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Effect of Yeast Assimilable Nitrogen Content on Fermentation Kinetics, Wine Chemical Composition and Sensory Character in the Production of Assyrtiko Wines

Stefania Christofi ¹, Seraphim Papanikolaou ¹, Maria Dimopoulou ¹, Antonia Terpou ^{1,2},
Ionel Bogdan Cioroiu ³, Valeriu Cotea ⁴ and Stamatina Kallithraka ^{1,*}

¹ Department of Food Science and Human Nutrition, Agricultural University of Athens, 75, Iera Odos, 11855 Athens, Greece; stefania.christofi@gmail.com (S.C.); spapanik@aua.gr (S.P.); maroula.dimo@hotmail.com (M.D.); aterpou@agro.uoa.gr (A.T.)

² Department of Agricultural Development, Agri-Food, and Natural Resources Management, School of Agricultural Development, Nutrition & Sustainability, National and Kapodistrian University of Athens, 34400 Evripos Union, Greece

³ Romania Academy—Iasi Branch, Research Centre for Oenology, 9 M. Sadoveanu Alley, 700490 Iasi, Romania; bogdan.cioroiu@gmail.com

⁴ Department of Oenology, Iasi University of Life Sciences, 3 M. Sadoveanu Alley, 700490 Iasi, Romania; vcotea@uaiasi.ro

* Correspondence: stamatina@aua.gr

Abstract: Two wild-type *Saccharomyces cerevisiae* yeast strains (Sa and Sb) were tested for white wine production using Assyrtiko grape of Santorini. A third commercial *Saccharomyces* strain was also studied for comparison reasons. Two concentrations of yeast extract and diammonium phosphate (DAP) were added to the must (150 and 250 mg/L) in order to evaluate the effect of nitrogen content on the final wine quality. Analytical methods (HPLC, GC-MS) and sensory analysis were employed to assess the quality of the wines. Fermentation kinetics were monitored throughout the experiment. By the second day of fermentation, all strains showed an approximate consumption of 70% of amino acids. Differences among strains were observed regarding inorganic nitrogen requirements. Sb strain resulted in higher concentrations of higher alcohols (1.9-fold) and ketones (5.6-fold) and lower concentrations of esters (1.2-fold) compared to the control, while Sa strain resulted in higher content of fatty acids (2.1-fold). Both indigenous strains scored better results in aroma quality, body and acidity compared to control. The overall evaluation of the data highlights the great potential of the indigenous *S. cerevisiae* strains as fermentation starters providing promising results in the sector of terroir wines.

Keywords: yeast assimilable nitrogen content (YAN); wine fermentation starters; indigenous *Saccharomyces cerevisiae*; wine volatile compounds; wine organoleptic quality; terroir wines



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1. Introduction

In the past few years, radical changes in global wine exports have occurred, creating a new era in production and consumption of wine [1]. Targeting national competitive advances, producers have focused on the production of terroir wines, using indigenous grape varieties and autochthonous yeast strains [2]. In the industrial wine sector, selected yeast and bacterial strains are employed for fermentation, targeting high yields of productivity, stress tolerance, unique aromatic characteristics and positive sensory attributes [3,4].

Growth and fermentation kinetics of yeasts are influenced by their nutritional requirements and are highly strain-dependent [5]. Nitrogen availability in grape must is a key parameter for the wine fermentation process, since N- is a substantial nutrient element for yeast growth. It affects the formation of yeast biomass, which, in turn, regulates fermentation duration and kinetics [5]. Grape must contains several different nitrogen sources

including amino acids, ammonium, and small peptides, although not all of these forms can be metabolized by yeasts. The ammonium and the α -amino acid nitrogen forms are described as the preferential forms, while utilization of small peptides in different environments is possible under specific conditions [6]. A minimum of 140 mg/L of assimilable nitrogen has been established as essential for yeast growth in must under anaerobic fermentation conditions [5,7]. The assimilation rate of nitrogen is strictly strain-dependent, and as a result, the selected strain plays a major role in alcoholic fermentation kinetics [8]. Different *S. cerevisiae* strains are characterized by unique nitrogen uptake requirements resulting in variable fermentation behaviors and unique wine organoleptic profiles [9]. Previous studies revealed that different yeast strains can show sufficient fermentation capacity even with limited amounts of nitrogen, while other strains require higher amounts and may result in stuck fermentations in case of nitrogen deficiency [5,10]. In order to avoid nitrogen deficiency during wine fermentation, it is a common practice to supplement musts with inorganic nitrogen mainly in the form of diammonium phosphate (DAP). This nitrogen source has been linked with significant effects on wine flavor [11–13]. More specifically, the type of nitrogen source directly affects the production of wine volatiles (esters, alcohols, and volatile fatty acids) as well as glycerol and organic acid composition [12–14]. Likewise, nitrogen-containing compounds are considered as important precursors of wines' volatile compounds, and as a result, the origin and availability of nitrogen significantly affects the final quality of wine [11].

The aim of the current study was to investigate the potential of two *S. cerevisiae* strains (previously isolated from Santorini Island), to produce wine with unique organoleptic characteristics. For this purpose, must from the Assyrtiko grape variety (indigenous to Santorini Island), were fermented with the autochthonous yeasts. Aiming to produce wines of high quality, the fermentation performance of the yeasts under two YAN contents (high and low) was evaluated, and their impact on wine sensory character was assessed.

2. Materials and Methods

2.1. Microorganisms and Growing Conditions

Commercial and autochthonous yeast strains were studied as starter cultures for wine-making. The yeast *Saccharomyces cerevisiae* Sa and Sb autochthonous strains from the private collection of the Agricultural University of Athens (Laboratory of Food Microbiology & Biotechnology, Department of Food Science and Human Nutrition, Athens, Greece), which had never been tested before regarding their fermentative activity, were used as starter cultures for fermentation of Assyrtiko grape must from Santorini Island (Greece). These strains were deposited to the private collection of the Laboratory of Food Microbiology & Biotechnology—Agricultural University of Athens, Greece, obtaining the code numbers FMCC Y73 and FMCC Y74, respectively. The identification of the yeast species was verified by sequencing of the D1/D2 domain of 26S rRNA gene [15] (data not shown). The commercial *S. cerevisiae* strain, iYeast[®] Passion Fruit (LaFood, Fasano, Italy) was chosen as a control inoculation, as this strain is suitable for the production of white wines with strong aromatic intensity.

Yeast strains were stored at $-80\text{ }^{\circ}\text{C}$ in vials with glycerol (30%) and were activated by adding 200 μL to 10 mL yeast peptone dextrose (20 g/L glucose, 10 g/L yeast extract and 10 g/L peptone) medium at $28\text{ }^{\circ}\text{C}$ for 24 h, while the purity of each strain was verified via optical microscopy [16]. Pre-cultures were performed in 250 mL flasks filled with 50 mL of medium (YPD medium: 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, $\text{pH} \approx 3.5$) previously autoclaved at $T = 115\text{ }^{\circ}\text{C}$ at 1.5 atm for 15 min.

2.2. Vinification

Wine was produced with Assyrtiko from Santorini Island (Santo Wines, Union of Santorini cooperatives, Pyrgos Santorini, Greece) in duplicate. The grapes were mechanically crushed and pressed at $8\text{ }^{\circ}\text{C}$. The must was left for 12 h for clarification at $4\text{ }^{\circ}\text{C}$ without any addition of enzymes. After clarification, free sulphur dioxide (SO_2) of the

must was adjusted to 10 mg/L, and filtration (0.45 µm filters) was applied to eliminate the presence of autochthonous yeast cells. The initial concentration of reducing sugars was 210 g/L, pH was 3.15, total acidity had a value of 5.2 g/L (expressed as tartaric acid), and yeast assimilable nitrogen (YAN) was 80 mg N/L (50 mg/L in the form of α -amino acids and the remaining 30 mg/L in the form of ammonium nitrogen). Enrichment of the musts with YAN was carried out with addition of equal contents of yeast extract and DAP (diammonium phosphate), until respective concentrations of 150 and 250 mg N/L were achieved. These two concentrations were chosen, as the minimum (150 mg N/L) to avoid sluggish or stuck fermentations and the most commonly found (250 mg N/L) for industrial fermentations [17]. The three yeast strains were inoculated at 10^6 cfu/mL to initiate alcoholic fermentations in 4-L vessels, at 18 °C in biological duplicates. The fermentation rate was monitored daily by recording the density of the must [18]. Sugar concentration (glucose and fructose) was determined through HPLC in a Waters Association 600E apparatus equipped with an RI detector (Waters 410, Midland, ON, Canada) [4]. The fermentations were monitored for 25 days, until the wild type strains reached the levels of dry wines (<4 g/L of sugars), and to avoid further oxidation of the produced wines even though the concentrations of sugars in fermentations contacted with the commercial strain were slightly higher.

2.3. Chemical Analysis of Must and Wines

Classic parameters of wines (free and total SO₂ contents, % vol., pH, titratable and volatile acidity, A_{420nm}) were determined according to the international methods of the International Organization of Vine and Wine (OIV) [19]. Nitrogen of α -amino acid was determined as described by Dukes and Butzke, (1998) [17], while ammonium nitrogen was determined according to Aerny (1996) [20].

Glycerol and acetic acid were quantified through high-performance liquid chromatography/HPLC (Waters Association 600E apparatus) with an RI detector (Waters 410, Midland, ON, Canada) and an ion exclusion column (Aminex HPX-87H, Bio-Rad, CA, USA). The column temperature was set at 65 °C with a flow rate of 0.8 mL/min. The mobile phase was H₂SO₄. For quantitative analysis, standard solutions of glycerol and acetic acid (Sigma-Aldrich Ltd., Taufkirchen, Germany) were prepared in pure water (Milli-Q, Merk, Taufkirchen, Germany) and wine samples were injected directly into the column. Determinations were performed by means of standard curves [16].

The amino-acid analysis was performed using a liquid chromatographic–triple-quadrupole mass spectrometric method. Analyses were performed on a Thermo Ultimate 3000 ultra-high pressure liquid chromatograph consisting of a 1000 pressure pump, degasser, PAL open-autosampler, and valve interface module. The detection system consisted of a Thermo Quantum Access Max triple-quadrupole mass spectrometer, and for the evaluation, a multiple reaction monitoring option was used. Chromatographic separation was performed in an Intradia Aminoacid (mixed mode, normal phase and ion exchange) column, particle size 3 µm (150 × 4.6 mm), thermostated at 45 °C through the binary gradient shown in Table 1 (phase A, 74:16:10:0.2 mixture of isopropanol, 25 mM ammonium formate, acetonitrile, and formic acid; phase B, 20:80 mixture of acetonitrile and 100 mM ammonium formate) and a flow rate 0.35 mL/min. For detection, triple-quadrupole mass spectrometry with spray voltage 3000 V, vaporizer temperature 350 °C, sheath gas pressure 35 psi, ion sweep gas pressure 0.0 psi, aux gas pressure 10 psi, capillary temperature 380 °C, and capillary offset 35 v was used. The target compounds were identified according to the retention times and the characteristic ions from molecular ion fragmentation.

Table 1. Eluent gradient for UPLC determination of amino acids.

Time (min)	0–12	12–17	17–19	19–31	31–31.5	31.5–35
Eluent A (%)	100	97	0	0	100	100
Eluent B (%)	0	3	100	100	0	0

The identification of the headspace volatiles of produced wines was conducted by means of gas chromatography/mass spectrometry (GC/MS) using the solid phase microextraction method (SPME). GC/MS analysis was performed on a GCMS-QP2010 Ultra (Shimadzu Inc., Japan) system set at 240 °C in split mode (split ratio 1/20) [21]. The conditions of headspace SPME sampling were as follows: 2 mL of wine sample, 7.5 mL of deionized water, 1 g ammonium sulfite, and 500 µL internal standard (1-octanol) were transferred into a 20 mL headspace vial fitted with a polytetrafluoroethylene (PTFE)-lined silicone septum. The sealed wine samples were initially stirred (250 rpm) in a tap bath ($T = 40\text{ °C}$) for 5 min (Thermodyne-Nuova II; stir speed 5) to achieve the appropriate temperature, and then the SPME fiber (DVB/CAR/PDMS, 2 cm; Sigma Aldrich, Germany) was exposed to the headspace for 30 min. The separation of wine volatile compounds took place in a DB-Wax capillary column (30 m \times 0.25 mm i.d., 0.25 µm film thickness, Agilent, USA). Helium was used as the carrier gas at a constant linear velocity of 36 cm/s. The oven temperature was set at 40 °C for 5 min and increased by 5 °C/min up to 180 °C, and subsequently by 30 °C/min up to 240 °C. The oven temperature was finally stated at 240 °C for 5 min. Source and interface temperatures were set at 200 °C and 240 °C, respectively. For each sample during the elution of ethanol, which was expected at 3.4–4.0 min [22], the filament was programmed to turn off targeting to extend its life, as ethanol was expected at much higher rates compared to other volatiles. Molecular identification of volatile compounds was carried out using AMDIS (v. 2.65) software based on retention time and mass spectra and peak area of each compound, respectively.

2.4. Sensory Analysis

Sensory assessment was performed, for all wines, by a group of 12 trained panelists with previous experience in wine analysis using the Pivot© profile sensory method [23]. Twenty-five mL of the samples and the pivot wine (prepared with the commercial yeast strain) were presented in a completely randomized order to each panelist, marked with three-digit code, at 16 °C. The wines produced with Sa and Sb were compared with pivot wine. All samples were expectorated, and tap water was provided for mouth-rinsing between samples. The attributes selected were grouped in two categories: olfactive descriptors (floral, fruity, reduction, vinegary, odor of oxidation) and gustative descriptors (sourness and mouthfeel as the perception of the body of wine). The panelists also performed an overall odor quality assessment. All samples were evaluated in triplicate.

2.5. Statistical Analysis

Analysis of variance (ANOVA) was performed using Statistica V.7 (Statsoft Inc., Tulsa, OK, USA) to determine whether the mean values of the parameters differed between treatments. Tukey's HSD was used as comparison tests when samples were significantly different after ANOVA ($p < 0.05$).

3. Results & Discussion

3.1. Effect of YAN Content on Fermentation Kinetics

The kinetics of alcoholic fermentations depended mostly on the yeast strains and secondarily on YAN concentrations. The *S. cerevisiae* strain Sa consumed half of the reducing sugars after 4 days of fermentation. In contrast, the commercial and Sb strains were characterized by a 48 h delay, consuming half of the concentration of reducing sugars on the 6th fermentation day. Furthermore, Sa managed to reach the levels of residual sugars (3.6 g/L) corresponding to dry wine on the 25th day, while the Sb and the commercial strain ended up with 4.0 and 4.5 g/L, respectively. Despite the initial, somehow higher total sugar consumption rate and subsequently ethanol production rate of the commercial strain compared to the new isolates (Sa and Sb), finally, the ethanol content of the three wines produced did not show any statistically important differences (final ethanol content $\sim 100\text{ g/L} = 12.9\% v/v$) (Figure 1). The conversion yield of ethanol produced per unit of total sugars consumed was c. 0.50–0.51 g/g (the absolute value of the slope of the line) for all

strains tested, that is, 98–100% *w/w* (Figure 2) of the maximum theoretical yield ($=0.51$ g/g), demonstrating the potential of the employed wild type strains for the production of wines containing high concentrations of ethanol [16]. No significant differences were observed regarding the conversion yield of ethanol between ferments of the same strain provided with different initial YAN. This outcome could indicate that the relatively low fermentation temperature and the yeast strain pose a more significant role compared to YAN [24,25].

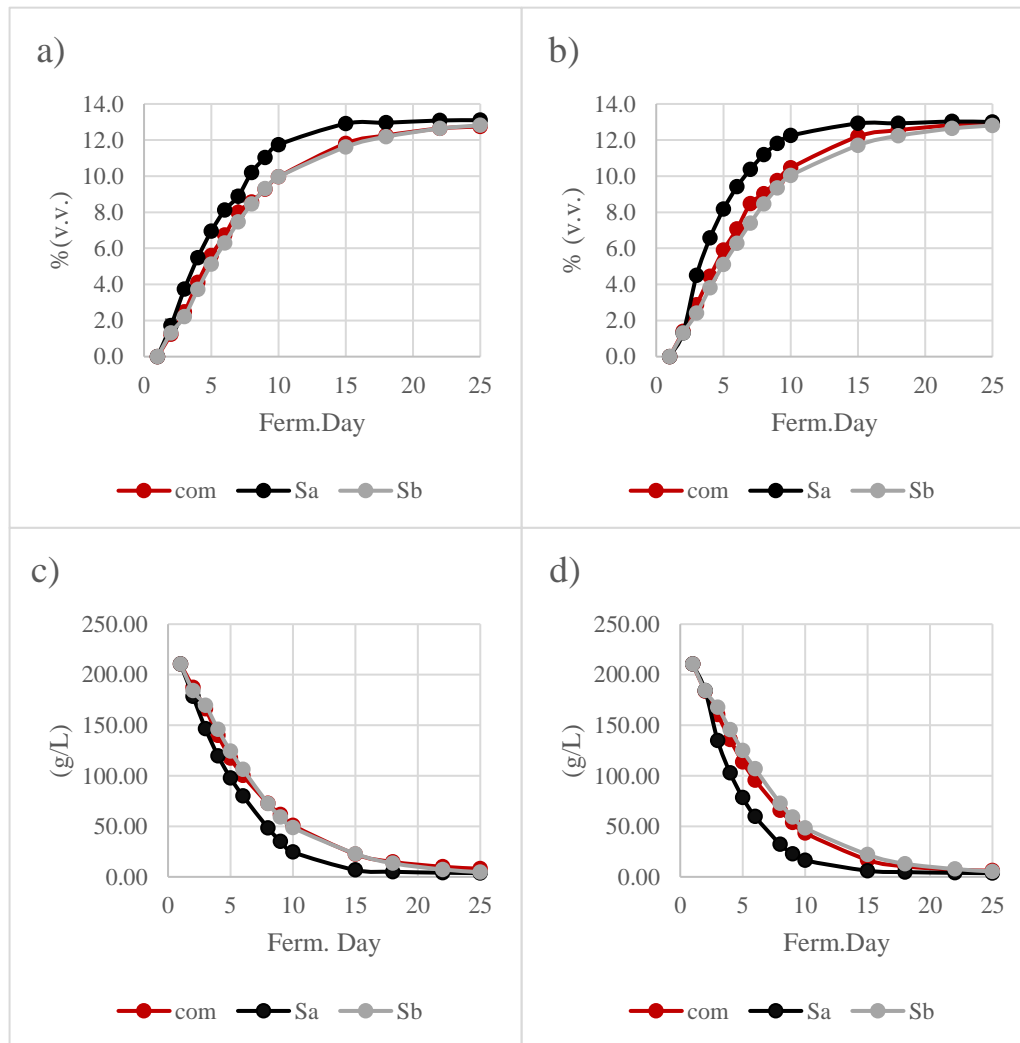


Figure 1. Production of ethanol by *S. cerevisiae* in fermentations with (a) low and (b) high concentrations of YAN and consumption of reducing sugars by *S. cerevisiae* in fermentations with (c) low and (d) high concentrations of YAN. Red, black and grey lines correspond to samples fermented with the commercial, Sa and Sb strains, respectively.

Apart from the differences in fermentation kinetics, the strains showed variations in the consumption of fructose and glucose (Figure 3). The commercial strain presented a clear preferential uptake of glucose. Once glucose was almost depleted, there was a sharp increase in fructose uptake. As previously reported, this outcome was expected on the growth face, where actively growing cells present a preferential affinity for glucose. After this stage, the consumption rates of both glucose and fructose were noted to be similar. Furthermore, in case of the existence of two separate sugar transport systems, these results may indicate that both systems are capable of transporting either sugar, but only if the preferred sugar is not present. In the case of a common transport system, competitive inhibition of fructose uptake by glucose could have taken place [26]. However, this pathway was not confirmed in the present study, as the two sugars were consumed

almost simultaneously at different rates (Figure 3). This could be an indication of two separate transport systems with distinct kinetic properties. Furthermore, no significant differences concerning glucose and fructose uptake were observed between fermentations with different initial YAN, as nitrogen content in both cases was sufficient and prevented reduction in glucose uptake capacity [27], while must sugar content did not pose a limiting factor in nitrogen availability and requirements [28].

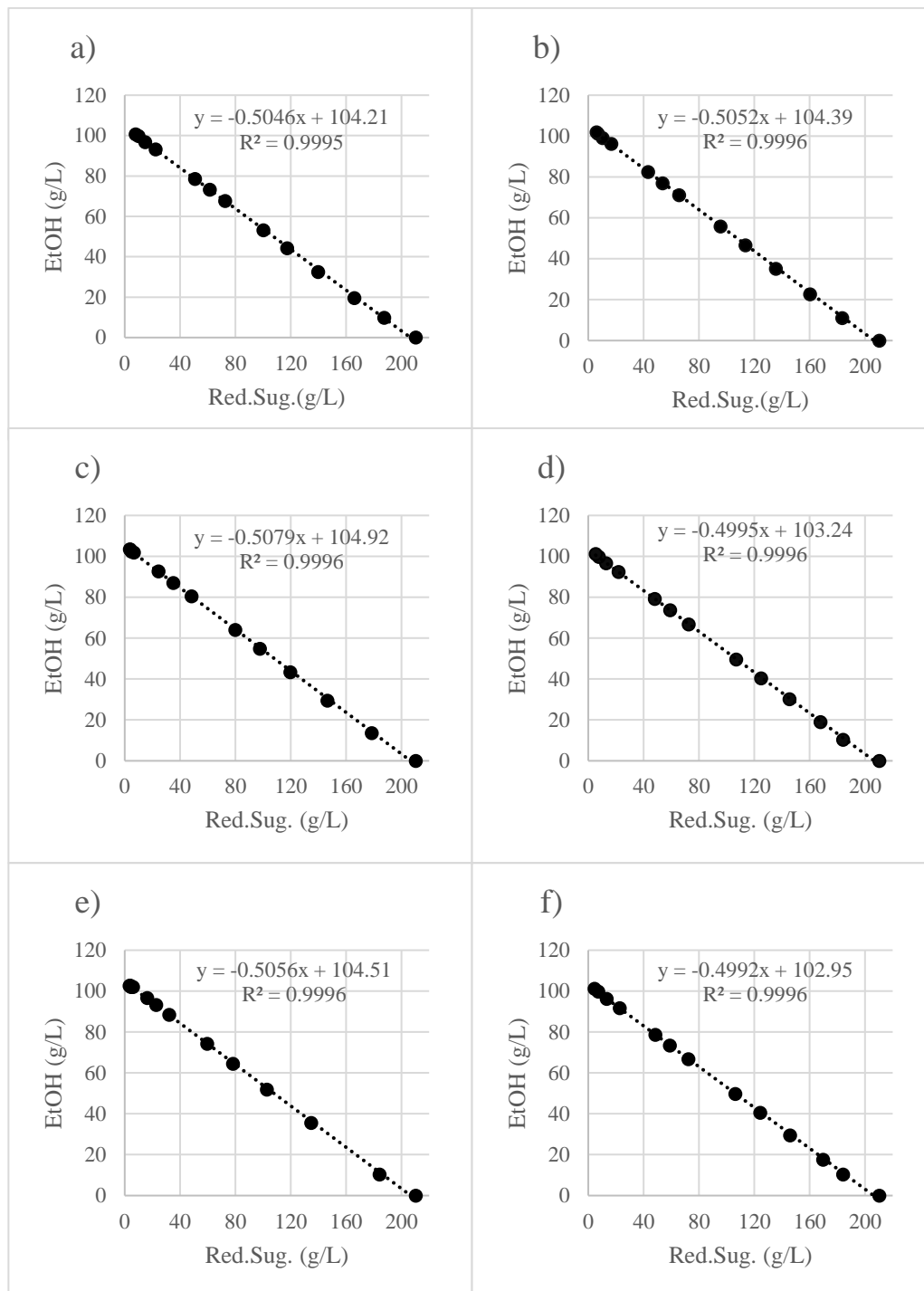


Figure 2. Conversion yield of ethanol produced per unit of total sugars consumed by the commercial strain in fermentations with (a) low and (b) high concentrations of YAN, Sa in fermentations with (c) low and (d) high concentrations of YAN, and Sb in fermentations with (e) low and (f) high concentrations of YAN.

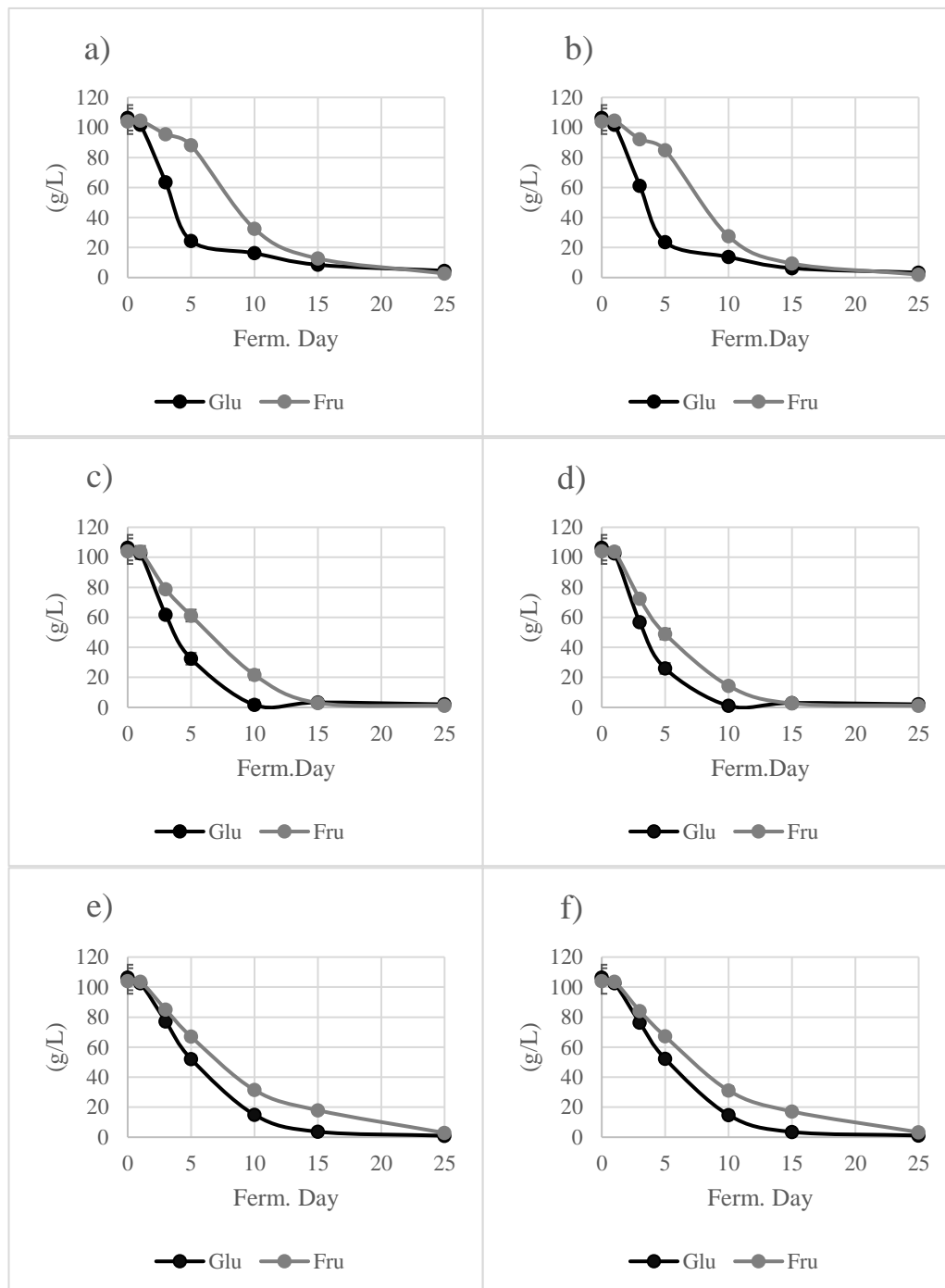


Figure 3. Consumption of glucose and fructose by commercial strain in fermentations with (a) low and (b) high concentrations of YAN, Sa in fermentations with (c) low and (d) high concentrations of YAN, and Sb in fermentations with (e) low and (f) high concentrations of YAN. Black and grey lines correspond to glucose and fructose consumption, respectively.

Regarding sugar utilization, differences in inorganic nitrogen requirements were observed among the different *S. cerevisiae* strains. Sa strain consumed ammonium nitrogen (NH_4^+) faster, especially in the case of ferments containing 150 mg N/L. In those cases, ammonia was exhausted by the 3rd day of fermentation (data not shown). Sb strain showed a completely different behavior regarding ammonium nitrogen consumption. In the case of musts containing 250 mg N/L, the NH_4^+ consumption speed was higher, even though complete exhaustion of ammonia was achieved on the 6th day of fermentation. On the

other hand, in musts inoculated with Sb, containing 150 mg N/L, ammonia was exhausted by the 5th day of fermentation (data not shown). Based on these observations, Sa strain seems to exhibit higher NH_4^+ requirements than the other strains examined, while Sb and commercial strains might be characterized by comparable NH_4^+ demands (Figure 4a).

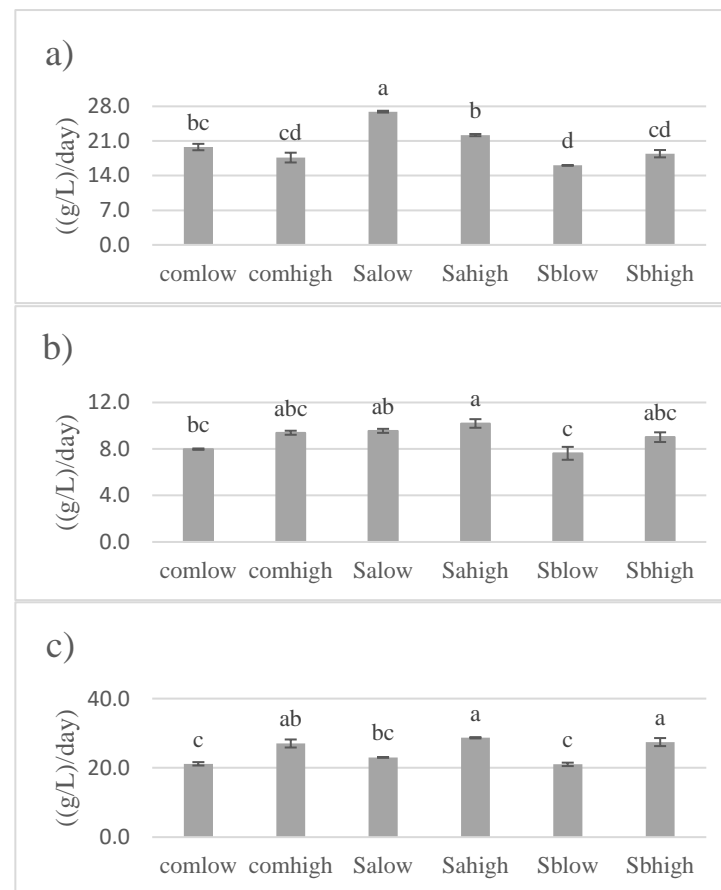


Figure 4. Utilization rate of (a) NH_4^+ , (b) FAN and (c) YAN by three different *S. cerevisiae* strains. The 'low' and 'high' correspond to fermentations with 150 mg N/L and 250 mg N/L of initial YAN, respectively. The 'com', 'Sa' and 'Sb' correspond to fermentations carried out with the commercial and Sa and Sb strains, respectively. Values followed by different letters in each column indicate significant differences ($p < 0.05$) among different samples.

In addition, all three *S. cerevisiae* tested strains exhausted approximately 70% of α -amino acids (FAN) by the second day of fermentation. More specifically, consumption of FAN in musts inoculated with the commercial, Sa and Sb strains, reached 69.1%, 73.7% and 59.6%, respectively. The FAN utilization followed NH_4^+ consumption in a nitrogen catabolite repression manner. The fastest consumption of NH_4^+ in ferments inoculated with Sa strain compared to the Sb strain, probably lead to a faster activation of the GAP1 (general amino acid permease) and GLN3 (activated when preferred nitrogen sources are not available) proteins. The activation of the specific proteins leads the metabolic pathway to non-preferred nitrogen sources (FAN) [29]. Once again, the Sb strain was characterized by the lowest nitrogen depletion speed, indicating the lowest FAN demand (Figure 4b). In general, the utilization rate was higher in the trials containing 250 mg N/L, compared to fermentations containing 150 mg N/L. Moreover, the consumption of the different amino acids by the ferments with lower initial YAN was not significantly different. In contrast, commercial strain musts enriched with 250 mg N/L YAN were characterized by the lowest utilization rate of leucine, valine, asparagine and glutamine, while the Sb strain presented the highest utilization rate for the same amino acids. The absence of

statistically important differences in ferments with lower initial YAN could be attributed to a possible derepression of external hydrolytic enzymes involved in amino acid utilization (e.g., asparaginase) and/ or polymerization of metabolic enzymes responsible for the synthesis of amino acids, due to nitrogen starvation, leading to altered or secondary functions [30,31]. The differences noted for leucine, valine, asparagine and glutamine could be the consequence of the membrane structural disparities of the three yeasts, leading to alternate permease activity. More specifically, the enzymatic activity of Bap2 permease (responsible for leucine and valine transfer) and Agp1 (which transports asparagine and glutamine in the yeast cell) [32] excreted by Sb strain might be higher in comparison with the other yeasts strains examined. Asparagine utilization and fermentation kinetics were inversely proportional, while glutamine affected yeast growth indirectly through its connection with asparagine, providing evidence that asparagine supports yeast growth [33]. Leucine cannot provide any positive impact on yeast growth, as also verified by previous studies [32,33]. Fermentations carried out with Sa strain with a low initial level of YAN contained the highest concentration of total amino acids after 25 days of fermentation, most likely due to extended yeast cell autolysis [34]. Further investigation of mannoprotein concentration could substantiate this assumption. None of the three examined strains presented the ability to degrade proline (no statistical differences were observed compared to the initial concentration (data not shown)); however, arginine content of the final wines was statistically different depending on the strain (Table 2). Since both amino acids include the proline pathway for their conversion to glutamate and NH_4^+ , arginine degradation probably followed the urea cycle [35,36]. Inhibition of arginine to proline utilization through Put 4 (the main transporter of proline uptake) and GAP1 endocytosis could also be another explanation [37]. The lower concentration of arginine in trials conducted with Sb compared to Sa strain could be explained through the more limited expression of URE2 protein, repressing the transcription of the genes responsible for proline, hence of arginine degradation under nitrogen catabolite repression [35].

Table 2. Concentration (ng/mL) of amino acids after 25 days of fermentation. Values followed by different letters in each column indicate significant differences ($p < 0.05$) among different samples.

YAN	Commercial	Sa	Sb	Commercial	Sa	Sb
	150 mg N/L			250 mg N/L		
4-Hydroxy-Proline	2.05 ± 0.39 ^a	1.54 ± 0.31 ^a	1.92 ± 0.39 ^a	1.20 ± 0.00 ^a	2.00 ± 0.79 ^a	2.02 ± 0.41 ^a
Proline	92.93 ± 5.20 ^{ab}	101.34 ± 0.25 ^{ab}	91.82 ± 0.80 ^{ab}	104.74 ± 0.00 ^a	97.95 ± 3.99 ^{ab}	87.79 ± 2.49 ^{ab}
Phenylalanine	0.76 ± 0.29 ^a	0.19 ± 0.02 ^a	2.02 ± 0.26 ^a	1.07 ± 0.04 ^a	1.00 ± 0.10 ^a	0.80 ± 0.00 ^a
Tyrosine	0.05 ± 0.01 ^a	0.54 ± 0.05 ^a	0.24 ± 0.02 ^a	0.77 ± 0.01 ^a	0.30 ± 0.03 ^a	0.12 ± 0.00 ^a
Aspartic acid	1.33 ± 0.10 ^a	2.41 ± 0.53 ^a	1.95 ± 0.3 ^a	3.57 ± 0.00 ^a	2.45 ± 0.50 ^a	1.73 ± 0.30 ^a
Glutamic acid	0.95 ± 0.08 ^b	0.24 ± 0.00 ^b	3.02 ± 0.84 ^{ab}	5.46 ± 0.04 ^{ab}	5.54 ± 0.19 ^{ab}	9.26 ± 0.65 ^a
Isoleucine	0.54 ± 0.04 ^a	0.26 ± 0.03 ^a	0.00 ± 0.00 ^a	0.52 ± 0.01 ^a	0.04 ± 0.00 ^a	0.00 ± 0.00 ^a
Tryptophane	0.19 ± 0.01 ^a	0.30 ± 0.00 ^a	0.09 ± 0.01 ^a	0.04 ± 0.01 ^a	0.10 ± 0.00 ^a	0.25 ± 0.09 ^a
Threonine	2.51 ± 0.01 ^a	1.73 ± 0.05 ^a	1.18 ± 0.07 ^a	1.26 ± 0.00 ^a	0.61 ± 0.06 ^a	0.94 ± 0.03 ^a
Leucine	0.57 ± 0.03 ^b	0.21 ± 0.02 ^b	0.13 ± 0.01 ^b	2.46 ± 0.00 ^a	0.43 ± 0.02 ^b	0.55 ± 0.04 ^b
Valine	1.38 ± 0.02 ^{ab}	0.98 ± 0.04 ^{ab}	0.45 ± 0.02 ^{ab}	1.50 ± 0.07 ^a	0.48 ± 0.07 ^{ab}	0.30 ± 0.01 ^b
Serine	1.11 ± 0.03 ^c	1.24 ± 0.03 ^c	0.95 ± 0.05 ^c	1.43 ± 0.03 ^{bc}	2.23 ± 0.02 ^a	1.94 ± 0.00 ^{ab}
Glutamine	22.37 ± 0.51 ^{ab}	68.30 ± 0.25 ^{ab}	8.85 ± 0.31 ^b	22.87 ± 0.00 ^a	50.66 ± 0.20 ^{ab}	7.43 ± 0.00 ^b
Alanine	17.72 ± 0.73 ^{ab}	33.58 ± 0.44 ^{ab}	4.87 ± 0.31 ^b	44.93 ± 0.00 ^a	12.45 ± 0.52 ^b	15.86 ± 0.00 ^{ab}
Asparagine	1.33 ± 0.28 ^{bc}	0.56 ± 0.27 ^c	0.27 ± 0.07 ^c	10.34 ± 0.00 ^a	2.45 ± 0.32 ^b	0.74 ± 0.00 ^c
Glycine	2.84 ± 0.40 ^{bc}	2.49 ± 0.10 ^c	4.25 ± 0.17 ^{ab}	5.41 ± 0.30 ^a	5.54 ± 0.44 ^a	5.04 ± 0.00 ^a
Cysteine	12.63 ± 0.06 ^a	12.64 ± 0.08 ^a	12.56 ± 0.01 ^a	12.54 ± 0.00 ^a	12.58 ± 0.03 ^a	12.60 ± 0.06 ^a
Histidine	1.72 ± 0.34 ^a	1.77 ± 0.29 ^a	1.25 ± 0.23 ^a	2.01 ± 0.08 ^a	1.93 ± 0.44 ^a	1.75 ± 0.00 ^a
Lysine	12.73 ± 0.30 ^a	12.89 ± 0.46 ^a	13.47 ± 1.01 ^a	15.19 ± 0.00 ^a	12.91 ± 0.49 ^a	13.08 ± 0.28 ^a
Arginine	1.54 ± 0.63 ^c	6.35 ± 0.45 ^b	2.53 ± 0.31 ^c	9.18 ± 0.00 ^a	5.99 ± 0.96 ^b	2.22 ± 0.22 ^c
Total	191.24 ± 5.22	263.48 ± 7.63	165.77 ± 3.01	360.08 ± 0.11	231.59 ± 6.71	178.38 ± 6.08

3.2. Total Acidity and Production of Secondary Metabolites

After the end of alcoholic fermentations, an average reduction of 20.1% in tartaric acid was observed (Table 3). No significant differences were observed between ferments containing different amounts of YAN. These results are in agreement with previous studies for *S. cerevisiae* and could be due to biochemical utilization or physical losses through decreased solubility or tartaric acid's adsorption to the surface of yeast cells [38]. Based on the absence of statistically important differences between different yeast strains, it is surmised that physiochemical phenomena have a greater impact on the alterations of tartaric acid concentrations.

Table 3. Chemical parameters of ferments after 25 days of fermentations with different *S. cerevisiae* strains. Values followed by different letters in each column indicate significant differences ($p < 0.05$) among different samples.

YAN (mg/L)	Must	Commercial			Sa		Sb	
	80	150	250	150	250	150	250	
EtOH % (v/v)	0.00 ± 0.00	12.76 ± 0.12 ^a	12.91 ± 0.01 ^a	13.10 ± 0.08 ^a	13.00 ± 0.00 ^a	12.82 ± 0.00 ^a	12.81 ± 0.17 ^a	
pH	3.15 ± 0.01	3.13 ± 0.01 ^a	3.14 ± 0.00 ^a	3.09 ± 0.01 ^b	3.09 ± 0.01 ^b	2.98 ± 0.00 ^c	3.00 ± 0.01 ^c	
Total Acidity (g tartaric acid/L)	5.21 ± 0.03	7.84 ± 0.03 ^b	7.68 ± 0.02 ^b	7.39 ± 0.03 ^c	7.50 ± 0.03 ^c	8.67 ± 0.02 ^a	8.56 ± 0.06 ^a	
Volatile Acidity (g acetic acid/L)	0.30 ± 0.01	0.53 ± 0.02 ^a	0.53 ± 0.00 ^a	0.37 ± 0.01 ^b	0.40 ± 0.01 ^b	0.31 ± 0.01 ^c	0.32 ± 0.02 ^c	
Residual Sugar (g/L)	210.05 ± 2.1	5.05 ± 1.9 ^a	3.95 ± 0.3 ^a	3.70 ± 1.4 ^a	3.65 ± 0.4 ^a	3.65 ± 0.2 ^a	4.35 ± 1.3 ^a	
Glycerol (g/L)	0.00 ± 0.00	6.25 ± 0.15 ^{abc}	6.00 ± 0.00 ^{bcd}	5.75 ± 0.15 ^{cd}	5.55 ± 0.05 ^d	6.75 ± 0.15 ^a	6.50 ± 0.00 ^{ab}	
Tartaric (g/L)	6.75 ± 0.1	5.4 ± 0.1 ^{abc}	5.2 ± 0.0 ^c	5.3 ± 0.1 ^{bc}	5.3 ± 0.0 ^{bc}	5.6 ± 0.1 ^a	5.5 ± 0.1 ^{ab}	
Citric (mg/L)	720 ± 0.0	180.0 ± 0.0 ^b	185.0 ± 0.0 ^b	190.0 ± 0.0 ^b	170.0 ± 0.0 ^b	365.0 ± 0.0 ^a	385.0 ± 0.0 ^a	

There were no differences in malic acid concentrations, nor was any lactic acid production noted (data not shown) even when, in some cases, an up to 22% consumption of malic acid could be observed, being mainly strain-dependent [38,39]. The ability to metabolize extracellular malic acid depends on an efficient system for malic acid transport (i.e., active import via a malate transporter) and a malic acid converting enzyme [40] that seemed to be absent in the investigated strains.

Reduction in citric acid concentration was also observed (Table 3). Fermentations carried out with the commercial, Sa and Sb strains presented a 74.5%, 74.8% and 47.6% reduction in citric acid content, respectively. Overexpression of CIT1 and CIT3 genes encoding mitochondrial citrate synthase, which catalyzes the first step in the tricarboxylic acid (TCA) pathway, and/or of the KGD1, KGD2 and LPD1 genes encoding the oxoglutarate dehydrogenase (OGDH) complex, which helps the formation of succinic acid and fumaric acid in commercial and Sa strains, could be a possible explanation for these results [29]. Absence of significant differences between ferments of the same strain with different YAN contents, constitutes indication of suppressed expression of GLN3 and URE2 genes associated with alternative nitrogen assimilatory pathways connected with the TCA cycle [35]. The more limited alterations in citric acid concentration in ferments carried out with Sb strain led to higher total acidity (TA) and lower pH, compared to wines fermented with the commercial and Sa strains. The observed relatively high TA (and low pH), were within the range commonly measured in wines produced from Assyrtiko grapes [41].

Apart from the higher TA values, a more moderated increase in volatile acidity (VA) was also achieved in Sb wines compared to the commercial and Sa wines (Table 3). These results are in line with previous works that demonstrated a positive correlation between acetic acid and glycerol production. As reported by Romano et al., 1994, the production of acetaldehyde and consequently, the production of acetic acid and other compounds such as acetoin and higher alcohols, is mainly strain-dependent [42]. The different strains examined could be categorized according to phenotypes as low, medium and high acetaldehyde producers, respectively. Moreover, accumulation of acetic acid in fermentations carried

out with the commercial strain, with the possibly suppressed expression of HAA1 gene (responsible for the adaptation of yeast cells upon exposure to toxic concentrations of acetic acid), could have led to decreased cell viability and retarded fermentations [43,44]. In addition, a slightly higher (however, not significantly important) increase in VA was observed in fermentations with 250 mg N/L compared to those with 150 mg N/L YAN, when carried out with the commercial and Sb strains, unlike with the Sa strain. Different research groups have proposed that acetic acid production is negatively correlated with nitrogen content; however, this was not observed for acetic acid content for the commercial and Sb strains [10,45]. Considering, though, the strain-dependent manner of acetic acid production and the absence of statistically important differences between ferments with discrete YAN concentrations, these results could be explained.

Glycerol is a significant by-product of alcoholic fermentation, as it contributes to the organoleptic properties of wine, increasing viscosity and sweetness [46]. In the current study, the highest production of glycerol was observed in musts containing 150 mg N/L YAN, for all examined *S. cerevisiae* strains (Table 3). These results are in agreement with previous studies, suggesting that nitrogen limitation increases glycerol production [45,47]. Independently from the YAN level, glycerol concentrations ranged between 5.8–6.8 g/L, which was within the range of previously reported data (4.2–10.4 g/L) [45,47–50]. However, the Sb strain was characterized by the highest glycerol and lower ethanol production, although the differences observed were not statistically significant. Such differentiations between strains regarding glycerol production could be attributed to the differences between the two pathways that are linked to glycerol formation. The low and high glycerol producing strains differ in glycerol-3-phosphate dehydrogenase activity. The gapN gene that encodes non-phosphorylating NADP⁺ is responsible for the enzymatic activity, and in strains where this gene is suppressed, ethanol production is lower, while glycerol yield is higher. Higher production of glycerol by the Sb strain could also be ascribed to the suppressed expression of the mhpF gene encoding acetylating NAD⁺ linked to acetaldehyde dehydrogenase and overexpression of the frdA gene encoding NAD⁺ linked to fumarate reductase [51]. Furthermore, the three strains presented different production kinetics (Figure 5). Glycerol production could be related to glucose consumption, since glycerol reached a plateau when glucose was exhausted. Aerobic production of glycerol in the presence of glucose, which may result in respiro-fermentative growth, is a possible explanation for the results observed [52].

3.3. Volatile Composition

Analysis of volatile compounds was performed by SPME GC/MS targeting to evaluate the volatile characteristics of wines produced by the two wild type yeast strains and compared with the commercial one (Figure 6). In line with the more limited increase in VA, Sb media were characterized by considerably higher production of higher alcohols and ketones compared to commercial and Sa media (Figure 6a,b). According to the results, it was observed that the lower the nitrogen content, the lower the production of higher alcohols in wine. However, as far as Sb strain is concerned, the opposite was observed. A possible explanation for this could be a better utilization of valine by this strain that intensifies the production of fusel alcohols [10,42,53,54]. The Sa and commercial strains produced similar contents of higher alcohols regardless of nitrogen content. Moreover, the Sb strain produced 1.9-fold higher amounts of alcohols and 5.6-fold higher amounts of ketones compared to the commercial strain; however, no significant differences were observed between Sa and the commercial strain. These results highlight the variations that might occur in the metabolic pathways of different yeast strains. The increased production of higher alcohols by the Sb strain evinces a less efficient uptake of nitrogen, as previously reported [55].

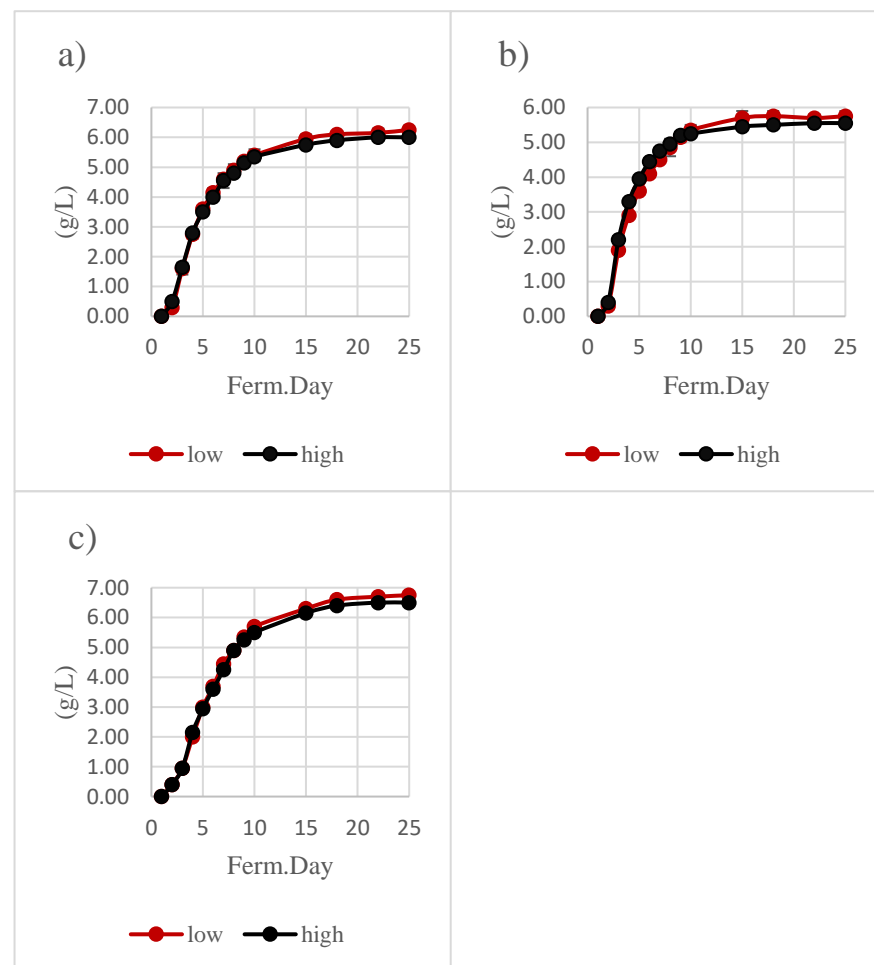


Figure 5. Production of glycerol by *S. cerevisiae* in fermentations with (a) commercial, (b) Sa and (c) Sb strains. Red and black lines correspond to samples fermented with low and high initial concentrations of YAN, respectively.

Furthermore, the Sb strain produced less fatty acids and esters compared to the other two strains, probably as a defensive mechanism to toxic ‘metabolic waste’ (Figure 6c,d) [55]. Lower concentrations of fatty acids could be linked to lower concentrations of acyl-coenzyme A (CoA), one of the factors that is directly related to ester production [56]. Deficient amounts of acyl-CoA could also result in lower concentrations of acetyl-coenzyme A (CoA), suppressing the TCA cycle in the Sb strain, as has been previously documented. Reduced activity of enzymes involved in the synthesis (alcohol acetyl transferases I and II) and hydrolysis (esterases) of esters in Sb media could be responsible for the insufficient ester production compared to the commercial and Sa wines. Lower expression of *ATF1* and *ATF2* genes encoding alcohol acetyl transferases I and II and overexpression of the *IAH1* gene encoding ester degrading enzyme could be another possible explanation [56]. In addition, lower concentrations of esters in Sb wines could be linked to leucine consumption [57]. As far as the different initial YAN contents are concerned, the three strains showed different patterns of ester and fatty acid production. In media with 150 mg N/L, the commercial and Sa strains resulted in lower amounts of fatty acids and higher amounts of esters compared to the wines containing 250 mg N/L. For the commercial strain, ester production was proportional to initial YAN.

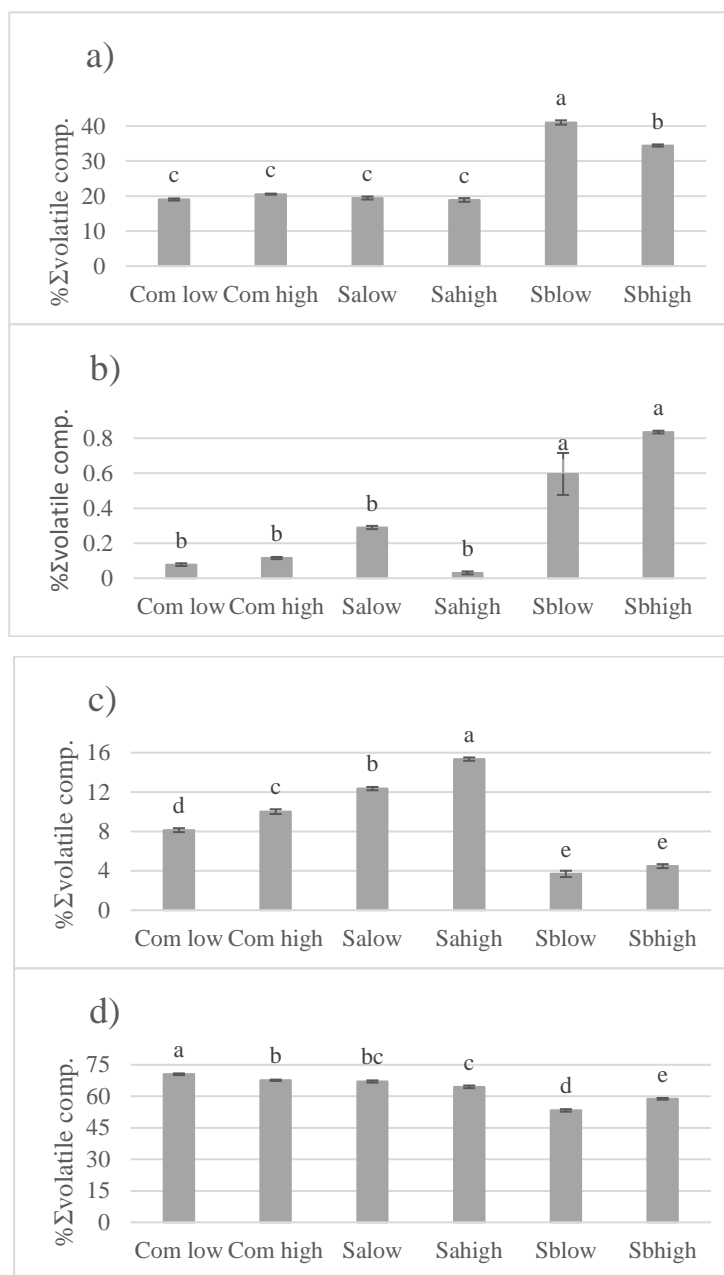


Figure 6. % Concentrations of the most important groups of volatile compounds: (a) higher alcohols, (b) ketones, (c) fatty acids and (d) esters. The 'com', 'Sa' and 'Sb' correspond to fermentations carried out with the commercial, Sa and Sb strains, respectively. Values followed by different letters in each column indicate significant differences ($p < 0.05$) among different samples.

3.4. Sensory Analysis

The results of sensory analysis did not show any significant differences concerning color intensity, indicating that the judges could not perceive any differences in color intensity among the pivot (control wine fermented with the commercial *S. cerevisiae* strain) and the rest of the samples (Figure 7). The results are in agreement with the results obtained for low molecular weight phenolic compounds and absorption at 420 nm (data not shown), with no statistically important differences observed among the samples. Moreover, in line with the results regarding VA, the Sa and Sb wines were clearly judged as less vinegary and oxidized, since the negative frequencies for these attributes were higher than the positives. The same wines were also judged as less reductive than the pivot, probably due to lower production of volatile sulfurous compounds [58]. Further investigation in this group of

volatile compounds is needed to support this assumption. Regarding floral attributes, the positive frequencies for the Sb strain were higher than the negative, indicating this wine was clearly judged as more floral than the pivot, while for the commercial strain, positive and negative frequencies were equal, showing similar floral notes with the pivot. These results are in line with the results obtained by GC-MS analysis regarding the contents of higher alcohols and ketones that are responsible for the floral and green aromas [4,59]. Wines fermented with the Sa strain were also characterized as more fruity than the pivot, probably due to the higher production of esters (Figure 7). Surprisingly, Sb wines were also characterized as more fruity even though their ester content was lower than that of the pivot, highlighting the importance of wine matrix in the perception of unique aromas. In addition, the overall aroma of the samples was judged as more pleasant than that of the pivot. Moreover, for wines fermented with the Sa and Sb strains, the positive frequencies for acidity were higher than the negatives. This was expected for the Sb wines due to the higher TA and lower pH, but not for Sa. The higher acidity rating of the Sa wine could be attributed to the lower concentration of residual sugars compared with the control, since they could also affect the perception of acidity [60]. The higher concentration of residual sugars in control samples probably affected the mouthfeel perception as well, since pivot was characterized as poorer than Sb and similar to Sa based on this attribute. The lower concentration of asparagine observed in Sb samples could also be another possible explanation for the observed lower ratings of mouthfeel, since this amino acid is positively related with mannoprotein production in wine [61]. Further investigation into mannoprotein production from the three strains is necessary to support this assumption.

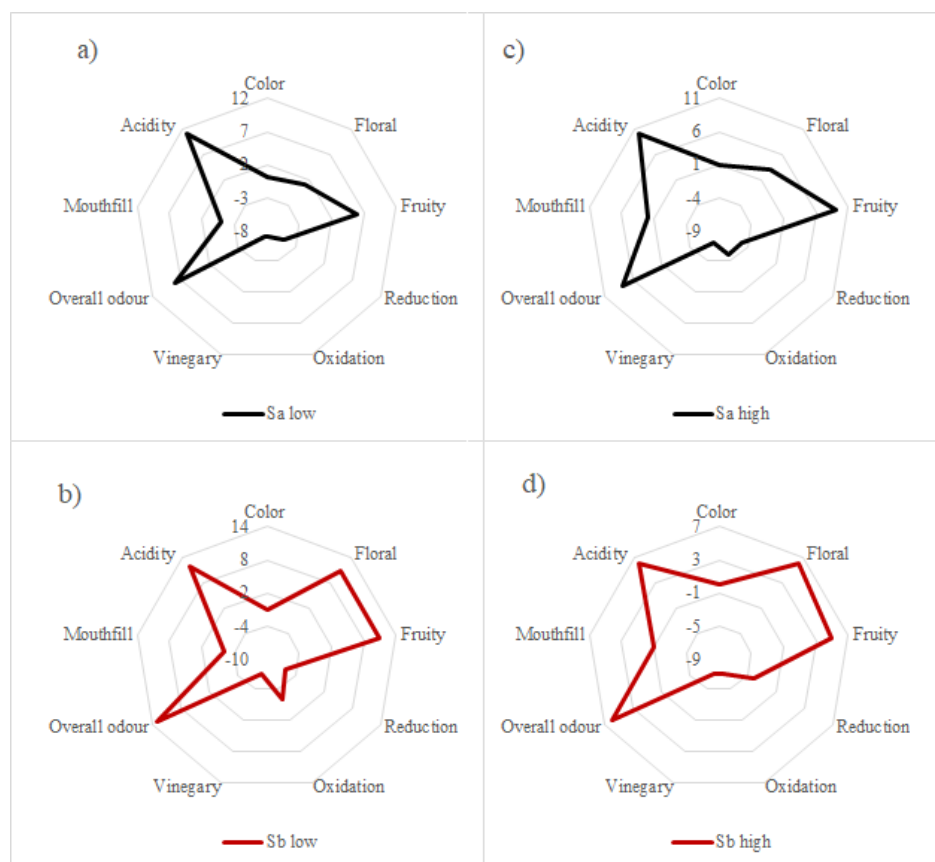


Figure 7. Pivot organoleptic analysis for samples fermented with (a) Sa strain and 150 mg/L initial YAN, (b) Sb strain and 150 mg/L initial YAN, (c) Sa strain and 250 mg/L initial YAN and (d) Sb strain and 250 mg/L YAN.

4. Conclusions

The three *S. cerevisiae* strains were characterized by different nitrogen (NH_4^+ and N α -amino acid) depletion rates in Assyrtiko must fermentation, resulting in different fermentation kinetics, production of secondary compounds, and eventually in different sensory profiles for each wine. Initial YAN concentration had a greater impact on the volatile profile of the produced wines. The organoleptic profile of Assyrtiko wine could be modulated, based on consumer preferences, by selecting the specific strain and the initial YAN content. The new indigenous strains examined seem to have the potential to be involved in large-scale wine production substituting the commercial strain, since they were characterized by lower VA and vinegary and oxidation scores and higher quality of overall aroma assessment. More specifically, the Sa strain combined with 150 mg N/L initial YAN could result in Assyrtiko wines with enhanced fruity character and body due to higher production of esters and glycerol, respectively. If a more floral profile with higher concentrations of higher alcohols is preferred, then the Sb strain with 250 mg N/L of initial YAN would be more suitable. Even though the production of glycerol in this case is more limited, the VA is lower and both higher alcohol and ester concentrations are higher.

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Informed Consent Statement: Panelists gave informed consent before participating in this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author (pending privacy and ethical considerations).

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