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Effect of Sequential Inoculation with Non-*Saccharomyces* and *Saccharomyces* Yeasts on Riesling Wine Chemical Composition

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Abstract: In recent years, studies have reported the positive influence of non-*Saccharomyces* yeast on wine quality. Many grape varieties under mixed or sequential inoculation show an overall positive effect on aroma enhancement. A potential impact by non-*Saccharomyces* yeast on volatile and non-volatile compounds should benefit the flavor of Riesling wines. Following this trend, four separate sequential fermentations (using the non-*Saccharomyces* yeasts *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, and *Lachancea thermotolerans* with *Saccharomyces cerevisiae*) were carried out on Riesling must and compared to a pure culture of *S. cerevisiae*. Sequential fermentations influenced the final wine aroma. Significant differences were found in esters, acetates, higher alcohols, fatty acids, and low volatile sulfur compounds between the different trials. Other parameters, including the production of non-volatile compounds, showed significant differences. This fermentation process not only allows the modulation of wine aroma but also chemical parameters such as glycerol, ethanol, alcohol, acidity, or fermentation by-products. These potential benefits of wine diversity should be beneficial to the wine industry.

Keywords: sequential inoculation; *Saccharomyces*; non-*Saccharomyces*; Riesling; aroma compound; *Torulaspora delbrueckii*; *Pichia kluyveri*; *Lachancea thermotolerans*

1. Introduction

Wine is considered to be one of the most complex aromatic products. It is composed of a large amount of aroma compounds and yet only a fraction of them are responsible for the bouquet. These compounds can be volatile, with these being the fragrant compounds, or non-volatile, with these being the compounds which are responsible for the taste sensations [1]. The final aromatic bouquet of the wine is the result of various factors: the raw material (variety, climate, vine management, and ripeness, etc.), the vinification choices, and the effect of microorganisms (yeast and lactic bacteria) [2]. In addition to the aromatic composition of the wine, it is important to note that each human has their own flavor perception. This perception is an interaction between three factors: the food properties, the in-mouth environment, and the psycho-social effects. As the aroma flavors lead to preferences for particular choices, the final bouquet of the wine plays a non-negligible role for the consumer taste [3].

Riesling is an aromatic grape variety which has been linked to various aroma compounds. Specific terpenes have been associated to this cultivar [1], such as monoterpenes, which are considered to be principally responsible for the floral and citrus character in wine [4]. The monoterpenes geraniol (rose and geranium notes) [5] and linalool (flower and lavender notes) [5] have been identified as typical for Riesling wines [6]. The thiols can also contribute to the bouquet of Riesling [7]. These compounds have a very low threshold, in the order of 1 ng/L, and express grapefruit, passion fruit, box tree, or black current notes [8].

Two different kinds of aroma can be distinguished: the varietal aromas that are grape-variety-specific and the by-products of the alcoholic fermentation that are aromas produced by yeast metabolism.

Varietal aroma compounds are intrinsic to the grape variety. Currently, only a few varieties have been linked to certain aroma compounds such as the Muscat cultivars and its relatives, like Riesling, with specific terpenes [1]; Sauvignon Blanc [9], with thiols, or Cabernet Sauvignon, with methoxypyrazines [10]. However, only some free forms of aroma compounds, such as monoterpenes or methoxypyrazines, are present in the grape juice. Most of the aroma compounds occur in their linked form, which makes them non-volatile and hence odorless [2]. However, the linked aromas can be liberated through several processes by specific enzymes [11], yeast [12–14], or lactic bacteria [15]. Even if the differences of the aroma compound amount between varieties can seem small, it can have an enormous impact on the final product.

Three families of aromas are known as by-products of the alcoholic fermentations. The esters are a result of various pathways that appear during the alcoholic fermentation as a by-product of the fermentation by yeast [16]. Their proportion in wines is yeast-strain-specific and depends on the fermentation conditions (temperature and pH), and evolves, positively or negatively, during wine aging [17]. The fatty acids can be separated into two groups: the straight chain fatty acids and the branched-chain fatty acids. They are both synthesized during the alcoholic fermentation through the yeast metabolism, but through different pathways [18]. The higher alcohols are synthesized by the yeast as an intermediate in amino acids metabolism. Thus, they are formed from various amino acids through two different pathways: the catabolic and the anabolic [18].

Since yeasts have an impact on the formation and the liberation of aroma compounds during alcoholic fermentation, yeast strains may play a role on the quantitative and qualitative production of these aromas. Nowadays, various strains are commercially available and promise aroma enhancement.

Only a few strains of non-*Saccharomyces* can complete alcoholic fermentation due to their sensitivity to high levels of ethanol. Moreover, they often produce undesirable secondary metabolites such as acetic acid, ethyl acetate, ethyl phenols, aldehydes, and acetoin [19,20]. Their oenological interest to the wine industry has been even less common given that they are SO₂ sensitive and their fermentation rate is low [21,22]. However, since climate change is impacting the level of sugar in grapes, non-*Saccharomyces* have become quite popular for their ability to reduce the level of alcohol [23,24]. Renewed interest is being shown for their ability to enhance certain aromas and produce more complex wines [12,25,26]. As their pure fermentation is not oenologically interesting, they must be used along with a *Saccharomyces* spp. The sequential inoculation target is then to imitate the spontaneous fermentation by providing at the early stage non-*Saccharomyces* and at the middle stage *Saccharomyces*, while avoiding the disadvantages of indigenous yeast, namely the production of off-flavors as well as sluggish or stuck fermentation [19,27,28].

Although some yeast strains can influence some specific volatile compounds to be over their sensory thresholds, wine is a complex matrix where individual compounds are occasionally difficult to perceive depending on the diversity of the matrix.

The aim of this work is to show the impact of non-*Saccharomyces* yeast on the wine's aroma modulation and to find the best strains to improve Riesling wine's flavor. To that purpose, four non-*Saccharomyces* yeasts were selected to be vinified under a sequential inoculation with a *Saccharomyces cerevisiae*. Their aroma profile was determined and compared to a wine fermented with only one *S. cerevisiae* strain.

2. Materials and Method

2.1. Yeast Strains

Commercial strains from different companies were used for this trial: *S. cerevisiae* Level 2[®] (Lallemand, Montreal, QC, Canada), *Torulasporea delbrueckii* Level 2[®] (Lallemand, Montreal, Canada), *Metschnikowia pulcherrima* Flavia[®] (Lallemand, Montreal, Canada), *Pichia kluyveri* FrootZen[™] (Hansen, Hørsholm, Denmark), and *Lachancea thermotolerans* Concerto[™] (Hansen, Hørsholm, Denmark).

2.2. Vinification

Riesling grape juice from Hochschule Geisenheim University (Germany) was used to lead the microvinification after autoclaving (115 °C, 15 min, 20 psi). Component concentration in the must were: sugar, 225.2 g/L; total dry extract, 237.8 g/L; pH, 2.9; total acidity, 8.7 g/L; tartaric acid, 4.7 g/L; acetic acid <0.1 g/L; malic acid, 3.0 g/L; ethanol <0.1 g/L; gluconic acid <0.1 g/L; glycerol <0.1 g/L; available primary amino acids 58.8 mg/L; ammonium 48 mg/L. In order to provide good conditions for the yeast to grow, the total nitrogen was adjusted to 250 mg/L with Vitamon[®] A (Erbslöh, Geisenheim, Germany) and nutrients were provided by Optimum White[™] (Lallemand, Montreal, Canada), 30 g/hL. Bactiless[™] (Lallemand, Montreal, Canada), 30 g/hL, was added to avoid any bacterial contamination.

Five assays, in quadruplicate, were carried out in 2-liter bottles with 1.8 L of juice following a similar methodology to that described before but adapted to the scale [29]. A single inoculation with *S. cerevisiae* Level 2[®] (10⁶ CFU/mL) was performed for the control trial (1) and sequential inoculation was performed for the other fermentations by inoculating first the juice with the non-*Saccharomyces* yeast strain (10⁶ CFU/mL) and at 48 h (*T. delbrueckii* Level 2[®] (2), *M. pulcherrima* Flavia[®] (3), and *L. thermotolerans* Concerto[™] (5) trials) or 96 h (*P. kluyveri* FrootZen[™] (4) trial) after with the *S. cerevisiae* Level 2[®] (10⁶ CFU/mL).

The inocula of the first inoculation were carried out in 100 mL of must and 1 mL of YEPD liquid media in a 250 mL Erlenmeyer flask. The YEPD liquid media was prepared with yeast extract (10 g/L), peptone from casein (20 g/L), and glucose (20 g/L), and its pH was adjusted to 6.5. Yeast extract and glucose were provided by Merck (Darmstadt, Germany) and peptone by Roth[®] (Karlsruhe, Germany). The solution YEPD-must was autoclaved at 121 °C for 30 min. It was inoculated with the dry yeast strains when the media was at ambient temperature under sterile conditions. Only the *P. kluyveri* FrootZen[™] strain was previously rehydrated. This strain was a frozen product. The frozen solution was rehydrated in the must in a 250-mL Erlenmeyer flask and incubated at 26 °C for 24 h before the inoculation of the YEPD liquid media. The inocula were cultivated in a chamber at 26 °C for 7 days. Their final concentration was 10⁸ CFU/mL. Forty-five milliliters of inocula per bottle were used to inoculate the juice. The bottles were sealed by fermenting bung in order to allow gas to be released and then stored at 20 °C. The inoculum for the second inoculation (*S. cerevisiae* Level 2[®]) was performed according to recommendations of the yeast producer. Five grams of dry yeast were rehydrated in 50 mL of 35 °C water for 20 min. The inoculation (3.6 mL/bottle) was carried out when the inoculum (10⁹ CFU/mL) was about 20 °C. At the end of the fermentation monitored by weight loss, the bottles were stored in a 4 °C room to let the yeasts settle down. Then, the young wines were racked after 48 h and filled in brown glass bottles, according to the same procedure. Furthermore, potassium disulfite (Merck, Darmstadt, Germany) was added to each sample in a concentration of 80 mg/L sulfur dioxide before the bottles were locked with screw caps. Afterwards, the filled bottles were again stored at 4 °C.

2.3. Fermentation Kinetics

Yeast strain kinetic growth was followed on two different agar media: a YEPD medium and a lysine medium [29]. The two media were chosen in order to differentiate *Saccharomyces* and non-*Saccharomyces* growth. The *Saccharomyces* yeast grows only on YEPD media whereas the non-*Saccharomyces* yeast grows on both. Aliquots were taken at regular intervals from the bottles of the quadruplicates of each

assay. They were plated on both media in three different dilutions and incubated in a chamber at 26 °C for 48 h before counting.

In order to estimate aliquot dilutions, their concentration in yeast (CFU/mL) was estimated by using a Thoma cell counting chamber (Marienfeld, Germany) under the microscope (objective x40). The Thoma cell counting chamber was also used to estimate the inocula concentration. The weight of each bottle was taken regularly to follow the fermentation progress. The fermentation progress was calculated by the difference of the initial weight at day 0 and the weight from the day.

2.4. Chemical Compounds Analysis

2.4.1. HPLC

Organic acids measurements were performed by HPLC following the method described by Schneider et al. (1987) [30], which was adjusted and improved by Semmler et al. (2017) [31]. Measurements were carried out on the initial juice and obtained wines by the Department of Microbiology and Biochemistry at Hochschule Geisenheim University (HGU).

2.4.2. FTIR

Fourier transform middle infrared spectroscopy (FT-MIR) was used to assess density, alcohol, extract, sugars, pH, glycerol, and SO₂ measurements on initial juice and obtained wines. The method applied followed Baumgartner et al. (2001) [32], Patz et al. (1999) [33], and the Standard Operating Procedure SOP-WG1-84 of the HGU Department of Beverage Research.

2.5. Volatile Compounds Analysis

Volatile compound analysis was conducted by the HGU Department of Microbiology and Biochemistry. Fatty acids, higher alcohols, and esters were measured according to the method described by Rapp et al. (1994) [34] and modified by Fritsch et al. (2017a) [35]. A gas chromatography and mass spectrometer were performed to assess these volatile compounds. Terpenes were determined by the application of solid phase extraction (SPE), gas chromatography, and mass spectrometry following the method of Schüttler et al. (2015) [6], modified by Fritsch et al. (2017b) [36]. Headspace injection and the use of gas chromatography and pulsed flame photometric detection were achieved to measure low volatile compounds following the publication Rauhut et al. (2005) [37], adapted and modified by Rauhut, Beisert (2017) [38].

2.6. Statistical Analysis

R software (version 3.4.1, R Foundation for Statistical Computing, Vienna, Austria, 2017) and its package R-commander were used to perform all the statistical analyses. The significance level $p < 0.05$ was chosen for all the applied tests.

3. Results and Discussion

3.1. Fermentation Kinetics

3.1.1. Yeast Population Kinetics

The yeast populations of the various fermentations processes are shown in Figure 1. Non-*Saccharomyces* yeasts started to decline between 2 to 4 days after the second inoculation with *S. cerevisiae*. Previous studies show similar trends on the decline of the non-*Saccharomyces* population shortly after inoculation with *Saccharomyces* spp. strain [39]. This phenomenon could have numerous explanations. It has previously been shown that yeast growth can be inhibited by yeast metabolites such as ethanol [40,41], medium chain fatty acids [42], and acetaldehyde [43]. Killer toxins produced during the exponential phase by specific strain can also have an inhibitory impact on some yeast

growth [44–46]. More recently, it was found that *S. cerevisiae* could secrete peptides who inhibit non-*Saccharomyces* yeast growth [47]. Other parameters such as nutrient limitation and possible physical changes such as temperature and redox potential could also inhibit yeast developing.

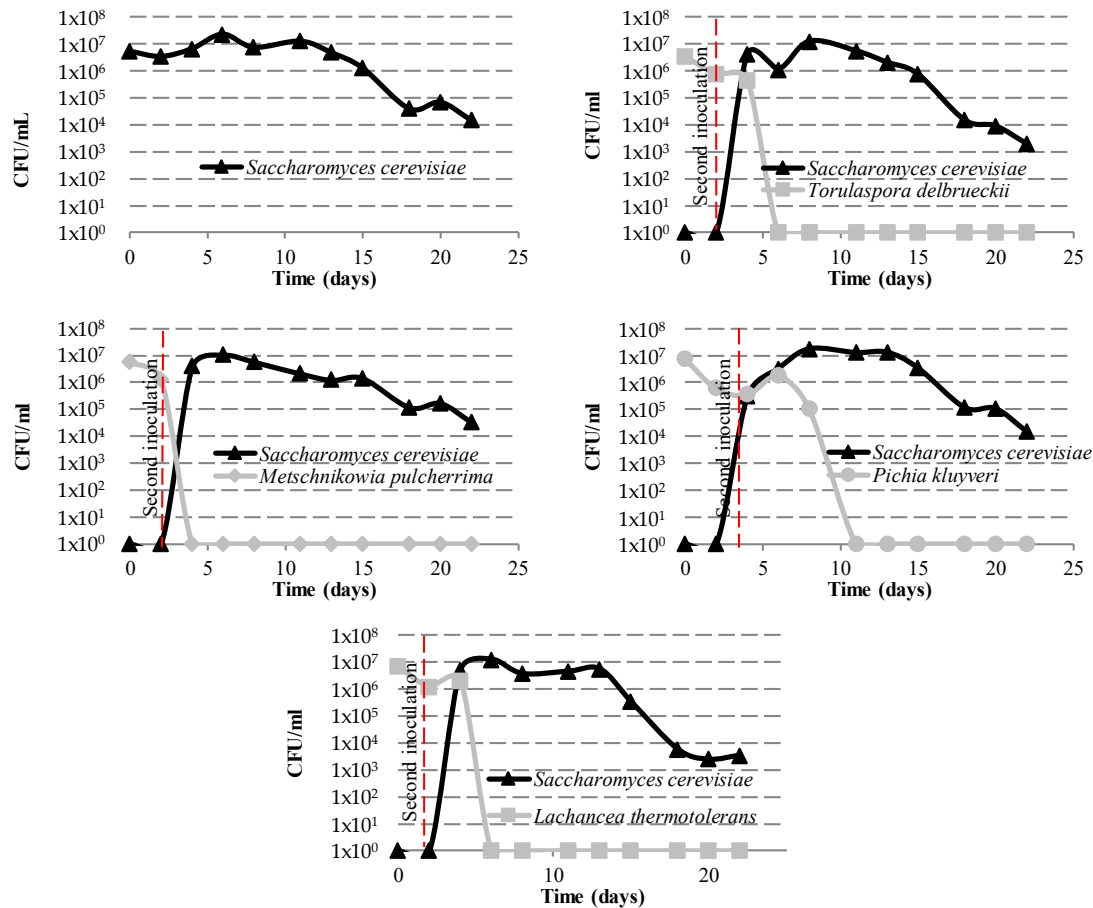


Figure 1. Population development of *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, and *Lachancea thermotolerans* during the fermentation process of the different trials.

3.1.2. Alcoholic Fermentation Kinetics

Figure 2 shows the alcoholic fermentation curves based on the weight losses of the replicates and trials during the fermentation process. For each assay, quadruplicates followed the same trend. The curves follow typical fermentation curves which have already been reported in the literature [48]. Fermentation dynamics show differences in the speed of sugar consumption. *S. cerevisiae* fermentation and *M. pulcherrima* sequential fermentation consumed sugars the fastest at the beginning of the alcoholic fermentation. However, other trials consumed the sugars faster during the following steps because all the fermentations reached the stationary phase at the same time. This difference of speed at the beginning could be attributed to the population density of *M. pulcherrima*, which declined immediately after the second inoculation, and the low fermentation rate and power of non-*Saccharomyces* species. As the other non-*Saccharomyces* populations declined later, their low fermentation power and rate, as has been reported by previous studies [49,50], could be associated with a slower fermentation process [51].

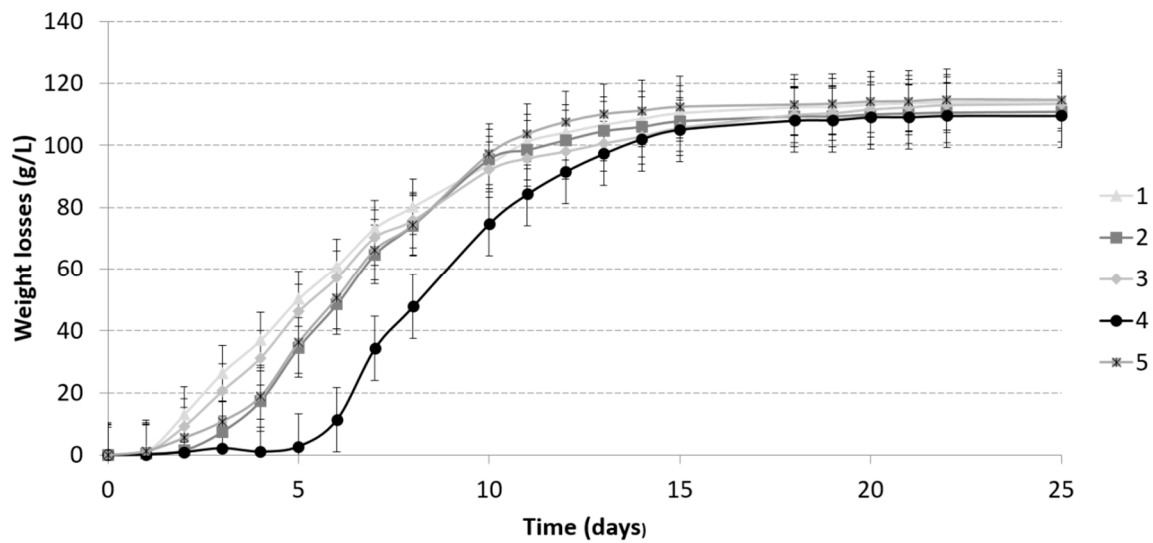


Figure 2. Alcoholic fermentation curve of *S. cerevisiae* Level 2® (1), and sequential fermentations with *S. cerevisiae* Level 2® and *T. delbrueckii* Level 2® (2), *M. pulcherrima* Flavia® (3), *P. kluyveri* FrootZen™ (4), and *L. thermotolerans* Concerto™ (5).

3.2. Chemical Monitoring

Tartaric acid was initially 5.2 g/L. Thus, all the fermentations show a decrease in tartaric acid in the wine between 0.48 and 0.77 g/L (Table 1). This phenomenon can be explained by the precipitation of tartaric acid as calcium or potassium tartrate [52]. Tartaric acid can also be degraded by yeasts by up to 23% [53]. In this trial, the *S. cerevisiae* degraded the tartaric acid by the smallest amount and the sequential inoculation with *T. delbrueckii*, *M. pulcherrima*, and *P. kluyveri* by the greatest amount. It seems that the latter used more tartaric acid for their metabolisms.

Table 1. Final analysis, before adding SO₂, of *S. cerevisiae* Level 2® (1), and sequential fermentations with *S. cerevisiae* Level 2® and *T. delbrueckii* Level 2® (2), *M. pulcherrima* Flavia® (3), *P. kluyveri* FrootZen™ (4), and *L. thermotolerans* Concerto™ (5).

Compounds	1	2	3	4	5
Tartaric acid (g/L)	4.72 ± 0.18a	4.38 ± 0.02b	4.43 ± 0.01b	4.43 ± 0.01b	4.63 ± 0.28ab
Malic acid (g/L)	2.28 ± 0.00d	2.21 ± 0.00c	2.10 ± 0.01a	2.21 ± 0.01c	2.17 ± 0.01b
Shikimic acid (mg/L)	50.19 ± 0.06b	49.94 ± 0.36ab	49.36 ± 0.25a	49.86 ± 0.34ab	49.64 ± 0.37ab
Lactic acid (g/L)	0.21 ± 0.01a	0.17 ± 0.01a	0.21 ± 0.01a	0.19 ± 0.00a	1.51 ± 0.04b
Acetic acid (g/L)	0.25 ± 0.03ab	0.21 ± 0.03a	0.30 ± 0.03b	0.23 ± 0.02a	0.31 ± 0.02b
Citric acid (g/L)	0.14 ± 0.02a	0.14 ± 0.01a	0.14 ± 0.00a	0.15 ± 0.02a	0.13 ± 0.01a
Residual sugars (g/L)	4.4 ± 0.25b	2.8 ± 0.23a	4.5 ± 0.30b	2.9 ± 0.16a	3.0 ± 0.11a
pH	3.1 ± 0.00a	3.2 ± 0.00b	3.2 ± 0.00b	3.2 ± 0.00b	3.1 ± 0.00a
Ethanol (% v/v)	13.20 ± 0.19b	13.17 ± 0.44b	12.98 ± 0.35a	13.04 ± 0.28a	12.96 ± 0.31a
Glycerol (g/L)	5.8 ± 0.04a	6.6 ± 0.04b	7.0 ± 0.05c	7.1 ± 0.05c	7.4 ± 0.07d

Average values of the quadruplicates ± standard deviation. The letters represent significantly different statistical groups ($p < 0.05$).

Previous studies have shown that some yeasts are able to consume malic acid such as *Issatchenkia orientalis*, *Saccharomyces* spp., or *Schizosaccharomyces* spp. By up to 45% of the initial level [19,54–57]. The malic acid levels from 2.10 to 2.28 g/L (Table 1) according to the assay are indeed lower than the initial malic acid level of the juice, which was 3.00 g/L. All the trials presented significant differences in their malic acid amount except for the sequential fermentation with *T. delbrueckii* and *P. kluyveri*, which can be seen to have had exactly the same production. The sequential fermentation with *M. pulcherrima* and the single fermentation with *S. cerevisiae* had the lowest and highest levels,

respectively. Thus, *M. pulcherrima* shows a higher ability to consume malic acid than the other strains and *S. cerevisiae* shows a lower ability to consume malic acid.

Shikimic acid can be a precursor of aroma compounds such as ethyl cinnamate or benzaldehyde. Differences of the shikimic acid amount are shown. The level in the wines was found to be between 49.4 and 50.2 mg/L (Table 1). The fermentation with *S. cerevisiae* and the sequential fermentation with *M. pulcherrima* have the highest and the lowest amounts of shikimic acid, respectively. Thus, *M. pulcherrima* could have a positive impact on the formation of aroma compounds from this precursor.

Lactic acid concentrations were between 0.17 and 1.51 g/L, with the sequential fermentation with *T. delbrueckii* and *L. thermotolerans* having respectively the lowest and the highest levels (Table 1). Previous studies have reported high production of lactic acid by *L. thermotolerans* during its growth phase and its ability to acidify the must [28,58–62]. Assay 5, fermented with *L. thermotolerans* and *S. cerevisiae*, shows significantly higher production of lactic acid than the other trials, whereas the other trials do not show significant differences in their lactic acid production.

The acetic acid production (Table 1) varied from 0.21 to 0.31 g/L, according to the assay. No statistical differences are shown between the single fermentation with *S. cerevisiae* and the other trials, as has already been reported [49]. The sequential fermentations fermented with *T. delbrueckii* and *P. kluyveri* produced significantly lower acetic acid compared to the other non-*Saccharomyces* yeasts, which differs from previous studies. Nevertheless, the total amount of produced acetic acid does not negatively impact the wine quality [63].

The ethanol level ranges from 12.96 and 13.20% vol. (Table 1). Some strains are known for their ability to produce less alcohol [64,65]. Similar to what has been reported in the literature, the *S. cerevisiae* batch produced the highest ethanol level, albeit one that is not significantly different from that resulting from the sequential fermentation with *T. delbrueckii* [49]. The lowest level of ethanol was produced by the sequential inoculation with *L. thermotolerans*, though this level is not significantly different from the one produced by the sequential inoculations with *M. pulcherrima* and *P. kluyveri*. Similar results have already been found in the literature, showing that these yeasts can be used to produce low-alcohol wines [59,65]. In this trial, *S. cerevisiae* fermentation produced the highest level of ethanol, which confirms its high fermentative purity.

The level of pH was found to be not drastically different from one wine to another and ranged from 3.1 to 3.2 (Table 1). However, the statistical analysis shows significant differences. The fermentation with *S. cerevisiae* and the sequential fermentation with *L. thermotolerans* have lower pH levels, whereas the sequential fermentations with *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri* have higher levels. Higher pH differences of *L. thermotolerans* with the other fermentations may be expected because of its high production of lactic acid [28,66,67]. Some studies confirm that even if lactic acid production is significantly higher for mixed fermentation with *L. thermotolerans* and *S. cerevisiae* than a single inoculation with the latter, the pH level can be significantly the same [58,59].

Glycerol production varies from 5.8 to 7.4 g/L for fermentation with *S. cerevisiae* and sequential fermentation with *L. thermotolerans*, having, respectively, the lowest and the highest production (Table 1). All of the other trials produced significantly higher levels of glycerol, which means that non-*Saccharomyces* have a positive impact on glycerol production. Higher production of this compound from sequential inoculation with non-*Saccharomyces* and *Saccharomyces* spp. has already been shown in the literature [28,68–71].

3.3. Volatile Compounds

3.3.1. Esters

The total esters produced were between 124,842 and 194,053 µg/L (Table 2). *P. kluyveri* sequential fermentation produced the highest level of esters and *L. thermotolerans* sequential fermentation produced an equally significant amount of esters. On the contrary, *T. delbrueckii* and *M. pulcherrima* sequential fermentations produced the lowest amount of esters.

Table 2. Volatile compounds measured after alcoholic fermentations of *S. cerevisiae* Level 2[®] (1), and sequential fermentations with *S. cerevisiae* Level 2[®] and *T. delbrueckii* Level 2[®] (2), *M. pulcherrima* Flavia[®] (3), *P. kluyveri* FrootZen[™] (4), and *L. thermotolerans* Concerto[™] (5).

Compounds	1	2	3	4	5
Esters					
Ethyl esters					
Ethyl lactate (mg/L)	nq	nq	nq	nq	52.26 ± 3.27
i-Ethyl butanoate (µg/L)	nd	nd	nd	nd	nd
Ethyl butanoate (µg/L)	432.19 ± 31.18b	308.35 ± 16.36a	332.37 ± 20.24a	278.46 ± 26.92a	304.86 ± 15.42a
Ethyl hexanoate (µg/L)	1876.06 ± 69.54bc	1798.42 ± 45.10b	1994.32 ± 45.97c	1551.1898.47a	1518.12 ± 28.25a
Ethyl octanoate (µg/L)	1472.32 ± 89.10ab	1367.63 ± 33.80a	1672.57 ± 186.55b	1440.66 ± 122.99ab	1364.97 ± 96.58a
Ethyl decanoate (µg/L)	442.63 ± 57.49a	437.23 ± 37.76a	553.12 ± 87.48a	546.90 ± 60.22a	581.35 ± 47.92a
Diethyl succinate (µg/L)	nq	nq	nq	nq	nq
Ethyl 2-hydroxy-4-methyl valerate (µg/L)	nq	nq	nq	nq	nq
Total ethyl esters (µg/L)	4223.20 ± 152.74a	3911.63 ± 18.66a	4552.38 ± 305.74a	3817.20 ± 282.13a	56031.95 ± 3282.57b
Acetates					
Ethyl acetate (mg/L)	159.18 ± 7.20b	135.02 ± 9.10a	115.53 ± 6.80a	184.01 ± 5.02c	127.97 ± 10.25a
Isoamyl acetate and 2-methyl butyl acetate (µg/L)	3927.41 ± 186.64a	4446.21 ± 205.56bc	3779.80 ± 90.54a	4751.71 ± 257.79c	4154.59 ± 163.59ab
Hexyl acetate (µg/L)	694.15 ± 29.53b	666.82 ± 11.07b	626.61 ± 38.94ab	590.44 ± 34.53a	592.86 ± 23.73a
Ethyl phenylacetate (µg/L)	nq	nq	nq	nq	nq
2-Phenyl-ethyl acetate (µg/L)	429.43 ± 9.15b	510.21 ± 16.05c	352.56 ± 14.12a	885.06 ± 28.39d	375.91 ± 8.12a
Total acetates (µg/L)	164229.49 ± 7358.44c	140647.20 ± 9289.39b	120290.10 ± 6811.04a	190236.09 ± 5279.61d	133095.47 ± 10391.01ab
Total esters (µg/L)	168452.69 ± 7369.92b	144558.83 ± 9282.59a	124842.48 ± 6791.85a	194053.29 ± 5518.21c	189127.42 ± 9985.27c
Higher alcohols					
3-Methyl-butanol and 2-methyl-butanol (mg/L)	188.62 ± 9.08a	230.96 ± 8.93b	212.96 ± 11.85ab	224.83 ± 15.01b	212.78 ± 10.67ab
2-Phenyl-ethanol (mg/L)	13.74 ± 1.20a	23.42 ± 0.95c	18.00 ± 0.60b	28.62 ± 1.39d	23.38 ± 1.00c
Hexanol (µg/L)	1131.24 ± 37.40b	1208.40 ± 53.76bc	1277.45 ± 71.62c	677.55 ± 15.71a	1415.87 ± 43.94d
Total higher alcohols (mg/L)	203.48 ± 9.95a	255.59 ± 9.61b	232.23 ± 11.84ab	254.14 ± 15.91b	237.58 ± 11.63b
Fatty acids					
Hexanoic acid (mg/L)	11.13 ± 0.30cd	10.78 ± 0.17bc	11.38 ± 0.14d	10.41 ± 0.15ab	9.90 ± 0.21ab
Octanoic acid (mg/L)	11.99 ± 0.20c	10.96 ± 0.25bc	11.56 ± 0.75c	10.31 ± 0.35b	9.05 ± 0.41a
Decanoic acid (µg/L)	4209.93 ± 141.19c	3975.70 ± 119.78bc	4233.22 ± 178.75c	3434.45 ± 229.12a	3718.80 ± 67.31ab
i-Valeric acid (µg/L)	1446.64 ± 12.53a	1493.16 ± 21.54a	1458.47 ± 20.22a	2093.35 ± 19.51b	1491.55 ± 19.36a
Total fatty acids (mg/L)	28.79 ± 0.38c	27.21 ± 0.39bc	28.64 ± 1.03c	26.26 ± 0.67b	24.15 ± 0.51a

Table 2. *Cont.*

Compounds	1	2	3	4	5
Terpenes					
Linalool oxide 1 (µg/L)	nq	nq	nq	nq	nq
Linalool oxide 2 (µg/L)	nq	nq	nq	nq	nq
Linalool (µg/L)	54.52 ± 1.96a	56.06 ± 1.16a	56.97 ± 1.49a	56.61 ± 2.17a	57.85 ± 2.54a
α-Terpineol (µg/L)	37.27 ± 0.64a	37.46 ± 0.89a	36.92 ± 0.61a	38.51 ± 0.90a	37.81 ± 0.60a
Total terpenes (µg/L)	91.80 ± 2.15a	93.52 ± 1.05a	93.89 ± 2.01a	95.12 ± 2.65a	95.65 ± 2.91a
Low volatile sulfur compounds					
H ₂ S	3.48 ± 0.28a	5.85 ± 0.15b	6.28 ± 0.29bc	7.65 ± 0.87cd	7.78 ± 0.82d
MeSH	nq	nq	nq	nq	nq

Average values of the quadruplicates ± standard deviation. The letters represent significantly different statistical groups ($p < 0.05$). Legend: nd, not detected; nq, not quantifiable (traces).

While previous studies are in accordance with this *M. pulcherrima* sequential fermentations result regarding the concentration of esters, others have found higher production of ester compounds than *Saccharomyces* spp. single fermentation [29,72]. Nevertheless, *M. pulcherrima* sequential fermentation produced the highest level of ethyl hexanoate and ethyl octanoate. These compounds confer apple peel and fruit flavors to the wine. Similar results have already been reported [14,73]. In previous studies, ethyl acetate production was reported higher for *M. pulcherrima* than the *S. cerevisiae* control [72,74]. In the present study, *Metschnikowia pulcherrima* produced the lowest amount of ethyl acetate (Table 2), as has been demonstrated by other authors [29].

T. delbrueckii produced higher levels of acetate compounds than the *S. cerevisiae* fermentation, producing higher concentrations of compounds such as isoamyl acetate, 2-methyl butyl acetate, and 2-phenyl-ethyl acetate. Other authors have reported a higher production of 2-phenyl-ethyl acetate [13,27,75].

Previous studies have highlighted the ability of *P. kluyveri* to produce ester compounds [76,77]. Total esters were produced the most by this strain. While its production of acetate esters was significantly higher than the *S. cerevisiae* control, its ethyl esters production did not differ. This result is not in agreement with the literature [29]. This difference could be explained by the length of time between the first and the second inoculation. If this is the case, a longer fermentation with *P. kluyveri* should improve the production of acetate ester. The different results can also be explained by the interaction between yeasts that can influence wine aroma [64,78]. The *P. kluyveri* trial also produced higher levels of isoamyl acetate, 2-methyl butyl acetate, and 2-phenyl-ethyl acetate than the other variants.

L. thermotolerans produced the highest level of ethyl esters. This high level is mainly related to the production of ethyl lactate. The trial produced the lowest amount of all the ethyl esters measured but it was the only producer of ethyl lactate (Table 2). However, the final concentration of ethyl lactate was lower than the sensory threshold of 60 mg/L [28,79]. Some studies have previously reported higher production of ethyl lactate by *L. thermotolerans* sequential fermentation than by *Saccharomyces* spp. single fermentation [59]. Ethyl lactate production might be favored by the formation of lactic acid which can produce ethyl lactate by an esterification reaction with ethanol [80]. Total esters were higher than the *S. cerevisiae* control. The total amount of esters is also influenced by the production of ethyl lactate. All the measured acetates were lower or equal to the *S. cerevisiae* control (Table 2). Thus, *L. thermotolerans* enhanced the milky and fruity flavors associated with ethyl lactate [28,79].

3.3.2. Higher Alcohols

Four higher alcohols were analyzed for the wines and shown in Table 2. Three of them were detected at levels above their odor threshold [5], with the exception of hexanol. *T. delbrueckii* sequential fermentation produced the highest level of higher alcohols, 256 mg/L (Table 2), but this trial was not significantly different to the other sequentially fermented assays.

P. kluyveri produced significantly more alcohols than the *S. cerevisiae* control. It produced significantly the highest level of 2-phenylethanol than the other trials, giving pleasant flavors such as honey, spice, rose, and lilac [5]. It also produced a significantly higher level of 3-methyl-butanol and 2-methylbutanol, which confer whiskey, malt, and burnt notes.

L. thermotolerans produced higher levels of higher alcohols in total than the *S. cerevisiae* control. In particular, it produced more of the 2-phenyl-ethanol, honey, spice, rose, and lilac fragrances than the *S. cerevisiae* control, and the greatest amount of the hexanol compound, resin, flower, and green fragrances [5]. While levels of both compounds are under their perception threshold, the level of higher alcohol contributes positively to the wine aroma [5].

3.3.3. Fatty Acids

Fatty acids were detectable via the analysis (Table 2). Four of them were analyzed and found in higher quantities relative to their odor threshold [5]. The total amount of fatty acid production ranged from 24 to 29 mg/L (Table 2). *S. cerevisiae* fermentation produced the highest levels of fatty

acids, followed by the sequential fermentation with *M. pulcherrima*. Both trials' levels were significantly equal according to the statistic. On the contrary, *L. thermotolerans* sequential fermentation produced the lowest levels.

P. kluyveri produced less fatty acids than the control. However, it produced significantly higher levels of i-valeric acid than other fermentations. This compound confers unpleasant notes such as sweat, acid, and a rancid flavor [5].

3.3.4. Terpenes

Four terpene compounds were analyzed but only two of them were quantifiable. Moreover, no significant differences were found between them. Levels of linalool, known for its flower and lavender notes [5], ranged between 55 and 58 µg/L (Table 2). This compound was produced in higher quantities than its perception threshold, 25.2 µg/L [5], and therefore contributed to the bouquet of the wine.

3.3.5. Low Volatile Sulfur Compounds

Among the analyzed low volatile sulfur compounds, only H₂S was detectable and quantifiable. Levels ranged from 3 to 8 µg/L (Table 2). According to the literature, the H₂S threshold varies across a wide range and a negative contribution to the flavor can already be expected at low microgram concentrations [81]. Thus, it could be sensorially detectable. *S. cerevisiae* fermentation produced a significantly lower amount of H₂S than sequential fermentations with non-*Saccharomyces*. On the contrary, *L. thermotolerans* produced the highest amount of H₂S. The high variability between strains and yeasts [49,82–84] and the limited information on H₂S production from sequential inoculation fermentation do not allow the confirmation of those results.

4. Conclusions

In this work, the ability of various yeast strains to enhance the aroma of Riesling wines was investigated. Five commercial wine yeast strains were chosen in order to compare their impact on the wine chemical composition. Four non-*Saccharomyces* species strains were selected due to their ability to produce specific aromas compared to a classic fermentation with a *S. cerevisiae*. Sequential inoculation allowed the modulation of the wines' non-volatile compound production. Significant differences between the trials of chemical parameters such as ethanol, pH, and glycerol were found in this study. Differences in volatile compounds were observed for esters, higher alcohols, fatty acids, and low volatile sulfur compounds. *T. delbrueckii* sequential fermentation produced the lowest acetic acid concentration and the highest concentration of higher alcohols. *M. pulcherrima* sequential fermentation produced the highest levels of ethyl hexanoate, ethyl octanoate, and shikimic acid. The *P. kluyveri* trial produced the highest concentrations of total esters, glycerol, and i-valeric acid. *L. thermotolerans* produced the highest concentrations of lactic acid, ethyl lactate, and H₂S.

Author Contributions: D.R., S.B. and O.D. developed the experimental design; O.D. performed the vinifications; O.D. and S.B. performed the formal data analysis and supervised the project; O.D., S.B. and D.R. wrote the article; D.R., S.F. and B.B. performed gas chromatographic analysis; C.-D.P. introduced to the FTIR analysis and assisted to data acquisition and statistical analysis. All authors discussed the results and contributed to the final manuscript.

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