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A consortium of different *Saccharomyces* species enhances the content of bioactive tryptophan-derived compounds in wine fermentations

Andrés Planells-Cárcel [a], Julia Kazakova [b], Cristina Pérez [a], Marina Gonzalez-Ramirez [b], M. Carmen Garcia-Parrilla [b], José M. Guillamón [a,*]

[a] Department of Food Biotechnology, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Avda. Agustín Escardino, 7, 46980 Paterna, Spain
[b] Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universidad de Sevilla, c/ Profesor García González 2, 41012 Sevilla, Spain

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ABSTRACT

In recent years, the presence of molecules derived from aromatic amino acids in wines has been increasingly demonstrated to have a significant influence on wine quality and stability. In addition, interactions between different yeast species have been observed to influence these final properties. In this study, a screening of 81 yeast strains from different environments was carried out to establish a consortium that would promote the improvement of indolic compound levels in wine. Two strains, *Saccharomyces uvarum* and *Saccharomyces eubayanus*, with robust fermentative capacity were selected to be combined with a *Saccharomyces cerevisiae* strain with a predisposition towards the production of indolic compounds. Fermentation dynamics were studied in pure cultures, co-inoculations and sequential inoculations, analysing strain interactions and end-of-fermentation characteristics. Fermentations showing significant interactions were further analyzed for the resulting indolic compounds and aroma profile, with the aim of observing potential interactions and synergies resulting from the combination of different strains in the final wine. Sequential inoculation of *S. cerevisiae* after *S. uvarum* or *S. eubayanus* was observed to increase indolic compound levels, particularly serotonin and 3-indoleacetic acid. This study is the first to demonstrate how the formation of microbial consortia can serve as a useful strategy to enhance compounds with interesting properties in wine, paving the way for future studies and combinations.

1. Introduction

In wine, there is a wide variety of molecules present in low concentrations that play crucial roles in stability, quality, and bioactivity. Understanding the composition and effects of these compounds is essential for comprehending the overall characteristics of quality and health benefits associated with wine consumption. Molecules such as polyphenols, naturally occurring in grapes, and flavonoids have been extensively investigated to assess their antioxidant properties. Nevertheless, recent investigations have directed their attention towards the metabolites generated by yeast through the metabolism of aromatic amino acids, which are considered potential bioactive compounds capable of enhancing the quality and stability of wines (Cordente et al., 2019). Among them, the well-known higher alcohols derived from the aromatic amino acids, such as tryptophol, 2-phenyl ethanol (2-PE) and tyrosol, as well as the more recently evidenced indolic compounds derived from the tryptophan metabolism, such as melatonin, serotonin or 3-indoleacetic acid (3-IAA) (Fernández-Cruz et al., 2016; Rodriguez-Naranjo et al., 2011). These indolic compounds are produced by yeasts during wine fermentation, as previously demonstrated (Fernández-Mar et al., 2012; Rodriguez-Naranjo et al., 2011). These metabolites are not exclusive to the main fermentative species *S. cerevisiae.* Other yeast species commonly developed during wine fermentation are also capable of producing these bioactive molecules (Fernández-Cruz et al., 2019; Jiao et al., 2022; Morcillo-Parra et al., 2020; Rodriguez-Naranjo et al., 2012). In fact, the synthesis of these compounds exhibits differences depending on the yeast strain, including species, metabolic state of the cell, and the amount of tryptophan and sugars in the medium (Fernandez-Cruz et al., 2019; Morcillo-Parra et al., 2020). Interestingly, when using mixed inocula of *Saccharomyces* and non-*Saccharomyces* strains, higher extracellular levels of melatonin were observed compared to single fermentations (Valera et al., 2019), suggesting a potential influence of the microbiota present during fermentation.

In addition, another group of molecules that, despite their low

* Corresponding author.
E-mail address: guillamon@iata.csic.es (J.M. Guillamón).

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concentration, have a major impact on wine quality are aroma compounds. Yeast metabolism determines the synthesis of volatile compounds such as acids, aldehydes, higher alcohols and their corresponding esters, which make up the "so-called" fermentative aroma of wines. Recently, our group have evaluated the simultaneous production of volatile compounds and bioactive molecules such as melatonin, tyrosol, and hydroxytyrosol, assessing a correlation between the synthesis of both type of molecules (Gonzalez-Ramirez et al., 2023). It is also well-known that yeast interactions can result in an increased repertoire of wine flavors and aromas (Kosel et al., 2017). Under winemaking conditions, various interactions occur among different yeast species naturally present in the must or subsequently added. These interactions can be direct or indirect, influencing the environmental conditions that, in turn, affect other species (Sieuwerts Sander et al., 2008). Several studies have demonstrated differences between pure yeast monocultures, mixed cultures, and final wine blends, indicating that the interactions occurring during fermentation significantly influence the metabolism of each strain, playing a crucial role in the ultimate wine profile (Howell et al., 2006; Naoufel et al., 2005; Roullier-Gall et al., 2020).

Winemaking could be a paradigm of microbial interactions because various microorganisms coexist and interact influencing the dominance of yeast and LAB species during alcoholic and malolactic fermentations. These microbial interactions determine the metabolic and analytical profiles of wine. Thus, in the future, rather than thinking about metabolism as a collection of individual reactions in a cell, we should understand metabolism as a dynamic, interconnected network of processes in which many strains/species collaborate. Under this paradigm, the main goal of this study was to design a consortium of yeast strains that enhanced the production of certain indolic compounds and modify the aroma profile of the wines in comparison with the monoculture fermentations.

2. Material and methods

2.1. Strains and media

The list of strains utilized in the screening assay can be found in the Supplementary Table 1. The origins of these strains vary and come from different collections held by the IATA-CSIC. All yeast strains were grown at the controlled temperature of 28 °C on modified yeast extract dextrose medium (YPD). Yeast extract peptone dextrose (YPD) medium, which contains 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract, was used for yeast propagation for 24 h in 15 mL sterile falcon tubes containing 5 mL of YPD under 150 rpm of agitation.

The screening assay was carried out in two different stages and two different media were used in each stage. Firstly, all the strains were grown in Yeast Nitrogen Base (YNB) without Amino Acids and Amonium (BD™ Difco™, USA) modified in its nitrogen content, which was made up of a mixture of ammonium and tryptophan (1:1) and at a final concentration of 300 mg N/L. Secondly, a selection of the most promising *Saccharomyces* melatonin producers were grown in a synthetic grape must (SM), such as described by Riou et al. (1997), but with some modifications as detailed. The SM contains 200 g/L reducing sugar (100 g/L glucose and 100 g/L fructose), malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4$ 0.25 g/L, $CaCl_2$ 0.16 g/L, NaCl 0.2 g/L, trace elements ($MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH_4)_6Mo_7O_{24}$ 1 mg/L), vitamins (myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, thiamine hydrochloride 0.25 mg/L, pyridoxine hydrochloride 0.25 mg/L and biotine 0.003 mg/L). The composition of the nitrogen sources in the SM was 40 % ammonium and 60 % amino acids, as described by Beltran et al. (2004), corresponding to 0.46 g/L ammonium chloride and 13.09 mL/L of an amino acid stock solution consisting of isoleucine (2.5 g/L), tyrosine (1.5 g/L), tryptophan (13.4 g/L), threonine (5. 8 g/L), aspartic acid (3.4 g/L), glutamic acid (9.2 g/L), leucine (3.7 g/L), glycine (1.4 g/L), histidine (2.6 g/L), glutamine (38.4 g/L), alanine (11.2 g/L), valine (3. 4 g/L), methionine (2.4 g/L), cysteine (1.6 g/L), phenylalanine (2.9 g/L), arginine (28.3 g/L), serine (6 g/L), lysine (1.3 g/L) and proline (46.1 g/L). The pH was adjusted to 3.31 with NaOH and filtered through 0.22 μm pore size membrane filters (Thermo Scientific). The resulting final yeast assimilable nitrogen (YAN; the sum of amino acid and ammonium concentrations, excluding proline) was 300 mg/L. This medium will be referred to as SM300.

All the yeast growths during the screening were carried out on 24-well plates (Greiner CELLSTAR®, Greiner Bio-One). In each well, a 1.5 mL of medium was introduced and it was inoculated to obtain a final cell density of $2{\cdot}10^6$ cells/mL for each strain, measured as $OD_{600} = 0.2$, in triplicate. The plates were incubated at 28 °C with gentle agitation on an orbital shaker (Mini–Shaker PSU-2 T, Biosan) at 300 rpm for 120 h. Samples were collected by centrifugation to separate cells and supernatant was stored in the freezer at −20 °C.

2.2. Fermentation trials

The fermentations were carried out in 100 mL bottles with 80 mL of SM300. Each fermentation was inoculated with $2{\cdot}10^6$ cells/mL of final cell density. Cocultures were inoculated with a 50:50 ratio of each strain and sequential fermentations were inoculated first with $2{\cdot}10^6$ cells/mL of *S. uvarum (Su)* or *S. eubayanus (Se)* and, after 24 or 48 h, they were inoculated with $2{\cdot}10^6$ cells/mL of *S. cerevisiae (Sc)*. Fermentations were incubating at 20 °C and 150 rpm agitation. Each fermentation was performed in triplicate. Implantation control of each strain was realized by ITS restriction analysis of 24 colonies of each fermentation and time (Guillamón et al., 1998). Using restriction enzyme *Hae*III, both *S. uvarum* and *S. eubayanus* show 3 bands (483, 230 and 128 bp) and *S. cerevisiae* shows 4 bands (311, 230, 172 and 128 bp), allowing to discriminate between species of each consortium. To monitor the development of the fermentations, 1.5 mL of sample was extracted every 24 h to measure the density using a Densito 30PX densimeter and the OD600nm with a UVmini-12 spectrophotometer. Samples were centrifuged 5 min at 4000 rpm to separate the cells, and the supernatant was stored at −20 °C previous to the analysis.

2.3. Determination of sugars and acids by UHPLC-IR

The amounts of sugars, acids, and alcohols of the final wines were determined by HPLC (Thermo Fisher Scientific, Waltham, MA, USA) with a HyperREZTM XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) equipped with a Hyper RETZM XP Carbohydrate Guard (Thermo Fisher Scientific). The sugars and alcohols were detected by a refraction index detector and the acids by a UV detector. The samples were filtered through a 0.22-μm nylon filter and diluted according to their estimated residual sugar amount. The analysis conditions were: 1.5 mM of H_2SO_4. 0.6 mL/min flux, a pressure of 35 bars, and an oven temperature of 50 °C. The concentrations of these compounds were determined by using the calibration curves of the corresponding standard compounds.

2.4. Determination of melatonin during the screening by UHPLC-Fluorescence

Only melatonin was determined during the screening by using a UHPLC coupled with fluorescence detector, the supernatant stored in the freezer was combined with methanol at a 50:50 ratio and filtered using nylon syringe filters with a pore size of 0.22 μm and a diameter of 13 mm. Chromatographic analysis was conducted using an AccucoreTM C18 column (4.6 × 150 mm, 2.6 μm; Thermo Fisher Scientific, Waltham, MA, USA) with mobile phases A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile). The flow rate was set at 0.8 mL/min, and the injection volume was 20 μL. The gradient program was as follows:

0–2 min, 80 % A, 20 % B; 2–7 min, a gradient from 20 % to 44 % B; 7–11 min, 10 % A, 90 % B; 11–17 min, 80 % A, 20 % B. The column temperature was maintained at 30 °C, and samples were kept at 10 °C. The excitation and emission wavelengths were set at 286 nm and 350 nm, respectively. A fluorescence detector equipped with an Acquity ARC core (Waters, Milford, MA, USA) (Waters 2575 Fluorescence), a quaternary pump, an autosampler, and a degasser was employed. Calibration curves were generated with melatonin standards.

2.5. Determination of indolic compounds in the final wines by UHPLC-MS-MS

Waters Acquity UHPLC (Milford, Massachusetts, USA) coupled to a Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer equipment was used to carried out the analysis of the content of 9 indolic compounds in the final wines. The software used was MassLynx MS. The column used was an Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm, Waters, USA). The chromatographic conditions consisted of two mobile phases, water with 0.1 % formic acid (A) and methanol with 0.1 % formic acid (B), with a gradient elution programmed. The flow rate selected was 0.3 mL/min and the injection volume was 5 μL. The gradient elution was programmed as follows: 95 % A, 5 % B (0–1 min); 90 % A, 10 % B (1–1.5 min); 80 % A, 20 % B (1.5–2 min); 70 % A, 30 % B (2–3 min); 60 % A 40 % B (3–4 min); 50 % A 50 % B (4–5 min); 0 % A 100%B (5–7 min). As shown, the 9 compounds were eluted in 7 min. The temperature of the column was set at 40 °C and 10 °C for the autosampler. For the MS-MS detection, the instrument was operated in ESI + mode. The ionization of the compounds was carried through a heated electrospray ionization source (HESI). The two most abundant fragments were selected for each metabolite, one as quantifier (most abundant) and the other as a qualifier ion, using the MRM method for selective quantification. Probe capillary voltage was 1.5 kV. Desolvatation and source temperature were 450 °C and 150 °C respectively. Source offset was 20 V. The source gas flow desolvation was 700 L/Hr and the cone 150 L/Hr. Nitrogen (Peak Scientific, UK) and Argon, used for collision-induced dissociation (CID), were coupled to the mass detector. The guidelines of the Association of Official Analytical 142 Chemist (AOAC, 2012) were used for the validation for the method. Samples were filtered through PTFE 0.45 μm prior to analysis.

2.6. Determination of volatile compounds by SPME/GC/MS analyses

Volatile compounds' extraction was performed using Headspace Solid Phase Microextraction (HS-SPME), employing a triple fiber of 1 cm DVB (divinylbenzene)/C-WR (carbon wide range)/PDMS (polydimethylsiloxane) (Agilent Technologies, Switzerland). For this purpose, 4 mL of each sample was transferred into 20 mL glass vials with 0.8 g of sodium chloride and 10 μL of 4-methyl-2-pentanol (0.75 mg/L) used as an internal standard, which were then placed in the autosampler. For the static headspace extraction, incubation lasted 40 min at 45 °C and 250 rpm agitation speed, and the fiber was then exposed to the headspace for 40 min, with a penetration into the vial of 22 mm. Once the adsorption finished, the fiber was desorbed for 180 s in the injector using the splitless mode. For the analyses of the samples, a Bruker 450 Gas Chromatograph was employed coupled to a Mass Spectrometer Bruker 300-MS. For the chomatographic analysis, the conditions followed are described in Ubeda et al. (2019). For identification purposes, linear retention indices (LRIs) were calculated after the injection of C10-C40 alkanes solution by applying the same conditions of sample analysis. Identification was made by matching the LRIs of each compound from the NIST standard library (version 2.0) with those found in the literature (Pherobase: www.pherobase.com; NIST Mass Spectrometry Data Center: LRI Odour database: www.odour.org.uk; accessed on March 2022).

2.7. Statistical analysis

All the experiments were carried out at least in triplicate. Data obtained were compared using ANOVA and Fisher Least Significant Difference (LSD) Method ($p < 0.05$) employing the InfoStat software (version 2017p, FCA Universidad Nacional de Córdoba, Argentina). Principal component analysis (PCA) was performed using the web server Clustvis (https://biit.cs.ut.ee/clustvis/), a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap (Metsalu and Vilo, 2015).

3. Results

3.1. Yeast screening for the production of tryptophan-derived compounds

To screen for higher indolic compounds yeast producers, a total of 81 strains from different species and genus were grown in an enriched YNB medium with tryptophan, to facilitate the production of these tryptophan-derived metabolites, and the extracellular concentration of melatonin was used as the representative metabolite of these pathways. This selection included *S. cerevisiae* strains from winemaking environments, all of which are theoretically adapted to the different stresses exerted during wine fermentation such as osmotic stress and the presence of ethanol. We also included specialized *S. cerevisiae* strains used in other types of traditional fermentative processes, such as tequila, cachaça, beer, etc., together with environmental *S. cerevisiae* strains (labelled as non-winery environment) and *Saccharomyces* strains from other species such as *S. kudriavzevii, S. uvarum, S. paradoxus,* and *S. eubayanus* (labelled as *Saccharomyces* non-*cerevisiae*). We completed the array of yeast strains assayed with the incorporation of different species found in the winery environment, such as *Zygosaccharomyces bailii, Pichia kudriavzevii, Torulaspora delbrueckii, Pichia membranaefaciens, Wicherhamiella pararugosa,* and *Candida cantarelli*, which are generically labelled as non-*Saccharomyces*.

All these strains are ranked in Fig. 1 in terms of melatonin production in the tryptophan-enriched YNB medium (melatonin concentration of each strain is shown in Supplementary Table 1). The highest melatonin production was observed in *S. cerevisiae* strains adapted to winemaking environments (green colour). *Saccharomyces* non-*cerevisiae* showed a trend to produce lower quantity of this molecule. In the case of non-*Saccharomyces* strains, we observed a greater variability in the quantities produced, depending on the species, but still lower than the wine *S. cerevisiae* strains.

The objective of this study was to generate a microbial consortium comprising diverse strains to increase indolic compounds and modified the aroma of the final wines. Despite their melatonin production in YNB, we focused our selection on the non-*cerevisiae* strains because most of these species have been mainly isolated from fermentation environments and have demonstrated a good fermentative capacity and aroma production (Su et al., 2019a, 2019b). This secondary screening was conducted in synthetic grape-must (SM) among various strains of *Saccharomyces* non-*cerevisiae* to combine high capacity of melatonin production and good fermentation activity.

The capacity of melatonin production in grape-must of the different tested strains is represented in Fig. 2. The species *S. uvarum* and *S. eubayanus* exhibited the highest quantities of melatonin, although with some exceptions. *S. paradoxus* strains yielded lower quantities than the other two species mentioned before, and, in the case of *S. kudriavzevii* strains, only one strain showed melatonin production in the grape-must. This result highlights the significance of conducting a screening, even within the same species, because of potential disparities among strains. Based on these results, strains A30 of *S. uvarum* and A32 of *S. eubayanus* were selected as candidates for the consortium. In addition, the selected strains were tested for their ability to complete fermentation and show good characteristics for a winery process.

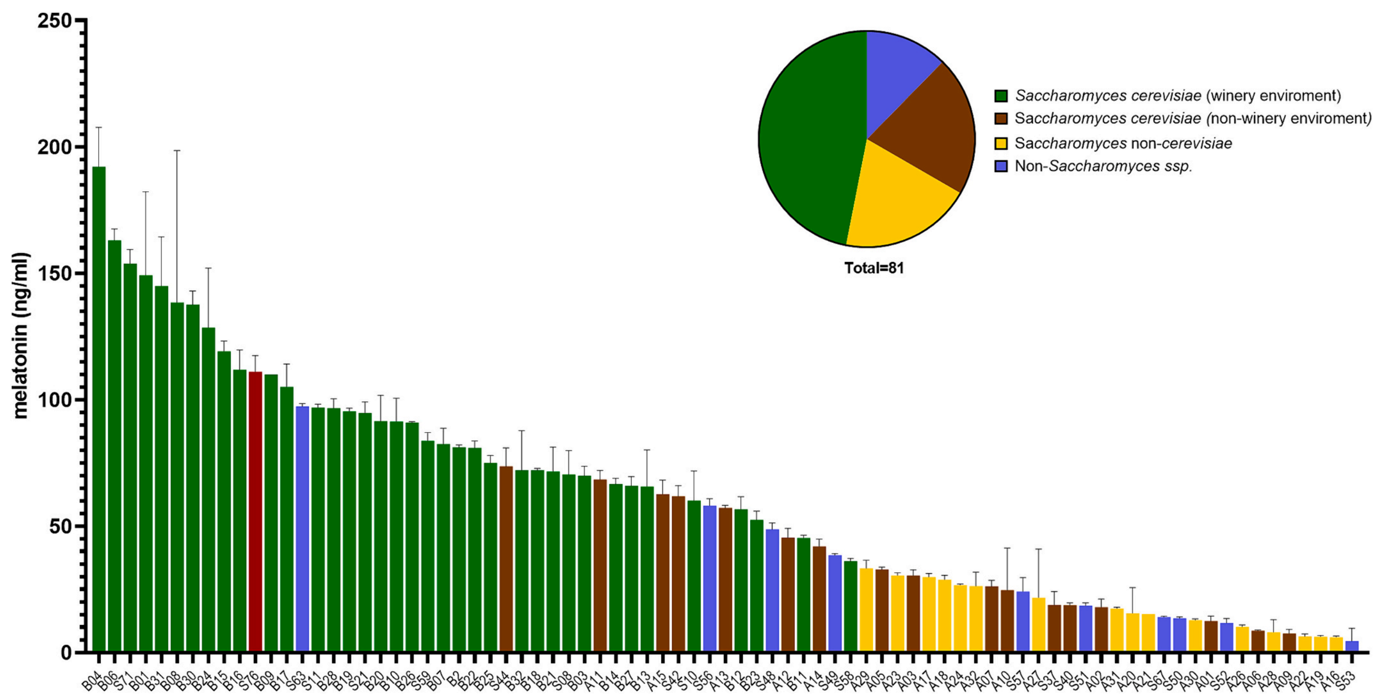

Fig. 1. Melatonin production levels for each strain from the screening. Samples were analyzed after 120 h of growth on YNB medium supplemented with tryptophan. The strains represented are: *Saccharomyces cerevisiae* from winery environment (green), *S. cerevisiae* from other environments (brown), *Saccharomyces* non-*cerevisiae* (yellow) and non-*Saccharomyces* yeast species (blue). Data were performed in triplicate.

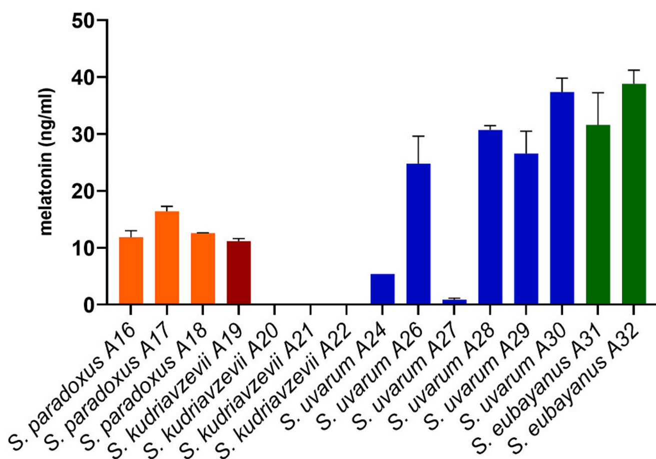

Fig. 2. Relative amounts of melatonin production for *Saccharomyces* non-*cerevisiae* strains. Samples were analyzed after 120 h of growth on synthetic must (SM). *S. paradoxus* (orange), *S. kudriavzevii* (red), *S. uvarum* (blue), *S. eubayanus* (green). Data were performed in triplicate.

3.2. Design of yeast consortium

The aim was to obtain wines enriched with a higher abundance of indolic molecules and aroma compounds, thus enhancing the overall quality, biostability and bioactivity of the wine. To achieve this, two pairwise consortia were designed: one comprising *S. cerevisiae* and *S. uvarum*, and another involving *S. cerevisiae* and *S. eubayanus*. Despite the good fermentation behavior of the selected non-*cerevisiae* strains, we considered it advisable to have a strain of *S. cerevisiae* in the consortium, which has proven to be more competitive under the harsh fermentation conditions, to ensure the completion of the process. Therefore, we selected the *S. cerevisiae* B04 strain (*Sc*), which was the highest melatonin producer in the YNB, in combination with either the *S. uvarum* A30 (*Su*) or the *S. eubayanus* A32 (*Se*).

These consortia were inoculated using two distinct approaches: either through co-inoculation, maintaining a ratio 1:1 of each strain, or via sequential inoculation, with the non-*cerevisiae* strain initially inoculated and followed by the *Sc* strain at 24 h. This approach has been implemented because of the strong competitive advantage of *S. cerevisiae*, which requires careful consideration in the overall process. The rapid and robust growth of *S. cerevisiae* can potentially outcompete and suppress the coexisting strain, disrupting the favorable microbial interactions that are critical for succeeding in our aim of enhancing metabolic interactions. As control fermentations, a pure culture of each of the strains participating in the consortia was inoculated in the same conditions that the mixed cultures. The fermentations were carried out at a temperature of 20 °C in an attempt to increase the competitiveness of the non-*cerevisiae* strains, due to their superior cryotolerance compared to the *S. cerevisiae* species (Su et al., 2019a, 2019b).

The kinetics of growth and fermentation activity, measured as OD and density, are shown in Fig. 3. The pure cultures of *Sc* and *Su* displayed a similar fermentative behavior and growth yield, ending-up the fermentations in 5 days, with densities below 998 g/L and complete sugar consumption (Fig. 3AB and Supplementary Table 2), whereas the pure culture of *Se* took one day longer to consume all the sugars of the SM, displaying a slower glucose consumption rate (Fig. 3C and Supplementary Table 2). Anyway, these results evidenced that the three strains are able to finish a wine fermentation by themselves. Regarding the mixed fermentations, the presence of both strains also shows good fermentation capacities. Curiously, the end of fermentations was even shorter in the case of coinoculation of *Sc* and *Se* (Figure 3DEFG and Supplementary Table 2).

In the mixed fermentations, we also determined the presence of each strain in three different stages of the process, which corresponded with initial (IF), middle (MF) and final fermentation (FF), plotted as implantation percentage in Fig. 4. This percentage represents the competitiveness of each strain throughout the fermentation process. In the case of the co-inoculated fermentations, we observed a rapid imposition of the *Sc* vs. the *Su* strain after 48 h of fermentation, which was kept until the end of fermentation. Conversely, *Se* strain showed a

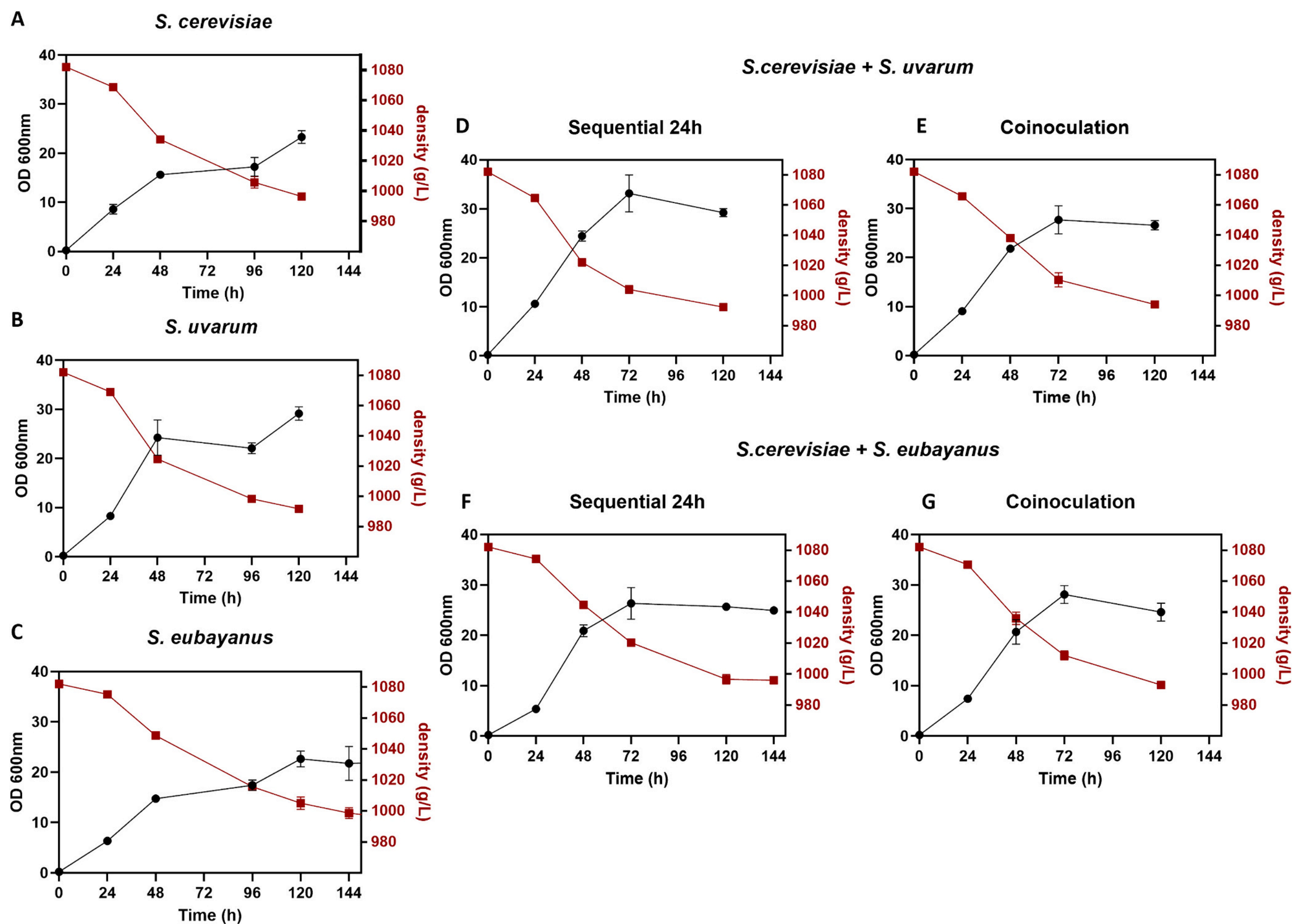

Fig. 3. Kinetics of pure and sequential fermentations of *S. cerevisiae* (*Sc*), *S. uvarum* (*Su*), and *S. eubayanus* (*Se*). Graphs show growth (OD_{600}) and density (g/L) for each fermentation assay. (A) Pure fermentation of *S. cerevisiae* (B) Pure fermentation of *S. uvarum* (C) Pure fermentation of *S. eubayanus* (D) Fermentation with sequential inoculation of *Su* + *Sc* (E) Co-inoculation fermentation of *Su* + *Sc* (F) Fermentation with sequential inoculation of *Se* + *Sc* (G) Co-inoculation fermentation of *Se* + *Sc*. Data were performed in triplicate.

co-existence with the *Sc* strain, with similar percentages in the first stages of the fermentation to end-up with a clear imposition of *Sc* at the end of the process.

In the case of sequential inoculations, the *Sc* strain was inoculated after 24 h of the non-*cerevisiae* inoculation. As expected, 100 % of the colonies analyzed belonged to the non-*cerevisiae* at IF (24 h), but the same result was also observed after 48 h of fermentation (24 h after *Sc* inoculation). This percentage in favor of the non-*cerevisiae* strains was also kept at the end of fermentation (FF), with 78 % and 72 % dominance for *Su* and *Se* respectively. Despite the greatest competitiveness of *Sc* during wine fermentation, its inoculation after one day of fermentation occurred with a large population size of the non-*cerevisiae* strains and *Sc* was unable to impose to *Su* or *Se*. In fact, we also carried out sequential inoculated fermentations in which the *Sc* strain was inoculated 48 h after the inoculation of *Su* or *Se*, and the percentage of implantation was 100 % in favor of *Su* and *Se* in all the stages of fermentation (data not shown). Therefore, the imposition capacity of *Sc* depends on the timing of inoculation regarding to the other strain in competence. The latter the *Sc* strain was inoculated the lower the chances of imposition of this strain are. A medium with a large population of the competitor strain and exhausted in key nutrients may explain this lack of dominance of the fittest species during wine fermentation.

3.3. Metabolic profile

Samples were collected at the end of fermentations to analyze the metabolic profiles of indolic compounds derived from tryptophan and volatile metabolites responsible for the aromatic profile of the wines. The objective was to identify differences in these profiles, demonstrating how the interaction between different strains generates a distinct profile compared to that originated from pure cultures. To achieve this, various indolic metabolites derived from tryptophan were analyzed using HPLC-MS/MS, and volatile metabolites responsible for the aromatic profile of the wines were analyzed using GC–MS. These analyses were carried out on both pure culture fermentations and sequential inoculations, where there was a greater presence and interaction of both species throughout the fermentation. We excluded the coinoculated fermentation because the dominance of *Sc* was the majority from the beginning of the process.

3.3.1. Indolic compounds

The Principal Component Analysis (PCA) conducted on the indolic compounds derived from tryptophan revealed distinct profiles for each fermentation type, with replicates exhibiting close proximity (Fig. 5). The dispersion observed among the replicates can be attributed to inherent biological variation to the three fermentation replicates. In the case of the consortium *Sc* + *Su* (Fig. 5A and B), Principal Component 1, accounting for 44.4 % of the total variability, effectively discriminated the pure *Sc* fermentations from the other samples. Principal Component 2, explaining 19.9 % of the total variability, successfully differentiated

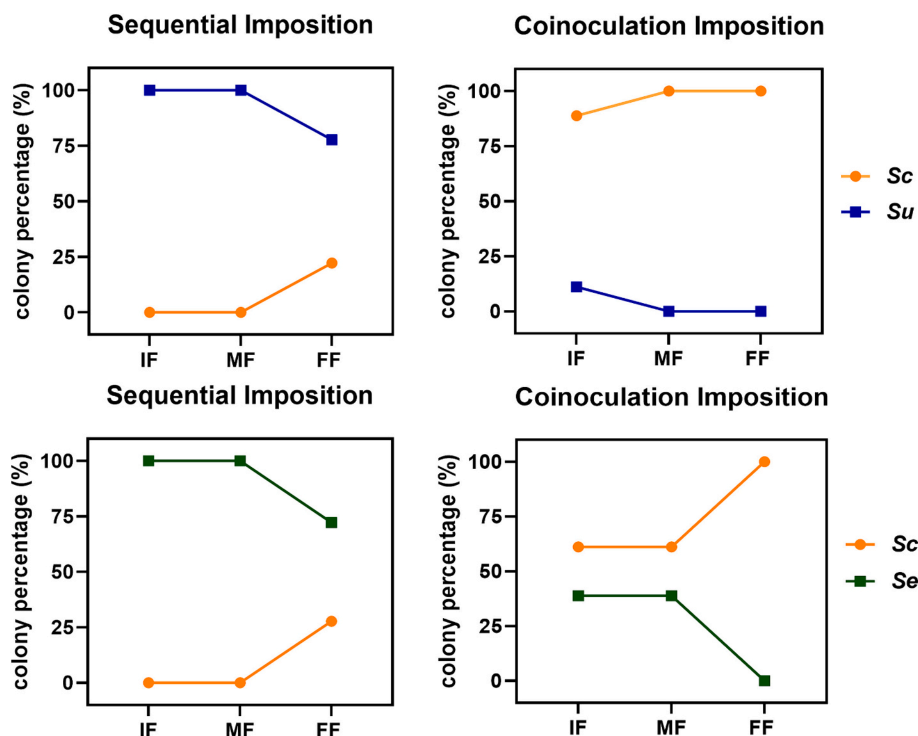

Fig. 4. Percentage of imposition of *S. cerevisiae* (*Sc*), *S. uvarum* (*Su*), and *S. eubayanus* (*Se*) at different fermentation stages: Initial fermentation (IF), mid-fermentation (MF) and final fermentation (FF) of sequential and co-inoculation fermentations.

the pure *Su* fermentations from the sequentially inoculated fermentations. Hence, the fermentations exhibited satisfactory discrimination with a cumulative variability of 64.3 % among the analyzed samples. Analysis of the loadings revealed significant contributions from key metabolites such as serotonin, 3-indoleacetic acid, and 5-hydroxytryptophan to the differentiation observed in Principal Component 1, indicating their contribution in fermentations involving the *Su* strain. In terms of Principal Component 2, the variables with the highest weight in distinguishing the sequential fermentation from the pure *Su* fermentation were identified as tryptophol and tryptophan.

Regarding the fermentations with *Sc* + *Se*, similar patterns emerged (Fig. 7C and D). The PCA revealed distinct profiles for each type of fermentation, with close proximity between replicates. In this case, Principal Component 1 accounted for a higher percentage of variance, reaching 62.7 % of the total, effectively separating the pure *Sc* fermentations from the rest, as previously observed. Meanwhile, Principal Component 2 explained 18.4 % of the variability and distinguished the pure *Se* fermentations from the sequentially inoculated ones. The observed differences are collectively explained by 81.1 % of the total variability among the samples. Notably, the compounds with higher loadings in Principal Component 1 are serotonin and 3-indolacetic acid, which were prominent in the fermentations involving the *Se* strain. Regarding the loadings of Principal Component 2, which separated the pure *Se* culture from the sequential inoculation, we observed that tryptophan carried a significant weight, followed by N-acetylserotonin.

The concentration values of these tryptophan-derived compounds were represented on a heatmap with hierarchical clustering for each *Saccharomyces* consortium assayed (Fig. 6). In both consortia, the non-*cerevisiae* strain grouped with the mixed culture fermentation, evidencing that *Su* and *Se* were impacting more significantly in the content of indolic compounds in the wines obtained by sequential inoculation. However, we also observed significant differences in some compounds and in the higher total content, due to the addition of all these molecules, in the mixed cultures compared to those obtained in the pure fermentations (Fig. 6 and Supplementary Table 3). This is the case of serotonin and 5-hydroxytryptophan, which showed a higher concentration in the *Sc* + *Su* fermentation than in the pure fermentations, and particularly noteworthy was the presence of indole-3-acetic acid, which was detected exclusively in the *Sc* + *Su* mixed fermentation, suggesting that the co-presence of both strains facilitates the production of this compound and demonstrates the metabolic interaction between the two species for the biosynthesis of these interesting molecules.

When comparing fermentations with *Se*, serotonin and indole-3-acetic acid also stood out by a higher concentration in the mixed fermentation *Sc* + *Se* (Fig. 6 and Supplementary Table 3). However, the remainder indolic compounds showed a lower concentration in the sequential inoculated fermentation. Again, the most paradigmatic compound as a result of metabolic interaction between the strains was the indole-3-acetic acid, which was not detected in the pure *Sc* fermentation and reached a concentration of 131.68 ng/mL in the *Se* fermentation. However, when both strains were present in the *Sc* + *Se* fermentation, the titer increased to 194.45 ng/mL, being the highest levels of all fermentations.

3.3.2. Volatile compounds

The volatile compounds were analyzed by GC–MS and a total of 34 volatile compounds were determined, and classified into 4 chemical groups, most of them esters, followed by alcohols and in minor amounts acids and ketones (Supplementary Table 4).

As for the indolic compounds, a PCA was carried out with the volatile metabolites associated with aromas of all the fermentations of each consortium of strains (Fig. 7). In the consortium *Sc* + *Su*, Principal Component 1 has 48.5 % of the variance and effectively differentiated the pure *Sc* fermentation from the others. Principal Component 2 has 21.9 % of the variance, however, in this case, one sample of the mixed *Sc* + *Su* fermentation cannot be differentiated of the pure *Su* fermentations and grouped together. Anyway, the fermentations exhibited satisfactory discrimination with a cumulative variability of 70.4 % among the analyzed samples. The *Su* pure fermentation and the sequential combination of *Sc* + *Su* have a similar volatile profile, with the *Su* strain more significantly impacting on the aroma profile of the

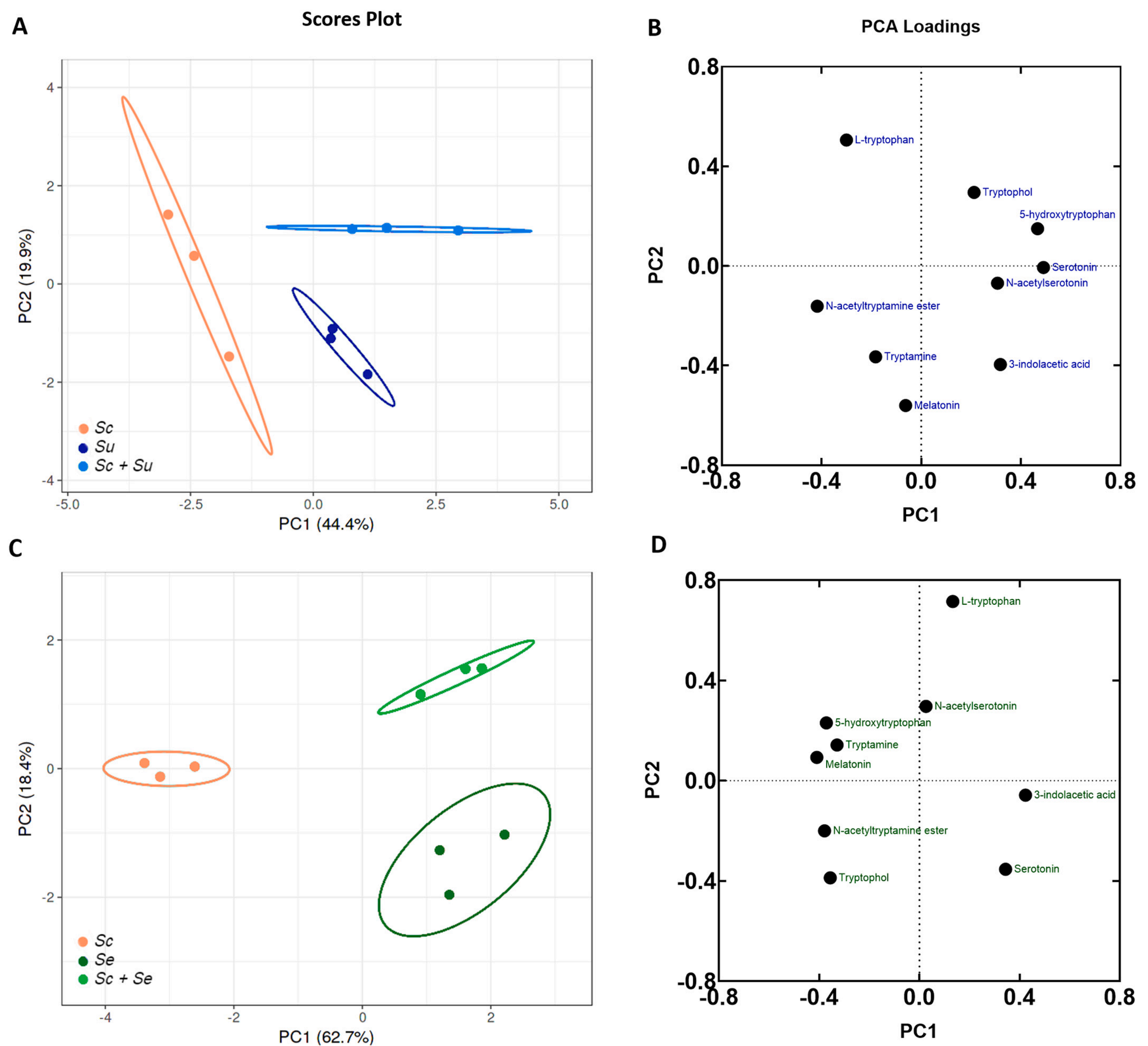

Fig. 5. Principal component analysis (PCA) of tryptophan-derived indolic compounds produced in fermentations. (A-B) PCA of pure and sequential inoculation fermentations of *S. cerevisiae* and *S. uvarum* (C-D) PCA of pure fermentations and sequential inoculation fermentations of *S. cerevisiae* and *S. eubayanus*.

mixed fermentation, as can be seen from the score plot (Fig. 7A). From the loadings plot it is possible to conclude that alcohols and esters are the volatile compounds that discriminate between the fermentative types. The *Sc* strain was the highest producer of ethyl esters, whereas the *Su* strain stood out by the great synthesis of alcohols and acetate esters, mainly of 2-phenyl ethanol and 2-phenyl-ethyl acetate. In the consortium *Sc* + *Se*, it can be seen that the variance explained by Principal Component 1 is 40.3 % and for Principal Component 2 it is 19.8 %, with a total variance of 60.1 %. Similarly to the consortium *Sc* + *Su*, the pure *Se* and the mixed *Sc* + *Se* fermentations were not clearly discriminated in the PCA. In this case, it is even more difficult to separate these two groups, which have similar aromatic profiles. Therefore, the presence of the *Se* strain clearly determined the final aroma in both fermentations. In the loading plot (Fig. 7B), it can be seen that the volatile compounds responsible for this classification are the acetate esters, determined in the case of fermentations with the presence of the *Se* strain, and the ethyl ester synthesis, determined by the presence of the *Sc* strain.

We also represented the concentration of 34 volatile compounds influencing and defining the aromatic profile of fermentations on a heatmap with hierarchical clustering (Fig. 8). Among these compounds, there are 15 esters, 11 alcohols, 5 acids, and 3 ketones. Curiously, when only pure fermentations were considered (Fig. 8A), *Sc* fermentations grouped with *Se* fermentations, with *Su* showing a more differentiated volatile profile. *Sc* exhibited higher quantities of ketones compared to non-*cerevisiae* strain pure cultures, except for acetoin, which is more prevalent in pure *Se* cultures. In terms of acids, medium chain fatty acids such as octanoic and decanoic acids predominated in the *Sc* culture, whereas isobutyric and isovaleric acids predominated in the *Se* and *Su* cultures respectively. Regarding to alcohols, *Su* fermentations stand out for their elevated presence of higher alcohols, specifically propylene glycol, 3-methyl-1-butanol, isobutanol, and 2-phenylethanol. In the case of *Sc* fermentations highlighted the concentration of 2-propyl-1-pentanol, 2-ethyl-1-hexanol and 3-ethoxy-1-propanol. The highest alcohol concentration in the *Se* fermentations was 1-octanol. Finally, the ester

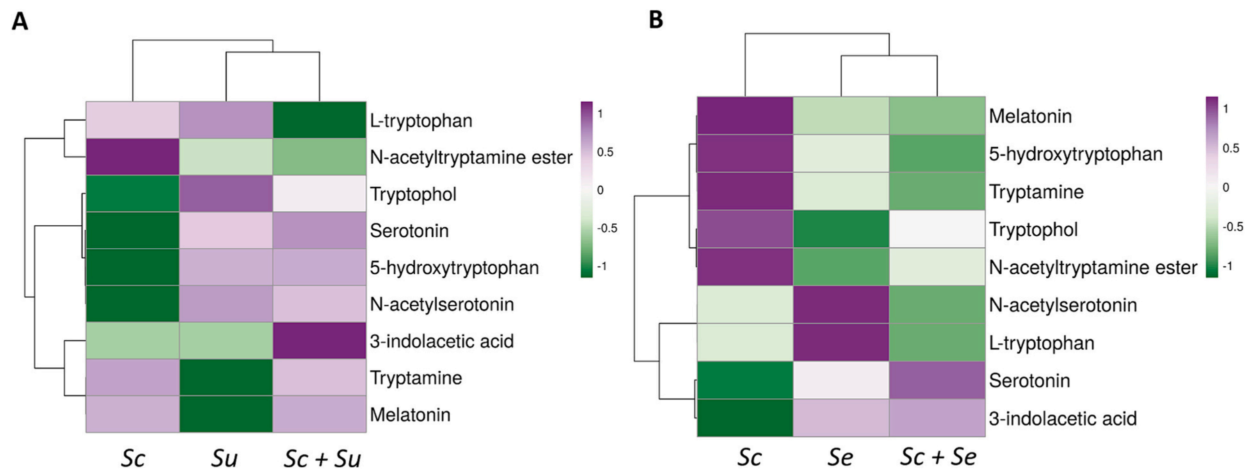

Fig. 6. Heatmap of tryptophan-derived indolic compounds produced in (A) pure and sequential inoculation fermentations of *S. cerevisiae* and *S. uvarum* (B) pure and sequential inoculation fermentations of *S. cerevisiae* and *S. eubayanus*.

concentration was mainly correlated with the synthesis of acids and alcohols in each of the strains. Accordingly, *Sc* fermentations increased esters of medium chain fatty acids (ethyl hexanoate, ethyl octanoate and ethyl decanoate), whereas pure *Su* fermentations exhibit high concentration in several acetate esters, such as 2-phenyl ethyl acetate, isoamyl octanoate, and hexyl formate. In the case of the pure *Se* culture fermentations, aromatic compounds are lower compared to the other two strains, but notable for the presence of some ethyl esters such as ethyl heptanoate, ethyl 9-decenoate, and ethyl 9-hexadecenoate, all of them imparting a pleasant fruity aroma.

Fig. 8B and C illustrate the heatmap of volatile compounds produced in mixed fermentations resulting from sequential inoculation at 24 h, alongside compounds produced in pure fermentations of each implicated strain. In the consortium of *Sc* with *Su*, the aromatic profile of *Sc* + *Su* fermentations appeared more similar to the *Su* aromatic profile, consistent with our PCA. Nonetheless, it is worth highlighting that *Sc* inoculation into a prior *Su* culture generates an alteration in the final aromatic profile, fostering synergy that increases compounds in higher levels than pure cultures. Some examples of these increased compounds are 2-phenylethanol, methionol, and R2-3butanediol among alcohols, and ethyl 9-hexadecenoate, ethyl 9-decenoate, and ethyl 2-phenyl acetate among esters. Regarding *Sc* + *Se* fermentation (Fig. 8C), its aromatic profile is quite similar to *Se*, although with decreased quantities of esters and alcohols overall, with the only exception of the ester ethyl 9-hexadecenoate, which shows a notable increase.

4. Discussion

Several studies and small screenings have been conducted among different wine strains to investigate their ability to produce melatonin during fermentation (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015). However, this is the first study to screen a large number of yeast strains and species, mainly associated with fermentative processes, for their ability to produce melatonin, a tryptophan derivative with recognised bioactive properties. The highest number of analyzed strains belong to *S. cerevisiae* because it is the main fermentative species. Within this species, there exists a great genetic diversity present in both natural and industrial environments, where these yeasts have evolved under different environmental pressures. According to our data, the adaptation of the *S. cerevisiae* strains to wine-related environment has enhanced the capacity to synthesise melatonin. The highest melatonin producers belong to this group of strains whereas the rest of *S. cerevisiae* strains isolated from wild and other fermentative processes showed a lower production. Melatonin synthesis in yeast have been associated with an antioxidant role (Vázquez et al., 2017; Bisquert et al., 2018; Sunyer-Figueres et al., 2020) and a protective molecule against ethanol stress (Sunyer-Figueres et al., 2021). Wine fermentation is a very stressful process which can promote the adaptation to this harsh environment by promoting the synthesis of these protective molecules against the multiple stresses. In other words, the differences between the responses of environmental and wine *S. cerevisiae* strains could be related to genetic differences shaped by human activity (domestication). Spor et al. (2009) also observed that populations adopted different strategies depending on their ecological niche. These authors found that forest and laboratory strains have a low reproduction rate in respiration and produce lower quantities of ethanol, suggesting that they store cell resources rather than secreting secondary products. Contrarily, wine strains of this species reproduce slowly, reach a small carrying capacity but have a big cell size in fermentation and a high reproduction rate in respiration with higher glucose consumption rates.

There are others *Saccharomyces* species that have been connected with fermentation processes, either as pure species, such as *S. uvarum* and *S. paradoxus*, or as one of the parental of hybrid strains, such as *S. eubayanus* and *S. kudriavzevii*, (González et al., 2006; González et al., 2007; Libkind et al., 2011). These non-*cerevisiae* strains have been proposed as alternative to *S. cerevisiae* to be inoculated in wine fermentations at low temperature due to their cryotolerant character (Su et al., 2019a; Su et al., 2019b). We also detected some non-*cerevisiae* strains that were able to synthesise melatonin in quantities over the average and also showed a good fermentation capacity. Two strains of *Su* and *Se* were selected with the aim of using them in a consortium with the selected *Sc* strain in order to enhance these compounds of interest and influence the final wine aroma profile.

The use of mixed inoculum has become a usual practice in wine production since several yeast species have shown high oenological potential (Rossouw and Bauer, 2016; Binati et al., 2019). Indeed, yeasts like *Saccharomyces* non-*cerevisiae* (Alonso-del-Real et al., 2017), non-*Saccharomyces* (Varela et al., 2016; Binati et al., 2020), and even artificial hybrids (Origone et al., 2020) are of interest, because their different metabolisms compared to *S. cerevisiae* brings diversity to quantitative and qualitative composition of final wine (for example, ethanol content, organic acids, aroma production, etc.). However, all these studies show that the use of these yeasts in combination with *S. cerevisiae* as wine starters is still a challenge due to the unpredictability and lack of reproducibility of the results. Among the different parameters that determine the competence of each of the species of the mixed inoculum, the inoculation procedure is of paramount importance. We conducted mixed fermentations with the simultaneous or sequential inoculation of yeasts, with various times between both inoculations (24 and 48 h). Co-inoculations with 50 % of each strain at the beginning of

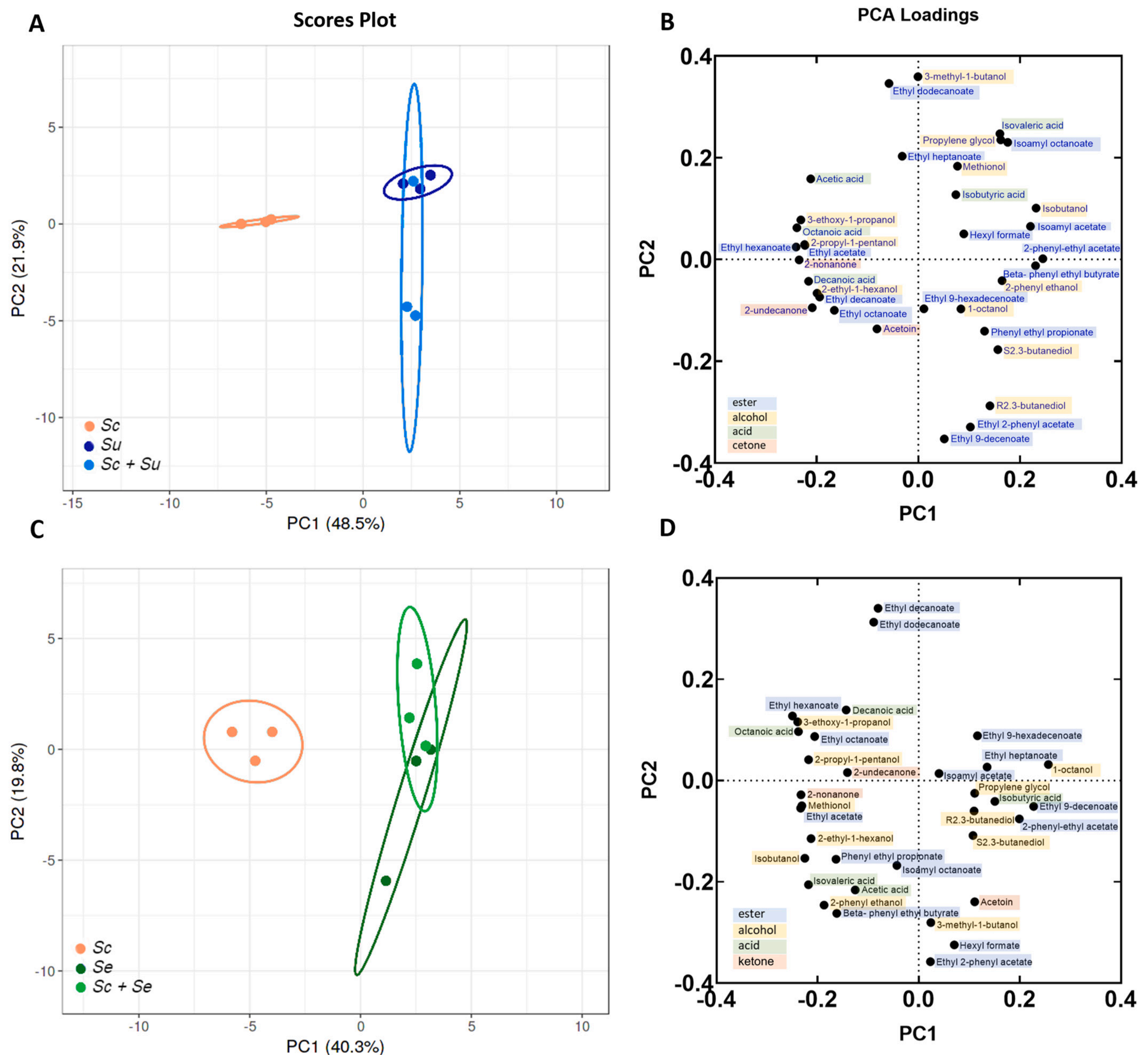

Fig. 7. Principal component analysis (PCA) of aromatic compounds produced in (A-B) pure and sequential inoculation fermentations of *S. cerevisiae* and *S. uvarum* (C-D) pure and sequential inoculation fermentations of *S. cerevisiae* and S. *eubayanus*.

the process favoured the rapid implantation of *Sc* that outcompeted to *Su* and *Se*, despite the fermentations were carried out to 20 °C to increase the competitiveness of the cryotolerant species *Su* and *Se* (Su et al., 2019a). It is well-known the highest competence of *Sc* during wine fermentation due to its ability to withstand high ethanol concentrations, low nutrient availability, and anaerobic conditions (Lax and Gore, 2023). However, we detected a co-existence of both strains when the *Sc* inoculation was delayed 24 h (sequential inoculation). Despite the fact that the same population size of each strain was inoculated, after 24 h, *Sc* had to face to a larger population of the non-*cerevisiae* strain and a growth medium depleted in some key nutrients such as vitamins and amino acids (Comitini et al., 2021). In fact, when the *Sc* inoculation was carried out 48 h after the *Se* or *Su* inoculation, we did not detect the presence of the *Sc* strain at any stage of the fermentation. It has been previously reported, in sequential mixed cultures, an insufficient initial amount of assimilable nitrogen can be entirely consumed by non-*Saccharomyces* yeasts before the inoculation of *S. cerevisiae*, resulting in incomplete fermentation (Taillandier et al., 2014). We also evidenced that temperature is the most important parameter governing the implantation of both *S. eubayanus* and *S. uvarum* in mixed cultures with *S. cerevisiae*, however, we also observed a better competition capacity of these non-*cerevisiae* in grape-musts with low nitrogen content because of their low nitrogen requirement (Su et al., 2019a).

As consequence of the percentages of residence of each species during wine fermentations, we focused our further analysis of indolic and volatile compounds on the sequential fermentation with inoculation of *Sc* after 24 h. These fermentations showed a co-existence of both species during the process, but with a higher percentage in favor of the non-*cerevisiae* strain. The PCA analysis of the indolic compounds clearly confirms this interaction between the two species, as the principal components allow the pure culture conditions to be distinguished. Non-*cerevisiae* pure culture and mixed culture fermentation showed similar

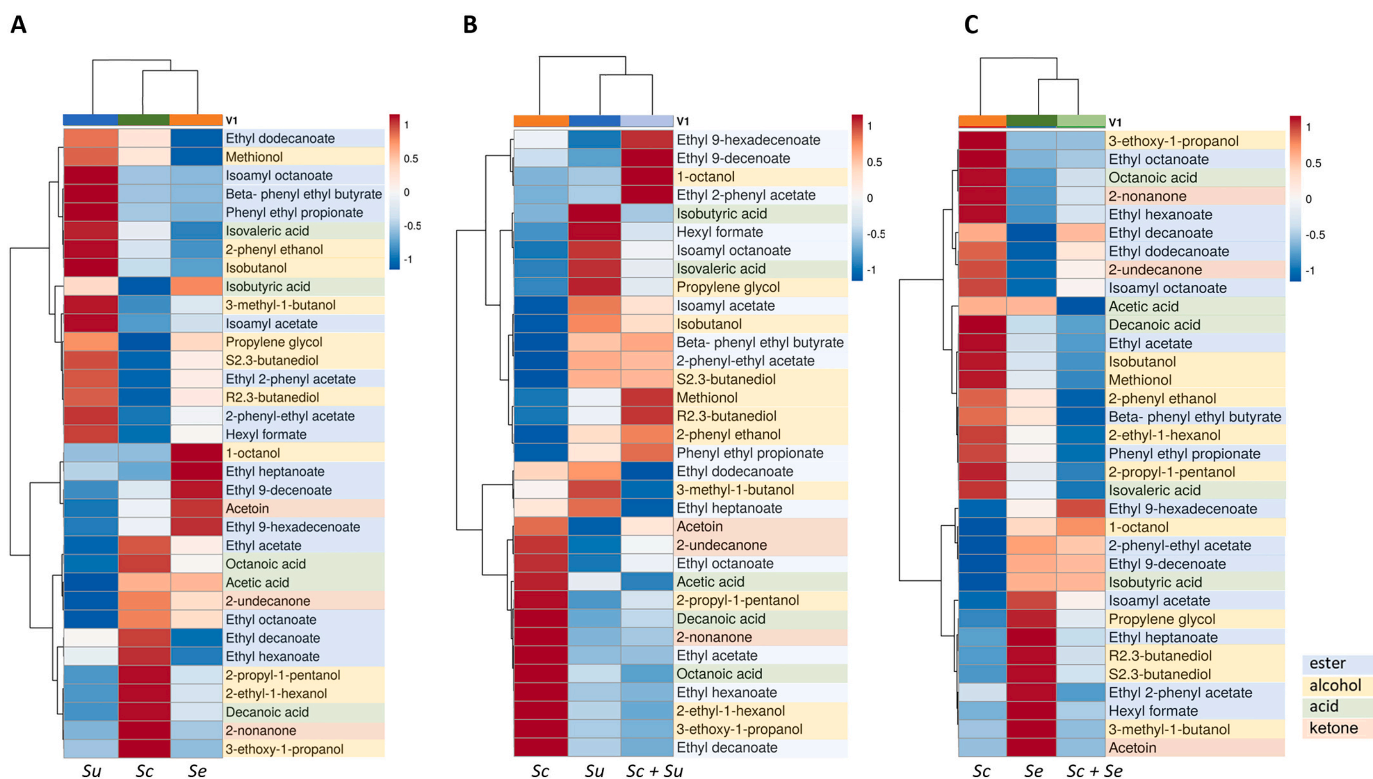

Fig. 8. Heatmap of aromatic compounds produced in (A) pure fermentations of *S. cerevisiae* (*Sc*), *S. uvarum* (*Su*), and *S. eubayanus* (*Se*) (B) pure and sequential inoculation fermentations of *S. cerevisiae* and *S. uvarum* (C) pure and sequential inoculation fermentations of *S. cerevisiae* and *S. eubayanus*.

indolic profile between them. However, the metabolic interaction can also be assessed, since the presence and concentration of some compounds were not only the average of the synthesis capacity of each strain, but a quantity that could only be explained because both strains have synergistically collaborated in the synthesis of the different precursors of the pathway, which was ultimately our main objective in this study. In the case of *Sc* + *Su*, the most paradigmatic compound is the indole-3-acetic acid, which was only detected in an important amount in the mixed fermentation. This compound was also enhanced in the *Sc* + *Se*. Another compound that increased in both mixed fermentations was serotonin. In absolute terms, the sum of all these indolic compounds yielded a significant higher concentration in the mixed fermentations in comparison with the three pure fermentations (Supplementary Table 3). An interesting data is these mixed cultures made a better use of the initial tryptophan of the SM because the residual concentration of this amino acid was significantly lower in the final wines of the mixed fermentations. This data could explain the higher yield of tryptophan-derived compounds when two *Saccharomyces* strains are interacting in the grape-must. In any case, our results clearly proved that the design of yeast strains selected on the basis of their synthesis capacity can be a good strategy to increase the concentration of these bioactive tryptophan-derived compounds in wines. Our previous results have shown that these compounds are promising as bioactives for the protection towards neurological disorders and angiogenesis, this last related to cardiovascular and cancer diseases. We demonstrated that serotonin, melatonin and some other related indolic compounds showed an inhibitory and destabilizing effect on Aβ peptide fibril formation and in the abnormal assembly of α-synuclein, which are related with Alzheimer's and Parkinson's diseases (Hornedo-Ortega et al., 2018; Gallardo-Fernández et al., 2019), and could effectively inhibit VEGF-induced VEGFR-2 activation and subsequent angiogenesis. In particular, indole-3-acetic acid showed the highest inhibitory effect, followed by 5-hydroxytryptophol, melatonin and serotonin (Cerezo et al., 2017; Cerezo et al., 2019).

In a previous study of our group (Su et al., 2019a), we determined the volatile profile produced by different *Saccharomyces* species during fermentations at different temperature and nitrogen content in the SM. Despite, the number of volatile compounds studies was much more limited (only 11) and the differences in the fermentation conditions and strains, there are some traits specific of the yeast species that are common to both studies. On one hand, *Sc* strain was clearly the highest producer of ethyl esters, mainly ethyl hexanoate, ethyl octanoate, and ethyl decanoate, which are usually responsible of the fruity aromatic characteristics of the wine. On the other hand, *Su* strain was the best producer of higher alcohols and their respective acetate esters. The correlation between the formation of acetate esters and their corresponding higher alcohol was previously described in wines by Cordente et al. (2012). In addition, in this study, *Se* was shown as the strain with higher capacity for the production of esters derived from long chain fatty acids, such as the ethyl 9-hexadecenoate (C18) and ethyl 9-decenoate (C12). In terms of metabolic interactions, the volatile analysis also evidenced the boosting of some compounds in the sequential fermentations, mainly of some esters that provides very pleasant aroma to the wines. This improvement in aroma as a result of the interaction between the two species should be validated by a sensory analysis of these wines.

5. Conclusions

The wine industry requires a high diversity of species and strains to inoculate its musts and obtain wines with improved stability, bioactivity and different aroma profiles that contribute to wine quality and differentiation from other products on the market. In terms of wine stability and bioactivity, it may be interesting to increase the synthesis of different molecules derived from aromatic amino acid metabolism, such as different indolic compounds, with interesting antioxidant properties and benefits for the health of the consumers. This study demonstrates for the first time that the combination of different *Saccharomyces* strains with good fermentation capacity is able to increase the production of

some interesting tryptophan-derived compounds in the final wines. Our results also underline that the interactions between the yeast strains lead to metabolic interactions and synergies, as shown by the significant increases of some molecules in the sequential inoculation fermentations. Moreover, the selection of cryotolerant *Saccharomyces* strains ensures that the fermentations start at low temperature (20 °C) and the subsequent inoculation of *S. cerevisiae* also guarantees the total consumption of sugars. It is well known that low temperature fermentations produce wines enriched in the main fermentative aromas (Torija et al., 2003; García-Ríos et al., 2017) and the results of this study also demonstrate the increase of some interesting aromas as a result of the interactions with the inoculated yeast. In conclusion, this study represents a proof of concept that the design of yeast consortia for the inoculation of grape musts is a useful strategy to increase the production of some molecules that contribute to the stability and bioactivity of wines and to obtain an enriched and differentiated aroma profile that determines the typicality of the wines and their competitiveness in a global market.

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CRediT authorship contribution statement

Andrés Planells-Cárcel: Writing – original draft, Validation, Methodology, Investigation. **Julia Kazakova:** Methodology, Investigation. **Cristina Pérez:** Methodology, Investigation. **Marina Gonzalez-Ramirez:** Writing – review & editing, Validation. **M. Carmen Garcia-Parrilla:** Writing – review & editing, Validation, Funding acquisition, Conceptualization. **José M. Guillamón:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Data availability

No data was used for the research described in the article.

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