Food Chemistry: Molecular Sciences 11 (2025) 100301

Contents lists available at ScienceDirect

Food Chemistry: Molecular Sciences

journal homepage: www.sciencedirect.com/journal/food-chemistry-molecular-sciences

Metabolic-inhibitor profiling links phenotype and transcriptome of *Lachancea thermotolerans* to wine fermentation chemistry

Samuel Jimena-López [a,1], Javier Vicente [a,1], Santiago Benito [b], Domingo Marquina [a], Antonio Santos [a,*]

[a] Department of Genetics, Physiology and Microbiology, Unit of Microbiology, Faculty of Biological Sciences, Complutense University of Madrid, 28040 Madrid, Spain
[b] Department of Chemistry and Food Technology, Polytechnic University of Madrid, 28040 Madrid, Spain

ARTICLE INFO

Keywords:
Lachancea thermotolerans
Chemical composition
Metabolic inhibitors
Lactic fermentation
Lactic acid
Transcriptome

ABSTRACT

We applied targeted metabolic inhibitors to 145 *Lachancea thermotolerans* strains to uncover fermentation traits with direct relevance to wine quality. Oxamate, a lactate dehydrogenase inhibitor, reduced lactic acid and total titratable acidity by 21% and 26%, respectively, while increasing succinic acid and pH without affecting ethanol levels, offering a promising strategy to fine-tune wine freshness and balance. Notably, industrial grape-associated strains (clusters C4–C6) maintained robust growth under oxamate stress, unlike wild strains, positioning oxamate resistance as a practical marker for selecting high-performing, acidifying yeasts for winemaking. Additional inhibitors such as metformin shifted redox metabolism, significantly enhancing glycerol (+25%) and acetic acid (+319%) production. Transcriptomic analyses showed that OXA alone, and even more so the DSF + OXA combination, repressed *LDH2* and upregulated *GPD1* and oxidative phosphorylation genes, whereas MET caused only moderate changes. This integrated phenomic-transcriptomic approach not only provides valuable tools for yeast screening but also defines a roadmap for optimizing wine composition through the precision selection of *L. thermotolerans* strains.

1. Introduction

Lachancea thermotolerans, formerly *Kluyveromyces thermotolerans* (Kurtzman, 2003), is a ubiquitous spherical-ellipsoidal yeast (Benito, 2018; Kurtzman, Fell, & Boekhout, 2011), commonly associated with winemaking environments but also found in a wide range of both natural and anthropogenic environments (Freel, Friedrich, Hou, & Schacherer, 2014; Kogan et al., 2023; Porter, Divol, & Setati, 2019; Robinson, Pinharanda, & Bensasson, 2016; Sipiczki, 2016; НауМова, Serpova, & НауМов, 2007). These diverse environments have contributed to the evolution and current diversity of the species L. *thermotolerans* (Hranilović, Bely, Masneuf-Pomarède, Jiranek, & Albertin, 2017; Vicente, Benito, Marquina, & Santos, 2025; Vicente et al., 2025).

L. thermotolerans possesses lactate dehydrogenase (LDH) enzymatic activity, which gives it the ability to carry out lactic fermentation. This acidification differs from that of malolactic bacteria in that it does not consume malic acid to produce lactic acid (Benito, 2020). Lactic acid production typically ranges from 1 to 9 g/L and contributes to total acidity in the range of 1–6 g/L (Benito, 2018). This lactic acid helps to decrease the pH by 0.1–0.5 units. These values depend on the strain, yeast inoculation, and the conditions under which fermentation occurs (Gobbi et al., 2013; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020; Vicente et al., 2021). Sequential inoculation with *S. cerevisiae* has yielded better results, as it allows *L. thermotolerans* to perform its fermentative activity without competition that could interfere with acidification (Vicente et al., 2022). Furthermore, the use of *L. thermotolerans* in fermentation has additional advantages beyond lactic acid production, such as higher concentrations of glycerol, polysaccharides, thiols, and terpenes, and lower concentrations of higher alcohols, ethanol, and acetaldehyde (Vicente et al., 2021).

However, *L. thermotolerans* also has disadvantages that hinder its use in the wine industry compared to other yeasts (Vicente et al., 2021). *L. thermotolerans* is a moderate fermentative yeast, unable to tolerate ethanol concentrations above 10%, which are typically exceeded in dry wines (Benito, 2020; Vicente et al., 2022). Additionally, this yeast cannot withstand sulfur dioxide concentrations above 20 mg/L (Benito,

* Corresponding author.
E-mail address: ansantos@ucm.es (A. Santos).
[1] Both authors contributed equally to this work

https://doi.org/10.1016/j.fochms.2025.100301
Received 16 May 2025; Received in revised form 29 August 2025; Accepted 11 September 2025
Available online 12 September 2025

2018), which is used as a corrective to improve the sanitary conditions of grapes. Therefore, its use is limited to grapes with good sanitary conditions or fermentations using chitosan or other sulfur dioxide alternatives that protect fermentation without inhibiting *L. thermotolerans* (Vicente, Baran, et al., 2022). Lastly, another disadvantage is its very slow fermentative activity below 20 °C, resulting in very low lactic acid production at these temperature ranges (Benito et al., 2015). However, most of these issues have been resolved through strain selection programs, and several commercial strains are now available for enological use.

Fig. 1 illustrates the main characteristics of the fermentative metabolism in *L. thermotolerans*. Three genes involved in this pyruvate to lactic acid conversion (*LDH1*, *LDH2*, *LDH3*) and two genes involved in the transport of lactic acid from the intracellular to the extracellular medium (*JEN1*, *ADY2*) have been identified (Sgouros et al., 2020; Gatto et al., 2020; Vicente et al., 2022). Although the exact lactate transport mechanism is not fully understood, these transporters (MCTs -monocarboxylate transporters-) are likely to consume ATP to expel lactate in a symport with protons. When the extracellular pH is low, this lactate is protonated, converting into lactic acid. Lactic acid can diffuse through the cell membrane into the cytoplasm, where the intracellular pH causes it to dissociate back into lactate, which the cell must then expel again (Sauer, Porro, Mattanovich, & Branduardi, 2010). The accumulation of lactic acid in the extracellular medium activates a negative feedback loop that inhibits the expression of genes encoding lactate dehydrogenase (Gatto et al., 2020). However, it has been found that the genes encoding lactate dehydrogenase have a greater influence on lactic acid accumulation than the genes encoding the transporters (Soares-Silva, Paiva, Diallinas, & Casal, 2007; Vicente et al., 2021).

Metabolic regulation plays a crucial role in metabolic engineering, enhancing microbial productivity by targeting key regulatory nodes in pathways such as fermentation (Kern, Tilley, Hunter, Legisa, & Glieder, 2007). The application of metabolic inhibitors is a valuable approach to elucidate the phenotypic diversity within microbial species, such as *L. thermotolerans*. By selectively inhibiting specific enzymes, researchers can redirect metabolic fluxes, thereby uncovering variations in metabolic pathways and end-product synthesis among different strains. This method not only enhances our understanding of microbial metabolism but also aids in identifying strains with desirable traits for industrial applications. Research has explored various inhibitors to refine the design and efficiency of microbial yeast cell factories (Lu, Shen, Liu, et al., 2023; Rocha et al., 2024).

In the context of *L. thermotolerans*, the use of metabolic inhibitors could shed light on its metabolic flexibility and phenotypic variability (Fig. 1). This approach allows for the assessment of how different strains adapt their metabolic processes in response to specific enzymatic blockages, thereby revealing the underlying genetic and regulatory mechanisms that contribute to phenotypic diversity. As stated before, studies have demonstrated that the production of lactic acid in *L. thermotolerans* is highly variable among strains. This variability is not solely attributed to the presence of LDH genes but also to differences in their expression levels and the regulation of associated metabolic pathways (Vicente, Benito, et al., 2025; Gatto et al., 2020). By employing metabolic inhibitors, researchers can dissect these pathways to determine the contribution of specific enzymes and regulatory nodes to the overall metabolic output.

Additionally, the use of inhibitors targeting other enzymes, such as those involved in respiratory metabolism (e.g., sodium azide inhibiting mitochondrial complex IV), can provide insights into the balance between fermentative and respiratory pathways in *L. thermotolerans*. This balance is crucial for understanding how different strains manage energy production and redox balance, which in turn affects their growth and metabolite production profiles (Tyibilika, Setati, Bloem, Divol, & Camarasa, 2024).

The main objective of this study is to advance our understanding of the metabolic and transcriptional diversity of *L. thermotolerans* by characterizing its chemico-phenotypic responses to selected metabolic inhibitors under winemaking fermentation conditions. By analyzing

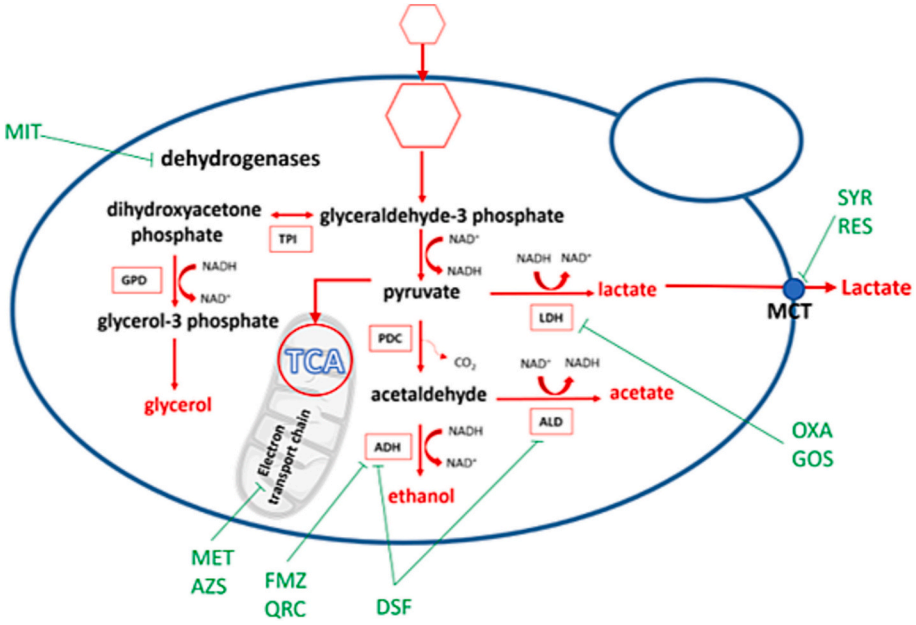

Fig. 1. Schematic representation of the main fermentative pathways in *Lachancea thermotolerans*, highlighting key enzymes and metabolic inhibitors used in this study. Pyruvate is decarboxylated by pyruvate decarboxylase (PDC) to acetaldehyde, which is then reduced to ethanol via alcohol dehydrogenase (ADH) or oxidized to acetate by aldehyde dehydrogenase (ALD). A portion of pyruvate is converted into lactate by lactate dehydrogenase (LDH), while glycerol is synthesized through the dihydroxyacetone phosphate pathway involving triosephosphate isomerase (TPI) and glycerol-3-phosphate dehydrogenase (GPD). Additional key enzymes include mitochondrial NADH dehydrogenase and cytochrome *c* oxidase (electron transport chain), as well as monocarboxylate transporters (MCTs: *JEN1*, *ADY2*) responsible for lactic acid export. The inhibitory effects of gossypol (GOS), oxamate (OXA), fomepizole (FMZ), quercetin (QRC), syrosingopine (SYR), reserpine (RES), metformin (MET), disulfiram (DSF), sodium azide (AZS), and methylisothiazolinone (MIT) on these pathways are illustrated. Adapted and modified from Hranilović et al. (2018).

strain-specific variations in growth kinetics and fermentation-related parameters, this work reveals how genotypic background and ecological origin influence the biochemical adaptability of this yeast. The approach offers novel insights into the modulation of key metabolic pathways—such as lactic acid and ethanol production—relevant to food fermentation chemistry, with implications for improving acidification and redox balance in wine production. The addition of inhibitors such as oxamate and metformin significantly altered wine-relevant chemical parameters. In *L. thermotolerans*, oxamate notably reduced lactic acid and total acidity (−21% and −26%, respectively), while increasing succinic acid and pH, suggesting redirection of carbon flux and acid metabolism. Metformin elevated acetic acid and glycerol levels (+319% and +25%, respectively), affecting both wine acidity and mouthfeel. Combined treatments (e.g., Disulfiram+Oxamate) revealed synergistic effects, intensifying metabolic shifts. These findings provide novel insights into the modulation of fermentation pathways via chemical intervention and underscore the potential of *L. thermotolerans* as a target for metabolic engineering in the development of wine yeasts with improved acidification profiles and tailored compositional outcomes.

2. Materials and methods

2.1. Yeasts strains, culture media and metabolic inhibitors

We utilized 145 strains of *L. thermotolerans* along with a single strain of *S. cerevisiae* (AWRI796) used as control. For comparative analysis, the strains were categorized based on their origin, either anthropized or wild, and grouped into six clusters (C1 to C6) according to genotypic studies (Vicente, Friedrich, et al., 2025). Clusters C1, C2, and C3 (Americas, Asia, or Canada-trees, respectively) primarily consist of wild strains from natural environments, whereas clusters C4, C5, and C6 (Europe/Domestic-1, Europe/Domestic-2, and Europe-mix, respectively) are predominantly composed of strains associated with enological settings. Detailed information on strain collection, genotypic clustering, and geographic origin is provided in the Supplementary Material (Table S1).

All strains were maintained on YMA agar Petri dishes (containing 10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L proteose peptone, and 15 g/L agar), derived from a cryopreserved stock in 25% glycerol. Synthetic Grape Must (SGM) was prepared following the methodology described by Henschke and Jiranek (1993) and Vicente et al. (2023).

Ten metabolic inhibitors (gossypol (GOS), oxamate (OXA), fomepizole (FMZ), quercetin (QRC), syrosingopine (SYR), reserpine (RES), metformin (MET), disulfiram (DSF), sodium azide (AZS) and methylisothiazolinone (MIT)) were used throughout the study. All of them were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for OXA, which was obtained from Med Chem Express (Monmouth Junction, NJ, USA). The stock solutions of the inhibitors, prepared in the most adequate solvent, are shown in Table S2, with most inhibitors dissolved in DMSO, except for RES, AZS, and MIT, which were dissolved in distilled water.

2.2. MIC determinations and analysis of growth parameters in the presence of inhibitors

All selected strains were precultured in 250 µL SGM using 96-well microplates for 16 h at 25 °C and 100 rpm and then diluted in fresh SGM to achieve a final OD_{600} of 0.01. These standardized precultures were subsequently used for both Minimum Inhibitory Concentration (MIC) determinations and growth parameters analysis.

Before assessing the growth parameters of the entire *L. thermotolerans* strain collection in the presence of metabolic inhibitors, a set of four representative *L. thermotolerans* strains was selected for MIC determination. These strains (Lt023, Lt033, Lt085 and Lt166) were chosen to encompass the genetic diversity of the previously identified clusters and their ability to produce lactic acid (Vicente, Friedrich, et al., 2025). Subsequently, growth kinetics studies were conducted on the complete collection of 145 strains.

MIC values for each metabolic inhibitor were assessed using a serial microdilution in SGM of each inhibitor (Table S2). Inhibitors were tested in quadruplicate by making twelve 1:2 serial dilutions in 96-well microplates using the Vialab robot arm with the VOYAGER-300 µL-8CH pipette (INTEGRA Biosciences, Zizers, Switzerland). Inoculated microplates using the same pipetting robot were incubated at 25 °C for 48 h and growth was assessed by determining the OD_{600nm} using a Varioskan Lux microplate reader (Thermo Scientific, Waltham, MA, USA). Additionally, negative controls were prepared in the same way, but without yeast inoculation. To determine the MIC of each inhibitor, growth efficiency at the end of the culture was calculated (Final OD_{600nm} - Initial OD_{600nm}).

With the aim of phenotyping the complete collection of 145 *L. thermotolerans* strains, fermentations were conducted in SGM to determine growth parameters in the presence of the inhibitors. The concentrations of inhibitors used were obtained from the MIC results, ensuring a representative inhibitory effect while allowing comparative growth assessments. Only those inhibitors that exhibited the highest inhibitory effect (AZS and DSF) or caused the greatest phenotypic differentiation (MET, MIT, and OXA) among *L. thermotolerans* strains were selected. To evaluate the impact of the five selected inhibitors on the growth parameters of the 145 strains, each compound was tested at 50% of its determined MIC value (Table S2): AZS (0.05 µM), DSF (1.95 µM), MET (31.3 mM), MIT (156.3 µM), and OXA (22.3 mM). When used, combination treatments employed each inhibitor at its same 50% MIC concentration as in single-agent assays. The OD_{600nm} was measured every 2 h starting from 12 h of cultivation up to 45–49 h depending on the inhibitor. The analysis of growth parameters (lag phase, maximum growth rate, and growth efficiency) for strains phenotyping was performed using the GrowthRates library (v0.8.4) in R (v4.4.0). Wilcoxon-Mann-Whitney test was applied to assess the significance of the growth parameters difference between the different groups of strains (Fig. 2).

2.3. Metabolic and transcriptional responses to inhibitors during fermentation

The strains *L. thermotolerans* Lt106 and *S. cerevisiae* AWRI796 were selected for these experiments. Precultures were prepared in 50 mL of SGM in 100 mL borosilicate bottles at 25 °C and 100 rpm for 24 h and used to inoculate 470 mL of SGM in 500 mL bottles at 10^4 cells/mL by triplicate. At 30 h, axenic cultures were divided into 40 mL aliquots in 50 mL borosilicate bottles and the necessary volume of each selected inhibitor (OXA, MET and DSF) or combination (DSF + MET and DSF + OXA) was added. The final concentration of each inhibitor was: OXA (22.3 mM), MET (31.3 mM), and DSF (1.95 µM). For the combination treatments (DSF + MET and DSF + OXA), each inhibitor was used at these same per-agent concentrations (i.e., 50% MIC). For the control condition, sterile distilled water was added up to the same volume as treatments.

The concentration of various metabolites related to alcoholic fermentation was determined using a Y15 enzymatic analyzer (Biosystems, Barcelona, Spain) for samples collected at 48 h and a Bacchus 3 MultiSpec (Tecnología de Difusión Ibérica, Madrid, Spain) infrared analyzer for final time-point samples (∼ 430 h). At 48 h, glucose-fructose, L-lactic acid, acetaldehyde, acetic acid, and glycerol were analyzed, while for the final time-point samples, ethanol, total acidity, and pH were additionally measured.

Transcriptomic analysis samples were collected 1.5 h after the addition of the inhibitors at their respective 50% MIC concentrations during the mid-exponential growth phase. Five samples from each of the three biological triplicates were taken in 2 mL tubes and immediately frozen using liquid nitrogen and stored at −80 °C until further processing. Total RNA was extracted from one of the samples stored at

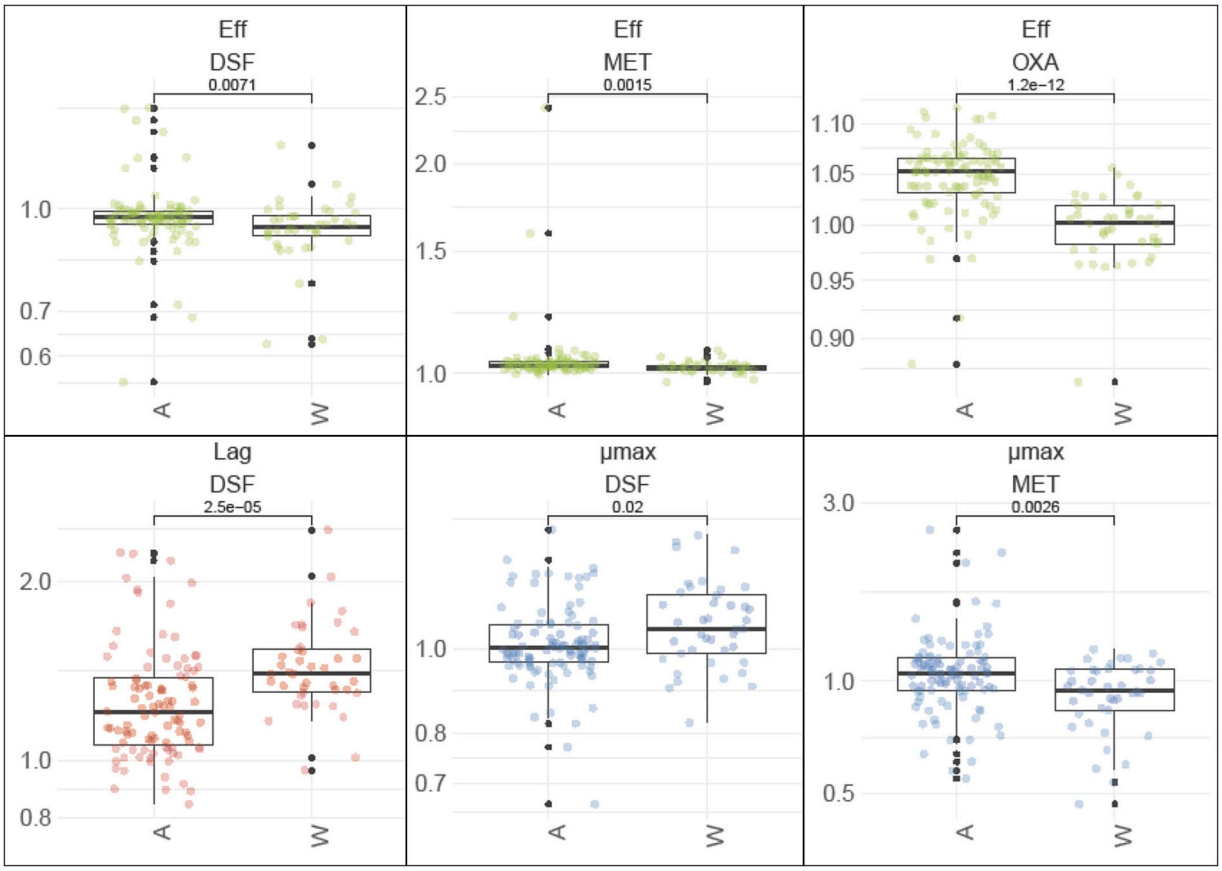

Fig. 2. Analysis of growth parameters in the presence of inhibitors in a collection of 145 *Lachancea thermotolerans* strains. Boxplots represent the mean values of the following growth parameters: proliferative efficiency (Eff), lag phase (Lag), and maximum growth rate (μ_{max}) for 145 *L. thermotolerans* strains cultivated in the presence of different metabolic inhibitors (AZS, DSF, MET, MIT, and OXA). The strains were classified into anthropized (A) and wild (W) categories based on previously determined genoclusters (Vicente et al., 2025). Growth parameters were normalized against control conditions in each experiment, and *p*-values are provided. This figure highlights only the most statistically significant results, while fig. S2 presents the complete dataset.

−80 °C using the Quick-RNA Fungal/Bacterial MicroPrep kit (Zymo Research, Orange, CA, USA). The quality of the RNA was evaluated with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and samples with an RNA Integrity Number (RIN) $\geq$ 7.5 were selected for further analysis. Directional mRNA libraries were prepared using polyA enrichment, and their quality was validated with a Qubit 3.0 (Thermo Fisher, Waltham, MA, USA) and a 2100 Bioanalyzer (Agilent Technologies, USA). The libraries were then sequenced on an Illumina NovaSeq X Plus sequencer (Illumina, San Diego, CA, USA) using 150 bp paired-end sequencing, with an output of 3 Gb per sample.

Illumina paired-end reads were quality assessed with FastQC and trimmed using Trimmomatic (v.0.39) (Bolger, Lohse, & Usadel, 2014). The HISAT2 pipeline (v.2.2.1) (Kim, Paggi, Park, Bennett, & Salzberg, 2019) was employed to align the reads to the indexed reference genome *L. thermotolerans* CBS 6340 (assembly ASM14280v1, obtained from https://www.ncbi.nlm.nih.gov/, last accessed September 2024) and *S. cerevisiae S288C* (R64) and to assemble the alignments into full-length transcripts. Counting of reads mapping to each genomic feature was performed using featureCounts (v.2.0.6) (Liao, Smyth, & Shi, 2014). The differential expression analysis was performed on read counts using the DESeq2 R package (v.1.44.0) (Love, Anders, & Huber, 2014). Library size normalization was performed with the default method implemented by DESeq2. Gene expression in each condition was compared to that in control samples (without inhibitor). Each gene was considered differentially expressed if their false discovery rate (FDR)- adjusted *P*-values were below 0.05. To conduct functional annotation of the DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using the enrichKEGG function from the clusterProfiler R package (v4.12.2) (Wu et al., 2021). The analysis included only those genes with an accumulated absolute log2 fold change greater than 1, focusing on metabolic pathways that showed altered expression relative to the control condition. The ranked "top-100" gene sets used for inspection are supplied as Supplementary Tables S7-S10.

3. Results

3.1. MIC determinations, growth parameters and cluster analysis

For MIC determination, a representative subset of four *L. thermotolerans* strains was selected, ensuring coverage of the species' genetic diversity and their ability to produce lactic acid (Vicente, Friedrich, et al., 2025). The tested inhibitors displayed substantial differences in their MIC values (Table S2), with AZS and DSF showing the highest inhibitory potency, with MICs of 0.1 μM and 3.9 μM, respectively.

The phenotypic characterization of 145 *L. thermotolerans* strains was then conducted by analyzing their growth parameters under winemaking-related conditions in the presence of inhibitors. Growth curves for each strain were obtained, and a Principal Component Analysis (PCA) was performed to simplify the study of growth parameters (Fig. S1). The lag phase duration (Lag) was primarily associated with the component explaining 99.84% of the variability, while growth efficiency (Eff) and maximum growth rate (μ_{max}) were mainly related to the component explaining 0.13% of the variability.

The global PCA, which integrates all experimental conditions, reveals a more balanced distribution of strains (Fig. S1, panel F). This

suggests that, despite the specific effects of each inhibitor, the overall variance in the dataset remains primarily structured around the three growth parameters in a balanced manner. Among these, lag phase exhibits the most pronounced projection, indicating that it serves as a more effective tool for differentiating strains across conditions. Variations in adaptation time significantly contribute to the observed diversity in *L. thermotolerans* responses to metabolic inhibitors.

To facilitate comparisons, strains were categorized based on their origin -anthropized or wild- and their previously defined genetic clusters (Table S2). Significant differences were observed in the resistance of different strains to DSF, MET, and OXA, which inhibit alcoholic fermentation, the Krebs cycle, and lactic acid production, respectively (Fig. S1). The most affected parameter was proliferative efficiency at the end of culture, which was consistently higher in anthropized strains compared to wild strains (Fig. 1 and Fig. S2). Regarding OXA, the most striking result was observed in proliferative efficiency, where wild strains exhibited significantly lower values compared to anthropized strains ($p = 1.2 \times 10^{-12}$). MET reduced efficiency in wild strains ($p = 0.0015$) and significantly decreased their growth rate ($p = 0.0026$), indicating that wild strains are more affected by MET in terms of growth capacity (Fig. S2). Lastly, DSF impacted overall growth by prolonging the lag phase of wild strains ($p = 2.5 \times 10^{-5}$). In addition, the maximum growth rate was slightly but significantly higher in wild strains compared to anthropized ones ($p = 0.02$). Within individual genetic clusters, the same was observed under DSF treatment. Strains from European clusters (clusters C4 to C6), primarily composed of anthropized strains from winemaking environments, exhibited a shorter lag phase and higher proliferative efficiency (Fig. S3).

While not absolute, the presence of the inhibitors aided in differentiating the distinct strain groups within the clusters based on the phenotypic analysis of their behavior. This differentiation allows us to predict that anthropized strains exhibit different growth patterns under inhibitor exposure compared to wild strains.

3.2. Fermentation kinetics and metabolite analysis

The application of inhibitors resulted in notable changes in the metabolite profiles of both Lt106 and AWRI796 strains at the initial (48 h) stages of fermentation (Table S3). In Lt106, the most significant effect was observed with OXA and DSF + OXA, which led to a marked reduction in lactic acid production (from 10.05 g/L in the control to 7.28 g/L and 7.69 g/L, respectively). These treatments also resulted in increased residual sugars, indicating a possible inhibition of fermentation efficiency. In AWRI796, which did not produce lactic acid, OXA and DSF + OXA significantly increased acetaldehyde levels (from 18.67 mg/L in the control to 25.00 mg/L and 26.67 mg/L, respectively), suggesting altered yeast metabolism under these conditions. On the other hand, glycerol and acetic acid levels remained relatively stable across treatments in both strains, indicating that these pathways were less affected by the inhibitors. Notably, DSF alone did not reduce residual sugar relative to the control at 48 h in either species (Table S3).

Considering the finalized fermentations, after approximately 430 h, no significant differences in fermentation kinetics were observed among treatments (data not shown). The impact of inhibitors and their combinations on the physicochemical parameters of the resulting wines resulted in notable changes in the fermentation chemical profiles of both Lt106 and AWRI796 strains (Table 1). In Lt106, the OXA and DSF + OXA treatments significantly reduced total acidity (from 13.97 g/L in the control to 10.32 g/L and 10.53 g/L, respectively), as well as lactic acid production, which decreased from 17.12 g/L in the control to 8.75 g/L and 8.67 g/L, respectively. Additionally, these treatments led to lower residual sugar levels, suggesting a more complete fermentation process. By contrast, under DSF alone, residual sugar in *L. thermotolerans* Lt106 was comparable to the control (5.30 vs 5.32 g/L; Table 1), indicating no material impairment of sugar consumption by DSF. In combinations, particularly DSF + OXA, residual sugar was lower than in the control, consistent with a rerouting rather than stalled uptake. In AWRI796, treatments with MET and DSF + MET resulted in a sharp increase in pH (from 3.57 in the control to 4.26 and 4.24, respectively) and a significant reduction in total acidity, indicating a shift in acid metabolism. Moreover, the OXA and DSF + OXA treatments increased succinic acid levels, particularly in AWRI796, suggesting a metabolic compensation mechanism.

In *L. thermotolerans*, metabolic changes were even more pronounced than in *S. cerevisiae*. OXA and DSF + OXA significantly reduced lactic acid production, which correlated with lower total acidity and increased pH (Table 1). OXA-treated fermentations exhibited lower residual sugar concentrations. Glycerol production increased in MET- and OXA-treated fermentations, both alone and in combination, while MET specifically increased acetic acid concentration. These results highlight the strong metabolic impact of the inhibitors, potentially affecting wine composition and sensory properties.

3.3. Transcriptomic responses to inhibitors

Table 2 presents the differential gene expression of *L. thermotolerans* and *S. cerevisiae* in response to various inhibitor treatments (DSF, MET, OXA, and their combinations) (raw data are deposited in the NCBI BioProject PRJNA1145308 (Tables S4-S6). For readability, we additionally provide ranked lists with the top 100 differentially expressed genes (up- and down-regulated) for each focal strain across the five inhibitor conditions (Tables S7–S10). OXA triggered the most pronounced transcriptional response, with *S. cerevisiae* showing 15% upregulated and 13% downregulated genes, while *L. thermotolerans* also exhibited significant differential expression. The combination of DSF and OXA further amplified this effect, suggesting a synergistic interaction between these inhibitors. MET treatment resulted in moderate gene expression changes, particularly in *L. thermotolerans*, where 1.7% of genes were upregulated and 2.6% were downregulated, whereas *S. cerevisiae* showed a comparatively lower response. DSF alone induced minimal changes in both species, with only a small fraction of genes differentially expressed. This minimal DSF-driven transcriptional effect aligns with the modest phenotypic impact observed at 48 h and 430 h (Tables S3 and 1). Overall, *S. cerevisiae* displayed a stronger transcriptional response than L. *thermotolerans*, particularly under OXA and DSF + OXA treatments, indicating a higher sensitivity or regulatory adaptation to these inhibitory conditions (Table 2).

Functional enrichment analysis indicated species-specific responses. Fig. 3 presents the KEGG pathway enrichment analysis inferred from the transcriptomic analysis under different inhibitor treatments. Each panel illustrates the accumulated fold change (FC) of differentially expressed genes associated with various metabolic pathways. Overall, *L. thermotolerans* underwent significant metabolic and biosynthetic shifts depending on the inhibitor applied, with OXA exerting the most pronounced transcriptional effect (Fig. 3, panel A). In response to DSF treatment, the most significantly enriched pathways included oxidative phosphorylation, carbon metabolism, and glycolysis/gluconeogenesis, indicating a metabolic adjustment to energy production. MET treatment had a broader impact, with notable enrichment in amino acid biosynthesis, carbon metabolism, and oxidative phosphorylation, suggesting metabolic adaptation and stress response mechanisms. OXA treatment induced the most extensive transcriptional reprogramming, affecting multiple biosynthetic pathways, including amino acid, secondary metabolite, and cofactor biosynthesis, along with various central carbon metabolic pathways. The combination DSF + OXA resulted in a similar expression pattern to OXA alone, with increased enrichment in pathways related to amino acid biosynthesis and metabolic processes. The DSF + MET combination primarily affected proteasome activity and oxidative phosphorylation, reflecting a potential stress-related proteolytic response.

Compared to *L. thermotolerans*, *S. cerevisiae* exhibited distinct transcriptomic responses under the same inhibitor treatments (Fig. 3, panel

Table 1
The concentration of various metabolites related to alcoholic fermentation from samples collected at approximately 430 h (final time). Means of the 3 replicates ± standard deviation for L. *thermotolerans* and *S. cerevisiae* in each treatment. *t*-test p-value: *,<0.05; **,<0.01; ***,<0.001.

Strain Lt106	Ethanol (%, *v*/v)	pH	Total acidity (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Residual sugars (g/L)	Glycerol (g/L)
Control	9,11 ± 0,43	3,12 ± 0,05	13,97 ± 0,52	0,16 ± 0,01	11,1 ± 0,53	0,58 ± 0,04	5,32 ± 0,88	2,64 ± 0,12
DSF	8,97 ± 0,99	3,14 ± 0,02	13,61 ± 0,2	0,11 ± 0,03	10,88 ± 0,22	0,59 ± 0,12	5,3 ± 0,46	2,53 ± 0,09
MET	9,78 ± 0,27	3,92 ± 0,01**	11,7 ± 0,12**	0,67 ± 0,01***	11,06 ± 0,25	1,02 ± 0,07**	3,79 ± 0,3	3,3 ± 0,21*
OXA	9,35 ± 0,48	3,35 ± 0,01*	10,32 ± 0,39***	0 ± 0**	8,75 ± 0,17*	1,7 ± 0,36*	3,2 ± 0,42*	2,91 ± 0,28
DSF + MET	9,61 ± 0,4	3,93 ± 0,02***	12,03 ± 0,29**	0,66 ± 0,05**	11,32 ± 0,3	1,16 ± 0,22*	4,25 ± 0,24	3,19 ± 0,22*
DSF + OXA	9,48 ± 0,32	3,37 ± 0,01*	10,53 ± 0,22**	0 ± 0**	8,67 ± 0,22**	1,52 ± 0,15**	2,25 ± 1,95	3,09 ± 0,14*
Strain AWRI796	Ethanol (%, v/v)	pH	Total acidity (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Residual sugars (g/L)	Glycerol (g/L)
Control	10,13 ± 0,37	3,57 ± 0,01	5,21 ± 0,23	0,48 ± 0,05	0,02 ± 0,03	0,56 ± 0,06	0 ± 0	4,49 ± 0,17
DSF	9,7 ± 0,14	3,56 ± 0,01	5,6 ± 0,26	0,65 ± 0,08*	0 ± 0	0,67 ± 0,07	0 ± 0	4,49 ± 0,14
MET	10 ± 0,17	4,26 ± 0***	4,07 ± 0,21**	0,84 ± 0,03*	0,74 ± 0,02***	1,04 ± 0,08***	0 ± 0	4,75 ± 0,23
OXA	10,07 ± 0,16	3,59 ± 0,01*	5,05 ± 0,24	0,23 ± 0,11*	0,24 ± 0,05**	1,37 ± 0,05***	0 ± 0	4,31 ± 0,14
DSF + MET	10,01 ± 0,16	4,24 ± 0,01***	4,29 ± 0,16**	0,87 ± 0,1**	0,74 ± 0,12**	1,06 ± 0,08**	0 ± 0	4,81 ± 0,15
DSF + OXA	9,97 ± 0,14	3,58 ± 0,01	5,08 ± 0,04	0,23 ± 0,06**	0,24 ± 0,06**	1,4 ± 0,02***	0 ± 0	4,6 ± 0,21

B). In *S. cerevisiae*, ribosome biosynthesis was significantly enriched across DSF, MET, and OXA treatments, suggesting strong regulation of the translation machinery, which was less pronounced in *L. thermotolerans*. While DSF and MET primarily affected energy metabolism in *L. thermotolerans*, *S. cerevisiae* showed a stronger impact on amino acid biosynthesis and anabolic pathways. OXA also induced broader transcriptional reprogramming in *S. cerevisiae*, affecting carbohydrate, fatty acid, and cofactor metabolism more extensively than in *L. thermotolerans*. The DSF + OXA combination led to pronounced metabolic shifts in both species, but *S. cerevisiae* displayed a stronger adaptive response in biosynthetic pathways. Overall, *S. cerevisiae* appears to prioritize translational and anabolic regulation, whereas *L. thermotolerans* primarily focuses on adjustments in energy metabolism.

Further analysis of fermentative and respiratory metabolism genes revealed similar expression patterns across treatments but species-specific responses (Fig. S4 and Fig. S5). The two treatments (OXA and DSF + OXA) produced the clearest expression pattern in both *S. cerevisiae* and *L. thermotolerans*, highlighting their strong impact on metabolic regulation in both yeast species.

In *L. thermotolerans*, OXA and DSF + MET treatments downregulated Krebs cycle, LDH, and ADH genes, while alternative ADH and ALD gene expression increased (Fig. S4). Notably, MET upregulated LDH genes (*KLTH0D00440g* and *KLTH0G19558g*), while DSF and DSF + OXA increased the expression of a different LDH gene (*KLTH0G19536g*). The transcriptional response of *L. thermotolerans* to OXA alone and in combination with DSF showed distinct expression patterns. While OXA treatment led to the upregulation of *ARO5 (KLTH0G04026g)*, *KLTH0D00594g*, and *ILV6 (KLTH0H12342g)*, the combined treatment also induced changes in genes such as *CCP1 (KLTH0F05170g)*, *URA1 (KLTH0A05104g)*, and *QCR10* (*KLTH0D06512g)*. These additional upregulated genes suggest an enhanced metabolic stress response, possibly due to the dual inhibition of lactate dehydrogenase and aldehyde dehydrogenase. Furthermore, genes *KLTH0G10406g* and *KLTH0G09636g*, which were downregulated in the OXA-only condition, exhibited a similar trend in the combined treatment, indicating persistent suppression of pathways dependent on LDH activity. However, the co-treatment seemed to broaden the impact on other metabolic pathways, as observed in the differential clustering of gene expression. These findings suggest that the addition of DSF intensified the metabolic stress in *L. thermotolerans*, potentially shifting its adaptation strategies beyond the response to OXA alone.

In *S. cerevisiae*, DSF-containing combinations led to a global reduction in ALD gene expression and increased ADH gene expression, while Krebs cycle-related genes expression showed mixed trends (Fig. S5). The transcriptional response of *S. cerevisiae* to OXA and OXA + DSF showed distinct patterns of gene expression. In the OXA treatment, genes such as *YNL117W*, *YMR034C*, and *YLL055W* were significantly upregulated, suggesting metabolic adaptations to compensate for LDH inhibition. These genes may have been involved in alternative NAD^+ regeneration pathways or cellular stress responses. In the OXA + DSF treatment, additional changes were observed, including the upregulation of *YJR137C*, *YHR208W*, and *YBR045C*, indicating a more pronounced metabolic disturbance. The presence of DSF, an inhibitor of aldehyde dehydrogenase, likely intensified cellular stress, forcing *S. cerevisiae* to activate broader compensatory mechanisms beyond those seen in the OXA condition alone. These results confirm that OXA + DSF elicited the most distinct transcriptional response, reinforcing the idea that dual inhibition of LDH and ALDH significantly impacts yeast metabolism.

4. Discussion

4.1. The phenotypic characterization, fermentation kinetics and metabolite analysis

The phenotypic characterization of *L. thermotolerans* under enological conditions has been previously explored, particularly in relation to its genotype and ecological origin (Banilas, Sgouros, & Nisiotou, 2016; Hranilović et al., 2018; Porter et al., 2019; Vicente, Friedrich, et al., 2025). However, the present study expands on these findings by utilizing a diverse set of metabolic inhibitors to assess their impact on metabolic pathways and growth parameters in order to provide a more comprehensive understanding of the metabolic regulation in this yeast. Moreover, we compared these effects between strains from anthropized and wild environments to gain deeper insights into the metabolic flexibility of this yeast. Additionally, a transcriptomic analysis was conducted to evaluate the molecular responses to these inhibitors, allowing comparisons with *S. cerevisiae*, a well-established model yeast in winemaking fermentation. We used pathway-targeted inhibitors because they provide rapid, reversible, and scalable perturbations across 145 strains, enabling uniform pathway biasing even in non-model yeasts for which genetics is impractical at panel scale. Applying each compound at 50% MIC ensured a partial, pathway-directed inhibition while preserving fermentative activity, allowing like-for-like comparisons among strains. In contrast, gene-regulation approaches would require strain-specific toolchains and are not readily deployable at this breadth.

The response to metabolic inhibitors varied among *L. thermotolerans* strains, highlighting potential metabolic adaptations. We observed that three of the tested inhibitors induced significant differences in proliferative efficiency, lag phase, and maximum growth rate between anthropized and wild strains. Each of these inhibitors affects key metabolic pathways: respiration, alcoholic fermentation, and lactic acid

Table 2
Differential expression analysis L. *thermotolerans* y *S. cerevisiae* comparing the different treatments against the control condition. Only genes showing a FDR (p-adjusted value) <0.05 are included.

Treatment	*L. thermotolerans* Lt106		*S. cerevisiae* AWRI796	
	UP-regulated	DOWN-regulated	UP-regulated	DOWN-regulated
DSF	3 (0,057%)	14 (0,23%)	15 (0,25%)	6 (0,098%)
MET	92 (1,7%)	135 (2,6%)	36 (0,59%)	8 (0,13%)
OXA	504 (9,6%)	606 (11%)	892 (15%)	806 (13%)
DSF + MET	80 (1,5%)	42 (0,8%)	170 (2,8%)	28 (0,46%)
DSF + OXA	404 (7,7%)	577 (11%)	788 (13%)	724 (12%)

production, three essential processes for regenerating redox cofactors required for glycolysis, energy production and cell survival (Duncan, Setati, & Divol, 2024; Shekhawat, Patterton, Bauer, & Setati, 2019). It has been hypothesised that lactic acid production in L. *thermotolerans*, in addition to providing an alternative redox system to ethanol production during fermentation, acts as an anthropization trait. Strains associated with winemaking environments exhibit higher ADH and LDH activity together with higher lactic acid production (Vicente, Benito, et al., 2025, Vicente, Friedrich, et al., 2025). Thus, inhibition of any of these pathways is expected to impact cell growth.

MET, a well-known inhibitor of NADH dehydrogenase activity in mitochondrial complex I (Kim & You, 2017), affects energy production and alters metabolic flux distribution. In this work, MET induced differential effects depending on the strain subset, with anthropized strains showing increased efficiency and maximum growth rate if compared to wild ones. This could be explained by the metabolic adaptation of these strains to fermentative environments, where the Crabtree effect dominates. Inhibiting residual respiratory metabolism, already playing a minor role under fermentative conditions, may enhance the fermentative carbon flux in anthropized yeasts, which exhibit an increased carbon flux towards ethanol and lactic acid production as alternative pathways (Vicente, Benito, et al., 2025). Studies in *S. cerevisiae* have demonstrated that overexpression of NADH dehydrogenase can counteract MET's antiproliferative effects (Wheaton et al., 2014), suggesting that LDH enzymes in *L. thermotolerans* may serve as a compensatory mechanism through their role as NADH dehydrogenases (Shekhawat et al., 2019, Shekhawat, Bauer, & Setati, 2020).

Similarly, DSF, which targets alcohol and aldehyde dehydrogenases (Barth & Benvenuto, 2015; Wright & Moore, 1990), significantly extended the lag phase in *L. thermotolerans*, with a more pronounced effect in wild strains compared to anthropized ones. Similar results have been reported in *Brettanomyces bruxellensis* and *S. cerevisiae*. Given its broad inhibitory effects on both alcohol and aldehyde dehydrogenases, DSF is expected to redirect metabolic fluxes towards alternative fermentative products beyond ethanol and acetate. The observed disparity between *L. thermotolerans* strains may be attributed to differences in fermentative enzyme pools, which could confer greater resilience to anthropized strains against fermentative pathway inhibition via lactic acid production. Additionally, oxidative stress induced by DSF may contribute to these differences, as a *S. cerevisiae* strain deficient in Cu, Zn-superoxide dismutase (Sod1p), the enzyme responsible for decomposing superoxide anions, has been shown to exhibit hypersensitivity to DSF (Kwolek-Mirek, Zadrag-Tecza, & Bartosz, 2011). In *L. thermotolerans*, anthropized strains have shown a positive correlation between stress response gene expression and LDH gene expression levels (Vicente, Benito, et al., 2025).

Further analysis of the metabolic impact of MET, DSF, OXA, and their combinations under fermentative conditions in a wine-related *L. thermotolerans* strain (Lt106) confirmed their effects on yeast metabolism, revealing substantial impacts on fermentation outcomes. As expected, OXA significantly reduced lactic acid production in *L. thermotolerans*, both alone and in combination with DSF. This is consistent with OXA's inhibitory action on lactate dehydrogenase, as previously observed in cancer metabolism studies (Moreno-Sánchez, Marín-Hernández, Del Mazo-Monsalvo, Saavedra, & Rodríguez-Enríquez, S., 2017). However, the observed metabolic shifts suggest that OXA's effects extend beyond LDH inhibition, potentially impacting glycolysis and other fermentative pathways (as evidenced by decreased lactic acid and increased succinic acid and glycerol). Transcriptomic data support this hypothesis, showing marked repression of *LDH2* as well as several glycolytic genes. Regarding other acidity-related parameters, MET and OXA significantly reduced total acidity and increased pH in L. *thermotolerans*. The effect of OXA is directly linked to LDH inhibition, whereas MET's influence, particularly its increase in acetic acid production at the end of fermentation, supports the idea of carbon flux deviation through alternative pathways.

A similar trend was observed in *S. cerevisiae*, although only DSF treatments clustered independently in ethanol concentration analyses. These results suggest that DSF might slightly reduce ethanol yield. Although this trend was not statistically significant, it is consistent with its role in aldehyde metabolism, as evidenced by an increase in acetaldehyde production when alcoholic fermentation is inhibited alongside respiration or lactic acid production. Overall, these findings indicate that the inhibition of major metabolic pathways in *S. cerevisiae* (i.e., alcoholic fermentation) impacts sugar consumption and the accumulation of metabolic intermediates (acetaldehyde) (Scott, Smid, Block, & Notebaart, 2021).

Both *S. cerevisiae* and *L. thermotolerans* demonstrate that the presence of complementary and redundant metabolic pathways enhances adaptability and enables more efficient carbon metabolism. This flexibility is necessarily associated with different production levels of various metabolites, whether final products such as ethanol and lactic acid or metabolic intermediates such as acetaldehyde, in each route. Such adaptations allow yeasts to withstand metabolic inhibition by different compounds. The redirection of metabolic fluxes towards specific pathways to maintain an appropriate ratio of redox intermediates during fermentation is essential for cellular viability (Duncan et al., 2024; Tyibilika et al., 2024). In other yeasts, such as *S. uvarum*, this equilibrium under specific fermentation conditions is linked to the expression of alternative pathways producing erythritol as a compensatory mechanism for redox balance (Albillos-Arenal, Minebois, Querol, & Barrio, 2023).

To translate these findings into winery-relevant actions, we propose an integrated approach: first, pre-screen growth under OXA 22.3 mM (≈50% MIC) to enrich for robust, enology-adapted performers; then validate top candidates in SGM fermentations for high lactic acid yield, acetic acid below target, and sensory-relevant markers. In our dataset, OXA reduced lactic acid by ~21–22% and total acidity by ~25–26%, increased pH, and left ethanol unchanged (Table 1), supporting its use as a selective pressure for screening (Table S2). To balance acetic acid, avoid conditions that elevated acetate at endpoint (e.g., MET ± DSF) and favor LDH-centric routing (OXA ± DSF in Lt106 reduced acetate to ~0 g/L; Table 1). Operational levers (oxygen supply, YAN, and temperature) can bias flux towards lactate over acetate; where appropriate, employ sequential inoculation with *S. cerevisiae* to fine-tune total acidity and redox. Finally, combine strain choice, especially anthropized clusters C4–C6, with dose/condition tuning to reach the desired pH/TA while maintaining mouthfeel (glycerol/polysaccharides), leveraging the shorter lag and higher proliferative efficiency observed for C4–C6 under inhibition (see Results, section 3.1) together with endpoint chemistry (Table 1).

4.2. Transcriptomic analysis and pathway modifications

The transcriptomic responses observed in *L. thermotolerans* and *S. cerevisiae* under the influence of metabolic inhibitors provide valuable insights into species-specific regulatory strategies and metabolic plasticity. Overall, *S. cerevisiae* exhibited a more robust transcriptional

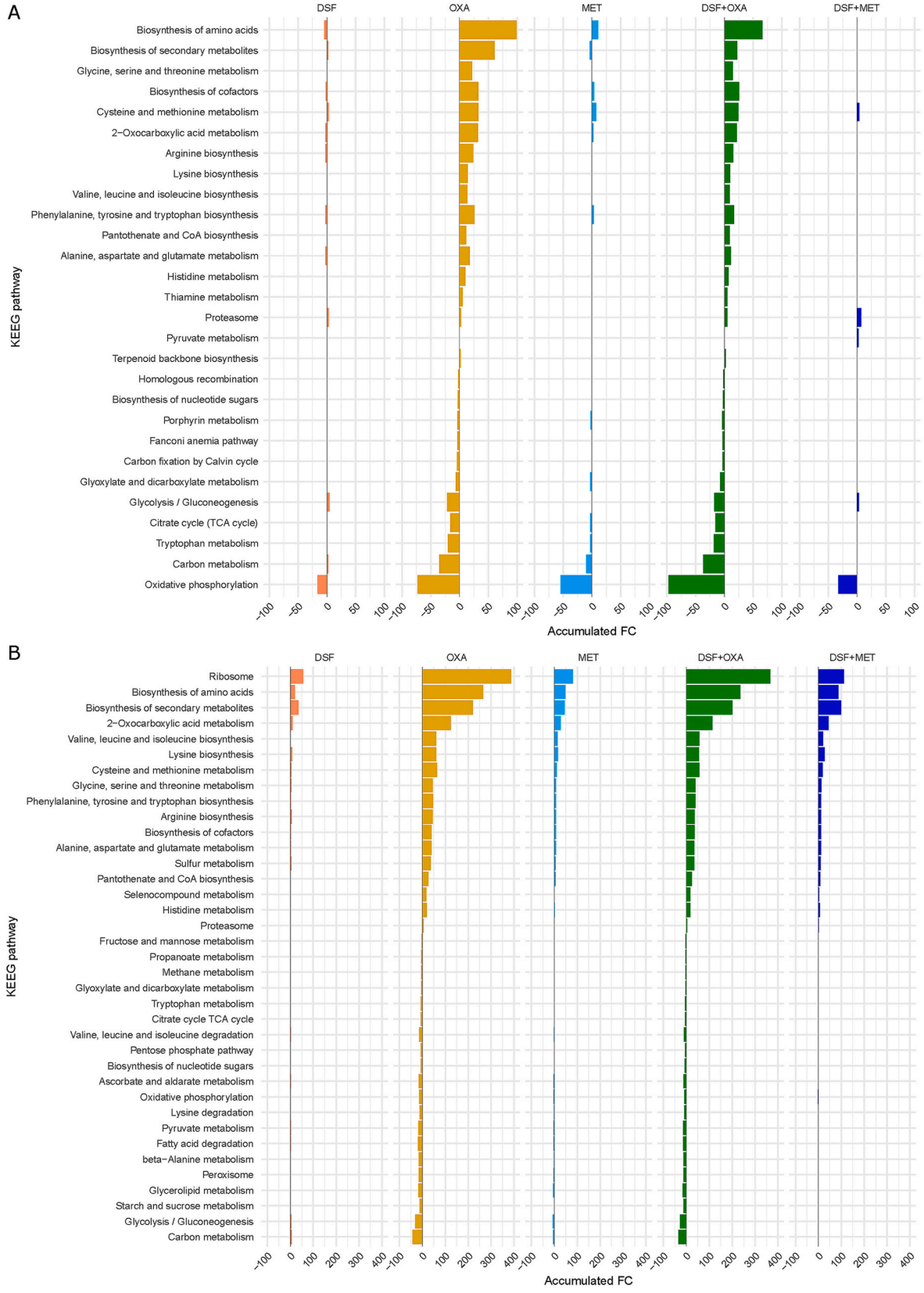

Fig. 3. Differential expression analysis (KEGG pathway enrichment analysis) under the different treatments in *L. thermotolerans* (A) and *S. cerevisiae* (B). The enrichment results are shown for five treatments: DSF, OXA, MET, DSF + OXA and DSF + MET. The y-axis represents the enriched metabolic pathways, while the x-axis indicates the accumulated fold change (FC) for each treatment. The bar colors correspond to the respective treatments. Differences in the modulation of key pathways, including amino acid metabolism, glycolysis/gluconeogenesis, carbon metabolism, and oxidative phosphorylation, highlight the distinct regulatory effects of the inhibitors on yeast metabolism.

reprogramming than *L. thermotolerans*, particularly in response to OXA and the OXA + DSF combination, suggesting heightened sensitivity or a broader regulatory network in adapting to inhibitory stress. These findings align with previous studies indicating the high metabolic flexibility of *S. cerevisiae* in response to environmental and chemical stressors (Gasch et al., 2000; Godard et al., 2007; Ibáñez et al., 2017). The strong enrichment of ribosome biogenesis and amino acid biosynthesis pathways across various treatments in *S. cerevisiae* underscores the organism's prioritization of anabolic processes and translational control under stress, a hallmark of its general stress response strategy (Causton et al., 2001). In contrast, *L. thermotolerans* appears to engage in more focused adjustments in energy metabolism, particularly within oxidative phosphorylation and central carbon metabolism pathways, echoing reports of its adaptation mechanisms in enological contexts.

OXA, a known inhibitor of lactate dehydrogenase (LDH), induced the most pronounced transcriptomic changes in both species. In *L. thermotolerans*, this included downregulation of genes encoding LDH, ADH, and TCA cycle components, while simultaneously upregulating specific aldehyde dehydrogenase (ALD) and alternative ADH isoforms. These patterns suggest metabolic rerouting to maintain redox balance, likely involving alternative NAD^+ regeneration mechanisms, an adaptation similarly observed in other non-*Saccharomyces* yeasts under metabolic constraint (Marsit et al., 2015).Interestingly, the combination of DSF and OXA led to an amplified transcriptional response, particularly in *S. cerevisiae*, where additional genes involved in NAD^+ homeostasis and stress signaling (e.g., *YHR208W*, *YJR137C*) were upregulated. This supports the hypothesis of a synergistic effect between LDH and ALDH inhibition, leading to significant perturbation of redox and carbon metabolism. Such compensatory transcriptional shifts have also been reported in *S. cerevisiae* exposed to combined metabolic or oxidative stresses (Morano, Grant, & Moye-Rowley, 2012), where stress-induced reprogramming enables survival despite impaired metabolic fluxes.

In contrast, DSF alone elicited only mild transcriptional changes in both species. This limited response may reflect lower cellular uptake, metabolic buffering, or redundancy in aldehyde detoxification pathways, especially in *L. thermotolerans*, which exhibits diverse ALDH gene families. MET also induced moderate effects, particularly in *L. thermotolerans*, with enrichment in amino acid biosynthesis and oxidative phosphorylation pathways, indicating a general stress response rather than a targeted inhibition. These results are consistent with previous reports of MET affecting mitochondrial metabolism and redox homeostasis in yeast models (Wu et al., 2016).

Notably, functional enrichment analyses revealed clear divergence in stress response strategies. While *S. cerevisiae* upregulated ribosomal and biosynthetic pathways, potentially linked to translational reprogramming, *L. thermotolerans* favored pathways related to energy metabolism and oxidative stress resilience, reinforcing its specialized ecological niche and metabolic profile. This distinction reflects broader evolutionary differences in the regulatory architecture of these two yeasts, with *S. cerevisiae* exhibiting a more complex and responsive gene regulatory network (Borneman et al., 2011).

Taken together, these findings highlight the complexity and specificity of yeast transcriptional responses to chemical inhibitors. They suggest that the dual inhibition of LDH and ALDH imposes significant metabolic stress, particularly in *S. cerevisiae*, requiring coordinated responses across redox, biosynthetic, and energy pathways. Such data contribute to a growing body of knowledge on yeast stress biology and may inform the development of selective metabolic inhibitors or adaptive yeast strains for industrial applications.

4.3. Integration of metabolite profiles and transcriptomic responses

The combination of metabolite analysis and gene expression data provides mechanistic insights into how *L. thermotolerans* modulates its fermentation metabolism under different inhibitory conditions. Notably, treatments with OXA and DSF + OXA led to significant reductions in lactic acid levels (−21% and −22%, respectively) and total acidity (−26% and −25%, respectively), accompanied by an increase in pH. These changes correlate with the downregulation of key genes involved in lactic acid production, including *LDH1* (KLTH0D00440g) and *LDH3* (KLTH0G19536g), and with the repression of respiratory genes such as *QCR10* (KLTH0D06512g), suggesting impaired redox balancing and energy metabolism under LDH inhibition. This suggests that inhibition of mitochondrial function via KLTH0D06512g repression may impact redox balance and acid metabolism in *L. thermotolerans*. The gene KLTH0D06512g in *L. thermotolerans* is annotated as homologous to *QCR10* in *S. cerevisiae*, suggesting a conserved function in mitochondrial electron transport. However, specific functional studies on KLTH0D06512g in *L. thermotolerans* are limited.

Treatments with MET and DSF + MET significantly increased acetic acid levels (up to +319%) and succinic acid concentrations (up to +100%), indicating redirection of metabolic fluxes. These changes are consistent with a partial repression of *ALD* and *ADH* isoforms, potentially limiting ethanol and acetate recycling and favoring accumulation of acidic by-products. In parallel, our transcriptomic analysis revealed that *GPD1* (KLTH0E04730g), a key gene in glycerol biosynthesis, was upregulated under oxamate (OXA) and DSF + OXA treatments (log2FC $\approx +1.39$), correlating with the observed increase in glycerol production under MET (+25%) and DSF-containing conditions. This suggests that, similar to *S. cerevisiae*, *L. thermotolerans* induces *GPD1* expression as a compensatory mechanism to enhance glycerol synthesis in response to specific metabolic stresses. This supports the notion of a compensatory mechanism to maintain NAD^+ regeneration via glycerol and acetic acid production. Although we did not directly measure the NADH/NAD^+ ratio, the upregulation of *GPD1* under LDH inhibition and the increases in glycerol/acids under MET or DSF-containing treatments support redox compensation via glycerol and organic acid pathways in *L. thermotolerans*.

Interestingly, despite the strong inhibition of lactic acid production by OXA, residual sugar levels were lower in OXA and DSF + OXA treatments, suggesting that carbon flux was redirected towards alternative fermentation routes rather than being associated with incomplete sugar consumption. Overall, these results highlight the metabolic plasticity of *L. thermotolerans* in response to specific enzymatic inhibitions and illustrate how transcriptional rewiring is reflected in the final chemical composition of fermented products.

5. Conclusion

This study provides novel insights into the metabolic regulation of *L. thermotolerans* through a comprehensive analysis of its phenotypic and transcriptomic responses to targeted metabolic inhibitors under wine fermentation conditions. By chemically perturbing key enzymatic nodes such as lactate dehydrogenase, aldehyde dehydrogenase, and mitochondrial complex I, we uncovered strain-specific shifts in central metabolic pathways that translated into measurable changes in wine-relevant chemical composition—including lactic acid, acetic acid, succinic acid, and glycerol levels. These metabolic outcomes were supported by transcriptional evidence, such as the downregulation of *LDH* and *QCR10* homologs under oxamate treatment, and the upregulation of *GPD1* in response to redox stress, highlighting the yeast's adaptive mechanisms for maintaining fermentation balance. The integration of chemical inhibition, metabolite profiling, and gene expression provides a powerful framework for dissecting the biochemical flexibility of food-related yeasts. These findings not only advance our molecular understanding of non-*Saccharomyces* fermentative metabolism but also support the strategic development of customized yeast strains to modulate wine acidity, aroma, and mouthfeel—addressing key challenges in modern food and beverage chemistry.

CRediT authorship contribution statement

Samuel Jimena-López: Writing – original draft, Investigation, Formal analysis, Data curation. **Javier Vicente:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Santiago Benito:** Writing – original draft, Investigation, Funding acquisition. **Domingo Marquina:** Supervision, Funding acquisition, Conceptualization. **Antonio Santos:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Funding for this research was provided by the LowpHWine Companies Consortia through the CDTI project LowpHWine (IDI-20210391) and the Spanish Ministry of Science and Innovation under the VinSeg-CalClim project (PID2020-119008RB-I00). Javier Vicente conducted this research under a fellowship from Complutense University of Madrid (CT58/21-CT59/21). Samuel Jimena conducted this research under an INVESTIGO (CT19/23-INVM-18) contract from the Spanish Ministry of Labour and Social Economy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2025.100301.

Data availability

Data will be made available on request.

References

Albillos-Arenal, S., Minebois, R., Querol, A., & Barrio, E. (2023). Understanding the role of GRE3 in the erythritol biosynthesis pathway in *Saccharomyces uvarum* and its implication in osmoregulation and redox homeostasis. *Microbial Biotechnology, 16*(9), 1858–1871. https://doi.org/10.1111/1751-7915.14313

Banilas, G., Sgouros, G., & Nisiotou, A. (2016). Development of microsatellite markers for *Lachancea thermotolerans* typing and population structure of wine-associated isolates. *Microbiological Research, 193*, 1–10. https://doi.org/10.1016/j.micres.2016.08.010

Barth, R., & Benvenuto, M. A. (2015). Ethanol and education: Alcohol as a theme for teaching chemistry. *En ACS Symposium Series*. https://doi.org/10.1021/bk-2015-1189

Benito, S. (2018). The impacts of *Lachancea thermotolerans* yeast strains on winemaking. *Applied Microbiology and Biotechnology, 102*(16), 6775–6790. https://doi.org/10.1007/s00253-018-9117-z

Benito, S. (2020). Combined use of *Lachancea thermotolerans* and *Schizosaccharomyces pombe* in winemaking: A review. *Microorganisms, 8*(5), 655. https://doi.org/10.3390/microorganisms8050655

Benito, S., Hofmann, T., Laier, M., Lochbühler, B., Schüttler, A., Ebert, K., Fritsch, S., Röcker, J., & Rauhut, D. (2015). Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces cerevisiae*. *European Food Research and Technology, 241*(5), 707–717. https://doi.org/10.1007/s00217-015-2497-8

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Borneman, A. R., Desany, B. A., Riches, D., Affourtit, J. P., Forgan, A. H., Pretorius, I. S., … Chambers, P. J. (2011). Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. *PLoS Genetics, 7*(2), Article e1001287. https://doi.org/10.1371/journal.pgen.1001287

Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., … Young, R. A. (2001). Remodeling of yeast genome expression in response to environmental changes. *Molecular Biology of the Cell, 12*(2), 323–337. https://doi.org/10.1091/mbc.12.2.323

Duncan, J. D., Setati, M. E., & Divol, B. (2024). The cellular symphony of redox cofactor management by yeasts in wine fermentation. *International Journal of Food Microbiology*. , Article 110966. https://doi.org/10.1016/j.ijfoodmicro.2024.110966

Freel, K. C., Friedrich, A., Hou, J., & Schacherer, J. (2014). Population genomic analysis reveals highly conserved mitochondrial genomes in the yeast species *Lachancea thermotolerans*. *Genome Biology and Evolution, 6*(10), 2586–2594. https://doi.org/10.1093/gbe/evu203

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., … Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell, 11*(12), 4241–4257. https://doi.org/10.1091/mbc.11.12.4241

Gatto, V., Binati, R. L., Lemos, W. J. F., Basile, A., Treu, L., de Almeida, O. G. G., Innocente, G., & Torriani, S. (2020). New insights into the variability of lactic acid production in *Lachancea thermotolerans* at the phenotypic and genomic level. *Microbiological Research, 238*, Article 126525. https://doi.org/10.1016/j.micres.2020.126525

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology, 33*(2), 271–281. https://doi.org/10.1016/j.fm.2012.10.004

Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J., & André, B. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Molecular and Cellular Biology, 27*(8), 3065–3086. https://doi.org/10.1128/MCB.01084-06

Henschke, P. A., & Jiranek, V. (1993). Yeasts-metabolism of nitrogen compounds. *Wine Microbiol. Biotechnol., 77*, 163.

Hranilović, A., Bely, M., Masneuf-Pomarède, I., Jiranek, V., & Albertin, W. (2017). The evolution of *Lachancea thermotolerans* is driven by geographical determination, anthropisation and flux between different ecosystems. *PLoS One, 12*(9), Article e0184652. https://doi.org/10.1371/journal.pone.0184652

Hranilović, A., Gambetta, J. M., Schmidtke, L., Boss, P. K., Grbin, P. R., Masneuf-Pomarede, I., … Jiranek, V. (2018). Oenological traits of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation. *Scientific Reports, 8*(1). https://doi.org/10.1038/s41598-018-33105-7

Ibáñez, C., Pérez-Torrado, R., Morard, M., Toft, C., Barrio, E., & Querol, A. (2017). RNAseq-based transcriptome comparison of *Saccharomyces cerevisiae* strains isolated from diverse fermentative environments. *International Journal of Food Microbiology, 257*, 262–270. https://doi.org/10.1016/j.ijfoodmicro.2017.07.001

Kern, A., Tilley, E., Hunter, I. S., Legisa, M., & Glieder, A. (2007). Engineering primary metabolic pathways of industrial microorganisms. *Journal of Biotechnology, 129*(1), 6–29. https://doi.org/10.1016/j.jbiotec.2006.11.021

Kim, D., Paggi, J. M., Park, C., Bennett, C., & Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology, 37*(8), 907–915. https://doi.org/10.1038/s41587-019-0201-4

Kim, J., & You, Y. (2017). Regulation of organelle function by metformin. *IUBMB Life, 69*(7), 459–469. https://doi.org/10.1002/iub.1633

Kogan, H. V., Elikan, A. B., Glaser, K. F., Bergmann, J. M., Raymond, L. M., Prado-Irwin, S. R., & Snow, J. W. (2023). Colonization of honey bee digestive tracts by environmental yeast *Lachancea thermotolerans* is naturally occurring, temperature dependent, and impacts the microbiome of newly emerged bees. *Microbiology Spectrum, 11*(2). https://doi.org/10.1128/spectrum.05194-22

Kurtzman, C. P. (2003). Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. *FEMS Yeast Research, 4*(3), 233–245. https://doi.org/10.1016/s1567-1356(03)00175-2

Kurtzman, C. P., Fell, J. W., & Boekhout, T. (2011). The yeasts: A taxonomic study. https://ci.nii.ac.jp/ncid/BB05482227.

Kwolek-Mirek, M., Zadrag-Tecza, R., & Bartosz, G. (2011). Ascorbate and thiol antioxidants abolish sensitivity of yeast *Saccharomyces cerevisiae* to disulfiram. *Cell Biology and Toxicology, 28*(1), 1–9. https://doi.org/10.1007/s10565-011-9200-z

Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics, 30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Love, M. I., Anders, S., & Huber, W. (2014). Differential analysis of count data – The DESeq2 package. *Genome Biology, 15*(550), 10–1186.

Lu, Z., Shen, Q., Liu, L., et al. (2023). Profiling proteomic responses to hexokinase-II depletion in terpene-producing *Saccharomyces cerevisiae*. *Engineering Microbiology, 3*(3), Article 100079. https://doi.org/10.1016/j.engmic.2023.100079

Marsit, S., Mena, A., Bigey, F., Sauvage, F. X., Couloux, A., Guy, J., Legras, J. L., Barrio, E., Dequin, S., & Galeote, V. (2015). Evolutionary advantage conferred by an eukaryote-to-eukaryote gene transfer event in wine yeasts. *Molecular Biology and Evolution, 32*(7), 1695–1707. https://doi.org/10.1093/molbev/msv057

Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics, 190*(4), 1157–1195. https://doi.org/10.1534/genetics.111.128033

Moreno-Sánchez, R., Marín-Hernández, Á., Del Mazo-Monsalvo, I., Saavedra, E., & Rodrıǵuez-Enrıǵuez, S.. (2017). Assessment of the low inhibitory specificity of oxamate, aminooxyacetate and dichloroacetate on cancer energy metabolism. *Biochimica Et Biophysica Acta. G, General Subjects (Print), 1861*(1), 3221–3236. https://doi.org/10.1016/j.bbagen.2016.08.006

Porter, T. J., Divol, B., & Setati, M. E. (2019). *Lachancea* yeast species: Origin, biochemical characteristics and oenological significance. *Food Research International, 119*, 378–389. https://doi.org/10.1016/j.foodres.2019.02.003

Robinson, H. A., Pinharanda, A., & Bensasson, D. (2016). Summer temperature can predict the distribution of wild yeast populations. *Ecology and Evolution, 6*(4), 1236–1250. https://doi.org/10.1002/ece3.1919

Rocha, G., Gómez, M., Baeza, C., Salinas, F., Martínez, C., & Kessi-Pérez, E. I. (2024). Phenotyping of a new yeast mapping population reveals differences in the activation of the TORC1 signalling pathway between wild and domesticated yeast strains. *Biological Research, 57*(1), 82. https://doi.org/10.1186/s40659-024-00563-5

Sauer, M., Porro, D., Mattanovich, D., & Branduardi, P. (2010). 16 years research on lactic acid production with yeast – Ready for the market? *Biotechnology & Genetic Engineering Reviews, 27*(1), 229–256. https://doi.org/10.1080/02648725.2010.10648152

Scott, W. T., Smid, E. J., Block, D. E., & Notebaart, R. A. (2021). Metabolic flux sampling predicts strain-dependent differences related to aroma production among commercial wine yeasts. *Microbial Cell Factories, 20*, 1–15. https://doi.org/10.1186/s12934-021-01694-0

Sgouros, G., Mallouchos, A., Filippousi, M., Banilas, G., & Nisiotou, A. (2020). Molecular characterization and enological potential of a high lactic acid-producing *Lachancea thermotolerans* vineyard strain. *Foods, 9*(5), 595. https://doi.org/10.3390/foods9050595

Shekhawat, K., Bauer, F. F., & Setati, M. E. (2020). The transcriptomic response of a wine strain of *Lachancea thermotolerans* to oxygen deprivation. *FEMS Yeast Research, 20*(7). https://doi.org/10.1093/femsyr/foaa054

Shekhawat, K., Patterton, H., Bauer, F. F., & Setati, M. E. (2019). RNA-seq based transcriptional analysis of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed-culture fermentations under anaerobic conditions. *BMC Genomics, 20*(1). https://doi.org/10.1186/s12864-019-5511-x

Sipiczki, M. (2016). Overwintering of vineyard yeasts: Survival of interacting yeast communities in grapes mummified on vines. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00212

Soares-Silva, I., Paiva, S., Diallinas, G., & Casal, M. (2007). The conserved sequence NXX [S/T]HX[S/T]QDXXXT of the lactate/pyruvate:H^+ symporter subfamily defines the function of the substrate translocation pathway. *Molecular Membrane Biology, 24*(5–6), 464–474. https://doi.org/10.1080/09687680701342669

Tyibilika, V., Setati, M. E., Bloem, A., Divol, B., & Camarasa, C. (2024). Differences in the management of intracellular redox state between wine yeast species dictate their fermentation performances and metabolite production. *International Journal of Food Microbiology, 411*, Article 110537. https://doi.org/10.1016/j.ijfoodmicro.2023.110537

Vicente, J., Baran, Y., Navascués, E., De la Oliva Santos, A., Calderón, F., Marquina, D., Rauhut, D., & Benito, S. (2022). Biological management of acidity in wine industry: A review. *International Journal of Food Microbiology, 375*, Article 109726. https://doi.org/10.1016/j.ijfoodmicro.2022.109726

Vicente, J., Benito, S., Marquina, D., & Santos, A. (2025). Subpopulation-specific gene expression in *Lachancea thermotolerans* uncovers distinct metabolic adaptations to wine fermentation. *Current Research in Food Science, 10*, Article 100954. https://doi.org/10.1016/j.crfs.2024.100954

Vicente, J., Friedrich, A., Schacherer, J., Freel, K., Marquina, D., & Santos, A. (2025). Whole-genome sequencing and phenotyping reveal specific adaptations of *Lachancea thermotolerans* to the winemaking environment. *Molecular Ecology, e17667*. https://doi.org/10.1111/mec.17667

Vicente, J., Kelanne, N., Rodrigo-Burgos, L., Navascués, E., Calderón, F., Santos, A., Marquina, D., Yang, B., & Benito, S. (2022). Influence of different *Lachancea thermotolerans* strains in the wine profile in the era of climate challenge. *FEMS Yeast Research, 23*. https://doi.org/10.1093/femsyr/foac062

Vicente, J., Navascués, E., Calderón, F., Santos, A., Marquina, D., & Benito, S. (2021). An integrative view of the role of *Lachancea thermotolerans* in wine technology. *Foods, 10*(11), 2878. https://doi.org/10.3390/foods10112878

Vicente, J., Ruiz, J., Tomasi, S., De Celis, M., Ruiz-De-Villa, C., Gombau, J., Rozès, N., Zamora, F., Santos, A., Marquina, D., & Belda, I. (2023). Impact of rare yeasts in *Saccharomyces cerevisiae* wine fermentation performance: Population prevalence and growth phenotype of *Cyberlindnera fabianii, Kazachstania unispora,* and *Naganishia globosa*. *Food Microbiology, 110*, Article 104189. https://doi.org/10.1016/j.fm.2022.104189

Wheaton, W. W., Weinberg, S. E., Hamanaka, R. B., Soberanes, S., Sullivan, L. B., Anso, E., … Chandel, N. S. (2014). Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife, 3*. https://doi.org/10.7554/elife.02242

Wright, C., & Moore, R. D. (1990). Disulfiram treatment of alcoholism. *The American Journal of Medicine, 88*(6), 647–655. https://doi.org/10.1016/0002-9343(90)90534-k

Wu, L., Zhou, B., Oshiro-Rapley, N., Li, M., Paulo, J. A., Webster, C. M., … Soukas, A. A. (2016). An ancient, unified mechanism for metformin growth inhibition in *C. Elegans* and cancer. *Cell, 167*(7), 1705–1718.e13. https://doi.org/10.1016/j.cell.2016.11.055

Wu, T. Z., Hu, E. Q., Xu, S. B., Chen, M. J., Guo, P. F., Dai, Z. H., … Yu, G. C. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation, 2*(3). https://doi.org/10.1016/j.xinn.2021.100141

НауМова, Е.С., Serpova, E. V., & НауМов, Г.И. (2007). Molecular systematics of *Lachancea* yeasts. *Biochemistry, 72*(12), 1356–1362. https://doi.org/10.1134/s0006297907120097