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Kinetic and metabolic behaviour of the pectinolytic strain *Aureobasidium pullulans* GM-R-22 during pre-fermentative cold maceration and its effect on red wine quality

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ABSTRACT

Pectinolytic yeasts can be applied to winemaking with the purpose of improving sensory and technological properties of wine because of their enzymes secreted during vinification. In this work, the autochthonous yeast-like pectinolytic strain from D.O. San Rafael viticulture region, *Aureobasidium pullulans* GM-R-22, was used in co-culture with *Saccharomyces cerevisiae* IOC 18-2007 in microvinification trials with Malbec must applying pre-fermentative cold maceration (PCM). *A. pullulans* remained viable during PCM and *S. cerevisiae* growth and fermentative kinetics were not affected in mixed culture with respect to pure *S. cerevisiae* culture. High pectinolytic activity (9.13 U/mg) was detected in mixed *A. pullulans* vinification during PCM, in which conditions of low temperature (8 °C), low pH (3.8) and high sugar concentration (250.6 g/L) governed. Mixed *A. pullulans* wine showed enhanced colour compared with pure *S. cerevisiae* wine, characterised by higher colour index and percentage of red colour, lower tonality and percentage of yellow colour, and negative values of b^* and h^* indicating more bluish and purplish tonalities. Moreover, filtration time and turbidity diminished by a 40% in mixed *A. pullulans* wine. The presence of GM-R-22 strain improved the production of desirable volatile compounds, such as esters and norisoprenoids, which displayed the maximum odour activity values (OAVs), whereas this strain reduced the total content of higher alcohols when compared to pure *S. cerevisiae* fermentation. Sensory analysis indicated that *A. pullulans* impacted on wine highlighting the violet hue, plum jam aroma, body and equilibrium that are distinctive features of Malbec variety. *A. pullulans* GM-R-22 seems to be promising for applying to low-temperature red winemaking as an adjunct culture to *S. cerevisiae* to improve the wine quality and vinification process.

1. Introduction

Argentina is the eighth largest wine producer in the world (OIV, 2016), and Malbec (*Vitis vinifera* L.) variety, originated in France, has well adapted to the soil and dry climate of this region becoming the emblematic cultivar of Argentinean viticulture production (INV, 2016). “San Rafael” D.O., a Western Argentinean wine-making region, presents a special microclimate that contributes to distinguish its vineyards and allows the production of some of Argentina's more highly rated Malbec wines, recognised worldwide.

Wine is the result of a complex process involving grape must, microorganisms and winemaking practices. Among microorganisms, yeasts (mainly *Saccharomyces cerevisiae*) are dominating because of their role in driving the alcoholic fermentation. The use of enzymes, especially pectinases, is a traditional oenological practice applied for improving technological process and wine quality (Belda et al., 2016; Cabeza et al., 2009; Piemolini-Barreto et al., 2014; Revilla and González-San José, 2003). Pectinases can help to attain higher juice yield and easier pressing, to enhance clarification and filterability of wine, to release more colour and flavour compounds entrapped in grape skins, thereby facilitating the winemaking process and making a positive contribution to wine sensory properties (Belda et al., 2016; Martín and Morata de Ambrosini, 2014). The addition of commercial pectinases, mainly produced by filamentous fungi, can be costly for oenological industry. In this sense, pectinases of indigenous yeasts have attracted attention from numerous research groups globally as an alternative to commercial pectinases (Belda et al., 2016; Maturano et al., 2012; Merín et al., 2011, 2015). It has been reported that at least 75% of oenological *S. cerevisiae* strains presented pectinolytic activity (Blanco et al., 1994); nevertheless, several studies have detected limited

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activity levels in this species (Blanco et al., 1997; Fernández-González et al., 2005; Merín et al., 2014; Merín and Morata de Ambrosini, 2015). Recently, there has been increasing interest in the application of non-*Saccharomyces* wine yeasts, since they can produce substantial sensory complexity in wines (Maturano et al., 2015; Mendoza et al., 2011; Rodríguez et al., 2010); however, the ability of these yeasts to secrete pectinases needs to be thoroughly studied.

Nowadays, winemakers are to introduce novel winemaking practices to enhance the quality of their products. One of the most used techniques for red wine production is pre-fermentative cold maceration (PCM) (Zott et al., 2008). This technique is based on the contact of skins, seeds and other solids with the must in a non-alcoholic setting, in order to favour the extraction of water-soluble compounds such as anthocyanins and aroma precursors (Sacchi et al., 2005). Considering that PCM consists of keeping the must at a low temperature for a certain time, cold-active pectinases might be used to intensify this process, supposing potential advantages such as their functionality during this low-temperature stage that could enhance even more the colour and flavour stability of wines.

Likewise, the wine industry is currently demanding new yeast strains to innovate and improve wine sensory features. In previous reports, we isolated and selected the strain *Aureobasidium pullulans* GM-R-22, an indigenous pectinolytic yeast-like organism able to display cold-active pectinase activity under wine-like conditions, and concluded that it has potential to be applied to the winemaking process (Merín et al., 2011; Merín and Morata de Ambrosini, 2015). Nevertheless, more specific information on the extent of the contribution of *A. pullulans*, a non-conventional species in winemaking, is required. Within this context, the increase in the knowledge about the physiological properties and the metabolic determinants of non-*Saccharomyces* yeasts is the only way to achieve their exploitation in the oenological industry (Belda et al., 2016).

This study examines the behaviour of *A. pullulans* GM-R-22 under microvinification conditions, analysing its growth and pectinase production during PCM, as well as its performance under co-culture with a commercial fermentative strain of *S. cerevisiae* during alcoholic fermentation of Malbec red must. Furthermore, the influence of the pectinolytic strain on chromatic and technological properties, and the aromatic profile and sensory characteristics of the resulting wines were evaluated.

2. Materials and methods

2.1. Microorganisms and growth conditions

Aureobasidium pullulans GM-R-22, previously isolated from the viticulture region D.O. (Denomination of Origin) San Rafael (Mendoza, Argentina), identified and selected on the basis of its ability to produce cold-active pectinases under oenological conditions (Merín et al., 2011; Merín and Morata de Ambrosini, 2015), was used throughout the study.

Saccharomyces cerevisiae IOC 18-2007 (Institut Œnologique de Champagne, France), commercial active dry yeast, was utilised as fermentation starter culture.

The pectinolytic strain is conserved in the Microbial Collection of the Laboratory of Biotechnology (FCAI-UNCuyo) of the SCCM-AAM (Argentine Association of Microbiology) under accession number CCBio-FCAI 020-R22. It was propagated in YPD (containing in g/L: yeast extract 10, peptone 20, glucose 20, pH 4.0) broth at 25 °C for 48 h at least three times prior to experimental use.

2.2. Preparation of starter cultures

Erlenmeyer flasks (100 mL) containing 50 mL of pasteurised grape juice, previously diluted 1:2 with sterile distilled water and adjusted to pH 4.0, were inoculated with 1 mL of culture in exponential phase of *A. pullulans* GM-R-22. Cultures were incubated at 25 °C during 48 h.

Commercial *S. cerevisiae* strain was inoculated at 200 mg/L of active dry yeast, prepared according to the supplier's specifications.

2.3. Preparation of the grape must

Must from *Vitis vinifera* L. cv Malbec, the emblematic red variety of Argentina, obtained from a winery of Rama Caída district (34.66° South latitude and 68.38° West longitude), in D.O. San Rafael wine region, during 2010 vintage, was used to carry out the microvinification trials. The grape must (reducing sugar 250.6 g/L, assimilable nitrogen 180 mg/L, titratable acidity 3.6 g/L of tartaric acid, pH 4.2) was adjusted to pH 3.8 with tartaric acid and exposed to heat treatment (90 °C for 15 min) according to Moreira et al. (2008). The grape must used in this research was steam-sterilised to eliminate its natural microbiota and inactivate its endogenous enzymes in order to study the behaviour of the pectinolytic strain. Subsequently, the must was treated with 80 mg/L sulphur dioxide (as sodium metabisulphite). Concentration of the preservative was chosen because of its lack of inhibitory effect on the yeasts employed in the fermentation trials previously tested (Merín and Morata de Ambrosini, 2015), with the purpose of emulating the winemaking conditions.

2.4. Microvinifications

Microvinifications were carried out in duplicate in 1-L Erlenmeyer flasks containing 800 mL of Malbec red must per replica, in the presence of skins, treated as previously explained (Section 2.3), and conducted in two stages: pre-fermentative cold maceration (PCM; 8 °C–6 days) followed by traditional alcoholic fermentation (TAF; 25 °C–10 days). Three inoculation strategies were applied: (1) pure culture of *A. pullulans* GM-R-22 (control of pectinolytic activity; Ap), (2) pure culture of *S. cerevisiae* IOC 18-2007 (control fermentation; Sc), and (3) mixed culture of both *A. pullulans* GM-R-22 and *S. cerevisiae* IOC 18-2007 (pectinolytic-treatment fermentation; Ap + Sc), with sequential inoculation: the pectinolytic yeast was inoculated at the beginning of PCM and the inoculation of the commercial *S. cerevisiae* strain gave rise to the TAF. Controls were inoculated each with the corresponding microorganism at their respective times of inoculation in the mixed culture. The purpose of the PCM practice in presence of the pectinolytic strain *A. pullulans* GM-R-22 was to enhance the extraction of water-soluble compounds from the solid parts of grape towards the must by means of the cold-active pectinases produced *in situ* by this strain. Monoculture of *A. pullulans* GM-R-22 was not a vinification properly said given that *A. pullulans* species is a non-fermentative microorganism, thus only two wines (Sc wine and Ap + Sc wine) were obtained and analysed.

The flasks were inoculated with the corresponding starter cultures to obtain an initial cell concentration of around 10^5–10^6 CFU/mL for studied yeasts. During the period of skin contact, vinifications were shaken for 20 min at 90 rpm twice a day. Microvinifications were conducted at a controlled temperature using cold chambers (8 °C) or stoves (25 °C).

2.4.1. Progress of the alcoholic fermentation

Vinification performance was monitored by daily measurements of temperature and weight loss of the flasks containing stoppers with a Müller valve that allows only CO_2 to escape from the system up to reach constant weight for two consecutive days. Fermentative power (% alcohol, v/v) was indirectly estimated by multiplying the CO_2 weight loss in grams by a stoichiometric factor of 1.3 (Ciani and Rosini, 1987).

At the end of the fermentations, wines were drained and a cold stabilisation (4 °C) was carried out for 10 days. Then, only free run juice was collected, transferred to glass bottles and kept at 12 °C until analysis.

2.4.2. Enumeration of microbial populations

Biomass determination was performed by counting viable cells on YPD agar that permits the morphological differentiation of both yeasts. Determination of *A. pullulans* GM-R-22 population was additionally carried out on Lysine agar (Oxoid), which is unable to support *S. cerevisiae* growth. The plates were incubated at 28 °C for 3–5 days.

2.4.3. Determination of pectinolytic activity

Pectinase activity was evaluated during the microvinifications on the must-wine centrifuged at 10,000*g* (15 min, 4 °C) and filtered (0.22 μm) 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 activity was detected at the corresponding enzyme-production temperature (8 or 25 °C) in each microvinification stage, following the protocol described 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 min under the assay conditions.

Total protein determination was performed according to Bradford (1976) using bovine serum albumin as standard.

2.5. Analytical determinations

2.5.1. General wine composition

Alcohol content was determined with an automatic wine analysis system "Alcolyzer Wine" (Anton Paar Alcolyzer, USA Inc.). Residual sugars, total and volatile acidity, total and free SO_2, density (15 °C/15 °C) and pH of wines were measured according to the Official Methods established by the International Organisation of Vine and Wine (OIV, 2005).

2.5.2. Chromatic parameters

Chromatic parameters of wines were determined measuring their absorbance on centrifuged (3000*g*, 5 min at 4 °C) samples, with 1-mm pathlength glass cells.

Classical colour indices were calculated measuring the absorbance (A) at 420, 520 and 620 nm. The following variable were then calculated: colour intensity (CI = A420 + A520 + A620), tonality (T = A420/A520), proportion of yellow colour (%Y = [A420/CI] × 100), proportion of red colour (%R = [A520/CI] × 100), proportion of blue colour (%B = [A620/CI] × 100), and proportion of red colour produced by the flavylium cations of the free and bound anthocyanins (%dA = [1 − (A420 + A620) / (2 × A520)] × 100) (Glories, 1984). Additionally, colour index (CIx = [(A420 + A520) / (A420/A520)] × 1000) was also calculated according to the INV (National Institute of Viticulture) for 2010 vintage, Argentina (Cabeza et al., 2009).

CIELAB parameters were determined by measuring the absorbance at 450, 520, 570 and 630 nm with the illuminant D65 and a 10° standard observer. L^* (lightness), C^* (chroma), h^* (hue angle), a^*(red-green colour intensity), and b^*(yellow-blue colour intensity) parameters were obtained using MSCV® software (Negueruela, 2005).

2.5.3. Technological parameters of clarification

Effect of the pectinolytic treatment on clarification parameters of wine was evaluated. Filterability was determined by measuring the time required for 10 mL of final product to pass through a 0.45 μm filter according to the method proposed by Fernández-González et al. (2005). Turbidity was measured through the absorbance at 600 nm of the final wine.

2.5.4. Volatile compounds profile

Determination of major volatile compounds (acetate and ethyl esters, higher alcohols, terpenes and norisoprenoids) and sulphur compounds of wines was carried out by gas chromatography–mass spectrometry (GC–MS) according to the protocol described by Jofré et al. (2010). The major volatile compounds were extracted from the head space using a solid phase microextraction (SPME) fibre of 100 μm PDMS (Supelco, Bellefonte PA, USA) according to the method proposed by Jiang and Zhang (2010). The method for the extraction of sulphur compounds was ultrasound assisted emulsification–microextraction (USAEME) according to the procedure described by Jofré et al. (2010).

Odour activity value (OAV) was calculated as the mean concentration of an aroma compound divided by its odour threshold value, published in the scientific literature (Welke et al., 2014).

2.6. Sensory analysis

Sensory descriptive analysis of young wines (one month after bottling) was carried out by a tasting panel that consisted of eight trained judges (Bianchi Winery and National Institute of Vitiviniculture, San Rafael Delegation). Wines were equilibrated at room temperature (22 °C) and 40 mL-samples were poured into randomly numbered wineglasses. 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: colour intensity, violet hue, aroma intensity, fruity aroma, floral aroma, toasted aroma, plum jam aroma, spicy aroma, vegetal character, astringency, body and equilibrium. At the second session the intensity of each descriptor was rated on a scale from 0 (not perceivable) to 5 (very strong).

2.7. Statistical analysis

Microvinifications were carried out in duplicate (biological duplicate) and microbiological and analytical determinations were performed in triplicate (analytical triplicate). Experimental data, presented as mean ± standard deviation, were subjected to ANOVA, and significant differences were determined by Fisher LSD test (significant level $\alpha = 0.05$) using the programme STATISTICA 7.0 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

In this study, we evaluated the kinetic behaviour of the microorganisms and the fermentation carried out with the commercial strain *S. cerevisiae* IOC 18-2007 in presence or absence of the pectinolytic strain *A. pullulans* GM-R-22 during microvinifications of Malbec grape must. Additionally, the effect of the pectinolytic strain on the technological process and the global quality of the red wine obtained more than on the chromatic and clarification parameters was study, taking into consideration that *A. pullulans* is a new species proposed to add to vinification.

3.1. Evolution of yeast microbial populations and fermentation kinetics

Fig. 1 shows the temperature profile (Fig. 1A) and the fermentation kinetics (Fig. 1B) during microvinification trials performed by pure culture of *S. cerevisiae* IOC 18-2007 (Sc fermentation) and mixed culture of the *S. cerevisiae* strain and the pectinolytic strain *A. pullulans* GM-R-22 (Ap + Sc fermentation). After PCM (8 ± 1 °C–6 days), the TAF (25 ± 1 °C) lasted 10 days; therefore, microvinifications finished in 16 days in both pure and mixed cultures (residual sugars < 0.9 g/L; Table 1). Significant differences were not observed between fermentative kinetics of both vinifications, exhibiting a fermentation rate of 0.36 g/h. Thus, no *A. pullulans* negative effect on *S. cerevisiae* fermentative kinetics was observed. These results are different from those reported for some fermentative non-*Saccharomyces* yeasts, like *Torulaspora delbrueckii*, *Kluyveromyces thermotolerans* and *Hanseniaspora uvarum/Kloeckera apiculata*, when they were co-cultured with *S. cerevisiae* strains, in which a reduction in fermentation rate was observed (Ciani et al., 2006; Mendoza et al., 2011).

As expected, no ethanol production was detected during PCM stage

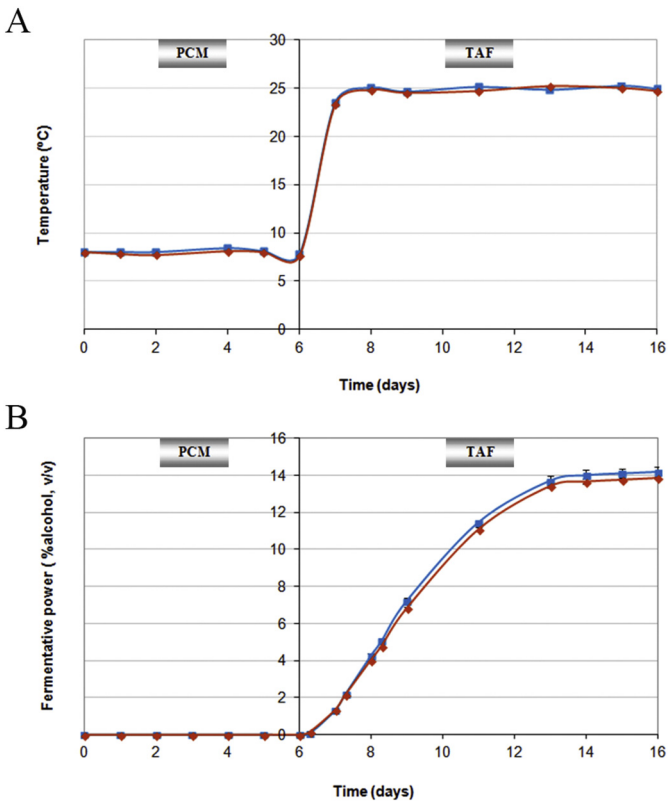

Fig. 1. Temperature profile (A) and fermentative power (B) of pure *S. cerevisiae* (■) and mixed *A. pullulans* (♦) fermentations during PCM (day 0 to 6) and TAF (day 6 to 16) of microvinifications with Malbec must.

Table 1
Physicochemical properties of Malbec wines obtained in absence (Sc wine) and presence (Ap + Sc wine) of the pectinolytic strain *A. pullulans* GM-R-22.

Analytical determination	Wines		
	Sc	Ap + Sc	Sig.
Density (15 °C/15 °C)	0.994 ± 0.001	0.995 ± 0.001	ns
Ethanol (%, v/v)	13.3 ± 0.4	12.7 ± 0.2	ns
Residual sugars (g/L)	0.79 ± 0.08	0.87 ± 0.06	ns
Volatile acidity (g/L acetic acid)	0.36 ± 0.03	0.32 ± 0.02	ns
Titratable acidity (g/L tartaric acid)	6.60 ± 0.12[b]	6.10 ± 0.10[a]	*
pH	3.81 ± 0.01	3.83 ± 0.02	ns
Free SO_2 (mg/L)	26.0 ± 1.55[a]	34.8 ± 2.14[b]	*
Total SO_2 (mg/L)	72.0 ± 3.67	76.8 ± 3.13	ns

Data are mean values of two experiments ± standard deviation.
[a,b] Mean values with different superscript letters within the same row are significantly different according to LSD Fisher test (*$p < 0.05$); Sig.: significance; ns: not significant.
Sc, *S. cerevisiae* IOC 18-2007; Ap, *A. pullulans* GM-R-22.

in either of the microvinifications (Fig. 1B), confirming the absence of microbial contamination as well as of must fermentation by the pectinolytic strain.

The evolution of microbial populations during microvinifications conducted by pure and mixed cultures of the studied microorganisms is shown in Fig. 2. *A. pullulans* GM-R-22, inoculated at the beginning of PCM stage at a final cell concentration of 1.94×10^5 CFU/mL, showed a decrease of 1 log cycle in cell density during the first day, maintaining this cell concentration approximately constant during the stage. After 6 days of vinification, when the commercial yeast *S. cerevisiae* was inoculated (beginning of TAF) and vinification temperature risen to 25 °C, *A. pullulans* population in mixed culture was 1.30×10^4 CFU/mL, but

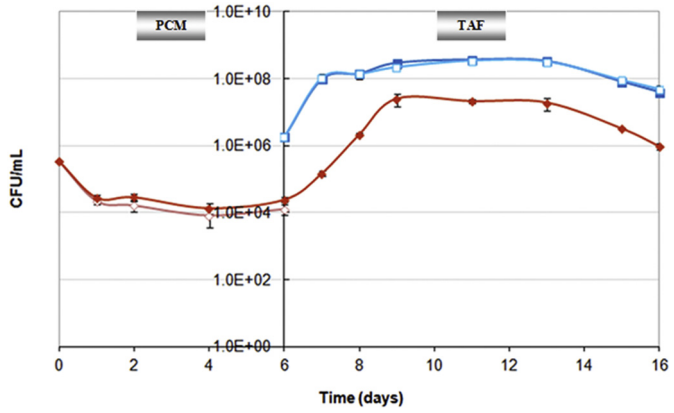

Fig. 2. Viable cell counts of commercial *S. cerevisiae* in pure (■) and mixed (□) cultures during TAF and *A. pullulans* GM-R-22 in pure (♦) and mixed (◊) cultures during PCM and TAF.

on the 7th day of vinification viable cells of the pectinolytic yeast were undetectable. Meanwhile, in pure culture the *A. pullulans* population started the exponential growth phase with a specific growth rate of 0.16 h^{-1}, reaching its maximal cell density (2.50×10^7 CFU/mL) on the 9th day of culture and starting the cell death phase on the 13th day, with a loss of 1 log cycle at the end of the incubation.

In microvinifications carried out both in absence and presence of *A. pullulans* GM-R-22, exponential growth of *S. cerevisiae* was observed on the first day of incubation with a specific growth rate of 0.25 h^{-1}, exhibiting the maximal cell population of 3.60×10^8 CFU/mL after 5 days of TAF (11 total days of vinification). After 7 days of TAF, *S. cerevisiae* started to decline with a loss of 1 log cycle at the end of the incubation. Therefore, this yeast in mixed culture did not modify its growth kinetics with respect to the pure culture, by the presence of the pectinolytic yeast.

S. cerevisiae growth and consumption sugar rates were similar in both mixed (Ap + Sc) and monoculture (Sc) fermentations suggesting the absence of an inhibitory effect of *A. pullulans* against *S. cerevisiae*. Previously, Merín and Morata de Ambrosini (2015) determined the killer phenotype of GM-R-22 and IOC 18-2007 strains. The neutral killer character of GM-R-22 strain is particularly important when *Saccharomyces* fermentation starters have a killer/sensitive phenotype, like the commercial strain used in this study.

Different non-*Saccharomyces* wine strains decrease their cell population and are not viable before finishing fermentation, while *S. cerevisiae* strains remain viable up to the end of the fermentation (Mendoza et al., 2011; Maturano et al., 2012, 2015; Rodríguez et al., 2010). In this work, *A. pullulans* was not detected after the inoculation of the fermentative yeast. This fact was probably due to the reducer environment caused by the fermentation process.

3.2. Kinetics of pectinase production during microvinification

Fig. 3 shows the pectinase activity in pure *A. pullulans* culture (Ap) and pure *S. cerevisiae* (Sc) and mixed *A. pullulans* and *S. cerevisiae* (Ap + Sc) fermentations during microvinification trials. In Ap + Sc fermentation, maximal pectinolytic activity was produced with a specific activity of 9.13 $U/mg_{protein}$ on the first day of PCM, maintaining a level of around 7 $U/mg_{protein}$ until the end of this stage. During TAF, a peak of activity was detected around the first and second day of fermentation (7th–8th day of vinification), when the fermentative power was 2.2–4.2% alcohol (v/v). From this point, the activity decreased up to reach a 50% and 30% of relative activity at 9 days (7% alcohol, v/v) and 13 days (13% alcohol, v/v) of vinification, respectively. Pure *S. cerevisiae* fermentation (Sc) showed basal levels of pectinolytic activity

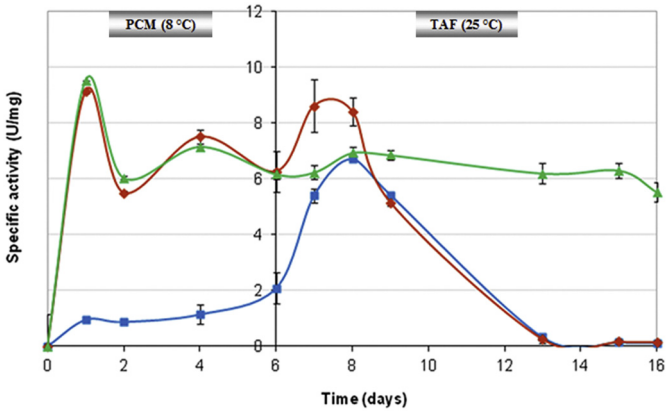

Fig. 3. Pectinase activity in pure *A. pullulans* culture (▲), pure *S. cerevisiae* culture (■) and mixed culture (♦) of both microorganisms.

(around 1 U/$mg_{protein}$) during PCM, although a significant increase of activity was observed during the first days of TAF until reaching the maximum at the 8th day of vinification (6.69 U/$mg_{protein}$) and then it decreased with a similar kinetics to that of mixed culture. Pectinolytic activity evolution in pure culture of *A. pullulans* GM-R-22 (Ap) during PCM was statistically similar to that in mixed culture. However, in pure culture the pectinolytic activity remained approximately constant around 7 U/$mg_{protein}$ up to the end of the incubation, unlike the other cultures in which the activity progressively decreased as the alcohol concentration increased.

These results exhibited that pectinolytic activity produced during microvinification in presence of *A. pullulans* was significantly higher than activity produced in its absence, attributing the production of this activity to the pectinolytic yeast. Previously, this strain was capable of producing significant level of pectinase activity at pH 3.5, 12 °C and 200 g/L glucose and 1 g/L pectin, in a semi-synthetic medium (Merín and Morata de Ambrosini, 2015). In this study, pectinase production by *A. pullulans* GM-R-22 was demonstrated under real red grape maceration conditions: low temperature (8 °C), low pH (3.8) and high sugar concentration (250.6 g/L). Biely et al. (1996) have reported that *A. pullulans* was able to produce pectinases at low acidic pH until 2.1. However, this is the first report on the pectinolytic activity production by *A. pullulans* at 8 °C and high sugar concentration (250.6 g/L) in red grape must. The high pectinase activity levels found during PCM are important for their possible contribution to colour extraction and stabilisation processes at this stage.

As regards the increase in pectinolytic activity at the 8th day of vinification both in Sc and Ap + Sc fermentations, it seems that *S. cerevisiae* IOC 18-2007 was responsible for this activity, since the activity peak observed is consistent with its inoculation. However, additional studies have shown significantly lower activity in its pure fermentations (Merín, 2012). Likewise, considering that pectinolytic activity was detected before the commercial strain inoculation, grape endogenous pectinases might also have produced this activity, indicating that these enzymes could be resistant to thermal denaturation or could have been reactivated in the complex matrix of grape must after the heat treatment. Certain polygalacturonases and polymethylestersases from different fruit and vegetable pulps do not reach the complete inactivation (Petruzzi et al., 2017) or are reversibly denaturalised by a heat treatment being then reactivated under different conditions (Thongsook and Barrett, 2005). Consequently, it is likely that the peak detected in Sc fermentation might have been due to the accumulated activity of both *S. cerevisiae* IOC 18-2007 pectinases and grape endogenous pectinases, probably activated by factors occurring during must fermentation, like pectin and SO_2 (Merín et al., 2015; Merín and Morata de Ambrosini, 2015).

3.3. Effect of pectinases produced by A. pullulans GM-R-22 on visual and technological parameters of young Malbec wines

3.3.1. General wine composition

Table 1 shows physicochemical properties of Malbec wines obtained in absence (Sc wine) and presence (Ap + Sc wine) of the pectinolytic strain. Sugars in pure and mixed cultures were completely consumed achieving the dryness of the must and standard ethanol concentrations (12–14%, v/v), without significant differences between samples. These results are directly related to the fermentation kinetics of both wines (Fig. 1B). In general, the resulting wines exhibited physicochemical characteristics of most regular wines, indicating that *A. pullulans* GM-R-22 did not significantly affect the general wine composition. Similar results using other non-*Saccharomyces* yeasts in winemaking have previously been observed (Maturano et al., 2012, 2015; Mendoza et al., 2011; Rodríguez et al., 2010).

However, slight differences were observed in two parameters. Titratable acidity in Ap + Sc wine was lower than in Sc wine, but no significant differences in pH could be observed between samples. Free SO_2 was significantly higher in Ap + Sc wine although total SO_2 concentration was statistically equal in both wines. Free SO_2 increase could be explained by the decrease in compounds susceptible to react with SO_2, like monomeric anthocyanins. As the result of the enzymatic treatment in aqueous phase (PCM), conversion of free anthocyanins into copigmented complex pigments, which are resistant to SO_2 binding (Ducasse et al., 2010), might have occurred, thus augmenting the free SO_2 concentration in the wine.

3.3.2. Chromatic and technological parameters

Table 2 shows significant differences in chromatic parameters of wine inoculated with the pectinolytic strain (Ap + Sc) with respect to Sc wine in colour index, tonality, %Y, %R and %dA, and in the CIELAB parameters h^* and b^*, in all the cases favouring red pigments extraction with respect to yellow pigments, as well as prevailing bluish or purplish tonalities, indicated by negative values of b^* and h^*, respectively.

Despite non-existence of significant differences in colour intensity between both wines, the maximum colour index was achieved in Ap + Sc wine (Table 2). This wine showed enhanced colour characterised by a higher percentage of red colour (63.2%) and lower of yellow colour (26.3%) compared with control wine, which showed

Table 2
Chromatic and technological properties of Malbec wines obtained in absence (Sc wine) and presence (Ap + Sc wine) of the pectinolytic strain *A. pullulans* GM-R-22.

Analytical determination	Wines		
	Sc	Ap + Sc	Sig.
Colour index (INV)	2040 ± 35[a]	2353 ± 94[b]	*
Colour intensity	1.180 ± 0.070	1.094 ± 0.059	ns
Tonality	0.524 ± 0.058[b]	0.417 ± 0.021[a]	*
%Y	31.7 ± 0.8[b]	26.3 ± 0.9[a]	*
%R	59.9 ± 0.6[a]	63.2 ± 0.9[b]	*
%B	9.8 ± 0.5	10.4 ± 0.4	ns
%dA	65.8 ± 0.65[a]	70.9 ± 1.29[b]	*
L^*	47.5 ± 3.8	51.4 ± 2.6	ns
C^*	55.3 ± 4.7	53.2 ± 2.7	ns
h^*	1.25 ± 0.07[b]	(−2.85) ± 1.01[a]	*
a^*	56.0 ± 4.2	54.0 ± 2.4	ns
b^*	1.24 ± 0.35[b]	(−2.57) ± 0.10[a]	*
Filtration time (min)	15.2 ± 0.35[b]	9.2 ± 0.30[a]	*
Turbidity ($A_{600\ nm}$)	341 ± 9[b]	206 ± 7[a]	*

Data are mean values of two experiments ± standard deviation.
[a,b] Mean values with different superscript letters within the same row are significantly different according to LSD Fisher test ($*p < 0.05$); Sig.: significance; ns: not significant.
Sc, *S. cerevisiae* IOC 18-2007; Ap, *A. pullulans* GM-R-22.

59.9% of red colour and 31.7% of yellow colour. These results confirmed the activity of pectinase enzymes detected under winemaking conditions (Fig. 3) and demonstrated the positive contribution of enzymatic treatment to the chromatic properties of red wines, as pointed out earlier in several studies (Ducasse et al., 2010; Martín and Morata de Ambrosini, 2014; Ortega-Heras et al., 2012; Piemolini-Barreto et al., 2014; Revilla and González-San José, 2003).

A similar study to ours, carried out by Belda et al. (2016), applying pre-fermentative cold soak at 12 °C and sequential inoculation of non-*Saccharomyces* pectinolytic yeasts, *A. pullulans* NS-O-82 or *Metschnikowia pulcherrima* NS-EM-34, or commercial pectinases, combined with *S. cerevisiae* Viniferm RVA, showed that only the wine obtained in presence of *A. pullulans* NS-O-82 produced a significant increase in anthocyanins content.

Regarding technological parameters, the filtration time and turbidity diminished significantly (by a 40%) in the wine inoculated with GM-R-22 strain with respect to Sc wine (Table 2). These results are consistent with the production of endo-polygalacturonase activity by *A. pullulans* GM-R-22 (Merín, 2012). Endo-polygalacturonases act at random along the chain of pectin poorly methylated, which is translated into a marked decrease in substrate viscosity for a low level of activity (Biely et al., 1996). This suggests an enzymatic degradation of pectin in grape must, which facilitates clarification and filtration processes.

Addition of pectinolytic yeasts or their enzymes during the vinification process has been informed to reduce filtration times or turbidity in resulting wines. Blanco et al. (1997) have reported that when wine fermentation was carried out using *S. cerevisiae* pectinolytic strains or their enzymes the filtration time was reduced by up to 50% and Belda et al. (2016) have found a reduction in filtration time of 48% in wine obtained with *S. cerevisiae* and the pectinolytic *A. pullulans* strain compared with wine attained only with *S. cerevisiae*, both consistent with the reduction in filtration time obtained in our study. Regarding turbidity, Piemolini-Barreto et al. (2014) reported that wine enzymatically treated were significantly clearer than controls, indicating that pectinolytic enzymes efficiently reduced wine turbidity by degrading pectic compounds present in grape must. Likewise, Belda et al. (2016) found that treatments in presence of pectinolytic yeasts exhibited a statistically significant decrease in turbidity compared with fermentation with *S. cerevisiae* as sole inoculum.

Although *A. pullulans* GM-R-22 population was not detected in the TAF, its secreted pectinases during PCM remained active in the fermentation medium. This fact is mainly supported by the pectinolytic activity detected during PCM and TAF (Fig. 3), and also by their positive contribution to the chromatic and technological properties of red wines (Table 2).

3.3.3. Aromatic profile of wines

Analysis of volatile compounds of wines was performed with the purpose of determining *A. pullulans* GM-R-22 effect on the aromatic profile of wine and the production of possible off-flavours. Table 3 shows the concentration obtained for each volatile compound detected in Malbec wines elaborated in absence (Sc wine) and presence (Ap + Sc wine) of the pectinolytic strain, as well as the odour threshold and descriptors for each aroma compound. Twenty-one individual volatile compounds have been studied in the wines, classified in four different chemical groups (ethyl and acetates esters, higher alcohols, terpenes and norisoprenoids). Sixteen of these compounds showed significant differences between the wines analysed, where almost all esters and all norisoprenoids were statistically higher in Ap + Sc wine, whereas the majority of higher alcohols were significantly higher in Sc wine.

Analysing by chemical groups, a significant increase in total esters (45.3 mg/L) was observed in wine inoculated with the pectinolytic strain (Ap + Sc) with respect to Sc wine (27.6 mg/L) (Table 3). This difference was mainly due to the increase in ethyl esters such as ethyl hexanoate and octanoate and diethyl succinate, related to fruity and fresh aromas, and to a lesser extent due to acetates, related to fruity and floral aromas (Peinado et al., 2004). These compounds are responsible for wine secondary aroma, produced during alcoholic fermentation (Cortés-Diéguez et al., 2015), thus these results would be related to the influence of *A. pullulans* metabolism on production of this aromatic fraction not only by contributing individually but also by modulating the genomic expression patterns of *S. cerevisiae* due to their coexistence during wine fermentation (Barbosa et al., 2015). It has been reported that grape-associated strains of *A. pullulans* produced a broad spectrum of volatile compounds typical of red wines, among which the esters ethyl acetate, benzoate and octanoate were present (Verginer et al., 2010). Likewise, epiphytic or environmental *A. pullulans* strains were able to produce higher alcohols like 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol (Davis et al., 2012; Di Francesco et al., 2015) and fatty acids (Gostinčar et al., 2008), both groups being precursors for ester formation mainly by a detoxification mechanism since esters are usually less toxic than their respective alcohol or acidic precursors (Belda et al., 2017). Increased ester concentrations in wines produced by mixed starters in comparison with pure cultures have been described earlier (Mendoza et al., 2011; Moreira et al., 2008).

Higher alcohols concentration was significantly lower in Ap + Sc wine (29.7 mg/L) than in Sc wine (45.9 mg/L), dominating 2-phenylethanol (Table 3) that is associated to honey and rose-like aroma, unlike the majority of higher alcohols commonly related to unpleasant herbaceous odour (Moreira et al., 2008). The significant decrease of 2-phenylethanol in the Ap + Sc wine could be explained by polysaccharidases production by *A. pullulans* GM-R-22 (Merín and Morata de Ambrosini, 2015), according to results reported by Louw et al. (2006), who informed that the reduction of this compound in red wines fermented with *S. cerevisiae* recombinant pectinolytic yeasts was related to the expression of genes that codify for pectinases, xylanases and glucanases.

Terpenes are compounds that constitute wine varietal aroma responsible for flowery, sweet and citric characters, which are present in the grape mainly as non-volatile *O*-glycosides precursors. Enzymatic activity on these glycosides intensifies the varietal aroma (Rodríguez et al., 2010). No significant difference was observed between both wines in total terpenes concentration or, excepting nerol, in each individual compound (Table 3), which is consistent with the absence of β-glucosidase activity in GM-R-22 enzymatic extract (Merín and Morata de Ambrosini, 2015). Similarly, Roldán et al. (2006) did not observe statistical differences in the terpene fraction when Palomino fino wine was treated with an enzymatic preparation containing pectinase, cellulase, quitinase and glucanase activities.

The varietal aroma also results from other compounds that arise from the grape, like norisoprenoids, where β-damascenone and β-ionone are important for being powerful odorants (Guth, 1997). In Ap + Sc wine, both norisoprenoids detected were significantly higher, β-damascenone associated with fruity (dried plum and blackberry), honey and caramel aromas, and β-methyl-ionone, the direct derivative of β-ionone, related to floral aroma (violet) (Peinado et al., 2004; Vilanova et al., 2017) (Table 3). The cause was probably the enzymatic action of the GM-R-22 pectinolytic system containing not only pectinase but also cellulase and hemicellulase activities (Merín and Morata de Ambrosini, 2015), contributing to the degradation of grape cell wall and to a greater releasing of these compounds into the wine.

Sulphur compounds, generally related to unpleasant aromas of onion, potato, cooked vegetable, cabbage, rubber (Jofré et al., 2010), were not detected in neither wine, demonstrating that *A. pullulans* GM-R-22 did not produce compounds associated to undesirable sulphur odours that could determine its exclusion in vinification.

Of all detected volatile compounds, 2-phenylethanol showed the highest concentration, followed by diethyl succinate, ethyl octanoate and ethyl hexanoate. Despite differences associated to distinct *terroir*, this aromatic analysis exhibited characteristic volatile compounds of Malbec wines that let us note similarities between wines produced in

Table 3
Volatile compounds studied in Malbec wines obtained in absence (Sc wine) and presence (Ap + Sc wine) of the pectinolytic strain *A. pullulans* GM-R-22, with their respective odour threshold, odour descriptor, concentration (mg/L) and odour activity value (OAV).

Aromatic compound	Odour threshold (mg/L)[c]	Odour descriptor[d]	Concentration (mg/L)			Odour activity value (OAV)[e]	
			Sc wine	Ap + Sc wine	Sig.	Sc wine	Ap + Sc wine
Esters							
Ethyl butanoate	0.020[f]	Strawberry, apple[g]	0.289 ± 0.010[a]	0.369 ± 0.013[b]	*	14.4	18.4
Ethyl hexanoate	0.008[g]	Fruity, green apple, brandy[g]	7.069 ± 0.072[a]	9.278 ± 0.061[b]	*	883.6	1159.8
Ethyl octanoate	0.005[h]	Fruity, sweet, pear[g]	8.524 ± 0.093[a]	10.588 ± 0.275[b]	*	1704.8	2117.6
Ethyl 2-hexenoate	NF	Fruity, pineapple[i]	0.131 ± 0.006	0.135 ± 0.008	ns	–	–
Diethyl succinate	1.200[g]	Light fruity, watermelon[g,j]	8.840 ± 0.127[a]	21.712 ± 0.147[b]	*	7.4	18.1
Butyl butanoate	0.100[k]	Sweet fruit, apricot[k]	1.236 ± 0.020[b]	0.713 ± 0.005[a]	*	12.4	7.1
Hexyl acetate	0.026[l]	Apple, cherry, pear, floral[g]	0.167 ± 0.004[a]	0.367 ± 0.012[b]	*	6.4	14.1
2-Phenylethyl acetate	0.250[f]	Floral, honey, tobacco[f,m]	1.352 ± 0.055[a]	1.879 ± 0.074[b]	*	5.4	7.5
Total esters			27.606 ± 0.183[a]	45.252 ± 0.395[b]	*		
Higher alcohols							
3-Methyl-2-butanol	NF	NF	1.828 ± 0.039[b]	0.087 ± 0.005[a]	*	–	–
4-Methyl-1-pentanol	50[n]	Almond, toasted[n]	0.242 ± 0.010	0.206 ± 0.012	ns	< 1	< 1
3-Methyl-2-hexanol	NF	NF	0.042 ± 0.002[a]	0.190 ± 0.007[b]	*	–	–
2-Ethyl-1-hexanol	8[n]	Floral, sweet fruity[n]	0.151 ± 0.003	0.152 ± 0.005	ns	< 1	< 1
2-Nonanol	0.058[o]	Green, cucumber[n]	0.035 ± 0.002	ND	*	< 1	< 1
2-Phenylethanol	14[f,h]	Rose, honey[g]	43.509 ± 1.021[b]	29.084 ± 1.269[a]	*	3.0	2.1
cis-3-Hexenol	0.400[f]	Green, bitter, unripe banana[n]	0.057 ± 0.003	ND	*	< 1	< 1
Total higher alcohols			45.864 ± 1.027[b]	29.715 ± 1.270[a]	*		
Terpenes							
Limonene	0.200[o]	Floral, green, lemon[o]	0.244 ± 0.015	0.180 ± 0.030	ns	1.2	< 1
Linalool	0.025[h]	Citrus, floral, sweet[g]	0.380 ± 0.014	0.414 ± 0.013	ns	15.2	16.6
Iso-limonene	NF	NF	0.086 ± 0.004	ND	*	–	–
Nerol	0.400[n]	Rose, lime[n]	0.185 ± 0.006[a]	0.355 ± 0.046[b]	*	< 1	< 1
Total terpenes			0.889 ± 0.007	0.946 ± 0.042	ns		
Norisoprenoids							
β-Damascenone	0.00005[f]	Honey, caramel, dried plum, blackberry[m,n]	0.467 ± 0.008[a]	0.736 ± 0.005[b]	*	9,340.0	14,720.0
β-Methyl ionone	NF	NF	ND	0.386 ± 0.010	*	–	–
Total norisoprenoids			0.467 ± 0.008[a]	1.121 ± 0.014[b]	*		
Sulphur compounds	–	–	ND[p]	ND[p]	–	–	–

[a,b]Mean concentration values of two experiments ± standard deviation with different superscript letters within the same row are significantly different according to LSD Fisher test (*$p < 0.05$); Sig.: significance; ns: not significant; ND: not detected; NF: not found in the literature; –: not corresponding.
[c] Odour threshold (mg/L) determined in 10–12% (v/v) ethanol and [d] odour descriptors, obtained from the literature: [f] Guth (1997); [g] Peinado et al. (2004); [h] Ferreira et al. (2000); [i] Qian and Wang (2005); [j] Farenzena and Tombesi (2015); [k] Takeoka et al. (1990); [l] Etiévant (1991); [m] Vilanova et al. (2017); [n] Welke et al. (2014); [o] Jiang and Zhang (2010).
[e] OAVs were expressed as the mean concentration of an aroma compound divided by its odour threshold value.
[p] Method detection limits: 0.36–1.67 μg/L.
Sc, *S. cerevisiae* IOC 18-2007; Ap, *A. pullulans* GM-R-22.

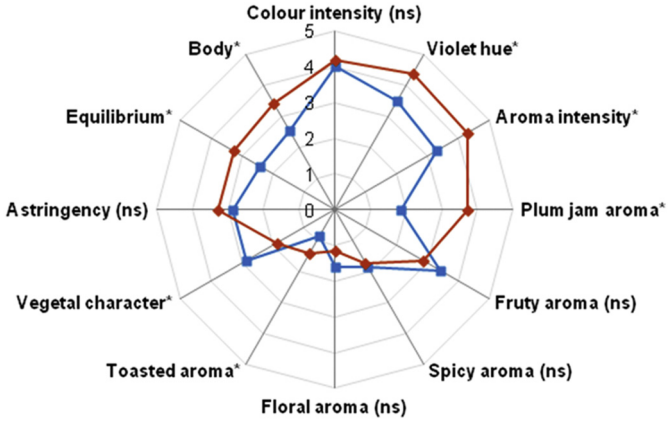

Fig. 4. Scores obtained from sensory analysis for wines fermented by pure *S. cerevisiae* culture (■) and mixed *S. cerevisiae* and *A. pullulans* GM-R-22 culture (♦). (*): significant difference between wines (ANOVA and LSD Fisher test, $p < 0.05$, n = 2); (ns): not significant.

different geographical regions, in term of major contributors of aroma compounds from this emblematic Argentinean wine (Farenzena and Tombesi, 2015; Goldner and Zamora, 2007; King et al., 2014; Mendoza et al., 2011).

Several volatiles were detected in each wine sample, however not all of them have the same impact on the overall wine aroma. The contribution of each volatile compound to the wine aroma can be evaluated qualitatively by means of its associate descriptor and quantitatively by means of its OAV (Jiang and Zhang, 2010). Eleven compounds of Malbec wine showed OAV > 1 (Table 3), which were deemed to contribute to wine aroma (Guth, 1997). Among them, esters were the class that includes the larger number of odorant components (64%). Furthermore, maximum OAVs were obtained for ethyl esters (ethyl octanoate and hexanoate) and β-damascenone in Ap + Sc wine. Previously, β-damascenone and ethyl octanoate OAVs have been found as the highest odoriferous values for red and white wines (Cortés-Diéguez et al., 2015; Jiang and Zhang, 2010; Welke et al., 2014).

3.4. Sensory analysis

Sensory analysis of young Malbec wines was carried out to assess the impact of *A. pullulans* GM-R-22 inoculated during PCM on the organoleptic quality of the fermented product. The main sensory descriptors of colour, flavour, mouth-feel and equilibrium attributes of wines were considered (Fig. 4). Wine inoculated with the pectinolytic strain did not show significant differences in the colour intensity but it showed significantly higher score for violet hue than Sc wine. These

results are in accordance with chromatic analysis carried out by the analytical method (Table 2).

The aromatic phase of wines exhibited significantly higher global aromatic intensity in Ap + Sc wine, with a noticeable increase in plum jam aroma and a lesser but significant increase in toasted aroma with respect to Sc wine (Fig. 4). However, no significant differences were obtained for fruity, floral and spicy aromas between both wines. These results are consistent with the volatiles profile, since the higher aroma intensity in the wine produced with *A. pullulans* is related to higher contents and OAVs of esters and norisoprenoids, like β-damascenone that is associated with dried plum and caramel aromas, and 2-phenylethyl acetate that is related to honey and tobacco aromas, features described for Ap + Sc wine in the sensory analysis.

Vegetal character was scored higher for Sc wine (Fig. 4) and this outcome could be associated with the detection only in this wine of 2-nonanol and *cis*-3-hexenol (Table 3), whose descriptors are green fruit and herbaceous. Finally, body, equilibrium and astringency mouth-feel attributes presented a significant augment in Ap + Sc wine, although the last attribute did not show statistical differences.

In conclusion, the presence of *A. pullulans* in the vinification, applied with the aim of improving chromatic and technological properties of Malbec wine, helped to highlight violet hue, but also plum jam aroma, body and equilibrium that are distinctive features of this wine variety.

This study contributes to the knowledge of pectinolytic production of non-*Saccharomyces* yeasts during low-temperature red vinification, particularly to the understanding of *A. pullulans* behaviour, a non-conventional species in winemaking. Previously, Belda et al. (2016) reported a first approach to the application of a strain of *A. pullulans* to red microvinification to improve clarification and phenolic extraction processes. To the best of our knowledge, the present study is the first work in proving the efficient enzymatic production of *A. pullulans* GM-R-22 during low-temperature vinification and assessing not only the chromatic and clarification properties but the overall quality of the wine, evaluating the impact of this *A. pullulans* strain on volatile compounds profile and sensory analysis of the final product.

The pectinolytic strain *A. pullulans* GM-R-22 seems to be promising for applying to red winemaking at low temperature in co-culture with *S. cerevisiae*. Microvinifications on non-pasteurised Malbec must and large-scale experiments are being carried out to confirm and validate these results. In future studies, the potential risks of the *A. pullulans* species on safety should be deeply investigated before its application to winemaking.

Conflict of interest

No conflict of interest declared.

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