MELATONIN METABOLISM IN YEAST CELLS DURING ALCOHOLIC FERMENTATION

María de los Ángeles Morcillo Parra

UNIVERSITAT ROVIRA i VIRGILI

Melatonin metabolism in yeast cells during alcoholic fermentation

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DOCTORAL THESIS
2019

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UNIVERSITAT ROVIRA i VIRGILI

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Doctoral thesis under the supervision of

Dr. María Jesús Torija

Dr. Albert Mas

Tarragona 2019

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WE STATE that the present study, entitled "**Melatonin metabolism in yeast cells during alcoholic fermentation**", presented by **María Ángeles Morcillo Parra** for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university.

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Dr. María Jesús Torija Martínez   Dr. Albert Mas Barón

Tarragona, May 2019

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A mis padres

A mi hermana

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How often have I said to you that when you have eliminated the

impossible, whatever remains, however improbable, must be the truth?

Arthur Conan Doyle

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Agradecimientos

Y ahora después de cuatro años, resulta difícil escribir los agradecimientos y poder expresar toda la gratitud y estima que tengo hacia todos los que habéis participado en el proceso de esta tesis.

No veo mejor manera de empezar que dando las gracias a las dos personas que han hecho posible que la melatonina acabe dando esta tesis. A mis directores. Al Dr. Albert Mas, por haber confiado en mí y darme la oportunidad de venir al grupo de Biotecnología Enológica, por transmitirme tu conocimiento y ayudarme en el inicio de mi carrera investigadora. A la Dra. María Jesús Torija, las palabras en este momento se me quedan cortas para agradecerte estos años luchando con la melatonina, por transmitirme tu entusiasmo por la investigación, por tu ayuda a nivel científico, pero también personal, por las risas y por tu positivismo en los momentos no tan bonitos. Gracias a los dos, porque no solo me habéis ayudado a crecer como científica sino también como persona. Sois un gran referente para mí. ¡Gracias!

Gracias a la Dra. Gemma Beltran, la otra *jefa*, por esas reuniones para trabajar con las células, por tus consejos y también por todos los cafés, reuniones y comidas en los que hemos reído tanto. Gracias a Carmen, la jefa que siempre viene a las cenas, por esas cervezas y por esos cafés intercambiando penas. ¡Gracias!

Gracias al Dr. Jose Manuel Guillamón, por acogerme en su laboratorio, pero sobretodo por la gran cantidad de consejos. Gracias a las Dra. Ana María Troncoso y Dra. María Carmen García por su hospitalidad en todas las reuniones y por los ánimos. También agradecer a todos los doctorandos tanto de Sevilla como de Valencia: Edwin, María Antonia, Ricardo y especialmente a Sara, la chica de las levaduras, por todas las risas que compartimos en el máster.

Dr. Florian Bauer, I want to thank you for hosting in your lab, transmit part of your knowledge and treat me as one of your team! Thanks to all the staff of the Institute for Wine Biotechnology for helping me always I needed and making me feel like home! Thanks to Thato, my *silly pocket*, to show me Stellenbosch and South Africa, to help me with all the lab staff, to suffer the melatonin world with me! My friend, we need to see each other soon! Thanks to all people of the Florian's lab, especially Rene, Junior, Kirti, Baghar, Mikkayla, Tristan, Zoë! Thanks to Tania, Lalo, Mihaela and Stoain, I really had many funny moments with all you! Thank you!

Gracias a todo el personal del departamento, secretarías, conserjes, que nos facilitáis tanto la vida. Gracias también a los técnicos, Braulio, Santiago, pero especialmente a Niurka y Rosa por

enseñarme todo lo que sé sobre líneas celulares. ¡Habéis sido unas profesoras estupendas! Gracias también a Irene y Sonia, las que han sufrido conmigo cuando el HPLC no quería funcionar- ¡Gracias por enseñarme a utilizarlo!

Una de las cosas *más mejor* que me llevo de esta época de mi vida es la gran cantidad de personas que he conocido y con las que he trabajado, algunas de ellas han pasado a formar parte de la categoría de grandes amigos. Sin vosotros, Bea, Jessica, Aitor y Adeline, esta tesis hubiese costado muchísimo más.

A Bea, mi topillo, desde nuestros inicios yendo andando a la uni o saliendo correr a las tantas de la mañana (aún sigo esperando esa carrera, quién sabe si en New York), pasaste de ser una compañera del laboratorio a ser una amiga esencial. ¡Gracias por todo, por los ánimos, pero sobre todo por esa alegría y locura que te caracteriza! A Jess, aún me acuerdo de nuestro viaje en autobús cuando yo acababa de llegar, ¿quién pensaría que iba a vivir contigo? Has sido un pilar muy importante en esta tesis. Por tus cenas al llegar a casa, tus bizcochos, nuestros chocolates y nuestras series raras. ¡Gracias! Ahora ya estás en la recta final y sólo puedo decir que ¡lo harás genial!

Y aunque aparecieron más tarde, no puedo dejar de agradecer a Adeline y Aitor. Adeline, mi compañera de *spinning*, gracias por tu sinceridad, por tu apoyo y por esos geles de proteínas ¿Qué hubiese sido de mí sin esa tinción? Ha sido un placer poder compartir este tiempo contigo y Burdeos está tan cerca, que seguro que nos tienes allí dando guerra pronto. ¡Ánimo con la recta final! Merci! A Aitor, porque este último año has sido un gran apoyo para mí. Gracias por todas esas tardes de cervezas para desconectar, por nuestros cotis, por los findes de semana en tu casa, por los domingos de peli, por ser más que un compañero de laboratorio. *Cuanto* más lo pienso, más creo que he encontrado a un amigo de los buenos. Eskerrik asko!

También me gustaría agradecer a toda la gente que ha ido pasando por el labo: Alba, Bea P., Chunxiao, Dani F., Flor, Gemma R., Isa, Jordi, Juan, Judith, Laura, María (la chica *Brett*), Mercè, Núria, Olga, Pere, Xiaolin, Yurena. ¡Gracias a todos! Jenny, la chica del estrés, por las penas y las quejas compartidas, por todo lo que reído contigo. Mª José, por todo lo que me has enseñado estos años y por estar siempre con una sonrisa para mí. Helena, por todas las series recomendadas y por alegrarnos los días con esas manualidades tan cuquis. Nurieta, por el apoyo y por todas esas conversaciones profundas. Dària, mi pupila, ha sido todo un placer poder compartir estos meses contigo en esta etapa final. ¡Ánimo con todo! Y cómo no, a las últimas incorporaciones que han hecho que estos meses de escritura sean mucho más animados: Miguel, Candela y Dani (*el de*

arriba), por las tardes en el apple, por los findes en casa de Aitor, por las tardes en el gym, por el cobre y sobre todo por todas las risas. ¡Sois *duliciosamente* geniales! Dani, tú también pasarás a ser A.D.-P.D. ¡¡Gracias, gracias y gracias!!

A mis compañeras de piso, Anna y Loren, por nuestras noches de gordis y por alegrarme los días de bajón. A Patricia, por nuestros cotilleos, tu alegría y por estar siempre ahí. Gracias a Sara y a Zafri, por nuestras quedadas y nuestras desconexiones, y también por todas las risas. ¡¡Gracias!!

A esas personas que no sabría qué hacer sin ellas. Víctor, porque contigo aprendí como hacer un buen *western,* por las rutas, las montañas compartidas estos años, pero también por las cervezas y las comidas. ¡¡Gracias!! A Pedro, por tus consejos, por todos los findes de desconexión, por ser un apoyo incansable en la ciencia, pero sobre todo personalmente. Pasaste a ser imprescindible en mi vida. Esta tesis tiene una gran parte vuestra. ¡¡Gracias!! ¡¡Ánimo con la etapa final, la tesis ya está en vuestras manos!!

A mi familia, sin ellos esto no hubiese sido posible. A mis tíos, Félix, Jose Ángel, Flor, Amalia, Paco, por nuestras celebraciones y especialmente, a Ana y Jose Luis, por acogerme todos esos findes, por nuestras comilonas y por ayudarme en todo. ¡¡Gracias!! Y finalmente, a mis padres, gracias por darme la oportunidad de hacer lo que más me gusta ¡¡ Gracias por apoyarme siempre!! Sois mi ejemplo a seguir, por vuestra constancia, esfuerzo y trabajo. Gracias también a la persona con la que comparto todo y que es la mejor hermana del mundo, ¡Teresita! Esta tesis también va dedicada a mis abuelos, Ángel, Amalia, Manuel y Mercedes.

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The present work in fulfilment of my PhD degree was carried out from 2015 to 2019 at the University Rovira i Virgili. This research was developed in the Oenological Biotechnology research group, Department of Biochemistry and Biotechnology. During these years, I held a predoctoral fellowship from the Ministry of Economy and Competitiveness of Spain (grant no. BES-2014-070542). Furthermore, in order to obtain the distinction of International Doctorate, I conducted a research at the Institute for Wine Biotechnology in Stellenbosch (South Africa) from April to July of 2016.

The work I carried out was part of the projects "Production of bioactive compounds derived from aromatic amino acids during alcoholic fermentation" and "Synthesis of bioactive compounds in fermented food by different yeast species", which were supported by the Ministry of Economy and Competitiveness of Spain (grant no. AGL2013-47300-C3 and grant no. AGL2016-77505-C3, respectively). The main objective of the projects was to understand the synthesis of bioactive molecules by yeast during alcoholic fermentation, especially melatonin and to develop faster detection methods for this indole compound. Melatonin modulates circadian rhythm and exerts multiple functions in human cells. Recently, melatonin has been detected in wine due to yeast metabolism during fermentation. However, little is known about its role and regulation in yeast cells.

With this framework, the hypothesis was as follows: Melatonin has a regulatory function in yeast cells, *Saccharomyces* and non-*Saccharomyces,* specifically during alcoholic fermentation.

To verify this hypothesis, the general objective was to study the synthesis profile of melatonin during alcoholic fermentation in different yeast species and to try to unravel the cause of its synthesis. This general objective was divided into the following specific objectives:

Objective 1. To study the effect of biotic and abiotic factors on the regulatory role of melatonin during alcoholic fermentation.

Melatonin is a bioactive compound that is synthesized by yeast during alcoholic fermentation. Additionally, melatonin can carry out an antioxidant role against oxidative stress or UV stress in yeast cells. However, little is known about the synthesis and role of melatonin during alcoholic fermentation. To fulfil this objective, three different experiments were carried out.

First, we studied the effect of melatonin supplementation on fermentation kinetics and the viability and species distribution in a set of fermentations performed with three combinations of inocula: *Saccharomyces cerevisiae* QA23, a mixed culture of four non-*Saccharomyces* strains

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(*Torulaspora delbrueckii, Hanseniaspora uvarum, Metschnikowia pulcherrima* and *Starmerella bacillaris*) and a mixed population of these four non-*Saccharomyces* strains together with *Saccharomyces cerevisiae* QA23. Yeasts were grown in synthetic grape must supplemented with different concentrations of melatonin (0.1, 0.5 and 1 g/L) in two nitrogen conditions: 100 and 300 mg N/L. Yeast growth was determined by plate counting in three media: YPD (rich medium for total yeast counts), lysine agar (selective medium for non-*Saccharomyces* species) and WL (Wallerstein Laboratory Nutrient Agar) (differential medium for the rapid identification of yeast species based on different colony morphologies). Moreover, extracellular melatonin was monitored in control fermentations (without melatonin supplementation) to determine the content of melatonin produced in each inoculum.

Second, we studied the effects of different nutrient and environmental conditions on melatonin production in synthetic medium in order to decipher which fermentation parameters yeast responds to by synthesizing melatonin. For that reason, first, we evaluated the effects of different *S. cerevisiae* strains (from different environments) on melatonin synthesis, and then, the effects of sugar (20 and 200 g/L) and nitrogen (100 and 300 mg N/L) concentrations, temperature (4, 12 and 28**°C**), inoculum amount (10^6, 10^7 and 10^8 cell/mL), and cell cycle synchronization (arrested cells) were analyzed only in *S. cerevisiae* QA23 strain. Intracellular melatonin content was measured during the first 4 h of fermentation by High Performance Liquid Chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

Third, we established the profile of melatonin synthesis in standard grape must in five yeast species, including *Saccharomyces* and non-*Saccharomyces* yeasts. Individual fermentations were carried out with *S. cerevisiae* QA23, *H. uvarum, M. pulcherrima, S. bacillaris* and *T. delbrueckii.* Melatonin was measured either intra- or extracellularly by HPLC-MS/MS.

The results of this objective are described in the following four chapters:

Chapter 1: Effect of melatonin and tryptophol addition on fermentation carried out by *Saccharomyces* and non-*Saccharomyces* yeast species under different nitrogen conditions. International Journal of Food Microbiology (2019) 289, 174-181.

Although this study was done with melatonin and tryptophol, only the melatonin effect is discussed in this thesis.

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Chapter 2: Effect of several nutrients and environmental conditions on intracellular melatonin synthesis in *Saccharomyces cerevisiae.* Manuscript in preparation.

Chapter 3: Glycolytic proteins interact with intracellular melatonin in *Saccharomyces cerevisiae.* The results have been submitted to Frontiers in Microbiology.

Chapter 5: Melatonin and glycolytic protein interactions are related to yeast fermentative capacity. The results have been submitted to Food Microbiology.

Objective 2. To decipher the fate of melatonin and its interactions in *Saccharomyces* and non-*Saccharomyces* yeasts.

Melatonin is an indolamine that not only interacts with its receptors but also with other proteins such as tubulin, calmodulin, calreticulin or sirtuins in mammalian cells. After evaluating the results obtained in Objective 1, we wanted to determine if melatonin interacts with any proteins in the period between its intracellular synthesis and extracellular secretion in yeasts. To achieve this aim, three different experiments were performed.

First, samples during this period were collected from fermentations carried out with different *Saccharomyces* and non-*Saccharomyces* strains (*T. delbrueckii, H. uvarum, S. bacillaris* and *M. pulcherrima*) in standard synthetic medium, and proteins were purified and subsequently identified by protein sequence analysis.

Second, to uncover the relationship between melatonin synthesis and yeast growth phases, a fermentation at low temperature (16**°C**) was performed to delay yeast growth and determine if the synthesis of melatonin and its interactions with proteins were also delayed. Therefore, intra- and extracellular melatonin were measured at different points in yeast growth phases, and additionally, proteins bound to melatonin were inmunopurified and identified by protein sequence analysis.

Third, to assess the importance of the proteins that interact with melatonin, fermentations with single mutants of these proteins were performed. For this reason, a collection of glycolytic mutants of the lab strain BY4742 was employed. Fermentations were performed at 28**°C** in standard synthetic grape must, and melatonin was quantified intra- and extracellularly. Afterwards, protein purification and identification were conducted.

The results of this objective are described in the following three chapters of this thesis:

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Chapter 3: Glycolytic proteins interact with intracellular melatonin in *Saccharomyces cerevisiae.* The results have been submitted to Frontiers in Microbiology.

Chapter 4: Melatonin synthesis and protein interactions in *Saccharomyces cerevisiae* during alcoholic fermentation are yeast growth-dependent. Manuscript in preparation.

Chapter 5: Melatonin and glycolytic protein interactions are related to yeast fermentative capacity. The results have been submitted to Food Microbiology.

Objective 3. To optimize a new method for the detection of melatonin in food samples.

Yeasts are able to synthetize melatonin during alcoholic fermentation either intra- or extracellularly in very low quantities (ng/mL). Although there are several methods to detect melatonin in yeast-derived samples, these methods have limited sensitivity or are technically complex to be adapted as routine techniques for the rapid detection of melatonin. Moreover, the main limitation that we had to mitigate during the previous objectives of this thesis was the difficulty in detecting melatonin due to its rapid appearance and disappearance during alcoholic fermentation; thus, we described a novel fluorescence method to detect melatonin in yeast-derived samples based on a β-lactamase cell assay. A human cell line that presented the melatonin receptor MTNR1B coupled with β-lactamase detection was used. Fluorescence detection was carried out by Fluorescence Resonance Energy Transfer. This new detection method was compared with a validated method (HPLC-MS/MS).

First, we optimized the BLA cell-based assay by checking several parameters that affect cell growth and melatonin detection (cell number per well, presence or absence of fetal bovine serum, stimulation time and cell adhesion before analysis) using melatonin standards within the range of linearity of the assay.

Then, we studied the effects of food matrices on the BLA cell-based assay by comparing the results with those of the HPLC-MS/MS method. To do so, a matrix-matched calibration curve was prepared with melatonin standards in different matrices: methanol/water mixture (40:60), Milli-Q water, white wine, grape must and the assay medium for the cell line. All calibration curves were analyzed by the BLA cell-based assay and the HPLC-MS/MS method. Finally, to validate the BLA cell-based assay, we analyzed melatonin content in 15 samples from alcoholic fermentation and compared the results with those obtained by HPLC-MS/MS.

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The results for this objective are described in Chapter 6: Determination of melatonin by a whole cell bioassay in fermented beverages. Results accepted in Scientific Reports.

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1. Yeast

Yeasts are eukaryotic and unicellular organisms that belong to the fungal kingdom. Given its similarity to other eukaryotic cells at the biochemical level, yeast has been used as a biological model system to study different mechanisms and metabolisms in eukaryotic cells (Pretorius, 2000). Yeast was observed under the microscope for the first time by Antonie van Leeuwenhoek, but it was 200 years later when Louis Pasteur recognized yeast as the living microorganism responsible for alcoholic fermentation. Since then, numerous studies have been performed with these microorganisms, particularly with regard to fermented food and beverages (processes as baking, brewing and winemaking) on one hand and biofuel and pharmaceutical products on the other (recombinant vaccines or enzymes) (Pretorius, 2017).

Yeasts are the simplest eukaryotic cells, and therefore, in 1996, *Saccharomyces cerevisiae* became the first eukaryotic genome to be fully sequenced, revealing that it encodes 16 chromosomes (12.1 Mbp in size) and 6000 genes, of which only 900-1200 are essential for cell survival (Zhang and Ren, 2015).

1.1. Yeast and winemaking

The central transformation during wine production is the transformation of fermentable sugars (mainly glucose and fructose) into ethanol and carbon dioxide (Ribéreau-Gayon et al., 2006). However, this metabolic process is very complex, and not only is alcohol produced but also volatile compounds, which have an impact on the aroma and flavor of wine (Pretorius, 2016; Swiegers et al., 2005). Yeasts are the prominent organisms of wine production and determine wine flavor and other qualities (Fleet, 2003). *S. cerevisiae* has been universally described as the *wine yeast*; however, this is not the only yeast species we encounter during the winemaking process (Pretorius, 2000).

1.1.1. *Saccharomyces* yeasts

S. cerevisiae presents the most efficient fermentative catabolism and is, traditionally, the preferred species to be inoculated as a starter in alcoholic fermentations (Pretorius, 2000) due to its ability to prevail along the winemaking process, although the environment presents hard conditions (low pH, increasing ethanol content, high osmotic pressure, the presence of SO_2, etc.). Moreover, *S. cerevisiae* is one of the best fermentation performers because this yeast species rapidly and

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efficiently transforms the sugars present in grape must into ethanol. Contrary to popular belief, *S. cerevisiae* yeast populations are extremely low on the surface of berries and are rarely isolated from intact berries and vineyard soils (Fleet, 2003; Valero et al., 2007). Nevertheless, this yeast species is a typical inhabitant of the winery environment (Pretorius, 2000).

1.1.2. Non-*Saccharomyces* yeasts

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 (Fleet, 2008), and they are naturally present on grape berry surfaces (Jolly et al., 2014). Originally, non-*Saccharomyces* yeasts were deemed to be responsible for microbe-related problems in wines due to their isolation from spoiled grape and wines. However, it was recently demonstrated that the presence of non-*Saccharomyces* yeasts during the early steps of alcoholic fermentation significantly contributes to improvement of the sensory qualities of wine. Non-*Saccharomyces* yeasts not only enhance wine complexity and flavor (Jolly et al., 2014, Padilla et al., 2016) but also contribute to wine stability, preventing the growth of spoilage microorganisms (Comitini et al., 2004; Mehlomakulu and Africa, 2015; Mehlomakulu et al., 2017; Villalba et al., 2016). Moreover, one of the most recent and interesting characteristics identified is their potential in lowering the ethanol content in wine (Contreras et al., 2015; Quirós et al., 2014; Varela et al., 2017). Consequently, research about non-*Saccharomyces* yeasts has substantially increased to uncover their role during wine production (Jolly et al., 2014; Pretorius, 2000; Varela and Borneman, 2017). In the last decade, studies have focused on the use of these yeasts as starter cultures for sequential or simultaneous inoculation with *S. cerevisiae* (Jolly et al., 2014; Varela and Borneman, 2017).

1.1.2.1. *Torulaspora delbrueckii*

T. delbrueckii was one of the first commercial non-*Saccharomyces* yeast to be released (Jolly et al., 2014) because its most important biotechnological application relies on its fermentative capacity; it is used for beer, wine and even bread fermentation (Varela and Borneman, 2017). *T. delbrueckii* was formerly classified as *Saccharomyces rosei*, suggesting a similar taxonomic lineage and function to *S. cerevisiae* (Bely et al., 2008). In fact, *Torulaspora* spp and *Saccharomyces* spp. are believed to have diverged evolutionarily approximately 100-150 million years ago (Hagman et al., 2014). Thus, *T. delbrueckii* is very close genetically to *S. cerevisiae* (Masneuf-Pomarede et al., 2016).

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T. delbrueckii has been described to influence the volatile composition and sensory profile in wines (Varela and Borneman, 2017) by increasing the amount of acetate esters and medium-chain fatty acids (Cordero-Bueso et al., 2013), terpenes such as α-terpineol and linalool (Cus and Jenko, 2013), "aroma intensity", "complexity" and "persistence" in red wines (Azzolini et al., 2015; Belda et al., 2016; Renault et al., 2015) and foamability and foam persistence (González-Royo et al., 2015). Moreover, recent studies have proposed the use of *T. delbrueckii* for solving oenological problems such as high acetic acid and ethanol concentrations (Benito et al., 2018). *T. delbrueckii* produces wines with lower concentrations of acetic acid (Bely et al., 2008) and ethanol (Contreras et al., 2014) and higher glycerol content (Belda et al., 2015).

In terms of genomic resources, the genome sequences of two strains of *T. delbrueckii* have been published (a type strain and one strain isolated from mezcal fermentation) (Gómez-Angulo et al., 2015; Gordon et al., 2011).

1.1.2.2.   *Hanseniaspora uvarum*

The apiculate yeast *H. uvarum* is the most abundant species recovered in the microbiota of grape berries. Thus, *H. uvarum* yeasts play an important role in wine quality (Jolly et al., 2014). Despite their low fermentative capacity, these yeasts improve wine volatile composition by increasing "tropical fruit", "berry", "floral" and "nut" aroma' characters (Hu et al., 2018) and the concentrations of acetate esters, 2-phenylethanol and α-terpineol (Garofalo et al., 2016). Nevertheless, *H. uvarum* also has been associated with the production of undesirable flavor compounds (volatile acidity, sulfur compounds, etc.), although high variability between strains exists, and some of them synthesize similar amounts to those of *S. cerevisiae* (Jolly et al., 2014). Recently, Langenberg and collaborators (2017) have attributed the low fermentative capacity of *H. uvarum* to a 10-fold-lower activity of the key glycolytic enzyme pyruvate kinase relative to *S. cerevisiae.*

Draft genome sequences of three *H. uvarum* strains are available, although only one belongs to the wine environment (from a wild Chardonnay fermentation) (Sternes et al., 2016). Nevertheless, single representative genomes of different *Hanseniaspora* species have been announced: *H. vineae, H. osmophila,* and *H. valbyensis,* among others (Giorello et al., 2014; Riley et al., 2016; Sternes et al., 2016).

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1.1.2.3.   Metschnikowia pulcherrima

M. pulcherrima is another yeast that is commercially available due to its impact on the concentrations of varietal aromas (terpenes and volatile thiols). Wines fermented using a combination of *M. pulcherrima* and *S. cerevisiae* have been described to have higher "citrus/grapefruit" and "pear" sensory descriptors (Benito et al., 2015). In addition, *M. pulcherrima* increases the concentration of mannoproteins (Domizio et al., 2014) and foam persistence (González-Royo et al., 2015). In addition to having a role in flavor profile, the presence of *M. pulcherrima* during alcoholic fermentation is associated with a lowering of ethanol production due to its respiratory metabolism (Canonico et al., 2016; Quirós et al., 2014; Ruiz et al., 2018; Varela et al., 2017). Moreover, notable increases in glycerol in sequential fermentation with this yeast have also been reported (Benito et al., 2015; Escribano et al., 2018; Ruiz et al., 2018). *M. pulcherrima* is known to have a lower fermentative capacity since it can respire between 40 and 100% of the sugar consumed (Quirós et al., 2014). On the other hand, *M. pulcherrima* is a strongly antifungal yeast due to the production of a precursor of the red pigment pulcherrimin, pulcherrimic acid, which provides this yeast with promising control against infamous plant pathogenic fungi such as *Botrytis* (Gore-Lloyd et al., 2018) but also against wine spoilage yeasts, such as *Brettanomyces* (Oro et al., 2014). However, this antimicrobial activity does not seem to affect the growth of *S. cerevisiae*, supporting the potential use of *M. pulcherrima* as a starter culture with *S. cerevisiae* (Oro et al., 2014). Recently, a draft genome sequence of a *M. pulcherrima* strain has been announced, although the origin of that strain is far from wine ecology: it was isolated from soil in a forest in Ireland (Venkatesh et al., 2018)

1.1.2.4.   Starmerella bacillaris (synonym Candida zemplinina/Candida stellata)

C. zemplinina has been confused with *C. stellata* for a long time because of their similarities (Masneuf-Pomarede et al., 2016). Therefore, it is possible that many studies about *C. stellata* are actually describing *C. zemplinina* (Varela et al., 2017). Currently, *C. zemplinina* is referred by its synonym *S. bacillaris* (Duarte et al., 2012). This species has been used for wine fermentation since it is described to reduce ethanol concentration (Englezos et al., 2016), enhance wine flavor and mouth-feel (Jolly et al., 2014) and reduce acetic acid formation in sweet wine (Rantsiou et al., 2012). *S. bacillaris* displays an interesting feature, a strong fructophilic character, resulting in a

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preferential consumption of fructose versus glucose (Englezos et al., 2015). Moreover, its potential use to produce wines with high glycerol content also has been addressed (Englezos et al., 2016; Zara et al., 2014). Recently, the genome data of three *S. bacillaris* strains, all of them isolated from wine environments, including the type strain of this species, has been published (Lemos Junior et al., 2017a; 2017b; Rosa et al., 2018).

1.2.   Life cycle

In any case, the activity of yeasts in the wine environment is closely related to the increase in population, that is, their reproduction. Reproduction in yeasts can be sexual and/or asexual; specifically, perfect fungi endure both reproductive cycles, whereas imperfect fungi only reproduce asexually. Sexual reproduction in yeast takes place via conjugation of two cells of different mating types. Then, the cell sporulates by meiosis, whose frequency depends on the yeast strain. On the other hand, the vegetative cycle is a sequence of events whereby a cell grows and divides into two daughter cells. The most common cell vegetative division in yeast occurs by budding, as in the case of *S. cerevisiae.* Other yeasts, such as *Schizosaccharomyces pombe*, reproduce by fission.
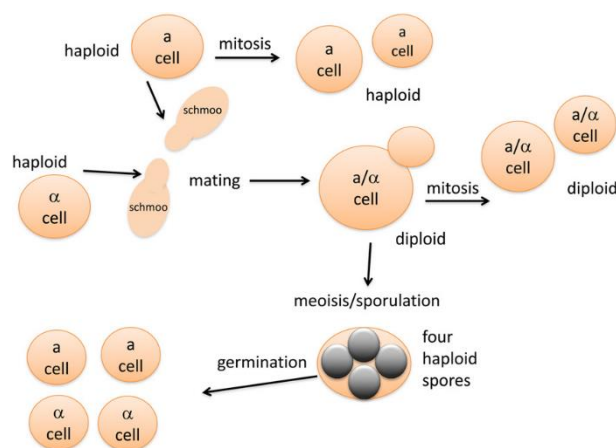
Figure 1. Life cycle of *S. cerevisiae* (Duina et al., 2014)

The life cycle of the budding yeast *S. cerevisiae* is illustrated in Figure 1. *S. cerevisiae* harbors genetic information in chromosome III that determines its mating-type identity (MAT locus). *S. cerevisiae* yeast cells can be either mating type a or α (MATa and MATα, respectively). Both type cells are haploid and, under the appropriate conditions, can mate with each other to generate MATa/MATα diploids. The diploid cells cannot mate but can reproduce by mitosis or can undergo meiosis to produce four haploid spores (MATa or MATα) when cells experience hard

conditions. *S. cerevisiae* exists either a haploid or diploid cell and, under adequate nutrient conditions, both haploids and diploids undergo a succession of rounds of vegetative growth and mitosis (Duina et al., 2014). Most laboratory strains are haploid or diploid, whereas industrial wine strains are predominantly diploid or aneuploid, and occasionally polyploid (Pretorius, 2017). During winemaking, the increase in population is mostly due to asexual reproduction.

1.3. Cell cycle

First, a cell undergoes a period of expansion, in which its volume increases. When the cell stops expanding, bud emergence occurs. During bud formation, the total volume of the mother and the bud is always constant; thus, the bud grows at the expense of the mother cell. Then, the bud separates as a single but smaller cell from the mother. Both the daughter and mother cells grow until they reach the same size (Posten and Cooney, 1993). By and large, throughout the two divisions, the genetic material is replicated because it must be segregated into the two cells. The cell cycle of yeast takes place every 90 min if conditions are optimal. The cell cycle is divided into four phases (Figure 2): G1, in which cells grow and prepare the machinery for DNA replication; S-phase or synthesis phase, when DNA is replicated into two copies; G2, in which cells prepare for mitosis and the reorganization of microtubules starts; and finally, M-phase or mitosis phase, when the content of the cell is distributed between the two future cells, and the nucleus and cytoplasm divide.
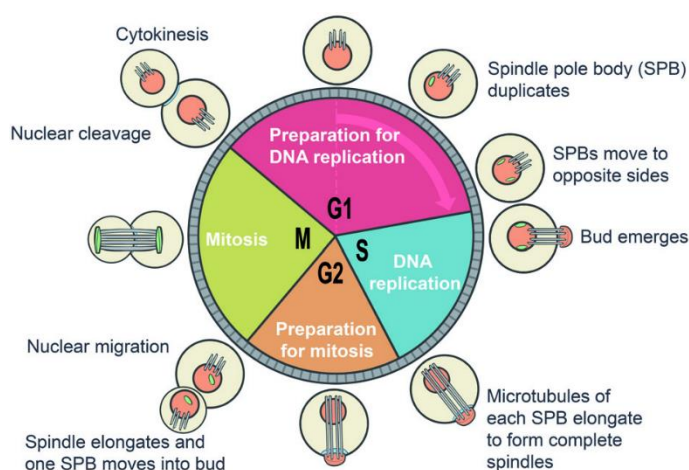
Figure 2. Cell cycle of *S. cerevisiae* (Pretorius, 2017)

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In *S. cerevisiae*, the vegetative cell pattern is controlled by cell type (a or α). Haploid cells bud following an axial pattern, in which both mother and daughter cells bud adjacent to the preceding division site. In contrast, diploid cells (a/α) normally exhibit a dipolar budding pattern, in which the daughter cell buds at the distal pole to the division site, and the mother cell buds near either pole (Madden and Snyder et al., 1998; Sloat et al., 1981).

1.4. Kinetics of yeast growth

Since the early 1960s, determination of microbial growth rate has been an important tool in microbial genetics, biochemistry, molecular biology and microbial physiology (Hall et al., 2014). *S. cerevisiae* exhibits a typical microbial growth curve (Figure 3), which consists of different phases: lag phase, exponential phase, deceleration phase and stationary phase. In addition to these phases, another can be added: the death phase, when there are no nutrients and cells begin to aggregate and die.

First, when yeast cells are inoculated in a new medium, they enter into the lag phase. During this period, cells are adapting to the new environment and preparing the machinery to grow at a higher rate, and thus, no population growth is noticed. Subsequently, the growth rate increases until a constant rate is achieved during the exponential phase. At the end of this phase, the deceleration phase occurs, and yeast cells reach maximal growth. Then, cells enter into the stationary phase, in which cell numbers no longer increase after a certain time. Afterwards, the death phase starts.
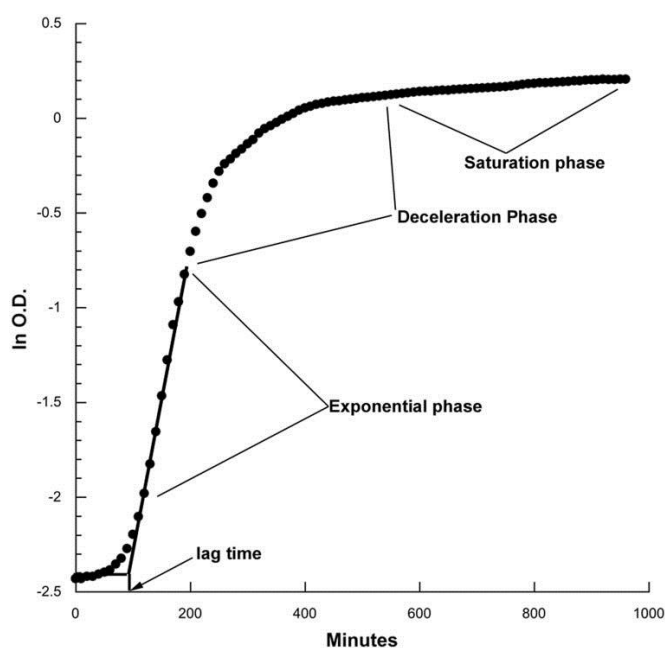
Figure 3. Typical microbial growth curve (Hall et al., 2014)

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For a long time, growth rates have been used by microbiologists to study different yeast properties, such as fermentation performance, phenotypic properties or regulation of gene expression, because the growth curve is affected when cells do not grow in optimal conditions (Hall et al., 2014). Different parameters can be measured during growth, such as the growth rate or the generation time, among others. Generation time explains the time required to double the population and, in the case of *S. cerevisiae*, is 90 min under optimal conditions.

2. Yeast metabolism

Metabolism refers to the biochemical assimilation (catabolic pathways) and dissimilation (anabolic pathways) of nutrients by a cell. These processes are mediated by enzymatic reactions, which have been studied in depth in yeast cells (Feldmann, 2012). Currently, most yeasts are known to utilize sugars as their main carbon source, although there are also yeasts that can utilize nonconventional carbon sources such as galactose or mannose. Regarding nitrogen metabolism, most yeasts are able to assimilate simple nitrogenous sources to synthetize amino acids, proteins and other nitrogen compounds. These two metabolisms (carbon and nitrogen) have been widely investigated.

2.1.   Carbon metabolism

Yeasts are chemoheterotrophic microorganisms, and they therefore obtain all energy and carbon from organic compounds. Collectively, yeast cells are able to grow on several carbon sources such as carbohydrates, organic acids, and amino acids, among others. However, the most common are carbohydrates; yeast can grow on monosaccharides, oligosaccharides and polysaccharides (Barnet et al., 2000). Carbohydrate uptake is carried out by transport proteins that are located in the plasma membrane. *S. cerevisiae* has a family of 20 genes encoding hexose transporters (HXT gene family) (Diderich et al., 1999). By and large, yeast cells can degrade sugar via two different metabolic pathways according to oxygen availability: respiration and fermentation, which share glycolysis as the first step in breaking down glucose. Both processes generate energy for cell activity (adenosine triphosphate, ATP; and reduced nicotinamide adenine dinucleotide, NADH).

2.1.1. Glycolysis

The glycolytic pathway is ubiquitous among yeast species. However, some differences have been observed depending on the yeast species due to the regulation of glycolytic enzyme gene expression (Querol and Fleet, 2006). In *S. cerevisiae*, glycolytic enzymes are expressed in a very

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high level during growth on glucose. Generally, glycolysis is a process in which glucose is oxidized to pyruvate, whereas NAD^+ is reduced to NADH, producing two ATPs as a result.

Glycolysis is composed of ten steps (Figure 4). First, hexokinase (*HXK1, HXK2*) phosphorylates glucose and fructose, consuming ATP. Phosphoglucose isomerase (*PGI1*) interconverts hexose-6-phosphate molecules in order to obtain fructose-6-phosphate molecules. Then, phosphofructokinase (*PFK1, PFK2*) adds a second phosphate group to fructose-6-phosphate to form fructose-1,6-phosphate, consuming another molecule of ATP. Aldolase (*FAB1*) cleaves fructose-1,6-phosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Both can be interconverted by triose phosphate isomerase (*TPI1*). At this point, yeast can metabolize dihydroxyacetone phosphate and glyceraldehyde-3-phosphate through glycolysis until pyruvate or reduce it until glycerol. Glycerol is also important for yeasts due to its role in osmotic stress protection. If we continue with glycolysis, glyceraldehyde-3-phosphate is oxidized to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (*TDH1*, *TDH2* and *TDH3*), while NAD^+ is reduced. Then, a phosphoryl group is transferred to ADP by phosphoglycerate kinase (*PGK1*) to form ATP and 3-phosphoglycerate. Afterwards, phosphoglycerate mutase (*GPM1*) generates 2-phosphoglycerate, which is transformed into phosphoenolpyruvate by enolase (*ENO1*, *ENO2*). Finally, a second phosphoryl transfer reaction occurs, mediated by pyruvate kinase (*PYK1*, *PYK2*), to form pyruvate and the second molecule of ATP. Glycolysis is not only an important source of ATP but also provides carbon skeletons for amino acid biosynthesis (Querol and Fleet, 2006). Once pyruvate is formed, this metabolite is degraded either through the respiration pathway, which is an oxygen-dependent reaction, or through alcoholic fermentation, which occurs in the absence of oxygen. (Figure 4).

2.1.1.1. Glycolytic enzymes

Glycolysis is an ancient biochemical pathway; however, glycolytic enzymes have been reported to be more complicated and multifaceted proteins rather than simple components of the glycolysis pathway (Kim et al., 2005). Unexpected functional roles of several glycolytic enzymes have been identified, especially for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) but also for enolase (ENO), hexokinase (HK), glucose-6-phosphate isomerase (GPI) and pyruvate kinase (PYK).

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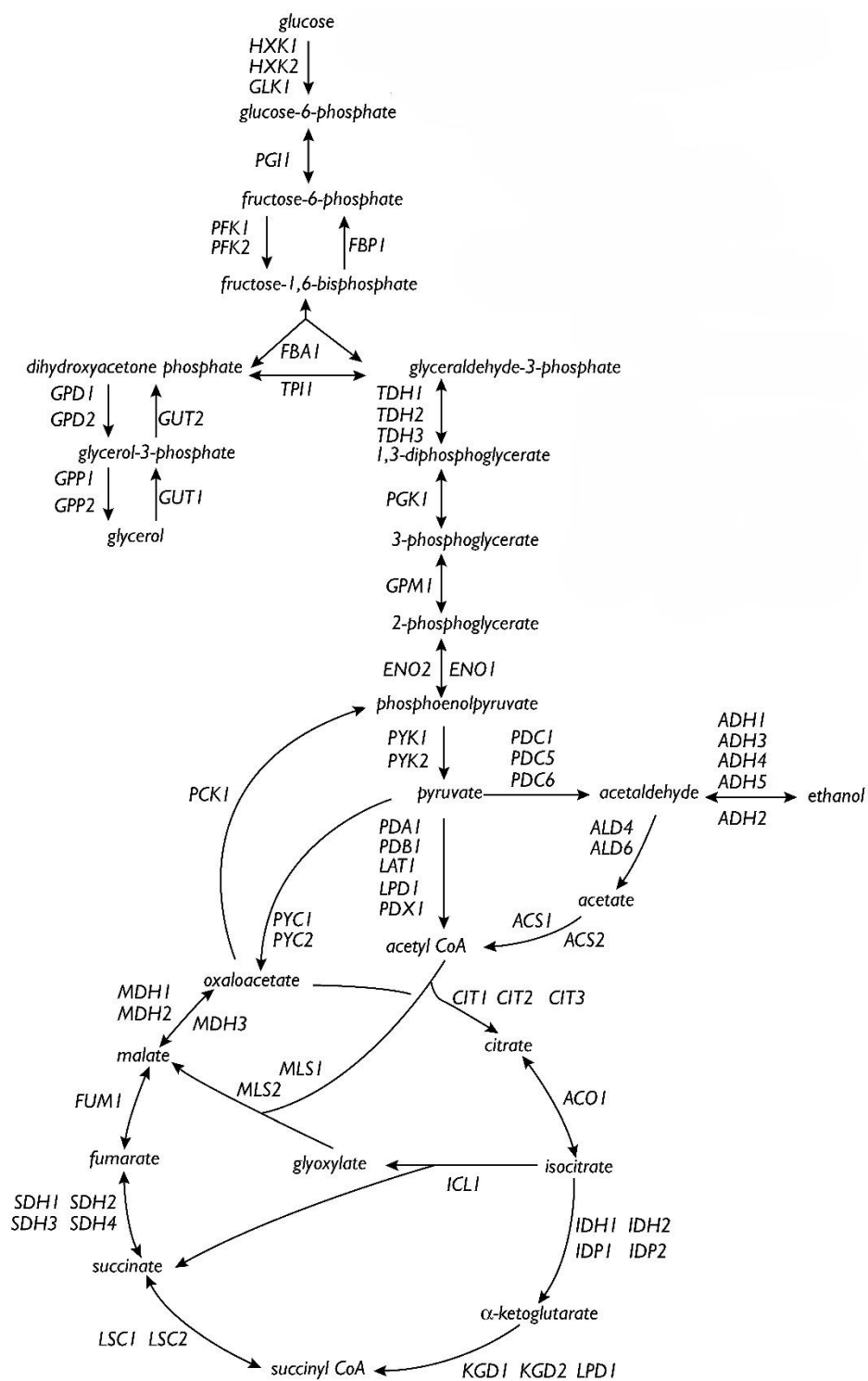
Figure 4. Glycolysis pathway and Krebs cycle in *S. cerevisiae* (Adapted from Dickinson and Schweizer, 2004)

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As an example, HK not only mediates the first step of glycolysis but also displays a transcriptional function involved in the repression of glucose metabolism genes in *S. cerevisiae* (Kim et al., 2005). GPI, in addition to its role during glycolysis (glucose-6-phosphate to fructose-6-phosphate), promotes cell motility and invasion in mammal cells (Watanabe et al., 1996), and the PYK2 enzyme was described to be insensitive to fructose-1,6-biphosphate, which is the most widely known regulator and activator of eukaryotic PYK and is active under conditions of very low glycolytic flux (Boles et al., 1997).

Nevertheless, the enzyme that has been described as a moonlighting protein is GAPDH. Thus, McAllister and Holland (1998) reported that the *TDH1* gene alone cannot support growth, whereas *TDH2* or *TDH3* can. Since then, many studies have reported that GAPDH presents more different functions than the metabolization of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate in several organisms (Sirover, 2011). In mammals, GAPDH is directly involved in transcriptional (Zheng et al., 2003) and posttranscriptional gene regulation (Bonafe et al., 2005; Zhou et al., 2008), vesicular transport (Tisdale et al., 2001; Tisdale et al., 2009), receptor-mediated cell signaling (Harada et al., 2007), chromatin structure (Demarse et al., 2009), maintenance of DNA integrity (Azam et al., 2008) and cytoskeletal structure via tubulin interaction (Sirover, 2011) (Figure 5).
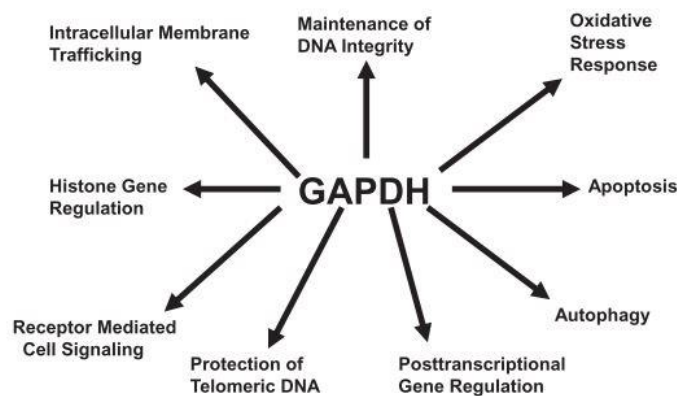
Figure 5. Functional diversity of GAPDH (Sirover, 2011)

Moreover, the GAPDH gene, as one of the most common *housekeeping* genes, is often used to normalize gene expression data (Sirover, 1999). However, in the ascomycete fungus *Neurospora crassa,* GAPDH has been reported to be circadian-regulated, suggesting that the circadian clock may be controlled through this gene regulation and affect cellular metabolism at a basic level (Shinohara et al., 1998). In *S. cerevisiae,* GADPH, a protein previously thought to be restricted to

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the cell interior, also has been described as a cell wall protein (Delgado et al., 2001). Indeed, GAPDH is part of the cell wall in other yeasts such as *Kluyveromyces marxianus* (Falcao Moreira et al., 1998) and *Candida albicans* (Gil-Navarro et al., 1997), and not only is GAPDH present in the cell wall, but the ENO and fructose biphosphate aldolase (FBA) enzymes are as well (Pardo et al., 1999; Edwards et al., 1999). The GAPDH enzyme is encoded by the *TDH1, TDH2* and *TDH3* genes in yeast cells. Tdh1p is the major polypeptide synthesized when cells enter stationary phase, whereas the *TDH3* gene is induced in response to heat shock (Boucherie et al., 1995). Moreover, GAPDH is also involved in wine interactions between microorganisms. Branco and collaborators (2014) revealed that GAPDH-derived peptides act as antimicrobial peptides that are active against a wide variety of wine-related yeasts (*Brettanomyces bruxellensis*) and bacteria (*Oenococcus oeni*).

Furthermore, glycolytic enzymes, including GADPH, PYK and ENO, are part of a complex on the surface of mitochondria in *Arabidopsis* and *Saccharomyces* cells (Enolase complex) (Brandina et al., 2006). The role of a glycolytic complex associated with mitochondria seems to be related to the transport of pyruvate towards mitochondria but also the process of tRNA import. The organization of enzymes into macromolecular complexes can be beneficial to catalytic efficiency and regulation (Brandina et al., 2006). Other authors also reported that glycolytic enzymes are functionally associated with mitochondria (Giegé et al., 2003; Graham et al., 2007).

2.1.2. Respiration

After pyruvate formation, the respiration pathway begins with the transport of pyruvate into the mitochondria (oxidative catabolism). Then, pyruvate is oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (*PDA1, PDB1, LAT1, LPD1* and *PDX1*), resulting in one molecule of NADH. Then, acetyl-CoA, in the presence of oxygen, is degraded by the citric acid cycle (TCA or Krebs cycle) into carbon dioxide and water, generating up to 36 molecules of ATP from one molecule of glucose, plus two ATP from glycolysis. In addition to acetyl-CoA, pyruvate can be transformed to oxaloacetate by pyruvate carboxylase (*PYC1, PYC2*).

The TCA cycle involves 8 steps (Figure 4). First, oxaloacetate reacts with acetyl-CoA to form citrate (the first intermediate in the cycle) by citrate synthase (*CIT1, CIT2* and *CIT3*). Then, citrate is used by aconitase (*ACO1*) to generate isocitrate. The next step of the TCA cycle is the oxidative decarboxylation of isocitrate to α-ketoglutarate, mediated by isocitrate dehydrogenase (NAD-specific, *IDH1, IDH2;* and NADP-dependent, *IDP1, IDP2*). Later, the α-ketoglutarate

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dehydrogenase complex and succinyl-CoA ligase (*KGD1, KGD2, LPD1* and *LSC1, LSC2*, respectively) catalyze the decarboxylation of α-ketoglutarate to succinate via succinyl-CoA. Succinate is transformed to fumarate by succinate dehydrogenase (*SDH1, SHD2, SDH3* and *SDH4*). The last steps of the cycle are mediated by fumarase (*FUM1)*, which converts fumarate into malate, and malate dehydrogenase (*MDH1, MDH2, MDH3*), which performs the oxidation of malate to oxaloacetate.

2.1.3. Alcoholic fermentation

As respiration, alcoholic fermentation starts when pyruvate is formed by glycolysis. However, in this case, pyruvate is cleaved to acetaldehyde and CO_2 by pyruvate decarboxylase (*PDC1, PDC5* and *PDC6*). The acetaldehyde formed can be reduced to ethanol by alcohol dehydrogenase (*ADH1, ADH2, ADH3, ADH4* and *ADH5*) or be metabolized to cytosolic acetyl-CoA via acetate by acetaldehyde dehydrogenase and acetyl-CoA synthase. This acetyl-CoA is used for the biosynthesis of lipids and amino acids. Although *S. cerevisiae* has 20 genes encoding alcohol dehydrogenases (Querol and Fleet, 2006), the two most important are the cytosolic enzymes encoded by the *ADH1* and *ADH2* genes (Lutstorf and Megnet, 1968).

In *S. cerevisiae*, the flux of pyruvate towards the respiratory or fermentative pathway has been widely studied. This flux mostly depends on the kinetic properties of pyruvate dehydrogenase and pyruvate decarboxylase (Querol and Fleet, 2006). Pyruvate dehydrogenase has a ten-fold higher affinity for pyruvate than pyruvate decarboxylase; however, its activity in the cell is low. Hence, at low rates of glucose (respiration), the intracellular pyruvate concentration is below the K_m of pyruvate dehydrogenase, and therefore, pyruvate will be preferentially metabolized via pyruvate dehydrogenase. In contrast, at high intracellular pyruvate concentrations (fermentation), pyruvate will be predominantly metabolized via pyruvate decarboxylase (van Hoek et al., 1998).

2.1.4. Crabtree effect

Some yeast species, such as *S. cerevisiae*, are able to grow either in aerobic or anaerobic conditions. These yeasts have the ability to adapt their metabolism depending on glucose availability. Thus, in the presence of high sugar concentrations (like in grape musts) and despite the presence of oxygen, *S. cerevisiae* can ferment these sugars and produce ethanol. This behavior is referred to as the Crabtree effect (de Deken, 1966). Yeast are classified as Crabtree-positive (Table 1) when they produce ethanol under aerobic conditions, resulting in a lower biomass production due to

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the preferential conversion of sugars into ethanol, and as Crabtree-negative (Table 1) when they are not able to display the Crabtree effect, and their metabolism is limited under oxygen-deprived conditions (Alexander and Jeffries, 1990). Ethanol is produced by Crabtree-positive yeasts as a tool to slow down and control the proliferation of other competitive microorganisms (Dashko et al., 2014; Pronk et al., 1996). In addition, most yeast species grow poorly under strict anaerobiosis, whereas *S. cerevisiae* is able to grow rapidly under this condition. This feature gives *S. cerevisiae* a natural advantage over non-*Saccharomyces* yeasts during alcoholic fermentation (Albergaria et al., 2016).

Table 1. Fermentation mode of some yeast species

Crabtree-positive	Crabtree-negative
Hanseniaspora guilliermondii	*Debaryomyces spp.*
Saccharomyces bayanus	*Hanseniaspora uvarum*
Saccharomyces cerevisiae	*Kluyveromyces marxianus*
Starmerella bacillaris	*Metschnikowia pulcherrima*[2]
Torulaspora delbrueckii[1]	*Pichia spp.*
Zygosaccharomyces bailii	*Rhodotorula spp.*

[1] Respiration process makes a higher contribution to metabolism in this species than in *Saccharomyces* (Albergaria et al., 2016; González et al., 2013)

[2] This species belongs to the respire-fermentative non-*Saccharomyces* group (Albergaria et al., 2016)

Nevertheless, according to recent studies (Quirós et al., 2014; Contreras et al., 2015), classification based on standard Crabtree assays has little predictive power for the behavior of yeast under wine growth conditions. Likewise, important differences are found among yeast strains belonging to the same species (Ciani et al., 2016).

2.2. Nitrogen metabolism

In addition to carbon metabolism, during alcoholic fermentation there are other compounds with remarkable importance such as nitrogen compounds. Nitrogen is the second most abundant nutrient in wine fermentation (Varela et al., 2004), and therefore, it plays a predominant role in the fermentation process. Yeasts can use a wide variety of nitrogen compounds, such as amino acids, ammonium ions and small peptides (called yeast assimilable nitrogen, excluding proline). Thus, the nitrogen composition of must can affect the fermentation performance in terms of biomass production and fermentation rate (Varela et al., 2004), since it is essential for yeast

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metabolism and growth. In fact, the absence of nitrogen during the fermentation process is one of the main reasons for stuck or sluggish fermentation (Bisson, 1999; Taillandier et al., 2007). Nevertheless, not all nitrogen sources support growth equally well. Thus, yeast cells present higher growth rates in the presence of good nitrogen sources (preferred) such as ammonium, glutamine and asparagine, rather than poor nitrogen sources (non-preferred), including urea and proline. Aromatic amino acids do not support a high growth rate and accumulate early in fermentation (Beltran et al., 2005).

Therefore, *S. cerevisiae* has developed a system known as Nitrogen Catabolism Repression (NCR), which selects the best nitrogen sources for growth. The NCR mechanism acts at two levels in order to reduce the proteins responsible for utilization and uptake of non-preferred nitrogen sources when preferred sources are available in the medium. The first consists of inactivation and degradation of non-preferred nitrogen source permeases, and the second consists of repression of genes that encode for non-preferred nitrogen source permeases (Magasanik and Kaiser, 2002; ter Schure et al., 2000). With this mechanism, yeast cells ensure use of the best nitrogen sources. However, the categorization between *good* and *bad* nitrogen sources depends on the carbon backbone resulting from the metabolism of these amino acids (Gutierrez et al., 2013). Thus, the transamination or deamination of *good* sources produces easily assimilable carbon compounds, whereas amino acids with a complex carbon backbone need to be detoxified or go through a complex metabolism, resulting in slow growth (Mas et al., 2014).

The first step for cells to use nitrogen compounds is internalization. On one hand, amino acids are transported across the membrane by permeases. *S. cerevisiae* contains 19 permeases for amino acids; however, only three are high-capacity permeases and are regulated by nitrogen: *AGP1* (high-Affinity Glutamine Permease), *GAP1* (General Amino acid Permease) and *PUT4* (Proline UTilization). The *GAP1* and *PUT4* permeases are active during growth in non-preferred nitrogen content and repressed when preferred nitrogen sources are available (Forsberg and Ljungdahl, 2001). Otherwise, *AGP1* is active in the presence of a preferred nitrogen source (Regenberg et al., 1999). Once inside the cell, amino acids can be used as building blocks in biosynthetic pathways or be deaminated to ammonium or transaminated to glutamate (Godard et al., 2007). Yeast cells transform any nitrogen molecule to glutamate or glutamine, and thus, these amino acids are used as nitrogen donors for the synthesis of all nitrogen-containing compounds in the cell (Magasanik, 1992).

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On the other hand, ammonium is incorporated into the cell by three permeases (*MEP1, MEP2* and *MEP3*). These permeases are only active when ammonium is present in low concentrations and are repressed in the presence of good nitrogen sources due to the NCR mechanism (Marini et al., 1997). Under glucose growth conditions, ammonium is metabolized by two reactions. First, ammonium is used to transform α-ketoglutarate into glutamate by glutamate dehydrogenase (*GDH1*). The second reaction is the synthesis of glutamine from ammonium and glutamate by glutamine synthase (*GLN1*). Moreover, when glutamine is the only source of nitrogen, a glutamate synthase (*GLT1*) metabolizes glutamine in glutamate. From this glutamate, ammonium can be released by glutamate dehydrogenase (*GDH2*). Alcoholic fermentation is a good example of NCR. Beltran and collaborators (2004) reported that when yeast cells are exposed to limited nitrogen conditions, the expression of *GAP1* and *MEP2* is highly activated, whereas under non-limited nitrogen, the expression of both genes decreases.

2.2.1. Production of fusel alcohols during winemaking

In grape must, the principal source of assimilable nitrogen in yeast is amino acids. Some amino acids (branched-chain amino acids (isoleucine, leucine and valine), aromatic amino acids (phenylalanine, tyrosine and tryptophan) and the sulfur-containing amino acid (methionine)) are mainly catabolized via the Ehrlich pathway in order to form a corresponding fusel acid or fusel alcohol. In this pathway, proposed by Ehrlich a century ago (Ehrlich, 1907), amino acids are taken up slowly throughout the fermentation process when preferred nitrogen sources are not available (Jones and Pierce, 1964; Beltran et al., 2005; Hazelwood et al., 2008; González et al., 2018b). The Ehrlich pathway consists of three steps (Figure 6). First, an aromatic amino acid is transformed into its α-keto acid by an aromatic amino acid aminotransferase (*ARO8, ARO9*). Then, the α-keto acid is decarboxylated to the corresponding aldehyde by a decarboxylase (*ARO10, PDC6, PDC5* and *PDC1*). Finally, depending on the redox state of the cell, the aldehyde can be metabolized to the aromatic alcohol (tryptophol, phenyl ethanol and tyrosol from tryptophan, phenylalanine and tyrosine, respectively) by an alcohol dehydrogenase (*SF1, ADH4, ADH5*) or the corresponding acid (indole acetic acid, phenyl acetic acid and 4-hydroxyphenyl acetic acid) by an aldehyde dehydrogenase (*ALD2, ALD3, ALD5*) (Mas et al., 2014). Some of these higher alcohols affect wine aroma, especially 2-phenylethanol, which is highly appreciated due to its scent of roses.

Moreover, these higher alcohols, tryptophol, phenyl ethanol and tyrosol, are produced and secreted by yeast into the extracellular medium and accumulate during alcoholic fermentation,

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being tryptophol one of the most commonly produced alcohols (González et al., 2018a) due to the yeast uptake of this amino acid early during fermentation (Beltran et al., 2005). Not only *S. cerevisiae* produces these higher alcohols; indeed, recent studies have provided evidence that other wine yeast species (*H. uvarum, S. bacillaris, M. pulcherrima, T. delbrueckii*, among others) also produce these compounds but in lower amounts (González et al., 2018a; Zupan et al., 2013). This lower synthesis is probably due to a different distribution of metabolic flux, including ethanol production, biomass and product formation, during fermentation in non-*Saccharomyces* strains (Ciani et al., 2000; Tofalo et al., 2012).

In addition, these compounds have been associated with cell signaling via modulation of physiological and morphological processes such as pseudohyphal growth (Mas et al., 2014). Recent studies have reported that higher alcohols can induce pseudohyphal and invasive growth either in *Saccharomyces* or non-*Saccharomyces* yeasts. However, cell signaling by aromatic alcohols to perform pseudohyphal and invasive growth seems to be a species-specific response, since different species exhibit different responses to these molecules (Chen and Fink, 2006; González et al., 2018a).

2.2.2. Production of indole compounds in wine

In addition to ethanol and fusel alcohols produced during winemaking, other metabolites derived from aromatic amino acids, such as melatonin and serotonin, are of special relevance for their bioactivity in higher organisms, in which their biosynthesis pathways are well known (Mas et al., 2014). Melatonin regulates circadian rhythm, and serotonin is a neurotransmitter and is known as the *happiness* hormone (Young et al., 2007). Both melatonin and serotonin have been found in either laboratory or commercial wines (Fernández-Cruz et al., 2017, 2019a; 2019b; Rodriguez-Naranjo et al., 2011a; Vigentini et al., 2015). Although these bioactive compounds are found in low quantities in wines, these levels could be enough because one characteristic of bioactive compounds is that a minimal concentration is required to be active (Hornedo-Ortega et al., 2016). Thus, given the importance of these compounds in humans, research on bioactive aromatic amino acid derivatives in yeast has increased.

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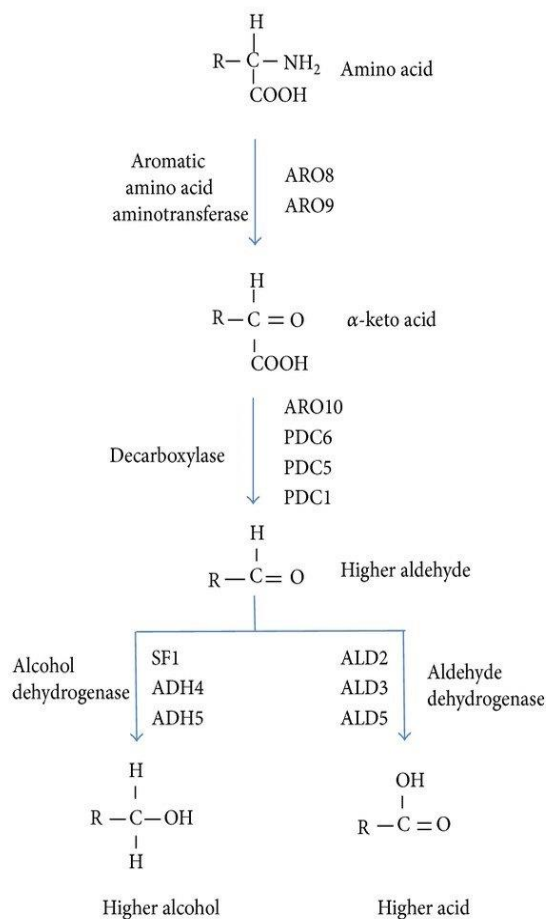
Figure 6. Ehrlich pathway (Mas et al., 2014)

Melatonin can be found in several and different types of wines. Table 2 shows a comparison of melatonin concentrations found across wines from different countries. Differences among wines depend on the yeast that performed the fermentation (Vigentini et al., 2015) but also on environmental features, which can affect the grapes and modify the nutrients available in the grape must (Querol et al., 2018; Wang et al., 2016). Melatonin synthesis largely depends on the concentration of tryptophan, reducing sugars and the growth medium (Rodriguez-Naranjo et al., 2012). However, little is known about melatonin metabolism in yeast cells, and thus, other organisms such as plants or mammals will be used as references.

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Table 2. Concentrations of melatonin in wine

Wine	Concentration (ng/mL)	References
Alaban, Sangiovese, Trebbiano (Italy)	0.4-0.6	Mercolini et al., 2012
Chardonnay, Malbec, Cabernet Sauvignon (Argentina)	0.16-0.32	Stege et a., 2010
Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Vijiriega and Tempranillo (Spain)	0.07-322.70	Fernández-Cruz et al., 2019a
Gropello, Merlot (Italy)	5.2-8.1	Vitalini et al., 2013
Cabernet Sauvignon, Merlot, Syrah, Tempranillo, Tintilla de Rota, Petit Verdot, Prieto Picudo and Palomino Fino (Spain)	5.1-420	Rodríguez-Naranjo et al., 2011b
Honggouo2 and Baiyuwang (China)	0.58-14.20	Wang et al., 2016
Nebbiolo (Italy)	0.038-0.063	Fracassetti et al., 2019
Tempranillo, Garnacha (Spain)	0.03-161.83	Marhuenda et al., 2016

3. Melatonin

Melatonin (N-acetyl-5-tryptamine) (Figure 7) is an indole amine that belongs to the family of methoxyindoles and is synthetized from the aromatic amino acid tryptophan. It is known as a neurohormone since it was discovered in the pineal gland of vertebrates and was originally believed that it was unique from vertebrates. However, over the last two decades, many studies have described the presence of melatonin in a wide range of invertebrates, plants, bacteria, nematodes and fungi (Hardeland and Poeggeler, 2003). Therefore, melatonin is considered a ubiquitous molecule because it is present in most living organisms.

Melatonin was first isolated and identified in the bovine pineal gland (Lerner et al., 1958) Their finding was that melatonin had the ability to change the color of the skin in amphibians and reptiles. Therefore, its name is composed of "*mela*", for its bleaching effect, and "*tonin*" because it derives from serotonin.

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In 1975, Lynch and collaborators described that melatonin exhibited a circadian rhythm in the human pineal gland, with higher concentrations in the night. Since then, melatonin has been related to multiple functions such as the regulation of circadian rhythm, synchronization of the reproductive cycle, anti-aging, antioxidant and anti-inflammatory activity, and modulation of neural, endocrine and immune functions (Eghbal et al., 2016; Romero et al., 2014). Hence, not only does melatonin develop different functions, but it is also distributed in all cells in the human body.
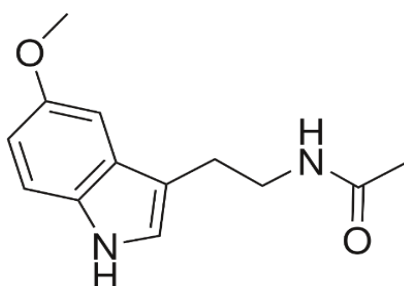
Figure 7. Chemical structure of melatonin

3.1. Melatonin synthesis

The classic melatonin synthetic pathway in animals was first deduced by Axelrod and Weissebach in 1960 (Axelrod et al., 1960; Weissebach et al., 1960). Biosynthesis of melatonin starts from the aromatic amino acid tryptophan, which is the principal precursor. Tryptophan is converted into melatonin in four consecutive enzymatic steps (Tan et al., 2016). Tryptophan is first hydroxylated to form 5-hydroxytryptophan by tryptophan-5-hydroxylase. Consequently, this product is decarboxylated to 5-hydroxytryptamine (or serotonin) under the catalytic action of an aromatic amino acid decarboxylase. Then, serotonin is acetylated to form N-acetylserotonin by arylalkylamine N-acetyltransferase, and finally N-acetylserotonin is methylated to melatonin by N-acetylserotonin O-methyltransferase. The classic melatonin biosynthetic pathway is illustrated in Figure 8.

As in humans, melatonin research in plants is a rapidly developing field (Arnao and Hernández-Ruiz, 2015). Melatonin has been described in many plants and derivatives (Paredes et al., 2009) at variable levels: from almost undetectable to higher concentrations, one order of magnitude higher than that in vertebrates (Hardeland et al., 2006; Hardeland, 2017). Melatonin synthesis depends on the availability of the precursor; the lowest concentration of tryptophan, the most limited the

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biosynthetic capacity of melatonin is. Plants and some microorganisms are able to synthesize tryptophan through the shikimic acid pathway according to certain requirements. Thus, tryptophan is first decarboxylated to tryptamine by tryptophan decarboxylase, and then tryptamine is hydroxylated to form serotonin by tryptamine-5-hydroxylase. The last two steps of the pathway are believed to be the same as those in animals (Figure 8).
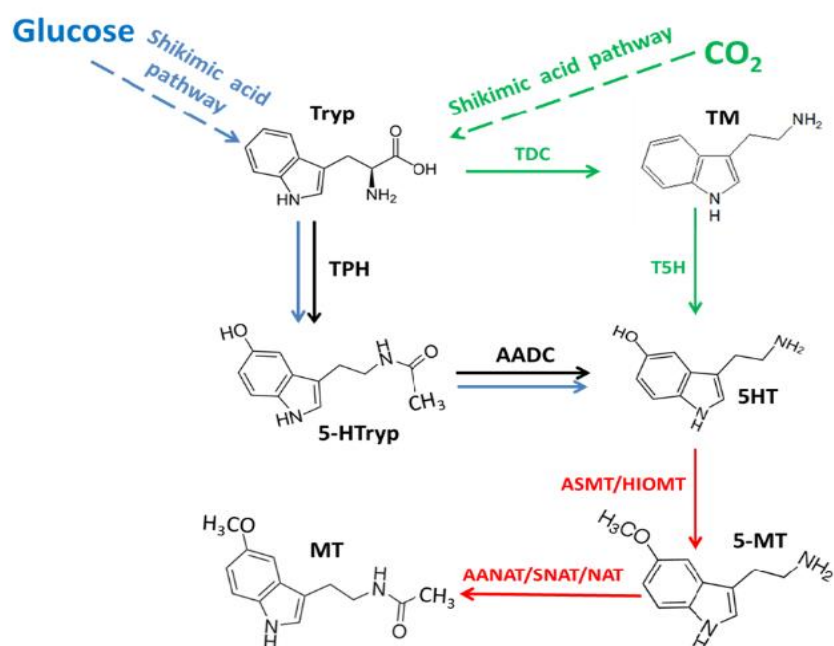
Figure 8. Hypothesized melatonin pathway in animals (black), plants (green) and yeasts (blue). Common steps in red. (Tan et al., 2016). Abbreviations: Tryp, tryptophan; TM, tryptamine; 5-HTryp, 5-hydroxytryptophan; 5HT, serotonin; MT, melatonin; 5-MT, N-acetylserotonin; TDC, tryptophan decarboxylase; TPH, tryptophan-5-hydroxylase; T5H, tryptamine-5-hydroxylase; AADC, aromatic amino acid decarboxylase; ASMT/HIOMIT, arylalkylamine N-acetyltransferase; AANAT/SNAT/NAT, N-acetylserotonin O-methyltransferase

However, the melatonin synthesis pathway in yeast is completely unknown. Only one gene has been described and characterized to be involved in melatonin production: *PAA1*, a polyamine acetyltransferase, homolog of the mammalian arylalkylamine N-acetyltransferase (Ganguly et al., 2001). Nevertheless, a recent study (Muñiz-Calvo et al., 2019) has proposed a putative melatonin biosynthetic pathway in *S. cerevisiae* that matches perfectly with the one detailed in plants (Figure 9). However, in yeast, alternative steps for the production of some precursors also have been uncovered. In fact, from serotonin, two intermediates are possible, 5-methoxy tryptamine and N-acetylserotonin, although the former is the preferred substrate to produce melatonin. In addition, the hydroxylation of tryptophan to 5-hydroxy tryptophan does not seem to happen in yeasts, despite their ability to transform this molecule into serotonin. Finally, some reversible steps have

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been proposed, since yeast can reconvert melatonin into N-acetyl-serotonin and 5-methoxytryptamine and serotonin into 5-hydroxy tryptophan (Muñiz-Calvo et al., 2019).
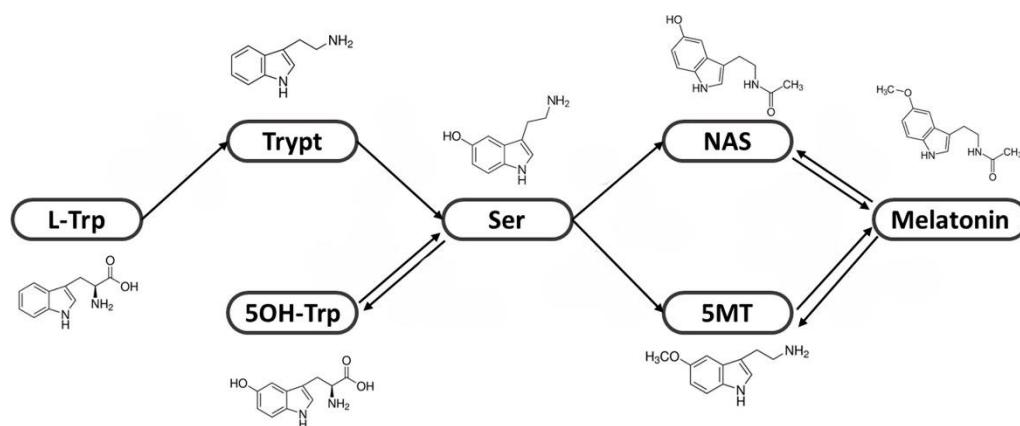
Figure 9. Hypothesized melatonin pathways in *S. cerevisiae* (Adapted from Muñiz-Calvo et al., 2019). Abbreviations: Trp, tryptophan; Trypt, tryptamine, Ser, serotonin, NAS, N-acetylserotonin; 5MT, 5-methoxytryptamine; 5OH-Trp, 5-hydroxytryptophan

3.2. Melatonin in yeast

3.2.1. Role in yeast

Melatonin is a ubiquitous molecule that has been described in all kingdoms. Previous studies indicated that the principal function of melatonin is mainly related to cell defense against oxidative stress (Tan et al., 2014). Indeed, this antioxidant role is highly conserved among melatonin-producing organisms (Hardeland and Poeggeler, 2003) and has been widely studied in mammals. However, until recently no evidence was available for yeast (Bisquert et al., 2018).

It is well known that oxidative stress is one of the main stressors that yeast cells encounter during ethanol fermentation (Auesukaree, 2017), and melatonin supplementation has been recently reported to alleviate yeast oxidative stress generated in stationary phase, decreasing intracellular reactive oxygen species and lipid peroxidation and thus allowing cells to better endure the stress (Vázquez et al., 2017, 2018). Additionally, melatonin activates antioxidant defense system genes, increases peroxisome accumulation and slightly raises catalase activity when cells are exposed to oxidative stress. This protective effect of melatonin takes place in both *Saccharomyces* and non-*Saccharomyces* yeasts (Vázquez et al., 2018). Furthermore, the presence of melatonin helps to enhance the performance of fermentation when non-*Saccharomyces* are present. Non-*Saccharomyces* yeasts, especially *S. bacillaris,* are able to persist until the end of fermentation when melatonin is available in low-nitrogen conditions (Valera et al., 2019).

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On the other hand, Bisquert et al. (2018) have also described the positive effect of melatonin in protecting yeast cells against UV radiation. The stress produced by UV radiation is closely related to oxidative stress since it is a significant source of reactive oxygen species and DNA damage (Farrugia and Balzan, 2012). Melatonin improves viability and growth performance after UV radiation stress. In fact, melatonin seems to prepare cells against upcoming stress by modifying the gene expression of antioxidant and DNA repair genes (Bisquert et al., 2018). Moreover, studies with high UV-exposed plant organs showed that melatonin can serve as a UV protector and promotes seed viability (Hardeland, 2016).

Therefore, as described in humans, melatonin in yeast might protect various biomolecules from damage caused by free radicals by acting as a direct scavenger, detoxifying reactive oxygen and nitrogen species (Anisimov et al., 2006; Reiter et al., 2001; Reiter et al., 2016) and indirectly increasing the activities of antioxidant defense systems (Vázquez et al., 2017).

3.2.2. Production in yeast

Despite the fact that the melatonin biosynthesis pathway and its role in yeast are still unknown, many studies have shown evidence for the presence of this indole during alcoholic fermentation. In 1999, Sprenger and collaborators conducted the first study of melatonin synthesis in fungi. In this work, the authors reported *S. cerevisiae* is able to synthesize melatonin from different precursors such as tryptophan, serotonin, N-acetylserotonin or 5-methoxytryptamine. Since then, most studies have focused on demonstrating the role of yeast in the biosynthesis of melatonin in a fermentative context. Rodriguez-Naranjo et al. (2011b) compared melatonin content before (initial must) and after (wine) alcoholic fermentation to establish the main role of yeast in its production. Differences were observed in melatonin content depending on the wine grape variety, varying from 74.13 to 423.01 ng/mL. Later, Rodriguez-Naranjo and collaborators (2012) used synthetic medium to study the best conditions for yeast to produce melatonin, which depends on the yeast growth phase and the medium composition, especially the concentrations of tryptophan and reducing sugars. Similar results were obtained by Fernández-Cruz and collaborators (2016), who outlined that melatonin was extracellularly detected on the second day of fermentation, at the end of the exponential growth phase, and its synthesis seems to be directly related to cell growth. Later, the same researchers stated that non-*Saccharomyces* yeasts also synthesized melatonin (Fernández-Cruz et al., 2017) and that putative intermediaries of the melatonin pathway are also formed during alcoholic fermentation (Fernández-Cruz et al., 2019a, 2019b). However, it is important to highlight that melatonin production seems to depend on the strain

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(Vigentini et al., 2015). Until recently, melatonin production had only been investigated in single-inoculum fermentations; however, Valera and collaborators (2019) examined melatonin synthesis by mixed inocula of *Saccharomyces* and non-*Saccharomyces* yeasts. Significant differences were highlighted between inocula: in *S. cerevisiae* single fermentation, melatonin appeared and disappeared very fast during exponential phase, while in mixed inocula with non-*Saccharomyces* participation, melatonin accumulated extracellularly in the medium.

All of these studies have only focused on extracellular melatonin; however, recent studies have detected melatonin intracellularly. Muñiz-Calvo and collaborators (2019) induced pulses with several putative intermediaries of the melatonin pathway to cells in different growth phases and observed intracellular melatonin after supplementation of the medium with N-acetyl-serotonin and 5-methoxytryptamine. Similar results were achieved by Fernández-Cruz and collaborators (2019b); in this case, intracellular melatonin was detected after a tryptophan pulse.

3.3. Detection methods in food

Given the importance of melatonin to human health, research on developing detection methods has significantly increased in recent years. Although taking samples of animal blood or urine is simple, non-destructive and can often be assayed directly to determine the presence of melatonin; other samples, such as those from plants or yeast cells, must normally undergo extraction and purification before indole analysis (Van Tassel and O'Neill, 2001, Fernández-Cruz et al., 2016). Moreover, it is important to keep in mind that melatonin occurrence in food samples is fairly low (at ng/mL level).

Melatonin has been detected by gas chromatography coupled to mass spectrometry (GC-MS) (Van Tassel et al., 2001). This method was compared with radioimmunoassay (RIA), with GC-MS being more sensitive. Melatonin can also be determined by enzyme-linked immunosorbent assay (ELISA), a method based on immunologic detection. This technique has been used in grape skin and beer with successful detection results (Iriti et al., 2006; Maldonado et al., 2009). In contrast, Rodriguez-Naranjo and collaborators (2011a) observed that an ELISA assay is not suitable for assaying melatonin in wines. In fact, the main immunoassay drawbacks were cross-reactions with molecules that mimic the melatonin structure or cross-react within the immunoassay (Paredes et al., 2009). To mitigate cross-reactions, Pape and Lüning (2006) developed an immunoprecipitation method in which melatonin was separated by high-performance liquid chromatography (HPLC) and then analyzed by ELISA. Muszynska and

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collaborators (2014) described a thin-layer chromatographic method coupled with densitometric detection to analyze the presence of melatonin and related indoles in garlic (*Allium sativum*) bulbs. Another method to determine melatonin is by chemiluminescence using potassium permanganate and formaldehyde. The advantages of this technique are speed, high sensitivity, low detection limit and a wide range of linear responses (Chen et al., 2003). Recently, Muñiz-Calvo and collaborators (2017) applied a solid-state electrochemical technique to detect and monitor the production of melatonin.

3.3.1. HPLC-MS/MS

Despite the fact that several of the above-described analytical methods have been used to detect melatonin in food, the most commonly used method is HPLC coupled to fluorescence or tandem mass spectrometry (MS/MS) detection. In melatonin determination, a chromatographic separation prior to detection is important to prevent any interference due to the complexity of the food matrices (Kocadagli et al., 2014). Many studies have employed this method to determine melatonin in food matrices because HPLC exhibits limits of detection and quantification that are very low (Table 3) relative to other techniques.

After a chromatographic separation of melatonin by HPLC, this molecule is often detected by a fluorescence detector (Mercolini et al., 2008; Rodriguez-Naranjo et al., 2011a). Recently, Mercolini and collaborators (2012) described a cheaper and more useful method in grape-related matrices. Melatonin was pretreated by performing a miniaturized microextraction with packed sorbent, which is faster than conventional solid phase extraction procedures. However, the most commonly used detection method in melatonin analysis is HPLC coupled to MS/MS because it allows obtaining an exact identification of the compound and accurate quantification in food samples.

The MS/MS detection has been used to determine the presence of melatonin in wine. The advantage of this technique is the simplified sample preparation (dilution or filtration) and the structural information provided by the MS detectors that unequivocally support identification (Kocadagli et al., 2014; Tudela et al., 2016). Melatonin can present up to eight isomers (Diamantini et al., 1998). No isomers were detected in oenological conditions but two were measured in laboratory conditions (Vigentini et al., 2015). Rodriguez-Naranjo and collaborators (2011a) observed that in wine, not only melatonin could be detected, but also one isomer. However, Gardana and collaborators (2014) uncovered that the compound believed to be a melatonin

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isomer by Rodriguez-Naranjo (2011a) was actually a tryptophan-derived compound, tryptophan ethyl ester (TEE). These findings emphasize the need for advanced analytical techniques to elucidate the indole-derived compounds involved in melatonin metabolism (Tudela et al., 2016). These authors developed a simultaneous determination of nine target indoles, including melatonin and TEE, in sparkling wines based on ultrahigh-performance liquid chromatography (UHPLC) coupled with MS/MS. In parallel, another simultaneous determination of melatonin-related compounds was developed to study their production throughout the fermentation process (Fernández-Cruz et al., 2016).

Table 3. Comparison of HPLC detection methods for melatonin

Method	LOD (ng/mL)	LOQ (ng/mL)	References
HPLC-fluorescence detection (HPLC-F)	0.01	0.03	Mercolini et al., 2008
HPLC-fluorescence detection (HPLC-F)	51.72	172.90	Rodriguez-Naranjo et al., 2011a
HPLC- ion trap spectrometer with electrospray ionization (LC-ESI-MS/MS)	0.13	0.44	Rodriguez-Naranjo et al., 2011a
HPLC- ion trap spectrometer with electrospray ionization (LC-ESI-MS/MS)	0.030	0.085	Gómez et al., 2012
MEPS extraction-HPLC-fluorescence detection (MEPS-HPLC-F)	0.02	0.05	Mercolini et al., 2012
HPLC-triple quadrupole mass spectrometer (LC-MS/MS)	0.0337	0.1123	Kocadagli et al., 2014
UHPLC- triple quadrupole mass spectrometer with electrospray ionization (UHPLC-MS/MS)	0.1	0.5 *	Vigentini et al., 2015
UHPLC- triple quadrupole mass spectrometer (UHPLC-MS/MS)	0.0047	0.0144	Fernádez-Cruz et al., 2016
UHPLC- triple quadrupole mass spectrometer (UHPLC-MS/MS)	0.0013	0.0023	Fracassetti et al., 2019

LOD: limit of detection; LOQ: limit of quantification; MEPS: microextraction by packed sorbent

*Authors did not calculate LOQ, but LLOQ (lower limit of quantification) as the lowest standard curve point used for quantification

Limits of detection (LOD) and limits of quantification (LOQ) are represented in Table 3. Regarding LOD values in melatonin, authors have reported different values for the determination of this indole by HPLC-MS/MS. Kocadagli et al. (2014) determined melatonin in red wine and

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obtained an LOD of 0.034 ng/mL with a triple quadrupole, whereas an LOD of 0.13 ng/mL was reported in wine by using an ion trap (Rodriguez-Naranjo et al., 2011a). In similar conditions, Gomez and collaborators (2012) obtained a much lower LOD (0.03 ng/mL) Finally, Fernández-Cruz et al. (2016) reported a lower LOD in synthetic must using a hybrid quadrupole-orbitrap mass spectrometer (0.0047 ng/mL). However, a recent study proposed a method based on UHPLC-MS/MS along with an SPE extraction procedure that obtained an even lower LOD in wine (0.0023 ng/mL; Fracassetti et al., 2019).

3.3.2. Cell lines as reporters

In addition to chromatographic methods, other techniques based on the activity of specific genes or enzymes, such as mammalian cell-based assays, have been widely used (Naylor et al., 1999). The principal advantages of these assays are their high sensitivity, reliability, convenience and adaptability to large-scale measurements (Naylor et al., 1999). To date, numerous reporter genes have been used for monitoring gene and enzyme activity. Indeed, the success of cell-based assays depends entirely on the quality of the reporter gene (Qureshi et al., 2007). The most widely known and used are luciferase, galactosidase (*lacZ*), green fluorescence protein (GFP) and β-lactamase. Despite the fact that luciferase-based assays provide good sensitivity and a wide dynamic range, the bioluminescence produced after the breakdown of D-luciferin is transient. Another important reporter gene is the *lacZ*-encoded enzyme β-galactosidase (Naylor et al., 1999). One of the drawbacks of this system is the crossing of the substrate through the plasma membrane. Given that fluorescence signal intensity depends on both enzyme and substrate concentrations, the assay is affected by the absence of substrate loading. In addition, the free fluorescent product of the reaction rapidly diffuses out of the cell. In spite of all of the shortcomings of the system, it has been used as a tool for studying protein-protein interactions in live cells (Rossi et al., 2000). GFP is another commonly used reporter system (Tsien, 1998) from the jellyfish *Aequora Victoria*. The main advantages of GFP are that it is a noninvasive system and does not require a substrate to be fluorescent. Nevertheless, the detection limit is very high (10^5 molecule/cell) because there is no enzyme amplification, and many molecules are needed to visualize the fluorescence (Qureshi et al., 2007).

The β-lactamase enzyme (BLA) is often used as reporter system for gene expression and enzyme activity. BLA is a 29-kDa enzyme that is active either as a monomer or when it is fused to a heterologous protein (Zlokarnik, 2000). There is no ortholog BLA gene in eukaryotes.

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Nevertheless, the BLA system as a reporter gene was not used efficiently until Zlokarnik and collaborators (1998) described the synthesis of a fluorogenic BLA substrate (CCF2 or CCF4). CCF2/4 is composed by two fluorescent dyes, 7-hydroxycoumarin-3-carboxamide and fluorescein, bridged by cephalosporin. CCF2/4-AM (acetoxymethylated) enters into the cell through the plasma membrane due to its lipophilic character, without inducing stress to the cell. As shown in Figure 10, once CCF2/4-AM enters inside the cell, endogenous esterases convert it to CCF2/4, which is negatively charged at physiological pH and therefore stays inside the cell. CCF2/4 is detected by fluorescence resonance energy transfer (FRET). When this molecule is intact, it is excited at 409 nm and produces green fluorescence (λ= 518 nm). When BLA cleaves its substrate, it separates the two fluorophores and causes loss of FRET, resulting in excitation of the coumarin and producing blue fluorescence (λ= 447 nm). In fact, if CCF2/4 is used as substrate, as few as 50 molecules of BLA can be detected within a cell (Zlokarnik et al., 1998). Furthermore, the BLA system provides high sensitivity due to the absence of endogenous BLA activity in mammalian cells.

BLA-based cell-based assays have been developed for various classes of gene families, including G-protein coupled receptors (GPCRs) (Kunapuli et al., 2003) such as melatonin receptors. In addition, these systems have been used to study protein folding (Bowden and Salmond, 2006, Camps et al., 2003), protease activity (Whitney et al., 2002), gene trapping (Lai et al., 2002, Whitney et al., 1998), RNA splicing (Hasegawa et al., 2003) and protein-protein interactions by fragment complementation (Lee et al., 2004, Spotts et al., 2002).
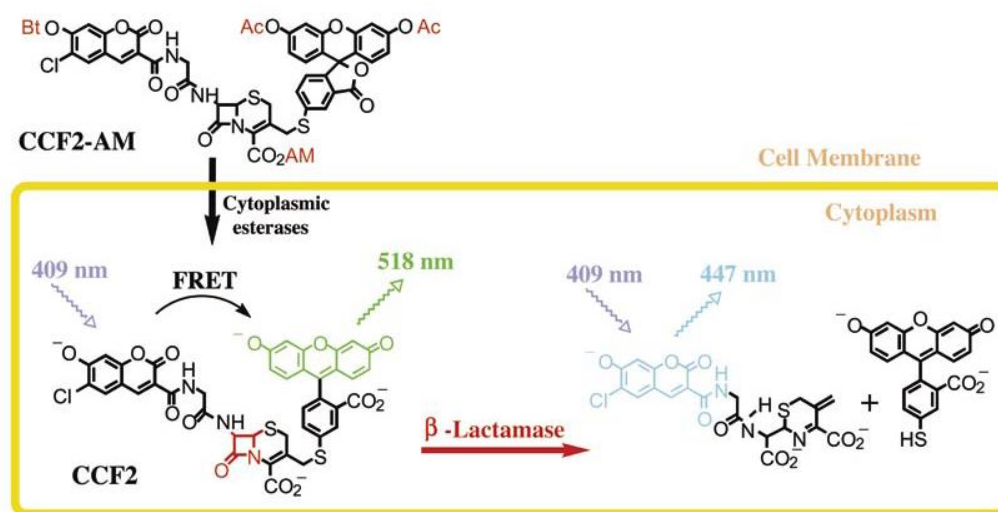
Figure 10. BLA reporter system (Qureshi, 2007)

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3.4. Known interactions in mammals

The different functions of melatonin in human cells suggest the existence of specific receptors. The existence of these binding sites was suggested in the early 1970s by kinetic studies using ^{3}H-melatonin (Cardinali et al., 1973). In fact, melatonin shows its function through four mechanisms (Emet et al., 2016): its binding to receptors in the plasma membrane, to intracellular proteins, to orphan nuclear receptors and the antioxidant effect itself.

3.4.1. Receptors

Melatonin-specific receptors are widely studied in mammals. Since Lerner (1958) reported the presence of melatonin in the pineal gland, many studies have shown the existence of interaction sites for the binding of this molecule. Dubocovich and collaborators (1988) described that melatonin bound not only to the pineal gland but also to other parts of the human body. At present, melatonin is formed in numerous organs and cells, such as the gastrointestinal tract, bone marrow, several leukocytes, membranous cochlea and, presumably, skin and other regions of the central nervous system (Hardeland, 2012). Finally, Reppert and collaborators (1994; 1995; 1997) reported that melatonin was associated with two transmembrane proteins that belong to the GPCR superfamily (MT1 and MT2) in mammals.

However, currently, three membrane receptors and one nuclear receptor for melatonin are known. The first one is a receptor type 1a (MT1 or MTNR1A), encoded in human chromosome 4, which consists of 351 amino acids (Li et al., 2013). The second is a receptor type 1b (MT2 or MTNR1B), encoded in human chromosome 11, which consists of 363 amino acids. These two are present in mammals. Another melatonin receptor (MTRN1C) has been described in fish, amphibians and birds but not in humans (Li et al., 2013) and exhibits a rhythm totally opposite to that of MT1 and MT2, with high levels in daytime and low levels at night time (Li et al., 2013).

Furthermore, melatonin also binds to the MT3 receptor, also known as the detoxification enzyme (quinone reductase 2) (Emet et al., 2016). This enzyme belongs to the reductase group and is involved in protection against oxidative stress by preventing electron transfer reactions of quinones (Pandi-Perumal et al., 2008). In addition, melatonin interacts with transcription factors of the retinoic acid receptor superfamily in the nucleus (Becker-André et al., 1997), specifically receptor-α (RZR/RORα) and GPR50, which is the orthologue of MTRN1C in humans. This gene is located on the X chromosome and consists of 618 amino acids (Li et al., 2013). Melatonin does

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not bind to this GPR50; however, when GPR50 dimerizes with MT1, it inhibits the melatonin signal on the receptor (Levoye et al., 2006).

3.4.2. Other compounds

Despite the existence of specific receptors for melatonin binding, other molecules also have been found to interact with this molecule: tubulin, calmodulin, calreticulin and sirtuins.

3.4.2.1. Tubulin

Tubulin is a globular protein that belongs to microtubules. In the early 1970s, melatonin was reported to affect microtubule or microfilament-dependent processes in living organisms (Cardinali et al., 1997). The hypothesis was that melatonin inhibited the effects of colchicine (a drug that does not allow division by binding to tubulin) in order to prevent the assembly of tubulin dimers into microtubules (Cardinali et al., 1997). However, melatonin had no effect on the *in vitro* assembly of microtubules (Cardinali and Freire, 1975). Many studies reported that the presence of melatonin increased the number of microtubule elongations and contact between cells (Benitez-King et al., 1990; Melendez et al., 1996; Cardinali et al., 1997). This finding supported the idea that the tubulin polymerization process is one of the targets of melatonin action. In addition to tubulin, melatonin also inhibited the expression of actin mRNA in the hypothalamus (Iovannna et al., 1990). Regarding microtubules, melatonin also binds to calmodulin (Benítez-King et al., 1993). Huerto-Delgadillo and collaborators (1994) suggested that at a low concentration of melatonin (10^{-9} M), the cytoskeleton is affected by melatonin antagonism to Ca^{2+}/calmodulin. However, at a higher concentration of the indole (10^{-5} M), nonspecific binding of melatonin to tubulin occurs.

3.4.2.2. Calmodulin

Calmodulin is a protein involved in Ca^{2+}-dependent signaling. A calcium signal is required for the activation of some proteins (Chin and Means, 2000). The relation between calmodulin and melatonin was described by Benitez-King and collaborators (1993). Calmodulin levels changed when cells were cultured with nanomolar concentrations of melatonin, which inhibited the calmodulin-dependent phosphodiesterase. Moreover, specific binding of ^{3}H-melatonin to a single site on calmodulin was observed, which seems to point towards a role for melatonin as an antagonist of calmodulin in cells (Benítez-King et al., 1996; Benítez-King, 2006; Cardinali et al., 1997).

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3.4.2.3. Calreticulin

Calreticulin is a ubiquitous and highly conserved Ca^{2+}-binding protein of the endoplasmic reticulum (Krausse and Michalak, 1997) that presents multiple functions: chaperone activity, control of intracellular Ca^{2+} homeostasis and regulation of cell adhesiveness by interactions with integrins (Macias et al., 2003). Using affinity chromatography and ligand blotting techniques, calreticulin was described to specifically bind melatonin (Macias et al., 2003). Calmodulin and calreticulin interacted with melatonin only in the presence of Ca^{2+} (León et al., 2000). This interaction could be of physiological importance in regulating the activity of a wide spectrum of nuclear receptors (Holaska et al., 2001).

3.4.2.4. Sirtuins

Sirtuins are class III histone deacetylase enzymes, which have been widely studied in humans due to their multiple functions, such as regulation of cell cycle, DNA repair, cell survival, and apoptosis. Sirtuins can also change the preference for glycolysis under aerobic conditions, mediated by *SIRT2*, which activates phosphoglycerate mutase activity (Mayo et al., 2017). In mammals, the SIR family includes seven members; however, only *SIRT1*, *SIRT2* and *SIRT3* have been related to melatonin.

Das and collaborators (2005) reported for the first time a relation between melatonin and sirtuins. Since then, many studies have reported that melatonin can increase *SIRT1* and *SIRT2* expression levels and can affect the function of sirtuins (Figure 11). Melatonin effects related to *SIRT1* are the reduction of mitochondrial oxidative damage, prevention of cell death, and induction of apoptosis in cancer cells, among others. Regarding *SIRT2* and *SIRT3*, melatonin effects are less studied and are associated with the reduction of oxidative stress or mitochondrial toxicity (Mayo et al., 2017).

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The interaction of melatonin in yeast cells has not been explored so far. However, some studies have led to the identification of the first *silent information regulator 2* (*SIR2*) (Blander and Guarente, 2004; Braunstein et al., 1993). In addition, other proteins encoded by SIR genes (*SIR3, SIR4*) are responsible for silencing at the mating-type loci and the integrity of telomeres (Smith and Boeke, 1997).
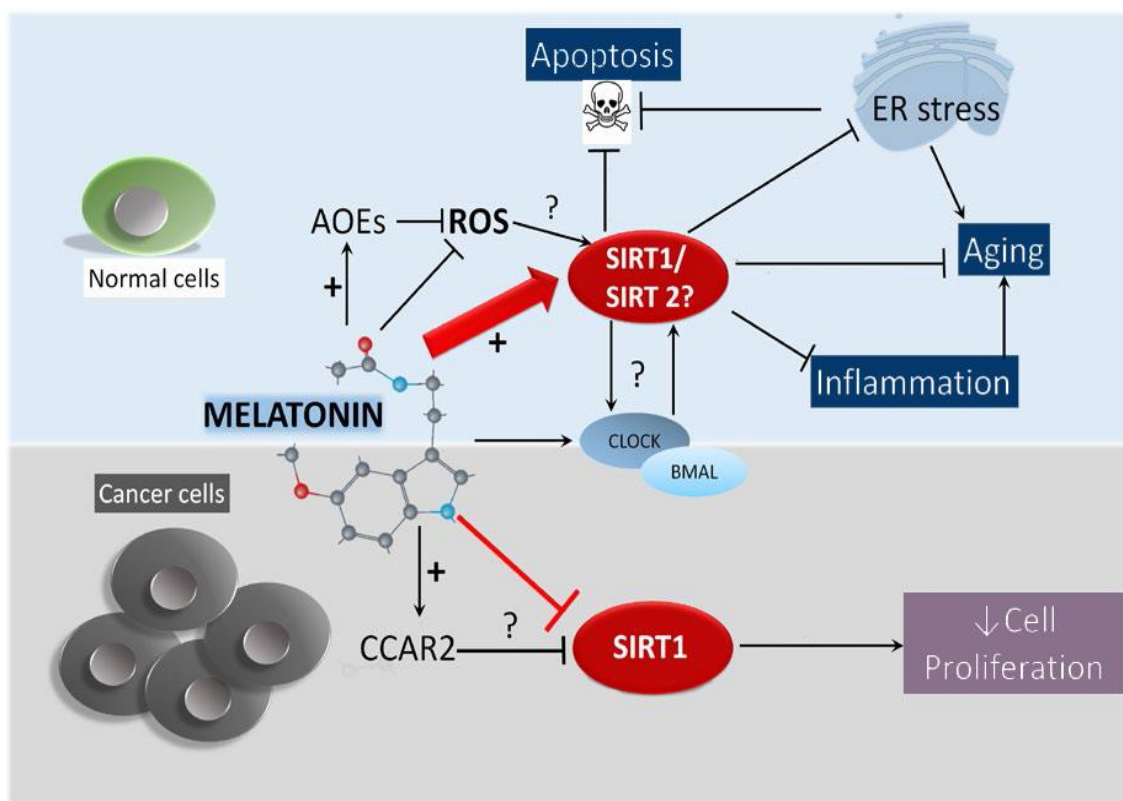
Figure 11. An overview of the actions of melatonin on SIRT1/2 reported to date (Adapted from Mayo et al., 2017)

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Results

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CHAPTER 1

Effect of melatonin and tryptophol addition on fermentation carried out by *Saccharomyces* and non-*Saccharomyces* yeast species under different nitrogen conditions

by María José Valera, María Ángeles Morcillo-Parra, Izabela Zagórska, Albert Mas, Gemma Beltran and María Jesús Torija†

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Chapter 1

Abstract

During wine fermentation, yeasts produce metabolites that are known growth regulators. The relationship between certain higher alcohols derived from aromatic amino acid metabolism and yeast signalling has previously been reported. In the present work, tryptophol or melatonin, which are putative growth regulators, were added to alcoholic fermentations. Fermentations were performed with three different inocula, combining *Saccharomyces cerevisiae* and four non-*Saccharomyces* yeast species, under two nitrogen conditions. The combinations tested were: (i) only *S. cerevisiae; (ii)* the mixture of four non-*Saccharomyces* species; and (iii) the combination of all five species together. The results revealed that the tryptophol and melatonin addition caused changes in fermentation kinetics, viability and species distribution during fermentation, but it was dependent on the nitrogen present in the media and the composition of the inocula.

Low nitrogen condition seemed to favour the presence of non-*Saccharomyces* species until mid-fermentation, although at the end of fermentation the imposition of *Saccharomyces* was higher in this condition. The presence of high concentrations of tryptophol resulted in limited growth and a delay in fermentation, noticeably significant in fermentations performed with *S. cerevisiae* inocula. These effects were reversed by the presence of non-*Saccharomyces* yeast in the medium. Low tryptophol concentration allowed faster fermentation with mixed non-*Saccharomyces* and *Saccharomyces* inocula. Moreover, in the absence of *S. cerevisiae*, a low concentration of tryptophol increased the presence of *Torulaspora delbrueckii* during fermentation with high nitrogen availability but not under low nitrogen conditions, when the population of *S. bacillaris* was higher than that in the control. The effects of melatonin were particularly evident at the beginning and end of the process, primarily favouring the growth of non-*Saccharomyces* strains, especially the first hours after inoculation.

Keywords: Wine, *Torulaspora delbrueckii, Metschnikowia pulcherrima, Hanseniaspora uvarum, Starmerella bacillaris*

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Introduction

Wine fermentation is a complex microbial process carried out by yeasts. These microorganisms produce metabolites that are growth regulators and modulate the quorum sensing response in yeast (Alburquerque and Casadevall, 2012, Zupan et al., 2013). Yeast catabolism results in the production of fusel alcohols, which are derived from amino acids through the well-known Ehrlich pathway (Eden et al., 2001). Yeasts convert amino acids through three enzymatic steps: transamination to form α-keto acid, decarboxylation to an aldehyde, and reduction to a fusel alcohol (Dickinson et al., 2003, Hazelwood et al., 2008). In the case of tryptophol, a fusel alcohol derived from tryptophan, biosynthesis starts with the amino group of tryptophan, which is transaminated into 3-indole pyruvate and subsequently decarboxylated to 3-indole acetaldehyde before undergoing final reduction to tryptophol depending on the redox state of the cell (Mas et al., 2014). Fusel alcohols such as tryptophol have been described as modulators of the quorum sensing response in yeast, particularly under low nitrogen conditions (Zupan et al., 2013). On the other hand, other metabolites derived from aromatic amino acids are considered valuable molecules as bioactive compounds (Mas et al., 2014). For example, melatonin (N-acetyl-5-methoxytryptamine) is also a tryptophan derivative. The biosynthesis of melatonin in yeast seems to be similar to that described in vertebrates. Tryptophan is hydroxylated into 5-hydroxytryptophan and decarboxylated to serotonin prior to its acetylation to N-acetylserotonin. Then, melatonin is finally synthesized by transmethylation (Mas et al., 2014, Sprenger et al. 1999). melatonin presents antioxidant activity in some organisms and, in humans, has been described as a hormone regulating circadian rhythms and reproductive functions (López et al., 2009; Serrano et al., 2010). Melatonin is synthesized by yeast during alcoholic fermentation, although its role remains unknown in these microorganisms (Gómez et al., 2012; Rodríguez-Naranjo et al., 2011).

Yeast metabolism presents variations based on the genetic characteristics of these microorganisms and environmental conditions. In complex environments such as wine fermentation, the interactions between different yeast species or even strains modulate their behaviour (Ciani and Comitini, 2015; Sadoudi et al., 2012). Indeed, different yeast strains in mixed cultures have either synergistic or antagonistic interactions, and this differential performance modifies the aromatic profiles of wines (Ciani and Comitini, 2015; Pérez-Nevado et al., 2006). A complex array of biological communication determines the interactions between microorganisms: killer toxins and antimicrobial compounds (Albergaria and Arneborg, 2016); nutrient limitation (Wang et al., 2016), which might result from rapid nutrient uptake; or the release of other compounds such as

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fatty acids or acetic acid (Sadoudi et al., 2012). The investigation of interactions between *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation is noteworthy, and understanding the modulation mechanisms performed by secondary metabolites derived from yeast activity is important to control this process (Ciani and Comitini, 2015).

Compounds such as tryptophol or melatonin, which have well-known intercellular communication activities in yeasts and other organisms, may play critical roles in the interactions between different species of yeast during alcoholic fermentation. To test this hypothesis, we performed a comparative analysis of mixed cultures of *Saccharomyces* and non-*Saccharomyces* strains during wine fermentation in the presence of secondary metabolites derived from tryptophan, tryptophol and melatonin. We analysed the induced changes in microbial succession in the synthetic must environment.

Materials and methods

Yeast strains

The following yeast species were used in this work: three commercial strains for wine production (*Saccharomyces cerevisiae* strain QA23®, *Torulaspora delbrueckii* strain Biodiva™ and *Metschnikowia pulcherrima* strain Flavia® (Lallemand Inc., Montreal, Canada)) and two strains isolated from the spontaneous fermentation of Priorat grape juice (Padilla et al., 2016) (*Hanseniaspora uvarum* strain CECT 13130 and *Starmerella bacillaris* strain CECT 13129). Overnight cultures were prepared in liquid YPD medium (2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract), grown at 28ºC and stirred at 120 rpm to be used as inocula.

Wine fermentations

To carry out fermentations, synthetic grape must was prepared as described by Riou et al. (1997) with some modifications. Two nitrogen concentrations, in terms of Yeast Available Nitrogen (YAN), were applied: 300 mg/L (180 mg/L derived from amino acids and 120 mg/L derived from NH_4Cl) and 100 mg/L (80 mg/L from amino acids and 20 mg/L from NH_4Cl). The sugar concentration in the synthetic must was 200 g/L, with the same proportion of glucose and fructose. The pH was adjusted to 3.3.

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Three different inocula were used to start the fermentations: i) *S. cerevisiae* QA23 (Sc); ii) a mixed culture of four non-*Saccharomyces* strains (*T. delbrueckii*, *M. pulcherrima*, *H. uvarum* and *S. bacillaris* (NSc)); and iii) a mixed population of these four non-*Saccharomyces* strains together with *S. cerevisiae* QA23 (ScNSc). The fermentations were inoculated with 2×10^6 cells/mL of each yeast species used.

For each fermentation, 200 mL of must were dispensed in a 250-mL opaque bottle. The effects of either tryptophol or melatonin were analysed for each inoculum and nitrogen concentration. Based on a previous study (González et al., 2018a), two different concentrations of tryptophol (Roche, Germany) (0.5 g/L and 0.1 g/L) and three different concentrations of melatonin (Roche, Germany) (1 g/L, 0.5 g/L and 0.1 g/L) were tested by adding them to freshly prepared must. Controls without any specific metabolite supplementation were included for each nitrogen condition and inoculum. The fermentations were carried out in triplicate at room temperature on an orbital shaker with a stirring rate of 120 rpm.

Wine sampling and yeast growth analysis during fermentation

Samples were taken every 24 h. Due to the different lengths of the fermentations, three stages were defined to compare them: beginning of fermentation (24 h after inoculation); end of fermentation, when the wines contained less than 2 g/L sugar; and the middle point of fermentation, which was considered the day that represented the median of the process. Yeast growth was determined by plate counting. Three media were used: YPD solid medium (YPD medium plus 1.7% (w/v) agar), a rich medium that was used for total yeast counts; lysine agar medium (Oxoid; USA), which is selective for non-*Saccharomyces* species; and Wallerstein Laboratory Nutrient Agar (WL) medium (Difco; USA), a differential medium that was used for the rapid identification of yeast species based on different colony morphologies (Figure S1.1). Additionally, when the morphology of a colony was not clear, amplification and subsequent restriction analysis of 5.8S ITS rDNA were performed directly from colony as described by Esteve-Zarzoso et al. (1999).

To calculate the dilution required for plating, samples were counted using a Neubauer chamber (0.0025 mm^2 and 0.100 mm deep). All plates were incubated at 28°C for 24-72 h before counting. All colonies that grew were counted, and the numbers of colonies per plate ranged from 20 to 200. However, in WL medium, the numbers of colonies counted were not greater than 50 due to difficulties distinguishing different morphologies when the colonies were too small.

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Chemical analysis of wines

The fermentation process was monitored daily based on density using a digital densitometer (Mettler Toledo, Portable Lab) as an indirect value of the sugar concentration. When density remained stable for at least two days, the fermentations were considered finished or stuck. The final wines were analysed to evaluate residual sugars, glucose and fructose using a specific enzymatic kit (Roche, Boehringer Mannheim, Germany) according to the manufacturer's instructions.

The melatonin and tryptophol concentration were analysed by performing liquid chromatography-mass spectrometry following the method described by Rodríguez-Naranjo et al. (2011) and González et al. (2018b), respectively. The system was based on a high performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6410; Agilent Technologies, Palo Alto, USA).

Statistical analysis

The variances of the results were statistically analysed by performing one-way ANOVA and Student's T test with a level of significance of 5% using IBM SPSS Statistics software.

Results

Effects of tryptophol addition on alcoholic fermentation and population dynamics

Alcoholic fermentation was strongly affected by tryptophol addition, although the effects were dependent on nitrogen conditions and the presence of different yeasts in the medium (Figure 1.1). When only *S. cerevisiae* was present in the medium (Sc), the highest concentration tested (0.5 g/L) produced a delay in fermentation that was significant throughout the entire process. The same effect was observed for the two nitrogen concentrations tested (Figure 1.1a and d). This effect clearly disappeared when *Saccharomyces* was inoculated together with other non-*Saccharomyces* strains (ScNSc), as shown in Figure 1.1b and e. Thus, alcoholic fermentations presented similar kinetics with one exception: when 0.1 g of tryptophol/L was added in the presence of 300 mg/L of YAN. In this case, fermentation proceeded faster than under the other two conditions. This effect was more noticeable when only non-*Saccharomyces* yeasts (NSc) were present in the medium (Figure 1.1c and f). In this case, at 300 mg/L of YAN, fermentations containing 0.1 g tryptophol/L

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were also significantly faster than the control, whereas a higher concentration of tryptophol (0.5 g/L) slowed down fermentation and even failed to consume all sugars. This effect disappeared in media with a low nitrogen concentration (100 mg/L of YAN).

The population dynamics of fermentation were analysed to determine the relationships between the yeast population and fermentation kinetics. Regarding the overall population, no significant variations were observed (Supplementary Table S1.1). As expected, non-*Saccharomyces* populations recovered on LYS plates were lower than the total population (counted on YPD medium) under ScNSc conditions. The results obtained with WL medium allowed us to discriminate between five species based on colony morphology, and *Saccharomyces* was the only species detected when it was inoculated individually (results not shown). In the mixed fermentation (NSc and ScNSc), there were several notable features (Figure 1.2): *M. pulcherrima* was hardly recovered on any plate, and, when it was recovered, it occurred practically only at the beginning of fermentation, while *H. uvarum* was only found at the beginning and mid-fermentation. When present, *S. cerevisiae* was predominant at mid and at the end of fermentation. Additionally, when *S. cerevisiae* was not present, *T. delbrueckii* normally was predominant, although *S. bacillaris* also had a significant presence (Figure 1.2b and d). However, there were certain deviations from this general pattern due to the presence of tryptophol. At low nitrogen concentrations in ScNSc, although *S. cerevisiae* imposed at the end of the process, *H. uvarum* was the main species detected in the middle of the process, when the highest concentration of tryptophol was in the medium. On the other hand, at high concentrations of nitrogen in the presence of 0.1 g/L of tryptophol, *T. delbrueckii* was the main species detected at the end of the process, resulting, as explained in fermentation kinetics, in a faster fermentation (Figure 1.1b and 1.2a). Similarly, in NSc fermentations in the same conditions (high nitrogen and 0.1 g/L of tryptophol), also the imposition of *T. delbrueckii* resulted in faster fermentations than the control, whereas the imposition of *S. bacillaris* at 0.5 g/L tryptophol resulted in stuck fermentations (Figure 1.1c and 1.2b).
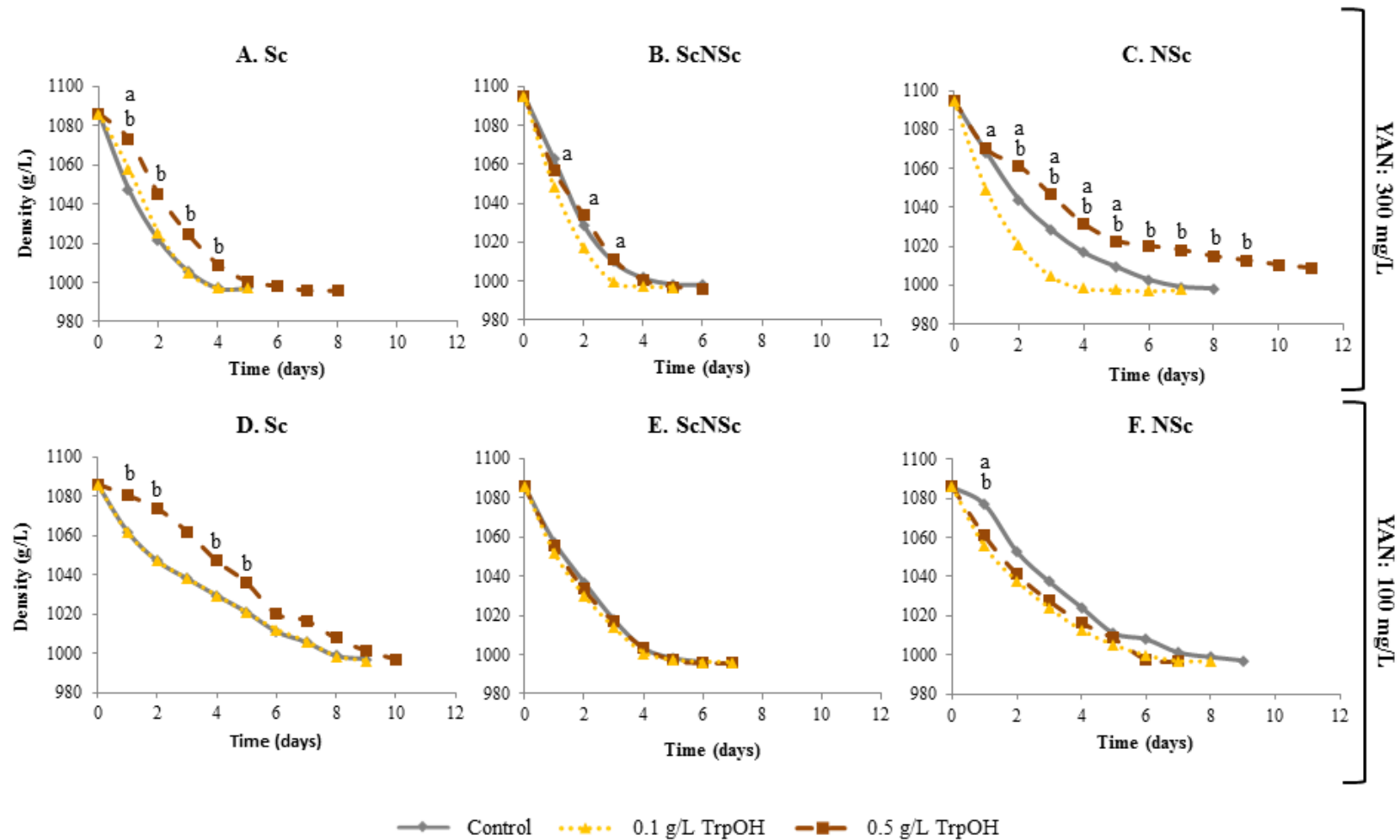
Figure 1.1. Fermentation kinetics with added tryptophol and control in synthetic must containing 300 mg/L (A, B, C) and 100 mg/L (D, E, F) of yeast assimilable nitrogen. Fermentations were inoculated with (i) *S. cerevisiae* (A, D), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B, E) and (iii) a mixture of four non-*Saccharomyces* species (C, F). Values are means of triplicate fermentations. Letters represent statistically significant differences.

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Figure 1.2. Distribution of yeast species at the beginning (24 h after inoculation), middle and end of the process in control fermentations and fermentations with tryptophol supplementation (0.1 g/L and 0.5 g/L). A) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 300 mg/L of yeast assimilable nitrogen (YAN) in the must; B) mixture of non-*Saccharomyces* in musts containing 300 mg/L of YAN C) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN; D) non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN.

Effects of melatonin addition on fermentation kinetics

The addition of melatonin exerted more limited effects than tryptophol in terms of fermentation kinetics. Although some significant differences were observed, those were normally restricted to single points during fermentations (Figure 1.3). With the NSc inoculum, most of the fermentations were significantly faster than the control when the highest concentration of melatonin was used. In general, in the absence of *S. cerevisiae,* the presence of melatonin appeared to help alcoholic fermentation because the control fermentation was always slightly delayed. This also happened in the presence of *S. cerevisiae* but only at high nitrogen concentrations. No significant differences were observed in the yeast population with the exception of specific time
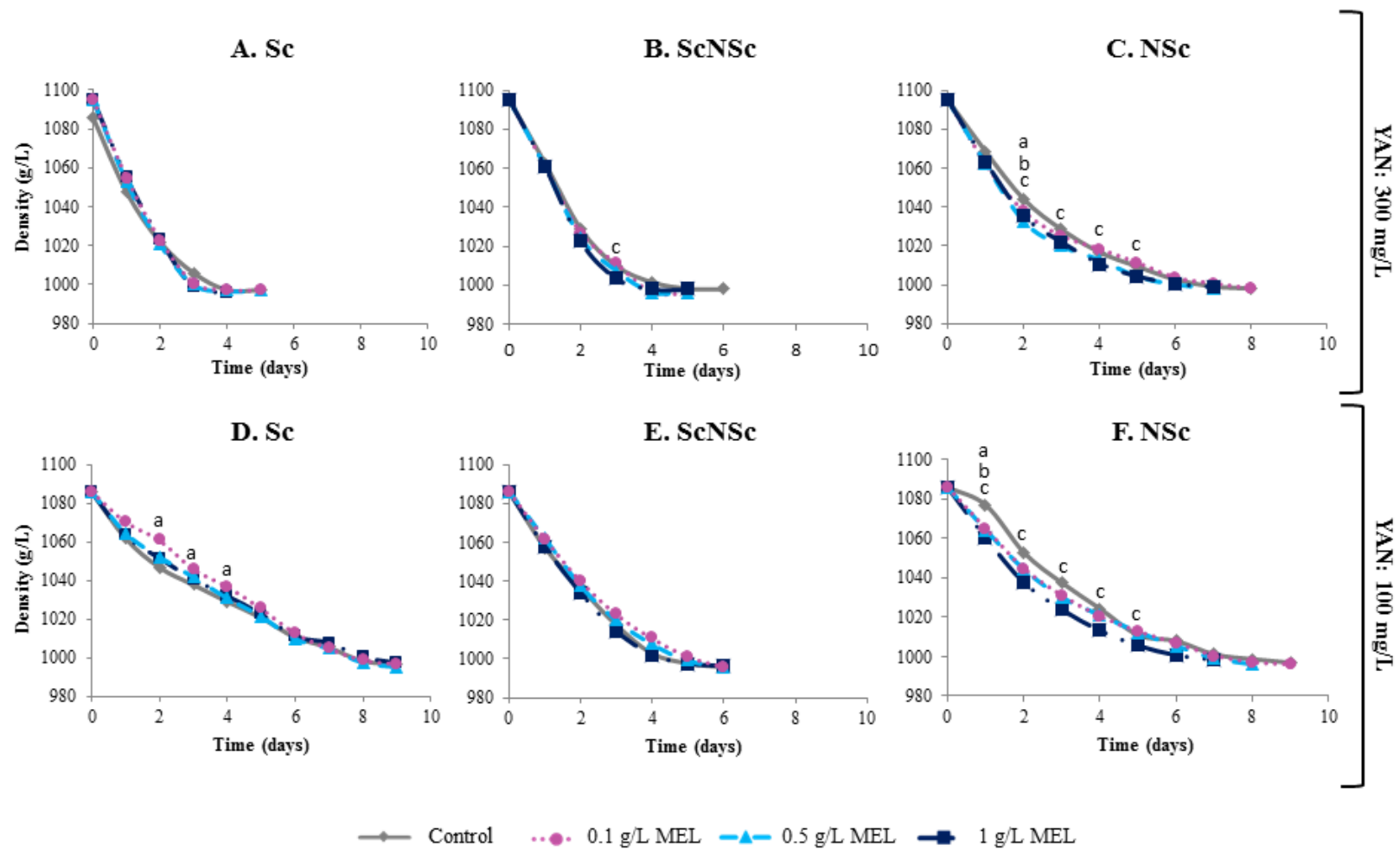
Figure 1.3. Fermentation kinetics with added melatonin and control in synthetic must containing 300 mg/L (A, B, C) and 100 mg/L (D, E, F) of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A, D), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B, E) and (iii) a mixture of four non-*Saccharomyces* species (C, F). Values are means of triplicate fermentations. Letters represent statistically significant differences.

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points, and there was no correlation with fermentation kinetics (Supplementary Table S1.1). When the population was analysed at the species level, similar observations to those obtained with tryptophol were detected (Figure 1.4). Remarkably, non-*Saccharomyces* species persisted at the end of the fermentation, in some cases comprising up to 50% of the total population, when melatonin was available in ScNSc fermentations with low nitrogen concentrations. Indeed, under low nitrogen concentrations, the proportion of *S. bacillaris* increased at the end of fermentation with melatonin supplementation.

Tryptophol and melatonin production during fermentation

Extracellular samples obtained from control fermentations for the three different inocula and two nitrogen concentrations were analysed for tryptophol and melatonin production at three different time points: the beginning, middle and end of fermentation.

In all the cases, higher amount of tryptophol was synthesized in the musts containing high concentration of nitrogen (Figure 1.5). At 100 mg/L of YAN, the tryptophol concentration was around 20 ppm, while it was between 50-70 ppm at 300 mg/L. The Sc inoculum produced the highest concentration of tryptophol (Figure 1.5a), while the presence of Non-*Saccharomyces* in the medium resulted in a lower amount of tryptophol, regardless the presence of *S. cerevisiae* (Figure 1.5b and c).

As in the case of tryptophol, the amount of melatonin in the medium was always lower in must containing 100 mg/L of YAN than in must containing 300 mg/L (Figure 1.6). However, the amounts of melatonin were much lower than those of tryptophol throughout the fermentation. Moreover, the proportion between the production of melatonin in a medium containing 100 and 300 mg/L of YAN was not maintained as it was in tryptophol. Depending on the inocula, melatonin presented the h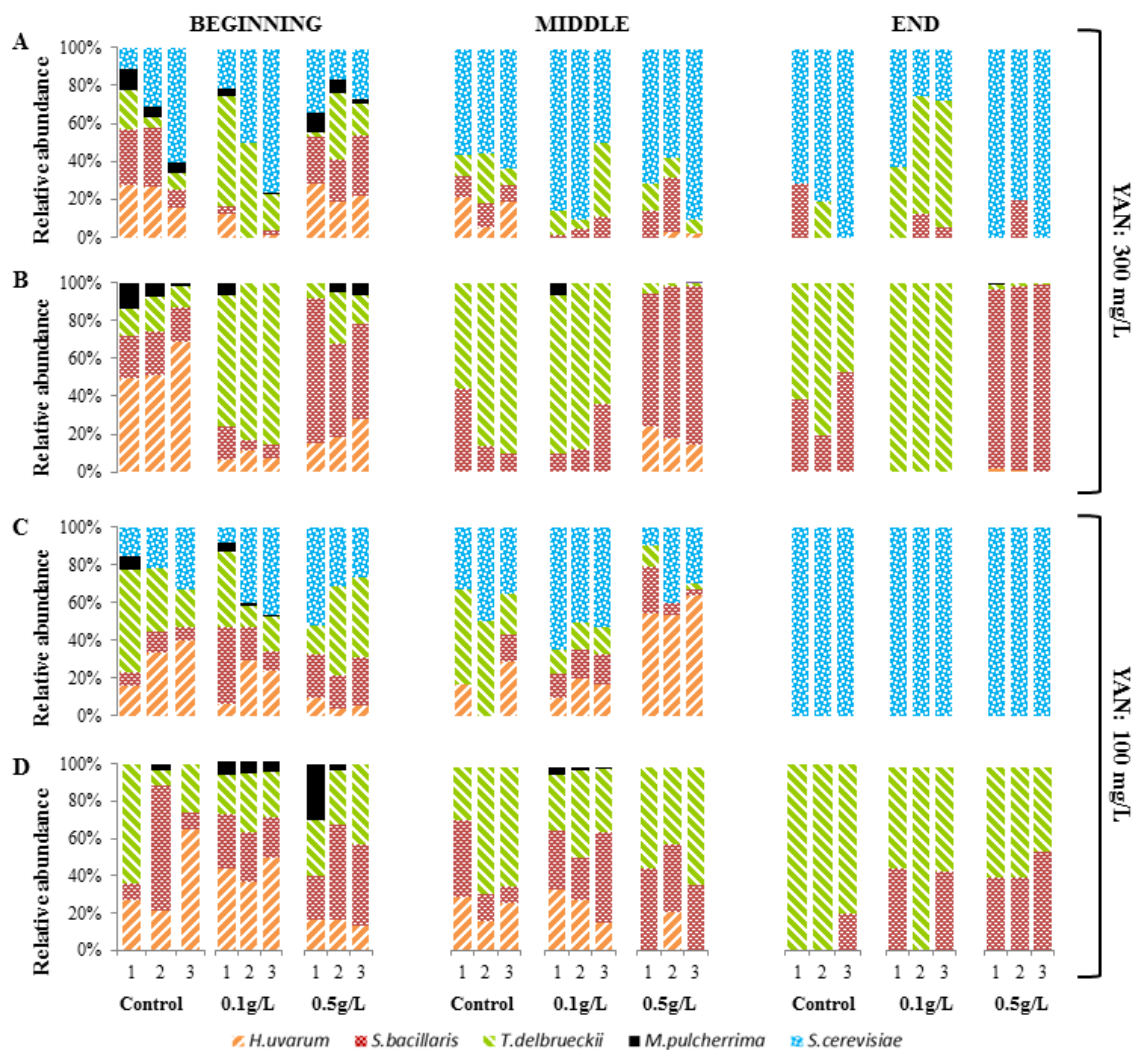ighest concentrations at different time points during fermentation regardless of the nitrogen concentration in the initial must. Sc-inoculated fermentations presented the maximum melatonin concentration at the beginning of fermentation, 24 h after inoculation (Figure 1.6a). Then, melatonin levels decreased progressively until the end of the fermentation. However, when inoculation was performed using the ScNSc mixture (Figure 1.6b), the melatonin concentration increased during fermentation. Finally, fermentations performed with the NSc inoculum (Figure 1.6c) presented maximum melatonin concentrations in the middle of fermentation, while melatonin levels decreased at the end. Moreover, in these fermentations,

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the melatonin contents were the lowest; and thus, the presence of *Saccharomyces* seems to promote a higher presence of melatonin in the medium.
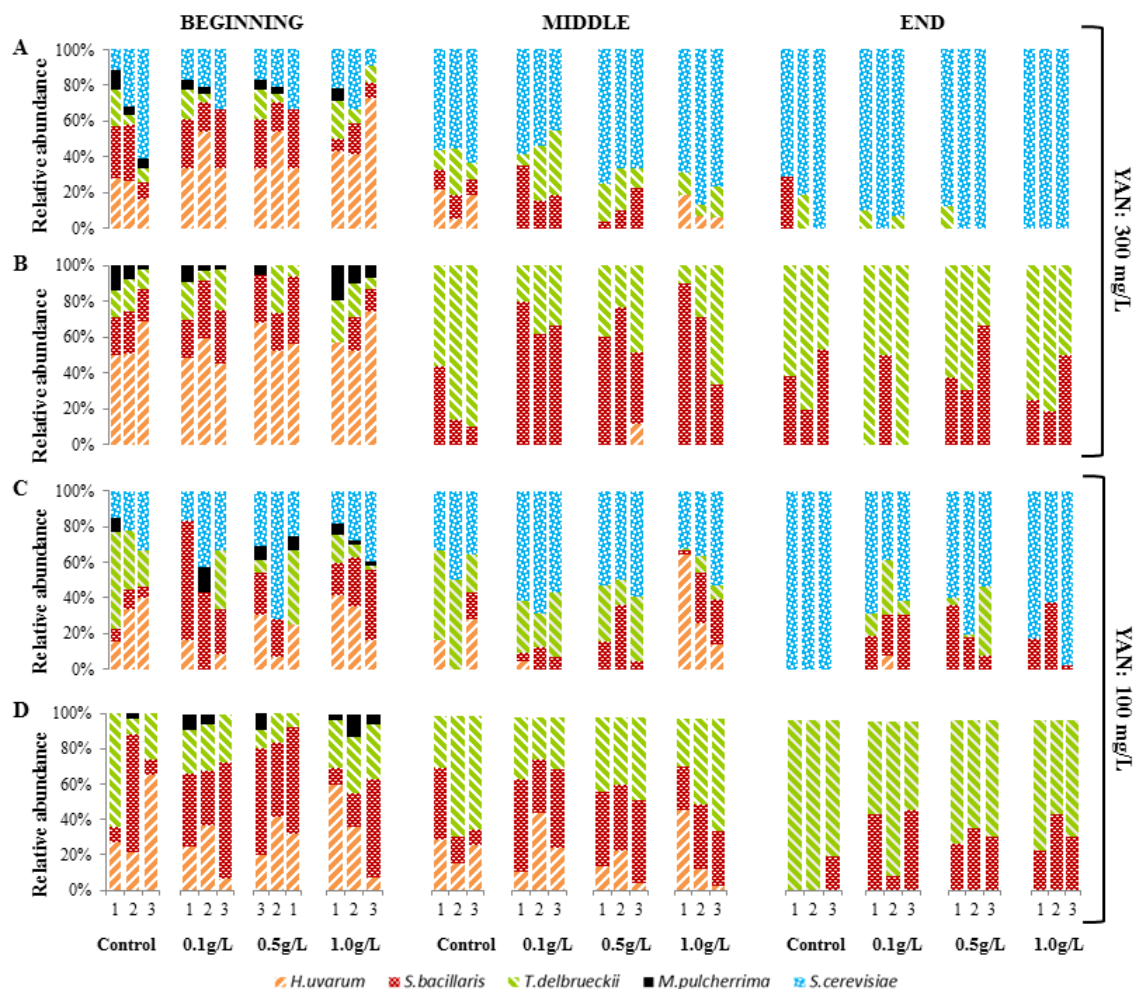
Figure 1.4. Distribution of yeast species at the beginning (24 h after inoculation), middle and end of the process in control fermentations and fermentations with melatonin supplementation (0.1 g/L, 0.5 g/L, 1 g/L). A) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 300 mg/L of yeast assimilable nitrogen (YAN); B) mixture of non-*Saccharomyces* in musts containing 300 mg/L of YAN; C) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN; D) non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN.
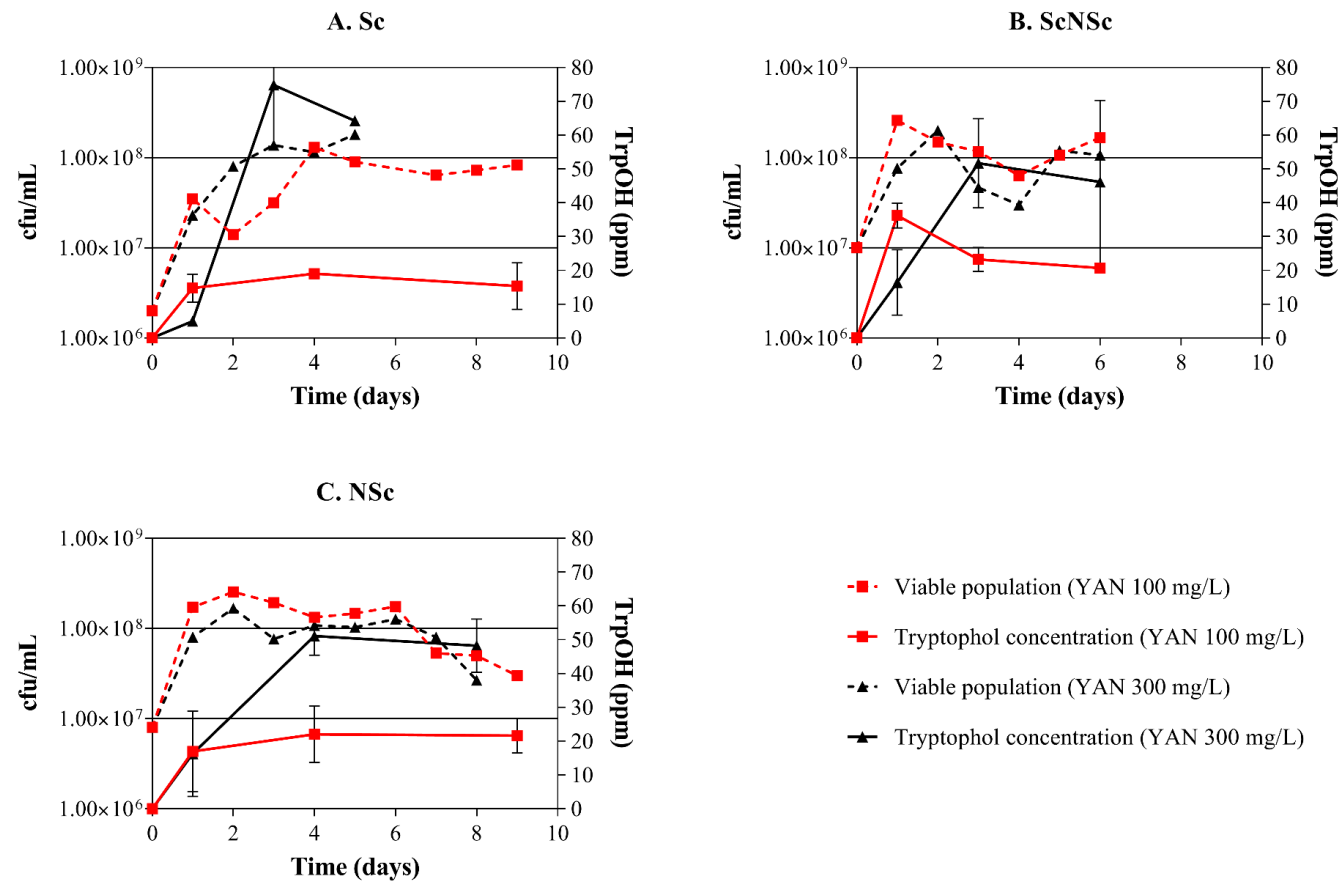
Figure 1.5. Yeast populations recovered on YPD medium during fermentation and tryptophol concentration produced at the beginning, middle and end of fermentation in control fermentations (unsupplemented with tryptophol) containing 300 mg/L or 100 mg/L of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B) and (iii) a mixture of four non-*Saccharomyces* species (C). Values are means of triplicate fermentations. Bars represent standard deviation.
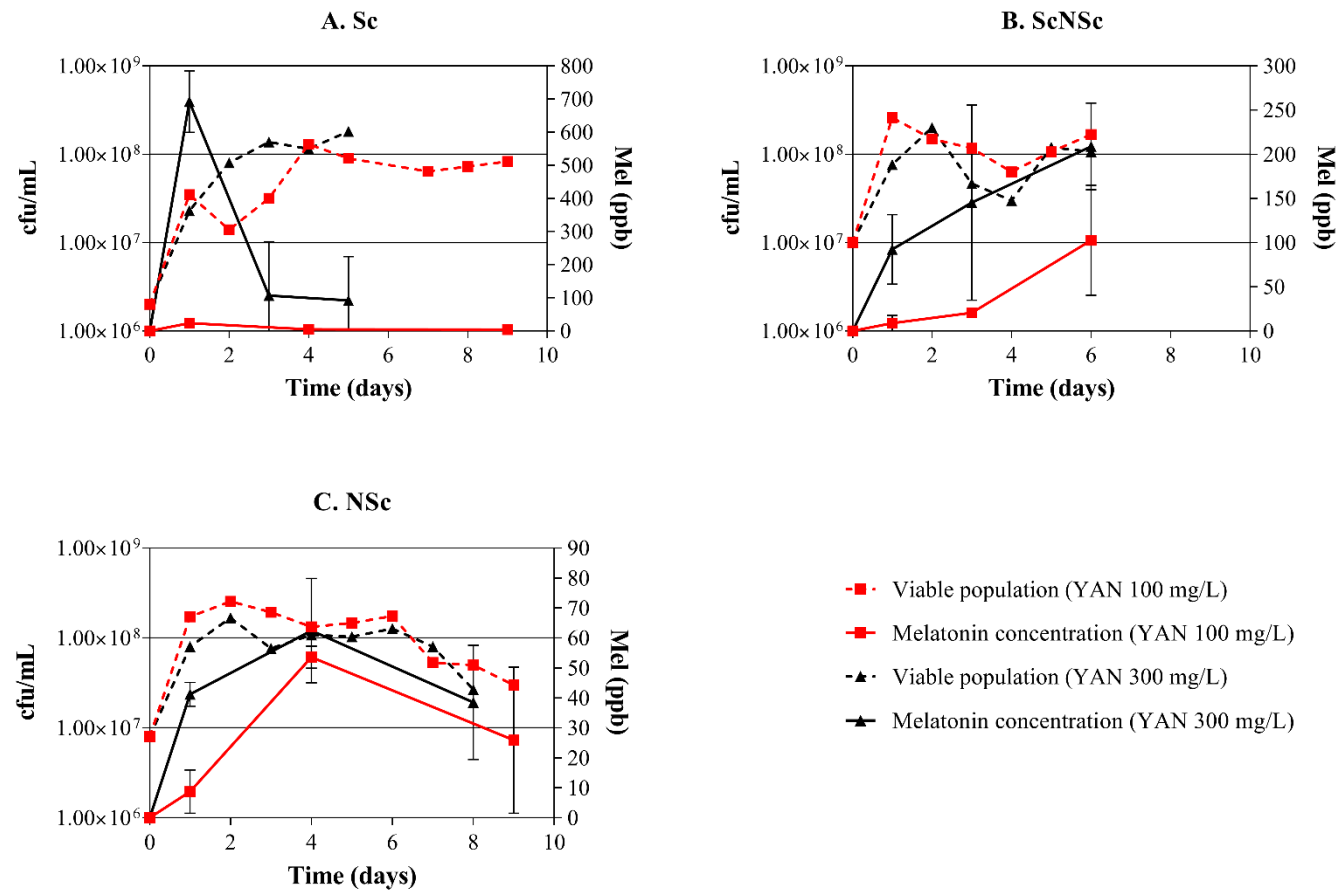
Figure 1.6. Yeast populations recovered on YPD medium during fermentation and melatonin concentration produced at the beginning, middle and end of fermentation in control fermentations (unsupplemented with melatonin) containing 300 mg/L or 100 mg/L of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B) and (iii) a mixture of four non-*Saccharomyces* species (C). Values are means of triplicate fermentations. Bars represent standard deviation

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Discussion

Alcoholic fermentation is the result of a complex microbial succession involving many interactions between different yeast species. The initial non-*Saccharomyces* yeast species, which are more abundant in grapes and grape must, have been considered undesirable for many years. To avoid their effects, fermentation control was favoured by the inoculation of selected strains of *S. cerevisiae*. However, in the recent years, good properties and contribution to the wine of non-*Saccharomyces* species has been described, turning the use of mixed cultures of *Saccharomyces* and non-*Saccharomyces* species as a good alternative for wine production (Ciani et al., 2010; Fleet, 2008; Jolly et al., 2014; Mas et al., 2016). The presence of *S. cerevisiae* ensures complete sugar consumption, while conversely, non-*Saccharomyces* species contribute to higher complexity, increasing the aromatic profile of wines (Comitini et al., 2011; Fleet et al., 2003). The growth of several species sharing the same environment causes competition for nutrients, but other interactions between yeast species remain unknown. In addition, additive effects are caused by the production of metabolites such as ethanol, killer toxins or fatty acids to induce the death of other sensitive yeasts (Ciani and Comitini, 2015; Pérez-Nevado et al., 2006; Wang et al., 2015). There are other additional mechanisms that contribute to this complex scenario, such as quorum sensing-like responses regulated by yeast metabolites, which some authors have identified as a possible mechanism of yeast interaction and not just morphological changes (Ciani and Comitini, 2015; González et al., 2018b).

Regarding competition for nutrients, the reduced availability of nitrogen in must causes slow or sluggish fermentation and limited biomass formation (Varela et al., 2004). Indeed, nitrogen concentration below 140 mg/L has been reported to limit the growth and fermentation rate of *S. cerevisiae* (Bell and Henschcke, 2005; Martínez-Moreno et al., 2012; Tesnière et al., 2015). Nitrogen was not a limiting substrate in the present study, because all fermentations with 100 mg/L of N finished successfully and the only fermentation that was halted contained a high nitrogen concentration. Nitrogen needs were likely covered by previous growth in YPD during inoculum preparation, permitting internal nitrogen accumulation (Lleixà et al., 2016). In general, non-*Saccharomyces* yeasts are considered high nitrogen consumers (Andorrà et al., 2010, 2012). However, nitrogen requirements are species-dependent. Thus, in the present work, the initial nitrogen concentration determined different fermentation kinetics, likely due to the differential sensitivities of yeast species to the lack of nitrogen. Padilla et al. (2016) reported that the *H. uvarum* strain used in our study was detected at the beginning and middle points of spontaneous

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fermentation and exhibited high population levels. However, Lleixà et al. (2016) showed that this *H. uvarum* strain was sensitive to low nitrogen conditions in fermentations performed with mixed yeast inocula. According to our results, *H. uvarum* was more persistent in time under low nitrogen conditions, although at the beginning of fermentations, it seems to be more competitive at high nitrogen concentrations. When fermentations were carried out with NSc inoculum and high nitrogen, *S. bacillaris* was able to survive until the end of the fermentation, which has been observed by other authors working with the same strain (Lleixà et al., 2016; Padilla et al., 2016). *S. cerevisiae* was the only species detected at the end of fermentation under low nitrogen conditions. However, at the midpoint of fermentation, the proportion of this species was reduced compared with the proportion observed under high nitrogen conditions. This is explained by the high proportion of *T. delbrueckii* found under low nitrogen conditions. Indeed, *T. delbrueckii* was able to complete fermentation in most processes performed only with non-*Saccharomyces* yeasts, even under low nitrogen conditions. Recent studies have reported that killer strains of *Torulaspora delbrueckii* can complete wine fermentations in single inoculation, although these fermentations were pretty slower than the ones performed with *S. cerevisiae* (Ramírez et al., 2016; Velázquez et al., 2015). Moreover, *T. delbrueckii* was recovered at the end of fermentation in the presence of *S. cerevisiae*, likely because *T. delbrueckii* strains have been characterized by their good fermentation capacity as well as their ethanol tolerance (Bely et al., 2008; Lleixà et al., 2016). A previous study reported that *T. delbrueckii* and *S. bacillaris* were able to maintain their culturability longer than *H. uvarum* upon inoculation with *S. cerevisiae* (Wang et al., 2016). Conversely, *M. pulcherrima* was unable to survive until the end of fermentation. This species is known to have a low tolerance to alcohol; some authors have reported that it is unable to survive in concentrations of 2-3% (v/v) of ethanol (Kunkee and Amerine, 1970). Our results agree with those of González-Royo et al. (2015), with a very low recuperation of this strain after inoculation.

The addition of tryptophol resulted in different effects on species distribution and fermentation kinetics depending on the added concentration. This compound has been highlighted as a quorum sensing molecule (Zupan et al., 2013). Quorum sensing-like responses in yeast have been mainly investigated to explain morphological changes, however, the involvement of this phenomenon in some yeast interactions cannot rule out (Ciani and Comitini, 2015). Our results confirmed that *S. cerevisiae* growth, similar to that of many other non-*Saccharomyces* species, is affected by the presence of tryptophol in the medium, as previously reported in single fermentations (González et al., 2018a). A high tryptophol concentration limited the fermentation

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performance of *S. cerevisiae*, although this effect was reversed by the presence of non-*Saccharomyces* yeast. On the other hand, low tryptophol concentration improved the fermentation performance in ScNSc conditions in high-nitrogen musts, although the major species at the end of these fermentations was *T. delbrueckii* instead of *S. cerevisiae*. Similarly, with NSc inoculum, low tryptophol supplementation also resulted in faster fermentations, being again particularly evident in samples obtained from high-nitrogen where *T. delbrueckii* was the only species detected at the end. On the other hand, high tryptophol concentrations under high nitrogen conditions favoured the growth of *S. bacillaris*, but the fermentation kinetics was slower. Control fermentations showed an intermediate situation, with the presence of both species and an intermediate kinetics. Thus, *T. delbrueckii* as the major species accelerated fermentation kinetics compared with the control, while high populations of *S. bacillaris* slowed down fermentation.

Melatonin, another tryptophan derivative, presents characteristics that point to a putative role as a signalling molecule during fermentation. In previous studies, melatonin was detected in extracellular medium during wine fermentations conducted by *S. cerevisiae*. This melatonin was pulsatilely released in the first hours of wine fermentation, but it also disappeared rapidly from the extracellular medium (Rodríguez-Naranjo et al., 2012). Moreover, in this study, the synthesis of melatonin has been related to the yeast growth phase and the concentration of reducing sugars, suggesting a role of melatonin as a growth signal. The effect of melatonin on fermentation kinetics was less evident than that observed for tryptophol, although the presence of high concentrations of melatonin (0.5 and 1 g/L), reduced fermentation time in mixed fermentations, especially in NSc conditions. Additionally, non-*Saccharomyces* viability was increased by the addition of melatonin in the first hours after inoculation when nitrogen concentration was high. Instead, under low nitrogen conditions, melatonin addition extended the survival of the non-*Saccharomyces* yeasts in presence of *S. cerevisiae* until the end of the fermentation and of *S. bacillaris* in NSc fermentations.

Tryptophol is produced by the Ehrlich pathway and thus, its synthesis is a way for cells to eliminate tryptophan and use nitrogen (Mas et al., 2014). This agree with the profile of synthesis obtained in this study, since its synthesis occurred during the first stages of the fermentation, when nitrogen is consumed, reaching the highest concentration in the beginning and middle of fermentation, under low and high nitrogen concentrations, respectively. Moreover, the higher is the nitrogen concentration, the higher is the tryptophol synthesis. However, in the case of

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melatonin, the concentration was rather low in comparison to that of tryptophol and the profile of synthesis was very different according to yeast populations. Furthermore, the lack of relation with the concentration of nitrogen present in the medium might point towards different role of this compound in yeasts.

In conclusion, tryptophol and melatonin addition caused changes in fermentation kinetics, viability and species distribution during fermentation. Additionally, their presence contributed in different ways to each yeast species studied. Few studies have been performed to describe yeast interactions during wine fermentation. Tryptophol and melatonin are tryptophan derivatives that have been previously identified or proposed to be growth regulators and associated with cell signalling. The present study focused on this putative role of these compounds as signalling molecules during wine fermentation. The conditions to perform the experiments were selected according to the limited information available about the impact of these molecules in the fermentation process. This is a first attempt to study the relation between tryptophan derivatives and interaction between yeast from oenological environments. Further studies must be carried out to elucidate the mechanisms in which they are specifically involved and how to apply these findings in winemaking industry.

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MELATONIN METABOLISM IN YEAST CELLS DURING ALCOHOLIC FERMENTATION
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Chapter 1

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Supplementary material

Supplementary Table 1.1: Yeast populations recovered in YPD or Lysine (Lys) medium (cfu/mL) from the different experimental conditions along the fermentation and total yeast population inoculated (t = 0) in each condition (cells/mL).

Fermentation point	Nitrogen and inoculum	Medium	Control	Tryptophol		Melatonin		
			0 g/L	0.5 g/L	0.1 g/L	1 g/L	0.5 g/L	0.1 g/L
Inoculation	300N[a]/100N[b] Sc[*]		2.00×10^6	2.00×10^6	2.00×10^6	2.00×10^6	2.00×10^6	2.00×10^6
	300N/100N ScNSc[**]		1.00×10^7	1.00×10^7	1.00×10^7	1.00×10^7	1.00×10^7	1.00×10^7
	300N/100N NSc[***]		8.00×10^6	8.00×10^6	8.00×10^6	8.00×10^6	8.00×10^6	8.00×10^6
Beginning	300N Sc	YPD	$7.15 \times 10^7 \pm 1.61 \times 10^7$	$1.43 \times 10^7 \pm 2.52 \times 10^6$	$7.37 \times 10^7 \pm 4 \times 10^6$	$1.23 \times 10^8 \pm 1.53 \times 10^7$	$1.20 \times 10^8 \pm 1.50 \times 10^7$	$9.80 \times 10^7 \pm 6.00 \times 10^6$
	300N ScNSc	YPD	$7.80 \times 10^7 \pm 2.66 \times 10^7$	$5.87 \times 10^7 \pm 3.19 \times 10^7$	$2.53 \times 10^8 \pm 3.19 \times 10^{7\dagger}$	$1.43 \times 10^8 \pm 1.15 \times 10^{7\dagger}$	$2.20 \times 10^8 \pm 2.65 \times 10^{7\dagger}$	$2.03 \times 10^8 \pm 6.66 \times 10^{7\dagger}$
		Lys	$4.33 \times 10^7 \pm 1.68 \times 10^7$	$1.57 \times 10^7 \pm 7.77 \times 10^6$	$2.07 \times 10^7 \pm 7.77 \times 10^6$	$1.47 \times 10^8 \pm 5.03 \times 10^{7\dagger}$	$1.17 \times 10^8 \pm 3.06 \times 10^{7\dagger}$	$1.63 \times 10^8 \pm 4.16 \times 10^{7\dagger}$
	300N NSc	YPD	$1.83 \times 10^8 \pm 7.09 \times 10^7$	$2.37 \times 10^7 \pm 6.81 \times 10^{6\dagger}$	$2.13 \times 10^8 \pm 6.35 \times 10^7$	$3.47 \times 10^8 \pm 4.73 \times 10^7$	$2.37 \times 10^8 \pm 7.57 \times 10^7$	$2.40 \times 10^8 \pm 4.58 \times 10^7$
		Lys	$1.07 \times 10^8 \pm 7.46 \times 10^7$	$1.50 \times 10^7 \pm 2.65 \times 10^{6\dagger}$	$2.23 \times 10^8 \pm 5.51 \times 10^7$	$5.67 \times 10^7 \pm 3.79 \times 10^7$	$1.13 \times 10^8 \pm 3.51 \times 10^7$	$1.93 \times 10^8 \pm 6.03 \times 10^7$

Supplementary Table 1.1: Continued.

Fermentation point	Nitrogen and inoculum	Medium	Control	Tryptophol		Melatonin		
			0 g/L	0.5 g/L	0.1 g/L	1 g/L	0.5 g/L	0.1 g/L
Middle	300N Sc	YPD	$1.38 \times 10^8 \pm 2.08 \times 10^7$	$9.00 \times 10^7 \pm 3.88 \times 10^7$	$2.30 \times 10^8 \pm 9.07 \times 10^6$	$2.30 \times 10^8 \pm 5.77 \times 10^6$	$2.63 \times 10^8 \pm 5.77 \times 10^6$	$2.07 \times 10^8 \pm 3.51 \times 10^7$
	300N ScNSc	YPD	$9.67 \times 10^7 \pm 6.22 \times 10^7$	$1.27 \times 10^8 \pm 4.82 \times 10^7$	$2.80 \times 10^7 \pm 2.08 \times 10^6$	$1.53 \times 10^8 \pm 5.03 \times 10^7$	$1.67 \times 10^8 \pm 3.21 \times 10^7$	$1.77 \times 10^8 \pm 2.08 \times 10^8$
		Lys	$3.33 \times 10^7 \pm 1.00 \times 10^8$	$1.27 \times 10^7 \pm 4.89 \times 10^7$	$2.43 \times 10^7 \pm 2.31 \times 10^6$	$4.67 \times 10^7 \pm 3.51 \times 10^7$	$9.00 \times 10^7 \pm 3.00 \times 10^7$	$6.00 \times 10^7 \pm 3.06 \times 10^7$
	300N NSc	YPD	$7.67 \times 10^7 \pm 3.21 \times 10^7$	$1.73 \times 10^8 \pm 8.39 \times 10^6$	$3.60 \times 10^8 \pm 7.02 \times 10^7$	$1.40 \times 10^8 \pm 5.77 \times 10^7$	$2.87 \times 10^8 \pm 4.73 \times 10^7$	$1.13 \times 10^8 \pm 2.08 \times 10^7$
		Lys	$6.93 \times 10^7 \pm 8.87 \times 10^7$	$1.40 \times 10^8 \pm 1.33 \times 10^7$	$2.23 \times 10^8 \pm 4.51 \times 10^7$	$6.33 \times 10^7 \pm 5.77 \times 10^7$	$1.53 \times 10^8 \pm 9.07 \times 10^7$	$1.17 \times 10^8 \pm 6.66 \times 10^7$
End	300N Sc	YPD	$1.81 \times 10^8 \pm 1.01 \times 10^7$	$7.00 \times 10^7 \pm 8.58 \times 10^{7\dagger}$	$2.03 \times 10^8 \pm 1.00 \times 10^7$	$1.93 \times 10^8 \pm 3.21 \times 10^7$	$1.92 \times 10^8 \pm 4.16 \times 10^7$	$1.60 \times 10^8 \pm 1.00 \times 10^7$
	300N ScNSc	YPD	$1.07 \times 10^8 \pm 8.66 \times 10^6$	$3.67 \times 10^7 \pm 5.77 \times 10^5$	$1.10 \times 10^7 \pm 4.82 \times 10^7$	$9.33 \times 10^7 \pm 1.15 \times 10^7$	$9.00 \times 10^7 \pm 1.00 \times 10^7$	$1.20 \times 10^8 \pm 1.00 \times 10^7$
		Lys	$4.00 \times 10^7 \pm 1.76 \times 10^7$	$1.33 \times 10^7 \pm 4.36 \times 10^6$	$1.67 \times 10^6 \pm 4.83 \times 10^7$	$< 10^7$	$< 10^7$	$< 10^7$
	300N NSc	YPD	$2.67 \times 10^7 \pm 7.78 \times 10^6$	$2.05 \times 10^7 \pm 1.17 \times 10^7$	$5.33 \times 10^7 \pm 5.77 \times 10^{6\dagger}$	$4.00 \times 10^7 \pm 1.00 \times 10^7$	$4.67 \times 10^7 \pm 2.89 \times 10^7$	$3.00 \times 10^7 \pm 2.52 \times 10^7$
		Lys	$1.00 \times 10^7 \pm 9.09 \times 10^6$	$1.98 \times 10^7 \pm 7.07 \times 10^7$	$4.33 \times 10^7 \pm 5.77 \times 10^{6\dagger}$	$2.00 \times 10^7 \pm 1.41 \times 10^7$	$1.03 \times 10^7 \pm 6.66 \times 10^7$	$4.33 \times 10^7 \pm 2.52 \times 10^7$
Beginning	100N Sc	YPD	$3.50 \times 10^7 \pm 5.57 \times 10^6$	$3.13 \times 10^6 \pm 1.43 \times 10^{6\dagger}$	$1.80 \times 10^7 \pm 4 \times 10^{6\dagger}$	$2.77 \times 10^8 \pm 1.50 \times 10^7$	$2.17 \times 10^8 \pm 1.00 \times 10^7$	$1.70 \times 10^7 \pm 6.56 \times 10^6$
	100N ScNSc	YPD	$2.61 \times 10^8 \pm 1.01 \times 10^8$	$7.90 \times 10^7 \pm 8.72 \times 10^6$	$1.21 \times 10^8 \pm 9.54 \times 10^6$	$1.67 \times 10^8 \pm 5.96 \times 10^7$	$1.30 \times 10^8 \pm 6.24 \times 10^7$	$1.33 \times 10^8 \pm 2.52 \times 10^7$
		Lys	$1.43 \times 10^8 \pm 5.77 \times 10^6$	$5.83 \times 10^7 \pm 1.23 \times 10^7$	$6.33 \times 10^7 \pm 1.61 \times 10^7$	$1.34 \times 10^8 \pm 8.49 \times 10^7$	$7.67 \times 10^7 \pm 3.06 \times 10^7$	$8.33 \times 10^7 \pm 2.08 \times 10^7$
	100N NSc	YPD	$1.72 \times 10^8 \pm 1.59 \times 10^8$	$8.70 \times 10^7 \pm 2.55 \times 10^7$	$1.65 \times 10^8 \pm 1.42 \times 10^7$	$2.30 \times 10^8 \pm 7.87 \times 10^7$	$1.57 \times 10^8 \pm 1.53 \times 10^7$	$2.47 \times 10^8 \pm 8.08 \times 10^7$
		Lys	$1.95 \times 10^8 \pm 2.04 \times 10^8$	$8.07 \times 10^7 \pm 2.00 \times 10^7$	$1.52 \times 10^8 \pm 1.74 \times 10^7$	$1.41 \times 10^8 \pm 5.21 \times 10^7$	$8.53 \times 10^7 \pm 2.31 \times 10^7$	$1.63 \times 10^8 \pm 4.04 \times 10^7$

Supplementary Table 1.1: Continued.

Fermentation point	Nitrogen and inoculum	Medium	Control	Tryptophol		Melatonin		
			0 g/L	0.5 g/L	0.1 g/L	1 g/L	0.5 g/L	0.1 g/L
Middle	100N Sc	YPD	$1.30 \times 10^8 \pm 7.51 \times 10^7$	$5.70 \times 10^7 \pm 1.15 \times 10^7$	$5.17 \times 10^7 \pm 9.07 \times 10^{6\dagger}$	$1.03 \times 10^8 \pm 2.69 \times 10^7$	$8.50 \times 10^7 \pm 2.00 \times 10^6$	$1.29 \times 10^8 \pm 1.05 \times 10^7$
	100N ScNSc	YPD	$1.49 \times 10^8 \pm 7.07 \times 10^7$	$1.27 \times 10^8 \pm 1.15 \times 10^7$	$1.38 \times 10^8 \pm 5.51 \times 10^7$	$1.16 \times 10^8 \pm 7.85 \times 10^7$	$2.10 \times 10^8 \pm 8.53 \times 10^7$	$1.30 \times 10^8 \pm 3.51 \times 10^7$
		Lys	$1.40 \times 10^8 \pm 7.00 \times 10^7$	$6.67 \times 10^7 \pm 1.53 \times 10^7$	$5.00 \times 10^6 \pm 3.00 \times 10^6$	$1.07 \times 10^8 \pm 5.34 \times 10^7$	$7.67 \times 10^7 \pm 3.06 \times 10^7$	$1.07 \times 10^8 \pm 1.53 \times 10^7$
	100N NSc	YPD	$1.33 \times 10^8 \pm 1.46 \times 10^8$	$1.24 \times 10^8 \pm 2.08 \times 10^7$	$1.39 \times 10^8 \pm 4.58 \times 10^7$	$1.89 \times 10^8 \pm 6.97 \times 10^7$	$2.57 \times 10^8 \pm 4.58 \times 10^7$	$2.33 \times 10^8 \pm 1.53 \times 10^7$
		Lys	$1.18 \times 10^8 \pm 2.23 \times 10^8$	$1.43 \times 10^8 \pm 3.21 \times 10^7$	$1.07 \times 10^8 \pm 1.53 \times 10^7$	$1.16 \times 10^8 \pm 3.82 \times 10^7$	$1.77 \times 10^8 \pm 3.06 \times 10^7$	$1.80 \times 10^8 \pm 4.58 \times 10^7$
End	100N Sc	YPD	$8.30 \times 10^7 \pm 5.29 \times 10^6$	$2.53 \times 10^7 \pm 4.84 \times 10^7$	$1.33 \times 10^7 \pm 1.53 \times 10^{6\dagger}$	$3.77 \times 10^7 \pm 7.77 \times 10^{6\dagger}$	$7.93 \times 10^7 \pm 2.53 \times 10^7$	$7.97 \times 10^7 \pm 7.51 \times 10^6$
	100N ScNSc	YPD	$1.67 \times 10^8 \pm 5.69 \times 10^7$	$2.37 \times 10^8 \pm 5.13 \times 10^7$	$4.00 \times 10^7 \pm 1.00 \times 10^7$	$8.89 \times 10^8 \pm 3.18 \times 10^7$	$1.67 \times 10^8 \pm 3.79 \times 10^7$	$7.33 \times 10^7 \pm 3.00 \times 10^7$
		Lys	$4.67 \times 10^7 \pm 7.69 \times 10^7$	$<10^7$	$<10^7$	$4.11 \times 10^7 \pm 3.19 \times 10^7$	$9.00 \times 10^7 \pm 1.00 \times 10^7$	$7.33 \times 10^7 \pm 1.15 \times 10^7$
	100N NSc	YPD	$3.00 \times 10^7 \pm 1.97 \times 10^7$	$9.67 \times 10^7 \pm 9.07 \times 10^7$	$8.00 \times 10^7 \pm 4.58 \times 10^7$	$1.25 \times 10^8 \pm 6.48 \times 10^{7\dagger}$	$1.55 \times 10^8 \pm 7.55 \times 10^{7\dagger}$	$8.00 \times 10^7 \pm 6.08 \times 10^7$
		Lys	$3.33 \times 10^7 \pm 2.58 \times 10^7$	$6.67 \times 10^7 \pm 1.15 \times 10^7$	$2.67 \times 10^7 \pm 1.53 \times 10^7$	$7.83 \times 10^7 \pm 4.20 \times 10^{7\dagger}$	$1.25 \times 10^8 \pm 6.51 \times 10^{7\dagger}$	$9.67 \times 10^7 \pm 4.51 \times 10^7$

[a] 300N corresponds to fermentations performed in must with 300mg/l of yeast available nitrogen (YAN)

[b] 100N corresponds to fermentations performed in must with 100mg/l of YAN

* Sc corresponds to fermentations inoculated only with *Saccharomyces cerevisiae*

** ScNSc corresponds to fermentations inoculated with *Saccharomyces cerevisiae* and the mixture of four non-*Saccharomyces* species

*** NSc corresponds to fermentations inoculated with the mixture of four non-*Saccharomyces* species.

†Statistically significant differences compared with control

Chapter 1

Supplementary Figure S1.1: Colony morphology of the five strains of the five species used in this study in WL medium after 48h of incubation at 28 °C (a); Colony morphology of *S. cerevisiae* and *T. delbrueckii* strains in the same medium after 72h of incubation at 28ºC (b).
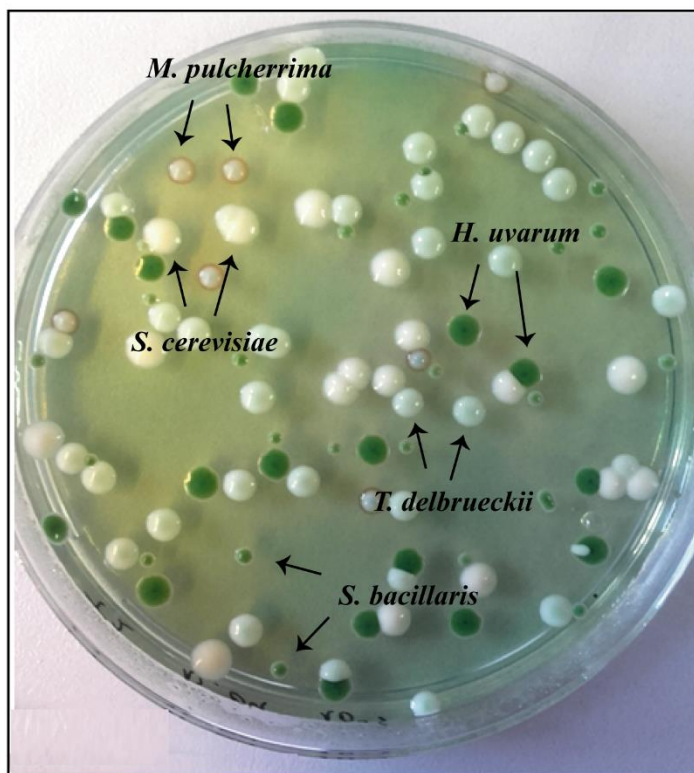
CHAPTER 2

Effect of several nutrients and environmental conditions on intracellular melatonin synthesis in *Saccharomyces cerevisiae*

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Manuscript in preparation

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Chapter 2

Introduction

Several factors, either biotic or abiotic, can affect the microorganisms present during alcoholic fermentation. Microbial changes modify fermentation performance and also the production of bioactive compounds. These microbial changes and their interactions are the main biotic factors. Abiotic factors are defined as environmental aspects that affect the winemaking process, such as nutrients (sugar and nitrogen), temperature and pH, among others. Sugar content in grape must is the first stress that yeasts have to deal with. During osmotic shock, yeasts modify the cell wall and the cytoskeleton as well as activate the synthesis of glycerol to reestablish the osmotic balance (Bauer and Pretorius 2000). Nitrogen is also an important and critical nutrient for yeast cells. In fact, low nitrogen levels can result in stuck or sluggish fermentation (Bison, 1999). Furthermore, temperature can also alter the development of fermentation, specifically yeast growth (Beltran et al., 2008; Torija et al., 2003).

Melatonin is a bioactive molecule that has been recently described to have a positive role against oxidative stress (Vázquez et al., 2017, 2018) and UV stress in yeast cells (Bisquert et al., 2018). In addition to its protective role, melatonin has been described to be produced by yeast during the winemaking process.

Since Sprenger and collaborators (1999) observed intracellular melatonin after tryptophan pulse when yeast cells were arrested in minimal medium, many studies have focused on finding this molecule in fermented beverages such as wine or beer (García-Moreno, 2013; Kocadgli, 2014; Rodriguez-Naranjo et al., 2011a; Vigentini et al., 2015). Rodriguez-Naranjo and collaborators (2011b) highlighted the role of yeast, specifically *Saccharomyces cerevisiae*, in the production of melatonin during the winemaking process. However, not only does *S. cerevisiae* produce melatonin but also several non-*Saccharomyces* strains are able to synthetize melatonin during alcoholic fermentation (Fernández-Cruz et al., 2017, 2019a, 2019b; Valera et al., 2019; Vigentini et al., 2015). Melatonin production depends on the availability of tryptophan, which is a precursor (Fernández-Cruz et al., 2019b; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015) but also some tryptophan derivatives (N-acetyl serotonin or 5-methoxytryptamine) were used as melatonin precursors (Muñiz-Calvo et al., 2019). However, other fermentation conditions as growth medium composition (reducing sugars or nitrogen content) and yeast growth phase can affect melatonin synthesis (Rodriguez-Naranjo et al., 2012; Valera et al., 2019). Moreover, melatonin content is positively correlated with the ethanol production rate during alcoholic fermentation, suggesting that melatonin could participate in alcoholic fermentation (Wang et al., 2016).

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Melatonin has been described in the extracellular medium during the exponential phase of growth (between the first and second day) (Fernández-Cruz et al., 2017, 2019a; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015) and at the end of alcoholic fermentation (at the stationary phase) (Valera et al., 2019) depending on the yeast species involved in the fermentation. Nevertheless, few studies about intracellular melatonin have been published. Some authors have provided evidence that melatonin is present intracellularly after precursor pulses (Fernandez-Cruz et al., 2019b; Muñiz-Calvo et al., 2019; Sprenger et al., 1999); however, in oenological conditions, intracellular melatonin only has been described in non-*Saccharomyces* yeasts (Fernández-Cruz et al., 2019b).

The aim of this study was (i) to assess the capacity of intra- and extracellular melatonin production by different strains of *Saccharomyces* yeasts and (ii) to study the effects of different nutrient and environmental conditions (sugar, nitrogen, temperature, inoculum and cell synchronization) on melatonin production in order to understand the conditions required for the production of melatonin by yeast.

Materials and methods

Yeast strain and inoculum preparation

In this study, we used three different strains of *S. cerevisiae*, QA23 (from wine), Instaferm (from bread baking) and Levucell SC20 (from animal nutrition), and one strain of *S. pastorianus*, Diamond (from beer). All yeasts were provided by Lallemand S.A. (Canada). Yeasts were rehydrated in water at 37ºC for 30 min and plated on YPD plates (1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) bacteriological peptone and 2% (w/v) agar (Panreac Quimica SLU, Barcelona, Spain)) for 48-72 h at 28ºC. Afterwards, the precultures were prepared in 50 mL of YPD broth (1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) bacteriological peptone) at 28ºC with a stirring rate of 120 rpm in an orbital shaker. Then, yeast cells were transferred into fresh minimal medium (1X yeast nitrogen base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) glucose, and 350 mM $(NH_4)_2 SO_4$ (Panreac Quimica SLU, Barcelona, Spain)) and cultured for 3 days at 28ºC and 120 rpm.

Alcoholic fermentation conditions

Synthetic grape must was prepared as in Beltran et al. (2004), but the aromatic amino acid (tryptophan, tyrosine and phenylalanine) concentration was modified. In this study, a five-fold

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increase in aromatic amino acids was used at the expense of the remaining amino acids to maintain the concentration of YAN (yeast assimilable nitrogen) at 300 mg N/L. This synthetic grape must (with 200 g/L sugars (1:1 glucose:fructose) and 300 mg N/L) was considered the standard medium for this study.

Two different experiments were designed. The first consisted of performing fermentations in standard medium using different *S. cerevisiae* strains. Yeasts (QA23, Instaferm, Levucell and Diamond) were inoculated at 2×10^6 cell/mL in 450 mL of synthetic must and incubated in 500-mL bottles with orbital agitation (120 rpm) at 28ºC. Sampling was done at different time points of the growth phases. For harvesting, 10 OD cultures were centrifuged at 7800 rpm for 5 min. Pellets were washed with Milli-Q water, frozen with liquid nitrogen and stored at -80ºC until melatonin analysis. Supernatants were stored at -20ºC until melatonin analysis was performed.

In the second experiment, different fermentation parameters were modified, using the conditions implemented with the QA23 strain in the first experiment (200 g/L, 300 mg N/L, 10^6 cell/mL and 28ºC) as a control fermentation. From precultures in YPD and YNB, the QA23 strain was inoculated in 45 mL of standard medium in 50-mL flasks. To study the effect of nutrients (sugar and nitrogen) on melatonin production, the final concentrations of sugars and assimilable nitrogen were decreased in some fermentors to 20 g/L and 100 mg N/L, respectively. To analyze the effects of environmental parameters, inoculum size was fixed at 10^6, 10^7 and 10^8 cell/mL, and fermentation temperature was adjusted to 4ºC or 12ºC. Cell cycle synchronization (arrested and not-arrested cells) was performed by adding alpha factor (5 µg/mL) before inoculation in grape must.

In all fermentations, samples (10 OD) for intracellular melatonin determination were taken every 10 min (except for synchronization, for which time points were every 20 min) and centrifuged at 7800 rpm for 3 min. After centrifugation, pellets were washed with Milli-Q water, frozen with liquid nitrogen and stored at -80ºC until melatonin analysis was performed.

Melatonin analysis

Intracellular metabolites were extracted by adapting the boiling buffered ethanol method previously described by Gonzalez et al. (1997). Briefly, 1 mL of a solution of 75% (v/v) boiling absolute ethanol containing 70 mM HEPES buffer (pH 7.5) was added to the cell pellet and incubated for 3 min at 80ºC. Then, the extract was first concentrated by evaporation at 45ºC in a SpeedBack (Concentrator plus, Eppendorf Ibérica, Madrid, Spain), and afterwards resuspended

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in 1 mL of Milli-Q water and centrifuged for 10 min at 5000 rpm to remove the insoluble particles. Finally, the supernatant was transferred to a new tube and stored at -20º C until use.

Intracellular melatonin samples were extracted with chloroform. Briefly, 50 µL of sample was mixed with Milli-Q water (1:1, v:v). Then, 10 volumes of chloroform were added, and the mixture was shaken for 1 h at 1200 rpm. The organic phase was dried under a flow of nitrogen gas and resuspended in 50 µL of a methanol and water mixture (40:60, v:v). Then, samples were centrifuged for 5 min at 14500 rpm and analyzed by performing liquid chromatography mass spectrometry (LC-MS/MS). The system was based on high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6410; Agilent Technologies, Palo Alto, USA), using an Agilent 150 x 2.1 mm i.d., 3.5 µM, Zorbax Sb-Aq column in a binary gradient consisting of (A) water and (B) methanol as solvents, both containing 0.1% (v/v) formic acid. The elution profile was 100% B (4 min), 10% B (6 min). The temperature was set at 40ºC, the flow rate was 0.4 mL/min and the injection volume was 7 µL. Melatonin quantification was performed using Agilent MassHunter WorkStation Software Quantitative Analysis Version B0104.

Results and discussion

Effect of strain

To determine whether melatonin production during alcoholic fermentation is a general trait of *Saccharomyces* strains or if instead it is a strain-dependent trait or linked to the strain origin, fermentations with four strains from different environments (QA23, a wine yeast; Instaferm, a baking yeast; Levucell, an animal food yeast; Diamond, a beer yeast) were carried out. In fact, some differences were observed in terms of growth during alcoholic fermentation (Figure 2.1). All yeast strains presented similar growth, except the Instaferm strain, which had less maximal growth. However, all growth phases occurred at similar time points. In terms of fermentation performance, Diamond and QA23 consumed all sugar after three days of fermentation, as observed in the study performed by Lleixà et al. (2019) under similar conditions, while Levucell and Instaferm needed one more day to consume all sugar. Both yeast strains are not used for ethanol production, which explains this slower fermentative behavior.

In addition to the fermentation kinetics, intracellular and extracellular melatonin were measured by LC-MS/MS. On one hand, melatonin was produced intracellularly during the lag phase of yeast growth (Figure 2.2), with QA23 showing the fastest synthesis (1 h), whereas the other

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Saccharomyces yeasts reached their maximum at 4 h. Even though melatonin peaks appeared at different time points (1 and 4 h), it is important to highlight that intracellular melatonin was observed during lag phase in all yeast strains, reinforcing the relationship of melatonin with the yeast growth curve and yeast adaptation to the medium, as previously described by Rodríguez-Naranjo et al. (2012). In fact, in that study, melatonin was proposed as a signal molecule in yeast cells, linked to yeast growth phases and medium adaptation, although only based on extracellular melatonin (Rodriguez-Naranjo et al., 2012). Although all yeast strains produced intracellular melatonin with a similar profile, melatonin quantities differed between strains, with Levucell and Diamond synthesizing more melatonin and presenting a basal concentration throughout all fermentations (Table S2.1).

Additionally, extracellular melatonin was evidenced during alcoholic fermentation in all *Saccharomyces* strains tested (Figure 2.2). The QA23 and Levucell strains reached their maximum level at 24 h, confirming previous results (Rodriguez-Naranjo et al., 2012; Valera et al., 2019). However, in the QA23 strain, the quantity of melatonin decreased from this point until 72 h, when a synthesis rebound was observed, whereas in the Levucell strain, no more melatonin was detected throughout fermentation. Conversely, Diamond exhibited its extracellular melatonin peak later, at 48 h. Finally, in the Instaferm strain, melatonin content progressively increased until the end of fermentation (at 7 days), being the only one that accumulated melatonin during fermentation.
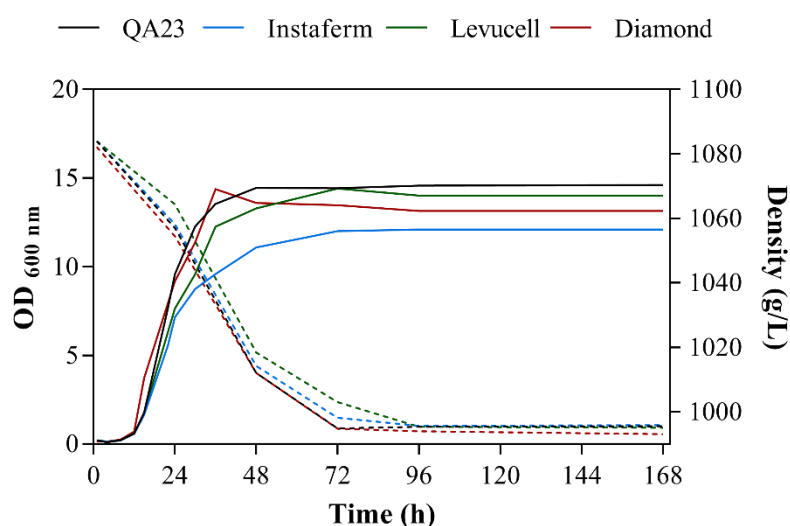

Figure 2.1. Fermentation kinetics of the different *Saccharomyces* yeast strains by monitoring density (dotted line) and population (solid line) throughout fermentation. *Saccharomyces* strains are represented by different line colors: QA23, black; Instaferm, blue; Levucell, green; Diamond, red.
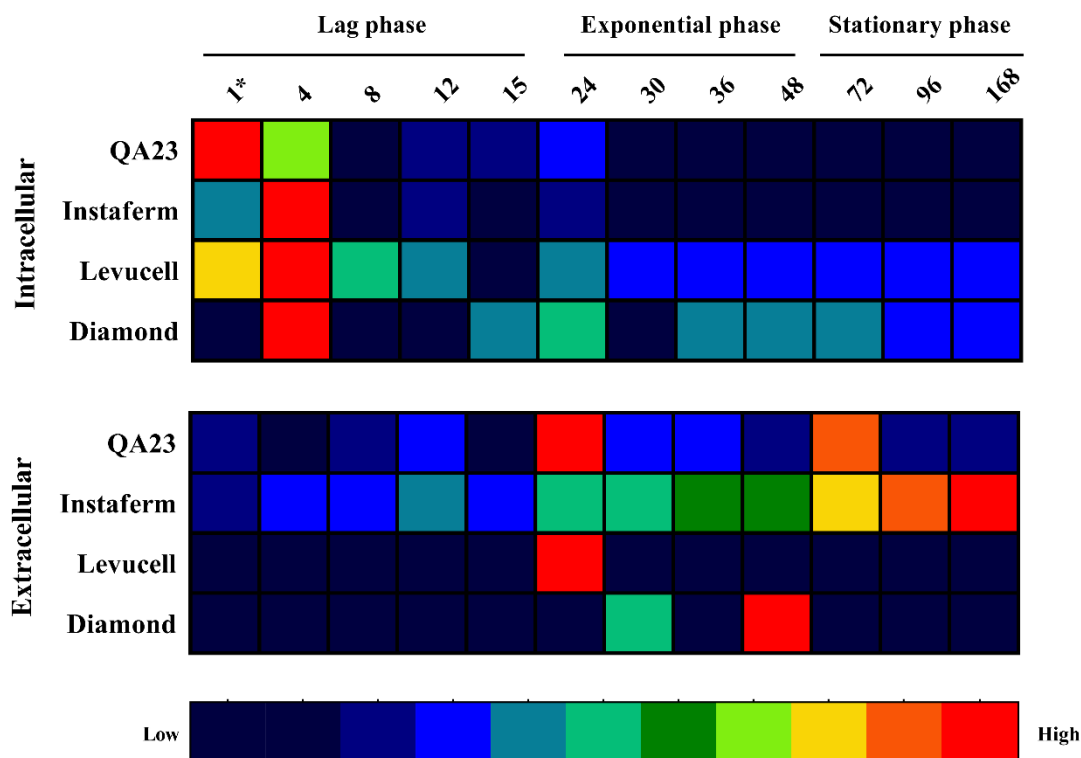
Figure 2.2. Heatmap of melatonin production by the four *Saccharomyces* strains during the different yeast growth phases. Concentrations were normalized to its maximum level. *Time is represented in hours.

These results were similar to those reported by Fernández-Cruz et al. (2016), in which six *Saccharomyces* strains produced melatonin at different points of alcoholic fermentation.

As intracellular melatonin synthesis occurred during lag phase, and we proposed that this synthesis was related to the adaptation of yeast cells to the medium, we modified different important fermentation parameters, such as sugar and nitrogen concentration, temperature or initial population, to determine if any of these parameters were responsible for triggering melatonin synthesis. Intracellular melatonin was monitored during the first 4 h of growth, covering the part of lag phase in which intracellular synthesis was detected in the previous experiment. The results were expressed as a heat map (Figure 2.3).

Effects of sugar and nitrogen

As explained in the previous experiment, QA23 in standard conditions (200 g/L sugar and 300 mg N/L) synthesized melatonin at 60 min (Figure 2.3a). However, in low-sugar conditions (20 g/L), intracellular melatonin was detected starting two hours after inoculation and increasing progressively until 150, 190 and 210 min, when the maximum melatonin concentration was

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quantified. In contrast, in low-nitrogen conditions, intracellular melatonin had a similar profile as that in standard conditions (50 and 80 min), appearing in a similar time frame, although it remained longer in the intracellular medium. Thus, glucose limitation affected melatonin synthesis during alcoholic fermentation. This result may highlight osmotic stress as being responsible for intracellular melatonin synthesis.

Effect of temperature

To elucidate the effect of temperature in melatonin production, alcoholic fermentations were performed at 4ºC and 12ºC, in comparison to standard fermentation carried out at 28ºC.

Intracellular melatonin was evidenced at all temperatures tested (Figure 2.3b). However, surprisingly, higher differences were observed at 12ºC than at 4ºC. Melatonin peaked at 240 min in 12ºC, whereas at the other two temperatures, the highest synthesis was observed at 60 min. Additionally, another peak of lower concentration appeared at 120 min at both low temperatures (4 and 12ºC). Temperature is an important factor during alcoholic fermentation that affects the development of fermentation, in which yeast growth was slowed down (Beltran et al., 2008; Torija et al., 2003). The results at 12ºC seemed to point towards a delay effect on melatonin synthesis due to the prolonged lag phase at low temperatures. In fact, Wang et al. (2016) observed that fermentations carried out at 16ºC delayed and decreased melatonin production in mulberry wines. However, the results at 4ºC did not support this delay effect; it may be that extreme temperatures such as 4ºC triggered other mechanisms responsible for this early melatonin synthesis.

Effect of the initial yeast population

To understand the effect of inoculum amount in melatonin production, fermentations with 10^7 and 10^8 cells/mL were carried out in comparison with the standard inoculation rate (10^6 cell/mL).

As mentioned above, in standard inoculation conditions, melatonin was detected at 60 min (Figure 2.3b). When the inoculum was increased, melatonin synthesis was delayed, at 140 min for 10^7 cell/mL and 180 min for 10^8 cell/mL. Therefore, the more inoculum we used, the greater the delay of the intracellular melatonin peak we obtained. Additionally, differences in melatonin content were observed and were higher in 10^7 cell/mL inoculum (Table S2.3). Given that high inoculum size causes less growth of cells (Carrau et al., 2010), this delay in melatonin synthesis may pinpoint melatonin as a signal molecule for yeast growth.

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Effect of cell cycle synchronization

Finally, as we obtained high variability in melatonin concentration between triplicates, we wanted to synchronize the cell cycle to have most cells in the same cell cycle phase and thus decrease this variability. Due to the impossibility of synchronizing QA23, the correspondent haploid was studied (QA23 ho-). Treatment with alpha-factor was used to synchronize the cell cycle in G1 phase.

As can be observed in Figure 2.3c, during growth in standard grape must, melatonin peaked in haploid cells that were non-arrested at 120 min (maximum content), which was later than the peak in diploid cells (60 min); however, small peaks of melatonin were observed during all experiments, specifically at 80 min. When cells were synchronized at G1 phase and inoculated in synthetic grape must, melatonin exhibited its maximum level at 100 min, but, as in cells that were not arrested, melatonin specifically peaked during the experiment at 60, 120 and 180 min, with 60 min being another important time point for melatonin synthesis. However, despite synchronizing 85% of cells, we did not achieve a clear decrease in melatonin detection variability, probably due to its fast appearance and disappearance and the impossibility of monitoring its synthesis in smaller periods.

Although differences were observed between strains, intracellular melatonin production by all *Saccharomyces* strains was evidenced during the lag phase of yeast growth, pinpointing a role for melatonin as a yeast growth signal molecule. Moreover, extracellular synthesis seems to be linked to the mid-end exponential or early stationary phases, reinforcing results that were previously reported (Fernández-Cruz et al., 2016; Valera et al., 2019). Finally, differences between strains can be attributed to its different isolation origin or uses, and therefore, to different adaptation mechanisms to the fermentation medium, as Vigentini et al. (2015) described for *Torulaspora delbrueckii* and *S. cerevisiae* strains.

When several conditions that affect the performance of alcoholic fermentation were tested, the intracellular melatonin peak was delayed in a low sugar concentration, a fermentation temperature of 12ºC and with a higher initial population. Nevertheless, in all conditions, melatonin appeared during lag phase and as a rapid signal molecule. It is also important to highlight that melatonin quantities were very different between conditions. Thus, the highest production occurred when the fermentation temperature was fixed at 12ºC; however, an initial

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inoculum concentration of 10^7 cells/mL and the low-nitrogen condition also seemed to promote melatonin synthesis (Table S2.2, S2.3 and S2.4).

Finally, despite all of the conditions studied, we were not able to clearly unravel which parameter triggers melatonin synthesis in yeast cells during alcoholic fermentation. The main drawback in achieving this goal was the great variability between triplicates, mainly due to the fast synthesis and disappearance of this molecule from the intracellular medium. Therefore, further studies are needed to understand why melatonin is produced by yeast and which conditions are related to its synthesis.
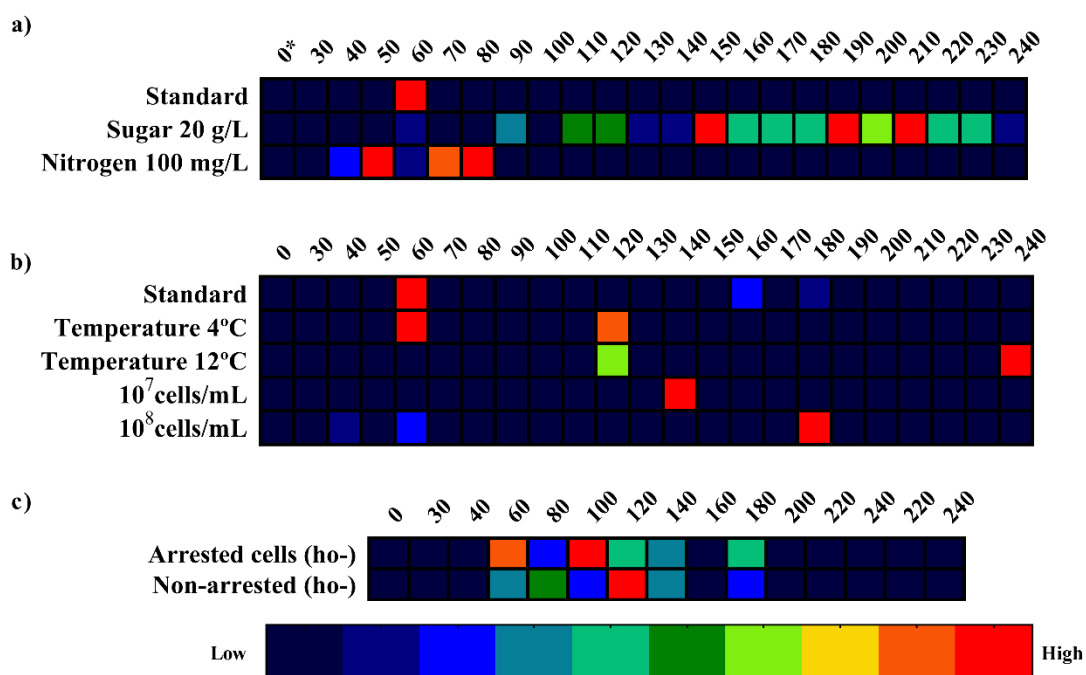

Figure 2.3. Heatmap of melatonin production in several fermentation conditions: (a) low sugar concentration (20 g/L) and low nitrogen concentration (100 mg N/L), (b) temperature and inoculum amount and (c) arrested and non-arrested cells. Concentrations were normalized to its maximum level. *Time is represented in minutes.

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Supplementary material

Supplementary Table 2.1: Intracellular and extracellular melatonin produced by the four different *Saccharomyces* strains during alcoholic fermentation. Data are expressed in ng/mL.

Yeast strain		Time (h)											
		1	4	8	12	15	24	30	36	48	72	96	168
QA23	I	1.38±0.15	0.90±0.16	0.40±0.04	0.20±0.07	0.26±0.00	0.28±0.04	n.d.	0.01±0.01	n.d.	n.d.	0.02±0.02	0.01±0.01
	E	0.16±0.00	0.13±0.02	0.16±0.03	0.31±0.02	0.27±0.22	1.35±1.69	0.28±0.24	0.36±0.28	0.15±0.00	1.09±1.34	0.25±0.07	0.18±0.08
Instaferm	I	0.48±0.01	1.47±1.17	0.28±0.01	0.25±0.08	0.07±0.09	0.21±0.04	n.d.	0.01±0.01	0.01±0.01	0.05±0.06	0.01±0.01	n.d.
	E	0.19±0.00	0.52±0.11	0.38±0.31	0.56±0.46	0.46±0.36	0.82±0.32	0.84±0.72	0.97±0.47	1.07±1.02	1.37±1.61	1.48±1.88	1.82±2.37
Levucell	I	51.80±0.01	67.44±4.91	27.57±3.02	25.97±1.54	20.16±2.35	22.70±2.08	17.53±1.61	17.03±0.05	19.17±1.48	16.67±0.23	17.63±1.78	18.89±0.25
	E	0.10±0.00	0.17±0.03	0.17±0.04	0.19±0.02	0.33±0.29	93.14±85.86	0.87±0.48	0.39±0.15	2.34±2.57	0.44±0.38	0.33±0.34	0.60±0.53
Diamond	I	46.97±0.50	52.56±4.31	26.08±3.37	20.54±3.15	19.57±5.83	21.38±3.40	15.77±2.24	17.95±1.09	18.33±0.58	17.26±023	12.82±2.92	12.73±0.28
	E	0.07±0.00	0.10±0.06	0.21±0.11	0.07±0.00	0.05±0.01	11.14±1.07	6.47±8.76	0.95±1.05	16.13±12.73	0.10±0.01	1.00±1.25	0.15±0.03

n.d.: not detectedt; I: Intracellular; E: Extracellular.

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Supplementary Table 2.2: Intracellular melatonin production by *S. cerevisiae* QA23 in a standard, low-glucose and low-nitrogen grape must. Data are expressed in ng/mL.

Time (min)	Standard	Low glucose	Low nitrogen
0	n.d.	n.d.	n.d.
30	7.39±12.81	0.55±0.45	0.13±0.22
40	n.d.	0.01±0.01	19.85±34.38
50	n.d.	n.d.	65.82±30.26
60	94.47±157.51	0.95±1.00	9.59±16.61
70	18.43±0.05	n.d.	61.44±104.41
80	6.92±0.52	0.01±0.01	69.24±26.14
90	2.18±3.77	3.19±4.49	0.07±0.13
100	n.d.	n.d.	n.d.
110	n.d.	4.11±1.00	n.d.
120	2.19±3.70	4.20±3.36	0.02±0.02
130	0.01±0.02	1.41±2.00	n.d.
140	n.d.	1.13±1.59	n.d.
150	2.16±3.74	7.77±5.15	0.11±0.19
160	n.d.	3.80±0.15	n.d.
170	n.d.	3.80±0.05	n.d.
180	2.30±3.98	3.34±3.38	0.08±0.14
190	0.05±0.01	7.45±9.26	n.d.
200	0.01±0.01	5.07±0.86	n.d.
210	6.52±11.30	8.18±6.85	n.d.
220	n.d.	3.56±0.12	0.32±0.56
230	n.d.	3.58±0.05	n.d.
240	2.29±3.86	0.82±1.42	0.17±0.29

n.d. not detected

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Supplementary Table 2.3: Effect of low temperature and inoculum size on intracellular melatonin production by *S. cerevisiae* QA23. Data are expressed in ng/mL.

Time (min)	Standard	4ºC	12ºC	10^7 cells/mL	10^8 cells/mL
0	n.d.	n.d.	n.d.	n.d.	n.d.
30	n.d.	n.d.	n.d.	n.d.	n.d.
40	n.d.	n.d.	n.d.	n.d.	0.13±0.02
50	n.d.	n.d.	n.d.	n.d.	n.d.
60	2.34±0.37	6.01±9.82	0.40±0.70	n.d.	0.24±0.03
70	0.01±0.02	0.01±0.01	n.d.	n.d.	n.d.
80	0.01±0.01	0.01±0.01	n.d.	n.d.	n.d.
90	n.d.	0.05±0.01	0.23±0.40	n.d.	n.d.
100	n.d.	n.d.	0.50±0.45	n.d.	n.d.
110	n.d.	n.d.	n.d.	n.d.	n.d.
120	n.d.	5.35±9.27	462.40±800.45	n.d.	n.d.
130	n.d.	n.d.	0.01±0.02	n.d.	n.d.
140	n.d.	n.d.	n.d.	24.33±2.92	n.d.
150	0.01±0.01	n.d.	2.78±0.70	n.d.	n.d.
160	0.56±0.19	n.d.	0.23±0.11	n.d.	n.d.
170	n.d.	n.d.	0.11±0.06	n.d.	n.d.
180	0.45±0.12	n.d.	27.70±1.02	n.d.	0.87±0.15
190	0.01±0.01	n.d.	0.01±0.01	n.d.	n.d.
200	n.d.	n.d.	n.d.	n.d.	n.d.
210	n.d.	n.d.	0.63±1.06	n.d.	n.d.
220	n.d.	n.d.	n.d.	2.06±0.52	n.d.
230	n.d.	n.d.	0.26±0.25	n.d.	n.d.
240	n.d.	n.d.	1328.92±0.69	n.d.	n.d.

n.d. not detected

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Supplementary Table 2.4: Comparison of intracellular melatonin production by arrested and non-arrested yeast cells. Data are expressed in ng/mL.

Time (min)	Non-arrested	Arrested
0	n.d.	n.d.
30	n.d.	n.d.
40	n.d.	n.d.
60	7.88±1.67	16.87±23.86
80	12.58±4.83	5.79±0.62
100	4.40±1.14	20.53±22.55
120	21.82±12.42	8.49±2.01
140	7.43±0.89	6.95±0.40
160	0.01±0.01	n.d.
180	4.96±0.37	8.79±11.19
200	n.d.	n.d.
220	n.d.	n.d.
240	n.d.	n.d.

n.d. not detected

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Glycolytic proteins interact with intracellular melatonin in *Saccharomyces cerevisiae*

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Abstract

Melatonin is a bioactive compound that is present in fermented beverages and synthesized by yeast during alcoholic fermentation. Many studies have shown that melatonin interacts with some mammalian proteins, such as sirtuins or orphan receptor family proteins. The aim of this study was to determine the intracellular synthesis profile of melatonin in *Saccharomyces cerevisiae* and to identify the proteins that may interact with this molecule in yeast cells. Melatonin from fermentation samples was analysed by liquid chromatography mass spectrometry, and proteins bound to melatonin were immunopurified by melatonin-IgG-Dynabeads. Melatonin was produced intracellularly in the lag phase of yeast growth and was exported to the extracellular media during the stationary phase. During this period, melatonin was bound to six proteins with molecular weights from 55 kDa to 35 kDa. Sequence analysis showed that most proteins shared high levels of homology with glycolytic enzymes. An RNA-binding protein was also identified, the elongation alpha factor, which is related to mitochondria. This study reports for the first time the interaction of melatonin and proteins inside yeast cells. These results highlight the possible role of melatonin as a signal molecule and provide a new perspective for understanding its role in yeast.

Keywords: melatonin, fermented beverages, glycolysis, GADPH, enolase, pyruvate kinase

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indole amine synthesized from L-tryptophan (Reiter, 1991) that presents antioxidant activity and has been associated with the regulation of circadian rhythm and reproduction in humans (Reiter et al., 2007; Galano et al., 2011; Serrano et al., 2001). Recently, melatonin has also been associated with a protective function against oxidative stress and UV radiation in *Saccharomyces* yeast (Vázquez et al., 2017, 2018; Bisquert et al., 2018).

Melatonin has been described in many organisms, including bacteria, algae, fungi, insects and plants (Harderland and Poeggeler, 2003). Several studies have also revealed the presence of melatonin in many fermented beverages, such as beer (Kocadağlı et al., 2014), fermented orange beverages (Fernández-Pachón et al., 2014) and wine (Mercolini et al., 2008; Stege et al., 2010; Rodriguez-Naranjo et al., 2011; Vitalini et al., 2013; Wang et al., 2016; Fernández-Cruz et al., 2017, 2018).

When the winemaking process is monitored, *Saccharomyces cerevisiae* produces significant amounts of melatonin and other methoxyindoles during standard yeast growth and alcoholic fermentation, which highlights the role of yeast in the production of melatonin in wine. The concentration of melatonin reaches its maximum between the first and the second day of fermentation (Sprenger et al., 1999; Arévalo-Villena et al., 2010; Rodriguez-Naranjo et al., 2012; Vigentini et al, 2015). Production also depends on precursor availability; tryptophan is essential as it is the principal precursor, and its presence increases and accelerates the synthesis of melatonin (Sprenger et al., 1999; Rodriguez-Naranjo et al., 2012). However, a recent study has detected melatonin produced from serotonin and 5-methoxytryptamine pulse (Muñiz-Calvo et al., 2019).

The different functions of melatonin in human cells suggest the existence of specific receptors. Many studies have associated melatonin with two transmembrane proteins that belong to the GPCR superfamily as the receptors of this molecule in the mammalian membrane (MT1 and MT2) (Reppert, 1997), which are encoded by *MTRN1A* and *MTNR1B* genes, respectively (Jockers et al., 2016).

In addition, melatonin has also been detected in cell nuclei. Previous studies with [3H] melatonin showed the existence of interaction sites for the binding of this molecule in the nuclei on orphan receptor family proteins (Becker-andré et al., 1994; Carlberg et al., 1995; Wiesenberg et al., 1995).

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Thus, other nuclear proteins, such as calreticulin, a ubiquitous protein that is involved in intracellular signaling pathways under Ca^{2+} binding, have been associated with melatonin interactions (Macías et al., 2003). Calreticulin is multifunctional and may play an important role in the modulation of a variety of cellular processes.

On the other hand, melatonin has also been associated with sirtuins, class III histone deacetylase enzymes that regulate the cell cycle, DNA repair, cell survival and apoptosis and have important roles in normal and cancer cells (Mayo et al., 2017). Sirtuin genes are highly conserved in organisms ranging from archaea to humans. In fact, the first sirtuin gene to be identified in yeast was the "silent information regulator 2" (*SIR2*) (Braunstein et al., 1993; Blander and Guarente, 2004), which is responsible for gene silencing at mating type loci, telomeres, or rDNA (Smith and Boeke, 1997). Nevertheless, only mammalian sirtuins have been associated with melatonin (Das, 2005).

Although several authors have demonstrated that melatonin is synthesized by yeast during alcoholic fermentation (Sprenger et al., 1999; Arévalo-Villena et al., 2010; Rodriguez-Naranjo et al., 2011, 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018; Muñiz-Calvo et al., 2019), its role inside yeast cells and what signals trigger its synthesis are unclear. The aim of this study was to determine the intracellular synthesis profile of melatonin in yeast during alcoholic fermentation and the fate of this molecule inside the cell before its excretion into the extracellular medium. To this end, we first analyzed the intracellular and extracellular melatonin levels produced by *S. cerevisiae* during fermentation conditions. We subsequently performed immunopurification melatonin-IgG-Dynabeads and identified a set of proteins that interact with melatonin inside the cell. This study reports for the first time the interaction of melatonin and proteins in yeast cells.

Material and Methods

Yeast strain and inoculum preparation

In this study, we used one strain of *S. cerevisiae,* QA23, from Lallemand S.A. (Canada). The yeast was rehydrated in water at 37ºC for 30 min and plated on YPD plates (1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) bacteriological peptone and 2% (w/v) agar (Panreac Quimica SLU, Barcelona, Spain)) for 48-72 h at 28ºC. Afterwards, the preculture was prepared in 50 mL of YPD

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broth (1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) bacteriological peptone) and shaken overnight at 200 rpm and 28ºC. Then, yeast cells were transferred into fresh minimal medium (1X yeast nitrogen base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) glucose, and 350 mM $(NH_4)_2 SO_4$ (Panreac Quimica SLU, Barcelona, Spain)) and cultured for 3 days at 28ºC and 120 rpm.

Alcoholic fermentation conditions

Synthetic grape must (SM) was prepared based on Beltran et al. (2004) with some modifications: the aromatic amino acid (tryptophan, tyrosine and phenylalanine) concentration was increased five-fold in relation to the regular concentration (González et al., 2018). These increased concentrations of aromatic amino acids occurred at the expense of the remaining amino acids to maintain the concentration of YAN (yeast assimilable nitrogen) (300 mg/L). A total of 450 mL of medium, inoculated with $2x10^6$ cells/mL, was placed in 500 mL bottles. Fermentations were performed in triplicate at 28ºC with continuous orbital shaking (120 rpm). Cell populations were evaluated by measuring the optical density (OD_{600nm}), and 10^8 cells were collected at different time points during yeast growth. Samples were centrifuged at 12000 rpm for 3 min at room temperature. The supernatant was stored at -20ºC for extracellular melatonin analysis, and the pellet was washed with distilled water, frozen in liquid nitrogen and stored at -80ºC for intracellular melatonin analysis.

Melatonin analysis

Intracellular metabolites were extracted by adapting the boiling buffered ethanol method previously described by Gonzalez et al. (1997). Briefly, 1 mL of a boiling solution of 75% (v/v) absolute ethanol containing 70 mM HEPES buffer (pH 7.5) was added to the cell pellet (10^8 cells). This mixture was incubated for 3 min at 80ºC. The extract was concentrated by evaporation at 45ºC in a SpeedBack (Concentrator plus, Eppendorf Ibérica, Madrid, Spain). The final intracellular content was resuspended in 1 mL of Milli-Q water and centrifuged for 10 min at 5000 rpm to remove the insoluble particles. The supernatant was transferred to a new tube and stored at -20ºC until use.

Intracellular and extracellular melatonin samples were extracted with chloroform. Briefly, 50 µL of sample was mixed with Milli-Q water (1:1, v:v). Then, 10 volumes of chloroform were added. Samples were shaken for 1 h at 1200 rpm. The organic phase was dried under a flow of nitrogen

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gas and resuspended in 50 μL of a mixture of methanol and water (40:60, v:v). Then, the samples were centrifuged for 5 min at 14500 rpm. Supernatants were transferred and analyzed.

Samples were analyzed by performing liquid chromatography mass spectrometry (LC-MS/MS) following the method described by Rodriguez-Naranjo et al. (2011) with some modifications. The system was based on a high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6410; Agilent Technologies, Palo Alto, CA, USA). Melatonin separation was performed using an Agilent 150 x 2.1 mm i.d., 3.5 μM, Zorbax Sb-Aq column. Chromatographic separation was performed using a binary gradient consisting of (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 40ºC. The flow rate was 0.4 mL/min. The injection volume was 7 μL. Melatonin quantification was performed using Agilent MassHunter WorkStation Software Quantitative Analysis Version B0104 by comparing the 233/174 transition MS data of the sample and the standard.

Protein purification

Samples from different fermentation times were purified by a Pierce™ Crosslinking Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, melatonin IgG-Dynabeads were prepared by crosslinking anti-melatonin rabbit IgG antibody to Dynabeads (LifeSpan BioSciences, Seattle, WA, USA). Then, 10^8 cells were resuspended in 1 mL of extraction buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% NP40; pH 7.4) and lysed by glass beads by applying three shaking cycles of 1 min in Mini Beadbeater-24 (BioSpec Products, Bartlesville, OK, USA) and 1 min on ice. Lysed cells were centrifuged at 3000 rpm and 4ºC for 10 min to remove insoluble particles. Melatonin IgG-Dynabeads were added to each lysate, and the suspension was rotated for 1 h at room temperature. The melatonin IgG-Dynabeads were collected with a magnet and washed three times with 500 μL of ice-cold extraction buffer. Isolated proteins were eluted from the melatonin IgG-Dynabeads by following manufacter's instructions and resolved by 12% SDS-PAGE. Proteins in the gel were visualized by Pierce™ Silver Stain kit (Thermo Fisher Scientific, Waltham, MA, USA). Image J software (https://imagej.nih.gov/ij/) was used to quantify the protein intensities in the SDS-PAGE analysis.

Protein digestion and analysis

Visible gel bands were excised from the silver-stained gel and washed with 25 mM ammonium bicarbonate (ABC) pH 8 and acetonitrile. Samples were reduced by adding 10 mM dithiothreitol

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for 1 h at 56°C and alquilated with 55 mM iodoacetamide for 30 min at room temperature in darkness. Then, the samples were washed with 25 mM ABC pH 8 and a mixture of 25 mM ABC and acetonitrile (50%, v:v). For digestion, gel bands were first rehydrated with sequencing-grade trypsin solution at a ratio of trypsin:protein 1:100 (w/w) (considering 50 µg of protein per band), covered with 50 mM ABC and incubated overnight at 37ºC. Extraction of peptides from gels was performed by incubating them with a mixture of acetonitrile (50%) and formic acid (5%) for 15 min and collecting the supernatant. The final extraction step was performed with acetonitrile (100%). Before mass spectrometry analysis, samples were desalted using HLB SPE (Waters, USA) and resuspended in 25 µL of 0.1% of formic acid.

Peptides were analyzed by Nano LC-(Orbitrap) MS/MS (LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher, San José, CA, USA)). Two microliters of sample (~1 µg of protein digest) was separated into a C18 reversed-phase (RP) nanocolumn (75 µm i.d.; 15 cm length; 3 µm particle diameter, NikkyoTechnos Co. LTD, Japan) coupled to a trap nanocolumn (100 µm i.d.; 2 cm length; 5 µm particle diameter, Thermo Fisher Scientific) using a 60 min acetonitrile gradient (A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid). The flow rate during the elution gradient was 300 nL/min. For real-time ionization and peptide fragmentation, an enhanced FT-resolution spectrum (resolution = 30000 FHMW) was used, followed by a data-dependent IT-MS/MS scan from the most intense ten parent ions with a charge state rejection of one using a CID fragmentation with a normalized collision energy of 35% and dynamic exclusion of 0.5 min.

Protein identification analysis and relative quantification

Tandem mass spectra were extracted and charge states deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific, Waltham, MA, USA). All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Mascot (v2.5) was set up to search SwissProt_2018_03.fasta database (557012 entries), restricting for *S. cerevisiae* taxonomy (7904 sequences) and for other mammals (13162 sequences) and assuming trypsin digestion. Three missed cleavages and an error of 0.8 Da for fragment ion mass and 10 ppm for precursor ion were allowed. Oxidation of methionine and acetylation of the N-terminal were specified as variable modifications, whereas carbamido methylation of cysteine was set as a static modification. The false discovery rate was set at 0.01. For proteins identified only with one peptide, visual verification of fragmentation spectra was performed.

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Results and discussion

Melatonin production

Fermentations with the QA23 strain were performed using SM, which ensured the reproducibility of the experiment, that was enriched in aromatic amino acids (five-fold normal concentration) because yeast growth conditions can influence levels of methoxyindoles (Sprenger et al., 1999). Different time points during the fermentation process were analyzed.

In addition to melatonin content determination, fermentation parameters such as density and yeast growth were measured. QA23 completed the fermentation with no residual sugars (<1 g/L, Figure 3.1a) in 3-4 days, similar to results achieved in previous studies using the same conditions (Lleixà et al., 2016; González et al., 2018). Intracellular melatonin was observed in the first hour of fermentation (lag phase, Figure 3.1b), reaching its maximum at 1.38 ng/mL per 10^8 cells. After this first peak, the intracellular levels of melatonin rapidly decreased, until the late exponential phase, when another peak of melatonin concentration was detected at 22 h (0.5 ng/mL). The first part of the growth curve (0-6 h) is expected to be crucial for yeast as only essential molecules are formed while the yeast is trying to adapt to the conditions of the new medium (Rodriguez-Naranjo et al., 2012).

In contrast, in the extracellular media, melatonin first appeared when yeast cells were in late exponential phase (22 h; Fig. 3.1b), confirming previous results of other authors that reported the detection of extracellular melatonin at 24 h (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Valera et al., 2019). At this point, metabolism is very active to support exponential yeast growth. After that, yeast cells entered the stationary phase, and the melatonin concentration decreased. When all sugars were almost consumed, extracellular melatonin peaked again at 72 h (1.15 ng/mL; Fig. 3.1b). A previous study reported that the maximum production of melatonin occurred at the end of alcoholic fermentation (Fernández-Cruz et al., 20118).

Although intracellular melatonin production occurred very quickly during the lag phase, probably due to the importance of some molecules in the yeast adaptation process to the new conditions (Rodriguez-Naranjo et al., 2012), the melatonin levels inside the cell rapidly decreased and could not be detected in the extracellular media until late exponential and stationary phase. Given the difference between intracellular production and extracellular detection of melatonin, a possible interaction with some molecules inside the cell, such as proteins, could be responsible for this gap in melatonin secretion.
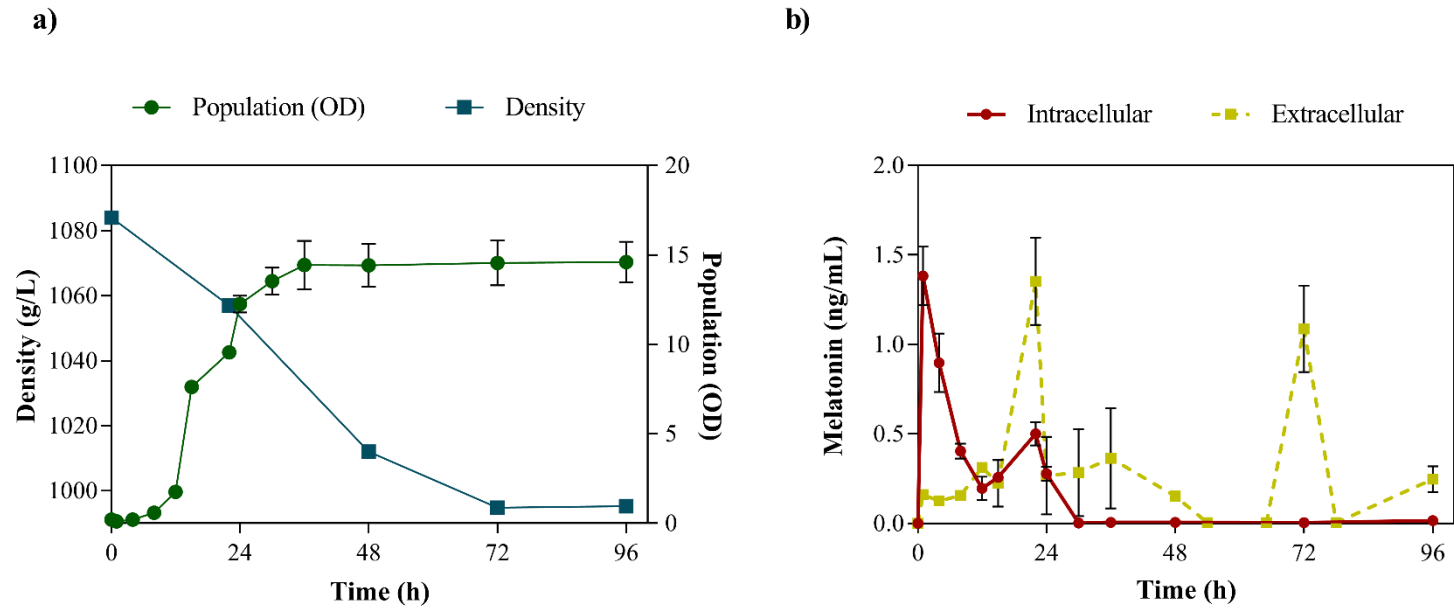
a)

b)

Figure 3.1. Fermentation kinetics of the QA23 yeast strain (a) by monitoring density (▪) and population (●) throughout the fermentation. Intra- (●) and extracellular (▪) concentrations of melatonin (b) during alcoholic fermentation performed by the QA23 *S. cerevisiae* strain. The intracellular concentration of melatonin is represented in ng/mL per 10^8 cells.

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Protein analysis

During alcoholic fermentation, several samples were collected to determine a possible interaction between melatonin and proteins. The proteins bound to melatonin IgG were purified from crude extract using a Pierce™ Crosslinking Magnetic IP/Co-IP Kit. After SDS-PAGE gel electrophoresis, proteins bound to the anti-melatonin antibody were excised and in-gel trypsin digested, and the resulting peptides were analyzed by Nano LC-(Orbitrap) MS/MS. The protein purification protocol was confirmed by gel electrophoresis of the total protein extract (TE) and the purified proteins (P) (Figure 3.2). Several protein bands with molecular weights ranging from 55 kDa to 35 kDa were bound specifically to melatonin.

The proteins bound to melatonin were purified at different time points during alcoholic fermentation (Figure 3.3). The detection of proteins bound to melatonin was inversely related with its intracellular concentration. No melatonin-binding proteins were detected during the first 8 h of fermentation (the lag phase of yeast growth; Figure 3.1), in which the first peak of intracellular melatonin was observed. Instead, coinciding with low levels of melatonin in the intracellular medium, six melatonin-binding protein bands appeared, presenting a stronger intensity at 20 h. Then, a clear decrease in the protein band intensity was observed, coinciding with the appearance of melatonin in the extracellular medium and with a small increase in intracellular melatonin. Once again, the decrease in intracellular melatonin overlapped with an increase in the protein band intensity at 30 h, which corresponded with the entrance in the stationary phase. During this phase, only very light bands were observed. The interaction of melatonin with these proteins seemed to follow a circadian rhythm-like. Although *S. cerevisiae* is
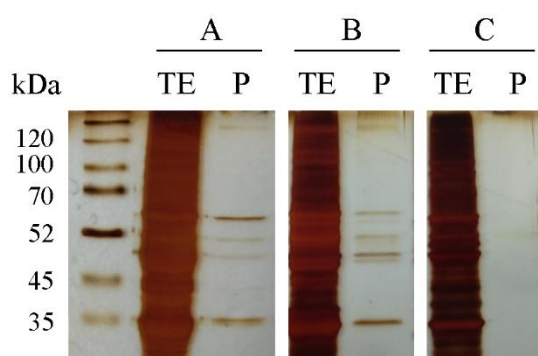

Figure 3.2. SDS-PAGE gel electrophoresis of total protein extracts before (TE) and after purification with melatonin IgG-Dynabeads (P) from three yeast samples (A-C). In all cases, the same total protein concentration was used. In samples A and B, several proteins bound to melatonin were observed, although with different intensities, while in sample C, no proteins were bound.

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not included in the circadian model organisms, Eelderink-Chen et al. (2010) demonstrated that yeast metabolism shows a systematic circadian entrainment. Given that melatonin is a hormone produced in the pineal gland in humans (Lerner et al., 1958) and a key regulator of human chronobiological and endocrine functions (Jahanban-Esfahlan et al., 2018), this interaction may indicate the role of melatonin as a signal molecule in yeast.

Mass spectrometry analysis allowed us to identify these melatonin-binding proteins. The results, shown in Table 3.1, were obtained from different independent experiments. Surprisingly, most of the proteins identified participate in the glycolytic pathway: pyruvate kinase 1 (Pyk2p; band a), enolase (Eno1p, Eno2p; band c), fructose biphosphate aldolase (Fba1p; band d) and glyceraldehyde-3-phosphate dehydrogenase (Tdh1p, Tdh2p, Tdh3p; band f). The interaction of melatonin with all these proteins could suggest the presence of a glycolytic complex. Brandina et al. (2006) demonstrated that all glycolytic enzymes were associated with mitochondria in yeast, providing evidence for the formation of a macromolecular complex involving enolase and other glycolytic enzymes bound to the mitochondrial surface. The formation of this glycolytic complex, the glycolytic metabolon, could allow and regulate the channeling of different substrates into the mitochondria, such as pyruvate towards the TCA cycle or t-RNA mitochondrial import (Brandina et al., 2006; Graham et al., 2007). In *Arabidopsis thaliana*, glycolytic enzymes have also been described to be present on the surface of the mitochondria, in addition to the cytosol, forming a complex that supports substrate channeling to the mitochondria (Graham et al., 2007). In addition, Giegé et al. (2003) observed that seven out of ten glycolytic enzymes of *Arabidopsis* cells were colocalized in the mitochondria.

Moreover, we identified an RNA-binding protein (Figure 3.3, band b), the cytoplasmatic translation elongation factor TEF1-A (Tef1p). Tef1p has also been reported to be part of the enolase complex in the mitochondria of *S. cerevisiae* (Brandina et al., 2006). Given that melatonin seems to be synthesized in the mitochondria (He et al., 2016), where it also develops its antioxidant function by preventing ROS toxicity (Acuña-Castroviejo et al., 2001; Okatani et al., 2002), melatonin may also act as a signal to activate the binding of the glycolytic complex. In addition, Cui et al. (2018) demonstrated that exogenous melatonin significantly altered the expression of glycolytic proteins, including enolase, fructose biphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase in wheat. Additionally, glyceraldehyde-3-phosphate dehydrogenase has been reported to be regulated on a daily basis by the circadian clock in *Neurospora*, an ascomycete fungus (Shinohara et al., 1998).

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Finally, alcohol dehydrogenase was also detected but with the lowest Mascot score (Fig 3.3, band "e" and Table 3.1). Geigé et al. (2003) detected low activity of this enzyme in mitochondrial extracts and hypothesized that a low proportion of alcohol dehydrogenase could be associated with mitochondria.

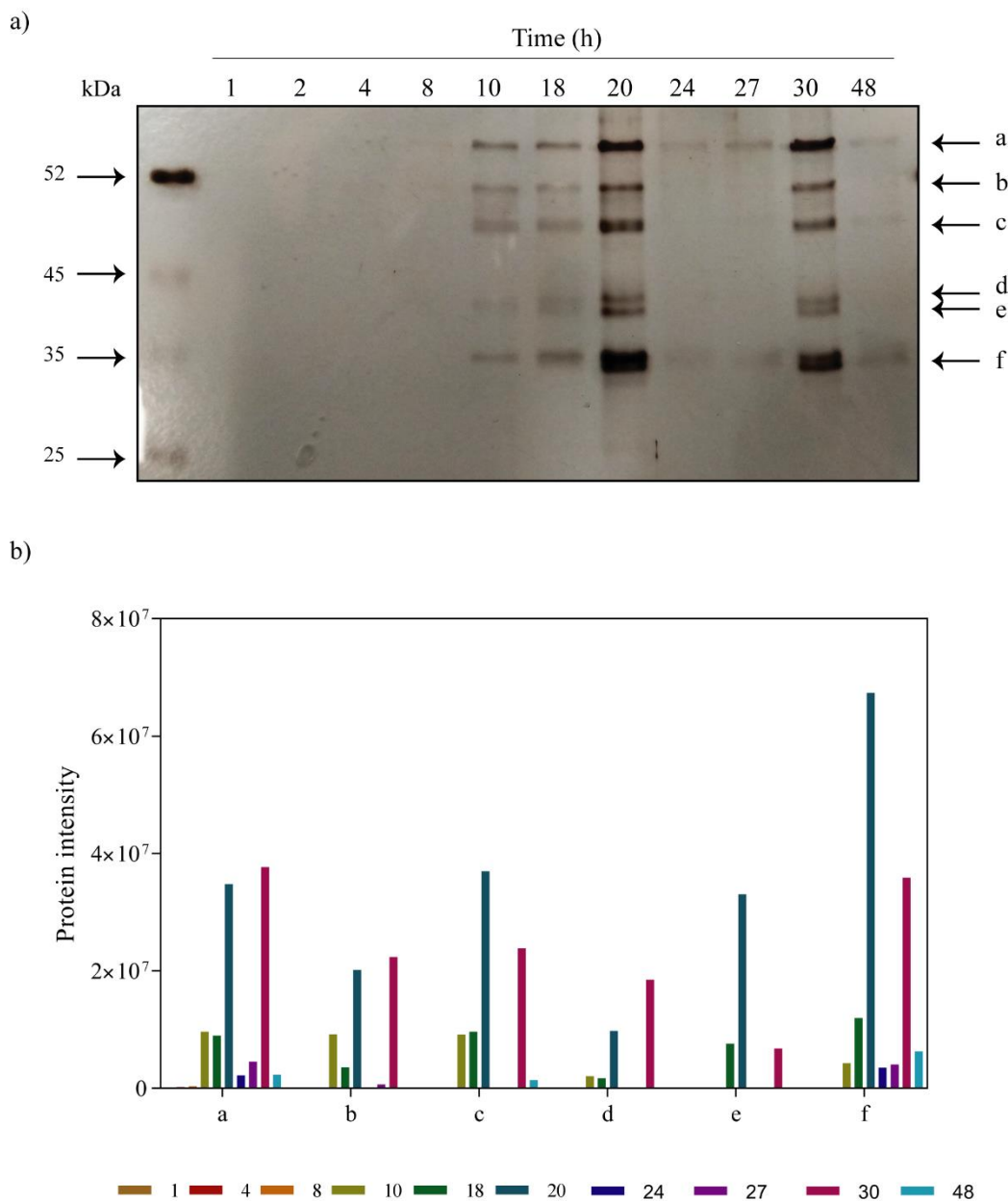Figure 3.3. Time course of purified proteins during alcoholic fermentation. (a) SDS-PAGE gel electrophoresis. Bands marked are proteins bound to melatonin: a. Pyruvate kinase 1; b. Elongation alpha factor; c. Enolase; d. Alcohol dehydrogenase; e. Fructose biphosphate aldolase; f. Glyceraldehyde-3-phosphate dehydrogenase. (b) Quantification of protein intensities performed using Image J software.

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Conclusions

Melatonin is produced in the lag phase of yeast growth and is exported to the extracellular media during the stationary phase in fermentation conditions. Between intracellular and extracellular concentration peaks, melatonin seems to be bound to several proteins in *S. cerevisiae*. Most purified proteins participate in the glycolytic pathway, suggesting a possible complex of glycolytic enzymes bound to melatonin. This interaction may highlight the role of melatonin as a signal molecule in yeast. In addition, the interaction with TEF1-A could indicate that melatonin is part of the cell signaling process in stress conditions in yeast and that this glycolytic complex might facilitate the export of this molecule. The interaction between these proteins and melatonin in *S. cerevisiae* seems to follow a circadian rhythm-like. Thus, this study reports for the first time the interaction of melatonin and proteins in yeast cells. Nevertheless, further studies are needed to understand the interaction between melatonin and yeast proteins to help elucidate the biological importance of melatonin in yeast.

Acknowledgments

Table 3.1. Identification of proteins purified from the SDS-PAGE gel using Nano LC-(Orbitrap) MS/MS.

Protein gel band	Gene name	Molecular weight (kDa)	Protein description (SGD database)	Sequence coverage, % number of peptides recovered by Nano LC-MS/MS	Mascot Score
A	PYK2	54.5	Pyruvate kinase 1	69.40	2048.70
B	TEF1	50.0	Translation Elongation Factor	56.55	1457.92
C	ENO1	46.8	Enolase	61.56	3294.52
	ENO2	46.9	Enolase	56.75	3343.08
D	FBA1	39.6	Fructose biphosphate aldolase	60.72	1551.78
E	ADH1	36.8	Alcohol dehydrogenase	56.32	797.83
F	TDH1	35.7	Triose-phosphate dehydrogenase	91.27	5885.33
	TDH2	35.8	Triose-phosphate dehydrogenase	74.40	4731.88
	TDH3	35.7	Triose-phosphate dehydrogenase	82.83	4219.04

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CHAPTER 4

Melatonin synthesis and protein interactions in *Saccharomyces cerevisiae* during alcoholic fermentation are yeast growth-dependent

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine synthesized from L- tryptophan (Reiter, 1991) that has been found in a wide range of invertebrates, plants, bacteria and fungi (Hardeland and Poeggeler, 2003; Tan et al., 2015). Thus, melatonin is considered a ubiquitous molecule in most living organisms (Tan et al., 2015).

Melatonin has been described in many fermented beverages such as beer or wine, pointing out the role of yeast metabolism in melatonin production during alcoholic fermentation (Fernández-Cruz et al., 2017; Rodriguez-Naranjo et al., 2012). The melatonin concentration reaches its maximum between the first and second day of fermentation in extracellular medium (Fernández-Cruz et al., 2016, 2017; Rodriguez-Naranjo et al., 2012). Nevertheless, intracellularly, melatonin appears in the lag phase of *Saccharomyces* yeast during alcoholic fermentation (Chapter 3). Production also depends on precursor availability; tryptophan is essential as it is the principal precursor, and its presence increases and accelerates the synthesis of melatonin (Rodriguez-Naranjo et al., 2012; Sprenger et al., 1999). However, a recent study has detected melatonin produced from serotonin and 5-methoxytryptamine pulse (Muñiz-Calvo et al., 2019).

Melatonin has been associated with different proteins in human cells. The most described interaction is with two transmembrane proteins that belong to the GPCR family, MT1 and MT2, which are the receptors of this molecule in the mammalian membrane (Reppert, 1997) encoded by the *MTRN1A* and *MTNR1B* genes, respectively (Jockers et al., 2016). Moreover, melatonin has been related to nuclear proteins such as orphan receptors (Becker-André et al., 1997; Carlberg and Wiesenberg, 1995; Wiesenberg et al., 1995), calreticulin (Macías et al., 2003) or sirtuins (Das, 2005; Mayo et al., 2017). Nevertheless, the ability of melatonin to interact with yeast proteins has not been explored so far. Recently, Morcillo-Parra et al. (Chapter 3 and 5) observed that glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GADPH; Tdh1p, Tdh2p and Tdh3p), enolase 1 (Eno1p), pyruvate kinase 1 (Pyk2p) and fructose bisphosphate aldolase (Fab1p), can interact with melatonin in *Saccharomyces* and non-*Saccharomyces* yeast, suggesting that melatonin may be a signal molecule to form a glycolytic complex.

The GADPH gene has been reported to be regulated on a daily basis by the circadian clock in *Neurospora*, an ascomycete fungus (Shinohara et al., 1998). In addition, gene disruption studies have shown that the *TDH1* gene alone cannot support growth in yeast and may therefore carry out a different function from those of the other two isozymes (*TDH2* and *TDH3*) (McAlister and

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Holland, 1985). Moreover, some studies indicate that GAPDH may be considered a moonlighting protein due to the observation of activities unrelated to these classical functions (Sirover, 2011). GAPDH is directly involved in transcriptional (Zheng et al., 2003) and posttranscriptional gene regulation (Bonafe et al., 2005; Zhou et al., 2008), vesicular transport (Tisdale, 2001), receptor-mediated cell signaling chromatin structure (Demarse et al., 2009) and the maintenance of DNA integrity (Azam et al., 2008). It has also been reported to be a cell wall protein (Delgado et al., 2001) and yield fragments secreted as antimicrobial peptides (Branco et al., 2014).

As part of the enolase complex in *S. cerevisiae*, Eno1p has been related to mitochondria. This complex is formed by different glycolytic proteins, such as enolase and GAPDH but also by RNA-binding proteins (Tef1p) (Brandina et al., 2006). Morcillo-Parra et al. (Chapter 3) also related all of these proteins to melatonin during alcoholic fermentation. In addition, the Pyk2p protein has been described to be insensitive to fructose-1,6-biphosphate and may carry out other functions not related to glycolysis (Boles et al., 1997).

Given that melatonin production occurs in the lag phase of yeast growth, and melatonin interaction with yeast proteins is highly observed in the exponential phase, two different experiments were designed. On one hand, to confirm that the melatonin-protein interactions were linked to the yeast growth phases, a fermentation at low temperatures was carried out in order to delay the growth curve and to determine whether melatonin-protein interactions were also delayed. On the other hand, to unravel the importance of these proteins in the synthesis of melatonin and in the interactions with this molecule, several knockout strains were examined from the "Yeast Mutant Collection" (*Δtdh1, Δtdh2, Δtdh3, Δeno1* and *Δpyk2*). To fulfill these objectives, we analyzed the intracellular and extracellular melatonin contents produced in the different conditions tested and performed immunopurification with melatonin-IgG-Dynabeads, identifying the proteins that interact with melatonin in each condition.

Materials and methods

Yeast strains and inoculum preparation

All *S. cerevisiae* strains used for these experiments are described in Table 4.1. Growth of yeast strains was carried out on YPD plates (1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) bacteriological peptone and 2% (w/v) agar (Panreac Quimica SLU, Barcelona, Spain)) for 48-72 h at 28ºC. Afterwards, the precultures were prepared in 50 mL of YPD medium (1% (w/v) yeast

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extract, 2% (w/v) glucose and 2% (w/v) bacteriological peptone) at 28ºC with a stirring rate of 120 rpm in an orbital shaker. Then, yeast cells were transferred into two different media depending on the experiment.

Table 4.1. Strains used in this work.

Strain	Genotype	Reference
QA23	MATa/MATα	Borneman et al. 2011
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Euroscarf
Δtdh1	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YJL052w::kanMX4	Euroscarf
Δtdh2	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YJR009c::kanMX4	Euroscarf
Δtdh3	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YGR192c::kanMX4	Euroscarf
Δeno1	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YGR254w::kanMX4	Euroscarf
Δpyk2	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YOR347c::kanMX4	Euroscarf

Growth phase delay experiment

To determine whether the interactions between melatonin and different glycolytic proteins were yeast growth phase-dependent, the QA23 strain was transferred into fresh minimal medium (1X yeast nitrogen base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) glucose, and 350 mM $(NH_4)_2 SO_4$ (Panreac Quimica SLU, Barcelona, Spain)) and cultured for 3 days at 28ºC and 120 rpm. Then, $2x10^6$ cell/mL QA23 were inoculated in 450 mL of synthetic must (200 g/L equimolar glucose:fructose) and incubated in 500-mL bottles with orbital agitation (120 rpm) at 16ºC.

Synthetic grape must was prepared as previously described in Beltran et al. (2004) but with some modifications: the aromatic amino acid (tryptophan, tyrosine and phenylalanine) concentrations were increased five-fold in relation to the regular concentrations (González et al., 2018). These increased concentrations of aromatic amino acids occurred at the expense of the remaining amino acids to maintain the concentration of YAN (yeast assimilable nitrogen, 300 mg/L). Fermentations were monitored by density and total population by OD (Abs at 600 nm).

Sampling was done at different time points of the growth phases. For harvesting, 10^8 cells were centrifuged at 7800 rpm for 5 min. The obtained pellet was washed with Milli-Q water, frozen with liquid nitrogen and stored at -80ºC until melatonin analysis was performed. The supernatants were stored at -20ºC until melatonin and protein analyses were performed.

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Glycolytic mutants experiment

To determine the importance of the proteins identified in a previous study as those that bound to melatonin during alcoholic fermentation (Chapter 3), mutants of these proteins (*Δtdh1*, *Δtdh2*, *Δtdh3*, *Δeno1* and *Δpyk2*) from the Yeast Mutant Collection constructed in the BY4742 background were studied. To perform alcoholic fermentations, these strains were inoculated in 40 mL of synthetic grape must, supplemented with histidine (35 mg/L), leucine (110 mg/L), lysine (120 mg/L) and uracil (40 mg/L), and incubated in 50-mL dark flasks with orbital agitation (120 rpm) at 28ºC. Fermentations were monitored by density and total population by OD (Abs at 600 nm). Samples were collected at different time points of the growth phases during the first 72 h of fermentation and centrifuged at 7800 rpm for 3 min. After centrifugation, pellets were washed with Milli-Q water, frozen with liquid nitrogen and stored at -80ºC until melatonin and protein analysis were performed.

Melatonin analysis

Intracellular metabolites were extracted using the method described in Gonzalez et al. (1997), with some modifications. Briefly, 1 mL of a solution of 75% (v/v) boiling absolute ethanol containing 70 mM HEPES buffer (pH 7.5) was added to the cell pellet and incubated for 3 min at 80ºC. The extract was evaporated at 45ºC in a SpeedBack (Concentrator plus, Eppendorf Ibérica, Madrid, Spain) and then resuspended in 1 mL of Milli-Q water and centrifuged for 10 min at 5000 rpm to remove the insoluble particles. The supernatant was transferred to a new tube and stored at -20ºC until use.

Intracellular melatonin samples were extracted with chloroform. Briefly, 50 μL of sample was mixed with Milli-Q water (1:1, v:v). Then, 10 volumes of chloroform were added. Samples were shaken for 1 h at 1200 rpm. The organic phase was dried under a flow of nitrogen gas and resuspended in 50 μL of a mixture of 40:60 methanol:water. Then, samples were centrifuged for 5 min at 14500 rpm. Supernatants were transferred and analyzed by liquid chromatography mass spectrometry (LC-MS/MS) following the method described by Rodriguez-Naranjo et al. (2011) with some modifications. The system was based on high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6410; Agilent Technologies, Palo Alto, USA). Melatonin separation was performed at 40ºC using an Agilent 150 x 2.1 mm i.d., 3.5 μM, Zorbax Sb-Aq column with an injection volume of 7 μL. Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) methanol as LC-grade solvents,

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both containing 0.1% (v/v) formic acid, at a flow rate of 0.4 mL/min and with the following elution profile: 100% B (4 min) and 10% B (6 min).

Protein analysis

Purification of the samples was performed by a Pierce™ Crosslinking Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Proteins were resolved by 12% SDS PAGE and visualized by a Pierce™ Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA). Image J software (https://imagej.nih.gov/ij/) was used to quantify the protein intensities in the SDS-PAGE analysis.

Protein digestion and analysis

Gel bands were excised and digested as described by Morcillo-Parra et al. (Chapter 3). Basically, samples were reduced with dithiothreitol at 56ºC and alkylated with iodoacetamide for 30 min. For digestion, gel bands were first rehydrated with sequencing-grade trypsin solution at a trypsin:protein ratio of 1:100 (w/w), covered with ammonium bicarbonate and incubated overnight at 37ºC. Peptides were extracted from gels by incubating them with a mixture of acetonitrile and formic acid for 15 min and then with acetonitrile (100%). Before mass spectrometry analysis, samples were desalted using HLB SPE (Waters, USA) and resuspended in 25 µL of 0.1% formic acid.

Peptides were analyzed by Nano LC-MS/MS (LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher, San José, CA, USA)) as described in Morcillo-Parra et al. (Chapter 3).

Protein identification analysis and relative quantification

Tandem mass spectra were extracted and charge states deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific, Waltham, MA, USA). All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Mascot (v2.5) was set up to search the SwissProt_2018_03.fasta database (557012 entries), restricting for *S. cerevisiae* taxonomy (7904 sequences) and for other mammals (13162 sequences) and assuming trypsin digestion. Three missed cleavages and an error of 0.8 Da for fragment ion mass and 10 ppm for precursor ion were allowed. Oxidation of methionine and acetylation of the N-terminus were considered variable modifications, whereas carbamidomethylation of cysteine was a static modification. The false discovery rate was set at 0.01. For proteins identified only with one peptide, visual verification of fragmentation spectra was performed.

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Results and discussion

Growth phase delay in melatonin production

To elucidate whether the synthesis of melatonin and its interactions with some glycolytic proteins were connected to the growth curve, alcoholic fermentations at low temperatures were carried out to delay yeast growth and extend the different growth phases. For this reason, fermentation temperature was adjusted to 16ºC.

QA23 increased its lag phase until 24 h after inoculation in synthetic grape must at 16ºC (Figure 4.1). However, when a higher temperature was used (28ºC), this yeast started growing 12 h after being inoculated in synthetic grape must (Lleixà et al., 2019). The effect of low temperature can also be observed in fermentation performance (Figure 4.1). The duration of the fermentation process was longer (seven days) relative to the duration with higher temperatures (three days; (Lleixà et al., 2019)). Thus, the development of fermentation at lower temperature affected yeast growth, as other authors have stated (Beltran et al., 2008; Torija et al., 2003).

Intracellular melatonin production was clearly delayed at 16ºC (Figure 4.2) relative to 28ºC (Chapter 3). Intracellular melatonin reached its maximum at 20 h after inoculation (1.00 ng/mL per 10^8 cells). Despite the fact that melatonin production was delayed, the intracellular melatonin peak was observed during the lag phase of yeast growth, which was also observed at 28ºC (Chapter 3). Later, melatonin was exported into the extracellular medium. We observed that melatonin appeared in the medium when cells were entering the late exponential phase or the beginning of
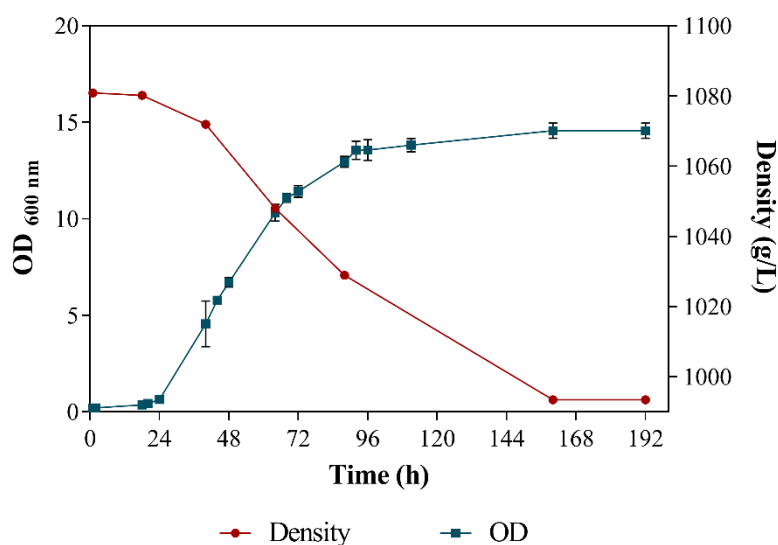

Figure 4.1. Fermentation kinetics of the QA23 yeast strain by monitoring density (●) and population (■) throughout fermentation.

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stationary phase (Figure 4.2, 0.66 ng/mL). Other authors achieved similar results, with the detection of extracellular melatonin during the exponential phase of *Saccharomyces* strains (Fernández-Cruz et al., 2017; Rodríguez-Naranjo et al., 2012; Valera et al., 2019). No melatonin was detected in the stationary phase, in contrast with a previous study that reported the maximum production of melatonin at the end of alcoholic fermentation (Fernández-Cruz et al., 2019a).

The gap between intracellular and extracellular melatonin production was described in *Saccharomyces* and non-*Saccharomyces* yeasts (Chapter 3 and 5). When the fermentation temperature was low, we observed that melatonin production presented a similar profile: the intracellular peak appeared, and later on, melatonin was observed in the extracellular medium (Figure 4.2). Given the gap between intracellular and extracellular melatonin, melatonin could be interacting with some proteins inside yeast cells.
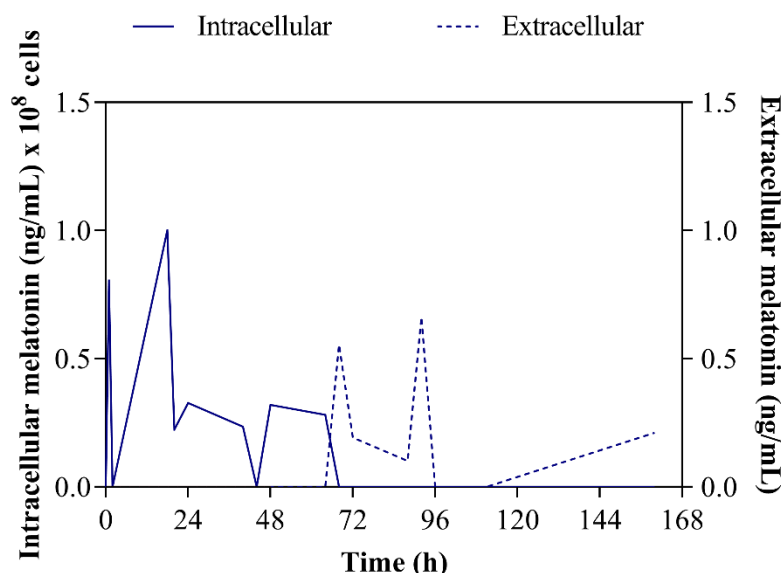

Figure 4.2. Melatonin production by QA23 during an alcoholic fermentation performed at 16°C. The solid line shows the intracellular content of melatonin (ng/mL per 10^8 cells), and the dotted line represents the extracellular amount of melatonin (ng/mL). *Three replicates were analyzed in all of the experiments, although only replicates that produced melatonin are represented.

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Growth phase delay in protein analysis

During alcoholic fermentation at low temperature, several samples were collected from the three phases of yeast growth, the lag, exponential and stationary phases, to determine when the interaction between melatonin and proteins took place.

In a recent study, Morcillo-Parra et al. (Chapter 3) reported that melatonin binds proteins during the exponential phase of yeast growth between intracellular and extracellular peaks of melatonin production. At low-temperature fermentations, proteins bound to melatonin appeared at the beginning of the exponential phase but were clearly delayed in time relative to fermentations at higher temperatures (Chapter 3). The strongest band intensity was at 40 h for a 50-kDa protein and at 64 h for two 60-kDa proteins during the exponential phase of growth (Figure 4.3). No melatonin-binding proteins were detected during the first 20 h of fermentation, coinciding with the lag phase of QA23. In addition, during the stationary phase, an intensity decrease of the protein bands was observed (Figure 4.3). Therefore, melatonin-protein interactions are linked to the exponential phase of yeast growth, when no melatonin is detected either in intracellular or in extracellular medium (Figure 4.2), as we have already observed at higher-temperature fermentations (Chapter 3).

Mass spectrometry analysis allowed us to identify four purified proteins (Figure 4.3 and Table 4.2), which coincided with some of those already uncovered in our previous study at higher temperature (Chapter 3). As in this previous study, most melatonin-binding proteins were from the glycolytic pathway: pyruvate decarboxylase 1 (Pdc1p; band a), pyruvate kinase 1 (Pyk2p; band b), enolase (Eno1p, Eno2p; band d) and glyceraldehyde-3-phosphate dehydrogenase (Tdh1p, Tdh2p, Tdh3p; band e). The interaction of melatonin with all of these proteins occurred during the exponential phase of yeast growth, providing more evidence for the formation of a glycolytic complex in yeast cells, which is probably related to channeling the glycolytic flux to a fermentative metabolism due to the inmunopurification of Pdc1p, a key enzyme for the development of alcoholic fermentation (van Hoek et al., 1998). These proteins have already been suggested to be involved in a macromolecular complex bound to the mitochondria in yeasts (Brandina et al., 2006) and other organisms (Giegé et al., 2003; Graham et al., 2007). Moreover, melatonin in yeast is thought to be synthesized in mitochondria (He et al., 2016), and this molecule also modifies the expression of glycolytic proteins, including enolase and glyceraldehyde-3-phosphate dehydrogenase in wheat (Cui et al., 2018). Therefore, all of these data provide more evidence that

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the role of melatonin during the alcoholic fermentation process is implemented through the glycolysis pathway.

Moreover, an RNA-binding protein, the cytoplasmatic translation elongation factor TEF1-A (Tef1p), was also identified (Figure 4.3, band c) as being one of the most strongly linked proteins to melatonin at the beginning of the exponential phase. This protein has been described to form part of the enolase complex in the mitochondria, which could allow and regulate the channeling of substrates such as pyruvate towards the TCA cycle or t-RNA mitochondrial import (Brandina et al., 2006; Graham et al., 2007).

Melatonin production in glycolytic gene mutants

To understand the role of these proteins in the interactions with melatonin, another set of fermentations was carried out using five knockout strains and their reference yeast (*Δtdh1, Δtdh2, Δtdh3, Δeno1, Δpyk2* and BY4742). These genes were chosen because their proteins have been described to interact with melatonin in *Saccharomyces* and non-*Saccharomyces* yeasts (Chapter 3 and 5). Other proteins have been described to bind melatonin, such as Tef1p or Pgk1p (Chapter 3 and 5). However, the knockout strains are inviable or exhibit chromosome instability, and therefore, we did not use these mutant strains in our study.

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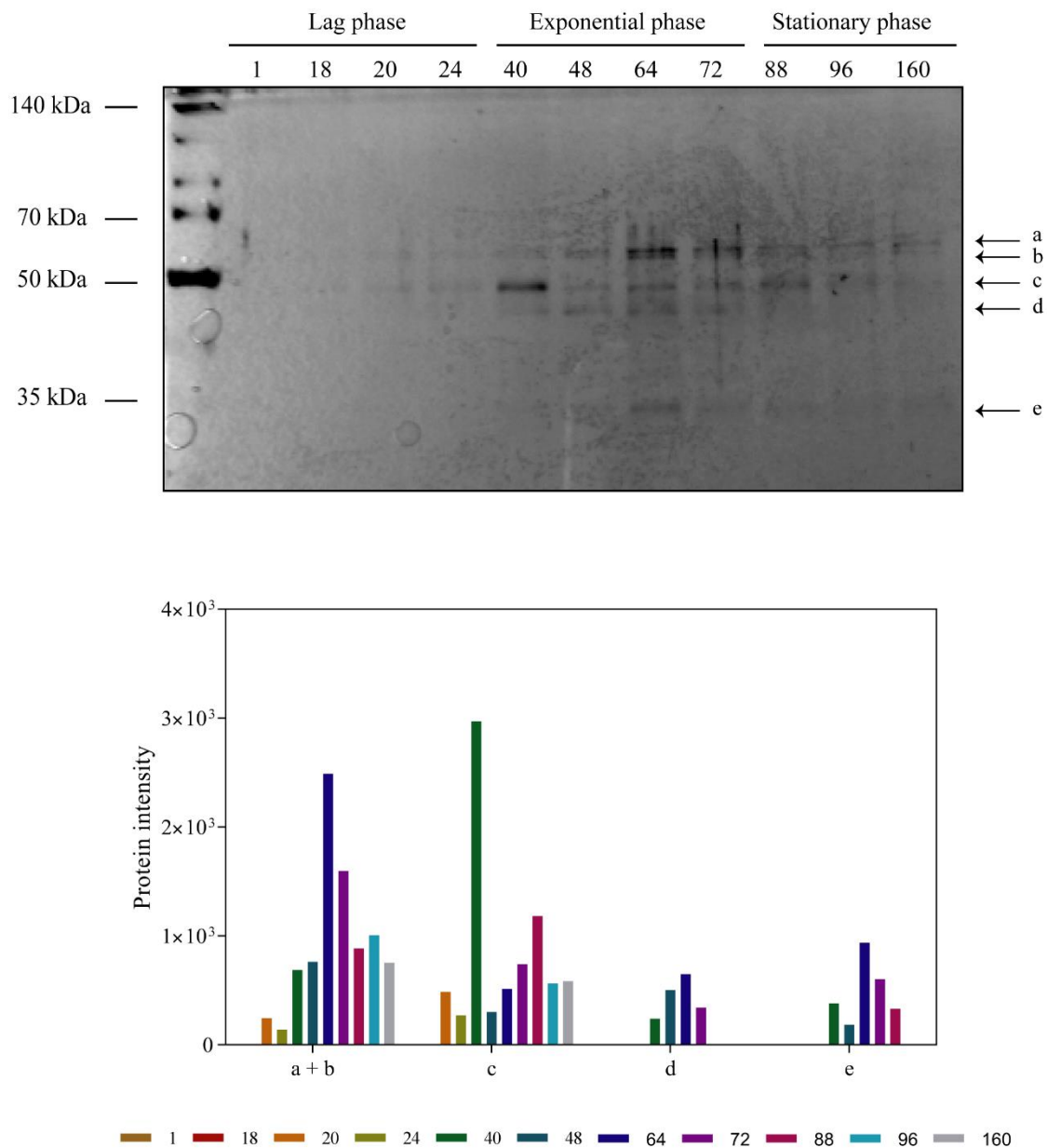Figure 4.3. Time course of purified proteins during alcoholic fermentation at low temperature. (a) SDS-PAGE gel electrophoresis. Bands marked are proteins bound to melatonin: a. pyruvate decarboxylase 1; b. pyruvate kinase 1 c. elongation alpha factor; d. enolase; e. glyceraldehyde-3-phosphate dehydrogenase. (b) Quantification of protein intensities performed using Image J software. *Time is represented in hours.

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Table 4.2. Identification of proteins purified from the SDS-PAGE gel using Nano LC-(Orbitrap) MS/MS.

Protein gel band	Gene name	Molecular weight (kDa)	Protein description (SGD database)	Sequence coverage, % number of peptides recovered by Nano LC-MS/MS	Mascot Score
A	*PDC1*	61.9	Pyruvate decarboxylase 1	53.11	1270.19
B	*PYK2*	54.5	Pyruvate kinase 1	66.60	1241.62
C	*TEF1*	50.0	Translation Elongation Factor	33.84	606.00
D	*ENO1*	46.8	Enolase	62.70	2015.76
E	*TDH1*	35.7	Triose-phosphate dehydrogenase	74.40	1510.88
	TDH2	35.8	Triose-phosphate dehydrogenase	77.41	1269.18
	TDH3	35.7	Triose-phosphate dehydrogenase	65.06	1525.19

On one hand, the population was measured (OD; Figure 4.4) to establish the three growth phases (lag, exponential and stationary phase) in the BY4742 background. All the strains presented similar growth at the beginning of fermentation, but the knockout strains of the glyceraldehyde-3-phosphate dehydrogenase (*Δtdh1, Δtdh2, Δtdh3*) had the lowest maximum population (Figure 4.4).

In relation to intracellular and extracellular melatonin content, the reference yeast, BY4742, produced intracellular melatonin in the first hour of fermentation (at 30 min, Figure 4.5a), reaching 4.85 ng/mL per 10^8 cells. Similar results were obtained with other *Saccharomyces* and non-*Saccharomyces* yeasts, which produced melatonin at the beginning of fermentation in the same conditions (Chapter 3 and 5). After the intracellular peak, melatonin was observed in the extracellular medium at 60 min, similar to Muñiz-Calvo et al. (2019), who reported the detection of melatonin after a few minutes in different media. We also detected another peak of extracellular melatonin at 24 h, confirming previous results of other authors that reported melatonin after the first day of fermentation (Fernández-Cruz et al., 2017; Rodríguez-Naranjo et al., 2012).
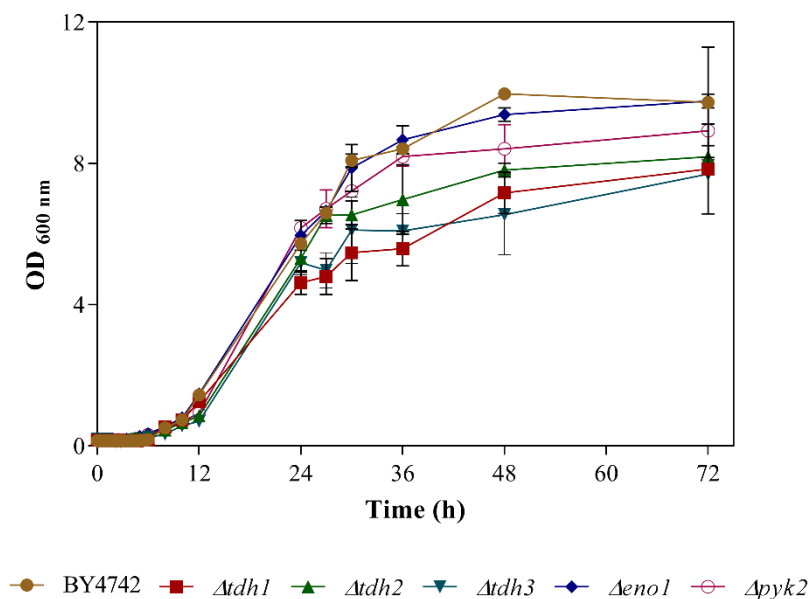
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Figure 4.4. Evolution of the populations (OD) of different glycolytic mutant strains throughout fermentation.

In the knockout mutant strains (*Δtdh1, Δtdh2, Δtdh3, Δeno1* and *Δpyk2*), intracellular melatonin production was different (Figure 4.5). It is important to highlight that we observed different production profiles for each biological replicate. Melatonin production exhibited high variability because it is a quick signaling process (min). For this reason, our results are presented for each biological replicate rather than as an average of three replicates. In all mutant strains, intracellular melatonin was detected in the lag phase of yeast growth, except in the *Δtdh3* strain, in which no melatonin was found at the analyzed time points (Figure 4.5d). Only *Δtdh2* presented the intracellular melatonin peak (1.22 ng/mL) at the same time as the reference strain (Figure 4.5c), while other mutant strains (*Δpyk2* and *Δeno1*; Figure 4.5e and f) also produced intracellular melatonin at similar times (between 30 and 90 min). *Δpyk2* reached its maximum of 0.48 ng/mL at 90 min, whereas *Δeno1* produced four times more than the reference strain (23.65 ng/mL) in the first hour of fermentation. The mutant with the later melatonin peak was the *Δtdh1* strain, after 3 h of fermentation (1.90 ng/mL; Figure 4.5b), although this peak still occurred during the lag phase as in the other strains. Therefore, all mutant strains, regardless of the lack of a glycolytic protein, produced melatonin intracellularly during the first hours of fermentation in a similar way to the reference strain, with the exception of the *Δtdh3* strain, in which no intracellular melatonin was detected at the time points studied (Figure 4.5d). However, the rapid synthesis of and decrease in melatonin from the intracellular medium make melatonin a rather elusive compound in terms of detection (Fernández-Cruz et al., 2019a, 2019b). For this reason, a lack of detection cannot fully

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ensure a lack of production. Intracellular melatonin has been described during the first phase of yeast growth because it can act as a signal molecule while the yeast is trying to adapt to the conditions of the new medium (Rodriguez-Naranjo et al., 2012). As a signal molecule, melatonin synthesis is quick (min), and more studies are needed to understand the effect that these proteins may have on melatonin metabolism.

In terms of extracellular melatonin during alcoholic fermentation, all strains presented similar melatonin production profiles, even the *Δtdh3* strain, although intracellular melatonin was not detected. The most similar strain to the reference strain was the *Δtdh1* strain (Figure 4.5), which produced melatonin in an up-and-down profile during the first hours of fermentation. In addition, BY4742 and its knockout strains accumulated melatonin in the extracellular media during fermentation as observed by Gomez et al. (2012). This result contrasts with those of other authors, in which this accumulation was not observed (Fernández-Cruz et al., 2019a; Rodriguez-Naranjo et al., 2012). However, this finding could reinforce the presence of melatonin in final wines (Mercolini et al., 2008; Vitalini et al., 2013). Valera et al. (2019) also observed that melatonin accumulated during fermentation when a mix of non-*Saccharomyces* strains was used. Nonetheless, more studies are needed to understand the accumulative metabolism of melatonin in the extracellular media in this laboratory strain.

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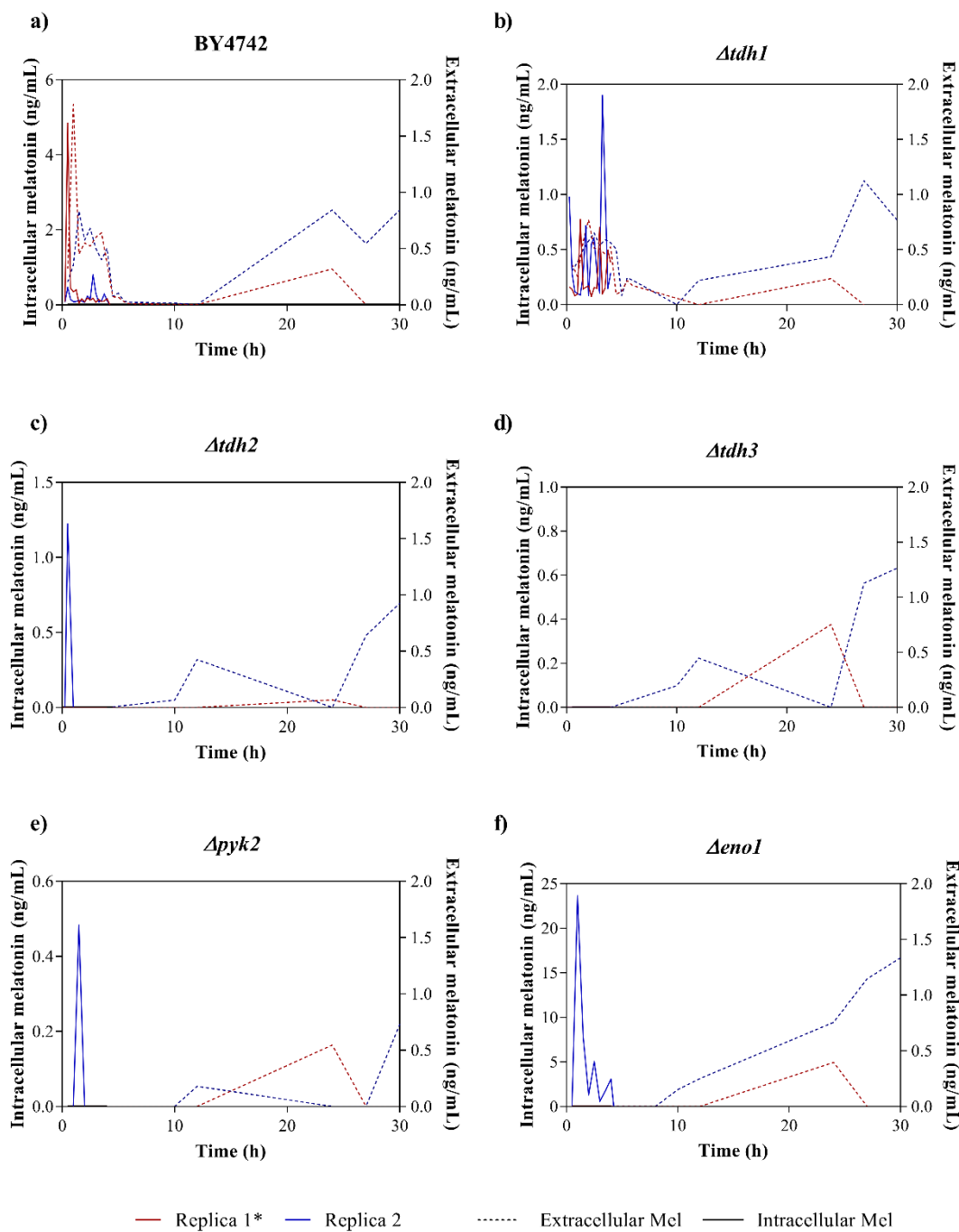
Figure 4.5. Melatonin production by BY4742 and the glycolytic mutant strains: (a) BY4742, (b) *Δtdh1*, (c) *Δtdh2*, (d) *Δtdh3*, (e) *Δpyk2* and (f) *Δeno1*. Solid lines show the intracellular content of melatonin (ng/mL per 108 cells), and the dotted lines represent the extracellular amount of melatonin (ng/mL).
* Three replicates were analyzed in all of the experiments, although only replicates that produced melatonin are represented.

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Protein analysis in glycolytic gene mutants

No differences in the number of protein bands were observed between BY4742 and all the knockout strains, except for the *Δeno1* strain, as shown in Figure 4.6a and S4.1. In fact, deletion of the *ENO1* gene pointed to a clear effect in melatonin-protein interactions since, in addition to the expected absence of the enolase band, only a few protein bands were purified from this mutant strain (Figure 4.6a). Moreover, the absence of this gene altered the accumulation of intracellular melatonin, given that the mutant exhibited the higher intracellular peak (Figure 4.5f). Eno1p is an important protein of the enolase complex described on the surface of yeast mitochondria (Brandina et al., 2006) and therefore may be crucial for the formation of the glycolytic complex. On the other hand, in the *tdh1*-knockout strain, the band identified as glyceraldehyde 3-phosphatase has decreased intensity relative to the reference strain (Figure 4.6). Although this low intensity was expected, it was only observed with the deletion of this isoform and not with the other two isoforms (*Δtdh2* and *Δtdh3*). This provided evidence that melatonin was mainly bound to Tdh1p. However, given that a faint protein band was still detected in the *Δtdh1* strain, melatonin may also be bound to Tdh2p and Tdh3p. Some studies indicate that Tdh1p may be considered a moonlighting protein due to the observation of activities unrelated to its classical functions (Sirover, 2011). Glyceraldehyde-3-phosphate dehydrogenase has been described to be involved in different cellular processes such as transcriptional and posttranscriptional gene regulation, vesicular transport or the maintenance of DNA integrity, among others (Sirover, 2011). In fact, gene disruption studies have shown that the *TDH1* gene alone cannot support growth in yeast and may therefore carry out a different function than those of the other two isozymes (*TDH2* and *TDH3*) (McAlister and Holland, 1985).

The fact that only the *tdh1* and *eno1*-knockout strains displayed differences relative to the reference strain uncovers a major role for these two proteins, glyceraldehyde-3-phosphate dehydrogenase and enolase, in melatonin interactions. Moreover, as both proteins are involved in the mitochondrial glycolytic complex, these interactions may suggest a role for melatonin as signal molecule for this complex. In terms of melatonin production, all strains presented the same synthesis profile, except *Δtdh3*, in which it was possible to detect melatonin extracellularly although intracellular melatonin was not detected, confirming that this mutant could also synthesize melatonin.
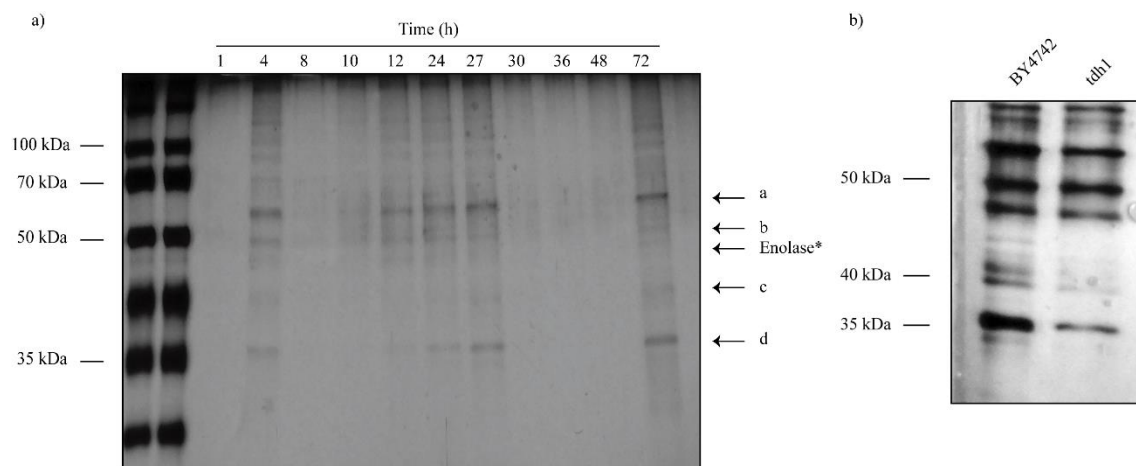
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Figure 4.6. BY4742 Inmunopurification of proteins by IgG Dynabeads. (a) SDS-PAGE gel electrophoresis of the time course of purified proteins during alcoholic fermentation in the *Δeno1* yeast strain. (b) Comparison of melatonin-binding proteins in the BY4742 and *Δtdh1* yeast strains at 24 h. *Eno1p did not appear.

Conclusions

Melatonin is produced in the lag phase of yeast growth and is exported to the extracellular medium during the stationary phase, regardless of the fermentation temperature. Thus, when the lag phase was extended (low temperature fermentation), melatonin production and secretion were delayed relative to regular fermentation, and therefore, its interactions with glycolytic proteins were delayed. This delay pinpoints a connection between melatonin metabolism and yeast growth phases.

When fermentation was performed by glycolytic knockout strains, melatonin presented a similar profile of intracellular production (during the lag phase of yeast growth), but its excretion into the extracellular medium resulted in the accumulative metabolism of this molecule. Only deletion of the *ENO1* and *TDH1* genes had any impact on the melatonin-protein interaction profile, pointing to a main role for these proteins in the interaction with melatonin, probably related to a mitochondrial glycolytic complex. Furthermore, this interaction may highlight the role of melatonin as a signal molecule in yeast. Nevertheless, further studies are needed to understand melatonin metabolism in yeast cells to help elucidate the biological importance of this compound in yeasts.

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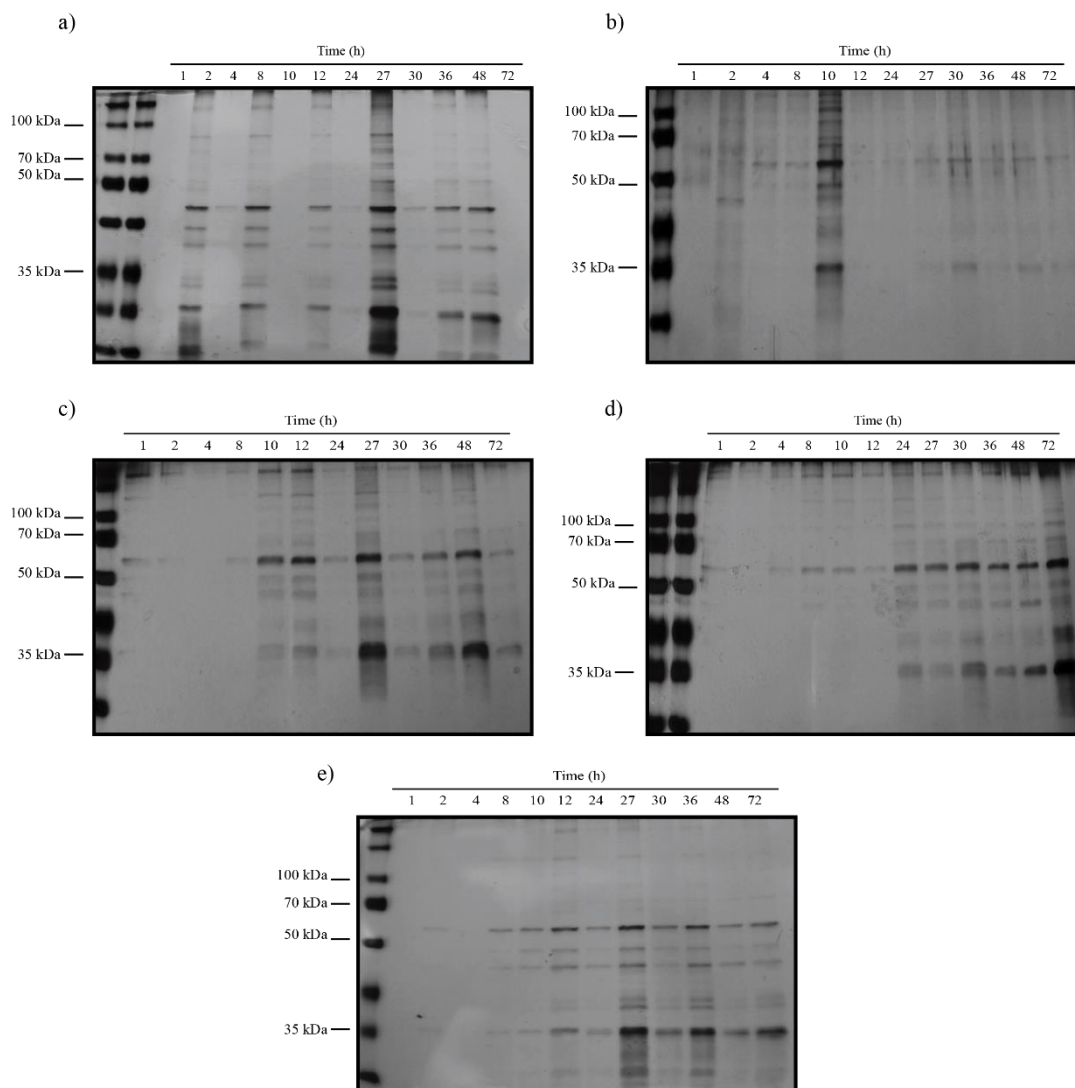
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Supplementary material

Supplementary Figure S4.1: Time course of purified proteins from glycolytic gene mutants during alcoholic fermentation. (a) BY4742, (b) *Δtdh1*, (c) *Δtdh2*, (d) *Δtdh3* and (e) *Δpyk2*.

CHAPTER 5

Melatonin and glycolytic protein interactions are related to yeast fermentative capacity

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Abstract

Melatonin is an indole amine that interacts with some proteins in mammals, such as calreticulin, calmodulin or sirtuins. In yeast, melatonin is synthetized and interacts with glycolytic proteins during alcoholic fermentation in *Saccharomyces cerevisiae*. Due to its importance as an antioxidant molecule in both *Saccharomyces* and non-*Saccharomyces* yeasts, the aim of this study was to determine the intracellular and extracellular synthesis profiles of melatonin in four non-*Saccharomyces* strains (*Torulaspora delbrueckii, Hanseniaspora uvarum, Starmeralla bacillaris* and *Metschnikowia pulcherrima*) and to confirm whether glycolytic enzymes can also interact with this molecule in non-conventional yeast cells. Melatonin from fermentation samples was analyzed by liquid chromatography mass spectrometry, and proteins bound to melatonin were immunopurified by melatonin-IgG-Dynabeads. Melatonin was produced in a similar pattern in all non-*Saccharomyces* yeast, with *M. pulcherrima* and *S. bacillaris* being the highest producers. However, melatonin only bound to proteins in two non-conventional yeasts, *S. bacillaris* and *T. delbrueckii*, which specifically had higher fermentative capacities. Sequence analysis showed that most proteins shared high levels of homology with glycolytic enzymes, but an RNA-binding protein, the elongation alpha factor, which is related to mitochondria, was also identified. This study reports for the first time the interaction of melatonin with proteins inside non-*Saccharomyces* yeast cells. These results reinforce the possible role of melatonin as a signal molecule, likely related to fermentation metabolism and provide a new perspective for understanding its role in yeast.

Keywords: melatonin, *Torulaspora delbrueckii, Starmerella bacillaris,* glycolysis, GADPH, fermentation.

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Introduction

Melatonin is an indole amine and is considered a ubiquitous molecule due to its presence in many biological kingdoms (Tan et al., 2015). Melatonin participates in the regulation of the human circadian rhythm (Reiter, 1993) and acts as an antioxidant via receptor-independent processes (Galano et al., 2011). Recently, melatonin has also been related with a protective function against oxidative stress and UV radiation in yeast (Bisquert et al., 2018; Vázquez et al., 2017; 2018).

Melatonin has been found in many fermented beverages, such as beer and wine (García-Moreno et al., 2013; Kocadağlı et al., 2014; Rodríguez-Naranjo et al., 2011; Vigentini et al., 2015). When the winemaking process is monitored, melatonin production significantly increases during alcoholic fermentation (Fernández-Cruz et al., 2017, 2019a, 2019b; Rodriguez-Naranjo et al., 2012), highlighting the role of yeast, particularly *Saccharomyces cerevisiae*, in the production of melatonin in wine. This production depends on precursor availability (tryptophan) (Rodriguez-Naranjo et al., 2012; Sprenger et al., 1999) but also, a recent study detected melatonin production after serotonin and 5-methoxytryptamine pulses (Muñiz-Calvo et al., 2019). In addition, intracellular melatonin is produced during the lag phase of *Saccharomyces* yeast in fermentation conditions (Chapter 3), while the presence of melatonin in extracellular medium has been described during the first and second day of fermentation (Fernández-Cruz et al., 2017; Rodríguez-Naranjo et al., 2012; Valera et al., 2019; Vigentini et al., 2015).

Due to its high fermentation capacity, *S. cerevisiae* is traditionally used to perform alcoholic fermentation in fermented beverages. However, in recent years, non-*Saccharomyces* yeasts are being investigated in depth (Contreras et al., 2015; Gonzalez et al., 2013; Jolly et al., 2014; Suárez-Lepe and Morata, 2012). Several studies have demonstrated that non-*Saccharomyces* yeasts can improve wine quality in terms of producing flavor, stabilizing wine color and promoting enzymatic activity (Jolly et al., 2014; Padilla et al., 2016b; Suárez-Lepe and Morata, 2012). Additionally, non-*Saccharomyces* yeasts can produce wines with lower alcohol content (Contreras et al., 2014; Quirós et al., 2014). In terms of melatonin production, previous studies have detected this molecule in different species of yeasts such as *Torulaspora delbrueckii* (Fernández-Cruz et al., 2017; Vigentini et al., 2015), *Metschnikowia pulcherrima* (Fernández-Cruz et al., 2017) and *Zygosaccharomyces bailii* (Vigentini et al., 2015). Recently, Valera et al. (2019) also studied the effect of mixed fermentations with *Saccharomyces* and non-*Saccharomyces* yeasts on melatonin

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production, which reached its maximum concentrations after 1 to 4 days of fermentation, similar to what happens in single inoculation studies (Fernández-Cruz et al., 2017; Vigentini et al., 2015).

The different functions of melatonin in human cells suggest the existence of specific receptors. Many studies have associated melatonin with two transmembrane proteins that belong to the GPCR superfamily, MT1 and MT2, acting as receptors in the mammalian membrane (Reppert, 1997), which are encoded by the *MTRN1A* and *MTNR1B* genes, respectively (Jockers et al., 2016). In addition, melatonin has been related to some nuclear proteins in human cells, such as orphan receptors (Becker-André et al., 1997; Carlberg and Wiesenberg, 1995; Wiesenberg et al., 1995) or calreticulin (Macías et al., 2003), a ubiquitous protein involved in intracellular signaling pathways under Ca^{2+} binding. Some studies have also related melatonin to sirtuins, which regulate the cell cycle, DNA repair, cell survival and apoptosis and have important roles in normal and cancer cells (Das, 2005; Mayo et al., 2017). However, the ability of melatonin to interact with yeast proteins has not been explored so far. Recently, we observed for the first time that glycolytic enzymes interact with melatonin in *Saccharomyces* yeast, suggesting that melatonin may be a signal molecule to form a glycolytic complex (Chapter 3). Therefore, the aim of this work was to determine the intracellular and extracellular melatonin production profiles during alcoholic fermentation by non-*Saccharomyces* yeasts and to determine if melatonin also interacts with glycolytic proteins in these non-*Saccharomyces* species, just as it has been recently described for *Saccharomyces* yeast.

Materials and methods

Yeast strains and inoculum preparation

Four strains of different non-*Saccharomyces* species (*Torulaspora delbrueckii* (Tdp) CECT 13135, *Hanseniaspora uvarum* (Hu4) CECT 13130, *Starmerella bacillaris* (Sb4) CECT 13129 and *Metschnikowia pulcherrima* (Mpp) CECT 13131) isolated from natural grape must from the Priorat Appellation of Origin (Catalonia, Spain) (Padilla et al., 2016a) were used. Yeast precultures were prepared in 50 mL of YPD medium (1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) bacteriological peptone (Panreac, Barcelona, Spain)) overnight at 28℃ with a stirring rate of 120 rpm in an orbital shaker. Then, yeasts were transferred into fresh minimal medium (1X Yeast Nitrogen Base without amino acids or ammonia (Becton, Dickinson and Company, Sparks, MD,

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USA), 2% (w/v) glucose, 350 mM $(NH_4)_2 SO_4$ (Panreac Quimica SLU, Barcelona, Spain)) and cultured for 3 days at 28**°C** and 120 rpm.

Alcoholic fermentation conditions

Fermentations were carried out in 450 mL of synthetic grape must (pH 3.3) as described in Beltran et al. (2004), but with some modifications. The aromatic amino acid (tryptophan, tyrosine and phenylalanine) concentrations were increased five-fold relative to the regular concentrations (González et al., 2018). These increased concentrations of aromatic amino acids occurred at the expense of the remaining amino acids to maintain the assimilable nitrogen at 300 mg/L. Fermentations were inoculated at $2x10^6$ cell/mL and cultured at 28**°C** with continuous orbital shaking (120 rpm). All fermentations were performed in triplicate. Cell populations were evaluated by measuring the optical density (OD_{600nm}), and 10 OD were collected at different time points during yeast growth. Samples were centrifuged at 12000 rpm for 3 min at room temperature. The supernatants were stored at -20**°C** for extracellular melatonin analysis, and the pellet was washed with distilled water, frozen in liquid nitrogen and stored at -80**°C** for intracellular melatonin and protein analysis.

Melatonin analysis

Intracellular metabolites were extracted by adapting the boiling buffered ethanol method previously described by Gonzalez et al. (1997), with some modifications. Briefly, 1 mL of a solution of 75% (v/v) boiling absolute ethanol containing 70 mM of HEPES buffer (pH 7.5) was added to the cell pellet and incubated for 3 min at 80**°C**. Extracts were concentrated by evaporation at 45**°C** in a SpeedBack (Concentrator plus, Eppendorf Ibérica, Madrid, Spain) and then resuspended in 1 mL of Milli-Q water, centrifuged for 10 min at 5000 rpm and stored at -20°C until use.

Intracellular and extracellular melatonin samples were extracted with chloroform. In brief, sample was mixed first with Milli-Q water (1:1, v:v) and then with 10 volumes of chloroform. Samples were shaken for 1 h at 1200 rpm, and the organic phase was dried by supplying nitrogen gas. Then, samples were resuspended in a mixture of methanol and water (40:60, v:v) and centrifuged for 5 min at 14500 rpm. Supernatants were transferred and analyzed.

Samples were analyzed by performing liquid chromatography mass spectrometry (LC-MS/MS, Agilent G6410; Agilent Technologies, Palo Alto, USA) using an Agilent 150 x 2.1 mm i.d., 3.5 µM,

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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), 10% B (6 min). The analysis temperature was set at 40℃, and the flow rate was 0.4 mL/min. The injection volume was 7 μL.

Protein purification

Samples from different time points of fermentation were purified by a Pierce™ Crosslinking Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA, USA). Melatonin IgG-Dynabeads were prepared by cross-linking anti-melatonin rabbit antibody IgG (LifeSpan BioSciences, Seattle, WA, USA) to the Dynabeads. A total of 10 OD were resuspended in 1 mL of extraction buffer and broken by glass beads by applying three shaking cycles for 1 min in a Mini Beadbeater-24 (BioSpec Products, Bartlesville, OK, USA), followed by 1 min on ice. Lysed cells were centrifuged at 14000 rpm and 4℃ for 10 min to remove insoluble particles. Melatonin IgG-Dynabeads were added to each lysate, and purification was performed following the manufacturer's instructions. The same amount of total protein from each sample was loaded and run in an SDS-PAGE gel. Immunopurified proteins were visualized in a stained gel by a Pierce™ Silver Stain kit (Thermo Fisher Scientific, Waltham, MA, USA). Image J software (https://imagej.nih.gov/ij/) was used to quantify the protein intensities in the SDS-PAGE analysis.

Protein digestion and analysis

Visible gel bands were excised from gels and washed with ammonium bicarbonate and acetonitrile. Then, samples were reduced with dithiothreitol and alkylated with iodoacetamide. For the digestion, gel bands were first rehydrated with trypsin sequencing-grade solution at a trypsin:protein ratio of 1:100 (w/w) (considering 50 μg of protein per band), covered with ammonium bicarbonate and incubated overnight at 37℃. Extraction of peptides from gels was performed by incubating them with a mixture of acetonitrile and formic acid for 15 min and collecting the supernatants. The final extraction step was done with acetonitrile. Before mass spectrometry analysis, samples were desalted using HLB SPE (Waters, USA). Peptides were eluted with a mixture of acetonitrile and formic acid. Eluates were dried in a Speed-Vac, and peptides were resuspended in 0.1% formic acid.

Peptides were analyzed by a Nano LC-(Orbitrap) MS/MS (LTQ-OrbitrapVelos Pro mass spectrometer (Thermo Fisher, San José, CA, USA)). Two microliters of sample were separated into a C18 reversed-phase nanocolumn coupled to a trap nanocolumn using a 60-min acetonitrile

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gradient. The flow rate during the elution gradient was 300 nL/min. For real time ionization and peptide fragmentation, an enhanced FT-resolution spectrum was used, followed by a data-dependent IT-MS/MS scan of the most intense ten parent ions with a charge state rejection of one using CID fragmentation with a normalized collision energy of 35% and dynamic exclusion of 0.5 min.

Protein identification and relative quantification

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific). All MS and MS/MS samples were analyzed using Mascot (Thermo Fisher Scientific; version 2.4.1.0). Three different workflows were set up using three different Mascot (v2.5) nodes: a first workflow with the *Torulaspora*.fasta database (56647 entries), a second one with the *Starmerella*.fasta database (118 entries) and a third one combining the *Starmerella* database and Swiss-Prot database filtered by Other Mammalia in taxonomy (13175 entries), assuming trypsin digestion in all three searches. Three missed cleavages and an error of 0.8 Da for fragment ion mass and 10 ppm for precursor ion were allowed. The false discovery rate was set at 0.01. For proteins identified only with one peptide, visual verification of fragmentation spectra was performed.

Results and discussion

Melatonin production

Strains of different non-*Saccharomyces* species, belonging to *T. delbrueckii, S. bacillaris, M. pulcherrima* and *H. uvarum*, were used to ferment synthetic grape must, which was enriched in aromatic amino acids to favor the synthesis of methoxyindoles (Muñiz-Calvo et al., 2019; Rodriguez-Naranjo et al., 2012). Different time points from the three phases of yeast growth were analyzed, as melatonin synthesis seems to be linked to yeast growth, at least in *S. cerevisiae* (Rodriguez-Naranjo et al., 2012).

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In addition to melatonin quantification, fermentation parameters (sugar consumption and population) were measured. No strain was able to consume all sugars in 96 h (Figure 5.1a). However, clear differences in sugar consumption and growth were observed between yeasts, with Tdp and Sb4 showing the highest population and sugar consumption at the end of the experiment (Figure 5.1b).
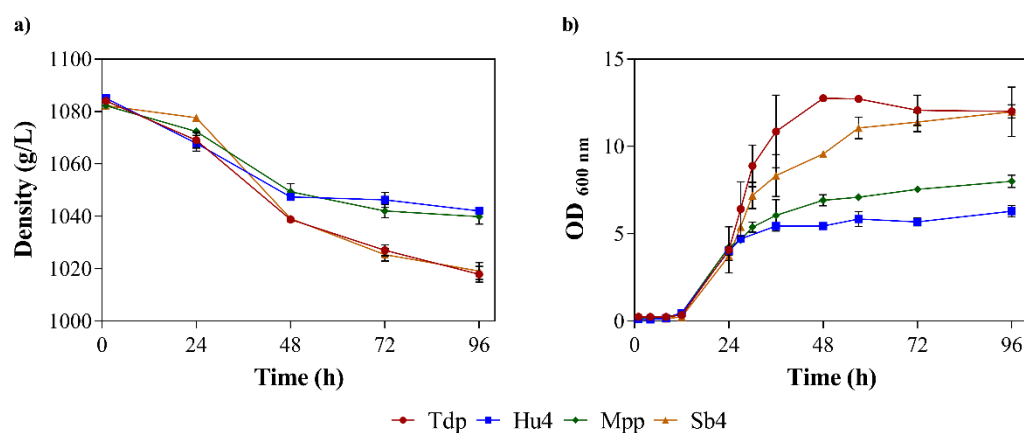

Figure 5.1. Fermentation kinetics of the non-*Saccharomyces* yeast strains determined by monitoring must density (a) and yeast growth (measured as OD_{600}) (b) throughout the fermentation.

Intracellular and extracellular melatonin content was determined by LC-MS/MS (Rodriguez-Naranjo et al., 2011). Given the large amount of data collected (Table S5.1) and to provide a better understanding, melatonin synthesis monitoring throughout each fermentation was represented in a heatmap (Figure 5.2). On one hand, intracellular melatonin was mainly detected in the lag phase, reaching its maximum at 4 h for all species, except for Mpp, which synthesized melatonin from the beginning of the fermentation. Similar results were achieved with *Saccharomyces cerevisiae* in similar conditions (Chapter 3), although in the case of *S. cerevisiae*, the detection of melatonin's peak was faster, in the first hour of fermentation. This synthesis of melatonin during the first part of the growth curve (0-6 h) has been reported as a strategy by which yeast adapt to new conditions (Rodriguez-Naranjo et al., 2012). Nevertheless, although a similar synthesis profile was observed for all species, important differences in melatonin quantity were detected between them. Mpp and Sb4 synthetized more melatonin and presented a higher melatonin basal level throughout all the fermentation (Table S5.1).

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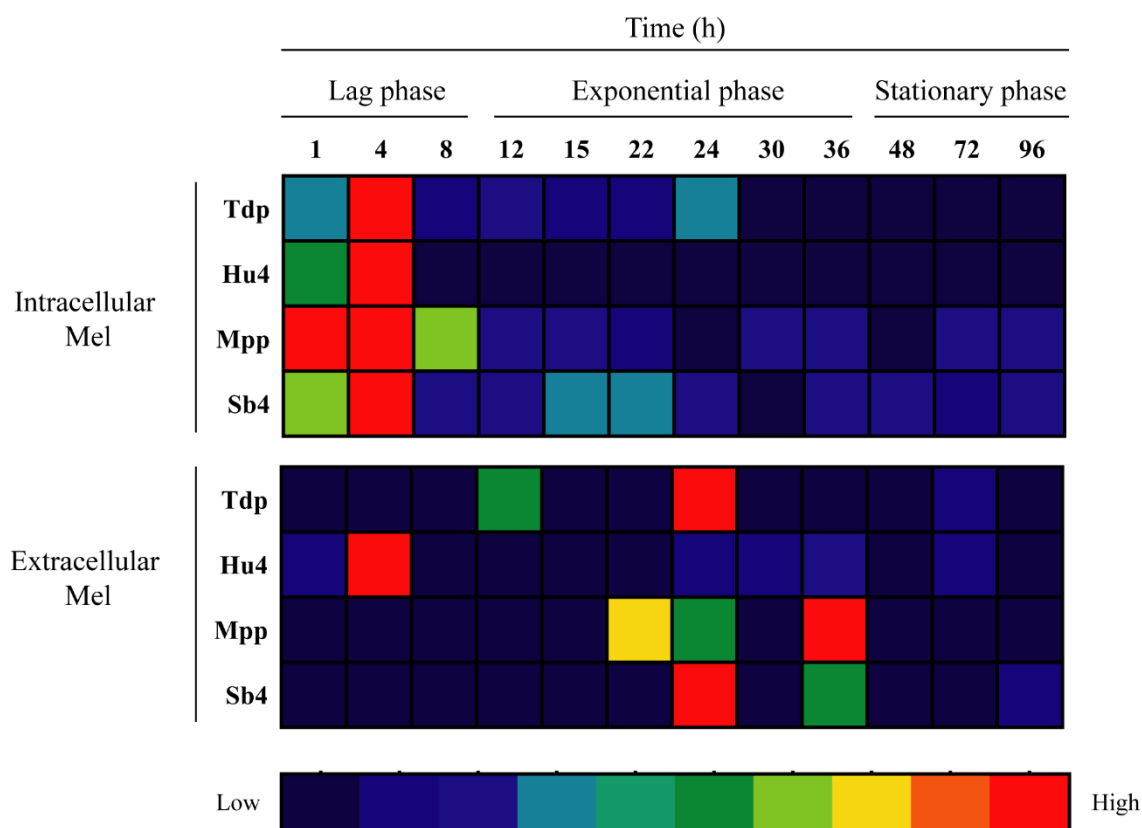María de los Ángeles Morcillo Parra

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Figure 5.2. Melatonin production by the four non-*Saccharomyces* strains during the different yeast growth phases. Concentrations were normalized with its maximum melatonin content. *T. delbrueckii* (Tdp), *H. uvarum* (Hu4) *M. pulcherrima* (Mpp*), S. bacillaris* (Sb4).

On the other hand, extracellular melatonin was observed in different growth phases depending on the species. Tdp and Sb4 showed the maximum melatonin concentrations at 24 h, in the exponential phase, as described previously in *S. cerevisiae* (Chapter 3). However, while Tdp started exporting melatonin at the beginning of the exponential phase (12 h), in the case of Sb4, the indole was detected in the late exponential phase (36 h). According to Vigentini et al. (2015), different strains of *T. delbrueckii* produced melatonin at different times of fermentation, between 24 and 72 h. Similarly, Mpp started exporting melatonin approximately 22 h, in the late exponential phase, although its maximum concentration was achieved at 36 h. Finally and exhibiting a very different behavior, Hu4 exported melatonin at 4 h in the lag phase. Instead, Fernandez-Cruz et al. (2017) observed melatonin in non-*Saccharomyces* yeasts on days 4 and 7. Nonetheless, as Vigentini et al. (2015) also showed, in this study, each species seemed to export extracellular melatonin at different growth points.

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The lag phase and exponential phase seem to be the most important stages in the intracellular and extracellular production of melatonin in most non-*Saccharomyces* yeasts, respectively; similar to what happens in *S. cerevisiae* (Chapter 3). The fact that the production of melatonin is linked to growth phase reinforces a possible role for this compound in the yeast adaptation process to the changing environment that is alcoholic fermentation (Rodriguez-Naranjo et al., 2012).

In a previous study, it was described that melatonin interacts with different glycolytic proteins in the time gap between the intracellular and extracellular detection (Chapter 3); therefore, in this study we also wanted to uncover if similar interactions were observed in non-*Saccharomyces* species.

Protein analysis

The interaction of melatonin with proteins was studied in fermentations carried out by 4 different non-*Saccharomyces* species in synthetic grape must. Several proteins, ranging from 70 kDa to 30 kDa, were purified from samples of Tdp and Sb4 (Figure 5.3 and 5.4), but no melatonin-binding proteins were detected in samples from Hu4 or Mpp (Figure S5.1 and S5.2). Therefore, melatonin-protein binding seems to be species-dependent. Additionally, the fact that proteins bound to melatonin were only detected in *T. delbrueckii* and *S. bacillaris* strains, and previously in *Saccharomyces,* seems to relate these interactions to higher growth and fermentative capacity of these yeasts. Both species have been reported to ferment a higher quantity of sugars, being in some cases able to complete fermentation and to survive high ethanol concentrations (Englezos et al., 2015, 2016; Quirós et al., 2014; Varela et al., 2017). In fact, the most important biotechnological applications of *T. delbrueckii* rely on its fermentative capacity; it is used for beer, wine and even bread fermentation (Varela and Borneman, 2017). Recent studies have revealed that the use of *T. delbrueckii* can help solve modern oenological problems (such as high acetic acid and ethanol concentrations) and, therefore, improve wine quality relative to individual *S. cerevisiae* fermentations (Benito, 2018). *T. delbrueckii* produces wines with lower concentrations of acetic acid (Bely et al., 2008) and ethanol (Contreras et al., 2014) and higher glycerol content (Belda et al., 2015). For all of these reasons, strains of *T. delbrueckii* are now widely used in winemaking in combination with *S. cerevisiae*. On the other hand, *S. bacillaris* presents an interesting trait for winemaking: its strong fructophilic character, consuming fructose in preference to glucose (Englezos et al., 2015). Moreover, its potential use to produce wines with reduced ethanol levels has also been studied (Giaramida et al., 2016; Magyar and Tóth, 2011). Fermentations with the

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other two species tested (*H. uvarum* and *M. pulcherrima)* were stuck after two days of fermentation (Figure 5.1a), and no proteins bound to melatonin were observed. *H. uvarum* has been widely described as a yeast species with lower fermentative power, being the most abundant yeast on the external surface of grape berries and predominating in the first stage of spontaneous alcoholic fermentation (approximately 48 h) (Fleet, 2003). Recently, the reduced capacity of *H. uvarum* for ethanol production, and therefore its lower fermentative capacity, has been attributed to the 10-fold-lower activity of the key glycolytic enzyme pyruvate kinase relative to *S. cerevisiae*, which is also the major factor in classifying this species as Crabtree-negative (Langenberg et al., 2017). *M. pulcherrima* is also known to exhibit lower fermentative capacity. Indeed, its presence during alcoholic fermentation has been associated with the lowering of ethanol production due to its respiratory metabolism, since under suitable aeration conditions, *M. pulcherrima* can respire between 40 and 100% of the sugar consumed (Quirós et al., 2014). A recent study (Valera et al., 2019) has reported that melatonin improves survival of non-*Saccharomyces* species in fermentation performed with mixed inoculum (co-inoculation of *Saccharomyces* and non-*Saccharomyces* or only non-*Saccharomyces)*. In fact, in the presence of melatonin, yeast cells improved their fermentative capacity, finishing the fermentation one or two days earlier. *T. delbrueckii* and *S. bacillaris* persisted until the end of the fermentation, whereas *M. pulcherrima* and *H. uvarum* only appeared at the beginning of fermentation but not at the end of the process, when melatonin was added to the synthetic must (Valera et al., 2019). Therefore, differences in the metabolism of sugars and enzyme activities could explain the differences in melatonin-protein interactions between non-*Saccharomyces* species.

The detection of proteins bound to melatonin was related to its synthesis profile. No melatonin-binding proteins were detected during the first 12 h of fermentation in any yeast (lag phase of all yeasts; Figure 5.1), during which the peak of intracellular melatonin was observed. Coinciding with low levels of the indole in the intracellular medium and highest accumulation of melatonin in the extracellular medium, several melatonin-binding protein bands were observed, with a stronger intensity at 24 h in Tdp (exponential growth, Figure 5.3 and 5.4). Similar results were obtained in a previous work (Chapter 3), in which melatonin was bound to different proteins during the exponential growth of *S. cerevisiae*. However, unlike what has been described in *S. cerevisiae*, a more intense interaction between melatonin and some proteins happened during the stationary phase, at 72 h in both species, and especially in Sb4, where this binding appeared practically only in this phase (72 and 96 h) (Figure 5.3 and 5.4). Indeed, melatonin-binding

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proteins seemed to follow a circadian rhythm-like at different time points of alcoholic fermentation, especially in Tdp. Given the properties of this molecule in humans (Jahanban-Esfahlan et al., 2018), these interactions described both in *Saccharomyces* and in some non-*Saccharomyces* provide more evidence for the role of melatonin as a signal molecule in yeast.

Protein identification

Melatonin-binding proteins were identified by mass spectrometry (Table 5.1 and 5.2). Most of the purified proteins were from the glycolysis pathway, similar to what we reported for *S. cerevisiae* in a recent study (Chapter 3). On one hand, although seven bands were observed in the Tdp protein gel (Figure 5.3 and Table 5.1), only four of them could be identified. Three of them coincided with proteins also identified in *S. cerevisiae*: pyruvate kinase (Pyk2p; band a), elongation factor 1 alpha (Tef1p; band b) and glyceraldehyde 3 phosphate dehydrogenase (Tdh1p; band f) (Chapter 3). The fourth was also a glycolytic protein: phosphoglycerate kinase (Pgk1p; band c). These similarities between *T. delbrueckii* and *S. cerevisiae* are not unexpected, since the strains are very close genetically (Masneuf-Pomarede et al., 2016). *T. delbrueckii* was previously identified as *Saccharomyces rosei*, suggesting a similar taxonomic lineage and function to that of *S. cerevisiae* (Bely et al., 2008). In fact, *Torulaspora* spp and *Saccharomyces* spp. are believed to have diverged evolutionarily approximately 100-150 million years ago (Hagman et al., 2014). Recently, Tondini et al. (2019) characterized the transcriptome of *T. delbrueckii* throughout alcoholic fermentation and observed that these three genes, *TDH2, PGK1* and *PYK2*, were upregulated at the exponential phase, whereas only the *TDH2* gene is highly expressed during the stationary phase. Furthermore, Tronchoni et al. (2017) showed that some glycolytic genes, including *PGK1*, were upregulated in *T. delbrueckii* (*S. cerevisiae* orthologues) at the exponential phase during a fermentation co-inoculated with *S. cerevisiae* and *T. delbrueckii*. Therefore, the binding of melatonin with the proteins encoded by these genes in the growth phase in which they are upregulated reinforces a possible signaling role for this molecule. Moreover, an RNA-binding protein (Tef1p) was also immunopurified. Tef1p has been related to a glycolytic complex on the surface of mitochondria in *S. cerevisiae* (Brandina et al., 2006).

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Figure 5.3. Time course of proteins bound to melatonin during alcoholic fermentation in *T. delbrueckii*. (a) SDS-PAGE gel electrophoresis. Bands marked are: a. pyruvate kinase; b. elongation alpha factor; c. phosphoglycerate kinase; d. uncharacterized protein; e. elongation alpha factor; f. glyceraldehyde-3-phosphate dehydrogenase. (b) Quantification of protein intensities performed using Image J software. (*) Uncharacterized protein.

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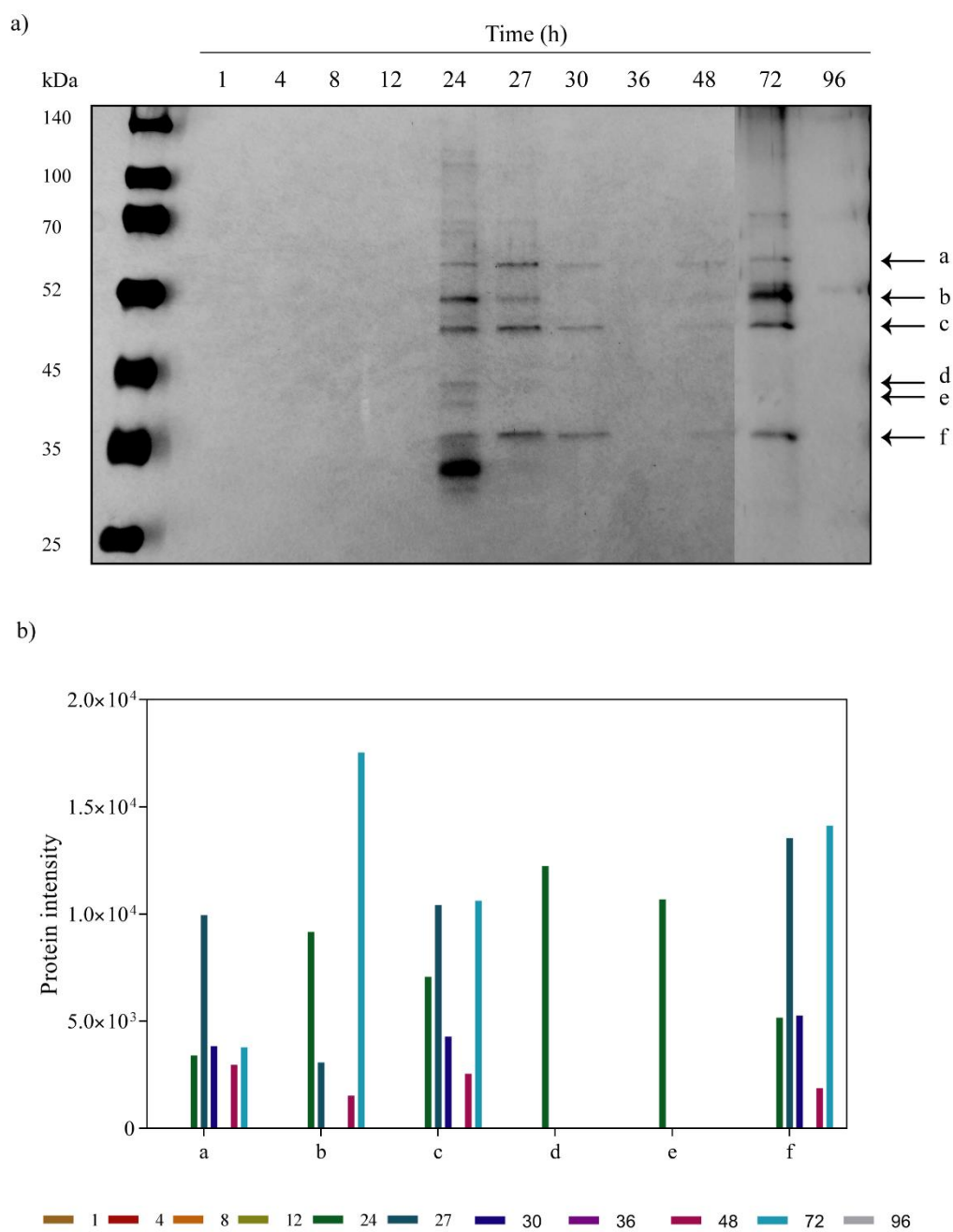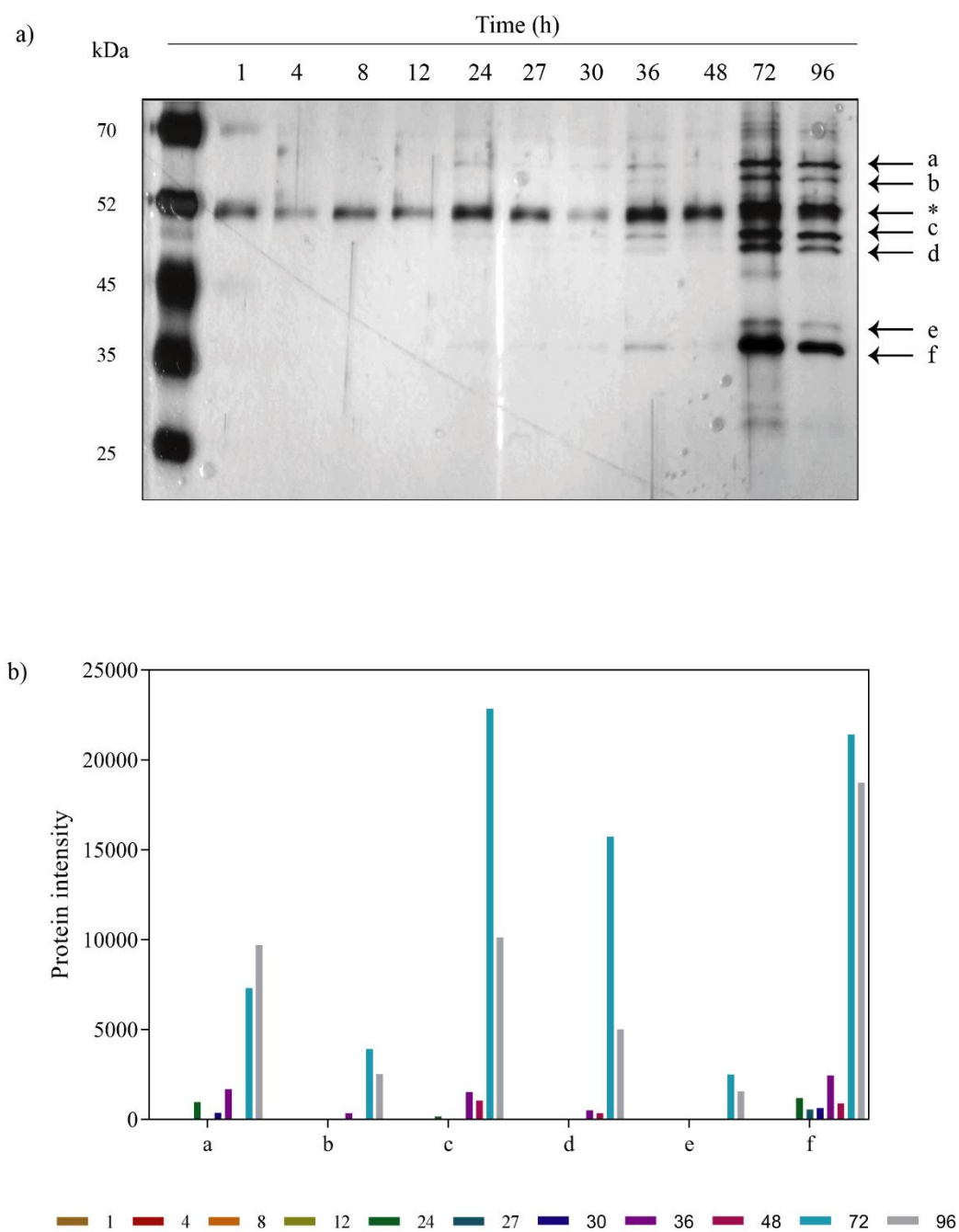Figure 5.4. Time course of proteins bound to melatonin during alcoholic fermentation in *S. bacillaris*. (a) SDS-PAGE gel electrophoresis. Bands marked: a. Pyruvate decarboxylase; b. glucose 6 phosphate isomerase; c. enolase; d. phosphoglycerate kinase; e. enolase fragment; f. glyceraldehyde-3-phosphate dehydrogenase. (b) Quantification of protein intensities performed using ImageJ software. (*) Rabbit IgG.

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On the other hand, six proteins interacted with melatonin during the fermentation process in Sb4 (Figure 5.4 and Table 5.2). Although *S. bacillaris* has been widely studied in terms of its technological properties during the fermentation process, especially for its fructophilic character (Englezos et al., 2015; Rantsiou et al., 2017), little is known about the genes involved in metabolic pathways such as glycolysis. Despite, recently, Rosa et al. (2018) reported *S. bacillaris* genome sequence, it was not available in UniProt Database and thus, in this analysis, protein sequences were compared to those of *Starmerella bombicola*. Despite the low coverage obtained, between 3-8%, tentative identification of the protein bands was possible. As in the other species, all proteins belonged to the glycolytic pathway (Figure 5.4 and Table 5.2): pyruvate decarboxylase (Pdc1; band a), glucose 6 phosphate isomerase (Pgi1p; band b), enolase (Eno1p; band c, e), phosphoglycerate kinase (Pgk1p; band d) and glyceraldehyde 3 phosphate dehydrogenase (Tdh1p; band f). Melatonin was reported to improve the cell growth viability of *S. bacillaris* when an oxidant stress was applied (Vázquez et al., 2018) and to extend the survival of *S. bacillaris* in mixed-inoculum fermentations (co-inoculation of *Saccharomyces* and non-*Saccharomyces* or only non-*Saccharomyces*), under low-nitrogen conditions (Valera et al., 2019).

Despite the fact that melatonin was synthesized during lag phase of yeast growth by all four non-*Saccharomyces* yeasts used in this study, only the *T. delbrueckii* and *S. bacillaris* strains bound proteins to melatonin. These two yeasts are described to be Crabtree-positive and to develop a fermentative metabolism when a high amount of sugar is present, despite oxygen availability (Mas et al., 2016). However, *H. uvarum* and *M. pulcherrima* are described as Crabtree-negative yeasts and prefer to develop an oxidative metabolism (Padilla et al., 2016a; Quirós et al., 2014). The *T. delbrueckii* and *S. bacillaris* strains also exhibited a greater fermentative capacity (Figure 5.1) than the strains of the other two species (*H. uvarum* and *M. pulcherrima*) and were closer to that of *S. cerevisiae*. On one hand, the formation of a glycolytic complex, the glycolytic metabolon, seems to regulate the channeling of different substrates into the mitochondria, such as pyruvate or t-RNA mitochondrial import (Brandina et al., 2006; Graham et al., 2007). On the other hand, melatonin has been related to mitochondria, where its synthesis occurs and its antioxidant activity is developed (Acuña-Castroviejo et al., 2001; He et al., 2016; Okatani et al., 2002). Given that glycolytic enzymes can associate to form a macromolecular complex near mitochondria (Brandina et al., 2006), melatonin may be acting as a signal molecule of this glycolytic complex in a fermentative capacity-dependent manner. Despite the lack of information at the gene, genome and protein levels for non-*Saccharomyces* yeasts, this study reports for the first time the

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interaction of melatonin and proteins in non-*Saccharomyces* yeasts. Nevertheless, further studies must be carried out to elucidate the biological importance of melatonin in yeast and characterize its interaction with glycolytic proteins.

Table 5.1. Identification of proteins purified from the SDS-PAGE gel using Nano LC-(Orbitrap) MS/MS in *Torulaspora delbrueckii.*

Protein gel band	Gene name in *S. cerevisiae*	Molecular weight (kDa)	Protein description (SGD database)	Sequence coverage, % number of peptides recovered by Nano LC-MS/MS	Mascot Score
A	*PYK2*	54.4	Pyruvate kinase 1	23.53	434.35
B	*TEF1*	49.9	Translation Elongation Factor	30.13	508.40
C	*PGK1*	44.6	Phosphoglycerate kinase	67.79	1051.02
D	-	39.2	Uncharacterized protein	-	-
E	-	36.7	Uncharacterized protein	-	-
F	*TDH1*	35.7	Triose-phosphate dehydrogenase	64.46	1934.02
G	-	28.0	Uncharacterized protein	-	-

Table 5.2. Identification of proteins purified from the SDS-PAGE gel using Nano LC-(Orbitrap) MS/MS in *Starmerella bacillaris.*

Protein gel band	Gene name in *S. cerevisiae*	Molecular weight (kDa)	Protein description (SGD database)	Sequence coverage, % number of peptides recovered by Nano LC-MS/MS	Mascot Score
A	*PGI1*	61.5	Glucose-6-phosphate isomerase	3.25	25.86
B	*PDC1*	49.7	Pyruvate decarboxylase	3.10	92.07
C	*ENO1*	46.8	Enolase	8.22	383.55
D	*PGK1*	44.1	Phosphoglycerate kinase	9.82	147.32
E	*ENO1*	46.8	Enolase fragment	3.88	91.25
F	*TDH1*	35.7	Triose-phosphate dehydrogenase	2.69	32.48

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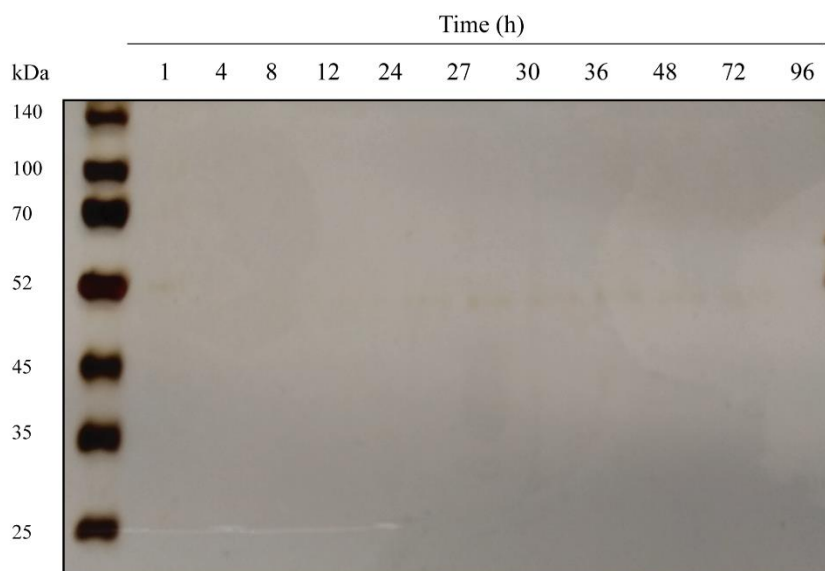
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Suplemmentary material

Supplementary Figure S5.1: Time course of purified proteins during alcoholic fermentation in *H. uvarum.*
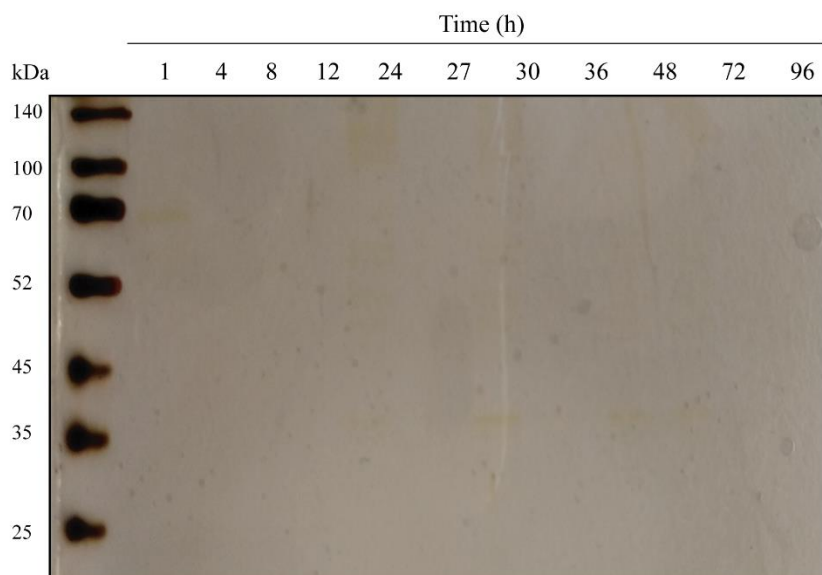
Supplementary Figure S5.2: Time course of purified proteins during alcoholic fermentation in *M. pulcherrima.*

Supplementary Table S5.1: Intracellular and extracellular melatonin produced by the four different non-*Saccharomyces* strains during alcoholic fermentation. Data are expressed in ng/mL.

Yeast strain		Time (h)											
		1	4	8	12	15	22	24	30	36	48	72	96
T. delbrueckii Tdp	I	0.47±0.02	1.52±1.10	0.28±0.01	0.42±0.32	0.26±0.18	0.17±0.02	0.47±0.34	0.02±0.02	0.01±0.01	0.03±0.02	0.05±0.06	0.02±0.02
	E	0,07±0.08	n.d.	n.d.	0,59±0.17	n.d.	n.d.	1,10±0.12	n.d.	0,04±0.02	0,09±0.02	0,13±0.05	n.d.
H. uvarum Hu4	I	0.47±0.01	0.94±0.18	0.06±0.01	0.04±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.01±0.01	n.d.	n.d.	0.02±0.01
	E	0.35±0.01	2.49±3.27	0.07±0.04	0.12±0.08	0.22±0.24	0.06±0.01	0.31±0.29	0.29±0.09	0.50±0.41	0.18±0.11	0.44±0.05	0.19±0.04
M. pulcherrima Mpp	I	61.93±0.01	62.45±11.71	41.73±16.60	15.17±4.21	13.54±0.89	9.94±0.50	12.45±3.56	15.28±4.74	17.83±4.47	12.30±1.67	15.32±2.84	12.71±5.08
	E	0.10±0.01	0.15±0.01	0.38±0.15	0.34±0.31	0.14±0.08	3.5±3.01	2.53±2.45	0.33±0.34	4.82±0.45	0.16±0.08	0.09±0.01	0.09±0.01
S. bacillaris Sb4	I	54.11±0.01	87.13±7.67	24.03±5.70	19.74±0.37	27.89±4.89	26.14±7.36	24.67±3.00	25.68±1.57	17.58±7.58	21.83±3.69	15.41±3.91	17.51±3.03
	E	0.08±0.01	0.23±0.01	0.09±0.01	0.12±0.02	0.07±0.02	0.09±0.05	3.42±4.55	0.07±0.0	2.01±2.75	0.20±0.20	0.08±0.01	0.54±0.01

n.d. not detected; I: Intracellular; E: Extracellular

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CHAPTER 6

Determination of melatonin by a whole cell bioassay in fermented beverages

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Abstract

Melatonin is a bioactive compound that is present in fermented beverages, such as wine and beer, at concentrations ranging from picograms to nanograms per mL of product. The purpose of this study was to optimize a novel fluorescent bioassay for detecting melatonin based on a cell line that contains the human melatonin receptor 1B gene and to compare these results with LC-MS/MS as a reference method. Conditions that could affect cell growth and detection (cell number per well, stimulation time, presence or absence of fetal bovine serum and adhesion of cells) were tested in the TANGO® cell line. Food matrices (wine and grape must) could not be directly used for the cell line due to low response. Therefore, for the determination of melatonin in food samples, an extraction procedure was required before conducting the assay. We demonstrated an improvement in melatonin determination by the cell-based bioassay due to increased sensitivity and specificity and improved quantification in complex matrices. Therefore, this method is a good alternative to determine melatonin content in some food samples, especially for those containing very low melatonin levels.

Key words: melatonin, cell line, LC-MS/MS, fluorescence, BLA activity

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine that has been identified within a wide range of invertebrates, plants, bacteria and fungi (Hardeland and Poeggeler, 2003; Tan et al., 2015). Therefore, melatonin is considered a ubiquitous molecule that is present in most living organisms (Tan et al., 2015).

Melatonin is a bioactive molecule that participates in many physiological processes in the human body, including the regulation of the circadian rhythm (Reiter, 1993) and as an antioxidant via receptor-independent processes (Galano et al., 2011; Reiter et al., 2007). Recently, melatonin has also been associated with a protective function against oxidative stress and UV radiation in yeast (Bisquert et al., 2018; Vázquez et al., 2017).

Melatonin has been found in many fermented beverages, such as beer or wine, at concentrations ranging from picograms to nanograms per mL of product (Kocadağlı et al., 2014; Rodriguez-Naranjo et al., 2011; Vitalini et al., 2013). In fact, during the winemaking process, the concentration of melatonin reaches its maximum between the first and second day of fermentation (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), highlighting the role of yeast in its production. Despite the occurrence of melatonin in fermented beverages is low, these concentrations have been described as contributing sufficiently to the dietetic intake to exhibit measurable effects (Hornedo-Ortega et al., 2016).

Melatonin can be determined by high-performance liquid chromatography (LC) with fluorescence (FL) (Arnao and Hernández-Ruiz, 2007; Mercolini et al., 2008; Yin et al., 2016), mass spectrometry (MS) (Fernández-Cruz et al., 2016; Iriti et al., 2006; Yang et al., 2002), gas chromatography and mass spectrometry (GC-MS) (Van Tassel et al., 2001), radioimmunoassay (RIA) (Van Tassel et al., 2001), enzyme-linked immunosorbent assay (ELISA) (Iriti et al., 2006; Kollmann et al., 2008; Maldonado et al., 2009) and immunoprecipitation (Pape and Lüning, 2006). Because melatonin production by yeast is very low (Fernández-Cruz et al., 2016; Rodriguez-Naranjo et al., 2012), these methods have limited sensitivity or are technically complex to be adapted as a routine technique for the rapid detection of the presence of melatonin in yeast-derived samples. For this reason, it is important to develop new methods for melatonin determination in fermented beverages.

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In this context, mammalian cell-based assays have been used with numerous reporter genes for monitoring gene or enzyme activity (Naylor, 1999), such as luciferase, β-galactosidase enzyme (lacZ) or green fluorescence protein (GFP). Nonetheless, these reporter technologies have several shortcomings as follows: bioluminescence of D-luciferin is transient, the lacZ system requires membrane permeabilization and despite the main advantages of GFP (non-invasive and no substrate requirement), its detection limit is very high because there is no enzyme amplification (Qureshi, 2007).

The β-lactamase enzyme (BLA) is another commonly used reporter system for gene expression and enzyme activity. Nevertheless, the effectiveness of the BLA system as a reporter gene was not used until Zlokarnik et al. (1998) reported the synthesis of a fluorogenic BLA substrate, CCF2/4. CCF2/4 is composed of two fluorescent dyes, 7-hydroxycoumarin-3-carboxamide and fluorescein, bridged by cephalosporin. With the CCF2/4 substrate, as few as 50 molecules of BLA can be detected within a cell (Zlokarnik, 1998). The BLA system also provides high sensitivity due to the absence of endogenous BLA activity in mammalian cells. BLA system could be used with several receptors (such as a melatonin receptor, MTNR1-B), where fluorescence detection is caused by fluorescence resonance energy transfer (FRET) system. When the BLA substrate is intact produces green fluorescence, whereas when the fluorophores are separated, the FRET fluorescence is lost and fluorescein generates blue fluorescence. Therefore, when there is no melatonin (Figure S6.1a), the BLA system is not activated and the CCF2/4 remain intact, producing green fluorescence. In contrast, when there is melatonin in the medium (Figure S6.1b), it is recognized by the MTNR1-B receptor, which activated the BLA reporter gene through a transcriptional factor. BLA enzyme cleaves CCF2/4, generating blue fluorescence. The relation between blue and green fluorescence allow quantifying melatonin, by using a standard calibration curve.

BLA-based assays are useful for studying signaling pathways in live cells (Zlokarnik, 2000), gene families including GPCRs (Kunapuli et al., 2003), protein folding (Fisher et al., 2006), adaptive and targeted gene evolution (Bowden and Salmond, 2006; Camps et al., 2003), protease activity (Whitney et al., 2002), gene trapping (Lai et al., 2002; Whitney et al., 1998), RNA splicing (Hasegawa et al., 2003) and protein-protein interactions by fragment complementation (Lee et al., 2004; Spotts et al., 2002).

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The aim of this work was (i) to develop a novel detection method of melatonin using a cell bioasssay based on BLA- assay associated to the human melatonin receptor 1B gene (MTNR1B) (ii) to compare this bioassay-method with LC-MS/MS, a validated method for melatonin detection.

Materials and methods

Chemicals and reagents

Melatonin (TLC grade, purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was provided by ChemLab (Zedelgem, Belgium), and methanol for liquid chromatography was supplied by J. T. Baker (Fisher Scientific, The Netherlands). Ultrapure water was obtained using a Milli-Q system (Millipore, Milford, MA, USA). Cell culture reagents were purchased from Gibco (Life Technologies, Carlsbad, CA, USA).

Melatonin standard stock solution was prepared before each use by dissolving a known amount of melatonin in absolute methanol (2 g/L). Standard solutions were prepared before each use by diluting melatonin standard stock solution in different chemical matrices.

Sample preparation

Samples with known concentrations (ranging from 0.001 to 100 ng/mL) of melatonin in different chemical matrices (synthetic must, white wine and assay medium (AM)) were analyzed. Melatonin was extracted by chloroform (Wolrab et al., 2016) with some modifications. Briefly, 50 µL of sample was mixed with 50 µL of Milli-Q water. Then, 500 µL of chloroform was added and vortexed for 1 min at room temperature. Samples were shaken at 1200 rpm for 1 h at room temperature. Organic phases from each sample were evaporated until dry under nitrogen gas. The residue was re-dissolved in 50 µL of a methanol/water mixture (40:60, v:v) or in 50 µL of AM (Dulbecco's-modified Eagle's medium (DMEM) supplemented with 1% dialyzed Fetal Bovine Serum (FBS), 0.1 mM non-essential amino acids (NEAA), 25 mM HEPES (pH 7.3), 100 U/mL penicillin and 100 µg/mL streptomycin) and centrifuged at 15000 rpm for 5 min at room temperature. Supernatants were analyzed using LC-MS/MS or the cell-based bioassay.

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Cell line

Human bone osteosarcoma epithelial cells containing the human melatonin receptor 1B (Tango MTNR1B-bla U2OS cell line) were obtained from Invitrogen (Carlsbad, CA, USA). The receptor is linked to a TEV protease site, and the Gal4-VP16 transcription factor is stably integrated into the Tango GPCR-bla U2OS parental cell line. Moreover, this parental cell line stably expresses a beta-arrestin/TEV protease fusion protein and the BLA reporter gene under the control of a UAS response element (TANGO^{TM} cell line).

Cell culture

TANGO cells were maintained in McCoy's 5A medium supplemented with 10% dialyzed FBS, 0.1 mM NEAA, 25mM HEPES (pH 7.3), 1mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL zeocin, 50 µg/mL hygromycin and 100 µg/mL geneticin. The cells were cultured at 37ºC in an incubator with 5% CO_2 in 75 cm^3 cell culture flasks. All experiments were performed with TANGO cells between passages 7 and 11.

Cell line optimization and analysis

The BLA cell-based bioassay was tested in several conditions: cell number per well (from 1.25 x 10^3 to 5 x 10^5), presence or absence of FBS, stimulation time and cell adhesion before analysis to optimize the analysis. After optimization, cells were washed with 10 mL of phosphate-buffered saline (PBS) and dispensed at a concentration of 2 x 10^4 cells per well on a 96-well black polystyrene plate with flat and clear bottom (Greiner Bio One, Austria) with 40 µL of staining solution, 50 µL of sample and 160 µL of AM. Cells were exposed to different known concentrations of melatonin for 24 h.

Melatonin determination was carried out according to the LiveBLAzer FRET-B/G Loading Kit (Invitrogen, Carlsbad, CA, USA). Fluorescence intensity was analyzed using a POLARstar Omega microplate reader (BMG LABTECH, Germany) with excitation and emission wavelengths of 390 and 450-520 nm, respectively. The results were calculated as a fluorescence ratio (FR) between blue fluorescence, representing the FRET system product as a result of melatonin presence, and green fluorescence from the FRET system substrate.

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LC-MS/MS analysis

The melatonin concentration was analyzed by performing liquid chromatography mass spectrometry following the method described by Rodriguez-Naranjo et al. (2011) with some modifications. The system was based on a high-performance liquid chromatograph coupled to a triple quadrupole mass spectrometer (Agilent G6410; Agilent Technologies, Palo Alto, USA). Melatonin separation was performed using an Agilent Zorbax Sb-Aq column (150 x 2.1 mm i.d., 3.5 µM). Chromatographic separation was performed using a binary gradient consisting of (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 flow rate was 0.4 mL/min. The injection volume was 7 µL.

Melatonin quantification was performed using Agilent MassHunter WorkStation Quantitative Analysis software version B0104 by comparing the 233/174 transition MS data of the sample and the standard.

The matrix effect was calculated as the percentage of the matrix-matched calibration slope (X) divided by the standard calibration slope (Y). If the ratio (X/Y x 100) is 100%, there is no matrix effect.

Results and discussion

In this study, we aimed to develop and optimize a new method for the detection of melatonin in food samples using a BLA cell-based bioassay. For this purpose, a validated method, LC-MS/MS, was used as a reference method to determine whether this methodology could be a good alternative to analyze melatonin in food samples.

Therefore, the first step was to implement the LC-MS/MS method developed by Rodriguez-Naranjo et al. (2011) to determine the LOD (limit of detection) and LOQ (limit of quantification) in different matrices.

LC-MS/MS

Matrix-matched calibration curves were prepared with melatonin standards (ranging from 100 to 0.05 ng/mL) in different matrices, such as a methanol/water mixture (40:60), Milli-Q water, white wine, grape must and AM. Samples were extracted by chloroform and injected into the LC-

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MS/MS system. Chloroform is one of the most common solvents used in indolamine extraction procedures, resulting in fewer residues that could affect melatonin determination by chromatography (Wolrab et al., 2016). In addition, liquid-liquid extraction is an inexpensive method that can be easily adapted to different types of biological samples (Muñoz et al., 2009).

Complex matrices (AM, wine and grape must) showed a signal suppression of the area response, resulting in a lower percentage of matrix calibration (28.6, 42.0 and 57.1%, respectively) (Figure 6.1 and Table 6.1). It is important to notice that even though melatonin presented good R^2 on calibration curves in complex matrices, only high concentrations (from 100 to 5 ng/mL) of melatonin were on the linearity of these curves. LOD and LOQ determination were obtained as 3 and 10 times the signal-to-noise ratio, respectively (Hubert et al., 2007). LOD (s/n = 3) was 1.03, 0.21 and 0.57 ng/mL, and LOQ (s/n = 10) was 3.10, 0.64 and 1.73 ng/mL for AM, wine and grape must matrices, respectively (Table 6.1). However, common solvents used for chromatographic analysis, such as a methanol/water mixture (40:60) or Milli-Q water, did not present practically matrix effect, and the LOD and LOQ were very similar (Table 6.1) due to the lack of primary or secondary metabolites that could interfere with chloroform extraction and chromatographic analysis. The LOD of our method on the determination of melatonin was similar to the values reported in previous studies (Rodriguez-Naranjo et al., 2011; Vigentini et al., 2015) but much
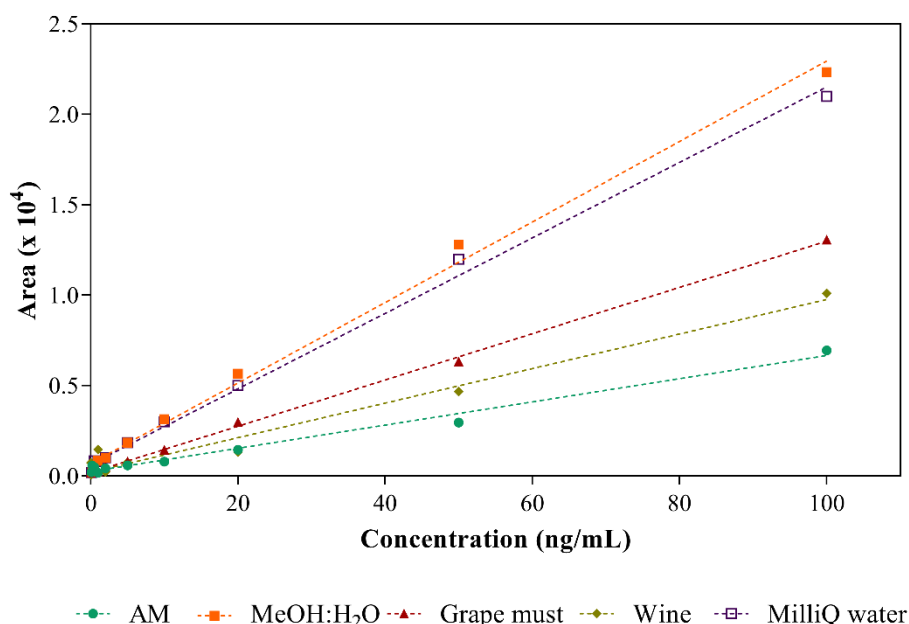

Figure 6.1. Matrix-matched calibration curves in different media: AM (Assay medium), MeOH:water mixture, Milli-Q water, grape must and wine. All samples were analyzed by triplicate.

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higher than the LOD obtained by Fernandez-Cruz et al. (2016), with an LOD of 0.0047 ng/mL and an LOQ of 0.0144 ng/mL.

Table 6.1. Calibration curve parameters for melatonin determination by LC-MS/MS *vs* BLA cell-based biosensor in different matrices. LOD (limit of detection), LOQ (limit of quantification).

Matrices	LC-MS/MS			Cell biosensor		
	LOD (ng/mL)	LOQ (ng/mL)	Matrix effect (%)	LOD (ng/mL)	LOQ (ng/mL)	EC50 (nM)
MeOH:water	0.17	0.53	100.0	n.d.	n.d.	n.d.
Milli-Q water	0.19	0.57	93.3	n.d.	n.d.	n.d.
Grape must	0.57	1.73	57.1	0.21	0.67	0.96
Wine	0.21	0.64	42.0	0.12	0.41	9.51
Assay medium	1.03	3.10	28.6	0.03	0.41	0.06

n.d. not determined

Cell-based method optimization

To optimize the BLA cell-based bioassay, several conditions that affect cell growth and detection, such as cell number per well, presence or absence of FBS, stimulation time and cell adhesion before analysis, were first studied in AM with melatonin standards (ranging from 100 ng/mL to 0.001 pg/mL) within the range of linearity described by the BLA cell-based bioassay.

Table 6.2. Effect of cell number on detection by BLA cell-based biosensor

Cell number per well	Maximum fluorescence ratio (FR)
1,250	<1
2,500	<1
5,000	<1
10,000	<1
20,000	6.1
50,000	5.6

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Regarding the cell number per well condition, concentrations of 2×10^4 and 5×10^4 cells per well were suitable for melatonin determination (with higher FR ratios), with the lowest concentration being the best (Table 6.2). Higher FR ratios implied that the calibration curve is better and quantification of melatonin in samples can be performed. Fewer cells per well were not sufficient to monitor melatonin content due to the low fluorescence ratio (FR<1). Other parameters studied included the presence or absence of FBS, a common supplement to *in vitro* and *ex vivo* cell cultures, and a stimulation time of 16 or 24 h (Figure 6.2). Longer stimulation times with melatonin (24 h) showed a higher signal detection of FR data and improved linearity ($R^2 = 0.9848$) (Figure 6.2a) than after 16 h of stimulation. In addition, FBS presence improved FR data (Figure 6.2b) due to its rich content of essential components, such as hormones, vitamins, protein transporters, and cell spreading and growth factors (Maurer, 1986). For cell adhesion, we compared the results using adhered cells or cell suspensions at the moment of melatonin addition, being better when cells were a suspension (data not shown).

Other fluorescent studies have been performed for melatonin determination using enzyme-linked immunosorbent assays (ELISA) in grape skin and beer samples with successful detection results (Iriti et al., 2006; Maldonado et al., 2009). However, according to Rodriguez-Naranjo et al. (2011), complex matrices, such as wine or other plant materials, did not provide a linear response by ELISA. Many molecules of the sample may interfere with ELISA determination by structurally mimicking melatonin or by cross-reactions within the immunoassay (Paredes et al., 2009), resulting in false-positives (Hernández-Ruiz and Arnao, 2008). According to Horwitz (1982), the appearance of either false-negative or false-positive results is typical of trace analysis. Therefore, the specificity of the MTNR-1B receptor used in this method for melatonin detection was determined by testing the response of different compounds related to tryptophan and its metabolism (tryptophan, serotonin, and tryptophan ethyl ester, Figure S6.2). No signals were detected with any of these compounds; therefore, the receptor presented a high specificity and no cross-reactions.

Food samples, such as wine or must, are complex matrices with different metabolites (ethanol and sugar molecules), which may interfere in BLA cell-based assays because of their effect on cell lines (Burg et al., 2007). Therefore, to validate the BLA cell-based bioassay for melatonin in food samples, we established the range of linearity in several chemical matrices, such as synthetic must, white wine, Milli-Q water and PBS, by diluting the melatonin standard stock solution in the different media. A low response was achieved when complex media were directly applied to the

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cells (Figure 6.3). The presence of sugar and/or ethanol considerably affected cell line growth. Ethanol increased cell volume and compromised cell viability (Figure 6.3b); while sugar resulted in decreased pH and cell volume due to osmotic effects (Figure 6.3c and d). Erickson et al. (2001) demonstrated similar results in chondrocytes; osmotic stress was caused significant cell volume changes. Cell volume decreases as extracellular osmolality increases, resulting in a logarithmic relationship (Finan and Guilak, 2010). Nevertheless, in a high concentration of sugar (200 g/L), similar to regular grape must, cells were not able to adhere to the surface (Figure 6.3e); thus, mammalian cells lost their viability, and no fluorescence signal was detected.

Therefore, for the determination of melatonin in food samples, an extraction step was required for sample preparation to eliminate non-specific interference prior to the detection of melatonin. To compare our results with melatonin determinations by LC-MS/MS, which was used in this study as reference method, the same extraction protocol with chloroform that is used in this chromatographic method was also applied to these samples.
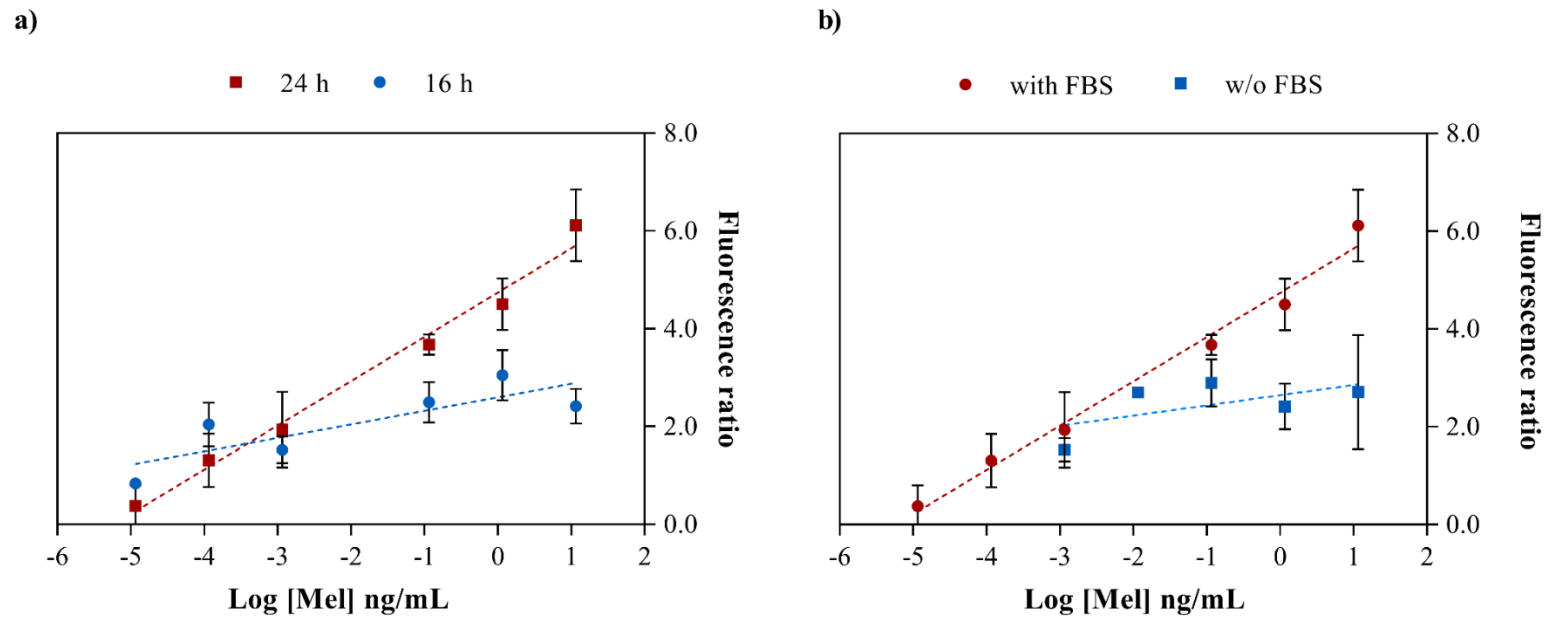
Figure 6.2. Effect of stimulation time with Mel (a) and presence of FBS (b) on efficacy of BLA cell-based assay.

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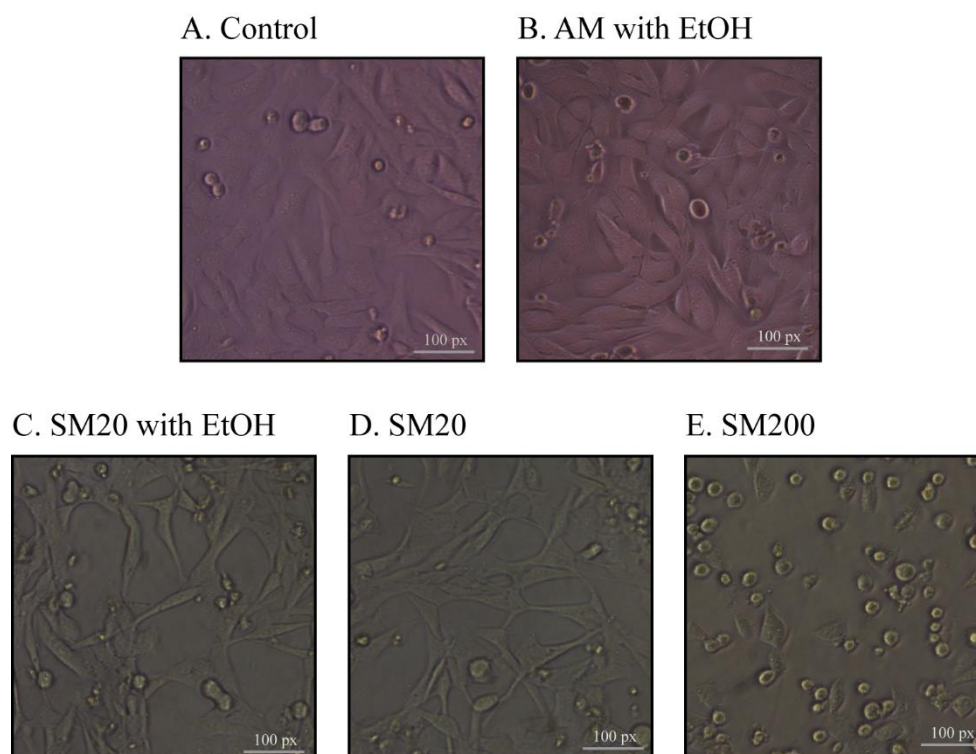María de los Ángeles Morcillo Parra

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Figure 6.3. Chemical matrix effect on growth of TANGO cells. a) Control (AM); b) AM with ethanol 12% (v/v); c) Synthetic must (SM) with ethanol 12% (v/v) and 20 g/L of sugar; d) SM with 20 g/L of sugar; e) SM with 200g/L of sugar.

Comparison of HPLC vs the cell-based bioassay

A range of several melatonin concentrations, from 100 to 0.01 ng/mL, were added to grape must and white wine, extracted by chloroform and resuspended in AM or a methanol/water mixture (40:60), depending on the method used for analysis, the BLA cell-based bioassay or LC-MS/MS, respectively.

Although a signal decrease was observed for both methods (Figure 6.4 *vs.* Figure 6.1 and 6.2, Table 6.1), the BLA cell-based bioassay showed increased sensitivity and quantification of melatonin in grape must and wine samples (LOD = 0.21 and 0.12 ng/mL and LOQ = 0.67 and 0.41 ng/mL, respectively). Another parameter that provides sensitivity information is the EC50 (refers to the concentration of melatonin which induces a half response of the bioassay), which was 0.06 nM in our cell assay with optimized conditions. Nevertheless, the EC50 increased in complex chemical matrices, such as grape must or wine samples (0.96 and 9.51 nM, respectively), due to the presence of primary or secondary metabolites, which could interact with the fluorescent assay system. Kunapuli et al. (2003) reported an EC50 of 2.2 nM with a BLA cell-based assay in mammalian cell

lines using a cell detection method similar to the BLA cell-based assay proposed in this study. Although in Figure 6.1 it was not appreciable because of the scale used, LC-MS/MS analysis showed a loss of linearity at 5 ng/mL of melatonin when complex matrices were used (Figure 6.4). However, the BLA cell bioassay maintained a good response even at these low concentrations in complex matrices, resulting in a higher sensitivity (Figure 6.4). Consequently, this method allows to quantify melatonin at low concentrations.

Sample analysis

Fermented beverages have been associated with the presence of bioactive compounds, which protect against cardiovascular and neurodegenerative diseases (Hornedo-Ortega et al., 2016). In particular, melatonin biosynthesis has been shown to be related to aromatic amino acid metabolism in yeast (Rodriguez-Naranjo et al., 2011; Vigentini et al., 2015). Therefore, to validate our fluorescent BLA cell-based bioassay, we analyzed melatonin content in 15 samples from alcoholic fermentation and compared with the results obtained by LC-MS/MS (Table 6.3). During this process, melatonin is produced by yeast in small quantities (from pg/mL to ng/mL) (Fernández-Cruz et al., 2019). The BLA cell-based bioassay detected melatonin in all samples analyzed in a range between 0.39 and 23.68 ng/mL. However, melatonin was detected only in five samples analyzed by LC-MS/MS. Surprisingly, those samples did not coincide with the samples that were determined to have higher concentrations of melatonin by the BLA cell-based bioassay. The concentrations of melatonin in most samples were very close to the LOD obtained in complex media, such as wine or grape must, which may justify the poor quantification in many of these samples (not quantified). Although the necessity to do a sample pre-treatment previous to the analysis of complex food samples, produced an important reduction of the signal response in both methods. This decrease was lower in the case of the BLA cell-based bioassay, allowing us to detect and quantify the low concentrations of melatonin synthesized by yeasts during the alcoholic fermentation. Conversely, in LC-MS/MS analysis, chloroform extraction drastically affected the melatonin determination in complex matrices (Figure 6.1), reducing the signal and producing the lack of detection in most fermented samples.
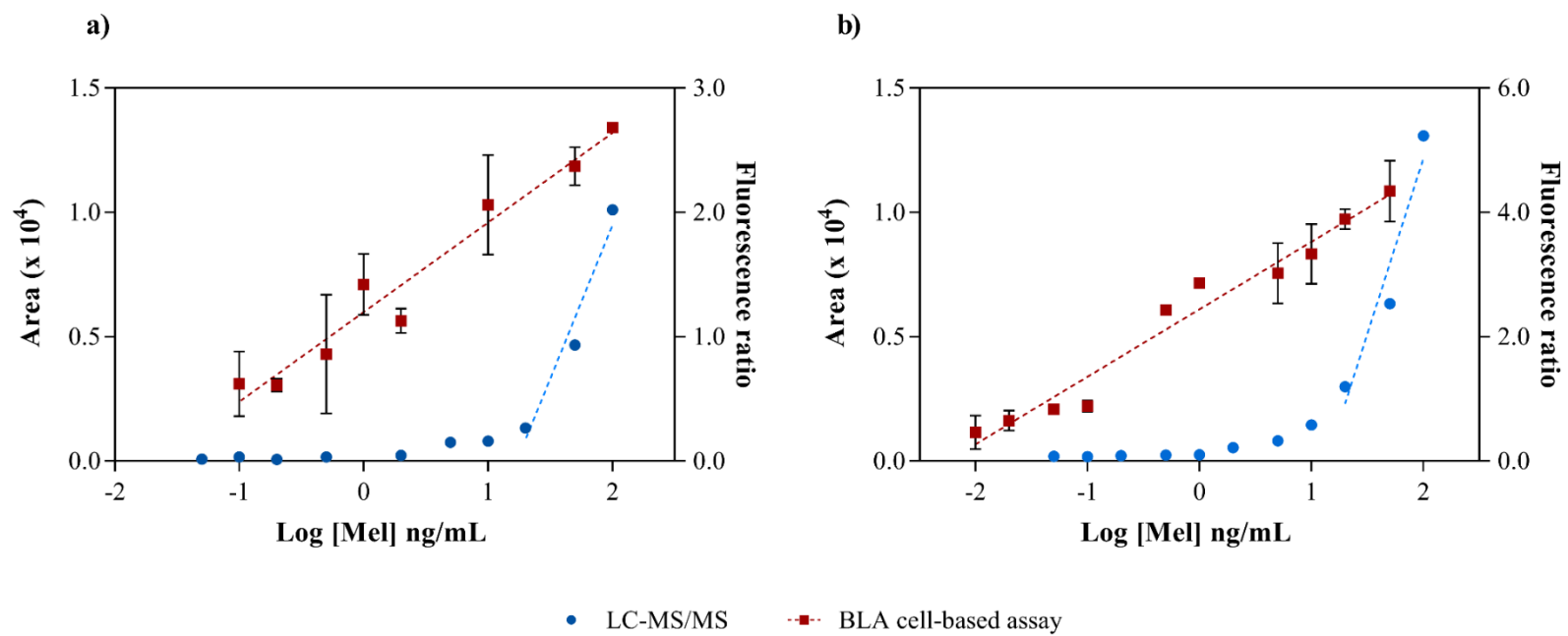
Figure 6.4. BLA cell-based assay and LC-MS/MS analysis comparison in two different matrices (a) wine and (b) synthetic must.

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Table 6.3. Mel quantification in samples from an alcoholic fermentation by BLA cell-based biosensor *vs* LC-MS/MS

Sample	Cell biosensor (ng/mL)	LC-MS/MS (ng/mL)
1	0.43	n.q.
2	0.58	n.q.
3	23.68	0.51
4	1.46	n.q.
5	1.39	n.q.
6	0.39	0.84
7	0.68	0.67
8	1.34	0.92
9	0.56	n.q.
10	5.06	n.q.
11	0.59	0.27
12	0.48	n.q.
13	0.99	n.q.
14	0.41	n.q.
15	1.43	n.q.

n.q. not quantified

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Conclusions

This investigation reports a comparative study on melatonin detection by two different methods, chromatographic analysis and a BLA cell-based bioassay. The study showed an improvement in melatonin determination by the BLA cell-based bioassay due to increased sensitivity and improved quantification. Although a noticeable reduction of the signal was observed when melatonin was analyzed in complex matrices, such as grape must or wine, due to the necessity of sample extraction prior to melatonin detection, this bioassay is a good alternative to determine melatonin content in food samples.

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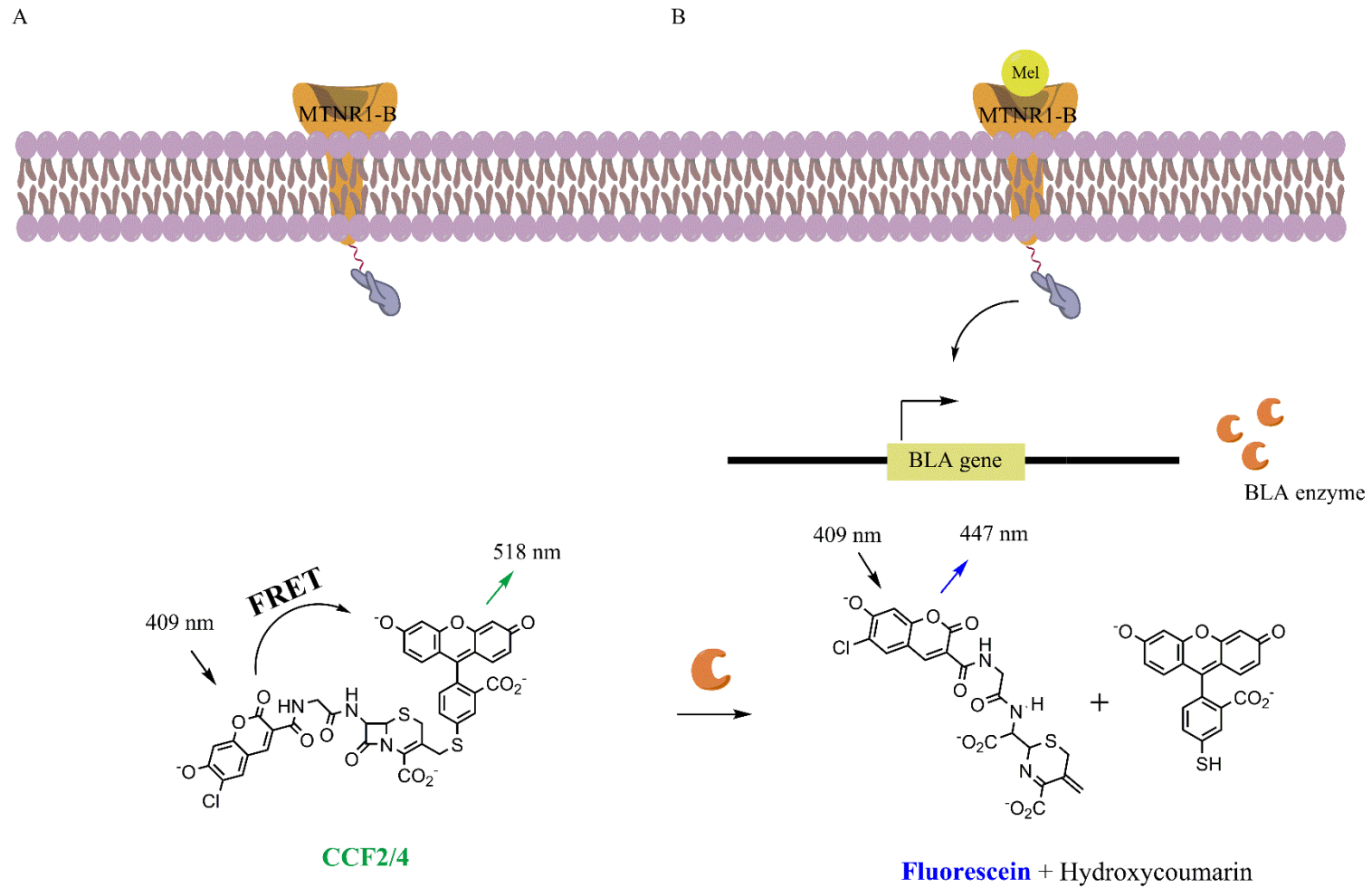
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Supplementary material

Supplementary Figure S6.1: Overview of BLA system mechanism in absence (A) or in presence (B) of melatonin in the medium.

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Supplementary Figure S6.2: Response of the MTNR-1B receptor to different compounds related to tryptophan and its metabolism. Mel (melatonina), TEE (tryptophan ethyl ester), Trp (tryptophan) and Ser (serotonin).
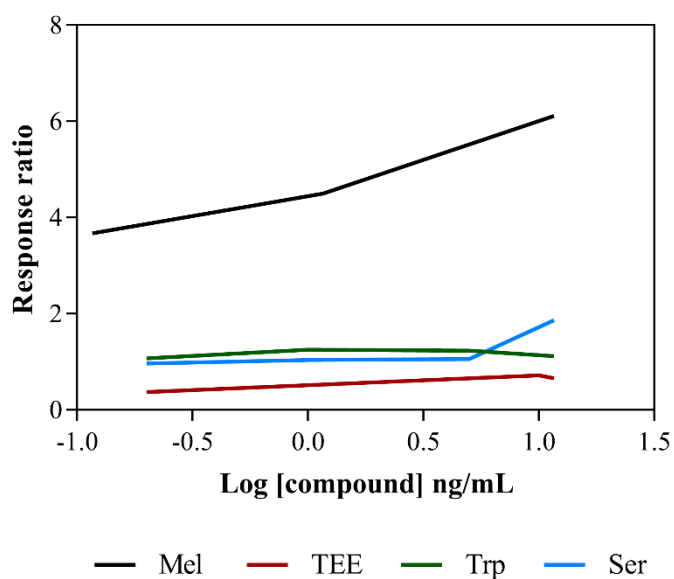
General discussion

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MELATONIN METABOLISM IN YEAST CELLS DURING ALCOHOLIC FERMENTATION
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The winemaking process consists of the transformation of sugars present in grape must into ethanol and carbon dioxide due to microorganism metabolism. Although *Saccharomyces cerevisiae* is considered the best candidate for performing alcoholic fermentation, interest in the utilization of non-*Saccharomyces* yeasts has increased in recent years (Mas et al., 2016). Non-*Saccharomyces* yeasts are found during the first stages of the process; however, during alcoholic fermentation, some factors, such as ethanol, anaerobic conditions or microbial interactions, facilitate the growth of *S. cerevisiae* to the detriment of other yeast species (Pretorius, 2000). In addition to ethanol, many other compounds are generated via microorganism metabolism derived from precursors present on grape must. A clear example is the aromatic amino acid tryptophan, from which yeast can generate tryptophol but also bioactive compounds such as serotonin or melatonin (Fernández-Cruz et al., 2019a; Muñiz-Calvo et al., 2019; Sprenger et al., 1999).

Melatonin is a bioactive molecule that has been widely studied due to its effects on human health. This molecule, in addition to its main function as a modulator of the circadian rhythm, has been associated with many other functions in mammals, among which its antioxidant activity stands out. Recently, a protective role of melatonin against oxidative and UV stresses has been described in yeast (Bisquert et al., 2018; Vázquez et al., 2017, 2018). Moreover, this compound has been reported in wines, ranging from pg/mL to ng/mL (Hornedo-Ortega et al., 2016; Rodriguez-Naranjo et al., 2011b), and its presence has been newly related to the activity of yeasts involved in alcoholic fermentation (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), such as *S. cerevisiae, T. delbrueckii, Z. bailii, H. uvarum, M. pulcherrima* (Fernández-Cruz et al., 2017, 2019a, 2019b; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015). However, little research has been carried out to analyze intracellular melatonin in oenological conditions.

Moreover, a valuable attribute of bioactive compounds is that minimal quantities are required for them to be active. However, this implies the necessity of having highly sensitive detection methods. Thus, lately, the development of new detection methods has increased in order to quantify the lowest possible content of melatonin, with the most commonly used method being high performance liquid chromatography (HPLC).

Considering all of these aspects, the hypothesis of this thesis was "Melatonin has a regulatory function in yeast cells, *Saccharomyces* and non-*Saccharomyces,* specifically during alcoholic

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fermentation", and to validate it, we studied melatonin synthesis in different yeast species and conditions to unravel the cause of its synthesis in yeasts.

Therefore, the first aim of this thesis was to evaluate the effects of different biotic and abiotic factors on melatonin metabolism.

Effects of biotic and abiotic factors on melatonin metabolism

Alcoholic fermentation performance can be affected by several factors that can change the production of bioactive compounds. Biotic factors are mainly caused by interactions between microorganisms during alcoholic fermentation, primarily in mixed populations (*Saccharomyces* and non-*Saccharomyces*), and abiotic factors are described as environmental or nutrition conditions that modify the development of fermentation (nutrients and temperature, among others). For that reason, our objective was to study the effect of adding melatonin to grape must on fermentation performance (Chapter 1) and to evaluate several conditions (different carbon and nitrogen concentrations, temperatures and inoculum sizes as well as synchronization of the cell cycle) for melatonin production (Chapter 2). First, the addition of melatonin extended the survival of non-*Saccharomyces* strains, specifically *Starmerella bacillaris* and *Torulaspora delbrueckii*, when high levels of the indole were used. *S. bacillaris* and *T. delbrueckii* were able to finish fermentation under low-nitrogen conditions even when *S. cerevisiae* was present, likely because *T. delbrueckii* strains are characterized by good fermentation capacity as well as ethanol tolerance (Bely et al., 2008; Lleixà et al., 2016). A previous study reported that *T. delbrueckii* and *S. bacillaris* remained longer than *H. uvarum* after inoculation with *S. cerevisiae* (Wang et al., 2016a). Conversely, *M. pulcherrima* was unable to survive until the end of fermentation. Some authors have reported that this species is unable to survive due to its low tolerance of ethanol and its low recuperation after inoculation (González-Royo et al., 2015; Kunkee and Amerine, 1970).

Melatonin has been described in many fermented beverages (Fernández-Cruz et al., 2016; Mercolini et al., 2008; Rodriguez-Naranjo et al., 2011a; Stege et al., 2010), and its presence has been related to yeast metabolism (Fernández-Cruz et al., 2017; Rodriguez-Naranjo et al., 2011b; Vigentini et al., 2015). In this study, we observed that all yeast species tested were able to produce melatonin extracellularly during the mid-late exponential phase or early stationary phase in a standard grape must (200 g/L sugar and 300 mg N/L). These findings are consistent with previous studies (Fernández-Cruz et al., 2017, 2019a; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), in which authors observed that melatonin production was species- and strain-dependent. In fact,

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the presence of a mixed population of non-*Saccharomyces* yeasts caused the accumulation of melatonin during alcoholic fermentation (Chapter 1), which may explain the presence of melatonin in wines (Marhuenda et al., 2016; Mercolini et al., 2012; Rodriguez-Naranjo et al., 2011a). Conversely, intracellular melatonin appeared at the beginning of the lag phase, when the synthesis of essential molecules has been described as an adaptation mechanism to new conditions (Rodriguez-Naranjo et al., 2012). Melatonin synthesis not only depends on precursor availability (Fernández-Cruz et al., 2019b; Rodriguez-Naranjo et al., 2012; Sprenger et al., 1999; Vigentini et al., 2015) but also on medium composition and growth phases (Rodriguez-Naranjo et al., 2012; Valera et al., 2019). We observed that melatonin increased its concentration when the temperature was adjusted to 12ºC but also delayed the appearance of a melatonin peak, as described by Wang et al. (2016b), in agreement with a delay in yeast growth produced by low temperature (Beltran et al., 2008; Torija et al., 2003). Melatonin concentration was also delayed in low-sugar conditions and with a higher initial population. The fact that melatonin appeared intracellularly and disappeared very fast reinforces the role of this indole amine as a signal molecule during alcoholic fermentation. Melatonin could induce a response to microorganism interactions and yeast growth during alcoholic fermentation.

To conclude this part, we demonstrated that all wine yeasts tested were able to produce melatonin either intra- or extracellularly, in a strain- and growth phase-dependent manner. Thus, intracellular melatonin appeared during the lag phase, and extracellular melatonin appeared in the late exponential or early stationary phase, indicating a delay between them. In addition to synthesis, we obtained evidence of a positive effect of the presence of melatonin on fermentation performance, mainly in non-*Saccharomyces* species.

Considering all of the results obtained in the first objective, in the second aim of the thesis, we wanted to decipher what happens with melatonin during the time gap between intracellular synthesis and extracellular secretion.

Melatonin fate during alcoholic fermentation

Melatonin has been related to yeast metabolism since Sprenger et al. (1999) observed melatonin production after tryptophan pulses. The rapid synthesis and the delay between intra- and extracellular melatonin content (shown in Chapter 2 and 3) suggested that melatonin could be bound to some molecules such as proteins. Thus, we checked whether melatonin interacts with proteins during this period and, if so, tried to identify them (Chapter 3 and 5). After melatonin

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synthesis in the intracellular compartment, melatonin was bound to several glycolytic proteins: glyceraldehyde-3-phosphate dehydrogenase, enolase and pyruvate kinase, among others. The interactions between melatonin and proteins only occurred in high-fermentative-capacity yeasts; *S. cerevisiae, S. bacillaris and T. delbrueckii* presented interactions with melatonin, while there was no evidence of interactions in *M. pulcherrima* or *H. uvarum*, which are described to be Crabtree-negative and develop an oxidative metabolism (Quirós et al., 2014; Varela and Borneman, 2017). In fact, pyruvate decarboxylase (*PDC1*), a key enzyme for channeling pyruvate to ethanol through alcoholic fermentation (van Hoek et al., 1998), was bound to melatonin in S. c*erevisiae.* Therefore, melatonin may have a role as a signal molecule to trigger fermentative metabolism.

The interaction of proteins with melatonin was inversely correlated with melatonin detection and was stronger in the exponential phase when melatonin was not detected as a free molecule, probably because it was bound to these proteins. Moreover, these interactions seemed to be related to yeast growth (Chapter 4), reinforcing the idea presented by Rodriguez-Naranjo et al. (2012). The increase in the duration of the lag phase caused a delay in melatonin synthesis as well as in the binding of melatonin to proteins. These results confirmed those of Wang et al. (2016b), who described a delay in melatonin production in mulberry fermentations at low temperature due to slower yeast growth. These interactions also seemed to follow a circadian rhythm-like. Although yeasts are not included among circadian model organisms, Eelderink-Chen et al. (2010) demonstrated that yeast metabolism shows a systematic circadian entrainment.

The interaction of melatonin with glycolytic proteins could strengthen the idea of the existence of a glycolytic complex related to mitochondria. Brandina et al. (2006) demonstrated that many glycolytic proteins are associated with mitochondria in yeast cells, suggesting the presence of a macromolecular complex bound to the mitochondrial surface. In addition, Graham et al. (2007) reported that glycolytic proteins are on the surface of mitochondria in addition to the cytosol in *Arabidopsis thaliana.* The authors suggested that the presence of these proteins near mitochondria could regulate the channeling of different substrates into the mitochondria, such as pyruvate towards the TCA cycle or t-RNA mitochondrial import (Brandina et al., 2006; Graham et al., 2007). However, glycolytic enzymes are very active during alcoholic fermentation in order to increase the flux of pyruvate to the fermentative pathway through pyruvate decarboxylase (Querol and Fleet, 2006). Therefore, the increase in glycolytic flux causes saturation of the respiration metabolism, and pyruvate is metabolized via pyruvate decarboxylase to ethanol (van Hoek et al., 1998). Thus, the interaction of melatonin, a molecule that has been reported to be synthetized on mitochondria (He et al., 2016), with glycolytic enzymes that could be forming a

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complex on the surface of mitochondria (Brandina et al., 2006) can evidence the role of melatonin as a signal molecule of this complex. Melatonin may be acting as a positive or negative regulator of the glycolytic complex in order to channel the flux to fermentative metabolism. Nevertheless, further studies are needed, such as measurement of the activity of glycolytic enzymes as well as their localization in the cell when they are interacting with melatonin.

In addition to glycolytic enzymes, an RNA-binding protein (Tef1p) was purified in *S. cerevisiae* and *T. delbrueckii*. Tef1p has been related to the enolase complex described by Brandina et al. (2006), and the fact that this protein was bound to melatonin as well as glycolytic enzymes seems to reinforce that it is related to the glycolytic complex during alcoholic fermentation, although its function is still unknown.

Several glycolytic proteins have been described to develop other functions in addition to glycolysis, such as enolase, pyruvate kinase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, formed by Tdh1p, Tdh2p and Tdh3p in yeast cells) (Kim et al., 2005). GAPDH has been reported to be present in the cell wall in addition to the cytosol in yeast (Delgado, 2001; Falcao Moreira 1998, Gil-Navarro, 1997), and Tdh1p alone cannot support growth (McAllister and Shinohara, 1985). Thus, GAPDH has been described as a moonlighting protein (Sirover, 2011). We studied the importance of some glycolytic proteins in the synthesis of melatonin and in the interactions with this molecule. Only the deletion of the *ENO1* and *TDH1* genes had an impact on the melatonin-protein interaction profile, pointing out a main role for these proteins in the interaction with melatonin, probably related to the mitochondrial glycolytic complex. Nevertheless, more studies with double mutants are needed to assess the implications of these proteins in the interactions with melatonin and the glycolytic complex.

In summary, we reported that the interactions of proteins with melatonin in *Saccharomyces* and non-*Saccharomyces* yeasts are related to fermentative metabolism. Most of the immunopurified proteins belong to the glycolysis pathway, and their interactions with melatonin could provide evidence for the presence of a glycolytic complex and this indole as a signal molecule of this complex.

Finally, throughout this thesis, we have dealt with the fact that melatonin is a very elusive molecule and is therefore difficult to detect, mainly because as putative signal molecule, its synthesis and mobilization are very rapid. For this reason, the last objective of this thesis was to optimize a detection method for melatonin in food samples.

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Detection methods for melatonin in food

Melatonin is an important molecule due to its regulatory and antioxidant functions described in humans (Reiter, 1993; Tan et al., 2015) but also in yeast cells (Bisquert et al., 2018; Vázquez et al., 2017, 2018). For this reason, since melatonin has been found in fermented beverages such as beer (Maldonado et al., 2009) and wine (Rodriguez-Naranjo et al., 2011a; Stege et al., 2010), and yeast has pointed out as the responsible for its production (Fernández-Cruz et al., 2017; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), most research has focused on demonstrating the presence of melatonin or synthesis by yeasts in a fermentative context. Thus, a wide range of detection methods and techniques have been described (explained in the Introduction of the thesis). Melatonin production by yeast is very low (Fernández-Cruz et al., 2016, 2019b; Rodriguez-Naranjo et al., 2011a), and most methods have limited sensitivity or are too technically complex to be adapted as routine techniques for the rapid detection of the presence of melatonin in yeast-derived samples. In addition, the results obtained in Chapter 2 showed that melatonin production and disappearance were very rapid (min), and melatonin did not accumulate in the medium in most of the tested conditions. Therefore, our purpose was to develop a continuous or semicontinuous method of melatonin detection to improve the monitoring of melatonin synthesis, reducing the variability between replicates due to noncontinuous sampling.

Optimization of a novel method based on a BLA cell assay was carried out. The method was based on the human melatonin receptor (MTNR-1B) coupled to FRET detection by the β-lactamase enzyme. In this analysis, must and wine samples could not be added directly to the cell line because these cells cannot endure the extreme conditions of such media (pH, sugar, ethanol content, etc.) (Burg et al., 2007), and thus, an extraction of the sample was needed. Therefore, the main problem of variability was not solved given the impossibility of using this method for continuous measurements. Nevertheless, a good and novel alternative method was developed to detect melatonin in food samples, achieving a lower limit of detection relative to a method based on HPLC, from which we obtained values similar to those reported by other studies (Rodriguez-Naranjo et al., 2011a; Vigentini et al., 2015). However, new methods came out in the last few years with much lower LODs (pg/mL) than the one we reported based on ultra-HPLC (Fernández-Cruz et al., 2016; Fracassetti et al., 2019). Some of these methods allow the quantification of not only melatonin but also related compounds such as serotonin and 5-methoxytryptamine, among others, at the same time (Fernández-Cruz et al., 2016; Tudela et al., 2016).

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Other fluorescence techniques, such as ELISA, have been used to detect melatonin with successful results in beer and grape skin (Iriti et al., 2006; Maldonado et al., 2009); however, complex matrices such as wine seem not to provide a linear response (Rodriguez-Naranjo et al., 2011a) due to the presence of many molecules that can interfere with melatonin determination (Paredes et al., 2009), resulting in false-positives (Hernández-Ruiz and Arnao, 2008). In our case, the MTNR-1B receptor exhibited a high specificity for melatonin, since no signal was emitted when any other compounds related to tryptophan and its metabolism (tryptophan, serotonin and tryptophan ethyl ester) were tested. In summary, we optimized a novel fluorescence method based on a BLA cell assay that allowed us to decrease the limit of detection of the HPLC used in this study to 0.03 ng/mL, facilitating the detection and quantification of melatonin in samples that were negative by the chromatographic method. Nevertheless, to better understand the synthesis profile of melatonin and avoid differences between replicates due to sampling, the development of a continuous method is still needed.

In summary, with the different experiments performed in this thesis, we demonstrated that (i) melatonin exhibits a positive effect on the performance of alcoholic fermentation, and (ii) melatonin is synthesized following a specific profile and interacts intracellularly with glycolytic proteins, depending on yeast growth phases. All of this evidence supports the initial hypothesis of melatonin as a signal molecule. In addition, we developed a good alternative for melatonin detection, although we cannot solve the variability problem because our method did not allow continuous detection. These results partially confirm our hypothesis: a signal molecule needs to undergo rapid synthesis and disappearance. Although we confirmed that there is a link between melatonin production, growth phases and the fermentative activity of yeasts that are Crabtree-positive, the exact mechanism and the triggering of melatonin synthesis are unclear and remain a challenge.

Open questions such as what triggers the synthesis of melatonin and its role in glycolysis and the fermentative response of yeast to the conditions of grape must remain unanswered and represent future challenges in understanding this process.

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General conclusions

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General conclusions

1. Melatonin is a very elusive molecule in yeast, which supports the idea of its role as a signaling molecule.

2. Melatonin addition causes changes in fermentation kinetics, viability and species distribution during fermentation.

    (a) *Starmerella bacillaris* and *Torulaspora delbrueckii* extend their survival when melatonin is present.

3. All yeast species, *Saccharomyces* and non-*Saccharomyces*, are able to produce melatonin.

    (a) Melatonin production depends on yeast growth phases.

    (b) Intracellular melatonin is detected during the lag phase of growth.

        i. Low sugar concentration, low temperature and high inoculum size delay the synthesis of melatonin in *S. cerevisiae*.

    (c) Extracellular melatonin is observed during the late exponential or early stationary phase of growth.

        i. In general, the tested *S. cerevisiae* strains do not accumulate melatonin throughout alcoholic fermentation, with the exception of Instaferm.

        ii. *M. pulcherrima* and *S. bacillaris* are the best non-*Saccharomyces* producers.

        iii. Mixed populations cause an accumulation of melatonin during alcoholic fermentation.

4. Melatonin is bound to proteins during alcoholic fermentation in yeasts with high fermentative capacity.

    (a) Melatonin interaction seems to follow a circadian rhythm-like.

    (b) Glycolytic enzymes interact with melatonin after intracellular melatonin synthesis

General conclusions

   i. Glyceraldehyde-3-phosphate is the main protein bound to melatonin in all yeasts.

   ii. Enolase in *S. cerevisiae* and phosphoglycerate kinase in non-*Saccharomyces* yeasts are proteins that are also commonly purified.

   (c) A RNA-binding protein is also bound to melatonin.

5. Glycolytic knockout strains present profiles of intracellular melatonin production similar to that of the tested wine yeast, but not in the secretion into the extracellular medium.

6. Deletion of the *ENO1* and *TDH1* genes has an impact on the melatonin-protein interaction profile, suggesting a pivotal role for these proteins in these interactions.

7. A novel BLA cell-based assay is used for melatonin detection in fermented beverages.

   (a) Melatonin analysis requires previous extraction with chloroform.

   (b) A lower limit of detection is achieved by the BLA cell-based assay than by LC-MS/MS.

Annex I

Materials and Methods

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Culture Media for yeasts

YPD (Yeast Peptone Dextrose)

Glucose	20 g/L
Yeast Extract	10 g/L
Bacteriological Peptone	20 g/L
Agar in case of Solid Medium	20 g/L

WL (Wallerstein Laboratory Nutrient)

WL Nutrient Agar	75 g/L

YNB (Yeast Nitrogen Base)

Glucose	20 g/L
Yeast Nitrogen Base without amino acids and ammonium sulfate	1.7 g/L
$(NH_4)_2SO_4$	350 mM

Lysine Agar Medium

Lysine Medium	66 g/L
Potassium Lactate	10 ml/L
Lactic Acid*	1 ml/L

*Added after boiling

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SM (Synthetic Must)

Glucose	100 g/L
Fructose	100 g/L
Citric acid	0.5 g/L
Malic acid	5 g/L
Tartaric acid	3 g/L
KH_2PO_4	0.75 g/L
K_2SO_4	0.50 g/L
$MgSO_2$ 7 H_2O	0.25 g/L
$CaCl_2$ 2 H_2O	0.15 g/L
NaCl	0.20 g/L
NH_4Cl	0.460 g/L
Total NH_4Cl YAN (mg N/L)	120 mg/L

Amino acids

Stock solution of aromatic amino acids (AAA)	
Tyrosine	1.95 g/L
Tryptophan	17.50 g/L
Phenylalanine	3.77 g/L
Stock solution of non-aromatic amino acids (AA)	
Isoleucine	3.25 g/L
Aspartic acid	4.42 g/L
Glutamic acid	11.96 g/L
Arginine	36.79 g/L
Leucine	4.81 g/L
Threonine	7.54 g/L
Glycine	1.82 g/L
Glutamine	49.92 g/L

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Alanine	14.56 g/L
Valine	4.42 g/L
Methionine	3.12 g/L
Serine	7.80 g/L
Histidine	3.38 g/L
Lysine	1.69 g/L
Cysteine	2.08 g/L
Proline	59.93 g/L

Add 50 mL from AAA stock solution**

Add 7.5 mL from AA stock solution**

Total amino acids YAN (mg N/L)	179.66 mg/L
Total YAN (mg N/L)	299.66 mg/L

Oligo-elements

Stock solution	
$MnSO_4 \cdot H_2O$	4.0 g/L
$ZnSO_4 \cdot 7\,H_2O$	4.0 g/L
$CuSO_4 \cdot 5\,H_2O$	1.0 g/L
KI	1.0 g/L
$CoCl_2 \cdot 6\,H_2O$	0.4 g/L
H_3BO_3	1.0 g/L
$(NH_4)_6Mo_7O_{24}$	1.0 g/L

Add 1 mL from stock solution**

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Vitamins

Stock solution	
Myo-inositol	2 g/L
Pantothenate calcium	150 mg/L
Thiamine hydrochloride	25 mg/L
Nicotinic acid	200 mg/L
Pyridoxine	25 mg/L
Biotine	3 mL (from stock 100mg/L)
*Add 10 mL from stock solution**	

*YAN: Yeast Assimilable Nitrogen

** Added after autoclave

All sugars, acids and minerals were mixed and autoclaved. Then, vitamins, oligoelements and amino acids were added as required, pH was adjusted at 3.3 and all preparation was filtered by 0.22 µM.

Low nitrogen must

*SM modified by reducing the nitrogen content to 100 mg N/L of YAN

NH$_4$Cl	0.154 g/L
AAA stock	16.67 mL
AA stock	2.52 mL
Total YAN (mg N/L)	99.89 mg/L

Low glucose must

*SM modified by reducing the sugar content to 20 g/L

Glucose	10 g/L
Fructose	10 g/L

Culture Media for TANGO MTNR1-B cell line

All cell reagents were purchased from Gibco (Life Technologies, Carlsbad, CA, USA).

Growth medium

McCoy's 5A Medium (modified) 1X	90%
Dialyzed FBS	10%
Non-Essential Amino Acids	0.1 mM
HEPES (pH 7.3)	25 mM
Sodium Pyruvate	1 mM
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Zeocin™	200 µg/mL
Hygromycin	50 µg/mL
Geneticin®	100 µg/mL

Assay medium

Dulbecco's-modified Eagle's medium (DMEM)	99%
Dialyzed FBS	1%
Non-Essential Amino Acids	0.1 mM
HEPES (pH 7.3)	25 mM
Penicillin	100 U/mL
Streptomycin	100 µg/mL

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Thawing medium

McCoy's 5A Medium (modified) 1X	90%
Dialyzed FBS	10%
Non-Essential Amino Acids	0.1 mM
HEPES (pH 7.3)	25 mM
Sodium Pyruvate	1 mM
Penicillin	100 U/mL
Streptomycin	100 µg/mL

Freeze medium

Recovery™ Cell Culture Freezing Medium	100%

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Melatonin determination by HPLC analysis

Chemicals and reagents

Methanol LC-MS grade was purchased from Sigma Aldrich (St. Louis, MO, USA) and formic acid was provided by ChemLab (Zedelgem, Belgium). Ultrapure water was obtained from a Milli-Q® system (MQ; Millipore, Milford, MA, USA). Chloroform was provided by Merck (Chloroform dried, Merck, Germany). Analytical standard of melatonin (TLC grade, purity ≥98%, Sigma Aldrich, St. Louis, MO, USA) was used.

Instrumental conditions

A 1200 HPLC Series Liquid Chromatograph coupled to a triple quadrupole mass spectrometer (Agilent G6490; Agilent Technologies, Palo Alto, USA) was used for melatonin determination. The chromatographic column was an Agilent Zorbax Sb-Aq (150 mm x 2.1 mm i.d., 3.5 µM). Chromatographic separation was performed using a binary gradient consisting of water (Solvent A) and methanol (Solvent B), both containing 0.1 % (v/v) formic acid. Flow rate was 0.4 mL/min. Elution profile was 100% B (4 min) and 10% B (6 min). A post run of 4 min was applied. Injected sample volume was 7 µL.

ESI conditions were drying gas temperature at 350ºC, a flow of 12 L/min and a nebulizer gas pressure of 25 psi. The capillarity voltage was set up at 2500 V. Acquisition was done in positive polarity. Triple quadrupole operated in multiple reaction monitoring (MRM) mode, applying a fragmentor voltage of 135 V and a cell accelerator voltage of 65 V. Quantitative and qualitative transitions of melatonin and the corresponding collision energy are showed in Table 1.

Table 1. MRM transitions and collision energy (CE) for melatonin.

Transition*	CE (V)
233.28→130	48
233.28→143	28
233.28→159	20
233.28→174	12
233.28→216	2

*Quantitative transition is in bold

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Sample preparation

For intracellular samples, a previous extraction of intracellular metabolites is needed. The boiling buffered ethanol method described by González et al. (1997)[1] was adapted. 1 mL of a boiling solution of 75% (v/v) absolute ethanol containing 70 mM HEPES buffer (pH 7.5) was added to the cell pellet (10^8 cells). This mixture was incubated for 3 min at 80ºC. The extract was concentrated by evaporation at 45ºC in a SpeedBack (Concentrator plus, Eppendorf Ibérica, Madrid, Spain). The intracellular content was resuspended in 1 mL of MQ water and centrifuged for 10 min at 5000 rpm. The supernatant was transferred to a new tube and stored at -20ºC until use.

Then, intracellular and extracellular samples were extracted with chloroform. 50 µL of sample were mixed with MQ water (1:1, v:v). Afterwards, 10 volumes of chloroform were added. Samples were shaken for 1 h at 1200 rpm. The organic phase was dried under a flow of nitrogen gas and resuspended in 50 µL of a mixture of methanol and water (40:60, v:v). Then, samples were centrifuged for 5 min at 14500 rpm. Supernatants were transferred to vials and analysed by LC-MS/MS.

Sample quantification

For the quantitative method validation, linearity, precision, accuracy and limits of detection and quantification were studied. Calibration curves by standard dilutions serial prepared in MQ water were prepared. Reproducibility was determined from relative standard deviation (%RSD) in the analysis of a spiked pooled sample. Limit of detection (LOD) was defined as the concentration to three times the signal/noise rate and limit of quantification (LOQ) was defined as the concentration to ten times the signal/noise rate. Linearity was defined as the lowest concentration giving a linear response. The obtained validation parameters of the method, which are showed in Table 2, allowed quantifying melatonin in the extract samples. Melatonin quantification was performed using Agilent MassHunter WorkStation Software Quantitative Analysis Version B0104 by comparing the 233/174 transition MS data of the sample and the melatonin standard.

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[1] Gonzalez, B., François, J., Renaud, M. (1997). A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. Yeast. 13, 1347–1356.

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Table 2. Retention time (Rt) and method validation parameters of reproducibility, detection and quantification limits (LOD and LOQ), linearity, accuracy and determination coefficient (R^2) for melatonin.

Rt (min)	Reproducibility (% RSD, n=10)	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)	Accuracy	R^2
5.8	4.1	0.17	0.53	2.68	102.3	0.994

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Protocols for cell line

Propagation Method

Passage or feed cells at least twice a week in order to maintain cell between 25% and 95% of confluence. Do not allow cells to reach 100% confluence.

To passage cells, all materials have to be pre-warming at 37ºC.

1. Aspirate the medium and rinse once in 10 mL PBS.

2. Add 2 mL 0.05% Trypsin/EDTA (in T75 flask) and swirl to coat the cells evenly. Incubate 3 min at 37ºC or until cells are detached (no more than 5 min).

3. Add 8 mL of Growth Medium to inactivate trypsin/EDTA.

4. Verify under a microscope that cells have detached and clumps have completely dispersed.

5. Centrifuge cells at 200 g for 5 min and resuspend in Growth Medium.

Freezing Method

To freeze cells, all materials have to be pre-warming at 37ºC to harvest.

1. Harvest the cells as described in Propagation Method.

2. After detachment, count the cells with a Neubauer chamber.

3. Centrifuged cells at 200 g for 5 min.

4. Resuspend in 4ºC Freeze Medium to a density of 2×10^6 cells/mL.

5. Dispense 1 mL aliquots into cryogenic vials.

6. Place in an insulated container for slow cooling and store overnight at -80ºC.

7. Transfer to liquid nitrogen the next day for storage.

Thawing Method

To thaw cells, all materials have to be pre-warming at 37ºC.

1. Rapidly thaw the vial of cells by placing at 37ºC in a water bath with gentle agitation for 1-2 min. Do not submerge vial in water.

2. Decontaminate the vial by wiping with 70% ethanol before opening in the cabinet.

3. Transfer the vial content drop-wise into 10 mL of Thawing Medium in a sterile 15-mL conical tube.

4. Centrifuge cells at 200 g for 5 min.

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5. Aspirate supernatant and resuspend the cell pellet in 10 mL fresh Thawing Medium.

6. Transfer content to the T75 culture flask.

7. Verify under a microscope that cells have completely dispersed.

8. Place the flask in the humidified 37°C/5% CO_2 incubator.

9. At first passage, switch to Growth Medium.

Cell assay

1. Harvest the cells as described in Propagation Method.

2. After detachment, count the cells with a Neubauer chamber.

3. Centrifuged cells at 200 g for 5 min.

4. Resuspend in pre-warmed Assay Medium to a density of 3.125 x 10^5 cells.

5. Add 160 µL of cell suspension to have 2 x 10^4 cells/well.

6. Add 40 µL of sample and incubate for 24 h in 37°C/5% CO_2 incubator.

7. At the end of the incubation, allow the plate to equilibrate to room temperature before proceeding to fluorescence measurement.

8. Prepare Substrate Loading Solution (see Table 3). This solution is stable during 12 h.

9. Add 40 µL of Substrate Loading Solution, mix and incubate at room temperature for 2 h.

10. Read blue fluorescence: excitation (~ 410 nm) and emission (~450 nm); and green fluorescence: excitation (~ 410 nm) and emission (~520 nm).

11. Calculate Response Ratio for positive and negative controls and experimental samples, following manufacturer's instructions (LiveBLAzer™ FRET- B/G Loading Kit, Invitrogen, Carlsbad, CA, USA).

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Table 3. Preparation of Substrate Loading Solution

Solution A*	6 µL
Solution B*	60 µL
Solution C*	934 µL
Final Volume	1000 µL

*Solutions from LiveBLAzer™ FRET- B/G Loading Kit (Invitrogen, Carlsbad, CA, USA)

Materials and methods

Protein purification

Protein purification was performed with Pierce Crosslinking Magnetic IP/Co-IP Kit (Thermo Scientific, USA). Melatonin antibody rabbit IgG was purchased from LifeSpan BioSciences (Seattle, WA, USA).

Table 4. Solutions used for protein purification

Lysis Buffer	25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, 5% glycerol, pH 7.4
20x Coupling Buffer (diluted)	10 mM sodium phosphate, 150 mM NaCl, pH 7.2
Disuccinimidyl suberate (DSS)	2 mg
Neutralization Buffer	Provided by manufacturer's (pH 8.5)
Elution Buffer	Provided by manufacturer's (pH 2.0)

Binding of Antibody to Protein A/G Magnetic Beads

1. Prepare 2 mL of 1x Modified Coupling Buffer for each immunoprecipitation by diluting 100 µL of 20x Coupling Buffer and 100 µL of Lysis Buffer with 1.8 mL of MQ water.

2. Prepare antibody solution by diluting 1:20 with 20x Coupling Buffer and Lysis Buffer in order to have a final concentration of antibody of 2-10 µg. To prepare 100 µL of antibody solution: mix 10 µL of antibody, 5 µL of 20x of Coupling Buffer, 5 µL of Lysis Buffer and 80 µL of MQ water.

3. Add 25 µL of beads into a microcentrifuge tube and place it on a magnetic stand to collect beads for 1 min. Discard the storage solution.

4. Add 500 µL of 1x Modified Coupling Buffer, gentle mix and incubate 1 min at room temperature on a rotating platform. Collect the beads on a magnetic stand and discard the supernatant. Repeat this step one time.

5. Add 100 µL of antibody solution to the beads, gentle mix and incubate on a rotating platform for 15 min (minimum) at room temperature. Gentle vortex the beads every 5-10 min during incubation.

6. Collect the beads with a magnetic stand and discard the supernatant. Save the flow-through to verify antibody binding.

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7. Add 100 µL of 1x Modified Coupling Buffer and vortex. Collect the beads with a magnetic stand and discard the supernatant.

8. Add 300 µL of 1x Modified Coupling Buffer and vortex. Collect the beads with a magnetic stand and discard the supernatant. Repeat this step one time.

Crosslinking of the antibody

If crosslinking is omitted, the antibody will co-elute with the antigen during the elution steps.

1. Prepare the DSS crosslinker by adding 217 µL of DMSO to a single tube of DSS. Dilute the DSS 1:100 in DMSO to make 0.25 mM DSS.

2. Prepare the crosslinking solution: add 2.5 µL of 20x Coupling Buffer, 4 µL of 0.25 mM DSS and 43.5 µL of MQ water to the beads.

3. Incubate the crosslinking reaction for 30 min at room temperature on a rotator. Vortex the beads every 10 min during incubation to ensure that the beads stay in suspension.

4. Collect the beads with a magnetic stand. Remove and save the flow-through to verify antibody crosslinking.

5. Add 100 µL of Elution Buffer to the beads and mix for 5 min at room temperature on a rotating platform to remove non-crosslinked antibody and quench the crosslinking reaction. Collect the beads with a magnetic stand and discard the supernatant.

6. Add 100 µL of Elution Buffer to the beads and vortex. Collect the beads with a magnetic stand and discard the supernatant. Repeat one time.

7. Add 200 µL of Lysis Buffer to the beads and vortex. Collect the beads with a magnetic stand and discard the supernatant. Repeat one time.

8. Proceed to the immunoprecipitation. The antibody-crosslinked beads can be stored at 4ºC.

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Lysis of yeast samples

To obtain crude cell extract for Magnetic beads purification:

1. Centrifuge at 7800 rpm for 5 min.

2. Wash cells once by suspending the cell pellet in MQ water. Centrifuge at 10000 rpm for 5 min. Samples need to be stored at -20ºC until purification analysis.

3. Add 500 µL of ice-cold Lysis Buffer to the cell pellet and transfer to a new tube with 500 µL of glass beads.

4. Rupture the cells by glass bead beating for 1 min in Mini BeadBeater-24 (bioSpec Products, Bartlesville, OK, USA)

5. Incubate 1 min on ice.

6. Repeat five times (5 shaking cycle times).

7. Centrifuge lysed cells at 13000 rpm for 10 min at 4ºC.

8. Transfer supernatant to a new tube for protein concentration determination and immunoprecipitation.

Immunoprecipitation

1. Add the lysate solution to the tube containing crosslinked magnetic beads and incubate for 1 h at room temperature or overnight at 4ºC on a rotator.

2. Collect the beads with a magnetic stand, remove the unbound sample and save for analysis.

3. Add 500 µL of Lysis Buffer to the tube and mix. Collect the beads and discard the supernatant. Repeat this step one time.

4. Add 500 µL of MQ water to the tube and mix. Collect the beads and discard the supernatant. Repeat this step one time.

5. Add 50 µL of Elution Buffer to the tube. Incubate for 5 min at room temperature on a rotator. Collect the beads and save the supernatant containing the target antigen. To neutralize the low pH add 5 µL of Neutralization Buffer for each 50 µL of eluate. Repeat this elution one time.

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Methods for working with proteins

Quantitative determination of protein content

Protein quantification of immunoprecipitated samples was carried out by using the Bradford protein assay. Method is based on the shift of absorbance in the dye Coomassie Brilliant Blue G-250 (absorbs at 595 nm), when the previously red form, Coomassie reagent (absorbs at 465 nm), changes and stabilizes into Coomassie blue.

1. Mix 1 µL of purified protein and 800 µL of Bradford Reagent (Sigma Aldrich, St. Louis, MO, USA) in a cuvette.
2. Incubate for 10 min at room temperature in the dark.
3. Measure the absorbance at 595 nm in a Spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience, United Kingdom)

Prior to the measurements, a calibration curve is generated with BSA. The lysis buffer used during the protein purification served as a reference. A direct correlation between extinction and protein concentration is only given in the range of linearity of the calibration curve. Samples above this range have to be diluted MQ water.

SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) preparation and running

The purified proteins were separated by their molecular weight by SDS-PAGE. Proteins were treated with SDS (sodium dodecyl sulphate), which neutralizes and covers them with negative charges. SDS-PAGE was carried out in gel electrophoresis chambers (Bio-Rad Laboratories, Munich, Germany).

Prior running the gel, crude extract of proteins was mixed with a 2x Sample Buffer and incubated at 95ºC for 5 min. Then, an equal amount of protein was loaded in 12% SDS-PAGE gels.

SDS-PAGE was prepared in a percentage of 5% and 12% of stacking and resolving gel, respectively (see Table 5). After mixing all components in a 50-mL tube, the resolving gel was poured between two glasses, which were previously cleaned with 70% ethanol and inserted into a stand. Isopropanol (Panreac Applichem, Barcelona, Spain) was poured on the top of the resolving gel to level the gel edge. After the resolving gel polymerized, isopropanol was decanted and all components for the stacking gel were mixed. SDS-PAGE gels were running for 2-3 h at 100V.

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Table 5. Solutions for SDS-PAGE

Ammonium Persulphate (APS)	10% (w/v) in MQ water
Acrylamide 37:5:1	Provided by Sigma Aldrich (St. Louis, MO, USA)
N, N, N', N'-Tetramethylene ethylene diamine (TEMED)	Provided by Bio-Rad Laboratories (Munich, Germany)
Resolving gel stock solution	1.5 M Tris-HCl, pH 8.8
Stacking gel stock solution	0.5 M Tris-HCl, pH 6.8
SDS	10% (w/v) in MQ water
Electrophoresis buffer (5x)*	0.025 M Tris Base, 192 mM Glycine, 0.1% SDS
Sample Buffer (5x)	300 mM Tris-HCl, 1% SDS, 50% glycerol, 0.05% bromophenol blue

*Dilute 200 mL of 5x stock in 800 mL of MQ water

Table 6. Composition of SDS-PAGE used in this study

	Stacking gel (4 mL)	Resolving gel (12mL)
Acrylamide	500 µL	3.6 mL
Resolving gel stock solution	-	3 mL
Stacking gel stock solution	1 mL	-
MQ water	2.5 mL	5.20 mL
SDS	40 µL	120 µL
TEMED	7 μL	6.5 µL
APS	40 µL	120 µL

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SDS-PAGE Stain

After running, the SDS-PAGE gel was stained with silver (Pierce Silver Statin Kit, Thermo Scientific, Waltham, MA, USA).

1. Wash the gel in MQ water for 5 min at room temperature. Repeat this step one time.

2. Fix the gel with Fixing Solution (30% (w/v) Ethanol, 10% (w/v) acetic acid) for 15 min at room temperature. Repeat this step one time. Gel may be kept in fixing solution overnight without affecting stain performance.

3. Wash the gel in 10% Ethanol for 5 min at room temperature. Repeat this step one time.

4. Wash the gel in MQ water for 5 min at room temperature. Repeat this step one time.

5. Prepare Sensitizer Working Solution by adding 50 µL of **Sensitizer** in 25 mL MQ water.

6. Incubate gel in Sensitizer Working Solution for exactly 1 min. Then, wash with two changes of MQ water for 1 min each.

7. Prepare Stain Working Solution by adding 500 µL of **Enhancer** in 25 mL **Stain**.

8. Incubate gel in Stain Working Solution for, minimum, 30 min.

9. Prepare Developer Working Solution by mixing 50 µL of **Enhancer** in 25 mL **Developer**.

10. Wash gel with two changes of MQ water for 20 seconds each.

11. Add Developer Working Solution and incubate until protein bands appear.

12. When the desired band intensity is reached, replace Developer Solution with 5% Acetic Acid. Wash gel briefly, replace with fresh solution and incubate for 10 min.

Reagents and components provided by the commercial kit are indicated in bold.

Materials and methods

Molecular techniques

RFLP-PCR of 5.8S-ITS- rDNA analysis

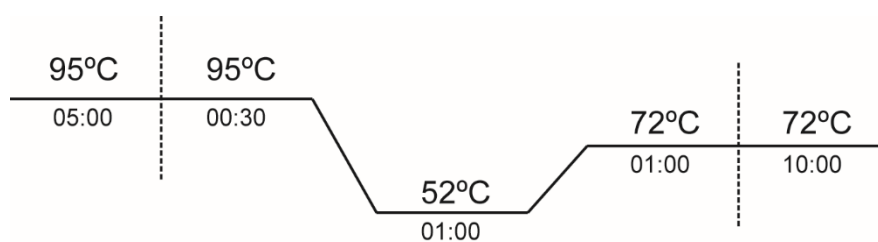For yeast identification, the protocol used was based on Esteve-Zarzoso et al. (1999)[2].

Table 7. Primers used

Primer	Sequence
ITS1	5'- TCCGTAGGTGAACCTGCGG-3'
ITS4	5'- TCCTCCGCTTATTGATATGC-3'

Table 8. PCR mix per reaction

Buffer 10X without Mg^{2+}	5 µL
MgCl$_2$	3 µL
Primer ITS1 (10 µM)	1 µL
Primer ITS4 (10 µM)	1 µL
dNTPs (10 mM each dNTP)	1 µL
Taq DNA polymerase	2.5 U
DNA	1 µL
MQ water	q.s.p. 50 µL

PCR program:

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To visualize PCR products, a 1.5 % (w/v) agarose gel electrophoresis containing ethidium bromide was used. The restriction of the PCR products with *Hinf*I, *Hae*III or *Cfo*I was performed with the following mixture (see Table 9). The restriction PCR mixture was incubated at 37°C and the visualization of the restriction profiles was performed in a 3% (w/v) agarose gel electrophoresis containing ethidium bromide.

Table 9. Restriction PCR product per reaction

Buffer (specific for each restriction enzyme)	2 µL
Restriction enzyme	1 µL
PCR product	10 µL
MQ water	7 µL

[2] Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.85 rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.

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Annex II
Other publications derived from this thesis

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The production of aromatic alcohols in non-*Saccharomyces* wine yeast is modulated by nutrient availability

by Beatriz González, Jennifer Vázquez, María Ángeles Morcillo-Parra, Albert Mas, María Jesús Torija† and Gemma Beltran

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Aromatic alcohols production

The production of aromatic alcohols in non-*Saccharomyces* wine yeast is modulated by nutrient availability

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ABSTRACT

Aromatic alcohols (tryptophol, phenylethanol, tyrosol) positively contribute to organoleptic characteristics of wines, and are also described as bioactive compounds and *quorum sensing* molecules. These alcohols are produced by yeast during alcoholic fermentation via the Erhlich pathway, although in non-*Saccharomyces* this production has been poorly studied. We studied how different wine yeast species modulate the synthesis patterns of aromatic alcohol production depending on glucose, nitrogen and aromatic amino acid availability. Nitrogen limitation strongly promoted the production of aromatic alcohols in all strains, whereas low glucose generally inhibited it. Increased aromatic amino acid concentrations stimulated the production of aromatic alcohols in all of the strains and conditions tested. Thus, there was a clear association between the nutrient conditions and production of aromatic alcohols in most of the wine yeast species analysed. Additionally, the synthesis pattern of these alcohols has been evaluated for the first time in *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Starmellera bacillaris*.

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1. Introduction

S. cerevisiae is a model microorganism used in many industrial applications due to its ability to synthesize ethanol from diverse sugar sources. For this reason, this budding yeast is commonly employed in several technological processes such as brewing, baking and winemaking. In the latter, alcoholic fermentation involves the succession and coexistence of a large diversity of yeast genera and species. At the beginning of fermentation, non-*Saccharomyces* yeast populations are high, and genera such as *Hanseniaspora, Issatchenkia, Starmerella (sym. Candida), Torulaspora, Metschnikowia* are commonly found. During fermentation, non-*Saccharomyces* species are replaced by *S. cerevisiae* due to its superior performance during this process and its ability to produce high ethanol concentrations (Fleet, 2003; Heard and Fleet, 1988). However, several studies recently confirmed the positive contributions of these non-*Saccharomyces* yeasts to wine: they are able to produce certain additional aromatic compounds that improve the flavour and bouquet (Fleet, 2003; Jolly et al., 2014; Lleixà et al.,

2016). Although ethanol is the major by-product of alcoholic fermentation, yeasts also produce other alcohols such as aromatic alcohols derived from the aromatic amino acids that are present in grapes. Tyrosine (Tyr), tryptophan (Trp) and 2-phenylalanine (Phe) are converted to tyrosol (TyrOH), tryptophol (TrpOH) and 2-phenylethanol (PheOH), respectively, via yeast metabolism through the Ehrlich pathway, particularly under nitrogen-limiting conditions (Ehrlich, 1907; Hazelwood et al., 2008). Those alcohols are largely used as additives in foods and beverages, and PheOH specifically is widely used in the cosmetics industry due to its rose-like scent (Fabre et al., 1998). Fusel alcohols positively influence the flavour and bouquet of wines and are also of interest due to their potential bioactivity for humans. TyrOH and TrpOH have been described as an antioxidant and a sleep inducer, respectively (Cornford et al., 1981; Giovannini et al., 1999). Moreover, recent studies in yeasts have demonstrated that these three aromatic alcohols are involved in growth regulation (Avbelj et al., 2015; Zupan et al., 2013) and are suggested to be quorum-sensing molecules (Dickinson, 1996; Lorenz et al., 2000). Many microbes use quorum-sensing communication to transmit information about population density and environmental conditions (Bassler, 2002; Fuqua et al., 1994); in yeast, filamentous growth has also been associated with these quorum-sensing molecules (Hornby et al., 2001; Kruppa,

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2009; Sprague and Winans, 2006).

In some yeast species, such as *S. cerevisiae*, the aromatic alcohols secreted into the extracellular medium are recognized by other cells and modulate their behaviour accordingly. Moreover, as mentioned above, nitrogen-limiting conditions increase the production of aromatic alcohols, leading to stronger filamentous growth in *S. cerevisiae*. In a recent study (González et al., 2017), we studied the behaviour of a collection of *S. cerevisiae* strains in the context of filamentous growth in the presence of different alcohols and observed higher effects for ethanol than for aromatic alcohols in terms of its ability to promote stronger filamentous growth under nitrogen-limiting conditions. On the other hand, studies performed with non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Pichia kudriavzevii* and *Pichia fabianii*, have reported that these yeasts also demonstrate changes in their growth mode under nutrient-limited conditions (nitrogen or carbon) or other stress factors (Pu et al., 2014; van Rijswijck et al., 2015). Therefore, the finding that aromatic alcohols exert different responses on morphogenesis in a yeast-dependent manner shows that these molecular signals may be species-specific (Chen and Fink, 2006).

The aim of this study was to evaluate the production of aromatic alcohols (TyrOH, TrpOH and PheOH) by four wild, non-*Saccharomyces* wine strains in different media (containing different concentrations of nitrogen and carbon) and compare them to a "model" *S. cerevisiae* wine strain.

2. Materials and methods

2.1. Yeast strains and inoculum preparation

The following wine yeast species were used in this study: commercial *Saccharomyces cerevisiae* QA23 (Lallemand, Canada) and four wild non-*Saccharomyces* isolates from the winemaking Priorat region of Spain, specifically Hv4 (CECT 13130, *Hanseniaspora uvarum*), Cz4 (CECT 13129, *Starmerella bacillaris* (sym. *Candida zemplinina*)), Mpp (CECT 13130, *Metschnikowia pulcherrima*) and Tdp (CECT 13135, *Torulaspora delbrueckii*) (Padilla et al., 2016). Yeast strains were taken from stocks preserved at $-80\,°C$ in glycerol and grown on YPD plates (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) agar) for 48–72 h at 28 °C. Then, cells were cultured for 24 h in 50 mL of YPD medium at 28 °C and 120 rpm and transferred into 250 mL of fresh minimal medium (1X Yeast Nitrogen Base without amino acids or ammonia, 2% (w/v) glucose, 10 mM $(NH_4)_2SO_4$) and cultured for 2 days at 28 °C and 120 rpm.

2.2. Fermentation conditions

Fermentation was performed in three different media based on synthetic must (Beltran et al., 2004), containing modified sugar and nitrogen concentrations: standard synthetic must (SM: 200 g/l sugars –100 g/L glucose and 100 g/L fructose- and 300 mg of N/L for yeast assimilable nitrogen (YAN)), low glucose must (LGM: 20 g of glucose/L and 300 mg of N/L for YAN) and low nitrogen must (LNM: 200 g of sugars/L –100 g/L glucose and 100 g/L fructose- and 100 mg of N/L for YAN) (Table 1). Fermentations were initiated by inoculating precultures in minimal medium to obtain an initial population of 2×10^6 cells/mL. To study the production of aromatic alcohols (TyrOH, TrpOH and PheOH) in each must, aromatic amino acids (Tyr, Trp and Phe), which are precursors of those alcohols, were added at two concentrations, standard concentration, based on the regular concentrations of aromatic amino acids in musts (1x) (Beltran et al., 2004), and five-fold increased (5x), see Table 1. The increased concentrations of aromatic amino acids occurred at the expense of the remaining amino acids to maintain the same final

concentration of nitrogen. Fermentations were performed in triplicate at 28 °C with continuous orbital shaking (120 rpm). Cell populations were evaluated daily by measuring the optical density (OD_{600nm}), and 1 mL of wine was stored to quantify the extracellular concentrations of TyrOH, TrpOH and PheOH by UHPLC after five days of fermentation for LGM and after seven days for SM and LNM.

Another set of alcoholic fermentations in SM and 5x aromatic amino acids was performed to associate the synthesis and secretion of aromatic alcohols with growth phases. Then, intracellular and extracellular concentrations of aromatic alcohols were analysed during the first 48 h (0 h, 12 h, 24 h, 48 h).

2.3. Aromatic alcohol analysis

Aromatic alcohols were detected and quantified by performing liquid chromatography triple quadrupole mass spectrometry (LCQqQ). For intracellular samples, prior extraction using boiling buffered ethanol (Gonzalez et al., 1997) was necessary. Briefly, a volume of culture corresponding to 10^7 cells was centrifuged at 5000 rpm for 10 min, and 1 mL of boiling absolute ethanol buffered with 2 mL of 1 M Hepes (pH 7.5) was added directly into the tube. Subsequently, the mix was incubated for 3 min at 80 °C, and the supernatant was evaporated at 45 °C using a SpeedVac (Thermo Fisher Scientific, USA). The residue was resuspended in 1 mL of sterile MilliQ water. Then, 100 μL of this extract or of the extracellular sample was diluted 1:10 in MeOH ($\geq$99.7% (LC-MS), Sigma Aldrich, USA), centrifuged at 15 000 rpm and maintained at 4 °C for 10 min. Samples were then serially diluted in MeOH to a final dilution of 1:100 for intracellular samples and 1:1000 for extracellular samples, and analysed by LCQqQ.

A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, USA) was used to evaluate TyrOH, TrpOH and PheOH. The chromatographic column was an Xbridge Shield RP18 (150 × 2.1 mm i.d. 3.5-μm particle size) (Waters). The mobile phases were 0.2% acetic acid ($\geq$99.7%, Sigma-Aldrich, USA) in water (MilliQ system, Millipore) (solvent A) and ACN (solvent B). The flow rate was 0.6 mL/min. To validate our quantitative method, calibration curves, linearity, precision, accuracy and the limits of detection and quantification were determined by analysing serial standard dilutions prepared in ultrapure LC-MS water and pooled samples spiked with standard solutions. The obtained validation parameters for the method for TyrOH ($\geq$99% Sigma-Aldrich, USA), PheOH ($\geq$98%, Sigma-Aldrich, USA) and TrpOH ($\geq$99%, Sigma-Aldrich, USA), permitted the quantification of studied compounds in extract samples.

2.4. Aromatic amino acid analysis

Final concentrations of Tyr, Phe and Trp were determined using an Agilent 1100 Series High-performance Liquid Chromatograph (Agilent Technologies, Germany) (Gómez-Alonso et al., 2007). Fifty microlitres from each sample was used in a derivatization reaction with diethyl ethoxymethylenemalonate (DEEMM). Separation was performed in an ACE HPLC column (C18-HL) with a particle size of 5 μm (250 × 4.6 mm) that was controlled thermostatically at 20 °C. Two eluents were used: eluent A contained 25 mM acetate buffer (pH = 5.8) with 0.02% sodium azide, and eluent B contained an 80:20 mixture of acetonitrile and methanol. The flow rate was 0.9 mL/min. The concentration of each compound was calculated using internal (L-2-aminoadipic acids, 1 g/l) and external standards.

2.5. Statistical analysis

Three biological replicates were performed for each experiment.

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Table 1
Reducing sugar, nitrogen and aromatic amino acid contents of the different synthetic musts used in this study.

Synthetic Must		[Aromatic amino acid]	Sugar concentration (g/L)		Nitrogen concentration			
					Aromatic amino acid (g/l)			Yeast assimilable nitrogen (YAN-mg nitrogen/L)
			Glucose	Fructose	Tyrosine	Tryptophan	Phenylalanine	aaa + NH₄Cl
Synthetic Must (standard)	(SM)	1x	100	100	0.020	0.174	0.038	300
		5x	100	100	0.098	0.871	0.189	300
Synthetic Low Glucose Must	(LGM)	1x	20	0	0.020	0.174	0.038	300
		5x	20	0	0.098	0.871	0.189	300
Synthetic Low Nitrogen Must	(LNM)	1x	100	100	0.007	0.058	0.013	100
		5x	100	100	0.033	0.290	0.063	100

Tukey's test was used to generate p-values. To better understand how different measured parameters influenced the production of aromatic alcohols, Principal Component Analysis (PCA) was performed using XLSTAT Software.

3. Results

3.1. Production of aromatic alcohols by different wine yeast species

In addition to aromatic alcohols, residual sugar and maximal growth were determined at the end of fermentation (Table 2). In LGM, all sugars were consumed by yeast. At high sugar concentrations (SM and LNM), only *S. cerevisiae* was able to consume all sugars present in the must, followed by *T. delbrueckii*. High sugar concentrations were present at the end of the process for the other three species, particularly under nitrogen restriction (LNM). In general, the increase in aromatic alcohol precursors affected the maximal growth and sugar consumption.

In SM, all strains tested were able to produce the three aromatic alcohols, although *S. bacillaris* produced much lower levels (Fig. 1A). Under the 1x condition, *S. cerevisiae* QA23 exhibited the maximum production of aromatic alcohols, together with the highest growth and total consumption of sugars (Table 2). TrpOH was the primary aromatic alcohol produced, followed by PheOH and TyrOH. Notably, synthetic must contains higher concentrations of Trp compared to Phe or Tyr (see Table 1). On the other hand, non-*Saccharomyces* species synthetized lower levels of aromatic alcohols; specifically, *S. bacillaris* produced the lowest concentrations (below 5 μM TyrOH and PheOH, and no detectable TrpOH). Among non-*Saccharomyces* species, *T. delbrueckii* presented the highest growth and sugar

Table 2
Maximal growth and sugar consumption achieved by each yeast species and in each medium. The results are expressed as the average; the standard deviation (SD) was calculated from three biological replicates. Statistical significance was calculated by comparing three different musts within the same strain; letters indicate significant differences (p-value < 0.05).

| Yeast strain	Must	[Aromatic amino acid]	Maximal growth		Consumed Sugar	
			OD₆₀₀ ₙₘ	SD	g/l	SD
S. cerevisiae QA23	SM	1x	11.47[c]	1.32	199.95[b]	0.00
		5x	9.22[c]	1.01	194.60[b]	1.60
	LNM	1x	7.64[b]	1.66	198.58[b]	1.00
		5x	7.25[b]	0.97	197.26[b]	1.75
	LGM	1x	4.80[a]	0.37	19.97[a]	0.04
		5x	4.20[a]	0.31	19.49[a]	0.35
H. uvarum Hu4	SM	1x	4.72[c]	0.14	145.40[c]	10.23
		5x	4.33[c]	0.24	136.75[c]	3.42
	LNM	1x	1.95[b]	0.38	78.43[b]	6.45
		5x	2.29[b]	0.36	81.55[b]	6.00
	LGM	1x	3.89[a]	0.46	19.93[a]	0.11
		5x	3.76[a]	0.12	19.90[a]	0.00
S. bacillaris Cz4	SM	1x	3.65[a]	0.33	123.66[c]	3.49
		5x	4.44[c]	0.25	127.40[c]	2.59
	LNM	1x	2.45[b]	0.35	71.85[b]	2.36
		5x	2.71[b]	0.32	81.35[b]	8.61
	LGM	1x	3.74[a]	0.16	19.913[a]	0.12
		5x	2.38[b]	0.39	19.653[a]	0.26
M. pulcherrima Mpp	SM	1x	3.67[b]	1.08	106.18[d]	0.06
		5x	6.57[c]	0.65	100.20[d]	7.80
	LNM	1x	3.36[b]	0.38	37.66[b]	4.88
		5x	5.36[a]	1.02	70.39[c]	15.29
	LGM	1x	4.49[a]	0.56	19.99[a]	0.00
		5x	5.72[a]	0.20	19.82[a]	0.26
T. delbrueckii Tdp	SM	1x	10.31[c]	0.40	176.34[c]	2.72
		5x	8.35[c]	1.58	177.82[c]	2.00
	LNM	1x	5.95[b]	0.66	107.81[b]	6.03
		5x	5.00[b]	0.79	93.03[b]	10.89
	LGM	1x	3.06[a]	0.50	19.99[a]	0.00
		5x	4.28[d]	0.08	19.95[a]	0.04

Aromatic alcohols production
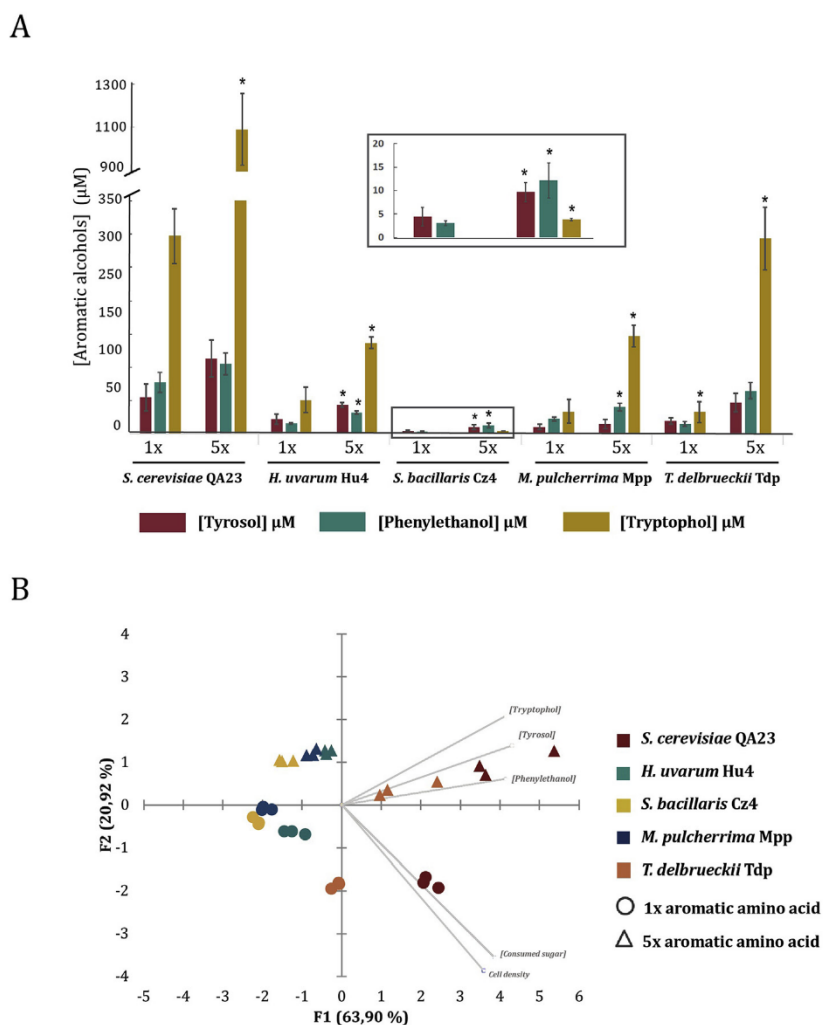
Fig. 1. Production of aromatic alcohols by different yeast species in standard must (SM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in SM (300 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05. **(B)** Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation.

consumption (although more than 20 g/l residual sugars was present after seven days of fermentation), *S. bacillaris* demonstrated the lowest growth, and *M. pulcherrima* demonstrated the lowest sugar consumption.

Higher concentrations of aromatic amino acids in the media (5x) resulted in higher production of aromatic alcohols, despite the lack of proportional increases in alcohols with increases in precursors (Fig. 1A). As observed in 1x condition, TrpOH was the major aromatic alcohol produced with the exception of *S. bacillaris,* which synthetized more PheOH, but at much lower levels. Moreover, although the differences were not statistically significant, the presence of higher concentrations of aromatic amino acids affected cell growth in all species: growth was lower for *S. cerevisiae,* *H. uvarum,* and *T. delbrueckii* but higher for *S. bacillaris* and *M. pulcherrima* (Table 2).

To better understand how different variables affected the strains, PCA was performed (Fig. 1B). This PCA analysis accounted for 90.17% of the variance: all variables were primarily explained by

component F1 (74.86%). Triplicates appeared together in the biplot, and PCA clearly separated *S. cerevisiae* and *T. delbrueckii* from the other species, which grouped together, primarily due to their superior growth and higher consumption of sugars. In those strains, samples from media containing different aromatic amino acid contents grouped separately based on the higher production of aromatic alcohols under the 5x condition. Two additional PCA were performed using only the data from the 1x and 5x conditions (Fig. S1). This PCA explains 92.23% and 91.7% of the variance under the 1x and 5x conditions, respectively. PCA generated three clusters: *S. cerevisiae,* *T. delbrueckii,* and the rest of the non-*Saccharomyces* strains.

3.2. Synthesis of aromatic alcohols under low nitrogen conditions

To analyse the effects of nitrogen concentration on the synthesis of aromatic alcohols, fermentations were carried out using low nitrogen synthetic must (100 mg of N/L) at two different

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concentrations of aromatic amino acids (1x and 5x).

In LNM, all strains grew poorly and, in general, fermented less sugar than in SM (Table 2), with the exception of *S. cerevisiae*, which consumed practically all sugars present in the media despite nitrogen limitation. As in SM, TrpOH was the main fusel alcohol synthesized, except in *S. bacillaris*. This strain did not secrete TrpOH under 1x condition and produced the other aromatic alcohols at only very low concentrations, despite consuming almost all of the precursors. The second aromatic alcohol most frequently secreted by non-*Saccharomyces* species was TyrOH, unlike *Saccharomyces*, which secreted PheOH (Fig. 2).

When the precursors of these aromatic alcohols were five-fold higher, growth and sugar consumption during fermentation were not significantly affected, with the exception of Mpp. In this case, higher concentrations of aromatic amino acids in the medium resulted in a clear increase in growth, and sugar consumption was doubled in comparison to 1x condition. In terms of the production of aromatic alcohols, a significant increase in their concentrations was observed after the addition of precursors. However, TrpOH was

the most relevant because the concentration of this alcohol was between 6 and 24 times greater than that under the 1x condition. In both cases, aromatic amino acids were practically exhausted, but production rates were higher under the 5x condition (Table 3 and Supplementary Table S1 for 5X).

PCA (Fig. 2B) indicated a variance of 87.29% (F1: 56.53%; F2:27.76%), and the variables were primarily explained by component 1. *S. cerevisiae* was clearly separated from non-*Saccharomyces* species largely due to its ability to generate a higher biomass and its elevated sugar consumption. Moreover, within this strain, samples from media containing different aromatic amino acid contents grouped separately due to the higher production of TrpOH and PheOH in 5x medium. In general, non-*Saccharomyces* species were also separated based on the concentration of aromatic amino acids (1x and 5x), primarily due to the higher amounts of TrpOH and TyrOH under the 5x condition, with the exception of the *S. bacillaris* Cz4 strain, which presented similar concentrations under both conditions.

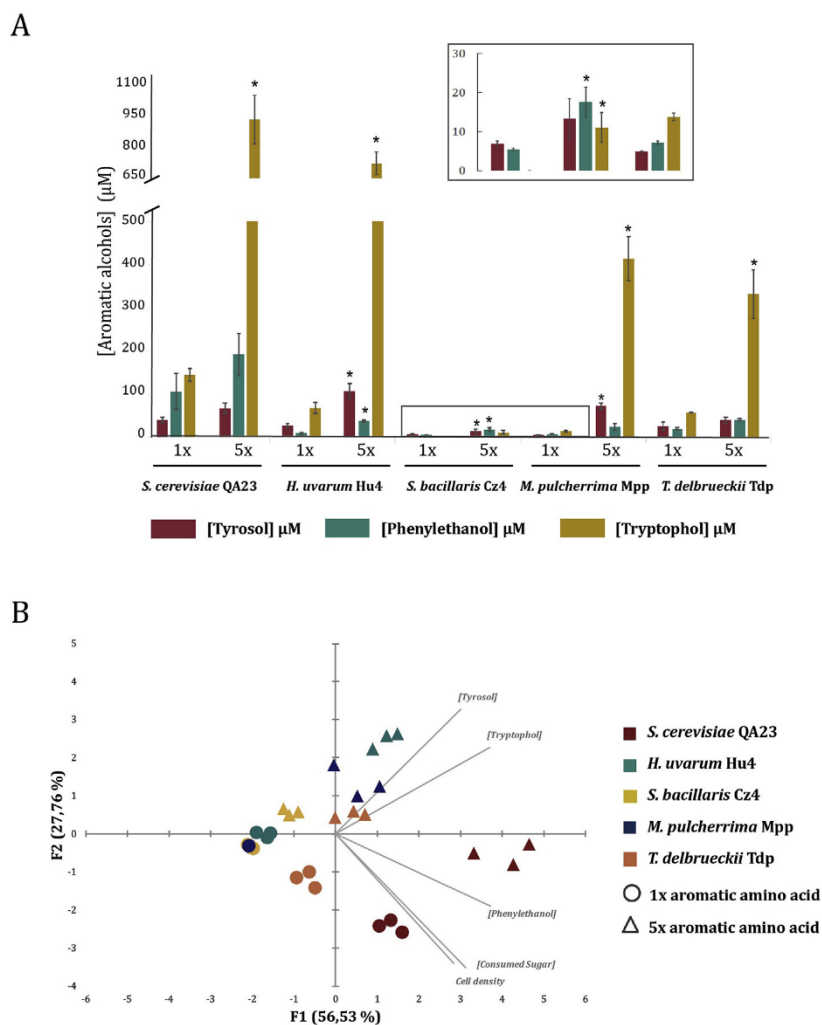LNM medium contained the same amount of sugars as SM, but

Fig. 2. Production of aromatic alcohols by different yeast species under nitrogen limitation (LNM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LNM (100 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x). (B) Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

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Table 3
Production yield of aromatic alcohols in SM and LNM. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account three biological replicates. In all cases, comparisons are within the same species; letters indicate significant differences (p-value < 0.05).

Yeast strain	Must	[TyrOH]/[Tyr]		[PheOH]/[Phe]		[TrpOH]/[Trp]	
		(μM/μM)	SD	(μM/μM)	SD	(μM/μM)	SD
S. cerevisae	SM	0.50^a	0.19	0.33^a	0.09	0.34^a	0.08
QA23	LNM	1.01^b	0.18	1.03^b	0.06	0.50^b	0.05
H. uvarum	SM	0.20^a	0.07	0.06^a	0.00	0.05^a	0.03
Hu4	LNM	0.72^b	0.14	0.10^b	0.03	0.23^b	0.05
S. bacillaris	SM	0.04^a	0.01	0.01^a	0.00	ND	—
Cz4	LNM	0.19^b	0.02	0.07^b	0.00	ND	—
M. pulcherrima Mpp	SM	0.09^a	0.04	0.09^a	0.01	0.03^a	0.02
	LNM	0.13^b	0.01	0.09^a	0.01	0.04^a	0.00
T. delbrueckii	SM	0.17^a	0.04	0.06^a	0.02	0.03^a	0.02
Tdp	LNM	0.71^b	0.26	0.25^b	0.04	0.19^b	0.00

the concentrations of YAN and aromatic amino acids were three-fold lower. Despite these lower amounts of precursors, the production of aromatic alcohols was similar in both media, indicating a higher yield of alcohol in LNM. Thus, the ratio between each aromatic amino acid and its respective aromatic alcohol was calculated (Table 3). For all strains, with the exception of *M. pulcherrima*, this rate of transformation was significantly higher in LNM than in SM, explaining why the final concentration of aromatic alcohols was similar despite the presence of fewer precursors. In *S. cerevisiae* QA23 grown in LNM under the 1x condition, all Tyr and Phe present in the medium were converted into their corresponding alcohols (ratios of approximately 1). In non-*Saccharomyces* species, the largest increase in this ratio occurred for TrpOH and TyrOH, corresponding to the higher production of these two aromatic alcohols by non-*Saccharomyces* species as opposed to *S. cerevisiae*, which synthesized more PheOH than TyrOH. When a PCA was performed using the data from SM and LNM, 90.21% of the variance was explained (F1: 50.20%, F2: 40.01%), being the amount of aromatic amino acid consumed and the ratio Tyr/TyrOH mainly explained by F1, and the rest of variables by F2. The uptake of aromatic amino acids was negatively correlated with alcohol transformation ratios, confirming that nitrogen limitation resulted in higher aromatic alcohol production ratios. Due to the differences in aromatic amino acid uptake, as well as growth and sugar consumption, the PCA clearly separated both media, and the latter two variables, together with aromatic alcohol production, were responsible for *Saccharomyces* forming a separate cluster versus non-*Saccharomyces* species.

3.3. Synthesis of aromatic alcohols at low sugar conditions

To analyse the effects of sugar concentration on aromatic alcohol synthesis, the same experiment was performed using a medium containing the same nitrogen concentration as SM but with low glucose content (20 g/l). All species depleted the sugars in LGM media (Table 2), and all were able to produce the three aromatic alcohols (Fig. 3) but at much lower concentrations than those obtained with SM or LNM. Increased amounts of precursors (5x condition) also induced a significant increase in aromatic alcohol production for all species, particularly *T. delbrueckii,* which produced almost no aromatic alcohols under the 1x condition but exhibited higher synthesis of aromatic alcohols under the 5x condition, as well as significantly better growth (Table 2). Interestingly, in this medium, *T. delbrueckii* produced PheOH as the main

aromatic alcohol, and *M. pulcherrima* produced the highest amount of TrpOH under either aromatic amino acid condition, even higher than *S. cerevisiae*. Notably, *M. pulcherrima* reached the highest cell density in this low glucose media, primarily under the 5x condition. Once again, *S. bacillaris* produced very low amounts of aromatic alcohols and only produced TrpOH under the 5x condition, despite exhibiting poor growth (Fig. 3; Table 2).

PCA accounted for a variance of 71.02% (F1: 49.30%, F2: 21.72%) (Fig. S2). All factors were primarily explained by the F1 component, with the exception of sugar consumed, which was associated with F2. In general, the 1x and 5x conditions clustered separately, but in LGM, *S. cerevisiae* was not separated from non-*Saccharomyces* species. Instead, *M. pulcherrima* grouped separately from the others due to its high growth and aromatic alcohol production, while *S. bacillaris* under the 5x condition was in the other cluster.

3.4. Synthesis and secretion of aromatic alcohols during fermentation

To study the synthesis and secretion of aromatic alcohols in different species during alcoholic fermentation and growth, fermentations in SM medium with 5x aromatic amino acids were repeated, and samples were obtained to analyse the intracellular and extracellular concentrations of these molecules during the first 48 h (Fig. 4). Initially, cells contained basal intracellular concentrations of fusel alcohols (from preculture growth), and then fusel alcohols increased considerably at 12 h, matching the early exponential phase. Then, during the period between the end of the exponential phase and the beginning of stationary phase, the intracellular concentration of aromatic alcohols decreased drastically, particularly for TrpOH and PheOH, likely due to their secretion into external media. Once in stationary phase, the intracellular levels of those alcohols maintained basal concentrations, similar to those observed at the beginning of the process. This decrease in intracellular levels coincided with the increase in aromatic alcohols observed in the extracellular medium, which slowed down when the cells were in mid-stationary phase. This profile was observed in *S. cerevisiae* (Fig. 4A), *H. uvarum* (Fig. 4B) and *M. pulcherrima* (Fig. 4D). However, in *T. delbrueckii*, PheOH demonstrated a peak at 12 h as in the other species, but TrpOH peaked at 24 h (Fig. 4E), likely due to the low population of this strain (1×10^6 cells/mL) in early exponential phase compared to the other strains ($>5 \times 10^6$ cells/mL) in mid-exponential phase. Therefore, the synthesis of PheOH coincided with early exponential phase, and that of TrpOH coincided with the mid-late exponential phase. Due to this delay in growth and thus aromatic alcohol synthesis, accumulation in the extracellular medium was also delayed, resulting in the detection of very low amounts of aromatic alcohols at 24 h outside the cells. Finally, in *S. bacillaris* (Fig. 4C), the intracellular accumulation of aromatic alcohols was very low (as expected given the low concentrations detected in the extracellular medium) and delayed due to the longer lag phase of this strain compared with the other strains. Moreover, in this strain, PheOH was the major aromatic alcohol that accumulated both inside and outside of the cell, unlike the other species.

4. Discussion

In this study, we analysed the synthesis of aromatic alcohols (TrpOH, PheOH and TyrOH), which may act as quorum-sensing molecules, by different wine yeast species during alcoholic fermentation. Additionally, we studied how the concentrations of aromatic amino acids, yeast assimilable nitrogen and sugar affected the production of these aromatic alcohols.

S. cerevisiae QA23 produced higher concentrations of aromatic

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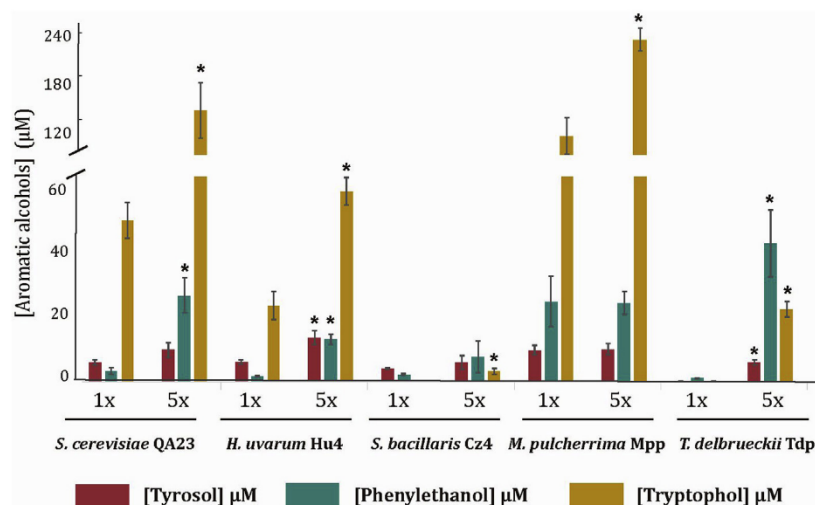
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Fig. 3. Production of aromatic alcohols by different yeast species in low glucose must (LGM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LGM (300 mg/l nitrogen and 20 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

alcohols, and its production yield was highest in must with nitrogen restriction. These findings agree with previous studies that showed an inverse correlation between initial nitrogen concentration and the production of fusel alcohols (Beltran et al., 2005; Carrau et al., 2008; Jiménez-Martí and del Olmo, 2008; Mouret et al., 2014), indicating that the closer the nitrogen concentration is to growth-limiting levels, the higher the yield of fusel alcohols. Indeed, the Ehrlich pathway may be activated to provide nitrogen from amino acids such as the aromatic amino acids, which are limited for protein synthesis and growth. Thus, higher levels of these alcohols may be a signal to modulate the regulation of yeast growth (Mas et al., 2014). Indeed, Chen and Fink (2006) reported that the production of these autosignaling alcohols is regulated by nitrogen; specifically, they are activated under nitrogen-poor conditions. On the other hand, the same authors stated that aromatic alcohol production is not affected by low concentrations of glucose; however, according to our results (Fig. 3 and Table S2), S. cerevisiae produced significantly lower concentrations of fusel alcohols in LGM than in SM, despite the same total nitrogen content in both media. There are different potential explanations for this low production. First, it may be related to low cell density (the OD$_{600nm}$ value in LGM was 4.80 ± 0.37 vs. 11.47 ± 1.32 and 7.64 ± 1.66 in SM and LNM, respectively). Additionally, cells at low density required lower protein synthesis, which may explain why cells consume fewer aromatic amino acids. Recent studies have associated the production of these alcohols with cell density, suggesting that high population density stimulates the synthesis of aromatic alcohols (Avbelj et al., 2015; Chen and Fink, 2006; Sprague and Winans, 2006; Wuster and Babu, 2009). However, in the case of low nitrogen (LNM), the production yield (with respect to the amount of precursor consumed) was higher despite a lower cell density than that in SM, indicating that cell density is not the sole variable that determines the synthesis of these alcohols. Second, the more rapid depletion of glucose in LGM may also be responsible for the low amounts of aromatic alcohols produced, as explained (Vidal et al., 2014). In their experiments, these authors observed that the accumulation of isoamyl alcohol halted after glucose exhaustion, despite the presence of residual leucine, its precursor, suggesting that leucine degradation through the Ehrlich pathway was strongly

dependent on the cytosolic availability of NADH and/or of the amino acceptor α-ketoglutarate. This was confirmed in glucose pulse experiments in the stationary phase that re-established the synthesis of the higher alcohol. Finally, differences in respiro-fermentative metabolism may also be partly responsible for this low accumulation; in C. albicans, the production of aromatic alcohols is higher under anaerobic growth than aerobic growth (Ghosh et al., 2008). In LNM and SM, the metabolism of S. cerevisiae is anaerobic due to the Crabtree effect; however, in LGM, a medium containing significantly lower amounts of sugar, S. cerevisiae metabolism is partially aerobic, which may result in decreased aromatic alcohol synthesis.

Aromatic alcohols are quantitatively the largest group of volatile components, and their presence, particularly phenylethanol, is essential for overall flavour quality. The Erhlich pathway is active in other yeast such as Kluyveromyces marxianus and Yarrowia lipolytica (Celińska et al., 2013; Fabre et al., 1998). Additionally, other non-conventional wine yeast, such as H. uvarum, Zygosaccharomyces bailii, T. pretoriensis (Zupan et al., 2013), S. bacillaris, M. pulcherrima and T. delbrueckii, which we reported here, are able to produce aromatic alcohols. However, in all cases, non-Saccharomyces species produce lower quantities than S. cerevisiae, indicating that the Erhlich pathway may not be as active in non-Saccharomyces species as in Saccharomyces under nitrogen-limiting conditions.

TrpOH was the major aromatic alcohol synthesized by most species. This may be attributable to the fact that Trp was present in higher concentrations than the other two aromatic amino acids in the media; however, the production yield of TrpOH was also higher for most species. Additionally, although Trp is a poor source of nitrogen, cells uptake this amino acid early during fermentation (Beltran et al., 2005; Henschke and Jiranek, 1993), which may favour the synthesis of TrpOH. The increase in precursors resulted in a general increase in the production of aromatic alcohols, which was consistent with previous studies (Ghosh et al., 2008; Gori et al., 2011), although the increases in alcohols were not truly proportional to the increases in precursors. Crépin et al. (2017) claimed that modulation of the production of targeted fermentative aromas was achieved by modifying the availability of exogenous amino
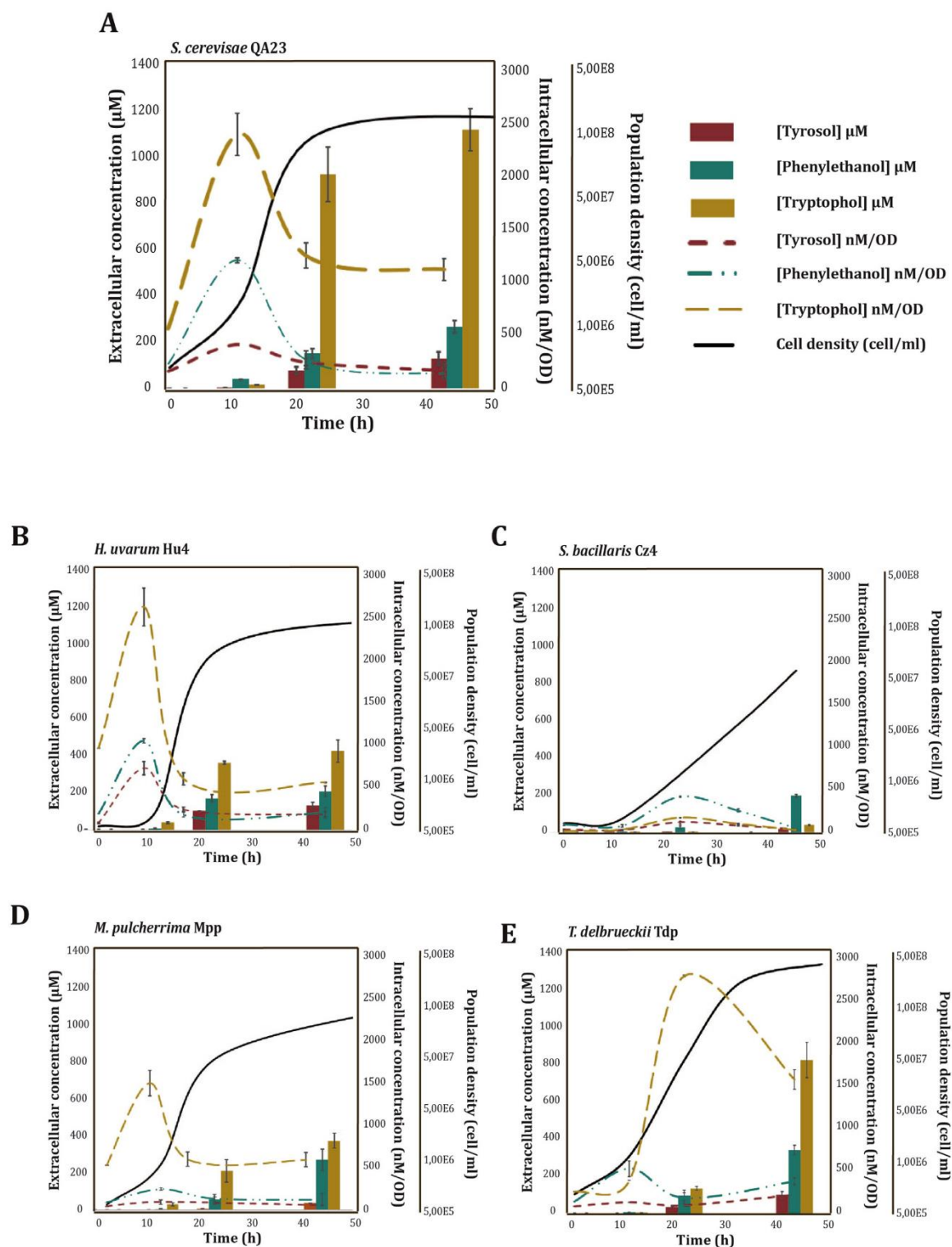
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Fig. 4. Monitoring the intra- and extracellular production of aromatic alcohols and cell populations during fermentation with different yeast species. Fermentations were carried out for 48 h at 28 °C in standard must (SM) supplemented with a five-fold increase in aromatic amino acids (5X). **(A)** *S. cerevisiae* QA23, **(B)** *H. uvarum* Hu4, **(C)** *S. bacillaris* Cz4 **(D)***M. pulcherrima* Mpp, and **(E)** *T. delbrueckii* Tdp. Aromatic alcohol concentrations are expressed as the average of biological triplicates.

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acids; the authors reported a low contribution of the carbon skeletons of consumed amino acids to the production of volatile compounds. However, these authors studied the synthesis of higher alcohols derived from branched chain amino acids, which primarily originate from sugar catabolism (Crépin et al., 2017), whereas aromatic alcohols are synthesized when their precursors are added to the medium (Ehrlich, 1907; Webb and Ingraham, 1963). Moreover, in *S. cerevisiae*, certain reactions in the aromatic amino acid biosynthesis pathway from glucose are subject to feedback inhibition (Helmstaedt et al., 2005). On the other hand, in *Saccharomyces*, the increase in alcohol precursors resulted in a lower cell density, which may be related to the high content of Trp in this medium, as the growth of *S. cerevisiae* is negatively affected when Trp is used as a unique nitrogen source (Gutiérrez et al., 2013).

The production of aromatic alcohols has been previously studied in other wine yeast species, such as *H. uvarum*, *S. bacillaris* (Sym *C. zemplinina*), *T. pretoriensis* and *Z. bailii*, in a medium similar to SM (Zupan et al., 2013). However, this production has not yet been studied in other yeast species of increasing interest for wine fermentation. Among the three aromatic alcohols, phenylethanol has been the most studied in fermentations, both in single and in coinoculated fermentations, due to its positive influence in the aroma (Belda et al., 2017; Benito et al., 2015; Gobbi et al., 2013; Parapouli et al., 2010; Sadoudi et al., 2012). In general, non-*Saccharomyces* species synthesize lower amounts of aromatic alcohols than *S. cerevisiae*. However, in different studies that have used coinoculation between non-*Saccharomyces* species and *S. cerevisiae* in fermentation, the resulting wines presented a higher contents of aromatic alcohols (Azzolini et al., 2015; Varela et al., 2016; Belda et al., 2017). The higher synthesis of these alcohols in *S. cerevisiae* may occur because non-*Saccharomyces* species differ from *S. cerevisiae* in the distribution of metabolic flux during fermentation and therefore differ in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Tóth, 2011; Milanovic et al., 2012; Tofalo et al., 2012).

Moreover, these species experience an elevated contribution of respiration to their metabolism; this is the case even for *T. delbrueckii*, which has been described as Crabtree-positive (Alves-Araujo et al., 2007). Indeed, Quirós et al. (2014) investigated the extent of respiration-fermentative metabolism in different yeast strains and reported that some species commonly found in oenological environments, such as *M. pulcherrima*, *Starmerella bombicola* and *T. delbrueckii*, respire between 40 and 100% of consumed sugar under suitable aeration conditions. Thus, an elevated respiratory metabolism may underlie lower aromatic alcohol production, as described for *C. albicans* (Ghosh et al., 2008). In fact, in this study, *C. albicans* cells grown anaerobically at 30 °C produced roughly twice as much PheOH, TrpOH and TyrOH as they do under aerobic conditions. As in *Saccharomyces*, the higher production of aromatic alcohols was detected under nitrogen-limiting conditions in non-*Saccharomyces* species. Another interesting aspect of our study is the finding that *S. bacillaris* produces all three aromatic alcohols, although at very low concentrations, unlike the observations of Zupan et al. (2013); one explanation for this may be the lower detection limits used in our method. Under the conditions used by Zupan et al. (2013), no aromatic alcohol synthesis was detected during alcoholic fermentation by *S. bacillaris*. *S. bacillaris* belonged to the *Candida* genus until 2012 (Duarte et al., 2012), and although it is currently included in another genus, it continues to share many features with *Candida*. Low levels of aromatic alcohols were secreted by *S. bacillaris*, but these were similar to the concentrations detected in *C. albicans* in SD medium (3 µM TyrOH (Chen et al., 2004)). Regarding *M. pulcherrima*, it is interesting that the synthesis of aromatic alcohols appeared to be favoured under low glucose condition. Furthermore, this species grew and underwent

significantly better fermentation when the aromatic amino acid contents were increased, unlike the other species. In a recent study performed using single nitrogen sources, Kemsawasd et al. (2015) observed that aromatic amino acids did not support well neither growth or fermentation performance in the species tested that included *M. pulcherrima*, although another strain was used. After *S. cerevisiae*, *T. delbrueckii* presents a better oenological profile; higher concentrations of aromatic alcohols in SM medium were also observed for other non-*Saccharomyces* yeasts. *T. delbrueckii* synthesized the fewest aromatic alcohols, with the exception of *S. bacillaris*, in other media containing low nitrogen and low glucose. Therefore, *T. delbrueckii* appears to be affected by nutrient limitation. Moreover, in LGM, the profile of accumulated aromatic alcohols changed, and PheOH was the major alcohol formed by *T. delbrueckii*.

When the intracellular synthesis of these aromatic alcohols was studied during SM fermentation, similar profiles were observed for *S. cerevisiae*, *H. uvarum* and *M. pulcherrima*. These yeasts synthesized aromatic alcohols during the early exponential phase, when the yeast population was increasing and the need for nitrogen was higher; therefore, the deamination and transamination of amino acids were essential steps to fulfil the nitrogen demands of cells. The transamination reaction, which is the first step in the Ehrlich pathway, is followed by decarboxylation, which is thermodynamically favoured (Henry et al., 2007). This decarboxylation pulls transamination toward complete utilization of the nitrogen-donating amino acid, resulting in the formation of the fusel alcohol or fusel acid, depending on the redox state of the cell (Hazelwood et al., 2008). We have observed that cells secrete those aromatic alcohols into the medium during the shift from exponential to stationary phase, when mechanisms required for the starvation period are induced, which is consistent with previous studies (Chen and Fink, 2006; Gori et al., 2011; Zupan et al., 2013). The secretion of these molecules at the end of the exponential phase indicates a connection to cell density, suggesting that aromatic alcohols are potential quorum-sensing molecules in these species, as previously suggested for *C. albicans*, *S. cerevisiae* and *D. hansenii* (Chen et al., 2004; Chen and Fink, 2006; Gori et al., 2011). According to Fabre et al. (1998), if Phe is added after the exponential growth phase, no bioconversion to PheOH is observed during stationary phase in *Kluyveromyces marxianus*. Compared to other yeasts investigated in this study, *T. delbrueckii* alone reached its highest levels of PheOH and TrpOH at two different times. PheOH may exert an auto-stimulatory effect on TrpOH production, as synergies between both alcohols have been observed in *S. cerevisiae* (Chen and Fink, 2006). Based on this result, *T. delbrueckii* undertakes growth phase-dependent differential regulation to synthesize these aromatic alcohols. This delay in synthesis, together with a low population during the first 12 h, also resulted in a delay in the extracellular secretion of the aromatic alcohols. In the case of *S. bacillaris*, very low intracellular accumulation and extracellular secretion were related to the deficient growth of this species.

In this study, we have demonstrated that an increase in precursor levels resulted in the higher accumulation of the resulting alcohols; therefore, these compounds may be increased under oenological conditions. Moreover, aromatic alcohols have been reported to possess quorum-sensing activity, and their effects, together with ethanol, on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). However, there is limited knowledge of the effects of these quorum-sensing molecules in non-*Saccharomyces* wine yeasts. Further studies investigating the roles of those aromatic alcohols in non-*Saccharomyces* yeast species will be necessary to understand yeast interactions and quorum-sensing mechanisms in wine yeasts.

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5. Conclusions

To summarize, we show that, in addition to the well-studied *S. cerevisae*, the Erhlich pathway is active in other yeasts that produce aromatic alcohols during alcoholic fermentation, although their regulation appears to be somewhat different than that of *S. cerevisiae*. *S. bacillaris* was the lowest producer of aromatic alcohols, whereas *S. cerevisiae* was the highest producer. Carbon and nitrogen availability as well as precursors influence the production of these alcohols; in particular, nitrogen depletion induced notable levels of aromatic alcohols. To date, the synthesis of these molecules by wine yeasts demonstrates high oenological potential, as *M. pulcherrima* and *T. delbrueckii* had not previously been evaluated. Here, these two species were able to synthesize considerable amounts of alcohols, which may regulate their growth. Aromatic alcohols also have many important biotechnological applications, and relevant concentrations of these compounds positively affect wine. In this study, we demonstrated that varying nutrient concentrations in must result in adjustments to the synthesis patterns of aromatic alcohols for all yeast species studied.

Funding sources

Acknowledgement

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.03.003.

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

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ORIGINAL PAPER

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

Edwin Fernandez-Cruz[1] · Beatriz González[2] · Sara Muñiz-Calvo[3] · María Ángeles Morcillo-Parra[2] · Ricardo Bisquert[3] · Ana Maria Troncoso[1] · Maria Carmen Garcia-Parrilla[1] · María Jesús Torija[2] · José Manuel Guillamón[3]

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 unequivocal 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-Methoxyindoles · Wine yeast

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

Edwin Fernandez-Cruz, Beatriz González and Sara Muñiz-Calvo contributed equally to this work.

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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 MLT 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 MLT 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, to ascertain the yeast metabolic origin of MLT and other related indolic compounds, we aimed to monitor the intracellular

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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 (SM). 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 to lower their detection limits, and to assess their occurrence in both 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.

Materials and methods

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).

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 $\sim 10^8$ cells/mL in salt medium (50 mM Na_2HPO_4, 0.5% NaCl, adjusted to pH 5.8 using citric acid) and incubated in Erlenmeyer flasks with orbital agitation (150 rpm) at 28 °C 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 ($\sim 10^9$ cells) was washed twice with distilled water and transferred to a microcentrifuge tube to be stored at − 80 °C until it was analysed.

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 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 fivefold 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 30 °C 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 28 °C 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 30 °C. 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).

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 80 °C and 3 min on ice. The extract was concentrated by the evaporation of the volume at 45 °C in a 5301 Concentrator plus/Vacufuge® plus (Eppendorf, Spain). The final intracellular content was resuspended in 1 mL of ultrapure milliQ water

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and centrifuged for 10 min at 5,000 g at 4 °C to remove insoluble particles. The supernatant was transferred to a new tube and stored at − 20 °C until used.

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 34 °C, 2000 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 − 18 °C until analysed.

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 × 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. 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.

Statistical analysis

All the experiments were carried out at least in triplicate. 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 indolic 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 (PCA).

Results and discussion

Intracellular indolic compounds after a tryptophan pulse to starved cells

The putative synthetic pathway of MLT in yeasts is completely unknown. 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. To highlight which metabolites significantly increased, we calculated the p values for each indolic compound and strain before and after the l-TRP pulse (Table S2). Moreover, to gain an overview, these values are represented in a heat-map as relative increases after this pulse (Fig. 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 Ehrlich 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 MLT 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

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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 1 mM L-TRP

Strain	Intracellular indolic compounds [ng/10^9 cells]		
	L-TRP	3IAA	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 $\pm$ SD of biological replicates ($n=3$)

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

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	$60.83 \text{ s} \pm 13.44$	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 $\pm$ 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 ester

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 was not 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 synthesise L-TRP EE should be done.
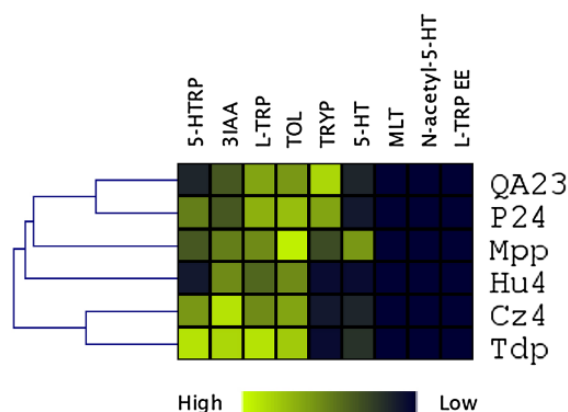
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Fig. 1 Effect of L-tryptophan (L-TRP) addition for 30 min on the intracellular production of the different indolic compounds in *Saccharomyces cerevisiae* (QA23 and P24) and non-*Saccharomyces* (Mpp, Hu4, Cz4, Tdp) starved cells

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 (Fig. 2). In this case, L-TRP and TOL represented more than 95% of the total metabolites for all the assayed conditions (Fig. 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, Fig. 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

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

Concentrations are indicated as mean ± SD of biological replicates ($n = 3$)

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 5-hydroxytryptamine, *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
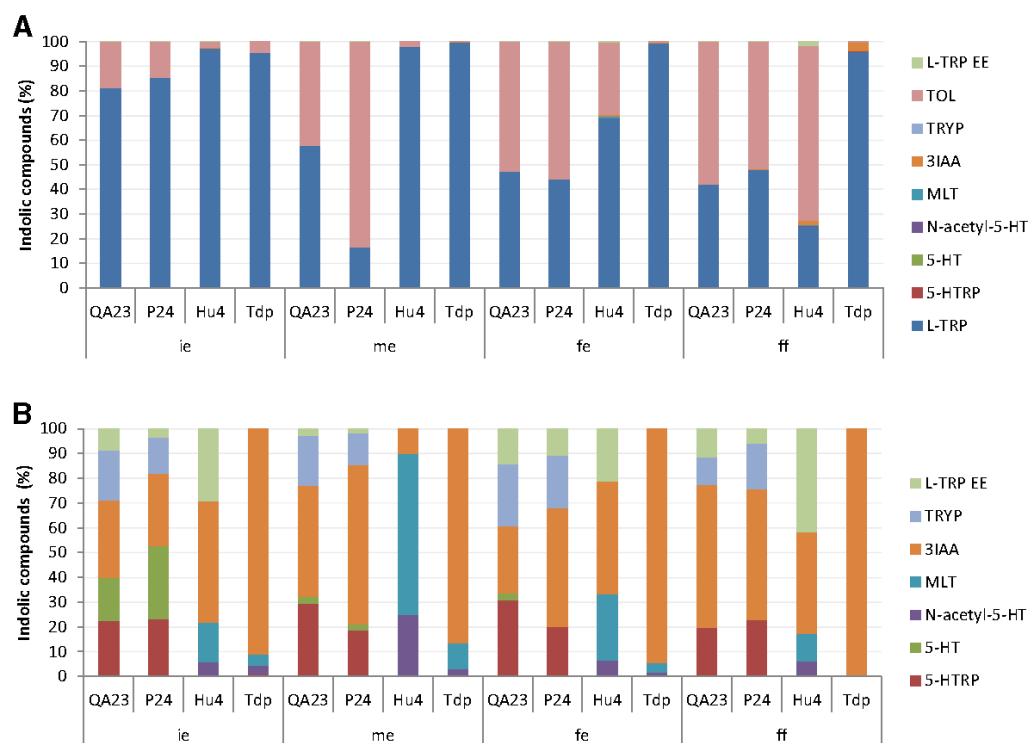
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Fig. 2 Progression of the detected indolic compounds across the different stages of alcoholic fermentation for studied yeast strains (**a**). Major compounds tryptophan (L-TRP) and tryptophol (TOL) were masked for better visualisation (**b**). *ie* initial exponential, *me* middle exponential, *fe* final exponential, *ff* final point

Ehrlich 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 (Fig. 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. Hu4 and Tdp were characterised by MLT and *N*-acetyl-5-HT production. For PC2, 5-HT and 3IAA 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*, Tdp was situated more in the upper right quadrant, which correlated with the positive loadings for PC2, whereas Hu4 was situated mainly in the lower right quadrant, which corresponded with negative loadings for PC2. This distribution can be explained by Tdp producing more 3IAA than Hu4 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.
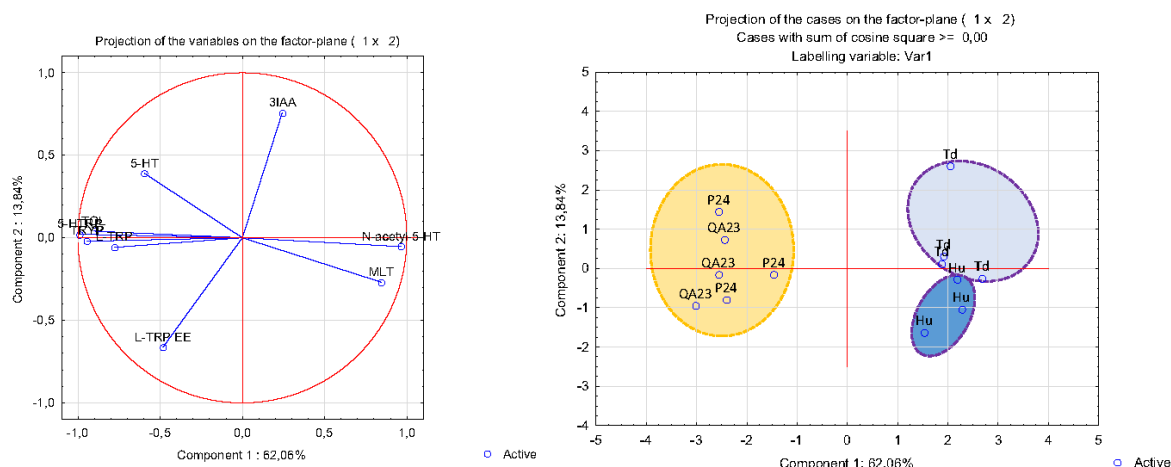
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Fig. 3 The statistical principal components analysis (PCA) of the effect of the different growth stages on the production of indolic compounds is *Saccharomyces* and non-*Saccharomyces* strains. Component 1 reflects 62.06% total variance (it negatively 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 posi-tively correlates with 3IAA 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 reflects differences in indolic profiles at the species level

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.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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