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# γ-AMINOBUTYRIC ACID EFFECT IN YEAST UNDER WINE-TYPE FERMENTATION

## TRABAJO FINAL DE MÁSTER

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# ABBREVIATION

ADY: Active Dry Yeast

GABA: γ-aminobutyric Acid

GAD: Glutamate Decarboxylase

GAT: GABA Transaminase

GC-MS: Gas Chromatography coupled Mass Spectrum

GM: Grape Must

IS: Internal Standard

HPLC: High Performance Liquid Chromatography

NCR: Nitrogen Catabolite Repression

OD: Optical Density

$OD_{max}$: Growth Efficiency

uOD: Optical Density Units

SSA: Succinate Semialdehyde

SSADH: Succinic Semialdehyde Dehydrogenase

SWM: Synthetic Wine Must

TCA: Tricarboxylic Acid

YAN: Yeast Assimilable Nitrogen

YNB: Yeast Nitrogen Base

$\mu_{max}$: Doubling Time

λ: Lag Phase

α-KG: α-ketoglutarate

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# ABSTRACT

Wine-making is a process increasingly controlled by human. Low nitrogen source grape musts are one of the most important problems in current oenology. Yeast assimilable nitrogen (YAN) consists of ammonium and α-amino acids; however yeast controls the nitrogen metabolism depending on the presence of preferred compound. This mechanism is called nitrogen catabolite repression (NCR). Nitrogen assimilation efficiency by wine yeast is the key for avoiding stuck fermentation on account of nitrogen source deficiency. The metabolism of γ-aminobutyric acid (GABA), a non-proteinogenic amino acid, has been elucidated but the role in wine yeast of this nitrogen source is still unknown. In this study we followed wine-type fermentation in synthetic wine must in absence and presence of GABA. The aim of this study is to detect differences in the kinetic and/or yeast metabolisms of the process promoted by GABA. We have analysed fermentation by *Saccharomyces* and non-*Saccharomyces* wine yeast. We determined the chemical composition of fermented media by high performance liquid chromatography (HPLC) and enzymatic reactions, and the yeast cell metabolome by gas chromatography coupled to mass spectroscopy (GC-MS).

# Introduction

Winemaking, oenology and human history are deeply intertwined. In the beginning, wine was considered a gift from nature, a gift from God to humans. Nowadays, the production of this aromatic and alcoholic beverage is considered fine art supported by scientific knowledge. High market demand promotes the innovation in this sector, which plays a relevant role in the economy of certain countries. New technologies allow producing varied style of wines, improving the process and the final product. New winemaking techniques fight efficiently with contamination problems, must poor in nutrient, production of off-flavours, as well as enhance wine bouquet (Pretorius 2000).

Grape must was originally fermented by a complex population of yeast located in the vineyard and in the cave atmosphere. However, the actual wine industry controls thoroughly which organisms are taking part in the process, and just few caves develop spontaneous fermentations. *Saccharomyces cerevisiae* is the mayor contributor in the grape juice transformation and so its features are the most studied among wine yeasts (Eldarov et al. 2016). However, new considerations have pointed the use of non-*Saccharomyces* yeasts as favourable for the development of complex aroma and desired flavours (Belda et al. 2017). Yeast metabolism is involved in any of the characteristic of wine, such as colour, mouth feeling, taste... That makes wine yeast research one of the most relevant oenological line studies, which expects to describe the perfect conditions for producing ethanol and carbon dioxide through their sugar source consumption (Pinu et al. 2014).

Wine yeast must be capable to survive in an adverse environment which gets worse along the fermentation. Initially, the acid (pH below 4) grape juice is sugar concentrated (200-300g/L) what causes high osmotic stress for cells. As the fermentation progresses, nutrient source decreases and ethanol is accumulated giving rise to toxicity. In this conditions, nutrient viability must be efficiently use by yeast. Moreover, nutrient viability in grape juice is changing each year promoted by the global warming, what makes necessary yeast metabolism researching. It is known that *S. cerevisiae* modulates it metabolism according the circumstances during the fermentation (Pinu et al. 2014). Wine yeast nutrient assimilation, especially *S cerevisiae*'s, has been studied deeply.

Nitrogen source abundance has a substantial impact in the progress of the fermentation and it results (Bell & Henschke 2005). Nitrogen deficient grape must triggers poor organoleptical wine, even it can cause stuck fermentation. Conversely, medium excess in nitrogen promotes microbial contamination and production of undesirables, such as off-flavours and toxins. In this

way, nitrogen assimilation influences clearly in the development of the fermentation process and in wine quality (Gutiérrez et al. 2013). Thus, nitrogen utilization by yeast has been studied by many groups of researching.

Yeast assimilable nitrogen (YAN) source consists of ammonium and α-amino acids, but with distinct preferences. The abundance of preferred YAN in the medium controls the transcription of gens related with the metabolism of the others. This regulation is known as nitrogen catabolite repression (NCR) (Hofman-Bang 1999). Yeast gets benefit with this metabolisms but it can also stimulate the production of toxins, such as biogenic amines (Zhao et al. 2014). High growth rates are been related with nitrogen sources based on ammonia, glutamate and glutamine, described as key components of yeast central nitrogen metabolism. Next nitrogen source preferred by yeast are arginine, alanine, aspartate, glycine and glutamate, above branched-chain and aromatic amino acids. Urea and proline have been detected as the poorer nitrogen sources (Bell & Henschke 2005). In this study, the role of γ-aminobutyric acid (GABA) was further investigated during wine fermentation.

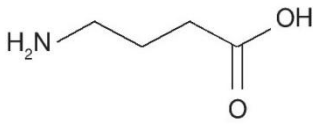



**Figure 1. γ-aminobutyric acid (GABA) chemical structure.**

GABA is a non-proteinogenic amino acid whose metabolism is conserved in the live world (Michaeli & Fromm 2015). The role of GABA shunt has been studied in plants and animals, however in yeast the objective of this metabolism is still unknown. In mammals the intracellular GABA low levels is related with clinical manifestations, acting this molecule as neurotransmitter, hormone and tropic factor in non-neuronal peripheral and endocrine systems. In plant, GABA has been found related with responses to biotic and abiotic stress, but also contributing in several cell regulations, such as cytosolic pH, nitrogen storage and osmoprotection (Bach et al. 2009). In bacteria and fungi, GABA pathway have been related with assimilation of exogenously supplied and excess glutamate (Cao et al. 2013).

GABA metabolism converts α-ketoglutarate (α-KG) into succinate, bypassing two steps of the tricarboxylic acid (TCA) cycle (see in Figure 2). GABA shunt involves three enzymes: a glutamate decarboxylase (GAD; EC 4.1.1.15), a GABA transaminase (GAT; EC 2.6.1.19) and a succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.16). GAD, GAT and SSADH are encoded by GAD1, UGA1 and UGA2 genes, respectively (Bach et al. 2009). Genes involved in

GABA uptake and degradations are regulated by NCR when preferred nitrogen source are present, however UGA1 and UGA2 and a specific transporter for GABA (UGA4) are upregulated by this compound.

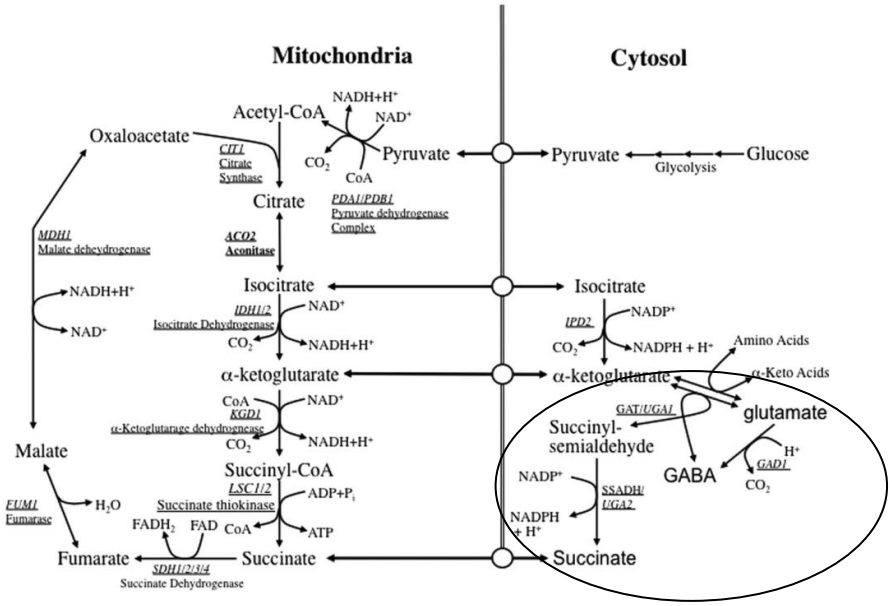



**Figure 2. Schematic model of GABA metabolism and TCA cycle. The diagram shows how GABA bypasses two steps in TCA cycle, which are regulated by α-ketoglutarate dehydrogenase and succinate thiokinase. The circumference covers GAD/GABA pathway** (Cao et al. 2013)**.**

GABA metabolic route or GAD/GABA pathway catalyses the decarboxylation of glutamate into GABA (GAD enzyme). GABA is converted into succinate semialdehyde (SSA) (GABA transaminase) and it is oxidized into succinate (SSA dehydrogenase). In higher eukaryotes SSA could be also reduced into γ-hydroxybutyric acid. The endogenous formation of GABA depends on the enzyme glutamate dehydrogenase in the cytosol, which catalyses the redox step from α-KG into glutamate, in both directions. Because of the α-KG origin from the glutamate and the succinate resulted from GABA metabolism, this route is considered a bypass of the TCA cycle (Bach et al. 2009).

Yeast GAD/GABA pathway research had been focused connecting the route with nitrogen regulation at transcriptional level. In *S. cerevisiae*, GAD enzyme in yeast has been found related with cell protection against oxidative stress promoted by $H_2O_2$ (Coleman et al. 2001). The most recent study of Bach et al. (2009) showed that the uptake extracellular GABA affects to the succinate source. Cao et al. (2013) study suggested that GABA shunt has a crucial role in *S. cerevisiae* thermo-tolerance. The heat damage protection would consist in the restriction of intracellular reactive oxygen intermediate (ROI) production by the flux of carbon from α-KG to

succinate through GABA shunt. Moreover this study revealed an alternative route of GABA degradation as previously described in higher eukaryotes.

The aim of this work is to study the effect of GABA addition in the growth medium on the kinetic parameters, fermentation products, amino acid consumption and intracellular metabolites of two types of wine yeasts, *Saccharomyces* and non-*Saccharomyces*. For this, we used gas chromatography coupled to mass spectrum (GC-MS), high performance liquid chromatography (HPLC) and enzymatic techniques for determining amino acids, intracellular metabolites and secondary products of the alcoholic fermentation.

# Materials & Methods

This study was carried out in 2017, between February 1st and May 31st.

## Yeast strains

The yeast strains used in this study are the following *Saccharomyces* and non-*Saccharomyces*. The strain *Saccharomyces cerevisiae* was EC1118 (Bio-Lallemand, Barcelona, Spain). Those non-*Saccharomyces* were *Torulaspora delbrueckii* TD291 (Bio-Lallemand, Spain), *Metschnikowia pulcherrima* MP346 (Bio-Lallemand, Spain), *Candida zemplinina* Cz3, *Schizosaccharomyces pombe* 622 and *Schizosaccharomyces pombe* 1197. EC 1118, TD291 and MP346 were in Active Dry yeast form. Cz3, 622 and 1197 were given grown in YPD dish from the PhD student Gemma Roca collection.

## Growth media

In this study we used a synthetic wine must (SWM) that mimics the amino acid and ammonium composition of the grape must reported by Pérez-Álvarez et al. (2016). Their study showed that climatic changes affect the nitrogen composition of the vineyard and consequently the growth of the plant and the maturity of the grapes. The synthetic wine must was prepared with 220 g/L glucose/fructose (Panreac, Barcelona, Spain), 5 g/L L-tartaric, 2 g/L L-malic and 0.5 g/L citric acids (Panreac), 0.67 g/L Yeast Nitrogen Base (YNB) w/o amino acid and ammonium (Difco, Sparks, USA), 300 mg/L proline (Sigma-Aldrich, Barcelona, Spain), 50 mg/L $NH_4Cl$ (Sigma-Aldrich), 300 mg/L proline, and amino acids as nitrogen source. The amino acid composition used were: L-glutamine, L-arginine, L-tryptophan, L-alanine, L-glutamic, L-serine, L-threonine, L-leucine, L-aspartic, L-valine, L-phenylalanine, L-isoleucine, L-histidine, L-methionine, L-tyrosine, L-glycine, L-cysteine, L-lysine, L-asparagine and citrulline, all of which were provided by Sigma-Aldrich. The tested concentration of GABA (Sigma-Aldrich) was 100 mg/L. More details of the SWM composition is found in Annex I. The fermentations were carried out in a 330 mL plastic bottle filled with 250 mL of SWM. The cap is provided with a micropipette tip filled with wool to allow $CO_2$ to escape and prevent oxygen from entering. This study also involved the medium for fermentation grape must (GM) (White Grenache harvested in 2016 in the Experimental Cellar of the Oenology Faculty of URV, Constantí, Spain), in which the yeast assimilable nitrogen was 47 mg/L of $NH_4$ and 78 mg/L of α-amino acids; the GM density was

1088 g/mL. It was stored at -20ºC until the experiment. The volume used in this case was 450 mL in 500 mL plastic bottle, with a micropipette tip filled with wool.

## Strain selection for fermentation

In the present study, EC1118, TD291, MP346, Cz3, 622 and 1197 were subjected to fermentation in micro volume in order to determine the growth parameters. The fermentations were performed in 96-well microplate (Brandplates, Wertheim, Germany). The strains were subjected to an increasing concentration of GABA (0, 20, 40, 60, 80 and 100 mg/L) in SWM. Five replicates were conducted for all the conditions studied. The media was prepared in 5mL of SWM. The initial yeast population inoculated was $10^6$ cell/mL. The fermentation temperature was set at 25ºC.

The yeast strains EC1118, TD291 and MP346 were inoculated after the rehydration of the cells (5 g of ADY strain in 5 mL water, incubated in water bath 30 minutes at 37ºC for EC1118 and 30ºC for MP346 and TD291). Nevertheless for Cz3, 622 and 1197 stains an overnight pre-cultured in liquid YPD media was carried out. Strains were inoculated at an initial OD of approximately 0.1 ($10^6$ CFU/mL).

The determination of Optical Density (OD) was monitored by a spectrophotometric FLUOstar Omega (BMG Labtech, Offenburg, Germany) at λ 600 nm. Microplates were subjected to shaking 60 second before the OD measurement and data was taken every 70 minutes over the enough time for cultures to reach the stationary phase.

Absorbance results were normalised and converted into the logarithm optical density (OD) via calibration curves and area under the curve was also calculated. As Optical Density is not a measure of the absorbance but a measure of the light scattered by the suspended yeast, plotting the log of OD against the time is proportional to the logaritm of cell concentration against the time. Growth parameters, variable growth efficiency ($OD_{max}$), doubling time ($\mu_{max}$) and lag phase (λ), were calculated by directly fitting OD measurements versus time by using the modified Gompertz mathematical (Zwietering et al. 1999):

$$y = A\exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\}$$

where A = ln ($OD_t$ /$OD_0$) is the asymptotic maximum ($OD_{max}$), $OD_0$ is the initial OD and $OD_t$ is the OD at time t; $\mu_m$ is the maximum specific growth rate and λ is the population lag time.

Growth data were obtained by non-linear regression procedure using the solver tool from Excel software (Microsoft Office 2010).

## Laboratory scale-winemaking

The fermentations were carried out with EC1118, TD296, Cz3 and 1197. The yeast strains fermented individually two conditions, control and tested GABA concentration (100 mg/L), three times each.

Two rounds of fermentation were performed in SWM. Firstly, EC1118 and TD296 were inoculated for the individual fermentation after the rehydration of the cells. For Cz3 and 1197 strains the inoculation was carried out after the pre-culture in YPD liquid medium, overnight and 48h respectively. Secondly, the next round of fermentations were inoculated with EC1118, Cz3 and 1197 pre-cultured, after rehydration and the YPD liquid medium overnight culture respectively. This pre-culture consisted in a rich medium glucose 80 g/L, $KH_2PO_4$ 5g/L, $MgSO_4$ $7H_2O$ 0.5 g/L, YNB (without amino acid and ammonium) 0.67 g/L, and $NH_4Cl$ 5 g/L. Anyway, the inoculate was $10^6$ cell/mL in a volume of 230 mL SWM, static and in semi-anaerobiosis at 25 ºC.

Initially each solution taking part in SWM were prepared and sterilised by autoclave. However, for the second round of fermentation with Cz3 and 1197, the amino acid solution was filtered instead of autoclaved, and the amount of $NH_4$ was increased until 100 mg/l.

The fermentations were followed by 600 nm absorbance (Ultropec 2100 pro UV, GE Healthcare, Barcelona, Spain) and density analysis with Densito 30PX Portable Density Meter (Mettle-Toledo, Barcelona, Spain).

A third rounf of fermentation was in grape must (GM). GM was inoculated by rehydrated EC1118 ADY at 25ºC, 450 mL in 500 mL plastic bottle. The conditions were the same as the SWM, static and semi-anaerobiosis at 25ºC. The process was followed by measuring the density with Densito 30PX Portable Density Meter (Mettle-Toledo). The kinetic curves were made by Excel program.

Cell samples and fermented medium were taken at the midpoint and at the end of the fermentation. The midpoint of the fermentation was considered in 1040 g/mL density, the end of the fermentation at 992 g/mL. The pellet of 50 uOD (five x $10^8$ cells) were frozen in cold

70% (v/v) of ethanol, pelleted again and stored at -20ºC until further analysis. After centrifuged the fermented medium at 6,000 rpm during 5 minutes, the supernatant was stored in 2 mL Eppendorf.

## Metabolite profiling of cell yeasts by gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry method (GC-MS) was used for metabolite profiling of EC1118 cells cultured in SWM, from rehydrated ADY and precultured ADY, and also cultured in GM, from rehydrated ADY. The protocol followed the methodology described by López-Martínez et al. (2014).

Briefly, the frozen cells were suspended in 400 µL methanol-water (1:1, v/v) with 10 µL of ribitol at 2 mg/mL added as an internal standard (IS). The metabolites were extracted using 0.5mm glass beads (BioSpec Products, USA) and a multitube bead-beater (BioSpec Products) using five cycles of 1 min/beat, followed by 30-s rest for cooling. Right after centrifugation, the supernatant was dried in a SC110 speed vacuum system SC110 (Savant Instruments, USA) for 4h. The derivatisation step was carried out by 50 µL of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich), followed by a treatment with 70 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich).

The GC-MS followed the method of López-Martínez et al. (2014). Briefly, the equipment used for the GC-MS was an Agilent Technologies Network GC system 6890N connected to an HP computer with the ChemStation software (Agilent Technologies, Böblingen, Germany), plus the mass selective detector (MSD, model 5975, Agilent Technologies). 4 µL of the cell extract was injected at a split ratio of 20:1. The carrier gas was helium was used at a constant flow of 1.0 mL/min. The column oven was programmed to increase 5 ºC/min from 80 ºC until 200 ºC and then to 300ºC at a rate of 25 ºC/min.

Data analysis was performed with the Agilent MSD Chemstation software. The metabolite profile had been created identifying the relative abundance of each compound detected. Every peak area had been corrected by the area peak of IS.

## Fermented product analysis

### High Performance Liquid Chromatography for amino acid determination

Amino acid content of samples was determined by High Performance Liquid Chromatography (HPLC) according to the method of Gómez-Alonso et al. (2007). Briefly, 400 µL of each sample was derivatised by 15 µL of diethylethoxymethylenemalonate (Fluka, Steinhein, Germany) in presence of 700 µL of borate buffer 1M (pH 9), 300 µL of methanol and 10 µL of 2-aminoadipic acid as the Internal Standard, *ca* 1 g/L. The treatment went on for 2 hours in water bath at 80 ºC.

The chromatography was performed on an Agilent 1100 Series HPLC (Agilent Technologies). The sampling was 50 µL carried out using a 4.6 x 250 mm, 5 µm ACE C18-HL column (Symta, Madrid, Spain).

Data analysis was performed in ChemStaion program for liquid chromatography (Agilent Technologies). The amino acids ($aa_x$) were identified by the retention time of the chromatograph peaks. Chromatographs of the SWM at time zero allowed establishing the K constant by the next equation:

$$K_{aa_x} = \frac{área\ de\ PI}{área\ aa_x}\ x\ [aa_x](\text{mg/L})$$

In this way the amount of amino acid were calculated from samples chromatographs by the next equation:

$$[aa_x](mg/L) = K_{aa_x}\ \frac{área\ del\ pico\ aa_x}{área\ del\ PI}$$

### Enzymatic kit for other compound in the media

Fermented SWM and GM were analyzed by enzymatic kits, of which ethanol kit (R-Biofarm, Roche, Pfungstadt, Germany) was performed manually and succinic acid (Megazyme, Bray, Ireland), glycerol (Biosystem, Barcelona, Spain), acetic acid (Biosystem), acetaldehyde (Biosystem), pyruvic acid (Biosystem) and glucose-Fructose (Biosystem) kits were performed automatically. The commercial kits were optimised by Dr. Isabel Araque for Miura One-Clinical Chemistry Multi Analyser (I.S.E., Guidonia, Italy).

## Statistical analyses

Growth parameters in the strain selection experience were compared between GABA concentrated fermentations, for each strain individually. ANOVA test with post-hoc Tukey HSD (honestly-significant-difference) calculator was used for comparing fermentation results. The cut-off level of significance was set to $\alpha \leq 0.05$.

Products results of enzymatic kits were statistically analyse with ANOVA test with post-hoc Tukey HSD ($\alpha \leq 0.05$). In this way, differences between the midpoint and the end of the fermentation, in control and GABA conditions, were highlighted. Kit enzymatic results of the end of the fermentation in SWM and GM were comparing by ANOVA test with post-hoc Tukey HSD ($\alpha \leq 0.05$) after data were normalised at 100 g of glucose/fructose consumed.

# Results & Discussion

## Effect of GABA addition on growth parameters of wine yeast strains

Wine yeast strains *Saccharomyces cerevisiae* EC1118, *Torulaspora delbrueckii* TD291, *Metschnikowia pulcherrima* MP346, *Candida zemplinina* Cz3, *Schizosaccharomyces pombe* 622 and *Schizosaccharomyces pombe* 1197 fermented individually synthetic wine must (SWM) with γ-aminobutyric acid (GABA). Testing different concentration of GABA in the media would show effects in kinetics curves. Results are shown in Figure 3, where growth curves for each GABA concentred condition are shown for each wine yeast strain studied.

MP346 and 622 wine yeast strains were initially rejected for the analysis and posterior fermentation because the growth curves detected were no favourable. We suppose that the composition of the SWM is not probably nutritive enough for both strains.

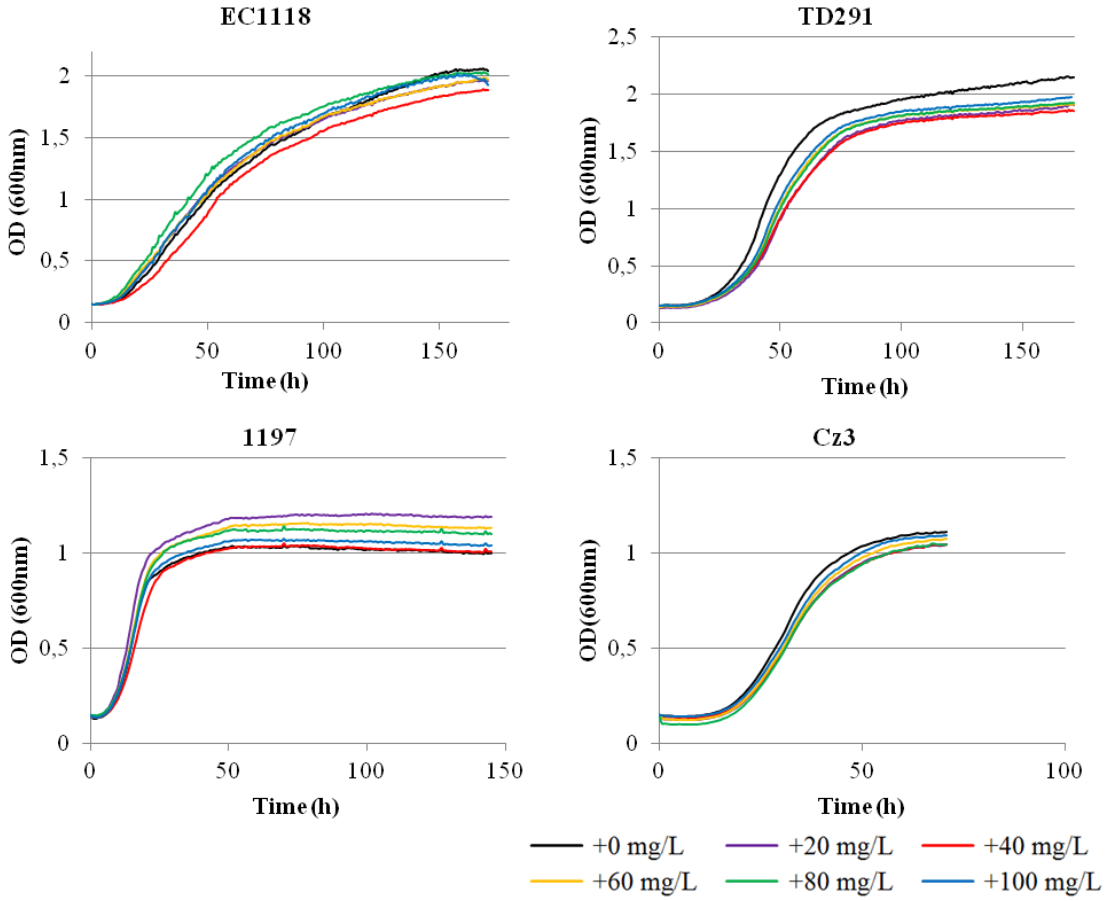



**Figure 3. Effect of increasing GABA concentration on wine yeast strain growth in SWM at 25ºC. Growth curves have been represented from the average OD of the five replicates performed for each GABA concentration fermentation in microplate.**

Between EC1118, TD291, Cz3 and 1197 growth curves there are notable differences (Table 1). However, GABA concentrated conditions were not apparently promoting changes in the development of the fermentation in each case. In order to confirm significantly differences between GABA curves, growth parameters (growth efficiency ($OD_{max}$), doubling time ($\mu_{max}$) and lag phase ($\lambda$)) were calculated by the modified Gompertz equation (Zwietering et al. 1999). The summary of the parameters is displayed in Table 1, empathizing statistically differences by ANOVA and Tukey (HSD) test ($\alpha < 0.05$) between conditions by each strain.

**Table 1 Effect of GABA addition in micro volume on the growth parameters of wine yeasts**

| GABA (mg/L) | | 0 | 20 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|---|---|
| EC1118 | OD max | 2.519±0.048 | 2.483±0.044 | 2.454±0.026 | 2.491±0.045 | 2.520±0.023 | 2.512±0.037 |
| | μ(h) | 0.048±0.005 | 0.049±0.008 | 0.045±0.003 | 0.048±0.008 | 0.056±0.008 | 0.051±0.010 |
| | λ(h) | 23.75±2.08 | 23.07±3.65 | 27.67±2.78 | 22.33±4.92 | 20.51±3.81 | 23.43±4.34 |
| TD291 | OD max[d] | 2.612±0.015[a] | 2.512±0.034[b] | 2.505±0.040[b] | 2.527±0.035[a,b] | 2.536±0.038[a,b] | 2.549±0.034[a,b] |
| | μ(h)[d] | 0.075±0.002[a] | 0.064±0.005[b] | 0.059±0.004[b] | 0.066±0.004[a,b] | 0.063±0.003[b] | 0.066±0.004[a,b] |
| | λ(h)[d] | 29.96±0.45[b] | 34.94±1.90[a] | 33.30±1.04[a,b] | 32.94±1.04[a,b] | 32.96±1.12[a,b] | 32.89±1.38[a,b] |
| 1197 | OD max | 1.912±0.044 | 2.062±0.054 | 1.914±0.072 | 2.074±0.041 | 1.999±0.089 | 1.949±0.021 |
| | μ(h) | 0.164±0.004 | 0.165±0.009 | 0.143±0.019 | 0.164±0.010 | 0.148±0.008 | 0.154±0.008 |
| | λ(h) | 11.26±0.87 | 10.30±0.34 | 12.33±1.83 | 10.57±0.10 | 11.02±0.76 | 11.23±0.36 |
| Cz3 | OD max | 2.000±0.041 | 1.925±0.086 | 1.933±0.041 | 1.918±0.069 | 1.912±0.123 | 1.982±0.067 |
| | μ(h) | 0.094±0.003 | 0.091±0.007 | 0.087±0.006 | 0.096±0.008 | 0.100±0.013 | 0.089±0.002 |
| | λ(h)[d] | 23.09±0.68[b] | 24.80±1.21[a,b] | 24.69±0.85[a,b] | 25.39±1.02[a] | 25.63±2.21[a] | 23.93±0.51[a,b] |

[a.b]: show significantly different groups distributed by Tukey (HSD) test for growth parameters indicated in each row.
[d]: mean growth parameter statistically different, by ANOVA test.

Statistical analysis highlights mainly an effect of GABA on TD291. *Torulaspora delbrueckii* is the non-*Saccharomyces* yeast most used in wine making for improving the complexity, decreasing glycerol and mannoproteins, even ethanol (Velázquez et al. 2015). However *T. delbrueckii* is not able to dominate under *S. cerevisiae* replacement, what became its main problem for winemaking. In this study we reveal that the presence of GABA in must reduces ability of *T. delbrueckii* for fermenting the synthetic must selected. We suggest that GABA effect in *T. delbrueckii* should be further study, with different strains and different synthetic and grape must.

## Rehydration *vs* pre-cultured growth of *S. cerevisiae* EC1118

High fermentation by EC1118 presented different evolution in the cases studied. Here we compared growth curves of EC1118 inoculated after rehydration from active dry yeast (ADY) stock and the one inoculated after pre-cultured in rich media. Growth results of pre-cultured EC1118 were not the expected. Instead of improving fermentation ability of EC1118 by pre-culturing in YPD followed by a rich medium, the results pointed out the opposite. Figure 4A shows total yeast populations calculated by the measurement of OD along the fermentations. Whereas the inoculum used for synthetic wine must (SWM) fermentation were not different in population, the curves displayed show that maxim OD reached by rehydrated EC1118 is higher than the one by the pre-cultured inoculum. In Figure 4B density curves of pre-cultured EC1118 suggest stuck fermentation, which was corroborated by the sugar composition of the products. Neither control nor GABA fermentation decreased in density under 992-995 g/mL using a pre-culture of EC1118 ADY stock.

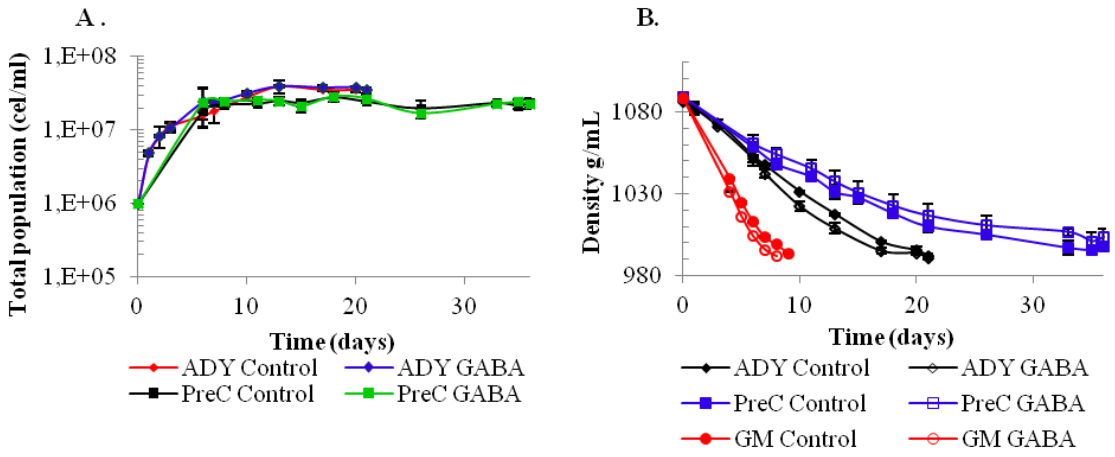



**Figure 4. A. Growth curve for fermentations by rehydrated (ADY) and precultured (PreC) EC1118 of the SWM. B. Density evolution in EC1118 fermentations.**

Grape must (GM) was fermented by rehydrated EC1118 ADY and it presented a fast fermentation at 24ºC in semi-anaerobic condition. In a low nitrogen source as grape must is, the addition of GABA gives extra yeast assimilable nitrogen, even when GABA assimilation is controlled by the nitrogen catabolite repression (NCR) (Bach et al. 2009). GABA addition in this GM fermentation has promoted drastic decrease of density what suggests this supplement was fast uptake by cells favouring alcoholic fermentation.

## Synthetic Wine Must is a suitable medium for non-*Saccharomyces*?

TD291, Cz3 and 1197 non-*Saccharomyces* wine strains were inoculated in SWM and cultured at 24ºC in semi-anaerobic condition. However SWM became a difficult media for these yeast strains' fermentations. Performing the density evolution curves (Figure 5) stuck fermentations were shown performed by TD291, Cz3 and 1197. Moreover 1197 yeast strain presented cell association promoted probably by the adverse conditions. In a second round of fermentation SWM was inoculated with Cz3 and 11197, individually, after pre-culturing the strains in rich medium. The objective of this treatment is to activate the fermentation mechanism in cells, however it did not show advantage for Cz3 or 1197, as well in EC1118 fermentations.

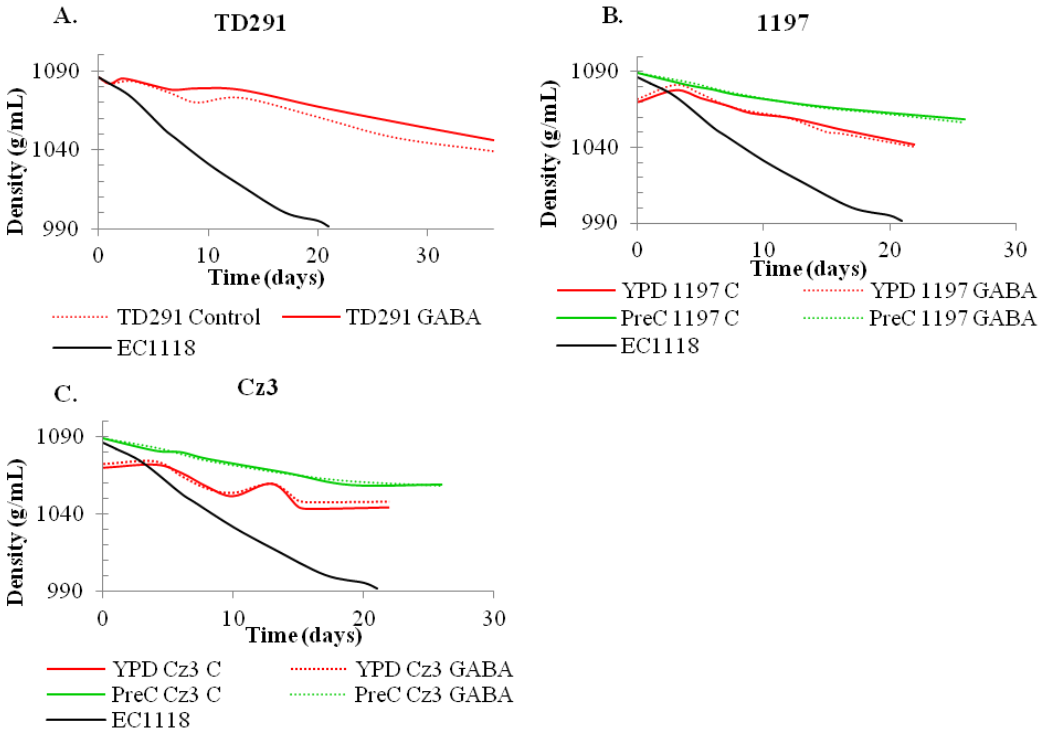



**Figure 5. Comparison of rehydrated EC1118 fermentation curve (EC1118) and non-*Saccharomyces* fermentation of SWM; A. SWM fermented by rehydrated TD291 ADY; B. SWM fermented by 1197 precultured in yeast extract peptone dextrose medium (YPD) medium and pre-cultured in rich medium (PreC);C. SWM fermented by Cz3 precultured in yeast extract peptone dextrose medium (YPD) medium and pre-cultured in rich medium (PreC).**

Cell and medium samples were taken of each fermentation conditions. Cell samples have been quencher with ethanol and they have been stored at -20ºC, with medium samples. We suggested a comparison between the initial status of strain metabolisms and the differences promoted by this adverse condition.

## Metabolite profiling by GC-MS of *S. cerevisiae* EC1118 cells

EC1118 cell samples taken at the midpoint and at the end of the fermentations were derivatised in order to analyse the yeast metabolome by gas chromatography and mass spectroscopy (GC-MS). Metabolites have been grouped in amino acids, sugar compounds, fermentation products and acids of Krebs cycle. Metabolite profile is shown in Figure 6 as group percentages of total compounds detected. Chromatography results have been normalised with the internal standard; the averages of three replicates are represented in Figure 6. The main intracellular metabolite group in percentage is the sugar total. At the end of each fermentation, as we expected, the percentage of this group decreases and tartaric percentage increase notably.

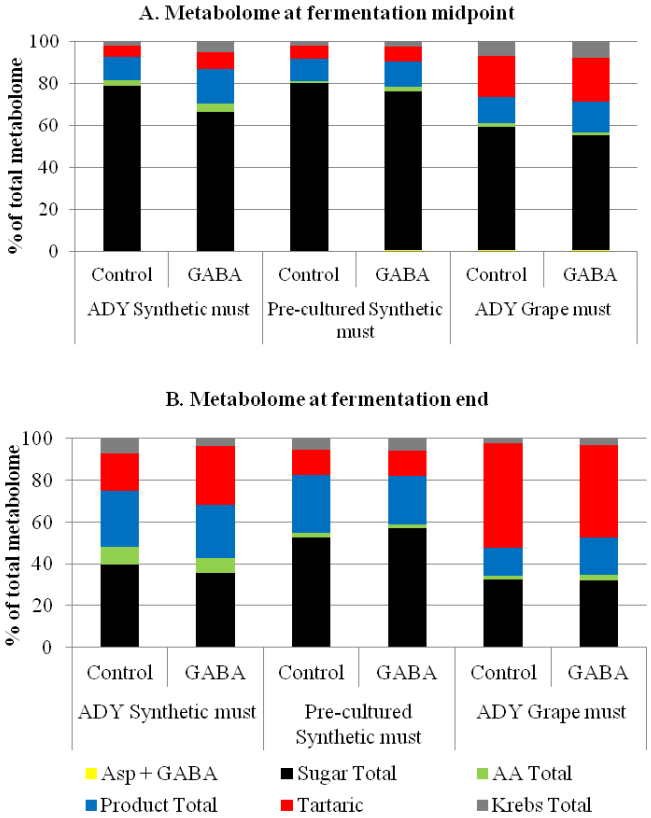
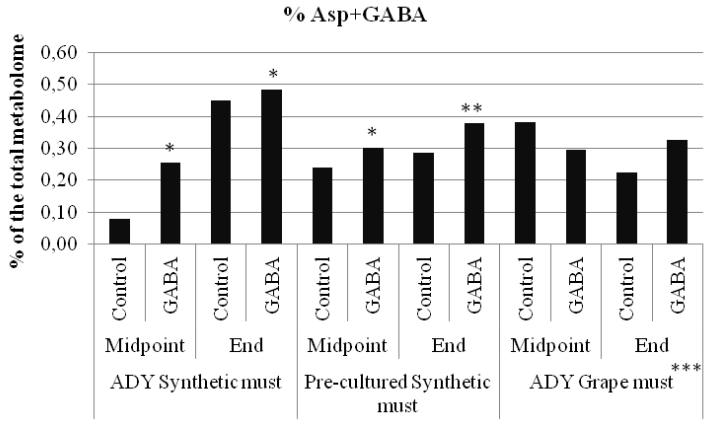



**Figure 6. Metabolite profile represented by percentages of compound groups; aspartatic acid plus GABA (Asp + GABA), sugar total (fructose, glucose, trehalose), amino acid total (AA total) (glucose, serine, glutamine, glycine, threonine, alanine, proline, valine, isoleucine, leucine, phenylalanine and ornithine), product total (pyruvic acid, glyceric acid, glyceraldehyde, glyoxylic acid, 2,3-butanediol, lactic acid, $PO_4$, glycerol and inositol), tartaric acid and metabolites of Krebs cycle (Krebs Total, succinic acid, malic acid and citric acid). A. GC-MS results for samples taken at the midpoint of the fermentation. B GC-MS results for samples taken at the end of the fermentation.**

Metabolome results are not giving any pattern that could be related with GABA effect. Comparing the results shown in Figure 6B, the most relevant analysis is the higher sugar component in cells at the end of the SWM fermentation after pre-culture them in rich media. Fermentation problems corroborated the high amount of sugar in the cells and also in the media, what means slow metabolism.

On the other hand, the metabolome of cell samples taken from GM fermentation showed no differences at the midpoint in between control and GABA conditions fermentation products. Nevertheless at the end of the process tartaric acid proportion is lower in GABA fermentation, increasing so fermentation products, acids of Krebs cycle and total of amino acids.

GABA chromatographic peak have been resolved with aspartic acid's (Asp) one. In Figure 7, peaks detected at GABA/Asp retention time are detailed in which cases mass spectroscopy detected GABA/Asp, just Asp or just GABA.




*: Mass spectroscopy described the peak as GABA and aspartic.
**: Mass spectroscopy described the peak as GABA
***: GABA was not detected by mass spectroscopy

**Figure 7. Percentages of grouped aspartic/ GABA peak in total metabolite profile studied.**

Rehydrated EC1118 which fermented supplemented-GABA SWM revealed intracellular GABA in those conditions. The difference in percentage of Asp/GABA retention time peak between control and GABA condition could be identified as GABA. In this way, GABA composition in rehydrated EC1118 is higher in the midpoint of the fermentation, than at the end of this process. GABA uptake mechanism have been described with general permeases, amino acid permease Gap1p and proline permease Put4p, and GABA specific transporter encoded by UGA4 (Cao et al. 2013). Meanwhile general permeases are regulated by NCR mechanisms, UGA4 is upregulated by GABA (Bach et al. 2009). We suggest GABA is taken by the rehydrated EC1118, but it is not consume until preferred nitrogen source have been assimilated.

The metabolome of pre-cultured EC1118 which fermented SWM described Asp/GABA retention time peak as just GABA, suggesting that GABA is accumulated inside the cell at the end of this process. SWM fermentations by pre-cultured EC1118 have been considered problematic, even considering stuck fermentations. We connect these results with some studies from the bibliography. The accumulation of GABA when cells are stressed have been described before, and other groups have suggested the role of GABA in stress tolerance limiting the production of intracellular reactive oxygen intermediates (ROIs) (Cao et al. 2013). Considering stuck fermentation, cells were under stress in the moment of the sampling and so, the accumulation of GABA could be related with cell stress tolerance.

Cell samples from grape must fermentation didn't display GABA presence in Asp/GABA retention time peak. Considering that grape is poorer in nitrogen source, we suggest that GABA was uptake and consumed from the beginning of the process. Future studies of metabolome could be focus in certain GABA shunt intermediates, such as glutamate, succinic semialdehyde and succinate, and the glyoxylate shunt pathway of Krebs cycle. We suggest selected ion monitoring (SIM) mass spectroscopy in order to identify and quantify GABA shunt intermediates, and even acids of Krebs cycle.

## Fermented product determination

### Estimation of amino acid consumption by HPLC

Samples of the fermented medium were analysed by high performance liquid chromatography (HPLC) for the amino acid fraction. HPLC results were normalised by the internal standard and amino acid concentrations were calculated. In Figure 8, the amino acids studied have been classified by percentages of consumption during the fermentation of SWM by rehydrated ADY EC1118.

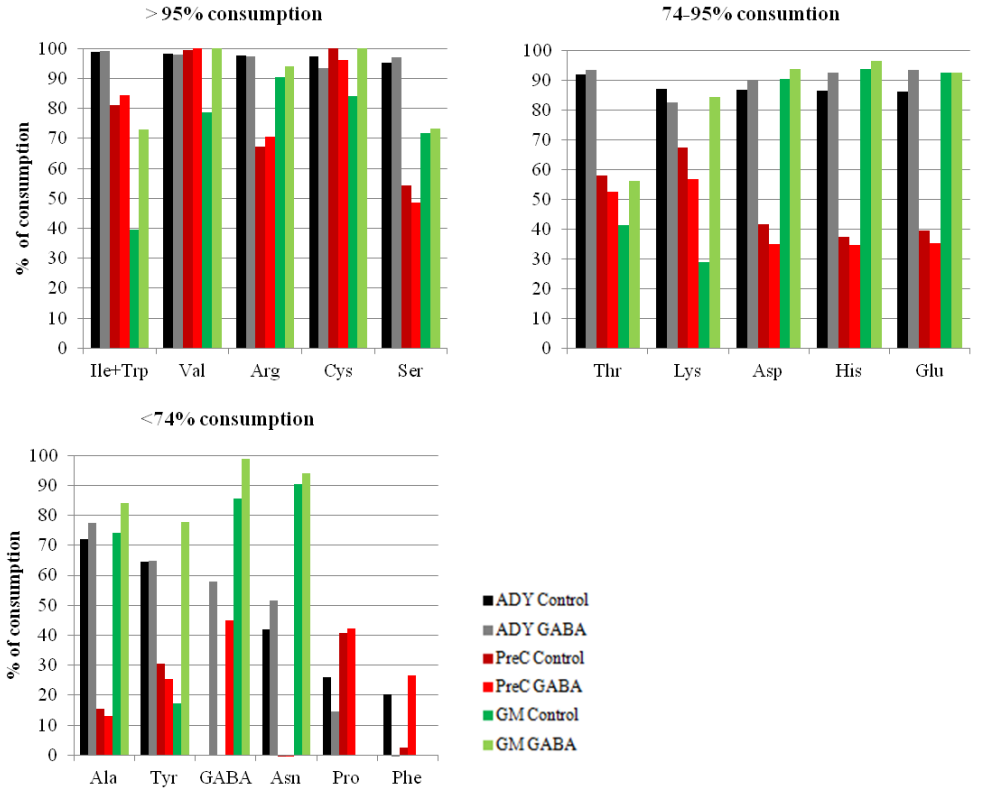



**Figure 8. Classification of amino acid in the medium according the percentage of consumption during fermentation. The amino acid group are: >95% consumed formed with isoleucine + tryptophan (Ile + Trp, HPLC peaks resolved just in one), valine (Val), arginine (Arg), cysteine (Cys) and serine (Ser); 95-74% consumed amino acids are threonine (Thr), lysine (Lys), aspartic acid (Asp), histidine (His) and glutamic acid (Glu); and amino acids <74% consumed were alanine (Ala), tyrosine (Tyr), γ-aminobutyric acid (GABA), asparagine (Asn), proline (Pro) and phenylalanine (Phe).**

Generally under GABA conditions the amino acid consumption is higher. We suggest that GABA is firstly an extra of nitrogen source, what promotes the process. Comparing SWM fermentations, the amino acid consumption by the pre-cultured EC1118 is lower. Considering the slow metabolism of the strain in this condition, higher presence of amino acid in the media is explained.

However fermented grape must revealed that the amino acid consumption is high for most of the amino acids, increased under the artificial GABA presence. HPLC results of GM confirm the conclusion reached by the kinetic analysis and metabolome. Grape must have been fermented in short period of time promoted with GABA addition, consuming mostly the nitrogen source available.

## Fermentation products analysed by enzymatic kits

The composition of the fermented results in glucose/fructose, succinic acid, glycerol, acetic acid, pyruvic acid, acetaldehyde and ethanol were determined by enzymatic kits. These compound concentrations are presented in Table 2**¡Error! La autoreferencia al marcador no es válida.**, Table 3 and Table 4 for SWM fermented by rehydrated EC1118 and pre-cultured EC1118, and for the fermentation of GM, respectively.

ANOVA test and Tukey (HSD) calculator showed differences in concentrations of acetic acid and acetaldehyde in fermented SWM at the midpoint and the end of the process by both rehydrated EC1118 and precultured EC1118. Fermented SWM by precultured EC1118 also presented differences in the concentration of glycerol between midpoint and the end of the process.

**Table 2. Fermentation products (g/L) of SMW fermentation by rehydrated ADY EC1118**

| | Midpoint | | End | |
|---|---|---|---|---|
| | Control | GABA | Control | GABA |
| Succinic acid | 0.56 ± 0.02 | 0.56 ± 0.02 | 0.53 ± 0.02 | 0.53 ± 0.02 |
| Glycerol | 4.98 ± 0.38 | 6.01 ± 0.69 | 7.75 ± 0.25 | 8.20 ± 0.93 |
| Acetic Acid | 0.74 ± 0.03[a] | 0.80 ± 0.03[a] | 0.98 ± 0.03[b] | 1.01 ± 0.03[b] |
| Pyruvic Acid | 0.21 ± 0.09 | 0.22 ± 0.04 | 0.20 ± 0.03 | 0.18 ± 0.09 |
| Acetaldehyde | 0.18 ± 0.02[a] | 0.15 ± 0.02[a] | 0.08 ± 0.02[b] | 0.08 ± 0.02[b] |
| Glucose / Fructose | n.d | n.d | 5.59 ± 3.98 | 1.90 ± 1.09 |
| Ethanol | 11.94 ± 2.11 | 38.93 ± 2.30 | 88.53 ± 7.32 | 97.55 ± 1.40 |

Tukey (HSD) analysis of the differences between the conditions with a confidence interval of 95%
[x]: groups from Tukey test in ANOVA for those significantly different
n.d: no determined

In Table 3 stuck fermentation is confirmed with the amount of glucose/fructose remained in the medium. Moreover fermented medium analysis showed that the alcoholic strength is higher than in the other cases studied. We supposed that the higher amount of ethanol became toxic for EC1118 and so stuck fermentation was the result. We suggested that ethanol toxicity can cause stress episodes in cells what supported the hypothesis of GABA role in stress tolerance.

**Table 3. Fermentation products (g/L) of SMW fermentation by precultured EC1118**

| | Midpoint | | End | |
|---|---|---|---|---|
| | Control | GABA | Control | GABA |
| Succinic acid | 6.11±0.35 | 6.45±0.97 | 5.81±0.18 | 6.06±0.07 |
| Glycerol | 5.42±1.63[a] | 5.38±0.30[a] | 6.03±0.52[b] | 7.73±2.87[b] |
| Acetic Acid | 0.34±0.01[a] | 0.33±0.01[a] | 0.45±0.02[b] | 0.43±0.01[b] |
| Piruvic Acid | 2.25±0.07 | 2.59±0.63 | 2.13±0.03 | 2.20±0.01 |
| Acetaldehyde | 1.51±0.03[a] | 1.39±0.07[a] | 1.10±0.05[b] | 1.03±0.10[b] |
| Glucose / Fructose | n.d | n.d | 15.40±2.97 | 22.80±12.38 |
| Ethanol | 47.97±3.18 | 49.99±6.07 | 101.78±2.37 | 108.61±12.32 |

Tukey (HSD) analysis of the differences between the conditions with a confidence interval of 95%
[x]: groups from Tukey test in ANOVA for those significantly different
n.d: no determined

Nevertheless fermented grape must results showed differences in succinic acid, glycerol, acetic, pyruvic acid, acetaldehyde and ethanol, see in Table 4. Glucose/fructose results at the end of the fermentation suggest that samples were taken before the process properly ended, even when the density was similar than in synthetic must fermented by rehydrated EC1118 ADY.

**Table 4. Fermentation products (g/L) of GM fermentation by EC1118**

| | Midpoint | | End | |
|---|---|---|---|---|
| | Control | GABA | Control | GABA |
| Succinic acid | 0.00±0.00[a] | 0.38±0.10[a,b] | 0.67±0.22[b] | 1.15±0.26[c] |
| Glycerol | 4.91±0.35[a,b] | 3.78±0.73[b] | 5.73±0.38[a] | 5.77±0.52[a] |
| Acetic Acid | 0.49±0.02[a] | 0.37±0.03[b] | 0.50±0.01[a] | 0.45±0.03[a] |
| Piruvic Acid | 0.69±0.03[a] | 0.72±0.04[a] | 0.83±0.01[b] | 0.69±0.04[a] |
| Acetaldehyde | 0.30±0.03[a] | 0.49±0.04[b] | 0.25±0.06[a] | 0.27±0.02[a] |
| Glucose / Fructose | n.d | n.d | 18.72±5.10 | 8.28±1.95 |
| Ethanol | 57.24±1.40[a] | 50.49±3.53[a] | 87.46±0.35[b] | 84.88±1.22[b] |

Tukey (HSD) analysis of the differences between the conditions with a confidence interval of 95%
[x]: groups from Tukey test in ANOVA for those significantly different
n.d: no determined

## Effect of growth medium composition and inoculum type on fermentation yield

In order to compare the three wine condition studied, the results of SWM, fermented by the rehydrated EC1118 ADY and by the precultured EC1118 ADY, and the grape must fermented by rehydrated EC1118 are shown in Table 5 as the efficiency of 100g of the sugar consumed at

the end of the process. Except for glycerol efficiency results, ANOVA test and Tukey calculator detected significantly differences between the wine conditions performed.

**Table 5. Yield of fermentation products (g) for 100g of the consumed sugar**

| | | ADY SWM | | PreC SWM | | GM | |
|---|---|---|---|---|---|---|---|
| | unit | Control | GABA | Control | GABA | Control | GABA |
| Sugar consumption | g | 214.4±3.9 | 218.1±1.1 | 204.6±3.0 | 197.2±12. 4 | 189.4±5.1 | 199.8±1.9 |
| Succinic acid | mg | 0.249±0.014$^{b,c}$ | 0.244±0.008$^{c}$ | 0.284±0.011 $^{a}$ | 0.308±0.018 $^{a,b}$ | 0.036±0.013$^{d}$ | 0.058±0.014$^{d}$ |
| Glycerol | g | 3.617±0.137 | 3.759±0.412 | 2.949±0.258 | 3.950±1.613 | 3.026±0.149 | 2.885±0.241 |
| Acetic | g | 0.457±0.028$^{a}$ | 0.465±0.012$^{a}$ | 0.222±0.011$^{b,c}$ | 0.219±0.017$^{c}$ | 0.264±0.011$^{b}$ | 0.225±0.011$^{b,c}$ |
| Piruvic Acid | mg | 0.092±0.015$^{b,c}$ | 0.084±0.005$^{c}$ | 0.104±0.003$^{a,b}$ | 0.112±0.007$^{a}$ | 0.044±0.002$^{b}$ | 0.035±0.002$^{b,c}$ |
| Acetaldehyde | mg | 0.037±0.001$^{b}$ | 0.037±0.001$^{b}$ | 0.054±0.002$^{a}$ | 0.052±0.008$^{a}$ | 0.014±0.003$^{c}$ | 0.014±0.001$^{c}$ |
| Ethanol | g | 41.267±2.813$^{b}$ | 44.731±0.783$^{b}$ | 49.762±1.842$^{a,b}$ | 55.296±7.898$^{a}$ | 46.192±1.066$^{a,b}$ | 42.476±0.608$^{b}$ |
| Yield products | g | 45.719±2.659$^{b}$ | 49.320±0.900$^{b}$ | 53.375±1.842$^{a,b}$ | 59.937±7.536$^{a}$ | 49.575±1.024$^{b}$ | 45.691±0.746$^{b}$ |

Tukey (HSD) analysis of the differences between the conditions with a confidence interval of 95%
$^{a,b,\ c\ or\ d}$: groups from Tukey test in ANOVA for those significantly different

We supposed from Table 5 that rich medium pre-culture of EC1118 promoted the increase of ethanol production, what caused at the same time the medium toxicity for yeast. Moreover, because of the stresses condition, the production of succinic acid has been revealed higher in SWM fermented by pre-cultured EC1118 ADY. Being succinate a component of GABA shunt, the high presence of succinic acid suggests that GABA metabolism could be activated under these adverse circumstances reported as stuck fermentation. This suggestion supports the hypothesis of GABA being part in stress tolerance yeast mechanism.

## Conclusion

Fermentation kinetic analyses concluded that the composition of synthetic wine must is not suitable for the non-*Saccharomyces* wine-type yeast strains used, TD291, Cz3 and 1197. Nevertheless TD291 growth parameters in GABA-supplemented fermentation suggested a negative effect on its behaviour in this wine fermentation type. The results of this work could suggest further deep studies on the GABA effect in different *Torulaspora delbrueckii* strains fermenting synthetic and grape must.

With regard to alcoholic fermentation carried out by *S. cerevisiae*, the results showed differences in growth behavior depending on the inoculum state. Pre-culture in rich media of EC1118 active dry yeast increased the synthesis of ethanol, what we could suggest promoting medium toxicity for yeast. Under the stress condition, the presence of GABA could be related with cell stress tolerance. Finally, when the EC1118 strain grows in grape must, the additional GABA in the medium allows the yeast to promote alcoholic fermentation by using it as a source of nitrogen.

Further studies on the yeast cell metabolism could be promoted by focusing on certain metbolites related to GABA metabolism, such as succinate and GABA by itself, in order to reveal the role of the GABA / GAD shunt. Futhermore the glyoxylate shunt of Krebs cycle could be studied.

## Acknowledgements

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**ANNEX I. Culture medium**

| Synthetic wine must (SWM) | | |
|---|---|---|
| **Solution** | **Compounds** | **Concentration (mg/l)** |
| **A** | Glucose | 110* |
| | Fructose | 110* |
| | L-Tartaric acid | 5* |
| | L-Malic acid | 2* |
| | Citric acid | 0.5* |
| **B** | YNB | 0.67* |
| **C** | Proline | 300 |
| **D** | $NH_4Cl$ | 50 |
| **E** | Glutamine | 260 |
| | Arginine | 115 |
| | Tryptophan | 75 |
| | Alanine | 90 |
| | Glutamic acid | 210 |
| | Serine | 80 |
| | Threonine | 60 |
| **F** | Leucine | 25 |
| | Aspartic acid | 45 |
| | Valine | 35 |
| | Phenyalanine | 25 |
| | Isoleucine | 25 |
| | Histidine | 40 |
| | Methionine | 10 |
| | Tyrosine | 10 |
| | Glycine | 10 |
| | Cysteine | 15 |
| | Lysine | 10 |
| | Asparagine | 10 |
| | Citrulline | 5 |
| **G** | GABA | 100 |

*unit: g/L