Simultaneous Inoculation of Malbec (*Vitis vinifera*) Musts with Yeast and Bacteria: Effects on Fermentation Performance, Sensory and Sanitary Attributes of Wines

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Summary

Malolactic fermentation has important consequences for the quality of wine. The present study analyzes the effect of timing of inoculation with bacteria on the performance of alcoholic and malolactic fermentation in Malbec musts from a warm climate wine growing region during two consecutive vintages, with a specific consideration for the sensory and sanitary wine parameters. In this study traditional vinification, where malolactic bacteria were inoculated after the completion of alcoholic fermentation, was compared with a simultaneous inoculation with yeast and bacteria. The experiment was made on pilot scale which closely reproduces winery conditions. The obtained results point out that simultaneous inoculation resulted in a reduction of total time of fermentation and a better control of the malolactic fermentation due to the early dominance of a selected bacterial strain. There were no negative effects on yeast population and alcoholic fermentation performance observed. Differences between the wine sensory attributes were no significant or they were in favour of simultaneous inoculations. No statistical differences in the biogenic amine levels between different timings of inoculation were found.

Key words: malolactic fermentation, *Oenococcus oeni*, *Saccharomyces cerevisiae*, inoculation timing, coinoculation, Malbec wine

Introduction

Malolactic fermentation (MLF), the enzymatic decarboxylation of L-malic acid to L-lactic acid, is an important secondary fermentation carried out by lactic acid bacteria (LAB), with *Oenococcus oeni* being the most suitable species to drive this fermentation (*1,2*). Malolactic fermentation has important consequences for the quality of wine, increasing microbiological stability and enhancing the flavour, and thus oenologists' efforts are directed towards a better control of how and when this fermentation takes place (*1,3,4*). Success of MLF depends on several physical, chemical and biological factors widely described, including ethanol concentration, pH, the presence of SO_2 and of other antimicrobial compounds, or nutrient depletion by yeasts (*1,4–10*). The introduction of *O. oeni* starter cultures for direct inoculation in wine has greatly simplified the management of this fermentation (*3,11*). Various studies have been carried out to determine the best time for bacterial inoculation (*3,4,12–14*).

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Some of them suggest a simultaneous fermentation (alcoholic and malolactic) by adding at the same time selected bacteria and yeast strains into the grape must. Early inoculation was extensively assayed in the 1980s along with the development of commercial bacterial starter cultures with little encouraging results (*15–17*). To avoid potential problems associated with simultaneous inoculation, the addition of bacterial cultures after the completion of alcoholic fermentation (AF) was massively adopted (*18–20*). Unfortunately, sequential inoculation with bacterial starter cultures has not always guaranteed successful induction of MLF, and problems have been reported from the industry (*2,21,22*). Consequently, this subject has recently been retaken and new studies have been done to determine the best time for inoculation with bacterial starter culture (*3,4,10,13,14*).

It was postulated that simultaneous inoculation of must with yeast and bacteria would allow a more successful induction of MLF due to a gradual adaptation of bacteria to increasing alcohol concentrations and to the benefit from higher nutrient availability present in grape musts, compared to the conditions at the end of AF (*2,4*). Several yeast and bacterial strain combinations have been used to study simultaneous AF and MLF with variable results (*3,4,10,12–14,23,24*). Most of the studies have been conducted under laboratory conditions which do not always allow a scale-up to winery conditions and successful prediction of a MLF in winery environment (*4,12,13,23*). Especially when studies are done in pasteurized juices or white musts, sanitary conditions are very different from the winery conditions, which could have an impact on the implantation of the starter culture, and consequently on the final sensory quality of the wines (*10,14*).

In Argentina, LAB inoculation after the completion of AF is still the practice most widely used, and wines are kept under ideal conditions in order to have the MLF successfully induced, but most of these conditions also increase the risk of spoilage by undesired microorganisms (*14*). Argentina belongs to the wine growing regions with warm climate, where musts and wines typically have pH values above 3.5, conditions favourable for the growth of lactobacilli and pediococci, and the prevention of spontaneous MLF is difficult. Especially red wines with high pH are most suitable for undergoing spontaneous MLF, mainly because of their potential to support the growth of spoilage bacteria with unpredictable impact on wine safety and quality (*e.g.* biogenic amine production, stuck fermentation) (*25*).

However, despite a possible advantage of simultaneous inoculation with yeast and bacteria in order to achieve an early dominance of the selected bacterial strain, this technique is little considered by Argentinean wineries, because oenologists fear the depreciation of wine quality due to the activity of LAB in musts, mainly related to an excessive acetic acid production as a consequence of heterofermentative sugar metabolism (C. Catania, personal communication).

This study was done to investigate the risk and/or the advantage of early bacterial inoculation under the Argentinean climate conditions for winemaking with special regard to security and wine quality. Studies were done in Malbec musts during two consecutive years and

traditional vinifications with LAB inoculated after completion of AF were compared with a simultaneous inoculation, where yeasts and bacteria were inoculated concurrently. Two commercial wine yeast strains and one malolactic bacterium strain were chosen for this study. The selection was done with respect to good compatibility between the microorganisms, but also with respect to the starter cultures commonly used in our region to ferment red wines. Malbec was chosen because it is the most important grape cultivar in Argentina, and it represents a typical example of a red wine production by vinification with malolactic fermentation.

Materials and Methods

Microorganisms

Two commercial active dry *Saccharomyces cerevisiae* strains (INTA MZA and ICV D80, Lallemand Inc., Canada) were used to ferment the musts. The yeast strain INTA MZA had previously been isolated from Mendoza vineyards (Argentina) and selected for Malbec fermentation in an earlier work from the laboratory (*26,27*). The freeze dried commercial *O. oeni* strain (Uvaferm Alpha, Lallemand Inc., Canada) was selected because of its good capacity to induce MLF in Malbec wines. Microorganisms were rehydrated according to the manufacturer's specifications.

Experimental design

This study was designed to closely reproduce, in pilot scale (1 hL), the real winery conditions and then to conduct the sensory analyses of the obtained wines. Experimental design included randomized complete block design with factorial arrangement of treatments. Two factors were included: yeast strain and bacterial inoculation timing. Each must was considered as one block with 4 treatments. Treatments (in triplicate) consisted in combining AF by *S. cerevisiae* Lalvin MZA or ICV D80 strains with MLF by *O. oeni* Uvaferm Alpha strain. Malolactic bacteria were inoculated either together with yeast (simultaneous treatment: SIM) or after the completion of AF (sequential treatment: SEQ).

Fermentations

Malbec grapes (*Vitis vinifera*) from Pedriel and Drummond, two important viticultural areas in Mendoza (Argentina), were hand harvested during 2004 (musts A and B_{04}) and 2005 (musts B_{05} and C) vintages. Grapes from vineyard B were collected in both sampling years, so they were identified as B_{04} and B_{05}. Musts A and C were harvested from two different vineyards during different vintages to respect the variation of the 'terroir' influence. The chemical composition of the musts is shown in Table 1. Grapes were crushed before 50 mg/L of total SO_2 and 30 g/hL of yeast nutrient (Fermaid K, Lallemand Inc., Canada) were added. Twelve 1-hL stainless steel tanks were filled with each must and yeast strains were added. Malolactic bacteria were inoculated either 12 h after yeast inoculation (SIM), when total and free SO_2 were 27 and 12 mg/L, respectively, or after the completion of AF (SEQ). The microorganisms were inoculated according to manufacturer's recommendations to

Table 1. Chemical composition of Malbec musts from Mendoza, Argentina

Parameters	2004 vintage		2005 vintage	
	Must A	Must B_{04}	Must C	Must B_{05}
pH	3.20±0.02	3.60±0.02	3.10±0.02	3.60±0.01
γ(assimilable nitrogen)/(mg/L)	90±2	199±3	112±4	126±3
γ(reducing sugars)/(g/L)	237.80±2.35	222.20±1.89	245.62±1.12	273.47±2.22
γ(titratable acidity)/(g/L)	7.5±0.3	5.9±0.1	8.3±0.2	7.2±0.2
γ(L-malic acid)/(g/L)	1.37±0.10	2.46±0.12	1.51±0.26	2.67±0.40

Data are means of triplicates±standard deviation

give cell counts of 10^6 CFU/mL for both commercial yeast and malolactic bacterial strains. Fermentations were done following the standard protocols for Malbec wine vinification. This includes a maceration period for 15–20 days at 24–26 °C and at the end of AF, the wines were settled, racked and then kept at 22 °C until the end of MLF. After MLF, the wines were physically and chemically stabilized, bottled without filtration and stored at 18 °C. The wines were considered to be dry and AF concluded when the reducing sugar level was below 2 g/L.

Yeast and bacteria enumeration

During fermentation, viable yeast and bacterial cell counts were determined using commercial culture media. Yeasts were enumerated by inoculating 0.1 mL of adequate dilutions (in 0.1 % peptone, if necessary) onto the plates of WL nutrient agar (Oxoid Ltd., UK) with the addition of chloramphenicol (50 μg/L) and erythromycin (70 μg/L). Plates were incubated at 28 °C during two days for colony development. Bacteria were enumerated by spread plating on MRS agar (Britania Labs S.A., Argentina) with the addition of tomato juice (15 % by volume) and natamycin (to suppress yeast growth) with a final concentration of 500 mg/L (Fermi-Stop, Rodán S.R.L., Argentina). Plates were incubated at 30 °C for 10 days under low oxygen conditions (GENbag Microaer, bioMérieux S.A., France).

Analytical methods

The progress of AF was monitored daily for decline in total soluble solids using the gravimetric method for density (*28*). Malolactic fermentation was monitored following L-malic acid degradation by enzymatic determination (Boehringer Inc., Germany). Initial free α-amino nitrogen in must was calculated by formol titration (*29*). Titratable and volatile acidity, pH, ethanol, sugar and SO_2 concentrations were determined by standard methods (*30*). Biogenic amines (histamine, tyramine, putrescine, cadaverine and phenylethylamine) were determined in the finished wines by HPLC (*31*).

Sensory analyses

Sensory analyses were performed to investigate the differences among treatments, always comparing within the same must and the same harvest year. Sensory analyses were done 4 months after bottling by 15 trained panelists from the Stable Sensorial Analysis Group be-

longing to Oenological Research Center from the National Institute of Agricultural Technology (INTA), Argentina. Panelists in this group are continuously trained in monthly sessions and at the Annual Sensory Descriptive Training Course. Wines were equilibrated at room temperature (22 °C) and 50-mL samples were poured into wine glasses ISO 3591 (*32*). Two consecutive sessions were done. Each tray contained four test wines from one must including all treatments. Replicates were done separately on different days. Sensory descriptive analysis was performed working on anonymous samples. For the sensory descriptive analysis, wine descriptors were selected by the panellists during the first session to allow discrimination between treatments (SIM and SEQ). At the following session, intensity of each descriptor was measured by means of a structured scale from 1 to 5 (*33,34*). The average rating of all panellists for each wine and each descriptor was obtained.

Statistical analyses

Statistical data analyses were done by analysis of variance (ANOVA) followed by LSD Fisher test to evaluate the significance of variation among mean values. All significance tests were conducted at p≤0.05 levels.

Results

AF finished in all musts between 10 and 14 days after yeast inoculation independently of the timing of LAB inoculations. No differences in duration of AF were observed between the two bacteria inoculation times in any of the yeast/bacteria pairings evaluated during both years studied (Table 2).

In wines with simultaneous treatments, MLF was completed in 10 to 26 days (Table 2), and the pH increased by 0.2–0.4 units compared to the initial values (Table 3). The only exception to this trend was must C simultaneously inoculated with INTA MZA yeast strain, where bacteria failed to reduce L-malic acid below 0.1 g/L, the level generally recognized as the threshold for a complete MLF (*12*). After 35 days, 0.38 g/L of L-malic acid still remained in wines of this treatment (Table 3). Treatments with simultaneous inoculation showed a reduced total fermentation time (AF+MLF) compared to sequential inoculations. However, the length of MLF itself in simultaneous treatments was similar or longer than their respective sequential treatments (Table 2).

Table 2. Time required to reach sugar concentrations below 2 g/L and L-malic acid concentrations below 0.1 g/L in Malbec musts fermented with two *S. cerevisiae* strains (INTA MZA and ICV D80) and with *O. oeni* strain (Uvaferm Alpha), comparing two inoculation times (SIM: simultaneous, SEQ: sequential)

Vintage year	Must	Yeast strain	Inoculation time	Time to complete AF/day	MLF time after LAB inoculation/day	Total fermentation time (AF+MLF)/day
2004	A	INTA MZA	SIM	14	10	14
			SEQ	14	14	28
		ICV D80	SIM	14	14	14
			SEQ	14	14	28
	B$_{04}$	INTA MZA	SIM	14	14	14
			SEQ	14	8	22
		ICV D80	SIM	14	26	26
			SEQ	14	21	35
2005	C	INTA MZA	SIM	12	NR	NR
			SEQ	12	14	26
		ICV D80	SIM	12	26	26
			SEQ	12	21	33
	B$_{05}$	INTA MZA	SIM	10	14	14
			SEQ	10	12	22
		ICV D80	SIM	10	22	22
			SEQ	10	26	36

AF: alcoholic fermentation; MLF: malolactic fermentation; LAB: lactic acid bacteria; NR: not reached
Values are means of three independent fermentation tank replicates

Table 3. Average of chemical analysis data±standard deviation recorded after MLF in Malbec wines fermented with two *S. cerevisiae* strains (INTA MZA and ICV D80) and *O. oeni* strain (Uvaferm Alpha), comparing two inoculation times (SIM: simultaneous, SEQ: sequential)

Vintage year	Malbec must	Yeast strain	Inoculation time	pH	γ(volatile acidity)/g/L	γ(L-malic acid)/g/L
2004	A	INTA MZA	SIM	$(3.42\pm0.01)^a$	$(0.41\pm0.04)^a$	≤0.04
			SEQ	$(3.40\pm0.04)^a$	$(0.40\pm0.03)^a$	≤0.04
		ICV D80	SIM	$(3.37\pm0.02)^a$	$(0.59\pm0.03)^a$	≤0.04
			SEQ	$(3.40\pm0.02)^a$	$(0.40\pm0.02)^b$	≤0.04
	B$_{04}$	INTA MZA	SIM	$(3.87\pm0.03)^a$	$(0.42\pm0.03)^a$	≤0.04
			SEQ	$(3.92\pm0.05)^a$	$(0.30\pm0.01)^b$	≤0.04
		ICV D80	SIM	$(3.86\pm0.03)^a$	$(0.41\pm0.02)^a$	≤0.04
			SEQ	$(3.89\pm0.03)^a$	$(0.32\pm0.02)^b$	≤0.04
2005	C	INTA MZA	SIM	$(3.45\pm0.01)^a$	$(0.41\pm0.01)^a$	$(0.38\pm0.07)^a$
			SEQ	$(3.47\pm0.01)^a$	$(0.40\pm0.01)^a$	$(0.06\pm0.04)^b$
		ICV D80	SIM	$(3.50\pm0.01)^a$	$(0.50\pm0.01)^a$	≤0.04
			SEQ	$(3.53\pm0.01)^a$	$(0.48\pm0.04)^a$	≤0.04
	B$_{05}$	INTA MZA	SIM	$(4.01\pm0.02)^a$	$(0.60\pm0.03)^a$	≤0.04
			SEQ	$(4.01\pm0.11)^a$	$(0.57\pm0.02)^a$	≤0.04
		ICV D80	SIM	$(4.00\pm0.08)^a$	$(0.49\pm0.16)^a$	≤0.04
			SEQ	$(4.04\pm0.05)^a$	$(0.36\pm0.02)^b$	≤0.04

Data are means of triplicates. Number with different letters within a same column from each yeast/bacteria pair differ at p<0.05 level (Fisher´s LSD test)

Viable yeast population was not significantly influenced by *O. oeni* addition in simultaneous treatments during active fermentation (Figs. 1–4). Considerable reduction of yeast populations was observed in simultaneous treatments with ICV D80 once AF was finished in two of the musts, compared to their respective sequential treatments (Figs. 1c and 4c).

Viable population of LAB did not decline after simultaneous inoculation. In the majority of treatments, *O. oeni* populations stayed constant or increased and reached peak populations above 10^6 CFU/mL (Figs. 1a and c, 2a and c, 3c, and 4a and c). Once more, fermentation from must C inoculated with INTA MZA/Uvaferm Alpha pair

represents the exception to this trend. LAB population decreased during the first days after inoculation, followed by a slow increase of bacterial population and consequently a slow rate of L-malic acid degradation. Malolactic fermentation failed to go to dryness during the evaluated time (35 days), as was reported above (Fig. 3a).
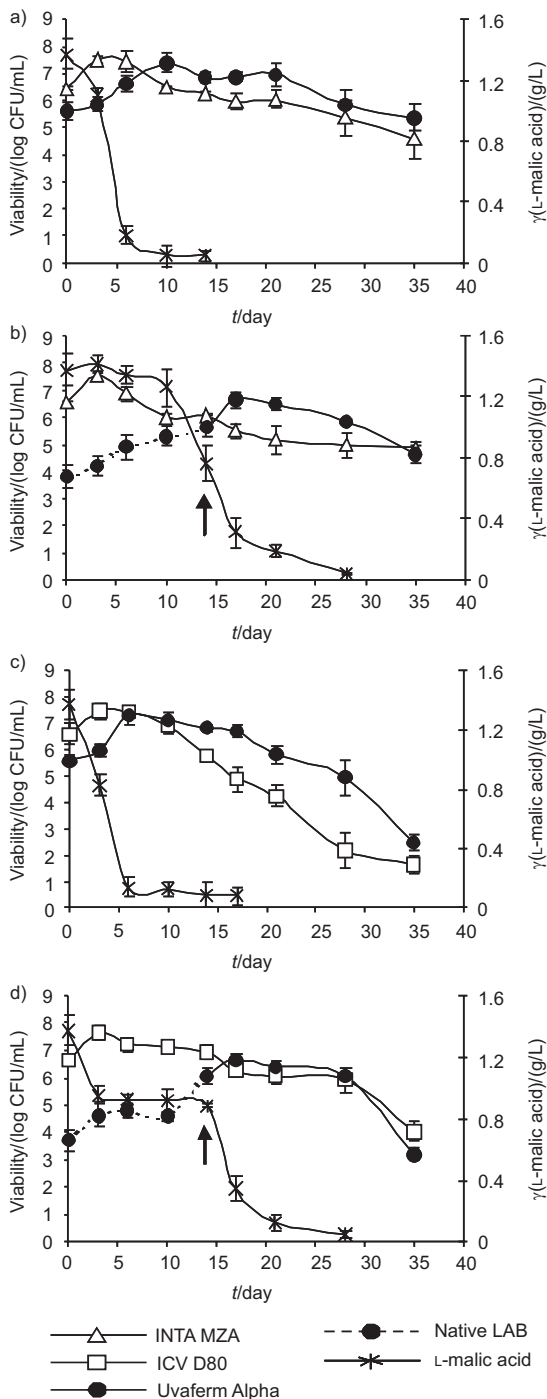
Fig. 1. Must A: kinetics of L-malic acid degradation and viability of *Saccharomyces cerevisiae* strains (INTA MZA and ICV D80) and *Oenococcus oeni* strain (Uvaferm Alpha) in simultaneous (a and c) and sequential (b and d) inoculations. Arrows indicate bacterial inoculation in sequential treatments. Error bars represent standard error
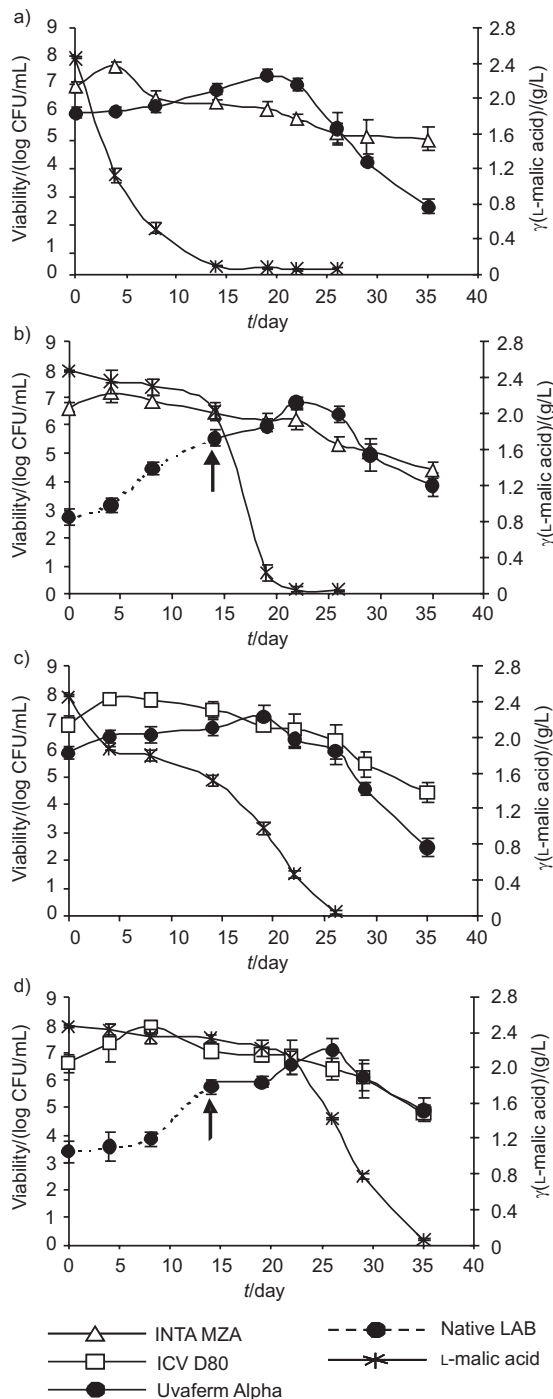
Fig. 2. Must B_{04}: kinetics of L-malic acid degradation and viability of *Saccharomyces cerevisiae* strains (INTA MZA and ICV D80) and *Oenococcus oeni* strain (Uvaferm Alpha) in simultaneous (a and c) and sequential (b and d) inoculations. Arrows indicate bacterial inoculation in sequential treatments. Error bars represent standard error

Bacterial viabilities were highly similar among simultaneous treatments, while different rates of L-malic acid degradation were recorded. In 5 of the 7 simultaneous treatments, which had undergone MLF, 80 % of the total L-malic acid were transformed during the first week (Figs. 1a and c, 2a, and 4a and c), while in the other two treatments, a slower MLF was observed (Figs.

2c and 3c). In addition, slow rates of L-malic acid degradation were also observed in their respective sequential inoculation treatments (Figs. 2d and 3d). This fact could suggest a certain incompatibility between yeast and bacteria in this pair in some must conditions. The addition of bacteria at the end of AF caused a change in the rates of L-malic acid degradation, which was different in every
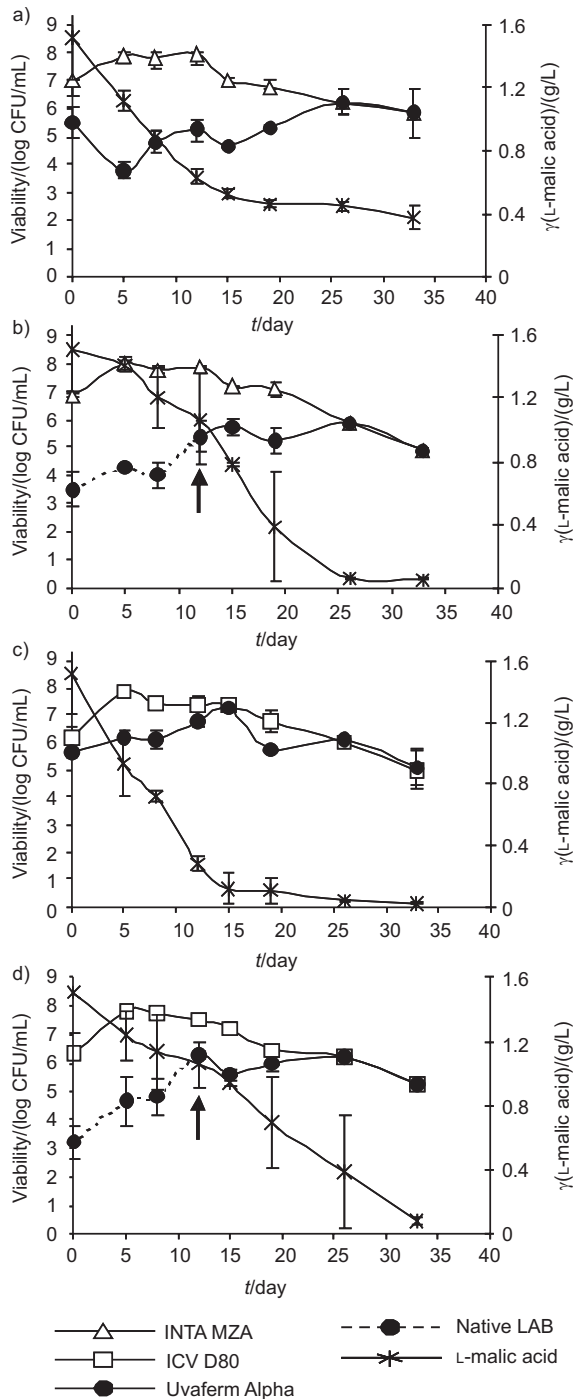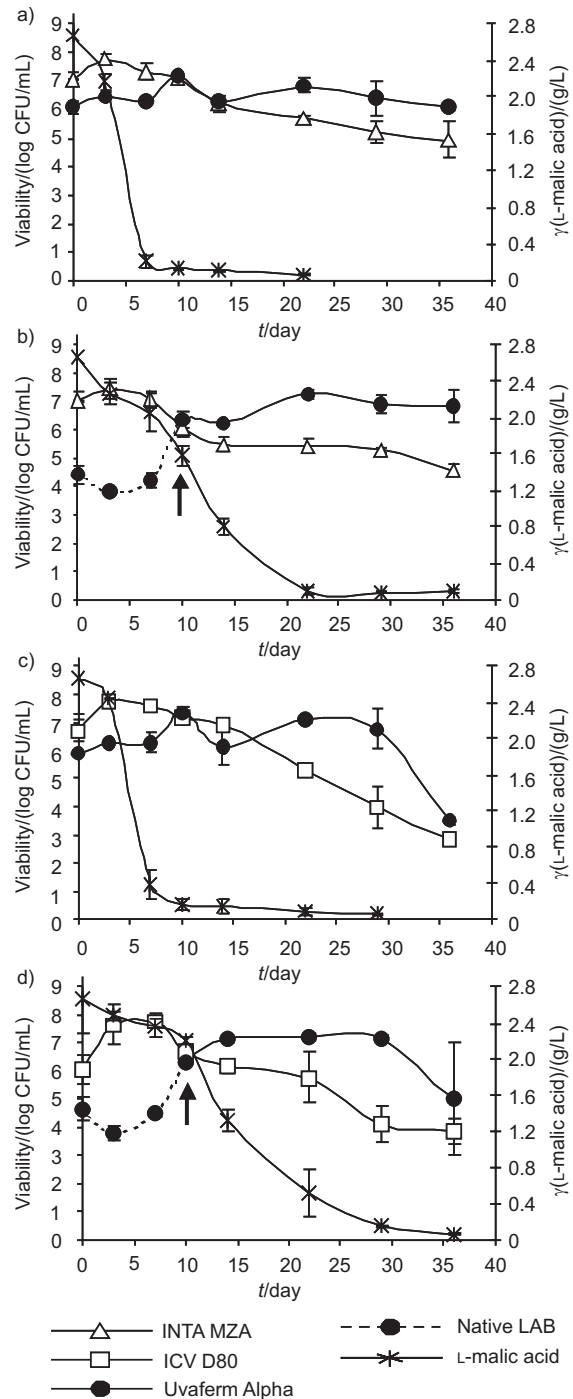

Fig. 3. Must C: kinetics of L-malic acid degradation and viability of *Saccharomyces cerevisiae* strains (INTA MZA and ICV D80) and *Oenococcus oeni* strain (Uvaferm Alpha) in simultaneous (a and c) and sequential (b and d) inoculations. Arrows indicate bacterial inoculation in sequential treatments. Error bars represent standard error

Fig. 4. Must B$_{05}$: kinetics of L-malic acid degradation and viability of *Saccharomyces cerevisiae* strains (INTA MZA and ICV D80) and *Oenococcus oeni* strain (Uvaferm Alpha) in simultaneous (a and c) and sequential (b and d) inoculations. Arrows indicate bacterial inoculation in sequential treatments. Error bars represent standard error

must. In 5 of 8 sequential treatments, 30–45 % of L-malic acid had already been metabolized at inoculation time (Figs. 1b and d, 3b and d and 4b).

Chemicoanalytical data of the finished wines after MLF are shown in Table 3. In all wines undergoing MLF, residual L-malic acid concentrations were below 0.1 g/L measured by the enzymatic test, always taking into account the exception in the trial with simultaneous inoculation of INTA MZA/Uvaferm Alpha pair in must C, already mentioned. Acetic acid concentrations were statistically higher in some treatments with simultaneous inoculation compared to the respective sequential inoculation. In spite of this, all measured values were well within the range normally found in Mendoza red wines and volatile acidity never exceeded 0.60 g/L (Table 3). No differences in ethanol and total and free SO_2 concentrations were recorded among treatments within the respective must (data not shown).

Biogenic amines (histamine, tyramine, putrescine, cadaverine and phenylethylamine), frequently found in wines with spontaneous MLF, were determined in the finished wines obtained from the 2005 vintage. Putrescine was the most abundant amine in all analyzed wines, in concentrations ranging from 5 to 9 mg/L (Table 4). Histamine was only detected at low concentrations in wines obtained from must B_{05} without distinction among the inoculation treatments, and levels were below the legal maximum for histamine in wine (*35*). Tyramine, cadaverine and phenylethylamine concentrations were under the detection limit of the method (0.05 mg/L for tyramine and cadaverine; and 0.1 mg/L for phenylethylamine) (data not shown). No statistical differences in the biogenic amine levels between the timings of inoculation were found (Table 4).

Table 4. Average of biogenic amine analysis data and standard deviation from Malbec wines fermented with two *S. cerevisiae* strains (INTA MZA and ICV D80) and with *O. oeni* strain (Uvaferm Alpha), comparing two inoculation times (SIM: simultaneous, SEQ: sequential) during 2005 vintage

Malbec must	Yeast strain	Inoculation time	γ(histamine) mg/L	γ(putrescine) mg/L
C	INTA MZA	SIM	<0.05	$(5.69\pm0.20)^a$
		SEQ	<0.05	$(5.84\pm0.84)^a$
	ICV D80	SIM	<0.05	$(6.40\pm1.78)^a$
		SEQ	<0.05	$(9.25\pm2.40)^a$
B_{05}	INTA MZA	SIM	$(1.97\pm0.47)^a$	$(5.41\pm0.16)^a$
		SEQ	$(1.84\pm0.25)^a$	$(5.31\pm0.30)^a$
	ICV D80	SIM	$(1.51\pm0.04)^a$	$(6.63\pm1.35)^a$
		SEQ	$(1.53\pm0.27)^a$	$(7.19\pm8.08)^a$

Data are means of triplicates. Number with different letters within a same column from each yeast/bacteria pair differ at p<0.05 level (Fisher´s LSD test)

For the sensory descriptive analysis (SDA), wine descriptors associated with MLF and typical Malbec wine flavours were selected: fruity, spicy, lactic, aroma intensity, bitterness, sweetness, astringency, concentration, violet colour and global colour intensity. Sensory

evaluation of all wines from 2004 vintage revealed not statistically significant differences for any treatment and combination regardless of the yeast strains employed and timing of LAB inoculation, always comparing within the same must. Similar results were obtained with wines from must C (2005), while wines from must B_{05} fermented with the yeast strain INTA MZA showed significant differences in 3 of the 10 sensory descriptors selected. In this last case, wines with sequential inoculation showed significantly lower values in fruity flavour, violet colour and global colour intensity compared to their simultaneous treatment (Fig. 5).
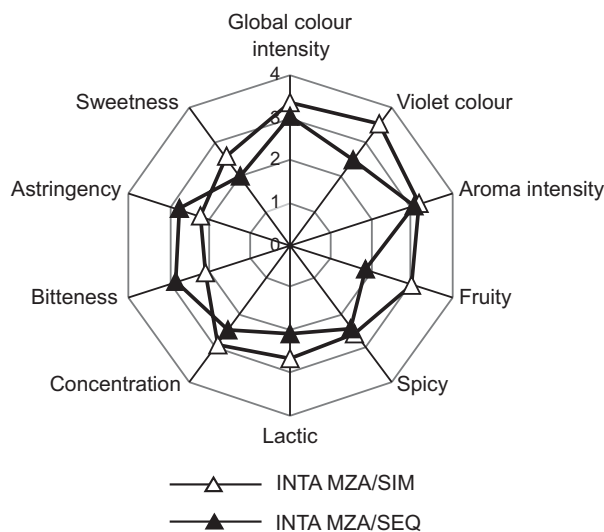

Fig. 5. Sensory descriptors of Malbec wines from must B_{05} fermented with *S. cerevisiae* strain (INTA MZA) and *Oenococcus oeni* strain (Uvaferm Alpha) in simultaneous (SIM) and sequential (SEQ) inoculations

Discussion

Malolactic fermentations of red wines with high pH are difficult to control, since wine conditions support the growth of native bacteria already during AF, which could have a negative impact on wine quality. Inoculation with selected bacterial cultures after AF does not always result in a dominance of the selected strain and the desired contribution. Simultaneous inoculation of LAB with the wine yeast will allow an early dominance of the selected strain and a better control over the outcome of MLF. Nevertheless, there is still the discussion of the risk to get excessive production of acetic acid due to the heterofermentative degradation of sugar when bacteria are inoculated in the must.

Although the benefits and risks in sequential and simultaneous AF and MLF remain controversial, early inoculation now begins to be more frequently used to ensure the success of MLF with selected bacterial strains (*1*). This work represents for the first time the impact of the timing of bacterial inoculation on the performance of AF and MLF in Malbec musts from a warm climate wine growing regions, comparing simultaneous inoculation of yeast and bacteria with traditional vinification protocol, where bacterial inoculation was done sequentially after the completion of AF. A specific consider-

ation was given to the sensorial and sanitary wine parameters. The experiments were made in pilot scale (experimental unit of 1 hL) which closely reproduces the winery conditions and allows sensorial evaluation.

Different researchers have suggested that simultaneous inoculation of yeast and bacteria could have a negative impact on the kinetics of the AF (17,36). The authors associated the inhibition of yeast growth with the high levels of acetic acid produced by LAB in the presence of available sugars in the must. In our experiments, a negative impact of the presence of bacteria on the performance of AF (duration 10–14 days of irrespective timing of inoculation) could not be found and levels of acid acetic never exceed 0.60 g/L. Our results confirm the findings of other research groups (3,10,13,14,24), showing the possibility of simultaneous induction of alcoholic and malolactic fermentations without excessive increase in volatile acidity. A reduction of yeast populations was observed in simultaneous treatments after AF had been finished, as described by King and Beelman (17) in a model grape juice/wine system where bacteria accelerated the yeast's death phase without any effect on AF.

Generally, a significant reduction in total fermentation time was observed when using simultaneous inoculation techniques compared to sequential inoculations. The time gained in the traditional inoculation practice was ranging between 7 and 14 days, depending on the must and the yeast involved. This represents an important advantage for the wineries not only for the process efficiency, but also for safety, because it avoids the presence of spoilage microorganisms and/or biogenic amines. Wines obtained after successful AF/MLF would immediately be ready for downstream treatments, such as racking, fining, and sulphur dioxide addition, allowing an early microbiological stabilization. In general, irrespective of the timing of inoculation, combinations with yeast strain INTA MZA resulted in faster performance of MLF (up to 14 days), compared to the wines inoculated with yeast strain ICV D80, suggesting a good compatibility of INTA MZA with the malolactic starter strain Uvaferm Alpha. Nevertheless, in one must this combination resulted in an incomplete L-malic acid degradation when bacteria and yeast were inoculated simultaneously. Sluggish MLF was directly correlated with an important die-off of the bacterial population upon inoculation (2 log) and bacterial population could not regain the critical number of 10^6 CFU/mL within the time frame of this experiment (35 days).

When slow MLF rates were recorded in simultaneous inoculation treatments, they were also observed in the respective sequential inoculations. Although the compatibility between yeast and malolactic bacteria is dependent of the specific strain combinations, the use of compatible strains will not always guarantee the success of MLF. As it was observed in our study, results obtained with the same yeast/bacteria pair were dependent on the must used. Successful induction of MLF depends not only on the must composition and vintage, but also on the yeast/bacteria pairing and timing of inoculation, as previously reported by other researchers (37,38).

On the other hand, partial degradation of L-malic acid was recorded in sequential treatments. The initial L-malic acid reduction before the inoculation with selected LAB was above 20 %, which is the maximum percentage that could be attributed to yeast metabolism (39,40). These results could indicate a partial native LAB L-malic acid degradation.

Although *O. oeni* implantation was not measured in this study an increase of LAB population was observed when commercial *O. oeni* strain was simultaneously inoculated. In our later studies, similar results were associated with 100 % dominance of the selected bacterial strain (41,42). This could suggest a better control over the spontaneous bacterial populations when simultaneous inoculation is done.

Biogenic amines were included in this study as a sanitary parameter to evaluate the advantage of simultaneous inoculation practices allowing an early dominance of the selected strain. High levels of biogenic amines in wine are mainly related to the development of spontaneous ML bacteria, frequently belonging to the genera *Pediococcus* and *Lactobacillus*, especially in red wines with high pH (25,43). No significant differences in the amine levels between the timings of bacteria inoculation were found in our studies, although in some sequential treatments considerable development of native bacteria was observed. Putrescine levels recorded in all wines could already have been present in the must. Some biogenic amines are normal constituents of grapes in variable amounts in different varieties of *Vitis vinifera*, frequently associated with excessive nitrogen nutrition and/or low potassium concentrations in soil, which increase polyamine levels in berries (44–46).

No evidence was found of simultaneous AF/MLF having a negative impact on the general quality of the inoculated wines with regard to the relevant chemical parameters and sensory attributes of wines. Sensory analysis is an important aspect that has been scarcely considered in earlier related works. When simultaneous and sequential treatments were compared, differences between wine sensory attributes were not significant or they were in favour of the simultaneous inoculations. Wines with simultaneous treatment showed enhanced sensorial attributes related to high quality wine like colour and fruity flavour. Many authors have described acetaldehyde degradation by malolactic bacteria leading to colour reduction in wine during MLF (47,48), which was not confirmed in our results.

Our study has shown that simultaneous inoculation results in an important reduction of total time (AF+MLF). Early inoculation with a selected bacterial strain allows the dominance of the selected strain and a better control over the spontaneous bacterial populations without any negative effect on the yeast population and performance of AF.

Conclusion

Simultaneous inoculation with yeast and bacteria could be an interesting winemaking practice with an easy protocol to carry out, which represents a real time saving tool for wineries and allows the best control over the sensorial and sanitary quality of wines from a warm climate wine growing regions.

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