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Multi-omics framework to reveal the molecular determinants of fermentation performance in wine yeast populations

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Abstract

Background Connecting the composition and function of industrial microbiomes is a major aspiration in microbial biotechnology. Here, we address this question in wine fermentation, a model system where the diversity and functioning of fermenting yeast species are determinant of the flavor and quality of the resulting wines.

Results First, we surveyed yeast communities associated with grape musts collected across wine appellations, revealing the importance of environmental (i.e., biogeography) and anthropic factors (i.e., farming system) in shaping community composition and structure. Then, we assayed the fermenting yeast communities in synthetic grape must under common winemaking conditions. The dominating yeast species defines the fermentation performance and metabolite profile of the resulting wines, and it is determined by the initial fungal community composition rather than the imposed fermentation conditions. Yeast dominance also had a more pronounced impact on wine metatranscriptome than fermentation conditions. We unveiled yeast-specific transcriptomic profiles, leveraging different molecular functioning strategies in wine fermentation environments. We further studied the orthologs responsible for metabolite production, revealing modules associated with the dominance of specific yeast species. This emphasizes the unique contributions of yeast species to wine flavor, here summarized in an array of orthologs that defines the individual contribution of yeast species to wine ecosystem functioning.

Conclusions Our study bridges the gap between yeast community composition and wine metabolite production, providing insights to harness diverse yeast functionalities with the final aim to producing tailored high-quality wines.

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Background

Originally, wine fermentations were spontaneous, and the native yeast communities present on the grape surface were responsible for completing the fermentation of grape musts. The grape surface constitutes a complex microbial ecosystem with yeasts, filamentous fungi, and bacteria, each influencing wine production differently [1]. Yeast communities play crucial roles in determining the chemical and sensory properties of the resulting wines [2–4]. A diverse yeast microbiome in grape musts can enhance the aromatic complexity of final wines [5–8]. The inherent microbial diversity in grape musts, coupled with the spontaneous nature of fermentations, implies limited control over population dynamics during wine fermentation, influencing its kinetics and final sensory output. In contrast to the spontaneity of natural fermentations, the wine industry introduced the standardization of wine fermentations through the inoculation of commercial yeast strains or consortia with predefined traits [9]. Regardless of the fermentation strategy followed, deciphering the molecular determinants of the individual contribution of wine yeast species to wine flavor is essential to advance in the targeted improvement of wine quality.

Several factors influence yeast communities associated with grape berries, subsequently shaping the diversity of fermenting yeast species in grape musts. Edaphoclimatic factors and vineyard management practices contribute to the complexity of these communities [10, 11]. After crushing grapes, these microbial populations have to cope with various environmental challenges during the fermentation of grape musts, such as high osmotic pressure, low pH, suboptimal temperatures for growth, increasing ethanol concentrations, and anaerobic conditions [12, 13]. This leads to a rapid succession of yeast populations where the initially wide fungal diversity is wiped out by the ethanol toxicity and is replaced by ethanol-tolerant fermentative yeasts, mainly *Saccharomyces cerevisiae* [14, 15]. By changing temperature, or adding sulfur dioxide (SO_2) or nitrogen nutrients, winemakers have the possibility to modify the fermentation kinetics and alter the performance of yeast species [16–18]. Understanding the influence of these conditions on yeast growth and fermentation processes will enhance future achievements in the oenological industry, facilitating a more rational use of yeast cultures, food additives, and nutrients in wine fermentations. However, to gain control and predict the metabolite profile of final wines, it is necessary to first comprehend the functional potential of wild yeast communities subjected to varying fermentation conditions.

In this study, we combined observational studies with laboratory fermentations to assess factors shaping yeast community composition associated with grape berries and to identify functional yeasts associated with wine metabolite production under different fermentation conditions. We integrated multi-omics data to understand the connection between the composition and function of fermenting yeast communities with the final metabolite profile of wines. In doing so, we aim to contribute to the ecological understanding of wine ecosystems and highlight the importance of preserving the complex dynamics even in controlled fermentation processes. To do so, we surveyed five distinct Spanish wine appellations, sampling grapes from vineyards under conventional and organic management. We also sampled at lower geographic scales to finely disentangle whether grape must and fungal variability were distance dependent (Supplementary Figure S1). We analyzed the metabolite and fungal community profiles of fresh grape musts, defining their initial variability, and subject them to spontaneous fermentations in four different fermentation conditions. Then, we inoculated the fermenting yeast communities in synthetic grape must, and studied the associations between their functional profiles, through meta-transcriptomic analysis and the final metabolite composition of the resulting wines.

Methods

Observational study design

A total of nine different locations were sampled from five different Spanish wine appellations (Supplementary Figure S1). The wine appellations were Ribera del Guadiana (RdG), Valdepeñas (VLP), La Mancha (LM), Madrid (M), and La Rioja (R). Within La Rioja, we sampled three locations (R1, R2, and R3; the later per triplicate, in three different rows within the same plot: R3A, R3B, R3C). This spatial survey was applied in parallel, sampling two vineyards within each location, under conventional and under organic farming. Contrary to conventional management, organic vineyards are restrictive with the use of inorganic fertilizers as well as phytosanitary products. The regulatory specifications outlining the criteria for conventional and organic classifications are established according to the European Union Regulation (EC) No. 1234/2007 (Official Journal of the European Union, 2007). The different patterns of fungal community and metabolite production during fermentation are largely dependent on grape variety [10]. Therefore, we only sampled grapes of Tempranillo variety, when possible, that is, in all cases except in conventional farming in RdG, where we could only sample grapes of Red Garnacha variety. Since the physical–chemical composition of Garnacha grape must differed significantly from that of Tempranillo grapes (Supplementary Figure S2), we removed it from the

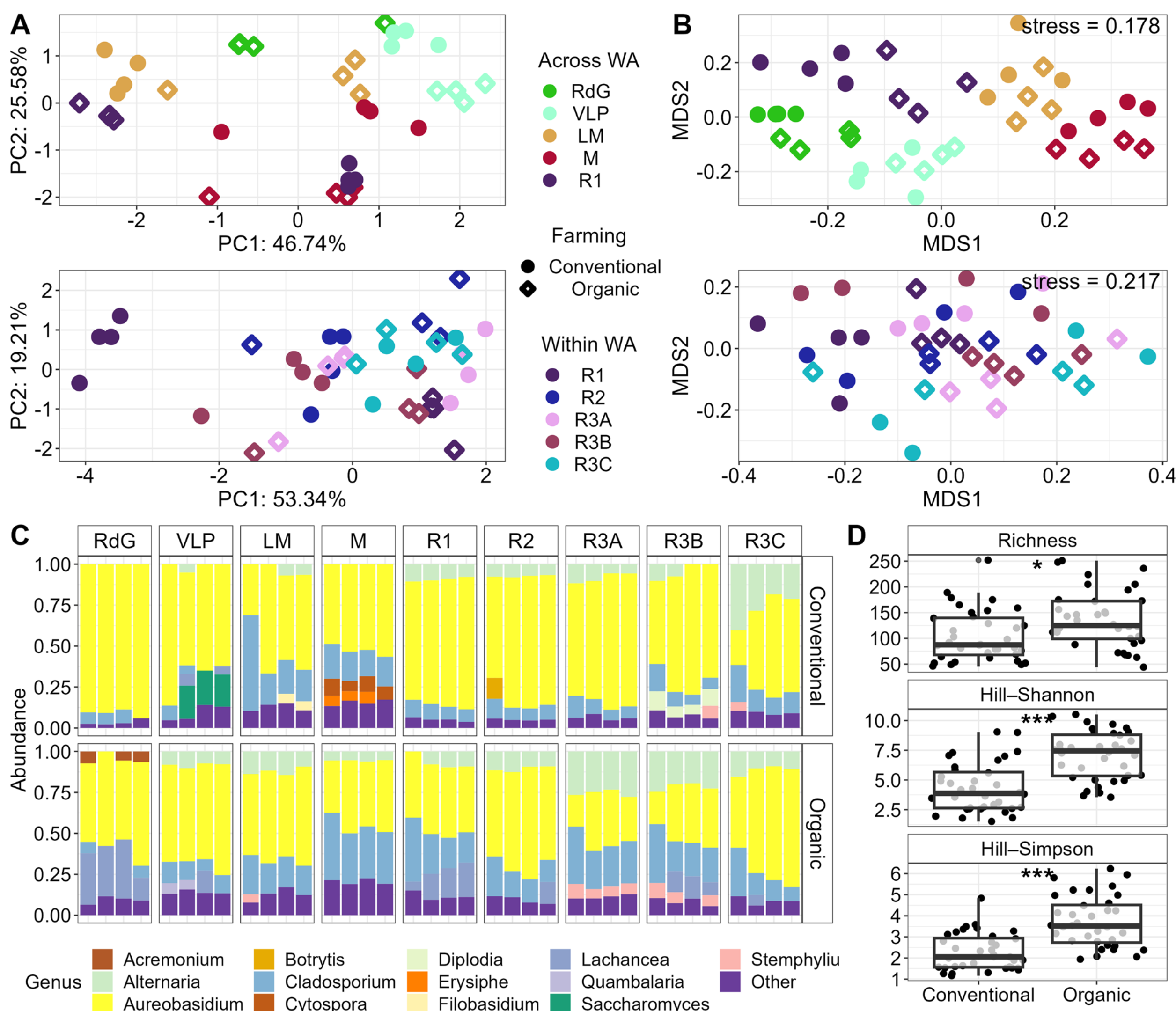

Fig. 1 Grape must composition and fungal community description of initial samples. **A** Principal component analysis (PCA) representing grape must composition diversity across wine appellations (WA) (upper panel, $n = 4$) and within the WA of La Rioja (bottom panel, $n = 4$). Raw data is detailed in Additional File 1. **B** Beta diversity of fungal communities based on a nonmetric multidimensional scaling analysis (NMDS, stress parameter indicated in the plot) from Bray–Curtis dissimilarity matrix calculated from genus abundance table. Colors represent the origin of the samples, whereas shapes represent farming practices. **C** Relative abundance of fungal genus shown for each replicate from each grape sample. "Other" include genus with relative abundance < 5%. **D** Alpha-diversity comparison between farming practices ($n = 36$). The analysis was performed using Hill-based indices with different diversity orders (q). We selected Richness ($q = 0$), Hill-Shannon ($q = 1$), and Hill-Simpson ($q = 2$) to account for the complexity of such communities. We performed Wilcoxon test to evaluate the differences between organic and conventional practices; asterisks denote the significance levels (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$)

analysis shown in Fig. 1A to better assess the impact of sampling location and farming practices.

Grape processing and grape must fermentations

We collected 3 kg of grapes (*Vitis vinifera* L.) in 5 bunches of grapes from 5 different grapevine plants, making a composite sample from each of the 18 sampling points. Upon arrival at the laboratory, and after ensuring that there was not visible damage or fungal rot on the berries, grapes were pressed under sterile conditions and macerated with the skins and pomace for 2 h. The resulting grape must, after removing the solid parts (skins and pomace), was dispensed into four sterile glass bottles, 200-mL grape must in 250-mL bottles. Immediately after filling the bottles, an initial sample was collected for DNA extraction and sequencing to assess the initial

fungal community composition. Then, these bottles were subjected to fermentation under different conditions: (i) control condition, fermented at 25 °C without supplemental addition of NH_4 or SO_2; (ii) low temperature condition, fermented at 18 °C without supplemental addition of NH_4 or SO_2; (iii) NH_4 condition, supplemented with 300 mg/L diammonium sulfate ($(NH_4)_2SO_4$); and (iv) SO_2 condition, adding 100 mg/L of potassium metabisulfite ($K_2S_2O_5$). Conditions iii (NH_4) and iv (SO_2) were fermented as 25 °C, the same as the control condition. We defined the end of wine fermentations as the point when the weight loss remained consistently below 0.01 g per day for two consecutive days. At this point, we collected samples for DNA extraction and sequencing to assess the final fungal community composition.

Experimental fermentations in synthetic grape must

To finely assess the impact of fermentation conditions on the transcriptional and metabolite patterns of wine yeasts, we replicated the fermentations using synthetic grape must (SGM), prepared as described by Ruiz et al. [19]. To assay the highest possible variety of fermenting yeast communities under experimental conditions, we extracted 2 mL of fermenting grape musts at the tumultuous stage (between 23 and 45% of total sugars consumed, Supplementary Table S1) from each control fermentation assay performed in the natural grape musts (Supplementary Figure S1). The fermenting yeast communities obtained were subsequently transferred as inoculum for the SGM assays. Specifically, the 18 samples collected from control grape must fermentations were frozen at −80 °C, and then after thawing, centrifugation, resuspension, and standardization of the optical density ($OD600_{nm}$) of the resulting pellet, they were used as seed communities to inoculate the experimental fermentations in 100-mL bottles containing 80 mL of SGM, in quadruplicate. These bottles were then subjected to the same fermentative conditions previously defined for grape must fermentations: (i) control, (ii) low temperature, (iii) NH_4, and (iv) SO_2 conditions (Supplementary Figure S1). In these experimental fermentations, we collected samples at the tumultuous stage of fermentations for DNA and RNA extraction for assessing the composition and transcriptional profile of fermenting yeast populations across samples and conditions assayed (see Supplementary Table S1 for details on the sugars consumed and yeast cells concentration at the time of sampling). As previously stated for the spontaneous fermentations, we defined the end of wine fermentations as the point when the weight loss remained consistently below 0.01 g per day for two consecutive days.

Fungal community assessment

We collected samples from grape musts and SGM fermentations for DNA extraction and ITS sequencing (Supplementary Figure S1). Grape musts were sampled immediately after starting the experiment (fresh grape must; $n=72$), and when fermentations stopped, even though sugars remained (fermented grape must; $n=70$), with the aim of studying the diversity patterns in fresh grape musts and the population dynamics of yeast communities from the initial to the final stages of wine fermentations. SGM fermentations were sampled at the tumultuous stage of fermentations ($n=59$), corresponding with the time of sampling for RNA-Seq analysis, to identify the dominant populations driving these experimental fermentations and to validate the latter taxonomic assignment of transcripts from RNA-Seq data.

Amplicon sequencing

We used the DNeasy PowerSoil Pro Kit (Qiagen) for DNA extraction following manufacturer's instructions. We checked DNA quality and quantity using a NanoDrop 2000 (Thermo Fisher Scientific, USA) and Qubit Fluorometer (Thermo Fisher Scientific, USA), respectively. Then, the diversity and composition of the fungal community were determined by amplicon sequencing. DNA sequencing was performed at the "López-Neyra" Institute of Parasitology and Biomedicine. For library preparation, we used ITS2_fITS7 forward (TCCTCCGCTTAT TGATATGC) and ITS4 reverse (GTGARTCATCGAATC TTTG) primers. Libraries were subsequently sequenced on Illumina® MiSeq instrument using 2 × 300 paired-end reads as per the manufacturer's instructions.

We obtained a total of 17098692 good quality sequences, averaging 62863 ± 20796 per sample (further quality analysis at https://github.com/Migueldc1/Wineteractions/). Sequence analysis was performed with *dada2* v1.24.0 [20] R package, after FastQC v0.12.1 [21] and MultiQC v1.18 quality assessment [22]. Dada2 identifies amplicon sequence variants (ASVs), allowing us to distinguish true biological variation from sequencing errors and PCR artifacts, and changes of one nucleotide can be detected [20]. This allows for more accurate and precise identification of unique microbial taxa and quantification of their abundances. Primers were removed using *cutadapt* v4.0 [23] and *Biostrings* v2.66.0 [24] R package to assess their orientation in the reads. After quality assessment, low-quality ends were deleted, and no mismatches were allowed when merging paired reads. Once chimeras were removed, we finally assigned taxonomy to our ITS reads using the UNITE v9.0 database [25].

Yeast community assessment and statistical analysis

We first addressed whether there were differences in alpha diversity among the different geographical origins and between farming practices. To do so, we used Hill-based diversity index in which the importance given to the relative abundance of each ASV can be varied [26]. This importance is determined by the diversity order (q). For instance, when $q = 0$, the relative abundance is not considered, and hence, the value equals the richness. When $q = 1$, each ASV is weighted according to their relative abundance (Hill-Shannon, equivalent to the exponential Shannon index), and when $q = 2$, more weight is given to abundant ASVs (Hill-Simpson, reciprocal Simpson index) [27]. We calculated the Hill-based alpha diversity using the *hillR* v0.5.2 R package [28]. To disentangle differences in alpha diversity caused by different farming practices and geographical origin, we used linear mixed models with the origin as a random factor and the farming practices as fixed factors, using the *nlme* v3.1–160 R package [29].

Differences in community composition across the studied geographical origin and farming practices (β-diversity) were evaluated with the *vegan* v2.6–6.1 R package [30]. We first calculated Bray–Curtis dissimilarity matrices from the ASV table collapsed at the genus level, to consider relative abundance of genera in our samples. Then, we performed multivariate permutational multivariate analysis of variance (PERMANOVA) to assess the effect of origin and farming on community composition and nonmetric multidimensional scaling (NMDS) to compress dimensionality into two dimensional plots. Code for statistical analyses is available at https://github.com/Migueldc1/Wineteractions.

Metabolite profiling

For initial fresh grape musts, we measured pH and concentration of nonvolatile compounds: sugar (glucose and fructose), organic (amino acid related) and inorganic (ammonium) nitrogen, and L-malic acid. Due to the increased metabolite complexity of wines after fermentation [31], we performed a wider metabolite profiling at the end of the fermentations, in both natural grapes and SGM assays, by analyzing the same nonvolatile compounds as in the fresh must, but also including ethanol, acetic acid, L-lactic acid, tartaric acid, citric acid, succinic acid, and glycerol, and the following volatile compounds: ethyl acetate, fusel alcohols (isopropanol, 1-propanol, 2-methyl-propanol, 1-butanol, 2-methyl-1-butanol, 1-hexanol, 2-ethyl-1-hexanol, 2-butanol, and 2-phenylethanol), fusel alcohol acetates (isobutyl acetate, isoamyl acetate, hexyl acetate, and 2-phenylethanol acetate), ethyl esters of fatty acids (EEFA: ethyl butanoate, ethyl octanoate, and ethyl dodecanoate), short-chain fatty acids (SCFA: propionic acid, isobutyric acid, butyric acid, valeric acid, and 2-methylbutanoic acid), and medium-chain fatty acids (MCFA: hexanoic acid, octanoic acid, and decanoic acid). The pH was measured using a pH meter (Crison pH Meter Basic 20, Crison, Spain), and the concentrations of nonvolatile compounds were measured using specialized enzymatic kits and the analyzer Y15 (Biosystems, Spain) following manufacturer's instructions. Gas chromatography-mass spectrometry (GC-FID) was used to measure the concentration of volatile compounds as previously described [32]. Raw data is detailed in Additional File 1.

Metatranscriptomic analysis

RNA extraction and sequencing

We collected SGM samples during the vigorous fermentation stage (see Supplementary Table S1 for details), and each 10-mL sample of the fermenting must was centrifuged at 7000 rpm and 4 °C for 5 min. Biomass was quickly frozen with liquid nitrogen and stored at − 80 °C until RNA extraction. RNA extraction protocol was carried out according to the specifications provided in the Quick-RNA Fungal/Bacterial MicroPrep kit (Zymo Research). RNA quality analysis, library preparation, RNA sequencing, and bioinformatics analyses were carried out at the Bioinformatics and Genomics Unit of The López-Neyra Institute of Parasitology and Biomedicine (IPBLN-CSIC, Granada, Spain). The quality of the RNAs was evaluated using Bioanalyzer (Agilent Technologies), and samples with RNA Integrity Number (RIN) $\geq$ 8.2 were selected for further analysis. Libraries were constructed using TruSeq™ Stranded mRNA sample preparation kit, according to Illumina's instructions. In addition, libraries quality was validated by Qubit dsDNA HS Assay Kit (Thermo Fisher) and 2100 Bioanalyzer (Agilent Technologies). Afterwards, these libraries were sequenced on an Illumina NextSeq High Output, producing 75-bp paired-end reads. We obtained a total of 15,720,876 ± 1,906,845 for further bioinformatic analysis (quality assessment can be found in the "RNA quality" file at https://github.com/migueldc1/Wineteractions/tree/main/Quality_Asessment/RNA).

Bioinformatic analysis

The quality assessment of the raw reads from RNA sequencing was performed with FastQC [21] and MultiQC software [22]. Overrepresented rRNA fragments were removed using sortmeRNA v4.3.6 software [33], retaining a total of 10,281,527 ± 3,618,915 non-rRNA reads for further analysis. The clean meta-transcriptomics sequence reads were used to assess the taxonomic composition of the SGM fermentations using Kraken 2 v2.1.3 [34] and Bracken v2.9 [35] and a custom database

built with the fungal genomes deposited in RefSeq and *Hanseniaspora* genomes from GenBank (including *Hanseniaspora guilliermondii*, *Hanseniaspora opuntiae*, *Hanseniaspora osmophila*, *Hanseniaspora uvarum*, and *Hanseniaspora vineae*). Besides, non-rRNA reads were assembled into contigs/transcripts with Trans-ABySS v2.0.1 [36] using 21, 29, 39, and 59 k-mers. We then quality checked the assemblies with assembly stats v1.0.1 and BUSCO v5.7.1 [37], using the *fungi_odb10* database, to assess how well represented is the functional composition of the active fungal community (all assembly statistics can be found in the "assembly-stats" file at https://github.com/migueldc1/Wineteractions/tree/main/Quality_Asessment/RNA). Functional annotation of transcripts was carried using the eggNOG-mapper v2.1.3 [38] with the DIAMOND aligner [39] against the eggNOG database obtaining ortholog annotation. We used the Burrows-Wheeler aligner with BWA v0.7.17-r1188 software to align the reads to the transcripts [40], as we are not performing the alignment against a reference genome. Finally, we used FeatureCounts v2.0.1 [41] to obtain the number of read counts per ortholog. Differently expressed (DE) orthologs among dominant yeasts were calculated accounting for different fermentative conditions and origins, using DESeq2 package v1.26.0 [42]. Here, we considered a yeast species as dominant when its relative abundance exceeded 90%, and for further differential expression analysis, we focused on samples dominated by *Hanseniaspora*, *Lachancea*, and *Saccharomyces*. An ortholog is considered DE when the false discovery rate (FDR) value is <0.05 and the absolute log2 fold change >1. After that, we used the GOseq v1.48.0 R package [43] to perform Gene Ontology enrichment analysis on the DE orthologs. Specifically, it focuses on the biological process category of GO terms. The resulting enriched GO terms were filtered to include only those with $FDR<0.05$. Code for bioinformatics and statistical analyses is available at https://github.com/Migueldc1/Wineteractions.

Results and discussion

Composition and structure of fungal communities in fresh grape musts

First, we conducted an observational study to identify the variability of yeast communities associated with grape berries across wine appellations, identifying the main factors influencing their composition. Climate is considered a pivotal determinant, influencing grape maturation and quality and, consequently, the associated yeast communities [44, 45]. Additionally, viticultural practices, such as conventional or organic farming managements, play crucial roles, with conventional farming supporting higher grape berry yields due to nutrient inputs and phytosanitary products [46]. Indeed, we observed that sample location, encompassing the environmental differences among localities, was the main factor defining the metabolite composition (Fig. 1A, Supplementary Table S2) and fungal diversity (Fig. 1B, Supplementary Table S3) of grape musts. We also observed differences in grape must physical–chemical and fungal composition between conventionally and organically managed samples, which were especially relevant at the local scale, which is within La Rioja appellation (Fig. 1, Supplementary Table S2, Supplementary Table S3), in agreement with previous findings [47]. These findings underscore the complexity of the grapevine ecosystem and the need for a nuanced understanding of both regional and local factors influencing yeast communities.

Concerning fungal community composition, the genus *Aureobasidium* dominated all communities, often accompanied by *Cladosporium* and *Lachancea* (Fig. 1C), consistent with their prevalence in vineyard ecosystems [15, 48]. We also identified a high prevalence of *Alternaria*, ubiquitous and predominant filamentous fungi in vineyards worldwide [49]. Samples from vineyards under organic management presented higher fungal diversity, considering phylotype richness and dominant phylotypes (Fig. 1D). This finding aligns with previous studies attributing the reduced diversity of grape-associated microorganisms in conventional managed vineyards to the adverse impact of phytosanitary products [47]. In this context, *Aureobasidium* could present higher resistance to these products and greater propensity to dominate the fungal communities of grapes from conventional farming regimes, as evidenced by its higher abundance in conventional vineyards (Wilcoxon, $p<0.001$). The relative abundance of *Cladosporium* and *Lachancea*, on their part, increased in organic vineyards (Wilcoxon, $p<0.001$). As previously described, *Saccharomyces* is rarely found in fresh grape musts [50]; in our case, it was only detected at relatively high abundances in conventional vineyards from Valdepeñas. Understanding the diversity of fungal species associated with grape berries is of great interest to infer the range of expectable fermenting communities across wine appellations.

Manipulating the fermentation environment had no effect on yeast population dynamics

We subjected the obtained fresh grape musts to spontaneous fermentation under four different conditions widely used in wine production (control condition, low temperature, high doses of nitrogen, and sulfite addition; see the " Methods" for further details). At the end of the fermentation (here defined as either when the community ceased sugar consumption or when all sugars were depleted), we characterized the fungal community

composition and the metabolite profile of the resulting wines. The contrasting fermentation conditions applied had no major effect on the yeast dynamics during the process, since we found no distinct trend in the dominance of yeast species across conditions (Supplementary Figure S3A). *Kluyveromyces*, *Lachancea*, and *Saccharomyces* dominated most fermentations, with the initial community variability within sampling location seemingly determining which yeast dominated the fermenting communities. Interestingly, *Kluyveromyces* dominated only in the location where Garnacha, rather than Tempranillo grapes, were sampled. This observation prompts questions and suggests the need for future research to understand how grape variety (i.e., the physicochemical composition of grape musts) influences the structure and dynamics of wine yeast populations.

The sampling location, a multifaceted factor encompassing the different physical–chemical composition of fresh grape musts and the subsequent dominant yeast population, showed the greatest explanatory power on the metabolite profile of the resulting wines, while the fermentation conditions showed a much lower but significant effect on it (Supplementary Figure S3B, Supplementary Table S4). The farming practices applied in the vineyard lost most of the already low explanatory power that it had on the metabolite profile of fresh grape musts (Supplementary Table S2, Supplementary Table S4), raising the question of whether or not the direct effect of farming practice on microbial diversity has any indirect effect on wine quality. Here, it is important to note that different assays showed significantly different fermentation kinetics, with only a fraction of them (those dominated by *Saccharomyces*) achieving complete consumption of fermentable sugars. This discrepancy should be considered when comparing the metabolite profiles of final wines, as the extent of sugar consumption affects the community's ability to produce and metabolize other compounds. Therefore, the interpretation of data focuses on whether distinct communities excelled in producing or consuming specific metabolites (e.g., lactic acid production when *Lachancea* dominates; malic acid consumption when *Kluyveromyces* dominates) or in fermenting sugars (Additional file 1). In addition, the significant differences in the initial chemical composition across fresh grape musts (Supplementary Table S2) prevented us from considering the fungal diversity or the fermentation condition as truly isolated variables in our study.

Metabolite production by different fermentative yeasts

To better assess the associations between the composition and function of fermenting yeast communities, and the impact of different fermentation conditions on it, we moved to a fully controlled experimental setup, which allow us to remove the masking effect of the different physical–chemical environment resulting from using different fresh grape musts. Thus, we recreated our previous spontaneous fermentations, transferring the fermenting yeast communities obtained at the tumultuous stage of the control grape must fermentations as seed communities to inoculate synthetic grape musts (SGM) (Supplementary Figure S1).

We investigated the composition and function of those fermenting yeast communities at the tumultuous stage of experimental SGM fermentations using RNA sequencing (RNA-Seq). To validate the taxonomic identification of transcripts from RNA-Seq data, we also conducted an ITS-amplicon sequencing on the same samples. We observed discrepancies in the abundance of certain taxa between ITS-amplicon sequencing and RNA-seq taxonomic assignment, particularly regarding the detection of *Hanseniaspora* (Supplementary Figure S4). DNA-based amplicon methods are subject to biases such as preferential primer binding (which can lead to biases for or against certain taxa) or an inability to differentiate between living and dead cells [51, 52], while RNA-Seq captures actively expressed genes, revealing the relative abundance of active community members. Based on these advantages and supported by certain metabolite features observed in samples where the dominant presence of *Hanseniaspora* was revealed by RNA-Seq data (e.g., higher concentrations of acetic acid and residual sugars), we considered the information provided by RNA-Seq as the most reliable method to define the taxonomy of active yeast populations and used it to categorize samples for further analysis (Fig. 2).

Our experimental fermentations (control, 18 °C, NH_4, and SO_2) were dominated by a handful of yeast genera frequently detected in wine yeast communities [15, 53], highlighting *Hanseniaspora*, *Lachancea*, and *Saccharomyces* (Fig. 2A). Performing our assays at laboratory scales and without the typical contamination of winery facilities likely reduced our chances of detecting native *Saccharomyces* strains, which are usually absent or present in very low abundances on grape surfaces but dominate the resident communities within wineries. As a result, this may have led to a greater number of fermentations dominated by non-*Saccharomyces* species in our study. Indeed, *Hanseniaspora* and *Lachancea* are frequently found in grape surface and initial phases of wine fermentation, contributing to the complexity of wine aroma [54, 55]. It is remarkable that about half of SO_2 treatments did not even start the fermentation, mostly in samples whose seed community were dominated by *Lachancea* or *Hanseniaspora* (Fig. 2A); so, this experimental condition could not be further assayed for these communities. In this sense, different yeast species exhibit

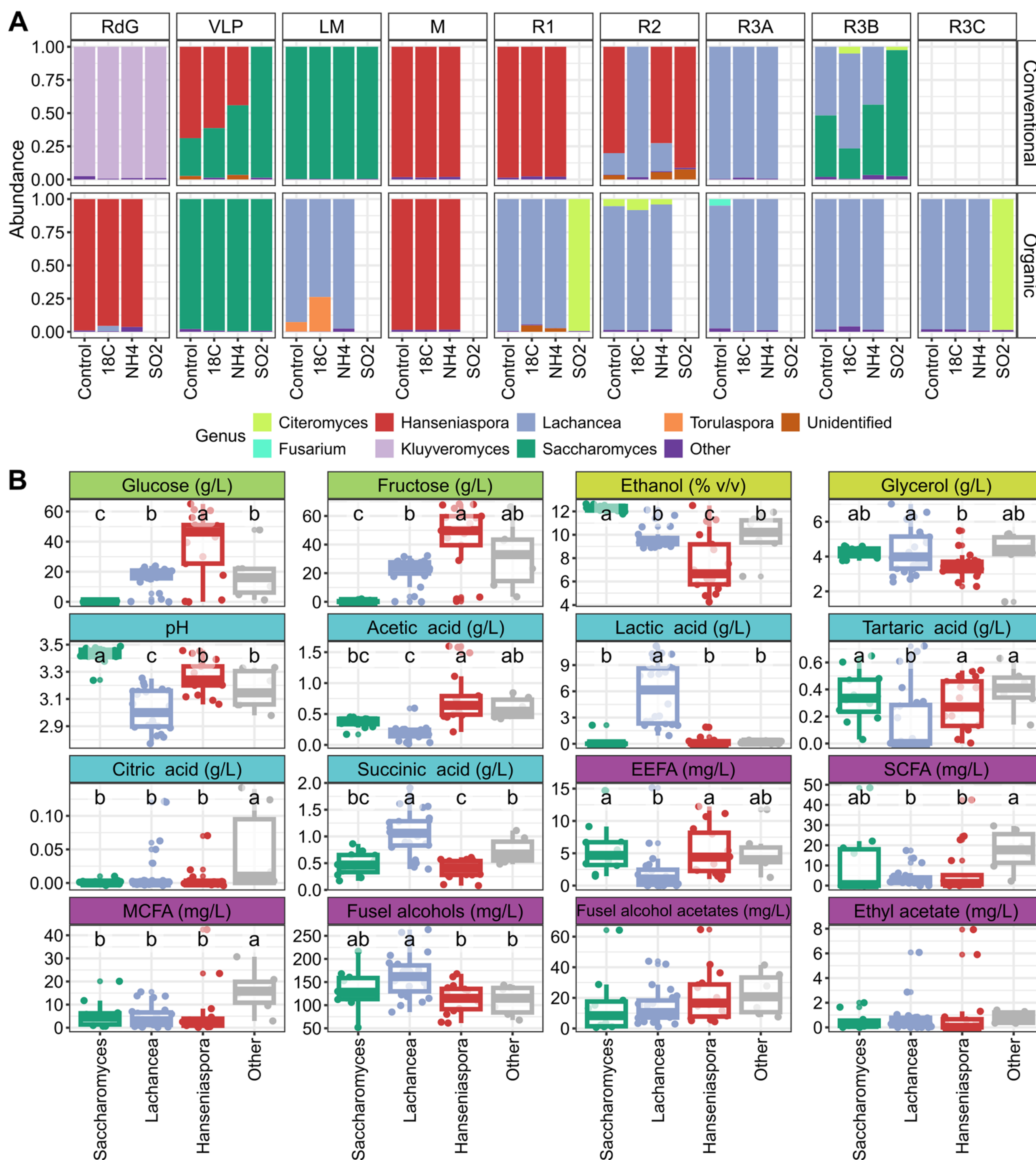

Fig. 2 Yeast community composition of synthetic grape musts during the most active fermentation stage and the resulting metabolite composition associated with the dominant yeast populations. **A** Relative abundance of yeast genus during fermentations assessed via RNA sequencing. "Other" include genus with relative abundance < 2.5%. The absence of bars for the conventional R3C samples indicates that they were inadvertently missed due to unforeseen factors unrelated to the experiment. Meanwhile, missing bars for certain SO_2 condition samples correspond to those that were discarded due to a lack of fermentation activity, indicated by no increase in turbidity (yeast cell proliferation) or weight loss for 5 days following inoculation. **B** Boxplot representing the metabolite composition of fermented synthetic grape must samples. Sugars, glucose, and fructose represent the remaining concentration after fermentation, whereas the rest of metabolites are produced during this process. Vertical axis indicates metabolite concentration. Raw data is detailed in Additional File 1. An ANOVA (analysis of variance) test and LSD (least square difference) test were conducted (a, b, c indicate significance groups, no marks if no significative differences are found). *Saccharomyces*, *Lachancea*, *Hanseniaspora*, and Other presented $n = 10$, $n = 21$, $n = 13$, and $n = 15$, respectively

varying sensitivity to this antimicrobial, commonly used in wines, with *Saccharomyces* usually displaying higher tolerances [56]. In the rest of fermentation conditions, communities dominated by *Hanseniaspora* and *Lachancea* left 47.37% ± 16.98% and 16.65% ± 6.54%, respectively, of residual sugars upon the natural cessation of fermentations (Fig. 2B, Additional File 1). This could be explained by their limited fermentative capacity and ethanol tolerance, preventing them to complete the wine fermentation process [57–59]. Apart from that, we did not observe a conserved metabolite pattern associated with the fermentation conditions imposed across samples (Supplementary Figure S5A, PERMANOVA: $R^2 = 0.062$, $p = 0.421$). We only found a general decrease in the final concentration of tartaric and succinic acids when adding diammonium sulfate as a nitrogen nutrient, NH_4 condition (Supplementary Figure S6, further discussion can be found in Additional File 2).

On the contrary, we found distinctive metabolite profiles associated with the dominant yeast carrying the fermentation process (Supplementary Figure S5B, PERMANOVA: $R^2 = 0.298$, $p < 0.001$). The substantial differences in the sugar consumption of fermentations, and hence ethanol production, in samples dominated by different yeast species would justify a lower concentration of aromatic compounds in fermentations dominated by non-*Saccharomyces* species (Fig. 2B, detailed description of differences in metabolite production at different fermentation conditions can be found in Additional File 2). Specific differences such as the higher L-lactic acid production by *Lachancea* yeasts or the increased concentrations of acetic acid production in presence of *Hanseniaspora* have made them especially relevant in wine sciences [60–62]. When studying the molecular basis of the individual contribution of yeasts to the chemical composition of wines, most works studied individual strains in the form of pure inoculum or in co-inoculation with *S. cerevisiae*, always leading to completed fermentations [63–65]. Here, we aim to investigate the impact of different fermenting yeast populations emerged from wild complex communities, which is closer to the reality found in spontaneous wine fermentations.

Transcriptomic profiles of fermenting yeast communities

We evaluated the transcriptomic profiles of the fermenting communities during the most active phase (tumultuous stage) of wine fermentation. The dominant yeast species also defined the meta-transcriptome of wine fermentations (Fig. 3A, PERMANOVA: $R^2 = 0.498$, $p < 0.001$), while fermentation conditions had a much lesser impact (Supplementary Figure S7, PERMANOVA: $R^2 = 0.037$, $p = 0.097$). Distinctive profiles emerged for samples dominated by *Saccharomyces*, *Lachancea*, or *Hanseniaspora*. We used the transcriptomic profiles of samples dominated by *Saccharomyces* (*Saccharomyces* from now on) as a control for comparative analysis, as it is the reference yeast in wine science [31]. *Hanseniaspora*-dominated samples (from now, *Hanseniaspora*) presented more differentially expressed orthologs (DEO) and higher accumulated log fold change (LFC) than fermentations dominated by *Lachancea* (hereafter referred to as *Lachancea*) (Fig. 3BC), 1019/7023.811 (DEO/LFC) compared to the 705/4094.062 of *Lachancea* (with 382 common DEO). Biological processes significantly enriched in *Hanseniaspora* samples were related with cell cycle and division (Fig. 3D). This altered cell cycle consists mainly of a decrease in the transcription of orthologs related with cell division accompanied by some transcriptomic response to the stressful environment, compared with *Saccharomyces* transcriptome (Supplementary Figure S8), which we attributed to fermentation conditions being suboptimal for community growth. The increasing ethanol conditions could be hindering the growth of this community, without provoking a strong stress response when compared with *Saccharomyces*. *Lachancea*, on its hand, showed significative enrichment in biological processes related with secondary metabolism and the production of alcohols and organic acids. These findings confirm the ability of *Lachancea* to promote the formation of higher alcohol esters, succinic acid, and reduced volatile phenols, and most importantly, L-lactic acid which represent a differential trait for this genus [54, 66], as demonstrated in this work in both natural grape must (Additional file 1) and SGM fermentations (Additional File 1; Fig. 2).

Saccharomyces showed significative differences in transcriptomic profiles under the different fermentation

(See figure on next page.)

Fig. 3 Differential expression analysis of *Hanseniaspora*- and *Lachancea*-dominated samples, with respect to *Saccharomyces* (*Saccharomyces*, *Lachancea*, *Hanseniaspora*, and Other presented $n = 10$, $n = 21$, $n = 13$, and $n = 15$, respectively). **A** Principal component analysis (PCA) representing and showing the different transcriptomic profiles colored by dominant yeast. **B** Venn diagram comparing differently expressed orthologs (DEO) between communities dominated by *Hanseniaspora* or *Lachancea* and *Saccharomyces*. **C** Histogram of absolute fold change (log2) expression. **D** GO enrichment analysis of the differentially expressed orthologs. Raw results from biological enrichment analyses can be found at https://github.com/migueldc1/Wineteractions/blob/main/Data/Meta-transcriptomics/

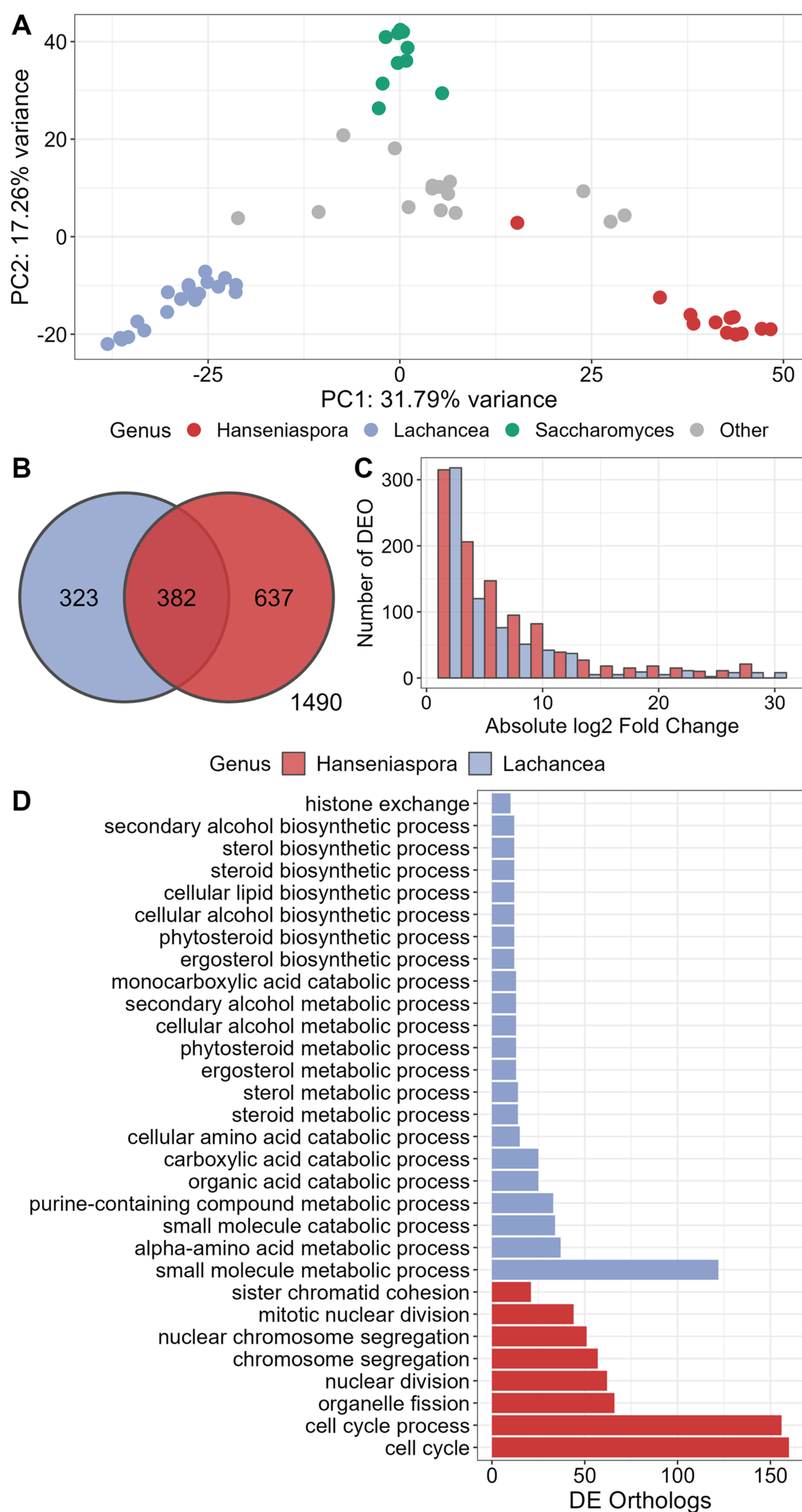

Fig. 3 (See legend on previous page.)

conditions assayed. These conditions were selected because they are part of common practices in wine production [67], so it seems reasonable that winemakers have designed different enological strategies that directly shape the performance of *Saccharomyces*, the main wine yeast. However, in a moment where spontaneous fermentations and the use of complex multispecies consortia are gaining importance in the wine industry, a deeper understanding of the ecological context in which non-*Saccharomyces* species thrive and their associated metabolic traits is needed [19].

Within the *Saccharomyces*-dominated samples, we observed significative effects of fermentative conditions on transcriptional profiles (Fig. 4). Low-temperature conditions caused the highest changes in transcriptomic profiles, presenting 147/329.17 DEOs and absolute accumulated log fold change, compared to 99/212.73 and 18/56.62 of NH_4 and SO_2, respectively (Fig. 4AB). Low temperature mainly affected metabolic processes related to amino acids and sulfur. Cold stress would slow growth and metabolic activity, and *Saccharomyces* is shown to respond by upregulating genes involved in sulfur assimilation and glutathione biosynthesis [68]. The biological enrichment obtained was linked to cold resistance and did not reveal any trend in metabolite production (Fig. 4C). The addition of NH_4 resulted in a significant modulation of the metabolism of organic nitrogen compounds and organic acids (Supplementary Figure S9). This translates in seemingly decreased concentrations of tartaric and succinic acids, as measured at the end of the fermentations. The presence of increased concentrations of ammonia could induce nitrogen catabolite repression, inhibiting the expression of genes related with the transport of amino acids [65]. Interestingly, metabisulfite addition (SO_2 treatments) presented little differences in transcriptomic profiles compared with control conditions, not revealing any enriched biological process. This response emerges as the result of the great tolerance of *S. cerevisiae* wine strains to metabisulfite, one of the best studied hallmarks of domestication in this species [69]. Metabisulfite addition and fermentation at lower temperatures help in avoiding microbial contaminations during wine fermentation [70–73], while the addition of ammonia prevents nitrogen limitation and premature fermentation stops [74, 75]. These practices are designed to ensure the safety and quality of final wines, while they seem to have a limited influence in the aroma of spontaneously fermented wines, even though they impact yeast metabolic activity.

Orthologs responsible for metabolite production

We further aimed to understand the connection between community functional potential and metabolite production by associating the transcriptomic and metabolite profiles of the experimental fermentations. Even though both profiles were assessed at different stages, we argue that at the tumultuous stage, the community is on its activity peak, revealing the most informative transcriptomic trends, whereas cumulative metabolite production was assessed at the end of fermentations in an attempt to exaggerate possible differences. To visualize the associations between ortholog expression and metabolite production, we used bipartite networks (Fig. 5A). Orthologs clustered into modules, each significantly associated with specific metabolites (Additional File 3). Interestingly, the accumulated expression levels within each module were influenced by the dominant yeast species, reflecting distinct transcriptional profiles associated with the diverse metabolite compositions of final wines. For instance, modules 1 and 2 showed higher accumulated expression in samples dominated by *Hanseniaspora* (Fig. 5B). These samples were characterized by higher residual sugars, acetic acid, and, seemingly, fusel alcohol acetates production (Fig. 2). The seemingly suboptimal fermentation conditions for *Hanseniaspora*, latter leading to a rapid fermentation halt, might be linked to increased ester production, such as fusel alcohol acetates [76]. In addition, *Hanseniaspora* species are also shown to produce high concentrations of acetic acid during wine fermentations [77]. Interestingly, although *Hanseniaspora* was not initially detected as a dominant yeast at the end of the natural grape must fermentations (Supplementary Figure S3), samples that later proved to contain large and active populations of *Hanseniaspora* by RNA-Seq (especially M-CONV and M-ORG; Supplementary Figure S4) actually produced high concentrations of acetic acid during those fermentations (Additional File 1). This reinforces our previous observations on the biases of ITS-amplicon sequencing in detecting *Hanseniaspora* and confirms acetic acid production as a specific trait of this yeast. The linear relationship found between the accumulated expression of module 2 and acetic acid production across species (Fig. 5B) suggests that the expression of this subset of orthologs is crucial for acetic acid production during wine fermentation. Module 3 was mainly related with *Saccharomyces*-dominated samples, only correlating with ethanol content, which is highest in these samples (Fig. 2, Fig. 5B). Module 4 was related with *Lachancea*-dominated samples and correlated with the production of L-lactic and succinic acids, as well as fusel alcohols (Fig. 5B). Consistently, the production of L-lactic and succinic acids was also significantly increased in natural grape must fermentations dominated by *Lachancea* (Fig. 2), and the expression of these orthologs may play a crucial role on their release.

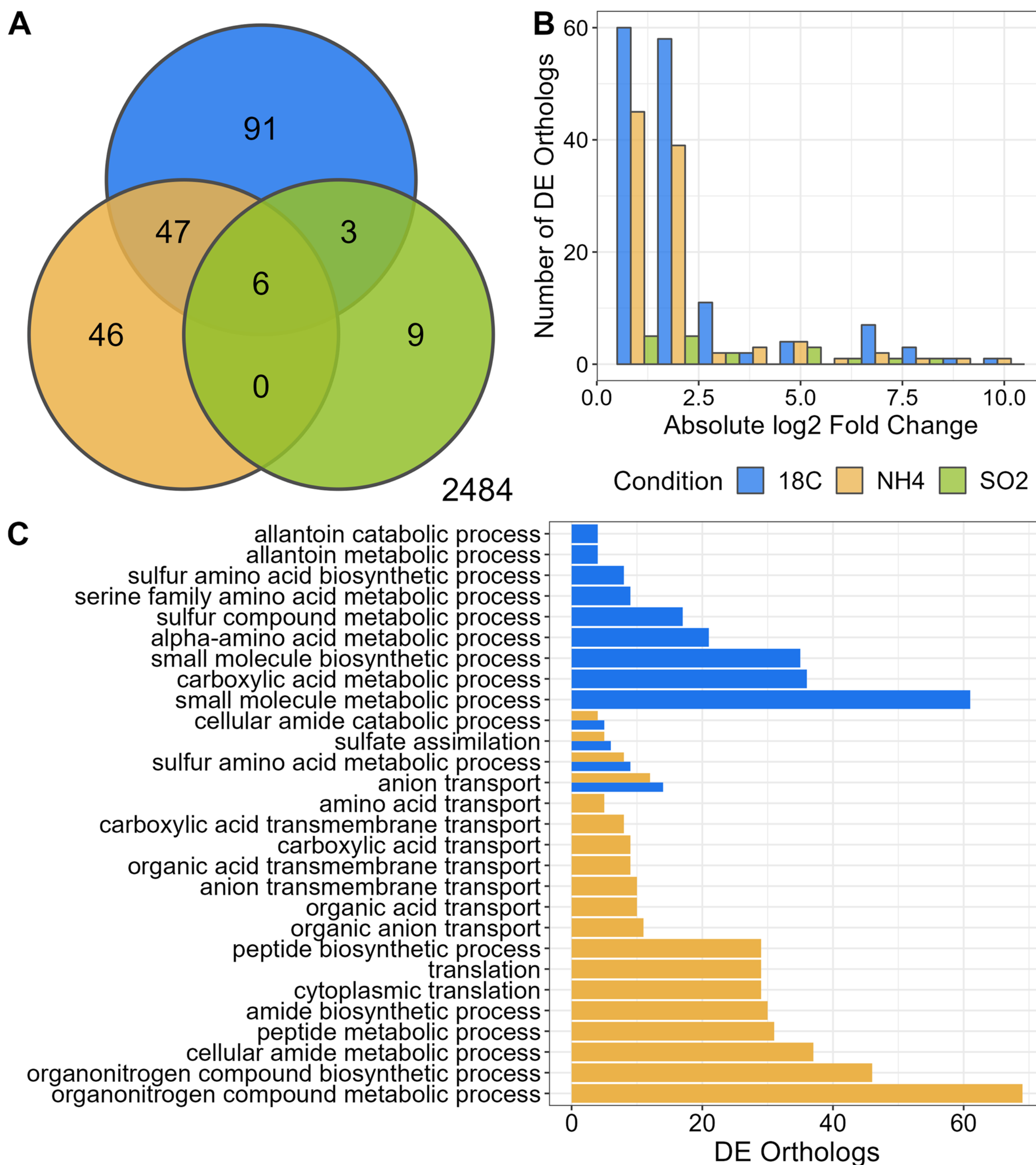

Fig. 4 Differential expression analysis of *Saccharomyces*-dominated samples comparing transcriptional profiles at different fermentative conditions ($n = 2$). **A** Venn diagram comparing differently expressed (DE) orthologs across conditions. **B** Histogram of absolute fold change (log2) expression between the experimental and control groups. **C** GO biological process enrichment analysis of the differentially expressed orthologs. Blue represents samples fermented at low temperature (18 °C), yellow samples with ammonia added (NH_4), and green samples supplemented with metabisulfite (SO_2). Raw results from biological enrichment analyses can be found at https://github.com/migueldc1/Wineteractions/blob/main/Data/Meta-transcriptomics/

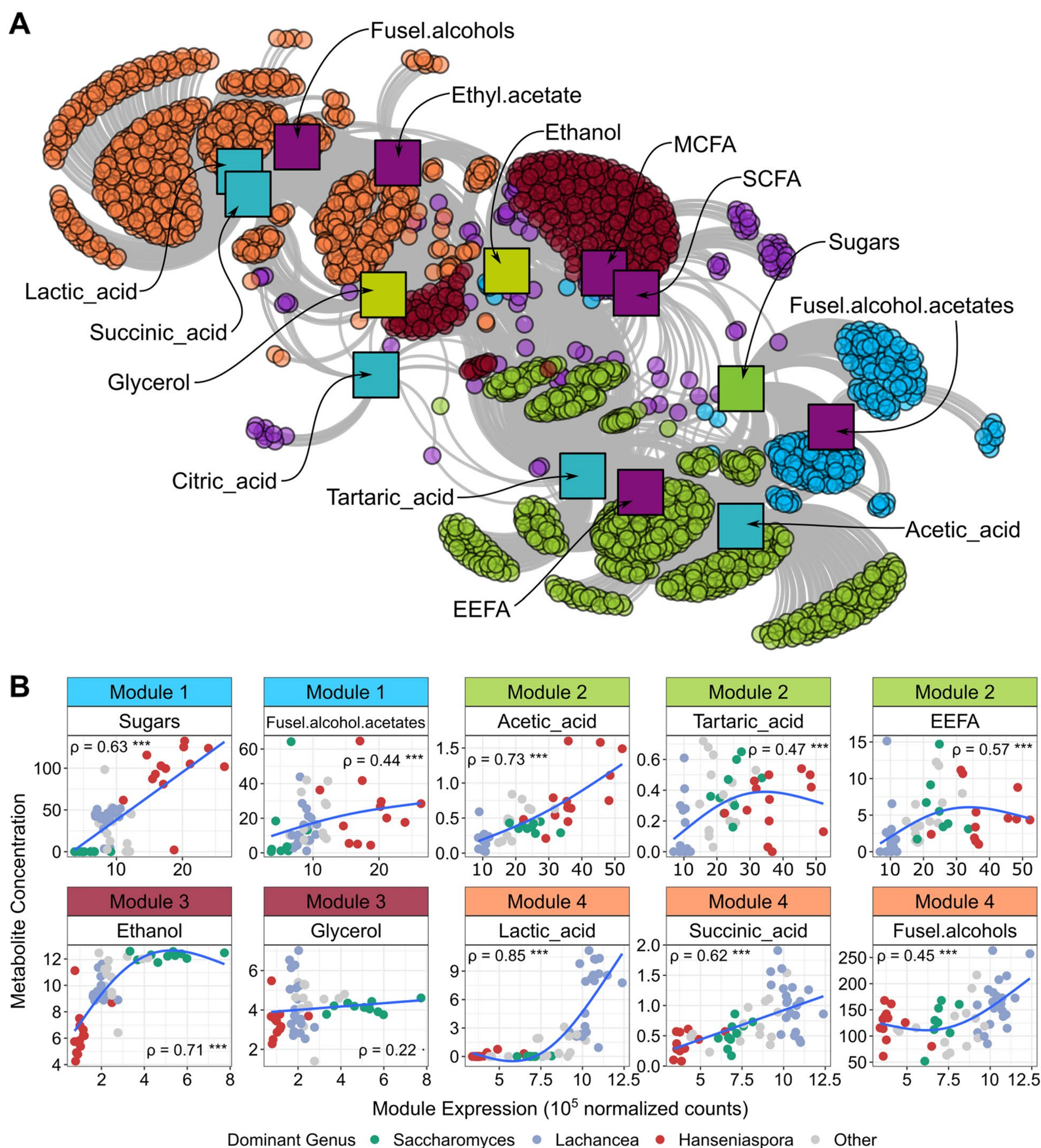

Fig. 5 Relationship between ortholog expression and metabolite production (*Saccharomyces*, *Lachancea*, *Hanseniaspora*, and Other presented $n=10$, $n=21$, $n=13$, and $n=15$, respectively). **A** Bipartite network showing the significant positive correlations between normalized ortholog expression and metabolite concentration. Circles represent orthologs and squares metabolites. Circle colors are indicative of module membership, i.e., orthologs associated with the same metabolites. Square colors represent the metabolite family. **B** Relationships between the total expression of ortholog belonging to a given module and metabolite concentration (raw data detailed in Additional File 3). Spearman's rank correlation coefficient is shown ($p<0.1$, *$p<0.05$, **$p<0.01$, ***$p<0.001$)

We only found significant differences across fermentative conditions in the accumulated expression of module 3 (Supplementary Figure S10). This module is mainly expressed by *Saccharomyces*, which is prevalent in SO_2-treated fermentations, explaining this result and reinforcing the idea that most traditional enological interventions have been developed with the aim of modifying the performance of *S. cerevisiae*. The patterns described here highlight differences in transcriptomic profiles among species, ultimately shaping the unique contribution of each yeast in the metabolic complexity of wine. In this sense, Belda et al. [78] proposed the adoption of synthetic biology to encapsulate the functional complexity of wine communities into a single cell. Our results offer crucial insights into identifying the specific set of orthologs that define the individual contributions of yeast species to the metabolite and sensory profiles of wines. However, further studies on the specific gene sequences and their transcriptional regulation in each species are necessary to address the limitations of working solely at the ortholog level.

Our study evaluating the fermentation process from an ecological point of view aims to escape from the classical enological approach. While this perspective offers valuable insights, it is not without limitations. Our fundamental goal was to assess the relationship between yeast community composition and function during fermentation with the final metabolite composition. Perhaps the major limitation we found is that several communities — those dominated by non-*Saccharomyces* species — were not able to consume all fermentable sugars, classically referred to as unfinished fermentations. In those cases, we focused on identifying specific functions provided by the dominant community members, as they can inform about the molecular mechanisms of yeasts contribution to wine flavor in multispecies fermentations. In this work, we focused on the consistent patterns found relating dominant yeast species with final metabolite production, representing a meaningful baseline to understand the intricate interplay between yeast communities and wine metabolite profile. Specific research questions need to be addressed to further investigate best practical applications of complex yeast communities in the wine industry. Our results contribute to the open debate about the actual possibilities of manipulating spontaneous fermentations through traditional enological interventions and to consider whether the exclusive avenue for crafting customizable wines lies in two alternative approaches: developing new precision methods to manipulate the composition and function of native wine yeast communities [79] or meticulously designing synthetic microbial consortia from the bottom up [19], which takes into account not only the best combination of species but also their inoculum ratio to ensure a perceivable contribution from non-*Saccharomyces* yeasts without compromising fermentation kinetics. In this context, prospective studies are needed to identify yeast species and strains that excel in specific functions such as producing specific wine metabolites, also considering the potential role of less prevalent and minor species isolated from grape musts [32].

Conclusions

In summary, our initial survey of fungal communities in fresh grape musts across diverse Spanish wine appellations revealed significant influences of biogeography and, at a lesser extent, viticultural practices on yeast community composition. We found higher fungal diversity in vineyards under organic management. Fermenting grape must under various contrasting winemaking conditions revealed minimal effects on population dynamics and metabolite production, as the location factor differentiated initial yeast communities and must composition. The transition to an experimental setup involved the inoculation of the obtained yeast communities in synthetic grape must, revealing that variations in metabolite profiles were associated with the dominant fermenting yeast rather than fermentation conditions. Transcriptomic analyses highlighted the different profiles across yeast species, surpassing the influence of fermentation conditions. Distinctive molecular responses were observed in samples dominated by *Saccharomyces*, *Lachancea*, and *Hanseniaspora*, emphasizing their roles in shaping wine metabolite composition. Fermentative conditions did, however, influence the performance of *Saccharomyces*-dominated samples, which are shown to be easily conditioned by oenological standard practices. Furthermore, specific orthologs were linked to metabolite production associated with different yeast-dominated community, offering valuable insights into the functional potential of diverse yeast communities. Our findings contribute to a nuanced understanding of the intricate interplay between yeast communities, environmental conditions, and fermentation dynamics, crucial for advancing both scientific knowledge and practical applications in the wine industry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01930-w.

Additional File 1. Raw data of metabolite composition of fresh (natural) and fermented (natural and SGM) samples.

Additional File 2. Supplementary discussion concerning fermented SGM metabolite composition.

Additional File 3. List of correlated metabolites and orthologs, indicating the ortholog module membership and KEGG annotation

Additional File 4: Supplementary material: Supplementary Figure S1. Schematic representation of the observational study and inoculated laboratory fermentations designs. A) Sampling map of the wine appellations surveyed and the sampling within La Rioja wine appellation. Colors represent different sampling locations: RdG (Ribera del Guadiana), VLP (Valdepeñas), LM (La Mancha), M (Madrid), R1-R3C (La Rioja). Shape indicates conventional (circle) and organic (diamond) farming managements. B) Observational study carried in fresh grape musts (GM). We first pressed grape samples and divided the grape must in bottles, per quadruplicate. Then, each replicate was fermented under different condition until they reached the tumultuous stage, consuming between 23-45% of sugars (Supplementary Table S1). C) We repeated the fermentations in laboratory conditions by inoculating synthetic grape must (SGM) with the fermenting yeast communities obtained from control replicates. The weight loss schematic graphic represents the fermentation stage sampled for numerous analyses, such as amplicon sequencing from DNA extraction, or RNAseq experiment from RNA extraction. In addition, the seed community indicates that control fermentations were used to inoculate the synthetic grape musts. A complete description of the sampling effort and data analysis can be found at https://github.com/migueldc1/Wineteractions/. Supplementary Figure S2. Grape must composition of initial samples. Principal Component Analysis (PCA) representing grape must composition diversity across wine appellations, including conventional RdG samples, i.e., Red Garnacha grapes (n= 4). Raw data detailed in Additional File 1. Supplementary Figure S3. Metabolite and yeast community profiles of fermented grape musts. A) Relative abundance of yeast genera at the final stage of grape must fermentations, as detected by ITS-amplicon sequencing. "Other" include genus with relative abundance < 2.5%. The missing bars indicates the loss of samples during fermentation, which occurred due to unforeseen factors unrelated to the experiment. B) Metabolite profile, including non-volatile and volatile compounds, at the end of the fermentation of fresh grape musts. Principal Component Analysis (PCA) representing metabolite profiles ($n = 4$). Raw data used for metabolite profiling can be found in Additional File 1. Supplementary Figure S4. Comparison of the taxonomic assignation achieved via ITS amplicon sequencing and meta-transcriptomics analysis. Relative abundance of fungal genus shown for each replicate from each grape sample. "Other" include genus with relative abundance. Supplementary Figure S5. Metabolite profile at the end of the fermentation of synthetic grape musts. Principal Component Analysis (PCA) representing metabolite profiles. Samples are colored based on A) fermentative conditions and (Control, 18°C, NH4, and SO2 conditions presented n= 17, $n = 17$, $n = 17$, and $n = 8$, respectively) B) dominant yeast (Saccharomyces, Lachancea, Hanseniaspora, and Other presented $n = 10$, $n = 21$, n= 13, and n = 15, respectively). Raw data detailed in Additional File 1. Supplementary Figure S6. Boxplot representing the metabolite composition of fermented synthetic grape must samples ($n = 18$). Sugars, glucose and fructose, represent the remaining concentration after fermentation, whereas the rest of metabolites are produced during this process. Vertical axis indicates metabolite concentration. An ANOVA test and LSD (Least Square Difference) test were conducted (a–c indicate significance groups). Raw data detailed in Additional File 1. Supplementary Figure S7. Principal Component Analysis (PCA) showing the different transcriptomic profiles colored by fermentative conditions (Control, 18°C, NH4, and SO2 conditions presented $n = 17$, $n = 17$, $n = 17$, and $n = 8$, respectively). Supplementary Figure S8. Transcriptomic analysis of Hanseniaspora dominated samples at the tumultuous stage of fermentation. Upper panel represent the accumulated expression change of selected differentially expressed orthologs (DEO) from enriched biological processes. Raw results from biological enrichment analyses can be found at https://github.com/migueldc1/Wineteractions/blob/main/Data/Meta-transcriptomics/. The lower panel represent the accumulated expression change of DEO related to different responses to stress (detailed in Supplementary Table S5). Expression fold change is calculated against Saccharomyces dominated samples. Supplementary Figure S9. Metabolite composition of fermented synthetic grape musts dominated by Saccharomyces ($n = 2$). Glucose and fructose represent the remaining concentration after fermentation, whereas the rest of metabolites are produced during this process. Vertical axis indicates metabolite concentration. Raw data detailed in Additional File 1. Supplementary Figure S10. Accumulated expression of the modules formed by orthologs in Figure 5 (Control, 18°C, NH4, and SO2 conditions presented $n = 17$, $n = 17$, $n = 17$, and $n = 8$, respectively). Analysis of Variance (ANOVA) test was carried out to test for different expression levels among fermentative conditions, and further Tukey post hoc test. (a-b indicate significance levels). Supplementary Table S1. Proportion of sugars consumed (%) and yeast cells concentration (CFU/mL; showed in brackets) in the sampling of yeasts communities at the tumultuous fermentation stage. Samples from Grape musts (only from the control condition assays) were used for collecting the seed communities lately used to inoculate the experimental fermentations in SGM. Samples collected from SGM were collected to extract DNA and RNA for ITS-amplicon sequencing and RNA-Seq analysis, respectively. Missing values correspond to samples that were unable to initiate the experimental fermentations in SGM. Supplementary Table S2. PERMANOVA results of fresh grape must physical-chemical composition comparing different origins and farming managements across and within wine appellations (WA). Supplementary Table S3. PERMANOVA results comparing initial fungal communities, present in fresh grape must, comparing different origins and farming managements across and within wine appellations (WA). Supplementary Table S4. PERMANOVA results comparing fermented grape must composition comparing across different origins, farming managements across and within wine appellations and fermentation conditions. Supplementary Table S5. Differently expressed orthologs related with stress response.

Acknowledgements

We thank the technical teams of the following wine growing companies for providing access to their vineyards and allowing the collection grape samples: Pago Los Balancines, Bodega de Las Estrellas, Bodega Dehesa de Luna, Bodega Bernabeleva, Bodega Jesús Díaz e Hijos, Bodegas Pernod Ricard.

Authors' contributions

MdC: Conceptualization; Data curation; software; formal analysis; investigation; methodology; writing – original draft. JR: Conceptualization; investigation; visualization; methodology; writing – original draft. BB-D: Validation; investigation; methodology; writing – original draft. JV: Conceptualization; Investigation; methodology. ST: Investigation; methodology. SI-G: Investigation; methodology. NR: Investigation; methodology. CRdV: Investigation; methodology. JG: Investigation; methodology. FZ: Investigation; methodology. AB: Investigation; methodology. LCT-C: Investigation; methodology. E-AL: Investigation; methodology. AS: Resources; investigation. IB: Conceptualization; resources; supervision; funding acquisition; validation; investigation; visualization; writing – original draft; project administration. All authors read and approved the final manuscript.

Funding

This work was supported by the grants PID2019-105834GA-I00 (acronym Wineteractions) and PID2022-138343NB-I00 (acronym INDUSYNCON) funded by MICIU/AEI/ 10.13039/501100011033 and by ERDF/EU. J. R. was the recipient of a postdoctoral contract from the Complutense University of Madrid (CT17/17-CT18/17). B. B.-D. was the recipient of a predoctoral contract from the regional government of Madrid (09-PIN1-00009.8/2022). J. V. was the recipient of a predoctoral grant from the Complutense University of Madrid (CT58/21-CT59/21). S. I.-G was the recipient of a FPU predoctoral grant (FPU21/06830) funded by MICIU. E.-A. L. was the recipient of a postdoctoral fellowship from the regional Andalusian government (POSTDOC_20 _00541). L. C. T.-C. was the recipient of a postdoctoral fellowship from the regional Andalusian government (POSTDOC_21 _00394).

Data availability

All the information supporting the conclusions of this article is included in the manuscript or supporting files (Supplementary material; Additional File 1; Additional File 2; Additional file 3). Raw sequencing data from this study are available in the NCBI Sequence Read Archive (SRA) repository: ITS PRJNA1047054 (https://www.ncbi.nlm.nih.gov/bioproject/1047054), RNA-seq PRJNA1047320 (http://www.ncbi.nlm.nih.gov/bioproject/1047320). All the code used during this work (https://github.com/Migueldc1/Wineteractions/

tree/main/Scripts), as well as the quality assessment of the raw fastq files (https://github.com/Migueldc1/Wineteractions/tree/main/Quality_Asessment) are available at github (https://github.com/Migueldc1/Wineteractions).

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 December 2023 Accepted: 11 September 2024
Published online: 15 October 2024

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