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Tailoring yeast and bacterial consortia to modulate wine fermentation profiles

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ABSTRACT

Wine results from complex interactions among microorganisms during fermentation, which produce a variety of metabolites, including secondary metabolites derived from aromatic amino acids (AADC) that influence wine quality, stability and bioactivity. Both yeast species and lactic acid bacteria (LAB) can contribute to the formation of these aromatic compounds, highlighting the need to study winemaking as a network of microbial interactions that shape wine's metabolic and analytical profiles. This study aimed to select yeast and LAB strains based on their potential to produce specific AADC compounds, such as tyrosol (TyrOH) and hydroxytyrosol (HT), and to design microbial consortia to enhance their production in wine.

Individual screenings of multiple strains of *S. cerevisiae,* non-*Saccharomyces* (non-*Sac*) and LAB were carried out in synthetic must enriched fivefold with aromatic amino acids, quantifying TyrOH and HT production. Two strains each of *S. cerevisiae*, *Zygosaccharomyces rouxii and Oenococcus oeni* were selected for their higher AADC production and tested in mixed fermentation strategies combining these microorganisms. Fermentation approaches included single fermentations or co-inoculation of non-*Sac* and LAB strains, followed by sequential inoculation of *S. cerevisiae*. Organic acids, microbial population dynamics and AADC production were monitored across different proposed consortia.

The combination of *S. cerevisiae* Lalvin CLOS and *Z. rouxii* CW96 produced the highest concentrations of HT. All co-inoculations with LAB completed malolactic fermentations efficiently, without increasing acetic acid levels. These results highlight the potential of controlled multi-species fermentations to modulate wine composition and support the development of microbial consortia aimed at improving functional and metabolic profiles.

1. Introduction

Wine is the result of a complex microbial ecosystem, shaped by interactions between yeast and bacteria during alcoholic and malolactic fermentation. Alcoholic fermentation (AF) is mainly carried out by diverse *Saccharomyces cerevisiae* strains. Malolactic fermentation (MLF) is carried out by lactic acid bacteria (LAB) and can occur before, during or after AF (Fleet, 2008). For centuries, spontaneous must fermentations relied on the indigenous microbiota present on grapes and in the winery environment. However, these fermentations were often unpredictable, and microbial-related issues, such as undesirable aromas and stuck or sluggish fermentations, were commonly attributed to the presence of non-*Saccharomyces* (non-*Sac*) yeasts (Ciani et al., 2010; Du Toit & Pretorius, 2000; Jolly et al., 2014). As a result, starter cultures of *S. cerevisiae* have been widely adopted since the 1970s for their predictable and reliable results, albeit at the expense of more complex aromatic profiles due to reduced microbial diversity.

Subsequent research has demonstrated that the negative perception of non-*Sac* yeast largely reflected the behavior of certain strains in uncontrolled spontaneous fermentations, whereas many non-*Sac* species and strains can be beneficial when used alongside *S. cerevisiae* under controlled conditions (Ciani & Maccarelli, 1997; Del Fresno et al., 2017;

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Padilla et al., 2016; Petruzzi et al., 2017). Although some non-*Sac* yeast are unable to complete AF on their own, they can contribute complex aromas and metabolites that influence wine sensory profiles (Ciani et al., 2010; Escribano et al., 2017; Jolly et al., 2014; Petruzzi et al., 2017). MLF is a secondary fermentation conducted by LAB strains that are tolerant to ethanol and low pH. *Oenococcus oeni* is the predominant bacterial species involved in MLF, but species of *Lactiplantibacillus, Leuconostoc and Pediococcus* can also be present. During MLF, LAB produce secondary metabolites that modulate microbial stability and enhance aroma and wine flavor (Bartowsky, 2005; Liu, 2002; Lonvaud-Funel, 1999).

Controlled mixed fermentations involving *S. cerevisiae* spp., non-*Sac* yeast and LAB have been extensively studied over the past few decades due to their attractive potential to modify the production of analytical compounds, reduce ethanol content and improve overall wine quality, stability and complexity (Du Plessis et al., 2017; Ferrando et al., 2020; Russo et al., 2020; Tristezza et al., 2016). Microbial interactions occurring during AF and MLF are increasingly recognized as species and strain-dependent, and the inoculation strategy adopted can significantly influence fermentation dynamics and final wine composition. Much research has focused on interactions between *S. cerevisiae* and non-*Sac* yeasts aimed at modulating AF (Andorrà et al., 2012; Bordet et al., 2020; Ciani et al., 2016; Nisiotou et al., 2018; Taillandier et al., 2014; Wang et al., 2022). Studies evaluating yeast-LAB interactions have also expanded, with many focusing on how *S. cerevisiae*-mediated AF conditions influence MLF (Izquierdo-Cañas et al., 2015; Jussier et al., 2006; Muñoz et al., 2014). More recently, studies have been exploring the interactions between non-*Sac* and LAB, with or without *S. cerevisiae*, revealing additional complexity in fermentation outcomes (Du Plessis et al., 2019; Nardi et al., 2019; Russo et al., 2020; Vicente et al., 2024). Microbial interactions can affect gene expression, enzymatic activity and nutrient competition, and may involve inhibitory mechanisms such as antimicrobial compound production or cell-to-cell contact. These interactions ultimately influence metabolite formation and shape the chemical and sensory profiles of wine (Balmaseda et al., 2018; Bartle et al., 2019; De Gioia et al., 2022; Fleet, 2003; Liu, Arneborg, et al., 2017).

Aromatic amino acids serve as precursors to important secondary metabolites during AF, produced as by-products of yeast metabolism. These include aromatic alcohols such as 2-phenylethanol, tyrosol (TyrOH) and tryptophol, as well as indolic compounds like melatonin and serotonin. These amino acid-derived compounds (AADC) are being studied for their potential as bioactive roles (Fernández-Mar et al., 2012). TyrOH and its derivative, hydroxytyrosol (HT), are present in wine, although HT typically occurs at low concentrations (Álvarez-Fernández et al., 2018; Di Tommaso et al., 1998; Rebollo-Romero et al., 2020). HT has been shown to influence wine aroma and sensory attributes. In red wines, it can enhance initial fruit intensity and contribute additional odor notes described as fruity, chamomile, popcorn-like and toasty notes (Raposo, Ruiz-Moreno, Garde-Cerdán, Puertas, Moreno-Rojas, Gonzalo-Diago, et al., 2016). In white wines, HT increases color intensity and shifts the aromatic profile by reducing varietal thiols and promoting secondary aromas such as chamomile, toasty and dried fruit notes (Raposo, Ruiz-Moreno, Garde-Cerdán, Puertas, Moreno-Rojas, Zafrilla, et al., 2016). Furthermore, HT presents some health promoting properties such as; antioxidant activity as a free radical scavenger, as well as its cardioprotective effects and antimicrobial properties against pathogenic bacteria harmful to humans (Cordente et al., 2019; Fernández-Mar et al., 2012; Mas et al., 2014).

In recent decades, significant progress has been achieved in the study of controlled mixed fermentations and their positive effects on the analytical and aromatic profile of wines. However, limited attention has been given to how interactions between different yeast and bacterial strains influence the production of bioactive compounds of interest. In this context, the aim of the present study is to select various strains of *S. cerevisiae,* non-*Sac* yeast and LAB based on their ability to produce AADC such as TyrOH and HT, in order to design a microbial consortium that enhances the production of these AADC in mixed sequential fermentations.

2. Materials and methods

2.1. Initial screening

2.1.1. Strains and media

2.1.1.1. Wine yeast. Five strains of *S. cerevisiae* were used for this study: Lalvin CLOS (ScCLOS, Lallemand, Spain), QA23 (ScQA23, Lallemand, Canada), AWRI 4235, AWRI 1742 (ScAWRI, Adelaide, Australia), and Red Fruit (ScRF, Enartis, Spain), along with forty-nine non-*Sac* isolates from a collection of the Universitat Rovira i Virgili (Table S1). Yeast strains were taken from stocks preserved at −80 °C in glycerol and grown on YPD plates (2% (*w/v*) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) agar) (Panreac Quimica SLU, Barcelona, Spain) for 48–72 h at 28 °C with shaking at 120 rpm in an orbital shaker. These pure cultures were used as pre-cultures for fermentation. Isolated colonies were cultured for 48 h in 25 mL of YPD at 28 °C with shaking at 120 rpm, then transferred to 50 mL of fresh minimal medium (1× Yeast Nitrogen Base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) glucose and 3.5 mM (NH_4) SO_4) (Panreac Quimica)) at 28 °C with shaking at 120 rpm. Non-*Sac* yeasts show reduced and strain-dependent performance when $(NH_4)SO_4$ is the only nitrogen source compared with *S. cerevisiae* (Roca-Mesa et al., 2020); therefore, non-*Sac* yeast were incubated in the minimal medium for 24 h, versus 72 h for *S. cerevisiae* in order to eliminate all intracellular nitrogen reserves.

2.1.1.2. Lactic acid bacteria (LAB). Bacterial strains used for MLF were selected from various collections at the Universitat Rovira i Virgili. *Oenococcus oeni* strains: MF1, MF2, MF6, CH11, 3P2, 2 T2 217T), *Levilactobacillus brevis* strains: 4111, 4246, *Lentilactobacillus hilgardii* 264, *Pediococcus pentosaceus* 4208 and *Lactiplantibacillus plantarum* 10w-11. Bacterial strains were taken from stocks preserved at −80 °C in glycerol and grown on modified MRS (Man-Rogosa-Sharp) plates (Panreac Quimica) (Margalef-Català et al., 2017). To obtain pre-cultures, isolated colonies were picked and grown in liquid MRS at 27 °C in a 10% CO_2 atmosphere for 3–5 days.

2.1.2. Small scale fermentations

AFs were performed in a modified synthetic must, as described by González et al. (2018) containing a five-fold increase (5×) in the concentrations of aromatic amino acids (tyrosine, tryptophan and phenylalanine) compared to the standard levels in regular must (1×) (Beltran et al., 2004). While the concentrations of aromatic amino acids were increased, the yeast assimilable nitrogen (YAN) was maintained at 300 mg/L by slightly adjusting the concentrations of the remaining amino acids (Table S2).

MLFs were carried out in the synthetic must previously mentioned and in a wine-like medium (WLM), both supplemented with 1 mM of TyrOH. The WLM, based of Bordas et al. (2015) with some modifications, contained (per liter): 2 g *L*-tartaric acid, 2.5 g L-malic acid, 0.28 g sodium acetate, 0.5 g citric acid, 1.25 g peptone, 1.25 casein amino acids, 0.6 g KH_2PO_4, 0.13 g $MgSO_4 \bullet 7H_2O$, 0.03 g $MnSO_4 \bullet H_2O$, 0.13 g $CaCl_2$, 0.45 g KCl, 5 mL glycerol, 0.4 g fructose, 0.25 g trehalose, 0.5 g L-cysteine-HCl, 12% (*v*/v) ethanol, with a final pH of 3.4. MLFs were incubated at 20 °C in a 10% (v/v) CO_2 incubator for 14 days.

For AF screenings, yeast strains were inoculated to an initial optical density (OD_{600nm}) of 0.2. For MLF screenings, precultures grown in MRS were inoculated in WLM at 0.6–1 OD_{600nm} units to achieve an initial concentration of 5% (v/v) (1×10^7 cell/mL).

AF screenings were monitored by daily weight loss measurements.

Since non-*Sac* strains typically cannot complete fermentations, a stopping point was set on day seven. MLF screenings were monitored by malic acid consumption using a Y15 multi-analyzer and the corresponding enzymatic kits (Biosystems SA, Barcelona, Spain).

To evaluate TyrOH and HT production, supernatant samples were collected and analyzed using ultra-high-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-QqQ/MS; Agilent 6490 Series) (see 'AADC analysis' below for details). Individual *S. cerevisiae* screenings were sampled once alcoholic fermentation was completed, while non-*Sac* screenings were sampled on days 2 (as inoculation of *S. cerevisiae* strains would be carried out after 48 h in sequential fermentations) and 7 (established end point). MLFs were sampled on days 1, 4, 7 and 14.

2.2. Mixed sequential fermentations

Based on the initial screening results, the *S. cerevisiae*, non-*Sac*, and LAB strains that produced the highest levels of TyrOH and HT were selected. These microorganisms were then used to form different consortia by combining various strains. These microorganisms were organized into different combinations for sequential inoculation in mixed fermentations, with the aim of evaluating which combination(s) could enhance the production of AADC, potentially due to microbial interactions.

Consortium labels (Table 1) encode the inoculation strategy using two operators. A "+" denotes co-inoculation (strains added simultaneously at time zero (t0)). A ";" denotes sequential inoculation, read left-to-right as successive inoculation events (strains to the left are added first; those to the right are added after 48 h). For combinations of more than two strains, "+" groups strains inoculated together within an event, and ";" separates distinct events.

2.2.1. Fermentation conditions and sampling

Sequential mixed fermentations performed in the synthetic must previously mentioned were initially inoculated with a non-*Sac* strain and/or a LAB strain, followed by the inoculation of a *S. cerevisiae* strain 48 h later. This design was replicated for all consortium combinations. The inoculation concentrations were 2×10^6 cells/mL for both *S. cerevisiae* and non-*Sac* strains, and 1×10^7 cells/mL for LAB strains.

Fermentations were monitored daily by measuring must density with an electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, Spain)), to determine the average and peak rates of sugar consumption as well as the area under the curve (AUC) (see section below). Malolactic fermentations were monitored daily by measuring malic acid consumption using a Y-15 multi-analyzer and the corresponding enzymatic kits (BioSystems).

Samples were taken at specific fermentation stages: on days 1 and 2, at mid-fermentation (MF), defined as the point when must density reached approximately 1040 g/L, and at advanced fermentation (AdF), when must density was around 1020 g/L. Once density dropped below 1000 g/L, sugar concentration was monitored using a Y-15 multi-analyzer with the corresponding enzymatic kits (BioSystems). The endpoint fermentation (EF) was defined as the point when sugar concentration (glucose and fructose) fell below 2 g/L.

To evaluate TyrOH and HT production in the different microbial consortia combinations, samples were taken at two AF stages (MF and EF). HT production from MLFs performed sequentially from wines of AF without LAB were also evaluated, samples were taken at endpoint. The samples were then analyzed using UHPLC-HRMS method described in AADC analysis section.

2.2.2. Derived kinetic parameters

Daily measurements of must density were used to quantify fermentation kinetics. From the density-time profiles, several kinetic descriptors were calculated for each replicate:

- Average rate (g/L/day): the mean rate of density decrease from the start of fermentation until the must first reached 1000 g/L.
- Peak rate (g/L/day): the maximum single-day decrease in density observed between consecutive measurements before reaching 1000 g/L.
- AUC (t_0-t_x) (g/L · day): the area under the density-time curve (trapezoidal rule) calculated over a fixed fermentation window defined within each experimental panel. The cutoff of this window corresponded to the average time at which single fermentations of the *S. cerevisiae* strains reached the 1000 g/L density threshold. A larger AUC indicates slower fermentation, as density remains higher for a longer period.
- Malic-acid consumption rate (g/L): estimated as the slope of a linear regression of malic-acid concentration over time. Absolute values were used so that higher positive values correspond to faster consumption.

2.2.3. Population dynamics

Population dynamics in mixed fermentations were monitored using two methods: viable colony counts by plating and cell quantification by real-time quantitative PCR (qPCR).

For viable colony counting, colony-forming units per milliliter (CFU/mL) were determined by plating samples collected at different fermentation time points (24 h, 48 h, MF, AdF and EF) onto three solid media: YPD for total yeast population, WLN (Wallerstein Laboratory Nutrient) (Difco Laboratories, Detroit, MI, USA) for morphological differentiation between *Saccharomyces* and non-*Sac* yeasts, and modified MRS for LAB growth, supplemented with 100 mg/L of nystatin (Panreac Quimica) to inhibit fungal growth.

For cell quantification using qPCR, DNA extractions were performed on 1 mL-samples collected from the same fermentation stages as in the plating method, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, as described by Andorrà et al. (2008), and strain-specific primers were used to quantify each microorganism. For *S. cerevisiae* quantification was performed using primers published by Hierro et al. (2006). For *Zygosaccharomyces rouxii* quantification, primers targeting the D1/D2 region of the 26S RNA were designed using the Primer-BLAST tool available on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). After confirming primer specificity for *Zygosaccharomyces* and the absence of amplification with *Saccharomyces DNA,* the following primer pair was selected: ZrF1 (5'-GATTCTGGGACTGGCCCT-3′) and ZrR1 (3'-TCGCTATCGGTCTCTCGC-5′). The amplification program for yeast consisted of an initial denaturation stage at 95 °C for 1 min, followed by

Table 1
Microbial consortia layout, with all combinations of the six selected microorganisms (*S. cerevisiae* (ScRF, ScCLOS), *Z. rouxii* (Zr95, Zr96) and *O. oeni* (OoMF6, Oo3P2) strains), including their respective two strain consortia combinations and single *S. cerevisiae* fermentation. Consortium labels encode the inoculation strategy using two operators: a "+" indicates co-inoculation, where strains are added simultaneously at time zero (t0)), and a ";" indicates sequential inoculation, read left-to-right as successive inoculation events (strains to the left are added first; those to the right are added after 48 h). For combinations of more than two strains, "+" groups strains inoculated together within the same event, while ";" separates distinct inoculation events.

Zr + Oo;Sc consortia	Zr;Sc consortia	Oo;Sc consortia	Single Sc
Zr95 + OoMF6;ScRF	*Zr95;ScRF*	*OoMF6;ScRF*	*ScRF*
Zr95 + Oo3P2;ScRF	*Zr95;ScRF*	*Oo3P2;ScRF*	*ScRF*
Zr96 + OoMF6;ScRF	*Zr96;ScRF*	*OoMF6;ScRF*	*ScRF*
Zr96 + Oo3P2;ScRF	*Zr96;ScRF*	*Oo3P2;ScRF*	*ScRF*
Zr95 + OoMF6;ScCLOS	*Zr95;ScCLOS*	*OoMF6;ScCLOS*	*ScCLOS*
Zr95 + Oo3P2;ScCLOS	*Zr95;ScCLOS*	*Oo3P2;ScCLOS*	*ScCLOS*
Zr96 + OoMF6;ScCLOS	*Zr96;ScCLOS*	*OoMF6;ScCLOS*	*ScCLOS*
Zr96 + Oo3P2;ScCLOS	*Zr96;ScCLOS*	*Oo3P2;ScCLOS*	*ScCLOS*

Zr95: Z.rouxii CW95; Zr96: Z. rouxii CW96; OoMF6: *O. oeni* MF6; Oo3P2: *O. oeni* 3P2; ScRF: *S. cerevisiae* Red Fruit; ScCLOS: *S. cerevisiae* Lalvin CLOS.

40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 35 s. For *O. oeni* quantification, primers published by Neeley et al. (2005) were used. The amplification program was identical to that used for yeast, with an additional extension step at 72 °C for 30s. Cycle threshold (Ct) values were obtained using the Relative Quantification application (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). All qPCR reactions were carried out in triplicate in a final volume of 20 μL using TB Green™ Premix Ex TaqTM II (Takara Bio Inc., Kusatsu, Japan), following the manufacturer's protocol, on a QuantStudio™ real-time PCR instrument (Applied Biosystems). For each species, standard curves were generated by plotting the average Ct values obtained from tenfold serial dilutions of DNA from pure cultures, ranging from 10^8 to 10^2 cells/mL for yeast and from 10^9 to 10^2 cells/mL for LAB, against the logarithm of the cell concentration (cells/mL).

2.2.4. Metabolite analysis

2.2.4.1. Chemical parameters of fermentation. At the AF endpoint, samples (1 mL per replicate) were centrifuged at 13,000 rpm for 2 min to obtain the supernatant. Concentrations of ethanol, glycerol, organic acids (malic and lactic acid), and residual sugars (glucose and fructose) were quantified following the protocol described by Quirós et al. (2010), using an Agilent 1100 HPLC (Agilent Technologies, Germany). The HPLC was coupled with a Hi-Plex H column (300 mm × 7.7 mm) installed in a 1260 MCT (Infinity II Multicolumn Thermostat). The column was maintained at 60 °C for 30 min, with a mobile phase of 5 mM H_2SO_4 flowing at 0.6 mL/min. The chromatograph was equipped with two detectors: a Multi-Wavelength detector (MWD, G1365B) and refractive index detector (RID, 1260 Infinity II) (Agilent Technologies). Calibration was performed using known concentrations of pure standards. Acetic and malic acid concentrations were determined using a Y-15 multi-analyzer with the corresponding enzymatic kits (BioSystems).

2.2.4.2. Aromatic amino acid derived compounds analysis. For the initial screening, samples from the small-scale fermentation (AF and MLF) were centrifuged; 800 μL of each sample was mixed with 200 μL of methanol (≥99.7%, Sigma-Aldrich, USA), filtered (0.22 μm), sent to Centre of Omics Studies (Universitat Rovira i Virgili-Eurecat, Reus, Spain) for TyrOH and HT quantification. Samples were analyzed using a UHPLC-MS/MS (1290 Series UHPLC coupled to a 6490 QqQ/MS, Agilent Technologies, Santa Clara, CA, USA). For HT, separation was performed on a Zorbax SB-Aq column (Agilent Technologies). For TyrOH, separation was carried out on an Acquity BEH C18 column (Waters Corporation, MA, USA). Samples were diluted (1,10 *v*/v) with 0.1% ammonia in water prior to analysis. The method followed that of Mazzotti et al. (2012). Analyses were performed in MRM mode under ESI negative ionization. All parameters followed the laboratory's validated protocol.

For mixed sequential fermentations, samples from AF and MLF were centrifuged to obtain the supernatant, and analyzed at the University of Sevilla. TyrOH and HT were quantified using the methodology described by Gonzalez-Ramirez et al. (2024), which is originally based on Álvarez-Fernández et al. (2018). Samples were analyzed using an Acquity UPLC system (Waters Corporation) coupled to a Xevo TQ triple quadrupole mass spectrometer (Waters Corporation), with chromatographic separation carried out on an Acquity UPLC BEH C18 column Detection was performed in multiple reaction monitoring (MRM) mode under negative and positive electrospray ionization, following the laboratory's validated protocol.

2.3. Statistical analysis

All experiments were performed in biological triplicate unless otherwise noted, and results are expressed as mean ± standard deviation (SD). Analyses and visualizations were conducted in R (R Core Team, 2024) using RStudio (Posit Team, 2024). Statistical analyses were selected based on data characteristics (sample size, variance homogeneity and distributional assumptions). Statistical significance was defined as Benjamini-Hochberg false discovery rate (FDR)-adjusted $p < 0.05$. unless otherwise stated.

For *S. cerevisiae* screenings, strain effects on TyrOH and HT concentrations were assessed using pairwise Welch's *t*-tests. For LAB screenings, HT concentrations were analyzed using linear mixed-effects models on $\log_1$p-transformed data, including day, strain, medium, (plus their interactions) as fixed effects and random intercepts for replicate nested within each strain-medium combination. Pairwise contrasts of estimated marginal means were FDR-adjusted.

Fermentation kinetics parameters (peak and average rates, and AUC) were evaluated as described in 'Derived kinetic parameters' section, intermediate values were interpolated where needed. Treatment groups were compared to their respective control using two-sided Welch's t-tests. Malic-acid consumption rates were estimated from linear regression of concentration versus time and compared across consortia using one-way ANOVA followed by Tukey's HSD. Ethanol, glycerol, organic acids, residual sugars and bioactive compounds (TyrOH and HT) were compared between consortia and single *S. cerevisiae* control using Welch's t-tests. Compact letter displays (CLDs) and asterisks were used where appropriate to denote significant differences in figures and tables.

3. Results

3.1. Individual screenings for Tyrosol and Hydroxytyrosol production

3.1.1. Yeast screenings

Individual screenings of *S. cerevisiae* and non-*Sac* strains were carried out under fermentative conditions in synthetic must to evaluate their capacity to produce TyrOH and HT (Fig. 1). All five *S. cerevisiae* strains completed AF (Fig. S1). The *S. cerevisiae* strains that produced highest concentrations of TyrOH and HT were Lalvin CLOS and Red Fruit, with concentrations of TyrOH at 31.3 and 30 ppm, respectively, and HT at 0.15 and 0.63 ppb, respectively. These two strains showed significant differences in HT production compared to the other *S. cerevisiae* strains (Fig. 1).

Non-*Sac* yeasts were not expected to complete fermentation, thus the process was halted on day 7 (Fig. S2 and Table S1). Of the 49 isolates studied, two strains of *Z. rouxii* (CW95 and CW96) showed the highest production of TyrOH and HT (Fig. 2). Strain CW95 produced the highest concentration of TyrOH on day 2 (13 ppm) but reached its peak HT concentration by day 7 (0.06 ppb). In contrast, CW96 strain produced the highest overall concentration of TyrOH (14 ppm) and HT (0.1 ppb) by day 7. However, these concentrations were lower than those observed in *S. cerevisiae* strains.

3.1.2. Lactic acid bacteria screenings

For LAB, two media (SM and WLM) supplemented with 1 mM of TyrOH, were evaluated to determine under which conditions the bacteria produced higher concentrations of HT (Fig. 3). Overall, MLF in SM yielded higher HT concentrations in most bacterial strains across all time points. Only *P. pentosaceus* 4208 produced a higher HT concentration in WLM (~ 4 ppb); however, this was still considerably lower than the concentrations achieved by *O. oeni* strains 3P2 and MF6 in SM. As shown in Fig. 3, these two strains produced on average the highest HT concentration on day 1 (9.96 ppb and 11.14 ppb, respectively). After MLF completion, HT levels slightly decreased. In contrast, in WLM, although the bacteria successfully completed MLF, HT production was very low and considerably lower than that observed in SM (Fig. S3).

3.2. Mixed sequential fermentations

Based on the initial screenings, two strains from each microbial group were selected for their ability to produce TyrOH and HT:

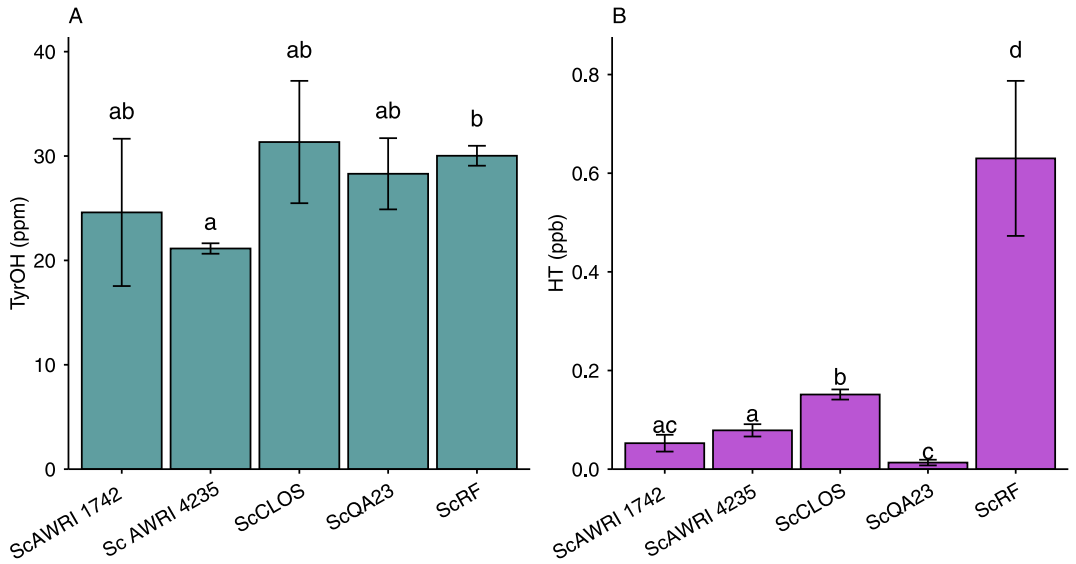


Fig. 1. Production of tyrosol (TyrOH) (A) and hydroxytyrosol (HT) (B) by *S. cerevisiae* strains at the endpoint of small-scale fermentation screenings, presented as mean ± SD for three replicates per strain. Letters above the bars indicate the results of pairwise Welch's t-tests with Benjamini-Hochberg FDR adjustment performed separately for each metabolite ($p < 0,05$).

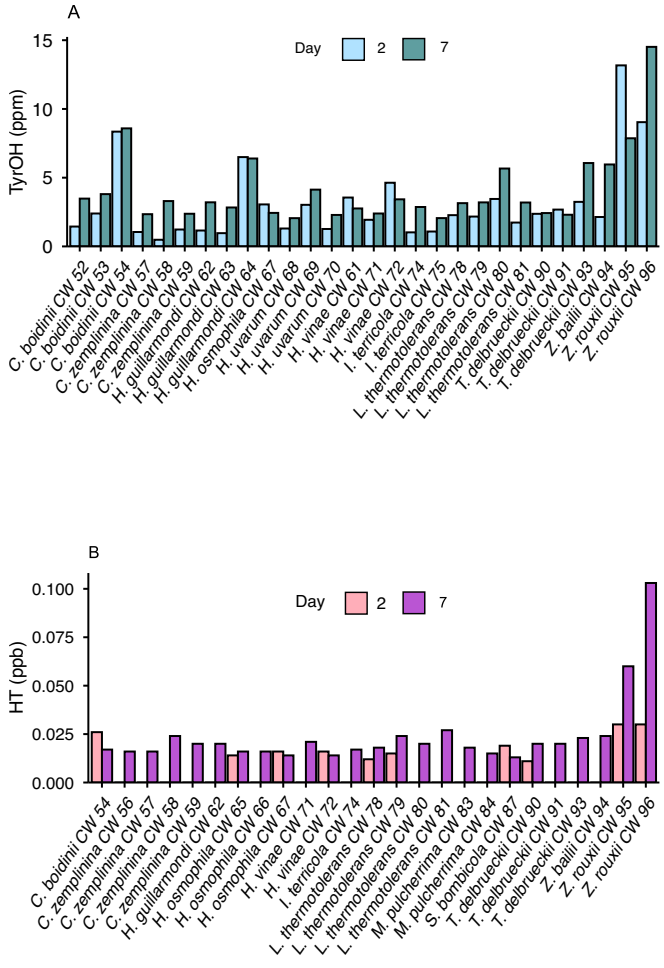


Fig. 2. Production of TyrOH (A) and HT (B) by non-*Saccharomyces* strains in small-scale fermentation screenings. Metabolite production was measured at two time points (day 2 and 7).

S. cerevisiae strains Red Fruit (ScRF) and Lalvin CLOS (ScCLOS), the non-*Sac* yeast *Z. rouxii* CW95 (Zr95) and CW96 (Zr96), and the LAB strains *O. oeni* 3P2 (Oo3P2) and MF6 (OoMF6). These selected strains were used to form three-strain mixed microbial consortia aimed at enhancing HT production (Table 1). Each three-strain consortium was compared with two combinations of two-strain consortia: *Z. rouxii; S. cerevisiae* (Zr;Sc), *O. oeni; S. cerevisiae* (Oo;Sc) and single *S. cerevisiae* (Sc) fermentation. This approach allowed for a comprehensive evaluation of how consortium composition influenced fermentation kinetics, population dynamics, oenological parameters, and secondary metabolite profiles.

3.2.1. *Fermentation kinetics*

Fermentation kinetics were assessed by monitoring must density loss over time (Fig. 4), reflecting sugar consumption by the different mixed microbial consortia. These results were compared with single *S. cerevisiae* fermentations to assess the potential impact of varying strains within the consortia on fermentation performance and kinetics.

Single fermentations with both *S. cerevisiae* strains proceeded faster than their respective mixed consortia, showing higher average rates and lower AUC (Fig. 4 and Tables 2 and 3). All fermentations completed sugar consumption, although fermentation endpoints varied depending on the strain combinations.

Because these were sequential fermentations, with *Z. rouxii* and/or *O. oeni* strains introduced 48 h before the *S. cerevisiae* strains, distinct consumption dynamics were observed. Fermentations initiated with *Z. rouxii* strains showed a slight, gradual sugar consumption initially, followed by a rapid increase after *S. cerevisiae* addition. In contrast, fermentations started with only *O. oeni* strains showed no early sugar consumption, remaining stable until *S. cerevisiae* strains were added. Upon inoculation, ScRF began consuming sugar immediately, whereas ScCLOS displayed a noticeable lag phase. The AUC of all fermentations was calculated, and cutoff points were established at day 8 for ScRF fermentations and day 9 for ScCLOS fermentations.

Peak and average fermentation rates varied among the mixed consortia. For ScRF, consortia containing Zr96 (Zr96 + OoMF6;ScRF and Zr96 + Oo3P2;ScRF) reached higher sugar consumption rates and completed fermentation by day 14. In contrast, consortia containing Zr95 were more variable: Zr95 + OoMF6;ScRF finished around day 12, whereas consortia Zr95 + Oo3P2;ScRF required up to day 18 to fully consume sugars. Two-strain consortia reflected similar trends, with Oo;Sc combinations generally fermenting faster than Zr;Sc combinations, and their rates closely resembling those of the single ScRF fermentation

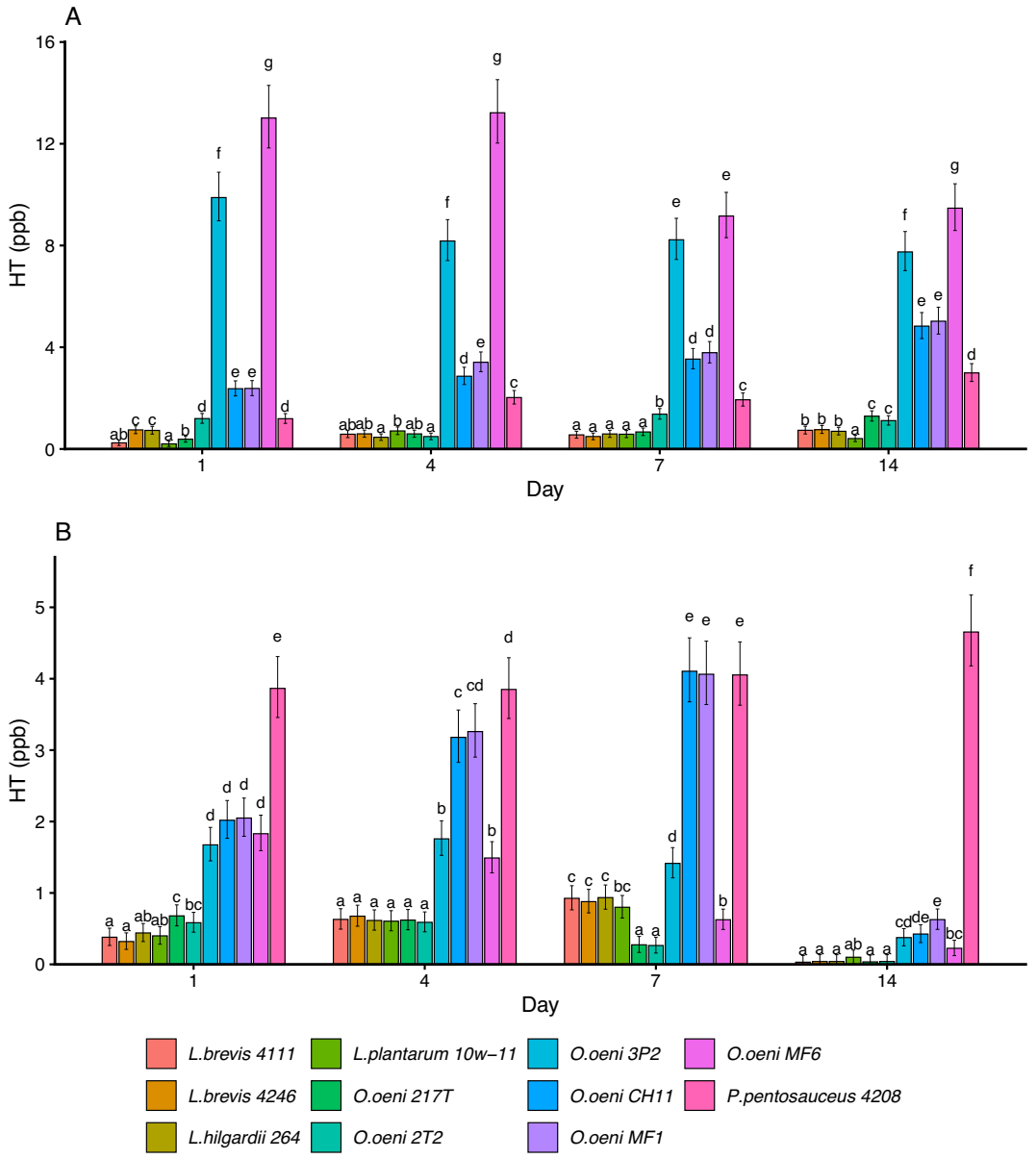


Fig. 3. Hydroxytyrosol (HT) production during malolactic fermentation screenings with different lactic acid bacteria strains in synthetic must (SM) (A) and wine-like medium (WLM) (B). Letters above the bars represent BH-adjusted compact-letter groups calculated within each Day × Medium panel ($p < 0.05$).

(Table 2).

ScCLOS fermentations were slower overall, with three-strain consortia requiring an average of 17 days to complete sugar consumption. Among these, consortia containing Zr95 presented the fastest kinetics, with higher average fermentation rates and lower AUC values, while fermentation combining only a LAB strain with ScCLOS exhibited the highest peak rates (Table 3).

3.2.2. *Malolactic fermentation*

By monitoring malic acid consumption over time, the rate of malic acid degradation was determined (Fig. 5), providing insights into how yeast presence and microbial interactions influence MLF dynamics.

Malolactic fermentation occurred simultaneously with AF whenever a LAB strain was present (Fig. 5). All three-strain consortia (Zr + Oo;Sc), as well as two-strain consortia Oo;Sc, successfully completed MLF. In contrast, the Zr;Sc consortia and single *S. cerevisiae* fermentations did not undergo MLF, as expected due to the absence of LAB.

In general, malic acid consumption rates were higher in consortia containing the ScRF strain compared to those with ScCLOS. For ScRF consortia, no significant differences were observed in malic acid consumption rate among those that carried out MLF. In contrast, for ScCLOS consortia, notable differences were observed: combinations presenting Zr96 exhibited the lowest malic acid consumption rates, whereas those containing only a LAB strain together with ScCLOS showed the highest rates (Fig. 5).

Malolactic fermentations with *O. oeni* strains were also conducted sequentially, following alcoholic fermentation, in wines obtained from Zr;Sc and Sc inoculations. After 14 days, none of the MLFs with either LAB strain (OoMF6 and Oo3P2) were able to complete malic acid consumption (Fig. S4). Most OoMF6 fermentations stopped at around 0.2–0.5 g/L of malic acid. In contrast, some Oo3P2 fermentations stalled with higher concentrations of malic acid (0.5–1.5 g/L).

3.2.3. *Population dynamics*

Yeast and LAB populations dynamics were monitored throughout both single and mixed AF (Fig. 6). Once inoculated in their designated times, all *Z. rouxii*, *O. oeni* and *S. cerevisiae* strains were able to grow in the fermentation medium, exhibiting distinct population dynamics. Both

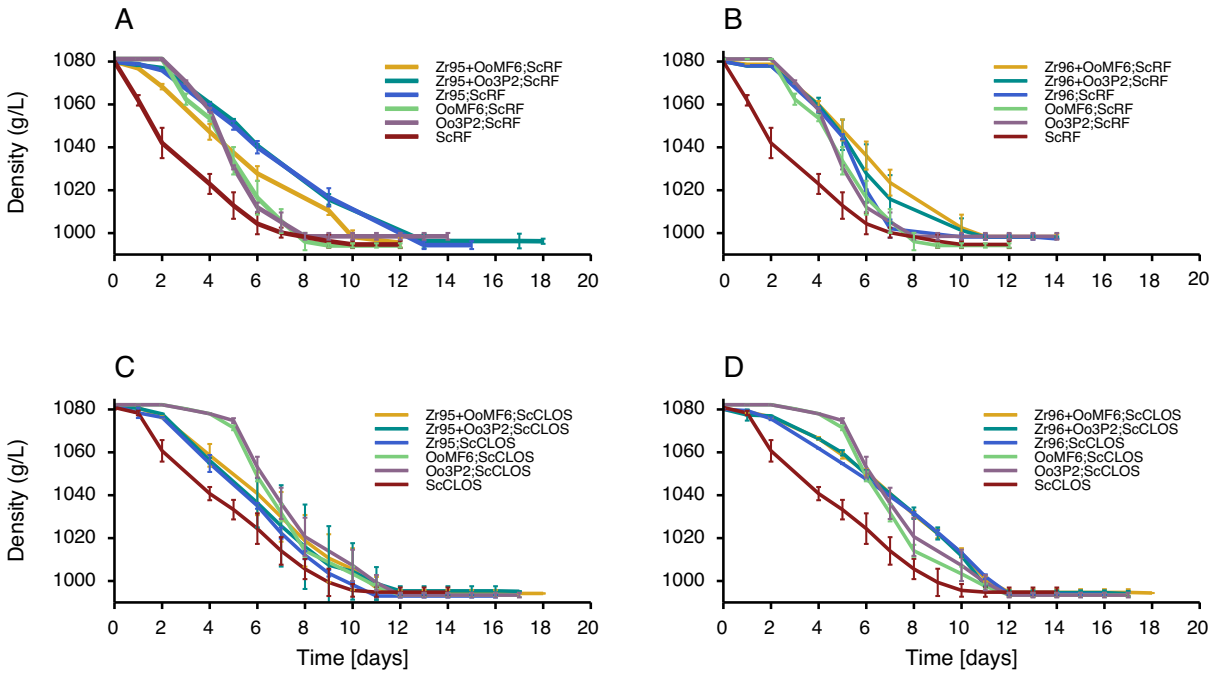


Fig. 4. Fermentation kinetics (density vs. time) in mixed sequential fermentations, combining different *S. cerevisiae* (ScRF, ScCLOS), *Z. rouxii* (Zr95, Zr96) and *O. oeni* (OoMF6, Oo3P2) strains. Lines represent the mean ± SD of biological replicates. Single inoculation of each *S. cerevisiae* strains was used as controls: ScRF (A and B) and ScCLOS (C and D). All strain combinations were compared against their respective *S. cerevisiae* single fermentation.

Table 2
Fermentation kinetics for mixed sequential consortia and single fermentations. Values are expressed as mean ± SD (units: g/L/day for rates; g/L· day for AUC). The average rate was calculated from the start of the fermentation until the must reached 1000 g/L; the peak rate represents the largest segment-wise density drop before 1000 g/L; AUC ($t_0 - t_8$) corresponds to the trapezoidal area under the density curve over the first 8 days. Asterisks denote Welch *t*-tests compared to the *S. cerevisiae* control, adjusted using the BH-FDR method across combinations.

Combination	Average rate (g/L)	Peak rate (g/L)	AUC (t_0 _ t_8)
ScRF	10.99 ± 2.37	20.99 ± 2.68	8216.57 ± 21.79
Zr95 + OoMF6; ScRF	8.00 ± 0.37 *	12.70 ± 2.4 **	8385.59 ± 17.96 ***
Zr95 + Oo3P2;ScRF	6.76 ± 0.53 *	11.50 ± 0.30 **	8462.14 ± 4.41 ***
Zr96 + OoMF6; ScRF	8.21 ± 0.01	14.82 ± 0.26 *	8414.91 ± 9.09 ***
Zr96 + Oo3P2;ScRF	7.91 ± 1.03	24.28 ± 9.16	8439.47 ± 49.11 **
Zr95;ScRF	7.30 ± 0.67 *	10.50 ± 0.44 **	8436.57 ± 34.11 **
Zr96;ScRF	9.78 ± 1.49	25.10 ± 0.69	8391.10 ± 6.84 ***
OoMF6;ScRF	10.80 ± 0.78	20.77 ± 4.35	8372.87 ± 24.70 ***
Oo3P2;ScRF	10.67 ± 0.73	26.70 ± 0.66	8377.95 ± 8.17 ***

$p < 0.05$ (*). $p < 0.01$ (**). $p < 0.001$ (***); "ns" = not significant (not shown).

Table 3
Fermentation-kinetics for mixed sequential consortia and single fermentations. Values are mean ± SD (units: g/L/day for rates; g/L· day for AUC). Average rate is calculated from the start until the must reaches 1000 g/L; Peak rate is the largest segment-wise drop in density before 1000 g/L; AUC $t_0 - t_9$ corresponds to the trapezoidal area of the density curve over the first 9 days. Asterisks denote Welch t-tests compared to the *S. cerevisiae* control, adjusted using the BH-FDR method across combinations.

Combination	Average rate (g/L)	Peak rate (g/L)	AUC ($t_0 - t_9$)
ScCLOS	9.18 ± 0.65	17.95 ± 5.46	9347.95 ± 27.28
Zr95 + Oo3P2; ScCLOS	8.70 ± 1.56	13.97 ± 2.34	9450.02 ± 66.82
Zr95 + OoMF6; ScCLOS	8.24 ± 0.68	12.93 ± 0.42	9454.37 ± 35.40 **
Zr95;ScCLOS	8.46 ± 0.17	12.70 ± 1.27	9431.62 ± 18.70 *
OoMF6;ScCLOS	7.86 ± 0.28 **	23.03 ± 2.20	9534.15 ± 9.80 **
Oo3P2;ScCLOS	7.62 ± 0.34 **	21.73 ± 3.86	9555.31 ± 26.10 *
Zr96 + Oo3P2; ScCLOS	7.39 ± 0.02 **	13.97 ± 1.16	9524.85 ± 11.71 **
Zr96 + OoMF6; ScCLOS	7.18 ± 0.13 **	12.13 ± 0.95	9525.23 ± 6.63 **
Zr96;ScCLOS	7.12 ± 0.05 **	14.10 ± 5.52	9514.33 ± 10.08 **

$p < 0.05$ (*). $p < 0.01$ (**). $p < 0.001$ (***); "ns" = not significant (not shown).

S. cerevisiae strains (ScRF and ScCLOS) displayed similar growth patterns, regardless of the presence of *Z. rouxii* and/or *O. oeni* strains, when compared with the corresponding *S. cerevisiae* monoculture. *S. cerevisiae* populations typically reached around 4–7 × 10^7 cells/mL, occasionally approaching 10^8 cells/mL. A slight decline in population, particularly in viable cells, was observed toward the end of the fermentations.

O. oeni strains did not display consistent population dynamics. Initial cell populations were approximately 10^7 cells/mL across all combinations, generally increasing to nearly 10^8 cells/mL after 24 h and remaining stable or slightly decreasing by 48 h. Following *S. cerevisiae* inoculation, *O. oeni* viable populations decreased rapidly to below 10^6 cells/mL by mid-fermentation in the ScRF combinations. From that point onward, *O. oeni* cells could no longer be quantified by viable plate count, thus were monitored solely by qPCR. In ScCLOS consortia, *O. oeni* populations maintained higher viable cell counts throughout AF compared with the ScRF consortia. *O. oeni* populations remained around 10^7 cells/mL until advanced fermentation, after which they declined below 10^6 cells/mL. The Oo;Sc fermentations did not show significant differences in population dynamic between the two strains (OoMF6 and Oo3P2), both maintaining approximately 10^7 cell/mL by the end of the process, except for the Oo3P2;ScCLOS combination, in which a marked decrease was observed, dropping below 10^6 cells/mL.

Z. rouxii populations exhibited distinct behaviors depending on the *S. cerevisiae* consortium in which they were present. *Z. rouxii* strains

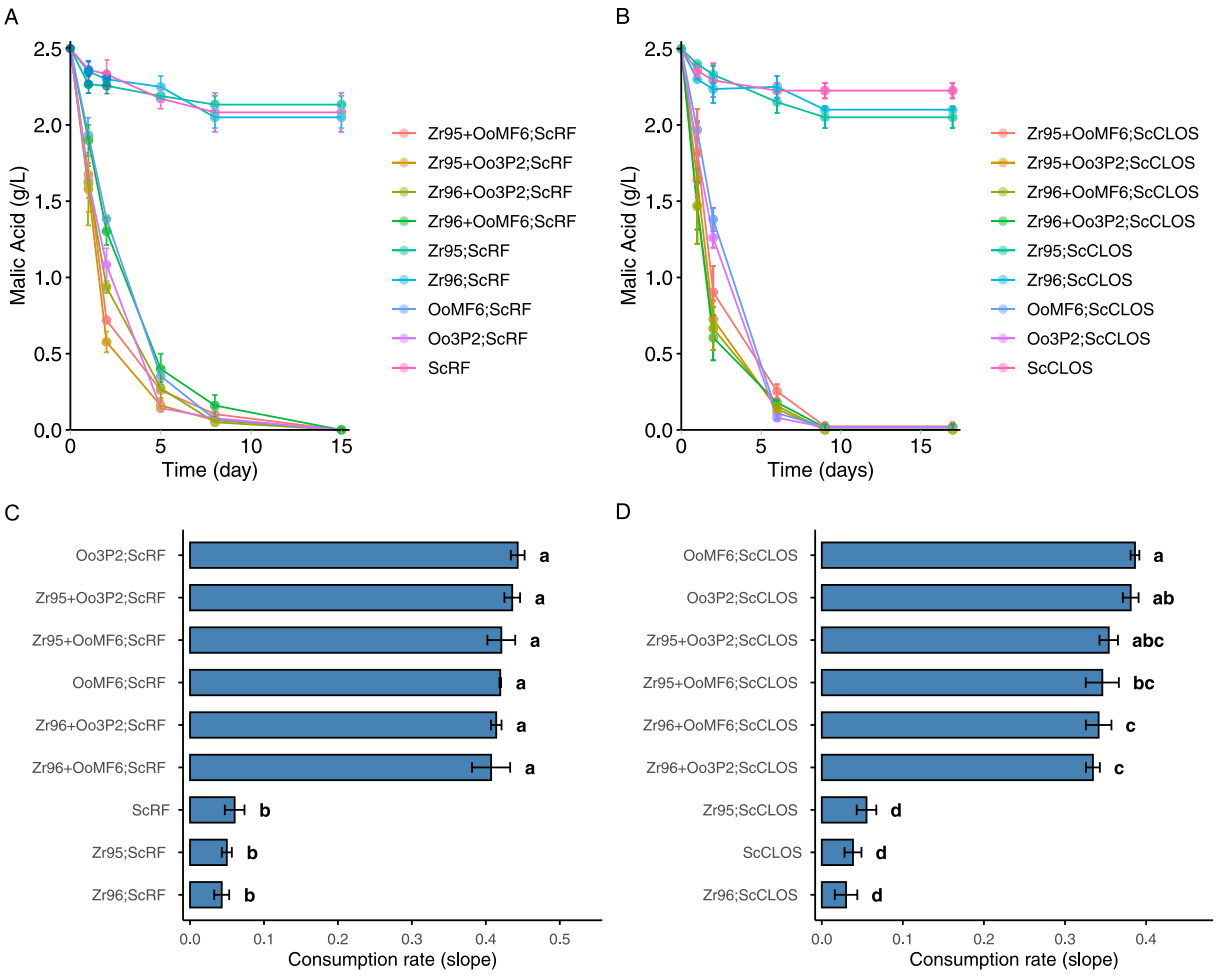


Fig. 5. Malic acid consumption (A and B) and rate (C and D) during MLF carried out simultaneously with AF in different microbial consortia combinations. Values represent mean ± SD. Different letters denote significant differences among consortia (Tukeys HSD $p < 0.05$).

showed good initial growth, starting at approximately 2×10^6 cells/mL and increasing to around 6×10^6 cells/mL at 48 h. However, similar to *O. oeni* strains, once *S. cerevisiae* was inoculated, *Z. rouxii* viable cell counts declined rapidly. The Zr95 strain experienced a more pronounced loss of viability, particularly in the Zr95 + Oo3P2; ScRF ($< 10^6$ cells/mL by AdF point) and Zr95 + OoMF6; ScCLOS ($\sim 10^6$ cells/mL at AdF point) consortia. In contrast, Zr96 maintained higher viability in both Sc combinations, with the most consistent populations observed in the ScCLOS consortia ($> 10^6$ cells/mL up to EF point).

3.3. Major fermentation metabolites

The composition of key oenological metabolites was assessed at EF point of AF to evaluate the impact of different microbial consortia on fermentation outcomes (Fig. 7, Tables S3 and S4). These parameters provided insights into differences between microbial consortia and helped assess how microbial interactions influence fermentation outcomes compared with single *S. cerevisiae* fermentations.

All fermentations were considered complete, with residual sugars below 2 g/L. Ethanol levels varied according to the *S. cerevisiae* strain used, and all comparisons were made relative to the corresponding single *S. cerevisiae* fermentation. In fermentations with ScRF (Table S3 and Fig. 7), significant ethanol decrease was observed in consortia containing Zr96 (10–10.2%). In ScCLOS fermentations (Table S4 and Fig. 7), the single *S. cerevisiae* fermentation yielded the highest ethanol concentration among all the combinations (10.9%), whereas significant decreases were observed in both consortia containing Oo3P2 with *a Z. rouxii* strain, as well as in Zr96;ScCLOS, with ethanol concentrations around 10.4–10.5%.

Malic acid consumption is directly related to lactic acid production, reflecting the MLF progress in the presence of *O. oeni*. In all the consortia containing *O. oeni* strains, malic acid was nearly completely consumed, accompanied by lactic acid production (Figs. 5 and 7, Table S3 and S4). However, lactic acid levels were higher than expected based on the initial malic acid concentration, indicating that *O. oeni* were also producing lactic acid from sugars. It is important to note that the HPLC analysis quantified both D- and L- lactic acid together. Finally, consortia containing both *O. oeni* and *Z. rouxii* strains exhibited higher lactic acid concentrations compared with consortia containing only *O. oeni*.

Acetic acid production can be attributed to both yeast and *O. oeni,* as this LAB exhibits heterolactic metabolism. Interestingly, the highest acetic acid concentration was observed in the single *S. cerevisiae* fermentations (ScRF: 0.83 g/L and ScCLOS: 0.73 g/L), while consortia containing *O. oeni* strains produced significantly lower levels. Glycerol production differed significantly between conditions, with the highest concentrations generally observed in consortia containing all three microorganisms. In particular, consortia with Zr95 and OoMF6 (Zr95 + OoMF6;ScRF and Zr95 + OoMF6;ScCLOS) exhibited the highest glycerol production, with 9 and 9.2 g/L, respectively.

3.4. Bioactive compound analysis

AADC concentrations, particularly TyrOH and HT, were analyzed at mid fermentation (Fig. S5 and S6) and endpoint (Figs. 8 and 9) to assess

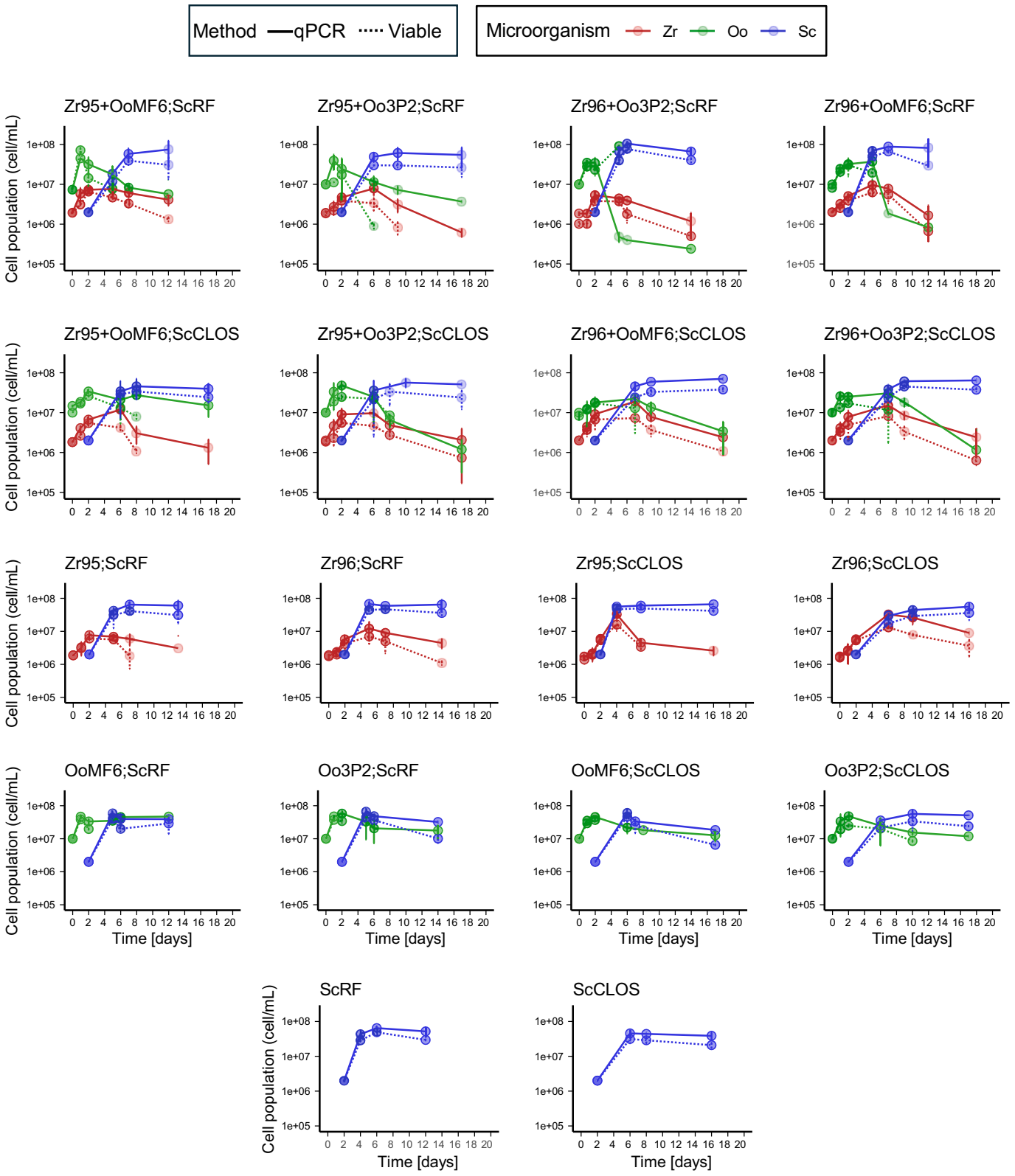


Fig. 6. Population dynamics of mixed consortia and single fermentations, combining different *S. cerevisiae* (ScRF, ScCLOS), *Z. rouxii* (Zr95, Zr96) and *O. oeni* (OoMF6, Oo3P2) strains, as indicated in Table 1. Cell populations were monitored over time (days) by qPCR (solid lines) and viable plate counts (dashed lines). Error bars represent SD of biological replicates.

whether combinations of the selected microorganisms could produce higher levels of these compounds compared with the individual screenings conducted at the beginning of this study.

Results at the end of the fermentation showed that, despite promising outcomes from individual screenings, consortia containing all three microorganisms did not necessarily yield higher concentrations of TyrOH or HT.

The highest TyrOH concentrations obtained in ScRF fermentations

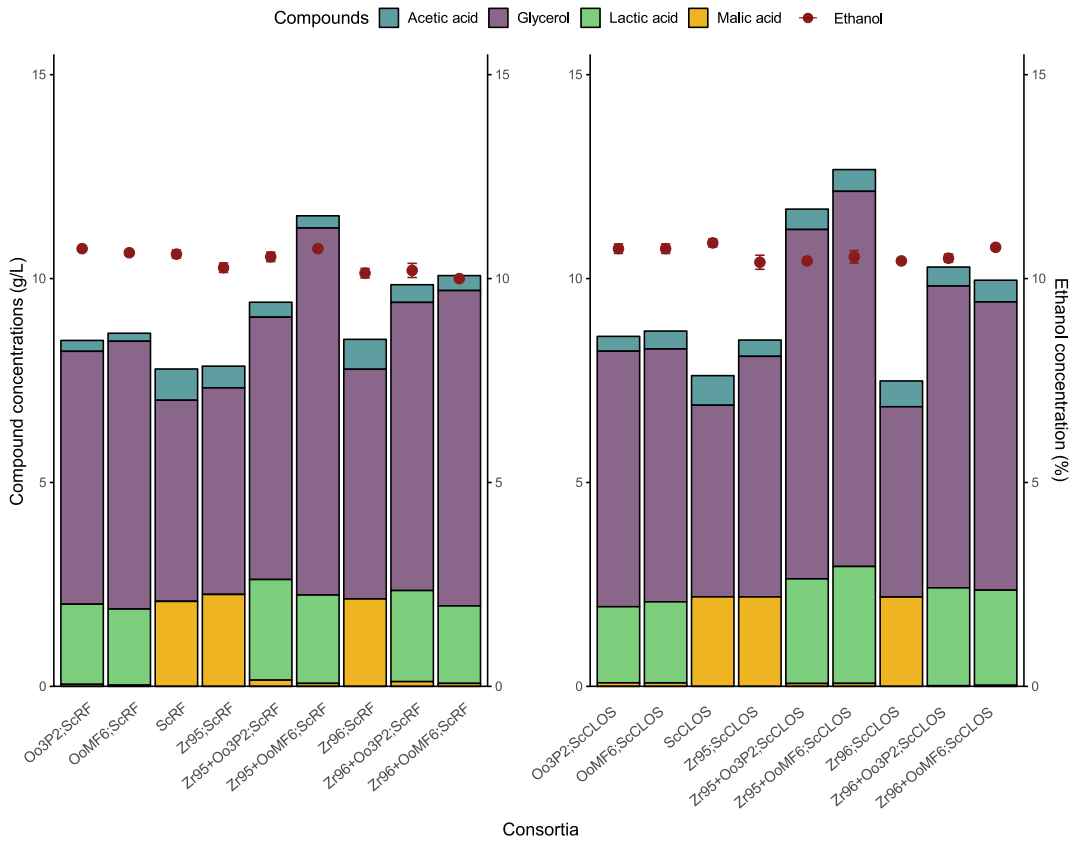


Fig. 7. Chemical parameters of fermentation in mixed sequential fermentations and their respective controls. A) Consortia involving *S. cerevisiae* ScRF and B) Consortia involving *S. cerevisiae* ScCLOS. Data represent mean ± SD from biological replicates (values provided in Supplementary Table S2 and S3).

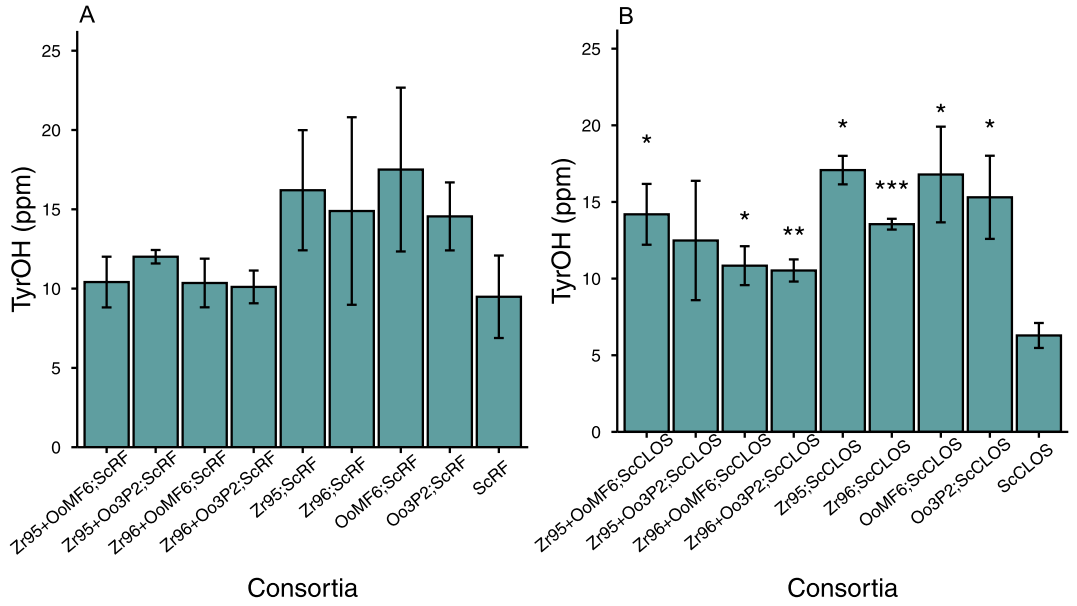


Fig. 8. Production of TyrOH (ppm) by mixed microbial consortia and single fermentations at the endpoint. (A) Consortia containing *S. cerevisiae* RF (ScRF). (B) Consortia containing *S. cerevisiae* CLOS (ScCLOS). Values represent mean ± SD from biological replicates. Asterisks (*) denote significant pairwise differences between each consortium and its corresponding single *S. cerevisiae* single fermentation (BH-FDR adjusted): $p < 0.05$ (*). $p < 0.01$ (**). $p < 0.001$ (***); "ns" = not significant (not shown).

(Fig. 8A) were found in combinations of *Z. rouxii* or *O. oeni* strains with *S. cerevisiae*. However, no significant differences were observed compared to the single ScRF fermentation, most likely due to high variability within the consortia replicates.

On the other hand, almost all fermentations involving ScCLOS (Fig. 8B) showed significant differences in TyrOH concentrations compared to the single ScCLOS fermentation, with the highest values observed in the combinations of Zr95;ScCLOS and OoMF6;ScCLOS.

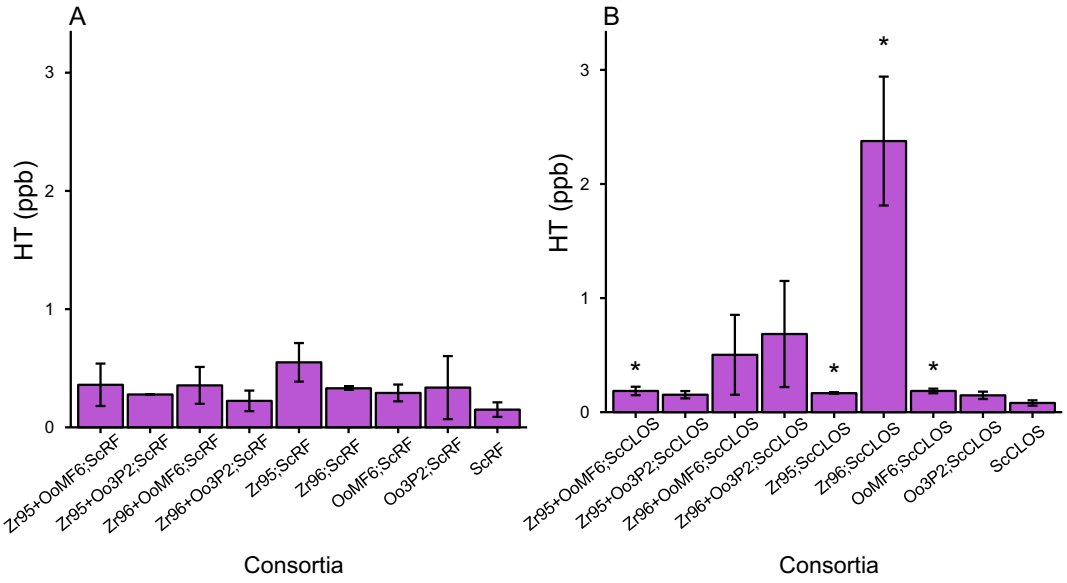


Fig. 9. Production of HT (ppb) by mixed microbial consortia and single fermentations at the endpoint. (A) Consortia containing *S. cerevisiae* RF (ScRF). (B) Consortia containing *S. cerevisiae* CLOS (ScCLOS). Values represent mean ± SD from biological replicates. Asterisks (*) denote significant pairwise differences between each consortium and its corresponding single *S. cerevisiae* single fermentation (BH-FDR adjusted): $p < 0.05$ (*). $p < 0.01$ (**). $p < 0.001$ (***); "ns" = not significant (not shown).

However, this trend did not extend to HT production (Fig. 9), where concentrations remained low in most consortia, with the notable exception of the Zr96;ScCLOS consortium, which had around 2.5 ppb.

Although some of these concentrations were higher than those detected in the initial yeast screenings, the presence of *O. oeni* in the consortia did not lead to an increase in HT levels, which still did not reach the concentrations observed in the initial LAB screenings.

MLF fermentations performed after AF in the combinations of Zr;Sc and single *S. cerevisiae* did not increase HT concentrations measured at the end of AF. Furthermore, in some cases, lower concentrations of HT were observed after 14 days of MLF (Fig. S7).

4. Discussion

Mixed fermentations involving *S. cerevisiae,* non-*Sac* and LAB are increasingly investigated for their potential to modulate wine composition (Bartowsky et al., 2015; Ciani et al., 2010; Ciani et al., 2016; Ciani & Comitini, 2015), yet their impact on AADC remains largely unexplored. In this context, the focus of this work was on the production of specific bioactive compounds, particularly TyrOH and HT. As previous research has shown that *S. cerevisiae* and several non-*Sac* species can naturally synthesize TyrOH (Álvarez-Fernández et al., 2018; Cordente et al., 2019; González et al., 2018), HT formation could theoretically occur if microorganisms with suitable hydroxylase activity are present. Accordingly, we aimed to develop microbial consortia including LAB strains to potentially increase HT production.

To this end, a preliminary screening was performed to assess the ability of a diverse collection of yeast and LAB to produce HT from TyrOH. Overall, *S. cerevisiae* produced higher concentrations of both compounds compared to non-*Sac* yeasts. Most strains in this collection had not previously been studied for this purpose. The *S. cerevisiae* strain, ScQA23, had been examined by González et al. (2018), and together with ScRF by Álvarez-Fernández et al. (2018). However, the results obtained in our study did not follow the same pattern, as the ScQA23 strain produced the lowest HT concentration compared with ScCLOS and ScRF. Among the non-*Sac* yeasts screened, *Z. rouxii* strains produced the highest concentrations of TyrOH and HT, yet this yeast species has not previously been studied in this context. To our knowledge, only one study has reported TyrOH production by a *Z. rouxii* strain in a broth fermentation context (Naylin et al., 2006), and no studies have documented its ability to produce HT. Although *Z. rouxii* is not commonly associated with wine fermentation, several of its physiological characteristics may help contextualize the behavior reported in this study.

Z. rouxii is a non-conventional yeast primarily associated with soy sauce fermentation. Like many *Zygosaccharomyces* species, it has often been considered a spoilage yeast, as it is typically isolated from stuck or sluggish fermentations and displays high tolerance to acidic and osmotic stress (Escott et al., 2018). Nevertheless, *Zygosaccharomyces* species are described as fructophilic yeasts capable of producing higher alcohols and are suitable for low-ethanol fermentations (Capece et al., 2022; Li et al., 2023; Solieri, 2021). These traits are consistent with the metabolic patterns observed in our study and support the potential use of *Z. rouxii* to enhance aromatic amino acid metabolism in mixed fermentations.

Several bacterial species from different genera, such as *Pseudomonas, Serratia* and *Halomonas*, have been reported to convert TyrOH into HT through enzymatic hydroxylation (Allouche & Sayadi, 2005; Bouallagui & Sayadi, 2006; Liebgott et al., 2007). However, since these genera are not suitable for winemaking, we only used these studies as a precedent to illustrate the hydroxylation mechanisms that bacteria may possess to transform TyrOH to HT. Moreover, several LAB species have been described as capable of producing HT through the hydrolysis of oleuropein (Santos et al., 2012). In our study, we observed that most LAB in synthetic medium were able to produce HT. Specifically, *O. oeni* strains, such as OoMF6 and Oo3P2, produced the highest HT level, suggesting that these LAB may possess a similar mechanism to hydroxylate TyrOH into HT. Based on these findings, we aimed to determine whether consortia including yeast and *O. oeni* could enhance the concentration of this bioactive compound in wine.

To this end, mixed sequential fermentations were conducted by first co-inoculating *Z. rouxii* with *O. oeni* strains, followed by the inoculation of *S. cerevisiae* strains. This approach was considered appropriate to allow the non-*Sac* and LAB strains to influence to wine composition during initial stages of AF, before introducing *S. cerevisiae*, which is expected to dominate and complete the fermentation. Co-inoculation strategies in which LAB starters are inoculated at the onset of AF, rather than sequentially after AF, have been shown to positively impact wine composition (Bartowsky et al., 2015; Virdis et al., 2021). Several studies have demonstrated that early or simultaneous inoculation of LAB

with non-*Sac* yeasts can facilitate MLF or modulate fermentation outcomes. For instance, Russo et al. (2020) reported that co-inoculation of *Starmerella bacillaris* with *O. oeni* enabled rapid MLF without negative impact on wine profiles. Likewise, Vicente et al. (2024) showed that introducing *Lachancea thermotolerans* and *O. oeni* prior to *S. cerevisiae* improved wine acidity and aroma complexity.

When dealing with mixed cultures, fermentation kinetics can follow different patterns. The presence of additional strains other than *S. cerevisiae* typically delays fermentation progression, even at initial stages when most nutrients are still abundant (Capece et al., 2022). In our study, we observed a similar trend in fermentations containing both *S. cerevisiae* and *Z. rouxii* strains, and even more markedly in mixed consortia including all three microorganisms, where sugar consumption was significantly slower than in single *S. cerevisiae* inoculation. This delay could be attributed to nutrient competition between *S. cerevisiae* and *Z. rouxii* strains. According to Ciani and Comitini (2015), when non-*Sac* species grow during the early stages of wine fermentation, they can consume amino acids and vitamins, thereby limiting the subsequent growth of *S. cerevisiae*. Consistent with previous studies, early growth of *Torulaspora delbrueckii* has been shown to affect nitrogen availability for *S. cerevisiae* and may slow fermentation (Roca-Mesa et al., 2022; Taillandier et al., 2014). Similarly, the delay in sugar depletion observed in the presence of *Z. rouxii* is consistent with an early competitive effect on nutrient availability and/or altered nutrient uptake between yeast species. Although nitrogen consumption was not directly quantified, *S. cerevisiae* ultimately dominated and completed all fermentations, albeit with some delay.

Interestingly, Leandro et al. (2014) identified the molecular basis of the fructophilic behavior of *Z. rouxii.* In our study, its early utilization of fructose may have contributed to altered sugar consumption patterns. Although mixed fermentations resulted in slower kinetics, this may not necessarily be a disadvantage. Capece et al. (2022) argue that, although rapid and reliable fermentation completion is of primary importance in the wine industry, slower fermentation kinetics may be beneficial for better retention of volatile compounds. Slower fermentation kinetics were also observed in Oo:ScCLOS consortia; however, in this case, the two-day period during which density did not decrease was due to the absence of any yeast in the medium. Importantly, the concentrations of compounds such as lactic or acetic acids were not significantly higher in these controls than with the three-strain microbial consortia. As mentioned previously, yeast-LAB compatibility relies on various factors, and the nature of this relationship is highly strain-dependent. Therefore, the presence or absence of *Z. rouxii* strains may contribute to differences in yeast-bacteria performance and, consequently, fermentation kinetics.

The population dynamics of the microbial consortia followed similar patterns regardless of the strains used. Both *O. oeni* strains lost viability almost immediately after the inoculation of *S. cerevisiae*, or shortly thereafter. In contrast, among the *Z. rouxii* strains, Zr96 generally maintained viability longer, up to the end of fermentation, whereas Zr95 remained viable only until mid- to late fermentation. Ciani and Comitini (2015) and Benito et al. (2019) indicated that loss of viability of non-*Sac* species in mixed fermentations is mainly related to ethanol production. Capece et al. (2022) reported high viability of a *Z. bailii* strain in sequential fermentations, they also observed, as in our study, that these fermentations proceeded more slowly. Comparatively, the loss of cell viability in *O. oeni* strains could also be attributed to ethanol shock, which leads to membrane fluidization followed by rigidification (Chu-Ky et al., 2005). Interestingly, most microbial consortia containing all three microorganisms exhibited higher *O. oeni* viability (up to mid-fermentation) compared to Oo;Sc consortia, where viability was maintained only up to 48 h. Englezos et al. (2019) proposed that in the presence of a non-*Sac* yeast, *O. oeni* could benefit from lower ethanol and higher nitrogen levels at the time of inoculation at the beginning of the AF. Similarly, the presence of *Z. rouxii* strains in mixed consortia may have altered fermentation kinetics and delay the ethanol production, which could contribute to the prolonged viability of *O. oeni* compared to fermentations carried out solely by *S. cerevisiae*.

Bartowsky et al. (2015) proposed that inoculating LAB at early stages of AF enables rapid and reliable vinification. In our study, all MLFs initiated simultaneously with AF were completed with either *O. oeni* strain, despite the relatively short viability of *O. oeni* populations (~ 5 days). By contrast, when MLF was attempted after AF in wines produced by single *S. cerevisiae* or mixed Zr:Sc fermentations (Figs. S6 and S7), none of the trials achieved complete malic acid degradation, and all fermentations stalled after a few days. Early LAB inoculation may be advantageous due to the higher availability of amino acids and other nutrients in fermenting must compared to finished wine, as well as lower ethanol concentrations and reduced levels of potential yeast-derived inhibitory compounds, thereby allowing MLF to proceed successfully (Englezos et al., 2019; Jussier et al., 2006; Sumby et al., 2019).

As mentioned previously, yeast-LAB compatibility depends on multiple factors, and is highly strain-dependent; consequently, interactions between yeast and LAB can influence both AF and MLF (Alexandre et al., 2004; Du Plessis et al., 2017; Sumby et al., 2019). The presence or absence of the *Z. rouxii* strains may therefore also modulate yeast-bacteria interactions and, in turn, fermentation kinetics. Indeed, several studies have demonstrated that non-*Sac* yeasts can affect LAB performance as reported for *S. bacillaris* and L. *thermotolerans* by Russo et al. (2020) and Vicente et al. (2024), respectively. Similarly, mixed fermentations involving *Hanseniaspora, Torulaspora* and *Metschnikowia* species have resulted in variable LAB growth and MLF outcomes (Du Plessis et al., 2017; Du Plessis et al., 2019). Together, these findings highlight the capacity of non-*Sac* yeasts to influence LAB performance, suggesting that *Z. rouxii* may likewise influence the fermentation environment when employed in mixed consortia.

Acetic acid is the primary contributor to volatile acidity, and its concentration should be maintained within acceptable limits. *O. oeni* is a heterofermentative species that converts sugar into lactic acid, acetate and CO_2 from sugars (Liu, 2002). Therefore, we initially expected consortia containing *O. oeni* strains to produce higher acetic acid levels; however, this was not observed in our study. The literature presents contradictory findings on this topic: some studies have reported that simultaneous MLF with AF does not affect acetic acid levels (Jussier et al., 2006; Rosi et al., 2003), while others describe slightly higher acetic acid production during early MLF with *O. oeni*, albeit below sensory thresholds (Englezos et al., 2019; Liu, Rousseaux, et al., 2017; Sumby et al., 2019). In our experiments, acetic acid concentrations were significantly higher in Zr;Sc and single *S. cerevisiae* fermentations, consistent with previous reports of elevated acetic acid production in mixed fermentations involving non-*Sac* and *Saccharomyces* wine strains (Ciani & Maccarelli, 1997; Ferrando et al., 2020).

Lactic acid is considered positive for the sensory perception of wine due to its contribution to soft mouthfeel. Its production is mainly attributed to LAB, which convert L-malic acid to L-lactic acid. In addition, *O. oeni* can produce D and L- lactic acid during heterolactic fermentation from sugars present in the initial must. *S. cerevisiae* strains also generate trace amounts of D-lactic acid during AF. Thus, overall concentrations of lactic acid are primarily attributed to LAB activity (Ferreira & Mendes-Faia, 2020).

In our study, lactic acid was produced in wines inoculated with *O. oeni* strain. Very small concentrations of lactic acid were detected in single *S. cerevisiae* or Zr;Sc consortia, consistent with the absence of MLF, as malic acid concentrations remained largely unchanged. Interestingly, the lactic acid levels detected were higher than expected based on the initial malic acid concentration, suggesting that part of lactic acid originated from sugar metabolism by *O. oeni* (Zhang et al., 2024). This is further supported by the earlier observation that fermentations lacking *O. oeni* produced very little lactic acid. Notably, consortia Zr95 + OoMF6;ScCLOS presented higher concentrations of lactic acid compared to Oo;Sc consortia, which may reflect strain-dependent effects associated with the presence of all three microorganisms.

Glycerol in wine is primarily produced through yeast metabolism,

and, although it does not directly contribute to aroma, it enhances the sweetness, viscosity and smoothness of the wine (Ciani & Ferraro, 1996). In our study, glycerol production appeared to be both strain-dependent and influenced by the composition of the microbial consortia. The highest concentrations were observed in consortia containing ScRF, particularly when all three microorganisms were present. Similarly, high glycerol levels were detected in ScCLOS consortia that include all three strains. In both *S. cerevisiae* consortia, glycerol production peaked in the combination containing Zr95 and OoMF6. According to Scanes et al. (1998), glycerol production during wine fermentation is affected by multiple environmental factors. While it is primarily associated with redox balancing and the response to hyperosmotic stress, it can also increase in response to oxidative stress. Moreover, some studies have reported that *Z. rouxii* strains are capable of producing glycerol as an adaptive mechanism to survive high osmolarity conditions, reflecting the osmophilic nature of this yeast species (Escott et al., 2018).

HT is a phenolic compound derived from TyrOH, which can be synthesized through the Ehrlich pathway (González et al., 2018), during AF by *Saccharomyces* and non-*Sac* yeasts (Álvarez-Fernández et al., 2018). In our study, we confirmed that different strains of *S. cerevisiae,* non-*Sac* and LAB were able to produce HT, with some LAB strains generating notable concentrations. However, when these microorganisms were combined into different microbial consortia to enhance the production of these bioactive compounds, we observed that although HT was produced, its concentration was significantly lower than that obtained in independent screenings. This reduction likely results from multiple factors, including strain-specific interactions within the consortia that influence aromatic amino acid precursor availability during fermentation, as well as the still incomplete understanding of the biosynthetic pathways involved. One possible explanation is that early inoculation of *O. oeni* limited its access to TyrOH, which is produced primarily during yeast growth; consequently, little substrate may have been available for subsequent hydroxylation. Conversely, when *O. oeni* was inoculated sequentially after AF, MLF frequently stalled, indicating that the metabolic environment generated by yeasts was not conducive to LAB activity. Notably, co-inoculation of *O. oeni* and *Z. rouxii* did not result in mutual inhibition, and *O. oeni* viability was prolonged in consortia containing *Z. rouxii*, suggesting that the presence of *Z. rouxii* may have contributed to a more favorable fermentation environment for LAB persistence. Nevertheless, the mechanisms underlying these behaviors remain unclear at this stage, and will need further investigation.

In conclusion, this study provides new insights into the behavior and phenotypic interactions of *S. cerevisiae, Z. rouxii*, and *O. oeni* during mixed fermentations, with particular focus on their potential to produce bioactive compounds TyrOH and HT. Our findings confirmed that both *S. cerevisiae* and non-*Sac* yeasts can synthesize TyrOH and HT, with *S. cerevisiae* strains generally yielding higher concentrations. Among the non-*Sac* species tested, *Z. rouxii* strains Zr95 and Zr96 exhibited notably unexpected high production of these compounds, despite limited prior evidence of such metabolic capability. Likewise, *O. oeni* strains OoMF6 and Oo3P2 demonstrated the ability to produce HT, supporting the hypothesis that LAB may possess enzymatic systems to hydroxylate TyrOH.

Mixed sequential fermentations revealed that the coexistence of these microorganisms influences fermentation kinetics, metabolic profiles, and microbial viability. Although HT concentrations in mixed fermentations were lower than those observed in independent screenings, the results highlight the complex interactions that can occur in multi-species fermentations and their potential for functional wine production. In particular, the combination of *Z. rouxii* Zr96 and *S. cerevisiae* ScCLOS emerged as a promising model for future studies on HT biosynthesis and microbial interactions.

Our findings represent a first step toward designing microbial consortia aimed at enhancing HT production, while also highlighting the complexity of strain-dependent metabolic outcomes that arise during mixed fermentations. Future research should apply molecular and systems-level approaches to elucidate the mechanisms underlying the phenotypic interactions observed in the most promising microbial consortia, clarify the enzymatic pathways associated in TyrOH and HT transformation. Further work should optimize inoculation strategies, and evaluate the impact of these microbial consortia on wine sensory and bioactive properties.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used ChatGTP (OpenAI) in order to assist in improving the readability and linguistic quality of the text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Andrea Silva: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Braulio Esteve-Zarzoso:** Methodology, Investigation. **Pedro García-Serrano:** Methodology, Investigation. **M. Carmen García-Parrilla:** Supervision, Project administration, Conceptualization. **Gemma Beltran:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **María-Jesús Torija:** Writing – review & editing, Supervision, Project administration, Methodology, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118486.

Data availability

Data will be made available on request.

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