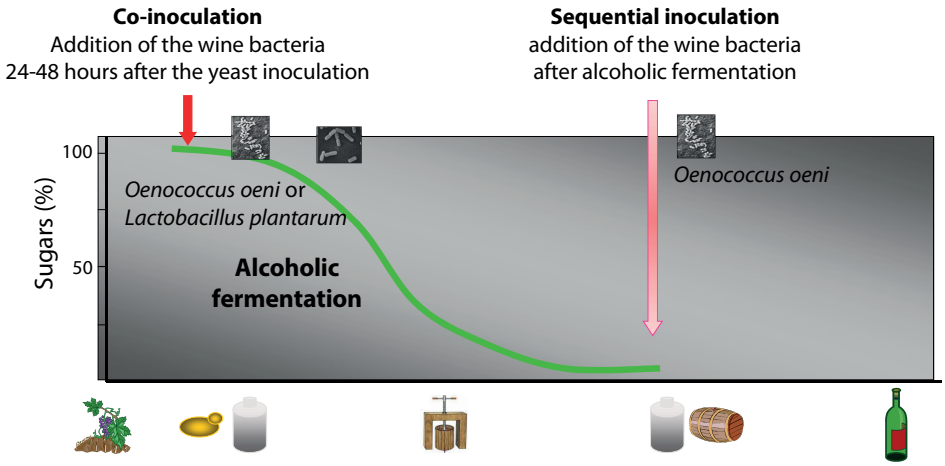

Figure 1. Timing of inoculation of wine bacteria

Benefits of co-inoculation with selected wine bacteria

Time and process management & security

Inoculation 24 or 48 hours after yeast addition ensures that the selected bacteria find all the key nutrients needed in the grape must, and can slowly adapt to the increasing alcohol content during the fermentation. This technique ensures higher survival rates and vitality of the inoculated bacteria, which results in better implantation and an early dominance of the MLF. This allows them to not only to outcompete over the indigenous LAB flora, such as heterofermentative *Lactobacillus* species, *Pediococci*, but also to limit the development of *Brettanomyces*.

Applying co-inoculation strategies, the wines inoculated with our selected bacteria will not contain biogenic amines such as histamine and tyramine, which can often occur during spontaneous MLF or delayed inoculation after the end of alcoholic fermentation.

Finally, the completion of MLF is faster compared to sequential inoculation, which allows wine stabilization to happen earlier, to avoid the potential development of microbial spoilage (Figure 2). The stable and clean wines are ready for commercial release earlier compared to wines produced with sequential or spontaneous MLF.

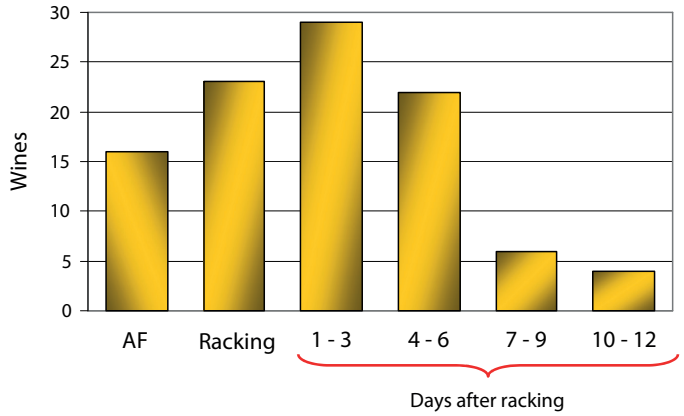

Figure 2. Frequencies of wines that terminated MLF before the end of alcoholic fermentation (before AF), at racking and 1-3, 4-6, 7-9, 10-12 days after racking

Biocontrol tools over growth of and wine spoilage by *Brettanomyces*

It has been shown that some wine bacteria (*O. oeni* and *L. plantarum*) have the ability to degrade hydroxycinnamic acids bound to tartaric ester present in the wine into their free form which are the precursors for volatile phenol production by the *Brettanomyces* yeast (Osborne et al 2012). Volatile phenols produced during contamination with *Brettanomyces* impart off-flavors in wine described a barnyard, or Band-Aid, medicinal.This study highlighted that some *O. oeni* wine bacteria clearly have the capacity to increase the level of coumaric acid (free form) in the wine. If *Brettanomyces* is present in the wine it will metabolize the coumaric acid resulting in an increase in the level of ethyl phenols the wine and subsequent wine spoilage (Figure 3).

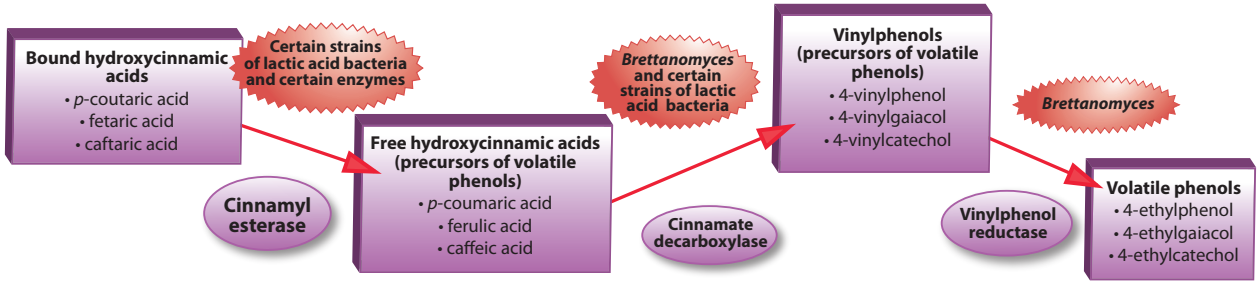

Figure 3. Production of volatile phenols

Their results showed that depending on the wine bacteria used to induce MLF, different concentrations of free cinnamic acids could be detected. Thus when using a selected wine bacteria, it should be cinnamyl esterase negative in order to avoid the production of the precursors of volatile phenols by *Brettanomyces*. All our selected wine lactic acid bacteria have been tested and confirmed that they are all cinnamyl esterase negative as shown in Figure 2

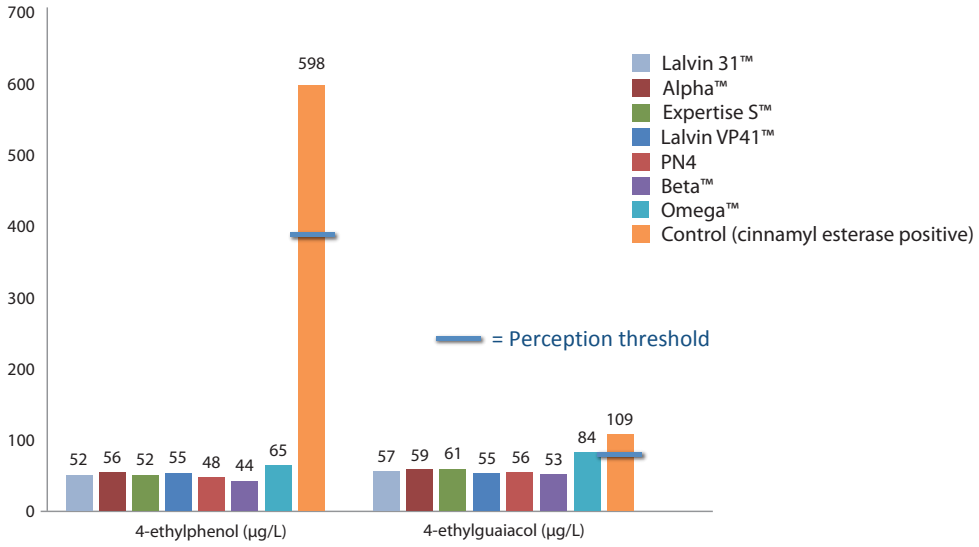

Figure 4. Volatile phenols production when different LAB strains are used for malolactic fermentation

Several studied from IFV (Institut Français de la Vigne et du Vin - France) have shown that co-inoculation with selected wine bacteria will reduce the lag time between alcoholic fermentation and malolactic fermentation and consequently the development of *Brettanomyces* during this sensitive period when the wine is unprotected.

In this study yeast and bacteria populations were monitored in a wine contaminated with *Brettanomyces* and inoculated for MLF with a selected bacteria (Figure 5) or as a spontaneous MLF (indigenous bacteria) (Figure 6) to demonstrate how lactic acid bacteria can affect the growth of *Brettanomyces*. When inoculated with selected wine bacteria, there was no *Brettanomyces* growth (even with high contamination) and moreover, the *Brettanomyces* population decreased as the population of the selected bacteria increased. In contrast, where

spontaneous MLF occurs, *Brettanomyces* population maintains the high contamination level until the 11th day (date of racking) and after racking, a regrowth of *Brettanomyces* was observed due to the slow development of spontaneous bacteria population. Final *Brettanomyces* levels are significantly different between the wines in co-inoculation and the control wines: there are 10 times more *Brettanomyces* cells in the control wine than in the co-inoculated wines. These results confirm the strong competition between our selected bacteria and *Brettanomyces*, due to the early dominance and an excellent viability of these bacteria. The interest to apply co-inoculation in order to control *Brettanomyces* growth has been acknowledged by the OIV in its good winemaking practices (OIV Oeno -264-2014).

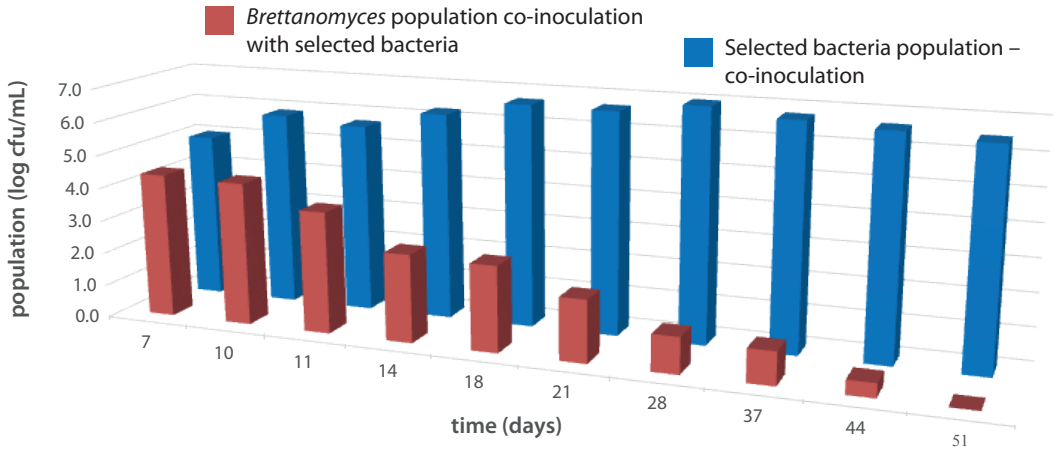

Figure 5. *Brettanomyces* growth during co-inoculation with wine bacteria in Pinot Noir (Burgundy, France)

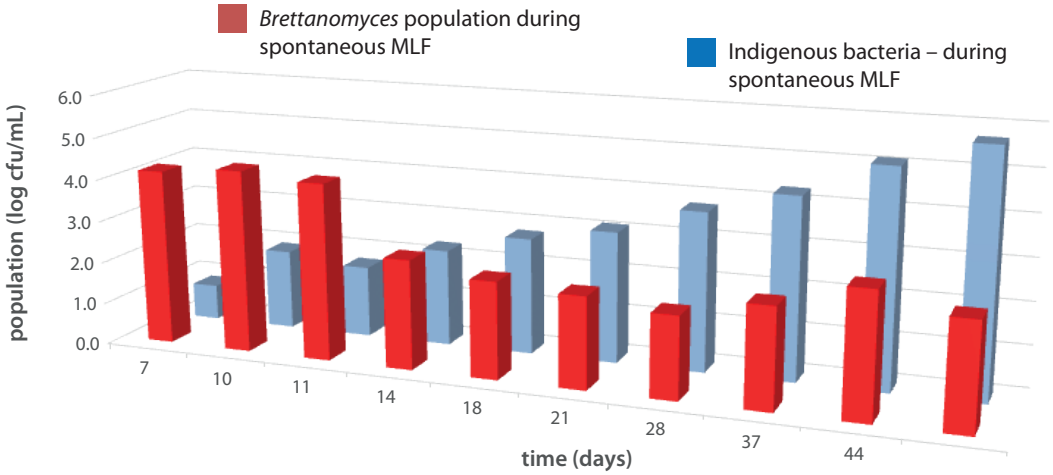

Figure 6. *Brettanomyces* growth during spontaneous MLF in Pinot Noir (Burgundy, France)

Sensory impact of timing of selected wine lactic acid bacteria inoculation

Timing of inoculation, interaction with the wine yeast, presence of precursors promoting the production of aroma compounds, pH and temperature conditions are all criteria that modulate the aromatic expression in wines. Choosing a wine bacteria and its timing of inoculation have become a key factor to take into consideration for developing a specific wine profile.

Using co-inoculation results in a different wine aroma profile compared to sequential inoculation with the same bacteria; wines are often perceived as fruitier, better balanced and with a fuller body compared to the wines inoculated at the end of alcoholic fermentation which is not always the case during sequetial inoculation (Figure 7). After MLF completion, the young wines present them selves as better integrated and harmonious.

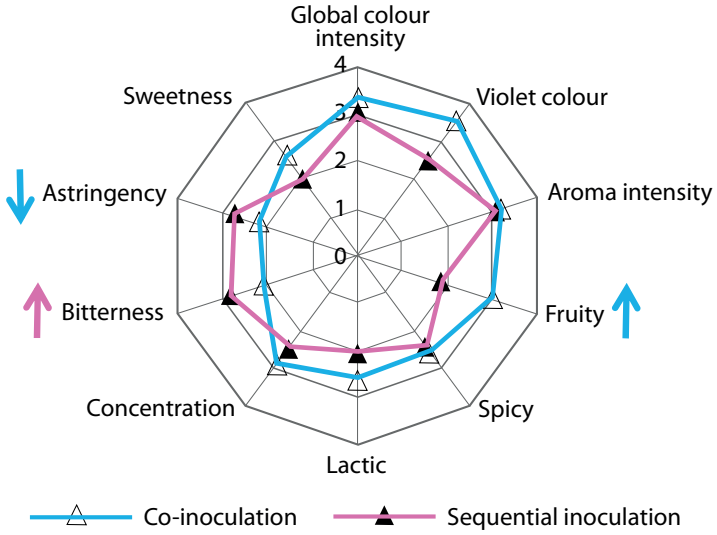

Figure 7. Sensory descriptors of Malbec wines fermentation with *S.cerevisiae* and *Oenococcus oeni* (Uvaferm Alpha™) in co-inoculation and sequetial inoculation.

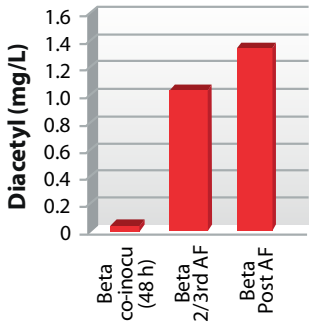

Figure 8. Impact of the timing of inoculation on diacetyl content in Chardonnay wines

Co-inoculation of selected yeast and wine LAB also has important stylistic implication in terms of diacetyl production. Our studies have shown that co-inoculation often results in more fruit-driven wine styles as opposed to lactic, buttery, nutty styles that are produced when MLF starts upon completion of alcoholic fermentation (sequential inoculation). On the contrary, co-inoculation will limit the production of diacetyl, and if diacetyl is produced it will be reduced in the presence of yeast to 2,3-butandiol, which has a much higher aroma threshold. Consequently the fruity character of white and rosé wines will be reinforced with a co-inoculation MLF strategy (Figure 8).

Volatile acidity management

Despite the heterofermentative metabolism of *Oenococcus oeni* and the theoretical risk of volatile acidity production in presence of sugars, numerous experiments (Semon *et al*., 2001; Rosi *et al*., 2003; Jussier *et al*., 2006) and many years of practical winery experience have shown that no significant amount of acetic acid will be produced from sugars during selected wine LAB growth and active MLF. No expressive differences in volatile acidity levels have been found in wines deriving from co-inoculation compared to with sequential inoculation. More recently, a study carried out by Zapparoli *et al*., (2009) in high alcohol wine matrices showed, that in Corvina and Rondinella varieties used for the production of Amarone wine, acetic acid levels were similar, or even lower when co-inoculation was applied compared to sequential inoculation. Certain well-known conditions (good management of the alcoholic fermentation: choice of a reliable yeast strain, good temperature control, and an adapted nutrition strategy) are essential to ensure a low level of acetic acid in the final wine.

Cost consideration

Co-inoculation practice means a shorter, reliable and complete MLF. This will significantly reduce the necessity to heat tanks or the cellar, a step that is necessary to start the MLF when a sequential inoculation or spontaneous are desired.

We carried out a study in Spain in 2016 which comprised of controlled malolactic fermentations using co-inoculation with Lalvin VP41™ in comparison with the spontaneous fermentation. The wines were kept at 20° C until MLF was completed. Figure 9 summarizes the experiment. The co-inoculation MLF was very fast (completed 5 days after the end of the AF), whereas the spontaneous tank started the MLF very late (completed 45 days after the end of the AF). In this long period of time, the energy consumption to heat the tank was significant, in the order of 150 kWh/hL.

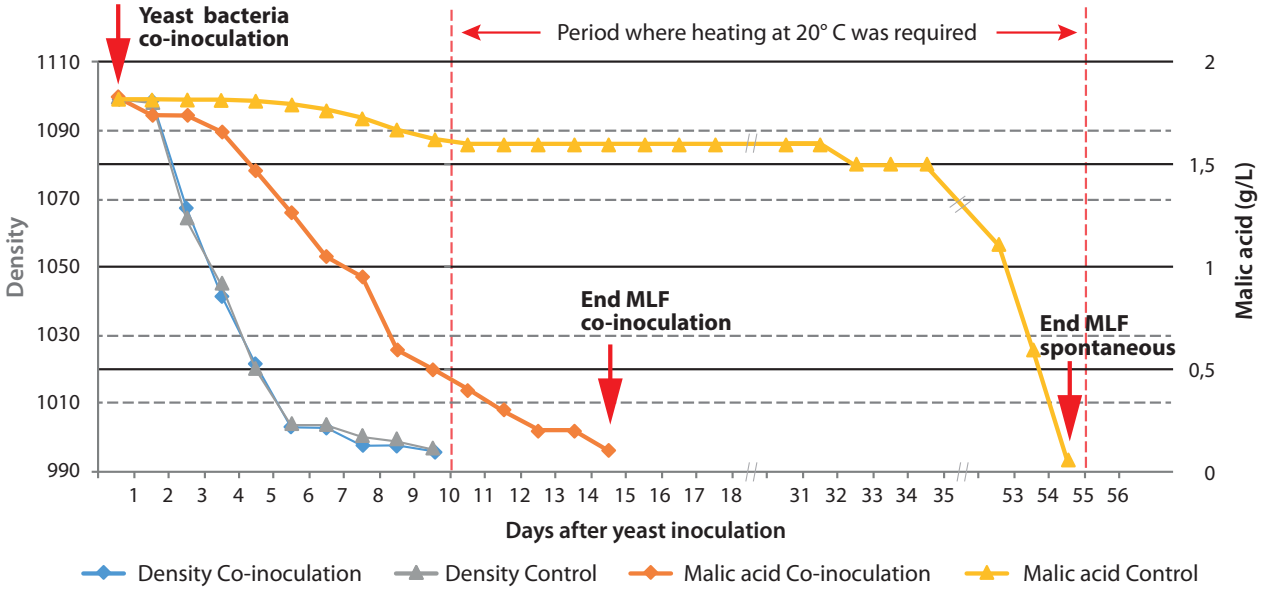

Figure 9. MLF on a Tempranillo (Spain) with 13.2% alcohol, – pH 3.5 showing differences in malic acid metabolism comparing co-inoculation with spontaneous MLF.

This energy expenditure for the spontaneous MLF trial had a calculated cost in the order of 10 € / hL (cost may vary depending on the price of KWH depending on the country, the power of heating equipment, the outside temperature, the volume of wine, the duration of the MLF); among other studies done for several years, this new result confirm that the energy cost is much higher than the cost of inoculation with a selected wine bacteria. In parallel with this energy expenditure, many other direct costs have been saved such as the analytical monitoring of wine and also hidden costs on the potential reduced wine quality. It is now well proven that inoculation with our bacteria provides guarantees on the quality of finished wines (biocontrol against the development of *Brettanomyces* or other undesirable lactic acid bacteria, no risk of production of biogenic amines and helps the wine retain its sensory style.

A new concept of selected *Lactobacillus plantarum* for the co-inoculation in high pH red must

Despite the infallible results showing no important increase in VA during co-inoculation with *O. oeni*, some winemakers still consider co-inoculation with *Oenococcus oeni* as risky because of their obligatory heterofermenative properties. They wrongly fear co-inoculation although this practice has more than proven itself to be a secure choice also for high pH reds (above 3,5- 3,6) in which the native microflora is more critical.

In the vinification of higher maturity grapes, resulting in higher pH musts, the use of a new generation of selected wine LAB starter culture consisting of *Lactobacillus plantarum* offers various advantages: *L. plantarum* has a homofermentative metabolism that means no acetic acid production from hexose sugars. A few *Lactobacillus* spp. can perform very efficiently under wine conditions and possess many favorable characteristics for MLF, especially in a high pH environment, as demonstrated in the patented application (EP1631657). The selection of a "good" wine *Lactobacillus plantarum* is not easy. Isolated at the University Catolica del Sacro Cuore in Italy,

our ML prime™ bacteria has proven being a very effective *L. plantarum* under wine conditions. It guarantees a fast and secure MLF within 3 to 7 days to complete, depending on the conditions. Due to a specific optimized production process, ML Prime™ expresses very high malolactic enzymatic activity, resulting in a fast MLF during alcoholic fermentation along with other interesting oenological properties. ML Prime™ is an unrivalled, effective option for co-inoculation and ultra-fast, risk-free MLF, even in normally sulfited musts. (Figure 10)

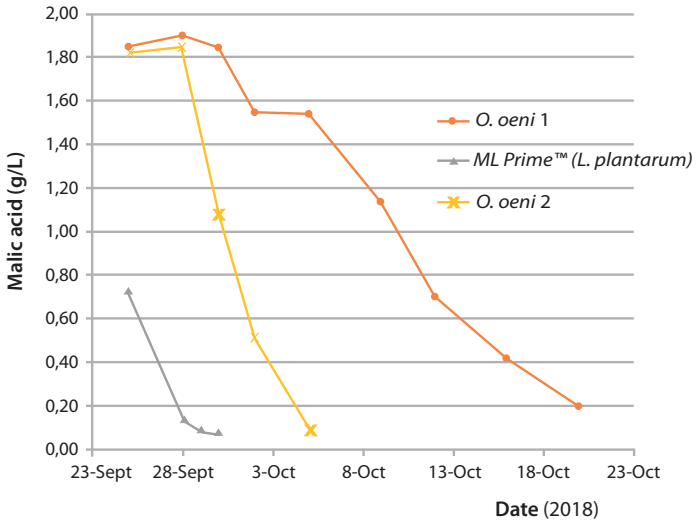

Figure 10. Malolactic fermentation with ML Prime™ compared to two strains of *Oenococcus oeni*

Conclusion

Co-inoculation is the practice of inoculating selected wine bacteria at the beginning of the winemaking process shortly after yeast inoculation. This technique has gained popularity not only because it assures a fast and complete malolactic fermentation, but also because there are numerous other advantages that are recognized by winemakers and wine professionals. In France and Spain for example, close to 50% of MLF is now conducted with co-inoculation. Co-inoculation plays a key role for a faster and more secure MLF process, an earlier wine stabilization, along with cost and energy saving. It limits the development of spoilage microorganisms and thus reduces off flavor compound production, ensuring wine quality.

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