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SHORT COMMUNICATION

# Simultaneous yeast–bacteria inoculum. A feasible solution for the management of oenological fermentation in red must with low nitrogen content

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**Abstract** The simultaneous inoculum of yeasts and bacteria is a feasible solution for improving fermentation in wines with a harsh chemical composition, capable of inhibiting microbial activity. Considering the risk of wine spoilage due to lactic bacteria, co-inoculum is suggested in white wines with a low pH. However, climate change has also caused problems in achieving malolactic fermentation in red wines, due to the high concentration of ethanol and the low nutrient content. In this work, 5 pairs of commercial oenological starters were tested in simultaneous fermentation, using 4 red musts with a low nitrogen content, and compared with a traditional winemaking process. The simultaneous inoculum caused a slowdown in the activity of yeasts, although no problems in the accomplishment of alcoholic fermentations were observed. More reliable malolactic fermentation was performed in the co-inoculum trials, while, in traditional winemaking, some failures in the degradation of malic acid were observed. Microbiological analyses agreed with these observations. No differences were found in yeast density during alcoholic fermentation, demonstrating the absence of negative interaction between the yeast and the bacteria. However, simultaneous fermentation is not without risks; the highest increases of acetic acid were noted in the co-inoculum trials. The addition of yeast and bacteria to must with a serious lack of nutrients would appear to be a promising alternative to traditional fermentation; however, careful control of the chemical composition of must is mandatory to obtain reliable microbiological activity in the first stages of winemaking.

**Keywords** Simultaneous fermentation · Lactic bacteria · Wine spoilage · Readily available nitrogen

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In recent years, simultaneous inoculum of yeast and bacteria cultures at the beginning of the winemaking have been proposed as a feasible solution for obtaining fast and reliable malolactic fermentation (MLF) in wines with a high acidity or a high ethanol concentration (Jussier et al. 2006; Zapparoli et al. 2009). Some reasons justifying this approach (Abrahamse and Bartowsky 2012) relate to the expectation that bacteria may adapt better to environmental conditions in must rather than in wine, the limiting factors increasing gradually according to the evolution of alcoholic fermentation (AF). At present, simultaneous fermentation has only been adopted in must with a low pH (Rodriguez and Thornton 2008) to avoid the risk of producing acetic acid due to the heterofermentative metabolism of the main species of lactic bacteria used to start MLF (Liu 2002).

In this work, we considered the possibility of improving MLF in red must/wine with a low nitrogen content, a feature frequently observed in the last few years, especially in the Mediterranean area, by yeast–bacteria co-inocula. A total of 40 microvinification trials (50 L for each trial) were carried out, comparing the behaviour of fermentation performed by co-inoculum (CO) with that of a traditional winemaking procedure (TW). Five pairs of commercial yeast and bacteria cultures were considered and the following active dry yeasts were used: Mycoferm Cru 05 (Ever srl, I), La Claire C58 (Perdomini

IOC spa, I), QD145, S6U, and ICV254 (Lallemand, CA, USA). Extremo IT06 (Ever srl, I), Malica (Perdomini IOC spa, I), PN4, Vp41, and V22 (Lallemand) were employed as MLF starters. Yeast and bacteria were inoculated following the supplier's suggestions as regards the quantity and rehydration protocol. Four different grape musts (cv. Cabernet Sauvignon, Merlot, Teroldego and Marzemino) were employed. In all the grape musts, the content of readily available nitrogen (RAN) was below 70 mg/L and the sugar concentration exceeded 210 g/L. The evolution of fermentation was followed by daily chemical analysis using FT-IR (WineScan; Foss, Denmark) and microbiological analysis (microscopic count for yeast quantification and plate count for bacteria enumeration) according to the OIV methods (2012).

Figure 1, bar graph, summarises the tests performed on Merlot grape must; the results were reported as the days necessary to achieve AF and MLF. The Merlot grape must showed the worst ratio between sugar content (220 g/L) and RAN (<30 mg/L) in the whole set of tests, and was therefore able to magnify the effects of stress exerted by environmental conditions on the microbiota. As expected, differences were observed between the different yeast/bacteria pairs in terms of the success and rate of fermentation. This evidence confirms the importance of careful selection of microbial starters for oenological fermentation, not only as regards the performance of each microorganism but also the interaction between different species operating in the same environment (Mendoza Lucia et al. 2010; Wells and Osborne 2011). In the tests performed by the first 3 pairs of microorganisms, we did not observe interference in the evolution of AF, due to the inoculum of bacteria in must, given that the AF rate of TW and CO trials was the same. On the contrary, lactic bacteria were only able to metabolise malic acid completely in must, while in wine (TW trials), MLF stopped prematurely, leaving a residue of malic acid (up to 0.5 g/L). The 4th yeast–bacteria pair was the most efficient in the whole set of tests because it degraded malic acid to a residual concentration below 0.2 g/L, both in must and wine. The 5th pair of microorganisms showed the highest susceptibility to the specific conditions of must. AF showed behaviour similar to other tests, but MLF was halted both in must and in wine.

A summary of data for the whole fermentation set is given in Fig. 1, point graph. The addition of bacteria to grape must during AF does not increase the speed of MLF, but rather guarantees a lower mortality for bacteria and therefore greater efficiency in the degradation of malic acid (Henick-Kling and Park 1994; Rosi et al. 2003). In the CO trials, MLF occurred in the majority of samples, with the single exception of Teroldego grape must, with malic acid residues between 0.8 g/L (5th pair) and 0.46 g/L (1st pair) 45 days after the start of fermentation. Of the five pairs of microorganisms, only the 5th did not show satisfactory activity in grape must, being unable to accomplish malic acid degradation in all the trials. The concentration of malic acid observed 45 days after the CO tests performed by the 5th pair was 0.89 g/L (Teroldego), 0.67 g/L (Merlot), 0.71 g/L (C. Sauvignon) and 0.49 g/L (Marzemino). TW of the 5th pair gave similar, or in some cases even worse, results. For example, in Cabernet Sauvignon, MLF was halted after consumption of only 1.6 g/L of malic acid. As observed for the Merlot data, the differences between the duration of alcoholic fermentation performed using the two different strategies were relatively small and not technologically relevant. These results agree with previous works, demonstrating that the risk of AF inhibition by lactic bacteria is remote (Alexandre et al. 2004), while, on the contrary, there is evidence of the production of substances toxic to bacteria by yeasts (Zapparoli et al. 2003; Comitini et al. 2005). This phenomenon is particularly clear in the case of AF carried out in difficult environments, as in the case of must with a low nitrogen content.

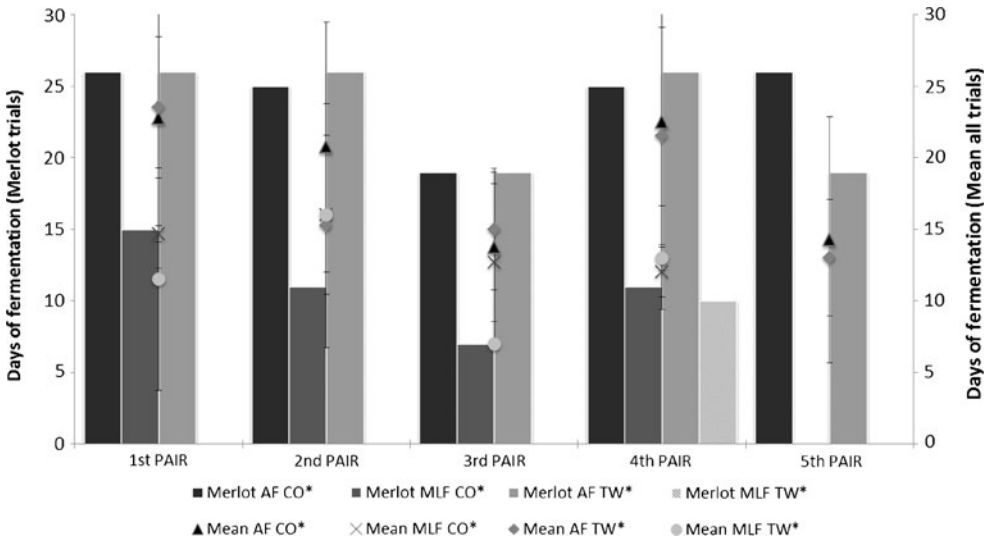


**Fig. 1** Time required by different pairs of yeast/bacteria to accomplish oenological fermentation. *Bar graph* (*left-hand vertical axis*) test performed on Merlot grape must. *Point graph* (*right-hand vertical axis*) mean ± SD of 4 tests performed on Teroldego, Marzemino, Merlot and Cabernet Sauvignon grape must. **CO* co-fermentation tests, *TW* traditional winemaking

The evolution of fermentation highlighted the role of RAN on the performance of yeast cultures (Fig. 1). Despite the fact that the differences between the RAN concentrations of the 4 musts were limited, around 10 g/L, there is a clear correlation between fermentative activity and nutrient availability, as suggested by Bell and Henschke (2005). In Teroldego must, which showed RAN around 70 mg/L, all AF occurred fully and no residual sugar was found in the wine. In Cabernet (RAN: 55 mg/L), Marzemino (RAN: 30 mg/L) and Merlot (RAN: < 30 mg/L), a mean of about 1.5 g/L sugar remained in the wine after 45 days of fermentation. Some halting of AF was observed, as, for example, in the case of the test performed using the 2nd yeast–bacteria pair on Marzemino must by subsequent inocula (6.6 g/L of sugar after 45 days). To conclude, observation of the evolution of fermentation performed by yeast and bacteria cultures in mixed or sequential inoculum showed the importance of must composition in reliable microbial transformation, over and beyond the specific characteristics of each microorganism (Bell and Henschke 2005).

Microbiological data (Table 1) provided an alternative point of view, helping to understand the development of fermentation and the interaction between yeast and bacteria. Measurement of live/dead yeast cells performed after 10 days may make it possible to detect any differences in the viability of yeast populations, due to the presence of lactic acid bacteria in co-fermentation. In reality, the concentration of yeast cells in CO tests appeared to be similar, or often higher, than that observed in TW. The difference between the counts would seem to be more related to the strain of yeast than to the grape must (Table 1). The concentration of native lactic bacteria in all grape musts was very low, around $10^2$ CFU/mL, as observed in studies regarding the microflora of grape and fresh must (Guzzon et al. 2011; Barata et al. 2012). After CO, the concentration of lactic bacteria increased, to around $10^6$ CFU/mL, with little difference between the different pairs, probably due to the specific characteristics of freeze-dried cultures. The main point of interest is that in CO tests the concentration of lactic bacteria remains constant, or in some cases increases up to $10^7$ CFU/mL for several weeks, allowing effective MLF. This behaviour would appears to be specific to MLF performed on grape must and is very different from the behaviour observed in wines, where the limiting factors (pH, $SO_2$, ethanol) often cause a quick decline in bacterial viability (Guerzoni et al. 1995; Guzzon et al. 2009)

Despite these promising results, simultaneous fermentation is not without risks. The increase in acetic acid was higher in the case of CO of yeast and bacteria, confirming

**Table 1** Yeast concentration (measured by microscopic counts of live/dead cells) after 10 days fermentation; acid acetic content of wine at the end of tests (45 days after yeast inocula)

| Must (malic acid content) | Pair | Yeast concentration after 10 days (CO trials) | | Yeast concentration after 10 days (TW trials) | | Acetic acid concentration in wine | |
|---|---|---|---|---|---|---|---|
| | | Live cells ($10^6$ CFU/mL) | Dead cells ($10^6$ CFU/mL) | Live cells ($10^6$ CFU/mL) | Dead cells ($10^6$ CFU/mL) | CO trials (g/L) | TW trials (g/L) |
| Teroldego (4.20 g/L) | 1st | 53.0 | 1.0 | 44.0 | 1.3 | 0.15 | 0.14 |
| | 2nd | 51.0 | 2.3 | 41.0 | 1.8 | 0.24 | 0.12 |
| | 3rd | 48.0 | 0.5 | 43.0 | 1.0 | 0.24 | 0.11 |
| | 4th | 61.0 | 0.8 | 49.0 | 1.0 | 0.34 | 0.22 |
| | 5th | 34.0 | 2.8 | 40.0 | 4.0 | 0.14 | <0.10 |
| Marzemino (2.19 g/L) | 1st | 180.0 | 7.0 | 60.0 | 4.5 | 0.31 | 0.25 |
| | 2nd | 130.0 | 13.0 | 75.0 | 1.0 | 0.31 | 0.52 |
| | 3rd | 65.0 | 8.0 | 46.0 | 9.0 | 0.42 | 0.23 |
| | 4th | 110.0 | 5.0 | 56.0 | 6.0 | 0.51 | 0.49 |
| | 5th | 60.0 | 12.0 | 44.0 | 9.5 | 0.22 | 0.22 |
| Cabernet Sauvignon (2.35 g/L) | 1st | 27.0 | 8.5 | 29.0 | 6.5 | 0.71 | 0.56 |
| | 2nd | 29.0 | 6.5 | 25.0 | 5.5 | 0.60 | 0.69 |
| | 3rd | 28.0 | 5.5 | 70.0 | 3.0 | 0.53 | 0.38 |
| | 4th | 26.0 | 1.1 | 16.0 | 7.0 | 0.95 | 0.71 |
| | 5th | 32.0 | 5.5 | 24.0 | 4.0 | 0.37 | 0.52 |
| Merlot (2.31 g/L) | 1st | 67.0 | 11.0 | 50.0 | 4.5 | 0.31 | 0.25 |
| | 2nd | 61.0 | 10.0 | 38.0 | 9.5 | 0.31 | 0.52 |
| | 3rd | 57.0 | 14.0 | 39.0 | 7.5 | 0.42 | 0.23 |
| | 4th | 80.0 | 5.6 | 43.0 | 3.0 | 0.51 | 0.49 |
| | 5th | 98.0 | 17.0 | 38.0 | 7.0 | 0.22 | 0.22 |

the potential risk of wine spoilage due to the activity of bacteria in a sugar-rich environment (Lonvaud-Funel 1999). In this case, the pH of grape must, close to 3.5, favoured the control of the heterofermentative activity of bacteria. However, considering the trend for pH increases observed in some wine regions, the risk of an excessive accumulation of acetic acid should not be underestimated.

To conclude, this work suggests that the simultaneous inoculum of yeast and bacteria in must with a critical chemical composition may be a promising alternative to traditional oenological approaches. However, careful control of the chemical and microbiological composition of must remain the main way of obtaining reliable microbiological activity in the first stages of winemaking.

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## References

Abrahamse CE, Bartowsky EJ (2012) Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. World J Microbiol Biotechnol 28(1):255–265

Alexandre H, Costello PJ, Remize F, Guzzo J, Guilloux-Benatier M (2004) *Saccharomyces cerevisiae* - *Oenococcus oeni* interactions in wine: current knowledge and perspectives. Int J Food Microbiol 93(2):141–154

Barata A, Malfeito-Ferreira M, Loureiro V (2012) The microbial ecology of wine grape berries. Int J Food Microbiol 153(3):243–59

Bell SJ, Henschke PA (2005) Implications of nitrogen nutrition for grapes, fermentation and wine. Aust J Grape Wine Res 11 (3):242–295

Comitini F, Ferretti R, Clementi F, Mannazzu I, Ciani M (2005) Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound(s) active against *Oenococcus oeni*. J Appl Microbiol 99(1):105–111

Guerzoni ME, Sinigaglia M, Gardini F, Ferruzzi M, Torriani S (1995) Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos* - Modelling of the malolactic activity. Am J Enol Vitic 46(3):368–374

Guzzon R, Poznanski E, Conterno L, Vagnoli P, Krieger-Weber S, Cavazza A (2009) Selection of a new highly resistant strain for malolactic fermentation under difficult conditions. S Afr J Enol Vitic 30(2):133–141

Guzzon R, Widmann G, Settanni L, Malacarne M, Francesca N, Larcher R (2011) Evolution of yeast populations during different biodynamic winemaking processes. S Afr J Enol Vitic 32(2):242–250

Henick-Kling T, Park YH (1994) Considerations for the use of yeast and bacterial starter cultures: $SO_2$ and timing of inoculation. Am J Enol Vitic 45:464–469

Jussier D, Dube Morneau A, Mira de Orduna R (2006) Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate chardonnay. Appl Env Microbiol 72(1):221–227

Liu SQ (2002) Malolactic fermentation in wine - beyond deacidification. J Appl Microbiol 92(4):589–601

Lonvaud-Funel A (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. Anton Leeuw Int J Gen Mol Microbiol 76(1–4):317–331

Mendoza Lucia M, de Nadra M, Maria C, Farias Marta E (2010) Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance partial characterization of inhibitory compounds produced by yeasts. Food Res Int 43(8):1990–1998

OIV (2012) Resolution OENO 17/2003 “Bacteriological control”. International Oenological Codex. OIV, Parigi (F)

Rodriguez SB, Thornton RJ (2008) Use of flow cytometry with fluorescent antibodies in real-time monitoring of simultaneously inoculated alcoholic-malolactic fermentation of Chardonnay. Lett Appl Microbiol 46(1):38–42

Rosi I, Fia G, Canuti V (2003) Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of *Oenococcus oeni*. Aust J Grape Wine Res 9:194–199

Wells A, Osborne JP (2011) Production of $SO_2$ binding compounds and $SO_2$ by *Saccharomyces* during alcoholic fermentation and the impact on malolactic fermentation. S Afr J Enol Vitic 32(2):267–279

Zapparoli G, Torriani S, Malacrino P, Suzzi G, Dellaglio F (2003) Interactions between *Saccharomyces* and *Oenococcus oeni* strains from Amarone wine affect malolactic fermentation and wine composition. Vitis 42(2):107–108

Zapparoli G, Tosi E, Azzolini M, Vagnoli P, Krieger S (2009) Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. S Afr J Enol Vitic 30(1):49–55