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Research Paper

Fermentation Performance of Selected Microorganisms in Malbec Musts under Two Different Wine Making Conditions

ABSTRACT

This work evaluates the fermentation performance of simultaneous cultures of selected indigenous *Saccharomyces cerevisiae* mc$_2$, *Kloeckera apiculata* mF and *Oenococcus oeni* X$_2$L in Malbec musts. Fermentations (3 L) were carried out under two conditions: 1-initial pH 5.5, 60.24 mg/L SO$_2$, 26°C) and 2-initial pH 3.8, 125 mg/L SO$_2$, 28°C), using different culture combivnations and *S. cerevisiae* IOC 18 to 2007 as a control. The final wine densities were 0.992 to 0.997 g/L (10 days). Micro-organisms grew in all conditions. Yeasts prevailed, especially *S. cerevisiae* (10^8 CFU/ml), while *O. oeni* reached a value of 10^7 CFU/ml. Total reducing sugars consumption was >80% and full malate consumption was observed in all musts inoculated with *O. oeni*. Ethanol formation corresponded to the Malbec variety (310 to 342 mM), while total acidity did not exceed the organoleptic quality limit (4.6 to 6.49 g/L). Glycerol production was approximately, 84.3 to 95.1 mM. Among the esters tested, ethyl acetate/2-phenyl ethyl acetate showed the highest concentration. The sensory analysis showed the highest scores for visual descriptors (fluency, color intensity and tonality), olfactory intensity, taste descriptors and general harmony. Thus, we proposed an inoculation condition using mixed yeasts-*O. oeni* culture and an optimal statistical point to improve the microbial performance in order to obtain quality Malbec wines, thus, enhancing the body and sensorial properties.

Key words: Malbec wine, fermentation, mixed yeasts culture, *Oenococcus oeni,* glycerol.

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INTRODUCTION

In the modern winemaking process, alcoholic fermentation (AF) is conducted by starters of selected *Saccharomyces cerevisiae* strains, in contrast to traditional spontaneous fermentations carried out by the indigenous microbiota present in grapes and wineries (Mercado et al., 2011). New fermentation technologies for optimizing quality and producing wines with particular flavor profiles are one of the worldwide trends in oenology (Romano et al., 2003; Escudero et al., 2007; Medina et al., 2013; Maturano et al., 2015). These monocultures ensure easy control and homogeneity of fermentations. However, end-products have often shown absence of flavor complexity, stylistic distinction or vintage variability (Lambrechts and Pretorius, 2000; Romano et al., 2003; Maturano et al., 2015). Thus, the use of mixed starter cultures instead of spontaneous fermentations would allow an improvement in wine quality with no risk of a stuck fermentations or wine spoilage (Ciani et al., 2003; Jolly et al., 2003; Rojas et al., 2003; Romano et al., 2003; Medina et al., 2013). Some non-*Saccharomyces* yeast can improve the fermentation behavior of *Saccharomyces* starter cultures, leading to a product with a more complex aroma (Romano et al., 1997; Ciani and Maccarelli, 1998; Egli et al., 1998; Henick-Kling et al., 1998; Medina et al., 2013; Maturano et al., 2015).

The transformation of grape juice into wine represents a complex process that involves the sequential development

Table 1: Fermentations conditions of Malbec musts.

Condition	Cultures	Physicochemical factors
1	SC-KA-OO	ST*: pH 3.8, 125 mg/L SO_2, 28°C
2	SC-KA-OO	SRM#: pH 5.5, 60.24 mg/L SO_2, 26°C
3	SC-KA	ST: pH 3.8, 125 mg/L SO_2, 28°C
4	SC-OO	ST: pH 3.8, 125 mg/L SO_2, 28°C
5	SC	ST: pH 3.8, 125 mg/L SO_2, 28°C
6	SC commercial+	ST: pH 3.8, 125 mg/L SO_2, 28°C

*Standard condition. #Surface response method. SC: *S. cerevisiae* mc_2, KA: *K. apiculata* mF, OO: *O. oeni* X_2L. +*S. cerevisiae* IOC18-2007 was used as control because it is widely used in local fermentations.

of members of its indigenous microbiota mainly constituted by yeasts and bacterial species (Barata et al., 2012). Grape must exert a strong selective pressure on the microbiota due to its low pH and high sugar content where only a few microbial species can proliferate, *S. cerevisiae*, *Kloeckera apiculata* and *Oenococcus oeni* being the best mainly under controlled conditions. Sulphur dioxide addition, anaerobic conditions during wine making, nutrients depletion and high ethanol levels enhance selective pressure (Henschke, 1997; Hansem et al., 2001; Barata et al., 2012).

In order to simulate standard wine making conditions in previous works we used *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L on the basis of their ability to grow and metabolize sugars when grown in simultaneous cultures in natural grape juice.

These conditions allowed the improvement in glycerol production and therefore in the organoleptic properties of the end-products (Ale et al., 2014b). Moreover, the effect of some physico-chemical factors involved in wine making allowed the selection of a statistical fermentation condition (initial pH 5.5, 60.24 mg/L SO_2 and 26°C) to improve glycerol synthesis (Ale et al., 2014a). Therefore, the aim of the present work is to evaluate the fermentation performance of selected micro-organisms in Malbec musts under two different wine making conditions.

MATERIALS AND METHODS

Micro-organisms

S. cerevisiae mc_2 (elliptic yeast), *K. apiculata* mF (apiculate yeast) and *O. oeni* X_2L were selected for their ability to drive AF efficiently, to produce glycerol and carry out MLF, respectively. Moreover, this microbial consortium improves glycerol production in a natural grape juice (NGJ) medium (Ale et al., 2014a, b).

Culture conditions

Growth media

S. cerevisiae mc_2 (SC) and *K. apiculata* mF (KA) were grown in YEPG medium (in g/L: yeast extract, 10; peptone, 20; glucose, 20), pH 5.5, for 24 h at 28°C in microaerophilic conditions (5% CO_2 enriched-chamber), while *O. oeni* X_2L (OO) was grown in MRS medium (de Man et al., 1969) supplemented with 150 ml/L natural tomato juice (MRStj), pH 4.8 for 24 h at 30°C in microaerophilia.

Pre-adaptation medium

Micro-organisms were grown in pure cultures in NGJ medium (17 ml/L), pH 5.5, previously treated by heating at 90°C for 10 min to prevent thermal decomposition of the grape juice. After 24 h at 28ºC, micro-organisms were inoculated into musts.

Musts

The Malbec variety was used to conduct vinification trials. Grapes (*Vitis vinífera* L.) were pressed by hand extrusion using sterilized latex gloves at 20°C and automatically dumped in flasks before microbial inoculation. Fermentations were carried out in 3-L glasses flasks containing 2.5 L Malbec must in the presence of skins (total reducing sugars 1,283 mmol/L, titratable acidity 0.42 g/L, total acidity 1.42 g/L and pH 4.22).

Musts inoculation and fermentation conditions

On the basis of the previous studies, two fermentation conditions were defined: 1) a statistical fermentation condition (SRM) in culture 1: initial pH 5.5, 60.24 mg/L SO_2 and 26°C (Ale et al., 2014 a); 2) a standard condition (ST) in cultures 2 to 6: initial pH 3.8, 125 mg/L SO_2 (as sodium metabisulfite) and 28°C. Table 1 shows simultaneous inoculation strategies. The flasks were aseptically inoculated with different potential starter cultures to get an initial cell density of $5×10^6$ CFU/ml for each yeast strain and/or *O. oeni* X_2L. Flasks were closed with cotton plugs to facilitate CO_2 escape and kept under static conditions with a round of agitation every 24 h. Fermentation was monitored by using a double scale mostimeter (Alla France®). A constant density value for

three consecutive days indicates the end of the fermentation.

Enumeration of microbial populations

Yeasts and *O. oeni* populations were determined by viable cell counts. In order to differentiate *Saccharomyces* and non-*Saccharomyces* yeasts from mixed cultures, samples were plated on YEPG medium supplemented with ethanol (120 ml/L), sodium metabisulphite (0.15 g/L) and chloramphenicol (1 g/L) for the elliptic yeast, while YEPG medium supplemented with cycloheximide (0.01% w/v) was used for the apiculate strain. The samples were also plated on MRStj (pH 4.8) supplemented with cycloheximide (0.1% w/v) to assess *O. oeni* X_2L growth.

Bottling of young wines

After fermentation, young wines were recovered and allowed to stand for 72 h for lees separation. Then, wines were bottled in 750-ml dark glass recipients, which were sealed with synthetic corks, leaving an air chamber of 3 cm in the neck. The bottles were stabilized by storing at 7°C for 60 days. Finally, sensory analysis and analytical determinations were carried out.

Analytical determinations

Enzymatic assays

Cell-free wine samples were obtained from each experiment and stored at -20°C until analytical determinations. Glucose, ethanol, glycerol and organic acids (total lactic, acetic and malic) were quantified using kits supplied by Boehringer-Mannheim, Inc. (Germany).

Chemical assays

Total reducing sugars and fructose were determided according to Ale et al. (2014a, b). Titratable acidity was measured using acid-base titration with standardized 0.1 M NaOH. Chemicals were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO (USA).

Gas chromatography

The analysis of main esters related to wine aroma was conducted by gas chromatography (GC). The extraction of volatile compounds from the wine samples was done by addition of 5 ml diethyl ether to 3 ml of each wine followed by vigorous shaking for 2 min and centrifugation (1,300 *g* for 5 min). The top solvent layer was then transferred to a vial (Mamede and Pastore, 2006). A GC (Agilent 6890N, Agilent Technologies, CA, USA) equipped with a flame ionization detector (FID) was used. An HP-5 column (length 30 m, i.d. 0.32 mm, thickness 0.25 μm) (Hewlett-Packard, CA, USA) was used to analyze the following esters: ethyl acetate, ethyl caproate, ethyl caprylate, isoamyl acetate, and 2-phenyl ethyl acetate according to Abeijón et al. (2009).

Sensory analysis

Sensory descriptive analysis of the young wines (2 months after bottling) was carried out by a tasting panel that consisted of three trained judges from the Bianchi winery and from the National Institute of Viticulture (Mendoza, Argentina). Wines were equilibrated at room temperature (22°C) and 50 ml-samples were poured into randomly numbered wine glasses. Two consecutive sessions were performed on different days. The selection of sensory descriptors was done by the panelists during the first session, taking into account the ones that allowed discrimination among treatments as previously analyzed in our group (Mendoza et al., 2011; Merín et al., 2014). During the second session, the intensity of each descriptor was rated on a scale from 0 (not perceivable) to 5 (very strong). The points evaluated were: 1**:** visual descriptors: fluency, clarity, tone, intensity; 2**:** olfactory descriptors: intensity, floral and fruity aromas; 3**:** taste descriptors: astrigency, body, complexity, floral and fruity flavors, bitterness, and 4**:** general harmony.

Statistical analysis

Experiments were performed in triplicate. One-way analysis of variance (ANOVA) was applied to the experimental data for the comparison of mean values for growth, metabolic behavior and sensory analysis (Fisher's test) while Tukey's test was used for multiple mean comparisons. All statistical analyses were performed with Infostat software version 7 (Universidad Nacional de Córdoba, Argentina).

RESULTS AND DISCUSSION

Evolution of growth and fermentation of wine microorganisms

The growth performance of selected micro-organisms in wine making conditions in Malbec musts was evaluated. *S. cerevisiae* mc_2, *K. apiculata* mF and *O. oeni* X_2L were grown in NGJ and inoculated into Malbec musts.

Two conditions were defined in order to enhance glycerol production by using these autochtonous cultures: a statistical condition RSM (1) with initial pH=5.5 and a
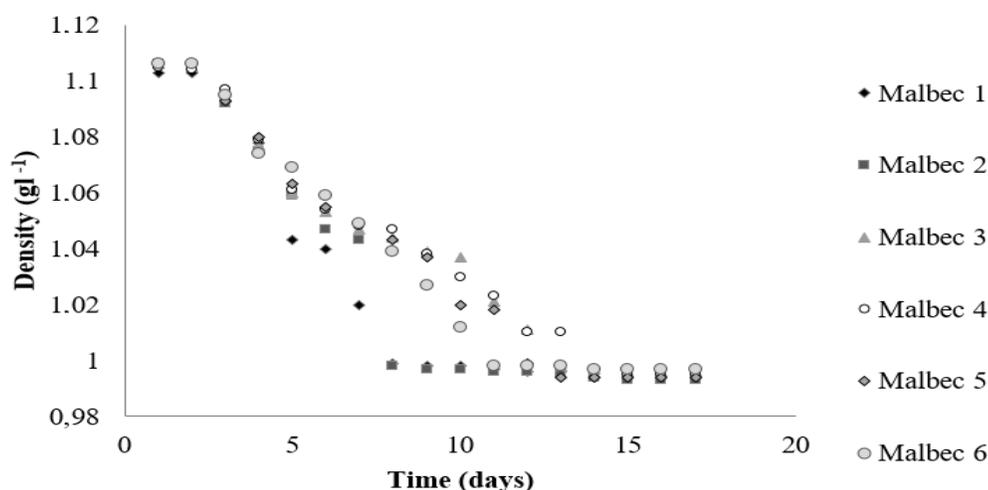
Figure 1: Density diminution during fermentation in Malbec musts.

Table 2: Growth parameters of wine micro-organisms in vinification assays.

Wine	Strain	K[1]	Max. growth[2]	Max. gr. day[3]	Relat. growth[4]	Fin. cell counts[5]	Cel. Viab.[6]
	SC	0.19*	8.58#	10	22.8&	7.52†	1.27‡
Malbec 1	KA	0.15	8.09	10	17.85	7.53	1.22
	OO	0.01	6.75	14	1.13	6.66	1.13
	SC	0.15	8.6	10	19.64	7.63	1.28
Malbec 2	KA	0.15	8.53	10	19.06	7.64	1.29
	OO	0.03	6.89	14	4	6.66	1.11
Malbec 3	SC	0.4	8.73	10	15	7.75	1.31
	KA	0.12	8.65	10	14.41	7.74	1.29
Malbec 4	SC	0.11	8.54	12	12.44	7.65	1.28
	OO	0.09	6.88	14	7.51	6.36	1.07
Malbec 5	SC	0.16	8.83	14	19.55	7.75	1.31
Malbec 6	SC	0.18	8.75	10	20.46	7.83	1.21

KA. *K. apiculata* mF, SC: *S. cerevisiae* mc2, OO: *O. oeni* X2L, [1]Growth rate (h-1), [2] Maximun growth (log CFU/ml), [3]Maximum growth day, [4]Relative growth: (%)= (N_t-N_0/N_0) x100, [5]Final cell counts (log CFU/ml),[6]Cell viability = log N_t/log N_0: N_0: initial viable cell counts; N_t: viable cell counts. condition 1: simultaneous inoculation of *S. cerevisiae* mc2, *K. apiculata* mF and *O. oeni* X2L at 26ºC/~60 mg/L SO2/pH 5.5; condition 2: simultaneous inoculation of *S. cerevisiae* mc2, *K. apiculata* mF and *O. oeni* X2L at 28°C/125 mg/l SO2/pH 3.8; condition 3: simultaneous inoculation of *S. cerevisiae* mc2 and *K. apiculata* mF at 28°C/125 mg/L SO2/pH 3.8; condition 4: simultaneous inoculation of *S. cerevisiae* mc2 and *O. oeni* X2L at 28°C/125 mg/L SO2/pH 3.8; condition 5: inoculation of a pure culture of *S. cerevisiae* mc2 at 28°C/125 mg/L SO2/pH 3.8; condition 6: inoculation of a pure culture of *S. cerevisiae* IOC 18-2007 (commercial yeast) at 28°C/125 mg/L SO2/pH 3.8.

standard condition (2) with initial pH= 3.8. Moreover, other 4 conditions (3 to 6) were defined only for microbial growth and analytical controls.

Although, pH 5.5 in condition 1 does not agree with the standard wine making processes, it was selected according to the predictive model (RSM) in order to obtain high glycerol levels and low acetate production and represents only the initial pH value of the simultaneous cultures since the cultures reached pH<4.0 at the end of exponential growth (Ale et al., 2014a).

Fermentation was considered finished on day 16, when the density of young wines was between 0.992 and 0.997 g/L for a minimum of 3 consecutive days (Figure 1). Musts incubated with three indigenous strains in condition 1 (Malbec 1) evidenced a faster decrease in density than the other musts assayed. A density diminution below 1.049 g/L was observed in all culture conditions since day 7 (Malbec 1 to 6), indicating that the fermentation process ocurred mainly during the first week.

Strains grew in all culture conditions (Malbec 1 to 6, Table 2) and reached maximum growth after 10 to 14 days while maximum biomass values were about 10[8] CFU/ml

for yeasts and 10^7 CFU/ml for *O. oeni* X_2L.

S. cerevisiae growth reached 8.58 log units after day 10 when cultured at 26°C, 60.24 mg/L SO_2 and pH 5.5 (condition 1), while growth in condition 2 was 8.6 log units (Table 2). Both growth values were lower than those observed for the mixed culture of yeast strains (8.73 log units, condition 3); however, this value diminished even more in the presence of *O. oeni* (8.54, culture 4). Maximum growth was found in *S. cerevisiae* in pure cultures (8.83, culture 5). On day 16 of vinification, *S. cerevisiae* viability was about 10^7 CFU/ml, with a relative growth of between 1.28 and 1.3 above the initial inoculum (Table 2).

K. apiculata grew up to 10^8 CFU/ml in co-cultures, showing 8.65, 8.53 and 8.09 log units for Malbec 3, 2 and 1, respectively. At the end of fermentation, cell viability was 1.22 to 1.29 (final cell counts 7.53 to 7.74). The relative growth values of the yeast strains in mixed cultures (Malbec 1, 2 and 3) were above 12%, with growth rates between 0.11 and 0.19 h^{-1} (Table 2).

It was observed that both yeast populations

maintained similar values (cell viability approximately, 1.2 to 1.3) at 16 days of fermentation. Similar results for total fermentative yeasts (36.7% of *Saccharomyces*, 21.4% of *K. apicultata* and 41.9% of other non-*Saccharomyces* strains) under pilot scale conditions at 24°C were reported by Maturano et al. (2015) where total yeast population used as fermentation control reached its maximum growth at day 6 (~8 log units) and diminished below 7 log units until the end of the assay (14 days) in Malbec musts under standard wine making conditions.

Our results showed that the lactic acid bacterium failed to reach 10^7 CFU/ml (Table 2), while growth rate values ranged from 0.01 to 0.09, maximum growth being reached at day 14. This behavior was different when the same strain was compared in simultaneous cultures with *S. cerevisiae* mc_2 and *K. apiculata* mc_1, where it reached values of 10^7 CFU/ml at about 4 to 5 days and then decreased without cell counts on day 14 (Mendoza et al., 2011).

In our work, only *S. cerevisiae* growth was

significantly higher in pure cultures with respect to mixed cultures 1 and 2 (Fisher's test, P>0.05). No significat differences were found in the growth pattern of the other micro-organisms with respect to the number of different strains present in musts.

All microbial strains exhibited similar growth patterns to those observed in a smaller scale (Ale et al., 2014b). Although, the elliptic strain prevailed in laboratory conditions (Ale et al., 2014b), its growth was 1 log unit above the one determined in this work.

Strains reached maximum population values after 10 days of fermentation while in laboratory conditions those values were reached at day 6 (Ale et al., 2014b). The number of viable cells obtained at this stage was in agreement with Mendoza et al. (2011), where *S. cerevisiae* mc_2 and *K. apiculata* mc_1 reached about 10^8 CFU/ml in

Malbec musts; however, in those conditions the maximum population value was found between 2 and 3 days of fermentation. In our work, the apiculate yeast prevailed over other microbial populations and remained with similar values after 4 to 6 days. In another mixed yeast culture, it was reported that the viability of an elliptic yeast strain remained higher than those of the non-*Saccharomyces* yeast (Fleet et al., 2003; Moreira et al., 2005; Ciani et al., 2006).

Analysis of substrates and products in young wines

Substrate consumption and products formation

Table 3 shows the patterns of sugars/malic acid consumption and products formation by a selected mixed culture. The strains used between 84 to 94% glucose (range= 497.32 to 553.46 mM in conditions 1 and 2, respectively), 75 to 90% fructose (range= 476.19 to 573.44 mM in conditions 5 and 1, respectively) and 6.59 to 9.56% pentoses (range= 6.43 to 9.32 mM in conditions 6 and 1, respectively). This sugar consumption represents 80.2 and 85.27% of total reducing sugars in conditions 1 and 2, respectively. When using the commercial yeast under standard wine making (condition 6), 505.13±2.2 glucose, 547.32±1.54 fructose and 6.43±0.98 pentoses consumption was observed, representing about 80.16% of total reducing sugars.

Overall, sugar consumption did not show significant differences (P<0.05) between different wines, with the exception of Malbec 5 (pure culture of *S. cerevisiae* mc_2) for glucose and fructose, and Malbec 4 (SC-KA) and 6 (commercial *S. cerevisiae*) for pentoses. Since total sugar content in musts (1,320.92 mM) was higher than in NGJ medium (Ale et al., 2014b), a modification in the microbial consumption pattern was expected, and in fact we observed that sugar utilization exceeded 85% in all fermentation conditions. This consumption was also observed by Maturano et al. (2015) in Malbec musts fermented with a mixed yeasts population (*S. cerevisiae* Lalvin D254= 2 x 10^7 CFU/ml and native yeasts= 2±0.25 × 10^3 CFU/ml) after 6 days.

Malic acid consumption for conditions 1 and 2 was 90.43 and 98.72%, respectively, and between 50 to 62% in musts without *O. oeni* X_2L inoculation (61.66 and 54.27% for conditions 5 and 6, respectively). This consumption was significantly lower (P<0.05) in Malbec 3, 5 and 6 than in the other conditions. The total consumption of malic acid under all conditions allowed us to infer a successful MLF, which represents an important point in the wine making process for quality wine (Versari et al., 1999). This malic acid diminution was observed in the acceptance by the jury since the values of bitterness/acidity were below 2.5.

Ethanol was the main product formed. Its synthesis was about 310 to 342 mM, representing an alcohol content of

Table 3: Substrate consumption and products formation in winemaking assays.

Condition	Strains	Sample	Glucose	Fructose	Pentoses	Malic acid	Acetic acid	Ethanol	Glycerol	Total acidity†
-	-	Must‡	589.36±1.24*#	634.12±1.02	97.44±1.6	16.73±1.22	24.32±0.52	8.12±1.47	1.02±0.24	1.42±0.34
1-RSM	SC-KA-OO	Wine	527.14±2.01¥a	573.44±2.88¥a	9.32±3.07¥a	15.13±1.23¥a	16.52±1.7Δa	332.1±1.02Δa	95.12±1.32Δa	5.02±0.22Δb
2-ST	SC-KA-OO	Wine	553.46±2.03a	563.95±2.22a	8.98±2.44a	16.22±1.33a	18.31±1.26a	342.23±0.87a	88.48±1.12a	6.08±0.12a
3-ST	SC-KA	Wine	544.83±3.02a	513.40±3.4a	8.62±1.72a	9.73±1.74b	16±2,41a	312.6±0.9b	87.55±0.42a	4.55±0.53a
4-ST	SC-OO	Wine	502.42±2.01a	505.64±3.13a	7.44±1.32b	15.23±1.58a	13.16±0.93b	310.8±0.88b	84.29±1.32a	6.49±0.12a
5-ST	SC	Wine	497.32±2.12b	476.19±1.43b	7.62±1.12a	9.33±0.94b	10.12±1.38b	318.6±0.9b	68.64±0.72b	5.35±0.62a
6-ST	SC com.	Wine	505.13±2.2a	547.32±1.54a	6.43±0.98b	9.08±0.91b	12.38±1.41b	327.98±0.32a	92.44±1.25a	5.47±1.15a

‡Compounds values in musts without inoculation, *Concentration expressed in mM. †Concentration expressed in g l^{-1} tartaric acid, #Values obtained are results of three independent determinations ± standard deviation. ¥Subtrates consumption, ΔProducts formation. Condition 1: simultaneous inoculation of *S. cerevisiae* mc$_2$ (SC), *K. apiculata* mF (KA) and *O. oeni* X$_2$L (OO)at 26ºC/~60 mg/L SO$_2$/pH 5.5; condition 2: simultaneous inoculation of *S. cerevisiae* mc$_2$, *K. apiculata* mF and *O. oeni* X$_2$L at 28°C/125 mg/L SO$_2$/pH 3.8; condition 3: simultaneous inoculation of *S. cerevisiae* mc$_2$ and *K. apiculata* mF at 28°C/125 mg/L SO$_2$/pH 3.8; condition 4: simultaneous inoculation of *S. cerevisiae* mc$_2$ and *O. oeni* X$_2$L at 28°C/125 mg/L SO$_2$/pH 3.8; condition 5: inoculation of a pure culture of *S. cerevisiae* mc$_2$ at 28°C/125 mg/L SO$_2$/pH 3.8; condition 6: inoculation of a pure culture of *S. cerevisiae* IOC 18-2007 (commercial yeast-SC com.) at 28°C/125 mg/L SO$_2$/pH 3.8. [a-b]: Different words indicate significant differences for each column (Tukey's test, $P≤0.05$).

between 11.25 to 12.55% v/v, a significantly higher ethanol production ($P<0.05$) being found in systems 1, 2, 3 and 6. As expected, ethanol contents were in accordance with Malbec variety and similar values were observed by other authors who detected 12.97±0.44 g/L (Mendoza et al., 2011) and 14.1±0.1 g/L (Maturano et al., 2015) in Malbec wines by simultaneous fermentation carried out with *Saccharomyces*/non-*Saccharomyces*/*O. oeni* and commercial *S. cerevisiae*+*O. oeni*, respectively.

Total acidity was between 4.6 and 6.49 g/L. The highest value was observed for condition 4, followed by condition 2 (6.08 g/L). The lowest value was detected for condition 3 (Table 3).

Volatile acidity was between 10.12 and 18.3 mM, which would represent an acetic acid content of 0.1 to 1.1 g/L. In our work conditions, only Malbec 2 (mixed culture of the three selected micro-organisms under standard winemaking conditions) exceeded the limit of organoleptic quality only by 0.1 g/L above the unit (Table 3). Acetic acid production was significantly lower ($P<0.05$) only in Malbec 4, 5 and 6.

These total acidity values (6.49 g/L; acetate= 1.23 g/L), which were similar in all conditions, were below the ones previously reported for Malbec wines (Mendoza et al., 2011). In our experimental conditions, in the fermentations performed using the predictive condition obtained from RSM (condition 1 or Malbec 1), total and volatile acidity were within the limits of organoleptic quality and therefore would not affect the sensory profile of the end-product. This was also supported by sensory analysis and the results were comparable to the ones obtained from musts fermentation with commercial yeast (Malbec 6). On the other hand, Malbec 2 exceeded the established range by 0.1 g/L. Our results are in agreement with Maturano et al. (2015), who observed that volatile acidity was 0.61±0.05 g/L in Malbec musts fermented with commercial *S. cerevisiae*.

Glycerol production was between 84.3 and 95.1 mM, highest production being observed in wines fermented at low temperature with the three selected micro-organisms (condition 1). These values were similar to those found in wines obtained by fermentation with commercial yeasts and in those fermented with mixed cultures under standard conditions (condition 2), and significant lower ($P<0.05$) only in Malbec 5 (Table 3). The presence of glycerol is in agreement with the expected profile for this variety (Rankine and Bridson, 1971; Ough et al., 1972; Nieuwoudt et al., 2004; Moro et al., 2007).

Profile of short chain esters

The production of esters in wines results from the dynamic process between alcohol acetyltransferase and esterases which promote synthesis and hydrolysis, respectively (Fukuda et al., 1998; Jyun-Liang and Wheeldon, 2014). Some volatile compounds (esters) that contribute significantly to wine flavor were determined only in conditions 1 and 2 (Table 4). With respect to ethyl acetate, highest value was detected in conditions 2 and 1 (18.73±0.23 and 7.15±0.33, respectively). 2-phenyl ethyl acetate was also found at higher

Table 4: Esters concentrations of Malbec wines.

Short chain esters	Malbec 1	Malbec 2
Ethyl acetate	$7.15 \pm 0.33^{a,1*}$	$18.73 \pm 0.23^{a,2}$
Isoamyl acetate	$0.05 \pm 0.01^{b,1}$	$0.05 \pm 0.01^{b,1}$
Ethyl caproate	$0.04 \pm 0.01^{b,1}$	$0.04 \pm 0.01^{b,1}$
Ethyl caprylate	$0.02 \pm 0.01^{b,1}$	$0.03 \pm 0.01^{b,1}$
2-Phenylethyl acetate	$0.45 \pm 0.03^{b,1}$	$0.27 \pm 0.01^{b,2}$

*mg/L. Values obtained using calibration curves of each compound for each sample analyzed in triplicate. [a,b] Means within a column with different superscripts differ (Fisher's test, $P < 0.05$). [1,2] Means within a line with different superscripts differ (Fisher's test, P<0.05). M: Malbec, condition 1: simultaneous inoculation of *S. cerevisiae* mc$_2$, *K. apiculata* mF and *O. oeni* X$_2$L at 26ºC/~60 mg/L SO$_2$/pH 5.5; condition 2: simultaneous inoculation of *S. cerevisiae* mc$_2$, *K. apiculata* mF and *O. oeni* X$_2$L at 28°C/125 mg/L SO$_2$/pH 3.
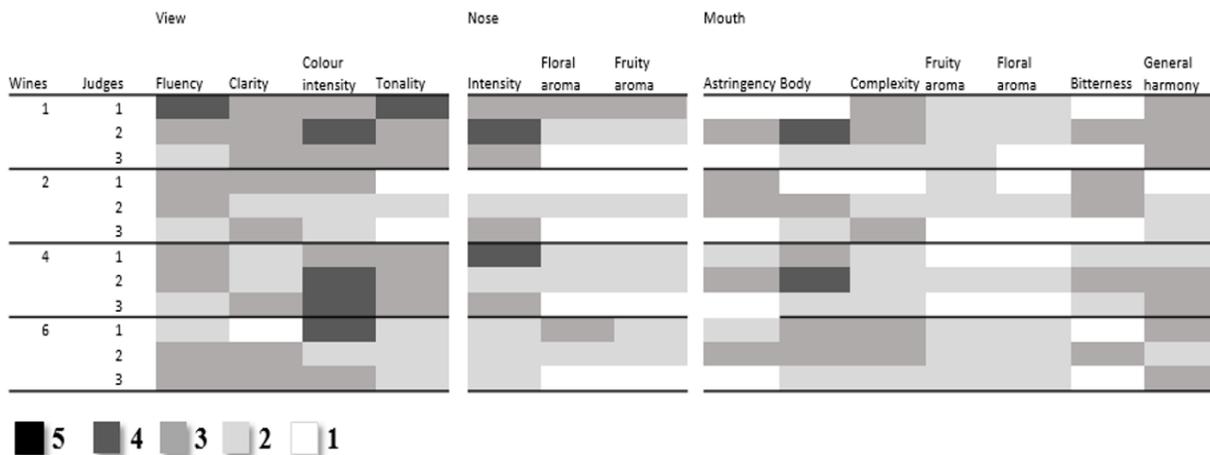
Figure 2: Visual panel of evaluation from judges. 5: very strong → 1: very low.

concentrations in conditions 1 and 2 (0.45±0.03 and 0.27±0.01 mg/L, respectively). Isoamyl acetate, ethyl caproate and ethyl caprylate were detected at low concentrations (0.02 to 0.05 mg/L) (Table 4).

The values found in this study (Table 4) for Malbec wines were lower than those reported by Mendoza et al. (2011) for the same wine variety produced by simultaneous fermentation of *S. cerevisiae* mc$_2$/*K. apiculata* mc$_1$/*O.oeni* X$_2$L. However, Fabani et al. (2013) reported intermediate concentrations of ethyl caproate and ethyl caprylate in Malbec wines inoculated with both commercial and autochthonous monocultures of *S. cerevisiae.*

Low/medium esters concentrations in wine are considered a desirable feature because high ethyl acetate concentrations (150 to 200 mg/L) can produce an aroma similar to organic solvents (Lambretchs and Pretorius, 2000). In our experimental conditions, ethyl acetate concentrations in Malbec 1 and 2 were below 80 mg/L, which was favorable for wine complexity (Lambretchs and Pretorius, 2000). In addition, the synthesis of ethyl and acetate esters could also be affected by the time of laboratory inoculation, simultaneous cultures being a more advantageous strategy than a sequential one (Knoll et al., 2012). The presence of non-*Saccharomyces* yeasts improves this characteristic (Maturano et al., 2012;

Medina et al., 2013). Moreover, pH 5.5 would favor the growth of the apiculate strain by increasing esters production as well as, glycerol synthesis (Ale et al., 2014a).

Sensory analysis

Figure 2 shows the visual profile assessment (scores 1 to 5) of the jury for young wines. Overall, highest scores were awarded for positive visual descriptors (fluency, color intensity and tonality) and olfactory intensity. With respect to the characteristics in the mouth (taste descriptors), positive scores in both body (scores 2 to 4) and general harmony (scores 2 to 3) of wines were determined. Wines fermented at low temperature (condition 1) had better scores for descriptor "preference" (Figure 2) and were comparable with wines obtained with commercial yeasts in standard wine making conditions.

Figure 3 shows the main descriptors analyzed in the visual evaluation of young wines. Among them, tonality and visual intensity showed a general mean of 3.33, with Malbec 4 significantly higher for intensity and both Malbec 1 and 4 for tonality as compared to other conditions for wine production.

With respect to olfactory descriptors, the highest score (4) was for Malbec 1 (intensity), while taste descriptors
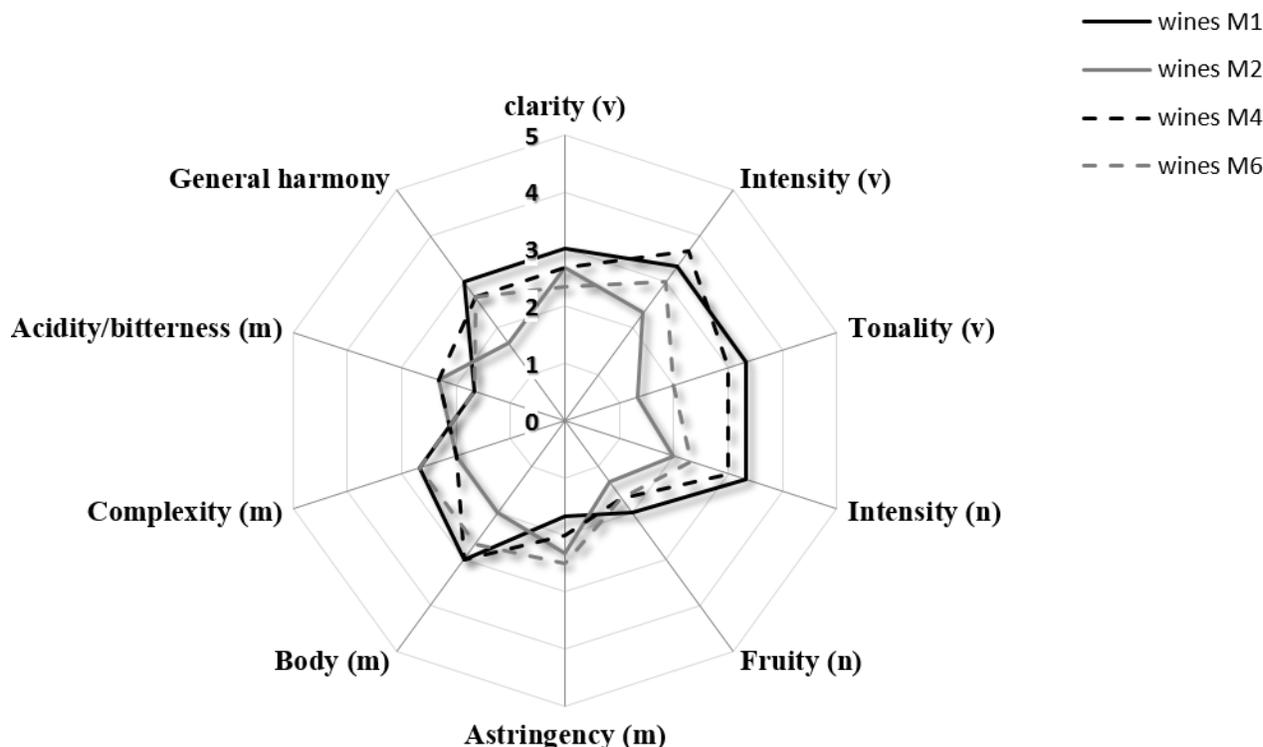
Figure 3: Main descriptors in sensory analysis. v: visual descriptors; n: olfactory descriptors; m: taste descriptors. [a,b] indicates significant differences between descriptors for each wine (1, 2, 4, 6).

(body and complexity in mouth) showed a general mean of 3.33. Overall, descriptors did not exceed score 3 for bitterness/acidity in the mouth even in Malbec 4, in which acetic acid concentration exceeded the highest organoleptic quality limit. The "body" attribute agreed with the results observed in the analytical determinations and is directly related to glycerol production.

The general mean for astringency was 2.6, while the general harmony of the wines had a mean score of 3, Malbec 2 being the only condition with a significantly lower difference. The descriptors evaluated did not show significant differences in their respective mean values (P≥0.05).

In practical terms, judges tested those wines produced by controlled alcoholic and malolactic fermentation with different combinations of the proposed starter culture (*S. cerevisiae* mc$_2$, *K. apiculata* mf and *O. oeni* X$_2$L) against traditional fermentation with commercial wine yeast (spontaneous malolactic fermentation, culture 6). Thus, Malbec 3 (*S. cerevisiae* mc$_2$-*K. apiculata* mF, spontaneous malolactic fermentation) and Malbec 5 (inoculation with a pure culture of S*. cerevisiae* mc$_2$) were only used for microbiological and analytical determinations. Visual descriptors were in agreement with Maturano et al. (2015), where color intensity and astringency had similar patterns to those obtained in this work. Medium/high astringency was also observed by Casassa et al. (2015) in fermentations of Malbec musts using commercial starters in simultaneous inoculation.

In our experimental conditions, bitterness was lower than the that observed by Maturano et al. (2015), who carried out fermentations of Malbec musts using commercial *S. cerevisiae* and Mendoza et al. (2011), who performed simultaneous fermentation of *S. cerevisiae* mc$_2$/*K. apiculata* mc$_1$/*O. oeni* X$_2$L.

Finally, fruit and floral aroma (directly related to esters production) scored low in the sensory analysis and did not exceed 2.7 (Figures 2 and 3).

Conclusion

The results obtained in this work, based on a laboratory scale research using an optimal statistical point (initial pH 5.5, 26°C and 60.24 mg/L SO$_2$, condition 1) (Ale et al., 2014a, b) as a fermentation condition against traditional fermentation (condition 2) allowed us to propose a microbial inoculation condition to achieve high wine production with roundness, appropriate alcohol content and acidity and acceptable visual, olfactory and taste characteristics.

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