



Identification of the potential inhibitors of malolactic fermentation in wines

Renata Vieira da MOTA^{1*}, Cintia Lacerda RAMOS², Isabela PEREGRINO¹, Neuza Mariko Aymoto HASSIMOTTO³, Eduardo PURGATTO³, Claudia Rita de SOUZA¹, Disney Ribeiro DIAS⁴, Murillo de Albuquerque REGINA¹

Abstract

This exploratory work aims to identify the potential inhibitors of lactic bacterial growth and to propose enological practices to guarantee the occurrence of spontaneous malolactic fermentation (MLF) in wines from traditional and double-pruning management harvests in southeast Brazil. One white wine from a summer harvest and one red wine from a winter harvest that failed to complete MLF were utilized as comparative models to identify inhibitor compounds to lactic bacteria. Wine composition, alcoholic-fermentation temperature and bacterial strain contribute to the success or failure of MLF. Temperatures below 12 °C during alcoholic fermentation decrease lactic bacterial metabolism and may impair the bacteria's growth after yeast cells lysis. A must pH below 3.2 in a summer harvest impairs bacterial growth, and the association of low pH with a free-SO₂ concentration above 10 mg L⁻¹ may inhibit MLF. For grapes with a high sugar content, harvested in the winter cycle, enologists should keep the alcohol content below 15% and control the alcoholic-fermentation temperature.

Keywords: *Vitis*; winemaking; lactic bacteria; malic acid; composition.

Practical Application: Wineries in the southeast region of Brazil have a busy post-harvest period, since they must attend to the demand of summer and winter harvests. Early and rapid MLF results in more efficient utilization of the tanks and, furthermore, reduces the risk of microbiological spoilage and allows for early commercialization of the wines.

1 Introduction

Malolactic fermentation plays an important role in reducing acidity and improving both the microbiological stability and the aroma profile of wines. The conditions required for the induction and appropriate course of MLF include initial temperatures of 20 °C to 25 °C, a free-SO₂ content below 10 mg L⁻¹, a total SO₂ concentration below 30 mg L⁻¹, a pH level between the range of 3.2 and 3.4, and nutrients, which are obtained from the sediment of yeast cells (Lasik, 2013). Spontaneous MLF, however, cannot be guaranteed due to the harsh environmental conditions present in wine. Malolactic fermentation may fail or occur many months after alcoholic fermentation is complete, which impairs the production processes and may cause wine-depreciation associated with the occurrence of spoilage or the production of toxic compounds (Iorizzo et al., 2016).

Growth studies with different strains of lactic bacteria in culture media indicated that temperature, acetaldehyde- and pyruvic acid-bound SO₂, low pH, high amounts of tannins, pesticide residue, high levels of ethanol and medium-chain fatty acids may impair bacterial growth and activity (Wells & Osborne, 2012; Lasik, 2013). The inoculation of resistant strains of lactic bacteria simultaneously with yeast or just after alcoholic fermentation

has been proposed as an alternative to reduce the duration of MLF (Suriano et al., 2015; Larena et al., 2016). However, using commercial strains to induce MLF is costly and not always successful; it depends on the geographical origin and adaptation to the winemaking conditions of each wine (Iorizzo et al., 2016).

Southeast Brazil emerged as a new fine-wine viticultural region due to the introduction of double-pruning management (Favero et al., 2011; Regina et al., 2011). Grapes from a winter harvest have higher levels of malic acid than those harvested in the summer (Mota et al., 2010), and MLF is an essential practice to guarantee the quality of the wine. Wineries in the southeast deal with two annual crops; therefore, rapid MLF is indispensable not only to guarantee the quality of the wine but also to optimize the utilization of tanks in the wineries.

As far as the authors know, there are no studies regarding the behavior of native strains of lactic bacteria from the vineyards of southeast Brazil. This initial exploratory work aims to identify the potential inhibitors of native lactic bacteria in traditional vinification (summer harvests) and winter wines (double-pruning management), and to suggest enological practices to ensure the occurrence of faster MLF.

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¹Núcleo Tecnológico EPAMIG Uva e Vinho, Caldas, MG, Brazil

²Departamento de Agronomia, Universidade Federal dos Vales do Jequitinhonha e Mucuri – UFVJM, Diamantina, MG, Brazil

³Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo – USP, São Paulo, SP, Brazil

⁴Departamento de Ciência dos Alimentos, Universidade Federal de Lavras – UFLA, Lavras, MG, Brazil

*Corresponding author: renata@epamigcaldas.gov.br

2.6 Wine composition

Physicochemical analyses consisted of alcohol, total titratable acidity (g L^{-1} tartaric acid), volatile acidity (g L^{-1} acetic acid), pH, sugars (g L^{-1} glucose), free and total SO_2 (mg L^{-1}), dry extract, and ashes (Amerine & Ough, 1980).

Total polyphenol indices (280 nm) were evaluated by spectrophotometry, and total flavanoid content by the Bate-Smith reaction (Ribéreau-Gayon et al., 2006). Total phenolics were measured using the Folin-Ciocalteu method (Amerine & Ough, 1980).

Phenolic compounds were quantified by both HPLC-DAD-MS (Shimadzu, Prominence, Japan) and an ion-trap MS model Esquire HCT (BrukerDaltonics, Germany) with an electrospray (ESI) mode. Mobile phase consisted of acetonitrile and a 0.5% aqueous solution of formic acid at 1 mL min^{-1} for 45 min in a Prodigy $5 \mu\text{m ODS}_3$ $250 \times 4.60 \text{ mm}$ column (Phenomenex Ltda, UK) at $25 \text{ }^\circ\text{C}$. Eluting compounds were detected by UV absorbance at 270 nm and 370 nm; thereafter the flux was reduced to 0.2 mL min^{-1} to pass through the ESI source. Positive-mode ESI ionization was applied for anthocyanins at 3,500 V, while a negative mode was applied for flavonols and phenolic acids at 3,000 V, both in a full 100 m/z to 1,000 m/z scan. Peaks were identified and quantified using an external standard calibration of quercetin and chlorogenic acid (Sigma Aldrich, EUA), and the results were expressed as the mg g^{-1} quercetin equivalent.

The presence of pesticide residues (cimoxanyl, phenamidone, dimetomorphe, metalaxyl, dithiocarbamate and cooper) as potential inhibitors of MLF was investigated in Chardonnay wines from Andradas and Caldas. AgroSafety, an external laboratory that is accredited by the Brazilian Department of Agriculture, performed the analyses.

Other potential inhibitors, such as acetaldehyde; decanoic and dodecanoic fatty acids; and pyruvic acid, were also investigated in Tempranillo (Vargem), Chardonnay (Caldas), Syrah (Itobi) and Chardonnay (Andradas) wines. The following external laboratories performed the analyses: Randon Laboratory (Caxias do Sul, Brazil), the Science and Food Quality Center at the Institute of Food Technology (ITAL, Campinas, Brazil), and the Food-Chemistry and Biochemistry Laboratory at the Faculty of Pharmacy, University of São Paulo (São Paulo, Brazil).

3 Results and discussion

As expected, spontaneous MLF was unpredictable. In the 20 wine samples that were investigated from different cultivars, vineyards and seasons, MLF lasted between 37 and 125 days and failed in two wines, namely Chardonnay (Caldas) and Tempranillo (Vargem). These two wines were used as models to identify the potential inhibitors of lactic bacteria.

There were no reports about direct influence of the temperature of alcoholic fermentation in MLF. Lasik (2013) notes that the appropriate conditions required for MLF induction include an initial temperature of between $20 \text{ }^\circ\text{C}$ and $25 \text{ }^\circ\text{C}$, falling to between $18 \text{ }^\circ\text{C}$ and $20 \text{ }^\circ\text{C}$ during the MLF process. Temperatures between $15 \text{ }^\circ\text{C}$ and $20 \text{ }^\circ\text{C}$ would stimulate MLF, while values above or below this range would reduce the population of active lactic bacteria.

Data presented in Figure 1 indicate that there is no clear correlation between temperature and the length of MLF.

Data from the same cultivar and viticultural region, however, show that a decrease in alcoholic-fermentation temperature increases the length of MLF (Figure 2).

Bokulich et al. (2013) observed that the microbial population correlates to specific climatic features, suggesting a link between a vineyard's environmental conditions and microbial patterns during wine fermentations. Therefore, the knowledge of native lactic bacteria from each viticultural region may contribute to the enhancement of MLF practices.

The indigenous lactic-bacteria population present in berries, must and wine were evaluated in the 2013 season in an MRS agar medium containing tomato juice. Lactic acid bacterial growth over $5 \times 10^4 \text{ FCU mL}^{-1}$ was observed in 63% of the berries, in 50% of the must samples and 14% of the wines after the

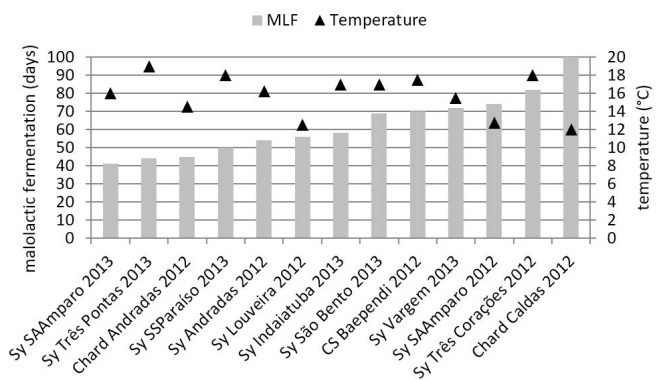


Figure 1. Lengths of MLF (days from running off to the complete degradation of malic acid) and the alcoholic-fermentation temperatures of wines from different regions in southeast Brazil that were harvested in summer or winter (double-pruning management). Sy = Syrah; Chard = Chardonnay and CS = Cabernet sauvignon.

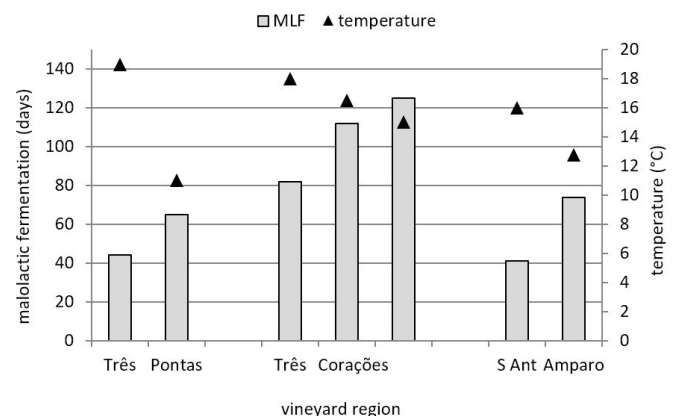


Figure 2. Lengths of MLF (days from running off to the complete degradation of malic acid) and alcoholic-fermentation temperatures of Syrah wines from Três Pontas, Três Corações and Santo Antônio do Amparo in the south of Minas Gerais State, Brazil, that were harvested in winter (double-pruning management).

run-off operation. No growth was observed in samples during MLF or in bottled wine; however, all the samples, apart from the Tempranillo (Vargem) and Chardonnay (Caldas) wines, displayed complete degradation of malic acid. Although not detected by spot plating, PCR-DGGE confirmed the presence of lactic bacteria in all samples with different bands according to the viticultural region (Figure 3).

Chardonnay (CH-CAL) and Bordô (FF-CAL) from vineyards in Caldas had similar bands, while Cabernet franc (CAB-FC), which is also from Caldas, had a different profile. Chardonnay and Bordô grapes were harvested in the summer season on December 20th, 2012 and January 8th, 2013, respectively, in high-humidity conditions (200 mm). Cabernet franc, although harvested in the summer season (February 20th, 2014), ripened in drier conditions (only 50 mm). Reguant et al. (2005) and Ruiz et al. (2010) mentioned the variable strains of lactic bacteria in different seasons. These authors observed high genotype variability in consecutive seasons in the same vineyard with the selection and adaptation of native strains.

The conditions found in wine, such as low pH values, high alcohol content and high SO₂ concentrations, compromise bacterial survival and growth (Pan et al., 2011; Iorizzo et al., 2016). Growth studies that were performed with isolated strains of lactic bacteria in media containing inhibitor compounds demonstrated that *Oenococcus* can grow at a pH < 3.5, while *Pediococcus* and *Lactobacillus* prefer a pH > 3.5. An ethanol concentration above 13% decreases the lactic-bacteria population with higher tolerances to *Oenococcus* (Edwards & Beelman, 1989). These findings are well known; however, most of the studies were performed in controlled conditions with pure bacterial strains and synthetic media.

To identify probable inhibitors, the composition of different wines from summer and winter harvesting was compared with the length of MLF (Table 1).

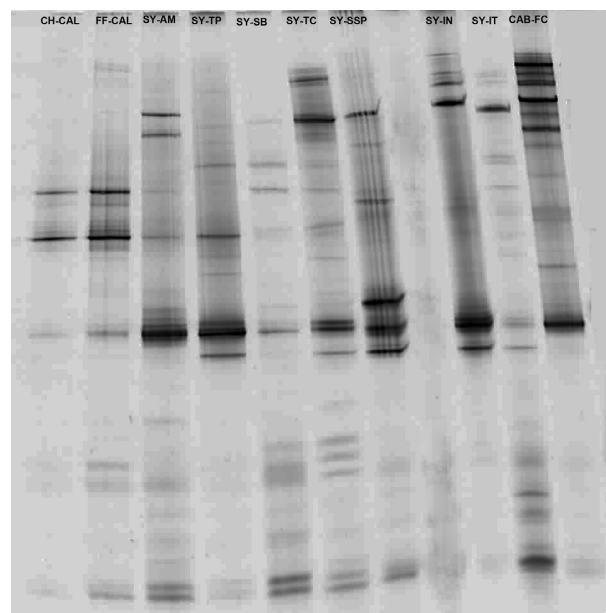


Figure 3. PCR-DGGE fragments of lactic bacteria found in berries of different cultivars and vineyards. CH-CAL = Chardonnay (Caldas); FF-CAL = Bordô (Caldas); SY-AM = Syrah (Santo Antônio do Amparo); SY-TP = Syrah (Três Pontas); SY-SB = Syrah (São Bento); SY-TC = Syrah (Três Corações); SY-SSP = Syrah (São Sebastião do Paraíso); SY-IN = Syrah (Indaiatuba); SY-IT = Syrah (Itaipava) and CAB-FC = Cabernet franc (Caldas).

Table 1. Lengths of MLF (days from running off to the complete degradation of malic acid) and chemical compositions of wines from different cultivars and viticultural regions in southeast Brazil that were harvested in the winter (double-pruning management) and summer (traditional) seasons.

Vineyard	Cultivar	Season*	MLF [†] (days)	Free SO ₂ (mg L ⁻¹)	pH	Sugars (g L ⁻¹)	Alcohol (%)
Caldas	Bordô	2012S	37	34.4	3.28	2.66	12.32
Andradas	Chardonnay	2012S	45	12.8	3.19	0.94	11.65
Divinolândia	Chardonnay	2012S	45	20.8	3.50	0.94	11.58
Caldas	Chardonnay	2012S	nd	16.0	3.20	0.94	11.66
Três Corações	Syrah	2012W	43	28.8	3.78	1.80	12.00
Andradas	Syrah	2012W	54	17.6	4.09	3.80	12.00
Louveira	Syrah	2012W	56	12.8	3.94	2.46	13.65
Baependi	Cab.sauvig	2012W	70	14.4	3.82	2.06	14.60
SAAmparo	Syrah	2012W	74	12.0	3.86	3.86	15.60
Três Corações	Syrah	2012W	82	9.6	3.92	8.80	15.00
SAAmparo	Syrah	2013W	41	28.8	3.64	2.46	14.00
Três Pontas	Syrah	2013W	44	28.0	4.21	3.92	14.50
SSParaíso	Syrah	2013W	50	20.8	3.92	2.46	14.00
Indaiatuba	Syrah	2013W	58	24.8	3.79	1.86	13.00
São Bento	Syrah	2013W	69	24.0	4.01	2.26	14.00
Vargem	Syrah	2013W	72	24.0	3.89	3.72	13.50
Vargem	Tempranillo	2013W	nd	15.5	4.05	4.19	16.00
Itobi	Syrah	2014W	56	19.2	3.84	2.80	15.20

*S = summer harvest; W = winter harvest; †nd = MLF failure.

There is a clear correlation between the length of MLF, the potential inhibitors and the season. Alcohol strength plays an important role in reducing lactic bacterial activity. Summer wines with alcohol contents < 12% completed MLF in 45 days even at a pH < 3.2.

Lasik (2013) mentioned that an alcohol concentration > 8% reduces bacterial growth but not bacterial activity, while a free-SO₂ concentration of 15 mg L⁻¹ and a pH < 3.5 impair bacterial activity. Malolactic fermentation of Chardonnay wines from Caldas failed in the 2012 summer season; however, the wine compositions indicated lower free-SO₂ and alcohol contents than Bordô wines from the same region and season. Red wines are fermented at higher temperatures, which may have contributed to bacterial growth and the success of MLF in Bordô wines. Comparing only Chardonnay wines, the high pH content of the Divinolândia sample counterweighted the high levels of free SO₂, and malic acid was degraded within 45 days. In the Caldas sample, however, the free-SO₂ content over 15 mg L⁻¹, associated with low pH and low temperature, may have impaired lactic bacterial growth and activity.

The evaluation of lactic bacterial behavior in wine is difficult due to the complex composition of the wine. Compounds such as acetaldehyde and medium-chain fatty acids released by yeasts may impair lactic bacterial growth and reduce the bacteria's activity especially when associated with alcohol, a low pH and a high SO₂ content (Carreté et al., 2002; Lasik, 2013). In culture media similar to wine, Wells & Osborne (2012) observed that acetaldehyde concentrations over 5 mg L⁻¹ and 10 mg L⁻¹ of pyruvic acid inhibited *Oenococcus oeni* at pH 3.50, while at pH 3.70, the concentrations had to be increased to 10 mg L⁻¹ for both compounds to have the same effect. Phenolic compounds may contribute to the activation or inhibition of bacterial growth depending on their structure, concentration or bacterial strain (García-Ruiz et al., 2008; Lasik, 2013). Pesticide residues are also mentioned as inhibitors of malic-acid degradation, and the presence of copper or dichlofluanid may impair MLF (Cabras et al., 1999; Carreté et al., 2002).

The presence of these potential inhibitors was searched in Chardonnay wines from Caldas and Andradas because of their similar compositions in alcohol, sugar and pH content. Piruvic acid was not detected in both samples, and the acetaldehyde concentration was higher in the Andradas samples (49.7 mg L⁻¹) than the Caldas samples (29 mg L⁻¹). These values are much higher than those mentioned by Wells & Osborne (2012) as inhibitors of lactic bacterial activity, but not enough to inhibit MLF in wines from the Andradas sample. White wines have low phenolic compounds, since there is no maceration step. Derived compounds of hidroxicinamic acids and catechin were higher in the Andradas sample than the Caldas sample: 82.5 µg mL⁻¹ and 60.64 µg mL⁻¹ of chlorogenic acid and 15.86 µg mL⁻¹ and 14.21 µg mL⁻¹ of catechin, respectively. The phenolic concentration was much lower than the toxic limit of 500 mg L⁻¹ mentioned by García-Ruiz et al. (2008). The lipid composition in both wines was below 0.10 g 100 mL⁻¹, which impaired the evaluation of fatty acids. Concerning pesticide residues, only dithiocarbamate was detected at 0.499 mg kg⁻¹ and 0.595 mg kg⁻¹ in the Caldas and Andradas samples, respectively. Therefore, none of the

potential inhibitors mentioned in the literature could explain the MLF failure in the Caldas samples.

However, alcoholic fermentation of the Caldas wine proceeded at 12 °C, while the Andradas tank fermented at 14.5 °C. A low temperature associated with a low pH and a free-SO₂ concentration above 15 mg L⁻¹ may be responsible for the observed inhibition of MLF. Reguant et al. (2005) relate a minimum amount of 10⁵ FCU mL⁻¹ *Oenococcus oeni* to the development of MLF. At the beginning of alcoholic fermentation, the lactic-bacteria population represents approximately 10² FCU mL⁻¹; this value increases at the end of alcoholic fermentation. Authors observed MLF failure in trials with bacterial growth no higher than 2 × 10³ FCU mL⁻¹ at the end of alcoholic fermentation.

Grapevines harvested in the winter season accumulate more sugar, anthocyanins and total phenolic compounds (Favero et al., 2011). Yeast fermentation normally occurred at temperatures below 20 °C, and the alcohol content exceeded 14% in most of the samples. Under these conditions, apart from the high pH of the must (above 3.60), MLF is unpredictable (Table 1). A high free-SO₂ concentration (28.8 mg L⁻¹) did not inhibit MLF; however, wines from the same cultivar and vineyard displayed a positive correlation between the length of MLF and the alcohol content.

Ramos (2013) observed the effect of glucose (2 to 10 g L⁻¹), ethanol (10% to 15%) and SO₂ (0-40 mg L⁻¹) in the inhibition of the following lactic bacteria in synthetic media: *Leuconostoc* spp., *Lactobacillus* spp., and *Oenococcus oeni*. There was no inhibitory effect of glucose or SO₂; however, an alcohol content over 13% inhibited *O.oeni*, and above 14%, all the strains were inhibited.

The Tempranillo wine sample displayed three inhibitor compounds at high concentrations: glucose (4.19 g L⁻¹), alcohol (16%) and acetaldehyde (62.5 mg L⁻¹). While the alcohol content of Syrah wine from Itobi was high (15%), the glucose and acetaldehyde contents were lower: 2.80 g L⁻¹ and 15.8 mg L⁻¹, respectively. The high glucose content (8.80 g L⁻¹) in Syrah wines from Três Corações also delayed MLF (82 days) compared to those from Santo Antônio do Amparo (3.86 g L⁻¹ and 74 days) and Itobi (2.80 g L⁻¹ and 56 days).

Wine is a complex medium for microbial growth, and composition, fermentation temperature, and microbial strain may contribute to either a delay in or an impairment of MLF.

Vineyards from different viticultural regions are a challenge for wineries; knowledge regarding both must composition and the temperature control of alcoholic fermentation should be taken into account to decrease the latent phase of lactic bacteria.

4 Conclusions

Native microflora adapts to the geographical origin.

Alcoholic-fermentation temperatures under 12 °C decrease the metabolism of lactic bacteria and, when associated with a pH below 3.2 and free SO₂ above 15 mg L⁻¹, may impair their growth and activity after the lysis of yeast cells.

Winter wines may experience delays in MLF due to high alcohol and residual sugar contents.

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