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Influence of Nutrient Supplementation on *Torulaspora Delbrueckii* Wine Fermentation Aroma

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Abstract: This study was performed with the aim of characterizing the fermentative performance of three commercial strains of *Torulaspora delbrueckii* and their impact on the production of volatile and non-volatile compounds. Laboratory-scale single culture fermentations were performed using a commercial white grape juice. The addition of commercial nutrient products enabled us to test the yeasts under two different nutrient conditions. The addition of nutrients promoted fermentation intensity from 9% to 20 % with significant differences ($p < 0.05$) among the strains tested. The strain diversity together with the nutrient availability influenced the production of volatile compounds.

Keywords: *Torulaspora delbrueckii*; volatile compounds; nutrients

1. Introduction

In the last few decades, several researchers have focused on characterizing the oenological potential of non-*Saccharomyces* yeasts [1–3]. Non-*Saccharomyces* species can contribute substantially to the quality of wines. Some yeast species can enhance and modulate the production of volatile aroma compounds thanks to specific enzymatic properties [4–8]. Others promote the production of glycerol, the reduction of acetic acid, and the release of mannoproteins, as well as contributing to color stabilization [9–12]. Detailed and extensive knowledge of the properties of these yeasts will ensure targeted use in order to achieve the desired wine quality and style.

Torulaspora delbrueckii is a ubiquitous yeast species frequently found in association with human activity and food processing. In particular, it has been found in close association with wine, beer, and bread [13,14]. *T. delbrueckii* was one of the first non-*Saccharomyces* yeasts to draw the attention of industrial producers of starter cultures and to be commercially available as active dry yeast, first as a component of different yeast blends and then in pure formulation [1,15,16]. Nowadays, the most popular dry yeast manufacturers have *T. delbrueckii* available in their catalogues [14,16].

After the first discoveries of *T. delbrueckii* in association with fermenting must, different research groups started investigating its oenological potential. Some studies comprehensively characterized the genetic diversity within *T. delbrueckii* strains focusing on its application in winemaking [17,18]. The high variability registered among different strains of *T. delbrueckii* [6,19] demonstrates the high genetic diversity that characterizes this species. It has been demonstrated that this genetic diversity is the result of a long process of domestication under the most diverse conditions in wine production

environments [13]. The availability of such high genetic diversity is very important and means that there is still an opportunity to search for improved characteristics.

Many studies have investigated the influence of *T. delbrueckii* under different fermentation conditions, using different inoculation strategies, for the production of red, white, and sparkling wines [20]. *T. delbrueckii* often shows a slower fermentation rate than *S. cerevisiae*; many studies reported longer fermentation durations and a longer lag phase, with wines frequently containing an unacceptable residual sugar content for dry wines [21,22]. *T. delbrueckii* was demonstrated to produce lower amounts of acetaldehyde and acetic acid in comparison with *S. cerevisiae*, confirming its high potential for application in wine production [9,22]. Regarding the production of glycerol, results from the literature are contradictory, showing sometimes higher [9,17,23] and sometimes lower production of glycerol than *S. cerevisiae* [24]. With respect to organic acids, and apart from acetic acid production, *T. delbrueckii* also demonstrated a certain activity of malic acid degradation, higher than the usual levels reported for *S. cerevisiae* [21,22,25,26]. *T. delbrueckii* is generally regarded as a low producer of aroma compounds; studies have reported an average production of higher alcohols of about 100 mg/L [17] and a lower production of esters than *S. cerevisiae* [4,17,26–28]. Confirmation of these findings comes from a demonstration of the low enzymatic activity of esterases in *T. delbrueckii* [6]. On the other hand, studies have reported slight activity of *T. delbrueckii* toward monoterpenes [29,30] and the precursors of volatile thiols [25,31,32] and therefore its ability to modulate the aroma profile of wines from grape varieties such as those from the Muscat family or Gewürztraminer, but also Sauvignon blanc, Riesling, or Verdejo [8].

The aim of this work was to compare the fermentation performance and aroma compound production of three commercial yeast strains of *T. delbrueckii* during the fermentation of a white grape juice under two different nutrient conditions.

2. Materials and Method

2.1. Yeast Strains

All fermentations were conducted with commercially available *Torulaspora delbrueckii* strains: Viniflora®Prelude™ (Hansen, Hørsholm, Denmark), Biodiva™ TD291 (Lallemand, Montreal, Canada), and Zymaflore®Alpha TD n. *Sacch* (Laffort, Bordeaux, France) (hereafter Prelude, Biodiva, and AlphaTd).

2.2. Juice Composition

A commercial white grape juice (Jacoby GmbH, Germany; http://www.jacoby.de/index.php/en/produkte?page=shop.product_details&flypage=flypage.tpl&product_id=47&category_id=83) was used to perform laboratory-scale fermentations. D-glucose and D-fructose were both added at a concentration of 10 g/L. The total amount of sugars in the juice after the addition was 168.9 g/L. The fermentations were performed under 2 nutrient conditions for each strain tested: (1) no addition of nutrients and (2) addition of 2 nutrients. Two commercial products by the Lallemand company (Montreal, Canada), Fermaid®E-blanc and OptiMUM white™, were used at a concentration of 0.4 g/L to provide nutrients. In the juice without the addition of nutrients, the concentration of ammonium was 81 mg/L and the primary amino nitrogen (NOPA) value was 118 mg N/L. The total yeast assimilable nitrogen content of the juice after the addition of nutrient preparations was 281 mg N/L, corresponding to a NOPA value of 137 mg N/L and an ammonium content of 144 mg N/L. The initial acid content was as follows: tartaric acid 2.33 g/L, malic acid 2.18 g/L, citric acid 0.11 g/L, lactic acid <0.1 g/L, and acetic acid <0.1 g/L. Primary amino nitrogen and ammonium concentrations were determined using an Evolution™220 spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany) and the commercial enzymatic kits K-PANOPA (Megazyme-Romer Labs, Bulzbach, Germany) and K-AMIAR (Megazyme-Romer Labs, Bulzbach, Germany) following the instructions of the manufacturer.

2.3. Microvinifications

All fermentations were conducted in 500 mL Schott–Duran® bottles following a previous protocol but adapted to the scale [33,34]. Each variant was performed in triplicate. All operations took place under aseptic conditions to avoid any contamination. According to the specifications of the protocol [33], 0.5 L of must sterilized (115 °C, 15 min) after nutrient and sugar correction were placed in a 1 L glass fermentation flask, leaving enough space for carbon dioxide emission. All inoculations were performed in 1 L flasks sealed with a fermentation lock filled with 98 % H_2SO_4 (Sigma-Aldrich, San Luis, USA), which allowed the release of CO_2 while avoiding microbial contamination. Three non-inoculated flasks were used to verify the success of the sterilization and aseptic conditions, as they did not ferment during the study. The volume of inoculum was 10 mL in each bottle. The inoculum was prepared by rehydrating 100 mg of the corresponding commercial strain product in 10 mL of sterilized water under laboratory sterile conditions. The number of cells was evaluated by cell counting using a Thoma counting chamber Blaubrand® (Brand, Wertheim, Germany) in a Leica DM 500 microscope (Wetzlar, Germany). The final concentration of inoculated cells in 500 mL was 0.5×10^7 cfu for Prelude samples, 1.2×10^7 cfu for Biodiva samples, and 3.8×10^7 cfu for AlphaTd samples. The bottles were closed with fermentation lockers previously filled with a solution of water and potassium metabisulfite at a concentration of 50 g/L to avoid contamination. The fermentations were carried out in an incubator at a controlled temperature of 25 °C for 15 days. The progress of the fermentations was followed by daily measurements of flask weights. On the 15th day, all bottles were moved to a refrigerator room at 4 °C for 8 days. stopping the alcoholic fermentation and settling down the wine, then the wine was racked. Under aseptic conditions, sterilized glass bottles were filled and closed with screw caps after the addition of a solution of potassium metabisulfite at a final concentration of 80 mg/L. The wines were kept refrigerated at 4 ºC until the chemical analyses were performed.

2.4. HPLC Analyses

The main oenological parameters of juice before fermentation were measured by means of high-performance liquid–liquid chromatography (HPLC) based on a previously reported methodology [35] with the following modifications [34]. Samples were prepared in 1.5 mL vials after centrifugation at 13,000 rpm for 5 min and subsequent dilution to 1:1 with distilled water, and 5 µL was successively injected into an Agilent Technologies 1100 series liquid chromatograph (Agilent Technologies GmbH, Germany) equipped with 2 detectors, a multi-wavelength detector, and a refractive index (RI) detector (Agilent Technologies GmbH, Germany). Eluting compounds were detected by UV absorbance at 210 nm by the multi-wavelength detector. The samples were analyzed using an Allure Organic Acid Column (250 mm × 4.6 mm inside diameter, 5 µm particle size, 60 A° pore size) from Restek GmbH (Bad Homburg, Germany) with a C-18 Security Guard Cartridge, 4.0 × 3.0 mm (Phenomenex GmbH, Aschaffenburg, Germany). The column temperature was set at 46 °C; the eluent consisted of distilled water with 0.0139% sulfuric acid and 0.5% ethanol (flow rate: 0.6 mL/min). For quantitative analysis of each compound, external standards of organic acids, sugars, and ethanol were used. The concentration of glycerol was determined with the same procedure but at a temperature of 29 °C.

2.5. Wine Composition

The concentration of low-volatile sulfur compounds was evaluated in the final wines by means of a headspace gas chromatograph (6890, Agilent Technologies, Germany) using a pulsed flame photometric detector (HS–GC–PFPD), according to the methodology described in a previous study [36]. The analysis was carried out as follows: 5 mL of sample at 4 °C was placed in a 10 mL headspace vial containing 1.7 g of NaCl and flushed with argon, which replaces oxygen and prevents the occurrence of oxidation reactions. As internal standards, methyl-iso-propyl sulphide and butyl methyl sulphide were used. The other reagents were 4 mg/L of 2,6-di-tert-butyl-4-methyl-phenol (antioxidant), 0.2 g/L of ethylene diamine tetraacetic acid (EDTA; chelating agent), and 500 mg/L of propanal (added to

bind SO_2). The procedure started with preheating the samples to 60 °C for 45 min under constant agitation, and then the headspace sampler (MPS 2 MultiPurpose Sampler, Gerstel, Mülheim an der Ruhr, Germany) injected 1 mL of the headspace into the cooled injection system (CIS 4, Gerstel, Mülheim an der Ruhr, Germany). The injection system operated with the following temperature program: first, a cooling step to −100 °C, then heating to 40 °C at a rate of 12 °C/s and held for 1 min, and finally, heating to 180 °C and held for 8 min. Subsequently, the analytes were moved to the column in 10:1 split mode. The column was an SPB-1 sulfur capillary column (30 m × 0.32 mm I.D., 4 µm film thickness; Supelco, Darmstadt, Germany). The temperature program of the column was as follows: 7 min at 29 °C, then 10.5 min at 180 °C, heating at a rate of 10 °C/min. The analytes passed through the column carried by helium (He), with an average speed of 20 cm/s. At the end, the detector (PFPD 5380, OI Analytical, College Station, TX, USA) worked at 250 °C.

The fermentative aroma compounds (higher alcohols, esters, fatty acids, etc.) of the resulting wines were evaluated by means of a gas chromatograph (HP 5890 Series II, Hewlett Packard, Palo Alto, California, Estados Unidos) coupled with a mass spectrometry detector (HP 5972 MSD, Hewlett Packard). The analysis was performed based on a previously reported methodology [37] with the following modifications [34]. First, samples needed to be prepared for the extraction: 10 mL of sample, 10 µL of each internal standard, 2 g of sodium chloride (NaCl), and 160 µL of 1,1,2-trichlorotrifluoroethane (Freon 113) were added to a 15 mL tube. The internal standards were 2,6-dimethyl-5-hepten-2-ol (DMH, 1219 µg/L) and isopropylbenzene (268 µg/L), and 1,1,2-trifluorotrichloroethane was the extraction reagent. Then, the solution was shaken for 20 min with an Intelli-Mixer (NeoLab) and was subsequently centrifuged at 3000 rpm for 8 min. The organic phase was removed with a glass pipette and dried with 50 mg of Na_2SO_4 on glass wool. Then, 2 µl of the extract was used for chromatographic analysis. The GC oven was equipped with a Gerstel MPS 2 Autosampler and CIS 3 cooled injection system. The sample was injected in splitless mode (initial temperature 30 °C, rate 12 °C/s to 230 °C, held for 4 min) into a VF-5MS capillary column (60 mm × 0.32 mm × 1 µm; Varian, Steinheim, Germany), and helium was used as a carrier gas (flow of 1 mL/min). A precise temperature program was applied: an initial temperature of 40 °C was held for 5 min, then raised to 125 °C at 3 °C/min, and then raised again to 200 °C at 6 °C/min and held for 14.2 min. The working temperature of the MS detector was 180 °C. Mass spectral data were acquired in a range of mass to charge ratio (m/z) of 35 to 250 and used to derive concentration values.

2.6. Statistical Analysis

Statistical analysis was performed using RStudio v. 1.1.414 software (© 2009–2016 RStudio Inc.). Parametric analysis of variance (ANOVA) was used when the assumptions of normal distribution and homogeneity of variances were satisfied (LSD.test function in agricolae package). Alternatively, in the case of non-normal distribution or heterogeneity of variances, non-parametric analysis was chosen (waerden.test function in agricolae package). Differences were considered significant at a p-value < 0.05.

3. Results

3.1. Fermentation Kinetics

Figure 1 shows the fermentation curves of each strain under the two nutrient conditions. It is evident that the addition of nutrients promoted higher fermentative activity. AlphaTd showed the highest activity, while Prelude and Biodiva had similar behavior. The values of residual sugar in the resulting wines followed these differences (Table 1): only wines fermented with AlphaTd and added nutrients were able to almost finish the fermentation, with less than 3.27 g/L of residual sugar on average. In the other cases, the amount of residual sugar varied from 17.49 to 67.15 g/L. Therefore, the range of ethanol produced ranged from 5.2% to 9.2%. AlphaTd, Biodiva and Prelude synthesized 6%, 24% and 30% more ethanol from fermentable sugars with nutrient suplementation than the regular controls. Acetic acid concentrations were less than 0.1 g/L in all cases, even though high variability was evident in the fermentation performance. The content of malic acid after 15 days of fermentation was lower than the

initial value in all cases, indicating malic acid degradation. The level of this degradation was moderate, with a rate ranging from 10.5% to 27.3%, corresponding to a consumption of 0.23 and 0.60 g/L of malic acid, respectively. The highest amount was consumed by AlphaTd under both nutrient conditions.

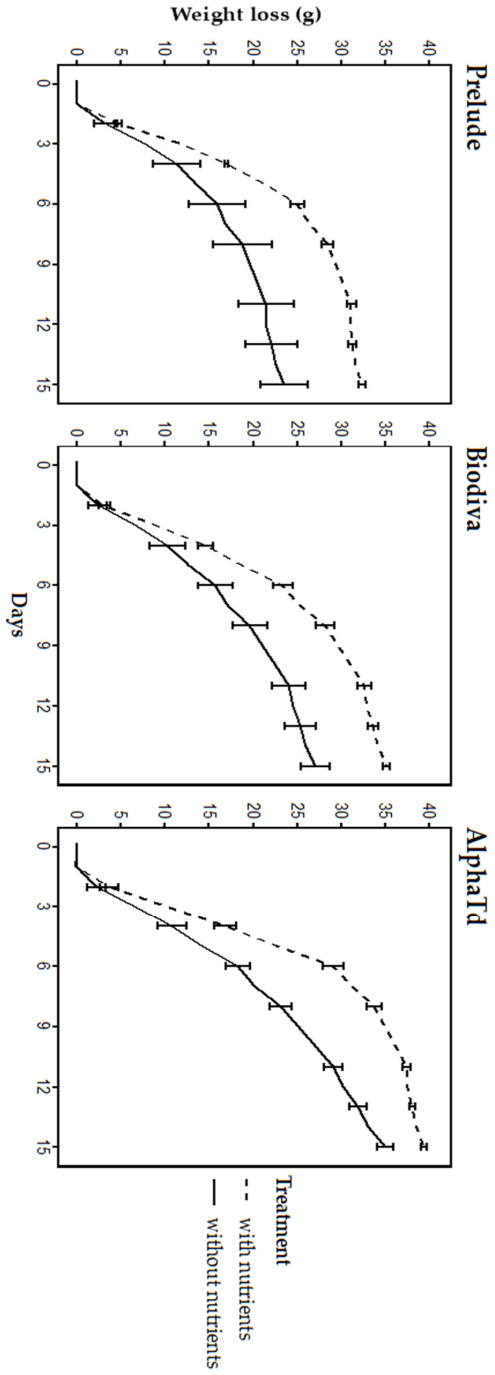

Figure 1. Fermentation kinetics of Prelude, Biodiva, and AlphaTd strains under the two nutrient conditions. Curves describe the loss of weight (g) throughout the 15 days of fermentation. Curves were plotted using the average values of triplicates; error bars represent standard deviations of each measurement. Solid lines represent fermentations conducted without the addition of nutrients; dashed lines represent fermentations with the addition of nutrients.

Table 1. Main fermentation parameters of wines resulting from grape juice fermentation by *T. delbrueckii* strains Prelude, Biodiva, and AlphaTd, in single cultures and the statistical differences.

Parameters	Yeast Strains and Nutrient Conditions					
	Prelude	**Prelude + Nutrients**	**Biodiva**	**Biodiva + Nutrients**	**AlphaTd**	**Alpha Td + Nutrients**
Residual glucose (g/L)	22.28 ± 4.62 a	9.49 ± 0.81 c	14.89 ± 1.84 b	5.29 ± 0.66 d	3.10 ± 0.35 e	n.q.
Residual fructose (g/L)	44.87 ± 5.11 a	24.05 ± 1.29 b	36.91 ± 4.05 a	16.32 ± 0.96 c	14.39 ± 2.54 c	2.27 ± 0.29 d
Ethanol (g/L)	40.60 ± 5.11 d	57.92 ± 0.99 c	48.35 ± 2.95 d	63.22 ± 0.74 b	65.57 ± 1.26 b	72.68 ± 0.09 a
Ethanol (%)	5.1 ± 0.65	7.3 ± 0.13	6.1 ± 0.37	8.0 ± 0.09	8.3 ± 0.16	9.2 ± 0.01
Ethanol yield	0.40 ± 0.01 d	0.43 ± 0.00 c	0.41 ± 0.00 d	0.43 ± 0.00 c	0.43 ± 0.00 b	0.44 ± 0.00 a
Acetic acid (g/L)	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Malic acid (g/L)	1.85 ± 0.02 b	1.78 ± 0.01 cd	1.95 ± 0.01 a	1.82 ± 0.01 bc	1.75 ± 0.06 d	1.60 ± 0.03 e
Lactic acid (g/L)	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Citric acid (g/L)	0.11 ± 0.01 a	0.10 ± 0.00 a	0.11 ± 0.01 a	0.10 ± 0.00 a	0.11 ± 0.01 a	0.11 ± 0.01 a
Tartaric acid (g/L)	2.20 ± 0.00 a	2.22 ± 0.01 a	2.21 ± 0.00 a	2.24 ± 0.02 a	2.22 ± 0.00 a	2.24 ± 0.02 a

Significant differences ($p < 0.05$) are indicated with different letters. All values are averages of triplicates ± standard deviation. n.q., not quantifiable.

3.2. Production of Volatile Compounds

Table 2 reports the concentrations of volatile compounds found in the resulting wines. Hydrogen sulfide was the only sulfur compound detected in the resulting wines, at concentrations ranging from 5.3 to 12.7 µg/L. Fermentations carried out by AlphaTd produced the highest amounts, while Prelude and Biodiva produced non quantifiable concentrations fermented without nutrients. AlphaTd produced 29% more hydrogen sulfide in the presence of nutrients.

Table 2. Volatile aroma compounds produced by three commercial strains of *T. delbrueckii* under two nutrient conditions.

Parameters	Yeast Strains and Nutrient Conditions					
	Prelude	**Prelude + Nutrients**	**Biodiva**	**Biodiva + Nutrients**	**AlphaTd**	**Alpha Td + Nutrients**
H_2S (µg/L)	n.q.	5.3 ± 0.61 c	n.q.	5.5 ± 0.49 bc	7.8 ± 1.93 b	12.7 ± 1.72 a
Higher alcohols						
Isobutanol (mg/L)	8.7 ± 0.58 d	14.0 ± 1.0 b	7.0 ± 1.00 d	12.0 ± 0.00 c	15.7 ± 1.53 b	18.7 ± 0.58 a
2-Phenyl-ethanol (mg/L)	8.3 ± 0.58 d	13.0 ± 0.00 b	10.7 ± 0.58 c	16.0 ± 1.73 a	12.0 ± 1.00 bc	16.7 ± 0.58 a
Isoamyl alcohol (mg/L)	32.0 ± 2.56 d	63.3 ± 4.16 b	31.7 ± 3.51 d	66.7 ± 0.58 b	53.3 ± 4.51 c	87.7 ± 3.51 a
Active amyl alcohol (mg/L)	3.3 ± 0.58 e	7.3 ± 0.58 c	2.7 ± 0.58 e	5.7 ± 0.58 d	9. 3 ± 1.53 b	14.3 ± 0.58 a
Total higher alcohols	52.3	97.6	52.1	100.4	81	137.4
Medium-chain fatty acids						
Hexanoic acid (mg/L)	5.45 ± 0.04 c	5.73 ± 0.01 b	5.41 ± 0.06 c	5.72 ± 0.08 b	7.26 ± 0.43 a	7.26 ± 0.27 a
Octanoic acid (mg/L)	1.95 ± 0.11 b	2.12 ± 0.03 b	1.85 ± 0.13 b	2.15 ± 0.07 b	4.96 ± 0.70 a	4.61 ± 0.44 a
Decanoic acid (mg/L)	1.67 ± 0.42 a	1.91 ± 0.12 a	0.99 ± 0.31 a	1.14 ± 0.26 a	1.78 ± 0.64 a	1.62 ± 0.19 a
Total medium-chain fatty acids	9.07	9.76	8.25	9.01	14	13.49
Acetate esters						
Amyl acetate (mg/L)	n.q.	n.q.	n.q.	n.q.	0.79 ± 0.83 a	1.15 ± 0.26 b
Isoamyl acetate (mg/L)	n.q.	n.q.	n.q.	n.q.	1.21 ± 0.23 b	3.12 ± 2.75 a
2-Phenyl ethylacetate (mg/L)	n.q.	0.014 ± 0.06 d	n.q.	0.017 ± 0.01 c	0.36 ± 0.36 b	0.68 ± 0.36 a
Ethyl acetate (mg/L)	n.q.	n.q.	n.q.	36.6 ± 9.94 c	93.9 ± 14.62 b	152.2 ± 15.51 a
Total acetate esters	0	0.014	0	36.617	96.26	157.15
Ethyl esters						
Ethyl butyrate (µg/L)	n.q.	n.q.	n.q.	35.2 ± 10.77 b	447.0 ± 51.47 a	493.0 ± 16.52 a
Ethyl hexanoate (µg/L)	n.q.	n.q.	n.q.	n.q.	458.3 ± 28.89 a	221.2 ± 87.74 b
Ethyl octanoate (µg/L)	n.q.	n.q.	n.q.	n.q.	465.9 ± 76.74 a	440.9 ± 107.22 a
Ethyl decanoate (µg/L)	179.4 ± 51.50 ab	194.5 ± 6.44 ab	99.9 ± 38.21 b	129.0 ± 21.53 b	276.5 ± 26.51 a	189.2 ± 48.82 ab
Ethyl propionate (µg/L)	372.6 ± 91.46 cd	502.1 ± 23.49 b	363.7 ± 16.98 cd	631.9 ± 28.23 a	266.4 ± 73.50 d	470.6 ± 26.31 bc
Total ethyl esters	552	696.6	463.6	796.1	1914.1	1814.9

Different letters show statistically significant differences ($p < 0.05$) among variants. All values are averages of triplicates ± standard deviation. n.q., not quantifiable.

Regarding fermentative aroma compounds, the total concentrations of higher alcohols among the three strains tested varied from approximately 40 to 137 mg/L. For all compounds, a higher fermentation intensity corresponded with a higher concentration, and under both nutrient conditions, the predominant higher alcohol was isoamyl alcohol, exceeding its sensory threshold (30 mg/L). AlphaTd, Biodiva and Prelude produced, respectively, 59%, 52% and 54% more total higher alcohols for the trails enriched with nutrients. It is worth noting that all strains fermented with nutrients produced 2-phenylethanol above its odor threshold, in a range of 13.0–16.7 mg/L (Table 2). The concentrations of four volatile medium-chain fatty acids were investigated, and the results are reported in Table 2. Except for decanoic acid, for which no significant differences were found among all strains and nutrient conditions, the AlphaTd strain was shown to be the highest producer of hexanoic and octanoic acids. Slight differences in total volatile medium-chain fatty acids were observed between the trials with added nutrients and the regular controls. The production of ester compounds seemed to be affected by the yeast strain and the nutrient condition. In fact, only AlphaTd produced hexyl acetate, isoamyl acetate, and amyl acetate, while Prelude and Biodiva produced very low concentrations of 2-phenylethyl acetate in fermentation with nutrients, during which Biodiva also produced ethyl acetate. In addition, it is worth mentioning the production of ethyl acetate by AlphaTd at higher concentrations. On the other hand, ethyl butyrate, ethyl hexanoate, and ethyl decanoate were produced only by AlphaTd under both nutrient conditions. The Prelude and Biodiva strains instead produced ethyl propionate and ethyl decanoate. AlphaTd did not increase the total ester concentration with nutrient addition, while Biodiva and Prelude increased the sum of total esters for the trials enriched with nutrients by 44% and 21%, respectively.

4. Discussion

4.1. Fermentation Parameters

Supplementation with nutrients promotes fermentation activity, with a higher consumption of sugar and higher production of ethanol [38]. Thus, the role of nutrients in the stimulation of fermentation kinetics is confirmed for *T. delbrueckii* in this experiment. The ethanol yield can be considered to compare the fermentative power among the strains tested in this trial. A transformation ratio of 16.83 g/L of sugar for 1% *v/v* of ethanol for *S. cerevisiae* [39,40] is generally accepted. This means that a normal ethanol yield would be approximately 0.48 *g/g*, while the values obtained in this trial ranged from 0.40 to 0.44 *g/g* (Table 1). These results agree with the low fermentative ability attributed to non-Saccharomyces yeasts [33]. The interesting aspect is the higher yield with the addition of nutrients. The same behavior has been described by other authors for T. delbrueckii [41] and other non-Saccharomyces yeast species [42], suggesting a role of nitrogen in the promotion of a higher rate of fermentation over other metabolic functions.

The low amount of acetic acid present in all wines, regardless of sugar consumption or nutrient conditions, is not surprising. In fact, the low production of acetic acid is one of the most interesting features of *T. delbrueckii*, widely reported in the literature even in association with *S. cerevisiae* [43]. Therefore, the application of *T. delbrueckii* in association with *S. cerevisiae* is an interesting oenological resource to keep the volatile acidity low [9].

Few wine yeast species have been studied for their ability to degrade malic acid. Among all non-Saccharomyces yeasts associated with winemaking, only Schizosaccharomyces pombe showed activity comparable to lactic acid bacteria [44,45]. On the other hand, *Torulaspora delbrueckii* has been described as only a moderate malic acid consumer. In this study, malic acid was consumed by all strains with a degradation rate ranging from 10.5% to 27.3%. These results are consistent with other findings from the literature [26,46]. Additionally, in this study, nutrient supplementation stimulated malic acid degradation by all strains in 9% (AlphaTd), 7% (Biodiva) and 4% (Prelude) respectively (Table 1). The malic acid degradation can be variable depending on the fermentation conditions, as

demonstrated by the fact that the same strain used in this study showed no malic acid degradation in lychee must fermentation [47].

4.2. Hydrogen Sulfide Production

Hydrogen sulfide formation in wines is undesirable due to its "rotten egg" and "cabbage" odor. Different odor thresholds have been reported in the literature. Some authors reported an odor threshold of 1–1.6 ng/L [48,49], while others reported a range of 11–80 µg/L [50–52]. Based on the different odor thresholds, the concentrations of hydrogen sulfide found in this experiment could be considered either highly detrimental for wine quality or still quite acceptable. The lack of nitrogen availability is considered one of the main causes of H_2S production by yeasts [53]; however, in this case, the fermentations with higher nitrogen availability resulted in a higher content of hydrogen sulfide (Table 2). Although the production of H_2S as a result of supplementing must with nitrogenous compounds has been described [49,54,55], in this trial this scenario does not explain why H_2S was also found in slightly increased amounts in wines fermented with AlphaTd with and without the addition of nutrients. It could be hypothesized that high sulfite reductase activity is the reason for H_2S production, through a mechanism previously described in the literature [53,56]. This hypothesis is consistent with the high sulfite reductase activity reported for many *T. delbrueckii* strains [6,57]. Furthermore, it can be assumed that this strain generally has a relatively high requirement for nutrients. Finally, the production of hydrogen sulfide from cysteine degradation cannot be excluded since it has been demonstrated that *T. delbrueckii* has high β-lyase activity and carries a full-length copy of the IRC7 gene [25]. IRC7 has been reported as the gene that encodes the enzymes that produce 4-methyl-4-sulfanylpentan-2-one from Cysteine-4-methyl-4-sulfanylpentan-2-one [8].

4.3. Fermentative Aroma Compound Production

Non-Saccharomyces yeasts are interesting for their ability to improve wine aroma complexity [8,33,58]. The total concentrations of higher alcohols observed were lower than the concentrations usually found in wines, i.e., 140–420 mg/L [59,60], but consistent with previous findings from the literature [17,21,34]. Non-Saccharomyces yeasts are usually indicated as being lower producers of higher alcohols than *S. cerevisiae* [1,7,33]. In particular, *T. delbrueckii* has been described as being able to lower the total amount of higher alcohols in sequential fermentation with *S. cerevisiae*. This effect can result in a more positive score in sensorial analysis compared with *S. cerevisiae* in pure fermentation due to the greater impact of varietal aromas that are not masked by higher alcohols [8,25]. Other studies performed with different *T. delbrueckii* strains showed the same pattern of higher alcohol production [17,61]. This aspect highlights the hypothesis that there is common behavior within the *T. delbrueckii* species, confirming the possibility of using aroma production profiles as species indicators. In this study, all strains showed increased production of higher alcohols in fermentations with added nutrients. However, the opposite behavior has often been reported, with lower production of higher alcohols under the condition of higher nitrogen availability [62,63]. Some authors have suggested that higher alcohols are produced in greater amounts when there is a higher demand of nitrogen to sustain protein synthesis and population growth [7]. This explanation could also be applicable to the results of this trial. In fermentation with higher nitrogen availability, yeasts might have produced more higher alcohols in response to higher fermentation activity or a larger population size. The strain variability in the production of higher alcohols reported in this study agrees with other studies, where differences up to 38% were observed depending on the studied *T. delbrueckii* strain in higher alcohols, such as 1 butanol [26]. Only a few studies reported concentration values of medium-chain fatty acids (MCFAs) produced by *T. delbrueckii* in pure fermentations and, in general, the concentration of each compound did not exceed 1 mg/L [17,26]. In this trial, however, the three strains of *T. delbrueckii* produced higher concentrations of MCFAs compared to those reported in the literature. On the other hand, isovaleric acid resulted in nonquantifiable amounts for all strains (data not shown). However, other studies reported that some strains of *T. delbrueckii* were high producers of this compound [26]. Considering each fatty acid

individually, all concentrations were below the sensory thresholds; however, the total concentrations were close to 10 mg/L for Prelude and Biodiva, while AlphaTd produced almost 14 mg/L of MCFAs. A direct influence of nitrogen availability on the production of MCFAs has been described in the literature, with a higher nitrogen content promoting a higher production of these compounds in *S. cerevisiae* [64,65]. In this trial, only the production of hexanoic acid, and only by Prelude and Biodiva strains, was shown to be influenced by the addition of commercial nutrient preparations. Therefore, we can deduce that the production of MCFAs by *T. delbrueckii* is influenced either by the yeast strain, as in the case of octanoic acid, or by the nutrient availability, as in the case of hexanoic acid (Table 2).

The variability registered in the production of esters was high among the three strains; in particular, many compounds were produced only by the AlphaTd strain. The presence of esters in wines is desired for their fruity notes; however, in this case, the production of high amounts of ethyl acetate, which can be unpleasant at concentrations above 100 mg/L [66], should be noted. This behavior has never been reported in the literature; on the contrary, *T. delbrueckii* has always been described positively for its high fermentation purity thanks to its low production of acetic acid and ethyl acetate [21,26]. On the other hand, 2-phenylethyl acetate and isoamyl acetate were also produced in high amounts, and these compounds are known to impart very pleasant fruity and floral notes, in particular of banana and rose; also, for these compounds, the concentrations found in this trial were higher than those reported in the literature for fermentations by *T. delbrueckii* strains in single cultures [26,28,67]. For all acetate esters, AlphaTd showed a higher concentration in fermentations enriched with nutrients. This phenomenon is well described in the literature for *S. cerevisiae* [64,68], as the addition of nutrients likely had a direct stimulating effect on ester production. In this regard, the lack of acetate ester production by Prelude and Biodiva strains could be explained by low fermentative activity and thus low enzymatic activity. Considering ethyl esters, AlphaTd was the only producer of ethyl butyrate, ethyl hexanoate, and ethyl octanoate, while all three strains under both nutrient conditions produced ethyl decanoate and ethyl propionate. It is known that ethyl ester production is dependent on the availability of corresponding fatty acid precursors. Therefore, any increase in production as a result of higher nitrogen availability should be ascribed to an increased synthesis of fatty acids [69,70], and in this trial, AlphaTd confirmed this dependency.

As it is not possible to compare the results with previous studies regarding *Torulaspora delbrueckii* and nutrient supplementation influence over aroma composition., further studies should be performed in the future using deuterated internal and/or external standards to validate the commented results.

5. Conclusions

T. delbrueckii's production of aroma compounds during alcoholic fermentation is subject to complex regulation. Both strain diversity and the availability of nutrients lead to significant differences in the production of volatile aroma compounds. The addition of nutrients increases the speed of fermentation for all strains, as well as ethanol production, malic acid degradation, and the formation of most volatile aroma compounds. Alpha strain produced higher concentrations of ethanol, higher alcohols, medium-chain fatty acids and esters than the other studied strains while also degrading more malic acid. The addition of nutrients increased the levels of higher alcohols and esters significantly for all the *T. delbrueckii* studied strains, while the increase in medium-chain fatty acids was moderate.

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