The Wine EXPERT
Practical Winemaking Information
LALLEMAND

CO-INOCULATION OF SELECTED WINE BACTERIA

What is co-inoculation?

Co-inoculation is the practice of inoculating selected wine bacteria at the beginning of the winemaking process shortly after yeast inoculation. This technique is gaining in popularity because not only will it secure the malolactic fermentation (MLF), but also because there are definite advantages that are recognized by winemakers and professionals. Malolactic fermentation, the enzymatic decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, is the important secondary fermentation conducted by wine bacteria (Versari *et al.*, 1999). There are different timing of inoculation possibilities with selected wine bacteria (figure 1), such as co-inoculation which is the inoculation of wine bacteria at the beginning of alcoholic fermentation (AF) shortly after yeast addition, inoculation at 2/3 of the alcoholic fermentation (early inoculation) and inoculation after the completion of AF (sequential inoculation).

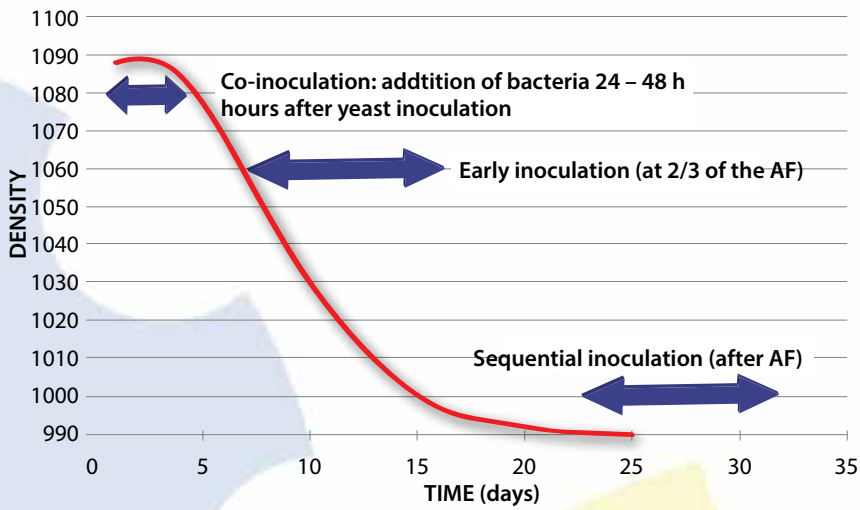

Figure 1: Different timing of inoculation of selected wine bacteria

How does it work?

Co-inoculation, where bacteria are inoculated briefly after yeast inoculation gives the selected wine bacteria a more favorable medium, mainly lower ethanol concentrations and a better nutrient availability. Since yeast grows more vigorously, ML bacteria activity will be suppressed during active AF, but the selected bacteria will acclimatize slowly to the increasing alcohol levels. Bacteria transition from the lag to the logarithmic phase of growth in a mixed culture with yeast coinciding with the start of the death phase of the yeast. This phenomenon may bring essential bacterial nutrients to the system as a result of yeast death and autolysis. Inoculation in the middle of alcoholic fermentation very often results in a more significant die-off of the selected ML bacteria, caused by the production of yeast-derived toxic compounds other than ethanol and sulfur dioxide during this highly active stage of AF. The most intense levels of yeast-induced antagonism by metabolites such as decanoic acid may be encountered at this stage. However under low pH conditions (< pH 3.15) inoculation at 1/3rd of alcoholic fermentation could be more favorable, because at this stage all sulphur dioxide added at crush will be bound and less active against the selected wine bacteria. Most compatible yeast strains for early inoculation strategies are low producers of SO_2, with a low to medium nitrogen demand and moderate fermentation kinetics.

Managing acetic acid production

When talking about the practice of co-inoculation, it is important to address the concern of possible production of acetic acid by the lactic acid bacteria. Inoculation of wine with malolactic starter cultures was traditionally practiced after the end of the alcoholic fermentation, when all fermentable sugars have been consumed by yeast and residual sugars are under 2 g/L so as to avoid the possible production of acetic acid and D-lactic acid, a situation which is referred to as "piqûre lactique" (Ribérau-Gayon *et al.*, 1975). However, inoculation of wine bacteria with selected yeast has been advocated in the USA since the early 1980's because it was felt the bacteria had a better chance of growing and acclimatizing in the absence of ethanol. The bacteria will not suffer from a shortage of nutrients nor will they be exposed to the toxic effects of alcohol. Previous experiments (Semon *et al.*, 2001; Rosi *et al.*, 2003; Jussier *et al.*, 2006) showed that significant amount of acetic acid will not be produced out of sugars during growth of MLB and active MLF. The trials conducted using simultaneous inoculation of bacteria with yeast (co-inoculation) always had no significant difference in the final acetic acid concentration. More recently, in a study done by Zapparoli *et al.*, (2009) in high alcohol wines showed that in Corvina and Rondinella varieties, which are used in the production of Amarone wine, the acetic acid levels were similar, or even lower in co-inoculation situation compared to sequential inoculation. For example, 0.19 g/L of acetic acid were measured in co-inoculation whereas 0.20 g/L were measured in sequential inoculation.

What are the benefits of co-inoculation? Secure and time saving

One of the more obvious advantages of co-inoculation is a better control over the winemaking process in terms of **time management and security of MLF completion**. Jussier *et al.*, (2006) observed a significant reduction in time for MLF from Chardonnay at a pH of 3.53 and ethanol over 13% (v/v) when co-inoculation was induced with respect to sequential AF/MLF. Under high alcohol conditions (16% and above), an important stress in winemaking, in the study of Zapparoli *et al.*, (2009), they not only showed that the MLF was completed successfully under difficult conditions, but that it was completed earlier than sequential inoculation (70 days, versus 112 days).

What are the benefits of co-inoculation? Sensory impact

Recent studies investigated the impact of co-inoculation on the **wine sensory quality**. It was shown (Knoll *et al.*, 2012, Costello *et al.*, 2012, Bartowsky *et al.*, 2011, Azzolini *et al.*, 2010) that selected wine bacteria have the potential to influence the aroma profile of wines by the production of volatile secondary metabolites and modify the grape or yeast derived metabolites such as ethyl esters, acetate esters, acids and alcohols. These sensory compounds are strongly influenced by the strain of wine bacteria used for MLF, as well as the timing of wine bacteria inoculation is very important for the wine aroma and flavor.

What are the benefits of co-inoculation? Risk management

The time between the end of alcoholic fermentation and the onset of malolactic fermentation is a critical period. Unstabilized wine is still at risk for aromatic deviations. Co-inoculation with selected *Oenococcus oeni* can help avoid the **production of potential spoilage compounds** by first reducing the **risk of spontaneous** MLF during alcoholic fermentation by suppressing wild bacteria, and at the same time conducting a more controlled MLF. This is especially important in red wine with a high pH where spontaneous MLF may occur during AF, causing stuck AF and rise in volatile acidity (VA) (Van der

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What are the benefits of co-inoculation?
Risk management

Merwe *et al.*, 2006). During co-inoculation, the microbiological activity of both yeast and bacteria helps to limit development of contaminating microorganisms such as hetero-fermentative *Lactobacillus* species, *Pediococcus*, and *Brettanomyces*. Consequently, the production of volatile phenols may be avoided. In a study done by Gerbaux *et al.*, (2009), it was shown that early inoculation of selected wine bacteria did not allow for the growth of *Brettanomyces*, even when it was intentionally inoculated in Pinot Noir wines from Burgundy (France).

Prof Maret du Toit

Maret is currently the Department head of Viticulture and Oenology, and Institute for Wine Biotechnology at Stellenbosch University (South Africa). She heads the research group focusing on the role of lactic acid bacteria (LAB) in winemaking, especially on the contribution of malolactic fermentation (MLF) on wine aroma, using lactobacilli as starter cultures as well as certain spoilage mechanisms associated with wine LAB. She is the author of 62 peer-reviewed scientific papers, 3 book chapters, 190 presentations at both international and national conferences, and graduated 33 master and 7 doctoral students.

A WORD FROM OUR EXPERT

Apart from alcoholic fermentation, malolactic fermentation (MLF) is a secondary fermentation conducted by lactic acid bacteria (LAB), firstly to reduce the acidity of wine and secondly to contribute to wine aroma.

Oenococcus oeni is currently still the best adapted starter culture for MLF, especially for low pH and high ethanol conditions and its contribution to wine aroma is well understood. MLF starter cultures can be inoculated at two stages of fermentation, namely sequential inoculation, but with higher alcohol levels due to climate changes, the pressure on the strains to perform under these conditions is becoming challenging. This has led to inoculation at another stage of fermentation, the co-inoculation of yeast and bacteria at the beginning of alcoholic fermentation. It is important that co-inoculation is done within 24 hours after yeast inoculation, otherwise alcohol and the competition from the actively fermenting yeast impacts on the inoculated MLF starter. A crucial factor is to ensure that the yeast and bacteria are compatible; therefore yeast selection needs to be considered carefully. The biggest question with regards to this technology is the potential production of acetic acid from sugars in the must. However, in the last 7 years of being involved in co-inoculation research it was never experienced that co-inoculation yielded significantly higher levels of acetic acid.

Co-inoculation has a number of advantageous. Firstly, the must contains all the necessary nutrients needed by the bacteria and therefore the addition of extra nutrients is not necessary. Secondly, the completion of MLF is faster compared to sequential inoculation, which means that SO_2 can be added sooner and the potential of microbial spoilage is reduced. Furthermore, with co-inoculation results in better implantation and out-competing of the natural LAB flora, which means the strain inoculated is the one that will dominate MLF. The other crucial factor is that there is no or limited alcohol present in the must which ensure higher survival rates and vitality of the inoculated strains. Wines made with a co-inoculation strategy has a different aroma profile than wines made with sequential inoculation, they are perceived as more fruity, balanced and a fuller body. After MLF the wines are also better integrated and in harmony at such an early stage.

Co-inoculation is a tool that can be used to ensure problems normally associated with some sequential inoculations are no longer part of the equation, as well as to diversify your wine style through the production of different aroma compounds or ratios of aromas in the final wine. This technology has also opened the opportunity for other wine LAB, such as *Lactobacillus plantarum* to be used in the future as MLF starter cultures, as the matrix and challenges are much less compared to sequential inoculation.

THE RESULTS

1. MLF Length and Reliability

Co-inoculation will shorten significantly the length of MLF compared to sequential or even more so, spontaneous MLF. In several studies, those results were consistently repeated. For example, figure 2 shows the results of various trials carried out in different varietals, over different vintages and conditions, as well as with different selected wine bacteria comparing co-inoculation to spontaneous MLF. In all cases, the length is significantly reduced. Not only co-inoculation shortens MLF but is also very reliable in a variety of situations

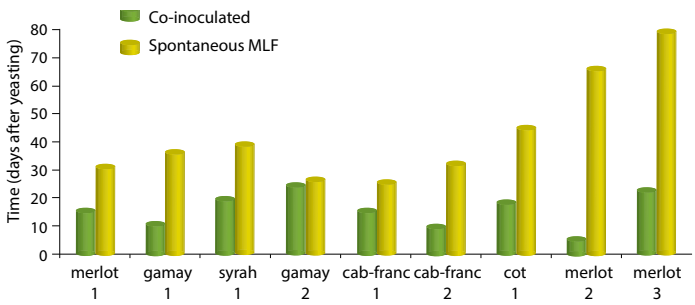

Figure 2 : Length of MLF in different varietals and different vintages with co-inoculation with seleted wine bacteria

Figure 3 shows the results of MLF length and completion under limiting conditions in a 2006 Amarone wine made out of partially dried grapes (pH 3.3, Alcohol 15.5% v/v, Total SO_2 50 mg/L). Zapparoli and Tossi (2006), could successfully achieve malolactic fermentation using co-inoculation techniques (bacteria inoculation 1 day after yeast) with VP41 MBR® culture compared to sequential inoculation and spontaneous MLF, which had not started after 90 days.

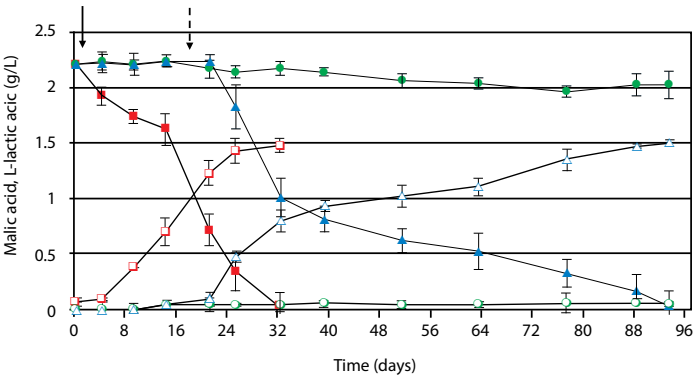

Figure 3: Malic acid consumption (solid symbols) and L-lactic acid production (hollow symbols) determined in trials co-inoculated with yeasts and bacteria (■ □), inoculated with bacteria post AF (▲ △) and not inoculated with bacteria (● ○). Continuous and dotted arrows indicate the bacteria inoculation times before and after AF, respectively. Values are means ± standard deviation (bars) of three independent trials.

Experiments done in collaboration with the University Catolica in Chile investigated the interest of co-inoculation techniques in high pH musts with higher alcohol potentials. In the 2005 vintage in Chile using 8 different lots of high pH (3.5 to 3.9) grapes (Carménère, Syrah, Merlot, Cabernet Sauvignon and Petit Verdot) with alcohol potential between 14 to 15 % vol. Co-inoculation of the bacteria 24 hours after yeast inoculation was compared to the control wines without bacteria inoculation.

Again the total length of malolactic fermentation was significantly reduced by co-inoculation of bacteria, clearly indicating the dominance of the selected wine bacteria. Total duration of MLF from the time of inoculation was about two times faster in the co-inoculated tanks compared with the spontaneous controls.

The reduction in MLF time and reliability of completion is an important advantage since it will reduce significantly the necessity to heat the cellar, which would be necessary when using sequential inoculation since it would happen later in the season, and consequently, the cellar (and wines) would need to be warmed up to start up the MLF. Another advantage is the possibility to have the wines earlier stabilized, which means that they are commercially ready faster compared to, for example if sequential or spontaneous MLF was used

2. Sensory impact

It has been observed that wines that have undergone simultaneous AF/MLF tend to be less buttery and are fruitier (Henick-Kling 1993; Bartowsky *et al.*, 2002; Jussier *et al.*, 2006; Krieger 2006; Massera *et al.*, 2009, Bartowsky *et al.*, 2011).

In a study done by Knoll *et al.*, (2012), it was shown that in Riesling wines with sequential MLF had the lowest concentrations of acetate esters and ethyl esters, most notably due to lower concentrations of acetic acid phenylethylester, acetic acid 3-methylbutylester, butyric acid ethylester, lactic acid ethylester and succinic acid diethylester. This might potentially result in decreased fruitiness sensation in wines with sequential MLF. The wines with the co-inoculation, on the other hand, had the highest concentration of fruity ethyl esters. In addition, changes in the ester concentrations were also affected by the bacterial strain used. *O. oeni* Lalvin VP41® seemed to produce higher concentrations of various fruity esters, such as propionic acid ethylester, butyric acid ethy-lester or lactic acid ethylester, associated with fruitiness, milky notes and mouthfeel, respectively (Figure 4).

THE RESULTS

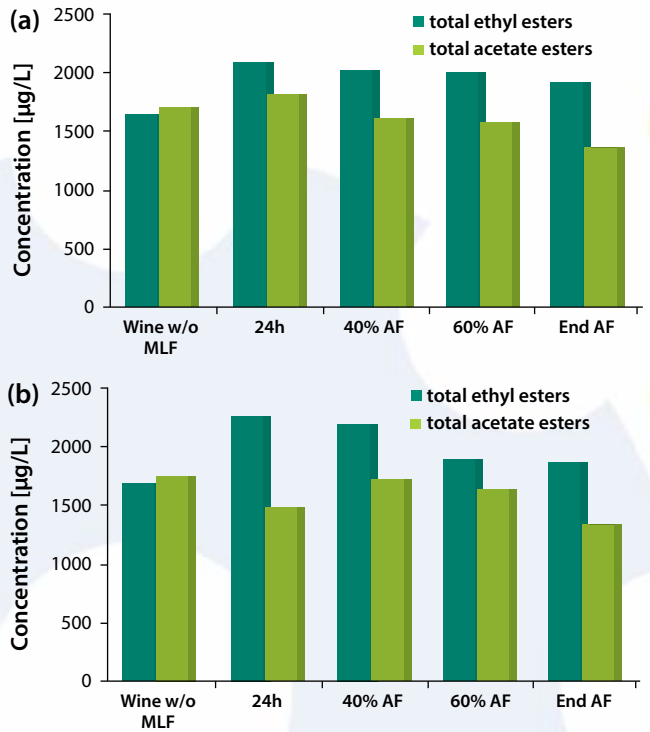

Figure 4: Average concentration of total ethyl ester and total acetate esters in (a) wines inoculated with VP41® and (b) PN4® at different inoculation time for the MLF.

Co-inoculation of selected yeast and MLB 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 result when MLF starts upon completion of alcoholic fermentation (sequential inoculation).

For example, Figure 5 shows diacetyl concentrations in a 2010 Chardonnay from Val de Loire (France). The selected bacteria Beta produces significantly less diacetyl in co-inoculation (48h) than in early inoculation (2/3 AF) or sequential inoculation (post AF). The impact of the ML strain on diacetyl production is not as strong in co-inoculation since the wines will show repeatedly low level of diacetyl with this technique, no matter which wine bacteria is used.

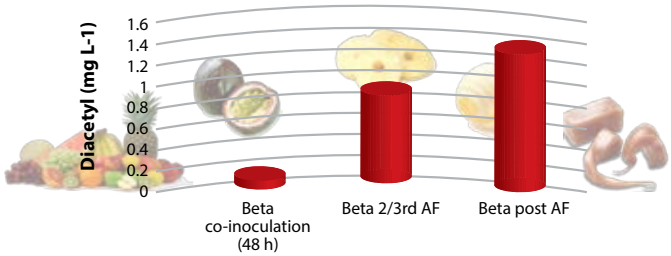

Figure 5: Diacetyl concentration in a 2010 Chardonnay (Val de Loire) with different timing of inoculation for MLF with Beta.

3. Management of undesirable compounds and undesirable indigeneous flora

In co-inoculation strategies, it was found that significantly less biogenic amines and no histamine and tyramine were produced compared to inoculation after the end of alcoholic fermentation in a trial done in collaboration with Stellenbosch University (du Toit *et al.*, 2007, van der Merve *et al.*, 2006) (Figure 6). The low concentration of putrescine and cadaverine also found in the wines with co-inoculation originate from the grape must.

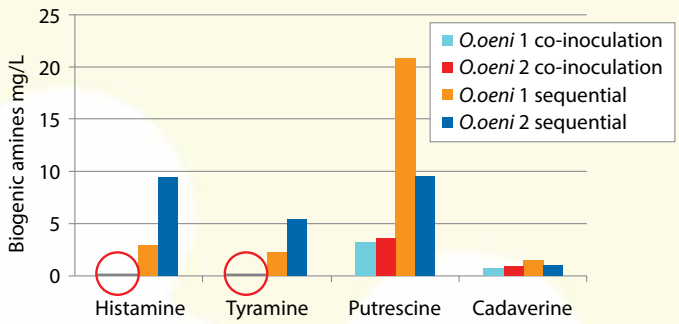

Figure 6: Biogenic amine levels in a 2006 Cabernet Sauvignon (South Africa) fermented with yeast Lalvin ICV D254 comparing co-inoculation with ML starter culture versus sequential inoculation.

Selected wine bacteria have always been screened during the selection procedures using genetic techniques to assure that the genes coding for the enzymes histidine decarboxylase or ornithine decarboxylase, which are responsible for the formation of biogenic amines, are not expressed. It was assumed that for inoculation post alcoholic fermentation (sequential), the spontaneous bacteria flora was responsible for the production of higher biogenic amine levels analyzed in these treatments. The implantation control using PCR (RAPD) with primers M13 techniques confirmed our findings. In high pH conditions we could achieve 100 % implantation for the co-inoculated bacteria and wine yeast treatments, whereas in all the selected bacteria post alcohol fermentation treatments other bacteria DNA profiles were also found.

Co-inoculation can also be a a useful tool to prevent formation of the unwanted volatile phenols 4-ethylphenol and 4-ethylguaiacol. The elimination, or drastic reduction, of these compounds results in superior wines. If the timing of alcoholic and malolactic fermentation is good, malolactic fermentation can be achieved immediately after alcoholic fermentation and if a gap between the end of AF and

THE RESULTS

the start of MLF is avoided, *Brettanomyces* can also be avoided, because the wine is stabilized earlier. Co-inoculation can become an efficient tool to prevent *Brettanomyces* development, and consequently, more winemakers are using this technique to fight against this contamination. Figure 7 shows the results of a Cabernet Franc trial from France, where the inoculation with MLB drastically reduced the population of *Brettanomyces* as well as the levels of volatile phenols in the wines.

Co-inoculation will not only help control *Brettanomyces*, but it will also limit the development of other undesirable species such as *Pediococcus* and *Lactobacillus*, especially in wines with pH higher than 3.5.

The use of co-inoculation allows for an earlier wine stabilization which prevents the development of contaminants and results in cleaner and more aromatic wines.

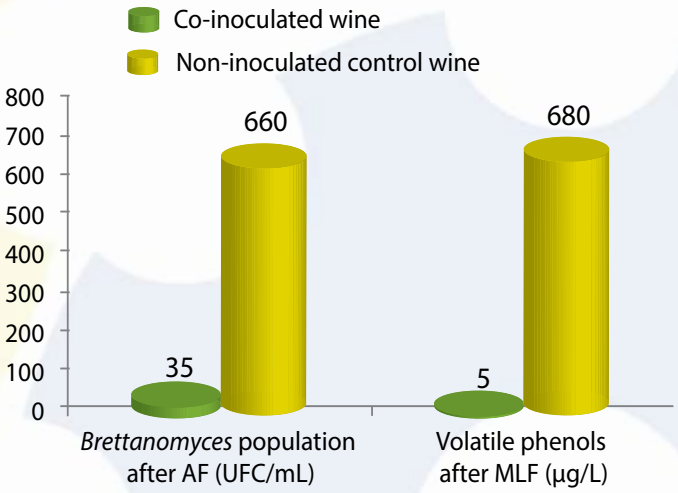

Figure 7: 2006 Cabernet franc: Analysis of Brettanomyces contamination and volatile phenols.

A QUICK SUMMARY

The practice of co-inoculation is becoming more popular. In France and Spain for example, close to 50% of MLF is now done via co-inoculation. The advantages are numerous, such as ensuring a faster more secure process and reducing time for the MLF. Co-inoculation is an important modulator in sensory development, and it helps limit the development of spoilage microorganisms and thus limits off flavor compound productions.

For example, a wine bacteria like the Enoferm Beta® can produce higher levels of diacetyl during sequential inoculation. Co-inoculation on the other hand, will reduce the production of diacetyl and consequently reinforces the fruity character of white wines. Timing of inoculation, interaction with yeast, the presence of precursors that promote the production of aromatic molecules, pH and temperature conditions are all criteria that modulate aromatic expression in wines. Choosing a wine bacteria has become a parameter to take into consideration for developing a specific wine profile.

Our next topic: " Acetaldehyde management during winemaking "