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The effect of indigenous non-*Saccharomyces* yeasts on the volatile profile of Maraština wine: Monoculture versus sequential fermentation

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ABSTRACT

Non-*Saccharomyces* yeasts are presented as a new promising tool in winemaking to enhance aroma complexity in fermentation with *Saccharomyces* yeasts. Indigenous yeasts are recognized for their better adaptation to environmental conditions and for highlighting the unique *terroir* impact on wine aroma characteristics. To study the individual impact of ten indigenous non-*Saccharomyces* yeasts on the primary metabolites and volatile compounds of wine from autochthonous Croatian Maraština variety, *Hyphopichia pseudoburtonii, Metschnikowia chrysoperlae, Metschnikowia sinensis/shanxiensis, Metschnikowia pulcherrima, Lachancea thermotolerans, Hanseniaspora uvarum, Hanseniaspora guilliermondii, Hanseniaspora pseudoguilliermondii, Pichia kluyveri,* and *Starmerella apicola* were inoculated in sterile grape juice in monoculture fermentations. Additionally, seven of them were also studied in sequential fermentation of sterile grape juice with commercial *Saccharomyces cerevisiae.* A targeted approach was used for the identification of volatile compounds via headspace solid-phase microextraction coupled with gas chromatography and a mass spectrometer. *P. kluyveri* and *H. uvarum* produced higher total concentrations of terpenes in sequential versus monoculture fermentation. Sequential fermentation of *M. chrysoperlae, L. thermotolerans*, and *P. kluyveri* with *S. cerevisiae* resulted in higher production of C_{13}-norisoprenoids. The esters concentration was higher in monocultures for *L. thermotolerans, H. uvarum*, and *H. guilliermondii*, whereas other isolates showed higher concentrations in sequential fermentations. The results highlighted different indigenous yeast metabolisms and provided promising insights into potential new non-*Saccharomyces* starter cultures as a first step in their selection, with several species characterized in terms of their potential effect on the aroma profile.

1. Introduction

The composition of volatile compounds defines the sensory characteristics and the quality of the wine. The majority of aromatic compounds contributing to the wine aroma are produced during the fermentation process by yeasts (Carpena et al., 2020). Yeasts are the core of the alcoholic fermentation ecosystem, responsible for converting grape sugars into ethanol, and carbon dioxide, while also producing a diverse array of volatile and non-volatile compounds (Jolly et al., 2014). The impact of each yeast strain is influenced by the demanding fermentation environment and various biotic factors, including initial cell density and the presence of other yeast strains (Bagheir et al., 2018). However, in recent years, there has been a re-evaluation of non-*Saccharomyces* yeasts in wine fermentations, often referred to as spoilage yeast. While *Saccharomyces cerevisiae* eventually dominates and completes the fermentation process, non-*Saccharomyces* yeasts, in the interim, can produce various metabolites contributing to the wine's aroma, particularly through the production of enzymes that release bound aromas (Mateo et al., 2016; de Ovalle et al., 2021). The varying enzymatic activity and polarities of yeast cell wall components can also affect polyphenols and wine colour (Zhang et al., 2021).

Data thus far have shown that species belonging to the non-*Saccharomyces* genera such as *Metschnikowia, Lachancea, Pichia, Hanseniaspora*, and *Starmerella* tend to enhance the aroma of wine in comparison with

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S. cerevisiae (Benito et al., 2015; Padilla et al., 2016; Rădoi-Encea et al., 2023; Miranda et al., 2023). For instance, *Metschnikowia pulcherrima* possesses intense extracellular enzymatic activity (Jolly et al., 2014) leading to the release of bounded terpenes and thiols, imparting a floral aroma to wine ferments (Morata et al., 2019; Hranilović et al., 2020; Varela et al., 2021). The timing of sequential inoculation is crucial, as prolonged exposure to *M. pulcherrima* can increase the concentration of ethyl acetate (Varela et al., 2017). Furthermore, *Lachancea thermotolerans* is recognized as an acidity regulator, lowering the pH value and producing acetic acid at low concentrations (Comitini et al., 2011; Hranilović et al., 2022). Additionally, in sequential fermentation with *S. cerevisiae, L. thermotolerans* can produce a higher concentration of monoterpenes, such as nerol and terpinen-4-ol, compared to wine ferments with *S. cerevisiae* (Beckner Whitener et al., 2016). *Pichia kluyveri* exhibits limited fermentation activity, yet it is recognized as the most promising non-*Saccharomyces* inoculant for thiol production (Dutraive et al., 2019; Borren and Tian et al., 2021). Also, an increase in esters, higher alcohols, and glycerols has been reported in sequential fermentation with *S. cerevisiae* (Lee et al., 2019; Dutraive et al., 2019, Vicente et al., 2021). *Hanseniaspora* species are the most abundant yeast on the grapes, with *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* being the most studied (Renouf et al., 2007). *Hanseniaspora* spp. are mostly characterized by an overall increase in aromatic complexity (Badura et al., 2023). Grape juice inoculated with *H. uvarum/S. cerevisiae* resulted in increased concentrations of acetate esters, especially isoamyl acetate, volatile acidity and higher alcohols. *H. guilliermondii* has also been shown to have an increased concentration of acetate esters, such as 2-phenylethyl acetate, associated with honey and rose aromas (Andora et al 2010; Martin et al., 2018).

Taken together, the majority of previous studies have focused on inoculating non-*Saccharomyces* and *S. cerevisiae* yeasts into grape must, which harbours a microbial community characterized by grape location (Bokulich et al., 2016; Beckner Whitener et al., 2017) or in synthetic grape juice (Wang et al., 2016; Bagheir et al., 2018; Vicente et al., 2024). However, our understanding is limited regarding the impact of specific indigenous non-*Saccharomyces* yeast on the aromatic profile of the wine, particularly in the Maraština variety. Additionally, the effects of interactions between single non-*Saccharomyces* and *S. cerevisiae* yeast, independent of the rest of the mycobiota, remain insufficiently explored. Maraština (*Vitis vinifera* L.) is an indigenous Croatian variety with the potential to produce high-quality wines and holds a significant position among white cultivars in the Dalmatia wine region (Maletić et al., 2015). It is characterised by relatively high concentrations of terpenes and norisoprenoids in a bound form, in addition to their free forms (Budić-Leto et al., 2020). Non-*Saccharomyces* yeasts can release these compounds through enzymatic activity, enhancing the wine's aromatic profile. Being an autochthonous or lesser-known variety, Maraština may have specific interactions with indigenous yeasts, which have adapted to the *terroir* over time. This offers a unique context for research, as indigenous yeasts can bring out specific aromatic qualities. The oenological characterization of non-*Saccharomyces* yeasts isolated from Maraština grapes indicated several isolates from various species within the genera *Hanseniaspora, Lachancea*, and *Metschnikowia* displaying the potential to serve as starter cultures capable of influencing aroma profiles (Milanović et al., 2023). We aimed to clarify the impact of indigenous non-*Saccharomyces* yeasts (*Hanseniaspora guilliermondii, Hanseniaspora pseudoguilliermondii, Hanseniaspora uvarum, Hyphopichia pseudoburtonii, Lachancea thermotolerans, Metschnikowia chrysoperlae, Metschnikowia pulcherrima, Metschnikowia sinensis/shanxiensis, Pichia kluyveri,* and *Starmerella apicola*) on the Maraština wine aroma profile and their interaction with *S. cerevisiae* in sterile grape juice by a targeted approach utilizing headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography to a mass spectrometer (GCMS–). This study encompasses two fermentation practices: monoculture fermentation of indigenous isolates and their sequential fermentation with *S. cerevisiae.* To the best of our knowledge, *M. sinensis/shanxiensis, M. chrysoperlae, Hyp. pseudoburtonii,* and *S. apicola* were examined for the first time within the context of wine production.

2. Materials and methods

2.1. Chemicals

The yeast extract, peptone, and bacteriological agar utilized in the preparation of yeast peptone dextrose (YPD) agar/broth, serving as growth media for the yeasts, were purchased from Biolife Italiana S.r.l (Milan, Italy). Additionally, bacteriological dextrose was supplied from Oxoid (Hampshire, UK). Lysine Agar (Biolife Italiana S.r.l, Milan, Italy) was used for the differentiation of non-*Saccharomyces* yeast populations from the *S. cerevisiae* starter strains. For GC-MS analysis, ethanol (99.8%), n-heptanol (99.9%), dichloromethane (99.8%), tartaric acid and methanol, were procured from Merck (Darmstadt, Germany). Diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) for adjusting nitrogen availability to yeast was supplied by VWR International (Pennsylvania, USA).

2.2. Yeast strains

The yeast isolates used in this study, including *Hyp. pseudoburtonii* N-11 (HP), *M. chrysoperlae* K-11 (MC), *M. sinensis/shanxiensis* P-7 (MS), *M. pulcherrima* K-6 (MP), *L. thermotolerans* P-25 (LT), *H. uvarum* Z-7 (HU), *H. guilliermondii* N-29 (HG), *H. pseudoguilliermondii* V-13 (HPG), *P. kluyveri* Z-3 (PK), and *S. apicola* VP-8 (SA), were sourced from the yeast collection of the Institute for Adriatic Crops (IJK, Split, Croatia), established in 2021. The yeasts were previously isolated from Maraština grapes and identified at the molecular level by sequencing the ITS1–5.8S-ITS2 rDNA region as previously described by Milanović et al. (2023). The resulting sequences were then compared to the ITS1–5.8S-ITS2 sequences of type strains deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Commercial strains *L. thermotolerans* Octave® (LT Octave), *M. pulcherrima* Flavia® (MP Flavia), and *S. cerevisiae* EC 1118 (SC) (Lallemand Inc., Montreal, QC, Canada), were used as controls in this study.

The indigenous yeasts, retrieved from glycerol stocks preserved at -80°C, were inoculated into YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose). Furthemore, isolates were subjected to two rounds of preculturing at 25°C with continuous agitation at 2000 rpm for 24 hours in an orbital incubator (Stuart SI500 - Incubator, Tec-Quipment Ltd, Nottingham, UK). The biomass was then collected by centrifugation (Hettich® Universal 320/320R centrifuge, Andreas Hettich GmbH & Co., Tuttlingen, Germany) at 1520 × *g* at 4°C for 5 min. The supernatant was removed following centrifugation, and the cell pellet was resuspended in sterile peptone water. The yeast cell concentration for inoculation was determined spectrophotometrically using a Varian Cary® 50 UV–Vis Spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA) at wavelengths of 600 nm. Commercial yeasts were rehydrated following the manufacturer's protocols. Inoculations were prepared under sterile conditions for all utilized yeast strains.

2.3. Laboratory-scale monoculture and sequential fermentations

Monoculture and sequential fermentations were conducted using Maraština grape must obtained from the vineyard in Plastovo (Skradin, 43°52′49″ N 15°55′29″ E), situated within the wine region Dalmatia (Croatia), on September 17th, 2022 in triplicate. The grapes were harvested at optimal maturity during the 2022 harvest, when the concentrations of glucose (118.53 g/L) and fructose (116.30 g/L) were equal (1:1). After destemming and crushing, the Maraština grape must (pH 3.35, total acidity 4.33 g/L expressed as tartaric acid) was treated with the addition of 50 mg/L of sulfur dioxide in a laboratory-scale 500 mL fermenter. Following optimal fermentation conditions, the yeast

assimilable nitrogen (YAN) concentration was adjusted to 250 mg/L of nitrogen by adding $(NH_4)_2HPO_4$. The grape must undergo cold stabilisation for 24 hours at 4°C. Subsequently, sterile filtration was carried out using PALL filters with a pore size of 0.45 μm to acquire the sterile grape must.

Yeast strains were inoculated at a concentration of approximately 10^7 cells/mL into Maraština grape must for monoculture and sequential fermentations. Monoculture fermentations were conducted using all ten non-*Saccharomyces* isolates. Moreover, seven non-*Saccharomyces* yeast strains chosen based on basic oenological properties and enzymatic activity previously reported by Milanović et al. (2023) were used for sequential fermentation with commercial *S. cerevisiae*. Furthermore, *S. cerevisiae* was inoculated at a concentration of approximately 5×10^6 cells/mL when fermentations reached 2–3 % *v/v* of ethanol. These seven strains included *M. chrysoperlae* K-11 (MC-SC), *M. sinensis/shanxiensis* P-7 (MS-SC), *M. pulcherrima* K-6 (MP-SC), *L. thermotolerans* P-25 (LT-SC), *H. uvarum* Z-7 (HU-SC), *H. guilliermondii* N-29 (HG-SC), and *P. kluyveri* Z-3 (PK-SC) isolates. Commercial yeast SC, MP Flavia, and LT Octave served as control trials for both experiments. Each strain's fermentation process was carried out in sterile Erlenmayer flasks with 500 mL capacity with porous cellulose sterile caps at 20°C.

The growth kinetics were monitored using two different agar media: YPD, to determine the total yeasts number and Lysine agar medium for the growth of non-*Saccharomyces* yeasts (Benito et al., 2015). Serial dilutions of the cell suspensions were carried out using peptone water (1g/L, w/v). Aliquots of 100 μL from each sample were then spread onto YPD and Lysin agar plates and incubated (Stuart SI500 incubator) at 25°C for 2–3 days. Colony-forming units were counted on plates containing between 30 and 300 colonies (Milanović et al., 2013). The results were expressed as the mean values of triple biological and double technical replicates, expressed in CFU per mL of each sample ± standard deviation. The fermentation kinetics were monitored by Fourier-transform infrared spectroscopy (FTIR Lyza 5000 Wine, Anton Paar GmbH, Graz, Austria) every two days for all fermentation trials. The fermentations were considered complete when the concentration of reducing sugars remained constant, typically below 5 g/L. At the end of fermentation, wine samples were stored at -80°C until the HS-SPME-GC-MS analysis.

2.4. Basic physicochemical analysis

The analysis included the determination of ethanol (% *v/v*), reducing sugars (g/L), pH, volatile acidity (g/L), total acidity (g/L), glucose (g/L), fructose (g/L), malic acid (g/L), lactic acid (g/L), relative density (20/20°C), total dry extract (g/L), and glycerol (g/L) using FTIR Lyza 5000 Wine (Anton Paar GmbH, Graz, Austria). The calibration of the FTIR method was carried out following the standard physicochemical OIV methods for wine in a laboratory accredited according to HRN EN ISO/IEC 17025:2017. Additionally, calibration for glucose, fructose, malic and lactic acid was done by enzymatic methods with L-malic acid, D-/L-lactic acid, and D-fructose/D-glucose assay kits (Megazyme, Wicklow, Ireland).

2.5. Colour determination and total polyphenols

Absorbance at wavelengths of 420, 520, and 620 nm was measured with a Varian Cary® 50 UV–Vis Spectrophotometer (Agilent Technologies Inc.). The colour intensity (I) was measured as the total absorbance at 420, 520, and 620 nm (I = A420 + A520 + A620), following the method described by Glories et al. (1984) and approved by the International Organization of Vine and Wine as an official method. The shade (N) was calculated as the ratio of absorbance at 420 nm to that at 520 nm (N = A420/A520). Additionally, the concentration of total polyphenols was determined by infrared spectroscopy using Lyza 5000 Wine (Anton Paar GmbH).

2.6. HS-SPME-GC-MS analysis

The determination of volatile compounds was conducted using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS), following a validated method outlined in a previous study (Škrab et al., 2021, 2024) with minor modifications. Each sample (0.5 mL) was spiked with 50 μL of 2-octanol (2.13 mg/L in ethanol) as an internal standard. After 10 minutes at 40 °C, samples were extracted for 30 minutes at the same temperature using solid-phase microextraction (SPME) with a 2-cm DVB/CAR/PDMS 50/30 μm fibre (Supelco, Sigma-Aldrich, Milan, Italy). Analysis was performed using an Agilent Intuvo 9000 GC coupled with a 7010B triple quadrupole mass spectrometer and a CombiPAL autosampler (Agilent Technologies Inc., Santa Clara, CA, USA). The volatiles were desorbed in the GC inlet at 250°C for 4 min in splitless mode, and the fibre was reconditioned between each sample for 5 min at 270°C (Carlin et al., 2016). The separation was performed using a DB-VAX (UI) column (30 m × 0.25 μm × 0.25 μm) packed with 100 % polyethylene glycol from Agilent Technologies Inc. The oven temperature was initially set at 50°C for 4 minutes, then ramped up at a rate of 3°C per minute until reaching 130°C, where it was held for 1 minute, and finally increased at a rate of 10°C per minute to 250°C for 1 minute. Helium with a purity of 99.9995 % was used as carrier gas with 1.2 mL/min flow. The MS parameters encompassed electron ionization at 70 eV using an ion source temperature of 230°C. The instrument operated in scan mode, with a quadrupole configured to scan the range from 40 to 300 m/z, and each scan took 150 milliseconds. MassHunter software (Agilent Technologies Inc.) was utilized to confirm the identified volatile compounds by injecting pure analytical standards. Finally, an analysis of obtained data was carried out, and the results were expressed as μg equivalents of the internal standard per L of a sample.

2.7. Odour activity values

To assess the individual contribution of each aroma compound in fermentations to the overall odour profile, the odour activity value (OAV) was computed (Sáenz-Navajas et al., 2015). This was achieved by dividing the concentration of a specific compound by its odour detective threshold (ODT), which was determined and obtained from literature data.

2.8. Statistical analysis

Statistical data analysis was performed using additional tool for Excel, the XLStat (Long Island, NY, USA) statistical and data analysis solution. Analysis of variance (ANOVA) was employed for statistical evaluation of volatile compounds and primary metabolites produced by yeast isolates. Furthermore, post hoc multiple comparisons were executed using Tukey's range test to pinpoint precise distinctions among the indigenous yeasts and controls. Principal component analysis (PCA) was also applied, to get a qualitative insight into the similarities and/or differences resulting from the influence of indigenous yeasts on the volatile groups of fermentation compared with those of commercial strains *S. cerevisiae, L. thermotolerans* and *M. pulcherrima* (controls). Investigating the clustering based on similar physical-chemical properties, hierarchical clustering was conducted by XLStat. Data for each strain were measured in triplicate. Thus, the data matrix for monoculture fermentation contained for each strain 3 × 12 data on physicochemical parameters, and 3 × 88 data on the concentration (μg/L) of volatile components in Maraština wines. Accordingly, the matrix had a format of 300 rows and 13 columns (ten indigenous non-*Saccharomyces* yeast isolates and three commercial yeasts (controls)). In the case of sequential fermentation, the number of rows remained the same (36 (12 × 3) rows related to physicochemical parameters, and 264 (3 × 88) to the concentration of volatile components). Still, the number of columns was 10 in total (seven indigenous non-*Saccharomyces* yeast isolates and

three commercial yeasts (controls). Additionally, a heat map was conducted using MetaboAnalyst v.5.0 (University of Alberta, Edmonton, AB, Canada) and involved the application of the Ward algorithm and Euclidean distance analysis.

3. Results and discussion

3.1. Monoculture fermentation with non-Saccharomyces isolates

3.1.1. Fermentation kinetics and primary metabolite production

The fermentation kinetics were verified through viable counts and summarised in Fig. 1. The fermentation progress was not uniform, concluding at different times when the concentration of reducing sugars remained constant, mainly below 5.0 g/L. Among indigenous yeasts, the MC isolate was the fastest, concluding fermentation in 18 days, concurrent with the commercial SC. In contrast, MP took 40 days to finish the fermentation, which was two and a half times longer than the control commercial MP Flavia strain. These results confirm previous studies that described MP as having low nitrogen consumption, resulting in slower fermentation performances (Roca-Mesa et al., 2020). The highest number of viable cells was observed for LT after 10 days of fermentation, reaching 11.68 log CFU/mL probably because of excellent nitrogen consumption which directly affects yeast growth (Prior et al., 2019). It is noteworthy that all indigenous yeasts showed a higher growth rate at the beginning of fermentation compared to the control yeasts. However, at the end of fermentation, the highest number of viable cells was recorded for SC (7.60 CFU/mL), followed by SA (7.56 CFU/mL), HPG (7.55 CFU/mL), and PK (7.20 CFU/mL), demonstrating their good tolerance to ethanol but without statistically significant differences compared to the rest of the utilized yeast.

The physicochemical parameters of Maraština wines produced by monoculture fermentations are presented in Table 1, with their monitoring during fermentation displayed in Supplementary Figure 1. All wines had similar ethanol concentrations, ranging from 12.31 % *v/v* (SA) to 12.74 % *v/v* (MS), except for MP (11.85 % *v/v*) and HPG (11.78 % *v/v*) showing statistically significant lower ethanol levels. Some ethanol reduction strategies in wine involve the use of *Metschnikowia*, either because of their low fermentative efficiency or the potential to induce a Crabtree-negative effect, allowing them to metabolize sugars through respiration instead of fermentation (Quiros et al., 2014). All indigenous yeasts produced Maraština wines with < 5.0 g/L of residual sugar, except HU (6.20 g/L). These results contradict previous research suggesting that non-*Saccharomyces* yeasts such as *H. uvarum* and *L. thermotolerans* struggle to complete fermentation under harsh conditions, resulting in low ethanol concentrations and fermentation stuck (Ciani et al., 2006). In the context of monoculture fermentations, previous studies have reported that non-*Saccharomyces* yeasts such as *H. uvarum* reached 4 % *v/v* of ethanol (Andorra et al., 2010), *L. thermotolerans* reached a range between 4–6 % *v/v* (Binati et al., 2020) same as *P. kluyveri* (Contreras et al., 2014). Interestingly, MP utilized glucose and fructose equally, while other yeast isolates appeared to be glucophilic, as higher residual fructose concentrations were measured at the end of fermentations. In a previous study conducted by Lee et al. (2019), *P. kluyveri, H. uvarum*, and *M. pulcherrima* isolates utilized fructose and glucose (< 1.0 g/L) without exhibiting specific favouritism during monoculture fermentations. Additionally, MS and MC had the fastest glucose utilization among indigenous yeasts, closely resembling the control fermentations conducted with MP Flavia and SC in terms of glucose utilization and ethanol production (Supplementary Figure 1). Fructose was utilized the fastest by MP Flavia and SC, followed by the indigenous MC and SA strains. Fermentations with HPG yeast produced the highest concentration (0.75 g/L) of volatile acidity, which along with PK (0.63 g/L), differed statistically from SC (0.42 g/L). The rest of the utilized indigenous isolates showed similar production of volatile acidity as control yeast SC. Non-*Saccharomyces* were often correlated with increasing volatile acidity (Jolly et al., 2014). However, our results

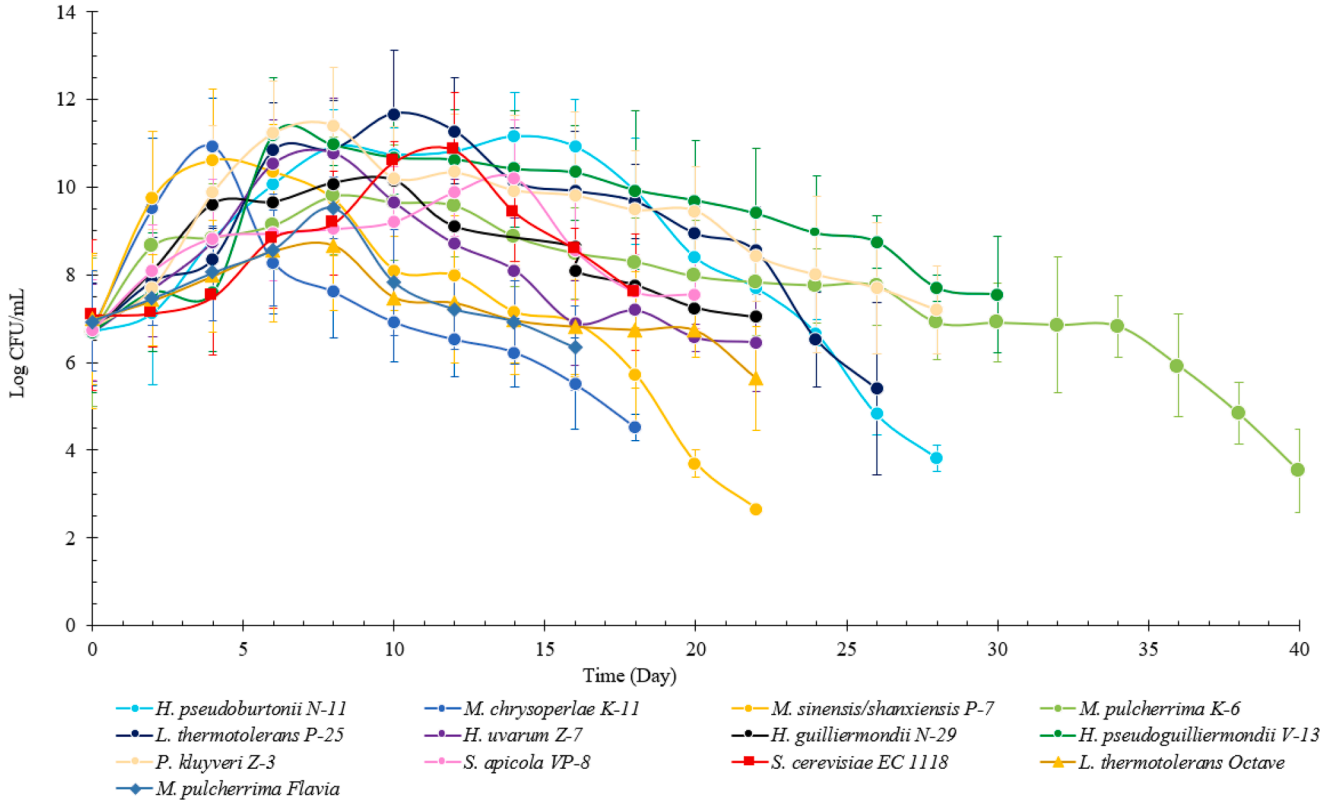

Fig. 1. Growth kinetics of 10 investigated non-*Saccharomyces* yeasts and three control strains (*S. cerevisiae, L. thermotolerans* Octave, and *M. pulcherrima* Flavia) during the monoculture fermentation.

Table 1
The physicochemical parameters of Maraština wines produced by monoculture fermentations with 10 indigenous non-*Saccharomyces* yeasts and three commercial strains (*S. cerevisiae* EC 1118, *L. thermotolerans* Octave, and *M. pulcherrima* Flavia).

Parameter	HP	MC	MS	MP	LT	HU	HG	HPG	PK	SA	SC EC 1118	LT Octave	MP Flavia
Relative density (20/20°C)	0.9918 ±0.00^{a}	0.9918 ±0.00^{a}	0.9918 ±0.00^{a}	0.9948 ±0.00^{b}	0.9938 ±0.00^{b}	0.9928 ±0.00^{a}	0.9918 ±0.00^{a}	0.9988 ±0.00^{c}	0.9948 ±0.00^{b}	0.9928 ±0.00^{a}	0.9936 ±0.00^{a}	0.9920 ±0.00^{b}	0.9928 ±0.00^{a}
Ethanol (% *v/v*)	12.40 ±0.26^{a}	12.65 ±0.05^{a}	12.74 ±0.05^{a}	11.85 ±0.15^{b}	12.06 ±0.14^{a}	12.43 ±0.24^{a}	12.50 ±0.27^{a}	11.78 ±0.71^{b}	12.38 ±0.04^{a}	12.31 ±0.02^{a}	12.44 ±0.10^{a}	12.41 ±0.13^{a}	12.37 ±0.03^{a}
Total dry extract (g/L)	23.63 ±2.06^{a}	20.83 ±0.45^{b}	22.27 ±0.76^{a}	26.67 ±0.80^{c}	24.07 ±1.76^{a}	24.30 ±3.54^{a}	22.17 ±0.64^{a}	27.80 ±0.14^{c}	22.93 ±0.31^{a}	23.30 ±0.00^{a}	21.93 ±0.45^{a}	25.33 ±3.12^{c}	22.43 ±0.50^{a}
Reducing Sugars (g/L)	4.0 ±0.31^{a}	3.3 ±0.51^{a}	4.3 ±0.90^{a}	5.3 ±0.06^{b}	5.0 ±0.95^{b}	6.2 ±2.55^{b}	4.3 ±0.21^{a}	5.9 ±1.13^{b}	4.2 ±0.70^{a}	4.5 ±0.14	2.7 ±0.20^{d}	7.1 ±2.82^{c}	2.2 ±0.06^{d}
Glucose (g/L)	0.53 ±0.12^{a}	0.47 ±0.06^{a}	0.50 ±0.10^{a}	2.87 ±0.21^{b}	0.70 ±0.27^{a}	0.50 ±0.14^{a}	0.47 ±0.06^{a}	0.85 ±0.07^{a}	0.50 ±0.10^{a}	0.50 ±0.00^{a}	0.43 ±0.06^{a}	0.57 ±0.21^{a}	0.50 ±0.00^{a}
Fructose (g/L)	3.43 ±0.23^{b}	2.87 ±0.42^{a}	3.83 ±0.81^{b}	2.90 ±0.17^{a}	4.33 ±0.87^{c}	5.50 ±2.26^{c}	3.80 ±0.20^{b}	5.00 ±0.99^{c}	3.67 ±0.67^{b}	4.00 ±0.14^{b}	2.30 ±0.17^{a}	6.30 ±2.52^{c}	1.87 ±0.12^{d}
Total acidity (g/L)**	6.23 ±0.05^{a}	6.28 ±0.07^{a}	6.24 ±0.26^{a}	6.60 ±0.07^{b}	6.74 ±0.70^{b}	5.97 ±0.04^{a}	6.38 ±0.25	6.92 ±0.25^{b}	6.47 ±0.21^{a}	6.85 ±0.00^{b}	6.87 ±0.16^{b}	6.16 ±0.03^{a}	6.99 ±0.14^{b}
Volatile acidity (g/L)*	0.56 ±0.14^{b}	0.54 ±0.11^{b}	0.53 ±0.06^{b}	0.50 ±0.06^{b}	0.67 ±0.07^{b,c}	0.60 ±0.03^{b,c}	0.58 ±0.08^{b}	0.75 ±0.33^{c}	0.63 ±0.04^{c}	0.46 ±0.02^{b}	0.41 ±0.05^{b}	0.48 ±0.02^{b}	0.29 ±0.02^{a}
pH	3.30 ±0.02^{a}	3.32 ±0.02^{a}	3.31 ±0.03^{a,b}	3.27 ±0.03^{a}	3.26 ±0.06^{a,b}	3.36 ±0.00^{b}	3.29 ±0.02^{a}	3.24 ±0.02^{a}	3.28 ±0.04^{a,b}	3.20 ±0.00^{a}	3.25 ±0.02^{a,b}	3.31 ±0.01^{a,b}	3.20 ±0.02^{a}
Malic acid (g/L)	0.35 ±0.07^{b,c}	0.15 ±0.04^{b}	0.09 ±0.08^{a}	0.22 ±0.05^{b}	0.27 ±0.15	0.20 ±0.11^{b}	0.17 ±0.11^{b}	0.43 ±0.14^{c}	0.31 ±0.10^{b,c}	0.33 ±0.09^{b,c}	0.47 ±0.08^{c}	0.18 ±0.04^{b}	0.46 ±0.04^{c}
Lactic acid (g/L)	0.67 ±0.38^{c}	0.28 ±0.03^{b}	0.28 ±0.03^{b}	0.14 ±0.02^{a}	0.24 ±0.07^{b}	0.30 ±0.11^{b}	0.18 ±0.04^{a}	0.65 ±0.35^{c}	0.39 ±0.35^{b,c}	0.19 ±0.07^{a}	0.13 ±0.02^{a}	0.21 ±0.02^{a,b}	0.15 ±0.02^{a}
Glycerol (g/L)	5.80 ±0.10^{a}	5.80 ±0.17^{a}	5.80 ±0.20^{a}	7.17 ±0.21^{b}	6.37 ±0.38^{a,b}	6.20 ±0.14^{a,b}	6.00 ±0.36^{a}	6.45 ±0.21^{a,b}	6.10 ±0.17^{a}	5.75 ±0.07^{a}	6.40 ±0.17^{a,b}	6.00 ±0.10^{a}	6.37 ±0.15^{a,b}

Data are representative mean ± standard deviation of three biological replications. Different letters in the column indicate a significant difference ($p < 0.05$). Abbreviations: HP –*Hyphopichia pseudoburtonii* N-11; MC- *Metschnikowia chrysoperlae* K-11; MS- *Metschnikowia sinensis/shanxiensis* P-7; MP- *Metschnikowia pulcherrima* K-6; LT- *Lachancea thermotolerans* P-25*;* HU- *Hanseniaspora uvarum* Z-7; HG- *Hanseniaspora guillermondii* N-29; HPG- *Hanseniaspora pseudoguilliermondi* V-13; PK- *Pichia kluyveri* Z-3; SA- *Starmerella apicola* VP-8; SC EC 1118 – *Saccharomyces cerevisiae* EC 1118; LT Octave- *Lachancea thermotolerans* Octave; MP Flavia- *Metschnikowia pulcherrima* Flavia;

* - expressed as acetic acid;

** - expressed as tartaric acid.

showed the contrary and highlighted the importance of the selection of yeast strains. The optimal concentration of acetic acid in wine is between 0.2 and 0.7 g/L, although levels from 0.7 to 1.2 g/L can be acceptable depending on the dry wine style according to Regulation (EU) No 1308/2013. In comparison with commercial strains SC and MP Flavia, indigenous yeast strains demonstrated better capability to transport and metabolise L-malate, especially MS (0.09 g/L). By this, indigenous yeasts produced a higher concentration of lactic acid where HP (0.67 g/L) and HPG (0.65 g/L) statistically differed from the rest of the used yeast. Previous studies indicated that non-*Saccharomyces* produce less fumaric acid, an inhibitor of malolactic fermentation, compared to *S. cerevisiae*. This reduction in fumaric acid levels can lead to an increase in lactic acid production (Cofran and Meyer, 1970; Hranilović et al., 2020). Another metabolite strongly associated with yeast metabolism is glycerol, which contributes to smoothness, and sweetness in wines when exhibited at 5.2 g/L. MP produced the highest concentration of glycerol (7.17 g/L) similar to LT (6.37 g/L), HPG (6.45 g/L) and control SC (5.75 g/L) and MP Flavia (6.37 g/L). Romano et al. (2022) characterized *M. pulcherrima* as a good producer of glycerol but also adding 60 mg/L of sulfur dioxide to the grape must can reduce glycerol production by one-third. Blanco et al. (2020) reported a twice smaller concentration of glycerol in pure *L. thermotolerans* ferments compared to fermentations with our LT isolate.

Regarding colour evaluation, in monoculture fermentations only the LT strain showed significant differences, exhibiting the highest colour intensity and a similar shade compared to SC (Supplementary Table 1). Other isolates had colour intensities comparable to SC. Cell adsorption is a strain-dependent phenomenon, making it possible to select yeasts with lower pigment adsorption rates than others (Morata et al., 2003). As with colour, differences in the composition of the cell wall, linked to oligosaccharides, glucans, and chitin, can affect polyphenols (Romano et al., 2024). All fermentations with indigenous yeasts resulted in statistically lower concentrations of total polyphenols (0.14–0.33 g/L) compared to SC fermentation (0.55 g/L), except for MP and SA, which had statistically similar concentrations as SC (Supplementary Table 1).

3.1.2. Volatile compound analysis by HS-SPME-GC–MS

A total of 58 volatiles were detected in Maraština wines produced by indigenous non-*Saccharomyces* yeasts in monoculture, as reported in Table 2. The OAV for compounds which exceed odour thresholds are listed in Supplementary Table 2.

The production of total terpenic compounds was the highest in fermentations performed by MP (11.12 μg/L) compared to the MP Flavia and SC control yeasts (6.55–8.21 μg/L), particularly evident for *cis*-rose oxide, linalool, α-terpineol, and β-citronellol. MP is a flavour-active yeast known for its excellent β-glucosidase activity, which reflects terpenic production (Benito et al., 2015; Milanović et al., 2023) and aligns with our results. Indeed, the linalool concentration was 6.11 μg/L, where linalool directly contributes to the aroma due to the low ODT (6 μg/L) with citrus notes (Supplementary Table 2). Furthermore, the positive impact of HPG isolate was observed through the production of geraniol (0.89 μg/L) which statistically differed from pure fermentation with SC. Yeasts can influence terpene content not only through the hydrolysis of terpene glycosides but also by direct biosynthesis of terpenes and transformation of certain terpenes into others, such as the conversion of geraniol to citronellol (Ugliano et al., 2006). Moreover, the MS isolate exhibited a significantly higher concentration of terpinen-4-ol (0.50 μg/L) compared to controls, MP Flavia and SC, while HU and PK

Table 2
Concentration (μg/L)* of volatile compounds in Maraština wines produced for ten indigenous non-*Saccharomyces* yeast isolates and three commercial yeasts (controls): *L. thermotolerans* Octave, *M. pulcherrima* Flavia, *S. cerevisiae* EC 1118 in monoculture fermentation..

Compound	HP	MC	MS	MP	LT	HU	HG	HPG	PK	SA	SC EC 1118	LT Octave	MP Flavia
1,4-cineole	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1,8-cineole	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis-Rose oxide	nd	0.03±0.01^{a}	nd	0.06±0.02^{a}	0.03±0.03^{a}	nd	nd	nd	nd	nd	0.02±0.00^{a}	nd	nd
trans-Rose oxide	0.22±0.08^{a}	0.14±0.08^{a}	0.25±0.26^{a}	0.24±0.05^{a}	0.15±0.10^{a}	0.19±0.13^{a}	0.30±0.04^{a}	nd	0.14±0.09^{a}	0.23±0.14^{a}	0.16±0.03^{a}	0.14±0.05^{a}	0.23±0.13^{a}
trans-Linalool oxide	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis-Linalool oxide	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Linalool	4.96±0.77^{a}	3.23±1.12^{b}	4.13±2.27^{a}	6.11±0.70^{a}	5.36±2.97^{a}	3.75±0.97^{a,b}	4.85±0.90^{a}	5.16±0.88^{a}	3.48±0.25^{b}	5.01±0.67^{a}	3.40±0.17^{b}	7.40±0.55^{c}	4.97±1.47^{a}
Terpinen-4-ol	0.33±0.19^{a}	0.21±0.10^{a}	0.50±0.60^{b}	0.46±0.13^{a,b}	0.25±0.05^{a}	nd	0.39±0.12^{a,b}	nd	nd	0.27±0.07^{a}	0.25±0.09^{a}	nd	0.20±0.10^{a}
Safranal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
α-terpineol	1.33±0.22^{a}	0.96±0.33^{a,b}	1.13±0.61^{a}	1.93±0.31^{a}	1.34±0.47^{a}	1.33±0.65^{a}	1.35±0.27^{a}	1.36±0.21^{a}	0.99±0.28^{a,b}	1.26±0.37^{a}	0.79±0.13^{b}	1.42±0.64^{a}	1.15±0.50^{a}
β-citronellol	1.07±0.17^{a}	1.27±0.38^{a}	1.25±0.69^{a}	2.00±0.40^{b}	1.32±0.86^{a}	0.80±0.43^{a}	1.44±0.29^{a,b}	nd	0.85±0.16^{a}	nd	1.55±0.23^{b}	nd	1.08±0.16^{a}
Nerol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Geraniol	nd	0.35±0.18^{a}	0.35±0.17^{a}	0.48±0.12^{a}	0.44±0.07^{a}	nd	0.57±0.10^{a,b}	0.89±0.01^{b}	nd	0.63±0.18^{a,b}	0.38±0.09^{a}	0.41±0.00^{a}	0.65±0.18^{a,b}
Eugenol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
trans-terpin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
β-ionone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
∑Terpenic compounds	8.03±1.33^{a,b}	6.17±2.17^{a}	7.61±4.61^{a}	11.12±1.40^{c}	8.89±4.41^{a,b}	6.25±1.92^{a}	8.89±1.54^{b}	9.07±3.45^{b}	5.62±0.29^{a}	7.54±1.23^{a}	6.55±0.51^{a}	9.63±0.71^{b,c}	8.21±2.29^{b}
TDN	0.49±0.05^{a}	0.38±0.06^{a}	0.40±0.32^{a}	0.49±0.08^{a}	0.49±0.36^{a}	0.52±0.23^{a}	0.70±0.07^{b}	0.58±0.18^{a}	0.49±0.22^{a}	0.59±0.28^{a}	0.48±0.06^{a}	0.74±0.17^{b}	0.50±0.23^{a}
Vitispirane	3.67±0.67^{a}	2.79±1.34^{a}	4.27±3.66^{a}	4.49±0.79^{a}	3.46±2.45^{a}	3.91±2.07^{a}	5.15±1.80^{b}	4.76±0.84^{a,b}	2.23±0.35^{a}	5.49±2.94^{b}	2.94±0.87^{a}	5.02±1.26^{b}	4.89±2.16^{a,b}
β-damascone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
β-damascenone	0.82±0.16^{a}	0.73±0.14^{a}	0.82±0.57^{a}	0.64±0.09^{a}	0.86±0.29^{a}	0.97±0.29^{a,b}	1.18±0.14^{b}	0.91±0.30^{a}	0.82±0.06^{a}	0.93±0.15^{a}	0.71±0.05^{a}	1.17±0.20^{b}	0.59±0.14^{a}
∑C_{13}-norisoprenoids	4.98±0.54^{a}	3.90±1.43^{a}	5.48±4.54^{a}	5.62±0.76^{a}	4.81±3.10^{a}	5.39±2.58^{a}	7.03±1.95^{b}	6.25±1.31^{a}	3.54±0.32^{a}	7.01±3.37^{b}	4.13±0.79^{a}	6.92±1.31^{a}	5.97±2.52^{a}
Furfurylthiol	0.85±0.03^{a}	0.98±0.08^{b}	0.96±0.02^{b}	0.91±0.15^{a}	0.91±0.02^{a}	0.93 ±0.02^{a,b}	0.88±0.05^{a}	0.92±0.09^{a,b}	1.70±1.02^{c}	0.85±0.09^{a}	0.90±0.18^{a}	0.90±0.07^{a}	0.91±0.05^{a}
∑Thiol	0.85±0.03^{a}	0.98±0.08^{b}	0.96±0.02^{b}	0.91±0.15^{a}	0.91±0.02^{a}	0.93 ±0.02a,^{b}	0.88±0.05^{a}	0.92±0.09^{a,b}	1.70±1.02^{c}	0.85±0.09^{a}	0.90±0.18^{a}	0.90±0.07^{a}	0.92±0.05^{a}
Ethyl acetate	2519.63 ±1311.97^{b}	597.56 ±227.26^{a}	1114.56 ±1221.67^{a,b}	2780.59 ±975.51^{b}	1610.89 ±661.36^{a,b}	2226.28 ±1571.29^{b}	2711.80 ±401.46^{b}	2213.80 ±1939.72^{b}	2634.95 ±1230.12^{b}	1125.75 ±865.09^{a,b}	680.90 ±7.09^{a}	1158.68 ±289.86^{a,b}	853.79 ±520.74^{a}
Isobutyl acetate	7.92±4.95^{b}	1.34±0.85^{a}	1.69±1.40^{a}	5.60±2.40^{b}	3.67 ±0.56a,^{b}	4.03±2.37^{b}	5.80±1.85^{b}	nd	8.02±3.22^{b}	2.44±1.90^{a}	1.60±0.38^{a}	1.26±0.31^{a}	2.98±1.69^{a}
Ethyl butyrate	30.45 ±9.45^{b}	16.88 ±8.88^{a}	26.76 ±26.00^{b}	32.93 ±15.26^{b}	26.40 ±17.21^{b}	29.83 ±24.58^{b}	44.36 ±14.85^{b}	20.13 ±12.44^{a,b}	36.44 ±21.85^{b}	26.32 ±20.50^{b}	27.62 ±3.67^{b}	18.31 ±5.33^{a}	31.74 ±18.88^{b}
Ethyl-2-methylbutyrate	0.57±0.03^{b}	0.21±0.09^{b}	0.11±0.04^{a}	1.21±0.79^{a}	0.32±0.24^{b}	2.55±3.45^{c}	0.40±0.07^{b}	0.49±0.01^{b}	0.65±0.07^{b}	0.39±0.34^{b}	0.85±0.35^{b}	0.31±0.11^{b}	1.28±0.80^{b,c}
Ethyl isovalerate	1.86±0.38^{b}	0.70±0.44^{a,b}	0.37±0.06^{a}	1.46±0.18^{b}	0.81±0.26^{a,b}	nd	nd	nd	1.88±0.75^{b}	0.76±0.60a^{b}	0.84±0.13^{b}	nd	2.55±1.59^{b}
Butyl acetate	6.24±3.72a	nd	nd	nd	nd	nd	nd	nd	3.14±0.09a	nd	nd	nd	nd
Isopentyl acetate	1388.26 ±892.47^{b}	158.64 ±115.27^{a}	214.99 ±169.69^{a}	335.71 ±142.14^{a}	742.05 ±187.46^{a}	899.80 ±64.98^{a}	2087.91 ±332.52^{b}	159.56 ±158.85^{a}	1820.68 ±976.71^{b}	352.30 ±318.62^{a}	258.46 ±24.82^{a}	134.39 ±54.48^{a}	728.36 ±409.41^{a}
Ethyl valerate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl caproate	492.31 ±106.77^{a}	304.14 ±167.22^{a}	453.70 ±447.38^{a}	331.74 ±131.53^{a}	347.13 ±106.79^{a}	606.83 ±402.12^{a}	938.51 ±382.24^{a}	349.76 ±136.10^{a}	584.60 ±326.49^{a}	533.90 ±423.79^{a}	428.80 ±77.09^{a}	405.73 ±109.54^{a}	522.87 ±284.05^{a}
Hexyl acetate	45.49 ±16.62^{b}	26.19 ±19.15^{b}	28.81 ±17.47^{b}	14.07 ±4.19^{a}	30.67 ±20.06^{b}	79.39 ±29.87^{b}	112.61 ±63.39^{b}	7.48±1.78^{a}	80.10 ±43.15^{b}	60.78 ±51.31^{b}	37.46 ±6.47^{b}	22.85 ±5.79^{a,b}	53.00 ±27.59^{b}
Ethyl heptanoate	0.60±0.21^{a}	0.37±0.17^{a}	nd	0.55±0.13^{a}	0.44±0.29^{a}	0.63±0.30^{a}	0.78±0.18^{a}	2.19±2.04^{b}	0.47±0.14^{a}	0.65±0.52^{a}	0.36±0.08^{a}	nd	nd

(*continued on next page*)

Table 2 (*continued*)

Compound	HP	MC	MS	MP	LT	HU	HG	HPG	PK	SA	SC EC 1118	LT Octave	MP Flavia
Ethyl caprylate	1867.05 ±355.83^{b}	1078.49 ±555.45^{a,b}	1319.35 ±1065.73^{b}	1103.21 ±237.93^{a}	1120.19 ±82.14^{a}	2251.13 ±1457.57^{b}	2582.44 ±548.74^{b}	1003.18 ±767.29^{a,b}	2102.12 ±1329.31^{b}	2146.35 ±1386.25^{b}	1412.00 ±146.58^{b}	1530.72 ±452.54^{b}	1875.22 ±903.08^{b}
Ethyl leucate	1.38±0.35^{a,b}	0.96±0.54^{a}	1.18±0.72^{a}	1.61±0.63^{b}	1.22±0.82^{a,b}	1.35±0.94^{a,b}	1.94±0.07^{b}	2.06±0.38^{b}	2.30±1.03^{b}	1.58±1.06^{b}	1.43±0.32^{b}	1.82±0.38^{b}	3.97±1.86^{b}
Diethyl succinate	55.70 ±24.18^{b}	10.03 ±5.16^{a}	7.35±4.25^{a}	13.72 ±3.53^{a}	10.61 ±11.56^{a}	27.15 ±0.57^{b}	84.58 ±9.76^{b}	44.86 ±31.91^{b}	23.17 ±17.19^{b}	65.50 ±13.80^{b}	48.84 ±20.56^{b}	17.74 ±5.66^{b}	35.20 ±12.90^{b}
Ethyl caprate	1187.17 ±416.68^{b}	242.92 ±119.89^{a}	186.10 ±110.09^{a}	336.87 ±71.69^{a}	246.58 ±255.51^{a}	654.37 ±58.97^{b}	1831.49 ±213.89^{b}	969.41 ±607.80^{b}	554.45 ±400.89^{b}	1398.26 ±443.51^{b}	1016.17 ±356.94^{b}	440.85 ±137.69^{a,b}	819.20 ±311.27^{b}
Methyl salycilate	nd	nd	nd	nd	nd	nd	nd	nd	0.12±0.01^{a}	nd	0.16±0.01^{a}	nd	nd
Ethyl phenylacetate	1.24±0.35^{b}	0.41±0.14^{a}	0.48±0.44^{a,b}	3.47±0.34^{c}	0.81±0.40^{b}	0.64±0.47^{b}	1.55±1.4^{b}	2.22±1.02^{b}	1.49±0.57^{b}	0.54±0.33^{a,b}	1.46±0.28^{b}	0.49±0.07^{a,b}	1.80±0.79^{b}
Phenylethyl acetate	1033.19 ±818.03^{b}	56.72 ±29.97^{a}	60.71 ±30.70^{a}	406.11 ±120.96^{b}	344.95 ±315.^{78a},b	390.67 ±198.22^{b}	959.75 ±616.43^{b}	140.89 ±88.50^{a,b}	1364.99 ±307.35^{b}	85.37 ±58.66^{b}	109.20 ±18.49^{a,b}	40.34 ±10.43^{a}	360.01 ±133.85^{b}
Ethyl laurate	477.62 ±322.39^{b}	10.04 ±4.34^{a}	10.96±5.84^{a}	57.56 ±9.97^{b}	51.34 ±63.75^{b}	9.45±7.25^{a}	259.72 ±201.59^{b}	205.56 ±67.00^{b}	133.43 ±114.19^{b}	41.93 ±3.92^{b}	57.03 ±38.58^{b}	3.11±1.75^{a}	24.99 ±7.37^{a,b}
Ethyl cinnamate	0.13±0.04^{a}	0.20±0.14^{a}	0.66±1.04^{b}	0.45±0.59^{a}	0.17±0.05^{a}	0.18±0.18^{a}	0.19±0.08^{a}	0.10±0.03^{a}	0.05±0.01^{a}	0.12±0.02^{a}	0.22±0.17^{b}	0.08±0.02^{a}	0.08±0.04^{a}
∑Esters	9114.73 ±3283.73^{b}	2505.54 ±1207.61^{a}	3427.87 ±3082.24^{a}	5426.24 ±1527.82^{a}	4538.28 ±14.82^{a}	7184.27 ±3426.71^{a}	11,625.04 ±925.94^{b}	5125.87 ±594.44^{a}	9352.84 ±4608.91^{b}	5842.99 ±3582.31^{a}	4083.35 ±662.16^{a}	3777.04 ±955.22^{a}	5317.23 ±2623.25^{a}
Isobutanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-hexanol	53.69 ±76.96^{b}	95.88 ±21.45^{b}	104.84 ±63.68^{b}	62.68 ±16.63^{b}	113.92 ±138.30^{b}	98.51 ±110.08^{b}	72.65 ±79.29^{b}	44.13 ±7.34^{b}	21.92±5.81^{a}	89.62 ±26.28^{b}	109.95 ±7.57^{b}	166.26 ±25.24^{c}	115.90 ±48.82^{c}
trans-3-hexenol	3.93±4.07^{b}	4.98±1.53^{b}	6.44±4.84^{b}	8.08±1.71^{c}	5.61±5.78^{b}	5.46±4.32^{b}	6.65±3.92^{b}	7.26±1.38^{b}	2.22±0.90^{a}	6.37±2.79^{b}	5.31±0.49^{b}	7.82±1.30^{b}	6.49±3.24^{b}
cis-3-hexenol	8.06±9.33^{a,b}	12.27 ±3.68^{b}	14.74±9.77^{b}	17.87 ±3.26^{b}	12.68 ±13.18^{b}	12.94 ±12.43^{b}	14.85 ±10.92^{b}	16.37 ±3.78^{b}	4.33±1.26^{a}	13.68 ±5.82^{b}	14.08 ±1.59^{b}	21.77 ±4.29^{b}	17.00 ±7.81^{b}
Compound	**HP**	**MC**	**MS**	**MP**	**LT**	**HU**	**HG**	**HPG**	**PK**	**SA**	**SC EC 1118**	**LT Octave**	**MP Flavia**
Benzyl alcohol	0.90±0.10^{a}	0.63±0.25^{a}	1.41±1.78^{a,b}	1.91±0.83^{b}	0.93±0.60^{a}	1.06±0.90^{a}	1.13±0.13^{a}	1.25±0.46^{a,b}	0.92±0.22^{a}	0.88±0.44^{a}	0.77±0.15^{a}	0.92±0.19^{a}	1.08±0.54^{a}
2-phenylethanol	1144.09 ±224.39^{a}	684.22 ±190.55^{a}	951.72 ±805.94^{a}	1793.53 ±702.30^{b}	1009.10 ±641.68^{a}	1121.63 ±1008.47^{a}	1280.54 ±161.26^{a}	1509.42 ±557.44^{b}	1471.75 ±744.56^{ab}	946.35 ±569.17^{a}	915.50 ±78.88^{a}	955.74 ±149.03^{a}	1460.40 ±763.85^{ab}
∑ Higher alcohols	1210.66 ±314.27^{a}	797.97 ±217.38^{a}	1079.15 ±885.68^{a}	1884.06 ±722.75^{b}	1142.22 ±799.54^{a}	1239.60 ±1136.19^{a}	1375.83 ±204.89^{a}	1578.43 ±570.40^{a,b}	1501.13 ±752.53^{a,b}	1056.90 ±604.49^{a}	1045.62 ±87.63^{a}	1152.50 ±177.63^{a}	1600.86 ±824.23^{a,b}
Acetic acid	828.43 ±239.82^{b}	616.39 ±95.59^{b}	948.58 ±858.40^{b}	827.97 ±385.44^{b}	653.93 ±679.81^{b}	1334.11 ±1363.26^{c}	853.93 ±83.18^{b}	831.12 ±352.23^{b}	902.09 ±214.72^{b}	430.74 ±264.73^{a,b}	252.20 ±24.61^{a}	942.08 ±478.62^{b}	145.60 ±93.74^{b}
Isobutyric acid	29.89 ±21.81^{b}	4.60±2.21^{a}	5.30±4.25^{a}	10.79 ±1.32^{a}	10.13 ±4.14^{a}	6.88±4.89^{a}	16.66 ±16.87^{a}	9.50±1.37^{a}	20.22 ±7.05^{b}	5.13±3.11^{a}	5.49±1.43^{a}	4.11±1.35^{a}	8.09±5.38^{a}
Butyric acid	2.64±0.96^{a}	2.26±0.58^{a}	2.61±2.20^{a}	4.05±0.89^{b}	2.67±1.94^{a}	2.85±2.23^{a}	5.35±2.89^{b}	3.31±2.11^{a}	3.83±0.19^{a,b}	2.20±1.44^{a}	3.20±0.92^{a}	2.36±1.04a	3.22±2.19^{a}
Isovaleric acid	111.53 ±33.25^{b}	23.94 ±11.69^{a}	18.87 ±11.03^{a}	34.28 ±6.78a	24.22 ±24.88^{a}	65.45 ±7.62^{ab}	173.58 ±21.37^{b}	90.41 ±49.92^{b}	55.44 ±39.95^{ab}	131.59 ±50.02^{b}	94.91 ±29.79^{b}	44.61 ±13.74^{a}	80.76 ±31.70^{ab}
Valeric acid	0.22±0.05^{a}	0.30±0.16^{a}	1.38±2.08^{b}	1.73±2.42^{b}	0.31±0.14^{a}	0.34±0.29^{a}	0.45±0.09^{a}	0.41±0.04^{a}	0.37±0.28^{a}	0.31±0.08^{a}	0.37±0.19^{a}	0.29±0.08^{a}	0.49±0.32^{a}
Hexanoic acid	65.02 ±30.56^{a}	73.87 ±22.28^{a}	76.83 ±51.19^{a}	57.14 ±26.88^{a}	71.63 ±30.95^{a}	99.75 ±80.36^{a,b}	137.84 ±30.52^{b}	59.68±7.80^{a}	92.65 ±21.74^{a}	81.75 ±22.11^{a}	72.53 ±11.28^{a}	73.91 ±39.29^{a}	72.91 ±46.93^{a}
Decanoic acid	18.42±4.48^{a}	38.87 ±37.92^{a}	16.62 ±13.51^{a}	16.32 ±14.92^{a}	10.45 ±3.81^{a}	15.59 ±17.58^{a}	62.50 ±51.61^{b}	91.08 ±114.49^{b}	80.66 ±66.26^{b}	12.04 ±13.13^{a}	57.38 ±42.11^{a,b}	59.27 ±66.53^{a,b}	12.00 ±9.93^{a}
Nonanoic acid	2.99±1.20^{a}	5.46±3.17^{a}	8.66±11.67^{a,b}	10.72 ±4.22^{b}	3.72±2.08^{a}	10.79 ±11.84^{b}	7.11±6.41^{a}	3.71±3.70^{a}	6.88±5.17^{a}	2.37±2.14^{a}	6.26±3.47^{a}	5.37±2.79^{a}	6.24±5.90^{a}
Octanoic acid	359.714 ±166.08^{a,b}	395.847 ±133.14^{a,b}	342.93 ±249.12^{a}	436.41 ±318.88^{a,b}	255.07 ±64.20^{a}	555.10 ±542.89^{a,b}	623.75 ±202.35^{b}	354.98 ±241.06^{a,b}	347.03 ±57.01^{a}	349.26 ±369.18^{a}	440.57 ±141.85^{a,b}	401.73 ±255.77^{a,b}	191.55 ±161.98^{a}
∑ Volatile acids	1418.85 ±462.63^{a,b}	1161.54 ±247.77^{a}	1421.78 ±1168.34^{a,b}	1399.40 ±664.87^{a,b}	1032.12 ±571.89^{a}	2090.84 ±2030.95^{b}	1881.17 ±326.55^{b}	1444.21 ±672.87^{a,b}	1509.16 ±147.36^{b}	1015.39 ±725.94^{a}	932.91 ±207.02^{a}	1533.71 ±824.73^{a,b}	520.85 ±337.47^{a}
Guaiacol	0.65±0.41^{b}	0.03±0.01^{a}	0.07±0.07^{a}	0.15±0.04^{b}	nd	nd	0.39±0.26^{a,b}	0.33±0.07^{a}	0.30±0.04^{a}	0.08±0.02^{a}	0.10±0.06^{a}	nd	0.08±0.05^{a}
4-vinylguaiacol	0.07±0.04^{a}	0.06±0.05^{a}	nd	0.42±0.31^{a,b}	0.05±0.03^{a}	nd	1.86±0.22^{b}	1.33±1.67^{b}	0.58±0.17^{a,b}	0.31±0.16^{a,b}	1.55±0.38^{b}	0.14±0.02^{a,b}	0.61±0.41^{a,b}

(*continued on next page*)

Table 2 (*continued*)

Compound	HP	MC	MS	MP	LT	HU	HG	HPG	PK	SA	SC EC 1118	LT Octave	MP Flavia
4-ethylphenol	0.15±0.02^{a}	0.12±0.05^{a}	0.20±0.23^{b}	0.18±0.05^{a,b}	0.13±0.05^{a}	0.18±0.16^{a,b}	0.23±0.04^{b}	0.16±0.08^{a,b}	0.10±0.01^{a}	0.14±0.06^{a}	0.13±0.04^{a}	0.13±0.04^{a}	0.14±0.08^{a}
4-ethyl guaiacol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
∑ Volatile phenols	0.85±0.45^{a,b}	0.20±0.09^{a}	0.28±0.29^{a}	0.74±0.38^{a,b}	0.25±0.01^{a}	0.19±0.15^{a}	2.48±0.33^{b}	1.82±1.68^{b}	0.68±0.38^{b}	0.54±0.24^{a,b}	1.78±0.45^{b}	0.27±0.06^{a}	0.80±0.50^{a,b}
Acetaldehyde	84.92 ±17.99^{b}	45.13 ±9.52^{a}	59.93 ±41.34^{a}	92.71 ±50.05^{b}	58.87 ±38.18^{a}	106.71 ±79.67^{b}	203.97 ±70.62^{c}	260.65 ±52.64^{c}	130.49 ±78.91^{b}	88.52 ±66.73^{b}	59.05 ±7.04^{a}	115.84 ±28.75^{b,c}	72.36 ±42.31^{b}
Octanal	1.39±0.26^{a,b}	1.08±0.29^{a}	0.77±0.10^{a}	1.78±0.65^{b}	1.62±1.49^{a,b}	nd	1.92±0.33^{b}	nd	2.86±0.67^{c}	nd	1.46 ±0.12^{a,b}	1.34±0.65^{a,b}	1.80±0.92^{b}
Benzaldehyde	23.84±5.48^{a}	16.78 ±7.25^{a}	31.67 ±35.39^{b}	28.47 ±6.43^{a,b}	19.14 ±11.07^{a}	28.90 ±23.64^{a,b}	30.34 ±4.72^{b}	24.45 ±10.42^{a,b}	20.16±5.06^{a}	22.42 ±11.55^{a}	20.03 ±3.30^{a}	30.22 ±6.66^{b}	25.56 ±12.58^{a,b}
Phenylacetaldehyde	6.10±0.97^{a}	3.51±0.58^{a}	4.32±3.04^{a}	16.13 ±8.66^{b}	5.52±3.20^{a}	4.77±3.38^{a}	6.53±0.83^{a}	12.80 ±9.31^{a,b}	9.49±2.75^{a}	5.25±3.55^{a}	5.13±1.41^{a}	5.53±0.90^{a}	12.78 ±6.08^{a,b}
∑ Aldehydes	115.79 ±24.29^{b}	66.51 ±8.28^{a}	96.44 ±79.23^{a,b}	139.09 ±54.30^{b}	85.14 ±53.94^{a}	141.04 ±105.74^{b}	242.12 ±69.24^{b}	297.90 ±72.37^{b}	162.04 ±78.93^{b}	116.48 ±81.42^{a,b}	85.68 ±11.38^{a}	152.49 ±36.59^{b}	112.48 ±61.86^{a,b}
Compound	**HP**	**MC**	**MS**	**MP**	**LT**	**HU**	**HG**	**HPG**	**PK**	**SA**	**SC EC 1118**	**LT Octave**	**MP Flavia**
2-aminoacetophenone	0.12±0.07^{a}	0.36±0.34^{a}	1.65±2.73^{b}	1.26±2.03^{b}	0.27±0.12^{a}	0.74±0.98^{a,b}	0.28±0.18^{a}	0.09±0.01^{a}	0.04±0.01^{a}	0.12±0.01^{a}	0.71 ±0.66^{a,b}	0.05±0.03^{a}	0.05±0.03^{a}
∑ Ketones	0.12±0.07^{a}	0.36±0.34^{a}	1.65±2.73^{b}	1.26±2.03^{b}	0.27±0.12^{a}	0.74±0.98^{a,b}	0.28±0.18^{a}	0.09±0.01^{a}	0.04±0.01^{a}	0.12±0.01^{a}	0.71 ±0.66^{a,b}	0.05±0.03^{a}	0.05±0.03^{a}
trans-whiskey lactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
γ-octalactone	0.39±0.07^{a}	0.25±0.06^{a}	0.35±0.30^{a}	0.36±0.05^{a}	0.24±0.01^{a}	0.39±0.30^{a,b}	0.49±0.08^{a,b}	0.40±0.01^{a,b}	0.27±0.03^{a}	0.57±0.02^{b}	0.28±0.05^{a}	0.39±0.14^{a,b}	0.34±0.16^{a}
cis-whiskey lactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
γ-nonalactone	0.10±0.01^{a}	0.09±0.01^{a}	0.14±0.13^{a}	0.18±0.04^{b}	0.10±0.04^{a}	0.13±0.09^{a}	0.13±0.03^{a}	0.18±0.06^{b}	0.12±0.01^{a}	0.10±0.04^{a}	0.10±0.01^{a}	0.11±0.01^{a}	0.16±0.08^{a,b}
γ-dodecalactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methlactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
δ-decalactone	0.09±0.06^{a}	0.09±0.04^{a}	0.11±0.08^{a}	0.13±0.07^{a}	0.08±0.01^{a}	0.07±0.07^{a}	0.10±0.01^{a}	0.16±0.07^{a}	0.10±0.02^{a}	0.08±0.02^{a}	0.09±0.02^{a}	0.10±0.03^{a}	0.13±0.07^{a}
γ-decalactone	0.08±0.01^{a}	0.08±0.01^{a}	0.08±0.08^{a}	0.17±0.05^{b}	0.10±0.06^{a}	0.08±0.05^{a}	0.19±0.13^{b}	1.54±1.98b	0.14±0.05^{b}	0.08±0.05^{a}	0.09±0.01^{a}	0.35±0.08^{b}	0.10±0.05^{a,b}
∑ Lactones	0.66±0.13^{a}	0.50±0.12^{a}	0.68±0.59^{a}	0.84±0.17^{a,b}	0.52±0.12^{a}	0.67±0.51^{a}	0.91±0.04^{a,b}	2.27±2.09^{b}	0.61±0.10^{a}	0.82±0.13^{a}	0.54±0.12^{a}	0.96±0.25^{a,b}	0.73±0.34^{a}
2-sec-butyl-3-methoxypyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzylmercaptan	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methionol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzothiazole	0.40±0.09^{a}	0.48±0.24^{a}	0.65±0.65^{a}	0.75±0.24^{a}	0.51±0.21^{a}	0.54±0.47^{a}	0.59±0.17^{a}	0.34±0.05^{a}	0.46±0.07^{a}	0.37±0.07^{a}	0.55±0.24^{a}	0.42±0.09^{a}	0.51±0.27^{a}
∑ Others	0.40±0.09^{a}	0.48±0.24^{a}	0.65±0.65^{a}	0.75±0.24^{a}	0.51±0.21^{a}	0.54±0.47^{a}	0.59±0.17^{a}	0.34±0.05^{a}	0.46±0.07^{a}	0.37±0.07^{a}	0.55±0.24^{a}	0.42±0.09^{a}	0.51±0.27^{a}

Tentative identification based on mass spectral pattern (*μg/L equivalent of 2-octanol internal standard). The value of volatile compounds is expressed as mean ± standard deviation (n=3). Different letters in the column indicate a significant difference ($p < 0.05$). Abbreviations: HP –*Hyphopichia pseudoburtonii* N-11; MC- *Metschnikowia chrysoperlae* K-11; MS- *Metschnikowia sinensis/shanxiensis* P-7; MP- *Metschnikowia pulcherrima* K-6; LT- *Lachancea thermotolerans* P-25; HU- *Hanseniaspora uvarum* Z-7; HG- *Hanseniaspora guillermondii* N-29; HPG- *Hanseniaspora pseudoguilliermondii* V-13; PK- *Pichia kluyveri* Z-3; SA- *Starmerella apicola* VP-8; SC – *Saccharomyces cerevisiae* EC 1118; LT Octave- *Lachancea thermotolerans* Octave; MP Flavia- *Metschnikowia pulcherrima* Flavia, nd- not detected.

isolates did not produce it. *Metschnikowia* genera are recognized by extracellular α-l-arabinofuranosidase which can modulate the concentration of terpinen-4-ol in MS fermentations.

As the second group of compounds belonging to the varietal aroma, C_{13}-norisoprenoids were an important group for investigating yeast differentiation. HG, the second most abundant *Hanseniaspora* spp., have been shown to exhibit increased concentrations of total C_{13}-norisoprenoids which statistically differed from other isolates and non-*Saccharomyces* controls, especially from control fermentations with SC. Additionally, individual C_{13}-norisoprenoids, such as 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), vitispirane, and β-damascenone, resulted in the highest concentration in HG wines. These results outlined this yeast as a good producer of C_{13}-norisoprenoids in monoculture fermentations which previously was pointed out as a good producer of ethyl esters, particularly isopentyl acetate and hexyl acetate (Moreira et al., 2008). Although the breakdown of carotenoids was previously associated exclusively with chemical, photochemical, and oxidase-coupled mechanisms, some studies supported the hypothesis of the involvement of a region-specific oxygenase in the formation of C_{13}-norisoprenoids (Lashbrooke et al., 2013). β-damascenone stands out particularly due to the highest OAV (23.62) which directly contributes to the wine aroma with a reminiscent note of honey (Supplementary Table 2).

Furfurylthiol was identified as the only thiol by applying the HS-SPME-GC-MS method described by Škrab et al. (2021). Previously, PK was reported as a good thiol producer (Vicente et al., 2021), which aligned with a significantly higher concentration of thiol (1.70 μg/L) produced by PK isolate compared to all used yeasts and control fermentation conducted with SC.

Quantitatively, esters were the most abundant group of secondary aroma compounds followed by acids and alcohols. The total concentrations of esters in wines produced by HG (11,625.04 μg/L), PK (9352.84 μg/L), and HP (9114.73 μg/L) differed significantly from those produced by SC (4083.35 μg/L), MP Flavia (5317.23 μg/L), and LT Octave (3777.04 μg/L) in single fermentations. Non-*Saccharomyces* yeast can produce more esters than *S. cerevisiae* whose synthesis and interconversion are based on enzymatic reactions. Among the significant compounds inside the ester profile, MP exhibited the highest concentration of ethyl acetate (2780.59 μg/L) compared to MP Flavia (853.79 μg/L) and SC (680.90 μg/L). Acetate concentrations may decrease by 30 % when non-*Saccharomyces* are not inoculated (Benito et al., 2015; Dutraive et al., 2019). A high concentration of ethyl acetate may induce negative effects on the quality of the wine when it exceeds 12,000 μg/L, as commonly observed for MP (Varela et al., 2016; Morata et al., 2019). Usually, acetate esters are produced by the esterification of ethanol and acyl-CoA intermediates (Wang et al., 2023). HU produced the highest concentration of ethyl-2-methylbutyrate (2.55 μg/L) which statistically differed from other isolates and all controls. One of the major characteristics of *H. uvarum* is its ability to increase the concentration of esters (Moreira et al., 2008, 2011). Furthermore, HG wines resulted in the highest concentration of isopentyl acetate (208.91 μg/L), ethyl butyrate (44.36 μg/L), and ethyl caproate (938.51 μg/L) which were quantified above ODT (Supplementary Table 2, Table 2). These compounds, at their present concentrations, contributed to fruity aromas such as apple and banana. Methyl salicylate is commonly detected in red wines (Poitou et al., 2021) and is found in bounded form. PK was the only native yeast, along with commercial SC which released methyl salicylate in the concentration of 0.12 μg/L. Also, HP excelled in the production of butyl acetate (6.24 μg/L), alongside PK as the only producer. The next significant compound among native and control SC yeasts was ethyl heptanoate produced in the highest concentration by HPG, while commercial non-*Saccharomyces* strains did not produce this compound, characterized by fruity notes.

Increasing concentration of higher alcohols in wine derived from the decomposition of amino acids and produced by yeasts through their metabolism (Hazelwood et al., 2008). The biggest impact on the higher alcohol profile in Maraština wines was observed for MP isolate. This yeast increased the concentration of total higher alcohol and individual ones, including *trans*-3-hexanol, *cis*-3-hexanol, benzyl alcohol, and 2-phenylethanol but without directly contributing to the aroma profile of wines. Likewise, another study showed that MP produced a higher content of higher alcohols (Zhang et al., 2022) mainly through the Erlich pathway. In general, higher alcohols display the most variable results, likely due to the significant differences observed in higher alcohol metabolism among *M. pulcherrima* strains (Vicente et al., 2020), resulting in the absence of a clear trend.

Volatile acids in wine primarily originate from two sources: the grape variety and yeast metabolism during fermentation. In terms of total volatile acids content, HU (2090.84 μg/L), HG (1881.17 μg/L) and PK (1509.16 μg/L) exhibited statistically significant differences from SC, with higher concentrations. The biggest impact was evident for HG affecting significant concentrations of butyric, isovaleric, hexanoic, and octanoic acid, while HU affected the concentration of acetic acid which was the highest one and statistically differed from the three controls. Andorra et al. (2010) reported an increase in the synthesis of ethyl acetate and acetic acid in pure fermentation with HU.

HG isolate had a similar effect on the volatile phenols profile as for volatile acids, resulting in the highest and statistically different concentrations of 4-vinylguaiacol and 4-ethylphenol compared to all control fermentations, while HU did not produce guaiacol, 4-vinylguaiacol, and 4-ethylguaiacol. These compounds are mainly produced through phenylpropanol and flavonoids pathways with phenylalanine ammonia lyase as a key enzyme (He et al., 2023). Accordingly, it is important to note that MC, MS, LT, and HU produced the smallest concentrations of total volatile phenols differed from SC.

Among miscellaneous compounds, the highest concentration of phenylacetaldehyde (16.13 μg/L) was detected in MP wines with an OAV of 4 and a reminiscent aroma of honey (Supplementary Table 2), statistically differing from SC and resembling that of commercial MP Flavia. HPG, with an acetaldehyde concentration of 260.65 μg/L, was the isolate with the highest concentration, statistically different from SC. The amount of acetaldehyde in wine can increase over time due to the activity of the yeast that forms film on the surface of the must (Fleet and Heard, 1993), which was the case in the fermentation with the HPG isolate. SA isolate exhibited statistically higher concentrations of γ-octalactone compared to the control SC strain.

3.2. Sequential fermentation of Maraština juice using non-Saccharomyces yeasts and Saccharomyces cerevisiae

3.2.1. Fermentation progress and primary metabolite production

The seven yeast isolates for sequential fermentations were chosen based on their superior oenological properties and enzymatic activity (Milanović et al., 2023). The strain-specific response of MC, MS, MP, LT, HU, HG, and PK yeast isolates in competition with *S. cerevisiae* was investigated in Maraština sterile must. The impact on the wine composition was assessed at the end of sequential fermentation, and results were compared to those obtained from sequential fermentation with *M. pulcherrima* Flavia (MP Flavia-SC), *L. thermotolerans* Octave (LT Octave-SC) and *S. cerevisiae* (SC) in monoculture fermentation.

To elucidate the interaction between selected non-*Saccharomyces* yeasts and SC in sequential fermentation, the growth kinetics were monitored by viable cell counting on YPD and Lysin agar (Fig. 2). SC was inoculated to achieve a final concentration of approximately 5×10^6 cells/mL (6.7 log CFU/mL) when fermentation reached 2–3% *v/v* of ethanol (Fig. 2). Due to different metabolisms and different resistance to demanding fermentation conditions, yeast isolates exhibited distinct fermentation kinetics. Among seven indigenous yeasts, MP-SC, HG-SC, and LT-SC demonstrated the fastest fermentation rates, completing fermentation in 16 days, the same as MP Flavia and LT Octave in sequential fermentations. MC-SC took the longest time, lasting for 24 days. It is evident from Fig. 2 that SC demonstrated a killer effect on

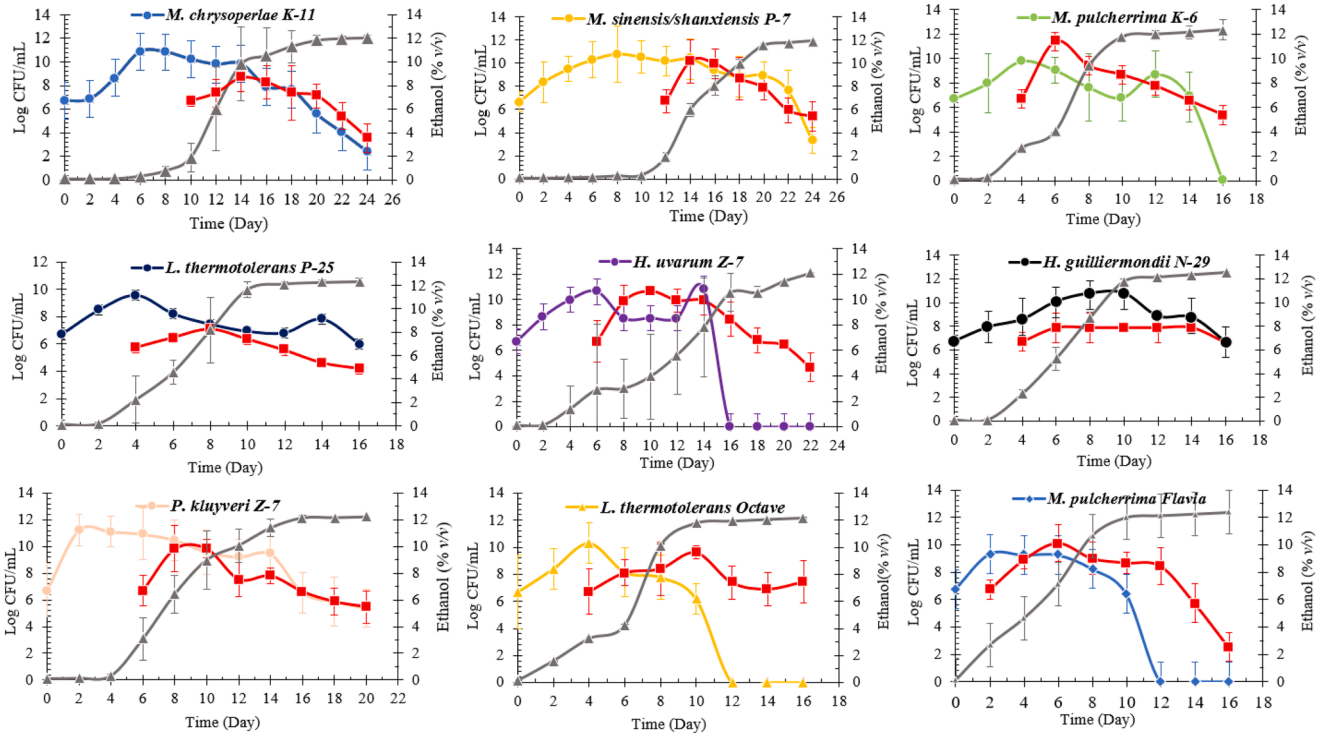

Fig. 2. Growth kinetics and ethanol production (—▲—) of seven selected non-*Saccharomyces* isolates, and two control strains, *L. thermotolerans* Octave and *M. pulcherrima* Flavia, during sequential fermentation with *S. cerevisiae* (—■—).

specific yeasts, as reported in earlier studies (Pérez-Nevado et al., 2006; Wang et al., 2016). *S. cerevisiae* secretes antimicrobial peptides that affect the intracellular space, pH, membrane permeability, and the abundance of other yeasts (Branco et al., 2014, 2015). In sequential fermentations performed with MP and HU, only SC cells were viable at the end, consistent with a previous study conducted on synthetic must by Wang et al. (2016) and Bagheri et al. (2018). Interestingly, even commercial yeasts MP Flavia and LT Octave serving as controls in interaction with SC were undetectable in fermentation after 12 days. HG and LT exhibited good competition, showing higher concentrations of

Table 3
Physicochemical parameter of Maraština wines produced by seven indigenous non-*Saccharomyces* yeasts and two controls strains (*L. thermotolerans* Octave, and *M. pulcherrima* Flavia) in sequential fermentations with *S. cerevisiae* EC 1118, and by monoculture fermentation with *S. cerevisiae* EC 1118.

Parameter	MC-SC	MS-SC	MP-SC	LT-SC	HU-SC	HG-SC	PK-SC	SC EC 1118	LT Octave-SC	MP Flavia-SC
Relative density (20/20°C)	0.9937 ±0.00^{b}	0.9928 ±0.00^{a,b}	0.9918 ±0.00^{a}	0.9918 ±0.00^{a}	0.9928 ±0.00^{a,b}	0.9918 ±0.00^{a}	0.9928 ±0.00^{a,b}	0.9918 ±0.00^{a}	0.9928 ±0.00^{a,b}	0.9918 ±0.00^{a}
Ethanol (% *v/v*)	12.03 ±0.16^{a,b}	11.91 ±0.05^{a}	12.35 ±0.12^{b}	12.32 ±0.34^{b}	12.14 ±0.12^{b}	12.51 ±0.07^{b}	12.21 ±0.12^{b}	12.44 ±0.10^{b}	12.15 ±0.08^{b}	12.08 ±0.03^{a,b}
Total dry extract (g/L)	25.03 ±1.91^{b}	23.03 ±0.85^{b}	21.63 ±0.61^{a}	20.87 ±0.55^{a}	22.50 ±1.31^{b}	20.53 ±0.12^{a}	23.53 ±0.40^{b}	21.93 ±0.45$^{a,\ b}$	22.40 ±0.17^{b}	20.97 ±0.21^{a}
Reducing sugars (g/L)	5.8±1.94^{b}	3.6±0.70^{b}	2.8±0.61^{a}	3.1±0.15$^{a,\ b}$	3.7±0.52^{b}	2.8±0.21^{a}	4.4±0.15b	2.7±0.20^{a}	3.0±0.17^{a,b}	2.1±0.15^{a}
Glucose (g/L)	0.73±0.15^{b}	0.77±0.12^{b}	0.37 ±0.06^{a}	0.63 ±0.32^{b}	0.50±0.17$^{a,\ b}$	0.33 ±0.06^{a}	0.50±0.10$^{a,\ b}$	0.43±0.06^{a}	0.37±0.12^{a}	0.37±0.06^{a}
Fructose (g/L)	5.07±1.89^{b}	2.93±0.61$^{a,\ b}$	2.47 ±0.55^{a,b}	2.57 ±0.06^{a,b}	3.23±0.29$^{a,\ b}$	2.53 ±0.15^{a,b}	3.87±0.15$^{a,\ b}$	2.30±0.17^{a}	2.67±0.12$^{a,\ b}$	1.83±0.06^{a}
Total acidity (g/L)*	6.99 ±0.03^{a,b}	7.10±0.12^{b}	6.68 ±0.09^{a}	6.57 ±0.13^{a}	6.74±0.33$^{a,\ b}$	6.56 ±0.12^{a}	6.92±0.06$^{a,\ b}$	6.87±0.16$^{a,\ b}$	7.15±0.13^{b}	6.44±0.03^{a}
Volatile acidity (g/L)**	0.77±0.08^{b}	0.87±0.05^{b}	0.42 ±0.03^{a}	0.40 ±0.05^{a}	0.81±0.28^{b}	0.43 ±0.05^{a}	0.50±0.01^{a}	0.41±0.05^{a}	0.50±0.03$^{a,\ b}$	0.45±0.03^{a}
pH	3.21±0.03^{a}	3.24±0.02^{a}	3.27 ±0.03^{a}	3.26 ±0.03^{a}	3.33±0.01^{b}	3.28 ±0.03^{a,b}	3.23±0.01^{a}	3.25±0.02^{a}	3.23±0.02^{a}	3.29 ±0.01^{a,b}
Malic acid (g/L)	0.35±0.07^{a}	0.40±0.04^{a}	0.42 ±0.03^{a}	0.28 ±0.12^{a}	0.40±0.01^{a}	0.38 ±0.02^{a}	0.43±0.03^{a}	0.47±0.08$^{a,\ b}$	0.50±0.01^{b}	0.48 ±0.03^{a,b}
Lactic acid (g/L)	0.21±0.03^{b}	0.23±0.01^{b}	0.19 ±0.02^{a,b}	0.25 ±0.09^{b}	0.23±0.01^{b}	0.15 ±0.02^{a}	0.13±0.03^{a}	0.13±0.02^{a}	0.20±0.01^{b}	0.13±0.02^{a}
Glycerol (g/L)	6.53±0.06^{b}	6.63±0.06^{b}	6.00 ±0.20^{a,b}	5.97 ±0.06^{a}	6.53±0.31^{b}	5.93 ±0.12^{a}	6.30±0.10b	6.40±0.17^{b}	6.30±0.17^{b}	6.67±0.12^{b}

Data are representative mean ± standard deviation of three biological replications. Different letters in the column indicate a significant difference ($p < 0.05$). Abbreviations: MC-SC – *M. chrysoperlae/S. cerevisiae*; MS-SC – *M. sinensis/shanxiensis/ S. cerevisiae;* MP-SC – *M. pulcherrima/ S. cerevisiae*; LT-SC – *L. thermotolerans/S. cerevisiae;* HU – *H. uvarum/ S. cerevisiae;* HG-SC – *H. guilliermondii/S. cerevisiae*; PK-SC – *P. kluyveri/ S. cerevisiae;* SC EC 1118- *S. cerevisiae;* MP Flavia-SC – *M. pulcherrima* Flavia/ *S. cerevisiae;* LT Octave-SC – *L. thermotolerans* Octave/ *S. cerevisiae;*

* - expressed as acetic acid;

** - expressed as tartaric acid.

viable cells at the end compared to SC, and reaching 12.32% and 12.51% *v/v* of ethanol, respectively. Great competition of LT with SC was reported by Bagheir et al. (2018) in synthetic must. In contrast, Moreira et al. (2008) reported that HG yeast showed viability till 9 % *v/v* of ethanol. Notably, all indigenous yeasts had better biomass evolution than SC, except native MP, which had similar growth kinetic as commercial MP Flavia strain in the first 10 days. The possible reason for better biomass evolution is the greater utilization of nutrients in the beginning (Andorra et al., 2010).

Table 3 represents the physicochemical characteristics of Maraština wines produced in sequential fermentation with selected indigenous isolates. In this study, MS-SC fermentation resulted in the lowest ethanol concentration (11.91 % *v/v*), while HG-SC produced the highest concentration (12.51 % *v/v*) (Fig. 2). Furthermore, only the MS-SC and PK-SC fermentations had 5.8 g/L and 4.4 g/L of residual sugars, respectively, while others resulted in Maraština wines being dry (< 4.0 g/L), with a preference for glucose over fructose (Supplementary Figure 2). The concentration of volatile acidity in MS-SC (0.87 g/L), HU-SC (0.81 g/L), and MC-SC (0.77 g/L) was statistically higher compared to that in other wines. The rest of the indigenous yeast in co-interaction with SC produced from 0.40 g/L to 0.50 g/L, similar to control sequential fermentations and SC in monoculture (0.41 g/L). This decrease could be attributed to yeast–yeast interaction or acetic acid co-metabolism, as Dos Santos et al. (2003) described. Additionally, fermentations with indigenous isolates showed better degradation of L-malic acid compared to controls SC, LT Octave-SC, and MP Flavia-SC. According to these results, the production of lactic acid was higher in inoculum with indigenous yeasts compared to SC and MP Flavia-SC. As excepted based on literature data (Benito et al., 2016; Hranilović et al., 2020), LT in fermentation with SC produced the highest concentration of lactic acid (0.23 g/L), even higher than the concentration produced by commercial LT Octave (0.20 g/L). This is due to its unique ability to produce lactic acid from sugar metabolism during alcoholic fermentation (Hranilović et al., 2020). Glycerol, as a desirable compound, was present in the concentration range from 5.97 to 6.67 g/L, where LT-SC and HG-SC produced significantly lower concentrations compared to others. Otherwise, the presence of competing microorganisms can affect glycerol production, with higher levels observed when the cellular concentration of *S. cerevisiae* is reduced (Comitini et al., 2011).

The chromatic characteristics of wine obtained from sequential fermentation did not show any statistical differences among yeast isolates or the control fermentation (Supplementary Table 3). However, the concentrations of total polyphenols varied among the different fermentations, ranging from 0.33 to 0.39 g/L, which were statistically lower than SC fermentation (0.55 g/L), except for MP-SC and LT-SC (Supplementary Table 3). As in monoculture fermentation, MP in sequential fermentation with SC exhibited a total polyphenol concentration comparable to that of the control fermentation with SC.

3.2.2. Volatile compound analysis by HS-SPME-GC-MS

In the process of sequential fermentation, alterations in non-*Saccharomyces* yeasts and *S. cerevisiae* often occur due to the inhibition and interactions of metabolites, interactions of gene expressions, and enzymatic activity (Wang et al., 2023). The volatile profile is reported in Table 4, while the OAVs for compounds which exhibited odour thresholds are presented in Supplementary Table 4.

The highest concentration of total terpenic compounds was produced by MP-SC fermentation, probably due to the good enzymatic activity of this indigenous isolate previously reported by Milanović et al. (2023). Terminal arabinoses are lysed by arabinosidase which releases the *β*-D-glycoside. Thereafter, terpenes are released by *β*-glucosidase (Wang et al., 2023). *M. pulcherrima* yeast tend to have higher glucosidase activity than control SC yeast as previously reported by Morata et al. (2019). Conversely, notable differences were observed in the production of *cis*-rose oxide (0.36 μg/L) and α-terpineol (1.41 μg/L) in MC-SC ferments compared to the monoculture fermentation with SC. Additionally, MC-SC was the only indigenous yeast that produced *cis*-rose oxide. HG-SC fermentations produced higher concentrations of α-terpineol (1.46 μg/L) and geraniol (0.74 μg/L) and statistically differed from SC pure fermentation.

The C_{13}-norisoprenoids profile of the PK-SC fermentation trial differed significantly from the all controls and yielded a significant concentration of vitispirane. Benito et al. (2015) indicated that employing sequential fermentation with *P. kluyveri* led to approximately a 30% improvement in overall perception compared to the *S. cerevisiae* control. This enhanced aromatic profile was attributed to a more pronounced fruity essence. Such flavours were likely associated with an elevation in thiol emission during the alcoholic fermentation process. MC-SC produced the highest concentration of TDN and differed from all used yeasts, thus demonstrating for the first time the effect of MC strain.

The effect of SC in sequential fermentation was evident due to the reduced variability and fewer significant differences between wines. However, the differing ester profile can mostly be attributed to the esterase activity of the PK isolate, as previously reported by Milanović et al. (2023). PK yeast in interaction with SC exhibited the highest concentration of ethyl acetate, isobutyl acetate, isopentyl acetate, ethyl caproate, hexyl acetate, ethyl caprylate, and phenylethyl acetate which differed from the Maraština wines produced by SC yeast, imbuing wines with a floral-rose note (Supplementary Table 4). Interestingly to note, only yeasts from *Hanseniaspora* genera in combination with SC produced ethyl heptanoate. Rojas et al. (2001) reported that ATF1 gene copy numbers of HU were relatively high and enhanced the activity of the alcohol acetyltransferase enzyme. The expression of this enzyme is crucial in determining the level of esters in fermentation. Conversely, LT-SC produced the lowest concentration of total esters, similar to fermentation led by commercial SC and LT Octave.

The content of higher alcohols was significantly affected by HG-SC fermentations through the production of *cis*-3-hexenol and by PK-SC fermentations which produced 2-phenyethanol in the highest concentration, statistically different in comparison with control SC fermentation. The 2-phenylethanol positively contributed to the wine aroma, imparting honey notes. Otherwise, HG-SC sequential fermentation has previously shown an increase in higher alcohols and a reduction of ethyl esters compared to SC fermentations (Barbosa et al., 2015), which aligned with our results. *H. guilliermondii* prefers amino acids as nitrogen sources, which, upon assimilation, are channelled into the production of higher alcohols and acetate esters (Seixas et al., 2023). The ability to produce a higher alcohol was strictly dependent on the yeast strain (Vicente et al., 2020) whose activity of the two anabolic synthesis pathways is affected by carbon and nitrogen sources fermentation medium (Jiang et al., 2019).

Volatile acids such as acetic acid, butyric acid, and isobutyric acid in fermentation with all indigenous isolates significantly differ from SC pure fermentations with higher concentrations. In comparison between the commercial LT strain and the indigenous one, the latter strain demonstrated a lower concentration of the mentioned acids. Additionally, MC-SC fermentations exhibited the highest concentrations of acetic acids, while there were no significant differences in total acid concentration between fermentations obtained with indigenous yeast and control trials.

Encouragingly, there was significantly lower production of volatile phenols in MS-SC fermentations compared to control fermentation with SC, as well as in other sequential fermentations, using indigenous or commercial strains. Only the HG-SC fermentations did not produce 4-vinylguaiacol, resulting in the lowest concentration of volatile phenols (0.35 μg/L). Hayasaka et al. (2010) reported a significant impact of non-*Saccharomyces* yeast on the content and types of volatile phenols due to *β*-glucosidase which can affect the structure of phenolic substance in wine through hydrolysis and destruction of bonds. In our study, the opposite was observed, so we can conclude that volatile phenols were produced through metabolic pathways of phenyl-propanol and flavonoids (He et al., 2023).

Table 4
Concentration (μg/L)* of volatile compounds in Maraština wines produced for seven indigenous non-*Saccharomyces* yeast isolates and two commercial yeasts: *L. thermotolerans* Octave and *M. pulcherrima* Flavia in sequential fermentation with *S. cerevisiae,* and in pure fermentation with *S. cerevisiae*.

Compound	MC-SC	MS-SC	MP-SC	LT-SC	HU-SC	HG-SC	PK-SC	SC EC 1118	LT Octave-SC	MP Flavia-SC
1,4-cineole	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1,8-cineole	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis-rose oxide	nd	nd	nd	nd	nd	nd	nd	0.02±0.00^{a}	nd	nd
trans-rose oxide	0.18±0.11	0.13 ±0.04	0.24±0.04	0.17±0.02	0.16±0.06	0.19±0.06	0.24±0.07	0.16±0.03	nd	0.21±0.10
trans-linalool oxide	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis-linalool oxide	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Linalool	3.30±1.31^{a}	3.91 ±0.24^{a}	4.30 ±0.79^{a}	4.27 ±1.13^{a}	3.97 ±1.01^{a}	3.44 ±0.34^{a}	4.74 ±0.24^{a}	3.40±0.17^{a}	5.78±1.09^{b}	4.08 ±0.97^{a}
Terpinen-4-ol	0.14±0.12^{a}	nd	nd	nd	nd	nd	nd	0.25±0.09^{a}	nd	nd
Safranal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
α-terpineol	1.41±0.86^{b}	0.99 ±0.18^{a,b}	1.42 ±0.34^{b}	1.43 ±0.24^{b}	0.99 ±0.25^{b}	1.46 ±0.30^{b}	1.26 ±0.56^{b}	0.79±0.13^{a}	1.53±0.33^{b}	1.34 ±0.46^{b}
β-citronellol	1.27±0.83^{b}	1.46 ±0.23^{b}	1.59 ±0.16^{b}	1.30 ±0.24^{b}	1.62 ±0.41^{b}	1.80 ±0.55^{b}	1.05 ±0.25^{b}	1.55±0.23^{b}	0.71±0.15^{a}	1.18 ±0.19^{b}
Nerol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Geraniol	0.32±0.09^{a}	0.23 ±0.07^{a}	0.34 ±0.07^{a}	0.42 ±0.14^{a}	0.26 ±0.06^{a}	0.74 ±0.23^{a}	0.45 ±0.18^{a}	0.38±0.09^{a}	0.63±0.15^{b}	0.33 ±0.12^{a}
Eugenol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
trans-terpin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
β-ionone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
∑Terpenic compounds	6.15±2.18^{a}	6.70 ±0.34^{a}	7.97 ±1.11^{a}	7.40 ±1.46^{a}	7.07 ±1.20^{a}	7.38 ±1.46^{a}	7.68 ±0.33^{a}	6.55±0.51^{a}	8.77±1.64^{a}	7.18 ±1.68^{a}
TDN	3.28±4.67^{b}	0.74 ±0.18^{a}	0.67 ±0.21^{a}	0.78 ±0.22^{a}	0.53 ±0.13^{a}	0.405 ±0.08^{a}	0.91 ±0.20^{a}	0.48±0.06^{a}	0.67±0.15^{a}	0.62 ±0.34^{a}
Vitispirane	2.46±1.71^{a}	2.37 ±0.42^{a}	4.19 ±1.50^{a}	3.62 ±1.66^{a}	3.80 ±1.54^{a}	2.87 ±1.297^{a}	6.94 ±1.96^{b}	2.94±0.87^{a}	3.53±1.40^{a}	3.25 ±1.65^{a}
β-damascone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
β-damascenone	0.70±0.28	0.77 ±0.03	0.55±0.06	0.69±0.21	0.71±0.20	0.62±0.08	0.76±0.03	0.71±0.05	0.71±0.09	0.55±0.11
∑C_{13}-norisoprenoids	6.44 ±6.40^{a,b}	3.88 ±0.24^{a}	5.41 ±1.77^{a}	5.09 ±1.46^{a}	5.04 ±1.82^{a}	3.89 ±1.33^{a}	8.61 ±1.77^{b}	4.13±0.79^{a}	4.91±1.58^{a}	4.41 ±2.01^{a}
Furfurylthiol	1.06±0.11^{b}	1.04 ±0.03^{b}	0.99 ±0.13^{b}	1.12 ±0.20^{b}	0.85 ±0.05^{a}	1.03 ±0.23^{b}	1.11 ±0.15^{b}	0.90±0.18^{a,b}	0.83±0.03^{a}	0.91 ±0.08^{a,b}
∑Thiol	1.06±0.12^{b}	1.04 ±0.03^{b}	0.99 ±0.14^{b}	1.12 ±0.20^{b}	0.85 ±0.05^{a}	1.03 ±0.23^{b}	1.11 ±0.15^{b}	0.90±0.18^{a,b}	0.83±0.03^{a}	0.91 ±0.08^{a,b}
Ethyl acetate	2635.38 ±1773.79^{a,b}	2189.43 ±48.23^{a,b}	1389.25 ±371.54^{a}	895.69 ±519.20^{a}	1379.04 ±687.51^{a}	10,723.00 ±438.54^{a}	3957.99 ±289.33^{b}	680.90 ±7.09^{a}	1293.04 ±584.51^{a}	1161.09 ±640.34^{a}
Isobutyl acetate	9.74±6.78^{b}	8.82 ±0.53^{b}	4.99 ±1.04^{a}	2.65 ±2.00^{a}	7.15 ±5.69^{a}	2.41 ±0.52^{a}	13.62 ±1.47^{b}	1.60±0.38^{a}	2.80±1.07^{a}	5.01 ±3.06^{a}
Ethyl butyrate	32.46 ±27.49^{b}	18.18 ±1.22^{a}	47.65 ±12.36^{b}	26.37 ±20.06^{b}	19.20 ±6.01^{a}	30.55 ±6.81^{b}	60.89 ±7.82^{b}	27.62 ±3.67^{a,b}	34.76 ±16.14^{b}	35.78 ±19.89^{b}
Ethyl-2-methylbutyrate	2.63±2.50^{b}	1.78 ±0.42^{b}	1.10 ±0.43^{b}	0.99 ±0.18^{a,b}	0.85 ±0.75^{a,b}	0.42 ±0.06^{a}	1.44 ±0.28^{b}	0.85±0.35^{a,b}	1.03±0.45^{b}	1.02 ±0.63^{b}
Ethyl isovalerate	4.48±4.85^{b}	2.31 ±0.60^{a}	2.71 ±1.29^{a}	1.74 ±1.17^{a}	1.904 ±1.59^{a}	1.09 ±0.15^{a}	3.31 ±0.55^{a,b}	0.84±0.13^{a}	2.34±1.20^{a}	2.41 ±1.38^{a}
Butyl acetate	5.04±0.36^{b}	3.81 ±0.16^{a}	nd	nd	4.70 ±2.44^{b}	nd	nd	nd	nd	nd
Isopentyl acetate	1782.91 ±1062.75^{b}	1623.28 ±52.13^{b}	788.30 ±175.19^{a,b}	506.08 ±455.86^{a}	1279.88 ±1035.88^{b}	344.17 ±201.51^{a}	2097.48 ±1280.26^{b}	258.46 ±24.82^{a}	360.00 ±162.36^{a}	833.93 ±483.41^{a,b}
Ethyl valerate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl caproate	443.29 ±382.46^{a}	218.55 ±1.38^{a}	676.10 ±228.55^{a}	361.15 ±288.46^{a}	285.72 ±124.62^{a}	504.02 ±210.37^{a}	1088.10 ±45.34^{b}	428.80 ±77.09^{a}	465.47 ±215.63^{a}	571.91 ±311.94^{a}
Hexyl acetate	70.03 ±41.69^{a}	52.16 ±1.15^{a}	74.31 ±19.72^{a}	59.76 ±52.44^{a}	77.29 ±32.14^{a}	61.56 ±24.57^{a}	194.55 ±36.05^{b}	37.46 ±6.47^{a}	43.69 ±20.17^{a}	74.91 ±40.90^{a}
Ethyl heptanoate	nd	nd	nd	nd	0.40±0.07	0.91±0.51	nd	0.36±0.08	nd	nd
Ethyl caprylate	1274.17 ±986.13^{a}	577.67 ±34.63^{a}	2051.04 ±650.96^{a}	1232.88 ±992.74^{a}	972.80 ±446.75^{a}	1461.97 ±283.14^{a}	3086.33 ±312.95^{b}	1412.00 ±146.58^{a}	1858.40 ±856.41^{a}	2125.85 ±1081.14^{a}
Ethyl leucate	2.33±1.71^{b}	2.21 ±0.10^{b}	2.32 ±0.66^{b}	2.77 ±1.20^{b}	1.45 ±0.57^{a}	1.92 ±0.38^{a,b}	2.67 ±0.34^{b}	1.43±0.32^{a}	2.66±0.68^{b}	3.27 ±1.27^{b}
Diethyl succinate	11.97 ±7.09^{a}	3.18 ±0.92^{a}	14.32 ±9.12^{a}	12.87 ±14.29^{a}	9.09 ±6.52^{a}	6.96 ±0.57^{a}	33.41 ±1.70^{b}	48.84 ±20.56^{b}	24.38 ±16.47^{a}	22.62 ±12.91^{a}
Ethyl caprate	287.30 ±161.46^{b}	81.63 ±23.04^{a}	353.34 ±213.92^{b}	314.88 ±335.08^{b}	225.50 ±156.13^{b}	176.68 ±14.15^{b}	800.12 ±40.15^{b}	1016.17 ±356.94^{c}	588.11 ±390.26^{b}	546.39 ±309.18^{b}
Methyl salycilate	nd	0.12 ±0.04^{a}	nd	nd	nd	nd	nd	0.16±0.01^{a}	nd	nd
Ethyl phenylacetate	3.18±2.58^{b}	3.82 ±0.12^{b}	1.46 ±0.36^{b}	1.32 ±0.32^{a,b}	2.72 ±1.76^{b}	0.86 ±0.14^{b}	1.71 ±0.01^{b}	1.46±0.28^{b}	1.16±0.34^{a}	1.52 ±0.66^{b}
Phenylethyl acetate	1391.56 ±812.87^{b}	1499.63 ±18.89^{b}	272.73 ±30.04^{a}	261.86 ±122.84^{a}	954.95 ±794.21^{b}	198.37 ±54.95^{a}	1851.15 ±157.54^{b}	109.20 ±18.49^{a}	74.71 ±18.90^{a}	279.89 ±139.04^{a}

(*continued on next page*)

Table 4 (*continued*)

Compound	MC-SC	MS-SC	MP-SC	LT-SC	HU-SC	HG-SC	PK-SC	SC EC 1118	LT Octave-SC	MP Flavia-SC
Ethyl laurate	79.82 ±125.51[b]	2.551 ±1.74[a]	8.853 ±5.60[a]	7.421 ±0.55[a]	7.268 ±2.54[a]	12.906 ±5.54[a]	95.593 ±37.81[b]	57.033 ±38.58[b]	7.24±5.28[a]	4.64 ±2.29[a]
Ethyl cinnamate	0.064 ±0.04[a]	0.044 ±0.00[a]	0.098 ±0.02[a]	0.08 ±0.02[a]	0.055 ±0.02[a]	0.075 ±0.03[a]	0.11 ±0.01[a]	0.223 ±0.17[b]	0.07±0.02[a]	0.08 ±0.04[a]
∑Esters	8034.85 ±4653.29[b]	6289.25 ±42.28[b]	5688.75 ±1574.42[b]	3688.17 ±2647.33[a]	5227.75 ±2699.80[b]	3876.25 ±859.11[a]	13,286.65 ±1555.50[b]	4083.35 ±662.16[a,b]	4759.84 ±2285.37[a,b]	5670.40 ±3044.82[b]
Isobutanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-hexanol	19.77 ±9.79[a]	20.29 ±0.40[a]	137.34 ±23.69[b]	157.70 ±20.31[b]	55.20 ±41.90[a]	184.40 ±102.53[b]	40.57 ±5.39[a]	109.95 ±7.57[b]	141.39 ±38.86[b]	110.42 ±42.72[b]
trans-3-hexenol	2.03±0.96[a]	2.03 ±0.12[a]	8.12 ±1.62[b]	8.23 ±2.03[b]	3.26 ±1.26[a]	9.30 ±5.26[b]	3.44 ±0.52[a]	5.31±0.49[a,b]	6.29±1.73[b]	6.72 ±2.95[b]
cis-3-hexenol	4.35±2.37[a]	4.43 ±0.10[a]	20.72 ±6.13[b]	17.83 ±1.21[b]	7.73 ±3.73[a]	24.19 ±16.57[b]	8.90 ±2.63[a]	14.0891.59[a]	19.09 ±6.22[a,b]	17.21 ±7.60[a,b]
Benzyl alcohol	0.79±0.43[a]	0.68 ±0.00[a]	1.05 ±0.26[a]	1.07 ±0.23[a]	0.70 ±0.16[a]	0.84 ±0.30[a]	1.40 ±0.01[b]	0.77±0.15[a]	0.80±0.26[a]	0.86 ±0.40[a]
2-phenylethanol	1229.77 ±742.29[b]	983.13 ±12.18[a]	1543.92 ±277.51[b]	1532.75 ±127.21[b]	900.56 ±248.41[a]	1407.31 ±540.44[b]	1963.65 ±18.75[b]	915.50 ±78.88[a]	1224.03 ±398.55[b]	1376.96 ±674.83[b]
∑ Higher alcohols	1256.70 ±755.31	1010.57 ±11.91	1711.14 ±305.29	1717.58 ±138.13	969.46 ±228.95	1626.03 ±663.91	2017.95 ±19.89	1045.62 ±87.63	1391.60 ±445.48	1512.17 ±728.34
Acetic acid	976.12 ±732.40[b]	1250.28 ±123.58[b]	592.58 ±331.91[b]	816.87 ±265.03[b]	949.18 ±848.53[b]	1213.38 ±1268.99[b]	723.20 ±149.13[b]	252.20 ±24.61[a]	550.58 ±204.44[b]	550.15 ±274.46[b]
Isobutyric acid	35.45 ±30.72[b]	62.3 ±5.74[b]	13.05 ±10.90[a,b]	19.22 ±13.27[b]	33.61 ±41.65[b]	10.31 ±6.02[a,b]	24.25 ±6.83[b]	5.49±1.43[a]	19.95 ±6.26[b]	14.61 ±5.80[a,b]
Butyric acid	6.43±4.26[b]	7.56 ±0.84[b]	3.86 ±2.18[a]	7.21 ±3.89[b]	3.76 ±3.39[a]	4.70 ±2.43[a]	24.25 ±6.83[b]	3.20±0.92[a]	5.30±1.66[a]	4.78 ±2.09[a]
Isovaleric acid	28.25 ±15.57[b]	7.925 ±2.65[a]	35.72 ±21.41[b]	31.22 ±33.87[b]	22.57 ±15.24[b]	17.98 ±1.39[b]	80.08 ±3.81[c]	94.91 ±29.79[c]	58.81 ±38.69[b]	55.06 ±31.10[b]
Valeric acid	0.31±0.19	0.40 ±0.22	0.36±0.13	0.74±0.53	0.27±0.14	0.34±0.25	0.62±0.23	0.37±0.19	0.41±0.06	0.35±0.13
Hexanoic acid	83.15 ±60.59[a]	59.53 ±24.13[a]	86.40 ±51.31[a]	194.34 ±99.02[b]	34.88 ±21.53[a]	75.36 ±31.34[a]	112.05 ±47.97[a,b]	72.53 ±11.28[a]	73.53 ±6.17[a]	88.93 ±29.77[a]
Decanoic acid	51.96 ±36.16[b]	54.79 ±25.22[b]	30.88 ±34.03[b]	54.96 ±50.50[b]	15.72 ±13.77[a]	125.82 ±170.14[c]	26.67 ±16.32[b]	57.38 ±42.11[b]	62.46 ±24.51[b]	96.80 ±90.54[b]
Nonanoic acid	3.38±1.86[a]	63.53 ±103.39[b]	11.66 ±0.08[a]	40.39 ±32.79[a,b]	7.52 ±5.25[a]	195.84 ±330.85[c]	15.90 ±14.09[a]	6.26±3.47[a]	124.68 ±186.13[b,c]	268.47 ±277.26[c]
Octanoic acid	310.14 ±284.84[b]	240.59 ±234.69[a,b]	506.47 ±364.89[b]	599.31 ±292.02[b]	122.17 ±82.72[a]	438.91 ±350.93[b]	338.92 ±77.07[b]	440.57 ±141.85[b]	388.85 ±129.99[b]	469.85 ±320.87[b]
∑Volatile acids	1495.18 ±853.19	1746.89 ±267.84	1280.98 ±759.06	1764.26 ±662.51	1189.66 ±928.77	2082.64 ±2158.46	1326.95 ±207.08	932.91 ±207.02	1284.55 ±114.14	1549.00 ±876.70
Guaiacol	nd	nd	nd	nd	nd	nd	0.17 ±0.06[a]	0.10±0.06[a]	nd	nd
4-vinylguaiacol	0.37±0.53[b]	1.00 ±0.27[b]	0.09 ±0.04[a]	1.80 ±2.98[b]	0.59 ±0.23[b]	nd	0.06 ±0.02[a]	1.55±0.38[b]	0.35±0.04[b]	0.19 ±0.07[b]
4-ethylphenol	0.10±0.05[a]	0.17 ±0.14[a]	0.19 ±0.03[a]	0.27 ±0.16[a,b]	0.12 ±0.05[a]	0.34 ±0.41[b]	0.24 ±0.10[a]	0.13±0.04[a]	0.26 ±0.16[a,b]	0.38 ±0.31[b]
4-ethyl guaiacol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
∑ Volatile phenols	0.56±0.46[b]	1.17 ±0.41[b]	0.25 ±0.07[a]	2.09 ±3.09[c]	0.52 ±0.40[b]	0.35 ±0.40[b]	0.45 ±0.12[b]	1.78±0.45[c]	0.612 ±0.19[b]	0.57 ±0.23[b]
Acetaldehyde	148.66 ±114.57[b]	176.79 ±18.65[b]	82.46 ±25.22[b]	81.59 ±13.40[b]	136.57 ±108.80[b]	106.10 ±67.99[b]	152.60 ±54.40[b]	59.05 ±7.04[a]	89.48 ±40.20[b]	79.78 ±46.87[a,b]
Octanal	nd	4.84 ±0.10[b]	nd	1.87 ±0.38[a]	nd	2.09 ±0.14[a]	nd	1.46±0.12[a]	2.66 ±1.12[a,b]	nd
Benzaldehyde	22.32 ±13.54[a,b]	22.52 ±1.35[a,b]	28.36 ±4.52[a,b]	26.88 ±5.93[a,b]	20.76 ±7.56[a]	21.27 ±5.39[a,b]	33.53 ±4.17[b]	20.03 ±3.30[a]	26.63 ±9.88[a,b]	23.84 ±11.46[a,b]
Phenylacetaldehyde	8.63 ±5.85[a,b]	6.58 ±0.04[a,b]	10.14 ±2.25[a,b]	11.50 ±0.53[a,b]	6.22 ±1.21[a,b]	8.12 ±2.51[a,b]	14.24 ±1.13[b]	5.13±1.41[a]	6.81 ±1.49[a,b]	9.28 ±4.63[a,b]
∑ Aldehydes	180.52 ±128.66[b]	209.12 ±19.98[b]	121.37 ±30.50[b]	121.21 ±12.97[b]	163.54 ±117.35[b]	136.88 ±74.30[b]	200.37 ±50.55[b]	85.68 ±11.38[a]	125.57 ±52.58[b]	113.17 ±61.47[b]
2-aminoacetophenone	0.03±0.01[a]	0.04 ±0.00[a]	0.06 ±0.02[a]	0.06 ±0.01[a]	0.03 ±0.01[a]	0.06 ±0.02[a]	0.07 ±0.02[a]	0.71±0.66[b]	0.06±0.02[a]	0.05 ±0.01[a]
∑ Ketones	0.03±0.01[a]	0.04 ±0.00[a]	0.06 ±0.02[a]	0.06 ±0.01[a]	0.03 ±0.01[a]	0.06 ±0.02[a]	0.07 ±0.02[a]	0.71±0.66[b]	0.06±0.02[a]	0.05 ±0.01[a]
trans-whiskey lactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Compound	**MC-SC**	**MS-SC**	**MP-SC**	**LT-SC**	**HU-SC**	**HG-SC**	**PK-SC**	**SC EC 1118**	**LT Octave-SC**	**MP Flavia-SC**
γ-octalactone	0.29 ±0.18[a,b]	0.22 ±0.09[a,b]	0.42 ±0.06[a,b]	0.50 ±0.09[b]	0.19 ±0.08[a]	0.37 ±0.26[a,b]	0.52 ±0.05[b]	0.28±0.05[a,b]	0.36 ±0.09[a,b]	0.42 ±0.22[a,b]
cis-whiskey lactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
γ-nonalactone	0.16 ±0.10[a,b]	0.18 ±0.10[a,b]	0.16 ±0.05[a,b]	0.24 ±0.13[a,b]	0.11 ±0.04[a]	0.22 ±0.20[a,b]	0.21 ±0.05[a,b]	0.10±0.01[a]	0.26 ±0.11[a,b]	0.29 ±0.18[b]
γ-dodecalactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methlactone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

(*continued on next page*)

Table 4 (*continued*)

Compound	MC-SC	MS-SC	MP-SC	LT-SC	HU-SC	HG-SC	PK-SC	SC EC 1118	LT Octave-SC	MP Flavia-SC
δ-decalactone	0.10±0.10[b]	0.04 ±0.01[a]	0.14 ±0.07[b]	0.15 ±0.05[b]		0.12 ±0.09[b]	0.11 ±0.03[b]	0.09±0.02[a,b]	0.10±0.05[b]	0.15 ±0.08[b]
γ-decalactone	0.19±0.15[a]	0.16 ±0.03[a]	0.14 ±0.06[a]	0.19 ±0.05[a]	0.08 ±0.03[a]	0.16 ±0.14[a]	0.18 ±0.02[a]	0.09±0.01[a]	0.41±0.05[b]	0.20 ±0.11[a]
∑ Lactones	0.74±0.53[a]	0.59 ±0.20[a]	0.86 ±0.21[a,b]	1.08 ±0.26[a]	0.41 ±0.14[a]	0.87 ±0.69[a]	1.02 ±0.14[b]	0.54±0.12[a]	1.13±0.26[b]	1.06 ±0.57[b]
2-sec-butyl-3-methoxypyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzylmercaptan	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methionol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzothiazole	0.44±0.22	0.39 ±0.03	0.48±0.12	0.61±0.30	0.32±0.10	0.47±0.09	0.54±0.04	0.55±0.24	0.45±0.16	0.42±0.13
∑ Others	0.44±0.22	0.39 ±0.03	0.48±0.12	0.61±0.30	0.32±0.10	0.47±0.09	0.542 ±0.04	0.55±0.24	0.45±0.16	0.42±0.13

Tentative identification based on mass spectral pattern (*μg/L equivalent of 2-octanol internal standard). The value of volatile compounds is expressed as mean ± standard deviation (n=3). Different letters in the column indicate a significant difference ($p < 0.05$). Abbreviations: MC-SC - *M. chrysoperlae* K-11/*S. cerevisiae*; MS-SC - *M. sinensis/shanxiensis* P-7/*S. cerevisiae;* MP-SC - *M. pulcherrima* K-6/*S. cerevisiae*; LT-SC - *L. thermotolerans* P-25/*S. cerevisiae*; HU-SC - *H. uvarum* Z-7/*S. cerevisiae*; HG-SC - *H. guilliermondii* N-29/ *S. cerevisiae*; PK-SC - *P. kluyveri* Z-3/ *S. cerevisiae*; SC EC 1118 – *Saccharomyces cerevisiae* EC 1118; LT Octave-SC – *L. thermotolerans* Octave/ *S. cerevisiae*; MP Flavia-SC – *M. pulcherrima* Flavia/*S. cerevisiae*. nd- not detected.

Among indigenous yeasts, MP in sequential fermentation resulted in the lowest concentration of acetaldehyde (82.46 μg/L), which is an undesirable compound. Conversely, MS in sequential fermentation had the highest concentration of acetaldehyde (176.79 μg/L) which statistically differed from pure fermentation with SC, as well as for octanal and total concentration of aldehydes. In general, aldehyde concentrations can be affected by the grape variety, yeasts, and available nutrients (Wang et al., 2023). Furthermore, PK in sequential fermentation exhibits the highest concentration of phenylacetaldehyde (14.24 μg/L), which positively contributes to wine aroma, respectively honey notes.

3.3. Multivariate analysis

In a projection of six groups (Terpenic compounds, C_{13}-norisoprenoids, Lactones, Aldehydes, Ketones and Others) identified in monoculture fermentation as parameters that defined the principal components PC1 and PC2, the first two principal components explained 53.15 % of the variability (Fig. 3a). The grouping of yeasts according to the observed parameters was evident and they were positioned in different quadrants. Control yeasts were positioned in the upper part of the biplot gravitating to positive PC2 values; in the 1st quadrant (LT Octave) and the 2nd quadrant (MP Flavia and SC). The biplot placed MP and HG isolates in the 1st quadrant indicating due to their good production of total terpenic compounds and C_{13}-norisoprenoids in monoculture. Similar production of volatile compounds as control yeasts SC and MP Flavia were shown by LT and MS isolates, while HP and HPG gravitated to positive PC1 values and production of lactones and aldehydes. To evaluate the differences/similarity of the investigated yeasts in sequential fermentation compared to control SC EC1118 strain, a principal component analysis was applied. Data set was the sum of volatile groups (Fig. 3b). The coverage of all variations in the observed data set was 57.53 %, In both cases, it is evident that indigenous strains were separated from three commercial controls. In Fig. 3b control yeasts may be seen in the upper part of the plot, with PC1 negative and PC2 positive. On the opposite side, MC-SC and PK-SC were positioned in the 4th quadrant due to the higher production of total C_{13}-norisoprenoids, esters, and aldehydes. Furthemore, LT-SC, MP-SC, and HG-SC gravitated to positive PC1 and PC2 values. Their positions were conditioned by the total concentrations of volatile groups including alcohols, lactones, and others.

3.4. Comparison of fermentation with monoculture and sequential fermentation

To gain insight into the qualitative changes of the observed parameters (presented in columns) based on fermentation practices and different yeasts, a heatmap is the best choice (Fig. 4). The upper part of the heatmap shows the sequential fermentation, where the lowest value for volatile phenols is evident for MP-SC (Σ Volatile phenols = 0.24708), while the highest value is observed for LT-SC (Σ Volatile phenols = 2.09164). Based on the colours of the control fermentations (names in green), regardless of the fermentation practices, the heatmap easily reveals which yeast resulted in similar values for the observed parameter; the more similar the colour (in the observed column for the observed parameter), the more similar the determined value.

Higher total concentrations of terpenic compounds were detected in monocultures for all yeasts, except for PK and HU, compared to their sequential fermentations. PK and HU were exceptions due to their low *β*-glucosidase activity (Milanović et al., 2023), and were unable to produce higher concentrations. The presence of SC yeast in sequential fermentation evidently affected the *β*-glucosidase activity of indigenous isolates. Conversely, PK and HU were the only two yeasts that produced the highest concentrations of thiols in interaction with SC rather than by themselves. This trend was also observed in the production of total volatile acids. The MC, LT, and PK yeasts in sequential fermentation with SC produced higher concentrations of C_{13}-norisoprenoids compared to their monoculture fermentations. Furthermore, the sum of esters, which contribute fruity-floral aromas, was detected in higher concentrations in monocultures for LT, HU, and HG, while the other four yeasts resulted in higher concentrations in sequential fermentation trials. MS, MP, and HU produced higher concentrations of higher alcohols in monoculture than in sequential fermentation, while the rest of the native yeasts produced higher concentrations in sequential fermentations. The heatmap also includes a dendrogram, with the observed parameters grouped into four clusters: (i) summarized volatile phenols and ketones, (ii) sums of terpenic compounds, aldehydes, C_{13}-norisoprenoids, and esters, (iii) sums of alcohols, lactones, and others, and (iv) sum of thiols and volatile acids.

In detail, the same compounds surpassed their odour threshold in both experiments, the next dataset was narrowed down to eleven compounds directly contributing to aroma for a heat map creation (Supplementary Figure 3). Interestingly, all seven yeasts applied in both

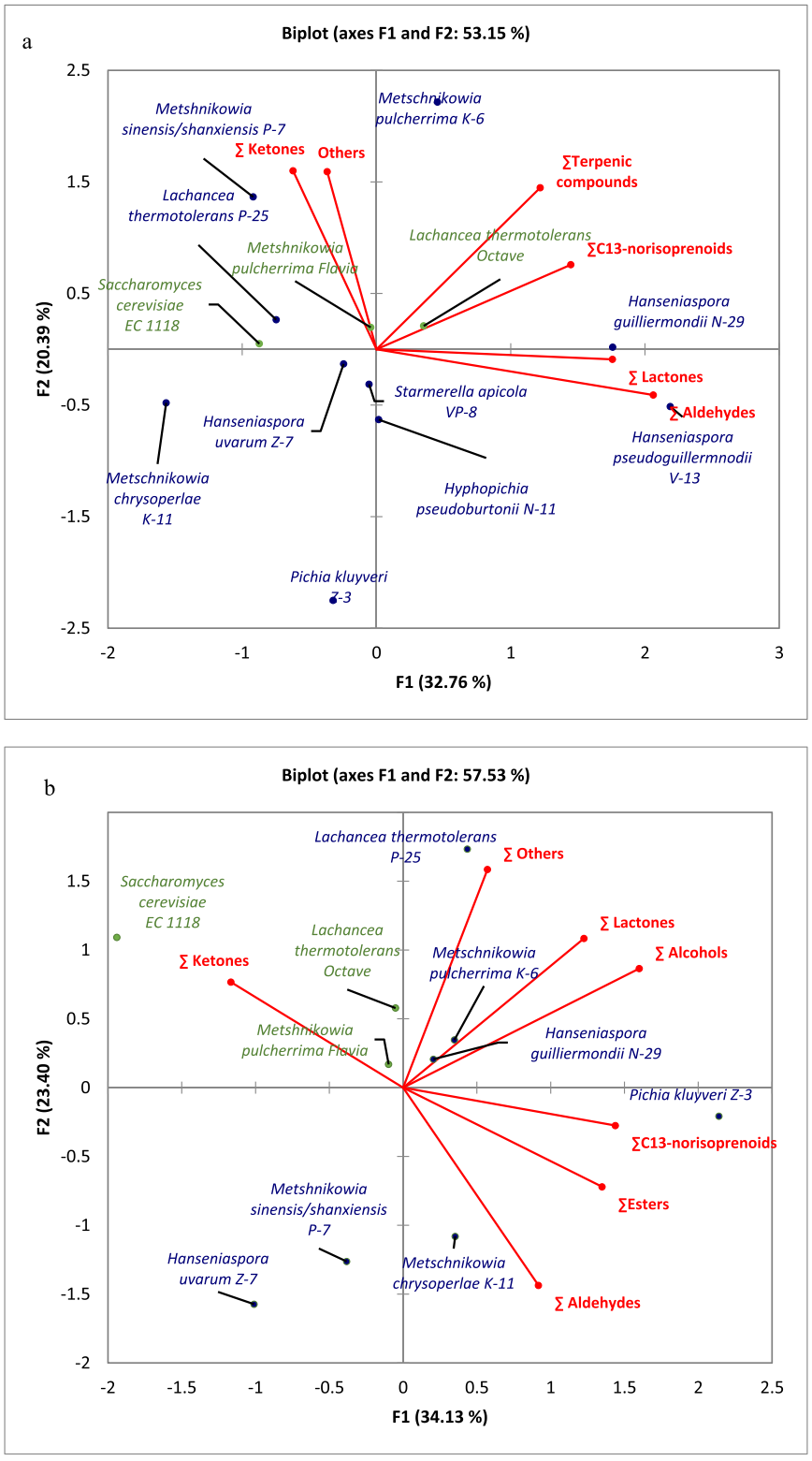

Fig. 3. Biplot of the principal component analysis for indigenous yeasts, control yeasts, and their developed volatile metabolites, categorized by chemical groups, in monoculture (a) and sequential fermentations (b).

fermentation practices exhibited similar profiles of the compounds with OAV higher than one, including *trans*-rose oxide, *β*-damascenone, ethyl caproate, ethyl caprate, phenylethyl acetate, ethyl butyrate, ethyl isovalerate, isopentyl acetate, isovaleric acid, octanoic acid and phenylacetaldehyde. Higher concentrations of *β*-damascenone were observed in a monoculture for all indigenous yeasts compared to the corresponding sequential fermentations. In contrast, ethyl isovalerate was detected in higher concentrations in wines produced by sequential fermentations. As for the other nine compounds, HG and MS showed better activity in monoculture fermentation, resulting in higher concentrations of compounds directly contributing to aroma, except for phenylacetaldehyde. HU isolate exhibited a similar behaviour in

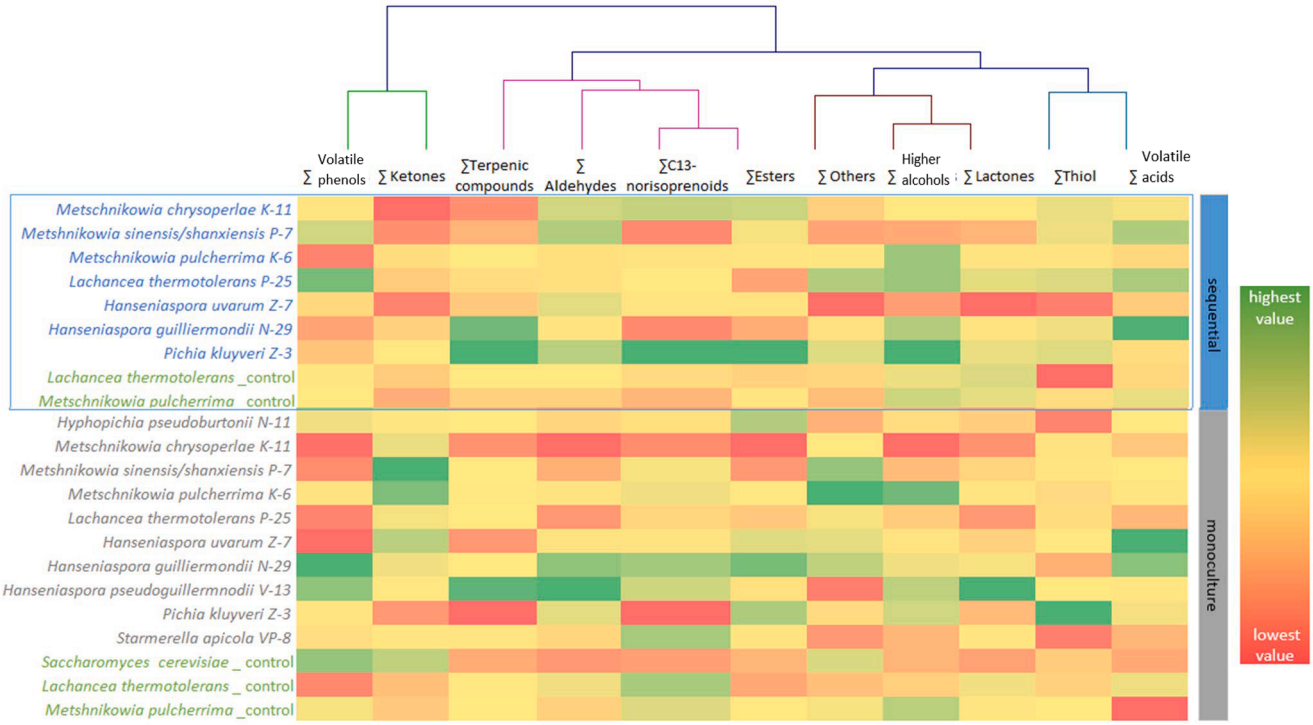

Fig. 4. Heatmap for summarised volatile groups developed during the monoculture and sequential fermentation, using different indigenous yeasts.

fermentation trials except for phenylethyl acetate whose concentration was higher in sequential fermentation with SC. The viable cells of HU were undetectable after 16 days of fermentation, so this increase in phenylethyl acetate may be caused by SC. Conversely, MC and PK isolates in interaction with SC, exhibited higher concentrations of odour-active compounds, except octanoic acid, whose concentration was higher in monoculture with these yeasts. Except for the previously mentioned β-damascenone, phenylethyl acetate was the compound detected in higher concentrations in LT monoculture compared to the corresponding sequential fermentation. The MP isolate behaved very similarly as LT isolate, with phenylacetaldehyde also differing between the two fermentation practices. Interestingly, increasing concentrations of acetic acid and acetaldehyde were among the main reasons for avoiding non-*Saccharomyces* yeasts in wine production. Results from this study showed various outcomes depending on the utilized strain. For example, MP, HU, PK, and HG isolates showed higher production of acetic acid in monoculture, while the other three yeasts, MC, MS, and LT, produced higher concentrations of acetic acid by sequential fermentation. Regarding acetaldehyde, only MP and HG showed higher production of this compound in monoculture. Notably, both MS and HU did not produce 4-vinylguaiacol independently, but in sequential fermentation, this compound was detected in the wine. According to our latest discoveries, it appears that the mutualistic relationship between *S. cerevisiae* and indigenous yeasts, as well as the antagonistic interactions observed within the sterile environment, could be attributed to species-specific interactions influenced by the presence of *S. cerevisiae.*

4. Conclusion

The effect of indigenous non-*Saccharomyces* yeasts on the aroma of wine, both in monoculture and in sequential fermentation with *S. cerevisiae*, was a subject of this research. The primary metabolites and volatile profile of Maraština wines were modulated, solely caused by the investigated yeasts, as the grape juice was initially sterilized. The comprehensive study on MC, MS, HP, and SA yeast strains in wine production has not been previously reported to our knowledge so this study exhibited with first results. Among monoculture fermentations, MP wines yielded the lowest ethanol level and exhibited high total terpenic production, PK exhibited high thiol and ester production, while HG increased the total concentration of C_{13}-norisoprenoids. Results obtained from seven sequential fermentations highlighted the interactions between species. Compared to the three controls, the most different profiles were observed in the PK-SC and MC-SC, where concentrations of total C_{13}-norisoprenoids and esters constituted the major differences. MP-SC fermentations decrease the volatile phenols production. Also, isolates did not affect the wine colour in sequential fermentations and they produced lower concentrations of polyphenols compared to SC control fermentations. The findings of this study illustrate the different volatile chemical profiles under the same winemaking conditions, demonstrating the biochemical role of each isolate in monoculture and sequential fermentations. This study represents a first step in untangling the interactions within the wine ecosystem, contributing to understanding the positive roles of non-*Saccharomyces* yeast in winemaking.

Ethical statement - studies in humans and animals

The study titled "The effect of indigenous non-*Saccharomyces* yeasts on the volatile profile of Maraština wine: Monoculture versus sequential fermentation" does not involve humans and animals.

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CRediT authorship contribution statement

Ana Boban: Writing – original draft, Methodology, Investigation,

Formal analysis, Data curation. **Urska Vrhovsek:** Writing – review & editing, Resources, Methodology. **Silvia Carlin:** Writing – review & editing, Formal analysis, Data curation. **Vesna Milanović:** Writing – review & editing, Methodology, Investigation. **Jasenka Gajdoš Kljusurić:** Writing – original draft, Formal analysis. **Zvonimir Jurun:** Formal analysis. **Irena Budić-Leto:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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