fermentation

MDPI

Article

Exploring Copper's Role in Metabolic Modulation of *Lachancea thermotolerans* in Winemaking Conditions

Scott Simonin [1,*], Valentina Bianconi [2], Federico Sizzano [2], Christine Monnard [2], Sylvain Schnée [3], Gilles Bourdin [2], Esteban Alfonso [1] and Benoit Bach [1]

[1] Changins, Viticulture and Enology, HES-SO University of Applied Sciences and Arts Western Switzerland, Route de Duillier 52, 1260 Nyon, Switzerland; esteban.alfonso@changins.ch (E.A.); benoit.bach@changins.ch (B.B.)

[2] Agroscope, Oenology Research Group, Department of Plant Production Systems, 1260 Nyon, Switzerland; valentina.bianconi@agroscope.admin.ch (V.B.); federico.sizzano@agroscope.admin.ch (F.S.); christine.monnard@agroscope.admin.ch (C.M.); gilles.bourdin@agroscope.admin.ch (G.B.)

[3] Agroscope, Mycology Research Group, Department of Plant Protection, 1260 Nyon, Switzerland; sylvain.schnee@agroscope.admin.ch

* Correspondence: scott.simonin@changins.ch; Tel.: +41-22-363-40-75

Abstract

The phenomenon of global warming has been demonstrated to induce the premature ripening of grapes, resulting in a concomitant increase in sugar and a decrease in acidity. This can result in the production of wines that are characterised by an imbalance in terms of alcohol content and acidity. The utilisation of lactic acid-producing *Lachancea thermotolerans* yeast in fermentation represents a promising strategy for reducing the pH of must without the need for chemical acidification. However, it is important to note that the fermentation rate and lactic acid production can be influenced by several factors present in the must. The present study focused on the effect of copper, used in organic vineyards to limit mildew, on biomass and lactic acid production by two *L. thermotolerans* oenological strains in laboratory-scale fermentations. The methodology used is based on an innovative integrative approach combining conventional microbiological, spectroscopic, cytometric and transcriptomic analyses. The results obtained demonstrated that the addition of copper increased lactic acid production in comparison with the control condition and this effect was strain-dependent, reaching up to 16.15 g/L for strain Lt1 under conditions of high copper concentration (20 mg/L). The monitoring of microbial populations demonstrated discrepancies in the fermentation kinetics in conditions exhibiting varying concentrations of copper and showed that an increased duration of cell generation is evident under conditions exhibiting elevated copper concentrations, particularly in the context of the Lt1 strain. Furthermore, the analysis of the samples using flow cytometry revealed the oxidative status of the two strains throughout the fermentation process. Transcriptomic data indicated that metabolic pathways involved in cell communication, ribosomal and cell wall activity of *L. thermotolerans* were modulated by the presence of copper. The findings of this study demonstrate strain-specific effects of copper on *L. thermotolerans*, which may be of interest to winemakers, particularly those engaged in organic viticulture who intend to use acidifying yeasts during warm vintages.

Keywords: *Lachancea thermotolerans*; copper fungicides; winemaking acidification; microbiology; flow cytometry; transcriptomic approach

Academic Editor: Maurizio Ciani

Received: 24 July 2026
Revised: 21 August 2026
Accepted: 26 August 2026
Published: 27 August 2026

1. Introduction

Lachancea thermotolerans is a species of non-*Saccharomyces* yeast that has been described as a major producer of lactic acid [1–5]. This species has been found in substantial proportions on grape berries and is part of the consortium of yeasts that are found during the process of winemaking. The majority of strains has the capacity to convert a proportion of the sugar into ethanol and another proportion into lactic acid, thanks to the enzyme lactate dehydrogenase present in its metabolism. The specific conditions of the fermentation process appear to have a significant impact on the amount of lactic acid produced [2,4,6–18]. *L. thermotolerans* has been observed to produce concentrations reaching up to 15 g/L and this process has been shown to result in a reduction in the pH level of grape must [4,10]. However, this yeast species exhibits a moderate sensitivity to increases in alcohol during the process of alcoholic fermentation, with an inhibition rate ranging from 7 to 10% alcohol. Consequently, in the majority of cases, it is unable to finish the alcoholic fermentation, resulting in another inoculation with a strain of *Saccharomyces cerevisiae* to complete the process [19–21].

Recent years have seen significant changes in the fermentation process [22–24], largely driven by the imposition of stringent regulations pertaining to the addition of chemical substances [25–27]. There is a noticeable tendency towards wines that have not undergone oenological interventions [28–31]. The modification of such behaviours necessitates superior microbiological control. Moreover, climate change can alter grape composition across vintages and consequently modify the conditions under which alcoholic fermentation occurs. This variability may affect fermentation kinetics and final wine quality, highlighting the importance of accurate monitoring of grape and wine composition [32]. In this context, the selection of robust yeast strains capable of maintaining consistent fermentative performance under variable environmental and compositional conditions is increasingly relevant. A more detailed characterisation of microbial starters may therefore contribute to improving fermentation stability and wine quality [33].

In particular global warming has exerted a substantial influence on the equilibrium between vine and wine, particularly by modifying the balance between the accumulation of sugar and the degradation of organic acids in grapes. [34,35]. The decline in the concentration of organic acid results in an increase in pH. The latter, a pivotal component in the stabilisation of wine, has undergone a marked increase [36,37]. The utilisation of the yeast species *L. thermotolerans* to produce lactic acid, a process which results in a decrease in pH, is a logical and rational approach to produce balanced wines in warn vintages [1,5].

The characterisation of the yeast *L. thermotolerans* remains a subject of limited comprehension, being influenced by a multitude of surrounding parameters [2,20]. One such factor is the concentration of copper in the must. Copper is one of the most abundant heavy metals in must and wine, with different origins including, but not limited to, fungicides residues, enrichment in soil, winemaking equipment, and exogenous addition [38]. Empirical evidence has demonstrated the efficacy of copper in mitigating the incidence of downy mildew in organic vineyards [38,39]. It is evident that environmental, health and social factors are once again exerting pressure on winegrowers to enhance the management of this input [25,38,40]. Recent analyses have demonstrated that copper concentrations in must can, on occasion, reach levels of up to 20 mg/L, particularly in vineyards that have undergone intensive copper-based treatments [41–43]. Excessive doses have already been shown to have a significant impact on microbial balance [38,44,45] and the presence of the substance in question has been revealed to diminish the physiological activity of certain yeast species [46–48]. The yeast species *S. cerevisiae* has been the subject of research in this field, and it has been demonstrated that this species exhibits resistance to copper within its species, with certain strains demonstrating a high degree of resistance [48–51].

In order to provide answers to the aforementioned issue, the focus has been directed towards the lactic acid production of two *L. thermotolerans* yeast strains in the presence of different copper concentrations. The contributions are of considerable interest within the oenological domain. The integration of methodologies, including biomass, fermentation kinetics, analytical data, flow cytometry and transcriptomic studies, facilitate a more profound comprehension of the mechanisms involved and shed light on the physiological and phenotypic characteristics of this species in greater detail. The practical applications of this research are of oenological interest, as it offers a more profound comprehension of the utilisation of *L. thermotolerans* as a yeast for the purpose of biological acidification.

2. Materials and Methods

2.1. Laboratory Experimental Design

Chasselas must from the Agroscope domain of Pully (Vaud, Switzerland, 2022: Total sugar 194 g/L and pH 3.55) was used for these experiments. The must was cleared, frozen at −20 °C and thawed for these tests. It was then placed in sterile 500 mL bottles with gas release, pasteurised for 20 min at 60 °C and returned to room temperature by cooling with running water [5]. Two independent biological replicates were prepared for each experimental condition. Copper sulphate was added to the must to obtain final concentrations of 0, 10, and 20 mg/L of Cu using a 2 g/L $CuSO_4$ stock solution. These concentrations were chosen in relationship to extreme data found in the existing literature on the subject [41]. Two commercial strains of *L. thermotolerans* were used for this experiment: Levulia^R Alcomeno, which is marketed by the AEB Group (Brescia–Italy) and referred to as Lt1; And Level^2 Laktia™, which is marketed by Lallemand Wine (Blagnac–France) and referred to as Lt2. The strain used as a control was an indigenous isolate strain of *S. cerevisiae* listed from our laboratory collection and listed as Sc1. Each yeast strain was inoculated at 2 × 10^6 CFU/mL, in accordance with the technical data sheets provided by the suppliers. Alcoholic fermentation was carried out at 20 °C in a temperature-controlled oven and monitored daily throughout the process.

2.2. Monitoring Microbial Population Kinetics

The microbial populations were monitored throughout the alcoholic fermentation process using a specific YPD agar medium (20 g/L glucose, 5 g/L yeast extract, 10 g/L peptone, 0.2 g/L chloramphenicol, 20 g/L agar). This medium facilitates the observation of the growth of yeasts and their capacity to form colonies under controlled conditions. The following days were selected for monitoring: days 0, 1, 2, 3, 4, 6, 8, 10 and 13. The sampling was performed in triplicate. The generation time (g) of the yeast strains on the first day will be used as an indicator of the impact of copper on their development. The first day was selected as it most accurately reflects the exponential growth phase, where growth conditions have not yet been restricted, thus enabling a more precise estimation of the intrinsic generation time. The generation time is expressed in number of generations (g) in order to provide a normalised measure of growth. The following equation is employed:

$$g = (t_{day0} - t_{day1}) \times \ln(2)/(\ln(n_{day1}/n_{day0}))$$

Factors: n: Population in CFU (Colony-Forming Unit)/mL; t: Time in days.

2.3. Biomass Production by Microorganisms

The mass of each fermentation sample was measured at predetermined time intervals (as outlined in Section 2.2) using an analytical balance. The decrease in sample weight over time was used as an indirect measure of CO_2 production, on the assumption that mass loss primarily resulted from CO_2 release during fermentative metabolism.

The production of carbon dioxide (CO_2) was calculated as the difference between the initial and recorded mass at each designated time point.

2.4. Analysis of Oenological Data by Fourier Transform InfraRed (FTIR) Spectroscopy

The analysis of oenological parameters using 15 mL samples was conducted at two distinct stages by means of Fourier transform infrared (FTIR) spectroscopy: must and finished wines. pre-fermentation and post-fermentation. The following parameters were measured: Tartaric acid (g/L), malic acid (g/L), acetic acid (g/L), lactic acid (g/L), glucose + fructose (g/L), glycerol (g/L), alcohol content (volume percentage) and pH.

2.5. Flow Cytominmetry (FCM)

The fermentation process was monitored on a daily basis by means of flow cytometry (FCM) in order to assess microbial physiological states at the single-cell level. Specifically, FCM was used to quantify cell viability, metabolic activity, oxidative stress, and intracellular redox status. In summary, 50 µL of fermenting must were diluted in 12 × 75 mm cylindrical tubes with 450 µL of phosphate-buffered saline (PBS). Subsequently, 500 µL of a mixture of fluorescent dyes was added to the tubes. The mix contained 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester, for general metabolic activity (CFDA-AM; Invitrogen, USA; final concentration, 2 µM), propidium iodide, to detect dead cells (PI; Fisher Scientific, Waltham, MA, USA; final concentration, 0.5 µg/mL); CellROX Deep Red, for oxidative stress (Fisher Scientific, USA; final concentration, 1 µM) and Thiol Tracker, for reduced Glutathione (GSH) (Fisher Scientific, USA; final concentration, 10 µM). Following a 20-minute incubation period at room temperature, the samples were analysed using a MACSQuant® 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with 405 nm, 488 nm, and 633 nm laser lines. The data were stored in the mqd format and analysed using FlowLogic software, version 8.1 (Inivai, Mentone, VIC, Australia).

2.6. Transcriptomic Analysis

The third day of fermentation was selected for this transcriptomic analysis as a representative time point of active fermentation. RNA-seq was performed on one biological sample for each experimental condition, resulting in four transcriptomic samples: Lt1 Cu0, Lt1 Cu10, Lt2 Cu0 and Lt2 Cu10. The transcriptomic data were therefore analysed at the individual-sample level. Because biological replicates were not available for the RNA-seq experiment, biological variance could not be estimated independently. Consequently, the transcriptomic analysis was considered exploratory and was primarily used to identify genes and pathways showing the strongest transcriptional changes associated with copper exposure. The 10 mg/L Cu condition was selected for transcriptomic analysis based on preliminary experiments indicating an increase in lactic acid production in Lt1. This concentration also allowed both *L. thermotolerans* strains to be included within a manageable experimental design.

The total RNA was extracted from 100 mL of culture immediately after sampling and using the SPINeasy RNA Kit for Yeast (MP Biochemicals, Irvine, CA, USA), in accordance with the manufacturer's instructions. The RNA concentration and integrity were measured using a 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). The RNA was of high quality, RIN > 7. The RNA samples (1 µg) were dispatched to Macrogen (Amsterdam, The Netherlands) for library construction and sequencing. The construction of cDNA libraries from total RNA was undertaken using the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA). The quality of the libraries was then verified using a TapeStation D1000 Screen Tape (Agilent Technologies, Santa Clara, CA, USA). Subsequently, the process of sequencing was conducted on an Illumina NovaseqX platform, utilising a

10B flow cell. This facilitated the generation of paired-end reads, characterised by a length of 150 base pairs.

Low-quality reads, adaptor sequences and other artefacts were removed using their standard preprocessing workflow, which includes adapter trimming and quality filtering based on Phred quality scores. The remaining clean reads were then aligned to the *L. thermotolerans* reference genome (CBS 6340—accession number GCF_000142805.1) using the HISAT2 software (version 2.2.1) [52]. The assembly and quantification of transcripts was conducted utilising the StringTie (version v2.1.3b) [53], with the expression profiles being reported as read counts.

The iDep (Integrated Differential Expression and Pathway Analysis) bioinformatics platform was used for the analysis of the RNA-seq data [54]. The clean data was entered into the tool, thereby enabling the performance of the following analyses. The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1289824. Genes showing a minimum two-fold change in expression were selected for exploratory analysis. Given the absence of biological replication, these gene lists were interpreted descriptively rather than as statistically validated differentially expressed genes. The resulting transcriptional profiles were subsequently explored using hierarchical clustering, PCA and pathway analysis [53–55].

2.7. Statistical Analysis

The generation time, cumulative CO_2 production (independently between the three strains), and oenological parameters (acetic acid, lactic acid, glucose + fructose, glycerol, pH and alcohol) were performed in triplicate and examined using XLSTAT© software (XLSTAT 2021.4.1). The analysis was conducted using an analysis of variance (ANOVA) with a Tukey post hoc test (confidence interval: 95%).

The FCM data were analysed using Prism 10 (Graph Pad, Boston, MA, USA). The analysis was conducted using an ANOVA with a Dunnett post hoc test right (confidence interval: 95%), as previously outlined. The FCM parameters included cell concentration and the ratio of the median fluorescence intensity (MFI) of the Thiol Tracker or CellROX probes. This ratio is indicative of the relative amount of GSH, which has been normalised for the background of oxidative stress at each time point.

3. Results

3.1. Microbial Population Kinetics and Analysis

The populations of *L. thermotolerans* and *S. cerevisiae* yeast strains were monitored throughout the fermentation process (Figure 1). The study observed divergent physiological behaviours between the various strains, contingent on the administered doses of copper.

For Lt1 yeast (Figure 1a), the trend of the curves showed a decline in population during the initial 48 h following the addition of copper, with the lowest population recorded for the highest copper concentration (Lt1 Cu20–day 2: 4.83×10^6 CFU/mL) and an increase in population in the absence of copper (Lt1 Cu0–day 2: 3.83×10^7 CFU/mL). After a period of two days, a stationary plateau was observed in the condition Lt1 Cu0. In contrast, the other two treatments containing copper exhibited a more gradual increase over time, reaching their maximum on day 10. At this juncture, a decline in the population of the Lt1 Cu20 treatment group was already becoming observable. It is evident that copper has a significant impact on the cellular growth of the Lt1 strain.

The Lt2 strain (Figure 1b) showed a similar response to the presence of copper in the medium. It is noteworthy that this strain exhibits a slightly reduced impact on its cellular development in the presence of copper.

With regard to the control strain Sc1 (Figure 1c), a familiar profile is observed in comparison to the first two strains. It appears that strain Sc1 was impacted by the addition of copper during the initial two days of fermentation, exhibiting growth patterns comparable to those observed in Lt1 and Lt2. Indeed, an impact of copper was observed in the two conditions where it was added (Sc1 Cu10–Sc1 Cu20), and the control condition, without copper addition (Sc1 Cu0), appears to have been less affected in terms of yeast growth. A stationary plateau was reached for Sc1 Cu0 and Sc1 Cu10 whereas the presence of 20 mg/L of copper in the Sc1 Cu20 condition has been observed to exert a significantly more pronounced effect on yeast populations, delaying the arrival of the stationary phase.

The yeast generation time corroborates the findings of the microbial kinetics (Figure 1d). The tests with the highest copper concentrations (Lt1 Cu20) induce a significant decrease in yeast development of Lt1 strain. The strain in question requires 74.55 h to double its population under conditions of 20 mg/L copper, whereas under conditions of 10 mg/L and 0 mg/L, the generation time is 13.54 and 5.11 h, respectively. In the case of strain Lt2, the presence of copper does not appear to exert an effect on yeast growth. The generation time was found to be 5.41, 8.04 and 9.59 h for concentrations of 0, 10 and 20 mg/L of copper, respectively. There is a negligible upward trend for this yeast strain when the amount of copper is increased, but this is not significant. For strain Sc1, copper exerted no significant influence on yeast development during the initial hours. The generation time was found to be 18.16, 27.45, and 27.45 for concentrations of 0, 10, and 20 mg/L of copper, respectively. As with Lt2, a slight increase in generation time was observed when copper was added, but this result was not statistically significant. The presence of copper at a concentration of 20 mg/L exerted a deleterious effect on the Lt1 strain.

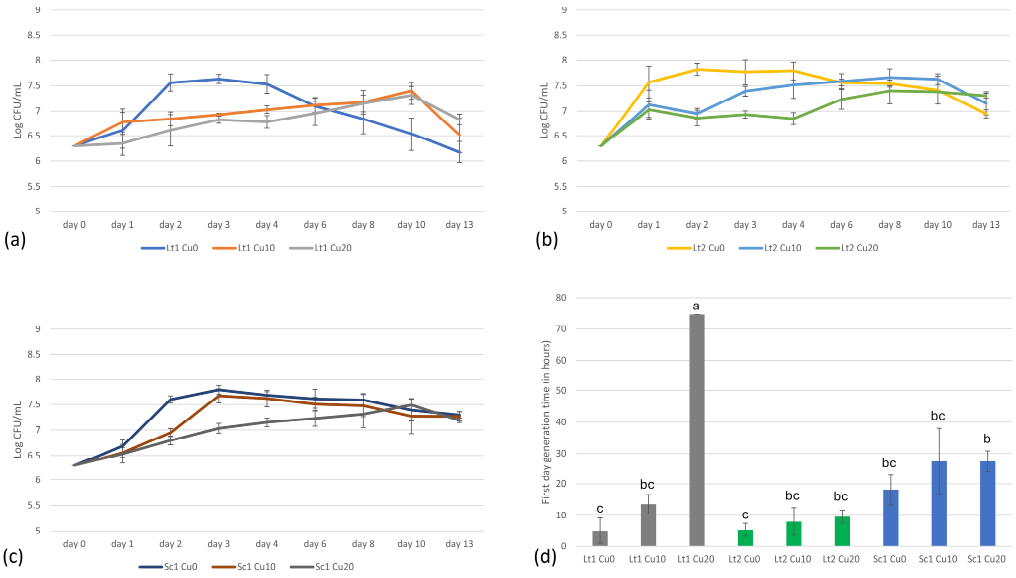

Figure 1. Kinetics of yeast populations Lt1 in (**a**), Lt2 in (**b**) and Sc1 in (**c**) throughout the fermentation process at 20 °C. The strains were exposed to copper at different concentrations: 0, 10 and 20 mg/L. The generation time was calculated on the first day of fermentation and is shown in (**d**). The gray section corresponds to yeast Lt1, the green section to yeast Lt2, and the blue section to yeast Sc1, with different concentrations for each color, and a, b, c represents the significant differences identified by ANOVA followed a Tukey's post hoc test ($p < 0.05$).

3.2. Total Cumulative CO_2 Production by Yeasts

The kinetics of CO_2 production in grams were monitored throughout the fermentation process for all three yeast strains (Lt1, Lt2, Sc1) (Figure 2). The loss in density was measured at several intermediate points, thus providing a total amount of CO_2 produced. As with

the population kinetics, trends were observed between the different strains, as well as depending on the dose of copper added. The Lt1 strain showed a notable increase in CO_2 production during the initial four days of fermentation in copper-free condition with 7.105 g on day 3 (Figure 2a). In contrast, in copper treated conditions, we observed a substantial decrease in CO_2 production (Lt1 Cu10: 1.98 g and Lt1 Cu20: 1.78 g–day 3). In the following days, CO_2 production increased under copper treated conditions, particularly at 10 mg/L going up to 8.29 g on day 6. CO_2 production at 20 mg/L remained lower than that observed under the other conditions throughout the process.

A comparable trend was observed for the Lt2 strain during the initial four days of fermentation (Figure 2b), analogous to the behaviour exhibited by Lt1. In the absence of copper, a high level of CO_2 production was achieved on the second day of fermentation with 7.95 g. Conversely, in conditions with copper, production was considerably lower (Lt2 Cu10: 2.33 g and Lt2 Cu20: 1.66 g). Subsequent to the initial four-day period, the Lt2 strain generates elevated levels of CO_2 that are comparable under both conditions, with and without the presence of copper.

For the control strain Sc1 (Figure 2c), the levels of CO_2 production were similar in the presence and absence of 10 mg/L of copper until the conclusion of the fermentation process. It is also evident that under the 20 mg/L copper condition, the Sc1 strain appears to be impacted by this concentration, with a tendency to produce considerably less CO_2 throughout the process.

The total cumulative CO_2 production is illustrated in Figure 2d. Despite the absence of substantial disparities between the two Lt groups, an observable downward trend in CO_2 production is evident when copper is present in the medium. The total cumulative CO_2 production of 26.98 g was observed for Lt1 Cu0, and 24.60 g for Lt1 Cu20. For the Sc strain, a significant difference was noted between the Sc1 Cu0–Sc1 Cu 10 and the Sc1 Cu20 conditions, with lower production in the 20 mg/L condition, showing an impact of copper on CO_2 production. It is imperative to provide support for these results, which demonstrate divergent physiological behaviour between the strains of *L. thermotolerans* and the reference strain *S. cerevisiae*, as well as a substantial effect of copper in these experiments.

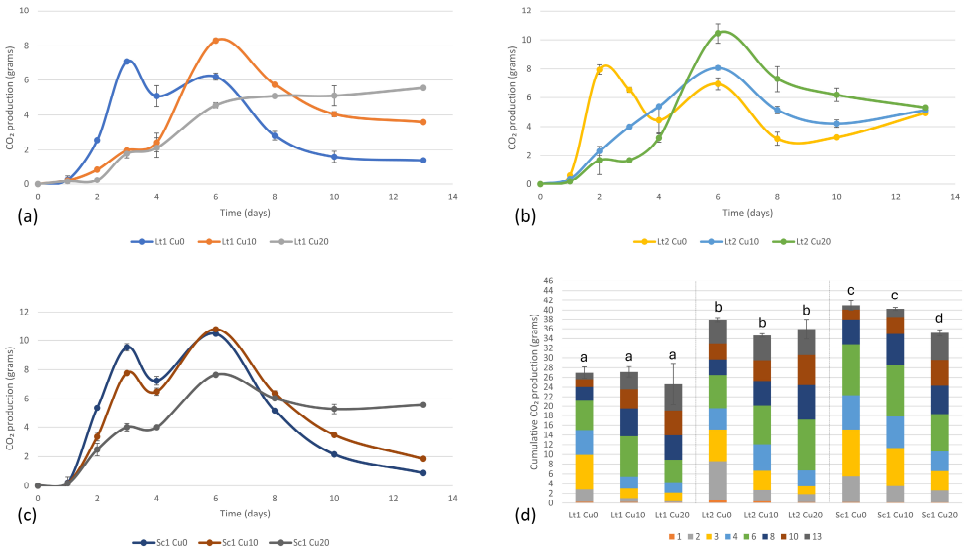

Figure 2. Kinetics of CO_2 production (grams) by yeast populations Lt1 (**a**), Lt2 (**b**) and Sc1 (**c**) throughout the fermentation process at 20 °C. The strains were exposed to copper at different concentrations: 0, 10 and 20 mg/L. Total cumulative production in grams of CO_2 was calculated and is observed in (**d**). 1, 2, 3, 4, 6, 8, 10, and 13 correspond to the days of analysis during fermentation, and a, b, c, d represents the significant differences identified by ANOVA followed a Tukey's post hoc test ($p < 0.05$).

3.3. Analysis of Oenological Parameters

The analysis of the classic oenological parameters was determined at the conclusion of the fermentation process by FTIR. The values are presented in Table 1. The levels of sugar, glucose and fructose, were found to be considerably elevated in the Lt1 conditions in comparison to the Lt2 and Sc1 conditions, indicating that the fermentation process had not been fully completed in the Lt1 conditions. For Lt1, glucose and fructose concentrations ranged between 34.65 and 47.05 g/L, while for Lt2 and Sc1, they were below 6.60 g/L. The quantities thus determined are consistent with the alcohol content observed in all the methods. In the Lt1 condition, there is a significantly lower quantity of alcohol in comparison to the Lt2 and Sc1 conditions. The alcohol quantities for Lt1 range from 7.90 to 8.45% vol, and for Lt2 and Sc1, the range is from 10.60 to 11.40% vol. Subsequently, the analysis of lactic acid production is contingent upon the strain and the presence of copper. Interestingly, the results suggest a strain- and dose-dependent effect of copper on lactic acid production. Lt1 demonstrated a marked variation in its production levels, contingent on the quantity of copper introduced. Lt1 produced 8.35, 13.45 and 16.15 g/L of lactic acid for 0, 10 and 20 mg/L of copper, respectively. For Lt2, the impact of copper appears to be negligible, with production values of 5.14, 4.05 and 3.20 g/L of lactic acid for the same concentration range. The pH also exhibits a similar trend, as it is contingent on the concentration of organic acid in the medium, and thus, to a certain extent, on the lactic acid produced. The pH level was found to be 3.55 for all Sc1 conditions, 3.33 to 3.39 for Lt2 conditions, and continued to decrease significantly for Lt1 conditions. A marked downward trend in pH is evident for conditions Lt1 Cu 10 and Lt1 Cu20, with values of 2.81 and 2.65, respectively. Finally, condition Lt1 Cu0 exhibited a pH of 3.12. With regard to the acetic acid results, no significant differences or trends emerge from these results. The results obtained for glycerol concentration exhibited a consistent trend, devoid of any statistically significant variations. A marginal decline in glycerol production was observed for Lt1 conditions, with values falling below 5.75. Conversely, for Lt2 and Sc1 conditions, values above 6.05 were recorded.

Table 1. Oenological parameter analysis at the end of the alcoholic fermentation process by means of Fourier transform infrared spectroscopy (FTIR). ^{a}, ^{b}, ^{c}, ^{d} represent the significant differences identified by ANOVA followed a Tukey's post hoc test ($p < 0.05$).

	Alcohol [vol.%]	pH	Acetic Acid [g/L]	Lactic Acid [g/L]	Glucose + Fructose [g/L]	Glycerol [g/L]
Lt1 Cu0	8.15 ^{b}	3.12 ^{bc}	0.29 ^{cd}	8.35 ^{bc}	47.05 ^{a}	4.95 ^{d}
Lt1 Cu10	8.45 ^{b}	2.81 ^{cd}	0.40 ^{b}	13.45 ^{ab}	34.65 ^{a}	5.55 ^{cd}
Lt1 Cu20	7.90 ^{b}	2.65 ^{d}	0.41 ^{b}	16.15 ^{a}	38.85 ^{a}	5.75 ^{bcd}
Lt2 Cu0	10.75 ^{a}	3.33 ^{ab}	0.42 ^{b}	5.14 ^{cd}	5.65 ^{b}	6.90 ^{ab}
Lt2 Cu10	10.60 ^{a}	3.39 ^{ab}	0.39 ^{b}	4.05 ^{cd}	6.60 ^{b}	7.10 ^{a}
Lt2 Cu20	11.00 ^{a}	3.39 ^{ab}	0.34 ^{bc}	3.20 ^{cd}	2.20 ^{b}	6.55 ^{abc}
Sc1 Cu0	11.40 ^{a}	3.55 ^{a}	0.19 ^{d}	0.10 ^{d}	0.15 ^{b}	6.05 ^{abcd}
Sc1 Cu10	11.40 ^{a}	3.55 ^{a}	0.38 ^{bc}	0.10 ^{d}	0.55 ^{b}	6.25 ^{abc}
Sc1 Cu20	11.00 ^{a}	3.55 ^{a}	0.59 ^{a}	0.10 ^{d}	5.15 ^{b}	7.10 ^{a}

3.4. Flow Cytometry

The FCM results recapitulate the patterns of results observed with microbiological analysis. The two-way analysis of variance (ANOVA) revealed that the supplementation of copper to the Lt1 fermentations had an impact on cell concentration; however, this effect did not attain statistical significance ($F = 4.83$; $p = 0.11$). In contrast, the interaction with time proved to be highly significant ($F = 19$; $p < 0.001$). Post hoc Dunnett's tests indicated

significant differences between the Cu0 condition and the Cu10 and Cu20 conditions at days 2 and 4 (Figure 3a). Statistical analysis of Lt2 showed a significant main effect of copper concentration ($F = 200$; $p = 0.0006$) as well as a significant copper × time interaction ($p < 0.0001$). Dunnett's test revealed significant differences between Cu0 and both Cu10 and Cu20 on days 1–4 (Figure 3b). In the Sc strain, the addition of copper resulted in a highly significant effect ($F = 4929$; $p < 0.0001$) accompanied by a significant interaction with time ($p < 0.0001$). Dunnett's test further confirmed significant differences between Cu0 and the higher concentrations on days 2, 3, 4, and 6 (Figure 3c). Oxidative stress and antioxidant response: The ANOVA revealed that, for Lt1, the addition of copper exerted a significant main effect on the Thiol Tracker/Cell ROX ratio ($F = 14.20$, $p = 0.03$). However, post hoc tests found no pairwise differences among fermentation conditions (Figure 3d). For Lt2, the main effect of copper was not significant ($F = 4.91$, $p = 0.11$), but the copper × time interaction was significant ($F = 3.23$, $p = 0.0091$); Dunnett's test (versus Cu0) detected significant differences on days 1 and 10 (Figure 3e). For Sc1, both the main effect of copper ($F = 34.42$, $p = 0.0085$) and the copper × time interaction ($F = 16.97$, $p < 0.0001$) were significant. Dunnett's test demonstrated that the Thiol Tracker/Cell ROX ratio was significantly higher in Cu20 than in Cu0 on days 4, 6, and 8 (Figure 3f).

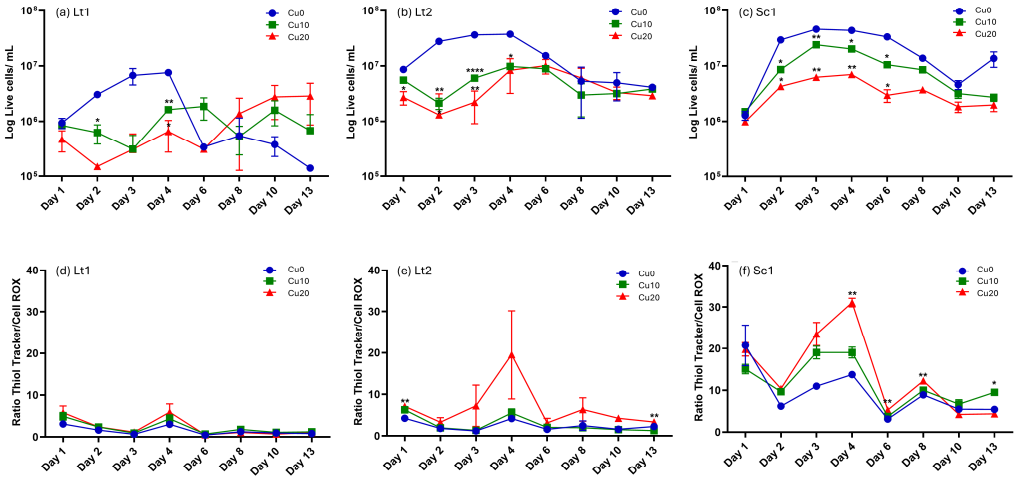

Figure 3. Flow cytometry analysis of cell growth and antioxidant response. (**a**) Lt1, (**b**) Lt2 and (**c**) Sc1, the population of Log Live cells/mL for the various strains associated with the copper dose (0, 10 and 20 mg/L). (**d**) Lt1, (**e**) Lt2 and (**f**) Sc1, the Thiol Tracker/Cell ROX ratio for the various strains associated with the copper dose (0, 10 and 20 mg/L). *, **, and **** correspond to *p*-values of ≤0.05, ≤0.01, and ≤0.0001, respectively.

3.5. Transcriptomic Analysis Metadata

A total of 5384 genes were identified after filtering of the RNA-seq data. Transcriptomic profiles were obtained for the four experimental conditions: Lt1 Cu0, Lt1 Cu10, Lt2 Cu0 and Lt2 Cu10. Because one transcriptomic sample was available for each experimental condition, the analysis was considered exploratory and focused on the overall patterns and magnitude of transcriptional changes rather than on statistically validated differential expression.

Hierarchical clustering revealed a clear separation between the samples according to copper exposure (Figure 4a,b). The two strains showed relatively similar transcriptional profiles under Cu0 conditions, whereas copper exposure resulted in a marked shift in gene expression profiles. The Cu10 samples clustered separately from the Cu0 samples, indicating that copper exposure was associated with a substantial change in the global transcriptional profile.

Principal component analysis (PCA) further supported this separation (Figure 4c). PC1 and PC2 accounted for 58.6% and 27.8% of the total variance, respectively, representing

86.4% of the total variance. Lt1 Cu10 was clearly separated from Lt1 Cu0, while Lt2 Cu10 also differed from the corresponding Cu0 condition, although the magnitude and position of the transcriptional shift differed between the two strains.

To further characterise the transcriptional response to copper, genes showing a minimum two-fold change between Cu10 and Cu0 conditions were examined. The global comparison of the two strains revealed distinct groups of genes with increased or decreased expression under Cu10 conditions (Figure 4d). To account for potential strain-dependent responses, the expression profiles were subsequently examined separately for Lt1 and Lt2 (Figure 4e,f). Both strains displayed extensive transcriptional changes, but the distribution and magnitude of these changes differed between Lt1 and Lt2.

Exploratory pathway analysis of the genes showing the strongest transcriptional changes identified several functional categories associated with copper exposure (Figure 4g). Pathways related to ribosomal activity were predominantly represented among the genes showing increased expression. Other pathways involved in cell wall organisation and synthesis of hydrogen sulphide were also identified. Conversely, several pathways related to cell wall morphogenesis, iron transport and cell division by meiosis showed decreased expression under copper conditions.

Importantly, genes encoding the lactate dehydrogenases previously identified in the genome of the strains (Gene IDs 8290491 and 8290832) did not show an evident increase in expression under copper conditions. Similarly, the genes previously associated with copper regulation (Gene IDs 8295329 and 8292522) did not display an evident transcriptional increase. Thus, the transcriptomic data did not provide direct evidence that the increased lactic acid production observed under copper exposure resulted from transcriptional up-regulation of lactate dehydrogenase genes.

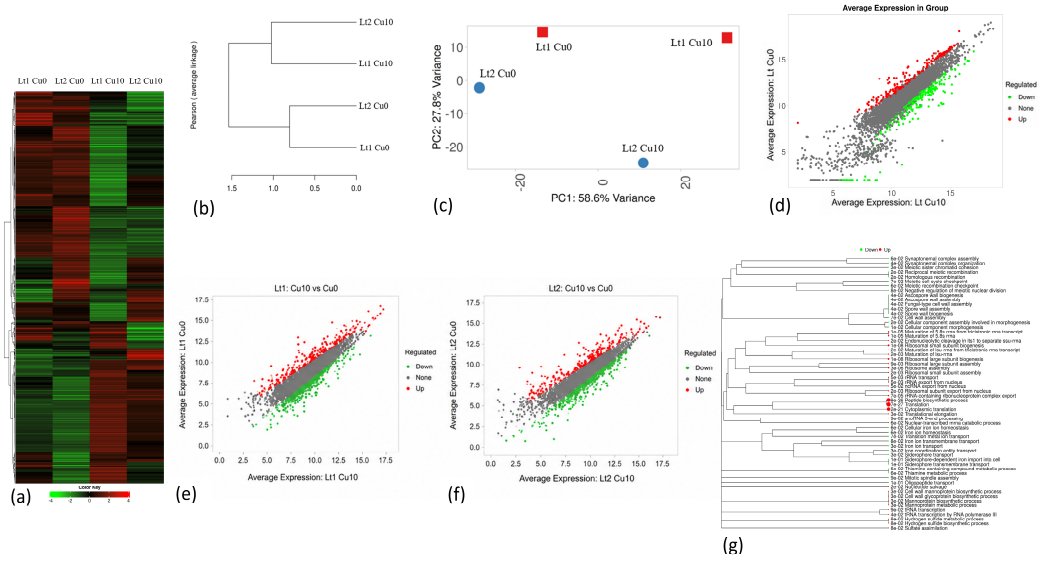

Figure 4. Transcriptomic analysis of *L. thermotolerans* strains Lt1 and Lt2 under copper exposure (**a**) Heatmap of the transcriptomic profiles of Lt1 Cu0, Lt2 Cu0, Lt1 Cu10 and Lt2 Cu10. (**b**) Hierarchical clustering based on Pearson correlation. (**c**) Principal component analysis (PCA). (**d**) Global comparison of gene expression between Cu10 and Cu0 conditions for the two strains. (**e**) Comparison of gene expression between Lt1 Cu10 and Lt1 Cu0. (**f**) Comparison of gene expression between Lt2 Cu10 and Lt2 Cu0. (**g**) Exploratory pathway analysis of genes showing the strongest transcriptional changes under copper exposure.

4. Discussion

In the present study, the impact of copper on *L. thermotolerans* lactic acid-producing yeasts was investigated. To our knowledge, this is the first study to comprehensively

examine the physiological, metabolic and transcriptomic responses of *L. thermotolerans* to copper exposure. It has previously been established that the incorporation of copper into vines exerts a substantial influence on yeast biodiversity [56,57], particularly with regard to the *L. thermotolerans* strain. The physiological responses exhibited by the two strains under investigation are found to be dependent upon the doses of copper administered, with significant disparities observed between the doses of 10 and 20 mg/L. Copper affected cell growth throughout the fermentation process, and more specifically on the cell generation times of the two strains, Lt1 and Lt2. The cellular behaviour exhibited by the yeast is dependent upon the copper content present in the must, which, in turn, is influenced by the specific strain used or selected by the producer. This observation is of considerable significance to the wine industry to manage its usage. A plethora of studies have previously reported on the impact of copper on various species, including *L. thermotolerans* [58,59], yet none of these studies have investigated the strain in question. The exogenous addition of a product invariably could exert an influence on total biodiversity and its equilibrium. Consequently, it is imperative to exercise prudence and give due consideration to the practice of yeast inoculation. Furthermore, analysis of the control strain, *S cerevisiae* Sc1, revealed the impact of copper on this fermentation yeast, both in terms of population kinetics and generation time, as previously documented in the existing literature [48]. This underscored the necessity for effective management of copper addition. Copper has been demonstrated to play a pivotal role in the dynamics of yeast populations during fermentation [48].

This idea was reflected in the CO_2 production observed during the fermentation process, particularly at the initiation stage. In our study, the two biomass of *L. thermotolerans* were impacted by the presence of copper during the initial days of fermentation, with a marked decrease in biomass observed in the presence of copper. The onset of fermentation metabolism was found to be relayed by the presence of copper in the medium. This observation has been documented in the existing literature [60], albeit not in these particular conditions and not with these two strains of *L. thermotolerans*. These results corroborate the findings of the study of population kinetics, which demonstrated the significant impact of the amount of copper associated with the utilisation of this particular acidifying yeast. The incorporation of copper has been demonstrated to induce a metabolic shift in the examined yeast strains [46,48,61], a phenomenon that was subsequently observed in the ensuing results.

Indeed, the results of the oenological parameters on the finished wine showed notable trends (Table 1). The Lt1 yeast strain was unable to complete fermentation, resulting in a substantial residual sugar concentration in the must and a concomitant decrease in alcohol content relative to both the second strain (Lt2) and the control strain (Sc1). Furthermore, the two strains of *L. thermotolerans* demonstrated divergent behaviour in response to varying doses of copper. The metabolism of Lt1 has been observed to produce significantly higher quantities of lactic acid when in contact with copper. The lactic acid concentration has undergone a transition from 8.35 g/L to 16.15 g/L, respectively from 0 to 20 mg/L of copper. This species has been observed to exhibit a high concentration of lactic acid, as previously documented in the extant literature [2,4]. Conversely, the *L. thermotolerans* Lt2 strain exhibited a relatively constant level of lactic acid production, regardless of the amount of copper administered. It is evident that the two strains tested showed divergent behaviours with regard to lactic acid production activity. It is also worthy of note that lactic acid production plays a pivotal role in the observed decrease in pH. The *L. thermotolerans* strain, under conditions of maximal lactic acid production (Cu20), showed a substantial decrease in pH, from 3.55 to 2.65, a reduction of 0.9 units. The analysis of this data suggested that acidification is a significant phenomenon in the Lt1 strain. In

addition, such marked changes in pH may influence copper speciation and bioavailability during fermentation [62]. However, these parameters were not assessed in the present study, and their possible influence on the observed strain-dependent response therefore cannot be determined. The second strain, Lt2, achieved a pH decrease of between 0.16 and 0.22 points. The *S. cerevisiae* strain exhibited minimal lactic acid production, with a yield of 0.1 g/L under all conditions, which is consistent with the data reported in the literature [63]. The outcomes for acetic acid and glycerol demonstrated no statistically significant variation. The only notable observation was the reduced glycerol production by Lt1, likely due to incomplete alcoholic fermentation by this strain. The yield of glycerol is contingent on the species, strain and the conditions of the experiment [64]. The results obtained demonstrated a variation in yeast metabolism depending on the strain and the fundamental copper conditions. An initial explanation has been proposed, employing the technique of flow cytometry (FCM).

The cell growth dynamics, as assessed by means of flow cytometry (FCM), are in alignment with the trends that have been observed in other parts of the manuscript. However, the data obtained from the FCM indicated that the addition of copper had an initial inhibitory effect on the growth of both the Lt1 and Lt2 strains, with a general recovery occurring after 2–3 days. To the best of our knowledge, no previous studies have described the behaviour of Lt under copper stress in flow cytometry, and further investigation is required to confirm the transient inhibition observed in this study. Conversely, Sc1 demonstrated a divergent response: no clear initial growth delay was detected, but biomass production diminished progressively with increasing copper concentration. This dose-dependent inhibition is consistent with the findings of [43,59], who reported proportional decreases in *S. cerevisiae* biomass up to 1.50 mM (approximately 95 mg/L) Cu^{2+}. At the molecular level, elevated copper concentrations are known to impair alcoholic fermentation in *S. cerevisiae* by generating reactive oxygen species (ROS) and inducing oxidative stress, which in turn damages membranes, proteins, and mitochondrial functions [65–67]. The combined effects of oxidative stress ultimately result in reduced viability, and in severe cases, sluggish or stuck fermentations. In this context, the results obtained demonstrate a copper-dependent increase in the ratio of Thiol Tracker fluorescence (reflecting intracellular reduced glutathione, GSH) to CellROX fluorescence (reflecting ROS levels). This trend was evident in Lt1 and Lt2, and particularly pronounced in Sc1, where the GSH/ROS ratio in the Cu20 condition was significantly higher than in the control on days 4, 6, and 8. These observations suggest the activation of an adaptive antioxidant response whose intensity is proportional to the copper concentration and differs between yeast species. Indeed, exposure to heavy metals has been demonstrated to upregulate glutathione biosynthetic genes (GSH1, GSH2) and sulphur-assimilation pathways, thereby enhancing the redox defence capacity [68]. The molecular data pertaining to the antioxidant pathway has been thoroughly analysed, and all metabolic pathways were studied by means of transcriptomic approach.

The transcriptomic analysis was restricted to 10 mg/L Cu based on preliminary evidence of increased lactic acid production in Lt1 and to keep the experimental design manageable. However, the absence of the 20 mg/L Cu condition limits interpretation of the transcriptional response at the highest copper exposure. This analysis provides an exploratory view of the molecular responses associated with copper exposure in *L. thermotolerans*. The global analyses showed that copper exposure was associated with substantial changes in transcriptional profiles in both strains. Hierarchical clustering and PCA consistently separated the Cu0 and Cu10 conditions, while the strain-specific comparisons indicated that the magnitude and distribution of transcriptional changes differed between Lt1 and Lt2. These observations are consistent with the strain-dependent physiological responses observed during fermentation.

The strongest transcriptional changes were associated with pathways related to ribosomal activities. Increased representation of ribosome-related functions may reflect changes in cellular protein synthesis and adaptation to environmental stress. Similar responses have been described in yeast exposed to adverse environmental conditions, in which regulation of ribosome biogenesis and protein synthesis is closely connected to growth and stress responses [69–72]. However, these transcriptional changes should not be interpreted as evidence of a specific metabolic redirection caused by copper. In particular, changes in ribosomal activity may also reflect differences in cellular growth state between the experimental conditions.

The transcriptomic analysis also highlighted pathways related to cell wall organisation. Changes in cell wall-associated pathways may represent an adaptive response to environmental stress. These observations are consistent with the physiological differences observed between the strains, particularly the longer generation times observed under elevated copper concentrations [73–76].

Sulphur-related pathways, including sulphate assimilation and hydrogen sulphide-associated processes, were also represented among the transcriptional responses. However, the present study did not directly measure H_2S production and therefore cannot establish a functional relationship between the transcriptional patterns observed here and H_2S accumulation. The hypothesis that H_2S could participate in a quorum-sensing-like mechanism under copper stress is therefore speculative and should not be considered a mechanistic conclusion of the present study. Future experiments combining transcriptomic measurements with direct quantification of H_2S and other sulphur metabolites would be required to test this hypothesis [77–80].

One of the most relevant observations of the transcriptomic analysis is the absence of an evident transcriptional increase in the lactate dehydrogenase genes identified in the strains. This finding is particularly relevant given the increased lactic acid concentrations observed under copper exposure, especially for Lt1. The present data therefore do not support a direct transcriptional activation of lactate production as the explanation for the increased extracellular lactic acid concentration [81]. Instead, the observed phenotype may result from a combination of physiological and metabolic effects associated with copper exposure. Copper exposure altered yeast growth kinetics, with the effect being more pronounced for the Lt1 strain. These physiological changes should be considered when interpreting the transcriptomic profiles, as differences in cellular growth and physiological state may contribute to the observed transcriptional responses. Therefore, the present transcriptomic data provide an exploratory view of the cellular response to copper and do not, on their own, allow a direct causal relationship to be established between transcriptional changes, growth inhibition, fermentation progression and lactic acid accumulation.

Overall, the transcriptomic data indicate that copper exposure is associated with broad changes in the transcriptional state of *L. thermotolerans*, involving processes related to ribosome activities, cell wall organisation and sulphur metabolism. These responses differed between the two strains and are consistent with the strain-dependent physiological effects observed during fermentation. However, the present data do not establish a direct molecular mechanism linking copper exposure to increased lactic acid production. Further studies combining transcriptomic, proteomic and metabolic approaches will be required to elucidate the mechanisms underlying these strain-dependent responses. Recent proteomic analysis of several *L. thermotolerans* strains, including Laktia™ (Lt2), revealed marked strain-dependent differences in the abundance of L-LDH and of proteins involved in central metabolism, stress response, redox balance, sulphur metabolism and cell-wall remodelling [82]. These findings suggest that substantial metabolic differences already exist among *L. thermotolerans* strains at the proteomic level. Copper exposure may further

accentuate these intrinsic differences, potentially contributing to the divergent lactic acid production and metabolic responses observed here. However, the mechanisms underlying this interaction remain unknown and will require further investigation.

Beyond lactic acid production, these strain-dependent metabolic differences may also have broader oenological implications. Potential implications for wine aroma should also be considered. Although volatile compounds and sensory attributes were not directly measured in the present study, copper exposure may influence yeast metabolism and sulphur-related pathways that are relevant to the formation of aroma-active compounds. Previous studies have shown that copper-based grape pest management can affect wine aroma [37], while non-Saccharomyces yeasts, including *L. thermotolerans*, can contribute to strain-dependent differences in wine volatile profiles [11,17]. The transcriptional changes observed in the present study, particularly those involving sulphur metabolism, may therefore have potential implications for wine aroma. However, these effects remain hypothetical in the present study and would require direct analysis of volatile compounds and sensory attributes to be experimentally confirmed.

Author Contributions: Conceptualisation, S.S. (Scott Simonin) and B.B.; methodology, S.S. (Scott Simonin), F.S., S.S. (Sylvain Schnée) and E.A.; validation, S.S. (Scott Simonin); formal analysis, S.S. (Scott Simonin), V.B., F.S., C.M. and E.A.; investigation, S.S. (Scott Simonin), V.B. and F.S.; resources, S.S. (Sylvain Schnée) and B.B.; writing—original draft preparation, S.S. (Scott Simonin), F.S., E.A. and B.B.; writing—review and editing, S.S. (Scott Simonin), V.B., F.S., S.S. (Sylvain Schnée), G.B., E.A. and B.B.; visualisation, S.S. (Scott Simonin); supervision, G.B. and B.B.; project administration, G.B. and B.B.; funding acquisition, B.B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors would like to thank the Swiss Federal Office for Agriculture (OFAG—Project number 124655) for providing financial support for this work.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

LT	*Lachancea thermotolerans*
FCM	Flow Cytometry
$CuSO_4$	Copper(II) sulphate
YPD	Yeast Peptone Dextrose
CFU	Colony-Forming Unit(s)
mL	Millilitre(s)
CO_2	Carbon Dioxide
FTIR	Fourier Transform Infrared Spectroscopy
CFDA	5(6)-Carboxyfluorescein Diacetate
GSH	Reduced Glutathione
DEG	Differentially Expressed Gene(s)
KEGG	Kyoto Encyclopedia of Genes and Genomes
HISAT	Hierarchical Indexing for Spliced Alignment of Transcripts

References

1. Benito, S. The Impacts of *Lachancea thermotolerans* Yeast Strains on Winemaking. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6775–6790. [CrossRef]
2. Gatto, V.; Binati, R.L.; Lemos Junior, W.J.F.; Basile, A.; Treu, L.; De Almeida, O.G.G.; Innocente, G.; Campanaro, S.; Torriani, S. New Insights into the Variability of Lactic Acid Production in *Lachancea thermotolerans* at the Phenotypic and Genomic Level. *Microbiol. Res.* **2020**, *238*, 126525. [CrossRef]
3. Petitgonnet, C.; Klein, G.L.; Roullier-Gall, C.; Schmitt-Kopplin, P.; Quintanilla-Casas, B.; Vichi, S.; Julien-David, D.; Alexandre, H. Influence of Cell-Cell Contact between *L. thermotolerans* and *S. cerevisiae* on Yeast Interactions and the Exo-Metabolome. *Food Microbiol.* **2019**, *83*, 122–133. [CrossRef]
4. Sgouros, G.; Mallouchos, A.; Filippousi, M.-E.; Banilas, G.; Nisiotou, A. Molecular Characterization and Enological Potential of A High Lactic Acid-Producing *Lachancea thermotolerans* Vineyard Strain. *Foods* **2020**, *9*, 595. [CrossRef]
5. Sizzano, F.; Bianconi, V.; Blackford, M.; Bieri, S.; Vuichard, F.; Monnard, C.; Amiet, L.; Spring, J.-L.; Dorsaz, E.; Pfenninger-Bridy, N.; et al. Use of *Lachancea thermotolerans* for the Bioacidification of White Grape Musts: Assays from the Bench to the Cellar Scale. *Fermentation* **2024**, *10*, 458. [CrossRef]
6. Ciani, M.; Comitini, F.; Mannazzu, I.; Domizio, P. Controlled Mixed Culture Fermentation: A New Perspective on the Use of Non-*Saccharomyces* Yeasts in Winemaking. *FEMS Yeast Res.* **2010**, *10*, 123–133. [CrossRef]
7. Ciani, M.; Comitini, F. Use of Non-*Saccharomyces* Yeasts in Red Winemaking. *Red Wine Technol.* **2019**, 51–68. [CrossRef]
8. Fleet, G.H. Yeast Interactions and Wine Flavour. *Int. J. Food Microbiol.* **2003**, *86*, 11–22. [CrossRef]
9. García, M.; Esteve-Zarzoso, B.; Arroyo, T. Non-*Saccharomyces* Yeasts: Biotechnological Role for Wine Production. In *Grape and Wine Biotechnology*; Morata, A., Loira, I., Eds.; InTech: Rijeka, Croatia, 2016; pp. 249–272. [CrossRef]
10. Hranilovic, A.; Gambetta, J.M.; Schmidtke, L.; Boss, P.K.; Grbin, P.R.; Masneuf-Pomarede, I.; Bely, M.; Albertin, W.; Jiranek, V. Oenological Traits of *Lachancea thermotolerans* Show Signs of Domestication and Allopatric Differentiation. *Sci. Rep.* **2018**, *8*, 14812. [CrossRef]
11. Hranilovic, A.; Li, S.; Boss, P.K.; Bindon, K.; Ristic, R.; Grbin, P.R.; Van der Westhuizen, T.; Jiranek, V. Chemical and Sensory Profiling of Shiraz Wines Co-Fermented with Commercial Non-*Saccharomyces* Inocula: Chemical and Sensory Impact of Mixed Yeast Inocula. *Aust. J. Grape Wine Res.* **2017**, *24*, 166–180. [CrossRef]
12. Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The Occurrence of Non-*Saccharomyces cerevisiae* Yeast Species over Three Vintages in Four Vineyards and Grape Musts from Four Production Regions of the Western Cape, South Africa. *S. Afr. J. Enol. Vitic.* **2017**, *24*, 35–42. [CrossRef]
13. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not Your Ordinary Yeast: Non-*Saccharomyces* Yeasts in Wine Production Uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef]
14. Roudil, L.; Russo, P.; Berbegal, C.; Albertin, W.; Spano, G.; Capozzi, V. Non-*Saccharomyces* Commercial Starter Cultures: Scientific Trends, Recent Patents and Innovation in the Wine Sector. *Recent Pat. Food Nutr. Agric.* **2020**, *11*, 27–39. [CrossRef]
15. Sadoudi, M.; Tourdot-Maréchal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacón, J.J.; Ballester, J.; Vichi, S.; Guérin-Schneider, R.; Caixach, J.; Alexandre, H. Yeast–Yeast Interactions Revealed by Aromatic Profile Analysis of Sauvignon Blanc Wine Fermented by Single or Co-Culture of Non-*Saccharomyces* and *Saccharomyces* Yeasts. *Food Microbiol.* **2012**, *32*, 243–253. [CrossRef]
16. Vicente, J.; Benito, S.; Marquina, D.; Santos, A. Fermentative Factors Shape Transcriptional Response of *Lachancea thermotolerans* and Wine Acidification. *npj Sci. Food* **2025**, *9*, 97. [CrossRef]
17. Zott, K.; Thibon, C.; Bely, M.; Lonvaud-Funel, A.; Dubourdieu, D.; Masneuf-Pomarede, I. The Grape Must Non-*Saccharomyces* Microbial Community: Impact on Volatile Thiol Release. *Int. J. Food Microbiol.* **2011**, *151*, 210–215. [CrossRef]
18. Zott, K.; Miot-Sertier, C.; Claisse, O.; Lonvaud-Funel, A.; Masneuf-Pomarede, I. Dynamics and Diversity of Non-*Saccharomyces* Yeasts during the Early Stages in Winemaking. *Int. J. Food Microbiol.* **2008**, *125*, 197–203. [CrossRef]
19. Vaquero, C.; Loira, I.; Heras, J.M.; Carrau, F.; González, C.; Morata, A. Biocompatibility in Ternary Fermentations With *Lachancea thermotolerans*, Other Non-*Saccharomyces* and *Saccharomyces cerevisiae* to Control pH and Improve the Sensory Profile of Wines From Warm Areas. *Front. Microbiol.* **2021**, *12*, 656262. [CrossRef]
20. Vicente, J.; Navascués, E.; Calderón, F.; Santos, A.; Marquina, D.; Benito, S. An Integrative View of the Role of *Lachancea thermotolerans* in Wine Technology. *Foods* **2021**, *10*, 2878. [CrossRef]
21. Vicente, J.; Vladic, L.; Navascués, E.; Brezina, S.; Santos, A.; Calderón, F.; Tesfaye, W.; Marquina, D.; Rauhut, D.; Benito, S. A Comparative Study of *Lachancea thermotolerans* Fermentative Performance under Standardized Wine Production Conditions. *Food Chem. X* **2024**, *21*, 101214. [CrossRef]
22. De Castro, M.; Baptista, J.; Matos, C.; Valente, A.; Briga-Sá, A. Energy Efficiency in Winemaking Industry: Challenges and Opportunities. *Sci. Total Environ.* **2024**, *930*, 172383. [CrossRef]
23. Dixon, T.A.; Williams, T.C.; Pretorius, I.S. Bioinformational Trends in Grape and Wine Biotechnology. *Trends Biotechnol.* **2022**, *40*, 124–135. [CrossRef]
24. Gonzalez, R.; Morales, P. Truth in Wine Yeast. *Microb. Biotechnol.* **2022**, *15*, 1339–1356. [CrossRef]

25. Baiano, A. An Overview on Sustainability in the Wine Production Chain. *Beverages* **2021**, *7*, 15. [CrossRef]
26. Cataldo, E.; Fucile, M.; Mattii, G.B. A Review: Soil Management, Sustainable Strategies and Approaches to Improve the Quality of Modern Viticulture. *Agronomy* **2021**, *11*, 2359. [CrossRef]
27. Varia, F.; Macaluso, D.; Agosta, I.; Spatafora, F.; Dara Guccione, G. Transitioning towards Organic Farming: Perspectives for the Future of the Italian Organic Wine Sector. *Sustainability* **2021**, *13*, 2815. [CrossRef]
28. Binati, R.L.; Felis, G.E.; Torriani, S. How to Reduce SO_2 Additions in Wine with the Aid of Non-Conventional Yeasts. In *Proceedings of the OIV 2024, Dijon, France, 14–18 October 2024*; International Viticulture and Enology Society: Villenave-d'Ornon, France, 2024. [CrossRef]
29. Salaha, M.I.; Kallithraka, S.; Marmaras, I.; Koussissi, E.; Tzourou, I. A Natural Alternative to Sulphur Dioxide for Red Wine Production: Influence on Colour, Antioxidant Activity and Anthocyanin Content. *J. Food Compos. Anal.* **2008**, *21*, 660–666. [CrossRef]
30. Simonin, S.; Alexandre, H.; Nikolantonaki, M.; Coelho, C.; Tourdot-Maréchal, R. Inoculation of *Torulaspora delbrueckii* as a Bio-Protection Agent in Winemaking. *Food Res. Int.* **2018**, *107*, 451–461. [CrossRef]
31. Zara, G.; Nardi, T. Yeast Metabolism and Its Exploitation in Emerging Winemaking Trends: From Sulfite Tolerance to Sulfite Reduction. *Fermentation* **2021**, *7*, 57. [CrossRef]
32. Fan, S.; Bindon, K.; Gilmore, A.; Jeffery, D. Fluorescence Spectroscopy for Grape and Wine Compositional Analysis and Quality Control. In *Adv Food Nutr Res*; Academic Press: Cambridge, MA, USA, 2025; Volume 115, pp. 45–130.
33. Wu, M.; Wang, J.; Wang, Y.; Xu, Y.; Sun, B. Traditional Fermented Food Microbial Ecosystems Oriented towards Industrial Standards: Multi-Dimensional Characterization and Artificial Regulation. *npj Sci. Food* **2026**. [CrossRef]
34. Sgubin, G.; Swingedouw, D.; Mignot, J.; Gambetta, G.A.; Bois, B.; Loukos, H.; Noël, T.; Pieri, P.; García de Cortázar-Atauri, I.; Ollat, N.; et al. Non-Linear Loss of Suitable Wine Regions over Europe in Response to Increasing Global Warming. *Glob. Change Biol.* **2023**, *29*, 808–826. [CrossRef]
35. van Leeuwen, C.; Sgubin, G.; Bois, B.; Ollat, N.; Swingedouw, D.; Zito, S.; Gambetta, G.A. Climate Change Impacts and Adaptations of Wine Production. *Nat. Rev. Earth Environ.* **2024**, *5*, 258–275. [CrossRef]
36. Mendes Ferreira, A.; Mendes-Faia, A. The Role of Yeasts and Lactic Acid Bacteria on the Metabolism of Organic Acids during Winemaking. *Foods* **2020**, *9*, 1231. [CrossRef]
37. Vicente, J.; Baran, Y.; Navascués, E.; Santos, A.; Calderón, F.; Marquina, D.; Rauhut, D.; Benito, S. Biological Management of Acidity in Wine Industry: A Review. *Int. J. Food Microbiol.* **2022**, *375*, 109726. [CrossRef]
38. Wang, J.; Ma, T.; Wei, M.; Lan, T.; Bao, S.; Zhao, Q.; Fang, Y.; Sun, X. Copper in Grape and Wine Industry: Source, Presence, Impacts on Production and Human Health, and Removal Methods. *Compr. Rev. Food Sci. Food Saf.* **2023**, *22*, 1794–1816. [CrossRef]
39. De Guidi, I.; Galeote, V.; Blondin, B.; Legras, J.-L. Copper-Based Grape Pest Management Has Impacted Wine Aroma. *Sci. Rep.* **2024**, *14*, 10124. [CrossRef]
40. Chen, J.; Jiang, Y.; Shi, H.; Peng, Y.; Fan, X.; Li, C. The Molecular Mechanisms of Copper Metabolism and Its Roles in Human Diseases. *Pflüg. Arch.-Eur. J. Physiol.* **2020**, *472*, 1415–1429. [CrossRef]
41. Sun, X.; Liu, L.; Ma, T.; Yu, J.; Huang, W.; Fang, Y.; Zhan, J. Effect of High Cu^{2+} Stress on Fermentation Performance and Copper Biosorption of *Saccharomyces cerevisiae* during Wine Fermentation. *Food Sci. Technol.* **2019**, *39*, 19–26. [CrossRef]
42. Sun, X.; Ma, T.; Han, L.; Huang, W.; Zhan, J. Effects of Copper Pollution on the Phenolic Compound Content, Color, and Antioxidant Activity of Wine. *Molecules* **2017**, *22*, 726. [CrossRef]
43. Sun, X.; Zhao, Y.; Liu, L.; Jia, B.; Zhao, F.; Huang, W.; Zhan, J. Copper Tolerance and Biosorption of *Saccharomyces cerevisiae* during Alcoholic Fermentation. *PLoS ONE* **2015**, *10*, e0128611. [CrossRef]
44. García-Esparza, M.A.; Capri, E.; Pirzadeh, P.; Trevisan, M. Copper Content of Grape and Wine from Italian Farms. *Food Addit. Contam.* **2006**, *23*, 274–280. [CrossRef]
45. Séralini, G.-E.; Douzelet, J.; Halley, J.-C. Copper in wines and vineyards taste and comparative toxicity to pesticides. *Food Nutr. J.* **2019**, *9*, 196.
46. Freitas, J.D.; Wintz, H.; Kim, J.H.; Poynton, H.; Fox, T.; Vulpe, C. Yeast, a Model Organism for Iron and Copper Metabolism Studies. *BioMetals* **2003**, *16*, 185–187. [CrossRef]
47. Salah, I.; Parkin, I.P.; Allan, E. Copper as an Antimicrobial Agent: Recent Advances. *RSC Adv.* **2021**, *11*, 18179–18186. [CrossRef]
48. Shi, H.; Jiang, Y.; Yang, Y.; Peng, Y.; Li, C. Copper Metabolism in *Saccharomyces cerevisiae*: An Update. *BioMetals* **2021**, *34*, 3–14. [CrossRef]
49. Grangeteau, C.; Roullier-Gall, C.; Rousseaux, S.; Gougeon, R.D.; Schmitt-Kopplin, P.; Alexandre, H.; Guilloux-Benatier, M. Wine Microbiology Is Driven by Vineyard and Winery Anthropogenic Factors. *Microb. Biotechnol.* **2017**, *10*, 354–370. [CrossRef]
50. Onetto, C.A.; Kutyna, D.R.; Kolouchova, R.; McCarthy, J.; Borneman, A.R.; Schmidt, S.A. SO_2 and Copper Tolerance Exhibit an Evolutionary Trade-off in *Saccharomyces cerevisiae*. *PLoS Genet.* **2023**, *19*, e1010692. [CrossRef]

51. Que, Z.; Wei, M.; Jiang, W.; Ma, T.; Zhang, W.; Zhao, Z.; Yan, Y.; Yang, Y.; Fang, Y.; Sun, X. Transcriptomic-Metabolomic Analysis Reveals the Effect of Copper Toxicity on Fermentation Properties in *Saccharomyces cerevisiae*. *J. Hazard. Mater.* **2024**, *475*, 134903. [CrossRef]
52. Kim, D.; Langmead, B.; Salzberg, S.L. HISAT: A Fast Spliced Aligner with Low Memory Requirements. *Nat. Methods* **2015**, *12*, 357–360. [CrossRef]
53. Pertea, M.; Pertea, G.M.; Antonescu, C.M.; Chang, T.-C.; Mendell, J.T.; Salzberg, S.L. StringTie Enables Improved Reconstruction of a Transcriptome from RNA-Seq Reads. *Nat. Biotechnol.* **2015**, *33*, 290–295. [CrossRef] [PubMed]
54. Ge, S.X.; Son, E.W.; Yao, R. iDEP: An Integrated Web Application for Differential Expression and Pathway Analysis of RNA-Seq Data. *BMC Bioinform.* **2018**, *19*, 534. [CrossRef] [PubMed]
55. Wang, L.; Feng, Z.; Wang, X.; Wang, X.; Zhang, X. DEGseq: An R Package for Identifying Differentially Expressed Genes from RNA-Seq Data. *Bioinformatics* **2010**, *26*, 136–138. [CrossRef] [PubMed]
56. Karimi, B.; Masson, V.; Guilland, C.; Leroy, E.; Pellegrinelli, S.; Giboulot, E.; Maron, P.-A.; Ranjard, L. Ecotoxicity of Copper Input and Accumulation for Soil Biodiversity in Vineyards. *Environ. Chem. Lett.* **2021**, *19*, 2013–2030. [CrossRef]
57. Pesce, S.; Mamy, L.; Sanchez, W.; Artigas, J.; Bérard, A.; Betoulle, S.; Chaumot, A.; Coutellec, M.-A.; Crouzet, O.; Faburé, J.; et al. The Use of Copper as Plant Protection Product Contributes to Environmental Contamination and Resulting Impacts on Terrestrial and Aquatic Biodiversity and Ecosystem Functions. *Environ. Sci. Pollut. Res.* **2024**, *32*, 2830–2846. [CrossRef]
58. Dönmez, G.; Aksu, Z. The Effect of Copper(II) Ions on the Growth and Bioaccumulation Properties of Some Yeasts. *Process Biochem.* **1999**, *35*, 135–142. [CrossRef]
59. Sun, X.; Liu, L.; Zhao, Y.; Ma, T.; Zhao, F.; Huang, W.; Zhan, J. Effect of Copper Stress on Growth Characteristics and Fermentation Properties of *Saccharomyces cerevisiae* and the Pathway of Copper Adsorption during Wine Fermentation. *Food Chem.* **2016**, *192*, 43–52. [CrossRef]
60. Arakaki, A.H.; Souza Vandenberghe, L.P.D.; Soccol, V.T.; Masaki, R.; Rosa Filho, E.F.D.; Gregório, A.; Soccol, C.R. Optimization of Biomass Production with Copper Bioaccumulation by Yeasts in Submerged Fermentation. *Braz. Arch. Biol. Technol.* **2011**, *54*, 1027–1034. [CrossRef]
61. Kirchman, P.; Botta, G. Copper Supplementation Increases Yeast Life Span under Conditions Requiring Respiratory Metabolism. *Mech. Ageing Dev.* **2007**, *128*, 187–195. [CrossRef]
62. Hasman, H.; Bjerrum, M.J.; Christiansen, L.E.; Bruun Hansen, H.C.; Aarestrup, F.M. The Effect of pH and Storage on Copper Speciation and Bacterial Growth in Complex Growth Media. *J. Microbiol. Methods* **2009**, *78*, 20–24. [CrossRef]
63. Peetermans, A.; Foulquié-Moreno, M.R.; Thevelein, J.M. Mechanisms Underlying Lactic Acid Tolerance and Its Influence on Lactic Acid Production in *Saccharomyces cerevisiae*. *Microb. Cell* **2021**, *8*, 111–130. [CrossRef]
64. Ivit, N.N.; Longo, R.; Kemp, B. The Effect of Non-*Saccharomyces* and *Saccharomyces* Non-*Cerevisiae* Yeasts on Ethanol and Glycerol Levels in Wine. *Fermentation* **2020**, *6*, 77. [CrossRef]
65. Howlett, N.G.; Avery, S.V. Flow Cytometric Investigation of Heterogeneous Copper-Sensitivity in Asynchronously Grown *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **1999**, *176*, 379–386. [CrossRef]
66. Shanmuganathan, A.; Avery, S.; Willetts, S.; Houghton, J. Copper-Induced Oxidative Stress in *Saccharomyces cerevisiae* Targets Enzymes of the Glycolytic Pathway. *FEBS Lett.* **2004**, *556*, 253–259. [CrossRef]
67. Liang, Q.; Zhou, B. Copper and Manganese Induce Yeast Apoptosis via Different Pathways. *Mol. Biol. Cell* **2007**, *18*, 4741–4749. [CrossRef]
68. Sun, J.; Xu, S.; Du, Y.; Yu, K.; Jiang, Y.; Weng, H.; Yuan, W. Accumulation and Enrichment of Trace Elements by Yeast Cells and Their Applications: A Critical Review. *Microorganisms* **2022**, *10*, 1746. [CrossRef]
69. Halbeisen, R.E.; Gerber, A.P. Stress-Dependent Coordination of Transcriptome and Translatome in Yeast. *PLoS Biol.* **2009**, *7*, e1000105. [CrossRef]
70. Matos-Perdomo, E.; Machín, F. Nucleolar and Ribosomal DNA Structure under Stress: Yeast Lessons for Aging and Cancer. *Cells* **2019**, *8*, 779. [CrossRef]
71. Shore, D.; Zencir, S.; Albert, B. Transcriptional Control of Ribosome Biogenesis in Yeast: Links to Growth and Stress Signals. *Biochem. Soc. Trans.* **2021**, *49*, 1589–1599. [CrossRef]
72. Steffen, K.K.; McCormick, M.A.; Pham, K.M.; MacKay, V.L.; Delaney, J.R.; Murakami, C.J.; Kaeberlein, M.; Kennedy, B.K. Ribosome Deficiency Protects Against ER Stress in *Saccharomyces cerevisiae*. *Genetics* **2012**, *191*, 107–118. [CrossRef]
73. García, R.; Bravo, E.; Diez-Muñiz, S.; Nombela, C.; Rodríguez-Peña, J.M.; Arroyo, J. A Novel Connection between the Cell Wall Integrity and the PKA Pathways Regulates Cell Wall Stress Response in Yeast. *Sci. Rep.* **2017**, *7*, 5703. [CrossRef]
74. Ribeiro, R.A.; Bourbon-Melo, N.; Sá-Correia, I. The Cell Wall and the Response and Tolerance to Stresses of Biotechnological Relevance in Yeasts. *Front. Microbiol.* **2022**, *13*, 953479. [CrossRef]
75. Ribeiro, R.A.; Vitorino, M.V.; Godinho, C.P.; Bourbon-Melo, N.; Robalo, T.T.; Fernandes, F.; Rodrigues, M.S.; Sá-Correia, I. Yeast Adaptive Response to Acetic Acid Stress Involves Structural Alterations and Increased Stiffness of the Cell Wall. *Sci. Rep.* **2021**, *11*, 12652. [CrossRef]

76. Sanz, A.; García, R.; Rodríguez-Peña, J.; Arroyo, J. The CWI Pathway: Regulation of the Transcriptional Adaptive Response to Cell Wall Stress in Yeast. *J. Fungi* **2017**, *4*, 1. [CrossRef]
77. Ferreira, V.; Franco-Luesma, E.; Vela, E.; López, R.; Hernández-Orte, P. Elusive Chemistry of Hydrogen Sulfide and Mercaptans in Wine. *J. Agric. Food Chem.* **2018**, *66*, 2237–2246. [CrossRef]
78. Hancock, J.T. Hydrogen Sulfide and Environmental Stresses. *Environ. Exp. Bot.* **2019**, *161*, 50–56. [CrossRef]
79. Huang, C.-W.; Walker, M.E.; Fedrizzi, B.; Gardner, R.C.; Jiranek, V. Hydrogen Sulfide and Its Roles in *Saccharomyces cerevisiae* in a Winemaking Context. *FEMS Yeast Res.* **2017**, *17*. [CrossRef]
80. Liu, H.; Xue, S. Interplay between Hydrogen Sulfide and Other Signaling Molecules in the Regulation of Guard Cell Signaling and Abiotic/Biotic Stress Response. *Plant Commun.* **2021**, *2*, 100179. [CrossRef]
81. Battjes, J.; Melkonian, C.; Mendoza, S.N.; Haver, A.; Al-Nakeeb, K.; Koza, A.; Schrubbers, L.; Wagner, M.; Zeidan, A.A.; Molenaar, D.; et al. Ethanol-Lactate Transition of *Lachancea thermotolerans* Is Linked to Nitrogen Metabolism. *Food Microbiol.* **2023**, *110*, 104167. [CrossRef]
82. Carrera, M.; Calo-Mata, P.; Barros-Velázquez, J.; Pazos, M.; Bañuelos, M.A.; Morata, A. Assessment of the Enological Potential of Several Strains of *Lachancea thermotolerans*. A Proteomic Approach. *Food Chem.* **2026**, *522*, 149983. [CrossRef]