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ORIGINAL PAPER

Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces cerevisiae*

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Abstract The production of nonvolatile and volatile compounds by different commercial non-*Saccharomyces* yeast strains in Riesling grape musts was monitored during fermentation, and the related changes in wine quality were noted. Sequential fermentations of *Saccharomyces cerevisiae* with *Pichia kluyveri*, *Lachancea thermotolerans*, or *Metschnikowia pulcherrima* were compared to a single fermentation using *S. cerevisiae* alone. The results from all developed analyses showed significant differences in several parameters including population kinetics, nonvolatile and volatile compounds, and sensorial parameters.

Keywords Riesling · *Saccharomyces cerevisiae* · Non-*Saccharomyces* · Pyruvic acid · Volatile compounds

Introduction

Several research groups have studied non-*Saccharomyces* yeast applications [1] in different grape varieties such as Sauvignon blanc [2, 3], Chenin blanc [3], Chardonnay [3, 4], Amarone [5], Muscat [6], Muscat d'Alexandrie [7], Debina [8], Macabeo [9, 10], Folle blanche [11], Bobal [12], Alvarinho, Loureiro, Trajadura, Pedernã, Azal branco, Avesso [13], Airen [14, 15], Pedro ximenez [16], Sangiovese [17], Pinot noir [18], Emir [19, 20], Syrah [21–23], Garnacha [24], and Tempranillo [25, 26]. In most cases, improvements in wine quality have been reported.

The presence of unselected *Saccharomyces* and non-*Saccharomyces* wild yeasts in fermentations has been traditionally associated with high levels of acetic acid and other off-flavors. Nevertheless, many researchers and winemakers are now aware of the positive influence of non-*Saccharomyces* in wine aroma complexity [1, 27–36]. Some fermentation traits justify interest in mixed fermentations, including ethanol reduction, glycosidase and β-lyase enzyme activities, and the release of interesting metabolites such as glycerol, pyruvic acid, and mannoproteins [37–40]. However, the difficulty with which non-*Saccharomyces* wine yeast finishes the alcoholic fermentation requires the development of combined fermentations with *Saccharomyces cerevisiae* to ensure that the fermentation finishes correctly.

Some studies have analyzed the use and influence of different non-*Saccharomyces* species on wine quality. Some of these yeast species are *Kloeckera apiculata* [41], *Hanseniaspora uvarum* [42], *Hanseniaspora vineae* [25], *Torulospora delbrueckii* [5, 26, 43], *Metschnikowia pulcherrima* [2, 43, 44], *Starmerella bacillaris* [45], *Zygosaccharomyces bailii* [46, 47], *Schizosaccharomyces pombe* [40], *Lachancea thermotolerans* [17], and *Hansenula anomala* [14].

The possibility of modulating the flavor and style of wine through different fermentation strategies has increased the interest in studying all possible combinations of non-*Saccharomyces* and *Saccharomyces* yeast strains [5]. In this sense, most studies developed fermentations with non-*Saccharomyces* strains alone, compared them with mixed fermentations that used simultaneous or sequential inoculation, and compared all of them with the alcoholic fermentation with *S. cerevisiae* by itself. Most of these studies

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reported sequential inoculation as the best option in terms of wine quality.

The present study contributes to a better understanding of the valuable application of selected commercial non-*Saccharomyces* strains to enhance Riesling wine quality. Therefore, recommended yeast strains for Riesling winemaking such as *Pichia kluyveri* FrootZen™ (Hansen, Hørsholm, Denmark), *Lachancea thermotolerans* Concerto™ (Hansen, Hørsholm, Denmark), and *Metschnikowia pulcherrima* Flavia® (Lallemand, Montreal, Canada) were selected to perform sequential fermentations and to verify their positive influence in wine quality from an analytical perspective. The study also allows comparison, for the first time, of different fermentation possibilities involving non-*Saccharomyces* in the Riesling variety. Our findings will allow winemakers to select non-*Saccharomyces* yeasts for Riesling fermentations according to personal objectives.

Materials and methods

Microorganisms

Commercial yeast products were used for the experimental fermentation of Riesling must: *Saccharomyces cerevisiae* EC1118 (Lallemand, Montreal, Canada), *Kluyveromyces thermotolerans* Concerto™ (Hansen, Hørsholm, Denmark), *Pichia kluyveri* FrootZen™ (Hansen, Hørsholm, Denmark), and *Metschnikowia pulcherrima* Flavia® (Lallemand, Montreal, Canada).

Vinification

All fermentations used the must of *Vitis vinifera* L. cultivar Riesling grapes grown at Hochschule Geisenheim University (Germany). Using a microvinification method similar to that described in the literature [48], 4 l of sterilized must (115 °C, 15 min) was placed in a 5-l glass fermentation vessel, leaving enough space for carbon dioxide emission. No sulfur dioxide was added. Constituent concentrations and conditions in the must were: sugar, 237 g/l; pH, 3.26; primary amino nitrogen (PAN), 147 mg/l; tartaric acid, 3.3 g/l; malic acid, 6.9 g/l; citric acid, 0.25 g/l; lactic acid and acetic acid <0.1 g/l. Vitamon® CE (0.6 g/l; Erbslöh, Geisenheim, Germany) was added to provide nutrition for the yeast.

Four assays were performed (in triplicate): (1) inoculation of the must with *S. cerevisiae* EC1118 alone (SC); (2) inoculation with *K. thermotolerans* Concerto™ (10^6 CFU/ml) followed by *S. cerevisiae* EC1118 (10^7 CFU/ml) 48 h later (KT); (3) inoculation with *P. kluyveri* (10^6 CFU/ml) followed by *S. cerevisiae* EC1118 (10^7 CFU/ml) 48 h later (PK); and (4) inoculation with *M. pulcherrima* (10^6 CFU/ml) followed by *S. cerevisiae* EC1118 (10^7 CFU/ml) 24 h later (MP). Yeast inocula were produced using 100 ml of sterilized must with 1 ml of yeast extract peptone dextrose (YEPD) liquid medium [49] containing about 10^6 CFU/ml (determined using a Thomas chamber). To reach this population, 100 µl of each yeast suspension was cultivated in 10 ml of YEPD at 25 °C for 24 h. This procedure was repeated three times before the final inoculation (1 ml of inocula). All inoculations were performed in 250-ml flasks sealed with a fermentation lock filled with 98 % H_2SO_4 (Panreac, Barcelona, Spain), which allowed the release of CO_2 while avoiding microbial contamination [50]. The temperature was maintained at 25 °C for 48 h. The development of inocula proceeded without aeration, oxygen injection, or agitation. All fermentation processes were carried out at 20 °C. Once the fermentation of sugars was complete (remaining glucose and fructose concentration <3 g/l), the wines were racked and stabilized for 7 days at 4 °C, concluding with the final product being bottled in 750-ml bottles. Potassium metabisulfite was then added to give a sulfur dioxide concentration of 50 mg/l, and the bottles were sealed and placed horizontally in a climate chamber at 4 °C for 3 weeks until sensory evaluation.

Analytical determinations of nonvolatile compounds

Glucose and fructose, L-lactic acid, acetic acid, glycerol, pyruvic acid, acetaldehyde, citric acid, malic acid, and PAN were all determined using a Y15 enzymatic autoanalyzer (Biosystems S.A., Barcelona, Spain) and its proper kits (http://www.biosystems.es). Ethanol, methanol, pH, free SO_2, and total SO_2 profile were determined following the methods in the *Compendium of International Methods of Analysis of Musts and Wines* [51].

Microvinification growth kinetics

During fermentations, aliquots were taken periodically under aseptic conditions and were diluted tenfold with sterile deionized water. Upon collection of each aliquot, the vessel was stirred manually to ensure that a representative sample was retrieved. Growth kinetics was monitored by plating 100 µl of the appropriate dilution on lysine media (non-*Saccharomyces* counts; [52]) and YEPD media (total yeast counts; [49]). Colonies were counted after growth at 30 °C for 48–72 h.

Analytical determinations of volatile compounds

Aromatic by-products of fermentation were determined following the method of Rapp et al. [53], with the following modifications. For extraction, 2 g of sodium chloride was added to 10 ml of wine, followed by 5 µl of internal

standard solution (2,6-dimethylhept-5-en-2-ol and 2,6-di-tert-butyl-4-methylphenol in ethanol; c = 1188 µg/l and c = 107 µg/l, respectively), and 100 µl of 1,1,2-trichloro-1,2,2-trifluoroethane. This mixture was agitated for 20 min and then centrifuged for 8 min (3000 rpm; 1700 *g*). The organic phase was removed and dried over anhydrous sodium sulfate. For gas chromatography–mass spectrometry, 2 µl was injected in splitless mode (1 min) at an injector start temperature of 30 °C, which then was increased to 230 °C at 12 °C/min and held for 4 min. The initial oven temperature was 40 °C, which was held for 5 min, then increased to 125 °C at 3 °C/min, further increased to 200 °C at 6 °C/min, and held for 14.2 min. The helium carrier gas (Linde Gas, Bingen, Germany) was supplied at a constant flow rate (1 ml/min). The temperature of the mass spectrometer interface was 210 °C, and the ion source temperature was 230 °C. Mass spectral data were acquired in scan mode, covering a mass-to-charge ratio range of m/z 35–250 in electron-impact mode at 70 eV. Terpenes were analyzed according to the literature methods [54, 55] with some modification [56, 57].

Analytical determinations of amino acids

Amino acids were analyzed by ultra-high-performance liquid chromatography using a JASCO (Tokyo, Japan) X-LCTM instrument equipped with a 3120-FP fluorescence detector. Gradients of solvent A (methanol/acetonitrile, 50:50, v/v) and B (sodium acetate/tetrahydrofuran, 99:1, v/v) were used in a C18 (HALO, USA) column (100 × 2.1 mm; particle size 2.7 µm) as follows: 90 % B (0.25 ml/min) from 0 to 6 min, 90–78 % B linear (0.2 ml/min) from 6 to 7.5 min, 78 % B from 7.5 to 8 min, 78–74 % B linear (0.2 ml/min) from 8 to 8.5 min, 74 % B (0.2 ml/min) from 8.5 to 11 min, 74–50 % B linear (0.2 ml/min) from 11 to 15 min, 50 % B (0.2 ml/min) from 15 to 17 min, 50–20 % B linear (0.2 ml/min) from 17 to 21 min, 20–90 % B linear (0.2 ml/min) from 21 to 25 min, and re-equilibration of the column from 25 to 26 min. Detection was performed by scanning in the 340- to 455-nm range. Quantification was performed by comparison against external standards of the studied amino acids. The different amino acids were identified by their retention times.

Sensory analysis

The final wines were assessed (blind test) by a panel of 13 experienced wine tasters; all were members of the staff of the Department of Microbiology and Biochemistry of the Hochschule Geisenheim University (Germany). Following the generation of a consistent terminology by consensus, several attributes were chosen to describe the wines. The tasters used a ten-point scale, from 0 (no defect) to 10 (very strong defect perceptible), to rate the intensity of 17 attributes: aroma intensity, aroma quality, oxidation, acetaldehyde, ethyl acetate, reduction, fruitiness, peach/apricot, citrus/grape fruit, pear, apple, general acidity, acetic acid, sweetness, bitterness, Riesling typicity, and overall impression.

Statistical analysis

All statistical analyses were performed using PC Statgraphics v. 5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p < 0.05$ for the ANOVA matrix F value. The multiple-range test was used to compare the means.

Results and discussion

Fermentation kinetics

Yeast population kinetics

Figure 1 shows the populations of the different yeast strains during the fermentation process. In all sequential fermentations, when *Saccharomyces cerevisiae* EC1118 was inoculated, all non-*Saccharomyces* started to decline quickly. Similar results have been reported before [5, 44], where non-*Saccharomyces* acted only during the first fermentation phase. In the present trial, all non-*Saccharomyces* yeasts disappeared on day 14. This phenomenon could be attributed to alcohol production by *S. cerevisiae* EC1118 and its higher stress resistance [29]. Some *S. cerevisiae* strains were also reported to secret antimicrobial peptides inhibiting non-*Saccharomyces* yeast growth [58]. This could explain the early disappearance of *L. thermotolerans*, which has been reported to tolerate up to 9 % v/v ethanol when it ferments by itself [59].

Sugar consumption kinetics

Saccharomyces cerevisiae EC1118 fermenting by itself (SC) (Fig. 2a) consumed the sugar the fastest, followed by *Kluyveromyces thermotolerans* Concerto™ fermentation (KT). This result is in accordance with the higher fermentative power of these two yeast species when compared with the non-fermentative *P. kluyveri* and *M. pulcherrima*.

Chemical monitoring

Acetic acid

Previous experiments with *L. thermotolerans* and *M. pulcherrima* reported significant reduction in acetic acid content [17, 60]. The trial results for the present study cannot

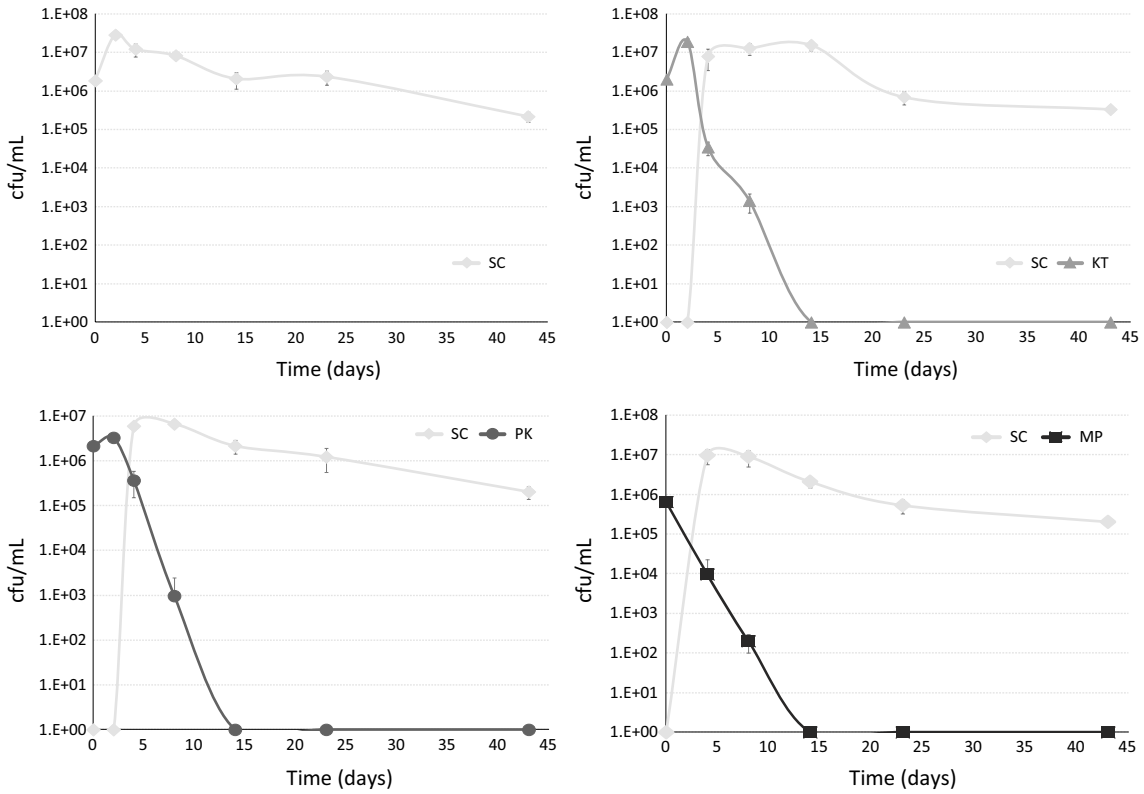

Fig. 1 Population development of *Saccharomyces cerevisiae* EC 1118 (SC), *Kluyveromyces thermotolerans* Concerto™ (KT), *Pichia kluyveri* FrootZen™ (PK), and *Metschnikowia pulcherrima* Flavia® (MP) during the different sequential fermentation processes

confirm an additional increase or decrease in this compound because no significant differences were detected (Fig. 2b; Table 1). With levels of 0.37 and 0.38 g/l (Table 1), the values are not excessive, and they did not negatively affect wine quality. The present results show that controlled use of at least some non-*Saccharomyces* in sequential fermentations does not cause an increase in acetic acid production.

Glycerol

Most glycerol was produced during the first days of fermentation (Fig. 2c). The SC fermentation gave the lowest level of glycerol, and only slight differences between the non-*Saccharomyces* strains could be found. The final levels of glycerol varied from 5.8 to 6.3 g/l (Table 1). Increased glycerol content is described as one of the main contributions of some non-*Saccharomyces* strains on wine quality [61] because it contributes positively to the mouthfeel. For *L. thermotolerans* and *M. pulcherrima*, an increasing content of glycerol is described in the literature [17, 60].

L-Lactic acid

Figure 2d shows that only *K. thermotolerans* Concerto™ (KT) produced 0.22 g/l of L-lactic acid (Table 1). Other authors [59, 62, 63] obtained stronger acidifications using mixed cultures of *L. thermotolerans* with the main objective of acidifying low-acid musts. In those studies, the *L. thermotolerans* population remained high for a longer time. The production of L-lactic acid is also linked to the viable cell concentration [60]. Because of the lack of acidity in musts from warm regions, it is recommended that the *S. cerevisiae* sequential inoculation be delayed until the desired acidity is achieved. Riesling musts from the Rheingau region normally do not need acidification, and production of high amounts of L-lactic acid would not contribute to improved wine quality. Therefore, the use of *L. thermotolerans* should be focused on contributions to other specific properties of wine.

Pyruvic acid

The highest levels of pyruvic acid were formed during the first days of fermentation (Fig. 3e). The non-*Saccharomyces* yeasts formed more pyruvic acid and more glycerol, both of which are derived from the glycerol pyruvate pathway [64, 65]. The maximum pyruvic acid concentrations were higher than those recorded in earlier works performed by selected *Saccharomyces cerevisiae* for their ability to produce pyruvic acid; these produced between 0.06

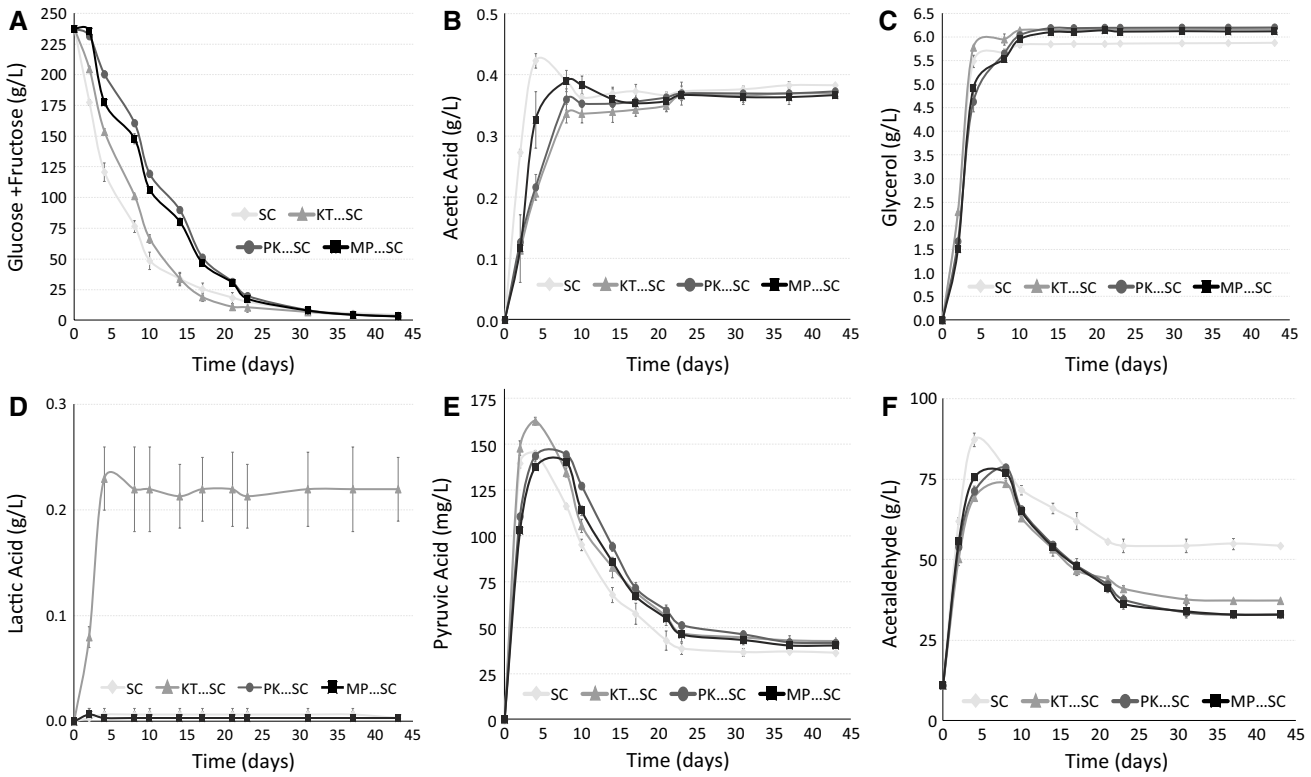

Fig. 2 Concentrations of (**a**) glucose + fructose (g/l); (**b**) acetic acid (g/l); (**c**) glycerol (g/l); (**d**) lactic acid (g/l); (**e**) pyruvic acid (mg/l); (**f**) acetaldehyde (mg/l). Parameters of the studied wines based on Riesling variety during fermentations performed by *S. cerevisiae* EC1118 (SC), and sequential fermentations with *S. cerevisiae* EC1118 and *K. thermotolerans* Concerto™ (KT…SC), *P. kluyveri* FrootZen™ (PK…SC), and *M. pulcherrima* Flavia® (MP…SC)

Table 1 Final analysis of *Saccharomyces cerevisiae* EC1118 (SC), sequential fermentation with *Saccharomyces cerevisiae* EC1118 and *Kluyveromyces thermotolerans* CONCERTO™ (KT…SC), *Pichia kluyveri* FrootZen™ (PK…SC) and *Metschnikowia pulcherrima* Flavia® (MP…SC)

Compounds	SC	KT…SC	PK…SC	MP…SC
L-Lactic acid (g/l)	0.00 ± 0.00b	0.22 ± 0.04a	0.00 ± 0.00b	0.00 ± 0.00b
Acetic acid (g/l)	0.38 ± 0.01a	0.37 ± 0.01a	0.37 ± 0.01a	0.37 ± 0.02a
Residual sugar (g/l)	4.65 ± 0.35ab	4.35 ± 0.49ab	4.50 ± 0.00a	4.20 ± 0.17b
Glycerol (g/l)	5.88 ± 0.02c	6.17 ± 0.03ab	6.21 ± 0.02a	6.12 ± 0.04b
Free SO_2 (mg/l)	<5	<5	<5	<5
Total SO_2 (mg/l)	56.00 ± 1.14a	44.00 ± 1.14c	54.50 ± 2.21ab	52.33 ± 1.15b
Alcohol (% v/v)	13.80 ± 0.01a	13.60 ± 0.01b	13.55 ± 0.04b	13.61 ± 0.02b
Methanol (mg/l)	61.50 ± 0.71a	62.00 ± 1.41ab	63.00 ± 0.00b	62.33 ± 0.58a
Acetaldehyde (mg/l)	53.50 ± 2.12 a	37.00 ± 0.00b	32.50 ± 0.71c	33.00 ± 1.00c
pH	3.39 ± 0,01a	3.38 ± 0.02a	3.39 ± 0,01a	3.40 ± 0,01a

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$)

and 0.13 g/l of pyruvic acid [66], rather less than 0.36 g/l (Fig. 2e) reached in this study by fermentation KT. Other non-*Saccharomyces* like *Schizosaccharomyces* have been described as higher producers of pyruvic acid than those shown in this study [40]. Higher levels of pyruvic acid are interesting for red wines because it contributes to color stability by reaction of malvidin with pyruvic acid to form the highly stable colored molecule vitisin A [23].

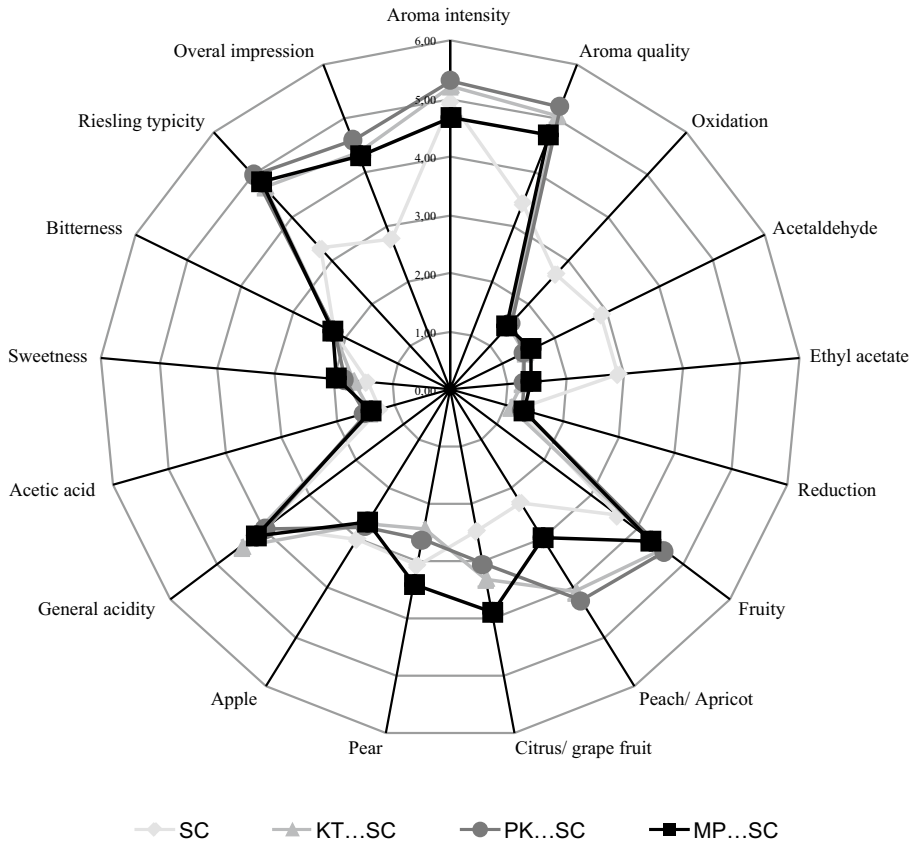

Fig. 3 Results of the sensorial analysis from bottled wines coming from different fermentation processes of *S. cerevisiae* EC1118 (SC), and sequential fermentations with *S. cerevisiae* EC1118 and *K. thermotolerans* Concerto™ (KT…SC), *P. kluyveri* FrootZen™ (PK…SC), and *M. pulcherrima* Flavia® (MP…SC)

Acetaldehyde

The acetaldehyde kinetics followed the same pattern as normally described, reaching a peak during the first fermentation phase (Fig. 2f). Acetaldehyde arises from the yeast metabolism of sugars and is partly reutilized [67]. SC fermentation produced more acetaldehyde than the others, with a final concentration of 54 mg/l (Table 1), which is under the sensory threshold of 100–125 mg/l [68]. The non-*Saccharomyces* fermentation produced less acetaldehyde, with values that varied from 32.5 mg/l (PK) to 37 mg/l (KT) (Table 1).

Alcohol

Some non-*Saccharomyces* yeasts are known for lower ethanol yields than *S. cerevisiae* [39]. Sugar consumption in those cases produces higher amounts of compounds other than ethanol, such as glycerol or pyruvic acid, or to increase the yeast biomass because of its reported lower Crabtree effect [69, 70]. Statistically significant differences were obtained in this study, where the alcohol levels varied from 13.55 to 13.80 % (v/v) (Table 1). These results are in agreement with several authors who confirmed the usefulness of non-*Saccharomyces* yeast in the production of lower concentrations of alcohol in wines [39, 71].

Previous studies have reported reduced ethanol content for sequential fermentations with *L. thermotolerans* [17]. The difference in this study is only just significant, being 0.2 % (v/v) lower than the *Saccharomyces* control. *M. pulcherrima* produced 0.19 % (v/v) less ethanol than *S. cerevisiae*. Other authors have reported differences of 0.35 % (v/v) [72], 0.28 % (v/v) [60], and 3.7 % (v/v), but under high aeration conditions [73].

Volatile aroma

The highest total concentration of higher alcohols was formed by SC fermentation (Table 2). Nevertheless, the total higher alcohols concentration remained below 300 mg/l, which is regarded as the minimum level for contributions to the general complexity of the wine [74]. Other authors have described non-*Saccharomyces* yeasts as lower producers of higher alcohols than *Saccharomyces cerevisiae* [8, 9, 17, 42, 75]. The KT fermentation was the second-best producer of 2-phenylethanol (Table 2) behind the SC fermentation. Other authors have reported higher production of this compound by this yeast species [17, 60].

In the present study, MP fermentation produced less total esters than the others, although the final levels of some specific esters such as ethyl octanoate were higher than in other trials (Table 2). In any case, high production

Table 2 Volatile compounds measured after fermentation of *Saccharomyces cerevisiae* EC1118 (SC), sequential fermentation with *Saccharomyces cerevisiae* EC1118 and *Kluyveromyces thermotolerans* CONCERTO™ (KT…SC), *Pichia kluyveri* FrootZen™ (PK…SC) and *Metschnikowia pulcherrima* Flavia® (MP…SC)

Compounds	SC	KT…SC	PK…SC	MP…SC
Esters				
Ethyl esters				
Ethyl lactate (mg/l)	9.76 ± 0.15b	14.27 ± 1.04a	8.65 ± 0.27c	8.57 ± 0.19c
Ethyl propanoate (μg/l)	75.92 ± 5.09a	73.25 ± 2.6a	76.64 ± 2.56a	65.3 ± 3.5b
i-ethyl butanoate (μg/l)	14.27 ± 0.12c	17.82 ± 0.21b	21.18 ± 0.43a	13.93 ± 1.04c
Ethyl butanoate (μg/l)	558.92 ± 19.75b	738.99 ± 26.72a	572.24 ± 4.44b	563.62 ± 12.82b
Ethyl hexanoate (μg/l)	1214.14 ± 34.63c	1409.47 ± 29.53a	1392.87 ± 22.91a	1327.43 ± 12.89b
Ethyl octanoate (μg/l)	1352.35 ± 16.08c	1247.72 ± 62.98d	1530.85 ± 14.5a	1466.46 ± 29.52b
Ethyl decanoate (μg/l)	625.39 ± 24.09b	732.73 ± 75.19a	716.54 ± 20.10a	681,6 ± 72.91aab
Total Ethyl esters (μg/l)	13600.99 ± 127.78b	18,489.98 ± 914.10c	12,960.32 ± 220.86a	12,688.34 ± 157.49a
Acetates				
Ethyl acetate (mg/l)	75.48 ± 9.99a	70.15 ± 4.57a	79.85 ± 10.29a	57.34 ± 3.72b
Isoamyl acetate (μg/L)	3,668.5 ± 241.6a	3336.16 ± 134.19a	3045.62 ± 45.58b	2441.25 ± 98.93c
2-Methyl butyl acetate (μg/l)	120.28 ± 8.63b	146.16 ± 5.96a	115.46 ± 3.8c	97.4 ± 2.59d
Hexyl acetate (μg/l)	501.1 ± 3.17a	377.44 ± 17.83b	508.92 ± 15.12a	485.24 ± 12.82a
2- Phenyl ethyl acetate (μg/l)	958.85 ± 17.34b	540.5 ± 14.5d	1238.42 ± 30.4a	825.51 ± 56.69c
Total acetates (μg/l)	80,728.78 ± 9660.15b	74,550.26 ± 4433.28b	84,758.42 ± 9987.84b	61,189.4 ± 3601.26a
Total esters	94,329.72 ± 8936.79b	93,040.24 ± 3989.27b	97,718.74 ± 9302.33b	73,877.74 ± 3278.11a
Higher alcohols				
i-Butanol (mg/l)	14.96 ± 2.34ab	17.55 ± 1.23a	12.2 ± 2.16b	9.69 ± 1.24bc
3-Methyl-butanol (mg/l)	151.37 ± 20.72a	129.41 ± 3.38a	127.52 ± 17.36ab	117.29 ± 7.23b
2-Methyl-butanol (mg/l)	16.54 ± 2.10ab	17.12 ± 0.79a	15.63 ± 1.57ab	14.63 ± 0.74b
2-Phenyl-ethanol (mg/l)	24.67 ± 1,44a	21.34 ± 1,56b	20.32 ± 3.46abc	19.10 ± 0.3c
Hexanol (μg/l)	1052.78 ± 103.22a	1202.34 ± 159.49a	549.60 ± 103.02b	721.9 ± 157.46b
Total higher alcohols (mg/l)	208.59 ± 17.83c	186.62 ± 2.92b	176.219 ± 14.93ab	161.43 ± 6.24a
Fatty acids				
Hexanoic acid (mg/l)	11.98 ± 0.97a	11.5 ± 0.72a	12.58 ± 0.92a	12.82 ± 0.26a
Octanoic acid (mg/l)	6.72 ± 0.18ab	5.92 ± 0.10c	6.91 ± 0.21a	6.55 ± 0.09b
Decanoic acid (mg/l)	3.1 ± 0.06b	3.39 ± 0.17a	3.19 ± 0.10ab	2.92 ± 0.13
Total fatty acids (mg/l)	21.8 ± 0.74ab	20.81 ± 0.55a	22.68 ± 0.71b	22.29 ± 0.21b
Terpenes				
Linalool oxide 1 (μg/l)	9.28 ± 0.41a	10.15 ± 0.74a	20.56 ± 0.30b	10.08 ± 0.74a
Neroloxide (μg/l)	22.66 ± 0.32a	21.38 ± 0.20b	22.24 ± 0.19a	21.91 ± 0.13c
Linalool oxide 2 (μg/l)	3.79 ± 0.08a	3.8 ± 0.09a	3.72 ± 0.00a	3.76 ± 0.04a
Vitispirane (μg/l)	3.62 ± 0.12a	3.45 ± 0.14a	3.52 ± 0.21a	3.6 ± 0.10a
Linalool (μg/l)	21.6 ± 0.12a	21.9 ± 1.30a	21.21 ± 1.05a	21.58 ± 0.20a
Hotrienol (μg/l)	47.65 ± 2.56a	57.35 ± 4.46b	68.38 ± 8.17b	57.25 ± 8.22b
α-Terpineol (μg/l)	14.84 ± 0.63ab	16.21 ± 0.89a	14.08 ± 0.49b	14.07 ± 0.64b
Nerol (μg/l)	220.11 ± 27.67a	267.87 ± 9.54b	161.93 ± 19.23c	162.15 ± 4.04c
β-Damascenone (μg/l)	13.19 ± 0.87a	13.31 ± 0.80a	14.24 ± 0.53ab	15.21 ± 0.83b
Geraniol (μg/l)	4 ± 1a	3 ± 0a	0 ± 0b	1 ± 0b
Total Terpenes (μg/l)	360.74 ± 21.89b	418.42 ± 7.89c	329.88 ± 14.13b	310.61 ± 4.63a

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$)

of esters by *M. pulcherrima* has been reported in the past [2, 9, 34, 60]. All fermentation variants produced 2-phenylethyl acetate higher than the threshold value [76]. PK fermentation formed the highest levels of 2-phenylethyl acetate (Table 2). PK and MP fermentations produced the highest levels of ethyl octanoate (Table 2). Other authors

Table 3 Amino acids measured after fermentation of *Saccharomyces cerevisiae* EC1118 (SC), sequential fermentation with *Saccharomyces cerevisiae* EC1118 and *Kluyveromyces thermotolerans* CONCERTO™ (KT…SC), *Pichia kluyveri* FrootZen™ (PK…SC) and *Metschnikowia pulcherrima* Flavia® (MP…SC)

Compounds	SC	KT…SC	PK…SC	MP…SC
Aspartic acid (mg/l)	10.85 ± 1.2a	11.55 ± 1.2a	18.15 ± 0.07b	19.03 ± 0.61c
Alanine (mg/l)	70.05 ± 2.76a	75.50 ± 3.39a	88.00 ± 2.83b	91.13 ± 1.21b
Arginine (mg/l)	37.50 ± 2.55a	41.15 ± 3.04a	61.90 ± 2.55b	66.07 ± 6.18b
Asparagine (mg/l)	43.20 ± 2.40a	39.70 ± 2.69a	52.15 ± 1.48b	53.17 ± 0.64b
Phenylalanine (mg/l)	10.50 ± 1.13a	10.75 ± 1.63a	19.15 ± 0.07b	20.33 ± 0.97c
Glycine (mg/l)	37.45 ± 1.20b	34.65 ± 1.34a	40.45 ± 1.20c	42.00 ± 0.20d
Tryptophan (mg/l)	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
Isoleucine (mg/l)	3.15 ± 0.35a	3.65 ± 0.49a	7.00 ± 0.14b	7.43 ± 0.78b
Lysine (mg/l)	3.95 ± 0.92a	7.20 ± 0.14c	5.70 ± 0.42b	6.47 ± 0.85b
Leucine (mg/l)	6.10 ± 0.01b	4.80 ± 0.14a	7.05 ± 0.64c	6.83 ± 0.76bc
Ornithine (mg/l)	36.55 ± 0.35a	32.05 ± 4.74a	36.05 ± 0.64a	36.27 ± 1.15a
Serine (mg/l)	3.6 ± 0.57a	5.50 ± 0.71b	9.60 ± 0.42c	10.70 ± 0.66c
Tyrosine (mg/l)	6.90 ± 0.57a	7.85 ± 1.06a	10.55 ± 0.92b	8.57 ± 1.08ab
Threonine (mg/l)	51.80 ± 0.14c	49.60 ± 0.01b	46.85 ± 2.19ab	46.50 ± 0.62a

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$)

reported *M. pulcherrima* as a good producer of pear-related esters such as ethyl octanoate [9, 34]. KT fermentation formed more ethyl butanoate than the others (Table 2), while KT and SC fermentations were the best producers of isoamyl acetate. *M. pulcherrima* has been described as a good producer of isoamyl acetate by some authors [2, 60], although others have reported huge strain variability [7] with respect to ester production. MP fermentation produced less ethyl acetate than the others, and no statistical differences were detected between the other fermentations. *Lachancea thermotolerans* has been reported to produce less ethyl acetate than *S. cerevisiae* [17]. KT fermentation was the only yeast to produce some ethyl lactate.

KT fermentations produced higher levels of total terpenes, hotrienol, and specially nerol (Table 2). Although PK fermentations did not produce higher total terpenes than the SC control, it produced the highest reported levels in hotrienol and linalool oxide. However, the total terpene concentrations were below the terpene perception threshold of 0.5–1 mg/l in all cases [77].

Final wine amino acid content

PK and MP fermentations gave higher final levels in aspartic acid, alanine, arginine, asparagine, phenylalanine, glycine, isoleucine, leucine, serine, and tyrosine than SC and KT fermentations (Table 3). KT fermentation gave a higher final level in lysine. SC and KT fermentations showed higher levels of threonine at the end of fermentation. The observed differences in isoleucine and leucine could explain the differences reported in higher alcohols, because they are precursors of 2-methylbutanol and 3-methylbutanol (Table 2). The statistical differences reported in phenylalanine and tyrosine show that [PK…SC] and [MP…SC] sequential fermentations increase the content of some biogenic amine precursors [78, 79].

Sensory evaluation

Fermentations involving non-*Saccharomyces* obtained higher scores in overall impression (Fig. 3). Fermentations involving *S. cerevisiae* by itself (SC) scored highest in ethyl acetate, acetaldehyde, and oxidation (Fig. 3), while SC wine gave the lowest score in aroma quality. Analytical data also show higher values of acetaldehyde for these fermentations. SC fermentation received the lowest scores in fruitiness, the related attributes of peach/apricot and citrus/grape fruit, and in Riesling typicity (Fig. 3). Fruitiness and aroma quality were similar for the non-*Saccharomyces* wines. This can be explained in terms of the elevated production of higher alcohols by *Saccharomyces*, which can mask the fruitiness of esters. Non-*Saccharomyces* scored higher in fruitiness even though they did not produce more esters than *S. cerevisiae* in all cases. *Metschnikowia pulcherrima* Flavia® showed the lowest ester production but was evaluated highest in citrus/grape fruit and pear. This may be explained by the lowest production of higher alcohols, which could mask fruitiness and its higher level in ethyl octanoate (Table 2). Fermentation involving *P. kluyveri* received the best scores in overall impression, although the final wine sensory profile was different from the other non-*Saccharomyces*. *M. pulcherrima*

fermentations scored higher in pear and citrus/grape fruit character, while *L. thermotolerans* and *P. kluyveri* scored higher in peach/apricot character.

Conclusion

Comparison of the results from the fermentation trials showed differences in several analyzed parameters such as pyruvic acid, glycerol, acetaldehyde, ethanol, higher alcohols, ethyl esters, 2-phenylethyl acetate, 2-phenylethanol, and terpenes. The wines fermented by non-*Saccharomyces* were preferred by the tasters; nevertheless, the sensory profiles were different depending on the different species used in the fermentations.

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Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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