

Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeasts

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In traditional winemaking, natural fermentation of grape juice is carried out by a sequence of different yeast species. The early stages are dominated by non-*Saccharomyces* yeasts and are replaced by *Saccharomyces cerevisiae* that finish the fermentation process. In this chapter was evaluated the kinetics and metabolic behavior of *Kloeckera apiculata* mc1 and *S. cerevisiae* mc2 in composite culture. In this condition, *K. apiculata* showed a higher viability through the fermentation; however the cell density of both yeasts decreased. This behavior was not due to ethanol concentration, killer toxins production or competition for assimilable nitrogenous compounds between both yeasts. Despite the consistent production of secondary products by single culture of *K. apiculata*, desirable concentrations of these compounds were observed in mixed culture. The influence of temperature and SO₂, on growth and metabolism of both wine yeasts was dependent of the culture type. Malolactic fermentation conducted by *Oenococcus oeni* is important enhancing wine quality, microbiological stability and flavour. The inclusion of *K. apiculata* mc1 as adjunct culture of *S. cerevisiae* mc2 during must fermentation improved the organoleptic characteristics of wines produced from vineyards in Argentina Northwest. In addition, sequential inoculation of *O. oeni* X₂L allowed better control on the sensory quality of the fermented product.

Keywords: non-*Saccharomyces* yeast; wine; flavor

1. Introduction

In traditional winemaking, natural (spontaneous) fermentation of grape juice is carried out by a sequence of different yeast species. The early stages are dominated by the growth of non-*Saccharomyces* yeasts, characterized by a low fermentative power [1]. The yeasts belonging to genera *Kloeckera/Hanseniaspora* or other genera such as *Candida*, *Pichia* and *Metschnikowia* start the fermentation [1, 2]. After 3-4 days these yeast die off, and are replaced by the highly fermentative yeast (*Saccharomyces cerevisiae*) that continue and finish the fermentation process [3]. However, some studies showed that non-*Saccharomyces* yeast survive during the natural and inoculated fermentations of grape juice for longer periods than previously thought.

Recently several groups have examined different non-*Saccharomyces* yeast strains as potential adjuncts to *S. cerevisiae* in an effort to modify wine flavor and improve product quality [4, 5, 6]. Apiculate wine yeasts (*Kloeckera apiculata/Hanseniaspora uvarum* and *Hanseniaspora guilliermondii*) have become an object of interest as they are frequently found in grapes and are also dominators of the early stages of must fermentation [7, 8]. In addition to ethanol and carbon dioxide, during the fermentation these yeasts release secondary products such as higher alcohols, esters, acids, carbonyl compounds important to the sensory characteristics of wines [9, 10, 11]. Therefore, the practical benefit of the physiological and metabolic properties of the non-*Saccharomyces* yeast could be important in winemaking [12, 13].

In this chapter, the kinetics and metabolic behavior of a selected non-*Saccharomyces* wine yeast, *K. apiculata* mc1 in composite culture with *S. cerevisiae* mc2 was evaluated. The influence of two physicochemical factors involved in winemaking, temperature and SO₂, on the growth and metabolism of the yeast cultures was also examined. In addition, differences in sensory characteristics of wines from Argentina Northwest inoculated simultaneously or sequentially with selected indigenous yeasts and lactic acid bacteria in microvinification conditions were studied.

2. Behavior of non-*Saccharomyces* yeasts in mixed starter culture with *S. cerevisiae*

In some cases, wine produced with pure yeast mono-cultures lack flavor complexity that may originate from good indigenous fermentations. The potential of non-*Saccharomyces* yeasts to enhance wine aroma intensity and flavour complexity is considerable. Some of these strains such as *K. apiculata*, *Pichia fermentans* and *Candida stellata* have been studied for their interesting organoleptic contributions [14, 15, 16]. Although some of these strains could improve the wine bouquet, most of them are not able to complete alcoholic fermentation (AF). For this reason incorporation of a *S. cerevisiae* strain with non-*Saccharomyces* strains was studied to overcome these shortcomings. The extent of flavour enhancement can be modulated by using different inoculation strategies [17].

Fifty-two strains of non-*Saccharomyces* yeasts were isolated from grape and must of Argentina Northwest. All strains were tested to study their oenological characteristics and enzymatic activities related to aroma. One of these yeasts, *K. apiculata* mc1, was selected for the optimal oenological properties to be tested as starter in wine fermentations (Table 1). *S. cerevisiae* mc2 was chosen as saccharomycetic yeast taking into account its high fermentative power.

Table 1. Evaluation of main oenological properties of selected yeast strains

Strains	Final biomass (cfu/mL)	Residual sugars (g/L)	Fermentative power (Ethanol % v/v)	Volatile acidity (g/L)	SO ₂ tolerance	H ₂ S production	β-glucosidase	Esterase (nmol/mg)
<i>K. apiculata</i> mc1	1x10 ⁶	18.1	8.7	0.58	++	-	+++	494.5
<i>S. cerevisiae</i> mc2	8x10 ⁷	1.8	13.5	0.42	+++	-	-	30.8

2.1 Growth of wine yeasts in pure and mixed cultures

The growth kinetics of *K. apiculata* mc1, non-*Saccharomyces* yeast isolated from Argentinian grape, and *S. cerevisiae* mc2 was evaluated during fermentation in basal grape juice medium [14]. *K. apiculata* in pure culture reached the maximal cell concentration of 3 x 10⁷ cfu/mL after the 3 days of incubation at 30 °C. From this time the yeast started the declination phase (Table 2). However, pure culture of *S. cerevisiae* grew for a longer period, reached its highest cell concentration (1.4 x 10⁸ cfu/mL) at 6 days of fermentation. In mixed culture both wine yeasts showed lower cell concentration than in pure culture. Also, the growth rate was 30 and 26% lower for *K. apiculata* and *S. cerevisiae*, respectively. These results are in accordance with Moreira et al.[18] who determined a specific growth rate of 0.38 h⁻¹ for pure cultures of *S. cerevisiae* and *H. uvarum*; whereas this value decreased when these yeast were grown in mixed culture (0.33 and 0.26 h⁻¹ for *S. cerevisiae* and *H. uvarum*, respectively).

Table 2. Growth and death kinetics of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

Yeasts cultures	Growth rate (h ⁻¹)	Day of maximal cell population	Maximal cell population (cfu/mL)	Relative growth (%) ^a	Death kinetics ^b	
					Curve shape	Rate (d ⁻¹)
K	0.20	3	3.0x10 ⁷	- 6.4	Exponential	0.51
Km	0.14	3	5.9x10 ⁶	4	Lineal	0.12
S	0.23	6	1.4x10 ⁸	29	Lineal	0.09
Sm	0.17	3	5.5x10 ⁷	9	Lineal	0.15

K and S: *K. apiculata* and *S. cerevisiae* in pure cultures

Km and Sm: *K. apiculata* and *S. cerevisiae* in mixed culture

^a Relative growth (%) = $(X_i - X_0/X_0) \times 100$. X₀: initial viable cell number; X_i: viable cell number after 10 days. Negative values indicate cellular death

^b Death kinetics were fitted using an exponential model $\log(N_t/N_0) = c + \exp(a + bt)$ and a lineal model $\log(N_t/N_0) = a + bt$. Modified from Mendoza et al. [14]

Furthermore, the authors reported that in composite culture the apiculate yeast remained viable during longer period than in pure culture and the elliptic yeast started to lose viability. As can see in Table 2 after 10 days of incubation of *K. apiculata* in co-culture condition, showed an increase of 4% of the relative growth with lineal death kinetic (death rate = 0.12 d⁻¹), while in pure culture this yeast showed a 6.4% loss of viability with exponential death kinetic (death rate = 0.51 d⁻¹). The different types of death kinetics of *K. apiculata* depending on whether the yeast grows as single or mixed culture, would reflect that the causes of the apiculate yeast death were not the same in both cultures. Nissen and Arneborg [19] found that the death kinetics of *Kl. thermotolerans* and *T. delbruekii* in mixed culture have not been the same. The relative growth of *S. cerevisiae* in pure culture increased 29%, while this value was 9% when the elliptic yeast was co-cultured. Pure and mixed cultures of the elliptic yeast showed lineal kinetic of death, being the death rate 0.09 and 0.15 d⁻¹, respectively [14].

2.2 Metabolic characteristics of wine yeasts in single and composite cultures

Mendoza et al [14] also examined the main fermentation metabolites produced by different cultures. The highest ethanol concentration was determined in pure cultures of *S. cerevisiae*. When the elliptic yeast was co-inoculated with *K. apiculata*, even if the AF was carried out with same rate than in pure culture of *Saccharomyces*, the ethanol

concentration was lower (Table 3). It is generally assumed that ethanol can reach concentrations leading to cell death of certain yeast species [7, 20]. Alcohol concentrations did not responsible of the lower biomass of both yeasts reached in co-inoculated cultures since in mixed trial a reduction of ethanol production related to the pure cultures was observed.

The high production of acetic acid is recognized as a common pattern in apiculate yeasts and so they have been considered for long time as spoilage yeasts [9]. Despite the consistent production of acetic acid in pure culture, *K. apiculata* did not cause an increase in volatile acidity in mixed cultures. Similar results were observed by Ciani et al. [21], showing that in mixed or sequential cultures of *H. uvarum*/*S. cerevisiae*, volatile acidity was lower than that seen in pure cultures of non-*Saccharomyces* yeast.

Glycerol is a wine constituent related to yeast metabolism which contributes to the sweetness, viscosity and smoothness of wine [16, 22]. In this study, the greater production of glycerol was related to non-*Saccharomyces* yeast and mixed culture.

Table 3. Metabolic characteristics of wine yeasts in single and composite cultures

Cultures	Ethanol (g/L)	Volatile acidity (g/L)	Glycerol (g/L)	Assimilable nitrogenous compounds consumed (mg/L)
<i>K. apiculata</i>	10.98 ± 0.20 ^a	0.98 ± 0.03 ^a	1.65 ± 0.04 ^a	225 ± 10.5 ^a
<i>S. cerevisiae</i>	11.46 ± 0.23 ^b	0.54 ± 0.05 ^b	1.21 ± 0.04 ^b	280 ± 7.8 ^b
Mixed culture	9.67 ± 0.17 ^a	0.68 ± 0.04 ^c	1.47 ± 0.08 ^c	255 ± 9.4 ^c

The initial yeast assimilable nitrogenous compounds were 545 mg/L (325 mg/L ammonia and 220 mg/L amino acids). Values are means ± standard deviations. Values displaying different superscript letters within each column are different according to the Tukey test. Modified from Mendoza et al. [14]

Pure cultures of *K. apiculata* exhibited lower consumption of the assimilable nitrogenous compounds than elliptic yeast. In co-culture conditions, utilization of these compounds (present in non-deficient concentrations: 545 mg/L) decreased with respect to *S. cerevisiae* in pure culture. Considering that in mixed trial less nitrogen was used than in pure culture (Table 3), the lower biomass of both yeasts in co-inoculation condition was not due to a competition for assimilable nitrogenous compounds.

2.3 Killer activity of wine yeasts

It is well known that during wine fermentations yeasts can produce, beside the ethanol, other toxic compounds, namely killer toxins [7]. Mendoza et al. [14] evaluated if these compounds produced by *S. cerevisiae* and *K. apiculata* were involved in the diminution of maximum cell population in mixed culture. The killer activity of the both yeast strains was tested against the reference killer toxins K1, K2, K4 and K10 (data not shown). Tests revealed that *S. cerevisiae* was killer sensitive against the reference killer toxins (phenotype K⁻ R⁺) and *K. apiculata* was killer neutral (K⁻ R⁻). Additionally, none of the strains were killer positive towards the killer sensitive strains. Pérez-Nevado et al. [23] studied the cellular death of two non-*Saccharomyces* wine-related yeasts in mixed fermentations with *S. cerevisiae* and the authors reported that the former strains were killer neutral, while *Saccharomyces* strain was killer sensitive against the classical killer toxin. On the other hand, when *S. cerevisiae* was seeded on *K. apiculata* lawn a zone of inhibition could be observed, however, cell death was absent, and the inhibition may be produced by metabolites other than yeast killer toxins [14].

The existence of *K. apiculata* mc1, a non-*Saccharomyces* yeasts isolated from wine during AF might be of technological interest. However, in wine biotechnology more specific information on the extent of its contribution is required. For the practical application of this biotechnological process, it is necessary to determine the influence of some of the fundamental fermentation parameters on the growth and metabolic activity of the microorganism involved.

3. Effect of temperature and sulfur dioxide on growth and metabolism of *K. apiculata* and *S. cerevisiae* cultures

The persistence of non-*Saccharomyces* fermentation species during fermentation may depend, however, upon many factors. The fermentation temperature is one of the important vinification factors that affect the rate of yeast growth and the AF. These changes determine the chemical and organoleptic qualities of the wine [1]. Addition of SO₂ to grapes or must to control oxidation reactions and restrict the growth of the indigenous yeast population is a well established practice in winemaking [24], allowing the subsequent inoculation with selected yeasts. Sulfur dioxide is highly toxic to most non-*Saccharomyces* yeasts, while strains of *Saccharomyces* in general are quite resistant to it [25, 26, 27, 28]. The total concentration of SO₂ in grape juice during fermentation consists of bound and free forms. At pH 3.0-4.0, normally

found during must fermentation process, free SO₂ exists mainly as bisulfite ion (94-98%) and only a very small proportion (2-6%) occurs in the molecular form, the main antimicrobial agent [27].

3.1 Influence of fermentation temperature on behavior of non-*Saccharomyces* and *Saccharomyces* yeasts

Mendoza et al. [29] studied the kinetic parameters as well as fermentative activity of *K. apiculata* mc1 and *S. cerevisiae* mc2 cultures at different incubation temperatures. The growth rate of both wine yeasts in pure and mixed cultures increased with increasing temperature until 30 °C (Table 4). At the temperatures assayed, maximal cell density of both strains were reached after 24 h, except for the elliptic yeast at 15 °C. Regardless of the culture type (pure or mixed) the highest biomass was achieved at 15 °C and 25 °C for *K. apiculata* and *S. cerevisiae*, respectively. Heard and Fleet [30] observed that *K. apiculata* grew and survived better in fermentations performed below 20 °C and dominated fermentations at 10 °C. However, *S. cerevisiae* exhibited higher cell population and kinetics at temperatures between 20 and 30 °C. Similar results have been obtained in cider production using mixed yeast starters [31]. Erten [32] has pointed out that *K. apiculata* dominated over *S. cerevisiae* and survived longer at low temperatures compared to fermentations conducted above 20 °C.

It is important to note that independently of the incubation temperature, non-*Saccharomyces* and *Saccharomyces* yeasts in co-culture conditions exhibited a lower growth rate and less final biomass than their respective pure cultures.

Table 4. Effect of fermentation temperature on growth kinetic parameters of *K. apiculata* and *S. cerevisiae* in single and composite culture

T (°C)	Growth kinetics							
	Rate (h ⁻¹)				Maximal cell population (cfu/mL)			
	Pure culture		Mixed culture		Pure culture		Mixed culture	
	K	S	K	S	K	S	K	S
15	0.14	0.09	0.10	0.07	1.1x10 ⁸	9.4x10 ⁷	4.7x10 ⁷	4.2x10 ⁷
25	0.17	0.20	0.12	0.16	6.5x10 ⁷	1.9x10 ⁸	2.8x10 ⁷	5.4x10 ⁷
30	0.20	0.21	0.14	0.15	2.8x10 ⁷	1.8x10 ⁸	6.0x10 ⁶	4.7x10 ⁷
35	0.07	0.17	0.02	0.09	1.1x10 ⁷	8.8x10 ⁷	8.9x10 ⁶	3.1x10 ⁷

K: *K. apiculata*, S: *S. cerevisiae*. Modified from Mendoza et al. [29]

In order to evaluate production of fermentation metabolites by yeast cultures at different temperatures, MANOVA statistical analysis were applied to data [29]. The metabolite concentrations showed significant differences under the different culture conditions and also for several fermentation temperatures ($P < 0.0001$). The interaction between culture type (pure or mixed culture) and temperature was statistically significant, suggesting that the effect of the fermentation temperature on the metabolite concentration depended on the culture condition. Principal components analysis (PCA) showed that the first two principal components accounted for about 98% of the total variation. PC1 was associated with the production of glycerol and acetic acid while PC2 with the ethanol concentration (Fig.1).

The highest concentrations of glycerol and acetic acid were obtained in pure cultures of *K. apiculata* at 25 and 30 °C, showing positive scores for PC1. While single cultures of *S. cerevisiae* produced the lowest amounts of these metabolites at 15 and 35 °C. At 30 °C the elliptic yeast displayed a similar production of the three fermentation metabolites when compared to 35 °C. At higher temperatures the production of ethanol decreased in cultures of the apiculate yeast, whereas an opposite behavior was observed in cultures of *S. cerevisiae*. In composite cultures at 15, 25 and 30 °C the yeasts produced intermediate concentrations of secondary products, similar to those observed for *K. apiculata*. At 35 °C mixed culture showed a different behavior, producing lower concentrations of secondary metabolites. Independently of the temperature, ethanol production in composite culture was lower than those observed in *S. cerevisiae* single cultures. These results are in disagreement with Toro and Vazquez [33], who determined in a mixed culture of a *Saccharomyces* and a non-*Saccharomyces* strain, that the final ethanol concentration was higher than in a pure culture of *S. cerevisiae*. Whereas Moreira et al. [18] reported that mixed starter cultures of *S. cerevisiae* and *H. uvarum* showed lower ethanol yield and ethanol productivity than pure cultures. It is interesting to emphasize the high production of volatile acidity as acetic acid in pure cultures of *K. apiculata* at 25 and 30 °C, compared to lower amounts produced by pure cultures of *S. cerevisiae*. Thus, it was confirmed the peculiar characteristic of little acetic acid production by *Saccharomyces* yeasts under the given conditions [34, 35].

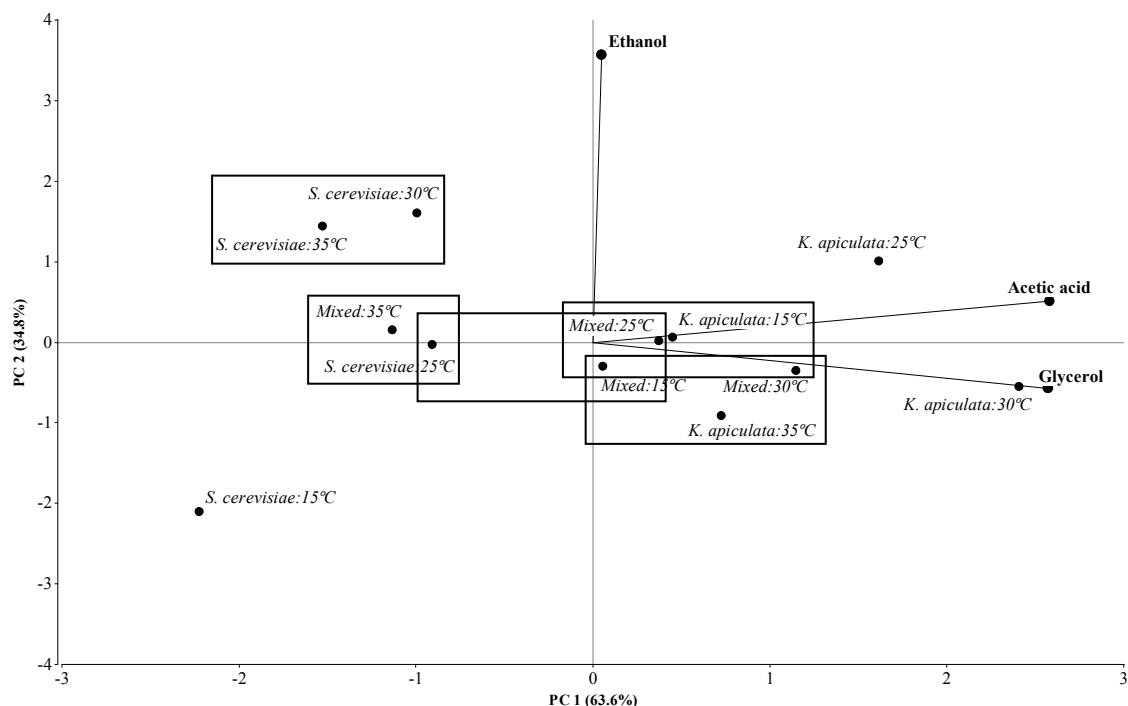


Figure 1. Biplot graph of the first two principal components for the production of ethanol, glycerol and acetic acid by *K. apiculata* and *S. cerevisiae* in single and composite cultures at 15, 25, 30 and 35°C. Values statistically similar according to Hotelling-Bonferroni test were grouped. Modified from Mendoza et al. [29].

Several studies reported that *K. apiculata* may produce higher acetic acid concentrations than *S. cerevisiae* [30, 31, 32]. However, despite the consistent production of volatile compounds in pure culture, *K. apiculata* did not produce an increase in volatile acidity in mixed culture [21, 32]. This behavior has a biotechnological importance since the increase in acetic acid to values higher than legal wine standards (1.1 g/L) could produce a sour-vinegar off odor.

3.2 Effect of sulfur dioxide on growth and metabolism of *K. apiculata* and *S. cerevisiae* cultures

Traditionally, SO₂ has been added to the must as an antioxidant and as an antimicrobial agent to suppress the growth and dominance of non-*Saccharomyces* species and selectively encourage the growth and dominance of *S. cerevisiae* [36]. Henick-Kling et al. [37] have indicated that musts treated with 20 mg/L sulfite produced no effects on the yeast population or the fermentation rate, whereas, 50 mg/L SO₂ produced inhibition of non-*Saccharomyces* yeasts.

Table 5 shows the effect of sodium metabisulfite addition on the growth kinetics parameters of pure and mixed cultures of *K. apiculata* and *S. cerevisiae* [29]. The growth rate of the apiculate yeast in single cultures decreased with increasing metabisulfite concentration. However, a slight increase in maximal cell population was observed in the presence of 50 and 100 mg/L metabisulfite. In mixed culture, the addition of SO₂ produced a similar effect on both growth kinetics parameters. The growth kinetics of *S. cerevisiae* in pure or mixed cultures was not affected by SO₂ addition; only a diminution in the biomass of the elliptic yeast was observed at the highest additive concentration.

The results showed little or no effectiveness of SO₂ regarding control of the non-*Saccharomyces* yeast, *K. apiculata* mc1, in pure and mixed cultures even if the theoretical concentration of molecular SO₂ (chemical form with antimicrobial activity) would be enough to produce the desired antimicrobial effect. Heard and Fleet [38] questioned the efficacy of SO₂ in controlling the initial growth of indigenous non-*Saccharomyces* yeasts. The authors demonstrated that 100 mg/L total SO₂ did not necessarily prevent growth of indigenous non-*Saccharomyces* species, especially in red wines. The inefficacy of SO₂ to inhibit *K. apiculata* mc1 means an important finding considering that nowadays many winemakers believe that growth of non-*Saccharomyces* yeasts also contributes to desirable sensory characteristics.

Table 5. Effect of sulphur dioxide on growth parameters of *K. apiculata* and *S. cerevisiae* in single and composite culture

SO ₂ (mg/L)	Growth kinetics							
	Rate (h ⁻¹)				Maximal population (cfu/mL)			
	Pure culture		Mixed culture		Pure culture		Mixed culture	
	K	S	K	S	K	S	K	S
0	0.20	0.22	0.14	0.17	3.0x10 ⁷	1.4x10 ⁸	5.9x10 ⁶	5.5x10 ⁷
50	0.16	0.22	0.15	0.16	3.2x10 ⁷	1.2x10 ⁸	6.1x10 ⁶	5.6x10 ⁷
100	0.15	0.22	0.14	0.16	3.8x10 ⁷	1.0x10 ⁸	6.3x10 ⁶	5.4x10 ⁷
250	0.14	0.21	0.13	0.15	1.9x10 ⁷	8.8x10 ⁷	3.4x10 ⁶	4.3x10 ⁷

K: *K. apiculata*, S: *S. cerevisiae* . Modified from Mendoza et al. [29]

Mendoza et al [29] applied MANOVA analysis, revealing that the culture conditions and SO₂ addition had significant effects on the fermentation products, with a lower influence for SO₂ addition ($P < 0.0001$ and $P < 0.001$ for culture conditions and SO₂, respectively). The interaction between culture and SO₂ was not significant ($P > 0.01$). In Figure 2 it can be observed that PC1 and PC2 explained 97.5% of the total data variance. PC1 was positively associated with glycerol and acetic acid production while ethanol concentration presented negative scores while PC2 was weakly associated with production of metabolites. Glycerol production divided *K. apiculata* and *S. cerevisiae* into two opposite groups. Pure cultures of the apiculate yeast obtained higher concentrations of this metabolite and also acetic acid than pure cultures of *S. cerevisiae*. However, the ethanol concentration was higher in single cultures of *Saccharomyces* displaying negative scores for PC1. In co-culture conditions the content of the three fermentation products showed intermediate values. Independently of the culture it was observed that in the presence of 50 and 100 mg/L SO₂ wine yeasts increased production of ethanol and acetic acid compared to non-supplemented medium or in the presence of 250 mg/L SO₂.

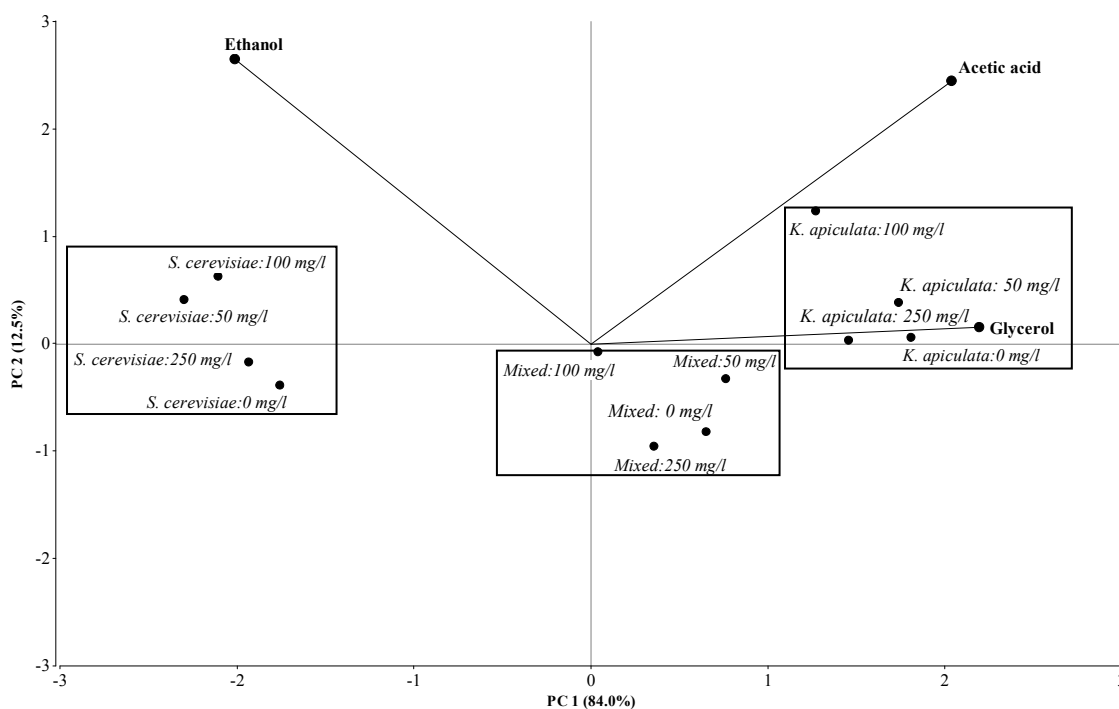


Figure 2. Biplot graph of PC1 vs. PC2 for ethanol, glycerol and acetic acid concentrations produced by *K. apiculata*, *S. cerevisiae* and mixed culture in presence of different levels of sulfur dioxide. Values statistically similar according to Hotelling-Bonferroni test were grouped. Modified from Mendoza et al. [29]

Fermentations at low temperatures could lead to a greater contribution of non-*Saccharomyces* populations. However, in composite culture *K. apiculata* can contribute to the final product at higher temperature. The results also showed little

efficacy of SO₂ to control *K. apiculata* mc1 growth in pure and mixed cultures. This finding has an important technological impact considering the interest of winemakers to maintain viability of certain non-*Saccharomyces* strains due to their contribution to the final product. It was emphasized the important role of temperature and SO₂ on the prevalence of composite yeast flora during vinification and the impact on their metabolic activities.

4. Microvinifications conducted by mixed yeast starter and *Oenococcus oeni*

Wine fermentations conducted by *S. cerevisiae* and non-*Saccharomyces* species, as explained earlier in this chapter, could lead to more complex organoleptic properties. Then, the use of mixed starter cultures would permit to improve wine quality and this way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage [9, 21, 39].

In winemaking process, malolactic fermentation (MLF) is an important secondary fermentation carried out by lactic acid bacteria (LAB), mainly *Oenococcus oeni* strains. The MLF which consists of the enzymatic decarboxylation of L-malic acid into L-lactic acid, is required during the vinification of most red wines and certain white and sparkling wines. This secondary fermentation diminishes wine acidity and improves taste, flavour and microbial stability [40, 41]. The MLF step is often difficult to accomplish due to the inadequate physico-chemical conditions of wine such as a high concentration of ethanol and nutrient depletion as well as some common inhibitory metabolites from yeasts such as SO₂ and fatty acids [42, 43, 44]. More recently it was reported that compound of peptidic or proteic nature produced by yeasts inhibited LAB growth [45, 46]. Mendoza et al. [47] found that the strain mc2 of *S. cerevisiae* inhibited wine LAB growth by synergistic effect between fermentation metabolites and peptidic compound of low molecular size (3-10 kDa). Although this yeast inhibited *O. oeni* X₂L growth, did not affect the malolactic activity.

Simultaneous inoculation of must with yeasts and bacteria would be beneficial regarding microbiological and technical aspects due to a low alcohol concentrations and higher nutrient content present in fermented grape musts compared with wines [48, 49]. However, some wine LAB strains can cause stuck AF or wines with increased concentrations of acetic acid that render them unacceptable for consumption [50, 51]. For this reason, sequential inoculation of bacterial cultures after the completion of AF is the strategy frequently adopted in winery [40, 52].

4.1 Evolution of microbial populations and fermentation kinetics in microvinifications

Mendoza and Fariás [53] carried out microvinifications in Malbec must from northwestern Argentina using different starter cultures and inoculation strategies. When the authors evaluated the yeast population in fermented must inoculated with pure culture of *S. cerevisiae* mc2, found that the maximal cell density (1.6×10^8 cfu/mL) was reached at 3 days of fermentation. In mixed culture with *K. apiculata* mc1, the biomass was lower than those observed in single fermentations. At 6 days of incubation *S. cerevisiae* began the declination phase showing a loss of 1 log cycle at the end of AF. *K. apiculata* in pure cultures showed the maximal cell density (5×10^7 cfu/mL) at the first day and immediately the apiculate yeast started the death phase with a decrease of the viable cells to 10^4 cfu/mL at the late stages of the process. Similar behavior was observed in mixed culture. Ciani et al. [21] evaluated the biomass evolution of multistarter trials of non-*Saccharomyces*/*S. cerevisiae* cultures and found that in mixed trials, non-*Saccharomyces* yeasts persist during the first stages of fermentation. Although *S. cerevisiae* kept its viability for a longer period than non-*Saccharomyces* strains in composite cultures, *Saccharomyces* yeasts did not reach cell population of pure cultures [18, 21]. Also, the influence of the inoculation timing of *O. oeni* X₂L on growth kinetics of the microorganisms involved and the evolution of the malic acid concentration during the microvinifications was evaluated. In regard to fermentations conducted by *K. apiculata* and *S. cerevisiae* without *O. oeni*, the yeast populations were not modified by bacterial inoculation (Fig. 3A). Previous studies indicated that viable yeast population was not negatively affected by the presence of the bacteria [46, 54]. In simultaneous fermentations, *O. oeni* exhibited a cell population of 2.2×10^7 cfu/mL at 3 days of incubation and the malic acid was fully removed at this time. During sequential inoculation of *O. oeni* after completion of AF (Fig. 3B), the bacterial cell population and growth rate were lower than those obtained in microvinifications simultaneously inoculated. However, it was observed complete depletion of malic acid at 5 days of incubation. Jussier et al. [55] reported that treatments with simultaneous inoculation of yeast and bacteria led to faster malic acid degradation. Other researches indicated that the malic acid utilization was similar in both inoculation strategies [54].

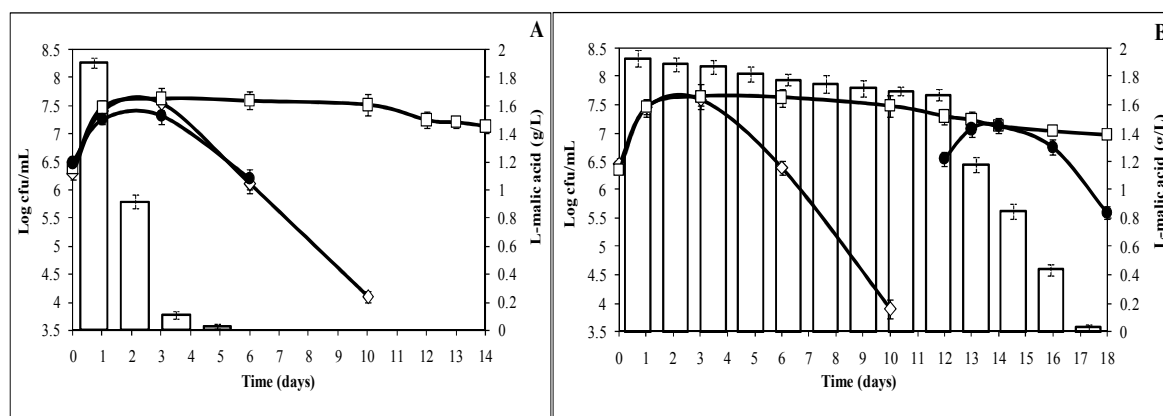


Figure 3. Malic acid consumption (bars) and evolution of cell population of *K. apiculata* mc1 (◇), *S. cerevisiae* mc2 (□) and *O. oeni* X₂L (●) with simultaneous (A) and sequential (B) inoculation during microvinification process.

On the other hand, it was studied the fermentation kinetics of AF conducted by pure or mixed starter cultures. Microvinifications carried out by single cultures of *K. apiculata* showed stuck fermentations at 3 days of incubation. It is widely acknowledged that non-*Saccharomyces* yeasts are weakly fermentative and produce only low amounts of ethanol [10]. Fermentations conducted by mixed cultures of non-*Saccharomyces* and *Saccharomyces* yeasts exhibited a similar kinetics than that observed in *S. cerevisiae* fermentations. Others authors reported that the inoculation of non-*Saccharomyces* yeasts can influence the kinetics of AF conducted by *S. cerevisiae* [21, 30]. In addition, *O. oeni* inoculation did not modify the fermentation kinetics.

4.2 General characteristics and sensorial analysis of young wines

In microvinifications conducted by *S. cerevisiae* in pure or mixed cultures, the sugars were completely consumed and the dryness of the must was achieved at the end of AF. The ethanol concentrations in these wines showed values of 12-13% (Table 6). However, products fermented by single culture of *K. apiculata* showed high residual sugars contents and low ethanol amount. The stuck fermentation was related to growth kinetics exhibited for this apiculate yeast. Similar behaviors were observed by Rodríguez et al. [56] who found that the wines obtained from monocultures and mixed fermentations with *S. cerevisiae* MMf9 inoculation showed the physical-chemical characteristics of most regular wines.

Table 6. Chemical characteristics of wines fermented by different starter cultures

	K	S	K + S	K + S + O (simultaneous)	K + S + O (sequential)
Ethanol (% v/v)	8.82±0.56 ^a	14.11±0.27 ^b	13.76±0.19 ^b	12.97±0.44 ^c	13.58±0.29 ^{bc}
Glycerol (g/L)	7.98±0.2 ^a	8.55±0.3 ^b	8.43±0.2 ^b	8.27±0.2 ^{ab}	8.16±0.3 ^{ab}
Volatile acidity (g/L)	0.79±0.04 ^a	0.45±0.03 ^b	0.58±0.03 ^c	1.23±0.05 ^d	0.61±0.02 ^c
Acetaldehyde (mg/L)	37.83±1.37 ^a	56.54±2.32 ^b	52.35±1.95 ^b	17.76±1.13 ^c	48.14±2.11 ^d
Titrateable acidity (g/L)	5.27±0.2 ^a	5.91±0.3 ^b	6.22±0.3 ^b	6.74±0.2 ^c	4.90±0.3 ^d
L-malic acid (g/L)	1.89±0.03 ^a	1.76±0.02 ^b	1.81±0.03 ^b	0.05±0.01 ^c	0.03±0.02 ^c
Ethyl acetate (mg/L)	198.81±5.69 ^a	20.2±0.92 ^b	79.7±1.31 ^c	76.9±1.64 ^c	68.3±1.26 ^d
Esters acetate (mg/L)	33.13±1.47 ^a	1.87±0.08 ^b	15.14±0.64 ^c	12.72±0.73 ^d	13.67±0.81 ^c
Ethyl esters (mg/L)	1.12±0.09 ^a	2.19±0.21 ^b	2.41±0.16 ^b	1.94±0.08 ^b	1.45±0.07 ^c

Values are means ± standard deviations. Values displaying different superscript letters within each column are different according to the Tukey test.

K: *K. apiculata* mc1; S: *S. cerevisiae* mc2; O: *O. oeni* X₂L. From Mendoza and Farias [53].

Wines obtained from different microvinifications showed similar glycerol contents (8-8.5 g/L). This by-product concentration contributes to the sweetness, viscosity and smoothness of wine [16]. Acetic acid becomes unpleasant at concentrations near its flavor threshold of 0.7-1.0 g/L and usually values between 0.2 and 0.7 g/L are considered optimal [57]. Volatile acidity levels were higher in products fermented by pure cultures of non-*Saccharomyces* yeast or mixed yeast starter cultures and bacteria simultaneously inoculated and so it could affect the wine quality and

organoleptic characteristics. It has been reported that at high sugar concentrations the mostly heterofermentative wine LAB can produce high acetic acid concentrations through the sugar metabolism [58, 59]. While other studies suggest that simultaneous fermentations did not affect acetic acid levels [54, 60].

Acetaldehyde is an important flavor-active compound of fermentation which achieves average values range from 40 mg/L and about 80 mg/L for red and white wines, respectively [61]. The acetaldehyde amount in the different wines was dependent of starter culture utilized in the microfermentation. It was observed an important decrease of this metabolite in wines fermented simultaneously by yeasts and *O. oeni*. Then, it would indicate that this bacterium was able to metabolize acetaldehyde in these conditions. Jussier et al. [55] indicated that simultaneous fermentations displayed the lowest overall acetaldehyde concentrations during AF, likely due to the degradation of this compound by bacteria.

Wines which the MLF was induced after the AF showed a titratable acidity notably lower than other products fermented by mixed yeast starter cultures. This behavior could be related to *O. oeni* inoculation that allows increasing the wine pH because of L-malic acid consumption by bacteria. The main value of MLF is the biological deacidification which induces an increase in pH, improves microbial stability and changes wine taste [62, 63].

It is generally described that esters make the greatest contribution to the characteristic fruity odours of wine fermentation bouquet [64, 65]. Our results indicated that products fermented by composite cultures of *K. apiculata* and *S. cerevisiae* showed higher concentrations of acetate esters with regard to wines obtained by *S. cerevisiae* monocultures, showing that non-*Saccharomyces* yeasts could improve the wine aroma (Table 6). Different species of genera *Hanseniaspora/Kloeckera* showed to be strong producers of ethyl acetate and 2-phenylethyl acetate [65, 66]. Ethyl acetate, the main ester in wine, can impart a sour-vinegar off odour when the threshold taste level was surpassed (150-200 mg/L). Whereas at the levels of 80 mg/L could contribute to the fruity notes and add to the general complexity [8]. *K. apiculata* produced high concentrations of ethyl acetate in single cultures, while in mixed fermentations it was observed a reduction in production of this ester. With respect to ethyl esters the genus *Saccharomyces* is the best producer of ethyl caprylate and ethyl caproate [57, 65]. Our assays revealed that ethyl esters levels in mixed fermentations were similar to those formed by *S. cerevisiae* pure culture. Independently of bacterial inoculation time, products fermented by *O. oeni* and yeast cultures showed similar composition of aroma compounds and it was only observed a slight decrease. Matthews et al. [67] indicated that LAB possess esterase activities that could potentially alter the ester profile of wine. Zeeman et al. [68] reported a decrease in some esters following MLF. These results further confirm that wine LAB produce hydrolytic esterases and suggest LAB could have an impact on wine aroma and so in wine quality. Whether or not these activities are indicative of potential ester synthetic capability is yet to be determined.

In order to evaluate the influence of each starter culture on organoleptic quality of fermented products, the sensory analysis of different young wines was carried out by the tasting panel consisted of 6 judges trained in wine tasting. Intensity ratings of main descriptors were scored on scale from 0 (not perceivable) to 5 (very strong) (Fig. 4). It was observed that products obtained from mixed cultures non-*Saccharomyces* and *Saccharomyces* yeasts showed higher qualification for fruity aroma and equilibrium-harmony descriptors with regard to wines fermented by monoculture of *S. cerevisiae*. Products fermented by single inoculation of *K. apiculata* were described as the most intense of all in fruity character but the values for astringency and global evaluation were low, being considered faulty wines.

Others authors reported the wines production using mixed starter cultures. Patagonian indigenous *S. cerevisiae* MMf9 and β -glucosidase producer *C. pulcherrima* V6 strains were assayed at laboratory-scale fermentations [56]. The results evidenced a positive impact on the wine aroma when CpV6 strain was adequately combined with ScMMf9 one, enhancing its fruity and floral aroma. Similar results for foreign wines produced by *S. cerevisiae* in co-culture with non-*Saccharomyces* yeasts belonging mainly to genera *Hanseniaspora* and *Candida*, these products presented the highest total concentration of higher alcohol, esters and terpenols and the strongest aroma [9, 39].

Products fermented simultaneously by yeasts and *O. oeni* showed the highest score for phenolic aroma and consequently the lowest global evaluation. Whereas wines that were sequentially inoculated with malolactic bacteria had the highest acceptance, with better fruity and floral aromas and high scores of global descriptor. These results are disagreement with those reported by Massera et al. [54] who found that wines with simultaneous treatment showed enhanced sensorial attributes related to high quality wine like color and fruity flavor.

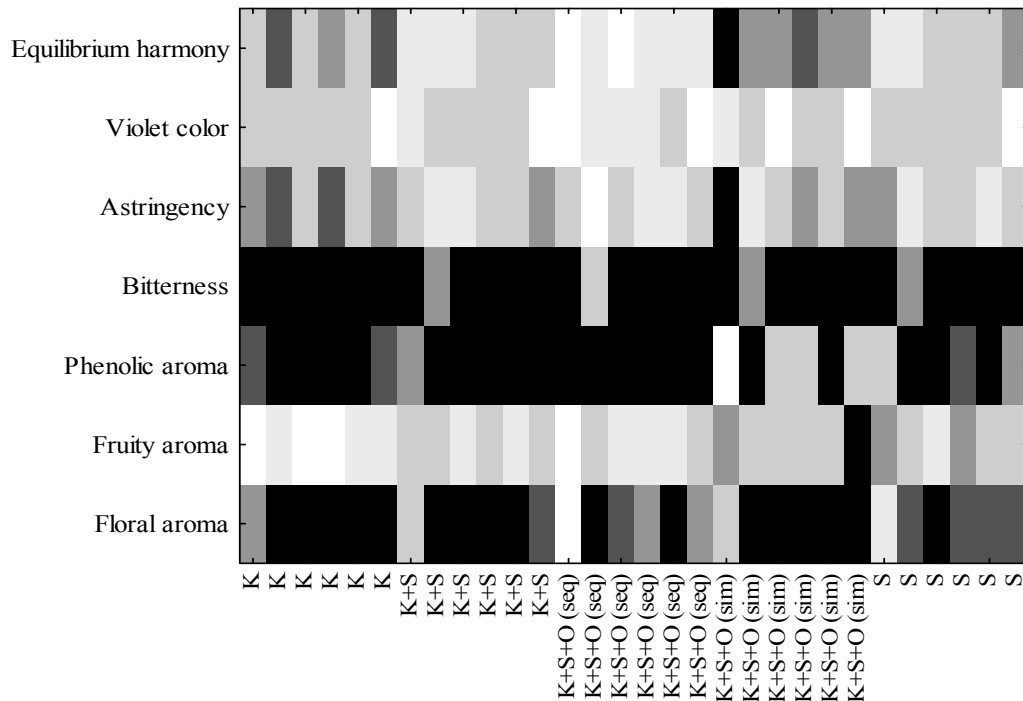


Figure 4. Diagram of the scores assigned by judges to each sensory descriptor of wines fermented by pure yeast cultures, mixed yeast culture or yeasts and malobacteria with simultaneous and sequential inoculation.

0 (■), 1 (■), 2 (■), 3 (■), 4 (■), 5 (□). K: *K. apiculata* mc1; S: *S. cerevisiae* mc2; O: *O. oeni* X₂L

In conclusion, we selected a non-*Saccharomyces* wine yeast, *K. apiculata* mc1, according to its adequate contribution to the wine organoleptic properties. In composite culture with a highly fermentative *S. cerevisiae* strain, both yeasts showed an appropriate kinetic and metabolic behavior. We proposed the inclusion of *K. apiculata* mc1 as adjunct culture of *S. cerevisiae* mc2 during must fermentation to improve the organoleptic characteristics of the fermented product. In addition, sequential inoculation of *O. oeni* X₂L after AF by yeast mixed culture allows better control on the sensory quality of wines produced from vineyards in Argentina Northwest.

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