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Potential of non-*Saccharomyces* yeast for improving the aroma and sensory profile of Prokupac red wine

Ivana Karabegović[1,*], Marko Malićanin[2], Bojana Danilović[1], Jelena Stanojević[1], Sandra Stamenković Stojanović[1], Nada Nikolić[1] and Miodrag Lazić[1]

[1] University of Niš, Faculty of Technology, Bulevar oslobodjenja 124, 16000 Leskovac, Serbia
[2] University of Niš, Faculty of Agriculture, Kosančićeva 4, 37000 Kruševac, Serbia

**corresponding author*: ivana.karabegovic@tf.ni.ac.rs

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ABSTRACT

This work aimed to analyse the effect of non-*Saccharomyces* yeasts on the volatile composition, aromatic profile and sensory properties of wine made from autochthonous Serbian grape variety Prokupac (*Vitis vinifera*). Fermentation was performed with two commercial yeasts, *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*, in pure and sequential fermentation with *Saccharomyces cerevisiae*. The standard analysis of produced wines indicated that the application of both non-*Saccharomyces* yeast (in pure or in sequential fermentation) significantly reduces the content of alcohol when compared to the control. The use of *M. pulcherrima* in both pure and sequential fermentation resulted in a higher content of different groups of polyphenols, anthocyanins and flavonoids or better colour characteristics compared to the wines obtained in fermentations with *T. delbrueckii*. HS-SPME GC/MS analysis revealed the presence of 27 different compounds in the wine samples, with higher alcohols and ethyl esters being the most dominant. It was proven that the presence of non-*Saccharomyces* yeasts in the initial stage of fermentation with later inoculation with *S. cerevisiae* contributes to increased complexity of the wine, as well as increased higher alcohol content and total extracts. Wines produced from sequential fermentation with *T. delbrueckii* showed floral and spice attributes, probably due to 1-pentanol and 2-phenylethanol detected in the wine, as well as to the large amount of ethyl acetate. The use of *M. pulcherrima* in pure fermentation resulted in wines with the lowest sensorial characteristics (*i.e.*, lacking fruity and floral aroma) probably due to the lowest relative contribution of ethyl esters. The control wine in this study (from pure fermentation with *S. cerevisie*), had the most intense 'red berries' and 'black berries' note and low intensity of spices or vegetable aroma, which may be explained by the highest relative contribution of ethyl octanoate and ethyl decanoate. The PCA results also clearly differentiated the analysed samples produced in the sequential, pure and control fermentation trials, leading to the conclusion that the aromatic profile of Prokupac wines produced in sequential fermentation was more complex compared to the wines produced in pure fermentation.

KEYWORDS

non-*Saccharomyces* yeast, aroma profile, sensory, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/3859

INTRODUCTION

Current trends in the modern wine industry focus on the production of more complex wines with improved quality, authentic taste and fresh, balanced and unique aromas. The unique sensory and aromatic attributes of wine are formed during the complex process of grape must fermentation when hundreds of different compounds are released into the wine. Their interactions and transformations during fermentation and aging result in the final aroma of wine (Molina *et al.,* 2007). Among them, higher alcohols, medium- and long-chain volatile fatty acids, esters, aldehydes and ketones are the most abundant (Molina *et al.,* 2007; Vilela, 2018). Higher alcohols are formed during the metabolism of sugar or amino acid through catabolic and anabolic pathways (Suárez-Lepe and Morata, 2012). Volatile fatty acids are synthesised in different yeast metabolism pathways depending on the chain length and branching type (Lambrechts and Pretorius, 2000), while the formation of esters is the result of the interaction of alcohols and by-products of yeast fermentation (Dutraive *et al.,* 2019; Molina *et al.,* 2007). Therefore, the type and amount of different compounds that constitute the aromatic profile of the wine is highly dependent on the type of yeast strain used in the process and the nutrients they use for growth (Seguinot *et al.,* 2020). For many years, *Saccharomyces cerevisiae* has been used as a primary yeast strain for the production of wines, due to its good fermentative properties and the ability to produce wines of uniform quality. However, wines made only with pure yeast culture often lack complex and distinctive compounds that contribute to the uniqueness of aroma and taste. Because each grape or a wine region is characterised by its own "microbial footprint" of specific non-*Saccharomyces* yeast strains, and such specificity can be transferred to wine, it follows that it should be possible to produce wines of the finest quality when those indigenous non-*Saccharomyces* yeast are used (Padilla *et al.,* 2016; Padilla *et al.,* 2017).

Non-*Saccharomyces* yeast have been proven to enhance overall wine quality, mainly because they provide greater complexity and variability of wine flavor and give a wealth of new aromas. In addition, some species have the ability to reduce the ethanol content of wine (Canonico *et al.,* 2019) and to help avoid problems in the winery, such as stuck fermentations (Vilela, 2018). Nevertheless, although they improve the quality of the wine, the industrial use of non-*Saccharomyces* yeasts is usually limited due to weak alcoholic fermentation ability. Therefore, they are usually combined with *S. cerevisiae* in a sequential or mixed fermentation process (Benito, 2018; Dutraive *et al.,* 2019; Lu *et al.,* 2016).

Of the different non-*Saccharomyces* strains, *Metschnikowia pulcherrima* and *Torulaspora delbrueckii* are of particular interest. *T. delbrueckii* was the first to be used and is still one of the most commonly used non-*Saccharomyces* yeast strains; it improves colour stabilisation and the mouthfeel properties of wine due to the release of large amounts of mannoproteins during wine aging and some important aroma compounds (Belda *et al.,* 2015; Jolly *et al.,* 2014). This yeast strain has been found to increase the amount of organic acids and higher alcohols in the wine, which, in a sensorial sense, enhances its fresh, floral and fruity aromas and overall complexity (Tataridis *et al.,* 2013). In addition, this species can be used for the production of wines with lower ethanol concentration and total volatile acidity (Benito, 2018; Renault *et al.,* 2009). On the other hand, *M. pulcherrima* is often used in sequential fermentation with other yeasts due to its ability to synthesise higher alcohols (particularly isobutanol and phenylethanol), different enzymes, terpenes and thiols, fatty acids and their ethyl esters derivatives, which contribute to the fruity aroma of wine *(*Morata *et al.,* 2019; Seguinot *et al.,* 2020).

M. pulcherrima also has the potential to limit competition in the fermentation medium due to the synthesis of antimicrobial compounds (Lu *et al.,* 2016). A recent study confirmed that a combination of *T. delbrueckii* and *M. pulcherrima* with *S. cerevisie* during sequential fermentation reduces the ethanol content and significantly influences the volatile profile of Chardonnay wines (Canonico *et al.,* 2019). Interesting research possibilities can arise from this, as it is still unknown how such yeast combinations could affect the wine made from other grape varieties.

For that reason, the aim of this work was to analyse the potential of non-*Saccharomyces* yeasts to improve the aromatic profile and sensory properties of wine made from autochthonous Serbian grape variety Prokupac (*V. vinifera*), as well as to determine the best formula for the production of quality wine that will respond to modern customer demands. To do so, the fermentation of Prokupac grape was performed with two starter cultures, *M. pulcherrima* and *T. delbrueckii*, in pure and in a sequential fermentation with *S. cerevisiae.*

Meanwhile, wine volatile compounds were determined using headspace solid-phase microextraction (HS-SPME) technique coupled with gas chromatography-mass spectrometry (GC/MS).

MATERIALS AND METHODS

The Prokupac grape variety (Variety VIVC number - 9734) was manually harvested (approximately 400 kg of grapes) from the vineyard located in the Tri Morave wine subregion (43°37' N, 21°34' E, continental climate, single Royat cordon vine training system) central Serbia in October 2018. The characteristics of the must were: 22.55 Brix°, 6.6 g/L total acidity, pH 3.34. Before yeast inoculation, the grapes were destemmed, crushed, equally divided and put into five steel microvinificators (50 L). *Torulaspora delbrueckii* (Biodiva™ TD291) or *Metschnikowia pulcherimma* (Flavia® MP346) (Lallemand, Canada) yeast strains were used for pure fermentations (25 mg/L), while *Saccharomyces cerevisiae* (ICV D254®, Lallemand, Canada, 25 mg/L) was used for the control fermentation. For sequential fermentations, *T. delbrueckii* or *M. pulcherimma* were used in the initial fermentation stages, while inoculation with *Saccharomyces cerevisiae* (ICV D254®, Lallemand, Canada, 25 mg/L) was carried out after °Brix reduction for 3 degrees. Potassium-metabisufite, pectolytic enzyme EXV and inactivated yeast cells preparation Opti-Red™ (Lallemand, Canada) were added to the must to obtain concentrations of 5, 2 and 30 mg/L respectively. In the first 24 h of fermentation, the cap was punched down every 3 hours. After the first day of fermentation, one delastage (rack-and-return of fermenting juice) per day was performed until the sugar content had dropped below 7 °Brix. At this stage of fermentation, a yeast nutrient Fermaid® E (Lallemand, Canada) was added (30 mg/L) and was continued with three daily punching-downs for each fermentation trial. Upon completion of the alcohol fermentation (sugar level below 4 g/L), the wine samples were racked off and sulphited (25 mg/L). Before bottling, wine samples were filtered using Seitz® filter plates K 100 (Pall Seitz®, Germany) and stored for about six months until analysis.

1. Standard wine analysis

Standard wine analysis was performed according to The International Organisation of Vine and Wine (OIV, 2012). Total phenols index, anthocyanins, flavonoids and flavan-3-ols were determined using the methods earlier described (Gambacorta *et al.,* 2011). All experiments were performed in triplicate and expressed as the mean value with standard deviation.

2. Fermentation kinetics

To track fermentation kinetics, the sugar content of the samples was measured every 24 h until depletion. The sugar content, expressed in Brix degrees (°Brix), was measured with a digital refractometer DR6000 (Krüss Optronic GmbH, Germany).

3. Extraction of volatile compounds in wines by headspace-solid phase microextraction (HS-SPME)

An SPME manual holder and fused silica fiber coated with Carboxen®/Polydimetilsiloxane (CAR/PDMS, 75 µm thickness) Supelco (Bellefonte, PA, USA) were used for the aroma compounds extraction by HS-SPME. The fiber was preconditioned according to the manufacturer's instructions. The extraction conditions given in the study of Fernandes and coworkers (2018) - with slight modifications - were used. Twenty milliliters of wine sample, 3 g of NaCl and a magnetic stirrer bar were placed in an amber glass bottle (30 mL), which was closed with a rubber septum and sealed with parafilm. The samples were heated at 55 °C and agitated for 15 min (pre-extraction). After pre-extraction, the fiber was inserted into the headspace of the sample for volatile extraction for 35 min at a constant temperature and with constant stirring. The fiber was then desorbed for 10 min in split/splitless inlet at 250 °C with 20:1 split mode and analysed by GC/MS and GC/FID.

4. Gas chromatography/mass spectrometry (GC/MS) analysis and gas chromatography-flame ionisation detection (GC/FID) of volatile compounds in wines

GC/MS analysis was performed on Agilent Technologies 7890B gas chromatography, coupled with a 5977A mass detector. Components were separated on a weakly polar, silica capillary column, HP-5MS (5 % diphenyl- and 95 % dimethyl-polysiloxane, 30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, USA). The previously mentioned GC/MS conditions (Fernandes *et al.,* 2018) - with slight modifications - were used. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was held at 40°C for 2 min, then increased to 250 °C at the rate of 7 °C/min,

and finally held at 250 °C for 2 min. The total run time was 34 min. Temperatures of the MSD transfer line, ion source and quadrupole mass analyser were set at 300 °C, 230 °C and 150 °C respectively. The ionisation voltage was 70 eV and mass detection was done in the Scan mode, in m/z range from 25 to 550.

Data processing was performed using MSD ChemStation (revision F.01.00.1903) in combination with AMDIS (revision 2.70) and NIST MS Search (version 2.0g) software (Agilent Technologies, USA). Retention indices of the components from the sample were experimentally determined using a homologous series of n-alkanes from C8-C20 as standards, which were analysed under identical GC/MS and GC/FID conditions. The compounds were identified by comparing the experimentally obtained linear retention indices (LRIexp) with those found in the literature (LRIlit, Adams, 2007), and their EI mass spectra with data from Willey 6, NIST11 and RTLPEST 3 mass spectra libraries.

5. Sensory analysis

The sensory evaluation of the produced Prokupac wine samples (ISO 6658, 2017; ISO 3591, 1977; OIV, 2015) was performed by eleven assessors who had extensive wine tasting experience and were officially certified for wine sensory analysis by the Serbian Ministry of Agriculture, Forestry and Water Management (6 females and 5 males aged from 29 to 51 years old). All the assessors had previously been trained in sensory profiling and had participated in the Prokupac wine sensory evaluation at least once a week (in a laboratory accredited and authorised by the Serbian Ministry of Agriculture for testing the quality of grape, wine and other products). Smell, taste and mouthfeel descriptors were defined by the assessors on consensus, while a short training session with reference standards was provided monthly for all the assessors to avoid any bias during the sensory evaluation. The analysis was performed in duplicate, and the intensity of each parameter was quantified by a ten-point intensity scale: from 0 (not detected) to 10 (very intense). Smell (spice, vegetable, red berries, black berries, floral, complexity, intensity, typicality and toasted), taste (harmony, acidity, astringency, fullness, complexity, duration, structure, intensity and typicality) attributes and colour intensity were evaluated for all analysed samples. The results are expressed as mean values in a spider chart (Microsoft Excel) and are summarised in Supplementary data (Tables S1 and S2).

6. Statistical analysis

Data analysis was conducted using the SPSS® 26.0 (IBM®, USA, trial version) and *STATISTICA 7* (StatSoft Inc., Tulsa, U.S.A., trial version) softwares. Shapiro–Wilk and Levene's tests were performed to evaluate the normality of data distribution and homogeneity of variances respectively. Statistical differences were determined by one-way ANOVA followed by Tukey's HSD test, while Kruskal-Wallis test with post-hoc Dunn's test were applied for variables that did not meet normality criteria. A significance level of 0.05 was used for all statistical analyses. Principal component analysis (PCA) was performed for the differentiation of samples based on the aroma profile of wines.

RESULTS

Sugar consumption profiles (Figure 1) indicated that fermentation kinetics of pure and sequential fermentations with non-*Saccharomyces* yeasts and *S. cerevisiae* varied depending on inoculated yeast strains.

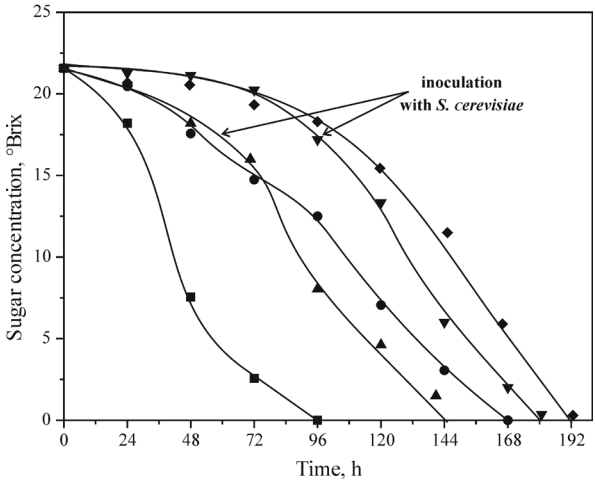

FIGURE 1. Utilisation of sugar during pure fermentation with T. delbrueckii (●), M. pulcherrima (♦), S. cerevisiae (■) and sequential fermentation T. delbrueckii + S. cerevisiae (▲) and M. pulcherrima + S. cerevisiae (▼).

During the pure fermentation with *S. cerevisiae* the total sugar was depleted within 96 h. The duration of the fermentation trials with non-*Saccharomyces* strains varied from 144 to 192 h for sequential fermentation with *T. delbrueckii* and pure fermentation with *M. pulcherrima*.

TABLE 1. Standard oenological parameters of Prokupac wines produced with *T. delbrueckii*, *M. pulcherrima* and *S. cerevisiae* in pure and sequential fermentation.

Parameter	Pure fermentations			Sequential fermentation with *S. cerevisiae*	
	S. cerevisiae	*T. delbrueckii*	*M. pulcherrima*	*T. delbrueckii*	*M. pulcherrima*
Alcohol, % vol	13.3±0.07[a]	12.6±0.02[b]	11.9±0.04[c]	12.9±0.03[d]	12.3±0.09[e]
Total extract, g/L	29.8±0.01[a]	31.8±0.17[b]	31.7±0.12[b]	31.3±0.18[c]	31.4±0.14[c]
Total acidity (as tartaric acid), g/L	6.53±0.06[a]	6.55±0.07[a]	6.0±0.05[b]	6.13±0.23[b]	6.20±0.20[b]
Volatile acidity (as acetic acid), g/L	0.57±0.015[a]	0.73±0.015[b]	0.76±0.046[b]	0.53±0.027[a]	0.55±0.015[a]
Reducing sugar, g/L	2.25±0.06[a]	2.21±0.05[a]	2.12±0.02[b]	1.81±0.03[c]	2.44±0.05[d]
Total polyphenols, g/L	1.22±0.070[a]	1.06±0.085[b]	1.05±0.055[b]	1.17±0.029[a]	1.22±0.006[a]
Total anthocyanins, g/L	0.26±0.006[a]	0.19±0.005[b]	0.23±0.003[c]	0.26±0.013[c]	0.27±0.006[a]
Total flavonoids, g/L	0.86±0.029[a]	0.58±0.021[b]	0.61±0.031[bc]	0.55±0.022[b]	0.64±0.006[c]
Colour intensity	0.46±0.022[a]	0.35±0.005[b]	0.46±0.007[a]	0.35±0.008[b]	0.48±0.024[a]
Colour tone	0.58±0.001[a]	0.60±0.004[a]	0.72±0.020[c]	0.55±0.013[b]	0.67±0.013[d]
Free SO_2, mg/L	25.0±0.04[a]	25.3±0.30[a]	25.5±0.27[a]	25.4±0.14[a]	25.1±0.47[a]
Total SO_2, mg/L	43.4±0.08[a]	45.6±0.20[b]	40.7±0.54[c]	43.4±0.27[a]	42.1±0.30[d]

*Different letters in the same row show significant differences according to the analysis of variance at $p \leq 0.05$ (Tukey's HSD test).

Within the 72 h time period, *S. cerevise* consumed almost all the sugar, while *M. pulcherima* had not even started the fermentation, as the sugar concentration was still at the initial level after 72 h. However, after co-inoculation with *S. cerevise,* the sugar level dropped significantly and fermentation was accelerated. As a result, the sequential fermentation with *M. pulcherima* was 10 h shorter than the pure fermentation. Similarly, a 20 h difference was noticed between the duration of sequential fermentation and that of pure fermentation of sugar with *T. delburecki*, indicating a high competition between *S. cerevisie* and non-*Saccharomyces* strains.

The standard chemical analysis of five Prokupac wines produced with different yeast strains in pure or sequential fermentations showed significant differences in alcohol content, total extracts, volatile and total acidity, the content of different phenolic compounds and colour characteristics (Table 1).

Compared to the control wine, the alcohol had been significantly reduced in the pure (5.3 and 10.5 % for *T. delbrueckii* and *M. pulcherrima*) and sequential (3 and 7.5 % for *T. delbrueckii* and *M. pulcherrima*) fermentations with non-*Saccharomyces* strains. All the wine samples were fermented to 'dryness' while the total dry extract was within a range of 29.8 g/L to 31.8 g/L. At the end of fermentation, total acidity expressed as tartaric acid was significantly lower in the wines produced with non-*Saccharomyces* strains (up to 8 %), which is in accordance with the literature data (Comitini *et al.,* 2011). Statistical analysis showed no significant differences in volatile acidity between the wines obtained in pure or in sequential fermentation with the *S. cerevisiae* strain. On the other hand, the volatile acidity of wines fermented with pure non-*Saccharomyces* strains was significantly different and can be considered to be high, but still not above the sensory threshold (approximately 0.8 g/L) (Benito *et al.,* 2019). The use of *M. pulcherrima* in both pure and co-inocultated fermentation resulted in higher content of different groups of polyphenols (total polyphenols, anthocyanins and flavonoids) or better colour characteristics compared to the wines obtained in fermentations with *T. delbrueckii*. The highest value of total flavonoids was reached in wine obtained in pure fermentation with *S. cerevisiae*, while a decrease of about 30 % in this group of compounds was observed in the fermentation trials with non-*Saccharomyces* strains.

The analysis of volatile compounds by HS-SPME GC/MS revealed the presence of 27 different compounds in the wine samples (Table 2).

TABLE 2. Relative contributions of volatile compounds (expressed as percentage of the peak area of each compound compared to the total area) in Prokupac wines produced with *T. delbrueckii*, *M. pulcherrima* and *S. cerevisiae* in pure and sequential fermentation identified by HS-SPME GC/MS

Parameter	Aroma descriptors	Pure fermentations			Sequential fermentation with *S. cerevisiae*		Kruskal-Wallis test
		S. cerevisiae	*T. delbrueckii*	*M. pulcherrima*	*T. delbrueckii*	*M. pulcherrima*	*p*-value
Alcohols							
Isobutyl alcohol	Alcoholic[a]	3.2±0.06a	5.9±0.14ab	1.9±0.15ac	4.3±0.07ab	1.6±0.08c	0.008
2-Butanol	Medicinal, wine-like[a]	/	0.5±0.1a	/	2.1±0.25b	/	0.014
3-Methyl-1-butanol	Cheese aroma[a]	27.9±4.58a	33.5±2.35ac	44.3±1.16b	28.8±2.16a	40.4±1.08bc	0.015
2-Methyl-1-butanol	-	14.2±0.84	10.2±1.09	10.4±1.26	6.9±0.38	12.6±0.97	0.067
1-Pentanol	Fruity, balsamic[a]	10.3±1.51a	11.4±0.74ab	12.1±0.93b	14.7±1.03c	11.7±1.69b	0.014
(S)-(-)-2 Methyl-1-butanol	-	3.3±0.66	1.3±0.26	4.1±0.82	5.7±1.14	3.2±0.64	0.052
n-Hexanol	Mowed grass[a]	0.8±0.16a	1.7±0.34b	2.1±0.42b	1.0±0.20a	1.9±0.38b	0.027
2-Phenylethanol	Floral, roses[a]	3.7±0.74ab	2.4±0.48a	2.7±0.54a	5.5±1.10b	4.0±0.80b	0.034
1-Propanol	Fresh,ripe fruit[a]	/	/	/	1.0±0.01a	0.8±0.04a	0.018
2,3-Butandiol	Butter, creamy [a]	/	/	0.9±0.11a	0.5±0.03a	/	0.010
Isohexanol		/	/	1.7±0.34a	1.4±0.28b	1.8±0.35a	0.0.8
2-Methyl-1-pentanol		1.4±0.12	/	/	/	/	/
Total area (%)		64.8	66.9	80.2	71.9	77	
Esters							
Ethyl acetate	Fruity, sweet[a]	4.2±0.84a	19.3±0.93b	/	8.8±1.76c	/	0.009
Ethyl hexanoate	Fruity, apple [a]	1.1±0.22a	0.4±0.08b	0.6±0.10ab	0.5±0.07b	0.9±0.02a	0.020
Ethyl isohexanoate	Fruity[b]	/	0.3±0.03a	0.7±0.04ab	1.0±0.07ab	1.5±0.09b	0.010
Diethyl succinate	Light fruity[a]	/	0.2±0.13	1.0±0.21	1.7±0.66	2.3±0.71	0.279
Ethyl octanoate	Pineapple, pear, fruity[a]	13.8±1.06a	3.1±0.61b	3.6±0.72b	6.5±1.33ab	6.8±1.36ab	0.015
Ethyl decanoate	Fruity, grape, oily[c]	5.9±1.03a	1.5±0.30b	1.9±0.41bc	5.1±1.12a	5.3±1.02ac	0.29
Ethyl dodecanoate	Flowery, fruity[a]	0.5±0.04	0.2±0.08	0.3±0.07	0.3±0.02	0.6±0.09	0.104
3-Methyl butyl acetate	-	/	1.2±0.22a	0.4±0.03a	0.2±0.01b	0.3±0.03a	0.010
Ethyl butanoate	Strawberry, apple[a]	0.2±0.04	/	/	/	0.2±0.06	0.184*
Ethyl 3-methyl pentanoate		1.2±0.09	/	/	/	/	/
Total area (%)		26.9	26.2	8.5	24.1	17.9	
Aldehydes							
Benzaldehyde	Almond [a]	0.9±0.06	0.4±0.02	2.2±0.9	0.9±0.01	1.7±0.09	
Acids							
Acetic acid	Acidic[a]	/	/	/	0.4±0.02	0.4±0.01	0.231
Octanoic acid	Unpleasant, cheese, fatty[a]	1.6±0.33a	/	/	1.2±0.09a	0.4±0.01b	0.049
Total area (%)		1.6	/	/	1.6	0.8	
Other							
4-Hydroxy-2-butanone	-	/	1.2±0.21a	2.0±0.38ab	2.0±0.34ab	2.5±0.55b	0.018
Isoamyl acetoacetate	-	/	/	1.1±0.43	/	/	/
Total area (%)		/	1.2	3.1	2.0	2.5	

Reported in [a]Peinado *et al.*, 2004, [b]Farenzena and Tombesi, 2015; Significant differences are indicated by different letters according to the Kruskal–Wallis test followed by Dunn's test with Bonferroni correction ($p < 0.05$).

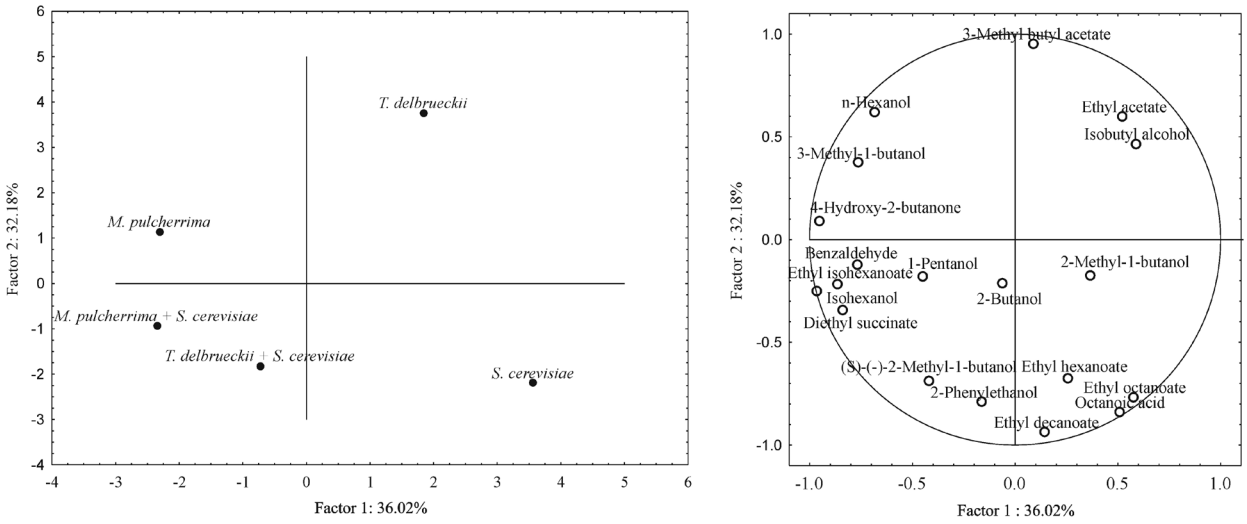

FIGURE 2. Principal component analysis (PCA) of the volatile compounds identified in the Prokupac wines produced in pure fermentation with *T. delbrueckii*, *M. pulcherrima*, *S. cerevisiae* and sequential fermentation *T. delbrueckii* + *S. cerevisiae* and *M. pulcherrima* + *S. cerevisiae* (only taking into account the volatile compounds with relative peak areas higher than 1 %).

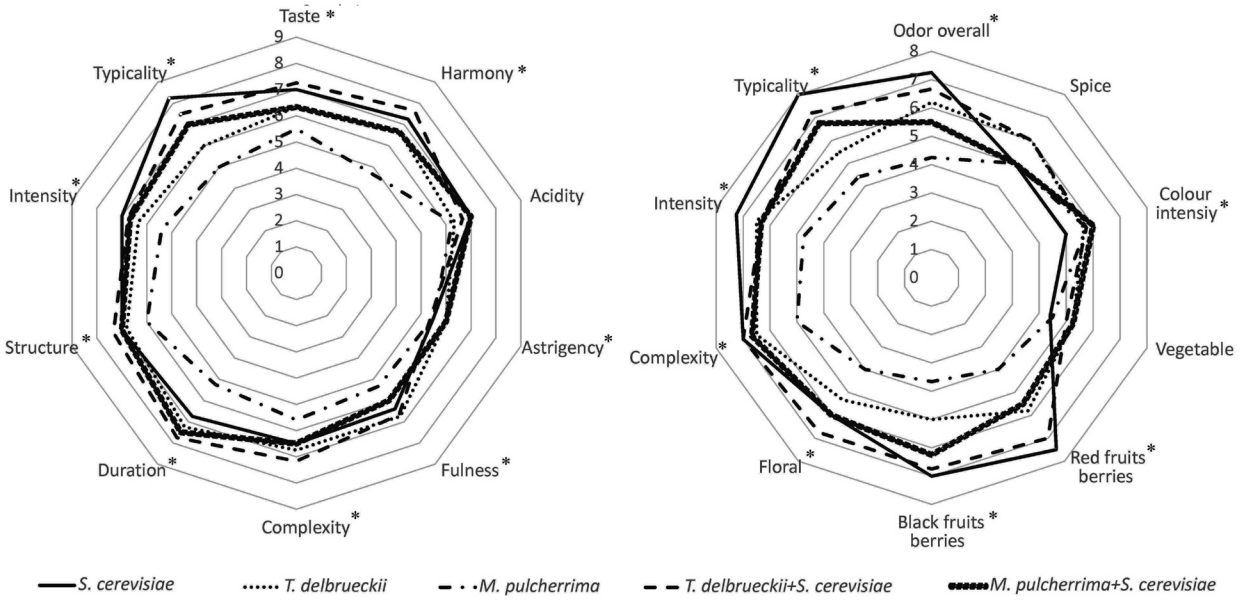

FIGURE 3. Taste and olfactory attribute scores of Prokupac wines produced with *T. delbrueckii*, *M. pulcherrima* and *S. cerevisiae* in pure and sequential fermentation.

Asterisks indicate significant differences between the means in attribute intensities ($p < 0.05$, Tukey's HSD test). Detailed results of the statistical analysis are summarised in Supplementary Data (Tables S1 and S2).

Volatile compounds are grouped according to their chemical structure (alcohols, esters, acetates, volatile acids and aldehydes). Alcohols and ethyl esters were the largest group of volatile compounds to be identified in all the wine samples.

The highest relative contribution of higher alcohols was found in the wine sample produced in pure fermentation with *M. pulcherrima*, being 23 % higher than in the control. The relative contributions of 3-methyl-1-butanol and 1-pentanol were higher in Prokupac wines produced in pure fermentations with *T. delbrueckii* and *M. pulcherrima* than in the control wine sample. In contrast, the relative contribution of 2-methyl-1-butanol was higher in the control wine. Ten different ethyl esters were detected in the analysed Prokupac wine samples, and their total relative contributions significantly differed among the wine samples.

Ethyl acetate was not detected in the samples produced with *M. pulcherrima*. However, the wine produced with *T. delbrueckii* contained 19.3 % (pure fermentation) and 8.8 % (sequential fermentation) of ethyl acetate, much higherthan in the control sample (4.2 %). Wine fermented with pure *S. cerevise* showed the highest relative contribution of ethyl octanoate and ethyl decanoate, which was double that of the sequential fermentation wines and four times that of the pure non-*Saccharomyces* fermentation wines.

All the identified volatile compounds with relative contribution above 1 % underwent a principal component analysis (PCA). PCA is a good way to visualise the differences in volatile composition between all produced Prokupac wines, as well as to identify the volatile compounds responsible for the greatest discrimination between samples (Figure 2). The first principal component (Factor 1) explained 36.02 % of the total variation, while the second principal component (Factor 2) explained a further 32.18 %. From the PCA results, it was possible to differentiate the samples produced in each of the sequential, pure and control fermentation trials.

The results of the sensory analysis (Figure 3) indicate that yeast strain or fermentation type (pure or sequential) have an impact on wine quality and sensorial characteristics due to the production of numerous specific metabolites which contribute to the aroma and sensory profile of wine. The wine sample produced in sequential fermentation with *T. delbrueckii* received the best overall score, being characterised as floral, spicy and fruity, full-bodied and as having more complex flavours than the other wine samples. However, the control wine sample scored better results for typicality, intensity, and red and black berry flavour notes. *S. cerevisiae* applied on Prokupac grapes clearly produced wine with good sensorial characteristics and fruity aroma. Wines made in pure *M. pulcherrima* fermentation scored significantly lower marks for most of the wine attributes.

DISCUSSION

The well-known high fermentative performance of *S. cerevisiae* was confirmed by the fermentation kinetics data, as well as by the significantly shorter time required to ferment almost all of the sugar in the grape must in the *S. cerevisiae* fermentation trial (Benito *et al.,* 2019; Escalante, 2019). In addition, co-inoculation with *S. cerevisiae* notably increased the consumption rate and accelerated the fermentation trials with non-*Saccharomyces* yeast. The acceleration happened just after the moment of inoculation in both sequential fermentations. A similar effect on the fermentation kinetics has been noticed in another study when the same non-*Saccharomyces* yeasts (*M. pulcherrima* and *T. delbrueckii)* were co-inoculated with *S. cerevisie* and *Saccharomyces bayanus* (Puškaš *et al.,* 2020). Inoculation with *S. cerevisiae* is, however, required to complete the fermentation. It is assumed that in the case of pure fermentation with non-*Saccharomyces* strains, wild indigenous *S. cerevisiae* significantly participates and is even responsible for the completion of the fermentation (Hranilovic *et al.,* 2020). Therefore, our results are in line with previously published data which state that there is high competition between *S. cerevisiae* strains (whether indigenous or commercial) and non-*Saccharomyces* yeast strains (Capece *et al.,* 2019).

A decrease in the final alcohol concentration (up to 1.4 vol %) in Prokupac wines produced in fermentation trials with non-*Saccharomyces* strains has also been previously reported for *M. pulcherrima* (Canonico *et al.,* 2019; Contreras *et al.,* 2014; Hranilovic *et al.,* 2020) and *T. delbrueckii* (Benito *et al.,* 2019; Dutraive *et al.,* 2019). This can be considered as a good strategy for reducing alcohol and obtaining better-balanced wines in the context of modified grape composition (more sugar and less organic acids in grape) under global warming. Knowing that non-*Saccharomyces* strains were on the whole unable to ferment the total amount of sugar, and that the level of sugar in all produced wines was below 4 g/L, it can be concluded that wild indigenous strains of S. cerevisiae were probably present in the must to finish off the fermentation. In the wines fermented with *T. delbrueckii,* the content of reducing sugars was slightly higher than in the wines produced by sequential fermentation with *S. cerevisiae,* which is in accordance with the results obtained for Tempranilo grape fermentation (Belda *et al.,* 2015).

The content of total dry extract found in all the fermentation trials in this study was much higher than those in recently published results regarding Prokupac wine produced by adding inactive-yeast-based products (Malićanin *et al.,* 2017) or selected aromatic herbs (Lakićević *et al.,* 2019). This is probably due to the ability of non-*Saccharomyces* strains to produce higher contents of different non-volatile compounds *(*Dutraive *et al.,* 2019), which are responsible for total extract

content and which positively affect wine taste sensations. A significantly lower total acidity of the wines produced with non-*Saccharomyces* strains is in accordance with the literature data (Comitini *et al.,* 2011). Higher contents of volatile acids in wines obtained in pure fermentations with *M. pulcherrima* and *T. delbrueckii* have also been obtained for Chardonnay wine (Jolly *et al.,* 2003), but otherwise they are mostly inconsistent with literature data (Bely *et al.,* 2008; Comitini *et al.,* 2011). However, it has been previously reported that *T. delbrueckii* can produce high levels of acetic acid (Jolly *et al.,* 2003). Furthermore, GonzálezRoyo and colleaugues indicated that the sequential fermentation of white wine with *M. pulcherrima* and *S. cerevisiae* resulted in similar values for volatile acidity, which is in accordance with the results reported in this paper (González-Royo *et al.,* 2015).

Higher total polyphenol, anthocyanin and flavonoid content, as well as better colour characteristics of wines produced with *M. pulcherrima* compared to those produced with *T. delbrueckii,* can be explained by the fact that *M. pulcherrima* is less able to adsorb anthocyanins in the cell wall. This property probably delayed the polymerisation process, resulting in wine with higher colour intensity and tonality compared to wine obtained using a different yeast, such as *T. delbrueckii* (Caridi *et al.,* 2004; Caridi *et al.,* 2015; Morata *et al.,* 2003). Furthermore, it has been reported that *M. pulcherrima* positively affects the formation of polymeric pigments in wines (Lappa *et al.,* 2020). The total polyphenols, anthocyanins and flavonoids observed in our study was several times higher than that in the Prokupac wine (vintage 2013 and 2014) produced with or without the addition of different aromatic herbs (Lakićević *et al.,* 2019), but significantly lower than the total polyphenols and anthocyanins observed in Prokupac wine in another recent study (Malićanin *et al.,* 2017). This could be explained by different viticultural and vinification practices, the duration of grape pomace contact or type of yeast strains (Caridi *et al.,* 2004). The highest amount of total SO_2 was detected in the wine produced only with *T. delbrueckii*. This result was in accordance with a higher total SO_2 content detected in red wine fermented only with *T. delbrueckii* than that in wines produced by sequential fermentation or only with *S. cerevisiae* (Belda *et al.,* 2015).

These results confirm the potential of studied yeast strains for modifying alcohol content, total dry extract and the content of phenolic compounds in Prokupac wine, thus improving its quality.

The analysis of the volatile profile of the wine showed significant differences between the samples produced by pure or sequential fermentation trials (Callejon *et al.,* 2010). Based on those differences it can be concluded that non-*Saccharomyces* yeast species can modulate the volatile aromatic profiles of wines. Knowing that fermented must was the same, it is possible to attribute the differences in wine composition to the yeast strains. The most abundant of the detected volatiles were primary alcohols, ethyl esters and fatty acid ethyl esters. As expected, primary alcohols were the most dominant group of volatile organic compounds. The total contribution of higher alcohols was greater in the wines produced with non-*Saccharomyces* yeast (in pure or in sequential fermentation with *S. cerevisiae*) than in the control, which is in accordance with the literature data (González-Royo *et al.,* 2015; Hranilovic *et al.,* 2020). A large amount of higher alcohols was also detected in sparkling wine and red wine produced with *M. pulcherrima* (Liu *et al.,* 2020) and with *T. delbrueckii* (Azzolini *et al.,* 2012). Among them, 2-methyl-1-butanol (alkyl alcohol) and 3-methyl-1-butanol (isoamyl alcohol) are the most dominant. Their presence in wine positively influences wine quality and contributes to its complexity, increasing fruity and flowery notes. Still, caution is needed here, since higher concentration of isoamyl alcohols may give wine unpleasant notes and fuel-like odours (Callejon *et al.,* 2010; De-La-Fuente-Blanco *et al.,* 2016; Pérez-Coello *et al.,* 1999), while alcohols like 2-methylbutan-1-ol, 3-methylbutan-1-ol, 2-methylpropan-1-ol, propan-1-ol, butan-1-ol may enhance the perception of solvent notes and attenuate the perception of characteristic fresh and jammy fruity aroma of red wine (Cameleyre *et al.,* 2015).

Both of the non-*Saccharomyces* yeasts used in this study showed a higher relative contribution of 1-pentanol and 1-propanol than did *S. cerevisiae*. The production of 1-pentanol, which is a primary alcohol responsible for the pleasant, almond, fruity or balsamic flavour of a wine, has also been previously confirmed in sequential fermentation with non- *Saccharomyces* yeasts (García-Carpintero *et al.,* 2011; Loira *et al.,* 2014). The formation of 1-propanol in sequential fermentation can be explained by the fact that non-*Saccharomyces* produce metabolites which are used by *S. cerevisiae* to form new aromatic compounds (Lappa *et al.,* 2020; Renault *et al.,* 2015).

S. cerevisie wines showed a higher relative contribution of 2-pehnylethanol (phenethyl alhocol), which belongs to the group of benzene derivatives that gives the wine a floral aroma (rose and lilac notes) (Pérez-Coello *et al.,* 1999). Nevertheless, it was once again demonstrated that sequential fermentation would be the best choice, as the relative contribution of 2-phenylethanol was double that in the pure fermentations. These results are also in accordance with previously published data which state that non-*Saccharomyces* yeast combined with *S. cerevisie* produce higher amounts of 2-phenylethanol in wine (Gobbi *et al.,* 2013).

Ethyl esters are the second largest group of volatile compounds in all the produced Prokupac wines. The type and the relative contribution of ethyl esters in wine is highly dependent on the yeast strain, as the majority of them are formed during the alcoholic fermentation. This type of compound positively affects wine quality, giving it fruity and fresh aromas (García-Carpintero *et al.,* 2011; Zhang *et al.,* 2018). Generally speaking, almost all the esters had a higher contribution in the wine made using *M. pucherrima* than in that produced from *T. delbrueckii*, with the exception of ethyl acetate, which is known to improve the quality of young wines (Cheng *et al.,* 2015) and to contribute to their desirable fruity aroma if present at low concentrations (up to 100 mg/L). At higher concentrations, it can give the wine an off-taste and an undesirable solvent/nail varnish-like aroma (Sumby *et al.,* 2010; Varela *et al.,* 2016). Ethyl acetate was the most abundant ester in wines produced as a result of pure fermentation using *T. delbrueckii*, as well as from sequential fermentation with both non-*Saccharomyces* strains, which is in accordance with previous research (Loira *et al.,* 2014; Tataridis *et al.,* 2013). Although *M. pulcherrima* alone should produce greater amounts of this compound according to literature data (Puškaš *et al.,* 2020; Varela *et al.,* 2016), our results show differently. This can be explained by the difference in grape varieties, composition of the must, yeast strain, inoculum size, moment of inoculation or nutrient availability. Actually, differences in the grape must composition can strongly influence the concentration of target aroma compounds, which is the reason for a fluctuation in the amount of certain compounds in wines produced with the same yeast strains (Seguinot *et al.,* 2020; Suárez-Lepe and Morata, 2012).

Another group of volatile compounds that were also present in all produced Prokupac wines are medium-chain fatty acid ethyl esters (ethyl esters of fatty acids with 6–12 carbon atoms). The greatest relative contribution of these compounds was in wines made in pure fermentations, followed by the wines produced in sequential fermentation with *S. cerevisie*. Their presence in wines in large quantities is common and desirable as they mostly give pleasant fruity candy- and perfume-like aromas (Hu *et al.,* 2018). However, it should be emphasised that the aroma of ethyl esters is related to their hydrocarbon chain length, and with the prolongation of the hydrocarbon chain the aroma changes from fruity to soap-like, while the aroma for the long chain ethyl esters (with 16–18 carbon atoms) could be described as being lard-like (Jackson, 2014). Type of yeast strain and fermentation conditions are the most important factors affecting the concentration of these ethyl esters in wine (Cheng *et al.,* 2015). Among them, ethyl octanoate and ethyl decanoate were the most present in the wines made from pure or sequential fermentations with *S. cerevisiae*. This is in accordance with the results of a previous study for Merlot wine production (Renault *et al.,* 2015). Esters production is strain-dependent (Renault *et al.,* 2009) and is the result of the enzymatic activity of yeasts (Jolly *et al.,* 2006), but it also depends on the fermentation conditions and the interaction of yeasts in sequential fermentation (Renault *et al.,* 2013, 2015). Given the existence of fatty acids ethyl esters, the low presence of free fatty acids can most likely be explained by their chemical esterification in the presence of ethanol and the low pH that occurs during fermentation.

As well as the group of compounds mentioned above, benzaldehyde, a representative of aromatic aldehydes, was also detected in the wines. Wine made by *M. pulcherrima* had a higher relative contribution of this compound, which can be explained by the ability of this microorganism to produce shikimic acid, a precursor for aroma compounds such is benzaldehyde (Dutraive *et al.,* 2019).

The PCA results (Figure 2) showed a considerable difference between the samples produced with non-*Saccharomyces* yeasts and the control. Factor 1 separated the control sample from the samples produced in the sequential fermentation; this was primarily due to the high production of volatile esters with generally floral and fruity odours, such as ethyl hexanoate and ethyl octanoate. The wines produced from the pure fermentation with *T. delbrueckii* were significantly different from the other wines and were characterised by a higher

relative contribution of ethyl acetate, isobutyl alcohol and 3-methyl-bytil-acetate. On the other hand, the wine produced using *M. pulcherrima* in the pure fermentation was distinct in terms of 3-methyl-1-butanol, *n*-hexanol and isohexanol. The wines produced from sequential fermentations were positioned on the negative lower quadrant, and Factor 1 distinguished them from the other samples by its higher levels of 1-pentanol, methyl-1-butanol and 2-phenylethanol. In addition, Factor 2 allowed the samples produced in the sequential fermentations to be differentiated, with a higher relative contribution of the above-mentioned volatile compounds than the control wines and wines produced using both of the non-*Saccharomyces* strains. It was observed that the control wine sample showed strong and positive correlations with the ethyl ethers (ethyl octanoate, ethyl decanoate, ethyl hexanoate), which have been described as being important contributors to fruity pleasant odours (Pineau *et al.,* 2009).

Finally, knowing the basic oenological parameters and aromatic composition of a wine is important for creating a unique sensory experience that is crucial for consumer acceptance and wine marketability. The presence of non-*Saccharomyces* yeasts in the initial stage of fermentation with later inoculation with *S. cerevisiae* increased levels of higher alcohols and total extracts in the wines. The wines produced from sequential fermentation with *T. delbrueckii* had floral and spicy attributes. Higher levels of ethyl esters, higher alcohol, total extract and polyphenolic compounds detected in this wine could be responsible for the increase in complexity, harmony structure and duration in comparison to other wines. Furthermore, the high levels of 1-pentanol (fruity, balsamic aroma) and 2-phenylethanol (flower, roses aroma) present in this wine most probably significantly affected its sensorial characteristics. The use of *M. pulcherrima* in the pure fermentation resulted in wines with the lowest sensorial characteristics, which were lacking in fruity and floral aroma. This is in accordance with the lowest relative contribution of ethyl esters detected in this wine, since they are responsible for the aroma type and are required in high-quality wines. The control wine in this study, produced from the pure fermentation with *S. cerevisiae,* had the most intense 'red berries', followed by 'black berries' note and low intensity of spices or vegetable aroma, which can be explained by the higher relative contribution of ethyl esters and higher alcohols. Both esters (ethyl octanoate and ethyl decanoate), found to be the most abundant in a control sample of Prokupac wine compared to other wine samples, have been previously identified as being responsible for the dominant red-berry and fresh-fruit aromas of the Merlot and Cabernet Sauvignon wines (Pineau *et al.,* 2009).

The influence of yeast strains and fermentation type (pure, mixed or sequential) on the sensory characteristics of wine is well-known (Jolly *et al.,* 2003; Martín-García *et al.,* 2020; Suárez-Lepe and Morata, 2012), and recently the advantage of sequential fermentations with *T. delbrueckii* or *M. pulcherrima* has also been confirmed (Belda *et al.,* 2015; Belda *et al.,* 2017; Loira *et al.,* 2014; Renault *et al.,* 2015; Varela *et al.,* 2016). A particular advantage of using non-*Saccharomyces* yeasts, was its positive influence on the intensity of wine colour, which is a very important characteristic for wines made from a grape variety with lower colour intensity. When compared to the previously published results of a sensory analysis carried out on Prokupac wine (Malićanin *et al.,* 2017), the wines obtained in this study from sequential fermentation mainly differ in their sensory profile for their spice attributes, which can be described as white pepper and cinnamon notes, or for their floral attributes, described as linden, acacia and rose notes. On the other hand, wines obtained from the pure fermentation with *M. pulcherrima* have much poorer sensory characteristics than the wine obtained in the sequential fermentation.

CONCLUSIONS

The modern wine industry is required to continually improve the quality of its wines and to enrich their aroma profiles in response to customer demands for different, new and more complex wines with fresh aromas and balanced taste. It therefore follows that wines made from high quality autochthonous and regional grape varieties are increasing in popularity due to their uniqueness and specificity. Since it has already been proven that the yeast strain plays a very important role in the creation of the unique and specific character of a wine, the use of non-*Saccharomyces* yeast in wine production may be a great opportunity that could open up many research possibilities.

S. cerevisiae is well-known for its high fermentative performance, which is why it has been irreplaceable for years. However, the presence of non-*Saccharomyces* yeasts in the initial stage of fermentation, along with later inoculation with *S. cerevisiae,* contributes to

a greater production of secondary metabolites, thus increasing the complexity of the wine and influencing the sensory experience. *T. delbrueckii* and *M. pulcherrima*, which were used in our study, were found to influence the increase in number of identified volatile compounds, higher alcohols and total extracts in the wines. A particular advantage of wines made using non-*Saccharomyces* was the improvement in colour intensity, which is important if the grape usually produces wines with lower colour intensity. As the composition of the fermented must remained the same, the differences found in the wine composition can be attributed to the added yeast strain.

In particular, the wines produced from the pure fermentation with *T. delbrueckii* had higher relative contribution of ethyl acetate, isobutyl alcohol and 3-methyl-bytil-acetate, while that resulting from the pure fermentation with *M. pulcherrima* had more 3-methyl-1-butanol, *n*-hexanol and isohexanol. Sequential fermentation wines contained higher levels of 1-pentanol, methyl-1-butanol and 2-phenylethanol, which all positively influence flavour and aroma. Control wine samples had a high relative abundance of ethyl ethers, which contribute to fruity pleasant odours. Since basic oenological parameters and the aromatic composition of wine contribute the most to the wine sensory experience, the fact that non-*Saccharomyces* yeast has potential for improving them – as established in our study – could be prove valuable in the development of modern viticulture practices.

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