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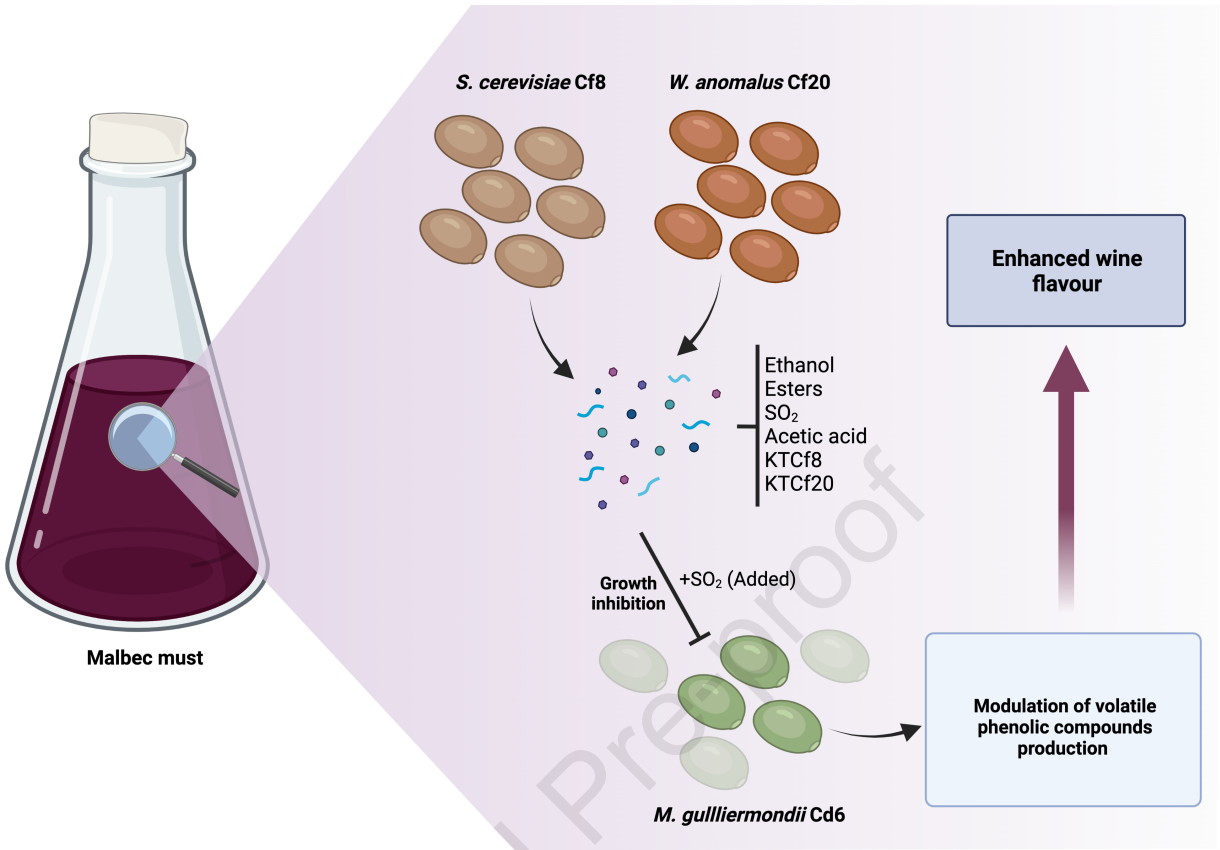

Killer yeasts used as starter cultures to modulate the behavior of potential spoilage non-*Saccharomyces* yeasts during Malbec wine fermentation

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Abstract

Yeast contamination is an important problem that affects wine production worldwide. In the present work, fermentative and biocontrol properties under winemaking conditions of the two killer strains, *Saccharomyces cerevisiae* Cf8 and *Wickerhamomyces anomalus* Cf20, were evaluated. *S. cerevisiae* Cf8 and its combination with *W. anomalus* Cf20 were able to effectively control the growth of *Meyerozyma guilliermondii* Cd6 at low SO_2 concentrations during Malbec must fermentation. Although the killer strain Cf8 alone exerted lower inhibitory activity, it modulated the growth of the strain Cd6, which positively influenced on wine aroma and complexity without being detrimental to product quality. Malbec wine produced by mixed culture Cf8 and Cd6 was the preferred one by the judges in the sensory analysis. To our knowledge, this is the first study made on red wines produced with indigenous killer yeasts from the Northwest Region of Argentina, as well as the first report of the modulation of potential spoilage yeasts into positive starters using killer yeasts in wine production. These results suggest that killer yeasts could be utilized as starter cultures to produce regional wines using low concentrations of SO_2.

Keywords: killer toxin; spoilage yeast; biocontrol agent; indigenous starters; Argentina wine

1. Introduction

Yeast contamination is a serious problem in winemaking process, which leads to important yield losses in wine industry. The main spoilage yeasts responsible for wine contamination belong to genera *Brettanomyces*/*Dekkera*, *Candida*, *Hanseniaspora*/*Kloeckera*, *Pichia*, *Meyerozyma*, *Schizosaccharomyces* and *Zygosaccharomyces* (Malfeito-Ferreira, 2019; Malfeito-Ferreira & Silva, 2019; Padilla et al., 2016). Their spoilage activities include ethanol consumption, fermentation arrestment, biofilm formation and production of undesirable compounds, which can be sub-classified in excessive volatile acidity (mainly acetic acid), high levels of volatile phenolic compounds, ethyl acetate or other esters (Malfeito-Ferreira, 2019; Sáez et al., 2010; 2011). Nevertheless, in the last years, it has been demonstrated that different non-*Saccharomyces* species, generally considered as spoilage yeasts, could have a desirable impact in winemaking under certain conditions of controlled growth and metabolism (Domizio et al., 2011; Padilla et al., 2017; Steensels et al., 2015). The development of spoilage yeasts in wine is often controlled through sulfur dioxide (SO_2). However, different yeast species and strains exhibiting high tolerance to this compound have been reported (Curtin et al., 2012). In addition, hypersensitivity to SO_2 in some wine consumers denotes the need to use other preservatives (Guerrero & Cantos-Villar, 2015; Vally et al., 2009). Chemical (e.g. sorbic acid and benzoic acid), physical (e.g. filtration, sanitization) and biological alternatives (e.g. chitosan) were tested with limited efficiency in controlling microbial contamination (Branco et al., 2021; Pinto et al., 2020; Suárez et al., 2007). Active phenolic compounds from plant extracts were also proposed to replace SO_2, as their effect has been demonstrated against acetic and lactic acid bacteria in wine preservation (García-Ruiz et al., 2012; Raposo et al., 2016).

For the last decades, killer toxins (KTs) produced by different yeast species have emerged as an interesting alternative (Branco et al., 2021; Comitini et al., 2021; Mehlomakulu et al.,

2015; Pinto et al., 2020). KTs are antimicrobial proteins that inhibit susceptible yeast strains, although producer strains remain immune to their own toxins (Lowes et al., 2000; Schmitt & Breinig, 2002). Several studies have reported the use of non-*Saccharomyces* KTs for inhibition of wine spoilage yeasts, as these toxins usually present broader inhibitory spectra and, in some cases, higher stability than KTs produced by *Saccharomyces cerevisiae* (Ciani & Comitini, 2011; Comitini et al., 2021; Fernández de Ullivarri et al., 2018; Villalba et al., 2016; Yamamoto et al., 1986). KT-producing *Tetrapisispora phaffii* and *Kluyveromyces wickerhamii* were able to control wine spoilage caused by the growth of *H. uvarum* and *Brettanomyces*/*Dekkera*, respectively (Ciani & Fatichenti, 2001; Comitini et al., 2004). The toxins named KwKt, PiKt, PMKT2 and KP6/KTs that are secreted by *K. wickerhamii*, *P. anomala*, *P. membranifaciens* and *Ustilago maydis*, respectively, inhibit the growth of *B. bruxellensis* and *D. bruxellensis* (Comitini et al., 2004; Mehlomakulu et al., 2015; Santos et al., 2009; 2011). Therefore, the use of killer strains and/or their KTs is a suitable option to reduce the addition of chemical preservatives and to control the growth of undesirable microorganisms during winemaking (Ciani & Comitini, 2011). In previous studies, we characterized the killer phenotype of *S. cerevisiae* Cf8 and *Wickerhamomyces anomalus* Cf20 and demonstrated their inhibitory activity against several wine spoilage yeasts (Fernández de Ullivarri et al., 2011; 2014; 2018). Wine production in Northwest Region represents approximately 6.7% of the total production of Argentina, being about 765,000 hl in 2022 (INV, 2023). Besides, Malbec (*Vitis vinifera* L.) is the red grape variety considered as the emblematic cultivar of Argentinean viticulture production and more than 50% of Malbec wine produced is exported (INV, 2023). In this work the aim was to evaluate the abilities of the killer strains, *S. cerevisiae* Cf8 and *W. anomalus* Cf20, to regulate the growth and metabolism of a potential spoilage non-*Saccharomyces* yeast under winemaking-like conditions during Malbec fermentation.

2. Materials and methods

2.1 Microorganisms and culture media

Strains, *S. cerevisiae* Cf8 and *W. anomalus* Cf20, previously selected for their fermentative and biocontrol properties under laboratory conditions (Fernández de Ullivarri et al., 2011; 2014; 2018), were used as KT producers. In addition, *Meyerozyma guilliermondii* Cd6 (formerly *Pichia guilliermondii*) was used because this species is considered a putative spoilage yeast of wines if its growth is not controlled; e.g., some strains produce high levels of acetate esters, acetaldehyde and 4-vinylphenol, and this species can produce high levels of citric acid from glucose (Benito et al., 2011; Lopes et al., 2009; Malfeito-Ferreira & Silva, 2019; Wrent et al., 2016). All these yeast strains were isolated from cellars from the Northwest region of Argentina (Cafayate, Salta). Furthermore, the strain *Oenococcus oeni* X_2L was used to conduct malolactic fermentation (MLF) according to previous works (Mendoza et al., 2011; Strasser de Saad & Manca de Nadra, 1987).

Yeasts were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), all components were obtained from Britania (Buenos Aires, Argentina), while *O. oeni* was cultured in MRStj (MRS medium supplemented with 150 mL/L natural tomato juice) that was purchased from Merck (Darmstadt, Germany). For solid media, broths were supplemented with 20 g/L agar (Britania).

For the killer activity assay, YPD-MB agar (YPD agar supplemented with 30 mg/L methylene blue) buffered at pH 4.2 was used. For the differential enumeration of yeasts, YNB (Difco, New Jersey, USA) agar plates supplemented with 20 g/L inulin or 20 g/L glycerol (Sigma Aldrich Co., St. Louis, MO, USA) were used as selective media for *M. guilliermondii* alone or *M. guilliermondii* and *W. anomalus*, respectively, whereas yeasts total count was performed in WLN (Merck).

Yeasts and *O. oeni* X_2L were maintained in YPD and MRS, respectively, supplemented with 20% glycerol at -80 ºC.

2.2 Pre-adaptation of microorganisms and grape must preparation for fermentation

Red grape must containing skins and seeds was prepared using Malbec variety grapes (*Vitis vinifera* L.) from DOC San Rafael, Mendoza, Argentina. Grapes were pressed by hand extrusion using sterilized latex gloves at 20 °C. The prepared must contained 230 g/L sugars, 4.2 g/L titratable acidity, 1.42 g/L malic acid, pH 4.1. After the crushing, 50 mg/L metabisulfite was added to the must.

Erlenmeyer flasks (100 mL) containing 80 mL of pasteurized grape juice, diluted 1:2 with sterile water and adjusted to pH 4.0, were inoculated with 2 mL (~10^8 CFU/mL) of cultures of each microorganism and incubated at 28 °C during 24 or 48 h for yeasts and bacterium, respectively. Cultures were then centrifuged at 10,000 × g for 10 min at 25 °C (Presvac, DCS-16RTV, Buenos Aires, Argentina) and cells were suspended in 5 mL of sterile grape juice.

2.3 Fermentation conditions

For alcoholic fermentation (AF), 800 mL of Malbec must in 1 L Erlenmeyer flasks were inoculated with pre-adapted cultures of *S. cerevisiae* Cf8 (Sc), *W. anomalus* Cf20 (Wa) and *M. guilliermondii* Cd6 (Mg) at 1×10^6 CFU/mL. After AF, *O. oeni* X_2L (Oo) was inoculated at 5×10^6 CFU/mL to conduct MLF, according to Mendoza et al. (2011). Vinifications were performed in duplicate with the following inoculum combinations: 1) Sc; 2) Sc+Wa; 3) Sc+Wa+Mg; 4) Sc+Mg; 5) Sc+Wa+Oo; 6) Sc+Oo; 7) Mg. In addition, a spontaneous fermentation was carried out as control of native yeasts from grape must (Supplementary Figure S1). Flasks were aseptically stoppered with a valve containing sulfuric acid to allow only CO_2 to escape from the system (Ciani & Rosini, 1987) and incubated at 25 ºC. Weight loss was monitored for several days until the end of the fermentation (constant weight for two

consecutive days). Fermentations were carried out under static conditions with a round of agitation every 24 h. After AF, skins and seeds were separated from wines, and MLF was carried out in the two combinations inoculated with *O. oeni* X_2L. Samples were taken every day for microbiological, analytical and colorimetric determinations. After AF and/or MLF, wines were statically rested for 24 h and then the liquid was carefully separated from sediments.

2.4 Microbial counts

Cell counts were performed by the serial dilutions method. For total yeasts counts during AF, samples were cultured on WLN agar whereas for differential enumeration the media YNB-I (inulin for *M. guilliermondii* Cd6) and YNB-G (glycerol for *M. guilliermondii* Cd6 and *W. anomalus* Cf20) were used. Plates were incubated at 25 ºC for 48 h and cell counts for *S. cerevisiae* (Sc), *W. anomalus* (Wa) and *M. guilliermondii* (Mg) were obtained according to the formulas $Sc = N_{WLN}-N_{YNB-G}$; $Wa = N_{YNB-G}-N_{YNB-I}$; $Mg = N_{YNB-I}$, where N is the count for each medium. The selected media allowed differential counts of yeasts at 48 h of incubation taking in account that *S. cerevisiae* Cf8 grew very slowly in YNB-Glycerol medium (after 4 days of incubation at 25 °C) and the killer strains (Cf8 and Cf20) were not able to utilize inulin under the tested conditions. For the count of *O. oeni* X_2L during MLF, samples were spread on MRStj agar supplemented with cycloheximide (100 mg/L) and incubated at 25 ºC for 5 days in a 5% CO_2 atmosphere.

2.5 Implantation of inoculated yeasts

The inoculated strains were genetically typified to confirm their ability to dominate the fermentation. For *S. cerevisiae* typing, amplification of inter-delta regions was carried out employing primers (delta12 and delta21) and protocols described by Legras and Karst (2003) whereas the non-*Saccharomyces* strains, Cf20 and Cd6, were characterized by RAPD-PCR using the M13 primer (Huey & Hall, 1989).

Ten colonies from each sample were randomly taken from enumeration plates of different media after 1, 3, 6 and 10 days of AF to obtain DNA from each culture. PCR products were separated by electrophoresis on 2% agarose gels. Comparison among the typing profiles obtained from colonies with those of the pure inoculated strains was performed to test the implantation.

2.6 Killer activity

Killer activity (KA) of wine samples was evaluated by a diffusion plate method in YPD-MB pH 4.2 using *M. guilliermondii* Cd6 (2×10^6 CFU/mL) as the sensitive strain (Fernández de Ullivarri et al., 2014). Wines samples (1 mL) were centrifuged twice at 8,000 × g, 10 min, 25 °C (Spectrafuge 24D, Labnet International, New Jersey, USA) to separate yeast cells from the wine, then 100 µL aliquots of the supernatant were seeded on the agar and plates were incubated for 48 h at 25 ºC. Heat-treated supernatants (100 ºC, 10 min) were used as negative controls of inhibitory activity produced by KTs present in the fermented musts. The diameter of the inhibition zones was measured with a caliper. KA was defined as arbitrary units (aU) per mL and was calculated using the formula: KA (aU/mL) = $10^{(D+5.64)/6.64}$, where D is the diameter of the inhibition zone in millimeters and 1 aU is the amount of toxin capable of producing a clear inhibition zone of 1 mm in diameter.

2.7 Analytical determinations

Glucose, fructose, glycerol, ethanol, acetic acid and malic acid were analyzed using enzymatic test kits (R-Biopharm AG, Darmstadt, Germany). Titratable acidity was measured with acid-base titration with standardized 0.1 M NaOH. Colorimetric determinations of wines were carried out measuring their absorbance on centrifuged (3,000 ×g, 5 min at 4 °C) samples, with 1-mm pathlength glass cells. The absorbance (A) of the samples was measured at 420, 520, and 620 nm in a spectrophotometer. Color intensity, tonality and red color pigments (% dA) were calculated according to the equations (*A420 + A520 + A620*), (*A420* /

182 *A520*) and [*A520* – (*A420* + *A620*) / 2] × (1/ *A520*) × 100, respectively (Glories, 1984; Pérez-
183 Magariño & González-San José, 2006).
184 Volatile compounds (esters and high alcohols) in wine samples were quantified by gas
185 chromatography (GC) using a flame ionization detector (FID) and an HP-5 column (length 30
186 m, i.d. 0.32 mm, thickness 0.25 µm), and following the protocol described by Mendoza et al.
187 (Mendoza et al., 2011). Odor activity value (OAV) was calculated as the mean concentration
188 of an aroma compound divided by its odor threshold value, published in the scientific
189 literature (Cortés-Diéguez et al., 2015; Welke et al., 2014).
190 *2.8 Sensory analysis*
191 Sensory descriptive analysis of the young wines (1 month after bottling) was carried out by a
192 tasting panel that consisted of ten trained judges (Facultad de Ciencias Aplicadas a la
193 Industria, Universidad Nacional de Cuyo and Instituto Nacional de Vitivinicultura, San
194 Rafael, Mendoza, Argentina). Wines were equilibrated at room temperature (18-20 °C) and
195 50 mL-samples were poured into randomly numbered wineglasses. To diminish the residual
196 effect between samples, judges washed their mouths with mineral water and ate unsalted
197 bread. The intensity of each descriptor was rated on a scale from 0 (not perceivable) to 5
198 (very strong). Fluidity, limpidity, color (intensity and tonality), floral, fruity, phenolic aroma
199 and others, astringency, bitterness, body, complexity and equilibrium-harmony were tested
200 (Noble et al., 1987; Stone et al., 2012).
201 *2.9 Statistical analysis*
202 After testing for normal distribution (Shapiro-Wilks test), homogeneity of variance (Levene's
203 test), and independence of the experimental data, ANOVA was performed and Tukey test
204 was carried out as post-hoc test for multiple mean comparisons, and $p \leq 0.05$ was considered
205 significant. Statistical analysis was performed with Infostat software (Version 2020, Infostat,
206 Córdoba, Argentina, https://www.infostat.com.ar).

3. Results and discussion

3.1 Fermentation kinetics, evolution of biomass and killer activity

Vinifications of Malbec musts were conducted with different starter cultures at 25 ºC. CO_2 release measurements indicated that almost all vinifications showed similar fermentation rates (Supplementary Figure S1). AF was completed after 11 days of incubation, with exception of the trial conducted with the pure culture of *M. guilliermondii*, whose fermentation rate and CO_2 production were lower than the other trials and its AF stuck after 6 days. Most of non-*Saccharomyces* wine-related species showed limited fermentation aptitudes which led to an incomplete AF in absence of a starter culture of *S. cerevisiae* (Ciani et al., 2006; Rodríguez et al., 2010).

During AF, cell populations of *S. cerevisiae* Cf8 and *W. anomalus* Cf20 were about 5×10^8 CFU/mL at day 1 in mixed trials. *S. cerevisiae* Cf8 was not inhibited by the presence of the killer strain *W. anomalus* Cf20, and its cell counts stayed at similar levels (10^8 CFU/mL) for 11 days (Figure 1A). However, *W. anomalus* showed a loss of viability after 6 days with cell counts about 10^6 CFU/mL at the end of fermentation (Figure 1B). In a previous study, it was demonstrated that *W. anomalus* Cf20 in co-culture with *S. cerevisiae* Cf8 showed a loss of viability whereas in pure culture the strain Cf20 did not lose viability during 10 days of Malbec fermentation (Fernández de Ullivarri et al., 2018). These results are consistent with previous studies of wine yeasts in mixed cultures, which reported that different non-*Saccharomyces* species only grow during the early stages of fermentation (Domizio et al., 2011; Padilla et al., 2017). Moreover, *W. anomalus* is not tolerant to high concentrations of ethanol (Passoth et al., 2006). In this study, a probable synergistic effect of ethanol and toxin Cf8 could also be responsible for the viability loss of strain Cf20 during fermentation process. Regarding the spontaneous fermentation, its kinetics was like those inoculated with

S. cerevisiae whereas the total yeasts count was lower during the first days showing a maximal population of 1×10^8 CFU/mL at 6 days (Supplementary Figure S2).

Moreover, typing analysis was performed to verify if inoculated strains were implanted in non-sterile grape must. *S. cerevisiae* Cf8 strain was able to implant in Malbec wine after 1 day of fermentation, being its profile the most abundant. For strains Cf20 and Cd6, dominance of their profiles was also found (Supplementary Figure S3).

On the other hand, the evolution of *M. guilliermondii* Cd6 microbial loads was evaluated in pure and mixed trials (Figure 2). At day 1, in mixed culture with *S. cerevisiae* Cf8 (Sc+Mg), the non-*Saccharomyces* yeast showed similar cell counts to pure culture (Mg). However, in the trial conducted by *S. cerevisiae* Cf8 and *W. anomalus* Cf20 (Sc+Wa+Mg), the cell population of the strain Cd6 was 2 log cycles lower. After 3 days, *M. guilliermondii* Cd6 lost viability in both mixed trials, possibly due to the presence of killer toxins and ethanol in these wines. At the end of each fermentation, a lower viability of the strain Cd6 was observed in wine Sc+Wa+Mg in correlation with its higher killer activity (4×10^4 aU/mL) respect to wine Sc+Mg (KA=3.6×10^3 aU/mL) (Figure 2). Thus, the toxin produced by *W. anomalus* Cf20 in mixed culture with *S. cerevisiae* could be responsible for the major effect on *M. guilliermondii* Cd6. It should be noted that the killer activity in both wines was maximal at 6 days and remained stable during the rest of the fermentation process. As shown in Figure 3, the KTs were produced during the wine fermentation conducted by Sc+Wa+Mg, with increasing levels as the fermentation progressed. These results point out the presence of *W. anomalus* Cf20 as adjunct starter of *S. cerevisiae* Cf8 in order to control potential wine spoilage from the beginning to the end of fermentation. Several authors have demonstrated the biocontrol potential of killer yeasts against wine undesirable yeasts in vitro (Błaszczyk et al., 2015; Comitini et al., 2004; Fernández de Ullivarri et al., 2014; Kuchen et al., 2019; Santos et al., 2011; Villalba et al., 2016). However, to our knowledge, only a few studies

have evaluated the biocontrol activity of wine yeasts during grape must fermentation (Branco et al., 2021; Comitini et al., 2021; Comitini & Ciani, 2011; Santos et al., 2011).

3.2 Chemical analysis of young wines

Table 1 shows the analytical profile of wines obtained using different starter cultures. Sugars were completely consumed in all musts and dryness was achieved at the end of AF, except for the must fermented by the pure culture of *M. guilliermondii* Cd6 (data not shown). Ethanol concentrations reached values of ~12.2%, being statistically similar in all wines. In general, wines obtained by mixed cultures of *S. cerevisiae* and non-*Saccharomyces* yeasts show lower ethanol content than those fermented only by *S. cerevisiae* (Ciani et al., 2016). However, glycerol concentrations were higher (~8.7 g/L) in wines fermented by starter cultures formulated with the strain *W. anomalus* Cf20. This species is capable of producing elevated amounts of glycerol and arabinitol in high-osmolarity and low-oxygen media, which accumulates in the cell interior and in the culture media (Passoth et al., 2006).

Values between 0.2 and 0.7 g/L are usually considered adequate for acetic acid, as the main component of volatile acidity, which becomes unpleasant at concentrations near its sensory threshold (> 0.75 g/L) (Ribéreau-Gayon et al., 2006). Fermentations conducted by pure cultures of *S. cerevisiae* Cf8 presented lower levels of volatile acidity than those conducted by mixed cultures. Wines named Sc+Wa and Sc+Wa+Oo showed levels slightly above desirable (0.84 g/L and 0.77 g/L, respectively). This might be due to the metabolism of *W. anomalus*, since this species is a producer of high acetic acid concentrations in musts (Passoth et al., 2006). However, the presence of *M. guilliermondii* Cd6 in both mixed fermentations (Sc+Mg and Sc+Wa+Mg) produced adequate levels of volatile acidity (0.40 and 0.46 g/L, respectively), which could mean that the presence of this strain is positive, something that is not always connected to it. Finally, as expected, wines where MLF took

place by the inoculation of *O. oeni* X_2L showed significantly lower titratable acidity as well as higher pH than wines produced without MLF.

With regard to colorimetrical analysis, the wines exhibited remarkable values of color intensity (1.4-2.4) with optimal ranges for tonality (0.55-0.62), similar to those found in market wines. Regarding the proportion of red pigments (% dA), the wines presented desirable values (> 45%) for this attribute, with exception of wine Sc+Wa. Most wines achieved a value for red pigments of about 60%, indicating that they presented a desirable bright red color.

Esters and higher alcohols play an important role on the sensorial profile of wines since they are widely responsible for their fruity and floral aroma (Escudero et al., 2007; Moio et al., 2004). Table 2 presents a breakdown of the concentrations of key aroma compounds found in wines that were fermented using both mixed yeast cultures and a monoculture of *M. guilliermondii* Cd6 (Mg). Notably, wines produced from Mg demonstrated elevated levels of ethyl acetate, reaching as high as 124.4 mg/L, which resulted in a pronounced solvent-like off-odor. It has been reported that ethyl acetate, at levels below 80 mg/L, contributes to fruity notes and general complexity (Romano et al., 2003; Sumby et al., 2010). Conversely, all wines fermented with mixed yeast cultures displayed significantly lower concentrations of ethyl acetate, ranging from 11.2 to 17.5 mg/L. Thus, non-*Saccharomyces* species present in the mixed cultures were able to produce desirable levels of this ester. The co-cultures that included Mg (Sc+Mg and Sc+Wa+Mg) consistently achieved higher yet desirable levels of ethyl acetate.

The second most abundant ester was 2-phenethyl acetate, which contributes to floral and fruity notes (Styger et al., 2011). The wine Mg showed an excessive concentration of this ester (23.25 mg/L). Wines elaborated with *W. anomalus* Cf20 presented higher values of this compound (7.61 mg/L) compared to Sc+Mg, in which a lower concentration was found (4.33

307 mg/L). Nevertheless, regarding ethyl caprylate, the highest concentrations (3.31 and 3.35
308 mg/L) were found in wines Sc+Wa+Mg and Sc+Mg, respectively, probably due to the
309 presence of *M. guilliermondii* Cd 6. Diverse studies have demonstrated that non-
310 *Saccharomyces* yeasts, including *M. guilliermondii*, are good esters producers, as their
311 production includes ethyl acetate, 2-phenethyl acetate and isoamyl acetate and diverse ethyl
312 esters (Mendoza et al., 2011; Rodríguez et al., 2010; Viana et al., 2008; 2009).
313 Regarding higher alcohols in wines, it is known that they derive from the glucides and amino
314 acids, which intervene directly on wine organoleptic characteristics. Compounds as 3-methyl-
315 butanol confer a desirable aroma if the concentrations are below 400 mg/L (Ribéreau-Gayon
316 et al., 2006). Regardless the starter cultures, wines showed adequate concentrations of 3-
317 methyl-1-butanol that ranged between 352 and 383 mg/L and low concentrations of trans-2-
318 hexen-1-ol.
319 Volatile compounds were detected at different concentration levels; nevertheless, not always
320 higher concentrations compounds had more impact on the overall wine aroma. The
321 contribution of each volatile compound to wine aroma can be evaluated quantitatively by
322 means of its odor activity value (OAV). Thus, to evaluate the most active odorants in Malbec
323 wines, the concentration of each volatile compound was correlated with its threshold value
324 and the OAVs were calculated (Table 2). The wine Mg showed excessively high OAVs for
325 ethyl acetate (OAV= 16.6) and 2-phenethyl acetate (OAV= 93), confirming the off-odor
326 production by the generation of these compounds in a disproportionated high concentrations.
327 The majority of volatile compounds showed OAV>1, which were deemed to contribute to
328 wine aroma (Guth, 1997). We found that the global aroma of all four mixed wines was
329 dominated by ethyl and acetate esters that conferred them with fruity notes. As shown in
330 Table 2, ethyl caprylate (OAV= 164-670), followed by 2-phenethyl acetate (OAV= 17-30),
331 ethyl caproate (OAV= 13-18) and isoamyl acetate (OAV= 9-15) contributed favorably to

wine aroma with fruity nuances. Ethyl acetate (OAV= 2) also contributed to a lesser extent to the overall fruity aroma. With regard to higher alcohols, 3-methyl-1-butanol (OAV= 12-13) showed high OAVs and also enhanced the fruity notes, whereas trans-2-hexen-1-ol was not found to be an active odorant (OAV<1).

3.3 Sensorial analysis of young wines

The sensorial analysis was carried out in order to evaluate the influence of each starter culture on the organoleptic quality of the obtained wines. General sensory descriptors related to sight, smell and taste of young wines were considered (Figure 4). It is worth noting that the wine Mg showed easily detectable spoilage characteristics, such as white biofilm on top of the liquid as well as strong solvent odor and sour flavor, possibly due to high concentrations of esters and citric acid production by the strain Cd6. For this reason, this wine was not evaluated by the panel. Wines conducted by Sc+Wa and Sc+Wa+Oo cultures were described as lacking in complexity in mouth possibly related to the higher concentrations of acetic acid (0.84 and 0.77 mg/L, respectively). This characteristic could be due to the cell concentration of *W. anomalus* Cf20 achieved during these fermentations (5×10^8 CFU/mL). Judges remarked the attributes of those wines obtained with the inoculation of *M. guilliermondii* Cd6 (Sc+Wa+Mg and Sc+Mg). These wines showed excellent violaceous red color as well as red fruits and dry plum aromas, this is probably related to higher esters concentrations. Malbec wine obtained by mixed culture Sc+Mg was the preferred one by the judges, showing the highest scores for all descriptors (Figure 4).

Several authors demonstrated that metabolism of yeasts in must might be reciprocally modulated in presence of other yeast species (Bely et al., 2003; Ciani et al., 2016; Comitini et al., 2011; Csoma et al., 2021). In addition, different non-*Saccharomyces* species that are generally considered as putative spoilage yeasts could have a desirable behavior during

fermentation under certain conditions (Malfeito-Ferreira & Silva, 2019; Steensels et al., 2015). It has been reported that most of the compounds normally produced at high concentrations by pure cultures of non-*Saccharomyces*, and which are considered detrimental to wine quality, do not reach threshold taste levels in mixed fermentations (Domizio et al., 2011; Mendoza et al., 2011; Rodríguez et al., 2010). In this context, the killer toxins produced during fermentation process could control the growth of *M. guilliermondii* Cd6 and consequently modulated its metabolism, improving some sensorial characteristics of the final product compared to wines where this strain was absent.

4. Conclusions

The present study suggests that killer yeasts are potential biocontrol agents in winemaking process using low concentration of SO_2. Killer strains utilized as starters during Malbec must fermentation controlled the growth of *M. guilliermondii* Cd6, a non-*Saccharomyces* species normally considered as a putative spoilage. Moreover, *S. cerevisiae* Cf8 alone was able to positively control *M. guilliermondii*, although its inhibitory activity was lower than it in combination with *W. anomalus* Cf20. The studied yeasts in this work demonstrated an adequate performance in non-sterile Malbec must, as a laboratory-scale approach of wine-making conditions, which validates its use for further studies in other musts and scales. Large-scale experiments should be carried out to confirm the behavior of the killer starter cultures proposed in this work. To our knowledge, this is the first study on red wines produced with indigenous killer yeasts from the Northwest of Argentina, a region with growing oenological relevance.

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617 **Figure captions**

618 **Figure 1.** Viable cell counts of *S. cerevisiae* Cf8 (A) and *W. anomalus* Cf20 (B) in pure
619 culture (●), mixed *S. cerevisiae*/*W. anomalus* culture (▲), mixed *S. cerevisiae*/*W.*
620 *anomalus/M. guilliermondii* culture (∆), mixed *S. cerevisiae/M. guilliermondii* culture (○).
621 Values represent the mean of two independent experiments. Linear vertical bars represent
622 standard deviation.

623 **Figure 2.** Viable cell counts of *M. guilliermondii* Cd6 (—) and killer activity (---) in Malbec
624 wines obtained using mixed *S. cerevisiae*/*W. anomalus/M. guilliermondii* culture (◆,◇),
625 mixed *S. cerevisiae/M. guilliermondii* culture (□,■) and pure *M. guilliermondii* culture
626 (▲,△).Values represent the mean of two independent experiments. Linear vertical bars
627 represent standard deviation.

628 **Figure 3.** Killer activity against *M. guilliermondii* Cd6 of Malbec must fermented by mixed
629 culture of *S. cerevisiae*/*W. anomalus/M. guilliermondii* after 1, 2, 3, 4 and 5 days in YPD-MB
630 pH 4.5 at 20 °C.

631 **Figure 4**. Cobweb graph of scores obtained from sensory analysis for wines fermented by
632 mixed *S. cerevisiae*/*W. anomalus*/*M. guilliermondii* culture (○); mixed *S. cerevisiae/W.*
633 *anomalus* (▲); mixed *S. cerevisiae*/*M. guilliermondii* culture (□); mixed *S. cerevisiae/W.*
634 *anomalus*/*O. oeni* X_2L (♦) in Malbec must at 25 ºC. (*): descriptors with significant
635 difference among all wines; (a): descriptors with significant difference for Sc+Mg; (b):
636 descriptors with significant difference for Sc+Wa+Mg; (c): descriptors with significant
637 difference for Sc+Wa+Oo (Tukey test $p < 0.05$).

638

639 **Table 1.** General characteristics of wines fermented by different starter cultures.

Analytical determinations*	**Starter cultures**					
	Sc	Sc+Wa	Sc+Wa+Mg	Sc+Mg	Sc+Wa+Oo	Sc+Oo
Residual sugars (g/L)	0.54 ± 0.08^{b}	0.84 ± 0.05^{c}	0.90 ± 0.04^{c}	0.50 ± 0.05^{b}	0.36 ± 0.04^{a}	0.59 ± 0.07^{b}
Ethanol (% v/v)	12.45 ± 0.2^{a}	12.01 ± 0.34^{a}	11.89 ± 0.23^{a}	12.47 ± 0.44^{a}	11.94 ± 0.41^{a}	12.36 ± 0.6^{a}
Glycerol (g/L)	6.73 ± 0.19^{a}	8.98 ± 0.25^{c}	8.75 ± 0.30^{c}	7.24 ± 0.26^{b}	8.41 ± 0.22^{c}	6.87 ± 0.23^{a}
Acetic acid (g/L)	0.34 ± 0.04^{a}	0.84 ± 0.04^{c}	0.46 ± 0.02^{b}	0.40 ± 0.06^{b}	0.77 ± 0.04^{c}	0.41 ± 0.04^{b}
Malic acid (g/L)	1.11 ± 0.02^{b}	1.27 ± 0.01^{c}	1.20 ± 0.03^{bc}	1.17 ± 0.03^{b}	0.05 ± 0.02^{a}	0.05 ± 0.02^{a}
Titratable acidity (g/L)	5.20 ± 0.31^{b}	5.80 ± 0.22^{b}	5.90 ± 0.26^{b}	5.40 ± 0.34^{b}	4.10 ± 0.23^{a}	4.00 ± 0.25^{a}
pH	3.77 ± 0.04^{b}	3.75 ± 0.03^{b}	3.77 ± 0.03^{b}	3.75 ± 0.02^{b}	3.82 ± 0.03^{a}	3.88 ± 0.02^{a}
Color intensity	1.70 ± 0.07^{a}	2.39 ± 0.16^{c}	1.50 ± 0.08^{a}	1.49 ± 0.26^{a}	1.89 ± 0.2^{b}	1.44 ± 0.16^{a}
Tonality	0.59 ± 0.03^{ab}	0.55 ± 0.02^{a}	0.61 ± 0.03^{b}	0.62 ± 0.04^{b}	0.55 ± 0.03^{a}	0.60 ± 0.08^{ab}
% dA	58.01 ± 0.43^{c}	43.57 ± 0.32^{a}	58.86 ± 0.35^{c}	56.61 ± 0.22^{d}	62.89 ± 0.57^{b}	58.66 ± 0.68^{c}

640 Data are mean values of two experiments ± standard deviation. Mean values with different superscript
641 letters within the same row are significantly different according to the Tukey test ($p \leq 0.05$).
642 Sc, *S. cerevisiae* Cf8; Wa, *W. anomalus* Cf20; Mg, *M. guilliermondii* Cd6, Oo, *O. oeni* X_2L.

643 *Evaluated in the finished wines

644 **Table 2.** Esters and higher alcohols concentrations (mg/L) and their odor activity values (OAV) in wines fermented by mixed starter cultures and

645 *M. guilliermondii* Cd6 in pure culture.

	Odor threshold (mg/L)	Starter cultures									
		Sc+Wa		**Sc+Wa+Mg**		**Sc+Mg**		**Sc+Wa+Oo**		**Mg**	
		Mean ± SD	OAV	Mean ± SD	OAV	Mean ± SD	OAV	Mean ± SD	OAV	Mean ± SD	OAV
Ethyl acetate	7.5	11.45 ± 0.09^{a}	1.5	12.84 ± 0.09^{b}	1.7	17.53 ± 0.08^{c}	2.3	11.21 ± 0.07^{a}	1.5	124.41 ± 1.13^{d}	16.6
Isoamyl acetate	0.03	0.35 ± 0.01^{b}	11.7	0.26 ± 0.01^{a}	8.7	0.44 ± 0.06^{c}	14.7	0.35 ± 0.05^{b}	11.7	0.38 ± 0.05^{b}	12.6
Ethyl caproate	0.014	0.18 ± 0.01^{a}	12.9	0.20 ± 0.02^{b}	14.3	0.25 ± 0.03^{c}	17.9	0.25 ± 0.01^{c}	17.9	0.17 ± 0.02^{a}	12.1
Ethyl caprylate	0.005	0.93 ± 0.05^{b}	186.0	3.21 ± 0.02^{c}	642.0	3.35 ± 0.03^{c}	670.0	0.82 ± 0.03^{a}	164.0	3.72 ± 0.05^{c}	744
2-phenethyl acetate	0.25	7.61 ± 0.08^{b}	30.4	7.14 ± 0.03^{b}	28.6	4.33 ± 0.03^{a}	17.3	7.33 ± 0.03^{b}	29.3	23.25 ± 0.53^{c}	93
3-methyl-1-butanol	30	383.54 ± 0.43^{d}	12.8	352.33 ± 0.62^{a}	11.7	365.24 ± 0.51^{c}	12.2	358.65 ± 0.71^{b}	12.0	369.11 ± 0.78^{c}	12.3
Trans-2-hexen-1-ol	0.4	0.12 ± 0.01^{a}	< 1	0.27 ± 0.03^{b}	< 1	0.34 ± 0.04^{c}	< 1	0.14 ± 0.02^{a}	< 1	0.29 ± 0.04^{b}	< 1

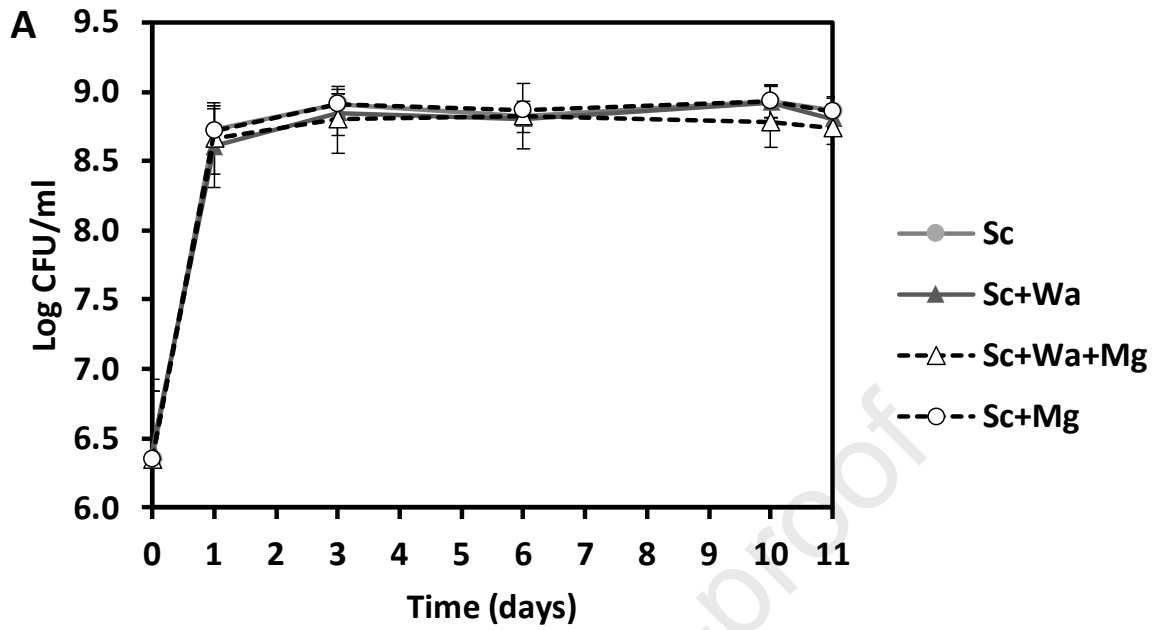
646 Data are mean values of two experiments ± standard deviation (SD). Mean values with different superscript letters within the same row are significantly

647 different according to the Tukey test ($p \leq 0.05$).

648 Sc, *S. cerevisiae* Cf8; Wa, *W. anomalus* Cf20; Mg, *M. guilliermondii* Cd6, Oo, *O. oeni* X_2L.

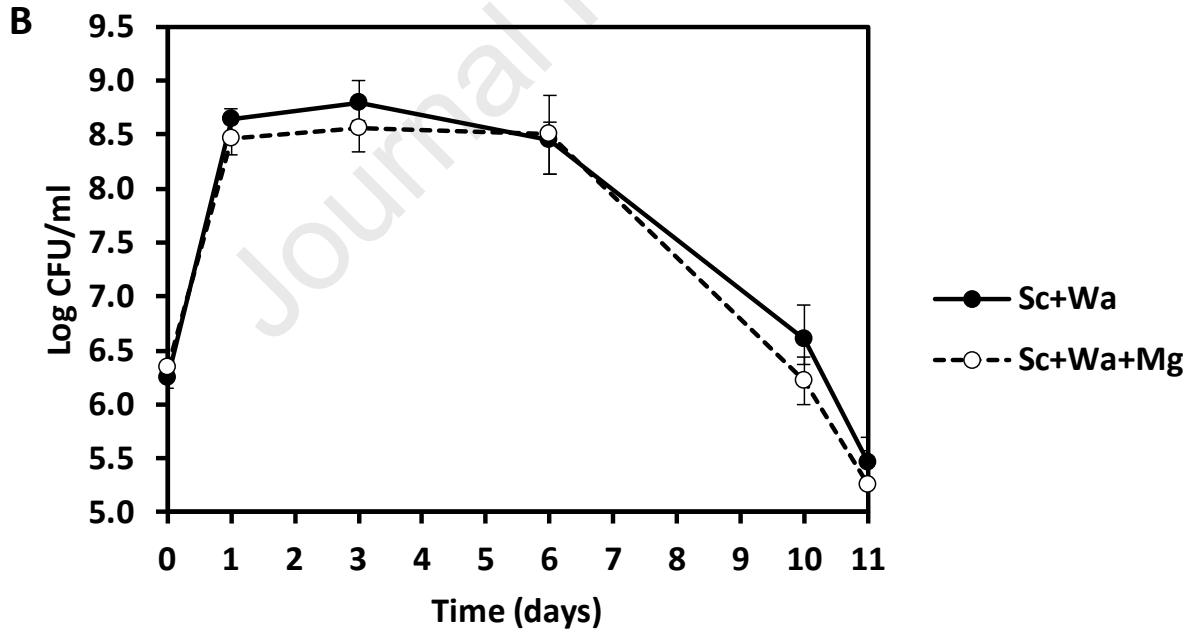
649

650 **Fig. 1A**



651

652 **Fig. 1B**



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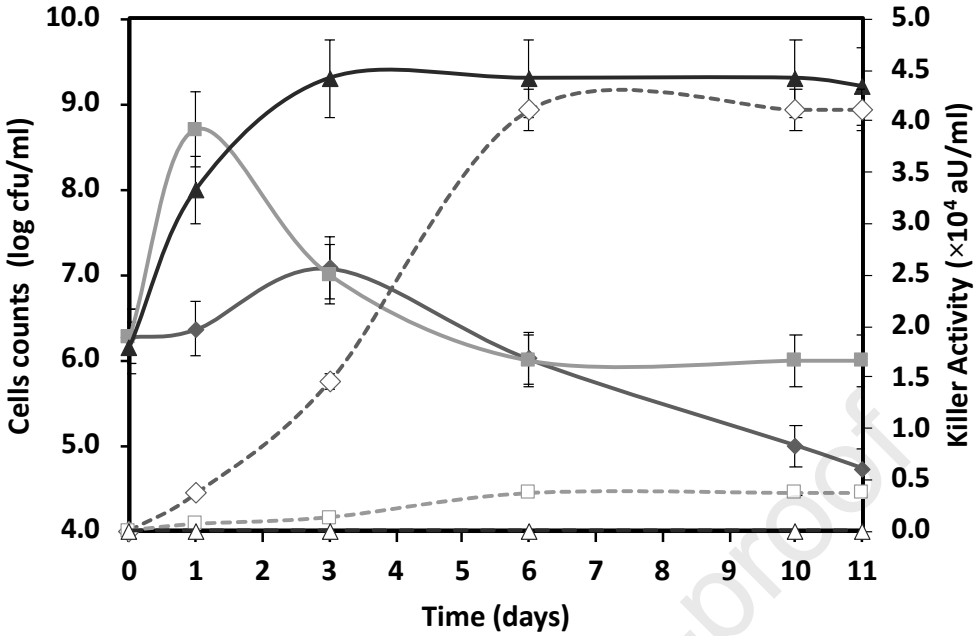
654

655 **Fig. 2**



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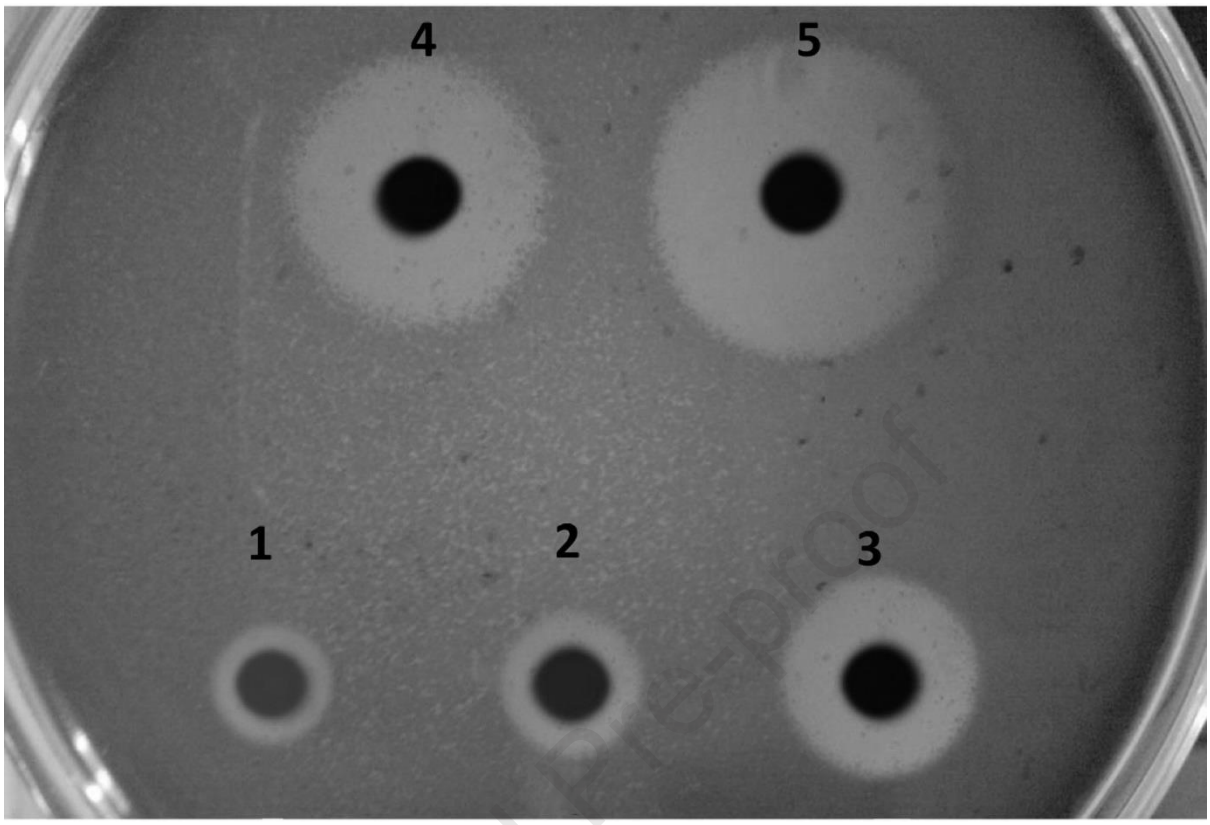
657

658 **Fig. 3**



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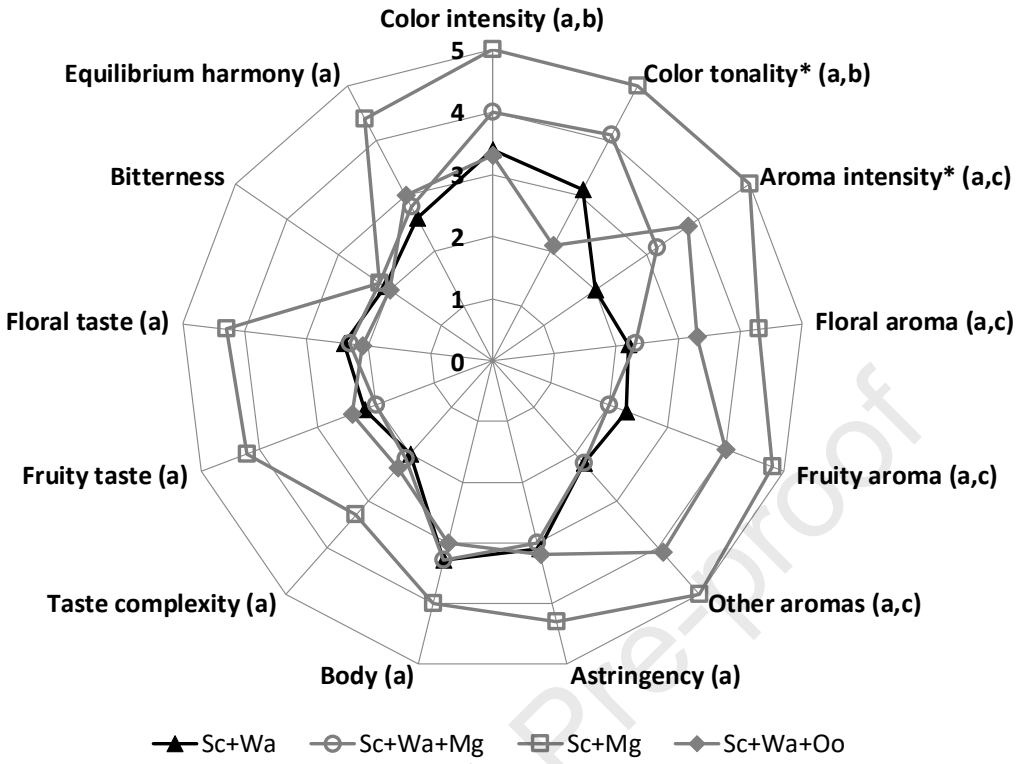
660

661 **Fig. 4**



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Highlights

- Killer yeast strains, Cf8 and Cf20, modulated the growth and metabolism of *M. guilliermondii* Cd6.
- Malbec wines produced by Cf8+Cd6 and Cf8+Cf20+Cd6 were the most appreciated after sensorial analyses.
- Killer yeasts could be used as starter cultures to elaborate regional wines.

Declaration of Interest Statement

Manuscript title: **Killer yeasts used as starter cultures to modulate the behavior of potential spoilage non-*Saccharomyces* yeasts during Malbec wine fermentation**

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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