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Exploring *Metschnikowia pulcherrima* as a Co-Fermenter with *Saccharomyces cerevisiae*: Influence on Wine Aroma during Fermentation and Ageing

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Abstract: Non-*Saccharomyces* yeasts, particularly *Metschnikowia pulcherrima*, are considered alternatives to SO_2 in winemaking, combating specific microorganisms. The sensory profile of the wine is contingent upon the type of yeast, the fermentation conditions, and the concentration and mode of application with *Saccharomyces cerevisiae* strains (whether pure or used in mixed/sequential co-fermentation). This study assessed the aroma in red wines produced with *S. cerevisiae* (Sc) and *M. pulcherrima* (Mp, non-Sc), incorporating variations in the method of addition and the inclusion or exclusion of SO_2. The enological parameters of the wines were slightly affected. Volatile compounds were analysed in the wines through gas chromatography–mass spectrometry (GC-MS) at three moments: at the end of malolactic fermentation (MLF) and after 6 and 9 months of bottle ageing. Sequential fermentation of Sc and Mp reduced the concentration of most identified alcohols and acids, which is favourable, as these compounds can yield undesirable aromas at high concentrations. Regardless of the yeast mixture and Mp dose, a majority of the acetate esters and ethyl esters were quantified at concentrations above their perception thresholds, thus enhancing the sensory quality of the wines. Sensory analysis of wines showed generally positive evaluations. Using non-*Saccharomyces* as an alternative to SO_2 improves the aromatic profile of wines.

Keywords: *Saccharomyces cerevisiae*; non-*Saccharomyces* yeast; *Metschnikowia pulcherrima*; sequential fermentation; volatile composition; red wine; ageing; bottles; SO_2

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1. Introduction

Wine fermentation is a complex biological process initiated by the activity of certain classes of carbohydrate-degrading microorganisms [1]. *Saccharomyces cerevisiae* (Sc) and non-*Saccharomyces* (non-Sc) yeasts, mainly belonging to the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Issatchenkia*, and *Kluyveromyces*, are commonly encountered during the early stages of alcoholic fermentation (AF). Nevertheless, *Saccharomyces cerevisiae* quickly overcomes them thanks to its higher ethanol tolerance and more competitive growth rate [2–4]. Throughout fermentation, sugars are primarily converted into ethanol and carbon dioxide, as well as sulphite and sulphur; excessive production of these compounds can lead to off-flavours and delay the onset of malolactic fermentation [4,5]. Once the AF process is finished, malolactic fermentation begins; at this point, it is also crucial to control microorganisms to avoid the growth of yeast and undesirable moulds that can spoil the wine, including mainly lactic acid bacteria (LAB) and acetic acid bacteria (AAB) [5,6].

To stabilise and extend the shelf life of wines, sulphur anhydride or sulphur dioxide (SO_2) has been allowed for many years as a food additive due to its antioxidant and antimicrobial

properties [5–12]. Despite the advantages of SO_2 in wine quality, its use has been limited because its ingestion in high concentrations can cause health problems in sensitive individuals and may even lead to potential organoleptic alterations, neutralising the aroma and producing undesirable aromas in wine [5–7,13]. For this reason, over the past two decades, the scientific and viticultural communities have developed new interest in implementing alternatives to reduce and replace SO_2 activity in winemaking [6,8,14]. Various scientific approaches have been proposed, ranging from physical treatments (microfiltration, pasteurisation, pulsed electric fields, high pressure, ultrasound, etc.) to chemical treatments (sorbic acid, lysozyme, dimethyl dicarbonate, chitosan, and colloidal silver) [5,13]. However, these treatments have several limitations, including high cost, deterioration of the sensory quality of the wine, and presenting only antimicrobial or antioxidant activity [8]. Therefore, biological treatments have gained great relevance in utilising microorganisms as a primary source of antimicrobial agents [6,11,15–17].

Non-Saccharomyces yeasts have been widely used as an alternative to reduce SO_2 dosage and improve wine's sensory characteristics [18,19]. In this sense, a recent study has demonstrated the potential of enological yeasts different from *Saccharomyces*; some of these non-*Saccharomyces* enological yeasts include *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Hanseniaspora vineae*, *Torulospora delbrueckii*, *Starmerella bacillaris*, and *Metschnikowia pulcherrima* [20]. Windholtz et al. [19] evaluated the antioxidant and antimicrobial properties of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* in grape must; they showed that these yeasts limit the growth of acetic acid bacteria, with a bioprotective effect comparable to the addition of sulphur dioxide. Similarly, Yao et al. [21] and Agarbati et al. [9] were able to improve the bioprotective character and olfactory intensity of wine made with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. Other characteristics explored have been the improvement in aromatic [22,23], phenolic [24,25], and nitrogen composition [26].

Thanks to its ability to produce antimicrobial compounds, *Metschnikowia pulcherrima* can be employed as a natural agent for biological control, partially or entirely replacing sulphur dioxide. Research has reported the antimicrobial activity of *M. pulcherrima* against undesirable pathogens, fungi, and yeasts. This antimicrobial action of *M. pulcherrima* is determined by the secretion of the pulcherrim protein [6,27].

Saccharomyces cerevisiae produces relatively low amounts of aromatic compounds and is considered reasonably neutral [28]. Consequently, non-Sc yeasts have gained importance because they possess some desirable enological characteristics that are absent in *S. cerevisiae*, such as the production of high levels of aroma compounds and the secretion of several enzymes that can enhance wine's complexity in controlled fermentation [16,29], so a wide variety of non-Sc yeast genetics are commercially available [30,31].

Quantitatively, the most abundant wine odorants are those generated during alcoholic fermentation, particularly higher alcohols, esters, and acids; moreover, the formation and accumulation of some volatile compounds require ageing time [32–34]. Non-Sc strains have been used to diversify the aromatic profile of wines, thus increasing the biosynthesis of fermentative aromas. However, to achieve correct fermentation, their use necessarily requires the performance of mixed fermentations (simultaneous or sequential) together with *S. cerevisiae* [35–37]. The implementation of this type of assay in enology has resulted in interesting data on the aromatic profile of wines [30,31,38–41].

It has been demonstrated that the addition of *M. pulcherrima* prior to *S. cerevisiae* substantially alters the profile of fermentative compounds produced during vinification [16,36]. For this reason, and with the goal of enhancing the chemical composition and sensory properties of red wine, the combined utilisation of Sc and non-Sc yeasts has been proposed [39]. It should be noted that most of the studies found in the literature have focused on the analysis of aromatic compounds at a single point in time, without considering the possible evolution of aromatic compounds over ageing time. Therefore, this work is novel in that it studies the wines' volatile compositions at three points in time: at the end of malolactic fermentation and at 6 and 9 months of bottle ageing. In addition, as mentioned above, the search for alternatives to SO_2 is a topic of great interest in enology. For these reasons, the objective of this study was to investigate the aromatic composition after the end of malolactic fermentation (MLF) and at 6 and 9 months

after the bottling of red wines elaborated with or without SO_2. This research involves the use of *M. pulcherrima* as an alternative to this additive in conjunction with *S. cerevisiae*.

2. Materials and Methods

2.1. Winemaking and Ageing in Bottles

The trial was carried out with approximately 450 kg of Tempranillo grapes hand-harvested at their optimal maturation stage and under perfect sanitary conditions. At the winery, the grape clusters underwent destemming and crushing. The resulting must was homogeneously distributed in eight tanks of 50 L. Four trials were conducted, each in duplicate. The initial must received the following additions: (i) 3 g/hL of total SO_2; (ii) 10 g/hL of *Metschnikowia pulcherrima* (AWRI 3050 Bioprotect, AB Mauri-ABBiotek, Toowoomba, Australia) non-*Saccharomyces* yeast; (iii) 3 g/hL of total SO_2; and (iv) 10 g/hL of *Metschnikowia pulcherrima* (AWRI 3050 Bioprotect). They were kept 4 days in cold storage at 8 °C. Subsequently, they were tempered (20 °C) and inoculated with: (i) and (ii) 20 g/hL of *Saccharomyces cerevisiae* yeast (Maurivin AWRI 796 (AB Mauri-ABBiotek); and (iii) and (iv) 50 g/hL of *Metschnikowia pulcherrima* (AWRI Bioprotect). In the latter two assays, when the ethanol content was 5%, 20 g/hL of *Saccharomyces cerevisiae* yeast (Maurivin AWRI 796) was inoculated. The names assigned to these 4 assays were: (i) SO_2+Sc; (ii) n-Sc_{10}+Sc; (iii) SO_2+n-Sc_{50}+Sc; and (iv) n-Sc_{10}+n-Sc_{50}+Sc.

The alcoholic fermentation (AF) was conducted at 20 °C, with daily monitoring of density and temperature evolution. Additionally, the glucose/fructose level was tracked at the end of AF. Throughout the fermentation process, the cap was punched down once a day to favour the contact between the marc and the must, thus promoting the extraction of compounds from the grape skins. After the AF was completed (when glucose/fructose were not detected), the wines were racked off and pressed. The resulting wines were transferred to 25 L stainless steel tanks, where lactic acid bacteria *Oeonococcus oeni* Pinnacle MaloSafe (ABBiotek) were inoculated, at 1 g/hL, to carry out the malolactic fermentation (MLF). This fermentation was developed at 20 °C. MLF progress was monitored by measuring malic and lactic acids content. Once finished (when malic acid was not detected), the wines underwent cold stabilisation (10 °C, during 1 month) and were bottled, remaining in the cellar bottle rack at controlled temperature and humidity (16 °C, 50–60%).

In the wines at the end of MLF and after 6 and 9 months of ageing in bottles, aliquots of each wine were taken and frozen at −20 °C for subsequent determination of their volatile compositions.

2.2. Analysis of General Parameters

Wines were characterised by measuring the alcoholic degree, pH, total acidity, volatile acidity, total anthocyanins, colour index (CI), and total polyphenol index (TPI) using the official methods established by the OIV [42]. Malic acid, lactic acid, yeast assimilable nitrogen (YAN), and total phenols were determined using Miura One enzymatic equipment (TDI, Barcelona, Spain).

As the vinifications were performed in duplicate, the results of these parameters are shown as the average of two analyses (n = 2).

2.3. Analysis of Wines' Volatile Compounds through GC-MS

The method employed for determining the wines' volatile compounds was detailed by Garde-Cerdán et al. [43]. In a 10 mL tube, 8 mL of wine (centrifuged at 3220× *g*, during 15 min, at 4 °C), 10 µL of internal standard (2-octanol, Sigma-Aldrich, Madrid, Spain), and a magnetic stir bar were added. Extraction of the wines' volatile compounds was performed by stirring the sample (during 15 min) with 400 µL of dichloromethane (Merck, Darmstadt, Germany). After cooling for 10 min at 0 °C, the organic phase was separated through centrifugation (5031× *g*, 10 min, 4 °C), and the extract was collected in a vial. Gas chromatographic determination of analytes was performed using a Gas Chromatograph (GC) with a Mass Detector (MS) (Agilent, Palo Alto, CA, USA). The volume of injection was of 2 µL. A VF-Wax 52 CB

(60 m × 0.25 mm i.d. × 0.25 µm) capillary column (Agilent) was used. The temperature of the injector was programmed from 40 °C to 250 °C, at 180 °C/min. The oven temperature was held during 2 min at 50 °C and then programmed to rise at 3 °C/min from 50 °C to 250 °C. The detector was operated at electronic impact mode (70 eV), with an acquisition range (m/z) from 29 to 260. The identification of volatile compounds was conducted using the NIST library and by comparing with the mass spectrum of available standards (Sigma-Aldrich). A semi-quantification was carried out, relating the areas of each volatile compound with the area and the known concentration of the internal standard (2-octanol). As the vinifications were performed in duplicate, the results of these parameters are shown as the average of two analyses ($n = 2$).

2.4. Sensory Analysis

The sensory analysis of the Tempranillo wines made with the different trials was carried out by 13 expert judges. The sensory analysis of the wine was carried out 9 months after bottling. The evaluation occurred in a specific testing area, adhering to the protocols set by the International Organization for Standardization. Before the assessment, panellists underwent training to acquaint themselves with sensory analysis terminology. The wines were presented in duplicate and were evaluated following the methodology outlined by Garde-Cerdan et al. [44] comparatively, using the random blind tasting system. The tasting sheet used has a scale determined by the OIV [45] to rate the wines from insufficient (40 points) to excellent (100 points). The sensory analysis also included a quantitative assessment of the olfactory and taste attributes. The judges scored each sensory attribute on a scale from its absence (0 points) to its maximum presence (10 points). The wine tastings were conducted randomly in clear glasses at room temperature and in individual blocks.

2.5. Statistical Analysis

The statistical elaboration of the data was performed using SPSS Version 21.0 (Chicago, IL, USA). Volatile compounds' data were processed using the variance analysis (ANOVA) ($p \leq 0.05$). The differences between means were compared using the Duncan, and the effect of the assay, the moment, and their interaction was analysed using a multifactor analysis and post hoc Duncan's multiple range test.

3. Results and Discussion

3.1. Enological Parameters of the Wines

The evaluation of the general parameters was carried out after MLF and after 6 and 9 months of bottling for each trial, as shown in Table 1. At the end of MLF, SO_2+Sc samples were characterised by a higher alcohol degree, total acidity, and lactic acid than the three other assays, except for the total acidity of n-Sc_{10}+Sc, which did not show differences from SO_2+Sc but was higher than SO_2+n-Sc_{50}+Sc and n-Sc_{10}+n-Sc_{50}+Sc. Additionally, n-Sc_{10}+Sc showed a lower lactic acid content than SO_2+Sc, but it was higher than the other trials. Varela et al. [3] also observed a reduction in the alcoholic degree of wines elaborated by sequential fermentations. In the rest of the enological parameters (Table 1), no differences were observed among the wines at the end of MLF. Some enological parameters were not measured after 6 and 9 months of bottling because they are parameters that should not change during bottling. After 6 months of bottling, the wines studied showed no differences among themselves in the general parameters. At 9 months after bottling, differences were only found among the wines studied in the OD values at 420 nm, 520 nm, and 620 nm. The n-Sc_{10}+Sc wines showed higher values than SO_2+n-Sc_{50}+Sc and n-Sc_{10}+n-Sc_{50}+Sc samples and similar values to those of the SO_2+Sc wines. However, no differences were observed in the CI of the wines (a parameter obtained from the sum of OD values cited above). The wines elaborated in the more traditional way (SO_2+Sc) showed the highest alcohol content. Therefore, the use of non-*Saccharomyces* yeasts is an interesting method to produce wines according to the interests of current consumers, who are moving towards lower alcohol consumption. Overall, no notable differences were found in the general parameters of the wines.

Table 1. Enological parameters of the wines at the end of malolactic fermentation (MLF) and after 6 and 9 months in bottles (assays SO_2+Sc, n-Sc_{10}+Sc, SO_2+n-Sc_{50}+Sc, n-Sc_{10}+n-Sc_{50}+Sc).

	End of MLF				6 Months in Bottles				9 Months in Bottles			
	SO_2+Sc	**n-Sc_{10}+Sc**	**SO_2+n-Sc_{50}+Sc**	**n-Sc_{10}+n-Sc_{50}+Sc**	**SO_2+Sc**	**n-Sc_{10}+Sc**	**SO_2+n-Sc_{50}+Sc**	**n-Sc_{10}+n-Sc_{50}+Sc**	**SO_2+Sc**	**n-Sc_{10}+Sc**	**SO_2+n-Sc_{50}+Sc**	**n-Sc_{10}+n-Sc_{50}+Sc**
Alcohol degree (% *v*/*v*)	13.75 ± 0.07 b	13.23 ± 0.25 a	13.05 ± 0.00 a	13.20 ± 0.00 a	-	-	-	-	-	-	-	-
pH	4.04 ± 0.06 a	3.95 ± 0.01 a	4.19 ± 0.03 a	4.11 ± 0.04 a	-	-	-	-	-	-	-	-
Total acidity (g/L) *	5.10 ± 0.11 b	5.23 ± 0.13 b	3.88 ± 0.19 a	3.94 ± 0.11 a	-	-	-	-	-	-	-	-
Malic acid (g/L)	n.d.	n.d.	n.d.	n.d.	-	-	-	-	-	-	-	-
Lactic acid (g/L)	2.62 ± 0.10 c	2.81 ± 0.01 b	2.29 ± 0.02 a	2.35 ± 0.19 a	-	-	-	-	-	-	-	-
Volatile acidity (g/L) **	0.50 ± 0.02 a	0.46 ± 0.06 a	0.51 ± 0.06 a	0.48 ± 0.11 a	0.46 ± 0.0 a	0.46 ± 0.06 a	0.52 ± 0.00 a	0.49 ± 0.15 a	0.49 ± 0.04 a	0.47 ± 0.10 a	0.56 ± 0.08 a	0.50 ± 0.16 a
YAN (mg N/L)	9 ± 2 a	8 ± 3 a	31 ± 31 a	11 ± 7 a	-	-	-	-	-	-	-	-
OD 420 nm	0.24 ± 0.01 a	0.23 ± 0.03 a	0.21 ± 0.00 a	0.21 ± 0.00 a	0.28 ± 0.02 a	0.27 ± 0.03 a	0.25 ± 0.00 a	0.25 ± 0.00 a	0.30 ± 0.02 ab	0.29 ± 0.03 b	0.26 ± 0.01 a	0.27 ± 0.01 a
OD 520 nm	0.30 ± 0.02 a	0.30 ± 0.05 a	0.23 ± 0.01 a	0.25 ± 0.01 a	0.36 ± 0.04 a	0.35 ± 0.06 a	0.30 ± 0.00 a	0.31 ± 0.01 a	0.37 ± 0.05 b	0.37 ± 0.06 b	0.30 ± 0.01 a	0.33 ± 0.00 a
OD 620 nm	0.07 ± 0.00 a	0.06 ± 0.01 a	0.05 ± 0.00 a	0.05 ± 0.00 a	0.11 ± 0.01 a	0.10 ± 0.02 a	0.10 ± 0.00 a	0.10 ± 0.00 a	0.12 ± 0.01 ab	0.12 ± 0.01 b	0.11 ± 0.00 a	0.11 ± 0.00 a
Colour intensity (CI)	6.11 ± 0.27 a	5.85 ± 0.88 a	4.92 ± 0.11 a	5.10 ± 0.06 ab	7.51 ± 0.74 a	7.21 ± 1.10 a	6.48 ± 0.08 a	6.64 ± 0.16 a	8.00 ± 0.74 a	7.73 ± 1.01 a	6.75 ± 0.20 a	7.15 ± 0.01 a
TPI	43.70 ± 0.69 a	41.66 ± 4.02 a	42.45 ± 0.66 a	41.40 ± 0.93 a	43.40 ± 0.42 a	41.50 ± 3.54 a	42.65 ± 0.78 a	40.75 ± 1.06 a	42.04 ± 0.49 a	40.15 ± 3.36 a	41.58 ± 0.75 a	39.96 ± 1.05 a
Total anthocyanins (mg/L)	553.3 ± 15.2 a	499.4 ± 67.1 a	574.8 ± 27.4 a	538.2 ± 6.1 a	226.1 ± 11.1 a	204.4 ± 35.9 a	255.0 ± 24.4 a	235.5 ± 5.5 a	73.1 ± 0.3 ab	66.1 ± 8.5 a	87.3 ± 1.9 c	84.5 ± 0.9 bc
Total phenols (mg/L)	1718.6 ± 63.7 a	1626.5 ± 48.9 a	1812.2 ± 130 a	1676.2 ± 70.4 a	1625.3 ± 15.3 a	1626.5 ± 146.1 a	1656.3 ± 52.2 a	1570.8 ± 70.2 a	1536.8 ± 11.2 a	1458.2 ± 122.8 a	1505.3 ± 26.1 a	1451.8 ± 19.9 a

* As g/L of tartaric acid. ** As g/L of acetic acid. YAN: yeast assimilable nitrogen; OD: optical density; TPI: total polyphenol index; n.d.: not detected; -: not analysed. All parameters are given as average values ± the standard deviations (n = 2). For each moment (end of MLF and 6 and 9 months in bottles), different letters indicate significant differences between samples ($p \leq 0.05$).

3.2. Volatile Compositions of the Wines

The results of the volatile compositions of the wines elaborated from the different trials (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc) after the end of MLF and after 6 and 9 months of bottle ageing are presented in Figures 1–4.

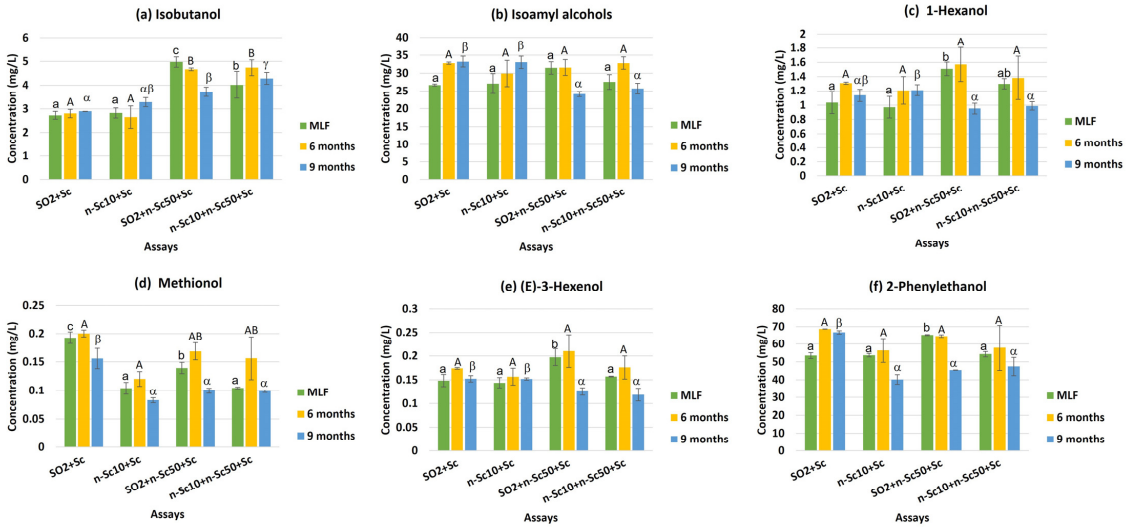

Figure 1. Alcohol concentrations (mg/L) of wines made with the different assays (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc). Different lowercase, uppercase, or Greek letters indicate significant differences between samples ($p \leq 0.05$) at the end of MLF and after 6 or 9 months in bottles, respectively.

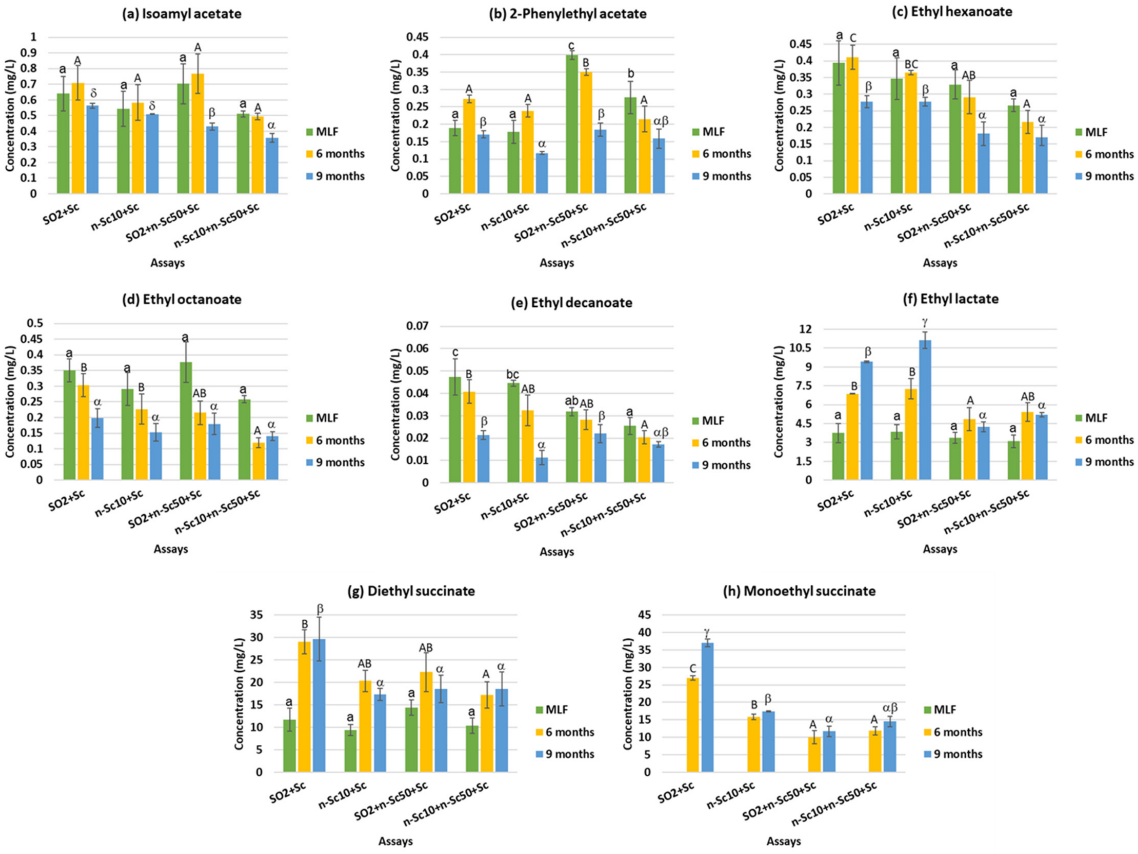

Figure 2. Concentrations of esters (mg/L) in wines made with the different assays (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc). Different lowercase, uppercase, or Greek letters indicate significant differences between samples ($p \leq 0.05$) at the end of MLF and after 6 or 9 months in bottles, respectively.

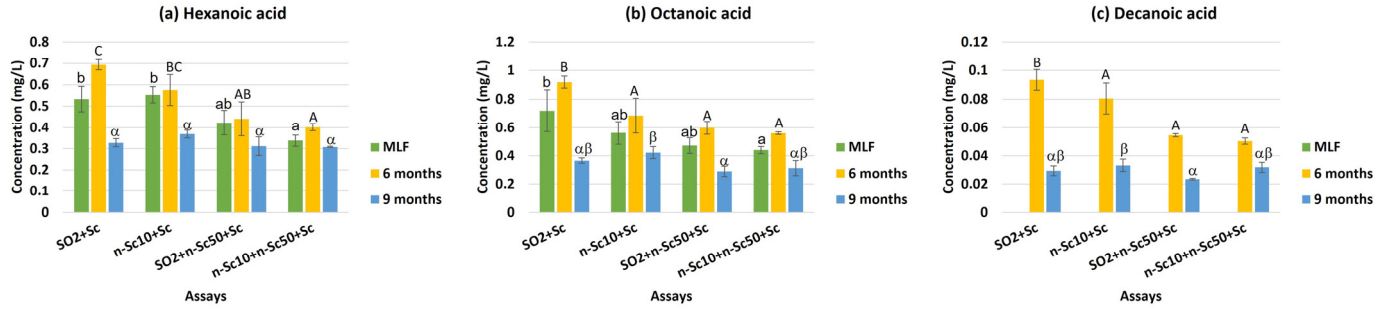

Figure 3. Acids concentrations (mg/L) in wines made with the different assays (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc). Different lowercase, uppercase, or Greek letters indicate significant differences between samples ($p \leq 0.05$) at the end of MLF and after 6 or 9 months in bottles, respectively.

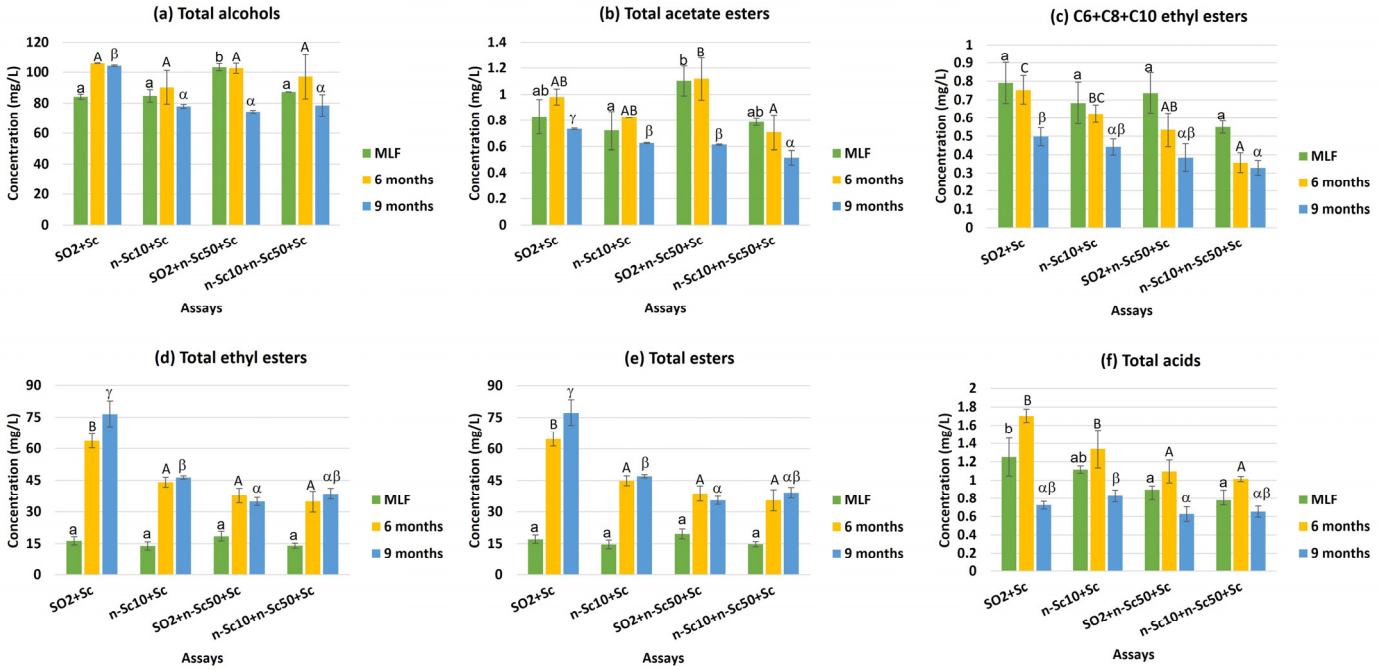

Figure 4. Concentrations of total alcohols, acetate esters, C6 + C8 + C10 ethyl esters, ethyl esters, esters, and acids (mg/L) in wines made with the different assays (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc). Different lowercase, uppercase, or Greek letters indicate significant differences between samples ($p \leq 0.05$) at the end of MLF and after 6 or 9 months in bottles, respectively.

Figure 1 shows the results of higher alcohols found in the different wines. In total, six compounds of this family were identified. At the end of malolactic fermentation (MLF), wines made with SO_2+n-Sc_{50}+Sc had higher contents of isobutanol, 1-hexanol, (E)-3-hexenol, and 2-phenylethanol (Figure 1a,c,e,f), showing significant differences compared to the other trials, except for 1-hexanol, which displayed similar levels in the samples treated with n-Sc_{10}+n-Sc_{50}+Sc. These results are consistent with the data reported by Prior et al. [46]. These authors observed that sequential fermentations with the aforementioned yeasts resulted in higher isobutanol concentrations compared to pure culture fermentation. Meanwhile, Escribano et al. [47], in their study on the fermentative behaviour of different non-*Saccharomyces* yeasts, also showed that *M. pulcherrima* produced a higher amount of isobutanol and 2-phenylethanol than *S. cerevisae*. As for the isoamyl alcohols content in the wines, there were no significant differences between trials at the end of MLF (Figure 1b). However, methionol concentration was significantly higher in the wines from the assay of SO_2+Sc (Figure 1d).

After 6 months of bottling, higher isobutanol content was observed in the SO_2+n-Sc_{50}+Sc and n-Sc_{10}+n-Sc_{50}+Sc assays (Figure 1a), providing evidence that isobutanol content was significantly higher when sequential fermentations were performed, i.e., when *M. pulcherrima* was applied at a concentration of 50 g/hL followed by *S. cerevisiae.*

There were no significant differences in isoamyl alcohols, 1-hexanol, (E)-3-hexenol, and 2-phenylethanol content (Figure 1b,c,e,f). On the other hand, higher levels of methionol were observed in the samples elaborated with SO_{2+}Sc (Figure 1d).

After 9 months of bottling, higher concentrations of isobutanol were observed in the n-Sc_{10}+n-Sc_{50}+Sc trials (Figure 1a). Contrary results were evident in the content of isoamyl alcohols (fatty and chemical notes) and (E)-3-hexenol, with higher concentrations obtained in the SO_2+Sc and n-Sc_{10}+Sc samples (Figure 1b,e). Similar behaviour was observed for methionol and 2-phenylethanol content (Figure 1d,f), because wines produced from sequential fermentations led to lower production of these compounds after 9 months of bottling. In contrast, Varela et al. [3] found that the mixed use of *M. pulcherrima* with *S. uvarum* contributed to the formation of 2-phenylethanol. However, it is important to note that the references found have investigated the aromatic composition of wines following fermentations with *M. pulcherrima* (whether pure, co-fermentations, or sequential). No references have been found regarding studies that assess how the aromatic composition of wines evolves in the bottle after employing *M. pulcherrima* in fermentations. It is known that the *S. cerevisiae* strain produces this alcohol through the bioconversion of L-phenylalanine via the Ehrlich pathway [41]. Although 2-phenylethanol decreased slightly after 9 months of sequential fermentation (Figure 1f), its content in the wines remains quantitatively more important compared to the other volatile compounds identified in this study. This could be due to the intervention of *M. pulcherrima* during the metabolic process of *S. cerevisiae*. Among all of the compounds identified in this family, 2-phenylethanol was the only one present in concentrations above the perception threshold (14 mg/L) [48,49], which was observed in all of the wines (independent of the time of analysis). This effect could be of great interest due to the rose, lilac, and honey descriptors associated with this compound in the wines.

On the other hand, 1-hexanol content in the wines was similar among the trials (Figure 1c), except for the n-Sc_{10}+Sc one, which had a higher content, demonstrating significant differences compared to the SO_2+n-Sc_{50}+Sc and n-Sc_{10}+n-Sc_{50}+Sc trials. This indicates that the sequential combination of yeast strains was better due to the herbaceous notes perceptible at elevated concentrations of 1-hexanol. The concentrations observed in all of the wines were below its perception threshold (8 mg/L) [50]. These results align with those reported by Zhang et al. [29].

One of the few common and sensory-relevant characteristics of non-*Saccharomyces* yeasts is the reduction of isoamyl alcohols levels [22,40]. In this work, differences at the end of MLF between samples were not observed. However, after 9 months of bottling, lower isoamyl alcohol content was obtained in the trials where a higher proportion of n-Sc yeast was used (Figure 1b). Zhang et al. [29] observed that a mixed fermentation of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* had positive effects on the formation of 1-hexanol, isoamyl alcohols, 2,3-butanediol, and 2-phenylethanol, because the combination of the strains did not increase the content of these alcohols. In addition to 2-phenylethanol, it was found that after 6 months of bottling, the concentration of isoamyl alcohols in the wines was above its perception threshold (30 mg/L) [49]. However, after 9 months, it was found that this concentration decreased significantly in those trials where n-Sc yeast sequences (50 g/hL) and Sc yeast were used. This trend is positive considering the perception of fatty and chemical notes associated with high concentrations of isoamyl alcohols.

Table 2 presents the results of the factorial analysis (assay, moment, and their interaction) of the wines' volatile compositions. The content of alcohols was affected by the assay factor, except for isoamyl alcohols. The concentration of isobutanol was lower in the SO_2+Sc and n-Sc_{10}+Sc wines than in the SO_2+n-Sc_{50}+Sc and n-Sc_{10}+n-Sc_{50}+Sc wines. In the case of 1-hexanol and (E)-3-hexenol, both C6 compounds, their content was higher in

the SO_2+n-Sc_{50}+Sc wines, while the concentration of methionol and 2-phenylethanol was higher in the SO_2+Sc wines (Table 2). As a result, the highest total alcohols content was found in the SO_2+Sc wines, and the lowest was in the n-Sc_{10}+Sc wines, with intermediate values in the other two wines, when a higher proportion of n-Sc yeasts was used (Table 2). As for the moment factor, its content in the wines, except for isobutanol, increased during the first 6 months in bottles, decreasing thereafter (Table 2). Interaction between the two factors studied was observed for all alcohols and their total content, expect for methionol (Table 2).

Table 2. Multifactor analysis of variance of volatile compounds (expressed as mg/L).

	Assay (A)				**Moment (M)**			
	SO_2+Sc	**n-Sc_{10}+Sc**	**SO_2+n-Sc_{50}+Sc**	**n-Sc_{10}+n-Sc_{50}+Sc**	**MLF**	**6 Months**	**9 Months**	**Interaction (A × M)**
Alcohols								
Isobutanol	2.81 a	2.91 a	4.45 b	4.34 b	3.63 a	3.71 a	3.54 a	**
Isoamyl alcohols	30.90 a	30.00 a	29.05 a	28.67 a	28.18 a	31.76 b	29.02 a	**
1-Hexanol	1.16 ab	1.13 a	1.34 b	1.22 ab	1.20 a	1.37 b	1.07 a	*
Methionol	0.18 c	0.10 a	0.14 b	0.12 ab	0.14 b	0.16 c	0.11 a	N.S.
(E)-3-Hexenol	0.16 ab	0.15 a	0.18 b	0.15 a	0.16 b	0.18 c	0.14 a	*
2-Phenylethanol	62.96 c	50.09 a	53.34 bc	53.20 ab	56.66 b	61.88 c	49.90 a	**
Total alcohols	**98.16 c**	**84.39 a**	**93.50 bc**	**87.71 ab**	**89.97 a**	**99.06 b**	**83.78 a**	**
Esters								
Isoamyl acetate	0.64 b	0.54 ab	0.63 b	0.45 a	0.60 b	0.64 b	0.46 a	N.S.
2-Phenylethyl acetate	0.21 b	0.18 a	0.31 c	0.22 b	0.26 b	0.27 b	0.16 a	***
Total acetate esters	**0.85 b**	**0.72 a**	**0.94 b**	**0.67 a**	**0.86 b**	**0.91 b**	**0.62 a**	**N.S.**
Ethyl hexanoate	0.36 c	0.33 c	0.27 b	0.22 a	0.33 b	0.32 b	0.23 a	N.S.
Ethyl octanoate	0.28 c	0.22 b	0.26 bc	0.17 a	0.32 c	0.22 b	0.17 a	N.S.
Ethyl decanoate	0.04 c	0.03 b	0.03 b	0.02 a	0.04 c	0.03 b	0.02 a	**
C6 + C8 + C10 ethyl esters	**0.68 c**	**0.58 b**	**0.55 b**	**0.41 a**	**0.69 c**	**0.57 b**	**0.41 a**	**N.S.**
Ethyl lactate	6.67 b	7.42 c	4.15 a	4.58 a	3.52 a	6.10 b	7.50 c	***
Diethyl succinate	23.47 b	15.66 a	18.41 a	15.38 a	11.47 a	22.20 b	21.02 b	N.S.
Monoethyl succinate	32.00 d	16.68 c	10.87 a	13.18 b	n.d. a	16.20 b	20.17 c	**
Total ethyl esters	**52.16 c**	**34.79 b**	**30.36 a**	**29.15 a**	**15.67 a**	**45.07 b**	**49.10 c**	***
Total esters	**53.00 c**	**35.51 b**	**31.31 a**	**29.82 a**	**16.53 a**	**45.98 b**	**49.72 c**	***
Acids								
Hexanoic acid	0.52 b	0.50 b	0.39 a	0.35 a	0.46 b	0.53 c	0.33 a	*
Octanoic acid	0.67 c	0.56 b	0.45 a	0.44 a	0.55 b	0.69 c	0.35 a	N.S.
Decanoic acid	0.06 b	0.06 b	0.04 a	0.04 a	n.d. a	0.07 c	0.03 b	***
Total acids	**1.23 b**	**1.09 b**	**0.87 a**	**0.82 a**	**1.01 b**	**1.29 c**	**0.71 a**	*

For each parameter and factor, different letters indicate significant differences between samples ($p \leq 0.05$). Interaction: *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$, and N.S., not significant ($p > 0.05$). n.d.: not detected.

The results of the esters identified in the four trials after MLF and after 6 and 9 months in bottles are shown in Figure 2. Esters are generated through yeast lipid and acetyl-CoA metabolism in alcoholic fermentation (acetate and ethyl esters), which is regulated by fatty acid and biosynthetic enzymes [31]. Yeast-derived esters are a class of volatile compounds with positive contributions to wine's aroma, introducing fruity and floral notes [41]. It was observed that once the MLF process was completed, no significant differences were found between the trials in terms of isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl lactate, and diethyl succinate content in the wines (Figure 2a,c,d,f,g). However, a significant increase of 2-phenylethyl acetate was evidenced in wines produced with SO_2+n+Sc_{50}+Sc (Figure 2b), followed by n-Sc_{10}+n-Sc_{50}+Sc. This increase in 2-phenylethyl acetate content was previously observed [22] in fermentations carried out with *M. pulcherrima*, which is interesting because this compound contributes to the wine bouquet. Varela et al. [3] also found that the mixed use of *M. pulcherrima* with *Saccharomyces uvarum* favoured the formation of 2-phenylethyl acetate at the end of alcoholic fermentation. Conversely, after the end of MLF, the ethyl decanoate content was higher in the samples SO_2+Sc and n-Sc_{10}+Sc (Figure 2e). At this stage of the fermentation process (MLF), monoethyl succinate was not identified in the wines made with the different assays (Figure 2h).

After 6 months of bottling, the isoamyl acetate content in the wines was similar across all trials, with no significant differences observed (Figure 2a). On the other hand, the

concentration of 2-phenylethyl acetate was significantly higher in the wines produced with SO_2+n-Sc_{50}+Sc (Figure 2b), showing differences with the rest of the trials. Moreover, it was observed that the content of ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, diethyl succinate, and monoethyl succinate in the wines (Figure 2c–h) was higher in the samples with a simpler approach, with significantly elevated levels in the SO_2+Sc and/or n-Sc_{10}+Sc tests.

Finally, after 9 months of bottling, the highest concentration of isoamyl acetate, ethyl hexanoate, and ethyl lactate in the wines was evidenced in the samples produced with SO_2+Sc and n-Sc_{10}+Sc (Figure 2a,c,f). Similar results were observed in the concentration of diethyl and monoethyl succinate, where higher levels of these compounds were achieved with the SO_2+Sc assay (Figure 2g,h). Nevertheless, the content of 2-phenylethyl acetate and ethyl decanoate in the wines was consistent across all tests, except in the samples tested with SO_2+Sc and SO_2+n-Sc_{50}+Sc (Figure 2b,e), which presented slightly higher concentrations. Furthermore, ethyl octanoate production in the wines was not altered by the different trials, as its content remained similar in all of them after 9 months of storage (Figure 2d).

Regarding the assay factor, in the case of esters, in general, the content of acetate esters and their total was higher in the wines in which SO_2 was added (Table 2). However, ethyl esters of fatty acids and their total showed a lower concentration in n-Sc_{10}+n+Sc_{50}+Sc wines, while ethyl esters of organic acids and their total showed, in general, a lower content in wines where higher doses of n-Sc yeasts were used (Table 2). Therefore, the concentration of total esters was higher in wines in which n-Sc yeast was not used and lower in wines in which higher doses of this yeast were used (Table 2). Regarding the moment factor, two different trends were observed: acetate esters and ethyl esters of fatty acids decreased their content during bottle ageing, while ethyl esters of organic acids increased their concentration during bottle ageing. Because the latter were the most abundant esters, the content of total esters increased in the wines with bottle ageing (Table 2). For esters, fewer interactions were observed than for alcohols only for 2-phenylethyl acetate, ethyl decanoate, ethyl lactate, and monoethyl succinate (Table 2).

Volatile fatty acids are formed by yeasts during fatty acid metabolism, contributing rancid, spicy, fruity, or cheesy odours to wine when above their thresholds [29]. Three fatty-acid-derived compounds were identified—hexanoic, octanoic, and decanoic acids (Figure 3)—the same aromatic compounds identified by Zhang et al. [29] in their study. First, after the end of MLF, a higher hexanoic acid content was observed in the SO_2+Sc and n-Sc_{10}+Sc assays (Figure 3a). The same pattern was present for octanoic acid content (Figure 3b). At this stage of the process, decanoic acid was not identified in the different assays (Figure 3c). Escribano et al. [47] also reported that *M. pulcherrima* produces a lower quantity of hexanoic acid than *S. cerevisae*. After 6 months of bottling, a higher concentration of hexanoic acid was observed in the wines treated with SO_2+Sc (Figure 3a), which was like the behaviour observed in the MLF stage. Similar results were observed in the content of octanoic and decanoic acids (Figure 3b,c), with higher content obtained in the samples tested with SO_2+Sc, showing significant differences from the rest of the tests. At the end of the experimental process (9 months), most of the wines showed no differences in the content of hexanoic, octanoic, and decanoic acids (Figure 3a–c). Considering the analysis time points (MLF, 6 months, and 9 months), it was found that the compounds belonging to this family increased until 6 months of bottling, with this effect being more noticeable in decanoic acid; then, regardless of the trials, the content of the acids decreased after 9 months of bottling. This effect is positive due to the undesirable notes that some compounds formed from volatile fatty acids can give when they are above their threshold of perception [29].

Regarding the factorial analysis, for the assay factor, the acid content was lower in the wines made using the highest doses of n-Sc yeast (Table 2). These compounds increased their content during the first 6 months of bottle ageing and decreased thereafter, as was observed for the alcohols (Table 2). Interactions were found between the two factors studied, with the exception of octanoic acid (Table 2).

Finally, the results of the totals by family are presented in Figure 4. After MLF, higher contents of total alcohols and total acetate esters were obtained with the SO_2+n-Sc_{50}+Sc assay (Figure 4a,b). However, similar levels of the latter family were obtained with the SO_2+Sc and n-Sc_{10}+n-Sc_{50}+Sc assays. On the other hand, the sum of C6 + C8 + C10 ethyl esters, total ethyl esters, and total esters (Figure 4c–e) did not show significant differences between the wines treated with the different assays. Regarding total acids, the highest content of this family was obtained in the samples treated with the SO_2+Sc assay, followed by n-Sc_{10}+Sc (Figure 4f). Higher alcohols can enhance the aromatic complexity of wines when present in concentrations below 350 mg/L [47]. In fact, lower levels of higher alcohols have been shown to amplify the perception of the varietal aroma of grapes. It is noteworthy that all of the samples examined in this study exhibited higher alcohol levels below 350 mg/L.

In the first 6 months of ageing, no differences were evident between the trials with respect to the content of total alcohols (Figure 4a); a similar outcome was observed for the content of total acetate esters (Figure 4b). Acetates are synthesised at higher concentrations than ethyl esters, and the ratio between the two, as well as the concentration at which acetates are produced, are mainly affected by the fermentation temperature, the nutrient content of the must, and the yeast strain, rather than the grape variety [51]. In contrast, the total concentration of C6 + C8 + C10 ethyl esters, total ethyl esters, total esters, and total acids presented in the wines was higher with the SO_2+Sc assay (Figure 4c–f). Similar effects were observed for total alcohols, total acetate esters, total ethyl esters, and total esters after 9 months of bottling (Figure 4a,b,d,e).

According to the literature, acetate esters tend to hydrolyse rapidly, resulting in a decrease during ageing [40]. These findings align with the observation in this study, where a notable decrease in isoamyl acetate, 2-phenylethyl acetate, and total acetate esters in the wines was observed after 9 months of bottling (Figures 2a,b and 4b and Table 2). It should be noted that acetate formation depends on the concentration of unsaturated fatty acids available in the medium and the carbon/nitrogen ratio [51]. In contrast, branched acid ethyl esters tend to increase continuously with ageing time [40], as confirmed by our results (Figures 2c–e and 4d and Table 2). During the ageing period, the ethyl esters increased with bottling time, with the most noticeable change occurring between MLF and 6 months of bottling.

On the other hand, it was demonstrated that at this fermentation time (9 months), the content of the sum of C6 + C8 + C10 ethyl esters was considerably reduced with any of the trials, with the n-Sc_{10}+n-Sc_{50}+Sc trial showing the lowest content (Figure 4c). Finally, the total acid content did not vary much from one trial to another (Figure 4f). Considering bottle ageing, it was observed that the total alcohol content increased significantly in the wines between MLF and after 6 months of bottling when the SO_2+Sc assay was used. Similarly, Garde-Cerdán et al. [43] found that the total alcohol content in Tempranillo wines increased after 6 months of fermentation, remaining constant until the end of the period studied (9 months) when Sc yeasts were used. Benito et al. [35] reported lower alcohol concentrations in Riesling grape must with the application of non-*Saccharomyces* yeasts, results that align with those evidenced in our work. The concentration of total alcohols decreased notably in the sequential fermentations at the end of bottling (9 months) (Table 2), which can be attributed to the fact that some non-*Saccharomyces* yeasts produce less alcohols than *S. cerevisiae*. Thus, Contreras et al. [52] showed lower ethanol concentration in Chardonnay and Shiraz wines sequentially inoculated with *M. pulcherrima* followed by a *S. cerevisiae*, as we observed (Table 1).

Some studies have shown that the production of aroma compounds depends on the grape variety and its nutritional composition. Hu et al. [31] reported that during sequential fermentation of *Hanseniaspora uvarum* and *S. cerevisiae,* the ethyl ester content increased in Ecolly white wine, while it decreased in Cabernet Sauvignon red wine.

3.3. Sensory Analysis of the Wines

Table 3 shows the results of the sensory analysis of the wines from the different trials (SO_2+Sc, n-Sc_{10}+Sc, SO_2+n-Sc_{50}+Sc, and n-Sc_{10}+n-Sc_{50}+Sc). The different wines obtained a total evaluation above 71 points, defined as "very good" according to the scale used. There were no significant differences in any of the attributes evaluated.

Table 3. Sensory evaluation of Tempranillo control wines (SO_2+Sc) and wines made with the different tests with *M. pulcherrima* (n-Sc_{10}+Sc, SO_2+n-Sc_{50}+Sc, and n-Sc_{10}+n-Sc_{50}+Sc).

		SO_2+Sc	**n-Sc_{10}+Sc**	**SO_2+n-Sc_{50}+Sc**	**n-Sc_{10}+n-Sc_{50}+Sc**
View	Cleannes	3.95 ± 0.79 a	4.13 ± 0.87 a	3.85 ± 0.75 a	4.04 ± 0.82 a
	Colour	7.62 ± 1.71 a	7.65 ± 1.56 a	6.99 ± 2.00 a	7.73 ± 1.74 a
Smell	Intensity	6.06 ± 1.43 a	6.31 ± 0.97 a	6.17 ± 0.95 a	6.00 ± 1.24 a
	Frankness	4.14 ± 0.77 a	4.36 ± 0.71 a	3.72 ± 0.98 a	3.88 ± 1.10 a
	Quality	12.55 ± 1.65 a	12.80 ± 1.78 a	11.43 ± 2.06 a	12.02 ± 2.56 a
Taste	Intensity	6.03 ± 1.11 a	6.05 ± 0.77 a	5.99 ± 1.21 a	6.06 ± 1.15 a
	Frankness	4.16 ± 0.89 a	4.17 ± 0.65 a	3.96 ± 0.83 a	4.01 ± 1.00 a
	Quality	16.15 ± 2.71 a	16.37 ± 2.29 a	14.96 ± 2.79 a	16.01 ± 2.86 a
	Persistence	6.17 ± 1.04 a	6.34 ± 0.83 a	6.22 ± 0.95 a	6.31 ± 0.93 a
Harmony		8.98 ± 0.90 a	9.20 ± 0.59 a	8.67 ± 0.93 a	8.97 ± 0.88 a
Total valuation		75.61 ± 10.04 a	76.97 ± 9.10 a	71.97 ± 10.41 a	75.02 ± 11.52 a

The mean values (n = 2) are shown with their standard deviation. Equal letters indicate that there were no significant differences among samples ($p > 0.05$).

Regarding olfactory and taste attributes (Figure 5), all wines were evaluated with more red fruit aromas, being more noticeable in the wine elaborated with SO_2+Sc and n-Sc_{10}+n-Sc_{50}+Sc, although without significant differences (Figure 5A). Varela et al. [53] demonstrated that Shiraz and Cabernet Sauvignon wines produced with *M. pulcherrima* yeast showed a higher intensity of some desirable aromas, such as red fruit and black fruit.

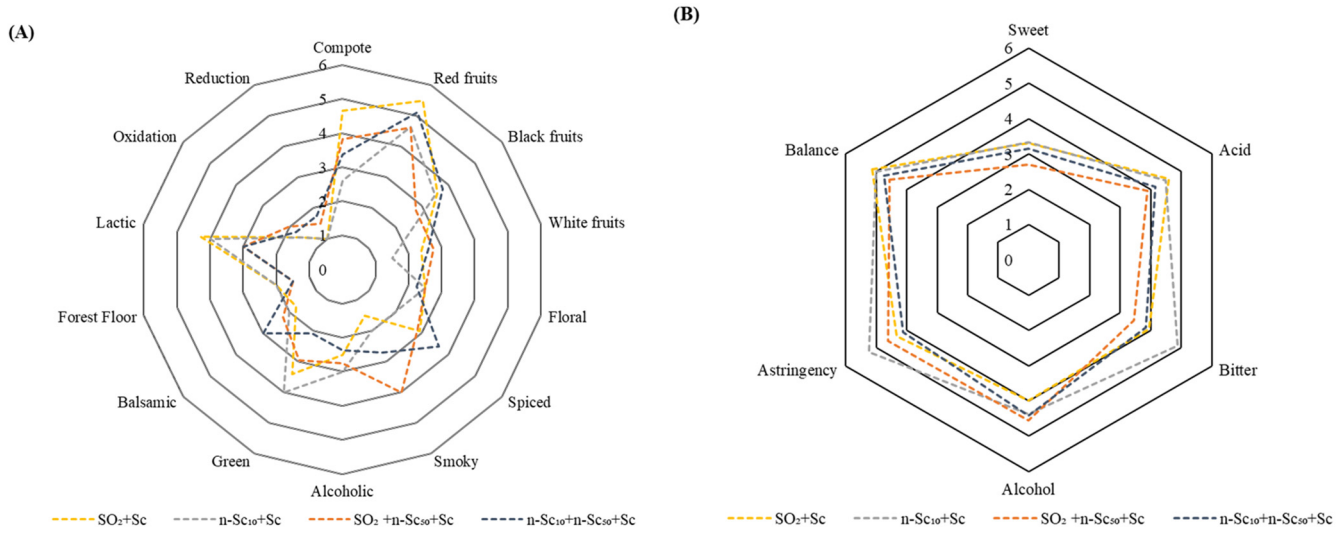

Figure 5. Polar coordinate (cobweb) graph of the mean intensity ratings of the sensory descriptors for the treatments SO_2+Sc, n-Sc_{10}+Sc, SO_2+n-Sc_{50}+Sc, and n-Sc_{10}+n-Sc_{50}+Sc. (**A**) Olfactory attributes. (**B**) Taste characteristics. At the origin, intensity = 0; at the perimeter, intensity = 6.

Likewise, the wines made with SO_2+Sc were also evaluated with a compote aroma, showing differences with n-Sc_{10}+Sc and n-Sc_{10}+n-Sc_{50}+Sc wines. Another aroma highlighted was the lactic aroma, showing greater intensity in the wines with conventional fermentation and in those wines where SO_2 was replaced by *M. pulcherrima* with doses of 10 g/hL, which implies greater roundness and smoothness of taste; there were no significant

differences from the rest of the assays (Figure 5B). On the other hand, all assays showed low scores in some negative descriptors (oxidation aroma, reduction in sensory attributes, and forest floor), results that agree with those reported by Varela et al. [53]. Similarly, less intensity of green notes was perceived in the wines made with n-Sc_{10}+n-Sc_{50}+Sc, which is positive.

In terms of taste characteristics (Figure 5B), all trials were perceived as having less sweetness. On the other hand, all wines were perceived as having medium-high intensity of acidity, bitterness, and astringency and with a greater sensation of balance. However, there were no significant differences between assays for the attributes evaluated; similar results were shown by Yao et al. [21] when they tested the potential of non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) on the physico-chemical and sensory properties of the wines.

4. Conclusions

The use of non-Sc yeasts in mixed fermentation with *S. cerevisae* strains has become a novel and important practice in winemaking. It allows for the reduction of the dose of SO_2 used during vinification and results in wines with greater aromatic complexity. It is worth noting that the majority of studies found have predominantly focused on analysing aromatic compounds at one time point, neglecting the potential evolution of these compounds over time. Hence, this study is pioneering in its approach of examining the volatile composition of wines at three distinct intervals: the end of MLF and after 6 and 9 months in bottles. Four trials were carried out (SO_2+Sc; n-Sc_{10}+Sc; SO_2+n-Sc_{50}+Sc; and n-Sc_{10}+n-Sc_{50}+Sc), each in duplicate.

The use of non-*Saccharomyces* yeasts led to minor variations in enological parameters, and the analysis of volatile composition and subsequent sensory evaluation of wines made with *M. pulcherrima* indicate positive contributions to both sensory profile and wine style, thus enhancing aromatic quality. It was found that the most important compound quantitatively was 2-phenylethanol, which contributes rose notes to the wine. The predominant impact was observed in the profile of volatile acids, compounds typically linked to undesirable aromas in wine. Nevertheless, the sequential co-fermentation of *M. pulcherrima* and *S. cerevisiae* reduced their levels after 9 months of bottling. The concentration of most of the compounds belonging to the esters showed concentrations above the perception thresholds, which is of new interest as these compounds contribute positively to the quality of the wines; moreover, 50% of the compounds identified in this study belong to this family. Sensory analysis of wines from different trials showed generally positive evaluations, with red fruit aromas being predominant. Taste characteristics included less sweetness and medium-high intensity of acidity, bitterness, and astringency, with all wines exhibiting a balanced sensation. Overall, *M. pulcherrima* yeast shows potential for shaping the volatile composition of wines, offering an alternative to SO_2.

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