Article

Differential Response of the Proteins Involved in Amino Acid Metabolism in Two *Saccharomyces cerevisiae* Strains during the Second Fermentation in a Sealed Bottle

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Abstract: The traditional method for sparkling wine making consists of a second fermentation of a base wine followed by ageing in the same bottle that reaches the consumers. Nitrogen metabolism is the second most important process after carbon and takes place during wine fermentation by yeast. Amino acids are the most numerous nitrogen compounds released by this process. They contribute to the organoleptic properties of the wines and, therefore, to their sensory quality. The main objective of this study is to compare the differential proteomic response of amino acid metabolism, specifically their proteins and their interactions in the G1 strain (unconventional yeast) during sparkling wine production *versus* the conventional P29 strain. One of the new trends in winemaking is the improvement of the organoleptic diversity of wine. We propose the use of unconventional yeast that shows desirable characteristics for the industry. For this purpose, these two yeasts were grown at sealed bottle conditions for the second fermentation (*Champenoise* method). No differences were obtained in the middle of fermentation between the yeast strains. The number of proteins identified, and the relationships established, were similar, highlighting lysine metabolism. At the end of the second fermentation, the difference between each strain was remarkable. Hardly any proteins were identified in unconventional *versus* conventional yeast. However, in both strains, the metabolism of sulfur amino acids, methionine, and cysteine obtained a greater number of proteins involved in these processes. The release of these amino acids to the medium would allow the yeast to balance the internal redox potential by reoxidation of NADPH. This study is focused on the search for a more complete knowledge of yeast metabolism, specifically the metabolism of amino acids, which are key compounds during the second fermentation.

Keywords: sparkling wine; protein; interact omics; amino acid metabolism; yeast; GO terms

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1. Introduction

"*Méthode champenoise*", also known as the traditional method, is a sparkling wine production method whereby wine undergoes a second fermentation process in the bottle to produce CO_2. During the aging of sparkling wines in contact with the yeast lees, the phenomenon of autolysis takes place. Through this process, nitrogen compounds are released that contribute to the organoleptic properties of the wines and therefore to their sensory quality.

Nitrogen is a key nutrient vital to yeast during wine fermentation, and its availability in grape is a key parameter for the progress of wine fermentation, affecting both fermentation kinetics and wine aroma formation. The shortage of these compounds can cause slow or stuck fermentations [1,2] or influence the formation of reduced sulfur compounds, such

as hydrogen sulfide [3]. Furthermore, the catabolism of nitrogen sources and the central metabolism of carbon is involved in the synthesis of fermentative aroma precursors in oenological yeasts [4,5].

Saccharomyces cerevisiae can grow on a wide variety of nitrogenous substrates; the metabolism of nitrogenous compounds depends on the yeast strain, its physiological state, and the physicochemical properties of the wine. Nitrogen metabolism is controlled by regulatory mechanisms that depend largely on the nature of the nitrogen sources present in the wine. These mechanisms act at the transcriptional level, as well as in the activity and degradation of enzymes and permeases [6]. The assimilable fraction of nitrogen by yeasts is mainly ammonia and amino acids in similar proportions, but they can also use other nitrogen sources, such as oligopeptides, polypeptides, proteins, amides, biogenic amines, and nucleic acids [7,8]. *S. cerevisiae* incorporates amino acids and small peptides (less than five amino acid residues) through an active transport process using specialized membrane proteins and a pH gradient [6,9]. The amount of these compounds in sparkling wines depends on the grape variety and the aging time [7,8]. To cope with these stressful conditions, which can cause nitrogen shortages, yeasts consume and metabolize these compounds as efficiently as possible during fermentation. The preferred compound for yeast is glutamate, which is stored for *de novo* amino acid biosynthesis. This is particularly relevant when the ethanol content increases, which inhibits amino acid absorption [9]. *S. cerevisiae* uses amino acids differentially; preferred nitrogen sources are alanine, arginine, asparagine, aspartate, glutamate, glutamine, and serine [7].

The amino acids present in sparkling wines have different origins: (i) they can come directly from the grape, without being metabolized by yeast during its growth, (ii) they can excrete at the end of fermentation, (iii) and they can also originate during the autolysis process. Generally, these amino acids are used by yeast for the formation of proteins or as a source of nitrogen by oxidative deamination; alternatively, an amino acid can be broken down and thus liberate nitrogen. During fermentation, yeast incorporates and metabolizes amino acids to grow and produce biomass. In this process, a series of volatile aromatic compounds are produced (esters, higher alcohols, volatile fatty acids, carbonyls, and sulfur compounds), which have a great impact on the organoleptic properties of wines. In addition, the nitrogen released can also be used for the biosynthesis of other nitrogenous cellular constituents, and its carbon structure can be excreted in wine or used as a carbon source for the biosynthesis of other compounds, such as higher alcohols [10,11], which constitute the main group of aromatic compounds in wine [7]. Nitrogen metabolism is very complex because its intermediaries are also shared among other metabolic pathways. Because of this, there is not yet a complete understanding of the impact of amino acids on the formation of different aromatic compounds.

Therefore, it is important to establish a fundamental dataset in order to build solid knowledge. This new study, along with the rest of the work carried out by our research group, seeks to address the lack of information that exists in multi-omics analysis by integrating the proteomics and metabolomics of *S. cerevisiae* during the second fermentation in the production of sparkling wines (cava). For this reason, in previous works, our research group has focused on the study of ester metabolism [12] and the autophagy process [13], as well as the effect of CO_2 overpressure on the aroma [14,15] during the second fermentation in the production of sparkling wines (cava). In addition, the novelty of using an unconventional yeast for the production of this type of wine was introduced. It is a flor yeast that, due to properties such as its high tolerance to ethanol and its flocculation capacity, could be a good candidate for use in the production of sparkling wine. In this way, the diversity, uniqueness, and typicality of sparkling wine yeasts could be improved.

The main objective of this study was to compare the differential proteomic response of amino acid metabolism, specifically their proteins and their interactions in the G1 strain (unconventional yeast) during sparkling wine production *versus* the conventional P29 strain. This relationship never been investigated before in sparkling wines, hence the

difficulty of this study and the relevance of its results. This knowledge is intended to serve as a guide for future research and ultimately to improve the quality of the wine.

2. Materials and Methods

2.1. Microorganisms and Fermentation Conditions

The microorganisms used were two strains of *S. cerevisiae*: the conventional P29 strain (CECT11770), a typical yeast in the production of sparkling wines, which was isolated from the designation of origin of the Penedès (DO) Barcelona (Northeast of Spain), and the G1 strain (ATCC: MYA-2451). The G1 strain is a flor yeast isolated from a flor "velum" under the biological aging of "Fino" Sherry wine of the designation of origin (DO) Montilla-Moriles in Córdoba (Southern of Spain).

The yeast strains were incubated for 5 days at 21 °C using a gentle agitation of 100 rpm for their growth in a pasteurized must of Macabeo grape variety, composed of 174.9 g/L of sugar, 18.5 °Bx, 3.6 g/L of total acidity, and 3.43 pH. When an ethanol content of 10.39% (*v*/*v*) was reached, the yeast cells were introduced into bottles with a standardized commercial base wine (Macabeo: Chardonnay (6:4), 10.21% (*v*/*v*) of ethanol, 0.3 g/L of sugar, pH 3.29, 5.4 g/L of total acidity, and 0.21 g/L of volatile acidity), 21 g/L sucrose, and 1.5×10^6 cells/mL. The second fermentation was carried out in a thermostatic chamber at 14 °C in bottles with a volume of 750 mL. During the second fermentation, the following samples were taken: at the middle of the second fermentation, MF (3 bar pressure), and at the end of the second fermentation, EF (6.5 bar pressure). In each sample, three bottles were opened for triplicate analysis.

Viable yeast cell counting was carried out using appropriate dilutions with Ringer solution. These were then plated in Sabouraud agar medium for 48 h at 28 °C, and all samples were analyzed five-fold.

2.2. Proteomic Analysis

Cells were collected from each bottle by centrifugation at 4500× *g* for 10 min using a centrifuge (Rotina-38, Kirchlengern, Germany), washing the pellet twice with cold sterile distilled water. Subsequently, the washed cell pellets were dissolved in 2 mL extraction buffer (100 mM Tris-HCl pH 8, 0.1 mM EDTA, 2 mM DTT and 1 mM PMSF) and a protease inhibitor cocktail. Lysis of cells was performed using a mechanical technique in a Vibrogen Cell Mill V6 (Edmund Bühler GmbH, Bodelshausen, Germany) using glass beads of 500 µm in diameter. Once the cells were disrupted, the protein extraction was removed. Glass beads as well as cell debris were discarded by centrifugation at 500× *g* for 5 min. Protein precipitation was carried out by overnight incubation at −20 °C after the addition of 10% *w*/*v* of trichloroacetic acid (TCA) and 4 vol of ice-cold acetone to the supernatant. After incubation, samples were centrifuged at 16,000× *g* for 30 min, and the protein pellet was then vacuum dried and resuspended in solubilization buffer. Protein concentration was estimated by Bradford (1976) and samples stored at −80 °C until protein analysis [16]. To perform this analysis, 500 µg of total protein for each condition and replica was loaded into an Agilent Technologies OFFGEL 3100 fractionator well tray. Previously, protein samples were solubilized in a Protein OFFGEL fractionation buffer containing urea, thiourea, DTT, glycerol, and ampholytic buffer. The aliquots were distributed in an Agilent Technologies OFFGEL 3100 fractionator (Santa Clara, CA, USA), in a tray with wells. Once the proteins were separated according to their isoelectric point, the fractions were collected from each well and their identification was carried out. For identification, protein fractions were analyzed on an LTQ Orbitrap XL mass spectrometer equipped with an Ultimate 3000 nano LC system at the Central Research Support Service (SCAI) of the University of Córdoba. Proteins had to be digested with trypsin beforehand. Finally, the identified proteins were quantified following the exponentially modified Protein Abundance Index, EmPAI, a method described by Ishihama et al. (2005) [17]. More detailed information is described in articles published by the research group [12].

2.3. Statistical Analysis

After identification, the proteins were filtered to take into account those involved in "amino acid metabolism", according to the Gene Ontology terminology (GO) of the *Saccharomyces* genome database (https://www.yeastgenome.org/, accessed on 15 February 2021), using the GO Slim Mapper tool. The selected processes were: (i) cellular amino acid metabolic process, (ii) amino acid transport, (iii) tRNA aminoacylation for protein translation.

The proteins involved in the "cellular amino acid metabolic process" were classified in different GO terms provided by the SGD database (https://www.yeastgenome.org/, accessed on 15 February 2021). This classification was made using the GO Term Finder tool, provided by the database. The GO terms are descriptive terms that allow relating each gene product with a molecular, cellular, and biological process context, providing a statistical value (p-value). This statistical study was performed with a level of significance (α) of 0.05.

The proteins discussed in this study were strain-specific proteins during the second fermentation in bottle.

2.4. Protein Network Reconstruction

The software STRING version 11 (available online, https://string-db.org/, accessed on 15 February 2021) was used to reconstruct an interaction network for proteins of interest, forming specific protein groups through an MCL (Markov Cluster Algorithm) clustering method. This algorithm accepts a parameter called 'inflation' that is indirectly related to the precision of the clustering. Data were previously normalized through the root square and auto-scaling methods [18].

3. Results and Discussion

The present study was carried out on two strains of *S. cerevisiae*: a conventional strain for the elaboration of this type of wine (P29 strain) and a non-conventional yeast (G1 strain) under real conditions of the second fermentation; the sample times were at the middle of the second fermentation (MF) and at the end of the second fermentation in a sealed bottle (EF).

For this, the total proteins identified in each strain were compared, and those different in each case were selected (from now on named as specific). They were then classified into three processes (amino acid transport, tRNA aminoacylation for protein translation, and cellular metabolism) according to the *Saccharomyces* Genome Database (SGD). Data are shown in Table 1. Finally, the proteins involved in the cellular metabolism process were classified in GO terms according to the SGD database, and the interactions between them were established using STRING software.

Table 1. Biological processes, specific proteins, and ratio of specific to the total proteins related to amino acid metabolism in *S. cerevisiae* P29 and G1 during two sampling times, at the middle of the second fermentation (MF) and at the end of the second fermentation (EF).

	Middle of the Second Fermentation, MF			
	P29 Strain		G1 Strain	
Biological Process	**Specific Proteins**	**Specific/Total Proteins**	**Specific Proteins**	**Specific/Total Proteins**
Cellular amino acid metabolic process (GO: 0006520)	Aat1p, Ald2p, Arg81p, Aro9p, Dal81p, Gcv2p, Gdh3p, Ilv6p, Leu9p, Lys1p, Lys4p, Met1p, Mis1p, Put1p, Snz3p, Ths1p, Uga2p, Uga3p, Ura7p, Utr4p, Xbp1p, Ydl168wp, Yml082wp, Ymr084wp	24/103	Aco2p, Adi1p, Aro10p, Bna4p, Cpa2p, Ehd3p, Gcv1p, Gfa1p, Gln4p, Gly1p, His5p, Idp1p, Lap3p, Lys21p, Mae1p, Met12p, Met16p, Mri1p, Nit3p, Pet112p, Ser1p, Sfa1p, Sno3p, Tum1p, Uga1p, Yhr112cp, Yhr208wp, Yll058wp	28/107

Table 1. *Cont.*

	Middle of the Second Fermentation, MF			
	P29 Strain		G1 Strain	
Biological Process	**Specific Proteins**	**Specific/Total Proteins**	**Specific Proteins**	**Specific/Total Proteins**
tRNA aminoacylation for protein translation (GO: 0006418)	Aim10p, Cdc60p, Ded81p, Dia4p, Dps1p, Msk1p, Slm5p, Ths1p, Wrs1p	9/23	Gln4p, Nam2p	2/16
Amino acid transport (GO: 0006865)	Avt7p, Bap2p, Lst4p, Put4p, Uga4p, Ydl119cp	6/9	Alp1p, Avt4p, Btn2p, Gnp1p, Ssy1p	5/8
	End of the second fermentation, EF			
	P29 Strain		**G1 Strain**	
Biological Process	**Specific Proteins**	**Specific/Total Proteins**	**Specific Proteins**	**Specific/Total Proteins**
Cellular amino acid metabolic process (GO: 0006520)	Ade3p, Adh3p, Arg1p, Arg7p, Arg8p, Arg80p, Arg82p, Aro1p, Aro2p, Aro3p, Asn1p, Asn2p, Bna1p, Car1p, Car2p, Cys3p, Cys4p, Dtd1p, Gdh1p, Gln1p, Gus1p, His4p, Hom6p, Hpa3p, Idh1p, Ilv6p, Kgd2p, Leu4p, Lpd1p, Lys12p, Lys20p, Mcm1p, Met17p, Mis1p, Mmf1p, Pro2p, Sam2p, Ser1p, Ser3p, Sfa1p, Shm2p, Uga1p, Yhr208wp	43/56	Gcv3p, Gfa1p, Idh2p, Idp1p, Met13p, Met8p, Sno3p, Snz1p, Ybr145wp	9/22
tRNA aminoacylation for protein translation (GO: 0006418)	Arc1p, Grs1p, Gus1p, Ism1p, Msk1p, Ses1p, Vas1p	7/8	Msm1p	1/2
Amino acid transport (GO: 0006865)	Npr2p	1/1		

3.1. Amino Acid Transport

Amino acids are involved in numerous metabolic pathways. *S. cerevisiae* can synthesize them *de novo*; they are also actively imported from the extracellular environment. This is less expensive in terms of energy; the estimated cost of *de novo* amino acid synthesis under aerobic conditions is between 9.5 ATP for glutamate and 75.5 ATP for tryptophan [19]. Yeast export of amino acids enables them to respond to different environments and take advantage of available resources. The amino acids present in the vacuole are histidine, lysine, and arginine, constituting 80 to 90%, while a similar fraction of glutamate and aspartate is present in the cytosol [20–22]. *S. cerevisiae* incorporates amino acids from the medium, making use of proteins known as amino acids permeases by a simport mechanism [23]. The multiplicity and diversity of transporters allows yeast to accumulate amino acids for biosynthesis and catabolism under multiple conditions and in a wide range of external concentrations [24].

Of the total transporters identified in *S. cerevisiae* (41 transporters) [19], 6 transporters (Avt7p, Bap2p, Lst4p, Put4p, Uga4p, Ydl119cp) in MF and 1 (Npr2p) in EF have been obtained in the P29 strain. While in the G1 strain, 5 (Alp1p, Avt4p, Btn2p, Gnp1p, Ssy1p) in MF and 0 in EF have been reported. In view of these results, it would not be expected to find a great difference between the two strains. However, the transporters identified in both types of yeast were different, although all transport the full range of amino acids from the cell exterior to the interior and from the cell interior to the vacuole or mitochondria.

Of the transporters identified in the P29 strain, Avt7p was the one with the highest protein content (0.028 mol%). Data are shown in Table 2. Yeast Avtp proteins can be subdivided into four main groups, defined as Avt1p, Avt2p, Avt3p/4p, and Avt5p/6p/7p [25]. Avt1p intervenes in the active absorption of amino acids in the vacuole, while Avt3p, Avt4p, and Avt6p are involved in the active exit of amino acids from the vacuole into the

cytosol. Avt7p is located in the vacuolar membrane of *S. cerevisiae* and participates in the vacuolar absorption of glutamine and proline and in the formation of spores in *S. cerevisiae*, which could be related to the Avt7p-dependent amino acid flux of vacuoles under the conditions of lack of nutrients [26]. On the other hand, the protein that had the highest protein content in the G1 yeast was Btn2p (0.022 mol%); this protein participates in the uptake of arginine [27].

Table 2. Quantification of specific proteins identified in each strain using the EmPAI method (mol%) in *S. cerevisiae* P29 and G1 during two sampling times: at the middle of the second fermentation (MF) and at the end of the second fermentation (EF).

MF						EF					
P29 Strain			G1 Strain			P29 Strain			G1 Strain		
Proteins	mol%	SD	Proteins	mol%	SD	Proteins	mol%	SD	Proteins	mol%	SD
Aat1p	0.024	0.0002	Aco2p	0.028	0.0003	Ade3p	0.007	0.0001	Gcv3p	0.427	0.004
Ald2p	0.010	0.0001	Adi1p	0.048	0.0005	Adh3p	0.078	0.0008	Gfa1p	0.033	0.0008
Arg81p	0.006	0.0001	Aro10p	0.010	0.0001	Arg1p	0.114	0.0011	Idh2p	0.081	0.001
Aro9p	0.028	0.0003	Bna4p	0.010	0.0001	Arg7p	0.055	0.0006	Idp1p	0.0465	0.0005
Dal81p	0.007	0.0001	Cpa2p	0.028	0.0003	Arg8p	0.013	0.0001	Met13p	0.051	0.001
Gcv2p	0.005	0.0001	Ehd3p	0.011	0.0001	Arg80p	0.039	0.0004	Met8p	0.069	0.0007
Gdh3p	0.057	0.0006	Gcv1p	0.013	0.0001	Arg82p	0.0187	0.0002	Sno3p	0.106	0.0011
Ilv6p	0.016	0.0002	Gfa1p	0.015	0.0001	Aro1p	0.004	0.0000	Snz1p	0.076	0.0006
Leu9p	0.048	0.0005	Gln4p	0.005	0.0003	Aro2p	0.150	0.0015			
Lys1p	0.052	0.0005	Gly1p	0.108	0.0011	Aro3p	0.276	0.0028			
Lys4p	0.015	0.003	His5p	0.060	0.0006	Asn1p	0.082	0.0008			
Met1p	0.015	0.0001	Idp1p	0.031	0.0003	Asn2p	0.036	0.0004			
Mis1p	0.005	0.0000	Lap3p	0.046	0.0005	Bna1p	0.039	0.0004			
Put1p	0.011	0.0001	Lys21p	0.138	0.0014	Car1p	0.134	0.0013			
Snz3p	0.016	0.0002	Mae1p	0.016	0.0002	Car2p	0.055	0.0006			
Ths1p	0.011	0.0001	Met12p	0.017	0.0002	Cys3p	0.113	0.0011			
Uga2p	0.011	0.0001	Met16p	0.022	0.0002	Cys4p	0.131	0.0013			
Uga3p	0.010	0.0001	Mri1p	0.014	0.0001	Dtd1p	0.114	0.0011			
Ura7p	0.017	0.0002	Nit3p	0.016	0.0002	Gdh1p	0.139	0.0014			
Utr4p	0.029	0.0003	Pet112p	0.009	0.0005	Gln1p	0.305	0.0031			
Yml082wp	0.008	0.0001	Ser1p	0.043	0.0004	Gus1p	0.041	0.0004			
Ymr084wp	0.019	0.0002	Sno3p	0.023	0.0002	His4p	0.093	0.0009			
Put4p	0.013	0.0001	Tum1p	0.015	0.0002	Hom6p	0.158	0.0016			
Uga4p	0.019	0.0004	Uga1p	0.081	0.0008	Hpa3p	0.116	0.0011			
Lst4p	0.012	0.0001	Yhr112cp	0.014	0.0001	Idh1p	0.079	0.0008			
Bap2p	0.012	0.0001	Yll058wp	0.008	0.0001	Ilv6p	0.068	0.0007			
Avt7p	0.028	0.0006	Yhr208wp	0.161	0.0016	Kgd2p	0.056	0.0006			
Ydl119cp	0.014	0.001	Alp1p	0.016	0.0002	Leu4p	0.041	0.0004			
			Gnp1p	0.009	0.0001	Lpd1p	0.011	0.0001			
			Avt4p	0.009	0.0001	Lys12p	0.326	0.007			
			Btn2p	0.021	0.0002	Lys20p	0.028	0.0003			
			Ssy1p	0.008	0.0001	Mcm1p	0.214	0.0021			
			Odc2p	0.015	0.0002	Met17p	0.054	0.0005			
						Mis1p	0.006	0.0001			
						Mmf1p	0.546	0.009			
						Pro2p	0.040	0.0004			
						Sam2p	0.205	0.003			
						Ser1p	0.017	0.0002			
						Ser3p	0.020	0.0002			
						Sfa1p	0.080	0.0008			
						Shm2p	0.039	0.0004			
						Uga1p	0.013	0.0001			

3.2. tRNA Aminoacylation for Protein Translation

Regarding tRNA aminoacylation for protein translation, more specific proteins were identified in the P29 strain than in the G1 strain: 16 proteins *versus* 3 proteins, respectively (Table 1, Figure 1). The formation of aminoacyl-tRNA (aa-tRNA) is an essential process in protein biosynthesis. Proteins of the aminoacyl-tRNA synthetase family can be classified into two groups, depending on the specificity of amino acids: (i) class I (specific for glutamine, glutamate, arginine, cysteine, methionine, valine, isoleucine, leucine, tyrosine, and tryptophan); (ii) class II (specific for glycine, alanine, proline, serine, threonine, histidine, aspartate, asparagine, lysine, and phenylalanine) [28–31]. At the middle of the second fermentation in the P29 strain, 9 proteins were identified (Aim10p, Cdc60p, Ded81p, Dia4p, Dps1p, Msk1p, Slm5p, Ths1p, and Wrs1p), and at the end of the second fermentation, 7 proteins (Arc1p, Grs1p, Gus1p, Ism1p, Msk1p, Ses1p, and Vas1p) were identified. These proteins are responsible for binding amino acids such as tryptophan (Wrs1p), threonine (Ths1p), asparagine (Ded81p, Slm5p), lysine (Msk1p), aspartate (Dps1p), serine (Dia4p, Ses1p), leucine (Cdc60p), methionine (Arc1p), glutamate (Arc1p, Gus1p), glycine (Grs1p), isoleucine (Ism1p), and valine (Vas1p). These results suggest that yeast may be activating its transcription and translation machinery to try to cope with cell death and autolysis, and they corroborate those previously obtained by our research group [30].

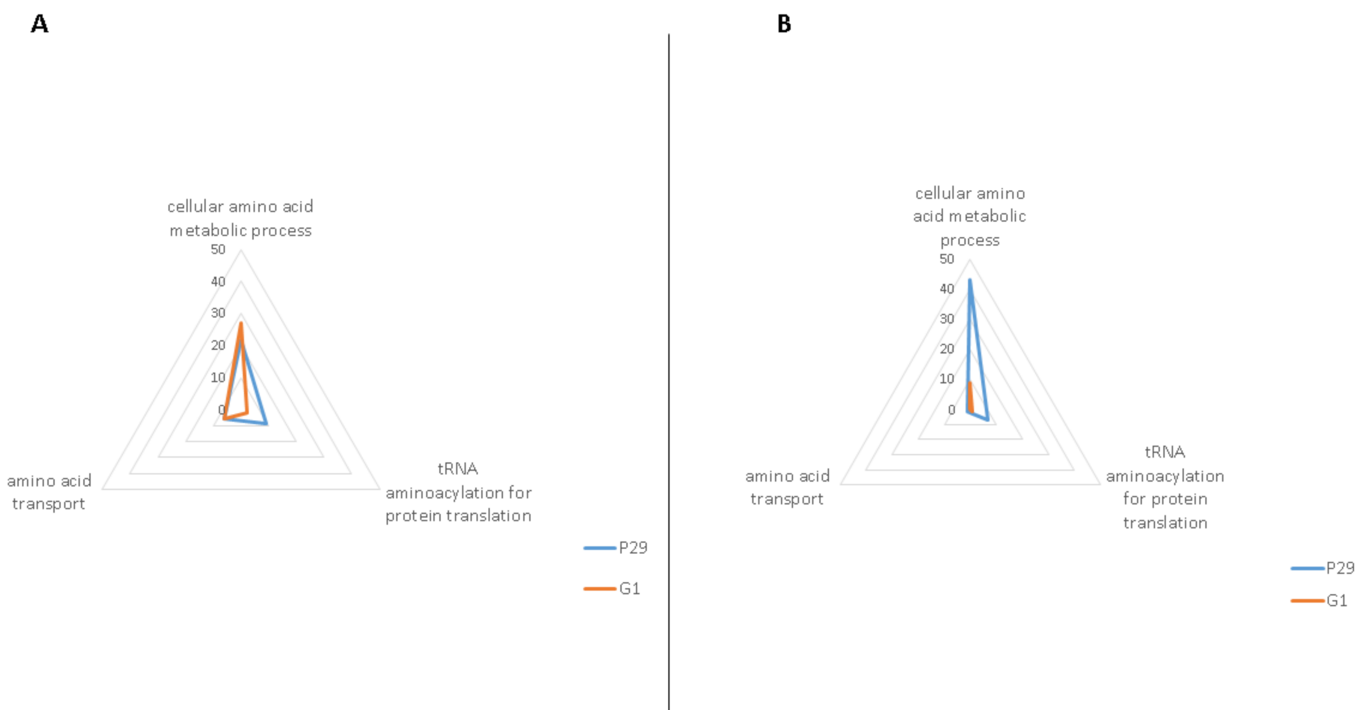

Figure 1. Total number of specific proteins related to amino acid metabolism in *S. cerevisiae* P29 (blue colour) and G1 (orange colour) during two sampling times: at the middle of the second fermentation, MF (**A**), and at the end of the second fermentation, EF (**B**).

On the other hand, the specific proteins identified in the G1 strain were Gln4p and Nam2p, which catalyze the binding of glutamine and leucine, respectively, the nuclear protein Pet112p, required to maintain rho$^+$ mitochondrial DNA [31], and Msm1p; this protein is a mitochondrial methionyl-tRNA synthetase. These results seem to indicate that the protein synthesis machinery in flor yeast (strain G1) is not working, though yeast autolysis may be occurring, which would help the survival of the rest of the population in this harsh condition [32].

3.3. Cellular Amino Acid Metabolic Process

In the P29 strain, a total of 67 specific proteins related to the cellular amino acid metabolic processes were obtained (24 in MF; 43 in EF), while in the G1 strain, 37 proteins were identified (28 in MF; 9 in EF). Data are shown in Table 1.

The metabolism of amino acids is affected by the pressure the yeast cells are subjected to in the second fermentation, carried out in the bottle, at both levels of the anabolism and catabolism of amino acids. In order to understand the possible interactions between the differential proteins from each strain involved in amino acid metabolism and to provide a better understanding of the GO terms obtained, a protein–protein interaction network map was created for sampling time (MF, EF) using STRING v11 [18]. Because metabolism is somewhat dynamic, these connections can be conceptualized as networks, and the size and complex organization of these networks present a unique opportunity to obtain a global visualization of the yeast genome during the second fermentation.

3.3.1. Cellular Amino Acid Metabolic Process at the Middle of the Second Fermentation

At the middle of the second fermentation, 24 specific proteins in the P29 strain and 28 specific proteins in the G1 strain were identified. These proteins are represented as nodes in Figure 2A,B, respectively. A total of 28 interactions (number of edges) were observed, with a *p*-value of PPI enrichment $<2.23 \times 10^{-12}$ in the P29 strain; while in the G1 strain, 36 interactions were obtained, with a *p*-value of PPI enrichment $<4.44 \times 10^{-16}$; such enrichment indicates that the proteins are at least partially biologically connected as a group.

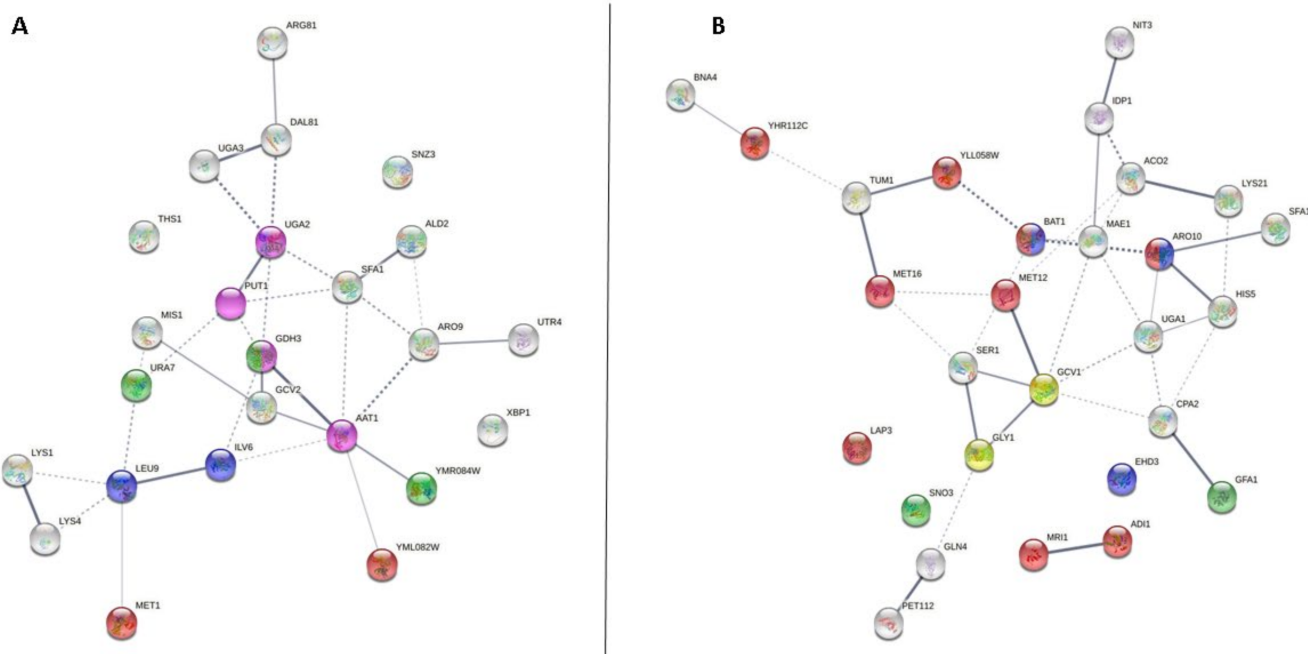

Figure 2. High confidence protein–protein interaction network map built using STRING v11 and based on the proteins of cellular amino acid metabolic process detected in *S. cerevisiae* P29 (**A**) and G1 (**B**) in the middle of the second fermentation. Proteins are shown as nodes, and the existence of interactions between them are represented by lines (the connection between nodes). Line thickness indicates the strength of the different interactions. Nodes with the same color represent specific clusters: sulfur amino acid metabolic process (red nodes), branched-chain amino acid metabolic process (light blue nodes), glutamine metabolic process (light green nodes), glycine metabolic process (yellow nodes), and glutamate metabolic process (light purple nodes).

In the P29 strain, the strongest interactions were observed between proteins belonging to lysine metabolism (white nodes) and the metabolism of branched chain amino acids (dark blue nodes). These amino acids are used for the synthesis of numerous volatile

aromatic compounds, such as higher alcohols. Furthermore, interactions were obtained between proteins belonging to different GO terms: Gdh3p-Aat1p, Uga3p-Dal81p, and Put1p-Uga2p. The Snz3p, Xbp1p, and Ths1p proteins showed no interaction with the rest of the proteins (Figure 2A). On the other hand, the strongest interactions present in the G1 strain were between proteins belonging to the metabolism of sulfur amino acids, the metabolism of lysine, and the metabolism of glycine. However, most proteins are involved with the metabolism of sulfur amino acids such as methionine and cysteine, which are necessary for yeast growth [33]. Lap3p, Sno3p, and Ehd3p did not show any interaction but were included in different GO terms (Figure 2B). In both strains, the metabolism of lysine was identified. This could be related to an antioxidant response by yeasts [34].

The main difference between both strains in the middle of the second fermentation is glutamate metabolism in the P29 strain and glycine and tryptophan metabolism in the G1 strain. These data are in agreement with the results obtained by Marsit et al. (2016) [6]. These authors reported an increase in the assimilation of various amino acids, particularly glutamate and glycine. Both amino acids are related to the synthesis of most amino acids. Furthermore, the reversible conversion of glutamate to α-ketoglutarate is an important branch point between carbon and nitrogen metabolism [6]. In addition, glutamate is one of the nitrogen sources that maintain a high growth rate [35]. During the early stages of fermentation, *S. cerevisiae* rapidly accumulates those amino acids necessary for protein synthesis and formation of volatile compounds such as higher alcohols and their respective ethyl and acetic esters [12], while the surplus is stored in the cellular vacuole, because an amino acid deficit causes a decrease in cell biomass [21]. During the fermentation process, bioactive compounds such as melatonin and serotonin may form because of the tryptophan metabolism, in addition to the higher alcohol tryptophol, which are compounds that have an impact on the sensory properties of the wine [22]. In this case, the identified protein belonging to the tryptophan metabolism, Bna4p (0.01 mol%), is a kynurenine 3-monooxygenase. This protein is required for the *de novo* biosynthesis of NAD^+ from tryptophan via kynurenine.

3.3.2. Cellular Amino Acid Metabolic Process at the End of the Second Fermentation

At the end of the second fermentation, 43 specific proteins were identified in the P29 strain and 9 specific proteins in the G1 strain. These proteins were represented as nodes in the P29 strain (Figure 3A) and G1 strain (Figure 3B). A total of 234 interactions (number of edges) were observed, with a p-value of PPI enrichment $<1.0 \times 10^{-16}$ in the P29 strain, while in the G1 strain, 4 interactions were obtained, with a p-value of PPI enrichment <0.00047; such enrichment indicates that the proteins are at least partially biologically connected as a group. In view of the results obtained, the P29 strain increased the number of proteins related to the cellular metabolism of amino acids at the end of the second fermentation, unlike the G1 strain.

In the P29 strain, the strongest interactions were observed between proteins belonging to the arginine metabolic process, the sulfur amino acid metabolic process, and the branched-chain amino acid metabolic process; those that obtained a greater number of proteins involved in their respective processes being the metabolism of arginine and sulfur amino acids (cysteine and methionine), 8 proteins in each process. Sulfur in methionine amino acid can be incorporated into cysteine. Furthermore, methionine has been shown to impact oxidative stress resistance and has the potential to be catabolized into α-ketoglutarate, which can directly enter the central carbon metabolism, providing additional support for why the demand for cell uptake was high. Glutamate, the most abundant supplemented amino acid, showed the highest uptake yield, and although no directly linked catabolic enzymes were significantly up-allocated, glutamate may be utilized metabolically for other transamination reactions because it is the major amino acid for this role [36]. On the other hand, Bna1p and Mmf1p did not interact with any protein. Bna1p is required for the *de novo* biosynthesis of NAD from tryptophan via kynurenine, and Mmf1p is required for the transamination of isoleucine. For the branched-chain amino acids (BCAA)

isoleucine, leucine, and valine and the aromatic amino acids (AAA) phenylalanine, tryptophan, and tyrosine, catabolism, via the Ehrlich pathway, is a way for the cell to salvage nitrogen through transamination reactions [37].

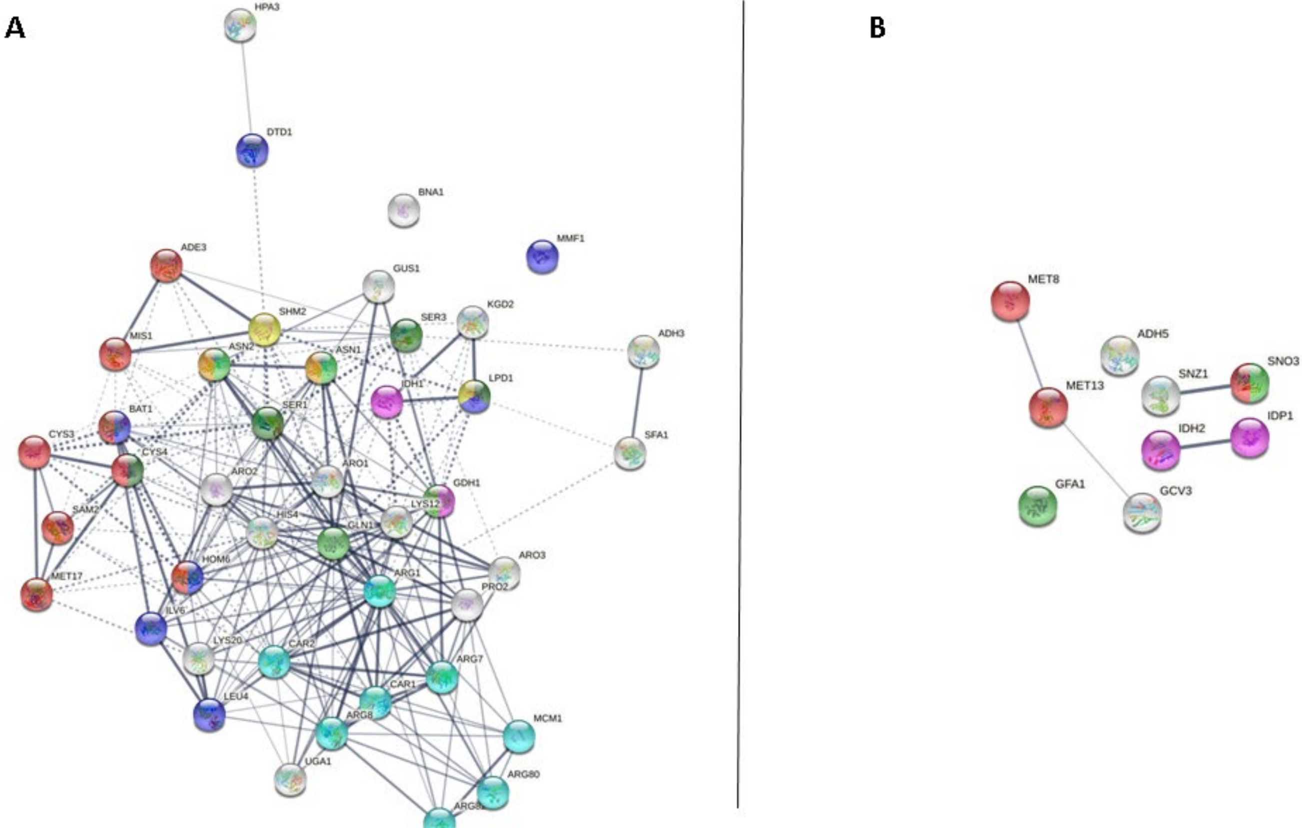

Figure 3. High confidence protein–protein interaction network map built using STRING v11 and based on the proteins of cellular amino acid metabolic process detected in *S. cerevisiae* P29 (**A**) and G1 (**B**) at the end of the second fermentation. Proteins are shown as nodes, and the existence of interactions between them are represented by lines (the connection between nodes). Line thickness indicates the strength of the different interactions. Nodes with the same color represent specific clusters: sulfur amino acid metabolic process (red nodes), branched-chain amino acid metabolic process (light blue nodes), glutamine metabolic process (light green nodes), glycine metabolic process (yellow nodes), glutamate metabolic process (light purple nodes), serine metabolic process (dark green nodes), arginine metabolic process (light blue nodes), and asparagine metabolic process (orange nodes).

In contrast, in the G1 strain, this strong interaction was between proteins involved with the glutamate metabolic process. However, Adh5p and Gfa1p did not interact. Adh5p is an alcohol dehydrogenase isoenzyme V and is involved in ethanol production, while Gfa1p converts fructose-6-phosphate to glucosamine-6-phosphate. This seems to indicate that the yeast is trying to maintain its cell growth and be able to cope with stressful conditions such as overpressure of CO_2.

Despite having a different number of proteins, both strains presented amino acids responsible for a high rate of cell growth [20], such as asparagine (P29 strain) and glutamate (G1 strain). Therefore, maintenance of cell viability should be expected in both strains during the second fermentation. The metabolism of these nitrogen compounds depends on the yeast strain, its physiological state, and the physicochemical properties of the wine and fermentation conditions [38]. This difference obtained with respect to the cellular metabolism of amino acids at the end of the second fermentation between both strains could be because they are different strains of yeast: a typical one for the elaboration of this type of wine (P29 strain) and a flor yeast (G1 strain) subjected to unusual CO_2 overpressure conditions for this type of strain. This suggests that the G1 strain at the end of

the second fermentation is stressed, viability is low, and its nitrogen metabolism is strong affected, stopping the biosynthesis of other nitrogenous cellular constituents as well as other compounds formed from its carbon skeleton [10].

The number of identified proteins was higher in the P29 strain compared to the G1 strain, which offered the possibility of establishing a greater number of interactions between these proteins. In both strains, the metabolism of sulfur amino acids, methionine, and cysteine obtained a greater number of proteins involved in these processes: 5 proteins in the P29 strain and 9 proteins in the G1 strain. In strain P29, glutamate metabolism in the middle of the second fermentation and arginine and sulfur amino acids metabolism at the end of the second fermentation stood out; while in the G1 strain, glycine and tryptophan metabolism stood out in the middle of the second fermentation and glutamate metabolism at the end of the second fermentation.

These results represent a first approach in the search for a greater and broader knowledge of the metabolism of yeast during the production of Spanish sparkling wine (cava). However, more research would be necessary, along with a metabolomic study where amino acids are quantified and an assay of enzymatic activities is performed, to reach more solid conclusions. The confirmation of these results by additional tests based on genomic, transcriptomic, and metabolomic activity assays could help achieve a better understanding of the metabolism of amino acids and, therefore, of yeast during the second fermentation in the production of sparkling wines.

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