Intracellular biosynthesis of melatonin and other indolic compounds in *Saccharomyces* and non-*Saccharomyces* wine yeasts

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Abbreviations: 5-HT: serotonin; 5-HTRP: 5-hydroxytryptophan; L-TRP: L-tryptophan; TRYP: tryptamine, N-acetyl-5-HT: N-acetyl-5-hydroxytryptamine; L-TRP EE: L-tryptophan ethyl ester; TOL: tryptophol; 3IAA: 3-indoleacetic acid; MLT: melatonin

Abstract

Certain bioactive compounds that derive from tryptophan have been determined in fermented foods, which suggests that the role of yeast is putative in origin. Melatonin is a neurohormone in humans that plays an important role in health. The activity of other compounds, such as 3-indoleacetic acid, has been recently highlighted, and interest in elucidating the conditions of their production has grown.

However, the biosynthesis of melatonin by yeasts remains unclear to a large extent. Therefore, this work was undertaken to demonstrate the unequivocally synthesis of melatonin and other compounds that derive from tryptophan metabolism by yeast by determining them in the intracellular compartment. By high resolution mass spectrometry and a validated method, tryptophan itself, melatonin, serotonin, N-acetyl-5-hydroxytryptamine and 3-indoleacetic acid, were identified in the intracellular compartment of *Saccharomyces* and non-*Saccharomyces* wine yeasts.

Keywords: exact mass, simultaneous determination, alcoholic fermentation, bioactive, 5-methoxindoles, wine yeast

1. Introduction

Melatonin (MLT), a neurohormone involved in circadian rhythm regulation, has been found in foods like strawberry, tomatoes [1] and cherries [2, 3], and in alcoholic beverages such as beer [4] and wines [5, 6]. The last-cited authors also monitored the whole winemaking process, and highlighted that melatonin was formed in the fermentation stage as a product of yeast metabolism [7]. This fermentative origin was later confirmed by the fermentation of other products, such as orange [8]. To date however, the synthesis pathway has not been unveiled in yeast and very little is known about the relevance of this molecule in its metabolism and physiology. Two independent studies have recently reported the protective role of intracellular melatonin against oxidative stress and UV radiation in *S. cerevisiae* [9, 10].

The MLT synthesis pathway in vertebrates uses tryptophan (L-TRP) as a precursor, which is converted into 5-hydroxytryptophan (5-HTRP), serotonin (5-HT) and N-acetyl-5-hydroxytryptamine (N-acetyl-5-HT) as intermediates. Other L-TRP-derived compounds with an indolic ring have been detected in wines, including L-tryptophan ethyl ester (L-TRP EE) [11], tryptophol (TOL) [12] , 3-indoleacetic acid (3IAA) [13] and tryptamine (TRYP) [14] . Thus in order to ascertain the yeast metabolic origin of MLT and other related indolic compounds, we aimed to monitor the intracellular synthesis of all these L-TRP-derived compounds and their evolution during either pulses of L-TRP to resting yeast cells or the alcoholic fermentation of synthetic must. To date, only the study of Sprenger et al. [15] has evidenced the presence of MLT in the intracellular compartment of the yeast *S. cerevisiae*. Since this article was published, analytical techniques have been extensively developed thanks to mass spectrometry advances that allow the unequivocal identification

of substances at relatively low concentrations. Fernández-Cruz et al. [16] recently developed and validated an analytical method by ultra high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC/HRMS) to monitor both MLT and related indolic compounds in order to lower their detection limits, and to assess their occurrence in culture medium and fermented products. In this study, we used this new validated analytical method to detect all these compounds intracellularly in yeasts of different species, which are all involved in wine fermentation. The intracellular detection of these molecules strongly reinforced the role played by yeasts in the final concentration of these bioactive molecules in fermented foods.

2. Materials and Methods

2.1. Yeast strains

Six yeast strains were used in this study. Two strains (QA23 and P24) belonged to *S. cerevisiae.* QA23 is a commercial strain and is marketed by Lallemand S.A. (Canada). P24 was also provided by Lallemand S.A. (Canada), but it has no commercial name as it is still going through its development stage. The other four strains are non-*Saccharomyces,* wild isolates from the winemaking *Priorat* region of Spain and belong to different yeast species: *Hanseniaspora uvarum* Hu4 (CECT 13130), *Starmerella bacillaris* Cz4 (syn. *Candida zemplinina*, CECT 13129), *Metschnikowia pulcherrima* Mpp (CECT 13131), and *Torulaspora delbrueckii* Tdp (CECT 13135).

2.2. Tryptophan pulses

Cells were grown overnight in YPD medium (2% (w/v) bacteriological peptone, 2% (w/v) glucose, 1% (w/v) yeast extract) and washed twice with distilled water before being transferred to other media. After this overnight growth, yeast strains were suspended at cell densities of ~ 10^8 cells/mL in salt medium (50mM Na_2HPO_4, 0.5% NaCl, adjusted to pH 5.8 using citric acid) and incubated in Erlenmeyer flasks with orbital agitation (150rpm) at 28ºC in complete darkness for 4 h. After this incubation period in salt medium, L-TRP was added at a final concentration of 1 mM. Control cells were not supplemented with any nutrient. Sampling was done at 30 min after adding this amino acid [15]. After this pulse, 10 mL of the culture were centrifuged (10 min at 4000 rpm). The obtained pellet (~ 10^9 cells) was washed twice with distilled water and transferred to a microcentrifuge tube to be stored at -80 ºC until it was analysed.

2.3. Fermentation conditions

Two strains of *S. cerevisiae* (QA23 and P24) and two strains of non-*Saccharomyces* species (*H. uvarum* CECT13130 and *T. delbrueckii* CECT13135) were used to test the intracellular synthesis of these indolic compounds under fermentation conditions. Fermentations were carried out in synthetic must (SM) (pH 3.3), as described by Riou et al. [17] , but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose). The assimilable nitrogen source was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in the amino acid form). The proportion of the different amino acids was the same as that previously described by Riou et al. [17] , except for L-TRP, whose concentration was increased 5-fold to boost indolic compound synthesis, but the same content in mg N/L terms (180 mg N/L) was kept. The population inoculated in SM came from an overnight culture in YPD at 30ºC and the cells were rinsed twice with sterile distilled water prior to transfer to SM. These

fermentations were performed in triplicate in laboratory-scale fermenters using 500-mL bottles filled with 400 mL of SM, which were fitted with closures that enabled carbon dioxide to escape and samples to be removed at 28ºC with continuous orbital shaking at 100 rpm. Yeast cell growth was determined by absorbance at 600 nm and by plating adequate dilutions on YPD agar by the end of fermentation. YPD plates were incubated for 2 days at 30ºC. Fermentation was monitored by measuring the density of the medium (g/L) in a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Sampling was done in the lag (1 h after inoculation), log (initial, medium and final exponential phases) and stationary phases (2 days after cells entered the stationary phase). The volume taken during each sampling was calculated to obtain 10^8 cells (approximately 10 OD_{600} units).

2.4. Intracellular metabolite extraction

Intracellular metabolites were extracted by adapting the boiling buffered ethanol method previously described by Gonzalez et al. [18] . Three mL of a solution of 75% (v/v) boiling absolute ethanol containing 70 mM (final concentration) of HEPES buffer (pH 7.5) were added to the cell pellet. This mixture was incubated for 3 min at 80ºC and 3 min on ice. The extract was concentrated by the evaporation of the volume at 45ºC in a 5301 Concentrator plus/Vacufuge® plus (Eppendorf, Spain). The final intracellular content was resuspended in 1 mL of ultrapure milliQ water and centrifuged for 10 min at 5,000 *g* at 4ºC to remove insoluble particles. The supernatant was transferred to a new tube and stored at -20º C until used.

2.5. Sample preparation

Samples were extracted as previously reported by Rodriguez-Naranjo et al. [6] with the following modifications. Briefly, C18 SPE cartridges (Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milliQ water. A 350-µL aliquot of the sample was loaded, which was followed by a washing step with 2 mL of a 10% v/v methanol/water solution. The analytes were eluted with 1 mL of methanol and then evaporated to dryness at 34ºC, 2,000 rpm in a vacuum concentrator (HyperVACLITE, GYOZEN, South Korea). The extract was reconstituted with 116 µL of water/methanol 1:1 (v/v) and stored away from light at -18ºC until analysed.

2.6. UHPLC/HRMS parameters

The UHPLC/HRMS analysis was carried out under the same conditions of a validated method reported by Fernández-Cruz et al. [16] in a UHPLC Dionex Ultimate 3000 system (ThermoScientific, San Jose, USA). This benchtop LC-MS/MS combines quadruple precursor ion selection with high-resolution, accurate-mass (HRAM) Orbitrap detection. All the devices were controlled by the Chromeleon Express Software. The column used for the analysis was a ZORBAX RRHD SB-C18 (2.1 x 100 mm, 1.8 µm particle size) with the corresponding guard column purchased from Agilent Technologies (Waldbronn, Germany). The UHPLC system was coupled to a Thermo Scientific Qexactive™ hybrid quadrupole-orbitrap mass spectrometer (Bremen, Germany). The target-MS^2 mode was set to run the analysis with the same parameters described by Fernández-Cruz et al. [16]. For identification purposes, the Xcalibur Software (version 3.0.63) was used. In order to quantitate, the TraceFinder™ Software (version 3.1) (Thermo Fisher Scientific, Waltham, MA) was applied. The mass characteristics of the compounds under study are shown in

Table S1, and include the exact mass of the protonated ion, calculated mass, Δmass ppm ranging between 0.004 and 2.55 ppm, MS/MS fragments with the molecular formula.

2.7. Statistical analysis

Differences in the intracellular indole concentrations after the L-TRP pulses were assessed by a directional Student's *t*-test to compare the concentration of each indole compound before and after the pulse. The 0.05 probability level was chosen as the maximum point of statistical significance throughout. The STATISTICA software V.7 (StatSoft, Inc, 2004) was used to perform the multivariate data analysis and the principal components analysis.

3. Results and Discussion

3.1. Intracellular indolic compounds after a tryptophan pulse to starved cells

The putative synthetic pathway of MLT in yeasts is completely unknown. In order to improve our knowledge about this route, we aimed to detect the intracellular synthesis of the different intermediates of this pathway (L-TRP, 5-HTRP, 5-HT, N-acetyl-5-HT and MLT), and other L-TRP-derived compounds (3IAA, TRYP, L-TRP EE and TOL), in different yeast strains and species, which all participate in wine fermentations.

In a similar experiment to that reported by Sprenger et al. [15] , we incubated yeast cells for 4 h in a non-proliferative medium, which were pulsed with L-TRP. After 30 min, intracellular metabolites of 10^9 cells were extracted for their analysis by UHPLC/HRMS. The absolute values of these compounds before and after the L-TRP pulses are shown in Tables 1 and 2, in which they are separated by higher concentrations (expressed as ng/10^9 cells) and lower concentrations (expressed as pg/10^9 cells), respectively. In order to

highlight which metabolites significantly increased, we calculated the P-values for each indolic compound and strain before and after the L-tryptophan pulse (Table S2). Moreover, to gain an overview, these values are represented in a heat-map as relative increases after this pulse (Figure 1). Hierarchical clustering divided the species into two major groups: cluster 1 grouped the strains of *S. cerevisiae*, *M. pulcherrima* and *H. uvarum,* with the two *S. cerevisiae* strains forming a sub-cluster. Cluster 2 comprised the *S. bacillaris* (Cz4) and *T. delbrueckii* (Tdp) strains.

The 3IAA compound was the metabolite synthesised at higher concentrations in all the strains, and Tdp and Cz4 presented the greatest accumulation. The other major synthesised compound was TOL, but at a much lower concentration. Both are synthesised via the Erlich pathway. While 3IAA is the higher acid that derives from L-TRP, TOL is the higher alcohol [19]. The synthesis of both metabolites is important because exogenous 3IAA and TOL induce morphological changes [20] and modulate the cell growth of wine yeast species, which suggests a possible role in microbial interaction during wine fermentation [21]. Among the metabolites detected at much lower concentrations, we found three intermediates of the MEL pathway of animals and plants: 5-HTRP, 5-HT and TRYP. TRYP accumulated in the *S. cerevisiae*, *M. pulcherrima* and *S. bacillaris* strains, but not in *T. delbrueckii* and *H. uvarum* ones. However, neither N-acetyl-5-HT nor MLT, these being the last products of the putative pathway, were detected in any strain. Sprenger et al. [15] reported high levels of methoxyindoles within the first 30 min after a L-TRP addition to starved cells. Our results indicate that, after a L-TRP pulse to starved cells, is favouring Ehrlich pathway over MLT biosynthetic pathway, which is more likely part of a secondary metabolism. Perhaps pulses with other intermediates of the route, such as 5-HT or other

assay conditions, not involving nitrogen starvation, would result in better yields of these secondary products.

We included in the analysis another compound, L-TRP EE, previously misnamed as MLT isomer, which has been extensively quantified in extracellular samples from SM and wine fermentations, but never detected intracellularly [11, 22, 23]. We were unable to detect it in any strain. The fact that L-TRP EE wasn't detected could be explained either because the conditions of the pulse experiments are very different to the ones during wine production or because its synthesis is the result of a spontaneous chemical esterification reaction in wines, without an enzymatic origin. In any case, more insight on yeast's ability to synthesize L-TRP EE should be done.

3.2. Intracellular synthesis of indolic compounds during wine fermentations

Recently, Fernández-Cruz et al. [23] reported the presence of the above-mentioned metabolites in wines fermented by different wine yeast strains. As our aim was to connect the presence of these compounds in wines with the metabolic activity of yeasts, we analysed the intracellular presence of these compounds in the same strains in different growth phases during SM fermentation (exponential and stationary phases). The concentration of the different compounds throughout fermentation is shown in Table 3. To provide a better understanding, we calculated the percentage of each compound in the different strains and fermentation stages (Figure 2). In this case, L-TRP and TOL represented more than 95% of the total metabolites for all the assayed conditions (Figure 2A). It is noteworthy that during fermentations, 3IAA was a minor compound, but, conversely, was the greatest metabolite synthesised after pulses. To make the visualisation of minor compounds easier, Figure 2B represents the percentage of these metabolites

detected at much lower concentrations (by removing L-TRP and TOL). The first striking result was that we were unable to detect N-acetyl-5-HT and MLT at any of the fermentation points of both the *S. cerevisiae* strains, while they were quantified practically throughout the fermentation in the non-*Saccharomyces* strains with levels varying from 0.12 to 0.93 ng/10^8 cells. Conversely, 5-HT and TRYP were detected in both *Saccharomyces* strains, but in none of the non-*Saccharomyces* strains used, and the accumulation of 5-HTRP was also greater in *Saccharomyces* compared to the non-*Saccharomyces* strains. The well-known differential capacity in taking up carbon and nitrogen sources from grape must between *Saccharomyces* and non-*Saccharomyces* can determine the differences observed in the intracellular synthesis of indolic compounds [24]. Recently, González et al. [25] have reported that the Erhlich pathway is alike active in non-*Saccharomyces* yeasts that produce aromatic alcohols during alcoholic fermentation, although their regulation appears to be somewhat different than that of *S. cerevisiae*. Fernández-Cruz et al. [23] detected the maximum MLT production by the *Saccharomyces* strains after 2 days of fermentation, whereas non-*Saccharomyces* took longer to reach the maximum MLT concentration (day 6).

Finally, L-TRP EE was intracellularly detected at very low concentrations in the *S. cerevisiae* and *H. uvarum* strains. Once again, the large differences between the external and intracellular concentrations could be explained either again by a non-biological (chemical) synthesis, favoured under wine conditions, where its precursors come in large quantities: ethanol and L-TRP, or because this compound is toxic for the cell and it is rapidly expelled out. Esterification and quick secretion of the formed esters have been proposed as a cell fatty acid detoxification in *S. cerevisiae* [26].

As an overview of the results, a multivariate statistical analysis was applied to the whole data matrix by considering the detected compounds as variables (Figure 3). The first principal component (PC1) accounted for 66.06% of total variance, while PC2 explained 13.84%. PC1 was characterised by MLT and N-acetyl-5-HT with the greatest positive loadings, while L-TRP, TRYP, 5-HTRP and TOL showed the highest negative ones. These compounds with positive and negative loadings in the PCA correlated with the non-*Saccharomyces* and the *Saccharomyces* strains, respectively. Hu and Td were characterised by MLT and N-acetyl-5-HT production. For PC2, 5-HT and 3-IAA showed positive loadings, while L-TRP EE exhibited negative ones. Although samples were clearly separated into two big groups corresponding to *Saccharomyces* and non-*Saccharomyces*, Td was situated more in the upper right quadrant, which correlated with the positive loadings for PC2, whereas Hu was situated mainly in the lower right quadrant, which corresponded with negative loadings for PC2. This distribution can be explained by Td producing more 3-IAA than Hu and, conversely, the opposite was observed for L-TRP EE. In any case, this multivariate statistical analysis reinforced the idea that *Saccharomyces* and non- *Saccharomyces* presented different metabolic activity for the indolic compounds under the studied growing conditions.

4. Conclusions

As far as we know, this is the first time that some of these metabolites have been intracellularly detected in different yeast strains. Our aim in this work was to prove the undoubted ability of different wine yeasts to synthesise different indolic compounds. The intracellular detection of all the intermediates of the MLT pathway described in vertebrates

and plants strongly supports the yeast origin of most of these indolic compounds in fermented beverages and foods, which reveals the potential of yeasts in the synthesis of bioactive compounds with added value in fermented products. This study clearly evidences that the synthesis of most of these indolic compounds strongly depends on the yeast strain/species and on the cell's metabolic state. However, as we are far from understanding how the metabolic route works in yeasts, we are now pulsing all these intermediates to different batches and continuous yeast cultures and analysing the products formed to unveil the sequential order of the substrates and products of this interesting metabolic pathway.

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Table S1. Mass characteristics for identification purposes.

Compound	R_T min	Exact mass $[M+H]^+$	Calculated Mass *(m/z)*	Molecular Formula	Δmass ppm	MS/MS fragments	Relative intensities
5-HT	1.26	177.1022	177.10269	$C_{10}H_{12}N_2O$	2.55	149.01244($C_{10}HN_2$)	100
5-HTRP	1.53	221.0921	221.09239	$C_{11}H_{12}N_2O_3$	1.46	175.08694($C_{10}H_{11}ON_2$)	100
L-TRP	4.08	205.0971	205.09715	$C_{11}H_{12}N_2O_2$	0.004	188.07057($C_{11}H_{10}O_2N$) 146.06015(C_9H_8ON)	100 29
TRYP	4.14	161.1073	161.10697	$C_{10}H_{12}N_2$	2.20	144.08079($C_{10}H_{10}N$)	100
N-acetyl-5-HT	5.23	219.1122	219.11201	$C_{12}H_{14}N_2O_2$	3.60	177.00673(C11HON2)	6.86
L-TRP EE	6.63	233.1284	233.12852	$C_{13}H_{16}N_2O_2$	0.30	216.10199($C_{13}H_{14}O_2N$) 174.09129($C_{11}H_{12}ON$)	100 24
TOL	7.09	162.0913	162.09164	$C_{10}H_{11}NO$	1.87	144.08104($C_{10}H_{10}N$)	100
3IAA	7.10	176.0706	176.07104	$C_{10}H_9NO_2$	2.50	130.06523(C_9H_8N)	100
MLT	7.17	233.1284	233.12852	$C_{13}H_{16}N_2O_2$	0.30	174.09125($C_{11}H_{12}ON$)	100

5-HT: serotonin; 5-HTRP: 5-hydroxytryptophan; L-TRP: L-tryptophan; TRYP: tryptamine, N-acetyl-5-HT: N-acetyl-5-hydroxytryptamine; L-TRP EE: L-tryptophan ethyl ester; TOL: tryptophol; 3IAA: 3-indoleacetic acid; MLT: melatonin

Table S2. The P-values of the comparison of the means for each indolic compound and strain before and after adding 1 mM L-tryptophan

	5-HTRP	3-IAA	L-TRP	TOL	TRYPT	SERO	MEL	NACSERO	L-TRP EE
QA23	0.00010357	0.00092524	1.1358E-05	0.00030773	3.7844E-05	1.0791E-05	n.i	n.i	n.i
P24	3.2167E-16	0.00309727	7.1358E-08	2.4896E-10	3.8906E-08	0.01295624	n.i	n.i	n.i
Mpp	5.6746E-14	2.6776E-06	5.8109E-08	7.1522E-06	6.7444E-06	1.0667E-18	n.i	n.i	n.i
Hu4	0.072464	0.01617441	0.0139287	0.00205023	n.i	n.i	n.i	n.i	n.i
Gz4	5.772E-08	0.00465218	0.00014551	0.00249176	0.09672938	0.01778494	n.i	n.i	n.i
Tdp	0.00059832	2.463E-06	1.5788E-12	1.2007E-07	n.i	2.2925E-07	n.i	n.i	n.i

n.i= not increased

Table 1. Concentrations of intracellular L-tryptophan (L-TRP), 3 indoleacetic acid (3IAA) and tryptophol (TOL) in the different yeast strains before and after (+) adding 1mM L-TRP

Strain		Intracellular indolic compounds [ng/10^9 cells]		
		L-TRP	3-IAA	TOL
QA23		802.88 ± 130.19	6618.00 ± 2079.58	22.52 ± 5.00
QA23	+	12747.35 ± 3942.87	40273.17 ± 19552.73	184.74 ± 80.80
P24		596.96 ± 54.39	16840.5 ± 19918.13	41.20 ± 34.85
P24	+	16038.78 ± 2921.30	48127.5 ± 9790.15	444.50 ± 24.42
Tdp		867.30 ± 1141.83	54303 ± 70915.99	37.50 ± 34.91
Tdp	+	59136.41 ± 3505.64	732656.5 ± 174376.77	1152.87 ± 220.23
Hu4		48.95 ± 45.46	21539.33 ± 34401.71	21.13 ± 17.33
Hu4	+	1461.73 ± 1345.40	128911.17 ± 101127.34	142.70 ± 78.57
Mpp		51.68 ± 10.83	1941.67 ± 1020.14	40.27 ± 6.61
Mpp	+	8990.70 ± 1655.16	88437.83 ± 24168.56	2360.90 ± 726.52
Cz4		383.21 ± 55.29	19006.75 ± 8540.48	22.60 ± 8.13
Cz4	+	8397.83 ± 2576.33	775384.00 ± 435367.43	218.16 ± 99.73

Results are expressed as mean ± SD of biological replicates (n=3)

Table 2. Concentrations of the intracellular indole minority compounds in the different yeast strains before and after (+) adding 1 mM L-tryptophan

Strain	Intracellular indolic compounds [pg/10^9 cells]					
	5-HTRP	5-HT	N-acetyl-5-HT	MLT	TRYP	L-TRP EE
QA23	139.00 ± 0.00	853.00 ± 23.82	n.d.	n.d.	n.d.	n.d.
QA23 +	948.50 ± 80.27	1308.00 ± 147.47	n.d.	n.d.	1304.17 ± 497.07	n.d.
P24	n.d.	681.00 ± 3.11	n.d.	170.33 ± 4.24	n.d.	n.d.
P24 +	934.83 ± 25.22	1037.17 ± 13.24	n.d.	60.83 ± 13.44	1167.67 ± 207.29	n.d.
Tdp	341.50 ± 6.36	894.00 ± 115.89	n.d.	n.d.	n.d.	n.d.
Tdp +	1711.67 ± 532.67	1700.33 ± 128.04	n.d.	n.d.	n.d.	n.d.
Hu4	n.d.	819.33 ± 4.50	n.d.	n.d.	n.d.	n.d.
Hu4 +	278.33 ± 1.41	692.67 ± 15.51	n.d.	n.d.	n.d.	n.d.
Mpp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mpp +	880.67 ± 39.90	993.83 ± 15.14	n.d.	n.d.	608.50 ± 189.23	n.d.
Cz4	n.d.	412.00 ± 7.55	n.d.	n.d.	n.d.	n.d.
Cz4 +	990.50 ± 110.84	1048.67 ± 99.51	n.d.	n.d.	147.83 ± 23.33	n.d.

Results are expressed as mean±SD of biological replicates (n=3) n.d= not detected; L-TRP = L-tryptophan; 5-HTRP = 5-hydroxytryptophan; 5-HT = serotonin, N-acetyl-5-HT = N-acetyl serotonin; MLT = melatonin; 3IAA = 3-indoleacetic acid; TRYP = tryptamine; TOL = tryptophol; L-TRP EE = L-tryptophan ethyl

Table 3. Concentration of the intracellular indolic compounds in ng/10^8 cells from the samples taken in the different alcoholic fermentation stages in two *Saccharomyces cerevisiae* strains (QA23 and P24) and in two non-*Saccharomyces* strains (Hu4 and Tdp).

Strain	Compound								
	L-TRP	5-HTRP	5-HT	N-acetyl-5-HT	MLT	3IAA	TRYP	TOL	L-TRP EE
QA23 ie	24584.64 ± 8348.46	1.95 ± 0.46	1.50 ± 0.81	n.d.	n.q.	2.75 ± 0.96	1.77 ± 0.48	5756.87 ± 1666.29	0.75 ± 0.28
QA23 me	5174.69 ± 1830.14	1.08 ± 0.06	0.07 ± 0.03	n.d.	n.q.	1.65 ± 0.16	0.73 ± 0.08	3780.78 ± 66.53	0.11 ± 0.06
QA23 fe	3841.20 ± 266.19	1.06 ± 0.01	0.06 ± 0.04	n.d.	n.q.	0.94 ± 0.19	0.93 ± 0.06	4295.66 ± 240.02	0.46 ± 0.01
QA23 ff	5936.66 ± 1309.64	1.16 ± 0.06	0.03 ± 0.01	n.d.	n.q.	3.31 ± 0.27	0.64 ± 0.10	8204.25 ± 982.44	0.66 ± 0.16
P24 ie	49027.84 ± 8874.80	5.46 ± 0.79	7.03 ± 1.49	n.d.	n.q.	6.98 ± 0.88	3.36 ± 0.31	8303.80 ± 527.15	0.88 ± 0.06
P24 me	1501.19 ± 198.34	1.10 ± 0.04	0.12 ± 0.08	n.d.	n.q.	3.80 ± 0.79	0.77 ± 0.08	7615.75 ± 1490.55	0.04 ± 0.03
P24 fe	3379.33 ± 459.49	1.06 ± 0.02	0.31 ± 0.23	n.d.	n.q.	2.57 ± 0.37	1.15 ± 0.27	4253.87 ± 561.80	0.57 ± 0.12
P24 ff	2425.52 ± 474.66	1.03 ± 0.01	0.03 ± 0.03	n.d.	n.q.	2.33 ± 1.29	0.81 ± 0.16	2605.30 ± 656.04	0.27 ± 0.07
Hu4 ie	3841.78 ± 3069.12	n.q.	n.d.	0.30 ± 0.20	0.93 ± 1.11	4.34 ± 2.73	n.d.	133.02 ± 48.38	1.70 ± 1.01
Hu4 me	703.13 ± 2.45	n.q.	n.d.	0.14 ± 0.01	0.34 ± 0.17	0.05 ± 0.01	n.d.	13.56 ± 0.07	n.d.
Hu4 fe	89.26 ± 0.63	n.q.	n.d.	0.13 ± 0.00	0.56 ± 0.26	0.95 ± 0.37	n.d.	36.74 ± 0.08	0.46 ± 0.01
Hu4 ff	15.31 ± 7.37	n.q.	n.d.	0.13 ± 0.01	0.28 ± 0.10	0.15 ± 0.04	n.d.	20.16 ± 0.19	1.04 ± 0.67
Tdp ie	2478.68 ± 1595.96	0.03 ± 0.01	n.d.	0.14 ± 0.00	0.17 ± 0.07	4.28 ± 1.09	n.d.	98.55 ± 16.38	n.d.
Tdp me	2529.59 ± 216.84	0.02 ± 0.00	n.d.	0.14 ± 0.01	0.66 ± 0.43	3.09 ± 1.76	n.d.	295.44 ± 51.35	n.d.
Tdp fe	1551.73 ± 166.32	0.02 ± 0.00	n.d.	0.13 ± 0.01	0.32 ± 0.21	7.62 ± 0.86	n.d.	654.20 ± 115.09	n.d.
Tdp ff	665.05 ± 230.83	0.04 ± 0.00	n.d.	0.13 ± 0.01	0.12 ± 0.03	22.63 ± 5.44	n.d.	842.79 ± 143.23	n.d.

n.d. = not detected; n.q. = not quantifiable; L-TRP = L-tryptophan; 5-HTRP = 5-hydroxytryptophan; 5-HT = serotonin, N-acetyl-5-HT = N-acetyl serotonin; MLT = melatonin; 3IAA = 3-indoleacetic acid; TRYP = tryptamine; TOL = tryptophol; L-TRP EE = L-tryptophan ethyl ester; ie = initial exponential; me = middle exponential; fe = final exponential, ff =finalized fermentation. Biological replicates are indicated as mean±SD of biological replicates (n=3) .

Figure 1. Effect of tryptophan addition for 30 min on the intracellular production of the different indolic compounds in *Saccharomyces cerevisiae* (QA23 and P24) and non-*Saccharomyces* (Mpp, Hu4, Cz4, Tdp) starved cells.

Figure 2. Progression of the detected indole compounds across the different alcoholic fermentation stages for the studied yeast strains (A). The major compounds tryptophan (L-TRP) and tryptophol (TOL) were masked for better visualisation (B). ie = initial exponential; me = middle exponential; fexp = final exponential, fp =final point

Figure 3. The statistical principal components analysis (PCA) of the effect of the different growth stages on the production of indolic compounds in *Saccharomyces* and non-*Saccharomyces* strains. Component 1 reflects 62.06% total variance (it negatively correlates with L-TRP, TRYP, 5-HTRP and TOL, and positively with N-acetyl-5-HT and MLT). Component 2 reflects 13.84% total variance (and positively correlates with 3-IAA and negatively with L-TRP-EE) and is arranged on two dimensions according to Components 1 and 2. The samples of Saccharomyces are grouped in the yellow ellipse, while the non-Saccharomyces are grouped in the blue ellipse, which reflect differences in indolic profiles at the species level.

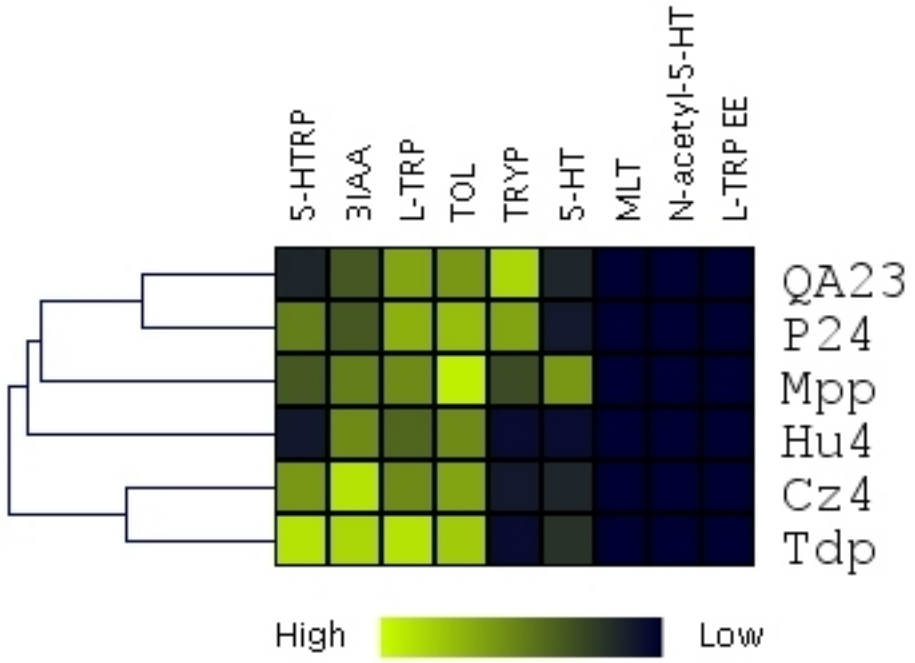


Fig 1: Effect of tryptophan addition during 30 min on intracellular production of the different indolic compounds in *Saccharomyces cerevisiae* (QA23 and P24) and non-*Saccharomyces* (Mpp, Hu4, Cz4, Tdp) starved cells.

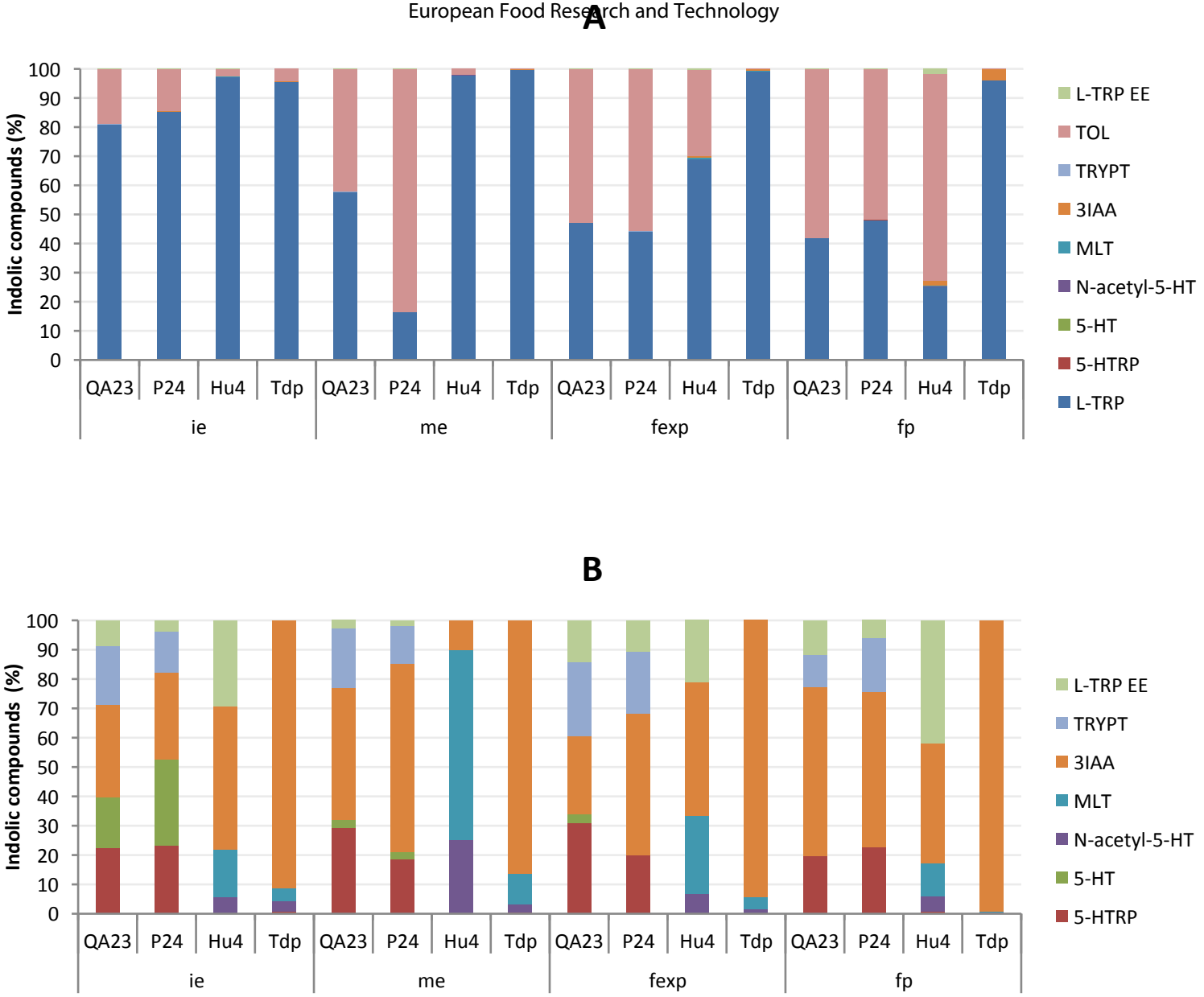
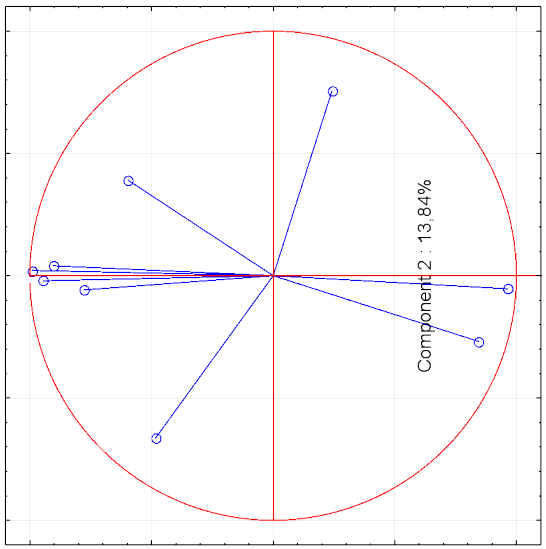
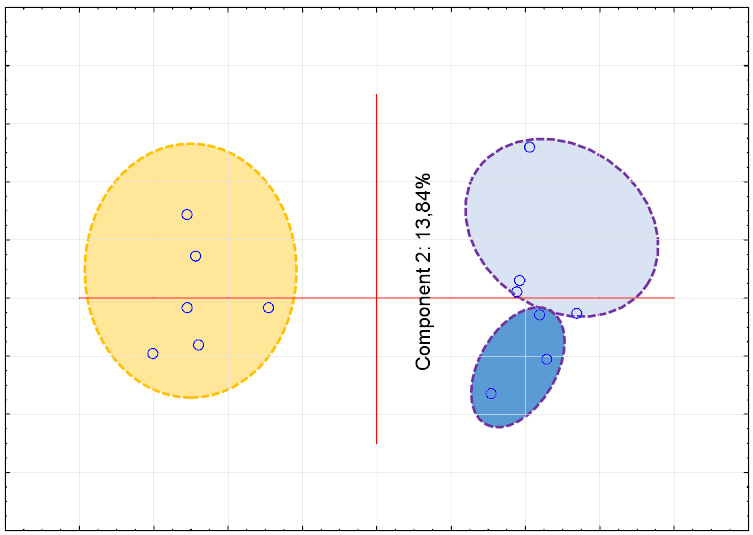


Fig 2: Progression of the detected indole compounds across the different stages of alcoholic fermentation for studied yeast strains (A). Major compounds tryptophan (L-TRP) and tryptophol (TOL) were masked for better visualization (B). ie = initial exponential; me = middle exponential; fexp = final exponential, fp =final point





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