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Melatonin-protein interactions are dependent on yeast carbon metabolism

Maria Angeles Morcillo-Parra[1,2], Sandra Martín-Esteban[1], Joan Almellones[1], Albert Mas[1], Gemma Beltran[1]✉ & Maria Jesus Torija[1]

Melatonin is an indole amine that is synthesized and interacts with glycolytic proteins during alcoholic fermentation in *Saccharomyces* and non-*Saccharomyces* yeasts depending on their yeast fermentative capacity. Due to its importance as a bioactive compound and antioxidant molecule, the aim of this study was to analyse the intracellular melatonin profile and melatonin-protein interactions in a large number of wine yeast species during alcoholic fermentation and respiration to determine whether these interactions were related to a specific carbon metabolism. Melatonin concentrations were analysed by liquid chromatography–mass spectrometry, and proteins bound to melatonin were immunopurified at different time points during yeast growth. Intracellular melatonin followed a similar pattern in all yeast species, with a peak in production during the lag phase and low or no melatonin detected in the exponential or stationary phase. However, melatonin was bound only to proteins in good fermentative yeasts, specifically during alcoholic fermentation but not during cellular respiration. The absence of this binding in some non-*Saccharomyces* yeasts could be related to their poor fermentative capacity. This study establishes for the first time a relationship between carbon metabolism and the interaction of melatonin with proteins in yeast cells, indicating that melatonin might play a regulatory role in glucose metabolism, as observed in human cells.

Keywords Melatonin, Glycolysis, Protein binding, Bioactive compounds, Wine

The glycolytic pathway is a central metabolic process in cells. The enzymes involved in this pathway have been evolutionarily conserved, although the mechanisms controlling carbon and energy metabolism—such as glycolysis, oxidative phosphorylation or alcoholic fermentation—have adapted to meet the specific needs of each species or cell type. In yeast, although sugar can be fully respired to carbon dioxide, maximizing energy transformation for ATP production through oxidative phosphorylation, it can also be rapidly converted to ethanol and carbon dioxide via alcoholic fermentation. Sugars such as glucose or sucrose have been described as fermentable carbon sources, but it depends on their concentration, since, for example, low amounts of glucose are respired in aerobic conditions by *Saccharomyces cerevisiae*[1]. On the other hand, carbon sources such as glycerol, ethanol or galactose are considered nonfermentable and can only be respired[2]. Hence, yeast can switch from fermentation to respiratory metabolism in response to the carbon source and its concentration. Some yeast species, such as *S. cerevisiae*, can grow under aerobic or anaerobic conditions. Indeed, in the presence of high sugar concentrations (such as grape must) and despite the presence of oxygen, *S. cerevisiae* mostly ferments these sugars and produces ethanol. This behaviour is referred to as the Crabtree effect[3,4].

In oenology, *S. cerevisiae* has the most efficient fermentative catabolism and is, traditionally, the preferred species for inoculation as a starter in alcoholic fermentation[5] due to its ability to prevail during the winemaking process, although the environment contains harsh conditions. In addition to *S. cerevisiae*, other yeast species coexist in the same habitat during some stages of the winemaking process. This group of yeasts is commonly known as non-*Saccharomyces* yeasts[6], and they are naturally present on grape berry surfaces[7]. Non-*Saccharomyces* yeasts not only enhance wine complexity and flavour[7,8] but also contribute to wine stability, preventing the growth of spoilage microorganisms[9–12]. Moreover, one of the most recent and interesting characteristics identified is its potential to lower the ethanol content in wine[13–16].

During alcoholic fermentation, yeasts synthesize a range of secondary metabolites, such as esters, volatile fatty acids, and higher alcohols, which contribute significantly to the flavour and aroma of wine[17]. Additionally, other

[1]Biotecnologia Enológica, Dept. Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Spain. [2]Present address: Laboratoire de Biologie Cellulaire et Moléculaire des Eucaryotes, UMR 8226, CNRS, Sorbonne Université, Institut de Biologie Physico-Chimique (IBPC), 75005 Paris, France. ✉email: gemma.beltran@urv.cat

minor metabolites derived from aromatic amino acids, such as melatonin, serotonin or hydroxytyrosol, are also produced. These compounds are of special relevance because of their bioactivity in higher organisms, in which their biosynthesis pathways are well known[18]. Despite the fact that the melatonin biosynthesis pathway and its role in yeast are still unknown, many studies have shown evidence for the synthesis of this indole during alcoholic fermentation, either by *S. cerevisiae* or non-*Saccharomyces* yeasts[19–22]. All these studies have focused only on extracellular melatonin. However, recent studies have detected intracellular melatonin after pulses with different melatonin pathway intermediaries, namely, tryptophan, N-acetyl-serotonin and 5-methoxytryptamine[23,24]. In fact, in previous studies, we detected intracellular melatonin under fermentative conditions using synthetic grape must, produced not only by *Saccharomyces* but also by non-*Saccharomyces* yeasts[25–27].

In *Saccharomyces cerevisiae,* intracellular melatonin production occurs quickly during the lag phase but decreases rapidly, with extracellular melatonin only detectable in the late exponential and stationary phases[25]. This discrepancy between intracellular production and extracellular detection suggested a possible interaction with intracellular molecules, such as proteins, which may account for the delay in melatonin secretion. Indeed, we recently described that glycolytic enzymes interact with melatonin in yeast strains that exhibit greater fermentative capacity, such as *S. cerevisiae*, *Torulaspora delbrueckii* and *Starmerella bacillaris*[25,26]. In contrast, yeasts with lower fermentative ability, such as *Metschnikowia pulcherrima* or *Hanseniaspora uvarum,* did not exhibit any interactions[26]. The interactions of melatonin with proteins have been widely studied in human cells, mainly with melatonin receptors (MTNR1A and MTNR1B)[28] although these interactions have also been related to other proteins, such as orphan receptors[29–31] or calreticulin[32].

However, the reason for melatonin binding to glycolytic proteins in yeast, particularly under fermentative metabolism, remains unclear. To elucidate this, the aim of this study was to analyse the intracellular melatonin profiles of a large number of wine yeast species during alcoholic fermentation and to determine whether melatonin-protein interactions are specific to certain yeast species and fermentation conditions. To this end, we first measured the intracellular melatonin concentration in several yeast species with different fermentative capacity, and subsequently analysed the melatonin-protein interactions in fermentative and nonfermentative carbon sources to establish whether there was a relationship between carbon metabolism and melatonin-protein interactions.

Materials and methods

Yeast strains and inoculum preparation

Several wild and commercial wine yeast strains belonging to the *S. cerevisiae* (3 strains) and non-*Saccharomyces* (16 strains) species were used in this study (listed in Table S1). These strains were chosen to represent a diverse array of yeast species, primarily originating from wine environments, and exhibiting varied alcoholic fermentation capabilities (Table S1). The starter cultures were prepared by growing each yeast strain in liquid YPD medium (1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) bacteriological peptone (Panreac, Barcelona, Spain)) overnight at 28 °C and 120 rpm in an orbital shaker.

Growth conditions

For alcoholic fermentation conditions, yeast starters were transferred into fresh minimal media (1X Yeast Nitrogen Base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) glucose, and 3.5 mM $(NH_4)_2\ SO_4$ (Panreac Quimica SLU, Barcelona, Spain)) and cultured for 3 days at 28 °C and 120 rpm. Fermentations were carried out as described by Morcillo-Parra et al[25]. Briefly, standard synthetic grape must (200 g/L sugars, 300 mg/L YAN, pH 3.3) with increased concentrations (5x) of aromatic amino acids (tryptophan, tyrosine, and phenylalanine), at the expense of the remaining amino acids, to maintain the assimilable nitrogen at 300 mg/L was used[33]. Fermentations were inoculated at 2×10^6 cell/mL and cultured at 28 °C with continuous orbital shaking (120 rpm).

For respiration conditions, yeast starters were transferred into YPG medium (1% (w/v) yeast extract, 3% (v/v) glycerol and 2% (w/v) bacteriological peptone (Panreac)). Respirations were carried out in 250 ml of YPG medium with a 2×10^6 cell/mL population and cultured at 28 °C with continuous orbital shaking (120 rpm).

For the carbon metabolism shift experiments, the yeast starter (2×10^6 cell/mL) was first transferred into 250 mL of YPE medium (1% (w/v) yeast extract, 1% (v/v) ethanol and 2% (w/v) bacteriological peptone (Panreac)) and cultured at 28 °C and 120 rpm for 48 h. Then, 5% (w/v) glucose was added to force the yeast to switch its carbon metabolism from respiration to fermentation. Cell populations were evaluated by measuring optical density (OD600 nm), and for intracellular melatonin and protein analyses, 10 OD units were collected at different time points during yeast growth. Then, the samples were centrifuged at 12,000 rpm for 3 min at room temperature. The pellet was washed with distilled water, frozen in liquid nitrogen and stored at -80 °C until use.

Melatonin analysis

Intracellular metabolites were extracted by adapting the buffered ethanol boiling method[34], as previously described by Morcillo-Parra et al.[25]. Then, intracellular melatonin was extracted with chloroform. Briefly, samples (50 µL) were mixed first with Milli-Q water (1:1, v:v) and then with 10 volumes of chloroform. The samples were shaken for 1 h at 1200 rpm, after which the organic phase was recovered and dried under a flow of nitrogen gas. Then, the samples were resuspended in a mixture of methanol and water (40:60, v:v) and centrifuged for 5 min at 14,500 rpm. The supernatants were subsequently transferred and analysed. Time points were selected to represent the lag, exponential and stationary yeast growth phases, as in previous studies[25,26].

The samples were analysed via liquid chromatography–mass spectrometry (LC–MS/MS; Agilent G6410; Agilent Technologies, Palo Alto, USA) using an Agilent 150 × 2.1 mm i.d., 3.5 µM, Zorbax Sb-Aq column. Chromatographic separation was performed using (A) water and (B) methanol as LC-grade solvents, both containing 0.1% (v/v) formic acid. The elution profile was 100% B (4 min) and 10% B (6 min). The analysis

temperature was set at 40 °C, and the flow rate was 0.4 ml/min. The injection volume was 7 µL. Melatonin was quantified using Agilent MassHunter WorkStation Software Quantitative Analysis Version B0104 and comparing the 233/174 transition MS data of the sample and the standard. The calibration curve was prepared with melatonin concentrations ranging from 0.001 to 100 ng/mL. Regarding the sensitivity of the method, the limit of detection was 0.17 ng/mL and the limit of quantification was 0.53 ng/mL as previously described in Morcillo-Parra et al.[35]. The samples were tested in triplicate, and the standard deviation was calculated.

Protein purification

Samples from different time points corresponding to yeast growth phases in fermentation and respiration conditions were purified by a Pierce™ Crosslinking Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA, USA) using anti-melatonin rabbit antibody IgG (LifeSpan BioSciences, Seattle, WA, USA)[25]. Cells (10 OD units) were resuspended in 1 mL of extraction buffer and lysed by glass beads by applying five shaking cycles for 30 s in a Mini Beadbeater-24 (BioSpec Products, Bartlesville, OK, USA), followed by 1 min on ice. Lysed cells were centrifuged at 14,000 rpm and 4 °C for 10 min to remove insoluble particles. Melatonin IgG-Dynabeads were added to each lysate, following the manufacturer's instructions. The same amount of total protein from each sample was loaded and resolved on a 12% SDS-PAGE gel. Immunopurified proteins were visualized on a stained gel with a Pierce™ Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Results and discussion

Intracellular melatonin synthesis during alcoholic fermentation

A selection of wine yeast species that can usually be found during the alcoholic fermentation process[36] were grown in synthetic grape musts to analyse their intracellular melatonin profile during alcoholic fermentation in relation to their fermentative performance. Although all the tested yeast species were able to grow under fermentative conditions, not all the strains exhibited active fermentative metabolism (Table 1, Figure S1). In fact, we observed three clear groups according to their alcoholic fermentation performance during the first 48 h of alcoholic fermentation (Table 1): (i) good fermentative yeasts, (ii) medium-good fermentative yeasts and (iii) low fermentative yeasts. As expected, *S. cerevisiae* wine strains (CLOS and QA23) consumed the most sugars during the first 48 h of alcoholic fermentation. However, some non-*Saccharomyces* strains and the laboratory strain of *S. cerevisiae* BY4742 were also able to consume significant amounts of sugar, mostly between 124 and 75 g/L, in 48 h (group ii of our classification, Table 1). These non-*Saccharomyces* yeasts have been described as good fermentative, and thus, they are able to persist during most of the fermentation process: *T. delbrueckii*, *S. bacillaris*[37,38], *Hanseniaspora vineae*[39], and *Lachancea thermotolerans*[38,40]. Additionally, the *Zygosacharomyces rouxii* strain also exhibited good fermentative performance, as already described for soy sauce fermentation[41]. This species is considered a spoilage yeast for food and beverages and is notably resistant to hyperosmotic stress; therefore, it is capable of spoiling products with a high concentration of sugars, such as fruit juices and concentrates, alcoholic beverages and honey.

On the other hand, other non-*Saccharomyces* species, such as *M. pulcherrima*, *H. uvarum*, *H. guillermondii* and *Candida boidinii,* were able to grow at the beginning of alcoholic fermentation but not to consume a significant amount of sugar (Table 1, Figure S1), which might indicate that these non-*Saccharomyces* yeasts

Yeast species	Strain	Initial density (g/L)	48 h density (g/L)	Consumed sugars (g/L)	Fermentative metabolism[a]
S. cerevisiae	QA23	1078.8 ± 0.0	1013.6 ± 2.1	151.3 ± 2.4	+++
	CLOS	1080.6 ± 0.0	1011.2 ± 6.1	154.1 ± 13.5	+++
	BY4742	1080.7 ± 0.0	1041.8 ± 1.0	86.3 ± 2.2	++
C. boidinii	10029	1081.2 ± 0.0	1068.6 ± 1.6	27.8 ± 3.6	+
	10035	1083.7 ± 0.0	1070.6 ± 3.1	27.9 ± 6.8	+
H. guillermondii	11139	1081.5 ± 0.0	1059.6 ± 0.6	48.1 ± 2.1	+
H. uvarum	37	1082.7 ± 0.0	1059.0 ± 0.2	52.5 ± 0.4	+
H. vineae	1471	1083.7 ± 0.0	1032.8 ± 2.1	112.9 ± 4.6	++
	T02/19AF	1082.7 ± 0.0	1020.6 ± 0.6	137.9 ± 1.4	+++
I. terricola	11139	1083.7 ± 0.0	1063.9 ± 3.0	44.0 ± 6.7	+
L. thermotolerans	Lt2	1080.5 ± 0.0	1042.9 ± 0.3	83.4 ± 0.6	++
	LAKTIA	1081.1 ± 0.0	1030.6 ± 0.4	113.2 ± 1.0	++
M. guillermondii	1020	1081.1 ± 0.0	1058.5 ± 3.3	50.1 ± 7.2	+
M. pulcherrima	FLAVIA	1082.7 ± 0.0	1062.1 ± 0.9	45.7 ± 1.9	+
S. bacillaris	11046	1082.4 ± 0.1	1036.2 ± 1.1	102.6 ± 2.3	++
	11109	1082.2 ± 0.1	1033.1 ± 0.8	109.2 ± 1.8	++
T. delbrueckii	BIODIVA	1079.5 ± 0.0	1036.7 ± 0.8	94.9 ± 1.7	++
Z. rouxii	1232	1081.2 ± 0.0	1042.8 ± 0.8	85.2 ± 1.8	++

Table 1. Alcoholic fermentation performance during the first 48 h. Yeasts were divided in three groups: (i) good fermentative yeasts, +++; (ii) medium-good fermentative yeasts, ++; and (iii) low fermentative yeasts, +. [a]Consumed sugar during the first 48 h: (+++) 200–125 g/L; (++) 124–75 g/L; and (+) 74–0 g/L.

presented a low fermentative capacity (iii group, Table 1). In fact, although *H. guillermondii* exhibited slow sugar consumption during the first 48 h, it was able to reach a similar grape must density after 7 days of alcoholic fermentation as yeasts belonging to the (ii) group (Figure S1), meaning that *H. guillermondii* had a delayed fermentative process in comparison to other yeast species. In contrast, *H. uvarum* exhibited a low fermentative capacity during the 7 days of the study. This species is known to be the most abundant yeast on the surface of grapes and is predominant during the first hours of alcoholic fermentation, although it disappears quickly afterwards due to its low fermentative capacity[37,38,42,43]. In fact, these two species, *H. uvarum* and *H. guillermondii,* were included in the faster-evolving lineage (FEL) of *Hanseniaspora* in a recent study on the evolution of this genus[44]. Instead, *H. vineae,* a species with good fermentative capacity, is included within the slower-evolving lineage (SEL)[44], which seems to indicate a relationship between this different genome evolution and the fermentative capacity of these species. *M. pulcherrima* has been intensely studied as a good candidate to be used in starter cultures to reduce ethanol concentrations in wines due to its respiratory metabolism under suitable aeration condition[45,46]. *M. pulcherrima* has been shown to be able to respire between 40 and 100% of the sugar consumed[13,47]. Finally, *C. boidinii* also presented low sugar consumption, similar to that observed for *H. uvarum* and *M. pulcherrima*. These results contrast with those obtained by other authors[16,48], possibly due to the different media and conditions used.

Regarding melatonin production during alcoholic fermentation, since its synthesis might be related to yeast growth[19,25], intracellular melatonin was measured in three different growth phases (lag, exponential and stationary phases). Intracellular melatonin was not detected in all yeast species analysed (Table 2), probably due to its rapid synthesis[26] and the lack of a continuous detection system for this compound[35]. Nevertheless, in most yeast species in which intracellular melatonin was detected, a similar pattern of synthesis was established, with a peak of production occurring during the lag phase, when yeast faces high osmotic stress due to the high sugar amount in the medium, and with low or no detection of melatonin in the exponential or stationary phases. These results agree with previous observations[19,25,26], reinforcing the idea that intracellular melatonin production is linked to the yeast growth curve and its adaptation to the medium. As an exception, in *S. cerevisiae* CLOS, a pick corresponding to melatonin was detected (but not quantifiable) during the exponential but not during the lag phase. Although most yeast species peaked intracellular melatonin during the lag phase, the concentrations obtained were very different, ranging from 19.19 ng/mL to 0.50 ng/mL, with *H. vineae* being the yeast species that produced the highest amount (19.19 ng/mL*10OD).

Furthermore, melatonin was also detected during the exponential phase in some strains, such as the laboratory strain *S. cerevisiae* BY4742, *M. pulcherrima* FLAVIA, *H. uvarum* 37 and *H. vineae* T02/19AF, with *S. cerevisiae* BY4742 being the maximum producer of melatonin (5.07 ng/mL*10OD) during the exponential phase. Finally, in *M. pulcherrima* FLAVIA, intracellular melatonin was also detected during the stationary phase, which is consistent with our previous results, in which a different *M. pulcherrima* strain produced intracellular melatonin throughout the fermentation process[26].

Melatonin-protein binding profile during alcoholic fermentation

During fermentation, several samples from different growth phases were collected and analysed for melatonin and protein binding. The proteins bound to melatonin IgG were immunopurified from the crude extract using a Pierce™ Crosslinking Magnetic IP/Co-IP Kit. The detection of proteins bound to melatonin under fermentative conditions was observed only for yeast species able to ferment most of the must sugars, belonging to good (+++) and medium-good (++) fermentative yeast group (Table 1), such as *S. cerevisiae, T. delbrueckii, S. bacillaris, H. vineae, H. guillermondii, Z. rouxii* and *L. thermotolerans* (Fig. 1, Figure S2). In these strains, no melatonin-protein interactions were observed during the lag phase, when melatonin accumulated within the intracellular

Yeast species	Strain	Intracellular melatonin (ng/mL * 10 OD)		
		Lag phase	Exponential phase	Stationary phase
S. cerevisiae	QA23	1.38[25]	0.50[25]	n.d.[25]
	CLOS	n.d	<LOQ	n.d
	BY4742	12.36	5.07	n.d
C. boidinii	10029	0.95	n.d	n.d
	10035	n.d	n.d	n.d
H. guillermondii	11129	1.15	n.d	n.d
H. uvarum	37	2.07	2.83	n.d
H. vineae	1471	4.98	n.d	n.d
	T02/19AF	19.19	n.d	n.d
I. terricola	11139	n.d	n.d	n.d
L. thermotolerans	LAKTIA	<LOQ	n.d	n.d
M. pulcherrima	FLAVIA	15.35	3.73	3.62
T. delbrueckii	BIODIVA	n.d	n.d	n.d
Z. rouxii	1232	0.84	n.d	n.d

Table 2. Intracellular melatonin production (ng/mL * 10OD) in fermentation conditions. n.d. not detected. LOQ limit of quantification.

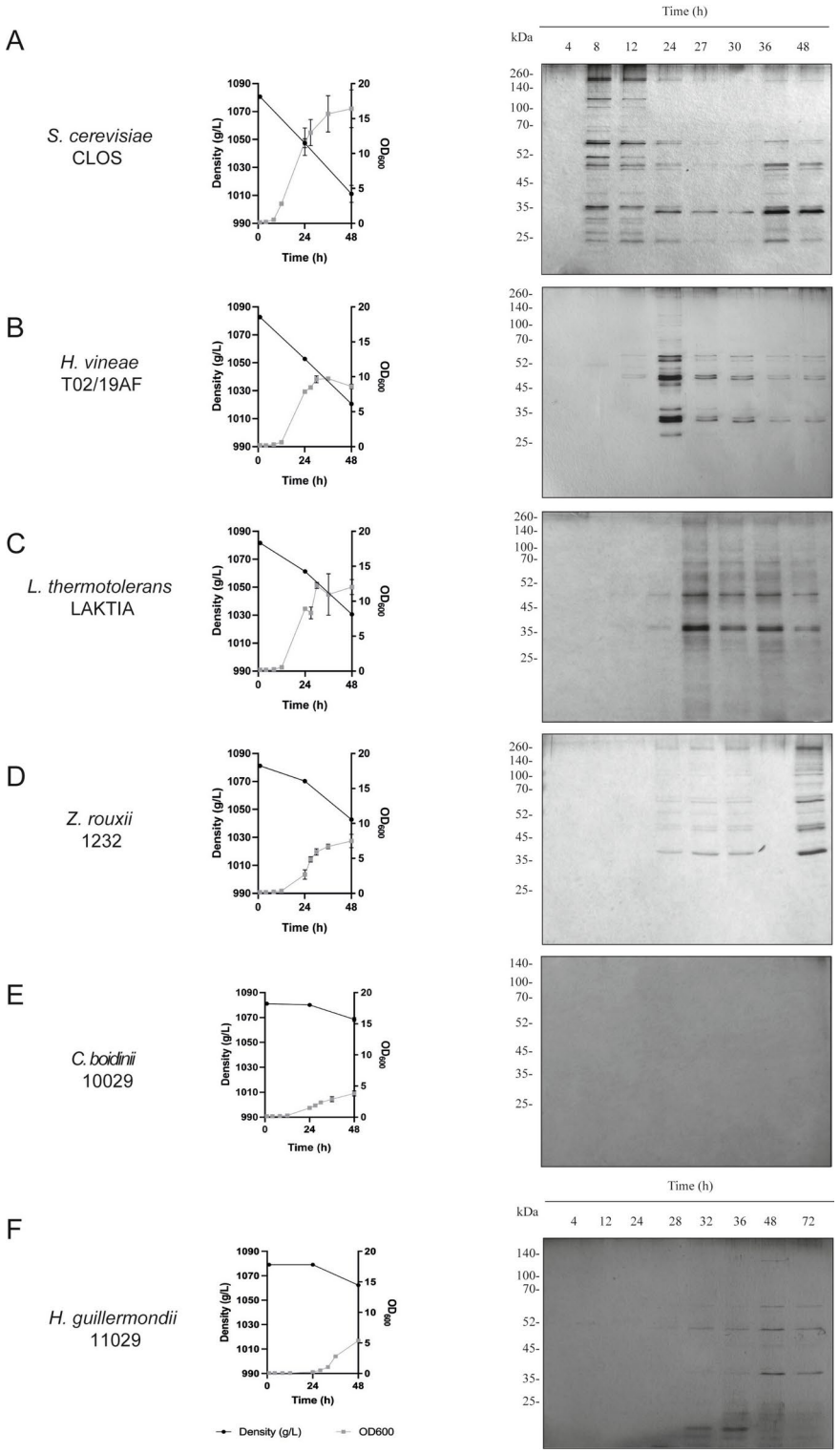

Fig. 1. Fermentation kinetics determined by monitoring must density (▪ black line, g/L) and cell growth (• grey line, measured as OD_{600}) and the time course of proteins bound to melatonin in (**A**) *S. cerevisiae* CLOS, (**B**) *H. vineae* T02/19AF, (**C**) *L. thermotolerans* LAKTIA, (**D**) *Z. rouxii* 1232, (**E**) *C. boidinii* 10,029, and (**F**) *H. guillermondii* 11,209. Full-length images of the gels are provided as Supplementary material.

compartment. Instead, melatonin predominantly bound to proteins during the exponential phase, when free intracellular melatonin was scarcely detected (Table 2). This binding pattern of melatonin mirrored previous observations[25,26], demonstrating an inverse relationship between melatonin binding to proteins and its presence as a free molecule. As different yeast species presented different growth profiles during alcoholic fermentation,

with longer lag and exponential phases, the melatonin-protein pattern was delayed in time in most non-*Saccharomyces* strains in relation to that obtained for *S. cerevisiae* (Fig. 1A). In fact, *H. guillermondii*, which has a delayed fermentative metabolism, exhibited the latest melatonin-protein interactions (starting between 36 and 48 h; Fig. 1F).

In yeast species described as low fermentative yeasts, such as *M. pulcherrima* and *H. uvarum*[13,42], and *C. boidinii*, melatonin was not bound to any protein (Fig. 1E, Figure S2). Even though these non-*Saccharomyces* yeasts produced intracellular melatonin, there was no binding of melatonin to proteins, indicating that this interaction might play a role in the development of alcoholic fermentation. The absence of such binding in these yeasts could be linked to their limited fermentative capacity. Understanding these different behaviours could provide insights into the regulation of carbon metabolism in non-*Saccharomyces* yeasts.

In addition, *I. terricola* and *M. guillermondii* did not show melatonin-protein binding, although, in these species, intracellular melatonin production was not detected in the samples. These two species have also been described as low ethanol producers, with the latter species having the poorest ethanol yield among all the strains analysed in a recent study[16].

Although we did not perform mass spectrometry identification of the proteins that interact with melatonin (Fig. 1, Figure S2), the protein profile and band sizes obtained were similar to those previously described in our studies[25,26], suggesting that these proteins might be glycolytic proteins previously identified to bind to melatonin. In fact, one of the strongest bands we observed in our immunoprecipitations was set at 35 kDa, which was previously identified as glyceraldehyde-3-phosphate dehydrogenase, either isoenzyme 1, 2 or 3[25,26].

Melatonin-protein interaction in relation to the yeast growth curve

To validate whether the interaction and binding of melatonin to proteins were related to the yeast growth curve and, more specifically, to the exponential growth phase, as we previously observed[25] (Fig. 1), we performed alcoholic fermentation at a low temperature (16 °C) to increase the lag phase and thus, delay entry into the exponential phase. For this experiment, we selected two good fermentative strains (*S. cerevisiae* CLOS and *S. cerevisiae* QA23) because they exhibited the earliest melatonin-protein interactions.

As expected, both *S. cerevisiae* strains increased their lag phase after inoculation in synthetic grape must at 16 °C but not to the same extent. *S. cerevisiae* CLOS extended the lag phase to 18 h compared to 8 h at 28 °C, while *S. cerevisiae* QA23 had a lag phase of 24 h at 16 °C compared to 12 h at 28 °C (Fig. 2). The effect of low temperature can also be observed in fermentation performance (Fig. 2). The duration of the fermentation

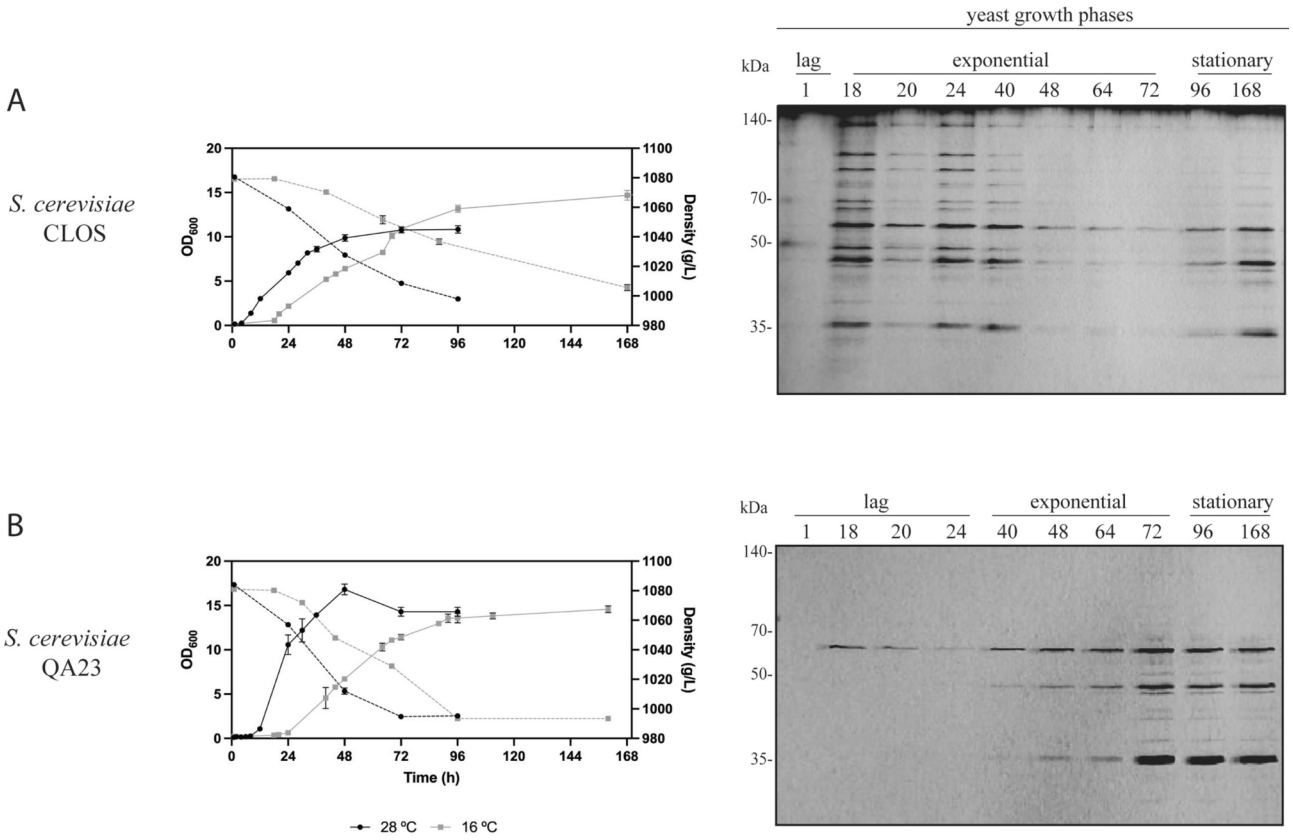

Fig. 2. Alcoholic fermentation at low temperature. Fermentation kinetics determined by monitoring must density (dashed line, g/L) and cell growth (straight line, measured as OD_{600}) at 28 °C (black) and 16 °C (grey) and the time course of proteins bound to melatonin in *S. cerevisiae* CLOS (**A**) and *S. cerevisiae* QA23 (**B**). Full-length images of the gels are provided as Supplementary material.

process was also longer at the low temperature (one to three days longer depending on the yeast strain used). At both temperatures, QA23 fermented better than CLOS, although adaptation to the medium was faster in the latter, with a shorter lag phase. Thus, as expected, a lower temperature affected yeast growth and, therefore, the development of fermentation, as other authors have stated[49–51].

Along with alcoholic fermentation at low temperature, the interaction of proteins with melatonin was also delayed in time in comparison with that at 28 °C (Fig. 1 and S2;[25]). However, we observed some differences between the strains. In CLOS, the strongest band intensity was at 18 h, just at the beginning of the exponential phase; however, in the QA23 strain, although some bands started appearing at the exponential phase, the strongest interaction was observed after 72 h in the late exponential phase (Fig. 2). No melatonin-binding proteins were detected during the lag phase (Figure S3), except for QA23, in which we observed a faint 65 kDa band beginning at 18 h (Fig. 2). In addition, during the stationary phase, a decrease in the intensity of the protein bands was observed in CLOS (Fig. 2).

Overall, our results indicate that the binding of melatonin to proteins is related to the exponential growth phase, which coincides with the absence of melatonin either intracellularly or extracellularly, confirming our previous results[25].

Intracellular melatonin synthesis and protein binding during cellular respiration.

To date, we have studied only melatonin and its interactions under fermentative conditions, and we wanted to determine whether there is a relationship between carbon metabolism and the possible role of melatonin in yeast. To this end, a set of selected wine strains (some that exhibited melatonin-protein binding under fermentative conditions and others that did not) were grown in glycerol, a nonfermentable carbon source, to achieve respiration conditions. All the strains tested were able to grow, although *S. cerevisiae* CLOS was the strain that presented the best growth in this medium (Fig. 3A). Intracellular melatonin synthesis was detected only in *S. cerevisiae* CLOS and *L. thermotolerans* LAKTIA (data not shown), indicating that both yeasts were also able to produce intracellular melatonin under these conditions. The large variability observed in melatonin detection was probably due to its rapid synthesis and disappearance[35]. In contrast, no melatonin-protein interactions were observed for any of the strains tested under respiratory conditions (Fig. 3), similar to what happened in poorly fermenting yeasts during fermentation conditions (Figure S4). In fact, some of these species are described as being better suited to respire rather than ferment sugars, even during winemaking[45,46]. These results reinforced the idea that melatonin could play a regulatory role during alcoholic fermentation.

Melatonin-protein interaction during carbon metabolism shifting.

To confirm that melatonin-protein binding was specific to fermentative metabolism, we performed a metabolism shift experiment. Three wine yeast species were selected: two strains of *S. cerevisiae,* CLOS and QA23, and one strain of *L. thermotolerans,* LAKTIA. The carbon source used was ethanol, another nonfermentable carbon source, which resulted in a better growth of the strains under respiratory conditions (data not shown).

Individually, yeast species were first grown in respiration medium (YPE), and after 48 h, a pulse of 50 g/L glucose was added to change metabolism to fermentation. As expected, during respiration, no melatonin-protein interactions were observed (Fig. 4). However, after the glucose pulse, a shift in metabolism from respiration to fermentation occurred (Figure S5), and some protein bands appeared bound to melatonin, suggesting a role for melatonin in modulating glucose metabolism through alcoholic fermentation. In fact, some studies have shown that melatonin can reprogram glucose metabolism in cancer cells by restoring glucose respiration, acting as an anti-Warburg effect molecule[52]. In addition, it has been reported that melatonin enhances glucose metabolism by reducing glucose levels in the plasma of goldfish[53]. Moreover, rare variants of the human melatonin receptor gene MTNR1B, which impair melatonin binding, have been associated with an increased risk of type 2 diabetes[54].

Even though all the yeast species exhibited good growth after glucose pulse, the *Saccharomyces* strains grew faster, with a more pronounced exponential phase, than did the *L. thermotolerans* strain. Indeed, the binding of melatonin to proteins also seemed to occur faster in *S. cerevisiae* strains, just after the pulse, in the mid-exponential phase, and remained till stationary phase. In LAKTIA, the strongest union occurred later, at the late exponential and stationary phases. The delay in the growth of *L. thermotolerans* after the pulse could explain the delay in melatonin-protein binding in this strain.

Although we did not identify these proteins by mass spectrometry (Fig. 4), the protein profile obtained was similar to that described previously (Fig. 1). We detected a strong band at 65 kDa in both *S. cerevisiae* strains, while in the *L. thermotolerans* strain, the strongest band was observed at 35 kDa, which might correspond to glyceraldehyde-3-phosphate dehydrogenase[26]. Nevertheless, further studies must be carried out to elucidate the biological importance of melatonin in yeast during alcoholic fermentation.

Conclusions

Our results provide evidence that melatonin is bound to proteins during fermentative metabolism but not during cellular respiration. This binding occurs during the exponential phase of yeast growth and occurs only in yeast strains with a high fermentative capacity, such as *S. cerevisiae* or *L. thermotolerans*. Therefore, as melatonin-protein interactions depend on the performance of alcoholic fermentation, these interactions are also strain specific. These interactions indicate the role of melatonin in alcoholic fermentation, mainly in sugar metabolism. Nevertheless, further studies are needed to understand the specific function as signal molecule on the alcoholic fermentation and its possible a role in modulating glucose metabolism in yeast.

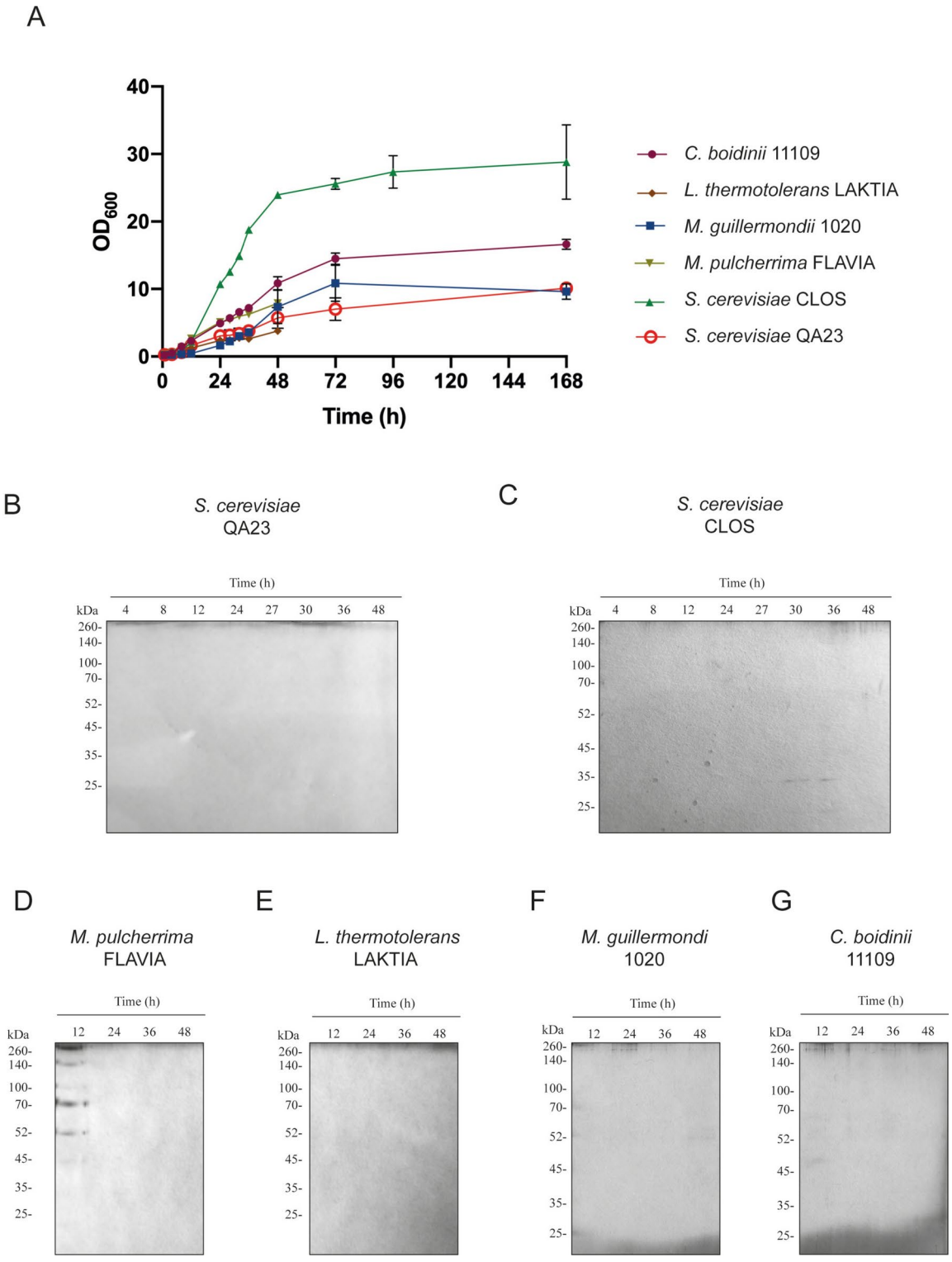

Fig. 3. Yeasts with high and low fermentative power capacity growing under respiratory conditions. (**A**) Growth kinetics determined by monitoring and yeast growth (measured as OD_{600}). Time course of proteins bound to melatonin in (**B**) *S. cerevisiae* QA23, (**C**) *S. cerevisiae* CLOS, (**D**) *M. pulcherrima* FLAVIA, (**E**) *L. thermotolerans* LAKTIA, (**F**) *M. guillermondii* 1020, and (**G**) *C. boidinii* 11,109. Full-length images of the gels are provided as Supplementary material.

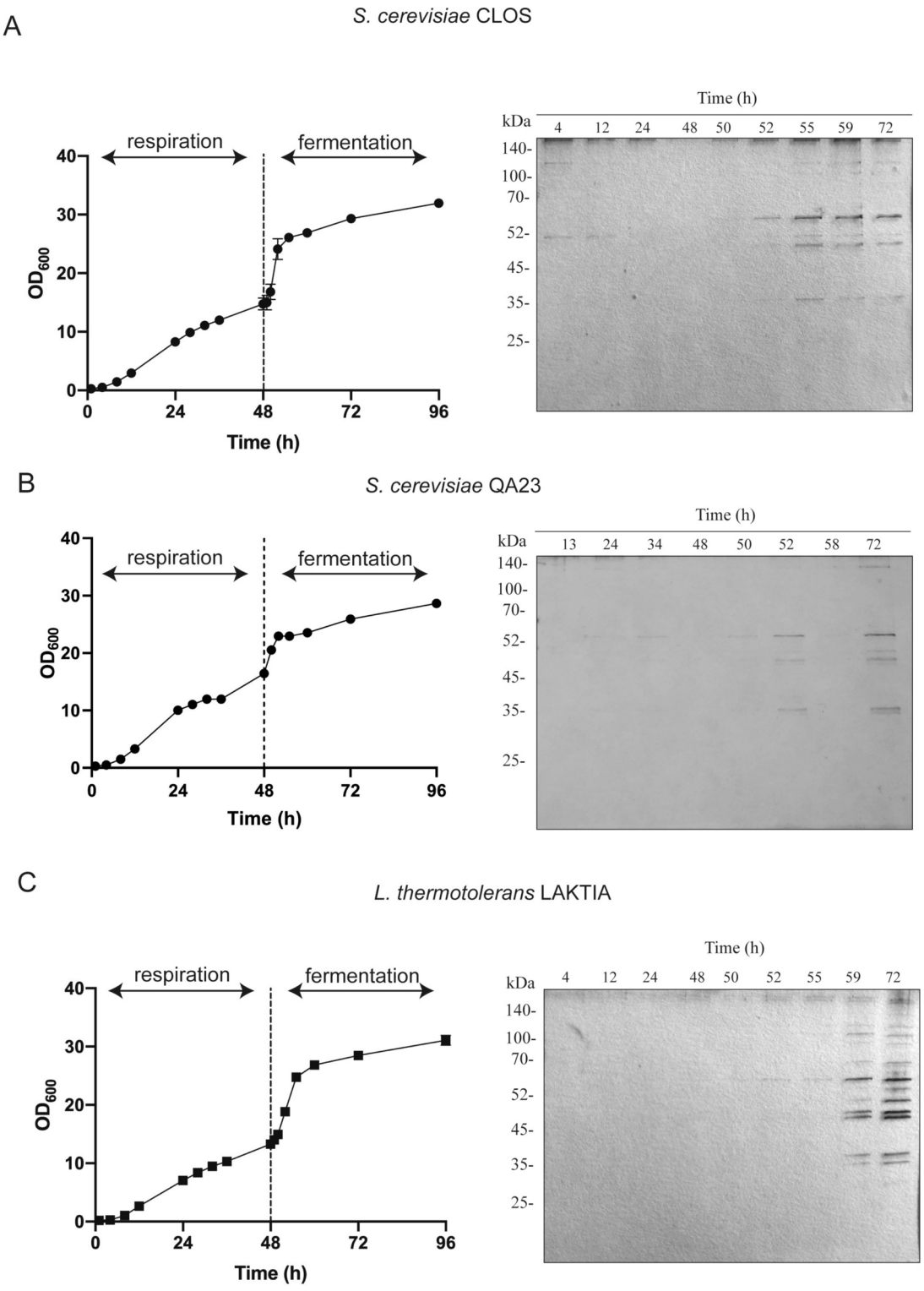

Fig. 4. Shifting metabolism from respiration to fermentation. Yeast cells were grown first in respiratory conditions (YPE), and after 48 h, 5% (w/v) of glucose were added to shift the metabolism to fermentation. Growth kinetics determined by monitoring yeast growth (measured as OD_{600}) and the time course of proteins bound to melatonin in (**A**) *S. cerevisiae* CLOS, (**B**) *S. cerevisiae* QA23, and (**C**) *L. thermotolerans* LAKTIA. Full-length images of the gels are provided as Supplementary material.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Verduyn, C., Zomerdijk, T. P. L., van Dijken, J. P. & Scheffers, W. A. Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. *Appl. Microbiol. Biotechnol.* **19**, 181–185 (1984).
2. Turcotte, B., Liang, X. B., Robert, F. & Soontorngun, N. Transcriptional regulation of nonfermentable carbon utilization in budding yeast. *FEMS Yeast Res.* **10**, 2–13 (2010).
3. De Deken, R. H. The Crabtree effect: A regulatory system in yeast. *J. Gen. Microbiol.* **44**, 149–156 (1966).
4. Dashko, S., Zhou, N., Compagno, C. & Piškur, J. Why, when, and how did yeast evolve alcoholic fermentation?. *FEMS Yeast Res.* **14**, 826–832 (2014).
5. Pretorius, I. S. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast* **16**, 675–729 (2000).
6. Fleet, G. H. Wine yeasts for the future. *FEMS Yeast Res.* **8**, 979–995 (2008).
7. Jolly, N. P., Varela, C. & Pretorius, I. S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **14**, 215–237 (2014).
8. Padilla, B. et al. Yeast biodiversity from DOQ priorat uninoculated fermentations. *Front. Microbiol.* **7**, 930 (2016).
9. Comitini, F., Pietro, N. D., Zacchi, L., Mannazzu, I. & Ciani, M. *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: Purification and characterization. *Microbiology* **150**, 2535–2541 (2004).
10. Mehlomakulu, N. N., Setati, M. E. & Divol, B. Non-*Saccharomyces* killer toxins: Possible biocontrol agents against *Brettanomyces* in wine?. *SAJEV* **36**, 94–104 (2015).
11. Mehlomakulu, N. N., Prior, K. J., Setati, M. E. & Divol, B. *Candida pyralidae* killer toxin disrupts the cell wall of *Brettanomyces bruxellensis* in red grape juice. *J. Appl. Microbiol.* **122**, 747–758 (2017).
12. Villalba, M. L., Susana Sáez, J., del Monaco, S., Lopes, C. A. & Sangorrín, M. P. TdKT, a new killer toxin produced by *Torulaspora delbrueckii* effective against wine spoilage yeasts. *Int. J. Food Microbiol.* **217**, 94–100 (2016).
13. Quirós, M., Rojas, V., Gonzalez, R. & Morales, P. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* **181**, 85–91 (2014).
14. Contreras, A. et al. The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **205**, 7–15 (2015).
15. Varela, C., Barker, A., Tran, T., Borneman, A. & Curtin, C. Sensory profile and volatile aroma composition of reduced alcohol Merlot wines fermented with *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Int. J. Food Microbiol.* **252**, 1–9 (2017).
16. Zhu, X., Navarro, Y., Mas, A., Torija, M.-J. & Beltran, G. A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield. *Microorganisms* **8**, 658 (2020).
17. Cordente, A. G., Curtin, C. D., Varela, C. & Pretorius, I. S. Flavour-active wine yeasts. *Appl. Microbiol. Biotechnol.* **96**, 601–618 (2012).
18. Mas, A. et al. Bioactive compounds derived from the yeast metabolism of aromatic amino acids during alcoholic fermentation. *BioMed. Res. Int.* **2014**, 1–7 (2014).
19. Rodriguez-Naranjo, M. I., Torija, M. J., Mas, A., Cantos-Villar, E. & Garcia-Parrilla, M. C. Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions. *J. Pineal Res.* **53**, 219–224 (2012).
20. Fernández-Pachón, M. S. et al. Alcoholic fermentation induces melatonin synthesis in orange juice. *J. Pineal Res.* **56**, 31–38 (2014).
21. Vigentini, I. et al. Yeast contribution to melatonin, melatonin isomers and tryptophan ethyl ester during alcoholic fermentation of grape musts. *J. Pineal Res.* **58**, 388–396 (2015).
22. Fernández-Cruz, E., Álvarez-Fernández, M. A., Valero, E., Troncoso, A. M. & García-Parrilla, M. C. Melatonin and derived l-tryptophan metabolites produced during alcoholic fermentation by different wine yeast strains. *Food Chem.* **217**, 431–437 (2017).
23. Fernandez-Cruz, E. et al. Intracellular biosynthesis of melatonin and other indolic compounds in *Saccharomyces* and non-*Saccharomyces* wine yeasts. *Eur. Food Res. Technol.* **245**, 1553–1560 (2019).
24. Muñiz-Calvo, S., Bisquert, R., Fernández-Cruz, E., García-Parrilla, M. C. & Guillamón, J. M. Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites. *J. Pineal Res.* **66**, e12554 (2019).
25. Morcillo-Parra, M. Á., Valera, M. J., Beltran, G., Mas, A. & Torija, M.-J. Glycolytic proteins interact with intracellular melatonin in *Saccharomyces cerevisiae*. *Front. Microbiol.* **10**, 2424 (2019).
26. Morcillo-Parra, M. Á., González, B., Beltran, G., Mas, A. & Torija, M.-J. Melatonin and glycolytic protein interactions are related to yeast fermentative capacity. *Food Microbiol.* **87**, 103398 (2020).
27. Morcillo-Parra, M. Á., Beltran, G., Mas, A. & Torija, M.-J. Effect of several nutrients and environmental conditions on intracellular melatonin synthesis in *Saccharomyces cerevisiae*. *Microorganisms* **8**, 853 (2020).
28. Reppert, S. M. Melatonin receptors: Molecular biology of a new family of G protein-coupled receptors. *J. Biol. Rhythms* **12**, 528–531 (1997).
29. Carlberg, C. & Wiesenberg, I. The orphan receptor family RZR/ROR, melatonin and 5-lipoxygenase: An unexpected relationship: Mini review. *J. Pineal Res.* **18**, 171–178 (1995).
30. Becker-André, M. et al. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *J. Biol. Chem.* **272**, 16707–16707 (1997).
31. Levoye, A. et al. The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J.* **25**, 3012–3023 (2006).
32. Macías, M. et al. Calreticulin-melatonin: An unexpected relationship. *Eur. J. Biochem.* **270**, 832–840 (2003).
33. González, B. et al. The production of aromatic alcohols in non-*Saccharomyces* wine yeast is modulated by nutrient availability. *Food Microbiol.* **74**, 64–74 (2018).
34. Gonzalez, B. & Franc, J. A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. *Yeast* **13**, 1347–1355 (1997).
35. Morcillo-Parra, M. Á., Beltran, G., Mas, A. & Torija, M.-J. Determination of melatonin by a whole cell bioassay in fermented beverages. *Sci. Rep.* **9**, 9120 (2019).
36. Padilla, B., Gil, J. V. & Manzanares, P. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* **7**, 411 (2016).
37. Lleixà, J., Manzano, M., Mas, A. & Portillo, M. C. *Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol.* **7**, 1959 (2016).
38. Roca-Mesa, H., Sendra, S., Mas, A., Beltran, G. & Torija, M.-J. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. *Microorganisms* **8**, 157 (2020).

39. Lleixà, J. et al. Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Front. Microbiol.* **7**, 338 (2016).
40. Prior, K. J., Bauer, F. F. & Divol, B. The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine. *Food Microbiol.* **79**, 75–84 (2019).
41. Wah, T. T., Walaisri, S., Assavanig, A., Niamsiri, N. & Lertsiri, S. Co-culturing of *Pichia guilliermondii* enhanced volatile flavor compound formation by *Zygosaccharomyces rouxii* in the model system of Thai soy sauce fermentation. *Int. J. Food Microbiol.* **160**, 282–289 (2013).
42. Fleet, G. Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **86**, 11–22 (2003).
43. Contreras, A. et al. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **80**, 1670–1678 (2014).
44. Steenwyk, J. L. et al. Extensive loss of cell-cycle and DNA repair genes in an ancient lineage of bipolar budding yeasts. *PLOS Biol.* **17**, e3000255 (2019).
45. Varela, C., Sengler, F., Solomon, M. & Curtin, C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **209**, 57–64 (2016).
46. Canonico, L., Galli, E., Ciani, E., Comitini, F. & Ciani, M. Exploitation of three non-conventional yeast species in the brewing process. *Microorganisms* **7**, 11 (2019).
47. Hranilovic, A., Gambetta, J. M., Jeffery, D. W., Grbin, P. R. & Jiranek, V. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing. *Int. J. Food Microbiol.* **329**, 108651 (2020).
48. Benavides, S., Franco, W., Ceppi De Lecco, C., Durán, A. & Urtubia, A. Evaluation of indigenous *Candida oleophila* and *Candida boidinii* in monoculture and sequential fermentations: Impact on ethanol reduction and chemical profile in Chilean Sauvignon Blanc Wines. *J. Fungi* **8**, 259 (2022).
49. Torija, M. J. et al. Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. *Int. J. Food Microbiol.* **85**, 127–136 (2003).
50. Beltran, G., Novo, M., Guillamón, J. M., Mas, A. & Rozès, N. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *Int. J. Food Microbiol.* **121**, 169–177 (2008).
51. Charoenchai, C., Fleet, G. H. & Henschke, P. A. Effects of temperature, pH, and sugar concentration on the growth rates and cell biomass of wine yeasts. *Am. J. Enol. Vitic.* **49**, 283–288 (1998).
52. Reiter, R. J., Sharma, R., Ma, Q., Rorsales-Corral, S. & de Almeida Chuffa, L. G. Melatonin inhibits Warburg-dependent cancer by redirecting glucose oxidation to the mitochondria: A mechanistic hypothesis. *Cell. Mol. Life Sci.* **77**, 2527–2542 (2020).
53. Watanabe, K. et al. Nocturnal melatonin increases glucose uptake via insulin-independent action in the goldfish brain. *Front. Endocrinol. (Lausanne)* **14**, 1173113 (2023).
54. Bonnefond, A. et al. Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nat. Genet.* **44**, 297–301 (2012).

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Author contributions

MAMP designed, performed, analyzed and discussed the results and wrote the manuscript. SME performed, analyzed and discussed the results. JA performed some experiments. GB, MJT and AM designed the experiments, discussed the results, and wrote the manuscript.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information

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Correspondence and requests for materials should be addressed to G.B.

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