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In-Depth Characterization of the Volatile Aroma Profile and Other Characteristics of White Wine Produced by Sequential Inoculation with a *Lachancea thermotolerans* Starter Yeast Strain

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Abstract: The yeast *Lachancea thermotolerans* has the ability to produce notable amounts of lactic acid and reduce alcoholic strength in fermentation, so it has a considerable potential for mitigating negative impacts of climate changes in winemaking. In this study, a treatment with *L. thermotolerans* and *Saccharomyces cerevisiae* in sequential inoculation was compared to a control *S. cerevisiae* monoculture fermentation of Malvazija istarska (aka Malvasia Istriana) white grape must. Standard physico-chemical parameters of the obtained wines were determined by the OIV methods. Targeted (GC/FID and GC/MS) and untargeted (GC×GC/TOF-MS) gas chromatographic techniques were combined for the analysis of volatile compounds. Phenolic compounds were analyzed by UPLC/QqQ-MS/MS, and proteins by RP-HPLC-DAD, while a sensory analysis of wines was performed by a panel of trained and certified tasters. *L. thermotolerans* co-fermentation treatment increased the concentration of lactic acid and decreased alcoholic strength. *L. thermotolerans* increased the concentrations of geraniol, β-ionone, isobutanol, isobutyric acid, ethyl isobutyrate, several major acetates, ethyl lactate, and diethyl succinate, followed by many minor compounds. This wine also contained more hydroxycinnamoyl tartrates, while control *S. cerevisiae* wine had higher levels of free hydroxycinnamates. The effects on PR proteins were minor. *L. thermotolerans* co-fermentation slightly enhanced the sensory perception of tropical fruit, herbaceous, tobacco, and buttery odor notes, as well as fullness of body. With the largest number of identified volatile compounds up to date and other results obtained, this study contributes to the better understanding of oenological and especially aromatic potential of *L. thermotolerans* in white wine production.

Keywords: *Lachancea thermotolerans*; climate change; acidity; volatiles; phenols; proteins; two-dimensional gas chromatography/mass spectrometry; Malvazija istarska

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1. Introduction

The winemaking community is facing many challenges connected with climate changes that affect viticulture and viniculture practices in many ways. The warming effect and consequent increase in temperature and occurrence of extreme weather, together with changes in rainfall amounts, change the short- and long-term climate structure. Among other consequences, new viniculture regions are emerging in countries of colder parts of Europe and America, while in many traditional grape-growing regions, earlier maturation results in grapes and wines with increased sugar and alcohol contents, respectively, at the same time lacking in acidity, contributing to altered and even unacceptable sensory profiles [1]. The number of scientific studies dealing with novel approaches to mitigate the mentioned negative effects of climate changes on wine quality is growing constantly. Some of these include the reduction of potassium ions that contribute to loss of acidity by sedimentation of tartrates, particular filtration techniques to reduce the initial concentration

of sugars in grape must, as well as dealcoholization of wine to decrease its alcohol level [2]. Special attention is focused on particular non-*Saccharomyces* yeasts as a potential solution for some of the abovementioned issues, since their use can significantly modulate the composition of wine. One such yeast is *Lachancea thermotolerans*, which inhabits different environments, such as grapes. Alongside its tolerance of high osmotic pressure [3], it has a moderate fermentative capacity and ethanol tolerance of around 5–9 vol % [4], so it has to be used in sequential inoculation or co-inoculation with *Saccharomyces cerevisie* or other strongly fermentative non-*Saccharomyces* yeasts, such as *Schizosaccharomyces pombe* or *Torulaspora delbrueckii* [5–7]. *Lachancea thermotolerans* has a special ability to synthesize L-lactic acid from sugars by the action of lactic acid dehydrogenase (LDH) enzymes during alcoholic fermentation and thus simultaneously decrease the production of ethanol [8], which is a feature that can be exploited to mitigate the negative effects of overripe grapes. Metabolic pathway of L-lactic acid synthesis is still not distinguished in detail, but certain studies showed a huge phenotypic divergence regarding lactic acid production among various investigated *L. thermotolerans* strains [9]. The activity of LDH enzymes is coded by three genes, *Ldh1*, *Ldh2*, and *Ldh3* [3]. When comparing the expression of the *Ldh* genes of high- and low-lactate-producing strains, Sgouros et al. [10] observed that only *Ldh2* was up-regulated in high-lactate-producing strains, while other *Ldh* genes were expressed at a similar level in both low- and high-lactate-producing strains. Given that lactic acid production is highly *L. thermotolerans* strain-dependent, wide ranges of increases in its concentrations were reported as a result of its activity, from 0 to 16 g/L [11–13]. Volatile acidity is another important oenological parameter that can be affected by the activity of particular *L. thermotolerans* strains, with the consummation of acetic acid in aerobic conditions as one of the proposed mechanisms [14]. Comitini et al. [15] observed a reduction in volatile acidity for about 50% after fermentation with pure *L. thermotolerans* culture in comparison with *S. cerevisiae* fermentation, while Gobbi et al. [16] reported a decrease in volatile acidity of 0.25 g/L after *L. thermotolerans* sequential inoculation. Some previous studies reported a reduced concentration of acetaldehyde in fermentation with *L. thermotolerans* in comparison with pure *S. cerevisiae* [11,17,18]. Glycerol production may also be enhanced by co-fermentation with *L. thermotolerans* and *S. cerevisiae* compared to *S. cerevisiae* fermentation in monoculture [5,19].

Lachancea thermotolerans, like other non-*Saccharomyces* yeasts, significantly affects the volatile aroma profile, which is one of the most important features that determines wine quality and distinctiveness. According to their origin, volatile compounds are often classified into varietal, fermentation, and aging aromas [20]. Varietal aroma compounds derive from grapes and are later transformed during pre-fermentation processes and fermentation. This class includes mainly terpenoids and norisoprenoids, while certain grape cultivars may also contain significant amounts of thiols and methoxypyrazines. The fermentation process yields numerous compounds, with a key impact on the aroma of all wines in general, including higher alcohols, fatty acids, and especially esters. Besides modulating the initial composition, the wine-aging process can produce particular other compounds and, in this way, further affect the volatile profile of wine. Several studies showed significant effects of the use of *L. thermotolerans* in fermentation on volatile aroma profile of wine, with strain-specific impacts, as well as the impact of inoculation timing. For example, in sequential fermentation, this species was shown to be able to increase the levels of particular higher alcohols and esters and decrease aldehydes and certain fatty acids [21]. Hranilović et al. [12] reported about higher production of isobutyric acid and ethyl esters in wines produced by sequentially inoculated *L. thermotolerans*, the same as Hranilović et al. [19] and Benito et al. [17] observed for ethyl lactate and isobutanol, respectively, and Vaquero et al. [22] for 1-propanol. Despite several valuable reports, the aromatic potential of *L. thermotolerans* has still not been distinguished well, probably because of the limited number of aromatic compounds that can be determined by conventional analytical techniques which have been mostly used in studies so far. In this way, many potentially important effects and compounds remained undiscovered. In this study, together with

conventional gas chromatographic techniques, comprehensive untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) was used to analyze the volatile aroma potential of *L. thermotolerans* in detail and compare it with that of an *S. cerevisiae* control. Two gas chromatographic columns with stationary phases of different polarity and different lengths were used, connected with a modulator which transfers the effluent from a primary column to additional separation in a shorter secondary column. Application of this technique results in mass spectra without interference and enhanced sensitivity and, consequently, a much larger number of identified volatile compounds [23,24]. Besides volatile compounds, this study addressed the repercussions of fermentation with *L. thermotolerans* on other important wine components, such as phenols and pathogenesis-related proteins which were investigated from this aspect poorly [25] or not at all up to date, respectively.

The aim of this study was to significantly deepen the level of knowledge about the influence of co-fermentation with *Lachancea thermotolerans* on the chemical composition of white wine. In addition to basic physico-chemical parameters and evaluation of sensory quality, the focus was especially directed towards detailed characterization of the composition of volatile aroma compounds using the currently most advanced analytical techniques, such as GC×GC/TOF-MS, as well as towards the first findings on the influence of *L. thermotolerans* on phenols and proteins originating from grapes. The experiment was performed with Malvazija istarska (*Vitis vinifera* L.) white grape must, which, in certain terroirs and growing seasons, results in wines with low acidity and high alcohol content, so the results may also have a practical significance.

2. Materials and Methods

2.1. Preparation of Yeast Inoculum

Lachancea thermotolerans (Levulia® Alcomeno) (*LEV*) was purchased from AEB s.p.a., (Brescia, Italy) and *S. cerevisiae* (Lalvin EC1118®) (*SCE*) was purchased from Lallemand Inc. (Montreal, QC, Canada). The yeasts were grown from rehydrated cultures on YPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 28 °C. After three days of incubation, single colonies were transferred into YPD broth (50 mL in 100 mL flasks) for overnight incubation at 24 °C and stirring at 120 rpm to reach concentrations around 10^8 cells/mL. Commercially available pasteurized grape juice (diluted at 50:50 (*v*/*v*) with deionized water to 100 mL in 300 mL flasks) was inoculated with a portion of fermenting YPD broth at 10^7 cell/mL and stirred overnight for additional incubation (24 °C and 120 rpm). Inoculation of grape juice from the experiment was performed directly from the liquid cultures. *Lachancea thermotolerans* was inoculated at 2×10^6 cells/mL, and when the alcohol level reached 2.0% vol., sequential inoculation of *S. cerevisiae* was performed at 1×10^6 cells/mL (*LEV* treatment). *Saccharomyces cerevisiae*, as a control, was inoculated in monoculture at 2×10^6 cell/mL (*SCE* control treatment). Cell density was determined by measuring optical density at 600 nm (OD600), using a Cary 50 UV/Vis spectrophotometer (Varian Inc., Harbor City, CA, USA).

2.2. Vinification

The grapes of Malvazija istarska (*Vitis vinifera* L.), the most important native white grape cultivar in Croatia, were handpicked from the experimental vineyard of the Institute of Agriculture and Tourism in Poreč, Istria, Croatia. All the equipment was carefully and thoroughly sanitized before use. The grapes (3280 kg) were destemmed, crushed, and pressed immediately after harvest using a closed-type pneumatic press of 500 L capacity with the pressures of 2×0.5 bar and 1×0.8 bar (Letina Inox d.o.o., Čakovec, Croatia). The obtained juice was sulfited and cold-settled with the aid of Endozym Rapid pectolytic enzymes at 2 g/hL (AEB s.p.a. Brescia, Italy) for 48 h at 10 °C. The grape must, after settling (2080 L), had a total acidity of 4.7 g/L, pH of 3.41 and 22.1 Brix°. Total acidity was adjusted by adding 1.3 g/L of tartaric acid to obtain the concentration of 6 g/L; after the addition, the pH was set to 3.27. A portion of the homogenized must was distributed in 5 L

demijohns equipped with an airlock and inoculated to start the fermentation, as described above. All fermentations were performed at 17 °C in triplicates. After 36 h, the grape must was supplemented with diammonium phosphate (Corimpex Service Srl, Romans d'Isonzo, Italy) at 30 g/hL. Sugar concentration was monitored daily by a portable density meter DMA 35 (Anton Paar, Graz, Austria). Control fermentation *SCE* lasted 23 days, while *LEV* fermentation lasted 27 days (reducing sugars < 4.0 g/L). After fermentation, wines were racked and left to spontaneously settle for 3 weeks, and then, after another racking, samples were taken for analysis. The concentration of free SO_2 was tracked continuously during the entire process and adjusted to 30 mg/L via the addition of potassium metabisulfite after fermentation, as well as before and after racking, and prior to sampling, if necessary.

2.3. Analysis

2.3.1. Standard Oenological Parameters

Standard physico-chemical parameters: Alcoholic strength by volume, total dry extract, total acidity, volatile acidity, and pH were determined according to the OIV methods [26]. Analysis of organic acids and glycerol was performed by high-performance liquid chromatography (HPLC) using an Agilent Infinity 1260 system equipped with a G1311B quaternary pump, a G1329B autosampler, a G1316A column oven, a G4212B DAD detector (for analysis of organic acids), and a G7162A RID detector (for analysis of glycerol) (Agilent Technologies, Santa Clara, CA, USA). Sample aliquots of 0.5 mL were diluted in 1.0 mL of ultrapure water, filtered through 0.45 µm PTFE filters, and then 10 µL was injected onto an Agilent Hi-Plex H column (300 mm × 7.7 mm, particle size 8 µm) with a PL Hi-Plex H guard (5 mm × 3 mm) (Agilent Technologies). The eluent used was 4 mM sulfuric acid with the flow rate of 0.5 mL/min at 70 °C. UV/Vis chromatograms were recorded at 210 nm. RID flow cell was maintained at 50 °C during analysis. Comparison of retention times and UV/Vis spectra to those of pure standards was used for identification, while quantification was performed using calibration curves. Standard solutions were prepared in 13 vol % of ethanol and pH 3.3.

2.3.2. Major Volatile Aroma Compounds

Direct injection gas chromatography with flame-ionization detection (GC/FID) was performed to analyze acetaldehyde, ethyl acetate, methanol, and major higher alcohols. A Varian 3350 GC (Varian Inc., Harbor City, CA, USA) was equipped with an Rtx-WAX capillary column (60 m × 0.25 mm i.d. × 0.25 µm d.f.) (Restek, Belafonte, PA, USA). Split ratio of 1:20 was applied. Prior to quantification using calibration curves, internal standard 1-pentanol was used for normalization. Other major volatile compounds were extracted by headspace solid-phase microextraction (HS-SPME) using a divinylbenzene/Carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS; StableFlex, 50/30 µm, 1 cm; Supelco, Bellafonte, PA, USA), and the analysis was carried out by GC/MS using a Varian 3900 GC coupled to a Saturn 2100T ion trap MS (Varian Inc.). The column used was the same as in the GC/FID analysis. Operation conditions and identification, quantification, and validation parameters were previously described by Lukić et al. [23].

2.3.3. Minor Volatile Compounds

Minor volatile aroma compounds were extracted via HS-SPME, using a DVB-CAR-PDMS fiber (StableFlex, 50/30 µm, 2 cm; Supelco, Sigma Aldrich, Milan, Italy). The samples were injected in splitless mode by a Gerstel MPS autosampler (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany) and analyzed via GC×GC/TOF-MS, using an Agilent 7890N GC (Agilent Technologies) connected to a LECO Pegasus IV time-of-flight MS (TOF-MS) (Leco Corporation, St. Joseph, MI, USA). The system was equipped with two columns of different dimensions and polarity connected by a modulator. The first-dimension column (30 m × 0.25 mm × 0.25 µm d.f. VF-WAXms) (Agilent Technologies) was held at 40 °C for 4 min, then increased to 250 °C at 6 °C/min, and then maintained at 250 °C for 5 min. The second-dimension column (1.5 m × 0.15 mm × 0.15 µm Rxi 17Sil MS) (Restek) was maintained

at temperatures of 5 °C higher than those applied for the first-dimension column throughout the analysis. Helium carrier gas flow rate was 1.2 mL/min. To acquire mass spectra in the 40–350 m/z range, EI mode with 70 eV was used. Baseline correction, chromatogram deconvolution, and peak alignment were conducted by LECO ChromaTOF software version 4.32 (Leco Corporation). Other operation conditions and identification and quantification parameters were reported previously by Carlin et al. [24] and Lukić et al. [23].

2.3.4. Phenolic Compounds

Phenolic compounds were analyzed by ultra-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC/QqQ-MS/MS). An Acquity UPLC system, connected to a Xevo TQ MS system with an ESI source, was employed for this purpose (Waters Corporation, Milford, MA, USA), according to the method by Vrhovsek et al. [27]. The samples were filtered through 0.2 µm PTFE filters and injected by an autosampler onto a reverse phase Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 µm) (Waters). Two mobile phases, water and acetonitrile, both containing 0.1% (v/v) formic acid, were employed. The specific multistep linear solvent gradients, conditions for MS/MS detection utilizing multiple reaction monitoring (MRM), and quantification details were described previously [27,28]. Data processing was performed using MassLynx 4.1 and Target Lynx 4.1. software (Waters Corporation).

Total phenolic content was determined using the Folin–Ciocâlteu colorimetric method. Cary 50 UV/Vis spectrophotometer (Varian Inc.) was used to measure the absorbance at 765 nm. The results were reported in mg/L of gallic acid equivalents (GAEs).

2.3.5. Analysis of Pathogenesis-Related (PR) Proteins and Determination of Protein Stability

The analysis of PR proteins was conducted using reversed-phase high-performance liquid chromatography (RP-HPLC), following the methods established by Marangon et al. [29] and Van Sluyter et al. [30]. The Agilent Infinity 1260 system (Agilent Technologies) was the same as for the analysis of organic acids and glycerol. Prior to injection, the samples were filtered through 0.45 µm PTFE filters, and 100 µL of each sample was injected into a C8 column (4.6 mm × 250 mm, particle size 5 µm, Vydac 208TP54) with a C8 guard (4.6 mm × 5 mm, particle size 5 µm, Vydac 208GK54), and the DAD detector was used for detection at 210 nm under conditions described previously [31]. The two solvents were A, 0.1% (v/v) trifluoroacetic acid (TFA) in 80% acetonitrile; and B, 0.1% TFA in 8% acetonitrile, using the gradient program reported in a previous study [31]. The flow was set at 1 mL/min at room temperature. Thaumatin-like proteins peaks were eluted between 9 and 12 min, while chitinases were eluted between 18.5 and 24.5 min [29]. The concentrations of PR proteins were determined using a calibration curve created with thaumatin from *Thaumatococcus daniellii* (Sigma, St. Louis, MO, USA), assuming a relative response factor equal to one.

Bentonite doses to achieve protein stability of wines were determined to the nearest 10 g/hL after testing with a variety of doses ranging from 50 to 200 g/hL. Increasing bentonite doses were added to the aliquots of wine in 100 mL glass cylinders, and the standard heat stability test, which included filtration of the sample, heating, and cooling, was applied [32,33], as described in detail in previous studies [31,34]. The minimal dose required for complete protein stabilization was defined as the amount at which the difference in haze produced, measured in nephelometric turbidity units (NTU), between a heated sample and an unheated control was less than 2 NTU. These measurements were performed using a nephelometric turbidity meter Hanna Instruments HI 83749 (Padova, Italy).

2.3.6. Sensory Analysis

The quantitative descriptive sensory analysis was performed by a panel of five trained and certified tasters (three females and two males aged between 30 and 50); a majority of them were members of the Croatian Enological Society and with extensive experience in sensory analysis of Malvazija istarska wine. The sensory panel is accredited according to the EN ISO/IEC 17025:2017 standard, ("General requirements for the competence of

testing and calibration laboratories") [35] for organoleptic (sensory) testing of wines, using the method prescribed by the Ordinance on Wine and Fruit Wine Sensory Testing from the "Official Gazette" No. 106/04, with all amendments concluding No. 1/15 [36], which was the official method for the assessment of wine sensory quality for release on the Croatian market at the time when the study was performed. Before sensory analysis, several preliminary training tests were performed. Qualitative (selection of descriptors) and quantitative (intensity of perception) criteria of the tasters were attuned by tasting representative samples of Malvazija istarska wine. Specific conditions were maintained to control and minimize the influence of any external elements, including noise, visual stimulation, and ambient odor. Wine samples stored at 11 °C were served in random order in standard wine-tasting glasses (ISO 3591:1977) [37] at room temperature of 20 °C. The tasters used a 10-point scale to rate the aroma or taste intensity of each descriptor (0–10, from not perceptible (0) to strongly perceptible (10)). The tasters also evaluated the varietal typicity of the investigated Malvazija istarska wines based on their experience using a 10-point structured scale (0–10; not typical (0) to very typical (10)). The 100-point OIV method was also applied to evaluate the overall quality of the produced wines.

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test ($p < 0.05$) were used to determine statistically significant differences between the two treatments (n = 3). ANOVA was performed with Statistica v. 13.2 software (StatSoft Inc., Tulsa, OK, USA). Hierarchical clustering analysis was performed by MetaboAnalyst v. 6.0 (http://www.metaboanalyst.ca, accessed on 20 August 2024).

3. Results and Discussion

3.1. Standard Oenological Parameters

Lachancea thermotolerans has an unusual and useful ability to partially convert fermentable sugars into L-lactic acid instead of ethanol during alcoholic fermentation [3]. In this study, as reported in Table 1, *LEV* wine had a mildly but significantly lower ethanol content (12.9 vol %) and increased concentration of L-lactic acid (0.86 mg/L) in comparison with *SCE* control wine (13.1 vol % and 0.08 mg/L, respectively). The increase in lactic acid concentration did not affect total wine acidity with a statistical significance, although a higher level was noted in *LEV* wine. Benito [11] reported about the changes in total wine acidity from 0 g/L to 5 g/L depending on the concentration of L-lactic acid produced as a result of *L. thermotolerans* activity, while the highest recorded concentration of lactic acid formed by *L. thermotolerans* under oenological conditions exceeded 16 g/L [13]. Hranilović et al. [8] observed a dichotomy between the performances of particular *L. thermotolerans* strains, with decreases in pH values from up to 0.5 units as a result of increased concentration of lactic acid on one side to concentrations comparable to *S. cerevisiae* control on the other. The performance of *L. thermotolerans* in lactic acid production and ethanol reduction was shown to be significantly affected by fermentation matrix and conditions. For example, the same strain under the same inoculation regime reduced the alcoholic strength by 1.6 vol % in sterile and only by 0.3 vol % in non-sterile conditions [10]. In this study, *LEV* wine had significantly increased the total dry extract without reducing sugars. Together with the content of alcohol, total dry extract can affect the viscosity of wine that contributes to the fullness of its body [38]. No significant difference in glycerol concentration was observed between the two investigated wines, although the concentration determined in *LEV* fermentation was slightly higher. Such a result was in line with the findings reported by Snyder et al. [39], Porter et al. [5], and Benito et al. [17], who noted a higher production of glycerol by a *L. theromotolerans* strain in sequential fermentation, although, in some cases, without a significant difference when compared to *S. cerevisiae.* In a recent study, no significant differences in glycerol concentrations were achieved by sequential inoculation and co-inoculation with *L. thermotolerans* in comparison to a *S. cerevisiae* control, although significant differences between different *L. thermotolerans* strains were observed [8].

Table 1. Standard physico-chemical parameters of Malvazija istarska white wine produced by fermentation with different yeasts.

Physico-Chemical Parameters	Treatment	
	SCE	***LEV***
Alcohol (vol %)	13.10 ± 0.08 [a]	12.88 ± 0.07 [b]
Total dry extract without reducing sugars (g/L)	17.87 ± 0.21 [b]	18.83 ± 0.40 [a]
Total acidity (g/L)	5.6 ± 0.1	6.0 ± 0.3
pH	3.21 ± 0.02	3.22 ± 0.03
Volatile acidity (g/L)	0.47 ± 0.03	0.45 ± 0.05
Citric acid (g/L)	0.37 ± 0.00 [a]	0.32 ± 0.00 [b]
Tartaric acid (g/L)	2.69 ± 0.02 [b]	2.73 ± 0.00 [a]
Malic acid (g/L)	2.03 ± 0.04	2.06 ± 0.06
Lactic acid (g/L)	0.08 ± 0.00 [b]	0.86 ± 0.14 [a]
Glycerol (g/L)	5.33 ± 0.10	5.54 ± 0.15

Abbreviations: *SCE*—*Saccharomyces cerevisiae* (control, monoculture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2. Volatile Aroma Compounds

In order to investigate the volatile aroma potential of the investigated *L. thermotolerans* strain, direct-injection targeted GC/FID and targeted GC/MS were combined with untargeted GC×GC/TOF-MS analysis. Three hundred seventy-three major and minor volatile aroma compounds were identified or tentatively identified, a number not reachable by conventional GC techniques alone. Conventional GC is based on the separation using a single column, while GC×GC uses two columns connected with a modulator, which collects the effluent from the first column every few seconds and focuses collected fractions into the secondary column, allowing an additional separation due to different characteristics of the stationary phases and column temperatures. Such a system ensures higher separation efficiency, enhanced sensitivity, and clearer mass spectra without interference. The results for each chemical class of volatile aroma compounds were sorted into separate tables in descending order based on their *F*-ratio values determined by one-way ANOVA; that is their differentiation potential (Tables 2–13).

3.2.1. Hydrocarbons

In the group of hydrocarbons (Table 2), 3-methylene-4-vinylcyclohex-1-ene and *cis*-2-methyl-7-octadecene had significantly higher concentrations in *SCE* wine. *Trans*,*trans*-2,6-dimethyl-1,3,5,7-octatetraene showed a tendency towards having a higher concentration in *LEV* wine, although without a significant difference (Table 2).

Table 2. Concentrations (µg/L) of hydrocarbons found in Malvazija istarska white wines produced using different yeasts determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
HY1	3-Methylene-4-vinylcyclohex-1-ene	MS	1672	-	11.195	0.053 ± 0.017 [a]	0.017 ± 0.007 [b]
HY2	*cis*-2-Methyl-7-octadecene	MS	1866	-	11.153	0.141 ± 0.014 [a]	0.096 ± 0.019 [b]
HY3	Azulene	MS, LRI	1754	1746	7.293	2.25 ± 0.20	1.83 ± 0.18
HY4	*trans*,*trans*-2,6-Dimethyl-1,3,5,7-octatetraene	MS, LRI	1456	1460	1.279	3.70 ± 0.356	4.34 ± 0.91

Table 2. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
HY5	1-Tetradecene	MS, LRI	1477	1444	0.393	2.69 ± 0.27	2.79 ± 0.06
HY6	Pentadecane	MS, LRI	1503	1500	0.108	1.04 ± 0.06	1.01 ± 0.19
HY7	1,3,5,5-Tetramethyl-1,3-cyclohexadiene ‡	MS	1405	1370	0.059	0.427 ± 0.019	0.445 ± 0.129
HY8	*trans*,*cis*-2,4-Dodecadiene	MS, LRI	1604	-	0.043	0.608 ± 0.142	0.628 ± 0.086

Abbreviations: Co.—compound's code. ID—identification of compounds: MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.2. Terpenoids

Terpenoids, normally found in wines, originate from grapes mainly as odorless, potentially volatile glycosidically bound (up to 95% of the total) or polyhydroxylated precursors, as well as free volatile terpenoids. To influence wine aroma, bound molecules have to be enzymatically and/or chemically cleaved to release volatile aglycons. Terpenoids are primarily affected by cultivar and growing condition; however, different yeast species and strains show varying enzymatic activities and may affect the release of volatile, odoriferous aglycons to different extents and proportions during fermentation, in this way affecting their concentration and impact on the aroma of finished wines.

Cis,*trans*-farnesol, geraniol, and menthol had a significantly higher concentration in *LEV* compared to *SCE* wine (Table 3). Zhang et al. [40] reported an increase in geraniol concentration in wines produced by sequential inoculation with commercial and indigenous *L. thermotolerans* strains with respect to a *S. cerevisiae* control. The majority of the other identified terpenoids showed lower concentration in *LEV* wine or no significant difference between the two investigated wines. The concentrations of major monoterpenols (other than geraniol), which are generally considered to exhibit a more significant influence on wine aroma, such as linalool, citronellol, α-terpineol, nerol, and hotrienol, did not differ between the treatments. Such results were in line with previous research published by Dutraive et al. [41] and Zhang et al. [40] in which no effect of *L. thermotolerans* was observed regarding linalool, citronellol, α-terpineol, and total terpenes concentrations. Escribano-Viana et al. [42] reported about the low β-glucosidase activity of various *L. thermotolerans* strains, suggesting a weaker impact on terpenoid concentrations in the corresponding wines.

Table 3. Concentrations (μg/L) of terpenoids found in Malvazija istarska white wines produced using different yeasts determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
TE1	*trans*-2-Pinanol	MS, LRI	1520	1522	151.843	3.80 ± 0.08 ^{a}	2.32 ± 0.19 ^{b}
TE2	Terpenoid n.i. I	MS	1779	-	112.763	0.587 ± 0.033 ^{a}	0.346 ± 0.021 ^{b}
TE3	Epoxyterpinolene	MS, LRI	1492	1486	112.467	1.33 ± 0.05 ^{a}	0.77 ± 0.08 ^{b}
TE4	Citronellol	S, MR, LRI	1766	1760	91.516	1.15 ± 0.07 ^{a}	0.58 ± 0.08 ^{b}

Table 3. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
TE5	Citronellyl acetate	MS, LRI	1666	1659	85.743	0.788 ± 0.088 [a]	0.305 ± 0.022 [b]
TE6	Carvone	MS, LRI	1741	1742	43.432	0.167 ± 0.027 [a]	0.06 ± 0.006 [b]
TE7	*trans*-β-Ocimene	S, MS, LRI	1250	1250	27.247	11.34 ± 1.51 [a]	5.30 ± 1.32 [b]
TE8	Cadalene	MS, LRI	2227	2226	26.201	0.192 ± 0.027 [a]	0.110 ± 0.006 [b]
TE9	*cis*-Calamenene	MS, LRI	1841	1840	21.188	0.272 ± 0.029 [a]	0.192 ± 0.007 [b]
TE10	*cis*-Alloocimene	MS, LRI	1382	1369	19.327	1.10 ± 0.09 [a]	0.74 ± 0.11 [b]
TE11	Neryl ethyl ether	MS, LRI	1482	1477	16.859	1.31 ± 0.07 [a]	0.78 ± 0.21 [b]
TE12	*cis*,*trans*-Farnesol	MS, LRI	2350	2351	16.818	0.112 ± 0.056 [b]	0.394 ± 0.105 [a]
TE13	Farnesene isomer I	MS, LRI	1672	1685	15.556	2.00 ± 0.25 [a]	1.27 ± 0.21 [b]
TE14	Estragole	MS, LRI	1679	1676	13.727	0.139 ± 0.014 [a]	0.099 ± 0.012 [b]
TE15	*p*-Menth-1-en-9-al	MS, LRI	1622	1629	13.159	1.13 ± 0.05 [a]	0.90 ± 0.09 [b]
TE16	α-Curcumene	MS, LRI	1785	1782	12.096	0.141 ± 0.027 [a]	0.082 ± 0.011 [b]
TE17	*trans*-Alloocimene	MS, LRI	1403	1400	11.921	1.15 ± 0.16 [a]	0.74 ± 0.13 [b]
TE18	Farnesene isomer II	MS, LRI	1754	1757	9.610	0.243 ± 0.062 [a]	0.118 ± 0.033 [b]
TE19	α-Ocimene	MS, LRI	1235	1245	9.279	10.10 ± 3.30 [a]	4.13 ± 0.79 [b]
TE20	Geraniol	S, MS, LRI	1847	1847	8.289	0.98 ± 0.26 [b]	1.46 ± 0.13 [a]
TE21	Menthol	MS, LRI	1641	1641	8.246	0.83 ± 0.06 [b]	1.04 ± 0.11 [a]
TE22	Limonene	S, MS, LRI	1193	1195	8.220	4.90 ± 1.63 [a]	2.12 ± 0.38 [b]
TE23	*cis*-Furan linalool oxide	S, MS, LRI	1445	1448	7.860	1.44 ± 0.09 [a]	1.06 ± 0.21 [b]
TE24	Nerol oxide	MS, LRI	1477	1473	7.843	4.35 ± 0.28 [a]	3.52 ± 0.43 [b]
TE25	β-Myrcene	S, MS, LRI	1160	1159	7.659	8.02 ± 3.10	3.00 ± 0.55
TE26	Terpenoid n.i. II	MS	1456	-	6.644	47.12 ± 3.13	30.50 ± 10.72
TE27	Dihydrolinalyl acetate	MS, LRI	1531	-	6.022	0.096 ± 0.093	0.400 ± 0.194
TE28	γ-Terpinene	MS, LRI	1245	1239	5.961	2.69 ± 0.93	1.30 ± 0.33
TE29	*trans*-Furan linalool oxide	S, MS, LRI	1471	1472	5.948	0.556 ± 0.037	0.487 ± 0.032
TE30	α-Calacorene	MS, LRI	1926	1928	4.899	0.434 ± 0.055	0.347 ± 0.04
TE31	Geranyl acetone	MS, LRI	1860	1856	3.999	4.31 ± 0.39	3.29 ± 0.80
TE32	Cyclomyral	S, MS, LRI	1722	-	3.855	1.21 ± 0.27	1.52 ± 0.03
TE33	*cis*-Ocimenol	MS, LRI	1691	-	3.158	0.304 ± 0.043	0.256 ± 0.019
TE34	4-Terpineol	S, MS, LRI	1604	1604	2.475	0.907 ± 0.06	0.643 ± 0.284
TE35	α-Phellandrene	MS, LRI	1174	1160	2.403	0.300 ± 0.136	0.170 ± 0.053
TE36	*cis*-Rose oxide	MS, LRI	1358	1350	2.035	0.224 ± 0.040	0.180 ± 0.036
TE37	α-Terpineol	MS, LRI	1704	1701	1.991	14.30 ± 1.23	15.57 ± 0.96
TE38	Nerolidol	MS, LRI	2040	2031	1.861	0.502 ± 0.176	0.644 ± 0.038
TE39	α-Bisabolene	MS, LRI	1736	1740	1.673	0.052 ± 0.020	0.067 ± 0.007
TE40	Ho-trienol	MS, LRI	1610	1612	1.635	11.41 ± 1.26	9.81 ± 1.77
TE41	Linalool ‡	S, MS, LRI	1542	1542	1.502	30.04 ± 3.89	33.01 ± 1.60
TE42	Dihydrolinalool	MS, LRI	1435	1420	1.493	2.14 ± 1.56	1.01 ± 0.32
TE43	Dihydromyrcenol	MS, LRI	1466	1455	1.365	1.90 ± 0.91	1.27 ± 0.19
TE44	Borneol	MS, LRI	1710	1714	1.154	0.296 ± 0.055	0.340 ± 0.044
TE45	β-Pinene ‡	MS, LRI	1146	1145	1.089	8.12 ± 0.67	8.62 ± 0.50
TE46	Terpenoid n.i. III	MS	1207	-	1.032	2.92 ± 0.68	3.36 ± 0.29
TE47	Linalool ethyl ether	MS, LRI	1324	1331	0.862	23.68 ± 4.73	19.27 ± 6.73
TE48	Nerol	S, MS, LRI	1804	1801	0.827	1.14 ± 0.23	1.26 ± 0.07
TE49	Neryl acetate	MS, LRI	1731	1733	0.557	0.408 ± 0.031	0.381 ± 0.057
TE50	Geranyl acetate	MS, LRI	1760	1759	0.059	1.28 ± 0.15	1.26 ± 0.09
TE51	3-Carene	MS, LRI	1155	1159	0.053	2.62 ± 2.35	2.29 ± 0.763

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.3. Norisoprenoids

Norisoprenoids in wine are mainly formed through biodegradation of carotenoids during pre-fermentation steps and fermentation. In this work, *LEV* wine showed a tendency towards higher concentration of an important odorant, *trans*-β-damascenone, although without a significant difference when compared to control *SCE* wine (Table 4). β-Damascenone is responsible for odours of stewed apple, dried plum, and honey. Another norisoprenoid with a high *F*-ratio, β-ionone, known for contributing with violet aroma in wine [43], was found in increased concentration in *LEV* wine. Particular other compounds from the group of norisoprenoids, such as an ionene isomer (n.i.), a vitispirane isomer, and 1,2-dihydro-1,5,8-trimethyl-naphthalene, as well as 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and *trans*-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB), had lower concentrations in *LEV* than in control *SCE* wine. The differences observed possibly arose from differential activity of β-glycosidases in the two investigated yeasts, as well as their possible interaction with carotenoid cleavage oxygenases from grapes.

Table 4. Concentrations (μg/L) of norisoprenoids found in Malvazija istarska white wines produced using different yeasts determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
NO1	Ionene derivative n.i.	MS	1525	1567	22.507	0.111 ± 0.009 [a]	0.034 ± 0.026 [b]
NO2	Vitispirane isomer II	MS, LRI	1537	1543	14.451	3.09 ± 0.24 [a]	1.89 ± 0.49 [b]
NO3	Ionene derivative n.i.	MS	1704	-	13.850	0.154 ± 0.014 [a]	0.102 ± 0.020 [b]
NO4	β-Cyclocitral	S, MS, LRI	1629	1630	12.866	0.313 ± 0.013 [a]	0.269 ± 0.017 [b]
NO5	β-Ionone ‡	MS, LRI	1916	1915	12.574	0.546 ± 0.054 [b]	0.727 ± 0.070 [a]
NO6	1,2-Dihydro-1,5,8-trimethyl-naphthalene	MS, LRI	1754	1751	11.728	1.84 ± 0.20 [a]	1.15 ± 0.29 [b]
NO7	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	S, MS, LRI	1722	1722	10.920	0.173 ± 0.065 [a]	0.025 ± 0.043 [b]
NO8	*trans*-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB)	MS, LRI	1835	1832	10.780	0.477 ± 0.153 [a]	0.166 ± 0.059 [b]
NO9	Norisoprenoid n.i.	MS	1697	-	6.330	0.730 ± 0.054	0.479 ± 0.164
NO10	Theaspirane isomer	MS, LRI	1536	1540	5.821	1.33 ± 0.14	1.07 ± 0.12
NO11	α-Ionene	MS, LRI	1559	1565	4.647	0.428 ± 0.070	0.243 ± 0.132
NO12	Damascenone isomer	MS	1741	-	4.127	0.152 ± 0.018	0.122 ± 0.019
NO13	trans-β-Damascenone	MS, LRI	1829	1829	2.982	21.65 ± 5.69	28.19 ± 3.26
NO14	α-Isomethyl ionone ‡	MS, LRI	1835	1848	1.319	0.702 ± 0.098	0.923 ± 0.318
NO15	*cis*-β-Damascenone	MS, LRI	1771	1774	0.339	1.95 ± 0.37	2.11 ± 0.30
NO16	Vitispirane isomer I ‡	MS, LRI	1521	1524	0.199	1.15 ± 0.29	1.27 ± 0.34
NO17	Safranal	MS, LRI	1654	1648	0.082	0.202 ± 0.017	0.198 ± 0.014

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.4. Carbonyl Compounds—Aldehydes and Ketones

As reported in Table 5, acetaldehyde, the most important wine volatile carbonyl yeast product was found in lower concentration in *LEV* than in *SCE* wine, which was in line with

the results reported by Benito et al. [17], while Vaquero et al. [22] reported the opposite. When present at low levels in wine, its contribution is often associated with fruity notes, while at higher concentrations, it is reminiscent of nuts and overripe apple [44]. A lower concentration of heptanal was also determined in *LEV* wine. Isobutanal, on the other hand, occurred only in *LEV* wine.

The ketones produced during vinification are generally considered yeast species and strain-specific. In this work, significant differences between the two investigated wines were observed for almost all of the identified ketones. Apart from an increase in acetoin and 3-(acetoxy)-4-methyl-2-pentanone concentrations in *LEV* wine, majority of other ketones were found in higher concentrations in *SCE* wine. Vaquero et al. [22] observed an increased level of acetoin in wine fermented with *L. thermotolerans* yeast when compared to *S. cerevisie*, while Ciani et al. [18] observed the opposite. It is known that acetoin production exhibits a high degree of variability, depending on the specific yeast strain used in fermentation [6]. It can be formed through several pathways from pyruvic acid via intermediates such as acetaldehyde, butanedione, and α-acetolactate.

Table 5. Concentrations (μg/L if not otherwise indicated) of carbonyl compounds, aldehydes and ketones, found in Malvazija istarska white wines produced using different yeasts determined by targeted gas chromatography with flame-ionization detection (GC/FID) ¤ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
	Aldehydes						
AD1	Heptanal	MS, LRI	1184	1187	99.080	4.41 ± 0.33 [a]	0.65 ± 0.57 [b]
AD2	Acetaldehyde (mg/L) ¤	S	<1100	714	31.333	18.05 ± 1.65 [a]	11.75 ± 1.04 [b]
AD3	Isobutanal	MS, LRI	<1100	833	3.999	0.000 ± 0.000	0.134 ± 0.116
AD4	Dodecanal	MS, LRI	1716	1713	2.826	1.24 ± 0.64	0.61 ± 0.06
AD5	Undecanal	S, MS, LRI	1608	1610	1.298	0.824 ± 0.931	0.212 ± 0.050
AD6	2-Nonenal	MS, LRI	1543	1540	1.191	0.583 ± 0.194	0.755 ± 0.193
AD7	Octanal	MS, LRI	1294	1281	0.414	0.282 ± 0.049	0.236 ± 0.113
AD8	Nonanal	MS, LRI	1399	1403	0.090	16.10 ± 1.49	17.78 ± 9.58
AD9	2,6,6-Trimethyl-1-cyclohexene-1-acrolein	MS	1933	-	0.085	0.171 ± 0.008	0.174 ± 0.016
AD10	Decanal	S, MS, LRI	1503	1504	0.017	5.47 ± 0.55	5.26 ± 2.67
	Ketones						
KE1	2-Nonanone	S, MS, LRI	1392	1392	379.548	220.1 ± 5.6 [a]	68.7 ± 12.3 [b]
KE2	2-Heptanone	MS, LRI	1179	1181	214.055	4.82 ± 0.35 [a]	1.67 ± 0.13 [b]
KE3	2-Undecanone	MS, LRI	1598	1598	192.430	9.90 ± 0.76 [a]	3.35 ± 0.31 [b]
KE4	Acetoin	S, MS, LRI	1282	1285	85.793	8.78 ± 0.54 [b]	12.41 ± 0.41 [a]
KE5	2-Dodecanone	MS, LRI	1710	1709	29.384	0.726 ± 0.07 [a]	0.491 ± 0.026 [b]
KE6	2-Decanone	MS, LRI	1498	1503	20.497	1.69 ± 0.10 [a]	1.29 ± 0.11 [b]
KE7	*p-tert*-Butylcyclohexanone	MS, LRI	1641	1645	13.685	0.467 ± 0.030 [a]	0.337 ± 0.053 [b]
KE8	3-(Acetoxy)-4-methyl-2-pentanone	MS	1466	-	8.470	0.332 ± 0.031 [b]	0.404 ± 0.029 [a]
KE9	3-Undecanone	MS, LRI	1570	1586	4.738	0.329 ± 0.036	0.264 ± 0.037
KE10	1-Hydroxy-3-methyl-2-butanone	MS	1450	-	2.247	1.12 ± 0.08	1.04 ± 0.036
KE11	6-Methyl-5-hepten-2-one	MS, LRI	1345	1343	0.007	0.776 ± 0.08	0.768 ± 0.124

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE—Saccharomyces cerevisiae* (control, pure culture); *LEV—Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.5. Alcohols

The concentration of the majority of alcohols with the highest *F*-ratio was significantly lower in *LEV* than in *SCE* wine, with the exception of *cis*-6-nonen-1-ol, 2-methyl-5-nonanol, 3-nonanol, 2-ethyl-2-(hydroxymethyl)-1,3-propanediol, and 6-methyl-5-hepten-2-ol (Table 6). *LEV* fermentation showed a tendency towards higher concentrations of some other minor alcohols, although without a significant difference. Among major alcohols, methanol and isobutanol were found in higher concentrations in *LEV* wine. Such a result for isobutanol was in line with previous findings by Vaquero et al. [22], while Hranilović et al. [8] reported variable concentrations of isobutanol produced by different *L. thermotolerans* strains under various inoculation regimes, although not significantly different from that found in control *S. cerevisiae* fermentation. 1-Propanol and isoamyl alcohol were found in lower concentrations in *LEV* than in *SCE* wine. The same trend for 1-propanol was reported by Vaquero et al. [22]. 1-Propanol, isobutanol, and isoamyl alcohol are known contributors to the aroma of all fermented alcoholic beverages. In total concentrations above 300 mg/L, they may have a negative influence with their medicinal and solvent-like odors [44]. 2-Phenylethanol, a carrier of a pleasant odor reminiscent of roses, was also found in lower concentrations in *LEV* than in *SCE* wine. The same was reported by Chen et al. [45], while Gobbi et al. [16] noticed an increased concentration in fermentation with *L. thermotolerans*. Hranilović et al. [8] observed variable concentrations of major higher alcohols in wines produced under sequential and co-inoculation regimes with different strains of *L. thermotolerans*; in some cases they were higher and in others lower than those found in control wine obtained via *S. cerevisiae* monoculture fermentation. The effects observed in this study suggest a different metabolism of higher alcohol amino acid precursors between *L. thermotolerans* and *S. cerevisiae* yeasts, while the discrepancies between different studies reveal apparent strain-specific effects, probably in interaction with other compositional characteristics and production conditions depending on the study. The concentrations of C_6-alcohols, which are mainly formed via the degradation of lipids catalyzed by hydroperoxide lyase and lipoxygenase enzymes in pre-fermentation steps did not differ between the treatments (Table 6).

Table 6. Concentrations (µg/L, if not otherwise indicated) of alcohols found in Malvazija istarska white wines produced using different yeasts determined by targeted gas chromatography with flame-ionization detection (GC/FID) ¤, targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡, and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
AL1	2-Heptanol	S, MS, LRI	1319	1312	1693.390	9.17 ± 0.05 ^{a}	2.12 ± 0.29 ^{b}
AL2	2-Nonanol	S, MS, LRI	1520	1518	1015.857	69.54 ± 2.12 ^{a}	15.87 ± 2.01 ^{b}
AL3	2-Undecanol	MS, LRI	1722	1723	756.958	5.27 ± 0.17 ^{a}	1.22 ± 0.19 ^{b}
AL4	3-Ethoxy-1-propanol	MS, LRI	1377	1379	194.427	23.99 ± 2.87 ^{a}	0.74 ± 0.29 ^{b}
AL5	1-Heptanol	MS, LRI	1456	1457	192.084	16.58 ± 0.54 ^{a}	10.08 ± 0.61 ^{b}
AL6	Isobutanol (mg/L) ¤	S, MS, LRI	1090	1098	167.389	14.49 ± 0,13 ^{b}	26.13 ± 1.55 ^{a}
AL7	3-Methylpentanol	S, MS, LRI	1329	1322	132.272	144.7 ± 16.1 ^{a}	35.9 ± 3.1 ^{b}
AL8	2-Phenylethanol (mg/L) ‡	S, MS, LRI	1891	1893	106.218	34.61 ± 2.05 ^{a}	20.84 ± 1.08 ^{b}
AL9	1-Propanol (mg/L) ¤	S	-	1035	103.811	23.53 ± 0.31 ^{a}	18.50 ± 0.80 ^{b}
AL10	4-Methylpentanol	MS, LRI	1314	1309	100.639	54.87 ± 7.33 ^{a}	12.27 ± 0.60 ^{b}
AL11	Isoamyl alcohol (mg/L) ¤	S, MS, LRI	1229	1229	93.326	164.9 ± 1.3 ^{a}	134.1 ± 5.4 ^{b}
AL12	1-Octanol	MS, LRI	1553	1558	67.072	34.09 ± 1.61 ^{a}	23.33 ± 1.61 ^{b}
AL13	*cis*-3-Octen-3-ol	MS	1450	1452	34.675	21.70 ± 0.44 ^{a}	17.27 ± 1.23 ^{b}
AL14	*cis*-6-Nonen-1-ol	MS, LRI	1716	1714	18.124	0.89 ± 0.04 ^{b}	1.11 ± 0.08 ^{a}
AL15	2-Methyl-5-nonanol	MS	1575	-	15.989	0.436 ± 0.015 ^{b}	0.497 ± 0.022 ^{a}

Table 6. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
AL16	3-Methyl-3-buten-1-ol	MS, LRI	1245	1244	13.885	0.731 ± 0.096 [a]	0.503 ± 0.044 [b]
AL17	1-Pentanol	MS, LRI	1245	1244	12.886	12.59 ± 1.30 [a]	9.09 ± 1.08 [b]
AL18	*cis*-2-Hexen-1-ol ‡	MS, LRI	1416	1413	11.083	17.54 ± 0.91 [a]	14.45 ± 1.33 [b]
AL19	1-Dodecanol	MS, LRI	1968	1973	9.806	1.90 ± 0.32 [a]	1.30 ± 0.07 [b]
AL20	3-Nonanol	MS, LRI	1492	1493	9.402	0.367 ± 0.008 [b]	0.400 ± 0.016 [a]
AL21	2-Ethyl-2-(hydroxymethyl)-1,3-propanediol	MS	1926	-	9.353	0.200 ± 0.039 [b]	0.275 ± 0.015 [a]
AL22	6-Methyl-5-hepten-2-ol	S, MS, LRI	1461	1460	8.053	0.154 ± 0.014 [b]	0.194 ± 0.02 [a]
AL23	1-Undecanol	MS, LRI	1865	1871	6.775	0.412 ± 0.095	0.254 ± 0.046
AL24	3-Octanol	S, MS, LRI	1392	1393	6.492	1.20 ± 0.04	1.13 ± 0.03
AL25	1,4-Butanediol	MS, LRI	1918	1911	6.251	1.11 ± 0.33	2.84 ± 1.15
AL26	*trans*-3-Hexen-1-ol ‡	MS, LRI	1366	1361	6.183	75.45 ± 2.49	68.08 ± 4.49
AL27	3,5-Dimethyl-4-heptanol	MS, LRI	1742	-	5.762	0.316 ± 0.047	0.251 ± 0.005
AL28	*trans*-2-Octen-1-ol	S, MS, LRI	1615	1618	5.471	1.66 ± 0.04	1.52 ± 0.09
AL29	2,3-Butanediol isomer	S, MS, LRI	1573	1576	4.078	383.4 ± 33.3	339.7 ± 17.3
AL30	1-Decanol	MS, LRI	1766	1767	3.672	5.83 ± 0.32	5.12 ± 0.56
AL31	2-Ethyl-1-hexanol	MS, LRI	1487	1490	2.938	12.08 ± 2.47	19.61 ± 7.19
AL32	1-Nonanol	S, MS, LRI	1660	1661	2.856	3.73 ± 0.93	4.75 ± 0.49
AL33	Methanol (mg/L) ¤	S	<1000	911	2.792	60.20 ± 1.73	69.40 ± 9.38
AL34	3-Ethyl-4-methylpentan-1-ol	MS	1466	1506	2.705	0.246 ± 0.133	0.097 ± 0.084
AL35	1-Hexanol (mg/L) ‡	S, MS, LRI	1356	1357	1.706	1.53 ± 0.044	1.46 ± 0.08
AL36	1,3-Propanediol	MS, LRI	1785	1789	1.530	0.460 ± 0.014	0.802 ± 0.479
AL37	*cis*-3-Hexen-1-ol ‡	S, MS, LRI	1389	1389	1.448	42.77 ± 2.01	46.16 ± 4.451
AL38	3-Ethyl-4-methylpentan-1-ol	MS	1509	1506	0.977	1.62 ± 0.10	1.54 ± 0.07
AL39	*cis*-4-Decen-1-ol	MS, LRI	1797	1797	0.224	0.162 ± 0.036	0.147 ± 0.041
AL40	2,3-Butanediol isomer	S, MS, LRI	1587	1584	0.209	4.07 ± 7.04	2.02 ± 3.23
AL41	2-Decanol	MS, LRI	1616	1621	0.176	0.726 ± 0.086	0.677 ± 0.186
AL42	2-Phenoxyethanol	MS, LRI	2147	2144	0.034	0.926 ± 0.768	0.837 ± 0.329
AL43	2-Methyl-2-buten-1-ol	MS, LRI	1319	1320	0.002	0.269 ± 0.038	0.268 ± 0.012

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.6. Acids

LEV wine was more abundant in isobutyric acid than *SCE* wine (Table 7). Saturated branched short-chain fatty acids are produced through the degradation of amino acids via the Ehrlich pathway [44], the same as their higher-alcohol analogues, so the results obtained for isobutyric acid and isobutanol (Table 6) indicated specific differences in valine metabolism among the two yeasts analyzed. Other particular branched-chain acids showed lower concentrations in *LEV* than in *SCE* wine. A number of minor acids were identified, but the differences in their concentration were not significant between the treatments. No significant differences were observed for the major linear medium-chain acids formed from acetyl-CoA through the fatty acid synthase (FAS) complex, such as hexanoic, octanoic, and decanoic acid, which are important contributors to wine aroma with their cheesy and fatty odors. A few previous studies reported a weaker production of fatty acids in co-fermentation with *L. thermotolerans* than in fermentation performed with *Saccharomyces cerevisiae* in monoculture [3,19,22].

Table 7. Concentrations (μg/L, if not otherwise indicated) of acids found in Malvazija istarska white wines produced using different yeasts determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
AC1	2-Methylbutyric acid	MS, LRI	1675	1674	140.473	61.10 ± 2.96 [a]	37.60 ± 1.74 [b]
AC2	Isovaleric acid	S, MS, LRI	1672	1675	70.105	181.2 ± 16.8 [a]	76.8 ± 13.5 [b]
AC3	Isohexanoic acid	MS, LRI	1810	1809	17.914	0.393 ± 0.049 [a]	0.249 ± 0.032 [b]
AC4	2-Methylpropenoic acid	MS, LRI	1697	-	16.205	0.148 ± 0.021 [a]	0.094 ± 0.009 [b]
AC5	Isobutyric acid	S, MS, LRI	1570	1570	8.101	1.95 ± 0.17 [b]	2.57 ± 0.33 [a]
AC6	Propanoic acid	S, MS, LRI	1537	1540	7.407	5.02 ± 0.54	3.75 ± 0.60
AC7	Tetradecanoic acid	MS, LRI	2696	2693	6.125	0.635 ± 0.098	0.494 ± 0.007
AC8	Hexanoic acid (mg/L) ‡	S, MS, LRI	1824	1828	3.675	6.74 ± 0.71	5.76 ± 0.52
AC9	Heptanoic acid	S, MS, LRI	1954	1955	2.922	4.63 ± 0.32	3.98 ± 0.57
AC10	Undecanoic acid	MS, LRI	2346	2359	2.833	0.039 ± 0.029	0.010 ± 0.009
AC11	Butyric acid ‡	S, MS, LRI	1617	1612	2.829	1.46 ± 0.08	1.31 ± 0.13
AC12	2-Propenoic acid	MS	1641	-	2.190	0.740 ± 0.023	0.890 ± 0.174
AC13	9-Decenoic acid	MS, LRI	2330	2335	1.861	13.41 ± 1.80	11.29 ± 2.00
AC14	Octanoic acid (mg/L) ‡	S, MS, LRI	2043	2042	1.696	7.10 ± 0.96	6.21 ± 0.69
AC15	Pivalic acid	MS, LRI	1581	1579	1.486	1.73 ± 0.31	1.43 ± 0.29
AC16	2-Ethylhexanoic acid	MS, LRI	1953	1960	1.321	3.71 ± 0.77	4.41 ± 0.73
AC17	3-Octenoic acid	MS	2102	-	0.700	1.67 ± 0.79	1.21 ± 0.55
AC18	Decanoic acid (mg/L) ‡	S, MS, LRI	2257	2258	0.425	2.60 ± 0.45	2.34 ± 0.52
AC19	Pentanoic acid	S, MS, LRI	1741	1751	0.305	3.24 ± 0.21	3.39 ± 0.44
AC20	Nonanoic acid	S, MS, LRI	2168	2168	0.057	21.43 ± 8.17	23.88 ± 15.82
AC21	*trans*-2-Hexenoic acid	MS, LRI	1968	1967	0.007	0.529 ± 0.042	0.525 ± 0.086
AC22	4-Methyl-3-pentenoic acid	MS	1595	-	0.004	1.50 ± 0.14	1.49 ± 0.44

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.7. Esters

Volatile esters, which are well-known contributors to the formation of the aroma and flavor character of wine, are mostly formed during fermentation and storage [44]. The results for esters identified in this study are presented in Table 8.

Ethyl esters are formed through several biosynthetic pathways, and it is considered that their concentrations in wine depend more on the precursor availability than on the activity of genes encoding the corresponding enzymes [46]. *LEV* wine had higher concentration of particular ethyl esters, including the ester of pyruvate, an important product of glycolysis and intermediate/precursor for the synthesis of volatile compounds [47], which could point to particular differences between *L. thermotolerans* and *S. cerevisiae* in the expression of genes that participate in the initial steps of yeast metabolism. *LEV* wine also contained increased amounts of certain esters with unknown sensory relevance, such as ethyl 3-hydroxyhexanoate, ethyl 9-decenoate isomers I and II, ethyl 3-hydroxybutyrate, ethyl 3-acetoxyoctanoate, ethyl hexanoate I and II, ethyl 2-butenoate, and ethyl 2-hexenoate II, as well as ethyl isobutyrate, an important contributor to wine aroma with its fruity odor. The increase in ethyl isobutyrate corresponded to several previous studies on *L. thermotolerans* [8,10,48] and was in line with the higher concentrations of its precursor formed in the Ehrlich pathway, isobutyric acid, in *LEV* wine (Table 7). The concentration of ethyl lactate, which is formed via the esterification of ethanol and lactic acid, was almost four times

higher in *LEV* than in *SCE* wine as a direct consequence of the higher concentration of lactic acid observed in the former wine (Table 1). Such an outcome was in line with previous studies on *L. thermotolerans* co-fermentation [8,19]. Ethyl lactate can have an influence on wine aroma with its buttery notes when present in high concentrations. The concentrations of important esters formed through the Ehrlich pathway from their amino acid precursors, such as ethyl 2- and 3-methylbutyrate, carriers of fruity notes, were higher in *SCE* than in *LEV* wine, suggesting a difference in their metabolism between the yeasts. This was in line with the higher concentration of isoamyl alcohol in *SCE* wine (Table 6) and with the fact that the mentioned esters and alcohol are formed from the same amino acid precursors, leucine and isoleucine. Concentrations of major linear medium-chain ethyl esters, such as ethyl hexanoate, octanoate, and decanoate formed from acetyl-CoA within the FAS complex, did not significantly differ between the two treatments, although a tendency towards a higher concentration of ethyl hexanoate in *SCE* and ethyl decanoate in *LEV* wine was observed. Benito et al. [17] reported an increase in the total amount of ethyl esters after sequential fermentation with *L. thermotolerans* in comparison with *S. cerevisiae* monoculture, while Escribano-Viana et al. [6] reported the opposite after monoculture fermentation with this yeast compared to *S. cerevisiae*. Hranilović et al. [8] observed inferior levels of linear medium-chain ethyl ester obtained after sequential inoculations with *L. thermotolerans*, although particular strains produced quantities comparable to those found in *S. cerevisiae* control wine. Such discrepancies confirm that these effects are strain-specific, although different conditions among the studies probably also had an influence.

Important odoriferous acetates, such as ethyl, isobutyl, butyl, and especially isoamyl acetate, were found in higher concentration in *LEV* wine (Table 8). Contrary to ethyl esters, it was previously found that the production of acetates is more dependent on the expression of alcohol acetyltransferase genes than on precursor concentrations [46,49]. A minor acetate, 3-methylheptyl acetate, also showed an elevated concentration in *LEV* wine. Hranilović et al. [50] reported an increase in acetate ester levels after sequential fermentation with *L. thermotolerans* in comparison with *S. cerevisiae* in monoculture, as well as variable results with some strains exceeding and some being comparable to the levels obtained by *S. cerevisiae* control [8]. Escribano-Viana et al. [6] reported a decrease in the concentration of acetates as a consequence of *L. thermotolerans* activity. Control *SCE* wine contained higher concentrations of particular minor acetates and 2-phenethyl acetate, an important wine odorant (Table 8).

Isoamyl lactate and ethyl phenyl lactate were strongly influenced by *LEV* fermentation, and their concentrations were significantly increased compared to those observed in *SCE* control wine, thus confirming the dependence of the formation of its esters on the availability of lactic acid. The result for isoamyl lactate was in agreement with that obtained by Zhang et al. [25], who reported an increase in its concentration achieved by different inoculation ratios for sequentially inoculated *L. thermotolerans* followed by *S. cerevisiae.* For ethyl phenyl lactate, which could also be considered a marker of *L. thermotolerans* activity, no information was found in the literature published to date, probably because previous studies on this topic used conventional analytical techniques with limited compound identification capabilities. Hexyl propyl oxalate was also increased by *LEV* treatment, the same as two esters of succinic acid, ethyl butyl succinate and a major compound, diethyl succinate. Succinic acid was not determined in this study; however, a negative influence of *L. thermotolerans* co-fermentation on its concentration was determined in a previous study [8]. Vicente et al. [21] also reported an increase in diethyl succinate concentration in a fermentation with *L. thermotolerans*. Isobutyl hexanoate showed a tendency towards a higher concentration in *LEV* wine, the same as some esters of dicarboxylic acids, such as diethyl malonate, diethyl malate, and diethyl 2-hydroxyglutarate, derived from α-keto acids. A larger number of other esters were found in higher concentration in *SCE* wine, including esters of higher alcohols and fatty acids, as well as methyl hexanoate and diethyl glutarate.

Table 8. Concentrations (μg/L if not otherwise indicated) of ethyl esters, acetate esters, and other esters found in Malvazija istarska white wines produced using different yeasts determined by targeted gas chromatography with flame-ionization detection (GC/FID) ¤, targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡, and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compound	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
	Ethyl esters						
EE1	Ethyl propanoate ‡	MS, LRI	<1000	949	2592.195	26.25 ± 0.37 [a]	13.61 ± 0.21 [b]
EE2	Ethyl 3-methylbutyrate ‡	S, MS, LRI	1065	1065	578.293	12.04 ± 0.42 [a]	5.27 ± 0.25 [b]
EE3	Ethyl acetylacetate	MS, LRI	1462	1466	261.142	0.409 ± 0.037 [a]	0.062 ± 0.006 [b]
EE4	Ethyl pyruvate	MS, LRI	1270	1267	163.902	8.06 ± 1.02 [b]	16.00 ± 0.33 [a]
EE5	Ethyl 3-hydroxydecanoate	MS, LRI	2104	2102	72.829	3.24 ± 0.32 [a]	1.26 ± 0.24 [b]
EE6	Ethyl 3-hydroxyhexanoate	MS, LRI	1685	1677	59.259	0.241 ± 0.019 [b]	0.343 ± 0.013 [a]
EE7	Ethyl lactate (mg/L) ‡	S, MS, LRI	1341	1341	52.936	11.83 ± 0.95 [b]	46.02 ± 8.08 [a]
EE8	Ethyl 2-methylbutyrate ‡	S, MS, LRI	1049	1049	39.279	3.94 ± 0.33 [a]	2.59 ± 0.19 [b]
EE9	Ethyl 9-decenoate isomer I	MS, LRI	1697	1697	16.676	43.45 ± 1.76 [b]	85.87 ± 17.91 [a]
EE10	Ethyl isobutyrate ‡	MS, LRI	<1000	965	14.930	19.67 ± 1.00 [b]	26.44 ± 2.86 [a]
EE11	Ethyl *cis*-11-hexadecenoate	MS, LRI	2281	2236	14.795	0.803 ± 0.097 [a]	0.358 ± 0.176 [b]
EE12	Ethyl 3-acetoxyoctanoate	MS, LRI	1897	1898	13.484	2.13 ± 0.14 [b]	2.94 ± 0.35 [a]
EE13	Ethyl 2-octenoate	MS, LRI	1559	1557	11.992	0.395 ± 0.013 [a]	0.296 ± 0.048 [b]
EE14	Ethyl 4-hexenoate I ‡	MS, LRI	1300	1292	10.357	0.824 ± 0.053 [b]	1.001 ± 0.079 [a]
EE15	Ethyl nonanoate	MS, LRI	1537	1535	9.558	7.98 ± 1.64 [a]	4.47 ± 1.09 [b]
EE16	Ethyl hexadecanoate	MS, LRI	2251	2241	9.538	21.3 ± 7.26 [a]	6.84 ± 3.61 [b]
EE17	Ethyl 9-decenoate isomer II	MS, LRI	1729	1712	9.365	0.491 ± 0.108 [b]	1.199 ± 0.386 [a]
EE18	Ethyl 3-hydroxybutyrate	MS, LRI	1520	1524	9.214	2.48 ± 0.20 [b]	2.91 ± 0.14 [a]
EE19	Ethyl octadecanoate	MS, LRI	2463	2464	8.266	0.323 ± 0.133 [a]	0.086 ± 0.052 [b]
EE20	Ethyl 2-butenoate ‡	MS, LRI	1153	1153	8.129	41.01 ± 1.11 [b]	45.86 ± 2.73 [a]
EE21	Ethyl 2-hexenoate II	MS, LRI	1361	1357	7.939	0.165 ± 0.037 [b]	0.303 ± 0.076 [a]
EE22	Ethyl butyrate ‡	S, MS, LRI	1030	1030	7.670	598.6 ± 19.5	520.3 ± 44.9
EE23	Ethyl 2-hydroxy-4-methylvalerate	MS, LRI	1542	1547	6.118	13.95 ± 1.38	17.16 ± 1.78
EE24	Ethyl heptanoate	MS, LRI	1340	1342	5.567	8.84 ± 0.50	6.62 ± 1.55
EE25	Ethyl tetradecanoate	MS, LRI	2054	2054	5.553	8.30 ± 1.91	4.17 ± 2.36
EE26	Ethyl hexanoate (mg/L) ‡	S, MS, LRI	1242	1236	4.635	1.40 ± 0.16	1.11 ± 0.17
EE27	Ethyl *trans*-2-butenoate	MS, LRI	1160	1158	3.651	19.35 ± 0.72	18.27 ± 0.67
EE28	Ethyl undecanoate	MS, LRI	1747	1739	2.757	0.551 ± 0.111	0.434 ± 0.052
EE29	Ethyl 2-hexenoate I	MS, LRI	1350	1357	2.422	14.68 ± 0.76	16.94 ± 2.40
EE30	Ethyl *cis*-3-hexenoate	MS, LRI	1307	1295	1.848	4.11 ± 0.79	4.76 ± 0.25
EE31	Ethyl dodecanoate ‡	S, MS, LRI	1843	1843	1.536	1.23 ± 0.37	0.88 ± 0.33
EE32	Ethyl *trans*-4-decenoate	MS, LRI	1672	1680	0.798	0.305 ± 0.064	0.443 ± 0.260
EE33	Ethyl nonanoate	MS, LRI	1495	1509	0.659	0.842 ± 1.181	0.256 ± 0.408
EE34	Ethyl decanoate (mg/L) ‡	S, MS, LRI	1637	1638	0.605	2.42 ± 0.48	2.89 ± 0.93
EE35	Ethyl 2-decenoate	MS, LRI	1766	1750	0.459	0.150 ± 0.002	0.132 ± 0.047
EE36	Ethyl 7-octenoate	MS, LRI	1482	1486	0.363	2.14 ± 0.49	1.84 ± 0.71
EE37	Ethyl 4-hexenoate II ‡	MS, LRI	1361	1357	0.318	0.842 ± 0.029	0.890 ± 0.143
EE38	Ethyl 4-hydroxybutyrate	MS, LRI	1804	1796	0.266	9.21 ± 2.66	8.40 ± 0.63
EE39	Ethyl octanoate (mg/L) ‡	S, MS, LRI	1435	1435	0.149	1.67 ± 0.39	1.53 ± 0.49
	Acetate esters						
AE1	Isobutyl acetate ‡	S, MS, LRI	1015	1009	440.677	111.7 ± 1.4 [b]	258.1 ± 12.0 [a]
AE2	3-Ethoxypropyl acetate	MS	1361	-	354.339	11.88 ± 0.45 [a]	2.37 ± 0.75 [b]
AE3	2-Ethyl-1-hexanyl acetate	MS	1480	-	101.131	14.84 ± 0.63 [a]	7.62 ± 1.07 [b]
AE4	Diol acetate n.i.	MS	1741	-	67.913	44.51 ± 5.82 [a]	15.90 ± 1.52 [b]
AE5	Isoamyl acetate (mg/L) ‡	S, MS, LRI	1133	1133	66.338	6.64 ± 0.24 [b]	8.69 ± 0.37 [a]
AE6	Butyl acetate	MS, LRI	<1100	1064	55.089	42.57 ± 2.40 [b]	63.81 ± 4.34 [a]

Table 8. *Cont.*

Co.	Volatile Aroma Compound	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
AE7	*trans*,*trans*-2,4-Octadienyl acetate	MS	1570	-	34.005	0.262 ± 0.026 [a]	0.134 ± 0.028 [b]
AE8	Isopropyl acetate ‡	MS, LRI	<1000	901	18.565	72.67 ± 2.89 [a]	61.17 ± 3.61 [b]
AE9	Octyl acetate ‡	MS, LRI	1481	1483	18.052	7.88 ± 1.40 [a]	3.47 ± 1.13 [b]
AE10	*cis*-6-Nonen-1-yl acetate	MS, LRI	1634	1634	14.909	0.852 ± 0.299 [a]	0.183 ± 0.021 [b]
AE11	Propyl acetate	MS, LRI	<1100	982	11.483	43.93 ± 0.45 [a]	28.59 ± 7.83 [b]
AE12	Ethyl acetate (mg/L) ¤	S, MS, LRI	<1100	890	10.734	26.33 ± 3.53 [b]	50.33 ± 12.19 [a]
AE13	2-Phenethyl acetate ‡	S, MS, LRI	1803	1801	10.173	455.0 ± 47.7 [a]	360.2 ± 19.3 [b]
AE14	3-Methylheptyl acetate	MS, LRI	1385	1395	8.379	0.852 ± 0.113 [b]	1.858 ± 0.591 [a]
AE15	Pentyl acetate	MS, LRI	1169	1185	6.820	8.29 ± 0.69	10.30 ± 1.13
AE16	*cis*-3-Hexenyl acetate	MS, LRI	1314	1308	3.529	268.2 ± 5.8	231.2 ± 33.5
AE17	Methyl acetate ‡	MS, LRI	<1000	813	2.736	22.40 ± 0.83	20.75 ± 1.51
AE18	1,3 Butanediol diacetate	MS, LRI	1785	1768	1.349	3.71 ± 4.24	0.87 ± 0.07
AE19	Heptenyl acetate	MS	1408	-	1.166	0.740 ± 0.242	0.530 ± 0.234
AE20	Hexyl acetate ‡	S, MS, LRI	1272	1272	0.047	436.9 ± 138.4	455.8 ± 60.3
	Other esters						
OE1	Propyl hexanoate	MS, LRI	1324	1319	92.313	3.04 ± 0.13 [a]	1.51 ± 0.24 [b]
OE2	Phenylethyl isobutyrate	MS, LRI	1888	1896	91.705	1.04 ± 0.07 [a]	0.43 ± 0.08 [b]
OE3	Pyruvic acid ester n.i.	MS	1779	-	75.225	3.68 ± 0.65 [a]	0.38 ± 0.11 [b]
OE4	Ethyl butyl succinate	MS, LRI	1797	1820	73.147	0.230 ± 0.018 [b]	0.424 ± 0.035 [a]
OE5	Isoamyl lactate	MS, LRI	1570	1572	66.426	2.36 ± 0.23 [b]	8.50 ± 1.28 [a]
OE6	Isoamyl isovalerate	MS, LRI	1298	1294	65.410	0.411 ± 0.046 [a]	0.186 ± 0.015 [b]
OE7	Isoamyl butyrate	MS, LRI	1266	1266	63.264	11.84 ± 0.49 [a]	6.33 ± 1.09 [b]
OE8	Phenethyl isovalerate	MS, LRI	1968	1983	45.331	2.32 ± 0.21 [a]	1.10 ± 0.23 [b]
OE9	Ethyl isoamyl succinate	MS, LRI	1903	1907	31.104	3.80 ± 0.17 [a]	2.90 ± 0.22 [b]
OE10	Propyl octanoate	MS, LRI	1520	1530	20.373	1.64 ± 0.16 [a]	0.98 ± 0.20 [b]
OE11	Isoamyl hexanoate	S, MS, LRI	1461	1458	19.946	27.12 ± 3.40 [a]	15.21 ± 3.13 [b]
OE12	Diethyl succinate ‡	MS, LRI	1677	1669	19.174	294.1 ± 22.3 [b]	363.8 ± 16.3 [a]
OE13	Hexyl propyl oxalate	MS	1525	-	18.498	1.01 ± 0.05 [b]	1.28 ± 0.10 [a]
OE14	Methyl hexanoate	S, MS, LRI	1179	1188	17.685	15.59 ± 1.83 [a]	8.47 ± 2.30 [b]
OE15	Diethyl glutarate	MS, LRI	1785	1780	16.773	0.210 ± 0.027 [a]	0.142 ± 0.011 [b]
OE16	Methyl 2-hydroxy-4-methylpentanoate	MS, LRI	1477	1470	15.092	0.862 ± 0.181 [a]	0.183 ± 0.243 [b]
OE17	Hexyl propanoate	MS, LRI	1345	1342	13.903	0.400 ± 0.016 [a]	0.120 ± 0.129 [b]
OE18	Butyl hexanoate	MS, LRI	1419	1416	12.007	0.084 ± 0.002 [a]	0.065 ± 0.009 [b]
OE19	Isoamyl octanoate	MS, LRI	1660	1657	11.764	33.31 ± 6.66 [a]	18.12 ± 3.81 [b]
OE20	Isoamyl butyrate ‡	MS, LRI	1262	1266	9.771	10.54 ± 1.61 [a]	6.67 ± 1.41 [b]
OE21	Ethyl phenyl lactate	MS, LRI	2281	2273	9.405	0.731 ± 0.054 [b]	1.054 ± 0.174 [a]
OE22	Isobutyl hexanoate	MS, LRI	1356	1357	7.571	2.29 ± 0.20	3.05 ± 0.43
OE23	2-Phenethyl octanoate	MS, LRI	2388	2373	7.189	1.88 ± 0.31	1.00 ± 0.49
OE24	Ethyl methyl succinate	MS, LRI	1635	1642	7.110	0.607 ± 0.058	0.491 ± 0.049
OE25	Isoamyl decanoate	MS, LRI	1866	1864	5.425	21.07 ± 3.82	11.86 ± 5.68
OE26	Diethyl malonate	MS, LRI	1581	1582	4.466	0.684 ± 0.037	0.751 ± 0.041
OE27	Propyl decanoate	MS, LRI	1729	1743	4.392	0.405 ± 0.035	0.284 ± 0.093
OE28	Methyl octanoate	MS, LRI	1397	1399	3.216	79.69 ± 3.40	65.51 ± 13.26
OE29	Propyl formate	MS, LRI	<1100	916	3.084	0.582 ± 0.480	1.658 ± 0.946
OE30	Isoamyl dodecanoate	MS, LRI	2069	2071	2.560	1.44 ± 0.81	0.51 ± 0.59
OE31	Diethyl fumarate	MS, LRI	1654	1647	1.830	0.179 ± 0.009	0.164 ± 0.016
OE32	Diethyl 2-hydroxyglutarate	MS, LRI	2161	2195	1.811	0.290 ± 0.022	0.503 ± 0.273
OE33	Isobutyl octanoate	MS, LRI	1553	1551	1.583	0.529 ± 0.087	0.658 ± 0.156
OE34	β-Phenethyl formate	MS, LRI	1797	1806	1.462	1.53 ± 0.20	2.03 ± 0.70
OE35	Ethyl hydrogen succinate	MS, LRI	2380	2367	1.272	76.88 ± 10.71	62.96 ± 18.49
OE36	Diethyl malate	MS, LRI	2047	2048	1.113	1.60 ± 0.12	1.89 ± 0.45

Table 8. *Cont.*

Co.	Volatile Aroma Compound	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
OE37	Methyl dodecanoate	MS, LRI	1810	1806	0.951	0.206 ± 0.029	0.173 ± 0.05
OE38	Isoamyl isobutyrate	MS, LRI	1188	1194	0.803	0.397 ± 0.014	0.354 ± 0.082
OE39	2-Ethyl-1-hexyl propanoate	MS	1452	-	0.730	1.40 ± 0.20	1.51 ± 0.08
OE40	Methyl decanoate	MS, LRI	1598	1599	0.507	6.70 ± 0.40	6.14 ± 1.30
OE41	Triethyl citrate	MS, LRI	2463	2461	0.002	0.089 ± 0.064	0.087 ± 0.014

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.8. Sulfur-Containing Compounds

In wines, sulfur-containing compounds originate from various sources, including yeast metabolism, more precisely catabolism and anabolism of the sulfur-containing amino acids methionine and cysteine and their derivative homocysteine through the Ehrlich pathway [47,51]. In this study, as reported in Table 9, dihydro-2-methyl-3(2H)-thiophenone, 3-hydroxyethyl-2-hydroxypropyl sulfide I and II, and 3-methionyl acetate had a higher concentration in *LEV* in comparison with the control *SCE* wine. The increased concentration of the acetate ester of methionol, the most abundant sulfur compound in this study, was in line with higher concentrations of abundant higher-alcohol acetates, such as isobutyl, butyl, and especially isoamyl acetate (Table 8), corroborating a possibility of higher activity of particular alcohol acetyltransferases in *L. thermotolerans* compared to *S. cerevisiae*. 2-Thiophenecarboxaldehyde, ethyl 3-(methylthio)propionate, methionol, and ethyl methanesulfonate concentrations were higher in *SCE* wine. Escribano-Viana et al. [42] reported about no activity of sulfite reductase involved in the biosynthesis of sulfur-containing compounds in *L. thermotolerans* strains, while, on the other hand, Comitini et al. [15] observed that all of the investigated *L. thermotolerans* strains showed sulfite reductase activity, suggesting that this characteristic is strongly strain-related. Other determined sulfur-containing compounds identified in this study showed no significant differences between the two investigated yeasts.

Table 9. Concentrations (μg/L) of sulfur containing compounds found in Malvazija istarska white wines produced using different yeasts determined by targeted one-dimensional gas chromatography/mass spectrometry (GC/MS) ‡ and untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
SU1	2-Thiophenecarboxaldehyde	MS, LRI	1704	1701	109.241	0.273 ± 0.027 ^{a}	0.105 ± 0.004 ^{b}
SU2	Ethyl 3-(methylthio)propionate	MS, LRI	1570	1571	95.263	2.72 ± 0.22 ^{a}	1.45 ± 0.07 ^{b}
SU3	Dihydro-2-methyl-3(2H)-thiophenone	MS, LRI	1512	1506	92.128	2.82 ± 0.08 ^{b}	3.31 ± 0.03 ^{a}
SU4	3-Hydroxyethyl 2-hyxdroxypropyl sulfide I	MS	1779	-	75.423	0.21 ± 0.18 ^{b}	1.71 ± 0.24 ^{a}
SU5	3-Hydroxyethyl 2-hyxdroxypropyl sulfide I	MS	1822	-	69.285	0.076 ± 0.010 ^{b}	0.297 ± 0.045 ^{a}
SU6	Methionol	S, MS, LRI	1722	1717	21.853	14.56 ± 1.21 ^{a}	10.50 ± 0.89 ^{b}
SU7	Ethyl methanesulfonate	MS	1691	-	8.972	2.53 ± 0.88 ^{a}	0.97 ± 0.18 ^{b}
SU8	3-Methionyl acetate	MS, LRI	1635	1627	7.876	2.67 ± 0.16 ^{b}	3.23 ± 0.31 ^{a}
SU9	Benzothiazole	MS, LRI	1962	1962	5.833	0.710 ± 0.026	0.609 ± 0.067
SU10	Sulfurol	MS, LRI	2305	2302	4.756	0.446 ± 0.083	0.301 ± 0.079
SU11	4-(Methylthio)-1-butanol	MS, LRI	1841	1812	4.753	0.450 ± 0.107	0.314 ± 0.016
SU12	Isothiocyanatocyclohexane	MS, LRI	1679	1670	4.142	0.793 ± 0.088	0.661 ± 0.071

Table 9. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
SU13	S-Ethyl octanethioate	MS	1525	-	0.889	12.88 ± 0.51	11.17 ± 3.09
SU14	Propyl ethynyl sulfoxide	MS	1559	-	0.831	1.07 ± 0.14	1.21 ± 0.22
SU15	2-Methyltetrahydrothiophen-3-one	MS, LRI	1531	1538	0.488	0.91 ± 0.89	1.49 ± 1.13
SU16	2-(Methylmercapto)benzothiazole ‡	MS, LRI	2433	2422	0.054	0.119 ± 0.004	0.117 ± 0.017

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.9. Furanoids and Lactones

Although furanoids and lactones are normally found in higher amounts in aged wines, they also occur in fresh young wines. In this study, as reported in Table 10, furfural and 4-(1-hydroxyethyl)-γ-butyrolactone were more abundant in *LEV* than in *SCE* wine. Several γ-lactones determined in this study showed a tendency towards higher concentrations in *LEV* wine, but control *SCE* wine contained higher levels of the most abundant ones. *SCE* wine contained higher concentrations of several δ-lactones as well, suggesting higher availability of their hydroxycarboxylic acid precursors and/or enzymatic activity in *S. cerevisiae* control fermentation. Two furanoids, 2-butyltetrahydrofuran and 2-pentylfuran, were also found in higher concentrations in *SCE* wine.

Table 10. Concentrations (μg/L) of furanoids and lactones found in Malvazija istarska white wines produced using different yeasts determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
FL1	δ-Dodecalactone	MS, LRI	2430	2423	139.385	0.364 ± 0.020 ^{a}	0.137 ± 0.027 ^{b}
FL2	2-Butyltetrahydrofuran	MS	1267	-	103.574	35.47 ± 2.41 ^{a}	15.66 ± 2.35 ^{b}
FL3	Furfural	S, MS, LRI	1466	1460	77.515	2.24 ± 0.17 ^{b}	3.42 ± 0.16 ^{a}
FL4	δ-Decalactone	MS, LRI	2197	2193	54.952	0.712 ± 0.067 ^{a}	0.362 ± 0.048 ^{b}
FL5	γ-Nonalactone	S, MS, LRI	2040	2046	38.203	4.63 ± 0.24 ^{a}	3.33 ± 0.28 ^{b}
FL6	γ-Dodecalactone	MS, LRI	2380	2384	27.196	0.243 ± 0.028 ^{a}	0.154 ± 0.009 ^{b}
FL7	γ-Butyrolactone	MS	1635	-	22.795	38.59 ± 2.96 ^{a}	29.38 ± 1.55 ^{b}
FL8	γ-Decalactone	MS, LRI	2154	2152	19.738	2.45 ± 0.22 ^{a}	1.43 ± 0.33 ^{b}
FL9	δ-Octalactone	S, MS, LRI	1976	1976	16.280	0.710 ± 0.033 ^{a}	0.521 ± 0.074 ^{b}
FL10	γ-Octalactone	MS, LRI	1926	1924	15.907	5.05 ± 0.34 ^{a}	3.56 ± 0.55 ^{b}
FL11	4-(1-Hydroxyethyl)-γ-butyrolactone	MS, LRI	2386	2431	10.144	1.33 ± 0.11 ^{b}	3.62 ± 1.24 ^{a}
FL12	2-Pentylfuran	MS, LRI	1229	1231	10.058	0.860 ± 0.075 ^{a}	0.694 ± 0.051 ^{b}
FL13	Mevalonic acid δ-lactone	MS	2551	-	5.846	0.213 ± 0.023	0.313 ± 0.068
FL14	γ-Crotonolactone	MS, LRI	1766	1758	5.706	0.475 ± 0.042	0.775 ± 0.214
FL15	γ-Hexalactone	MS, LRI	1710	1710	3.908	2.99 ± 0.48	1.96 ± 0.77
FL16	2-Hydroxy-γ-butyrolactone	MS	2076	-	2.989	0.11 ± 0.19	1.08 ± 0.96
FL17	γ-Heptalactone	MS, LRI	1815	1811	2.910	0.334 ± 0.124	0.481 ± 0.083
FL18	4-Ethoxy-γ-butyrolactone	MS, LRI	1735	1728	1.606	0.207 ± 0.022	0.224 ± 0.003
FL19	γ-Undecalactone	MS, LRI	2235	2235	1.392	4.66 ± 0.31	8.57 ± 5.74
FL20	α-Methyl-γ-crotonolactone	MS, LRI	1729	1726	0.944	0.186 ± 0.007	0.202 ± 0.029
FL21	δ-Lactone n.i.	MS	1879	-	0.817	0.106 ± 0.048	0.081 ± 0.005

Table 10. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
FL22	δ-Hexalactone	MS, LRI	1804	1798	0.610	0.659 ± 0.079	0.599 ± 0.108
FL23	5-Methyl-5-hydroxyhexanoic acid lactone	MS	1141	-	0.607	1.40 ± 1.28	0.73 ± 0.77
FL24	γ-Valerolactone	MS, LRI	1616	1617	0.477	0.231 ± 0.130	0.283 ± 0.012
FL25	Ethyl 2-furoate	MS, LRI	1629	1628	0.390	26.15 ± 1.50	27.26 ± 2.69
FL26	Solerone	MS, LRI	2076	2096	0.010	1.28 ± 0.19	1.25 ± 0.45

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.10. Benzenoids

1H-indole was the only benzenoid found in a higher concentration in *LEV* wine (Table 11). Benzenoids with the highest *F*-ratios were mostly much more abundant in control *SCE* wine, including particular benzenoids from the phenylalanine metabolism and their derivatives, such as ethyl 2-phenylacetate, ethyl phenethyl ether, and 2-phenylacetaldehyde. This, together with the higher concentration of 2-phenyethanol, implies a greater expression of the responsible genes in *S. cerevisiae* yeast. Besides the transformation of amino acid precursors and inter-conversions of benzenoids during fermentation, Martin et al. [52] reported about the possibility of de novo synthesis of some of these compounds by *Hanseniaspora vineae* (which is also a non-*Saccharomyces* yeast) without the presence of their corresponding precursors from grapes.

Table 11. Concentrations (μg/L) of benzenoids found in Malvazija istarska white wines produced using different yeasts determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
BE1	Ethyl 2-phenylacetate	MS, LRI	1791	1788	104.731	13.75 ± 1.21 ^{a}	6.25 ± 0.40 ^{b}
BE2	Ethyl phenethyl ether	MS	1526	-	52.663	0.877 ± 0.018 ^{a}	0.597 ± 0.064 ^{b}
BE3	4-Ethyl-*m*-xylene	MS, LRI	1377	1373	33.844	1.35 ± 0.06 ^{a}	0.76 ± 0.17 ^{b}
BE4	Durene	MS, LRI	1445	1435	32.607	5.30 ± 0.25 ^{a}	3.54 ± 0.47 ^{b}
BE5	2-Phenylacetaldehyde	S, MS, LRI	1654	1656	29.681	50.80 ± 7.53 ^{a}	25.94 ± 2.41 ^{b}
BE6	Styrene	MS, LRI	1258	1262	16.226	9.75 ± 0.44 ^{a}	5.78 ± 1.65 ^{b}
BE7	Ethyl *o*-methylbenzoate	MS, LRI	1747	1751	15.650	0.17 ± 0.03 ^{a}	0.10 ± 0.02 ^{b}
BE8	Cardene	MS, LRI	1259	1269	15.234	7.94 ± 0.56 ^{a}	6.10 ± 0.60 ^{b}
BE9	Ethyl benzoate	MS, LRI	1672	1680	14.827	6.90 ± 0.27 ^{a}	5.54 ± 0.55 ^{b}
BE10	*o*-Xylene	MS, LRI	1179	1189	12.771	2.04 ± 0.30 ^{a}	1.20 ± 0.27 ^{b}
BE11	Methyl salicylate	MS, LRI	1785	1789	9.929	1.83 ± 0.23 ^{a}	1.38 ± 0.09 ^{b}
BE12	1H-Indole	MS, LRI	2455	2454	9.200	0.80 ± 0.04 ^{b}	2.20 ± 0.80 ^{a}
BE13	*p*-Isopropenylphenol	MS	2455	-	7.899	0.066 ± 0.019 ^{b}	0.118 ± 0.026 ^{a}
BE14	3,3-Dimethoxy-1-phenylpropane-1,2-dione	MS	1471	-	7.601	4.36 ± 0.91	2.85 ± 0.29
BE15	2,4,6-Trimethylbenzoic acid	MS	2714	-	7.567	0.065 ± 0.021	0.143 ± 0.044

Table 11. *Cont.*

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	*LEV*
BE16	*p*-Cymene	MS, LRI	1276	1273	6.634	5.79 ± 0.53	6.67 ± 0.25
BE17	Ethyl phenyl ketone	MS, LRI	1735	1744	6.622	0.167 ± 0.016	0.203 ± 0.019
BE18	2-Methylnaphthalene	MS, LRI	1860	1856	5.436	0.242 ± 0.022	0.207 ± 0.014
BE19	*m*-Di-*tert*-butylbenzene	MS, LRI	1435	1436	5.351	0.358 ± 0.206	0.08 ± 0.03
BE20	4-Ethylbenzaldehyde	MS, LRI	1716	1714	5.185	1.29 ± 0.16	1.79 ± 0.35
BE21	4-Phenylbutenone	MS, LRI	1997	2032	5.072	0.310 ± 0.069	0.448 ± 0.081
BE22	3-Methylacetophenone	MS, LRI	1785	1786	5.013	0.280 ± 0.016	0.243 ± 0.024
BE23	*p*-Methoxyanisole	MS, LRI	1747	1752	4.565	0.80 ± 0.11	1.09 ± 0.21
BE24	3-Ethylacetophenone	MS	1841	-	4.459	0.299 ± 0.033	0.580 ± 0.228
BE25	Phenylacetic acid	MS, LRI	2560	2560	4.414	0.620 ± 0.032	0.463 ± 0.125
BE26	4-Acetylbenzaldehyde	MS	2235	-	4.410	0.85 ± 0.13	1.32 ± 0.36
BE27	3-Phenylbutyric acid	MS	2628	-	4.187	0.036 ± 0.019	0.297 ± 0.220
BE28	4-Ethylacetophenone	MS, LRI	1872	1867	3.875	0.215 ± 0.044	0.444 ± 0.197
BE29	Methyl benzoate	MS, LRI	1629	1624	3.397	0.133 ± 0.003	0.152 ± 0.018
BE30	4-Methylacetophenone	MS, LRI	1766	1763	3.327	0.183 ± 0.031	0.240 ± 0.044
BE31	Benzonitrile	MS, LRI	1610	1614	3.272	1.11 ± 0.29	1.55 ± 0.31
BE32	Benzoic acid	MS, LRI	2438	2432	3.001	5.11 ± 0.60	10.32 ± 5.18
BE33	2,5-Dimethylcrotonophenone	MS	1997	-	2.462	0.171 ± 0.032	0.210 ± 0.029
BE34	1-Phenyl-3-phenethylundecane	MS	1954	-	2.451	0.839 ± 0.135	0.578 ± 0.255
BE35	2-Phenylpropionic acid	MS	2542	-	2.156	0.014 ± 0.012	0.055 ± 0.048
BE36	*p*-Ethylstyrene	MS, LRI	1459	1462	1.732	0.157 ± 0.189	0.013 ± 0.022
BE37	Benzyl acetate	MS, LRI	1735	1739	1.040	0.313 ± 0.029	0.291 ± 0.024
BE38	2-Methylbenzaldehyde	MS, LRI	1629	1622	0.977	0.845 ± 0.074	0.910 ± 0.086
BE39	1-Phenylhexane	MS, LRI	1525	1524	0.965	1.05 ± 0.24	1.19 ± 0.09
BE40	α,α-Dimethylbenzenemethanol	MS, LRI	1766	1770	0.922	0.106 ± 0.033	0.147 ± 0.066
BE41	Benzyl alcohol	S, MS, LRI	1879	1877	0.679	2.64 ± 0.12	2.79 ± 0.29
BE42	1,2,3,4-Tetramethylbenzene	MS, LRI	1503	1505	0.676	0.641 ± 0.026	0.621 ± 0.033
BE43	α-Phenyldiethyl ether	MS	1482	-	0.600	1.01 ± 0.08	0.92 ± 0.19
BE44	1-Methylnaphthalene	MS, LRI	1897	1893	0.429	0.146 ± 0.018	0.163 ± 0.041
BE45	Benzaldehyde	MS, LRI	1525	1538	0.205	4.6 ± 0.72	4.88 ± 0.77
BE46	3-Methylbenzoic acid	MS	2532	-	0.185	0.179 ± 0.058	0.209 ± 0.104
BE47	Octyl benzene	MS, LRI	1741	1741	0.157	1.47 ± 0.32	1.35 ± 0.38
BE48	4-Methylbenzaldehyde	MS, LRI	1655	1655	0.131	0.486 ± 0.079	0.503 ± 0.011
BE49	1,2,3-Trimethylbenzene	MS, LRI	1345	1344	0.095	0.556 ± 0.101	0.578 ± 0.064
BE50	2-Ethyl-*o*-xylene	MS, LRI	1366	1362	0.041	0.974 ± 0.120	0.944 ± 0.229
BE51	2-(4′-Methylphenyl)-propanal	MS	1408	-	0.033	0.531 ± 0.057	0.517 ± 0.128
BE52	3-(1-Methylethyl)benzoic acid	MS	2642	-	0.022	0.031 ± 0.019	0.033 ± 0.012
BE53	*p*-Xylene	MS, LRI	1137	1149	0.004	2.63 ± 0.30	2.71 ± 1.87
BE54	Acetophenone	S, MS, LRI	1660	1660	0.001	3.24 ± 0.54	3.21 ± 1.46

Abbreviations: Co.—compound's code. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.11. Volatile Phenols

2,3,6-Trimethylphenol showed a significantly higher concentration in *LEV* than in *SCE* wine, while for other volatile phenols, significant differences were not determined (Table 12). Vinylphenols and ethylphenols are considered the most important volatile phenols in wine. They are formed in alcoholic fermentation by decarboxylation of ferulic and *p*-

coumaric acid by yeast hydroxycinnamic acid decarboxylases, respectively [44]. Higher levels of ethylphenols are indicative of *Dekkera/Brettanomyces* spoilage and can impart wine with negative odors. Several non-*Saccharomyces* yeasts, including *L. thermotolerans*, were previously found to produce lower levels of vinylphenols than *S. cerevisiae* [53].

Table 12. Concentrations (µg/L) of volatile phenols found in Malvazija istarska white wines produced using different yeasts determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
VP1	2,3,6-Trimethylphenol	MS, LRI	2004	2028	13.048	0.066 ± 0.010 [b]	0.116 ± 0.022 [a]
VP2	Phenol	S, MS, LRI	2011	2012	4.977	4.08 ± 0.26	5.08 ± 0.74
VP3	4-Vinylphenol	MS, LRI	2393	2406	3.362	0.586 ± 0.202	0.308 ± 0.168
VP4	*p-tert*-Amylphenol	MS	2413	-	3.257	0.193 ± 0.051	0.111 ± 0.060
VP5	4-Ethylphenol	MS, LRI	2177	2181	3.014	0.306 ± 0.166	0.479 ± 0.046
VP6	2-Ethylphenol	MS, LRI	2076	2071	1.701	0.103 ± 0.049	0.048 ± 0.052
VP7	4-Vinylguaiacol	S, MS, LRI	2197	2196	1.363	0.707 ± 0.163	0.531 ± 0.204
VP8	Guaiacol	MS, LRI	1866	1869	0.318	0.076 ± 0.019	0.083 ± 0.010
VP9	*o*-Cresol	MS, LRI	2011	2011	0.257	0.087 ± 0.004	0.089 ± 0.007
VP10	Thymol	MS, LRI	2183	2187	0.000	0.103 ± 0.020	0.103 ± 0.022

Abbreviations: No.—number of compounds. ID—identification of compounds: S—retention time accordant with that of a pure standard; MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. MS—compound that were tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE—Saccharomyces cerevisiae* (control, pure culture); *LEV—Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.12. Other Compounds

The concentration of other identified compounds did not significantly differ between the two treatments (Table 13).

Table 13. Concentrations (µg/L) of other compounds found in Malvazija istarska white wines produced using different yeasts determined by untargeted two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) sorted by decreasing Fisher's *F*-ratio.

Co.	Volatile Aroma Compounds	ID	LRI_{exp}	LRI_{lit}	*F*-Ratio	Treatment	
						SCE	***LEV***
OC1	*cis*-5-Hydroxy-2-methyl-1,3-dioxane	MS, LRI	1498	1494	3.994	0.242 ± 0.297	0.587 ± 0.036
OC2	1-Octen-3-ol, methyl ether	MS	1411	-	3.767	0.000 ± 0.000	0.154 ± 0.137
OC3	(3-Methylphenyl) methanol, 2-methylpropyl ether	MS	1968	-	0.703	0.500 ± 0.12 0	0.416 ± 0.127
OC4	Dimethylmaleic anhydride	MS, LRI	1741	1755	0.205	0.118 ± 0.015	0.130 ± 0.044
OC5	Glutaconic anhydride	MS	1997	-	0.065	1.91 ± 0.09	1.88 ± 0.17

Abbreviations: Co.—compound's code. ID—identification of compounds: MS—mass spectra accordant with that from NIST 2.0, Wiley 8, and FFNSC 2 mass spectra databases from electronic libraries or the literature; LRI—linear retention index accordant with the index from the literature. Compounds with only MS in the ID column were considered tentatively identified. LRI_{exp}—experimental linear retention index; LRI_{lit}—linear retention index from the literature. *SCE—Saccharomyces cerevisiae* (control, pure culture); *LEV—Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.2.13. Hierarchical Clustering Analysis

Hierarchical clustering analysis was performed to summarize and better visualize the main differences in volatile compound profiles between *LEV* and *SCE* wines (Figure 1). A reduced dataset was used with a total of 67 variables, comprising 30 compounds with the

highest *F*-ratios which had higher concentration in *LEV* wine, 30 compounds with the highest *F*-ratios which had higher concentration in *SCE* wine, and seven additional compounds for which statistically significant differences were determined by one-way ANOVA which are often cited amongst the key wine odorants. *LEV* wine was characterized by higher concentrations of several important odorants, including geraniol, β-ionone, isobutanol, isobutyric acid, ethyl isobutyrate, isobutyl acetate, isoamyl acetate, ethyl acetate, ethyl lactate, and diethyl succinate, followed by numerous compounds from various chemical classes with, to date, an unknown but possibly important contribution to wine sensory quality. The profile of control *SCE* wine was distinguished by higher levels of other impact compounds, such as citronellol, acetaldehyde, 2-phenylethanol, propanol, isoamyl alcohol, 2-methylbutyric acid, isovaleric acid, ethyl 2-methylbutyrate, ethyl 3-methylbutyrate, and 2-phenethyl acetate, also accompanied by a number of other compounds. While the differences in major odorants suggest a probable significant impact on the sensory profiles of the investigated wines, the abundance in minor and trace compounds, not studied from this aspect before but significantly affected by yeast species in this study, implies the need to investigate their sensory relevance and possible impact on wine aroma.

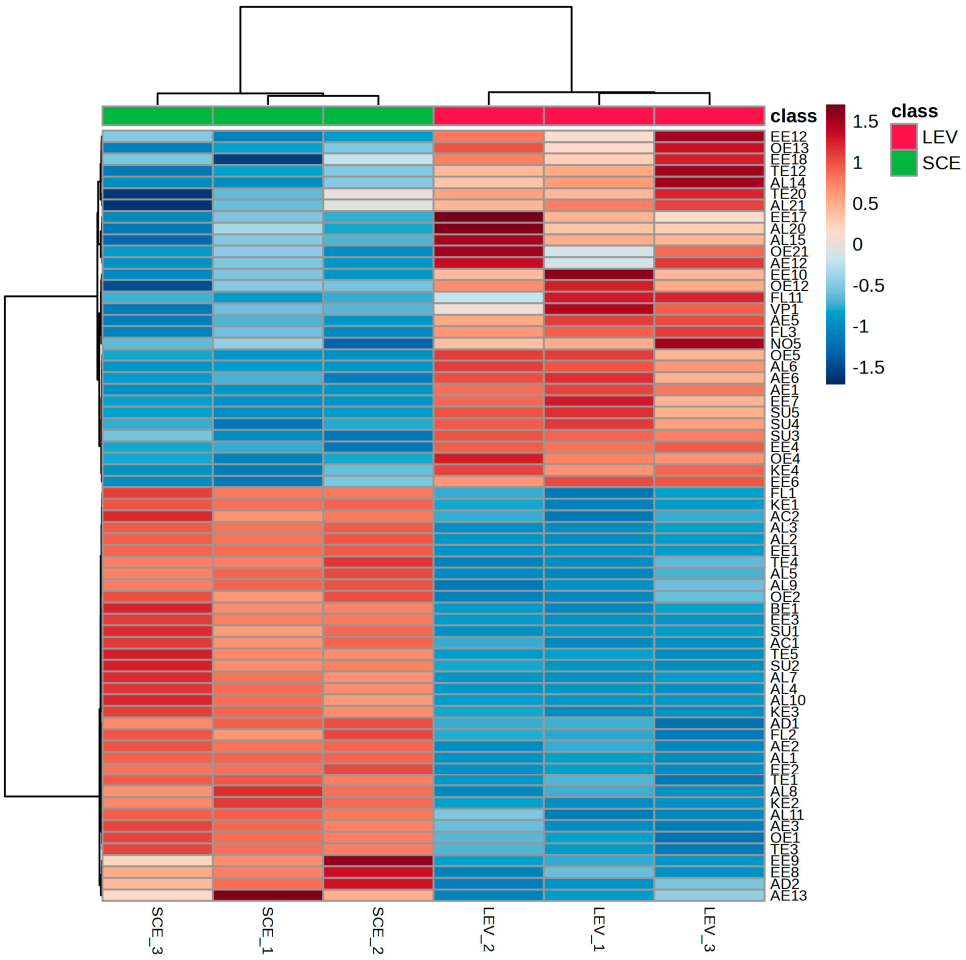

Figure 1. Hierarchical clustering analysis of Malvazija istarska wines produced by monoculture fermentation with *Saccharomyces cerevisiae* (*SCE*, control treatment) and *Lachancea thermotolerans* (*LEV*, sequentially inoculated, fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol) based on GC/FID, GC/MS, and GC×GC/TOF-MS volatile compounds analysis data. Compounds' codes correspond to those in Tables 2–13. The rows of the heatmap correspond to compounds, while the columns represent samples. The colors within the heatmap cells reflect the abundance of each compound (using normalized values), with dark blue indicating low, pale colors representing medium, and dark red signifying high abundance.

3.3. Grape Phenolic Compounds

The effects of the two investigated yeasts on grape phenolic compounds are reported in Table 14. Among hydroxybenzoic acids, the most significant differences were observed for 2,5-dihydroxybenzoic acid, followed by *p*-hydroxybenzoic acid, both found in higher concentrations in the control *SCE* wine. Both free forms of hydroxycinnamic acids and their esters with tartaric acid were significantly affected, but with opposite directions. Free *p*-coumaric and caffeic acid were found in higher concentrations in *SCE*, while all three major hydroxycinnamoyl tartrates, *trans*-caftaric, *trans*-fertaric, and *trans*-coutaric acid, were more abundant in *LEV* wine. Such results imply distinct differences between the activity of certain enzymes between the two yeasts, such as higher activity of cinnamyl esterases responsible for the release of free hydroxycinammic acids from their tartrate esters [54] in *S. cerevisiae*, as well as different activity of decarboxylases that catalyze the transformation of free *p*-coumaric and ferulic acid into 4-vinylphenols [42,55]. Besides that, the differential adsorption of grape phenols on the surface of yeast cells between different yeasts observed previously [56] could have also had an effect. *Trans*-resveratrol, a stilbene important because of its known antioxidant activity, was less abundant in *LEV*, while among flavanols, quercetin showed a higher concentration in this wine. From the group of flavan-3-ols, only procyanidin B1 and epigallocatechin showed significant differences, with a higher amount of the former found in *LEV* wine and that of the latter in *SCE* wine. Catechol had almost double the concentration in control *SCE* compared to *LEV* wine. The total phenolic content was slightly higher in the *LEV* treatment wine, implying a possibility of a higher degree of adsorption of phenols, including large molecules such as tannins, on *S. cerevisiae* yeast cells.

Table 14. Concentrations of phenolic compounds (mg/L) obtained by ultra-performance liquid chromatography/mass spectrometry (UPLC/QqQ-MS/MS) sorted by compound class and descending Fisher's *F*-ratio and concentration of total phenols (mg/L gallic acid equivalents) in Malvazija istarska white wines produced using different yeasts.

Phenolic Compounds	*F*-Ratio	Treatment	
		SCE	***LEV***
Hydroxybenzoic acid derivatives			
2,5-Dihydroxybenzoic acid	100.993	0.715 ± 0.077 ^{a}	0.262 ± 0.009 ^{b}
p-Hydroxybenzoic acid	10.571	0.439 ± 0.077 ^{a}	0.293 ± 0.012 ^{b}
Protocatechuic acid	0.639	0.565 ± 0.058	0.668 ± 0.214
Vanillic acid	0.424	0.106 ± 0.022	0.096 ± 0.012
Syringic acid	0.221	0.422 ± 0.128	0.370 ± 0.145
Hydroxycinnamic acid derivatives			
p-Coumaric acid	493.014	1.27 ± 0.04 ^{a}	0.44 ± 0.05 ^{b}
trans-Caftaric acid	138.458	0.179 ± 0.032 ^{b}	0.804 ± 0.086 ^{a}
trans-Coutaric acid	31.520	0.491 ± 0.072 ^{b}	0.797 ± 0.061 ^{a}
Caffeic acid	27.638	2.24 ± 0.11 ^{a}	1.75 ± 0.12 ^{b}
trans-Fertaric acid	12.844	2.45 ± 0.19 ^{b}	2.85 ± 0.04 ^{a}
Ferulic acid	5.108	0.498 ± 0.040	0.601 ± 0.067
Other acids			
4-Aminobenzoic acid	4.055	0.066 ± 0.009	0.082 ± 0.011
Stilbenes			
trans-Resveratrol	30.043	0.115 ± 0.008 ^{a}	0.080 ± 0.007 ^{b}
cis-Resveratrol	6.092	0.026 ± 0.014	0.053 ± 0.013
Flavan-3-ols			
Procyanidin B1	32.423	1.33 ± 0.28 ^{b}	2.73 ± 0.32 ^{a}
Epigallocatechin	10.543	0.019 ± 0.005 ^{a}	0.005 ± 0.005 ^{b}
Epicatechin	3.120	0.245 ± 0.068	0.360 ± 0.091
Gallocatechin	2.120	0.188 ± 0.010	0.159 ± 0.032
Catechin	1.117	1.41 ± 0.14	1.23 ± 0.25
Procyanidin B2 + B4	0.425	0.158 ± 0.055	0.239 ± 0.207

Table 14. *Cont.*

Phenolic Compounds	*F*-Ratio	Treatment	
		SCE	***LEV***
Flavonols			
Quercetin	21.766	0.097 ± 0.001 [b]	0.133 ± 0.013 [a]
Kaempferol	1.201	0.000 ± 0.000	0.005 ± 0.007
Miscellaneous			
Catechol	9.748	0.681 ± 0.084 [a]	0.376 ± 0.147 [b]
Phlorizin	5.502	0.039 ± 0.002	0.065 ± 0.019
Total phenolic content		196.9 ± 5.0 [b]	206.7 ± 2.6 [a]

Abbreviations: *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.4. Proteins and Protein Stability

The changes in the concentration of major soluble grape and wine proteins, pathogenesis-related (PR) thaumatin-like proteins (TLPs), and chitinases responsible for the formation of haze in white wine were mostly non-significant (Table 15). Only thaumatin-like protein 2 was found in significantly lower concentration in *LEV* wine. This difference was apparently not sufficient to achieve a change in protein stability, since the bentonite doses required to achieve protein stability of the two wines were the same. Chitinases were not affected, so it was assumed that the two investigated yeasts did not differ with respect to the content of cell wall chitin, a substrate for these PR proteins. In a recent study, particular *Saccharomyces paradoxus* strains were found to have increased availability of chitin and show a potential to adsorb chitinases and improve wine protein stability [57]. Available information about the interaction of non-*Saccharomyces* yeasts and PR proteins is generally rather scarce, so further research is needed.

Table 15. Concentrations of pathogenesis-related (PR) proteins (mg/L) determined by reversed-phase high-performance liquid chromatography with diode array detection (RP-HPLC/DAD) in Malvazija istarska white wines produced using different yeasts and bentonite doses (g/hL) required to achieve protein stability of the wines.

PR Proteins and Bentonite Dose	Treatment	
	SCE	***LEV***
Thaumatin-like protein 1	12.41 ± 1.13	13.33 ± 0.42
Thaumatin-like protein 2	12.32 ± 0.43 [a]	10.35 ± 0.26 [b]
Thaumatin-like protein 3	12.33 ± 0.92	11.61 ±0.54
Thaumatin-like protein 4	32.03 ±2.28	29.32 ± 0.64
Chitinase 1	29.17 ± 0.92	28.04 ±2.78
Chitinase 2	22.95 ± 0.54	22.52 ± 2.10
Bentonite dose	90 ± 0	90 ± 0

Abbreviations: *SCE*—*Saccharomyces cerevisiae* (control, pure culture); *LEV*—*Lachancea thermotolerans* (sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol). Different superscript lowercase letters in a row represent statistically significant differences among two investigated wines determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

3.5. Sensory Analysis

Particular differences between the intensities of main aroma group attributes and taste attributes of the two investigated wines were determined by quantitative descriptive sensory analysis, although, in most cases, without statistical significance (Figure 2a). Aroma group and taste attributes, as well as specific odor descriptors for which statistically significant differences were found, are shown in Figure 2b. *LEV* wine was characterized by

increased tropical fruit notes, specifically passionfruit-like odor, which could be tentatively ascribed to the increased levels of particular acetates determined in this wine (Table 8). The occurrence of this odor nuance is often associated with the contribution of volatile thiols, which were not analyzed in this study, but the possibility that these compounds may have had an effect should not be excluded. The slightly but significantly higher intensity of buttery odor in *LEV* was possibly related to the higher concentration of ethyl lactate, known to contribute with buttery notes, and possibly other esters of lactic acid with unknown sensory relevance found in this wine, such as isoamyl lactate and ethyl phenyl lactate. Higher intensities of herbaceous and tobacco odors were also observed in *LEV* wine. On the other hand, more intense muscat-like and citrus odors observed in control *SCE* wine were probably related to higher concentrations of several terpenoids found in this wine, such as citronellol, limonene, and many other minor compounds, despite the fact that the difference in linalool concentration, which usually exhibits the greatest contribution to Malvazija istarska flavor among major monoterpenols [58], was not significant (Table 3). *SCE* wine was described by slightly higher intensity of the overall floral odor. The perception of acidity was not altered by *LEV* with respect to control *SCE* wine. However, *LEV* wine was described as having a fuller body and higher viscosity, which was possibly a direct consequence of higher concentrations of lactic acid and total dry extract found in this wine (Table 1), which is known to contribute to such attributes. No significant differences between the two wines were observed either regarding Malvazija istarska varietal typicity or the overall quality assessed by the 100 points OIV grading method.

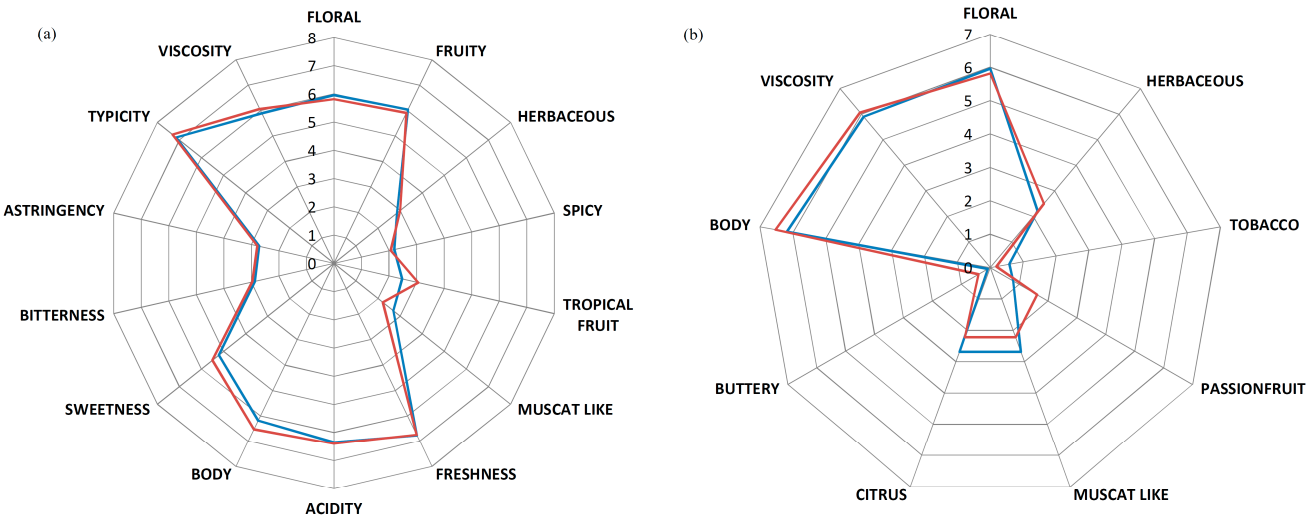

Figure 2. Sensory profiles of Malvazija istarska wines produced by *Saccharomyces cerevisiae* (*SCE*; control, pure culture, blue line) and *Lachancea thermotolerans* (*LEV*; sequentially inoculated; fermentation finished by *S. cerevisiae* (*SCE*) inoculated at 2 vol % ethanol, red line) obtained by quantitative descriptive sensory analysis: (**a**) intensities of main aroma group and taste attributes; and (**b**) intensities of main aroma group and taste attributes and specific odor descriptors for which statistically significant differences between the wines were determined by one-way ANOVA and least significant difference test (LSD) at $p < 0.05$.

4. Conclusions

The results of this study showed that sequential inoculation with the investigated *L. thermotolerans* fermentation starter followed by *S. cerevisiae* can produce significant effects on white wine composition and quality when compared to *S. cerevisiae* monoculture fermentation. The bioacidification effect of *L. thermotolerans*, together with the reduced alcoholic strength, was confirmed to be a prominent feature of this yeast, useful in mitigating the negative influence of climate changes in winemaking. This is especially important for grape varieties such as Malvazija istarska, which, in certain terroirs and growing seasons, produce wines with lower acidity and higher alcohol content. These effects were milder

than for some other strains in previous reports, confirming that the studies on the selection of *L. thermotolerans* strains with desired oenological performance are of utmost importance. Future research should also prioritize investigating how the complete physico-chemical composition of starting grape/must material, in combination with various vinification conditions, affect the performance of this yeast. This will enable more precise management of its activity to achieve the desired outcomes in winemaking. The comprehensive GC×GC/MS-TOF analysis, complemented by conventional GC techniques, provided an in-depth characterization of the changes in the volatile aroma profile of wine as affected by *L. thermotolerans* as a starter, with more than 370 identified volatiles. Although the levels of a number of compounds were lower after *L. thermotolerans* co-fermentation, the investigated starter produced significant increases in the concentration of several known key wine volatile aroma compounds, followed by numerous compounds from various chemical classes with to date unknown, but possibly important contribution to wine sensory quality. On the other hand, for a number of volatiles, no significant effects were observed. Particular phenolic compounds from grapes were significantly affected, while the observed marginal effect on proteins and no effect on protein stability suggest that the used *L. thermotolerans* strain is not a promising candidate for use for such purposes. In sensory terms, the wines of the two treatments were generally described as similar, albeit *L. thermotolerans* co-fermentation slightly enhanced the perception of particular positive sensory attributes and descriptors, meaning that bioacidification and ethanol reduction were complemented by positive side effects on wine quality. With the largest number of identified volatile compounds reported up to date and other results obtained, this study contributes to the better understanding of oenological and especially aromatic potential of *L. thermotolerans* in white wine production. Given the significant number of differentiating compounds whose sensory relevance remains unknown, it is crucial for future studies to delve deeper into understanding their potential impact.

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