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Exploring the Potential of Non-Conventional Yeasts in Wine Fermentation with a Focus on *Saccharomycopsis fermentans*

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Abstract: Despite the increasing number of publications on non-conventional yeasts (NCYs), many areas in this field remain poorly understood, making the examination of these strains important for determining their potential in wine fermentations. The amino acid metabolic pathways involved, particularly the catabolic Ehrlich pathway but also anabolic pathways such as the leucine biosynthesis pathway, are crucial for producing high-value aroma compounds that contribute to the final flavour of wine. We examined the potential use of *Saccharomycopsis fermentans* in wine fermentations. We selected mutant strains resistant to the toxic compound trifluoro-leucine (TFL), verified mutations in the SfLEU4 gene, and characterized the ability of the resulting strains to contribute to fermentation bouquets. Resistance to TFL relieves feedback inhibition in the leucine biosynthesis pathway and resulted in increased leucine biosynthesis. Concomitantly, the *S. fermentans* TFL-resistant mutants generated increased amounts of isoamyl alcohol and isovalerate during wine fermentation. Selection of TFL-resistant strains thus provides a generally applicable strategy for the improvement in NCYs and their utilization in co-fermentation processes for different grape must varieties.

Keywords: Ehrlich pathway; grape must; trifluoro-leucine-resistant mutants; *LEU4*

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1. Introduction

Wine fermentations involve complex biochemical processes to achieve the conversion of sugars to ethanol, carbon dioxide, and other volatile compounds by employing yeasts, specifically by *Saccharomyces cerevisiae*. Research has explored the roles of wine yeast strains and crucial parameters such as temperature, pH, oxygen levels, and the addition of nitrogen sources in the fermentation process and their impact on the final product [1,2]. Among the various secondary metabolites produced by yeasts during wine fermentations, active volatile flavour and aroma compounds crucially contribute to the wine bouquet [3]. Higher alcohols and esters, in particular, have a significant impact on the sensory attributes of wine [4].

During the early stages of fermentation, the presence of non-conventional yeasts (NCYs) can also contribute to the sensory characteristics of wines. Yeasts belonging to the genera of *Hanseniaspora*, *Pichia*, *Torulaspora*, *Kluyveromyces*, and *Metschnikowia* were found to dominate early stages of spontaneous wine fermentations due to their dominance in the vineyard. Crabtree-positive yeasts like *S. cerevisiae* take over only several days later [5,6]. *S. cerevisiae* proliferates under winemaking conditions, particularly due to its ability to grow anaerobically and its tolerance to high levels of sugar, sulphur dioxide, and heat stress [7].

The use of NCYs in wine fermentations may result in lower-alcohol-content wines with increased complexity due to the formation of a variety of higher alcohols and aromatic esters [8].

The Ehrlich pathway comprises a set of reactions resulting in the degradation of several amino acids into higher alcohols and further into their respective esters or acids. The pathway involves the transamination of an amino acid to its corresponding keto acid followed by decarboxylation to produce an aldehyde, which is then reduced to an alcohol. These alcohols can then be reduced into acetate esters or oxidized into corresponding acids [9]. In this way, leucine degradation leads to isoamyl alcohol; its ester, isoamyl acetate; or its acid, isovalerate. The production of isoamyl alcohol during fermentation is an important factor in determining the final flavour and aroma of a beverage [10,11]. The leucine biosynthetic pathway provides the starting compound for the derived aroma products. This pathway and its involved genes in *Saccharomyces cerevisiae* are shown in Figure 1. Studies have shown that the overexpression of the *LEU4* gene in yeast strains can significantly increase the production of isoamyl alcohol during wine fermentation, providing a potential strategy for the improvement in wine flavour [12]. Previous studies have demonstrated that resistance to 5,5,5-trifluoro-DL-leucine (TFL) is attributed to a mutation in the *LEU4* gene, specifically the mutant allele *LEU4fbr*, which is correlated with the resistance of α-isopropyl malate (a-IPM) synthase to leucine feedback inhibition, as reported by Baichwal et al. [13]. The *LEU4* genes of different *S. cerevisiae* mutants exhibiting resistance to TFL have been extensively characterized in previous studies by Cavalieri et al. [14]. These mutations primarily affect the R-region, which is responsible for leucine feedback inhibition.

NCYs are less studied. They may contribute to more complex aromas. Therefore, the use of non-conventional yeast, such as *S. fermentans*, may offer alternative options for the production of unique flavour profiles in wine through manipulation of the Ehrlich pathway and especially the leucine biosynthesis pathway [15]. In co-fermentation cultures, different inoculation approaches are utilized, with sequential inoculation being the prevailing method. In this approach, the NCY starter culture is initially introduced, allowing the NCY to exert its effects independently of *S. cerevisiae*. The addition of *S. cerevisiae* culture typically occurs after one to seven days or once a certain level of sugar consumption has been reached, depending on the NCY strain type. Alternatively, both starter cultures can be added simultaneously at the onset of fermentations. Another influential factor in the outcome of the wine is the ratio of *S. cerevisiae* to NCY strains [16]. Comparative studies of these inoculation modalities have revealed notable differences in the final quality of various alcohol beverages, emphasizing the intricate and unpredictable nature of the interaction between these distinct yeast strains [17,18].

To the best of our knowledge, there has been no investigation into the utilization of *Saccharomycopsis* yeasts in grape winemaking, particularly with *Saccharomycopsis fermentans* and its TFL-resistant mutants. This study focused on exploring the capacity of *Saccharomycopsis fermentans* to metabolize sugar with reduced ethanol production, as well as its impact on the aromatic characteristics of grape wine. Several other *Saccharomycopsis* species have been studied and have shown potential in producing interesting aroma products in different alcohol beverages [19–24]. One of the most popular NCY strains from *Saccharomycopsis* species is *Saccharomycopsis fibuligera*, which is present in various types of fermentation starters and is recognized for its ability to secrete α-amylase, β-glucosidase, and acid protease with high efficiency, which contributes to desirable flavours in the wine [25,26]. Despite the increasing number of publications on NCYs, many areas in this field remain poorly understood, especially regarding *S. fermentans*, requiring detailed examinations of this strain to determine its potential in wine fermentation. In previous studies of *S. fibuligera*, it was evident that varying sugar utilization patterns occurred in brewer's wort, enabling the production of both non-alcoholic and alcoholic beers [27–29]. In our preliminary single fermentations of *S. fermentans* in grape must, we also established that it generates very low levels of alcohol and still consumes some sugar from the must. This has led to the

conception of co-fermentation to reduce the alcohol level in the final must while preserving full flavour.

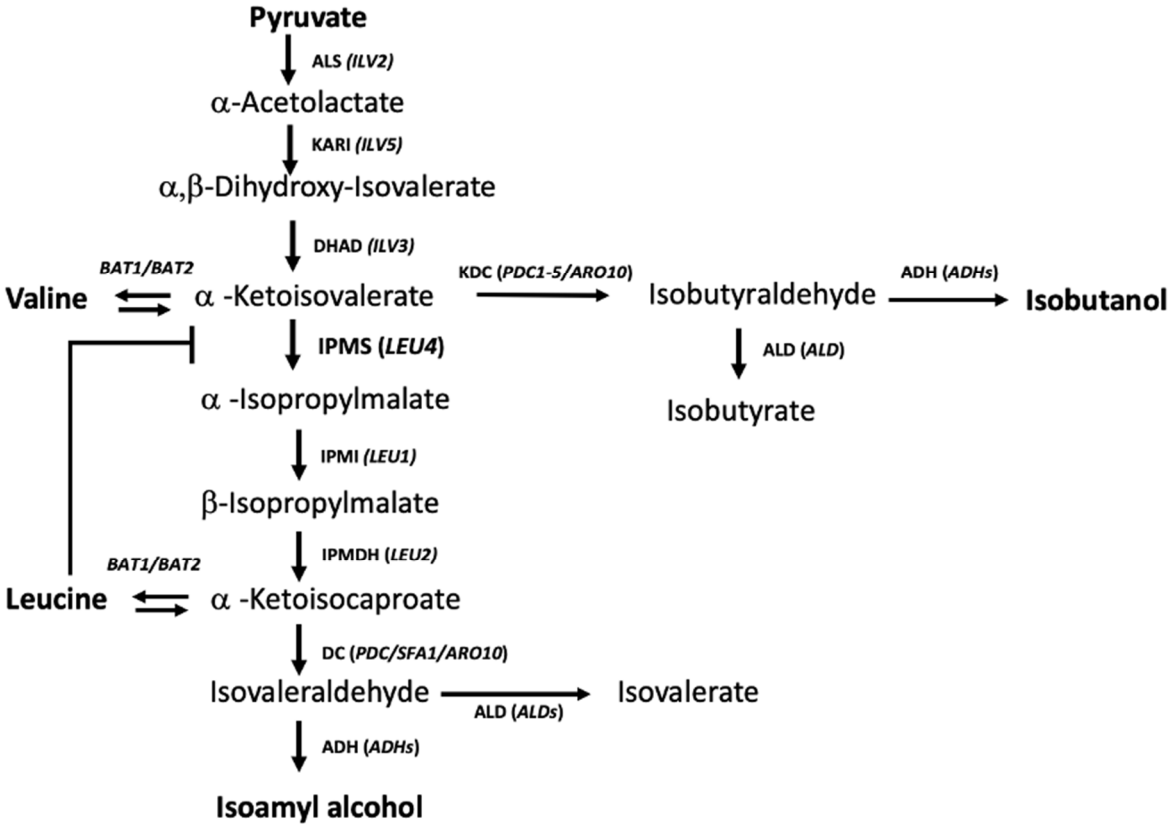


Figure 1. The process of isoamyl alcohol production and the regulation of the expression of the genes involved. Following the conversion of glucose into pyruvate and subsequently into α-ketoisovalerate, the enzyme α-isopropyl malate synthase (IPMS) becomes essential in producing both isoamyl alcohol and leucine. This is because IPMS is responsible for the feedback inhibition that results from leucine. Meanwhile, α-ketoisovalerate is also responsible for isobutanol production, with the help of alcohol dehydrogenase (ADH1). Isovaleraldehyde has another product in the form of isovalerate through aldehyde dehydrogenase (ALDH1, ALDH2).

2. Materials and Methods

2.1. Strains and Media

The yeast strains used in this study are shown in (Table 1). The *Saccharomyces cerevisiae* wine strain EC1118 was used for single fermentations and sequential co-fermentations. Rich medium (YPD, 20 g/L Bacto-peptone, 10 g/L yeast extract, 20 g/L glucose) was used for the propagation of yeast cells prior to fermentations.

Table 1. Strains used in this study.

Code Name	Strain Name	Description	Nucleotide Exchange	Amino Acid Residue in *S. cerevisiae*
EC1118	*Saccharomyces cerevisiae*	Wild-type wine strain	—	—
SFE	*Saccharomycopsis fermentans*	Wild type	—	—
G058, TFL1	*Saccharomycopsis fermentans*	Thr514Lys	ACA–AAA	Val522
G059, TFL2	*Saccharomycopsis fermentans*	His534Pro and Ala545Thr	GCT–ACT, CAC–CCC	His541, Ala551
G236, TFL3	*Saccharomycopsis fermentans*	Ser541Tyr	TCC–TAC	Ser547
G060, TFL4	*Saccharomycopsis fermentans*	Ser511Tyr	TCT–TAT	Ser519

The EC1118 strain is commercially available, SFE is wild-type CBS 7830, and TFL strains were obtained in this study. The *S. cerevisiae* residues correspond to ScLeu4p. Mutated positions are highlighted.

2.2. Trifluoro-Leucine (TFL)-Resistant Strain Selection

Wild-type *S. fermentans* was used for the selection of 5,5,5-trifluoro-DL-leucine (TFL)-resistant colonies. *S. fermentans* was inoculated in a Complete Supplement Mixture minus leucine (CSM-LEU) medium containing 20 g/L glucose, 1.7 g/L Yeast Nitrogen Base (YNB) without ammonium sulphate, 0.69 g/L CSM-LEU, 1 g/L asparagine, and 20 g/L agar. For the selection of TFL-resistant mutants, 150 µg/L TFL (5,5,5-trifluoro-DL-leucine, Sigma, Taufkirchen, Germany) was used according to Takagi et al. [30] on CSM-LEU plates. Twenty fast-growing colonies were picked and re-streaked on TFL selection spot assay plates with 150–300 µg/L TFL. The four fastest-growing colonies were chosen for further experiments.

2.3. PCR Amplification and Sequencing of LEU4 Amplicons

Publicly available sequences of the *LEU4* gene of *S. cerevisiae* and *S. fermentans* were aligned and compared in the lab before the PCR amplification. Over 70% identification was observed. TFL-resistant colonies were analysed for potential mutations in the *S. fermentans LEU4* gene. To this end, the *LEU4* ORF was amplified with two sets of primers (LEU4_U1–LEU4_M2 and LEU4_M1–LEU4_D1). The forward primer for the first part was 5′-GATTCAAGATTTTTGAAGAGAT-3′ and the reverse primer was 5′-CTTGCAGAAAACCGTTCATAAG-3′; as for the second part, the forward primer was 5′-AAACCTTTTCACGTTCAGTTAT-3′ and the reverse primer was 5′-AATCGAATATTTCT GCTCAAAT-3′. Primers were deduced according to the published draft genome sequence that is available at GenBank under accession number JNFW00000000 [21]. PCR products were sequenced (Starseq, Mainz, Germany). DNA sequence analyses were carried out using Geneious (Prime version 2019.2.1) software (Biomatters Ltd., NZL-1010 Auckland, New Zealand). Amino acids and nucleotide exchange sites were identified in each TFL-resistant mutant (see Table 1).

2.4. Fermentation Conditions

Lab-scale fermentations were carried out in triplicate with a standard pasteurized white wine Riesling, Chardonnay, and Müller-Thurgau musts of the indicated vintages derived from the university vineyards. These different types of musts were selected to potentially replicate and upscale the results of the lab-scale fermentations in the university winery setting. Riesling and Chardonnay fermentations were carried out in 250 mL Erlenmeyer flasks with 25 mL of must and Müller-Thurgau fermentation was carried out in 250 mL cylinder tubes with 50 mL of must. Musts were supplemented with the addition of 0.4 g/L Optimum-White (inactivated yeast product; according to the supplier's instructions; Lallemand, Vienna, Austria) and 0.3 g/L Vitaferm Ultra F3 (multi-nutrient complex containing nutrients such as amino acids, fatty acids, minerals, sterols, vitamins, etc.; according to the producer; Erbslöh Geisenheim GmbH, Geisenheim, Germany). *Saccharomycopsis fermentans* strains were inoculated at a density of 2×10^6 cells/mL, while the EC1118 strain was inoculated at a density of $OD_{600} = 0.5$. The single fermentation temperature was set to 18 °C, and cultures were incubated with constant stirring at 150 rpm for 11 days with only *S. fermentans* and TFL-resistant strains and the EC1118 strain as a control sample. Aliquots were then sampled and used for aroma profile analyses. EC1118 was added on the 12th day of the single fermentation. Sequential co-fermentations were run over a course of 23 days using the same conditions to allow EC1118 to finish fermentation. These sequential inoculation conditions were established through previous lab-scale fermentations trials. Daily measurements of weight loss were recorded, and at the end of the co-fermentation, residual sugars and ethanol content were measured via high-performance liquid chromatography (HPLC), and volatiles were analysed through gas chromatography and mass spectrometry (GC-MS).

2.5. Analytical Methods of Must Analysis

At the end of the fermentation process, high-performance liquid chromatography (HPLC) using an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) coupled

with an autosampler, a multi-wavelength (MWD), and a refractive index (RID) detector and a binary pump was utilized to analyse various compounds, such as fructose, glucose, ethanol, and organic acids. Quantitative analyses were conducted following the methodology outlined in Schneider et al. [31] and adapted as indicated in Scansani et al. [32] and are briefly described in the following: an Allure Organic Acids Column (length 250 mm, inner diameter 4.6 mm, and particle size 5 µm) from Restek (Bad Homburg v. d. Höhe, Germany) was used to separate the compounds. The organic acids were measured with the MWD (at a wavelength of 210 nm). The RID was applied for the detection of carbohydrates, organic acids, and ethanol. Purified water containing 0.5% ethanol acidified with 0.0139% concentrated sulphuric acid (95–97%) was used as the isocratic eluent. The flow rate was set at 0.6 mL/min using two column temperatures (29 °C and 46 °C). Chemstation software (Agilent, Germany) was used for analysis, integration, and determination of the concentrations of the individual analytes [32]. Headspace solid-phase microextraction (HS-SPME; multipurpose sampler MPS2 (Gerstel GmbH & Co. KG, Mülheim a. d. Ruhr, Germany)) alongside gas chromatography and mass spectrometry (GC-MS; 7890A and MS 5977B, Agilent Technologies, Waldbronn, Germany) was utilized to determine the presence of aroma compounds (higher alcohols, esters, fatty acids, etc.), following the analytical approach described in Tarasov et al. [33]. The process of sample preparation involved weighing 1.7 g of p.a. grade NaCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) into a 20 mL brown, glass headspace vial. Subsequently, 5 mL of the sample was added to the vial, along with 10 µL of each internal standard solution (1-octanol at 600 mg/L and cumene at 52 mg/L). The vial was then securely sealed using a magnetic screw cap. SPME extraction was performed with a 1 cm fibre (65 µm polydimethylsiloxane/divinylbenzene (Supelco)) for 20 min (incubation temperature: 40 °C, incubation time: 10 min). The sample was transferred to the GC with a cooled injection system (CIS-4, Gerstel, Mülheim an der Ruhr, Germany). Calibration was carried out with a model wine (10% (*v*/*v*) solution of ethanol in water, 3 g/L tartaric acid, adjusted to pH 3). A gas chromatography column of 60 m (length) × 0.25 mm (internal diameter) × 1 µm (film thickness) (Rxi-5Sil, Restek, Bellefonte, PA, USA), together with particular GC-MS software and technical settings (temperature program: 30 °C (1 min), 12 °C/s to 240 °C (4 min); split ratio 1:10), was used. Agilent MassHunter Workstation software was used for instrumental control, data acquisition, and analysis of the qualitative and quantitative data.

The following chemicals were used for the mobile phase: Purified water was taken from the TKA Thermo Scientific GenPure (Dreieich, Germany). Ethanol (ROTISOLV HPLC Gradient Grade) from Roth (Karlsruhe, Germany). Sulphuric acid (95–97%) was obtained from Merck (Darmstadt, Germany).

As reference substances for the analysis, we used citric acid anhydrous (puriss p.a. > 99.5%) and L-(+)-tartaric acid (puriss. > 99.5%) from Fluka (Buchs, Switzerland); D-(−)-fructose (>99%), D-(+)-glucose (≥99.5%), L-(−)-malic acid (97%), and-L(+)-lactic acid lithium salt crystalline from BioChemica; shikimic acid (>99%) from Sigma Aldrich (Seelze, Germany); ethanol (ROTISOLV HPLC Gradient Grade) from Roth (Karlsruhe, Germany).

The following chemicals were used as reference compounds for the analysis and were purchased from Sigma Aldrich (Seelze, Germany): hexanoic acid (>99%), octanoic acid (≥99%), decanoic acid (99.9%), i-valeric acid (99.5%), i-butanol (99.8%) (isobutanol), 2-methylbutanol (≥99%) (active amyl alcohol), 3-methylbutanol (≥99%) (isoamyl alcohol), hexanol (99.9%), 2-phenylethanol (≥99%) (phenylethyl alcohol), acetic acid phenylethylester (99%), acetic acid ethylester (>99.8%), acetic acid 2-methylbutylester (≥95%), acetic acid 3-methylbutylester (99.7%), benzeneacetic acid ethylester (99.8%), propionic acid ethylester (99%), i-butyric acid ethylester (99.8%), butyric acid ethylester (99%), lactic acid ethylester (98%), hexanoic acid ethylester (≥99%), succinic acid diethylester (99.8%), octanoic acid ethylester (99.8%), decanoic acid ethylester (99.8%), 2-methylbutyric acid ethylester (99%), and 2-methylbutyric acid methylester (99%). For the internal standards, we used octanol (99.8%) and cumene (99.9%). Acetic acid hexylester (99%) was acquired from VWR (Darm-

stadt, Germany). 2-Hydroxy-4-methylvaleric acid ethylester (>98%) was obtained from TCI (Eschborn, Germany).

The amounts of residual amino acids present in the Riesling must single fermentations were measured prior to sequential inoculation via post-column derivatization with ninhydrin and detection at 440 nm and 570 nm using maintenance-free LED photometers with ARACUS amino acid analyser (membraPure GmbH, Hennigsdorf, Germany). Sample preparation and analysis were carried out following the methodology outlined in Krause et al. [34]. All measurable amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine) were purchased from Sykam (Eresing, Germany).

3. Results

3.1. Verification of Obtained TFL-Resistant Mutants

S. fermentans strains are haploid heterothallic yeast strains. These strains are characterized by having a single set of chromosomes (haploid) and being unable to mate and form diploid cells (heterothallic) without a compatible mating partner. This makes them useful for controlled genetic modifications. We were able to select mutants resistant to TFL, a known toxic leucine analogue, from the wild-strain *S. fermentans*. Initial selection of *S. fermentans* cells on TFL plates identified several colonies of spontaneously resistant cells. The sequencing of the *LEU4* gene site for the chosen *S. fermentans* TFL-resistant mutants was carried out and compared to the wild-type *S. fermentans*. The draft genome sequence of *S. fermentans* was obtained previously [21]. The *S. cerevisiae LEU4* gene is encoded by YNL104C. The *S. fermentans LEU4* homologue is located on scaffold 1 of the draft genome sequence [21]. *SfLEU4* is placed between the *S. cerevisiae* homologues of *YLR106C* and *YNL241C*. The SfLEU4 open reading frame is 1782 bp in length and encodes a protein of 593 amino acids. In *S. cerevisiae*, *YOR108W/LEU9* encodes a paralogue of *LEU4* as a result of its whole-genome duplication. The SfLEU4 gene bears 64.5% and 65% sequence identity with *ScLEU4* and *ScLEU9*, respectively. The SfLeu4 protein shares 62.8% sequence identity with ScLeu4p and 64.1% with ScLeu9p. Since ScLEU4 provides major a-isopropylmalate synthase activity and Leu9 is a minor isoenzyme, we annotated the *S. fermentans* gene as *SfLEU4*.

The alignment of DNA and the amino acid sequence was performed to identify sites of mutation in the TFL-resistant strains and resulting amino acid sequence changes thereof. Mutated positions are listed in Table 1 and compared with mutations identified in the *S. cerevisiae LEU4* gene of TFL-resistant strains [15,35,36].

3.2. Fermentation Performance

Three different types of grape musts were selected for fermentation trials: Riesling, Müller-Thurgau, and Chardonnay. The chosen varieties were selected because Chardonnay usually has a high nitrogen content, while Riesling and Müller-Thurgau have lower levels of yeast available nitrogen. These varietal distinctions were expected to yield variations in yeast fermentation kinetics and flavour output. Single fermentations were carried out over 11 days, and sequential co-fermentations were initiated on the 12th day with the additional inoculation of EC1118. These fermentations were continued for an additional 23 days. Summary fermentation curves were plotted based on mass loss by combining the single and co-fermentation phases (Figure 2). These curves demonstrated that the contribution of *S. fermentans* strains to overall sugar consumption was much lower than that of the EC1118 wine yeast in all single fermentations. After the addition of the EC1118 strain to these single fermentations to start co-fermentation, the sugar consumption of all samples increased drastically. In most single fermentations, the TFL2 sample outperformed all other *S. fermentans* strains. In the Riesling and Müller-Thurgau co-fermentations, the TFL2 sample showed the best performance, while in Chardonnay, the TFL1 sample was better. In general, the fermentation process of Chardonnay exhibited a slower rate of activity in comparison

to the other two musts, despite the fact that the Chardonnay must had a higher nitrogen content, which was expected to enhance the fermentation process. Even EC1118 single fermentation demonstrated a slower rate in Chardonnay compared to Müller-Thurgau and Riesling. Overall, Müller-Thurgau co-fermentation showed more vigorous fermentation activity.

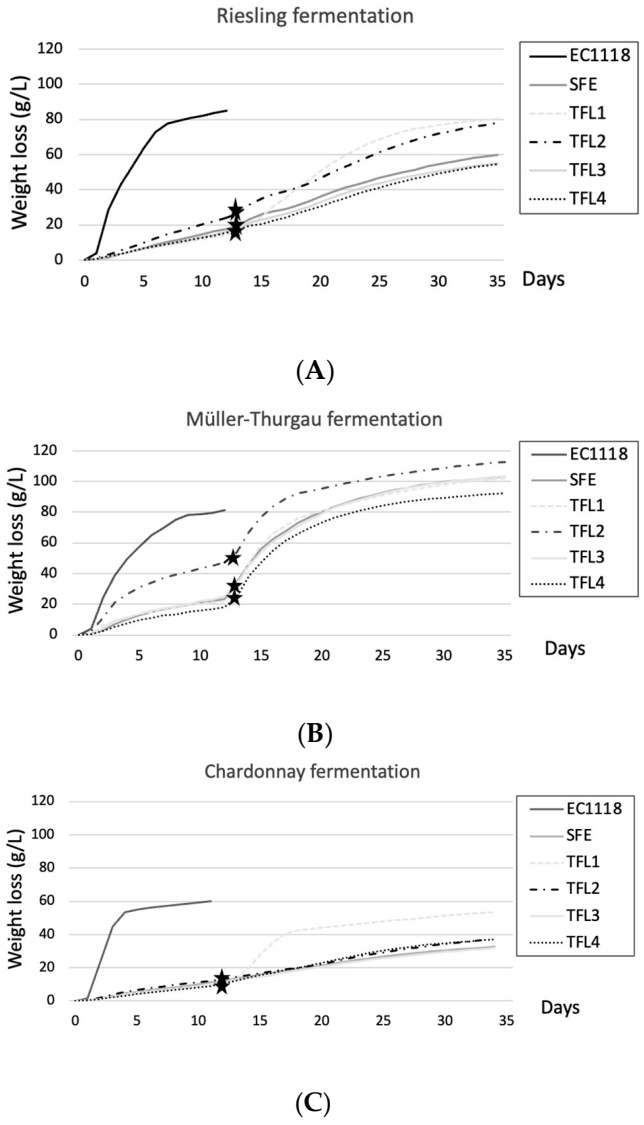


Figure 2. Fermentation curves based on CO_2 release (weight loss). Combined weight loss curves of single and co-fermentations as one sequential fermentation. Fermentation curves of three different musts, (**A**) Riesling, (**B**) Müller-Thurgau, and (**C**) Chardonnay, with (★) indicating when co-fermentation started on day 12 after the addition of EC1118.

3.3. Amino Acid Concentrations after Single Fermentation of Riesling Must

We analysed the amino acid composition of Riesling must before and after fermentations. The concentrations of amino acids involved in the Ehrlich pathway are listed in Table 2. As expected, most of the amino acids were metabolized by the yeast strains during the single fermentation, with EC1118 showing the highest degree of utilization overall. TFL resistance alleviates leucine's allosteric inhibition of *LEU4*, resulting in an excess of leucine production. This leucine overflow is catabolized via the Ehrlich pathway, as shown in the leucine pathway in Figure 1. Our amino acid measurement showed a higher concentration of leucine in the musts fermented with TFL-resistant strains compared to *S. fermentans*. As a consequence of *LEU4* inhibition, valine production is expected to decrease, which

is reflected in the utilization of valine from must by three TFL strains (Figure 1, Table 2). Interestingly, the levels of several other amino acids also involved in the Ehrlich pathway, such as phenylalanine, isoleucine, tyrosine, and tryptophan, were significantly elevated in the TFL2 sample; in the case of phenylalanine and tryptophan, the levels present in must were well above normal concentrations. This suggests that TFL resistance also induces changes in other amino acid biosynthesis pathways.

Table 2. List of selected amino acid measurements.

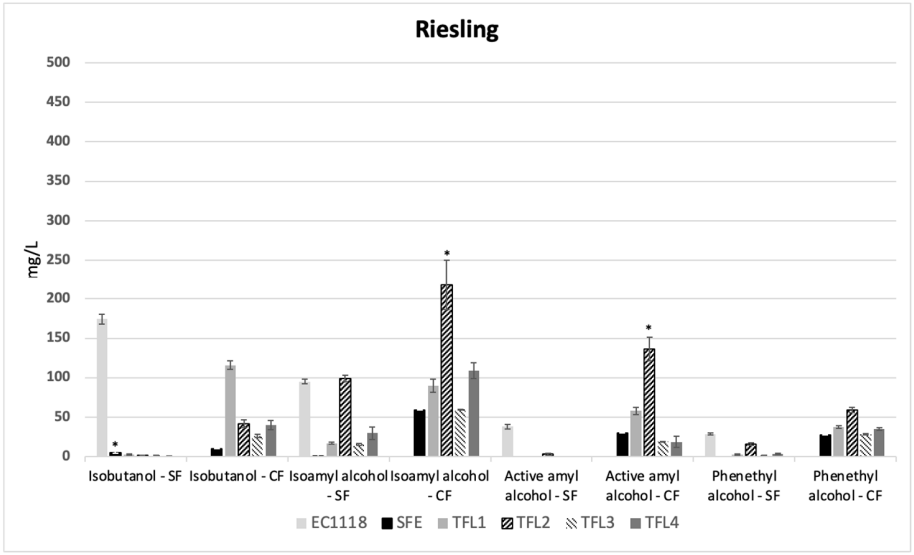
	Valine	Leucine	Isoleucine	Phenylalanine	Alanine	Methionine	Glycine	Glutamine	Threonine	Tyrosine	Tryptophan	Serine	Total
EC1118	0.2	0.4	0.3	0.3	6.2	0.2	0.7	0.3	1.1	0.2	0.1	0.7	21.9
SFE	**1.6**	0.4	0.5	2.1	2.6	0.1	1.2	0.1	1.2	0.1	<0.1	0.3	126.1
TFL1	<0.1	**3.2**	0.6	1.3	5.4	0.4	1.9	0.7	1.7	0.1	<0.1	0.3	171.2
TFL2	0.2	**3.2**	**2.2**	**7.7**	2.6	0.3	**5.8**	1.1	**10.0**	**3.2**	**1.7**	0.6	196.9
TFL3	0.1	**2.8**	0.4	0.4	5.1	0.2	1.9	0.7	1.3	0.1	<0.1	0.7	172.3
TFL4	1.5	**2.9**	0.7	2.3	4.1	0.8	2.2	1.2	3.5	0.6	<0.1	0.8	201.2
Riesling Must	9.2	7.2	4.9	6.6	77.3	0.9	2.4	<0.1	30.3	3.9	<0.1	27.5	589.7

Residual amino acid measurements after the single fermentation, concentration in mg/L. Significant increases are highlighted (see also Table S2).

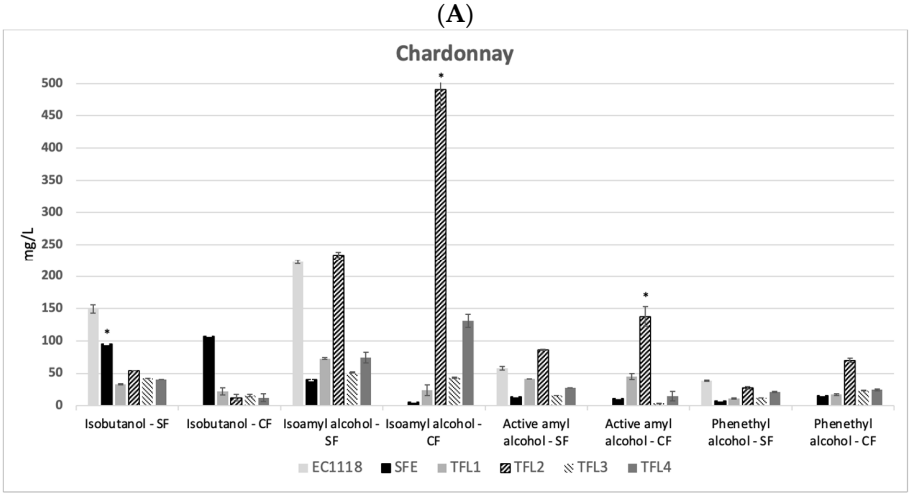
3.4. Aroma Analysis of Fermentations

We conducted standard analyses (HPLC and GC-MS) of aroma compounds, including for esters, higher alcohols, and acids. Amino acid degradation via the Ehrlich pathway generates higher alcohols such as isoamyl alcohol, active amyl alcohol, isobutanol, and phenethyl alcohol, which are important aroma compounds that contribute to the sensory characteristics of wine. We clearly observed the elevation of all these aroma compound levels in all co-fermentation samples compared to the single fermentations. The difference between wild-type *S. fermentans* and TFL-resistant mutants was also observed in the levels of isobutanol and isoamyl alcohol, which corresponds with the results of amino acid measurements, where valine and leucine levels are in accordance with the alleviation of the feedback inhibition of Leu4 (Figure 3). Although all strains generated various compounds, our findings show that EC1118, which is a prominent wine production strain, produced less isoamyl alcohol in comparison to the TFL2 sample in all single fermentation musts. Moreover, their joint effort in co-fermentation doubled isoamyl alcohol levels in all three musts.

The aroma profiles of single fermentations and co-fermentations showed significant differences. The most prominent difference could be observed in the levels of isovaleric acid, which differed significantly between the EC1118 strain and other strains, as well as across the different must types (Table 3). The increase in isovaleric acid levels in TFL-resistant mutants compared to wild-type *S. fermentans* can be attributed to the loss of feedback inhibition of *LEU4* and subsequent overproduction of leucine, as one of the products of the leucine biosynthetic pathway is isovalerate. Overall isovaleric acid is not one of the desired aroma compounds in wine.



(**A**)



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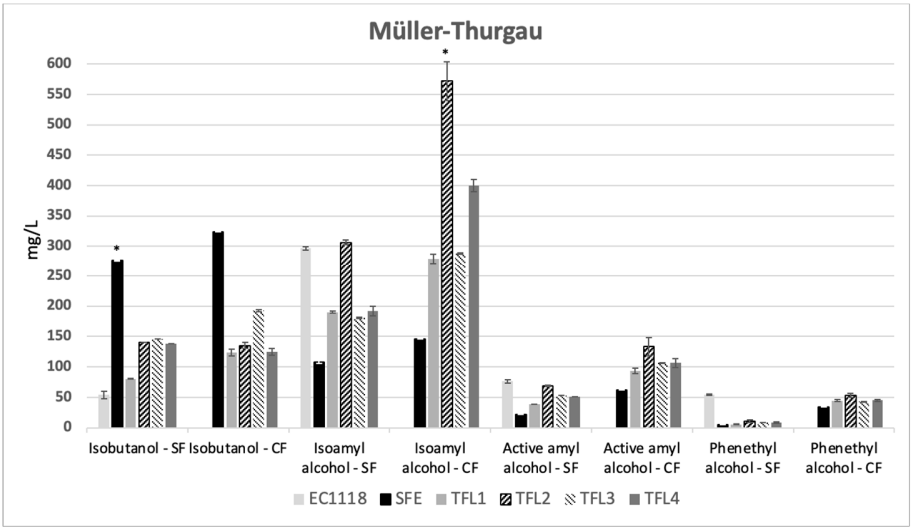


(**C**)

Figure 3. Bar charts with the amounts of selected higher alcohol compounds (in mg/L) in single fermentations (SFs) and co-fermentations (CFs) of different must types (**A**–**C**). Higher alcohol compounds were quantified via GC-MS at the end of each fermentation. (*) indicates a significant increase.

Table 3. Acids generated at the end of each fermentation *.

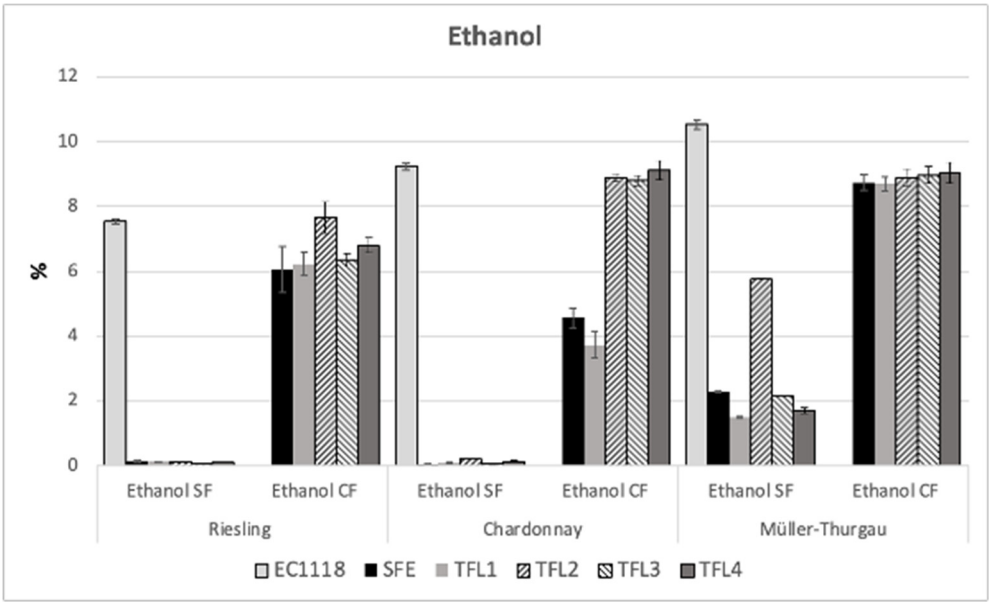
Must Type	Strain	Tartaric Acid (g/L)		Malic Acid (g/L)		Shikimic Acid (mg/L)		Lactic Acid (g/L)		Acetic Acid (g/L)		Citric Acid (g/L)		Isovaleric Acid ** (mg/L)	
		SF	CF	SF	CF	SF	CF	SF	CF	SF	CF	SF	CF	SF	CF
Riesling	EC1118	4.5		2.7		23.9		<0.1		5.4		<0.1		3	
	SFE	6.8	6.1	1.9	<0.1	28.6	23.3	<0.1	<0.1	<0.1	<0.1	<0.1	0.4	20	116
	TFL1	6.7	6.2	1.6	<0.1	29.9	23.3	<0.1	<0.1	<0.1	<0.1	0.1	0.3	76	302
	TFL2	6.8	6.2	<0.1	<0.1	29.4	21.9	<0.1	<0.1	<0.1	0.1	0.2	0.3	118	486
	TFL3	6.8	6.0	1.4	<0.1	29.0	22.5	<0.1	<0.1	<0.1	<0.1	0.1	0.3	80	419
	TFL4	5.4	5.6	1.9	<0.1	28.3	23.5	<0.1	<0.1	<0.1	<0.1	0.1	0.3	73	329
	Must	6.2	6.2	3.9	3.9	29	29.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	nd	nd
Chardonnay	EC1118	3.6		3.5		46		<0.1		6.9		<0.1		4	
	SFE	3.8	3.5	3.7	<0.1	55	50.8	0.17	<0.1	<0.1	0.3	<0.1	0.2	9	80
	TFL1	3.5	3.6	3.5	0.8	54	40.4	0.14	<0.1	<0.1	0.1	<0.1	0.2	34	71
	TFL2	3.5	3.3	3.1	<0.1	48	51.0	0.18	<0.1	<0.1	<0.1	<0.1	0.2	33	289
	TFL3	3.4	3.5	1.6	<0.1	55	49.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.2	29	253
	TFL4	3.4	3.5	2.4	0.6	54	51.2	<0.1	<0.1	<0.1	0.2	<0.1	0.2	50	206
	Must	3.3	3.3	4.6	3.8	54	52.9	<0.1	<0.1	<0.1	<0.1	<0.1	0.2	nd	nd
Müller-Thurgau	EC1118	4.4		1.8		25		0.16		0.36		<0.1		5	
	SFE	5.4	5.1	1.6	1.5	16	13.4	0.48	0.3	<0.1	0.1	<0.1	0.1	3	9
	TFL1	5.5	5.2	1.3	1.2	26	15.1	0.44	0.3	<0.1	0.4	<0.1	0.1	11	19
	TFL2	5.4	5.1	1.1	1.2	25	6.7	<0.1	0.2	0.36	0.1	<0.1	<0.1	20	21
	TFL3	5.4	5.1	1.3	1.6	25	14.2	0.69	0.4	<0.1	0.2	<0.1	0.1	8	14
	TFL4	5.3	5.1	1.3	1.5	25	14.8	0.40	0.4	<0.1	0.1	<0.1	0.1	10	19
	Must	5.6	5.0	1.6	1.6	28	26.0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	nd	nd

* This table contains the HPLC results for measured acid concentration generated during the fermentation of three grape varieties, Riesling, Chardonnay, and Müller-Thurgau, after both single fermentation (SF) and co-fermentation (CF) processes. ** Isovaleric acid was measured via GC-MS. nd: not detected. See also Table S3.

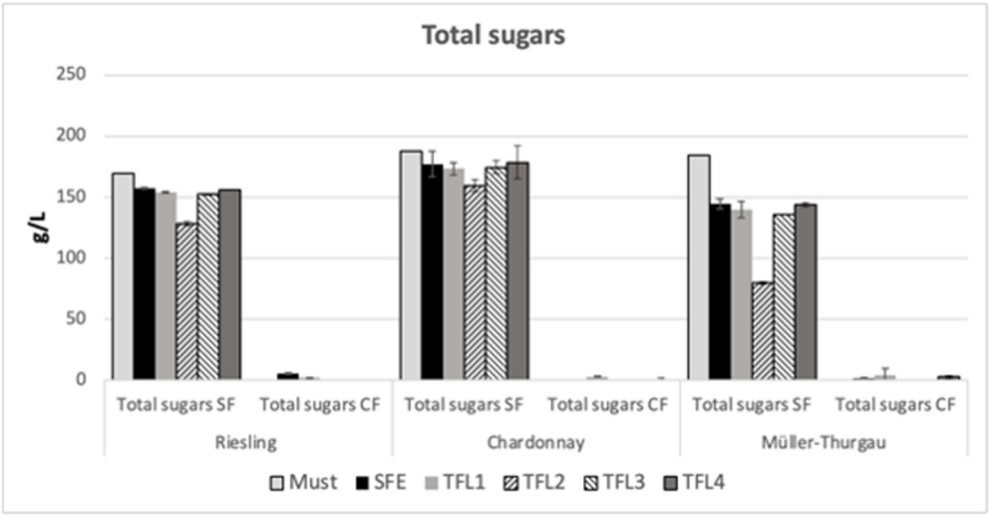
3.5. HPLC Results of Ethanol and Sugars

For more detailed analyses of the fermented wines, we determined the residual sugars and final ethanol content using HPLC. The alcohol produced at the end of the fermentations can be observed in Figure 4A, where it can be noted ethanol was typically not present in the pure must. The total sugar concentration of residual glucose + fructose is shown in Figure 4B, and pure must was used as an indicator of initial sugar levels compared to the consumed sugar levels after each fermentation. EC1118 completely consumed all sugars within the first 5–7 days. It was observed that *S. fermentans* strains did not produce ethanol in a single fermentation of Riesling must, but rather reduced sugars by an average of 10–18 g/L, with a maximum reduction of 42 g/L, as observed in the TFL2 sample. Similar results were obtained in Chardonnay single fermentations, where no significant ethanol production was observed in *Saccharomycopsis* strains, but there was a reduction of 10–15 g/L in sugar levels, with the TFL2 strain showing the highest reduction at 28 g/L, consistent with the earlier results for Riesling must. Interestingly, in Müller-Thurgau must single fermentations, there were low amounts of ethanol observed in the *S. fermentans* strains, with an overall reduction in sugars of around 40–50 g/L, and the TFL2 strain again showed the highest reduction at 105 g/L.

In all co-fermentations, EC1118 managed to carry the fermentation to completion and utilized all remaining sugars. EC1118 finished co-fermentations with an average ethanol production of 6–7% in Riesling must. Co-fermentation with *S. fermentans* strains reduced ethanol levels by 1–2% in Riesling must. As a consequence of the high performance of TFL2, alcohol reduction in this sample was minimal compared to other *S. fermentans* strains. The reduction in ethanol in the Chardonnay fermentation was slightly different. Low reduction was observed not only in TFL2, but also in the TFL3 and TFL4 samples, while *S. fermentans* and TFL1 showed higher ethanol reductions at 4.5% and 5.5%, respectively. The co-fermentation of Müller-Thurgau showed high ethanol production in all samples, including EC1118, which had the highest ethanol production among the controls. As a result, there was a reduction in ethanol of 1–2% in *Saccharomycopsis* strains.



(**A**)



(**B**)

Figure 4. Bar charts with the HPLC measurements of (**A**) ethanol at the end of fermentation and (**B**) total sugar concentrations of glucose + fructose at the end of single fermentations (SFs) and co-fermentations (CFs) in different must types. Pure must was used as a control indicator for initial sugar amounts to compare the consumption after each fermentation. EC1118 utilized all sugars within the first week of fermentation (**B**).

4. Discussion

Saccharomycopsis fermentans is a non-conventional yeast which, to the best of our knowledge, has not previously been used in grape must fermentations. In our study, we focused on *S. fermentans*, which is a haploid yeast, making it easier to modify this strain through non-GMO methods [21]. This has been demonstrated in previous studies which focused on TFL-resistant mutants in *S. cerevisiae* [30]. Interestingly, the mutation sites observed in our four *S. fermentans* TFL-resistant mutants displayed a close similarity to those reported in previous studies on *S. cerevisiae* [35,37]. Our results also reveal mutations accumulated within the R-region of the IPMS enzyme (Leu4). IPMS catalyses the condensation of acetyl-CoA and α-ketoisovalerate, leading to the formation of 2-isopropylmalate, which is further converted to leucine [10]. Specifically, the amino acid exchange observed in TFL2 and TFL4

at the His534Pro and Ser511Tyr sites, respectively, correspond to the mutations previously reported in *S. cerevisiae*, namely His541Pro and Ser519Thr (see Table 1).

To further explore the implications of our findings, TFL-resistant mutants and wild-type *S. fermentans* were compared with respect to isoamyl alcohol production during single fermentations of various must types. The results show that TFL-resistant mutants produced significantly more isoamyl alcohol, phenethyl alcohol, and active amyl alcohol in single fermentations compared to the wild-type *S. fermentans* across all must types [38]. This is likely due to the leucine biosynthetic pathway with a negative feedback loop that was disrupted by mutation, leading to leucine accumulation. Interestingly, the increase in phenethyl alcohol was observable in all TFL2 single fermentations in all musts compared to wild-type *S. fermentans*. This may be due to the efficient mutation and overall high metabolic activity demonstrated by the TFL2 strain, which helps to elevate other higher alcohol productions in the Ehrlich pathway [39]. In a study by Vuralhan et al. [40], yeast cultures were grown with phenylalanine, leucine, or methionine as a nitrogen source, resulting in high levels of the corresponding fusel alcohols and organic acids, indicating utilization of the Ehrlich pathway and suggesting the involvement of *ARO10*-dependent common enzyme activity. These results correspond to our elevated IPMS activity, where α-ketoisovalerate is highly activated for leucine production and isobutanol side production is limited, but *ARO10* is utilized [41]. Additionally, we observed that wild-type *S. fermentans* produced more isobutanol than all TFL-resistant mutants. This was also due to the leucine biosynthetic pathway, as the undisturbed negative feedback loop in wild-type *S. fermentans* should accumulate valine, which is the precursor of isobutanol [42,43].

The *S. fermentans* strains studied produced lower levels of esters, which might have had a negative impact on wine quality due to the oxidative, rusty, and sweaty notes that are associated with it [42]. Volatile acid production was also high for the *Saccharomycopsis* yeasts, with isovaleric acid values being much higher compared to the reported acceptable odour thresholds [31], which can be explained by the high metabolic activity in all TFL strains. Oxygen is crucial in the Ehrlich pathway and can control the generation of desired fusel alcohols and fusel acids [36]. Our results suggest that oxygen exposure led to a shift in the Ehrlich pathway towards producing more fusel acids in all *Saccharomycopsis* yeast fermentations. The Riesling and Chardonnay fermentations especially exhibited significantly higher levels of isovaleric acid compared to the Müller-Thurgau fermentation. This difference could potentially be attributed to the utilization of cylindrical tubes during the Müller-Thurgau fermentation process, which provides less head space and, therefore, limits the availability of oxygen intake during fermentation.

In parallel to the laboratory-scale fermentation, upscale fermentations were conducted using Riesling must in a winery setting, and the resulting wine was subjected to sensory evaluation by a panel of experts. The results from this evaluation indicate that with certain refinements, this type of wine could be interesting for consumers (Table S1 in the Supplementary Materials). This suggests that better adjustment of fermentation properties and optimization can reduce alcohol concentration in wine, due to demand from consumers [44].

5. Conclusions

To the best of our knowledge, this is the first study on *S. fermentans* and grape must fermentation. In this study, we demonstrated the feasibility of utilizing TFL selection in a non-conventional yeast. Single fermentations and co-fermentations of *Saccharomycopsis* TFL-resistant mutants with a wine yeast provided insight into the behaviour of *S. fermentans* in small-scale grape must fermentations. Such studies may also be useful for the generation of flavour-adapted beverages with other non-conventional yeasts enhanced through TFL selection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090786/s1, Table S1: results of simple descriptive test of winery fermentation of Riesling must (upscale), Table S2: amino acid measurements, Table S3: complete dataset of the measured acids with statistical values.

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