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Co-inoculation of a pectinolytic *Aureobasidium pullulans* strain and *Saccharomyces cerevisiae* for low-temperature red fermentation: A strategy to enhance the colour and sensory properties of Malbec wines

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ABSTRACT

Low-temperature fermentation is employed to improve aroma production and retention in red wines but at the expense of polyphenol and pigment extraction. In this study, the strategy of co-inoculating the pectinolytic strain *Aureobasidium pullulans* GM-R-22 with a commercial *Saccharomyces cerevisiae* for the low-temperature fermentation (LTF, 17.5 °C) of Malbec red grapes was evaluated at pilot scale, and compared with a traditional fermentation (TF, 25 °C), to compensate for the lower extraction of colour and polyphenols at low maceration temperature. The effects of this strategy on the chromatic, phenolic, technological, antioxidant and sensory properties of Malbec wines were assessed during fermentation and up to 12 months of bottle storage. *A. pullulans* (Ap) grew and remained viable during 4 days of LTF, without affecting the fermentation and growth kinetics of *S. cerevisiae* (Sc), and significantly higher levels of pectinase activity (about 4.5-fold) were detected in LTF (Ap + Sc) compared to LTF (Sc) and TF (Sc) vinifications. At the end of the fermentation, LTF (Ap + Sc) wine showed higher colour intensity (CI), total anthocyanins (TA), total polyphenolic index (TPI) and a^* with a lower L^*, b^* and filtration time (25 %) than LTF (Sc) wine. After 12 months of bottle storage, CI, TPI, TA and monomeric, copigmented and polymeric anthocyanins were significantly higher in LTF (Ap + Sc) than in LTF (Sc) and statistically equal to TF (Sc), although the CIELAB parameters and the antioxidant activity showed no statistical differences compared to control wines. Sensory analysis showed that the LTF (Ap + Sc) wine had greater colour intensity and violet hue than the LTF (Sc) wine, reaching the colour attribute scores of the TF (Sc) wine, and was particularly characterised by a spicy aroma, unlike the wines fermented in the absence of *A. pullulans*. This is the first study to demonstrate the performance of *A. pullulans* in pilot-scale fermentation and the efficiency of its pectinases in improving the extraction and stability of red wine colour fermented at low temperatures, approaching or achieving the levels of chromatic and phenolic parameters of the traditionally fermented Malbec wine.

KEYWORDS: anthocyanins, *Aureobasidium pullulans*, low-temperature red fermentation, pectinolytic yeasts, Malbec wine

INTRODUCTION

Argentina ranks fifth among wine-producing countries with a production of 10.9 million hectolitres in 2024 (OIV, 2024), of which about 80 % is elaborated in the Mendoza province. In particular, the "San Rafael" DO (Denomination of Origin) viticulture region, located in the south of Mendoza, presents a special microclimate that contributes to the distinction of its vineyards and allows the production of highly rated wines. Malbec (*Vitis vinifera* L.) is the red grape variety that is considered the emblem of Argentina. It has the highest production in the country and more than 60 % of its wines are exported to important markets such as the United Kingdom, the United States, Brazil, Canada, Mexico and Germany (INV, 2023).

Wine is the product of a complex process entailing grapes, microorganisms and oenological practices. Yeasts are the most significant microorganisms since they are responsible for alcoholic fermentation, the main reaction in the conversion of grape must into wine, producing ethanol, CO_2 and flavour compounds (Martín *et al.,* 2024). For red wines, colour is one of the most important parameters, as it is the first characteristic to be perceived by the tasters and is directly related to its quality (Martínez-Moreno *et al.,* 2023). Traditionally, red winemaking involves a long maceration period to extract nutrients, phenolic compounds and other constituents from the pulp, skins and seeds towards the grape juice, which occurs simultaneously with alcoholic fermentation at 22–28 °C (Massera *et al.,* 2021). Red fermentation at low temperatures (15–20 °C) has recently been proposed to enhance the aromatic profile of young red wines. Some studies suggest that low-temperature fermentation modifies the sensory profile and increases the aroma intensity of the wines (Kanellaki *et al.,* 2014; Massera *et al.,* 2021). However, the downside of this fermentation strategy is the slow diffusion of phenolic compounds from the skin and pulp into the juice (Sacchi *et al.,* 2005; Delić *et al.,* 2024). An alternative approach to overcome such limitations is the use of active pectinolytic enzymes under fermentation conditions (Martín & Morata de Ambrosini, 2014; Merín & Morata de Ambrosini, 2015; Merín & Morata de Ambrosini, 2018).

The use of pectinases in winemaking is a traditional oenological practice used to improve the technological process and wine quality (Osete-Alcaraz *et al.,* 2022). Pectinases are involved in breaking down the pectin in the cell walls of the grape skin, resulting in key benefits such as increased juice yield, more efficient pressing, improved colour extraction in red wines, enhanced flavour, and easier wine clarification and filtration (Martín & Morata de Ambrosini, 2014; Benucci *et al.,* 2018; Rollero *et al.,* 2018; Osete-Alcaraz *et al.,* 2022). Currently, the use of yeasts capable of producing pectinolytic enzymes during vinification as an alternative to commercial pectinases is in the spotlight of worldwide research (Belda *et al.,* 2016; Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020; Onetto *et al.,* 2020; Longhi *et al.,* 2022a). In this sense, besides the prominent role of *Saccharomyces cerevisiae* in fermentation, diverse non-*Saccharomyces* yeasts, both fermentative and dominant grape epiphytes, can provide products and effects that are of great value for achieving a quality final product (Fazio *et al.,* 2023; Martín *et al.,* 2024).

The wine industry is now demanding new yeast strains to improve wine sensory characteristics and differentiate wine styles (Fazio *et al.,* 2023). In this context, the use of the non-conventional winemaking yeast *Aureobasidium pullulans*, a predominant pectinolytic species on the grape surface and in the very early stages of fermentation (Merín *et al.,* 2015; Sternes *et al.,* 2017; Onetto *et al.,* 2020), may offer alternative options for the production of distinctive and more complex wines. This species, of great biotechnological interest, has been reported to secrete a wide range of extracellular enzymes such as pectinases, cellulases, xylanases, proteases, and glycosidases (Merín *et al.,* 2011; Merín & Morata de Ambrosini, 2015; Belda *et al.,* 2016; Longhi *et al.,* 2022b). However, few studies have been carried out to understand the impact of this species or its enzymes on red grape maceration (Longhi *et al.,* 2022b) or even during fermentation (Belda *et al.,* 2016; Onetto *et al.,* 2020; Longhi *et al.,* 2022a). Previously, a strain of *A. pullulans*, namely GM-R-22, was isolated from the grape surface of the San Rafael DO wine-growing region and shown to have cold-active pectinase activity under wine-like conditions (Merín *et al.,* 2011; Merín & Morata de Ambrosini, 2015). Recently, this strain has shown positive effects on clarification efficiency, phenolic and colour extraction and sensory properties of Malbec wines elaborated with different red winemaking approaches, both in the absence (Merín & Morata de Ambrosini, 2018) and presence of endogenous grape microbiota (Merín & Morata de Ambrosini, 2020); however, these vinifications were performed at laboratory scale, so its specific role in wine production and quality in larger scale production, closer to real winemaking conditions, remains understudied.

Wine technology is constantly being updated to produce high-quality wines that meet current consumer trends. Therefore, our study hypothesised that the combination of both approaches, on the one hand, the low-temperature red fermentation used to improve wine aroma production and retention and, on the other hand, the employment of a pectinolytic non-*Saccharomyces* strain applied to compensate for the lower colour and phenolic extraction at low maceration temperature through its enzymatic action, leads to obtain improved and differentiated Malbec wines. This work aimed to co-inoculate the pectinolytic strain *A. pullulans* GM-R-22 with a commercial strain of *S. cerevisiae* for the low-temperature fermentation (LTF, 17.5 °C) of Malbec red grapes for enhancing the extraction of colour and phenolic compounds at low temperature. In the present study, the growth kinetics and enzymatic production of the pectinolytic strain in simultaneous inoculation with *S. cerevisiae* were studied during low-temperature red fermentation carried out at pilot scale, and the effects of this strategy on the chromatic, phenolic, technological, antioxidant and sensory properties of Malbec wines were evaluated during fermentation and up to 12 months of bottle storage, and compared with the characteristics

of a traditional fermentation (TF, 25 °C) in the absence of the pectinolytic strain.

MATERIALS AND METHODS

1. Materials

1.1. Microorganisms, inocula and growth conditions

Aureobasidium pullulans GM-R-22, an indigenous strain previously isolated from wine grape surface of the viticulture region DO (Denomination of Origin) San Rafael (Mendoza, Argentina), and a producer of cold-active pectinases under low-temperature winemaking conditions (Merín *et al.,* 2011; Merín & Morata de Ambrosini, 2015; Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020) was used in this study. This strain is conserved in the *Biodiversidad San Rafael (Mendoza) Culture Collection* of the Laboratory of Biotechnology (FCAI-UNCuyo) affiliated with the FELACC (Latin American Federation for Culture Collections) as institutional partner SI-66. *Saccharomyces cerevisiae* IOC 18-2007®, purchased from Institut Œnologique de Champagne (France), was utilised as a fermentation starter culture. The strain GM-R-22 was propagated in YPD (containing in gL^{-1}: yeast extract 10, peptone 20, glucose 20, pH 4.0) broth at 25 °C for 48–72 h at least three times prior to experimental use.

Pre-adaptation of microorganisms for fermentation was as follows: Sterilised Malbec grape juice (diluted 1:2 with distilled water and adjusted to pH 3.80) was inoculated at 2 % (v/v) with a pre-culture grown in YPD broth-pH 4.0 of *A. pullulans* GM-R-22 in exponential phase and incubated at 20 °C for 72 h. Commercial *S. cerevisiae* strain was inoculated at 200 mgL^{-1} of active dry yeast and prepared according to the supplier's specifications. All reagents were analytical grade and were purchased from Sigma-Aldrich, Merck and Oxoid.

1.2. Preparation of the grape must

Malbec red grapes (*Vitis vinifera* L.) were obtained from a commercial vineyard located in Rama Caída district (34.66° South latitude and 68.38° West longitude) in DO San Rafael wine region (Mendoza, Argentina). Grapes were immediately crushed and destemmed in the experimental cellar of the Facultad de Ciencias Aplicadas a la Industria (FCAI-UNCuyo). The resulting must in the presence of skins (reducing sugar 257.7 gL^{-1}, yeast assimilable nitrogen [YAN] 230 mgL^{-1}, titratable acidity 3.82 gL^{-1} of tartaric acid, pH 3.92) was adjusted to 5.5 gL^{-1} tartaric acid and sulphited (50 mgL^{-1} SO_2).

2. Pilot scale winemaking

2.1. Experimental design

Vinifications were carried out in biological duplicates in 30 L food-grade plastic tanks containing 20 L of Malbec must per replica. Three different vinification treatments were applied: (i) co-inoculation of *A. pullulans* and *S. cerevisiae* in low-temperature fermentation for red winemaking (LTF; 17.5 ± 2.5 °C) constituting the pectinolytic treatment [LTF (Ap + Sc)], (ii) inoculation of *S. cerevisiae* in low-temperature fermentation for red winemaking (LTF; 17.5 ± 2.5 °C) constituting the control treatment [LTF (Sc)], and (iii) inoculation of *S. cerevisiae* in traditional fermentation (TF, 25.0 ± 1.5 °C) referred to as the general control [TF (Sc)].

2.2. Winemaking procedure

Grape must (skins, seeds, flesh and juice) was inoculated with the pre-adapted cultures of *A. pullulans* and *S. cerevisiae* to obtain a cell concentration of around 2×10^5 or 2×10^6 CFU/mL, respectively. Vinifications were conducted at a controlled temperature using cold (20 °C) or room (25 °C) chambers. Progress of the alcoholic fermentation was monitored by daily measurements of temperature and mass density (gmL^{-1}) at 20 °C using the gravimetric method (Iland *et al.,* 2000). The end of alcoholic fermentation was considered when at least a mass density of 0.995 gmL^{-1} was reached and the reducing sugar was depleted to less than 2.0 ± 0.1 gL^{-1}. During the period of skin contact, cap management consisted of two daily punch downs (morning and afternoon, 1 min each). After the alcoholic fermentation had finished, the wines were racked twice, physically stabilised (4 °C) for 20 days, bottled (750-mL glass bottles) without filtration and stored at 12 °C in darkness until analysis.

2.3. Enumeration of microbial populations

Viable yeast counts were carried out by the serial dilutions method on YPD agar for total yeasts and on Lysine agar (Oxoid) for *A. pullulans* GM-R-22, which is unable to support *S. cerevisiae* growth, and where the pectinolytic strain is clearly differentiated from other microorganisms by its colony morphology. The count can be ascribed to the *A. pullulans* GM-R-22 strain because this species naturally occurs in sound grapes at about 10^1–10^4 $CFUmL^{-1}$ (Barata *et al.,* 2012), which counts much lower than the cell concentration inoculated in this study. The plates were incubated at 28 °C for 3–5 days.

2.4. Determination of total pectinolytic activity

Pectinase activity was evaluated during fermentation on the must-wine centrifuged at 10,000 *g* (15 min, 4 °C), called enzymatic extract, by measuring the amount of reducing groups released from a pectin dispersion (0.25 % citrus pectin in 50 mM citric-citrate buffer, pH 3.8) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). The reaction mixture (enzymatic extract/substrate ratio: 1/10, v/v) was incubated for 15 min at the corresponding enzyme-production temperature (20 or 25 °C) in each vinification technique, according to the method proposed by Merín and Morata de Ambrosini (2015). One pectinase unit (U) is defined as the enzymatic activity that releases 1 µmol of reducing groups per minute under assay conditions. Determinations were performed in triplicate.

3. Wine analytical determinations

3.1. General wine composition

Ethanol (alcohol content, % v/v), residual sugars (gL^{-1}), total and volatile acidity (gL^{-1} tartaric acid and gL^{-1} acetic acid, respectively), pH, density (20 °C/20 °C), dry extract (gL^{-1}) and glycerol (gL^{-1}) of wines were determined at bottling using an ALPHA FT-IR Wine Analyser (Bruker Optik Gmbh, Ettlingen, Germany).

3.2. Filterability of wines

The effect of the pectinolytic treatment on the clarification was evaluated through the filterability of wines at bottling. Filterability was determined by measuring the time required for 25 mL of the final product to pass through a 0.45 µm filter according to the method proposed by Fernández-González *et al.* (2005).

3.3. Spectrophotometric parameters

Chromatic and polyphenolic parameters of wines were determined during the fermentation process, at bottling and throughout bottle storage (12 months of ageing). Samples were centrifuged (10,000 *g*, 5 min at 4 °C) before analyses and absorbance (A) was measured with 1-mm path-length glass cells for classical colour indices and CIELAB (*Comisión Internacional de L'Eclairage*) parameters, as well as with 1-cm path-length cells for total phenols.

Classical colour indices colour intensity (CI) and tonality (T) were calculated according to Glories (1984). The CIELAB parameters, L^* (lightness), a^* (red colour intensity) and b^* (yellow colour intensity) were obtained using MSCV® software (Grupo de Colour, Universidad de La Rioja, Spain). The CIELAB colour difference (ΔE^*) was calculated by using the following equation: $\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$, where the difference ($\Delta$) is calculated for each independent variable between the enzymatically treated wine and control wines.

Total anthocyanins (TA) (mgL^{-1}) were determined based on the method of Puissant-León, which consists of measuring A at 520 nm after incubation of a wine sample for 30 min in 0.1 N HCl (1:40 ratio, v/v), according to Merín and Morata de Ambrosini (2015).

Monomeric (MA), copigmented (CA) and polymeric (PA) anthocyanins were determined using the colorimetric effects of SO_2 and acetaldehyde on anthocyanins according to the method proposed by Levengood and Boulton (2004). Absorbance (A) at 520 nm was measured with 1-mm path-length glass cells after adjusting the pH to 3.6 and A_{acet}, A_{SO2} and A_{20} were assessed. Colour caused by monomeric anthocyanins ($MA = A_{20} – A_{SO2}$), copigmented anthocyanins ($CA = A_{acet} – A_{20}$) and polymeric anthocyanins ($PA = A_{SO2}$) was determined for each sample and expressed as absorbance units at 520 nm referred to 1-cm of optical path-length.

The total polyphenolic index (TPI) was determined according to Glories (1984), after measuring the absorbance at 280 nm of a 100 times diluted wine sample using deionised water. Additionally, total polyphenols content (TPC) was determined according to the classic Folin–Ciocalteu (FC) method (Chen *et al.,* 2015) and expressed in mg gallic acid equivalents (GAE) per L of sample, at 12 months of bottle storage.

3.4. HPLC-DAD analysis of anthocyanins

HPLC analysis of anthocyanin compounds of wines was carried out at 12 months of bottle storage on a Shimadzu (Kyoto, Japan) LC10 HPLC chromatograph, equipped with a SPD-M10Avp UV/Vis Photodiode Array Detector, fitted with Shimadzu software, and a LiChrospher 100 RP-18 reversed-phase column (4.6 mm × 250 mm, 5 µm particle size) (Merck, Darmstadt, Germany) equipped with a precolumn (RP-18; 2 mm × 20 mm, 30 to 40 µm particle size) according to the protocol and procedure reported by Cabeza *et al.* (2009) and the quantification was performed according to Merín and Morata de Ambrosini (2020).

3.5. Antioxidant activity

Antioxidant activity (AA) was determined using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$) radical scavenging method, according to the modified technique by Brand-Williams *et al.* (1995) at 12 months of bottle storage. The reaction mixture was prepared with 100 µL of sample (1:10 dilution) and 2.9 mL of $DPPH^•$ solution (0.03 $mgmL^{-1}$), the reaction time was set at 25 min and spectrophotometric readings were performed at 515 nm. All determinations were carried out at room temperature, and the results are expressed as antioxidant capacity in mg of GAE per L of the sample.

4. Sensory analysis of wines

Descriptive sensory analysis of wines was performed three months after bottling by a tasting panel that consisted of ten trained judges (six males and four females), with ages ranging between 25 to 65 years old, all of which had extensive experience in wine sensory analysis. Wines (approximately 30–40 mL samples) were presented at 16–18 °C in ISO randomly numbered wine glasses (ISO, 1977), labelled with a three-digit code. Two consecutive sessions were performed on different days. The selection of sensory descriptors was done by the panellists by consensus during the first session, choosing the following sensory properties: fluidity, limpidity, colour intensity, violet hue, aroma intensity, fruity aroma, floral aroma, spicy aroma, astringency and body. In the second session, the intensity of each descriptor was rated on a scale from 0 (not perceivable) to 5 (very strong). To minimise sensory carryover, panellists were asked to rinse their mouths with mineral water and eat a cracker between samples following a sip and spit procedure.

5. Statistical analysis

Vinifications were carried out in duplicate (biological duplicate) and microbiological and analytical determinations were performed in triplicate (analytical triplicate). After

testing for normal distribution (Shapiro–Wilks test), homogeneity of variance (Levene's test) and independence of the experimental data, one-way analysis of variance (ANOVA) and the Fisher's LSD test with a significant level $\alpha = 0.05$ were applied to the data (presented as mean ± standard deviation) using Infostat software v2020 (Universidad Nacional de Córdoba, Argentina).

RESULTS AND DISCUSSION

1. Kinetics of fermentation, yeast growth and pectinase production during pilot-scale winemaking

Temperature profile and fermentative kinetics during the alcoholic fermentation of Malbec wines are shown in Figure 1A and 1B, respectively. Red fermentation performed at low temperature (LTF, 17.5 ± 2.5 °C) was conducted by the commercial strain *S. cerevisiae* IOC 18-2007 in the presence (LTF [Ap + Sc]) or absence (LTF [Sc], control) of the pectinolytic strain *A. pullulans* GM-R-22. Additionally, a traditional fermentation for red winemaking (TF, 25.0 ± 1.5 °C) was carried out in the absence of the pectinolytic strain (TF [Sc]) and was referred to as the general control. Average fermentation temperatures for LTF (Ap + Sc) and LTF (Sc) were 17.6 °C and 17.8 °C, respectively, and for TF (Sc) was 24.5 °C (Figure 1A). LTF (Ap + Sc) displayed a slightly lower fermentation rate than LTF (Sc) during the maceration period; however, both alcoholic fermentations reached the same level of mass density in 16 days (0.9900 ± 0.0005 gmL^{-1}). In accordance with the higher fermentation temperature, TF (Sc) lasted 10 days to finish the alcoholic fermentation (Figure 1B).

The presence of *A. pullulans* slightly reduced the rate of fermentation conducted by *S. cerevisiae* in LTF.

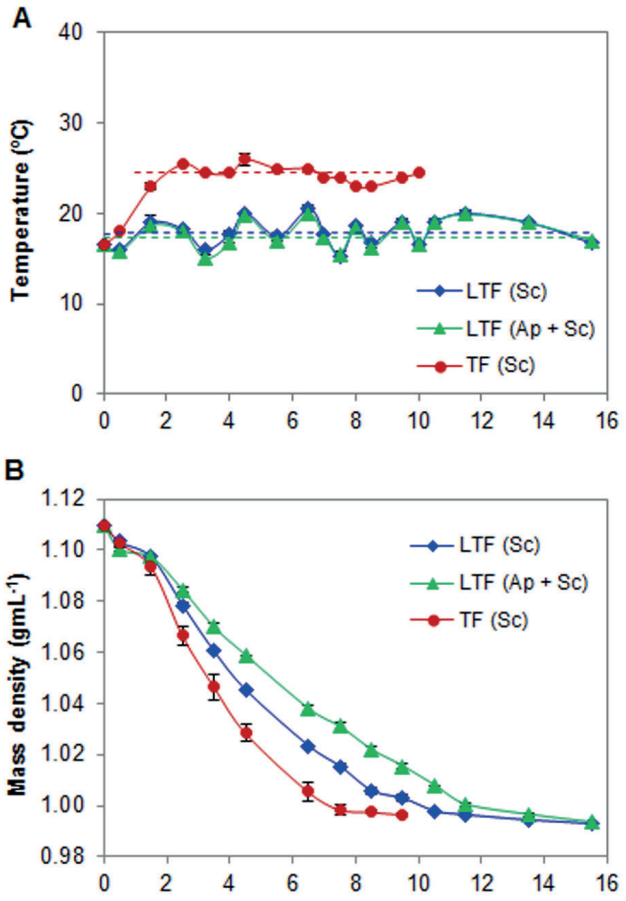

FIGURE 1. (A) Temperature profile and (B) kinetics of alcoholic fermentation (mass density, gmL^{-1}) conducted by S. cerevisiae IOC 18-2007 (Sc) in the absence or presence of *A. pullulans* GM-R-22 (Ap) under low-temperature fermentation (LTF) technique, LTF (Sc) (◆) or LTF (Ap + Sc) (▲), respectively; or in traditional fermentation (TF) in the absence of A. pullulans GM-R-22, TF (Sc) (●), referred to as the general control. Values represent the mean of two independent vinifications. Linear vertical bars represent standard deviation.

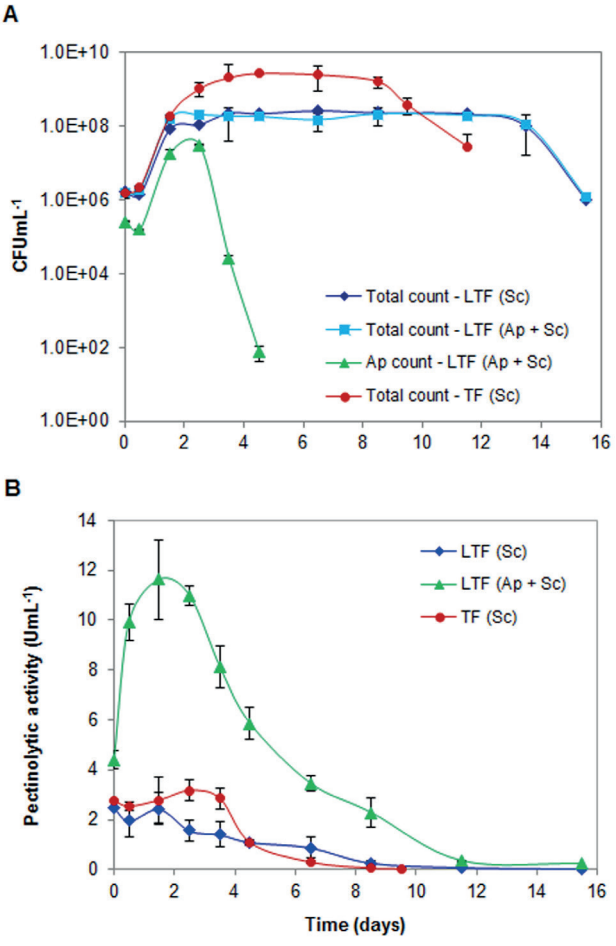

FIGURE 2. (A) Evolution of total yeasts population (viable cell counts, $CFUmL^{-1}$) during LTF (Sc) (◆), LTF (Ap + Sc) (■) and TF (Sc) (●), and evolution of *A. pullulans* GM-R-22 population in LTF (Ap + Sc) (▲). (B) Pectinase activity (UmL^{-1}) during LTF (Sc) (◆), LTF (Ap + Sc) (▲) and TF (Sc) (●). Values represent the mean of two independent vinifications. Linear vertical bars represent standard deviation.

These results are in concordance with those reported by Mendoza *et al.* (2011). These authors reported that the inoculation of non-*Saccharomyces* yeasts can affect the kinetics of the alcoholic fermentation carried out by *S. cerevisiae* and a reduction in fermentation rate is often observed. However, the length of the fermentation process at low temperatures was the same for both vinification trials.

Figure 2A shows the evolution of total yeasts and *A. pullulans* population during the fermentation trials. In LTF (Ap + Sc), the pectinolytic strain started the exponential growth after 12 h of lag phase, reaching the maximal cell concentration (3.15×10^7 CFUmL^{-1}) on the 3rd day of fermentation. Afterwards, the pectinolytic strain initiated the death phase rapidly decreasing its viable cell counts up to approximately 10^2 CFUmL^{-1} after 4.5 days of fermentation. Other studies have reported the predominance of *A. pullulans* on the grape surface, in fresh grape juice and at very early stages of spontaneous fermentation (Barata *et al.,* 2012; Merín *et al.,* 2015; Sternes *et al.,* 2017; Onetto *et al.,* 2020), but this species was not detected at day 4 of fermentation, unlike in the present study. However, it should be taken into account that *A. pullulans* was inoculated at a higher cell density than its natural occurrence in grapes, so it could have remained viable for a longer period during fermentation. It is also interesting to note that the *A. pullulans* GM-R-22 strain reached a significantly higher maximum cell density (1 log cycle higher) than that in our previous laboratory-scale study (Merín & Morata de Ambrosini, 2020). The daily punch downs in the large-scale assay likely provided more dissolved oxygen, allowing greater cell growth.

On the other hand, *S. cerevisiae* IOC 18-2007 (predominant in the total count) did not significantly modify its cell density in the absence or presence of *A. pullulans* in LTF, reaching a statistically comparable maximum concentration (around 2.50×10^8 CFUmL^{-1}, Figure 2A). These results are in line with previous ones obtained by our group, where no significant differences in the growth kinetics of this *S. cerevisiae* commercial strain were observed between the LTF in the presence or absence of *A. pullulans* (Merín & Morata de Ambrosini, 2020). With respect to TF, the total yeast population was higher than that of LTF, reaching a maximum cell density of 2.75×10^9 CFUmL^{-1}. The maximum population of *S. cerevisiae* IOC 18-2007 was significantly higher in the pilot-scale fermentation than in the previous laboratory-scale work (Merín & Morata de Ambrosini, 2020); however, it was similar to the maximum population of *S. cerevisiae* SR1 in vinification experiments with 3 L of Malbec must reported by Longhi *et al.* (2022a).

Figure 2B shows the course of pectinase activity during fermentation. The production of pectinolytic enzymes in LTF (Ap + Sc) was maximal on day 2 (11.6 UmL^{-1}). Thereafter, the activity decreased with increasing ethanol concentration. Control fermentations carried out in the absence of *A. pullulans* displayed comparatively low levels of pectinolytic activity throughout the fermentation, which could be attributed to the endogenous pectinase activity of the grape and indigenous yeasts present in the must, as suggested by Rollero *et al.* (2018). In any case, the fact that the pectinolytic activity detected in the fermentation inoculated with *A. pullulans* was significantly higher than the pectinase activity tested in the control fermentations indicates that this activity can be ascribed to the pectinolytic strain assessed, confirming that it is capable of producing active pectinases under oenological conditions, as observed in previous studies (Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020). This was validated in the present work on a pilot scale.

2. Effects of *A. pullulans* on physicochemical, chromatic, polyphenolic and antioxidant properties of Malbec wines

2.1. Chemical composition, phenolic, chromatic and filtration properties of wines at bottling

Table 1 shows the physicochemical composition of Malbec wines obtained with the different cultures and vinification techniques at bottling. Sugars in the three fermentations were completely consumed, achieving must dryness, although a slight difference was observed between the two LTF wines, and standard ethanol concentrations (around 14.5 %, v/v) were obtained in the three wines. The wines exhibited the oenological characteristics of most regular wines, and the *A. pullulans* strain had no effect on density, ethanol, total acidity, pH and glycerol with respect to its control, showing the same behaviour as that in different red winemaking techniques at laboratory scale (Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020). Similar Malbec wine compositions have been observed previously with other non-*Saccharomyces* yeasts in mixed fermentations (Mendoza *et al.,* 2011; Fanzone *et al.,* 2020; Longhi *et al.,* 2022a). A particularly interesting result was the reduction of volatile acidity, mainly due to acetic acid, in the wine made with *A. pullulans* compared to both controls, although not statistically significant for LTF (Sc) wine. In our previous works, a similar reduction was observed in laboratory-scale winemaking (Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020). We can hypothesise that *A. pullulans* can metabolise acetic acid in the presence of glucose, as previously shown for some wine strains of *Zygosaccharomyces bailii*, *S. cerevisiae* (Vilela-Moura *et al.,* 2011) and *Saccharomyces uvarum*, which metabolise acetic acid faster than their production during fermentation, resulting in low acetic acid concentration (Kelly *et al.,* 2020).

Table 1 also presents the chromatic and phenolic indices of Malbec wines at bottling. The CI, TA and TPI were significantly higher in wine inoculated with the pectinolytic strain, LTF (Ap + Sc), than in its control, LTF (Sc), whereas no statistical differences were found for these parameters between LTF (Ap + Sc) and TF (Sc) wines ($p > 0.05$, LSD test). For further comprehension of the colour composition of wines, the CIELAB parameters were also examined (Table 1). L^* and b^* exhibited a significant decrease, whereas a^* was significantly increased in LTF (Ap + Sc) wine with respect to LTF (Sc) wine. The L^* value is the vertical axis and defines the lightness, the property according to which each colour can be considered equivalent to a member

of the greyscale, between black and white, taking values within the range of 0−100, respectively (Gordillo *et al.,* 2014). This interpretation can be seen in Figure 3, which shows the position of the three wines in the colour plane (*a*b**) of CIELAB space (Figure 3A) and *L** (lightness) values in the vertical axis (Figure 3B). Therefore, the LTF (Ap + Sc) wine was more vivid and displayed higher intensity due to an increase in *a** and a decrease in *L**, and had less contribution to yellow tonalities, indicated by lower values of *b**, with respect to LTF (Sc) wine. These results can be explained by the higher levels of phenolic compounds, especially anthocyanins that are responsible mainly for red pigments, detected in LTF (Ap + Sc) wine at the end of fermentation. It has been reported previously that Malbec wines elaborated at low temperatures with *A. pullulans* enzyme-treatments improved their phenolic and chromatic parameters TPI, CI, *L**, *a** and *b** (Longhi *et al.,* 2022a; Longhi *et al.,* 2022b; Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020). Likewise, these results are in accordance with those reported for other pectinolytic non-*Saccharomyces* yeast co-inoculated with *S. cerevisiae* to improve the quality of wines, such as *Metschnikowia pulcherrima* (Belda *et al.,* 2016), *Metschnikowia fructicola* (Benucci *et al.,* 2018), *Kluyveromyces marxianus* (Rollero *et al.,* 2018) and *Torulaspora delbrueckii* (Longhi *et al.,* 2022a).

Moreover, the CIELAB colour difference *ΔE** between LTF (Ap + Sc) and LTF (Sc) wines and LTF (Ap + Sc) and TF (Sc) wines were 6.27 and 7.80 units, respectively (Table 1). These values are visibly detectable because they are higher than the estimation of 2.7 CIELAB units which is the threshold for the human eye to distinguish between the colour of wines when trained tasters use standardised wine-tasting glasses (Martínez *et al.,* 2001).

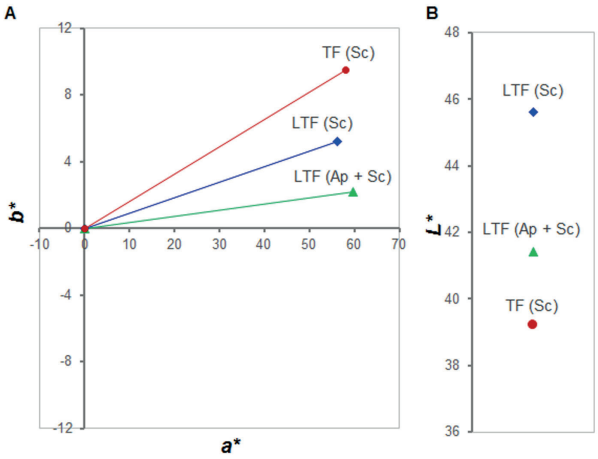

FIGURE 3. CIELAB parameters of Malbec wines LTF (Sc) (◆), LTF (Ap + Sc) (▲) and TF (Sc) (●) wines at bottling. (A) *a*b** diagram. (B) Lightness (*L**).

TABLE 1. Physicochemical, chromatic and technological parameters at bottling of Malbec wines obtained with LTF (Sc), LTF (Ap + Sc) and TF (Sc) processes.

Analytical determination	LTF vinification		TF vinification
	LTF (Sc) wine	LTF (Ap + Sc) wine	TF (Sc) wine
Density (20 °C/20 °C)	0.9896 ± 0.0001a	0.9901 ± 0.0001a	0.9902 ± 0.0003a
Ethanol (%, v/v)	14.40 ± 0.00a	14.40 ± 0.14a	14.55 ± 0.35a
Residual sugars (gL^{-1})	1.08 ± 0.04a	2.10 ± 0.42b	1.40 ± 0.01ab
Volatile acidity (gL^{-1} acetic acid)	0.53 ± 0.05ab	0.46 ± 0.01a	0.58 ± 0.01b
Total acidity (gL^{-1} tartaric acid)	5.50 ± 0.14a	5.60 ± 0.28a	5.55 ± 0.07a
pH	3.79 ± 0.05a	3.87 ± 0.08a	3.80 ± 0.07a
Dry extract (gL^{-1})	26.80 ± 0.00a	28.08 ± 0.14b	29.20 ± 0.00c
Glycerol (gL^{-1})	11.05 ± 0.21a	10.55 ± 0.21a	11.70 ± 0.14b
Colour intensity (CI)	1.303 ± 0.006a	1.505 ± 0.084b	1.629 ± 0.064b
Tonality (T)	0.519 ± 0.004a	0.504 ± 0.028a	0.523 ± 0.010a
TA (mgL^{-1})	468.4 ± 1.9a	500.7 ± 21.8b	496.2 ± 19.3ab
TPI	41.90 ± 0.85a	47.35 ± 0.50b	50.15 ± 1.63b
*L**	45.60 ± 0.07b	41.40 ± 0.71a	39.20 ± 1.20a
*a**	56.15 ± 0.08a	59.77 ± 1.92b	58.00 ± 0.20ab
*b**	5.21 ± 0.21b	2.21 ± 1.32a	9.47 ± 0.87c
*ΔE**	-	6.27[1]	-
	-	7.80[2]	-
Filtration time (smL^{-1})	48.0 ± 1.7b	36.2 ± 0.3a	68.4 ± 6.1c

Data are mean values of two biological replicates ± standard deviation. Mean values with different letters within the same row are significantly different ($p < 0.05$) according to the LSD Fisher test between the wines.

LTF: low-temperature fermentation (17 °C); TF: traditional fermentation (25 °C). Sc, S. cerevisiae IOC 18-2007; Ap, A. pullulans GM-R-22.

[1] ΔE* (LTF [Ap + Sc] wine - LTF [Sc] wine); [2] ΔE* (LTF [Ap + Sc] wine - TF [Sc] wine). ΔE* values greater than 2.7 CIE-Lab units indicate that two wines have colour differences which could be perceived by the human eye.

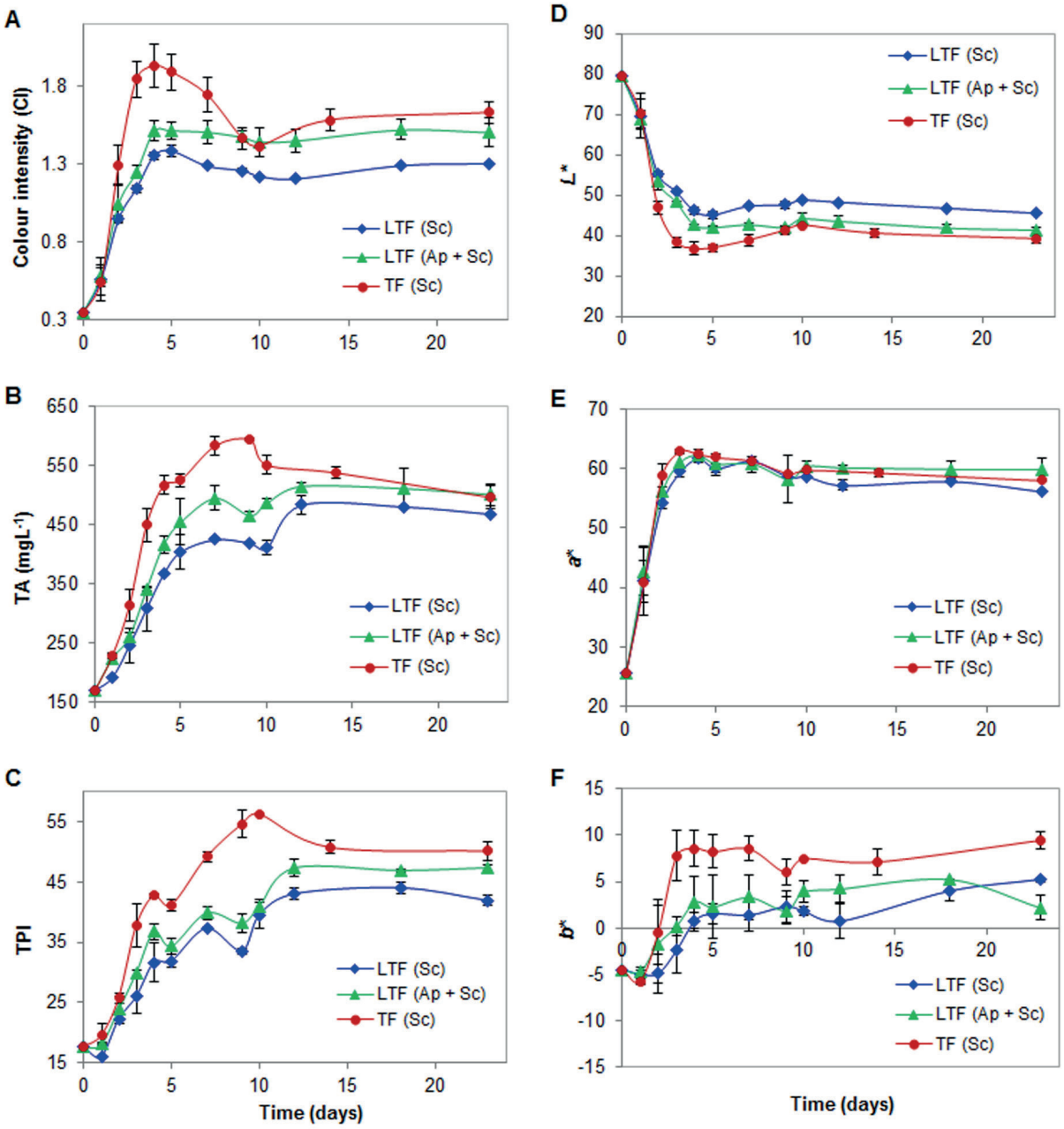

FIGURE 4. Development of CI (A), TA (B), TPI (C) and the CIELAB parameters *L** (D), *a** (E) and *b** (F) of Malbec wines elaborated with LTF technique in the absence (◆) or presence (▲) of *A. pullulans* GM-R-22, respectively, and TF technique in the absence of *A. pullulans* GM-R-22 (●, general control) during fermentation. Values represent the mean of two independent vinifications. Linear vertical bars represent standard deviation.

The filterability of wines at bottling is shown in Table 1. Wine inoculated with *A. pullulans* evidenced a significant reduction of 24.6 % and 47.1 % regarding LTF and TF control wines, respectively. These results are in agreement with previous outcomes demonstrating reductions of 30–40 % in filtration times of red wines elaborated with *A. pullulans* GM-R-22 (Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020), suggesting that the inoculation of this pectinolytic strain can improve clarification and filtration processes, giving a cleaner appearance to the wine. This filterability improvement is likely to occur due to the production of endo-polygalacturonase enzymes by the GM-R-22 strain, as suggested previously (Merín & Morata de Ambrosini, 2018), which markedly decreases the grape pectin viscosity. The application of pectinolytic yeasts during the vinification process has been previously reported to significantly reduce the filtration time of the attained wines (Fernández-González *et al.,* 2005; Belda *et al.,* 2016).

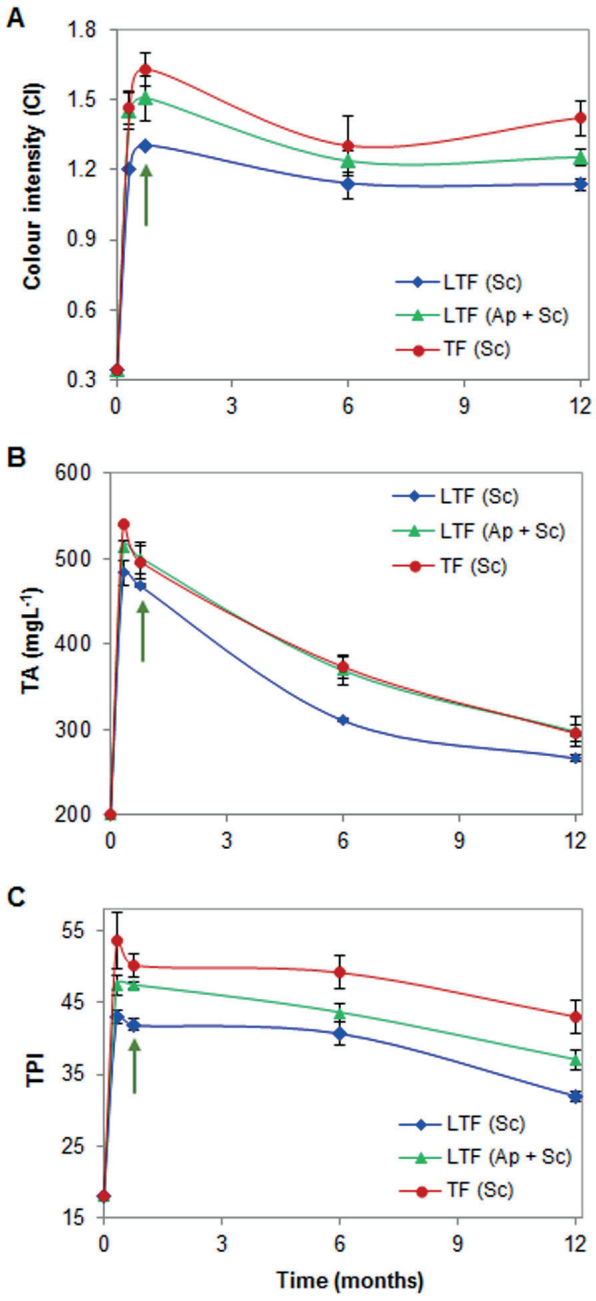

FIGURE 5. Evolution of CI (A), TA (B) and TPI (C) of Malbec wines elaborated with LTF technique in the absence (◆) or presence (▲) of *A. pullulans* GM-R-22, respectively, and TF technique in the absence of *A. pullulans* GM-R-22 (●, general control) during 12 months of bottle storage. Values represent the mean of two independent vinifications. Linear vertical bars represent standard deviation. The arrow indicates the bottling time.

2.2. Development of total anthocyanins, polyphenolic and chromatic indices of wines during fermentation

The development of the main polyphenolic and chromatic characteristics of Malbec wines in the presence or absence of *A. pullulans* from crushing to the end of fermentation is shown in Figure 4. The wines elaborated at low temperatures in the presence of the pectinolytic strain showed positive statistical differences with respect to control wine in CI, TA and TPI from the fourth day up to the end of fermentation (Figures 4A–C). These indices were significantly higher in TF (Sc) wine than in LTF wines, especially during the first week of fermentation, but the indices in LTF (Ap + Sc) wine reached statistically equal levels at the end of the fermentation process. The CIELAB parameters displayed a similar behaviour throughout fermentation, showing improved values in LTF (Ap + Sc) wine with respect to the control for L^*, a^* and b^* at the end of the process (Figures 4D to 4F). Previously, we observed the same results for Malbec grapes fermented at low temperatures (18–20 °C) in co-culture with *A. pullulans* and *S. cerevisiae* (Merín & Morata de Ambrosini, 2020), validating the present outcomes at the pilot scale.

2.3. Evolution of total anthocyanins, polyphenolic and chromatic indices of wines during storage in bottle

Figure 5 shows the colour and polyphenols evolution during 12 months of bottle storage in terms of CI, TA and TPI in the *A. pullulans*-treated wine and both control wines. After bottling, CI decreased up to the 6th month maintaining the differences between wines reached at bottling (Figure 5A). From this point onwards, CI remained stable in LTF (Ap + Sc) and LTF (Sc) wines being statistically higher in the former, whereas this index showed a rising trend towards the 12 months of storage period in TF (Sc) wine. TA concentration showed a marked decrease during the first 6 months of bottle storage, especially in LTF (Sc) wine showing a significantly lower concentration (266.75 ± 3.86 mgL^{-1}) than the values of the other wines, 297.70 ± 14.57 mgL^{-1} in LTF (Ap + Sc) wine and 295.88 ± 7.28 mgL^{-1} in TF (Sc) wine, at 12 months of storage (Figure 5B). Anthocyanins are the major grape pigments in red and black grapes and the compounds responsible for colour in red wine (Echave *et al.,* 2021). During storage, a reduction in TA concentration is expected due to oxidation reactions or pigment formation from anthocyanins such as pyranoanthocyanins and polymeric anthocyanins (Martínez-Moreno *et al.,* 2023). Regarding the pectinolytic treatment, it seems that pectinases contribute to the extraction of anthocyanins and other phenols from grape skin, which was related to the increment of CI. These results are in contrast to the previous ones obtained in low-temperature red winemaking in the presence of *A. pullulans* at the laboratory scale, in which there were no significant differences in TA and CI between this wine and its control at 9 months of bottle storage (Merín & Morata de Ambrosini, 2020). In the present study, the pectinolytic treatment compensated for the lower total anthocyanin extraction and colour intensity obtained at lower fermentation temperature, approaching or reaching the levels of these parameters in the wine obtained with traditional red vinification.

Regarding TPI, the significant difference observed at bottling in LTF (Ap + Sc) wine with respect to its control was reduced at the 6th month, but this difference was significantly higher at 12 months of storage (Figure 5C). TPI in TF (Sc) wine was the highest during the ageing period studied. Higher

temperatures generally lead to increased phenolic extraction due to the increased permeability of the hypodermal cells and solubility of certain phenolics (Sacchi *et al.,* 2005). The higher TPI values of wines obtained at low temperatures in the presence of *A. pullulans* were likely to be achieved because pectinolytic and related polysaccharidase enzymes favour tissue degradation and dissolution of grape cell wall contents, including anthocyanins and other phenolic compounds such as flavan-3-ols. Therefore, the pectinolytic treatment at least partially counterweighted the lower phenolic extraction obtained at the lower fermentation temperature. These outcomes are similar to own previous results obtained at the laboratory scale in terms of relative values and differences between wines (Merín & Morata de Ambrosini, 2020); nevertheless, the absolute TPI values on the larger scale assessed in this study were significantly higher. In contrast, Martín and Morata de Ambrosini (2014) and González-Neves *et al.* (2010) found in laboratory trials that pectinases produced a high extraction of certain phenolic compounds, especially anthocyanins, whereas the extraction was not sufficient to have a significant effect on TPI. It seems that small volumes of must subjected to small-scale winemaking increased the fixation of polyphenols on grape skins and seeds (González-Neves *et al.,* 2010).

Figure 6 exhibits the evolution of monomeric (MA), copigmented (CA) and polymeric (PA) anthocyanins in the three wines during fermentation and 12 months of storage. At bottling, MA was significantly higher in LTF (Ap + Sc) wine than in both controls (Figure 6A). These outcomes put in evidence the effect of pectinases and related polysaccharidase enzymes on the extraction of free anthocyanins from skin cells by degrading the cell wall components during winemaking, thus reaching the highest levels in LTF (Ap + Sc) wine at the end of fermentation. During the bottle ageing period, MA declined constantly in the three wines maintaining the significant differences between the *A. pullulans*-treated wine and its control, and resulting statistically equal to the level of the traditionally fermented wine. Monomeric anthocyanins are unstable and, during wine maturation and ageing, their concentration in red wines decreases continuously due to different mechanisms, such as their adsorption by yeast, their degradation and oxidation, their precipitation with proteins, polysaccharides or condensed tannins, and the progressive and irreversible formation of more complex and stable anthocyanin derived pigments (Martínez-Moreno *et al.,* 2023).

CA in LTF (Ap + Sc) wine was the highest at bottling, but it displayed a slight drop in the 6th month of storage from which it started to increase up to the 12th month, exhibiting the highest level, statistically the same as that of the TF (Sc) wine and greater than that of the LTF (Sc) wine (Figure 6B). These results showed that CA slightly declined during the storage period studied. The colour of young red wines is stabilised due to the interaction of anthocyanins with other colourless wine polyphenols through non-covalent copigmentation reactions (Escribano-Bailón *et al.,* 2019). Consequently, the pectinolytic treatment positively impacted the concentration

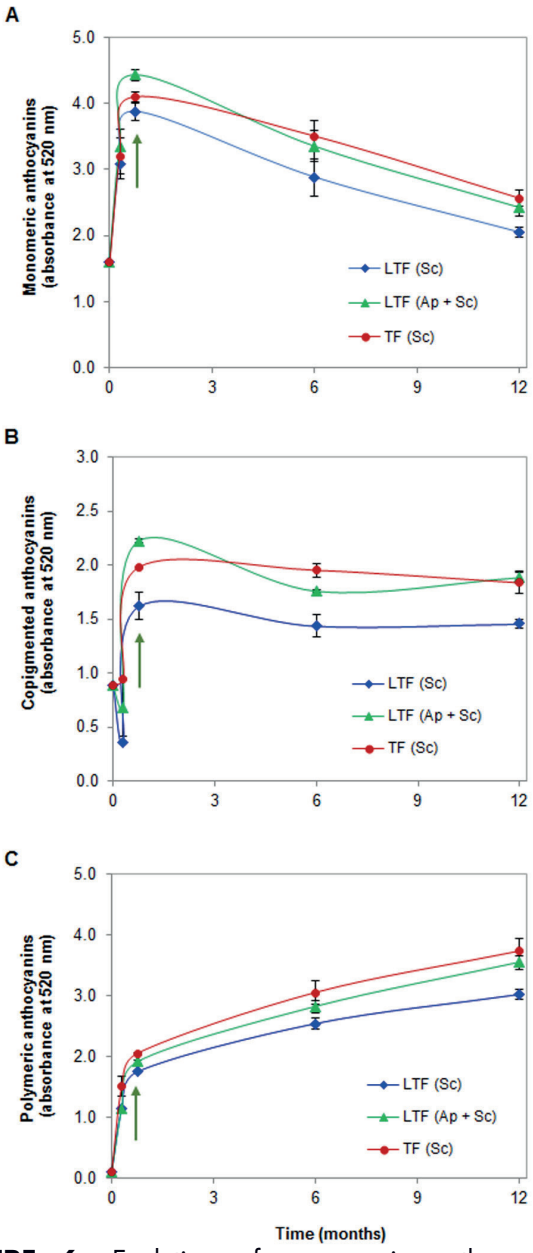

FIGURE 6. Evolution of monomeric anthocyanins (A), copigmented anthocyanins (B) and polymeric anthocyanins (C) of Malbec wines elaborated with LTF technique in the absence (◆) or presence (▲) of *A. pullulans* GM-R-22, respectively, and TF technique in the absence of *A. pullulans* GM-R-22 (●, general control) during 12 months of bottle storage. Values represent the mean of two independent vinifications. Linear vertical bars represent standard deviation. The arrow indicates the bottling time.

of copigmented anthocyanins, which may be considered as a storage form of anthocyanins to form future polymeric pigments.

PA showed a similar level in the three wines at bottling (Figure 6C), from which LTF (Ap + Sc) wine exhibited a continuous increase along the ageing period, reaching a statistically equal level to that of TF (Sc) wine, and a significantly higher value than that of LTF (Sc) wine at

TABLE 2. Global phenolic and colour parameters, and antioxidant activity of Malbec wines obtained with LTF (Sc), LTF (Ap + Sc) and TF (Sc) processes at 12 months of bottle storage.

Analytical determination	LTF vinification		TF vinification
	LTF (Sc) wine	LTF (Ap + Sc) wine	TF (Sc) wine
TPI	32.00 ± 0.71a	37.05 ± 0.92b	42.95 ± 2.33c
TPC (mg GAE L^{-1})	658.31 ± 0.71a	668.96 ± 0.18b	675.47 ± 5.14b
AA (mg GAE L^{-1})	489.0 ± 36.6a	490.5 ± 29.2a	516.1 ± 19.2a
TA (mgL^{-1})	266.75 ± 3.86a	297.70 ± 14.57b	295.88 ± 7.28b
L*	49.00 ± 1.13b	45.75 ± 0.92ab	43.65 ± 2.33a
a*	44.25 ± 0.59a	47.62 ± 1.98a	52.36 ± 1.09b
b*	6.80 ± 1.34a	8.21 ± 1.29ab	12.54 ± 1.75b
ΔE*	-	4.70[1]	-
	-	6.77[2]	-

Data are mean values of two biological replicates ± standard deviation. Mean values with different letters within the same row are significantly different ($p < 0.05$) according to the LSD Fisher test between the wines.

LTF: low-temperature fermentation (17 °C); TF: traditional fermentation (25 °C). Sc, S. cerevisiae IOC 18-2007; Ap, A. pullulans GM-R-22.

[1] ΔE* (LTF [Ap + Sc] wine - LTF [Sc] wine); [2] ΔE* (LTF [Ap + Sc] wine - TF [Sc] wine). ΔE* values greater than 2.7 CIE-Lab units indicate that two wines have colour differences which could be perceived by the human eye.

TABLE 3. Anthocyanins composition of Malbec wines elaborated with LTF technique in the absence (LTF [Sc]) and presence (LTF [Ap + Sc]) of *A. pullulans* GM-R-22 and Malbec wine elaborated with TF in the absence of GM-R-22 strain (TF [Sc]), at 12 months of bottle storage.

Compound (mgL^{-1})	LTF vinification		TF vinification
	LTF (Sc) wine	LTF (Ap + Sc) wine	TF (Sc) wine
Delphinidin-3-O-glucoside	5.36 ± 0.28a	5.97 ± 0.26b	5.14 ± 0.20a
Cyanidin-3-O-glucoside	0.11 ± 0.02b	0.03 ± 0.01a	0.10 ± 0.00b
Petunidin-3-O-glucoside	14.62 ± 0.72a	17.46 ± 0.50b	13.64 ± 0.47a
Peonidin-3-O-glucoside	3.73 ± 0.15a	4.97 ± 0.13b	5.22 ± 0.19b
Malvidin-3-O-glucoside	200.29 ± 2.67a	226.18 ± 3.06b	190.45 ± 3.71a
Peonidin-3-O-acetylglucoside	1.55 ± 0.03b	1.84 ± 0.03c	1.11 ± 0.04a
Malvidin-3-O-acetylglucoside	30.11 ± 1.21b	32.59 ± 0.82b	25.79 ± 0.70a
Peonidin-3-O-p-coumaroylglucoside	0.99 ± 0.03a	1.42 ± 0.13b	0.78 ± 0.02a
Malvidin-3-O-p-coumaroylglucoside	9.95 ± 0.20a	11.03 ± 0.26b	9.83 ± 0.38a
Total glycosylated	224.11	254.61	214.55
Total acetylated	31.66	34.43	26.89
Total coumaroylated	10.93	12.45	10.61
Sum of anthocyanins	266.70	301.50	252.06

Data are mean values of two biological replicates ± standard deviation. Mean values with different letters within the same row are significantly different ($p < 0.05$) according to the LSD Fisher test between the wines.

the end of storage. Ageing transforms the wine colour from dark to bright red by alterations and polymerisations of anthocyanins evolving towards more stable structures (Echave *et al.,* 2021). Polymerisation results in the chromophore of the anthocyanin being protected from water and nucleophilic attack, oxidation or other chemical modifications, such as pH changes in the wine and the bleaching of sulphur dioxide (Echave *et al.,* 2021). As the red wines aged, monomeric anthocyanin concentrations shifted into polymeric stable pigment compounds during the 12 months in the bottle. However, the pectinolytic treatment enhanced MA, CA and PA in the wine elaborated at low temperatures, equalling the parameters of traditionally fermented wine.

The global phenolic and colour parameters in Malbec wines at 12 months of bottle storage showed that, in general, the differences between wines were maintained throughout the storage time (Table 2). Significant differences were observed for TPI, in concordance with TPC, TA and *ΔE** between LTF (Ap + Sc) wine and LTF (Sc) wine after ageing. Regarding antioxidant activity (AA), there were no significant differences between wines studied at 12 months of bottle storage. These

results suggest that the difference in TPI and TPC could be attributed more to anthocyanins than to other polyphenolic compounds commonly associated with antioxidant capacity, such as stilbenes (Longhi *et al.,* 2022a).

2.4. Anthocyanin profile of Malbec wines

The most important anthocyanin compounds identified in wine samples by HPLC-DAD at 12 months of bottle storage are summarised in Table 3. They were grouped into non-acylated glucosides (5), acetyl-glucosides (2), and coumaroyl-glucosides (2). In all the wines analysed, malvidin-3-O-glucoside, its derivatives and petunidin-3-O-glucoside were the predominant anthocyanins with concentrations of malvidin-3-O-glucoside around 75 %. LTF (Ap + Sc) wine showed the maximum level of the sum of anthocyanins and a significant increase in most of the anthocyanins identified (7 out of 9) with respect to the control wines. Among them, all the 3-O-glucosides, except for cyanidin-3-O-glucoside, were higher in LTF (Ap + Sc) wine than in LTF (Sc) wine, with increments varying from 11 to 33 %, and acetylated and p-coumaroylated derivatives of peonidin-3-O-glucoside were larger with increments of 19 and 44 %, respectively, and showed even higher increments with respect to TF (Sc) wine. It seems that the enzymatic treatment extracted more anthocyanins, especially peonidin-3-O-glucoside and its derivatives, similar to previous results obtained with this strain (Merín & Morata de Ambrosini, 2020). The effect of pectinolytic treatment on anthocyanins extraction from grape skin is in accordance with earlier studies where pectinases have been applied (Cabeza *et al.,* 2009; Martín & Morata de Ambrosini, 2013; Osete-Alcaraz *et al.,* 2022).

It can be seen that a higher content of anthocyanins in LTF (Ap + Sc) wine than in LTF (Sc) wine, determined by HPLC (Table 3), was correlated with significantly higher levels of TA and MA (see Figure 5 and Figure 6). However, the significant difference observed in the sum of anthocyanins determined by HPLC compared to TF (Sc) wine did not correlate with the TA or MA measured spectrophotometrically. This behaviour is in agreement with that observed in the study by Fanzone *et al.* (2020), which could be explained by the fact that HPLC-DAD analysis detects only free anthocyanins, whereas spectrophotometric analysis overestimates their total amount, including other red pigments (Canals *et al.,* 2008). Curiously, the lack of correlation between TA and MA with anthocyanins by HPLC-DAD was observed in TF (Sc) wine, but not in LTF wines. Probably, the overestimation of anthocyanins could be greater in the wine obtained with traditional red winemaking, because at higher fermentation temperatures the red pigments involved in this measurement are higher than those extracted at lower temperatures.

3. Descriptive sensory analysis

A descriptive sensory analysis was carried out by a trained panel to determine the effects of the low-temperature red winemaking and the pectinolytic treatment on Malbec wine quality (Figure 7). In particular, this analysis was conducted (i) to verify colour differences between LTF (Sc) and TF (Sc) wines, (ii) to evaluate the effect of *A. pullulans* enzymatic treatment on colour attributes to confirm our hypothesis and (iii) to assess the overall Malbec wines quality influenced by these technological approaches.

In the visual phase, the LTF (Ap + Sc) wine scored significantly higher than the LTF (Sc) wine for colour intensity and violet hue, while it was statistically equal to the TF (Sc) wine. There was no statistical difference in the fluidity and limpidity of the three wines, which showed acceptable values. With regard to aroma attributes, aroma intensity showed a higher score in the

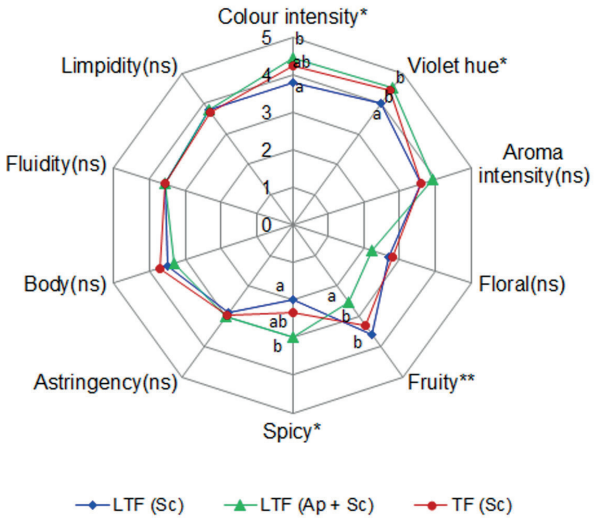

FIGURE 7. Scores obtained from sensory analysis for Malbec wines elaborated with LTF technique in the absence (◆) or presence (▲) of *A. pullulans* GM-R-22, respectively, and TF technique in the absence of GM-R-22 strain (●, general control). Values represent the mean of two independent vinifications. Differences for each attribute between wines were considered significant when $p < 0.05$ according to the LSD Fisher test, n = 10, with significance levels of $*p < 0.05$; $**p < 0.01$; ns, not significant. Distinct lower-case letters next to each wine marker indicate significant differences.

wine elaborated with *A. pullulans*, although not statistically supported, with significantly higher scores for spicy notes and lower scores for fruity notes than the LTF (Sc) wine. Judges highlighted clove, liquorice, spice, and quince for the *A. pullulans* wine (data not shown). The floral character was higher in the control wines, although not statistically different. For mouthfeel attributes, body and astringency showed no statistical differences between the wines. The sensory analysis showed that the Malbec wines were similar in terms of visual and mouthfeel attributes, except for the chromatic properties, which were improved in the wine fermented at low temperature in the presence of *A. pullulans*, reaching the colour characteristics of the traditionally fermented wine. From an aromatic point of view, the LTF (Ap + Sc) wine was particularly characterised by a spicy aroma, suggesting that spicy nuances were enhanced by the presence of *A. pullulans* in the winemaking process. These outcomes are consistent with our previous results on the sensory analysis of Malbec wines produced with this pectinolytic strain at laboratory scale (Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020), validating on a larger scale the influence of *A. pullulans* on the colour and aroma characteristics of Malbec wine.

TF (Sc) and LTF (Sc) wines scored the same in terms of aroma intensity, but the judges described a different aromatic profile for each. The former was characterised by plum, blackberry and raisin nuances, while the latter was represented by balsamic, minty (peppermint) and pronounced fruity notes (data not shown). These results confirmed our hypothesis of fermenting red grapes at a lower temperature than traditionally used to improve or specifically distinguish the aroma profile of a red wine. On the other hand, this approach proved to reduce the colour intensity and the violet hue of the wines. Therefore, this study demonstrated that it is necessary to apply another technique to increase the colour extraction of red wines fermented at low temperatures. In the present work, the pectinolytic strain *A. pullulans* GM-R-22 was co-inoculated with *S. cerevisiae* to produce pectinases during fermentation, and the results proved the efficiency of this pectinolytic approach in terms of colour and aroma properties of the wine. Similar results were reported by Longhi *et al.* (2022a), who elaborated Malbec wine with pre-fermentative cold maceration in the presence of a multienzyme extract produced by an indigenous strain of *A. pullulans*. The use of this extract had a positive effect on the visual aspects, such as colour intensity and tonality, and on the aromatic load, such as floral, complex, peppery and spicy aromas.

CONCLUSION

The strategy of co-inoculating the pectinolytic strain *A. pullulans* GM-R-22 and a commercial strain of *S. cerevisiae* for the low-temperature fermentation of Malbec red grape must produced intensely coloured Malbec wines with a high degree of polymerisation and higher polyphenolic content, comparable to wine fermented at traditional temperatures, but with notable spicy notes in their aroma profile. *A. pullulans*—the main pectinolytic species on the grape surface—has been shown to improve the technological and sensory properties of wines (Belda *et al.,* 2016; Longhi *et al.,* 2022a; Longhi *et al.,* 2022b; Merín & Morata de Ambrosini, 2018; Merín & Morata de Ambrosini, 2020; Onetto *et al.,* 2020). However, this is the first report proposing the use of *A. pullulans* in low-temperature red wine fermentation at the pilot scale, demonstrating the ability to grow in red grape must and produce pectinolytic enzymes, without affecting the fermentation and growth kinetics of *S. cerevisiae* and proving the efficiency of its pectinases in improving the extraction and stability of red wine colour and wine filtration. The promising results obtained in this study suggest that this strategy can be extrapolated to industrial winemaking. The impact on the aromatic profile of their wines should be further investigated.

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