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Evaluating the impact of *Torulaspora delbrueckii* and amino acid concentration on the nitrogen metabolism of *Oenococcus oeni*

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ABSTRACT

This study investigates the impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* inoculation strategies and amino acid supplementation on nitrogen metabolism and malolactic fermentation (MLF) performance in *Oenococcus oeni*. *Oenococcus oeni* has low demand of nitrogen and typically prefers peptides over amino acids. In this work we studied the nitrogen metabolism (proteins, peptides, amino acids and biogenic amines (BA)) of three *O. oeni* strains. Higher initial amino acid concentrations generally accelerated MLF, particularly with the PSU-1 strain, which exhibited stuck fermentation in some conditions with low initial amino acid concentration related with coinoculation of yeasts. Nitrogen metabolism in *O. oeni* showed a preference for peptide-bound amino acids, and gene expression analyses highlighted a general upregulation in response to increased amino acid concentrations. BA production, particularly cadaverine and putrescine, was strain-dependent and associated with the presence of the *odc* gene. Besides, the production of 2-phenylethylamine was related with alcoholic fermentation (AF) inoculation strategy. Overall, the study demonstrates the complexity of nitrogen metabolism in *O. oeni*, emphasizing that it is influenced by both the yeast species used in AF and the initial amino acid composition, highlighting the importance of peptide composition, offering a potential tool for optimizing MLF in winemaking.

1. Introduction

Oenococcus oeni is the main lactic acid bacteria (LAB) species carrying out the malolactic fermentation (MLF) in wine (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). This process usually takes place after alcoholic fermentation (AF) undergone by oenological yeasts, transforming grape must into wine (Liu, 2002). During the winemaking process, which involves AF and MLF, nutrients from must are used to enable microbial growth (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). The high fermentative capacity of wine yeasts demands large amount of nutrients to support their growth and metabolic activity. Thus, after AF, wine is depleted from the most simple and easy assimilable nutrient sources such as sugars, free amino acids, or vitamins (Balmaseda, Bordons, Reguant, & Bautista-Gallego, 2018). Besides, AF greatly transforms the physicochemical characteristics of wine by increasing the concentration of some microbial inhibitor compounds as

ethanol, sulfur dioxide, medium chain fatty acids or several acids that decrease the pH value.

In this sense, the current trend of using non-*Saccharomyces*, for instance, *Torulaspora delbrueckii*, in winemaking, modulates the chemical composition of wine, which is known to be dependent of the inoculation strategy (Balmaseda, Rozès, Bordons, & Reguant, 2024; Martín-García, Balmaseda, Bordons, & Reguant, 2020). *T. delbrueckii* is reported as an interesting starter for red winemaking due to the organoleptic modulation (Benito, 2018), for instance, polyphenolic composition (Balmaseda, Rozès, Bordons, & Reguant, 2021b; Ruiz-de-Villa et al., 2023b), even for rosé wine production (Ruiz-de-Villa et al., 2023a). Moreover, the use of this yeast can increase the residual free amino acids after AF (Martín-García et al., 2020; Ruiz-de-Villa et al., 2023b).

Then, once AF is finished, MLF usually occurs in the newly developed matrix, which is wine. Under the restrictive wine conditions, *O. oeni* is

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able to grow and participate in the winemaking process due to its particular and well adapted metabolic activities (Bech-Terkilsen, Westman, Swiegers, & Siegmund, 2020). It is specialised in the use of alternative energy sources different from sugars. Indeed, the main energy for *O. oeni* in wine is L-malic acid, which is the substrate of MLF (Liu, 2002).

Apart from carbon, nitrogen sources are also necessary for the bacterium to develop. Even if *O. oeni* is considered as a fastidious bacterium due to its large autotrophies, the nitrogen demand of the bacterium is very low (Remize, Augagneur, & Guzzo, 2005). Studies on this topic revealed the low consumption of amino acids and the ability of the bacterium to grow without most of them, a characteristic that appeared to be strain dependant (Remize et al., 2006). Indeed, wines after MLF usually present higher concentration of free amino acids than after AF (Alcaide-Hidalgo, Moreno-Arribas, Polo, & Pueyo, 2008). This is not because of an increase in amino acid content by *O. oeni*, but because of the hydrolysis of peptides present in wine from yeasts and subsequent release of individual amino acids (Manca de Nadra, Fariás, Pueyo, & Polo, 2005).

Nitrogen composition in wine is mostly composed of proteins, peptides and free amino acids (Ribéreau-Gayon et al., 2006). Protein concentration is low and remains with not much variation due to a low consumption of wine microorganisms. As introduced before, the amino acid concentration in wine is also very low, around 20 mg N/L. Besides, the largest source of nitrogen in wine are peptides (Alcaide-Hidalgo et al., 2008; Martínez-Rodríguez, Carrascosa & Polo, 2001). Peptides can represent up to 100 mg N/L in finished wine, and they are the preferent nitrogen source for *O. oeni* in wine.

Another nitrogenous compound found in wine are biogenic amines (BA). BA are polyamines derived from the decarboxylation of individual amino acids (Landete, Ferrer & Pardo, 2007; Lonvaud-Funel, 1999). Their concentration can represent up to 20 mg/L, and they can have a negative effect in the health of the consumer (Restuccia, Loizzo, & Spizzirri, 2018). These compounds can be produced by yeasts or LAB, being more likely related with LAB. From all BA, those more abundant in wine are histamine, cadaverine, 2-phenylethylamine, putrescine and tyramine (López et al., 2012; Restuccia et al., 2018). Besides, variable but little concentrations of BA, mainly putrescine and cadaverine, can come from grape berries (Bover-Cid, Izquierdo-Pulido, Marineé-Font, & Vidal-Carou, 2006; Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994).

As peptides are the main nitrogen source of nitrogen in wine, the understanding of the peptidic metabolism of *O. oeni* is important (Remize et al., 2005). The peptidase activities of *O. oeni* are largely described in literature, and they seem to be dependant of the fermenting strain (Manca de Nadra et al., 2005; Remize et al., 2006). Besides, they are affected by other exogenous parameters as pH. Those peptides, that are the preferent nitrogen source of *O. oeni* in wine, are the result of the metabolic activities of the previous fermenting yeasts. Thus, the use of different AF inoculation strategies will have an impact in the peptidic fraction, and consequently, in the nitrogen source of the MLF.

In recent years, several studies have assessed the impact of *T. delbrueckii* on *O. oeni* (Balmaseda et al., 2024), primarily focusing on general wine characteristics without specifically addressing the role of nitrogen metabolism. The use of *T. delbrueckii* has generally been associated with enhanced MLF performance by *O. oeni*, as it reduces toxic compounds such as medium-chain fatty acids (Belda et al., 2017), sulfur dioxide (Balmaseda et al., 2021b), or ethanol (Belda et al., 2015, 2017; Marcon et al., 2018), while increasing stimulating compounds like mannoproteins (Balmaseda, Aniballi, Rozès, Bordonos, & Reguant, 2021) or nitrogen-related compounds (Martín-García et al., 2020). However, these microbial interactions are multifactorial, making it difficult to attribute the effects to specific compounds. In this study, we aimed to evaluate whether the use of *T. delbrueckii* influences wine nitrogen concentration and, consequently, impacts *O. oeni*'s nitrogen metabolism. To this end, fermentations were conducted with different alcoholic fermentation (AF) inoculation regimes (coinoculation and

sequential inoculation) in musts with two amino acid concentrations. MLF was then carried out using three *O. oeni* strains, and nitrogen compound concentrations were quantified before and after MLF. Finally, we analysed the relative expression (RE) of genes associated with nitrogen metabolism in *O. oeni* PSU-1.

2. Materials and methods

2.1. Microorganisms

The yeast strains used were *T. delbrueckii* Biodiva (Lallemand, Inc., Montreal, Canada) (TdB), *T. delbrueckii* Viniferum NS-TD (Agrovin S.A., Alcazar de San Juan, Spain) (TdV) and *S. cerevisiae* Lalvin-QA23 (Lallemand, Inc., Montreal, Canada) (ScQA23). For MLF, *O. oeni* PSU-1 (ATCC BAA-331) (PSU-1), *O. oeni* 217^T (CECT217 = ATCC 23279^T) (217T), and *O. oeni* Enolab 4783 (4783) were selected. Yeasts were maintained on YPD plates (20 g/L glucose, 20 g/L bacto-peptone, 10 g/L yeast extract, 20 g/L agar, w/v, Panreac Química SLU, Castellar del Vallès, Spain) and the bacteria on MRSmf plates (55 g/L MRS broth (BD™ Difco™, Fisher Scientific, Madrid, Spain), 4 g/L DL-malic acid, 5 g/L fructose, 20 g/L agar), and all of them were stored at 4 °C.

2.2. Experimental fermentations

Fermentations were performed with natural concentrated Airén must (Mostos S.A., Tomelloso, Spain) diluted with sterile Milli-Q water to a density of 1085 ± 1 g/L. Must was supplemented with 0.4 g/L (w/v) of Nutrient Vit Nature™ (Lallemand, Spain) and pH was adjusted to 3.60 with 10 mol/L NaOH. Then, the must was sterilised using 1 mL/L of dimethyl dicarbonate (ChemCruz®, USA) and stored overnight at 4 °C. Sterility was confirmed by plating on YPD and MRSmf plates. The fermenting must amino acid composition was determined by HPLC according to Balmaseda, Rozès, Bordonos, and Reguant (2021a), and a second must was prepared with twice the initial amino acid concentration. The initial must (N1) was supplemented with a solution of the appropriate mixture of amino acids before sterilization to obtain the second must (N2). The concentration of amino acids in each must can be found in Suppl. Table 1.

To inoculate the fermentations, different strategies were employed in the two musts (N1 and N2). First, for sequential inoculation, each *T. delbrueckii* strain was inoculated for a population of 2 × 10⁶ colony forming units per mL (CFU/mL), and after 48 h of initial inoculation, *S. cerevisiae* QA23 was inoculated at the same population. Second, for coinoculated fermentations, each *T. delbrueckii* strain was inoculated together with *S. cerevisiae* QA23 in must for a population of 2 × 10⁶ CFU/mL, respectively. Finally, a control fermentation with *S. cerevisiae* as sole starter was also performed. Fermentations were carried out in triplicate in 1 L flasks containing 950 mL of must, statically at 20 °C. YPD agar plates were used to calculate the total viable yeast cells, and lysine agar medium (Oxoid LTD., Basingstoke, UK) was used to quantify the non-*Saccharomyces* yeasts after incubation at 28 °C for 48 h. AF was considered finished when the sugar concentration was <2 g/L. Then, wines were centrifuged at 10,000×g for 10 min. Samples of each replicate after AF were frozen at -20 °C. Then, replicates were blended, filtered (Merck Millipore Steritop™ Sterile Vacuum Bottle-Top Filters, Madrid, Spain) and transferred to 3 sterile flasks. Each flask was inoculated with one of the *O. oeni* strains for a population of 2 × 10⁷ CFU/mL. Then, the inoculated wine was divided into six 50-mL tubes, each containing 50 mL, and incubated under the same conditions as AF. Samples were taken every 24 h to monitor the consumption of L-malic acid, and bacterial population. Samples were plated on MRSmf and incubated at 27 °C in a 10% CO₂ atmosphere for 7–15 days. MLF was considered finished when the L-malic acid was <0.1 g/L. Three tubes were taken when half of the initial L-malic acid concentration was consumed (t_{1/2}), and the other three were taken when MLF was considered as finished (t_f), for gene expression analyses and wine

characterization after MLF, respectively.

Cell pellet of *O. oeni* PSU-1 at $t_{1/2}$ was collected. 50 mL of wine were centrifuged at $4250 \times g$ for 20 min at 4 °C. The resulting pellet was washed with 10 mmol/L Tris-HCl pH 8.00, prepared with diethyl pyrocarbonate-treated water (DEPC), and then frozen in liquid nitrogen and kept at -80 °C until RNA extraction.

2.3. Wine characterization

To monitor AF and MLF, density and L-malic acid were measured with an electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, Spain)) and the multianalyzer Miura One (TDI, SL, Gavà, Spain), respectively. Miura One was also used to determine the end of AF by quantifying the residual glucose + fructose concentration.

Wines were characterized after AF and MLF. pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain), and total and volatile acidity were measured by Fourier Transform Infrared Spectroscopy (FTIR Analysis by FOSS®, Nanterre, France).

Proteins were quantified using the KDS/BCA assay. Proteins from 500 μ L of wine samples after AF and MLF were precipitated, freeze-dried and resuspended as described in Gazzola, Vincenzi, Pasini, Lomolino, and Curioni (2015). Briefly, 5 μ L of SDS 10% (w/v) were added to the sample and heated at 100 °C for 5 min. After that, 125 μ L of KCl 1 mol/L were added, and the mixture was incubated at room temperature for 2 h. Samples were then centrifuged ($12,000 \times g$, 15 min, 4 °C) and washed twice with KCl 1 mol/L and once with Milli-Q water. Then, the pellet was freeze-dried and resuspended in 500 μ L of Milli-Q water. Proteins were quantified using the BCA-200 Protein Assay kit (Thermo Fisher, Waltham, USA) following the manufacturer's instructions at 562 nm.

2.4. Amino acid and peptide analyses of wines

Amino acid composition of wines was analysed by HPLC as described in Gobert, Tourdot-Maréchal, Morge, and Sparrow (2017). Briefly, samples were derivatised with AccQTag™ Ultra Derivatization kit (Waters, Milford, USA), according to the manufacturer's instructions. Chromatographic separation was performed in a C18 reverse-phase column (AccQ-Tag™ Ultra Column, 3.9×150 mm) with a fluorometric detector. L-alpha-amino-n-butyric acid (0.1 mmol/L, Sigma-Aldrich) was used as an internal standard. 2 μ L of sample were injected onto the column and the chamber was maintained at 37 °C.

Wine peptides were quantified as amino acid concentration. 500 μ L of HCl 6 mol/L were added to 500 μ L of wine sample in a glass vial that was vacuum sealed. Then, the vial was maintained at 110 °C for 24 h. After, vials were opened, and samples dried with N₂ gas at 37 °C. The dried pellet was resuspended with 500 μ L Milli-Q water and samples were derivatised and analysed as explained above.

Peptide-bound amino acids were calculated as the difference between the total amino acid concentration after hydrolysis and the free amino acid concentration. For amino acid analyses calculations, proline was discarded from the summary of amino acid concentration. Also, Met and Cys were discarded from peptide amino acid concentrations, as they can be degraded during hydrolysis. The summary of Asn and Asp, and Glu and Gln are expressed as Asx and Glx, respectively, due to a partial conversion of Asn into Asp and Gln into Glu during the hydrolysis (Gobert et al., 2017).

2.5. Biogenic amines analyses

Cadaverine, histamine, 2-phenylethylamine, putrescine and tyramine were quantified also by HPLC following Gómez-Alonso, Hermosín-Gutiérrez, and García-Romero (2007) with the modifications and conditions described in Bonnin-Jusserand, Grandvalet, Rieu, Weidmann, and Alexandre (2012). BA were identified according to the retention times and UV-visible spectral characteristics of the derivatives of the corresponding standards and were quantified by the internal

standard method with 2,4,6-trimethylphenethylamine hydrochloride (2 mg/mL, Sigma-Aldrich).

2.6. RNA extraction and RT-qPCR

For RNA extraction, a cell pellet of *O. oeni* PSU-1 was defrosted and washed with 10 mM Tris-HCl DEPC water. High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for the extraction following manufacturer's instructions changing the cell lysis for lysozyme dissolved in 10 mmol/L Tris-HCl buffer DEPC, at 50 mg/mL during 30 min at 37 °C (Balmaseda et al., 2021). Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). Extracted RNA was stored at -80 °C until analysis.

Some genes related with nitrogen metabolism of *O. oeni*, reported as differentially expressed in previous literature were selected to determine their relative expression (RE) in the different wines (Suppl. Table 2). OligoPerfect Primer Designer (Thermo Fisher) online tool was used for primer design. RT-qPCR was performed according to Balmaseda et al. (2021) as described by Olguín, Bordons, and Reguant (2010). Four constitutive genes (Margalef-Català, Araque, Bordons, Reguant, & Bautista-Gallego, 2016) were evaluated as internal controls. From those, *gyrA* and *gyrB*, which presented the less variation between samples, were selected. The $\Delta\Delta Ct$ method was used to calculate the RE of each gene. We used two reference conditions for determining (i) the impact of *T. delbrueckii*, with the RE in *S. cerevisiae* wine as reference condition for each initial must (N1 and N2), and (ii) the effect of doubling the initial amino acid concentration, with the gene expression in N2 wines referred to N1.

2.7. Statistical analysis

All the statistical analyses of the results were performed using the statistics software XLSTAT version 2020.2.3 (Addinsoft, Paris, France). The analysis of variance was carried out by ANOVA with a subsequent Tukey HSD test, to determine the significant differences between the samples: the confidence interval used was 95 % and the statistical level of significance was set at $p \leq 0.05$. Next-Generation Clustered Heat Map (NG-CHM) Builder (Ryan et al., 2019) with hierarchical clustering using the Euclidean distance metric with the ward agglomeration method was used for the analyses of amino acid composition, BA production and genes' RE.

3. Results and discussion

3.1. Fermentations

The duration of AF was extended using *T. delbrueckii* (Fig. 1). Control fermentation with *S. cerevisiae* as sole starter took almost half of the duration of the coinoculation and sequential inoculations (Fig. 1, Table 1). In this experiment, supplementation with twice the amino acid concentration (must N2) did not reduce the duration of AF, even if an increase in sugar consumption rate was observed (Table 1). This increase was more noticeable in Sc wine and was not observed in TdV wine. In general, the use of *T. delbrueckii* Viniferum resulted in the slowest AF, both in coinoculation and sequential inoculation (Table 1).

The viability of *T. delbrueckii* was longer maintained in sequential inoculations where *S. cerevisiae* was inoculated 48 h after the beginning of the AF (Fig. 1). In this sense, no positive effect of doubling the amino acid content was observed in *T. delbrueckii* viability. Indeed, the nitrogen demand of *T. delbrueckii* is similar to that of *S. cerevisiae* (Roca-Mesa, Sendra, Mas, Beltran, & Torija, 2020), and also show a similar fermentative behaviour (Su et al., 2020). The AF with *S. cerevisiae* as sole starter presented the highest viable yeast population during the fermentative process, mainly by the end of it (Fig. 1). The other inoculation strategies had a lower total viable yeast population, similar in both coinoculation

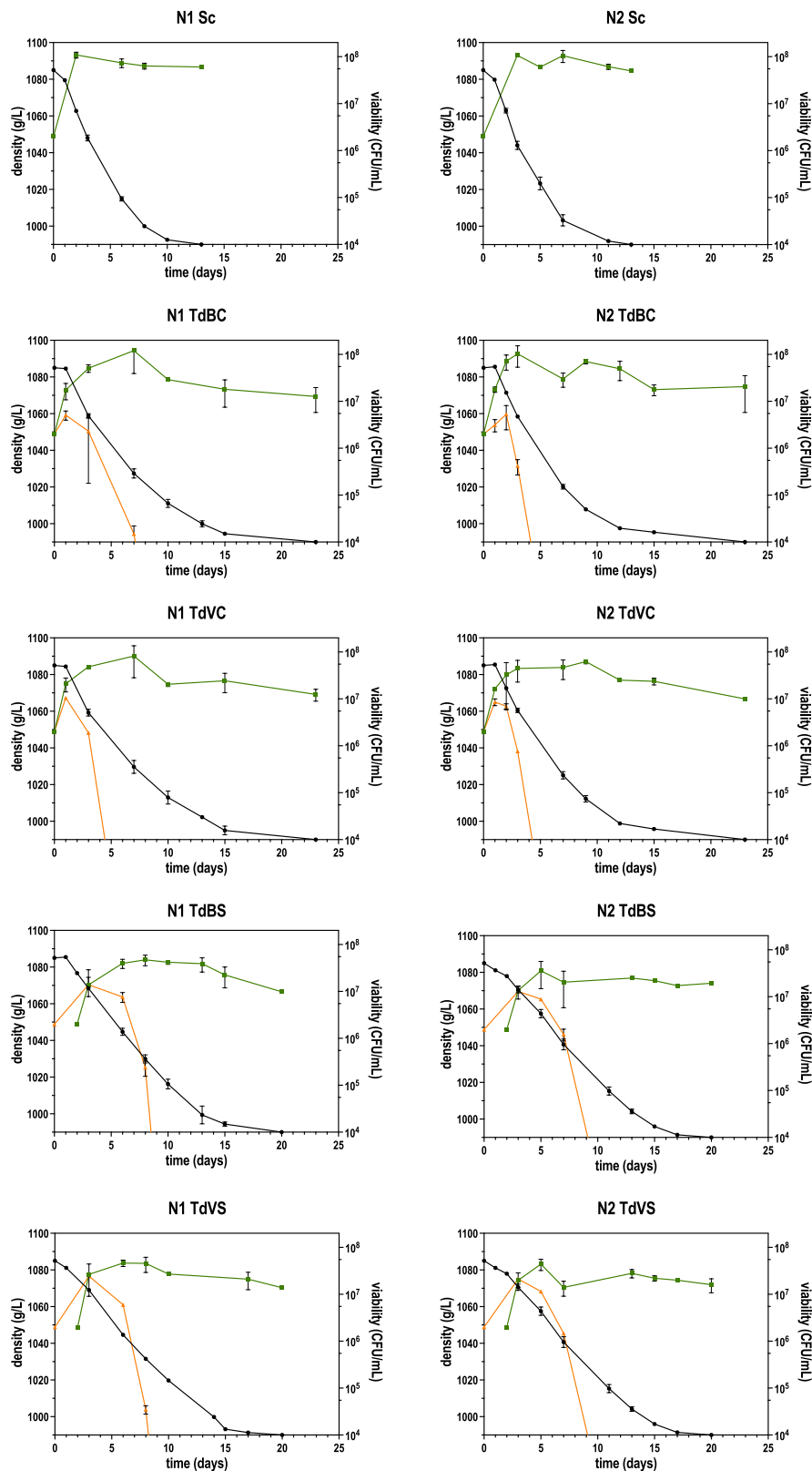


Fig. 1. Alcoholic fermentation dynamics where density decrease (black) and yeast viability are represented for the two used species: *S. cerevisiae* (green) and *T. delbrueckii* (orange) in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates \pm SD. Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Biodiva- *S. cerevisiae* QA23 and *T. delbrueckii* Viniferm- *S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Alcoholic (AF) and malolactic (MLF) fermentation duration and consumption rate of sugar and L-malic acid, and bacterial viability. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva), TdV (*T. delbrueckii* Viniform) with C (coinoculation with *S. cerevisiae*) or S (sequentially inoculated with *S. cerevisiae*) refer to the obtained fermented wines in the two must with standard amino acid concentration (N1) and supplemented with twice the initial concentration (N2). PSU-1, 217 and 4783 refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* 217T, or *O. oeni* Enolab 4783 were inoculated.

		Fermentation/Total process duration (days)				Consumption rate ^a (g/L/day)				Viability mid MLF ^b (log CFU/mL)			Viability end MLF (log CFU/mL)			
		AF	PSU-1	217T	4783	AF [§]	PSU-1	217T	4783	PSU-1	217T	4783	PSU-1	217	4783	
N1	Sc	13	-	5/18	5/18	10.47 ± 0.08 ^{ab}	- ^{FB}	0.36 ± 0.01 ^{bB}	0.38 ± 0.01 ^{bB}	6.65 ± 0.01 ^{EB}	7.45 ± 0.11 ^{abA}	7.4 ± 0.03 ^{abCA}	<4 ^{EB}	7.05 ± 0.01 ^{EB}	7 ± 0.02 ^{CB}	
	TdB	23	-	10/33	9/32	8.02 ± 0.23 ^{bb}	- ^{FA}	0.22 ± 0.01 ^{dB}	0.31 ± 0.01 ^{CB}	7.06 ± 0.06 ^{dB}	7.54 ± 0.06 ^{aA}	7.15 ± 0.1 ^{bcdB}	<4 ^{EA}	7.5 ± 0.04 ^{abA}	7.05 ± 0.06 ^{CB}	
	TdV	23	-	14/37	10/33	7.77 ± 0.37 ^{bb}	- ^{FA}	0.15 ± 0 ⁰	0.29 ± 0 ⁰	7.38 ± 0.45 ^{abCA}	7.51 ± 0.01 ^{abA}	6.89 ± 0.09 ^{deB}	<4 ^{EA}	6.65 ± 0.25 ^{BB}	6.87 ± 0.03 ^{CB}	
	TdB	20	-	5/25	5/25	7.71 ± 0.11 ^{ba}	- ^{FB}	0.3 ± 0.01 ^{CB}	0.3 ± 0 ⁰	7.14 ± 0.01 ^{bcdB}	7.43 ± 0.04 ^{abCA}	7.38 ± 0.06 ^{abCB}	<4 ^{EB}	7.04 ± 0.06 ^{CB}	7.32 ± 0.03 ^{BA}	
	TdV	23	3/26	4/27	4/27	7 ± 0.05 ^{ba}	-	0.57 ± 0 ⁰	0.55 ± 0.01 ^{aA}	0.34 ± 0.04 ^{bb}	n	n	n	7.47 ± 0.06 ^{abB}	7.54 ± 0.02 ^{abA}	7.68 ± 0.05 ^{aA}
N2	Sc	13	8/21	5/18	5/18	12.54 ± 0.53 ^{aA}	-	0.53 ± 0.02 ^{ba}	0.41 ± 0.03 ^{deA}	0.66 ± 0.03 ^{aA}	7.51 ± 0.01 ^{aA}	7.54 ± 0.05 ^{aA}	7.52 ± 0.01 ^{aA}	7.17 ± 0.01 ^{EA}	7.47 ± 0.01 ^{CB}	7.39 ± 0.01 ^{CA}
	TdB	23	-	7/30	4/27	9.13 ± 0.09 ^{ba}	- ^{FA}	0.39 ± 0.03 ^{deA}	0.36 ± 0.01 ^{deA}	7.48 ± 0.17 ^{abA}	7.45 ± 0.04 ^{abA}	7.48 ± 0.08 ^{abA}	<4 ^{EA}	7.53 ± 0.09 ^{ba}	7.49 ± 0.03 ^{BA}	
	TdV	23	-	4/31	4/27	8.61 ± 0.17 ^{ba}	- ^{FA}	0.43 ± 0.02 ^{cdA}	0.35 ± 0.09 ^{deA}	7.2 ± 0.1 ^{dA}	7.32 ± 0.02 ^{bcdB}	7.46 ± 0.05 ^{abCA}	<4 ^{EA}	7.26 ± 0.04 ^{deA}	7.27 ± 0.04 ^{dA}	
	TdB	20	4/24	6/26	6/26	7.36 ± 0.41 ^{ca}	0.43 ± 0.01 ^{cdeA}	0.35 ± 0 ⁰	0.34 ± 0 ⁰	7.58 ± 0.05 ^{aA}	7.49 ± 0.04 ^{abCA}	7.28 ± 0.01 ^{cdA}	7.5 ± 0.01 ^{ba}	7.54 ± 0.03 ^{ba}	7.05 ± 0.01 ^{FB}	
	TdV	20	4/24	6/26	4/24	6.73 ± 0.15 ^{CB}	0.5 ± 0.01 ^{bcB}	0.41 ± 0.03 ^{deB}	0.43 ± 0 ⁰	7.51 ± 0.01 ^{ab}	7.54 ± 0.05 ^a	7.54 ± 0.05 ^a	7.69 ± 0.01 ^{aA}	7.56 ± 0.01 ^{ba}	7.56 ± 0.01 ^{BB}	

^{a-g} Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences between values of the three strains in the same wine. Uppercase letters correspond to differences among the values of the same *O. oeni* strain in the different wines. [§] For AF, lowercase letters correspond to differences between values of the different AF strategies in the same must. Uppercase letters correspond to differences among the values of the same AF strategy in the different wines.

-: no finished MLF.

n: no data.

^a Calculation based on consumption rate of sugar as density (AF) and L-malic acid (MLF) considering the period of exponential decrease of these values.

^b Viability was determined by plating when half of the initial [L-malic acid] was consumed ($t_{1/2}$) and when MLF was considered as finished (t_f) when [L-malic acid] < 0.1 g/L.

and sequential inoculation, always higher than 10^7 CFU/mL. The competition of starter cultures, and specifically with these two yeast species, is a well-reported characteristic (Belda et al., 2015, 2017; Martín-García et al., 2020). The high demand of nutrients, together with the production of some metabolites, could explain the viability differences observed in these fermentations (Roca-Mesa et al., 2020).

The obtained wines were then inoculated with the three different *O. oeni* strains to perform MLF. Compared to AF, larger differences in MLF were observed, and doubling the initial amino acid concentration had a positive effect (Fig. 2, Table 1). MLF in N1 wines showed a general delay when it was performed in the wines coinoculated with *T. delbrueckii* and *S. cerevisiae*. Besides, the PSU-1 strain caused stuck fermentations in all conditions except in TdV wine. The best condition for the other two *O. oeni* strains was also TdV wine (Fig. 2, Table 1).

In general, N2 reduced the duration of the MLF process (Fig. 2, Table 1). Regarding to the PSU-1 strain, all fermentations finished except for the TdB and TdV wines, which were also stuck. Sequential inoculation (TdB, TdV) for PSU-1 was the most convenient AF strategy since it finished in half the time of that observed in Sc wine. Moreover, the delays in coinoculated wines observed in N1 disappeared in N2 with the other two strains. The MLF durations were reduced as the differences did.

Viability of the three *O. oeni* strains was maintained around 10^7 CFU/mL during the fermentative process, except for those stuck MLFs that lost their viability and were not able to finish the fermentation (Table 1). A higher population concentration was observed in those wines coming from N2. Besides, it is also interesting to point out that the TdV wines from N1 showed a population lower than 10^7 CFU/mL with 217T and

4783 strains, still enough to conclude MLF.

General oenological parameters did not show large differences in the tested conditions (Suppl. Table 3). L-malic acid in N1 wines was significantly lower in Td wines, both in coinoculated and sequential AF, than in Sc wines. In N2 wines, the decrease of L-malic acid at the end of AF was observed only in Td wines with sequential inoculation. Also, a significant increase in ethanol content was observed in coinoculation with *T. delbrueckii* in N2. *T. delbrueckii* is usually reported as a yeast, which can reduce the alcoholic content of wines (Benito, 2018). Nevertheless, the fermenting medium and the inoculation strategy are crucial to achieving that reduction (Martín-García et al., 2020).

The observed results in this study agree with the general tendency described for this yeast (Balmaseda et al., 2024), such as the L-malic acid decrease in *T. delbrueckii* wines. It has been reported that volatile acidity tends to decrease in sequential AF but increases under co-inoculation conditions. Similarly, total acidity also shows an increase (Sadoudi et al., 2012). Other behaviours such as the increase in ethanol content when coinoculating with *S. cerevisiae*, are not the general trend observed (Balmaseda et al., 2024), but are still reported by some authors (Marcon et al., 2018). The review of Balmaseda et al. (2024) also gathered some data about nitrogen-related compounds, which were enhanced in *T. delbrueckii* wines, maybe favouring MLF performance. Nevertheless, the reported data only discussed the YAN, ammonium and NOPA (alpha-amino nitrogen) composition, not emphasizing the whole nitrogen composition of wines.

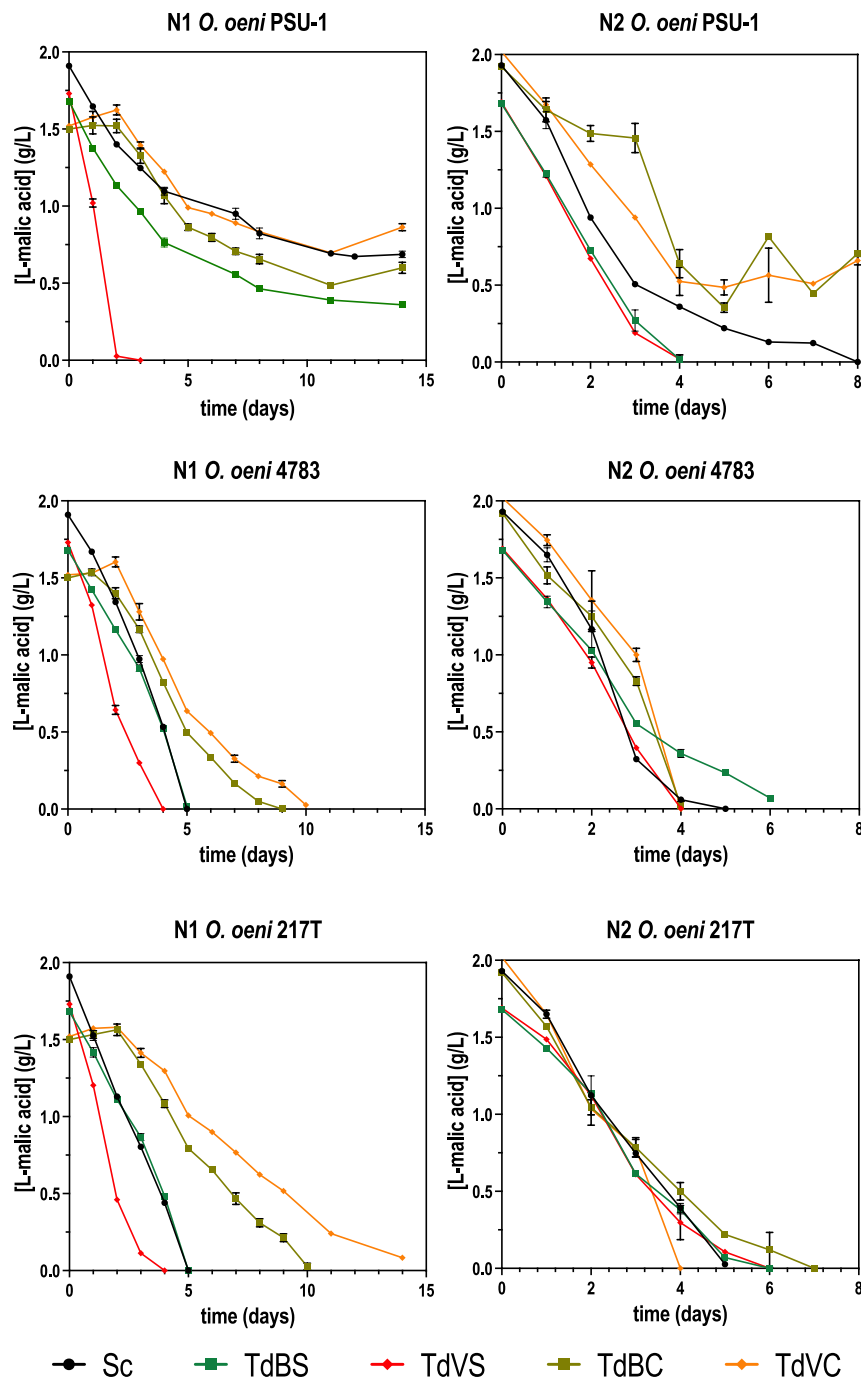


Fig. 2. Malolactic fermentation dynamics where L-malic consumptions of *O. oeni* PSU-1, 217T and 4783 in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2) are represented. Values shown are the mean of triplicates \pm SD. Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Biodiva-*S. cerevisiae* QA23 and *T. delbrueckii* Vinifer-*S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively.

3.2. Free and peptide amino acid concentrations

The different preferences and demands of nitrogen by the fermenting yeasts will determine the amino acid composition found in wine (Roca-Mesa et al., 2020). Moreover, the use of *T. delbrueckii* has been previously linked with higher free amino acid availability in wine after AF (Martín-García et al., 2020). In this study the different inoculation strategies produced wines with different amino acid compositions in both free and peptide amino acids (Fig. 3). In wines coming from N1, the concentration of free amino acids was an average of 20 mg N/L whereas

the peptidic fraction resulted in a higher nitrogen reservoir, representing an average of around 40 mg N/L. In contrast, the remaining free amino acids after AF in N2 were higher than quantified in N1 (Fig. 3). In these wines, the peptidic fraction was similar to N1 wines except for TdBS where the peptide amino acids were higher than the free fraction and higher than observed in TdBS from N1. In general, a slight decrease in the peptide amino acid concentration was observed when compared with N1 wines after AF. In this sense, we showed that the addition of free amino acids in must cause an increased the total amino acid composition by only increasing the free amino acid concentration, with no changes in

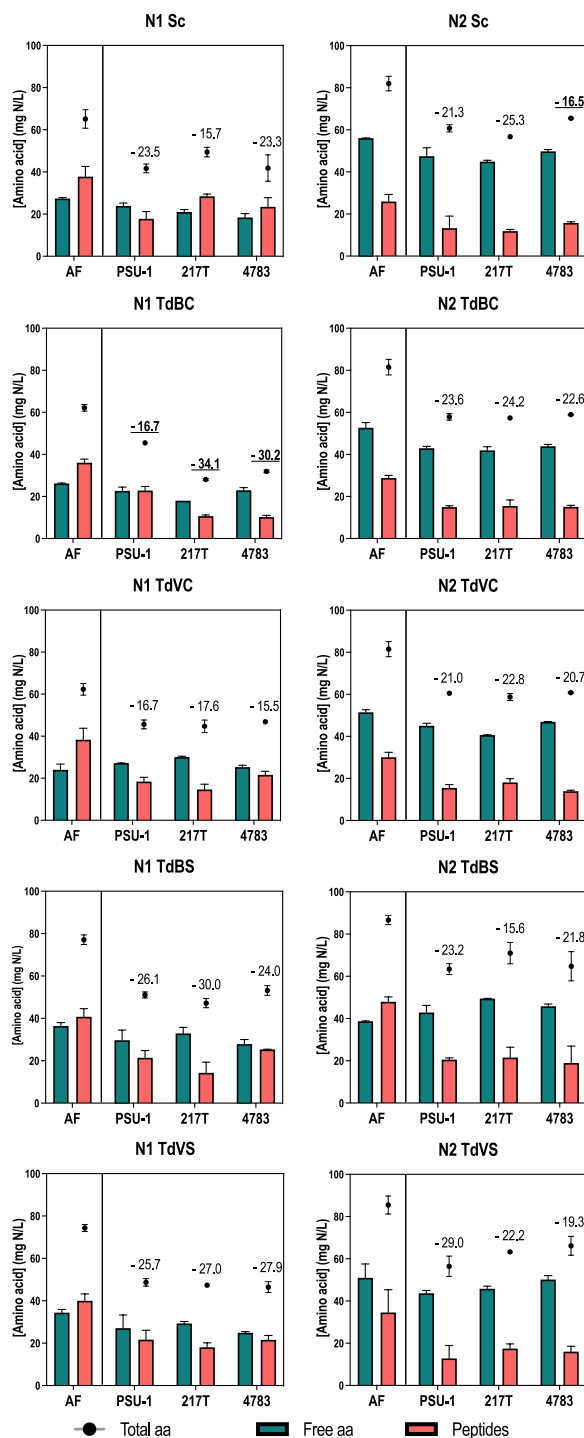


Fig. 3. Total amino acid (free and peptide amino acids) concentration (mg N/L) of wines before (AF) and after MLF with *O. oeni* strains (PSU-1, 217T and 4783) in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates \pm SD. The number above total amino acid concentration (black dot) after MLF (PSU-1, 217T and 4783) refers to consumed total amino acid concentration during MLF. Underlined and bolded values are significantly different between wines after MLF ($p \leq 0.05$ according to a Tukey HSD post-hoc comparison). Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Bio-diva- *S. cerevisiae* QA23 and *T. delbrueckii* Viniferms- *S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively.

peptide abundance (Fig. 3). Besides, in N1, the only wine significantly different from the control condition (Sc wine) was TdBS in concordance with Martín-García et al. (2020) and Ruiz-de-Villa et al. (2023b), since free amino acids concentration was enhanced.

It is well known that free amino acids are essential for yeasts during AF (Ribéreau-Gayon et al., 2006). They are largely consumed, and high amino acid demands are related to high fermentative capacity (Roca-Mesa et al., 2020). Nevertheless, peptide composition in wine by different yeasts has not yet been reported. In this study, the use of *T. delbrueckii* modified the amino acid concentration of peptides (Suppl. Fig. 1). In N1 wines, the concentration of Glx, Arg, Asx and His increased with respect to *S. cerevisiae* wine after AF. Nevertheless, the composition varied in N2 wines, where the composition of TdBS and TdVC wines was very different from the others due to an increased total amino acids concentration, mainly due to Glx and Ala (Suppl. Fig. 1).

Total amino acid concentration decreased after MLF (Fig. 3). During this process, *O. oeni* consumed amino acids. It is interesting to note that *O. oeni* consumed the amino acids coming from peptides, and the free amino acid concentration remained similar (Fig. 3, Suppl. Fig. 1). *O. oeni* has a vast set of peptidases that release free amino acids to the media during MLF (Balmaseda, Rozés, Bordons, & Reguant, 2022; Margalef-Català et al., 2016). Indeed, this bacterium can grow with peptides as sole nitrogen source, proving the importance of these nitrogen source in the metabolism of *O. oeni* (Remize et al., 2006). The amino acid consumption patterns were mainly affected by the fermenting medium. The three tested strains consumed similar total amino acid concentrations. We only observed a different consumption pattern in the 4783 strain in N2 Sc wine, and in N1 TdBC wine, all strains behaved differently (Fig. 3). Besides, the total concentration of each amino acid was similar in coinoculated wines (TdBC, TdVC), and Sc, but different from sequentially inoculated wines (TdBS and TdVS) (Suppl. Fig. 1). In stuck MLFs, there was also consumption in total amino acid concentration (Fig. 3), which can be related to the first fermentative stages where *O. oeni* was still viable and metabolically active (Fig. 2, Table 1). In this sense, Sc wine and coinoculated wines from N1 presented higher demands of amino acids than sequentially inoculated ones.

The consumption of amino acids during MLF was mainly due to a reduction in peptide concentration (Fig. 3). In this sense, the total consumption of nitrogen was similar in all wines, regardless of the initial concentration of the peptidic fraction, which was different. Fig. 4 shows the comparison of the amino acid consumption profiles during MLF in the different wines by the three different *O. oeni* strains. Overall, a consumption of every amino acid was observed. Moreover, a general higher consumption was observed in N2 wines (Fig. 4B) regarding N1 wines (Fig. 4A). This is interesting to point out since there was a higher concentration of available free amino acids in N2 regarding N1 wines (Fig. 3).

The consumption patterns of peptide amino acids in N1 wines were clearly dependent on the yeast inoculation strategy. A high consumption of Arg, Glx and His, together with a low general consumption of the rest of the bounded amino acids, grouped the wines sequentially inoculated with *T. delbrueckii* and *S. cerevisiae* (Cluster I, Fig. 4A). Meanwhile, the wines inoculated only with Sc, and all the wines coinoculated with the two yeast species clustered apart (Cluster II, Fig. 4A).

The consumption patterns of N2 wines were more heterogeneous. Although some similarities according to the yeast inoculation strategy were observed, there was not such a clear relationship as in N1 wines (Fig. 4B). All wines were clustered together in Cluster II except for TdBS wines, grouped in Cluster I. Interestingly, TdBS presented the highest peptide concentration from all conditions (Fig. 3), which also presented the highest consumption of bound amino acids (Fig. 4B). In N2 wines, the most consumed amino acids were Glx, in mainly all conditions, and Arg and Thr in TdBS wines.

Altogether, the preference for peptides by *O. oeni* under oenological conditions was confirmed. In this study we could observe the

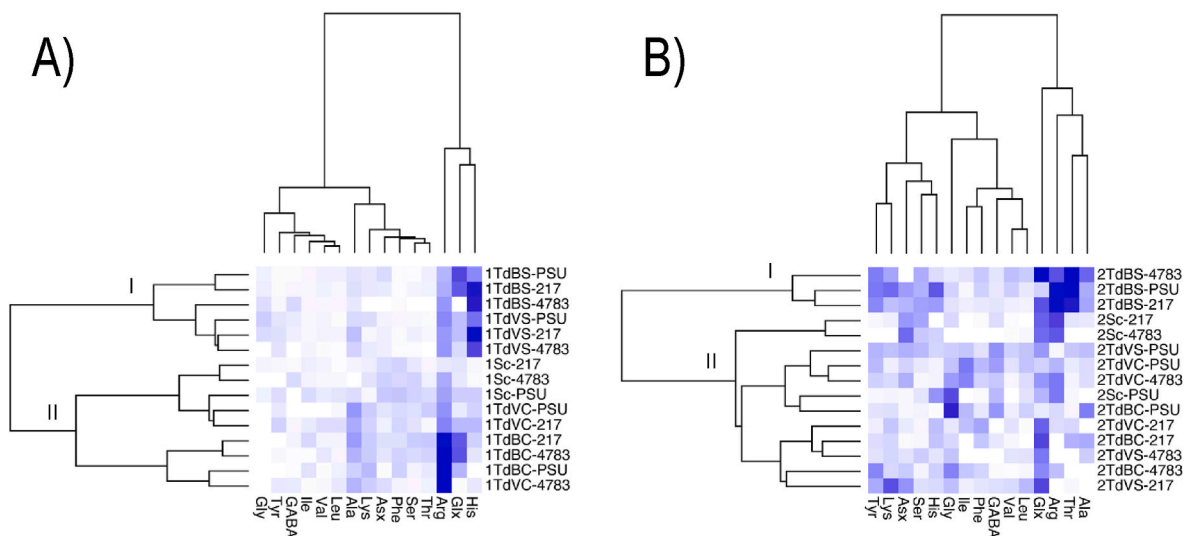


Fig. 4. Heat map and clusterization of amino acid consumption (mg N/L) of wine peptides. A) Consumption in N1 must. B) Consumption in N2 must. Increasing color intensity means higher amino acid consumption. The total length of the dendrogram correspond to 100% of similarity. Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Biodiva- *S. cerevisiae* QA23 and *T. delbrueckii* Viniferm- *S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

consumption patterns of *O. oeni* in wines fermented with different AF strategies. Nitrogen metabolism is one of the main metabolisms affected by wine like conditions (Balmaseda et al., 2022; Margalef-Català et al., 2016), and also reported to be affected by the use of non-*Saccharomyces*. We observed a complex metabolism, which was also influenced by the initial amino acid concentration. Indeed, high amino acid concentrations were also responsible for an increased peptide consumption. Further research is needed to better understand this complex metabolism under oenological conditions.

3.3. Biogenic amines

From the five biogenic amines (BA) studied, cadaverine, 2-phenylethylamine, putrescine and tyramine, were detected, but not histamine (Table 2). It is worth nothing that no BA content was detected in must, except for an average of 3–4 mg/L of putrescine (data not shown). That is why the amount of putrescine detected after AF was not a consequence of the inoculated fermenting yeasts, as it has been observed in other studies (Calisto et al., 2017). Overall, the production of BA was not related to the higher availability of its amino acid precursor or to its increased consumption.

The production of BAs was low in this work, except for putrescine (Table 2). Only wines fermented with *O. oeni* 4783 showed an increased putrescine content. This strain is characterized by the presence and expression of the *odc* gene (Franquès et al., 2018). Indeed, *O. oeni* 4783 is the only tested strain owning this gene in the study. It is interesting to point out that some ornithine decarboxylases can use, apart from ornithine, lysine as a substrate, and consequently produce cadaverine (Romano, Trip, Lonvaud-Funel, Lolkema, & Lucas, 2012). In this sense, it was also observed that there was a significant increase in cadaverine in wines after MLF fermented with *O. oeni* 4783. Thus, the increased cadaverine content in 4783 fermented wines should be related to a non-specific substrate ODC enzyme. The high production of putrescine in *O. oeni* 4783 wines clustered all those wines in the same group (Table 2). The production of putrescine only depended on the fermenting *O. oeni* strain (presence or absence of *odc* gene), regardless of the AF inoculation or the initial amino acid concentration. Little amounts of cadaverine were also detected after AF in concentrations of 0.11–0.21 mg/L in all wines after AF, which were maintained after MLF in *O. oeni* PSU-1 and 217T fermented wines (Table 2). Indeed, cadaverine can be detected after AF and usually does not increase after MLF

(López et al., 2012).

2-phenylethylamine increased after AF, and no changes were observed at the end of MLF (Table 2). 2-phenylethylamine appears after AF and usually remains unchanged after MLF (López et al., 2012). Interestingly, its concentration was increased in N1 wines and was dependent on the inoculation strategy. Lower concentrations were detected in wines sequentially coinoculated with *T. delbrueckii* in contrast to sequentially inoculated and control wines (Table 2). Tyramine content exhibited heterogeneous behaviour, it was detected in some wines after AF or MLF, and no correlation could be found with the detected concentration (Table 2).

Overall, we saw that the use of *T. delbrueckii* did not have a negative impact on BA concentration and indeed produced wines with a lower 2-phenylethylamine concentration regarding the control wine.

3.4. Relative expression (RE) of nitrogen related genes in *O. oeni* PSU-1

To better understand the nitrogen metabolism of *O. oeni* under oenological conditions, we selected some genes to study their RE (Suppl. Table 2). From the three strains used, we selected *O. oeni* PSU-1 as the representative strain, since the three of them had a similar amino acid consumption pattern and also due to its different MLF performances. The selection of genes was based on previous works where the genes classified in the Clusters of Orthologous Groups (COG) for amino acid transport and metabolism exhibited differential expression in terms of mRNA or protein abundance (Balmaseda et al., 2022; Margalef-Català et al., 2016; Olguín et al., 2015). These genes encode for two peptidases (OEOE_RS08595 and OEOE_RS2735), a peptide transporter (OEOE_RS02110), an amino acid/polyamine/organocation transporter (OEOE_RS05625) and the glutamine synthase (OEOE_RS04565).

We studied their RE, considering two possible effects: (i) *T. delbrueckii* impact and (ii) amino acid supplementation (Fig. 5). In all conditions, *O. oeni* PSU-1 exhibited an upregulation of the studied genes, whose significant upregulations (RE > 2) are coloured in Fig. 5.

Considering the effect of *T. delbrueckii*, *O. oeni* exhibited a very increased transcriptional response in TdBS wine of Must 1, which was also clustered very far from the other conditions (Fig. 5A). TDBC wines from N1 and N2 were clustered together and had a very low response. The other conditions had significant upregulation and were clustered together. The hierarchical clustering of the RE of each gene revealed a similar response pattern in the two transporters.

Table 2

Biogenic amine (BA) concentration (mg/L) in obtained wines the two must with standard amino acid concentration (N1) and supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates \pm SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva), TdV (*T. delbrueckii* Viniferum) with C (coinoculation with *S. cerevisiae*) or S (sequentially inoculated with *S. cerevisiae*) refer to the obtained fermented wines. P, 217T and 4783 refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* 217T, or *O. oeni* Enolab 4783 were inoculated.

	Cadaverine		Phenylethylamine		Putrescine		Tyramine		Total BA	
	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2
Sc	0.13 \pm 0.02 ^{aA}	0.11 \pm 0.01 ^{aA}	0.75 \pm 0.05 ^{bcdB}	0.53 \pm 0.02 ^{defA}	3.52 \pm 0.42 ^{aA}	4.53 \pm 0.34 ^{aA}	n.d. ^{aA}	1.17 \pm 0.54 ^{bB}	4.4 \pm 0.48 ^{aA}	6.33 \pm 0.18 ^{cdeB}
Sc-P ^a	0.17 \pm 0.04 ^{aA}	0.1 \pm 0.01 ^{aA}	0.77 \pm 0.02 ^{dB}	0.51 \pm 0.01 ^{cdefA}	4.11 \pm 0.02 ^{aA}	4.34 \pm 0.15 ^{aA}	n.d. ^{aA}	0.72 \pm 0.01 ^{bB}	5.1 \pm 0.04 ^{abA}	5.68 \pm 0.14 ^{abcdB}
Sc-217T	0.14 \pm 0.01 ^{aA}	0.12 \pm 0.01 ^{aA}	0.74 \pm 0.08 ^{bcdA}	0.58 \pm 0.01 ^{fA}	4.46 \pm 0.55 ^{aA}	4.64 \pm 0.21 ^{aA}	n.d. ^{aA}	1.16 \pm 0.15 ^{bB}	5.34 \pm 0.64 ^{abcA}	6.51 \pm 0.06 ^{deA}
Sc-4783	0.51 \pm 0.08^{cA}	0.47 \pm 0.02^{fA}	0.79 \pm 0.08 ^{dB}	0.52 \pm 0.02 ^{cdefA}	11.27 \pm 0.95^{bA}	11.67 \pm 0.65^{cA}	n.d. ^{aA}	0.95 \pm 0.05 ^{bB}	12.56 \pm 1.11^{dA}	14.08 \pm 1.29^{hA}
TdBC	0.16 \pm 0.07 ^{aA}	0.13 \pm 0.01 ^{abA}	0.38 \pm 0.17 ^{abA}	0.39 \pm 0.01 ^{abcdeA}	4.76 \pm 0.75 ^{aA}	4.97 \pm 0.16 ^{aA}	1.51 \pm 0.36 ^{deB}	n.d. ^{aA}	6.82 \pm 1.35 ^{bcA}	5.49 \pm 0.17 ^{abcdA}
TdBC-P ^a	0.12 \pm 0.01 ^{aA}	0.11 \pm 0.01 ^{aA}	0.28 \pm 0.01 ^{aA}	0.25 \pm 0.02 ^{aA}	5.05 \pm 0.3 ^{aA}	4.12 \pm 0.16 ^{aA}	1.83 \pm 0.13 ^{EB}	n.d. ^{aA}	7.28 \pm 0.45 ^{cB}	4.48 \pm 0.19 ^{aA}
TdBC-217T	0.13 \pm 0.01 ^{abB}	0.1 \pm 0.01 ^{aA}	0.29 \pm 0.01 ^{aA}	0.43 \pm 0.2 ^{bcddefB}	5.06 \pm 0.1 ^{aA}	4.74 \pm 0.04 ^{aA}	1.68 \pm 0.43 ^{deB}	n.d. ^{aA}	7.15 \pm 0.33 ^{bcB}	5.99 \pm 0.12 ^{bcdA}
TdBC-4783	0.39 \pm 0.03^{bcA}	0.24 \pm 0.04^{cA}	0.4 \pm 0.02 ^{abcA}	0.38 \pm 0.15 ^{abcdeA}	11.39 \pm 0.61^{bA}	11.35 \pm 0.56^{cA}	1.21 \pm 0.05 ^{cdb}	n.d. ^{aA}	13.4 \pm 0.71^{dA}	11.97 \pm 0.67^{gA}
TdVC	0.13 \pm 0.01 ^{aA}	0.14 \pm 0.01 ^{abA}	0.4 \pm 0.01 ^{abcA}	0.37 \pm 0.01 ^{abcdA}	4.68 \pm 0.03 ^{aA}	4.79 \pm 0.04 ^{aA}	1.97 \pm 0.05 ^{bb}	n.d. ^{aA}	5.2 \pm 0.01 ^{abcA}	7.26 \pm 0.01 ^{eb}
TdVC-P ^a	0.11 \pm 0.01 ^{aA}	0.12 \pm 0.02 ^{aA}	0.39 \pm 0.01 ^{abA}	0.32 \pm 0.06 ^{abA}	4.53 \pm 0.18 ^{aA}	4.72 \pm 0.17 ^{aA}	n.d. ^{aA}	n.d. ^{aA}	5.03 \pm 0.18 ^{abA}	5.16 \pm 0.21 ^{bcA}
TdVC-217T	0.12 \pm 0.02 ^{bA}	0.13 \pm 0.01 ^{abA}	0.28 \pm 0.01 ^{aA}	0.4 \pm 0.05 ^{abcdeA}	4.83 \pm 0.05 ^{aA}	4.84 \pm 0.01 ^{aA}	n.d. ^{aA}	n.d. ^{aA}	5.23 \pm 0.08 ^{abcA}	5.37 \pm 0.05 ^{abcdA}
TdVC-4783	0.37 \pm 0.01 ^{bcA}	0.32 \pm 0.02^{deA}	0.28 \pm 0.01 ^{aA}	0.36 \pm 0.01 ^{abcB}	11.22 \pm 0.3^{bA}	11.29 \pm 0.45^{cA}	1.38 \pm 0.01^{deB}	n.d. ^{aA}	13.25 \pm 0.32^{dA}	11.97 \pm 0.44^{gA}
TdBS	0.17 \pm 0.01 ^{aA}	0.18 \pm 0.01 ^{bA}	0.88 \pm 0.06 ^{dB}	0.52 \pm 0.01 ^{cdefA}	4.47 \pm 0.53 ^{aA}	4.33 \pm 0.27 ^{aA}	0.63 \pm 0.15 ^{bcB}	n.d. ^{aA}	5.9 \pm 1.12 ^{abcB}	5.03 \pm 0.27 ^{abA}
TdBS-P ^a	0.21 \pm 0.01 ^{abB}	0.1 \pm 0.01 ^{aA}	0.9 \pm 0.02 ^{dB}	0.3 \pm 0.01 ^{abA}	4.67 \pm 0.13 ^{aA}	4.28 \pm 0.05 ^{aA}	n.d. ^{aA}	n.d. ^{aA}	6.27 \pm 0.11 ^{abcB}	4.69 \pm 0.1 ^{aA}
TdBS-217T	0.21 \pm 0.02 ^{abB}	0.12 \pm 0.01 ^{aA}	0.94 \pm 0.01 ^{dB}	0.53 \pm 0.04 ^{efA}	4.99 \pm 0.22 ^{aA}	4.58 \pm 0.05 ^{aA}	n.d. ^{aA}	n.d. ^{aA}	6.13 \pm 0.25 ^{abcB}	5.24 \pm 0.01 ^{abcA}
TdBS-4783	0.7 \pm 0.04^{dB}	0.32 \pm 0.01^{dA}	0.88 \pm 0.01 ^{dB}	0.41 \pm 0.01 ^{abcdeA}	12.11 \pm 0.48^{bb}	9.63 \pm 0.33^{bA}	0.54 \pm 0.13 ^{abB}	n.d. ^{aA}	14.01 \pm 0.08^{dB}	10.36 \pm 0.31^{fA}
TdVS	0.13 \pm 0.04 ^{aA}	0.11 \pm 0.01 ^{aA}	0.63 \pm 0.26 ^{abcdA}	0.45 \pm 0.04 ^{bcdefA}	4.64 \pm 0.01 ^{aA}	4.53 \pm 0.11 ^{aA}	1.48 \pm 0.21 ^{deB}	n.d. ^{aA}	6.92 \pm 0.52 ^{bcB}	5.09 \pm 0.15 ^{abA}
TdVS-P	0.16 \pm 0.01 ^{abB}	0.11 \pm 0.01 ^{aA}	0.78 \pm 0.01 ^{dB}	0.43 \pm 0.01 ^{abcdeA}	4.77 \pm 0.02 ^{abB}	4.45 \pm 0.01 ^{aA}	1.49 \pm 0.03 ^{deB}	n.d. ^{aA}	7.21 \pm 0.02 ^{bcB}	4.97 \pm 0.01 ^{abA}
TdVS-217T	0.21 \pm 0.01 ^{abB}	0.12 \pm 0.01 ^{aA}	0.77 \pm 0.03 ^{cdB}	0.42 \pm 0.01 ^{abcdeA}	4.63 \pm 0.01 ^{aA}	4.25 \pm 0.07 ^{aA}	1.44 \pm 0.03 ^{deB}	n.d. ^{aA}	7.06 \pm 0.08 ^{bcB}	5 \pm 0.34 ^{abA}
TdVS-4783	0.69 \pm 0.01^{dB}	0.38 \pm 0.02^{cA}	0.59 \pm 0.22 ^{abcdA}	0.44 \pm 0.02 ^{abcdeA}	11.6 \pm 0.44^{bA}	10.74 \pm 0.46^{bcA}	1.51 \pm 0.14 ^{deB}	n.d. ^{aA}	14.39 \pm 0.37^{dB}	11.21 \pm 0.01^{fgA}

^{a-g} Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences between values of wines from the same must. Uppercase letters correspond to differences among the values of the inoculation condition from the two musts. Values in bold are significantly different from the value at the end of AF in the same wine (before MLF).

n.d.: not detected.

^a wines with stuck MLF at least in one of the musts.

When comparing the RE of the studied genes in N2 compared to N1 wines, we observed a general upregulation of all genes in all conditions (Fig. 5B). Indeed, an increase in amino acid consumption was observed in N2, mainly due to the consumption of peptides (Fig. 4). The exception was TdBS, which did not present any differentially expressed genes. This is related to the very high expression of each gene in N1 wine, that minimized the differences among N2 wines. In this comparison, the REs of the studied genes clustered Sc together with TdBC, and TdVC and TdVS, reporting a similar response to amino acid supplementation in Sc and TdB wines, which was more different in TdV wines. In this comparison, the expression patterns of the genes were clustered based on their function: the two peptidases together, the two transporters together, and glutamine synthase (Fig. 5B).

The general upregulation observed in this work could indicate that the studied mechanisms (peptidases, peptide and amino acid transporters, and glutamine synthase) are a general stress response to wine in *O. oeni* as described in the previous works (Balmaseda et al., 2022; Margalef-Català et al., 2016; Olguín et al., 2015). These mechanisms are generally upregulated in wine conditions, and their expression increases

during the fermentative process. Thus, it demonstrates that peptide composition and utilization are key factors in *O. oeni* survival under oenological conditions. Nevertheless, the survival of *O. oeni* depends on other mechanisms, as the increased transcriptional response of the studied genes, related to the nitrogen metabolism, did not ensure the completion of MLF in some wines (Fig. 2).

4. Conclusion

Nitrogen metabolism in *O. oeni* has been a recurrent topic addressed in the literature. Nevertheless, the high heterogeneity of results with no clear findings makes it difficult to understand the role of nitrogen compounds, especially amino acids, in *O. oeni* metabolism and MLF performance. In this study, yeast coinoculation was the least favourable strategy for MLF. Amino acid supplementation in must (N2) enhanced MLF performance for some *O. oeni* strains, yet it could not prevent stuck fermentation in wines inoculated with PSU-1 strain under coinoculation regime of the two yeasts. We were able to relate the nitrogen consumption of *O. oeni* to a decrease in peptide amino acids. The *O. oeni*

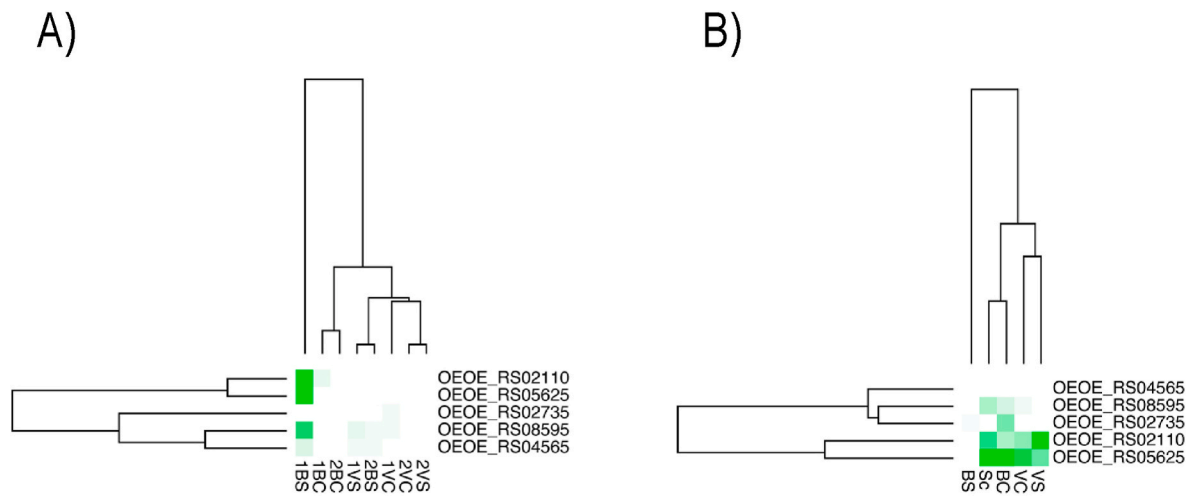


Fig. 5. Heat map and clusterization of the relative expression of some nitrogen related genes in *O. oeni* PSU-1. A) Effect of *T. delbrueckii*: RE is calculated with *S. cerevisiae* as control condition, where the gene expression of *O. oeni* PSU-1 in mixed fermentation is referred to *S. cerevisiae* in Must 1 (1) and *S. cerevisiae* in Must 2 (2). B) Effect of amino acid supplementation: RE is calculated with the gene expression of *O. oeni* PSU-1 in each wine of N2 referred to the gene expression in each wine in N1. The total length of the dendrogram correspond to 100% of similarity. Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Biodiva- *S. cerevisiae* QA23 and *T. delbrueckii* Viniferm- *S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively.

amino acid consumption pattern was greatly influenced by the yeast inoculation strategy in lower nitrogen content wines (N1); however, this influence was not so clear in wines with higher free amino acid concentration (N2). BA production was highly dependent on the *O. oeni* strain but was not influenced by the amino acid concentration in the must. Cadaverine and putrescine increased after MLF were related to the presence of *odc* gene. Also, the inoculation of *T. delbrueckii* did not affect the BA content of wines. In general, the RE of the studied genes responded to the increased amino acid concentration, but it did not show an enhanced amino acid metabolism. All the changes observed reflect the complex metabolism of *O. oeni*, which heavily influenced not only by the specific *O. oeni* strain, but also by the fermenting yeasts. Future research should address the differences in peptide composition depending on the yeast species/strain and their utilization by *O. oeni*.

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CRediT authorship contribution statement

Aitor Balmaseda: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Nicolas Rozès:** Writing – review & editing, Supervision, Funding acquisition. **Albert Bordons:** Writing – review & editing, Supervision. **Hervé Alexandre:** Writing – review & editing, Supervision, Resources, Methodology. **Cristina Reguant:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.116838>.

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