PROTECTIVE EFFECTS OF BIOACTIVE COMPOUNDS AGAINST FERMENTATION-ASSOCIATED STRESSES IN SACCHAROMYCES CEREVISIAE

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UNIVERSITAT ROVIRA i VIRGILI

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Doctoral thesis directed by

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Departament de Bioquímica i Biotecnologia

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Departament de Bioquímica i Biotecnologia

Facultat d'Enologia

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Catalunya

We STATE that the present study, entitled "**Protective effects of bioactive compounds against fermentation-associated stresses in *Saccaromyces cerevisiae***", presented by **Mercè Sunyer Figueres** for the award of the degree of Doctor with International Mention, has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia of this university.

Tarragona, September 5th 2021

Doctoral Thesis Supervisors

Dr. Gemma Beltran Casellas   Dr. Mª Jesús Torija Martínez

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Aristòtil ensenya

als alexandres a no conquerir

cap altra Pèrsia que la raó i la lògica

i comprova que li fan tant de cas

com en temps de la vella Macedònia

M. SUNYER MOLNÉ

A muns pares

i al Pablo

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OBJECTIVES AND OUTLINE OF THE THESIS

This doctoral thesis was performed from 2017 to 2021 in the *Biotecnologia Enològica* research group, at the *Departament de Bioquímica i Biotecnologia* in the *Facultat d'Enologia* of *Universitat Rovira i Virgili*.

This doctoral research was supported by doctoral fellowships from the *Universitat Rovira i Virgili*, the *Generalitat de Catalunya* and the *Ministerio de Universidades* (Spain) (grant numbers 2016PMF-PIPF-8, 2017FI_B_00607 and FPU17/05475, respectively). Furthermore, in order to obtain the distinction of International Doctorate, I conducted a mobility stay at Martin Ott's group of the *Institutionen för biokemi och biofysik* in *Stockholms universitet* (Sweden), which was funded by an Erasmus+ grant from the European Union.

The present thesis was performed within the framework of the SYNBIOFERM project entitled "Metabolism and protective effects of indole compounds in yeast of food interest, AGL-2016-77505-C3-3-R (financially supported by the *Ministerio de Economía y Competitividad* (Spain))". This project derived from the results obtained by the BIOACTIYEAST project entitled "Production and physiological effects of bioactive compounds derived from the aromatic amino acids in yeast populations, AGL2013-47300-C3-1-R (financially supported by the *Ministerio de Economía y Competitividad*)" and continues with the current CONSORWINE project "Design of microbial consortia to improve the stability and bioactivity of wines, PID2019-108722RB-C33 (financially supported by the *Ministerio de Ciencia e Innovación* (Spain))". The general aim of these projects was to increase the knowledge of the synthesis and detection of some key metabolites derived from the metabolism of aromatic amino acids in yeast, such as melatonin and hydroxytyrosol (HT), and to enhance the production of these metabolites to improve the stability and bioactivity of wines. One of the specific objectives was to determine the metabolic role of these bioactive molecules during alcoholic fermentation and the physiological and metabolic conditions in which they are synthesized by yeasts. Melatonin and HT have beneficial pleiotropic effects in humans, which mainly involve their powerful antioxidant activities, that are exerted through direct and indirect mechanisms. The antioxidant role of melatonin in yeast has been recently revealed, and melatonin seems to act not only by direct radical scavenging mechanisms but also by

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modulating the cell antioxidant stress response. During alcoholic fermentation, yeasts are exposed to several stresses, such as osmotic, acid or ethanol stresses, that influence their growth and eventually affect their fermentation performance. Although there is very little information regarding the reason why yeasts synthesize melatonin during alcoholic fermentation, it is known that some organisms produce melatonin to cope with environmental stresses. This may also be the case of yeasts, which synthesize melatonin to protect against fermentation-associated stresses. On the other hand, the physiological effects of HT in yeast, which is also produced during alcoholic fermentation, are completely unknown; however, HT could also have an antioxidant role, as does in other organisms.

Therefore, the working hypothesis of the present thesis was: **Melatonin and hydroxytyrosol have protective functions against stresses associated with alcoholic fermentation in *Saccharomyces cerevisiae*.**

To validate this hypothesis, the general objective was to study the possible role of melatonin and HT against oxidative stress and other fermentation-related stresses in the yeast *S. cerevisiae*. In the case of melatonin, the main objective was to determine the molecular mechanisms of the previously reported protective role against oxidative stress, as well as to determine its possible protective role against other fermentative stresses, such as ethanol stress. In the case of HT, the aim was to decipher whether, like melatonin, this bioactive molecule has a role as an antioxidant in *S. cerevisiae*, as observed in other organisms. To achieve this general objective, the thesis was divided into the following specific objectives:

Objective 1. To deepen the understanding of the melatonin protection mechanisms against oxidative stress in *S. cerevisiae*

Melatonin exerts a protective role against oxidative stress in both yeast and mammalian cells through direct and indirect mechanisms, such as scavenging free radicals and regulating the endogenous antioxidant cellular response. In mammals, melatonin is also involved in the regulation of other cellular pathways, with mitochondria being its

principal target. Therefore, we wanted to more deeply understand how the molecular mechanisms are affected by the presence of melatonin.

For that purpose, in Chapter I, we performed a transcriptomic study in a commercial wine strain of *S. cerevisiae* (QA23) supplemented with melatonin (5 µM) in the presence and absence of oxidative stress induced by 2 mM H_2O_2. After analysing the main up- and downregulated genes, we validated the effect of melatonin on the lipid composition and mitochondria. Later, in Chapter II, we improved the knowledge on the effect of oxidative stress and melatonin on mitochondrial performance during fermentative growth. The functionality of mitochondria was characterized by monitoring the mitochondrial abundance, oxygen consumption, activities of electron transport chain complexes, mitochondrial protein abundance, membrane potential and mitochondrial morphology.

Objective 2. **To evaluate the protection of melatonin against stresses other than the oxidative stress associated with fermentation**

To fulfil this objective, we first determined the effects of other stresses associated with fermentation, such as osmotic (induced by sugar, salt and sorbitol), acid and ethanol stresses, on *S. cerevisiae* growth. Then, we carried out a screening process to determine whether melatonin supplementation affected the growth or recovery of a commercial *S. cerevisiae* (QA23) wine strain exposed to glucose, NaCl, sorbitol, a low pH or ethanol, to trigger osmotic, acid and ethanol stresses (Chapter III).

Furthermore, in Chapter IV, we improved the knowledge on the effect of ethanol stress by applying different ethanol concentrations (6 – 14%) for short- and long-term periods of exposure to two strains of *S. cerevisiae* (the lab strain BY4743 and commercial wine strain QA23). We assessed the following effects: i) viability, by monitoring cell growth, recovery and mortality; ii) antioxidant shielding, by measuring reactive oxygen species accumulation and lipid peroxidation; and iii) endogenous antioxidant activity, by quantifying catalase and superoxide dismutase (SOD) activities. Afterwards, we determined whether the presence of melatonin protects *S. cerevisiae* cells during ethanol stress by evaluating the same indicators in both *S. cerevisiae* strains, in the presence and absence of melatonin under exposure to ethanol for different periods of time.

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Objective 3. To evaluate the possible antioxidant role of hydroxytyrosol in *S. cerevisiae*

To meet this goal, in Chapter V, we evaluated the effect of HT supplementation on the commercial wine strain of *S. cerevisiae* (QA23) in the presence and absence of mild oxidative stress induced by 2 mM H_2O_2.

First, we determined the effect of HT on yeast growth in different media (YPD, YNB and synthetic must), and after, we monitored the protective role of HT on cells exposed to oxidative stress by measuring reactive oxygen species accumulation, the degree of lipid peroxidation and the enzymatic activities of catalase and SOD.

Thus, these objectives were developed in the following five chapters:

Chapter I. **Transcriptomic insights into the effect of melatonin in *Saccharomyces cerevisiae* in the presence and absence of oxidative stress.** *Antioxidants* 2020, 9; 947.

Chapter II. **Effects of oxidative stress and melatonin on the mitochondria of *Saccharomyces cerevisiae* under fermentative conditions.** Manuscript in preparation.

Chapter III. **Effects of melatonin on the growth of *Saccharomyces cerevisiae* under fermentation-associated stresses.**

Chapter IV. **Protective effects of melatonin on *Saccharomyces cerevisiae* under ethanol-stress.** These results have been submitted to *Frontiers in Microbiology*.

Chapter V. **Protective role of hydroxytyrosol as an antioxidant in *Saccharomyces cerevisiae*.**

INTRODUCTION

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1. BUDDING YEAST *Saccharomyces cerevisiae*

Humans have been using and domesticating yeasts since ancient times, without even being aware of its existence; it seems that yeasts were already used in 10,000 BC to make bread, in 7,000 BC for beer production and in 6,000 BC for wine production (Pretorius, 2000; Nielsen, 2019). Winemaking spread from the Caucasus and the Fertile Crescent westward to the entire Mediterranean basin following Greek and Phoenician trade routes and colonizations, but it was not until the rise of the Roman hegemony that wine production reached its peak in terms of scale and quality (Pretorius, 2000), being the area around *Tarraco* (currently Tarragona), one of leading wine-growing regions of the Empire (Járrega Domínguez, 1995; Revilla, 2002; Prevosti and Martín, 2009).

Although the first to observe yeasts (using a self-designed microscope) was Antonie van Leeuwenhoek (1680) and the first to show that yeasts are living organisms was Charles Cagnard de la Tour in 1837, it was not until 1863 that Pasteur demonstrated that yeast is the main catalyst in the fermentation of wine and beer, which revealed that microbial activity ocurred during fermentation and, in addition, definitively rejected the previous idea that fermentation was a strictly chemical process (Pretorius, 2000; Ribéreau-Gayon et al., 2006; Madigan et al., 2012). This allowed the first isolation, by Emil Christian Hansen in 1883, of a pure yeast culture responsible for beer fermentation, which was used to improve the quality of beer and was also a cornerstone for developing fundamental research on yeast and establishing it as a model organism for the study of eukaryotic cells. All this established the basis for research on yeast and alcoholic fermentation (Ribéreau-Gayon et al., 2006; Nielsen, 2019; Steensels et al., 2019).

Saccharomyces is the most important commercial yeast since it is the main yeast in bakery, brewery and winery industries (Madigan et al., 2012). However, this importance is not only because of the social, cultural, and economical impacts of the products made with it, but also because *Saccharomyces* is the most commonly used yeast species in fundamental research and as a cell factory (Nielsen, 2019). Although other yeast species have similar applications, when the term "yeast" appears throughout this introduction, we will be mainly referring to the *Saccharomyces cerevisiae* species.

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Saccharomyces cerevisiae (which comes from the Latin *sugar fungus* and *of beer*) is a unicellular fungi ascomycete with spherical morphology. It normally reproduces asexually by budding but can also reproduce sexually or form filaments. *S. cerevisiae* has the cellular structure of a eukaryotic cell: a plasma membrane surrounds the cytoplasm, which contains a nucleus delimited by a membrane and several organelles, including the endoplasmic reticulum, the Golgi apparatus, the mitochondria and the vacuoles. Moreover, a rigid cell wall covers the plasma membrane (Ribéreau-Gayon et al., 2006; Aranda et al., 2011).

1.1. Applications of *S. cerevisiae*

S. cerevisiae has several advantages over other eukaryotic organisms (Figure 1). First, its cultivation is easy, rapid, and economical due to the short generation times and the ability to proliferate indefinitely, which enables us to obtain a large mass of cells; there are no ethical dilemmas associated with its use, and it is generally regarded as safe (GRAS) (Akram et al., 2020). Second, *S. cerevisiae* has a small genome with a structure that easily undergoes molecular genetic modifications. It was the first eukaryotic genome completely sequenced, which occurred in 1996 (Goffeau et al., 1996), and since then, the genome has been intensively studied. This has resulted in well-defined genetic knowledge and the creation of an elaborate toolbox for genetic and phenotypic investigations, including collections of genome-wide knockouts (which facilitate large-scale screenings), and regulated knockdowns or fluorescence-tagged versions of most gene products (Borneman and Pretorius, 2015; Kavšček et al., 2015; Malina et al., 2018; Steensels et al., 2019; Akram et al., 2020). These characteristics have motivated research on *S. cerevisiae*, which has offered many tools for further studies.

1.1.1. Yeast as a model

In the last twenty years, three studies based on classical genetics using this organism have won the Nobel Prize in Physiology or Medicine (Nielsen, 2019). Although this award is far from reflecting world research, it does highlight the important role of yeast research in understanding the fundamental principles of cell biology. The success of yeast as a model relies on a balance between its simplicity of cultivation and handling and the high

degree of similarity to human cells. Many cellular processes are conserved between yeast and human cells, and are based on the homology of several proteins, the preservation of signal transduction processes, protein–protein interactions, or regulatory hierarchies. Therefore, yeast is an ideal model for studying human health and disease genes and pathways, for testing drug targets, or for human therapeutics (Figure 1). Moreover, yeast cells are not only used as a model for human pathologies but also as a model for different applications, such as in the field of systems biology (Nielsen, 2019; Akram et al., 2020).
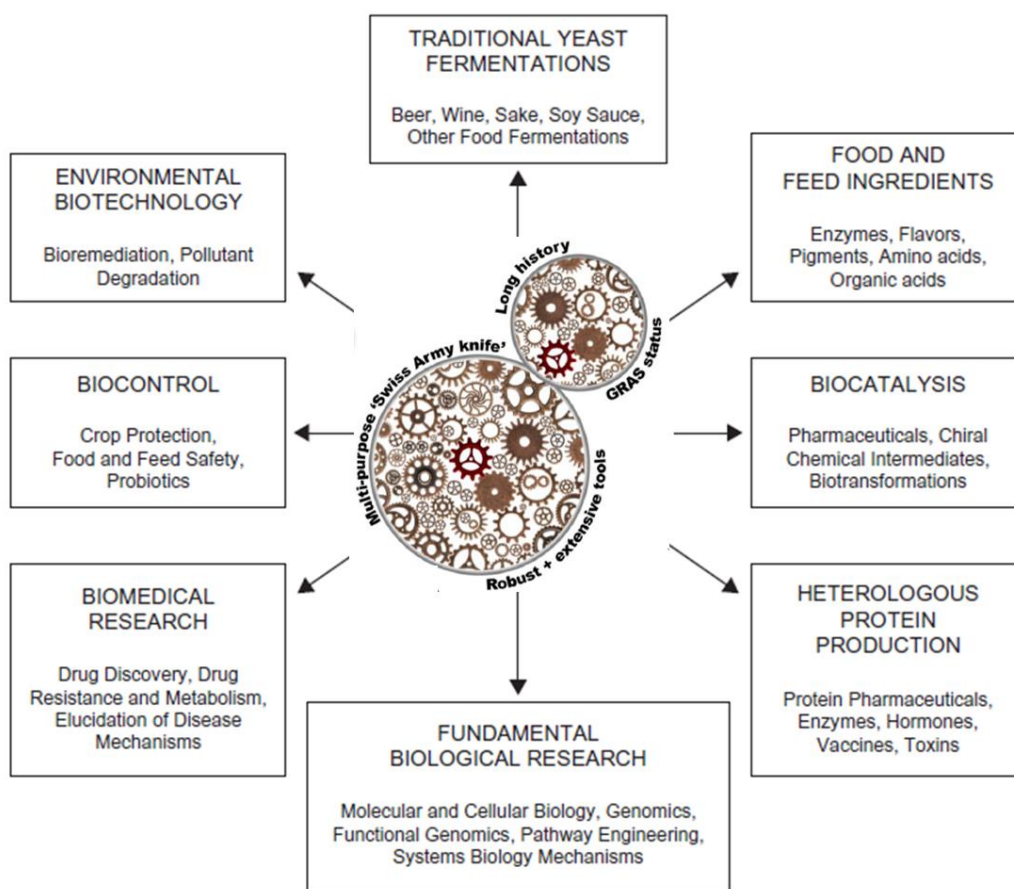
Figure 1. Traits of *S. cerevisiae* that favors its wide applications, and some of the disciplines in yeast biotechnology. GRAS stands for generally regarded as safe. (adapted from Johnson and Echavarri-Erasun, 2011; Walker and Pretorius, 2018).

Therefore, *S. cerevisiae* has been used for the study of several processes, but it is especially relevant for mitochondrial studies, since its ability to grow in fermentation gives it the ability to tolerate mutations that disrupt oxidative phosphorylation and even result in a complete loss of mitochondrial DNA (Rutter and Hughes, 2015; Malina et al., 2018; Akram et al., 2020).

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1.1.2. Yeast as a cell factory

As chemical and natural biosynthetic pathways are limited, there is a growing interest in bioengineering microorganisms to obtain products in a simpler and unlimited method, with higher yields, which is referred to as a cell factory (Yu et al., 2018; Malcı et al., 2020). Yeast is one of the main cell factories because its metabolism and genetics are well understood, which enables genetic manipulation. From this manipulation, many valuable biochemical products can be obtained, which have wide applications in the agricultural, food and beverage, cosmetic or pharmaceutical industries (Kavšček et al., 2015; Jin and Cate, 2017; Yu et al., 2018; Nielsen, 2019; Malcı et al., 2020). Indeed, *S. cerevisiae* plays an essential role in bioethanol production, mainly due to its highly efficient ethanol production and its relatively high tolerance to fermentation-associated stresses (Auesukaree, 2017). Thus, there has been an increasing amount of research on yeast, not only to improve yeast performance and to obtain higher ethanol production but also to generate ethanol from lignocellulose products, such as grass, wood or algae, which do not require crops or land that is valuable for the food industry (Ma and Liu, 2010a; Jayakody et al., 2015; Takagi and Kitagaki, 2015; Jin and Cate, 2017; Hu et al., 2019).

For all these reasons, yeast has been considered a tool to solve some of the most concerning problems of the world, not only regarding health but also regarding the produce of limited, industrial and energy goods (Figure 1).

1.2. Yeast in winemaking

Winemaking is the process of transforming grapes into wine (Figure 2), and one of the main steps involves alcoholic fermentation, which converts grape sugars into ethanol. Wine fermentation is a complex process in which several microorganisms, such as yeasts, bacteria and filamentous fungi (Aranda et al., 2011), are present and interact. However, the main active components are yeasts, which carry out alcoholic fermentation to convert the sugars of grape must in ethanol, CO_2 and other secondary products (which have an impact on the aroma and flavour of wine). Since the earliest yeast studies, countless investigations have focused on understanding the ecology, biochemistry, physiology and

molecular biology of the yeasts involved in the fermentation process and on selecting specialized yeast strains to improve wine quality (Pretorius, 2000; Fleet, 2003, 2008).
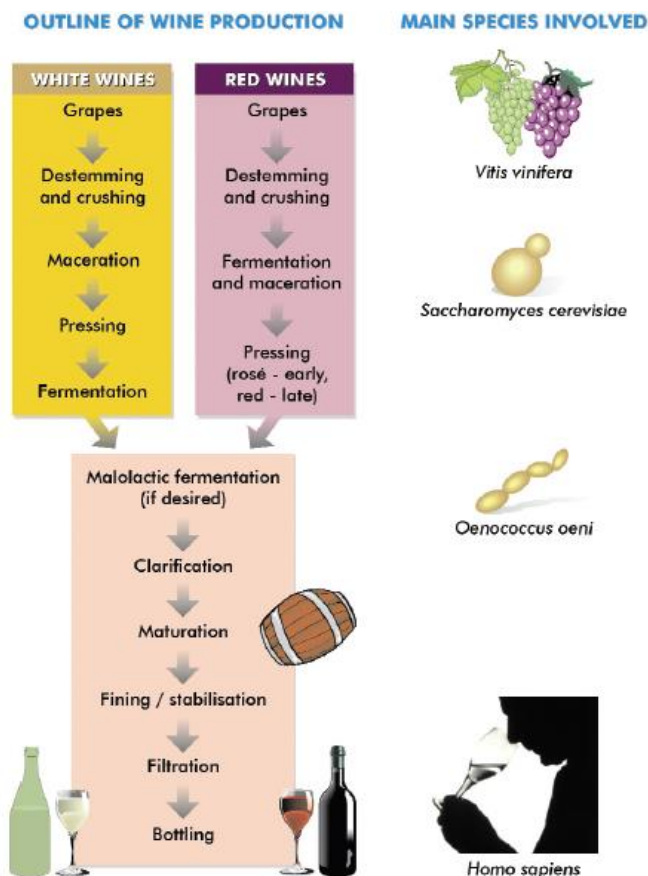
Figure 2. The main steps in wine production that need to be considered in an integrated grape-to-wine research approach (Pretorius and Høj, 2005).

The yeasts of the genus *Saccharomyces* and, in particular, the strains of *S. cerevisiae* are the main yeasts responsible for carrying out alcoholic fermentation, since they are best equipped to take advantage of the restrictive winemaking conditions. For this reason, *S. cerevisiae* is known as "the wine yeast". The key factors involved in the imposition of *S. cerevisiae* are the following: foremost, its ability to grow and ferment sugars vigorously to produce ethanol, which occurs regardless of the presence or absence of oxygen due to its Crabtree-positive carbon metabolism. Second, *S. cerevisiae* is well-adapted to the harsh environment changes (caused by anaerobiosis, sulfiting, high osmotic pressure, low pH and increasing ethanol concentrations), since it can sense, react and adapt its physiology

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to the conditions of stress during alcoholic fermentation (Pretorius, 2000, 2016; Ribéreau-Gayon et al., 2006; Mas et al., 2016; Matallana and Aranda, 2017).

Although *S. cerevisiae* predominates during fermentation, in the initial steps, other yeast species that are encompassed in the term "non-*Saccharomyces*" are present in sequential succession. They contribute to the organoleptic richness of the final wine, which has a positive impact on wine complexity. Some examples of these non-*Saccharomyces* are species belonging to the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Kluyveromyces* or *Torulaspora* (Fleet, 2008; Padilla et al., 2016).

The usual strategy employed to start fermentation involves inoculating the must with selected yeast in the form of active dry yeast (ADY), usually *S. cerevisiae*, to ensure complete fermentation and a reproducible final product. However, in recent years, the selection and use of autochthonous *S. cerevisiae* as starters as well as non-*Saccharomyces* yeasts in mixed fermentations has gained interest because these yeasts can contribute substantially to wine properties, such as the organoleptic quality, giving the wine complexity and authenticity (Ribéreau-Gayon et al., 2006; Aranda et al., 2011; Mas et al., 2016; Mateo and Maicas, 2016; Pretorius, 2016).

1.3. Yeast carbon metabolism

The primary concern of living organisms is the uptake and metabolization of nutrients to generate energy and biomass. *S. cerevisiae* is a facultative anaerobe with a chemoorganotrophic metabolism; thus, it obtains energy from the degradation of organic nutrients (Madigan et al., 2012). Therefore, the utilization of nutrients by yeast is an essential factor for successful fermentation and survival, and cell growth and nutrient uptake rates are very important for yeast to outcompete other organisms in wild growth conditions (Ribéreau-Gayon et al., 2006; Shimizu and Matsuoka, 2019).

S. cerevisiae can degrade carbohydrates through two metabolic pathways: alcoholic fermentation and respiration, which have glycolysis in common (Madigan et al., 2012). Hexoses (mainly glucose and fructose) are transported across the plasma membrane to the cytosol, where glycolysis takes place. During glycolysis (or the Embden-Meyerhoff

pathway), glucose metabolization occurs through a series of reactions (Figure 3) that result in the formation of two molecules of pyruvate, H_2O and adenosine triphosphate (ATP), and the reduction of one molecule of nicotinamide adenine dinucleotide (NAD) for each hexose molecule. ATP is a molecule that stores chemical energy that can be transformed into other forms of energy necessary for cell growth. NAD is a coenzyme that accepts electrons from a substrate that is oxidized, and thus, NAD is reduced to NADH. Pyruvate is the starting point of both alcoholic fermentation and respiration (Figure 3) (Ribéreau-Gayon et al., 2006; Aranda et al., 2011).
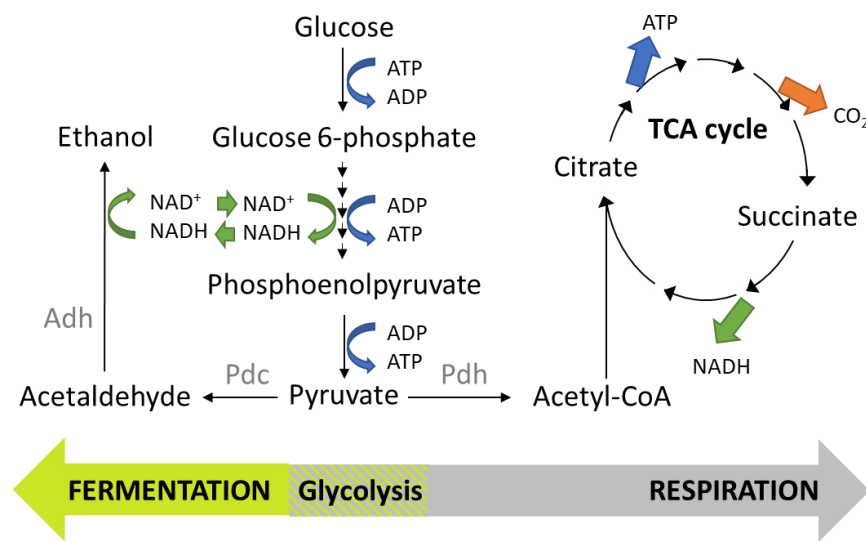
Figure 3. Schematic representation of the yeast carbon metabolism: the glycolysis is the common trunk between the fermentation and respiration pathways. Adh, alcohol dehydrogenase; Pdc, pyruvate decarboxylase; Pdh, pyruvate dehydrogenase. The ATP, NADH and CO_2 yields are not represented quantitatively (adapted from Ribéreau-Gayon et al., 2006; Wang et al., 2015; García et al., 2016).

1.3.1. Alcoholic fermentation

Alcoholic fermentation consists of glycolysis plus two additional reactions that convert pyruvate to ethanol in the cytoplasm. In the first reaction, pyruvate is decarboxylated to acetaldehyde and carbon dioxide by pyruvate decarboxylase (Pdc), and in the second reaction, acetaldehyde is reduced to ethanol by alcohol dehydrogenase (Adh) (Figure 3). In the latter reaction, NADH is recycled to NAD^+, which is reduced during glycolysis, ensuring that the cofactor needed for glycolysis activity remains present

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(Aranda et al., 2011). The enzyme Adh has several isoforms, but the most important are cytosolic Adh1 and Adh2. Adh1 is more proficient in the excretion of ethanol and Adh2 is more proficient in ethanol uptake (Pfeiffer and Morley, 2014). As there is no ATP production in this part of the pathway, the net ATP generation during alcoholic fermentation is 2 ATP (formed during glycolysis) for each hexose molecule (Ribéreau-Gayon et al., 2006).

1.3.2. Respiration

For the respiration pathway, the pyruvate generated by glycolysis is transported to the mitochondria and undergoes a process of oxidative decarboxylation by pyruvate dehydrogenase (PDH), which leads to acetyl-CoA. This molecule enters the TCA cycle (tricarboxylic acid cycle, also named citric acid cycle), where it is completely oxidized to CO_2, through eight oxidative and hydrolytic reactions, which require cofactors in the oxidized forms of NAD or flavin adenine dinucleotide (FAD) as reducing equivalent acceptors (Figure 3). Each complete cycle produces one molecule of ATP and two molecules of CO_2. Moreover, three hydrogen ions are transferred to three NAD^+ molecules and two to a FAD molecule, resulting in 8 electrons in total in the form of NADH and $FADH_2$, which enter the electron transfer chain (ETC) in the mitochondria, and in a process called oxidative phosphorylation (OXPHOS) (explained in Section 3.1.1), which yields three or two ATP molecules per pair of electrons (Ribéreau-Gayon et al., 2006; Baccolo et al., 2018). Therefore, OXPHOS produces 28 ATP molecules, so the overall ATP obtained from the respiration of a glucose molecule is 36–38 molecules. Moreover, TCA can also be fueled through some of its intermediates (Ribéreau-Gayon et al., 2006; Baccolo et al., 2018).

1.3.3. Regulation between fermentation and respiration metabolism

The regulation between respiration and fermentation, in most organisms, is driven by the Pasteur effect: organisms metabolize carbon sources through respiration unless the final electron acceptor, usually oxygen, is absent. In that case, species that can adapt to anoxic environments switch their metabolism to fermentation. This is based on the rule that cells carry out the most beneficial energy metabolism, and respiration produces more

ATP (18- or 19-fold) and biomass yield than does fermentation (Ribéreau-Gayon et al., 2006; Pfeiffer and Morley, 2014). However, the fundamental characteristic of *S. cerevisiae* during wine production is its ability to ferment glucose even under aerobic conditions. This is explained by the Crabtree effect, in which the presence of high glucose concentrations represses respiratory flux (Piškur et al., 2006; Ribéreau-Gayon et al., 2006; Hammad et al., 2016). As this effect has substantial relevance for winemaking, research is important not only to understand the regulatory mechanisms but also to decipher the advantages of using a less energetically beneficial metabolism for yeast cells (Pfeiffer and Morley, 2014). Despite this, yeast requires oxygen for some of its processes, so a situation of "semianaerobiosis" must be maintained because the development and fermentation of yeast is impossible in the total absence of oxygen (Ribéreau-Gayon et al., 2006). Therefore, the yeasts that participate in must fermentation are classified depending on their ability to employ or not the Crabtree effect as Crabtree-positive or Crabtree-negative.

There are two principal hypotheses to explain why Crabtree-positive yeasts favour fermentation over respiration. On the one hand, the make-accumulate-consume strategy is based on the rapid depletion of carbohydrates and accumulation of ethanol, which is toxic to other microorganisms but can be respired by *S. cerevisiae* (Piškur et al., 2006). On the other hand, the most widely accepted hypothesis is the rate/yield trade-off, which states that ATP production is quicker (higher rate) but less efficient (lower yield) in fermentation than in respiration. Upon competition, having a higher ATP rate could confer a selective advantage to yeast, which could overcome the decrease in yield (Pfeiffer and Morley, 2014).

The regulation of the Crabtree effect, respiration and fermentation relies on a complex network of transcription factors, which is only partially known (Fendt and Sauer, 2010; Hammad et al., 2016). During fermentation, when the metabolic flux into the TCA cycle is low, the respiratory pathway is repressed. Thus, mitochondria degeneration occurs along with the low expression of genes encoding enzymes of the TCA, ETC and OXPHOS, which are driven by different signalling pathways (Figure 4) (Ribéreau-Gayon et al., 2006; Pfeiffer and Morley, 2014; Zhang et al., 2017a). However, when glucose is

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depleted, yeast cells switch from fermentation to respiration metabolism, and use ethanol as a carbon source. This adaptation requires some time, during which the cell does not grow (known as diauxic shift) and activates the TCA cycle, ETC and OXPHOS genes, among others.

The transition between fermentation and respiration is controlled by different signalling pathways with several transcription factors, such as the HAP complex or Rtg1/3p (elements of the retrograde pathway, explained below), which are derepressed by the absence of glucose (Figure 4). The HAP complex is activated by heme and has an essential role in the yeast growth in nonfermentable carbon sources, since it induces the transcription of several genes of the TCA cycle, ETC and OXPHOS (Fendt and Sauer, 2010; Zhang et al., 2017a). As heme synthesis is regulated by oxygen availability, it acts as an intermediate in the signalling mechanisms to sense oxygen levels and is also essential for ATP production in the ETC by modulating the HAP complex. In fact, a study suggested that heme plays a role in the Crabtree effect, since its level is regulated not only by oxygen but also by the carbon source (Fendt and Sauer, 2010; Zhang et al., 2017a). Fructose-1,6-biphosphate also participates in the establishment of the Crabtree effect, since it inhibits the mitochondrial respiratory rate at the level of complexes III and IV of the ETC, modulating metabolism from complete respiration to respiration-fermentation without using transcription factors (Hammad et al., 2016; Malina et al., 2018; Shimizu and Matsuoka, 2019).
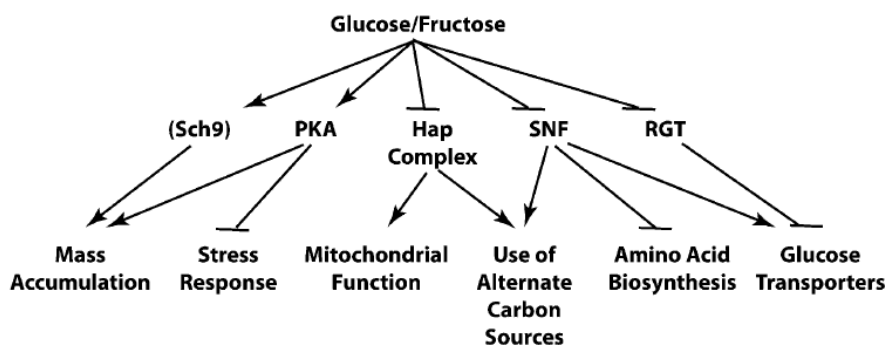
Figure 4. An overview of glucose signaling pathways. Different signaling networks respond to availability of fermentable sugars and regulate distinct, albeit overlapping, functions that optimize growth under the particular nutrient status of the cell. Sch9 appears to respond directly to sugar availability, but the mechanistic connection is not well defined (Broach, 2012).

1.3.4 Conversion of grape juice to wine

Grape must is a complex medium that contains all the necessary nutrients to ensure the vital functions of yeast, but its variable composition influences growth and dynamics, and the availability of some nutrients can act as a limiting growth factor (such as nitrogen). The efficient utilization of these nutrients determines the kinetics and completion of the fermentation, and they have an impact on the organoleptic profile of wine (Figure 5). The most abundant components are water and monosaccharides (mainly glucose and fructose), but there are also organic acids, nitrogen compounds, polyphenols, mineral salts, lipids, vitamins, and inhibitors (sulfites, pesticides). The main biochemical reaction that occurs during alcoholic fermentation is the conversion of sugars into ethanol and carbon dioxide. However, yeast uses and forms other substrates and metabolites, such as the assimilation, biosynthesis and degradation of amino acids or the formation of higher alcohols and esters (Figure 5) (Ribéreau-Gayon et al., 2006; Aranda et al., 2011).
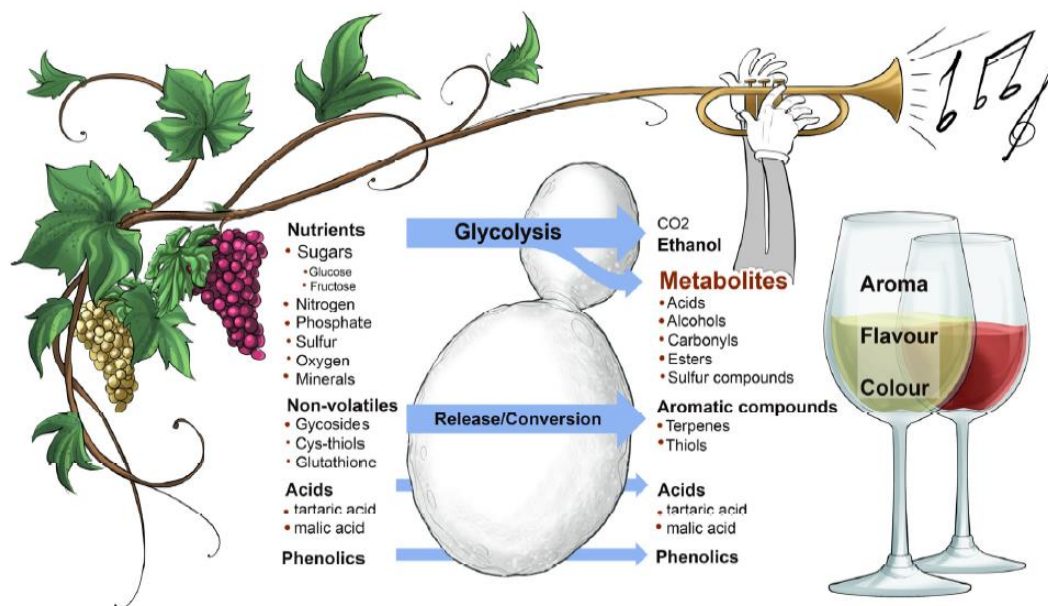
Figure 5. Metabolic conversion of grape juice to wine by the action of the yeast *S. cerevisiae*. Wine yeast convert grape nutrients into ethanol, carbon dioxide and secondary metabolites through its glycolytic metabolism. The compounds synthesized or modified by yeast can impart aromas that can diminish or enhance the quality of wine (Pretorius, 2016).

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1.5. Yeast growth

The growth curve of *S. cerevisiae* consists of three phases. First, there is a lag phase in which there is no growth as cells acclimate to the media, allowing them to adapt to the expression of specific genes. Second, there is an exponential phase, where the maximum increase in population occurs. Third, when some nutrients are limiting or there is a toxic substance in the medium, yeast slow down their growth and reach the stationary phase, in which the total population does not increase but they are metabolically active (Figure 6). Moreover, there may be a fourth phase in which there is more cell death than duplication, resulting in the population decreasing.

During the stationary phase, cells are stressed by a lack of nutrients and by the accumulation of toxic metabolites, which leads to an increase in oxidative stress and activation of mechanisms for stress resistance (Chen et al., 2005; Galdieri et al., 2010; Owsiak et al., 2010; Nussbaum et al., 2014). Thus, physiological, biochemical and morphological changes are triggered to ensure survival during periods of shortage, as explained below.

Additionally, as reported before, when yeasts are grown in a fermentable medium as glucose, most of them exhibit an additional phase, the diauxic shift, which is a transition period between glucose and ethanol as substrates for growth. Once glucose becomes scarce, yeasts enter this diauxic phase as they readjust their metabolism to use the ethanol produced as a carbon source. After this adaptation period, yeasts grow back exponentially until ethanol is depleted then finally enter the stationary phase.
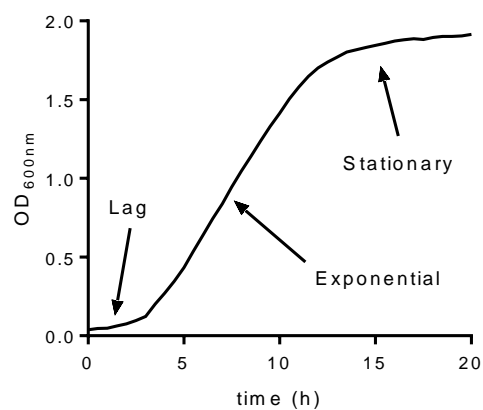
Figure 6. Growth curve of *S. cerevisiae*

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2. FERMENTATION-ASSOCIATED STRESSES

The winemaking process is like an obstacle course as there are many stresses that must be overcome. Stress consists of perturbations that can alter the internal cellular environment by disturbances of metabolic fluxes and chemical gradients, destabilization of cellular structures, or reduction of enzyme activities, leading to general cellular instability (Hohmann and Mager, 2003). In the fermentation process, yeasts must face stresses related to the must composition (high osmotic pressure, low pH/acidic stress, and additions of sulphites or copper), physicochemical processes (temperature, must aeration) or metabolism products (ethanol and other toxic products, oxidative stress or nutrient starvation) (Figure 7) (Pretorius, 2000; Ribéreau-Gayon et al., 2006; Auesukaree, 2017; García-Ríos and Guillamón, 2019; Walker and Basso, 2020). Moreover, other fermentation-related processes are also affected by different stresses, such as the production of ADY, which involves oxidative, osmotic and thermal stresses and desiccation (Figure 7) (Ribéreau-Gayon et al., 2006; Matallana and Aranda, 2017).
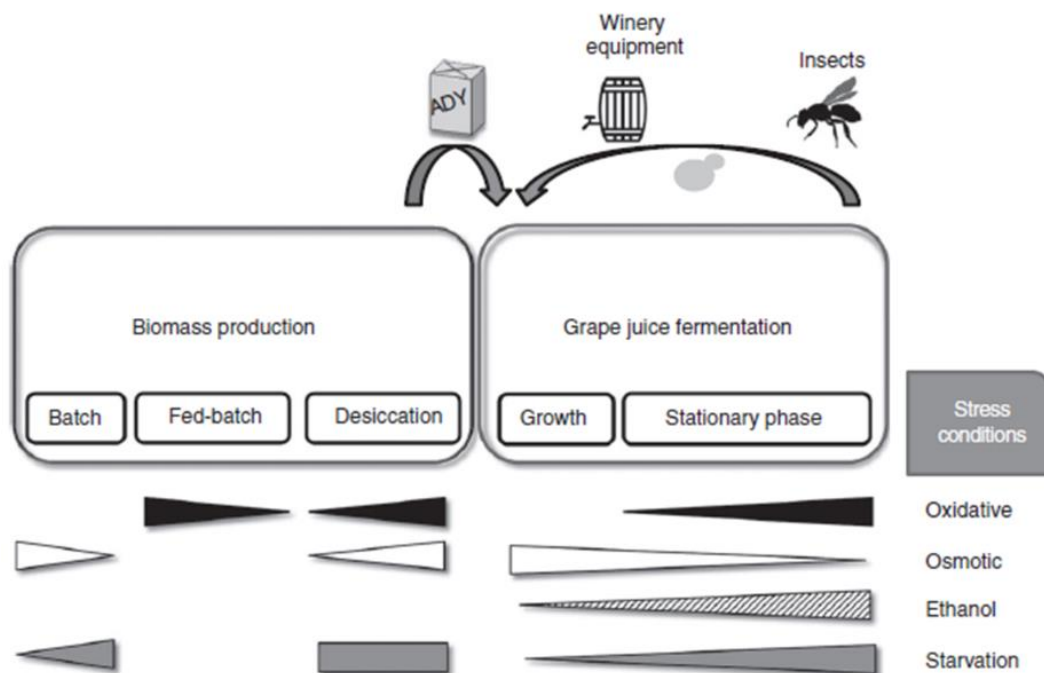
Figure 7. Stress response during the biotechnological use of wine yeast strains. The main winemaking stages, biomass production and grape juice fermentation are indicated by boxes. Arrows show the transitions of yeasts from different steps of the process. The profiles of the main conditions are indicated and show their increase or decrease in the stages. ADY, active dry yeast (adapted from Matallana and Aranda, 2017).

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These stresses affect yeast kinetics, reducing their growth and survival rates and their metabolic activity, thus compromising fermentation performance and causing sluggish, or in extreme cases, stuck fermentations (Ribéreau-Gayon et al., 2006; García-Ríos and Guillamón, 2019; Walker and Basso, 2020). As a consequence, wines have low ethanol and/or high residual sugar, which increases the risk of spoilage or undesired aromatic compounds due to the growth of unwanted microorganisms (Ribéreau-Gayon et al., 2006; Walker and Basso, 2020).

Therefore, yeast cells have mechanisms to protect and maintain internal homeostasis against variable external conditions, which results in a better performance of cellular processes (Hohmann and Mager, 2003). In the case of winemaking, there is a correlation between high stress tolerance and good fermentative capacity (Matallana and Aranda, 2017), so one of the objectives of fermentation studies is to increase yeast tolerance to those stresses to ensure that fermentation completes and the product quality is good. In fact, over the centuries, yeasts have developed responses to protect themselves from stress and have acquired tolerance against stress. In parallel, strains with better tolerance to different stresses have been selected, resulting in a wide diversity of species and strains (Auesukaree, 2017). A good example is the *S. cerevisiae* species, which has strains adapted to ferment musts with high sugar concentrations, in different temperature ranges or with high ethanol concentrations (Mas et al., 2016). Indeed, as mentioned above, one of the reasons for the imposition of this species during alcoholic fermentation is its ability to sense, react and adapt its physiology to the stresses inherent to the fermentation environment, which gives it an advantage over other yeasts (Auesukaree, 2017; Matallana and Aranda, 2017).

Therefore, understanding the mechanisms that confer protection against stress is pivotal for stress mitigation. Indeed, there are different strategies that can be applied to mitigate the stress of yeast cells: directly relieve the stress; select strains with improved oenological characteristics or higher stress tolerance; improve yeast cells by adaptive evolution (exposing the cells to a mild stress to increase their tolerance to a stronger stress) or by using genetically modified organisms (GMO) procedures (the construction of a genetically modified yeast with higher stress tolerance); or supplement the medium

with molecules that have protective effects on specific stress targets (Auesukaree, 2017; García-Ríos and Guillamón, 2019; Walker and Basso, 2020).

Table 1. Processes enriched by genes upregulated or downregulated during the environmental stress response (ESR) (from Gasch et al., 2000).

Processes activated by ESR	Metabolite transport, fatty acid metabolism, carbohydrate metabolism maintenance of the cellular redox potential, detoxification of reactive oxygen species, autophagy, protein folding and degradation, cell wall modification, DNA-damage repair, secretion, vacuolar and mitochondrial functions, intracellular signaling, intern osmolyte synthesis, longevity
Processes downregulated by ESR	Protein synthesis (related with decrease in translation initiation and arrest of cell cycle and growth), cell wall biosynthesis, cytoskeletal and chaperonin functions, protein glycosylation and secretion, amino acid and pyruvate metabolism, nucleotide biosynthesis, DNA replication, mRNA decay..

2.1. Environmental stress response

During fermentation, exposure to different stresses can be simultaneous or sequential, often resulting in a synergistic toxic effect. Yeast has stress-specific responses, which are generally regulated at the transcriptional level through stress-specific transcription factors. Nevertheless, these stress response pathways work in a coordinated manner, making it easier for yeast to cope with combined stresses (Jamieson, 1998; Costa and Moradas-Ferreira, 2001; Teixeira et al., 2011; Walker and Basso, 2020). This coordination relies on the fact that there is considerable overlap between the different stress responses that allow mechanisms to be triggered by several stresses, causing the phenomenon of cross protection or acquired stress resistance, in which cells exposed to a mild stress of one kind become resistant to higher doses of other stresses (Hohmann and Mager, 2003). For example, the protection conferred by a mild thermal shock triggers the resistance to heat shock, ethanol, high salt concentration, oxidative stress and radiation, and yeast tolerance to ethanol, sorbic acid or low pH gives protection to heat stress (Stanley et al., 2010). This response is coordinated by the environmental stress response (ESR), which encompasses several stress signalling pathways and integrates information from many stress responses, such as heat and osmotic shock, oxidative and ethanol stress, nutrient starvation, DNA damage and extreme pH (Figure 8) (Teixeira et al., 2011; Auesukaree, 2017; Matallana and Aranda, 2017). Moreover, there is a general

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stress response of the fermentation process, called the fermentation stress response (FSR), which regulates the ESR genes and other important genes in the vinification environment, such as those involved in glucose uptake, nitrogen regulation or vacuolar function and detoxification (Teixeira et al., 2011).

The principal transcription factors that mediate the ESR genes are Msn2/4 (Hohmann and Mager, 2003), which are hyperphosphorylated under stress conditions and migrate to the nucleus (Auesukaree, 2017; Matallana and Aranda, 2017). There, they bind the stress response elements (STRE) located in the promoters of stress-induced genes, regulating the transcription of 900 genes related to a wide variety of processes (Table 1) (Gasch et al., 2000). The induction of these genes is not in response to any environmental change but seems to represent a response to suboptimal environments (Gasch, 2003), so the induction is usually not required for the stress already applied but for long-term cellular protection against future stresses (Teixeira et al., 2011; de la Torre-Ruiz et al., 2015; Shima and Nakamura, 2015). Moreover, most Msn2/4p target genes are redundantly or coordinately regulated by other transcription factors, such as Yap1p and Hsf1p, which are specific for oxidative stress and heat-shock, respectively (Takagi and Kitagaki, 2015). Indeed, pathways such as the cell wall integrity (CWI) or high-osmolarity glycerol (HOG) pathways, which respond to osmotic stress, induce the transcription factors Msn2/4, which activate the genes that are under Msn2/4 regulation.
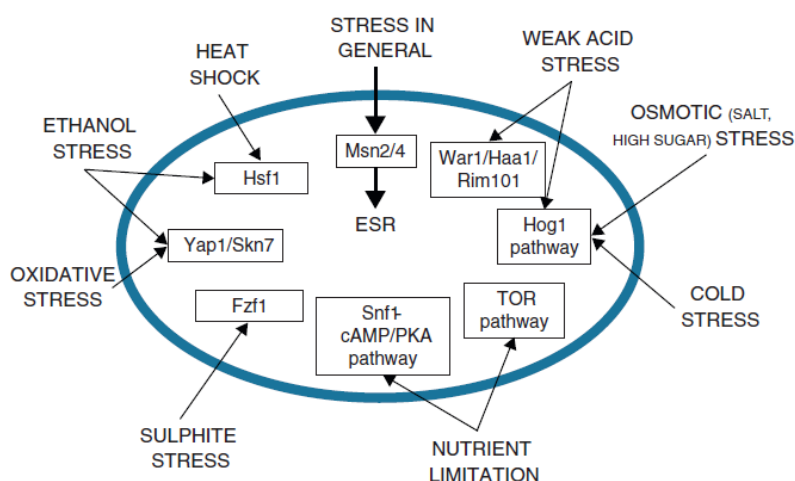
Figure 8. Main signaling pathways controlling yeast adaptive response to food-relevant stresses. Cross-talk between these pathways in each stress response was not considered in this simplified figure (Teixeira et al., 2011).

2.2. Specific stress responses

To understand the coordinated stress response, it is essential to study how each stress affects the cell, how it responds and what resistance mechanisms it has. For that, it is pivotal to distinguish between two types of cellular mechanisms: (i) the mechanisms of stress response, which are physiological and molecular responses to changes in the environment, and (ii) the mechanisms that give resistance to the cell against the external condition, which results in strains that are more tolerant to the stress (Bauer and Pretorius, 2000; Ma and Liu, 2010a; Teixeira et al., 2011). Next, we will explain the yeast responses to the main fermentation-associated stresses.

2.2.1. Osmotic pressure

Sugar (glucose and fructose) concentrations in musts range between 170–220 g/L, so the first drawback encountered by yeast in fermentation is osmotic stress (hyperosmolarity), which in high concentrations could inhibit growth and impair fermentation performance. Therefore, the ability of yeast to adapt to high osmotic pressure is crucial for fermentation performance and involves substantial gene expression reprogramming (reviewed in Aranda et al., 2011; Auesukaree, 2017).

The first effect of osmotic stress on yeast cells is the loss of intracellular water, which causes the cells to shrink and lose turgidity (Adya et al., 2006). This dehydration results in plasma membrane depolarization and permeabilization (Simonin et al., 2007). The main response of cells to this hyperosmolarity is the accumulation of compatible solutes, mainly glycerol (Blomberg and Adler, 1989; Westfall et al., 2008; Saito and Posas, 2012), to increase the internal osmolarity and thus equilibrate the osmolarity between intracellular and extracellular spaces, restoring turgor pressure and preventing the loss of intracellular water from cells (Figure 9) (Hohmann, 2002; Auesukaree, 2017; García-Ríos and Guillamón, 2019). Other compatible solutes, such as trehalose, have been reported to protect cells against osmotic stress (Martínez-Montañés et al., 2010); however, it is likely that trehalose does not protect yeast cells from glucose-induced osmotic stress during alcoholic fermentation, since its trehalose biosynthesis is inhibited under high glucose conditions (Apweiler et al., 2012; Kitichantaropas et al., 2016). Additionally, to respond to

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hyperosmolarity, yeast closes the glycerol exporter Fps1p and changes the physiochemical structure of the cell wall and plasma membrane (Posas et al., 2000; Saito and Posas, 2012; García-Ríos and Guillamón, 2019).
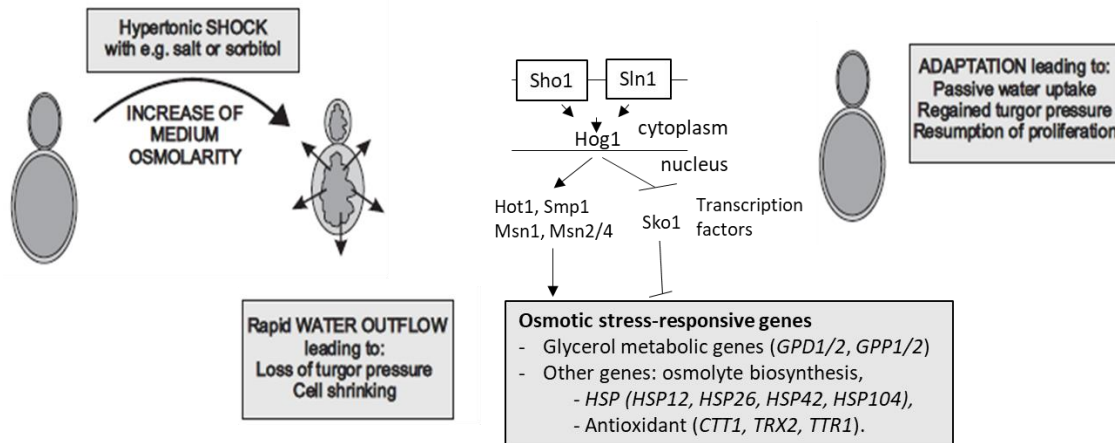
Figure 9. Basic features of the response of *S. cerevisiae* to hyper-osmotic shock. The simplified scheme of the osmotic stress signal transduction through the HOG pathway is illustrated (adapted from Tamás and Hohmann, 2003; Auesukaree, 2017).

The increase in glycerol synthesis is regulated by the HOG pathway, which controls osmoregulation (Figures 8, 9) (reviewed in Tamás and Hohmann, 2003; Saito and Posas, 2012) and senses changes in cellular turgor pressure to regulate the activities of transcription factors (among them, Msn2/4) (Figure 9). Thus, these transcription factors stimulate the expression of osmotic stress-responsive genes related to glycerol import and synthesis (Brewster et al., 1993) and to antioxidant and chaperone functions (Posas et al., 2000; Rep et al., 2000; Koziol et al., 2005), which increase glycerol accumulation and provide protection against oxidative damage and protein denaturation, respectively (Figure 9) (reviewed in Saito and Posas, 2012; Auesukaree, 2017; Matallana and Aranda, 2017; García-Ríos and Guillamón, 2019).

2.2.2. Low pH

The optimal pH for yeast growth is between 4 and 6, but yeast can also grow and ferment in the pH of grape must, which usually ranges between 2.8 and 4.2. However, a pH below 2.8 can result in sluggish or stuck fermentations. Resistance to this stress

depends on the temperature, presence of oxygen, and yeast strain (Aranda et al., 2011; Liu et al., 2015; Walker and Basso, 2020).

If a strong inorganic acid is added to the must, it dissociates in the media and generates a high concentration of protons, but it diffuses poorly across the membrane. Instead, if the acid is weak, it remains undissociated in the must, and this undissociated form can enter by passive diffusion into the yeast cell and dissociate inside the cell, as the intracellular pH is higher, generating a proton and the respective counteranion (Figure 10) (Carmelo et al., 1997). These protons decrease the intracellular pH, causing a reduction in metabolic activities (such as glycolysis), which affects signal transduction, protein interactions and the cell division rate. In addition, the accumulation of anions increases internal turgor pressure, which inhibits cell growth (Figure 10) (reviewed in Teixeira et al., 2007).
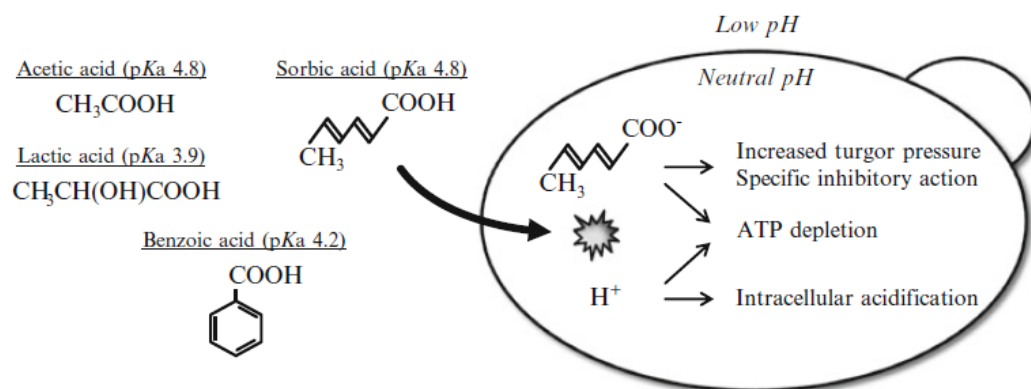
Figure 10. Weak organic acid stress. Diffusional entry and dissociation of sorbic acid in a cell is depicted as an example of weak organic acid stress. At lower pH values, sorbic acid exists substantially in the undissociated state, resulting in a large diffusional flux of acid into cells that, in turn, causes weak organic acid stress via the dissociation-mediated generation of protons and anions as a result of the near-neutral pH inside the cell (Sugiyama et al., 2015).

The cell response consists of increasing the ATPases to pump out protons to the extracellular media (H^+-ATPase, located in the plasma membrane) and to the vacuole lumen (V-ATPase, located in the vacuole) (Alexandre et al., 1996; Holyoak et al., 1996; Carmelo et al., 1997; Fernandes et al., 2003; Teixeira et al., 2005; Makrantoni et al., 2007),

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causing a decrease in the intracellular ATP pool, which is incremented by the inhibition of glycolytic enzymes (Figure 10) (Holyoak et al., 1996; Sugiyama et al., 2015). The cell also activates transporters involved in the efflux of propionate, sorbate, benzoate or acetate and exports anions through multidrug-resistance transporters (reviewed in Mira et al., 2010). Moreover, a low pH affects the cell wall structure and alters the lipid organization of the plasma membrane, affecting plasma membrane integrity (Alexandre et al., 1996). This results in an increase in cell permeability to ions and other small metabolites, which contributes to the acidification and dissipation of the electrochemical potential of the plasma membrane (Stevens and Servaas Hofmeyr, 1993; Mira et al., 2010). The cell responds by remodelling the cell wall and plasma membrane to restrict the diffusion of weak acids (Carmelo et al., 1996, 1997; Holyoak et al., 1996).

The responses to weak acids, such as acetic, benzoic, lactic, propionic and sorbic acids, induce specific pathways for pH (Rim101) (Mira et al., 2009) and for weak acid stress (War1, Haa1) (Kren et al., 2003; Fernandes et al., 2005; Frohner et al., 2010; Mollapour and Piper, 2012; Tanaka et al., 2012; Inaba et al., 2013; Sugiyama et al., 2014; Kim et al., 2019) but also activate the ESR and the HOG pathway (specific for osmotic stress) (Figure 8) (Teixeira et al., 2011).

2.3. Oxidative stress

S. cerevisiae, as an aerobic organism, is subjected to the so-called "oxygen paradox": it depends on oxygen for its survival during cellular respiration, but this aerobic metabolism leads to the formation of reactive oxygen species (ROS), which are toxic to the cell (Davies, 1995). ROS are generally free radicals, that is, molecules that contain one or more unpaired electrons, which are generated upon the partial reduction of oxygen by electrons that escape from the electron carrier systems, so ROS can be produced in all metabolic processes that use oxygen as an electron donor (mainly in the ETC in the mitochondria) (Murphy, 2009). Moreover, ROS can also be produced due to exposure to exogenous agents, such as ionizing radiation, redox-cycling chemicals or heavy metals (reviewed in Halliwell, 2006; Baccolo et al., 2018; Malina et al., 2018).

The main ROS species in the cell are superoxide ($O_2^{\bullet-}$), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), among others (Figure 11). Free radicals are highly reactive and unstable molecules. Superoxide is the first ROS formed, as oxygen can readily accept single electrons in a nonenzymatic transfer, but it is quickly converted to secondary toxic agents, such as ·OH, or undergoes dismutation to H_2O_2 (Figure 11). The hydroxyl radical is highly reactive and reacts immediately with molecules close to it, indiscriminately oxidizing DNA, proteins and lipids as soon as it is formed, and is the ROS species that causes the most damage and genomic instability. H_2O_2 is not a radical as it has an even number of electrons, so it is more stable and can be present at higher intracellular concentrations. However, H_2O_2 is still toxic, not only because of its lipophilicity, which allows it to cross lipid-rich membranes and spread potential damage, but also because it can react with transition metals (iron, copper) to yield ·OH by the Fenton reaction (Figure 11) (reviewed in Halliwell, 2006; Morano, 2012; Reiter et al., 2017; Baccolo et al., 2018; Malina et al., 2018). Therefore, oxidative stress occurs when the concentration of ROS increases beyond the antioxidant buffering capacity of the cell (Jamieson, 1998).
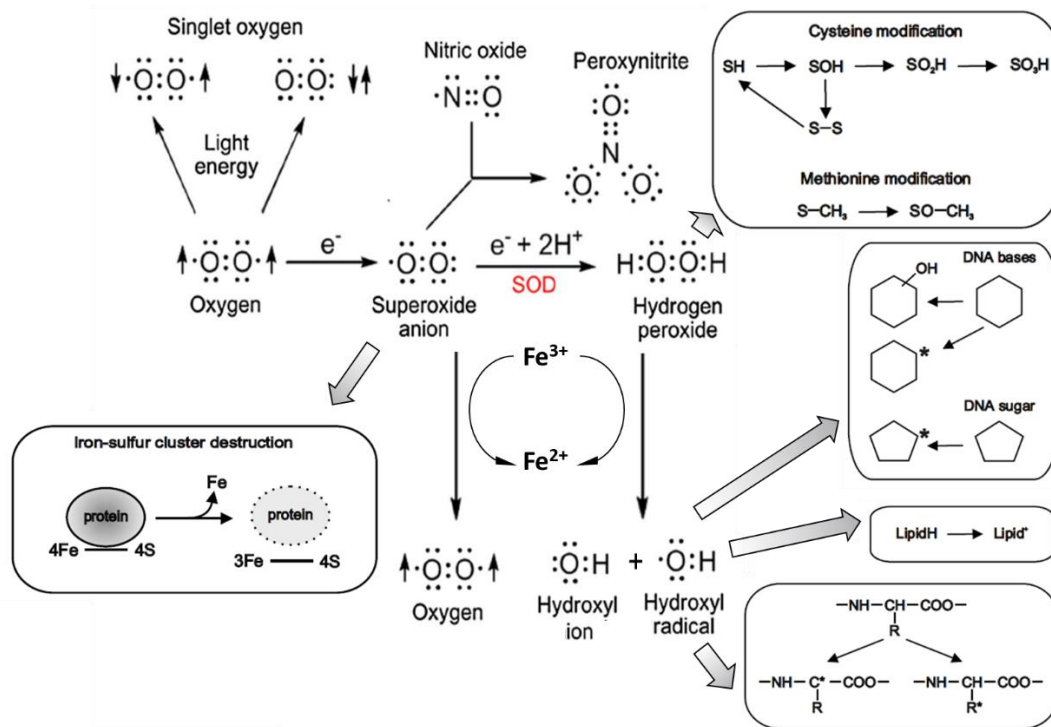
Figure 11. Formation and detoxification of the main reactive oxygen species (ROS) in the cell. The wide gray arrows represent the reactivity of ROS towards biological molecules. SOD stands for superoxide dismutase (adapted from Toledano et al., 2003; Reiter et al., 2017).

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Although alcoholic fermentation is an anaerobic metabolism, it is normally performed under semianaerobic conditions, since a low oxygen concentration is required to synthesize some fatty acids and sterols, and to maintain yeast in optimal conditions for efficient fermentation (Andreasen and Stier, 1953, 1954). Therefore, small amounts of ROS may be generated during the process. However, oxidative stress during alcoholic fermentation is mainly caused by the cells entering the stationary phase. The entrance to this phase is particularly important because it involves nutrient limitation (mainly nitrogen but also lipids and vitamins) and ethanol toxicity, which causes an increase in oxidative stress and the activation of mechanisms for stress tolerance. Therefore, in the stationary phase, the pathways that depend on nutrient availability, such as targets of rapamycin (TOR)/Sch9, Ras/cAMP or activated protein kinase (AMPK), are repressed, and Msn2/4 are activated, inducing stress-responsive and antioxidant genes and preparing the cell for the increase of oxidative stress (reviewed in Costa et al., 1997; Moradas-Ferreira and Costa, 2000; de la Torre-Ruiz et al., 2015; Matallana and Aranda, 2017). Thus, when nutrients are limited, correct and tight regulation of nutrient signalling pathways ensures a prolonged lifespan, preventing sluggish or stuck fermentations (García-Ríos and Guillamón, 2019).

In addition, there are fermentation-related processes carried out under respiratory conditions, such as industrial processes of biomass propagation and dehydration to obtain active ADY, that also cause oxidative stress to yeast cells (Pérez-Torrado et al., 2005). Moreover, osmotic, ethanol and heat-shock stresses also induce the accumulation of ROS and thus oxidative stress. Interestingly, the response to oxidative stress has been identified as among the most important responses for fermentation performance (Figure 7) (Aranda et al., 2011; Picazo et al., 2015; Matallana and Aranda, 2017; Burphan et al., 2018; García-Ríos and Guillamón, 2019).

2.3.1. Effects of ROS on yeast

As mentioned above, ROS react with cellular molecules (such as lipids, amino acids or proteins), oxidizing them and causing damage to cellular components (Figure 11). Hydroxyl radicals attack lipids, initiating a chain lipid peroxidation reaction that results in products and byproducts that can react with other biological molecules, forming adducts

with DNA bases and proteins. Lipid peroxidation causes changes in the composition of the lipid layer, which correlates with membrane disintegration (Wolff et al., 1986; Ayala et al., 2014). ROS also promote protein carbonylation, triggering chemical modifications that lead to important changes in the charge state of proteins, which may account for protein aggregation (Davies, 1987; Davies and Delsignore, 1987). When this aggregation affects enzymes, it can disrupt several cellular processes. ROS can also modify DNA, causing single- and double-strand breaks, direct base modifications, or DNA cross-linking (reviewed in Cadet et al., 1997). Therefore, all the products obtained by ROS oxidation can provide feedback to the oxidation state of the cell, leading to the production of more ROS species, which results in molecular, physiological and functional impairment, eventually causing cell death (reviewed in Jamieson, 1998; Costa and Moradas-Ferreira, 2001; Toledano et al., 2003).

2.3.2. Yeast responses to counteract oxidative stress

Cells have evolved and developed antioxidant defence mechanisms to protect their cellular components against oxidative stress. These mechanisms consist of enzymatic and nonenzymatic systems capable of neutralizing free radicals before they affect essential cell elements, maintaining the cellular redox state and repairing damage to molecules prone to oxidation, such as the thiol residues of proteins. Moreover, yeasts have developed a highly regulatory signalling to face these stresses (Jamieson, 1998; Costa and Moradas-Ferreira, 2001). The response to oxidative stress is regulated by transcriptional reprogramming and is mainly carried out by the specific transcription factors of oxidative stress (Yap1, Skn7) and the ESR (Msn2/4), which, as mentioned above, regulate the expression of some oxidative stress-responsive genes (such as *CTT1*) (Figure 12) (reviewed in Jamieson, 1998; Morano et al., 2012; Picazo and Molin, 2021).

Yap1 (basic leucine zipper transcription factor) promotes the transcription of genes encoding antioxidant and antioxidant-associated molecules, especially those involved in the glutathione system (*GSH1*, *GSH2*, *GLR1*) (de la Torre-Ruiz et al., 2015). Under physiological conditions, Yap1 is exported to the cytoplasm, but upon exposure to oxidative stress, Hyr1 catalyses the formation of disulfide bonds in Yap1, which inhibits its

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nuclear export and causes it to accumulate in the nucleus, where it upregulates the expression of some genes (Figure 12) (Harshman et al., 1988; Delaunay et al., 2000; Moradas-Ferreira and Costa, 2000; Auesukaree, 2017). Skn7 is a nuclear protein only activated by oxidative stress and is involved in the upregulation of heat-shock protein (HSP) genes in cooperation with Hsf1 (Raitt et al., 2000). Moreover, Skn7 has a role in regulating cell wall synthesis, the cell cycle and the osmotic stress response (Auesukaree, 2017). Both transcription factors are regulated by redox changes and coordinately control the transcription of several oxidative stress-responsive genes, such as the following: catalases (*CTA1*, *CTT1*), superoxide dismutases (SOD; *SOD1*, *SOD2*) and thioredoxin or glutathione systems (*TRX1*, *TRR1*, *TSA1*, *AHP1*, *GPX2*, *GSH1*, *GSH2*, *GLR1*) (Figure 12) (reviewed in Morano et al., 2012; de la Torre-Ruiz et al., 2015).
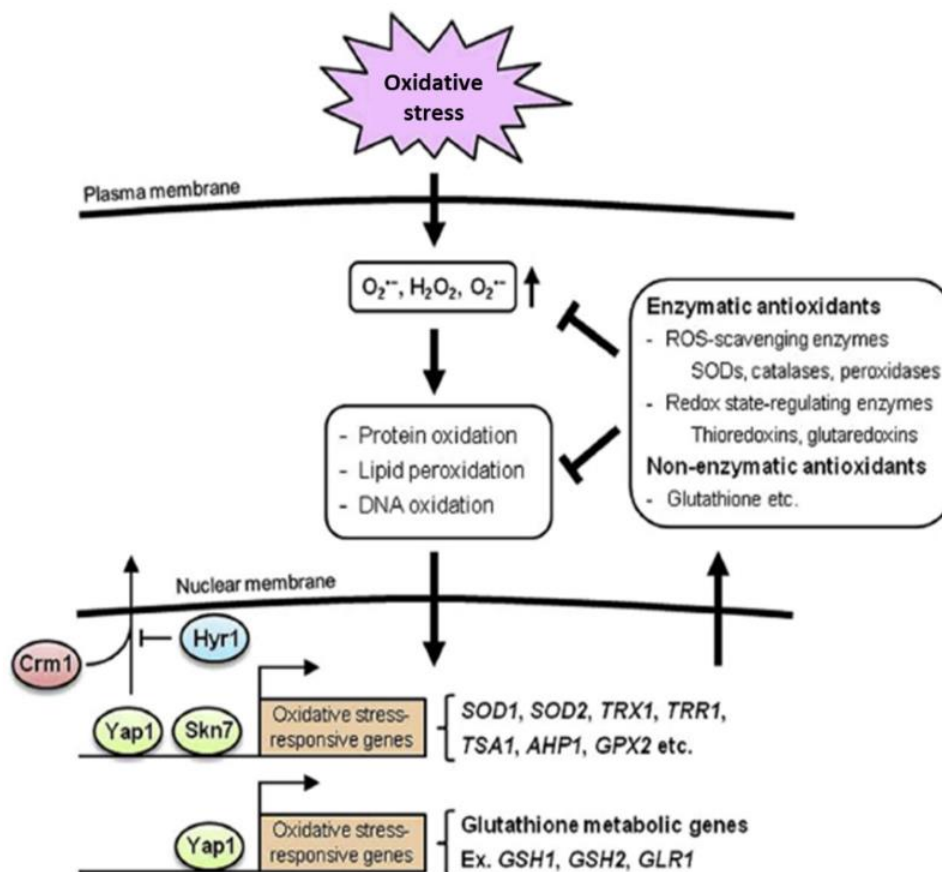
Figure 12. Cellular effects of oxidative stress and molecular mechanisms of oxidative stress response in yeast (adapted from Auesukaree, 2017)

Moreover, it is important to note that ROS levels appear to be decisive for different biological outcomes, since they cause cellular damage and promote senescence at high concentrations, whereas at low concentrations, they can be signalling molecules and induce cell proliferation and adaptation to oxidation (reviewed in de la Torre-Ruiz et al., 2015; Sies and Jones, 2020).

2.3.3. Enzymatic antioxidant defence mechanisms

Oxidative damage activates the synthesis of enzymes capable of detoxifying the cell. Antioxidant enzymes can be divided into the following categories: ROS scavengers, including SOD, catalase and peroxidase, and regulators of intracellular redox balance, including thioredoxins and glutaredoxins (Figure 13) (Jamieson, 1998; Auesukaree, 2017).

Superoxide dismutases (SOD) catalyse the conversion of superoxide anions into H_2O_2 and O_2. *S. cerevisiae* possesses two SODs that are classified according to their subcellular localization and metal cofactor, which are cytosolic (Cu/Zn-SOD), encoded by the *SOD1* gene and mitochondrial (Mn-SOD), encoded by the *SOD2* gene. Mn-SOD appears to be the primary scavenging enzyme of $O_2^{\bullet-}$ produced during respiration, while Cu/Zn-SOD appears to scavenge not only superoxide anions from the mitochondrial intermembrane space but also those generated externally and cytosolically (reviewed in Jamieson, 1998; Herrero et al., 2008; Auesukaree, 2017).

Catalases catalyse the breakdown of H_2O_2 into O_2 and H_2O. *S. cerevisiae* has two such enzymes, catalase A and catalase T. Catalase A is located in the peroxisome (Cta1p) and mainly scavenges H_2O_2 produced from beta-oxidation of fatty acids (Hiltunen et al., 2003), whereas catalase T is located in the cytoplasm (Ctt1p) and copes with cytosolic H_2O_2 (reviewed in Jamieson, 1998; Toledano et al., 2003; Morano et al., 2012).

While SODs and catalases use the properties of their metal groups for their activity, **glutathione and thioredoxin peroxidases** use the active site of cysteine thiols to reduce inorganic and organic peroxide into the corresponding alcohols and water. Therefore, they require glutathione and thioredoxin as thiol electron donors to reduce H_2O_2 (both) and organic (glutathione) or alkyl (thioredoxin) hydroperoxides. Gutathione peroxidases (Gpx1, Gpx2, Gpx3), located in several cell parts, protect membrane lipids from

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peroxidation, as they reduce phospholipid hydroperoxides. Thioredoxin peroxidases (which are also called peroxiredoxins) are located in the cytoplasm (Tsa1, Tsa2, Ahp1), nucleus (Dot5) and mitochondria (Prx1) and reduce peroxides and peroxinitrites (reviewed in Jamieson, 1998; Herrero et al., 2008; Auesukaree, 2017).

There are also other enzymatic systems, such as glutathione and thioredoxin reductases, which form part of the glutathione or thioredoxin systems (explained below). These reductases have a role in maintaining the redox capacity of the cell. Additional systems are methionine reductase, Apn1 endonuclease, or enzymes of the pentose phosphate pathway (Jamieson, 1998; Herrero et al., 2008).
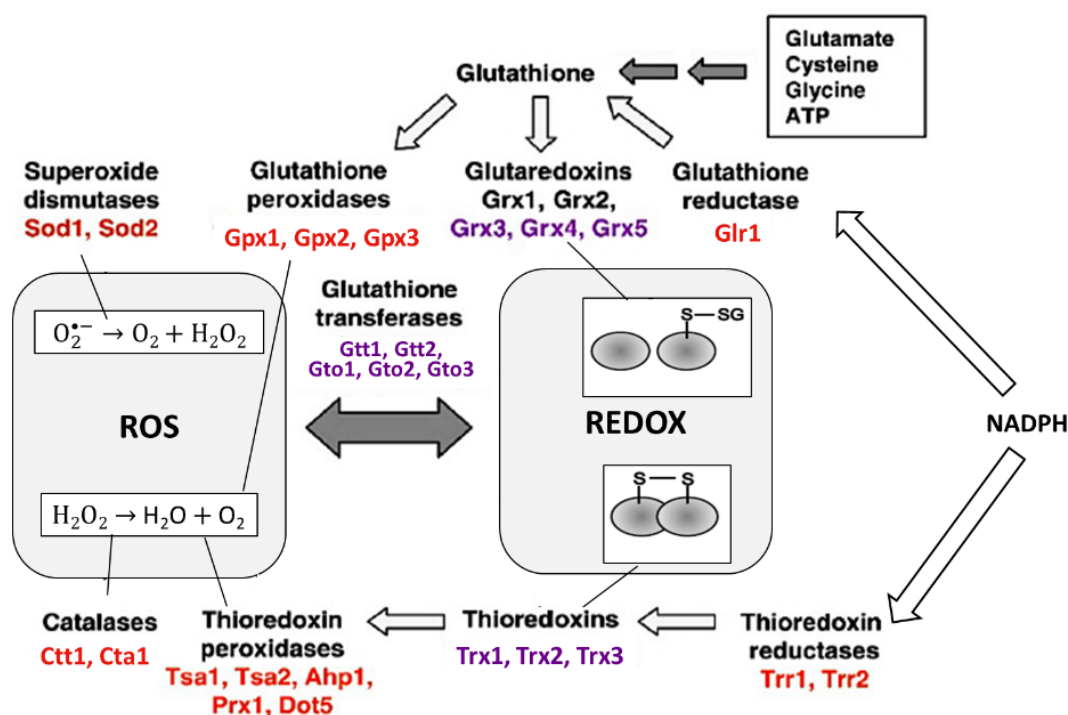
Figure 13. Primary interrelationships between different enzymatic (red) and non-enzymatic (purple) systems involved in detoxification and the control of the redox state in *S. cerevisiae*. The flow of electrons from NADPH to oxidized substrates is indicated (adapted from Toledano et al., 2003; Herrero et al., 2008).

2.3.4. Non-enzymatic antioxidant defence mechanisms

The *S. cerevisiae* antioxidant system also includes small molecules, such as glutathione, glutaredoxins or thioredoxins, that directly scavenge ROS and thus remove

oxidants from solution and are involved in redox balance (reviewed in Jamieson, 1998; Picazo and Molin, 2021).

Glutathione (tripeptide γ-L-glutamyl-L-cystinylglycine; GSH) is the most abundant radical scavenger and is therefore an important antioxidant molecule for yeast. In the glutathione cycle, reduced glutathione (GSH) is oxidized to the disulfide form (GSSG), which is reduced back to GSH by NADPH-dependent glutathione reductase, Glr1 (Figure 13). Due to its importance as an antioxidant in yeast, it is crucial to maintain a high ratio of reduced/oxidized glutathione in cells, which is achieved with a system that encompasses enzymatic and nonenzymatic defences (glutathione reductase, glutathione peroxidase and glutaredoxins) (reviewed in Jamieson, 1998).

Thioredoxins (TRXs) and glutaredoxins (GRXs) are small thiol oxidoreductases involved in regulating the redox state of protein groups, as they reduce protein thiols using their redox-active cysteines. The thioredoxin system contains thioredoxins, thioredoxin reductases and thioredoxin peroxidases. *S. cerevisiae* has three thioredoxin systems composed of thioredoxins, Trx1, Trx2 (cytoplasmic) and Trx3 (mitochondrial), and two thioredoxin reductases (cytoplasmic Trr1 and mitochondrial Trr2). *S. cerevisiae* has several glutaredoxins located in various compartments including the following: cytoplasmic Grx1/8, nuclear Grx3/4, mitochondrial Grx5, cis-Golgi Grx6/7, and cytoplasm/mitochondria-colocalized Grx2 (reviewed in Morano et al., 2012). GRXs receive their reducing ability from glutathione reductase and glutathione, as they form part of the glutathione system (Figure 13). GRXs may act redundantly with TRXs in some functions, such as DNA synthesis or sulfur metabolism (Luikenhuis et al., 1998; Hohmann and Mager, 2003; Herrero et al., 2008; Auesukaree, 2017).

There are other molecules that can protect yeast against oxidative stress, such as amino acid-derived polyamines, ascorbic acid or metallothioneins (Jamieson, 1998; Herrero et al., 2008).

2.4. Ethanol stress

The ability to produce high ethanol concentrations provides a selective advantage to *S. cerevisiae*, but at the same time, ethanol is the main stressor encountered during

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fermentation, which can cause a reduction in the growth rate and cell viability, often causing sluggish or stuck fermentations (Pretorius, 2000; Auesukaree, 2017; García-Ríos and Guillamón, 2019). Therefore, *S. cerevisiae* has developed mechanisms to tolerate, respond and adapt to this stress.

2.4.1 Effect of ethanol on yeast

Although *S. cerevisiae* possesses inherent tolerance to ethanol, some toxic effects have been associated with ethanol accumulation. In general, ethanol affects cell physiology by inhibiting cellular division, decreasing the growth rate and increasing mortality (reviewed in Ma and Liu, 2010a; Stanley et al., 2010; Vamvakas and Kapolos, 2020). Due to its structure, ethanol is soluble in both lipid and aqueous phases. For this reason, ethanol can be incorporated into cell membranes, which are its main target, and can also penetrate inside cells, where it mainly affects mitochondria and proteins and, to a lesser extent, the endoplasmic reticulum, the vacuole and the cell wall (Figure 14) (Teixeira et al., 2009; Stanley et al., 2010; Zyrina et al., 2017).

Initially, ethanol disturbs membranes by intercalating itself into the hydrophilic interior of the lipid bilayer, which causes dissipation of the electrochemical transmembrane potential, loss of membrane integrity and an increase in fluidity and permeability (Lloyd et al., 1993; Marza et al., 2002). Therefore, all these changes result in a loss of transport control through the membrane, since there is a passive influx of ions (mainly protons) that produces intracellular and vacuole acidification (Figure 14) (Salgueiro et al., 1988; Teixeira et al., 2009). High ethanol also affects the uptake of several molecules, such as glucose, ammonia and amino acids, causing leakage of nucleotides, amino acids and potassium, which results in altered metabolism (Piper, 1995).

Once inside the cell, the principal toxicity mechanism of ethanol is an oxidative burst produced mainly by ROS species generated in the mitochondria. This oxidative stress induced by ethanol causes lipid peroxidation (Gupta et al., 1994; Du and Takagi, 2007; Kitagaki et al., 2007; Fierro-Risco et al., 2013; Pérez-Gallardo et al., 2013; Gharwalova et al., 2017; Jing et al., 2020), enzyme inactivation, mitochondrial disruption and DNA damage, as mentioned above (Yang et al., 2012; Pérez-Gallardo et al., 2013). Moreover,

ethanol decreases water activity and therefore its bioavailability, resulting in protein denaturalization. This is especially relevant for key proteins, such as glycolytic enzymes, since a reduction in their activity or dysfunction causes altered metabolism (reviewed in Hallsworth et al., 1998; Stanley et al., 2010; Auesukaree, 2017). In addition, ethanol can be converted to acetaldehyde, which has been proposed to be largely responsible for ethanol toxicity (Aranda et al., 2011). In fact, ethanol is a chaotropic compound, that is, a compound that increases the general entropy of a solution, which in a biological system results in disordered and unfolded macromolecules and the disruption of biological membranes, causing generalized, nonspecific toxicity in living systems (Timson and Eardley, 2020).
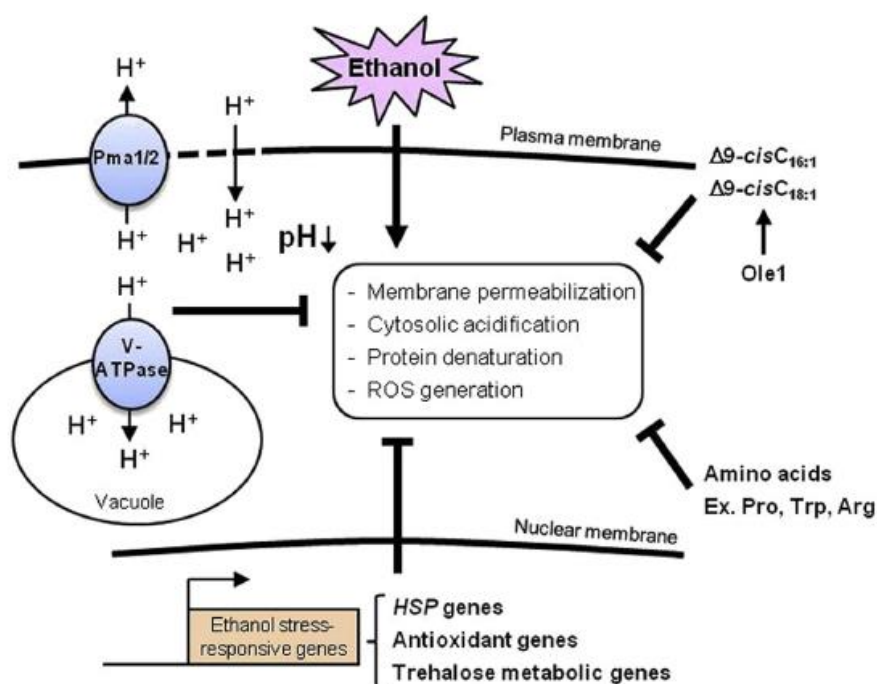
Figure 14. Deleterious effects of ethanol on yeast cells and cellular adaptive response to ethanol stress (Auesukaree, 2017).

2.4.2. Yeast responses to ethanol stress

It is well known that yeasts have developed responses to counteract the toxic effect of ethanol, which are mainly based on activating protective mechanisms and producing different compounds. Nevertheless, these mechanisms are not fully understood (Vamvakas and Kapolos, 2020).

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As ethanol toxicity implies oxidative stress, yeast responds by increasing the levels of ROS scavengers and activating the enzymatic mechanisms related to the antioxidant response, with catalases and SODs having a pivotal role (Figure 14) (Costa et al., 1993; Du and Takagi, 2007; Wang et al., 2014a; Cheng et al., 2016; Gharwalova et al., 2017; Vamvakas and Kapolos, 2020). However, contrary to the antioxidant response, mitochondrial SOD seems to be more important against ethanol than cytoplasmic SOD (Costa et al., 1997; Zyrina et al., 2017). This could be because mitochondrial ROS are the primary source of damage (Pérez-Gallardo et al., 2013; Zyrina et al., 2017; Jing et al., 2020). Upon ethanol stress, yeast cells also upregulate the genes of metabolic pathways that are involved in the regeneration of NADH and NADPH, which are important for the maintenance of redox equilibrium and, therefore, for yeast metabolism (Alexandre et al., 2001; Chandler et al., 2004; Ma and Liu, 2010a, 2010b). Additionally, ethanol induces mitophagy, a type of selective autophagy of mitochondria, which removes redundant, damaged or dysfunctional mitochondria. Mitochondrial clearance contributes to a decrease in mitochondrial ROS accumulation and is thereby another mechanism to protect cells from ROS damage (Jing et al., 2020).

To protect proteins from denaturalization, cells trigger mechanisms to maintain a functional protein conformation, such as the accumulation of HSP and trehalose (Figure 14) (Alexandre et al., 2001; Fujita et al., 2006; Trevisol et al., 2011; Wang et al., 2014a). HSPs are molecular chaperones that assist in folding newly synthesized proteins, refolding misfolded proteins, and disaggregating protein aggregates (reviewed in Auesukaree, 2017; García-Ríos and Guillamón, 2019). The upregulation of HSP chaperones is also induced by heat stress (Ma and Liu, 2010a; Stanley et al., 2010), suggesting that these proteins have a similar role in the response to both stresses, that is, preventing protein aggregation and assisting the subsequent refolding. However, ethanol stress also activates HSPs that act in several cellular localizations (cytoplasm, nucleus, mitochondria), with roles other than chaperones, such as peptidases, hydrolases, cell wall remodelling, enhancing ATP generation, facilitating functions of transcription factors or regulating H$^+$-ATPase (reviewed in Ma and Liu, 2010a). In addition, cells mitigate the effects of chaotropes by producing compatible solutes, which differ in response to different stresses

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(Timson and Eardley, 2020). Under osmotic stress, the compatible solute is glycerol, but under ethanol stress, the role of this molecule is unknown, and cells seem to induce the synthesis and accumulation of trehalose (Mansure et al., 1994; Ogawa et al., 2000; Kaino and Takagi, 2008; Cray et al., 2015). Trehalose has the following main roles: on the one hand, it stabilizes the structure of proteins and works, together with HSP, for protein reparation and good protein folding (Ding et al., 2009; Ma and Liu, 2010a); on the other hand, trehalose reduces membrane permeability and prevents the influx of excess salts during dehydration.

As the plasma membrane is the cellular component most affected by ethanol stress, cells respond to this stress by remodelling their membrane. The main change is in the lipid composition, increasing the unsaturatty faty acids (UFAs) and ergosterol content (Ma and Liu, 2010a; Auesukaree, 2017; Vamvakas and Kapolos, 2020; Lairón-Peris et al., 2021), since high amounts of ergosterol and UFAs, especially oleic and palmitoleic acid, are associated with higher ethanol tolerance in yeast (reviewed in Ma and Liu, 2010a; Auesukaree, 2017). This increase in membrane integrity also prevents protons from entering inside the cell. However, it is still necessary to eliminate protons that have already entered the cell to restore the normal intracellular pH. Therefore, the cell induces the H^+-ATPases of plasmatic and vacuole membranes to pump protons outside of the cell or inside the vacuole (Vamvakas and Kapolos, 2020). There are additional mechanisms that are activated under ethanol stress, such as a decrease in the RNA synthesis rate, accumulation of proteins, or remodelling of the cell wall to be less sensitive to digestion (reviewed in Ma and Liu, 2010a; Stanley et al., 2010).

2.4.3. Ethanol stress response signalling

The adaptation of cells to ethanol results from the coordinated action of the following stress responses: the ESR and the specific responses to oxidative stress and heat-shock stress (Figure 8) (reviewed in Stanley et al., 2010; Teixeira et al., 2011). First, Msn2/4 controls different key proteins for ethanol resistance, such as trehalose metabolism genes (reviewed in Ma and Liu, 2010a; Teixeira et al., 2011). As ethanol triggers an oxidative burst, yeast cells induce the response to oxidative stress through

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Yap1 activation by mitochondrial SOD (Zyrina et al., 2017). Nevertheless, its role seems to be less essential in this case than in the response to oxidative stress, indicating that other mechanisms are involved in the response to ethanol stress (Bleoanca et al., 2013). The response is similar to the heat-shock response because both induce changes in the composition of the lipid membrane and the induction of specific proteins, such as HSP and H^+-ATPase. Moreover, high ethanol concentrations also cause osmotic stress, suggesting that specific targets of the HOG pathway could also be involved in the response to ethanol stress (Alexandre et al., 2001; Ma and Liu, 2010b; Vamvakas and Kapolos, 2020).

Moreover, there are some specific responses to ethanol stress, mostly activated by mitochondrial dysfunction, because mitochondria have a dual role in ethanol tolerance, since they are the main site of ROS production, but at the same time, they are required to activate stress-responsive genes (González et al., 2017; Zyrina et al., 2017; Jing et al., 2020). Additionally, some of the signalling pathways of the ethanol response are correlated with the nutrient availability response. Protein denaturalization and intracellular acidification cause inhibition of the cAMP/Protein Kinase A (PKA) pathway as follows: in normal conditions, the HSP70 family can interact with Cdc25p to activate the Ras2/PKA pathway. When there is ethanol stress, HSP are required to participate in the refolding of denaturalized proteins to restore native protein conformation for normal functions. This decreases the interactions between HSP and Cdc25p, reducing the activity of the cAMP-PKA pathway, and Msn2/4 and Yap1p can be translocated to the nucleus, where they activate stress responses (reviewed in Ma and Liu, 2010a).

3. MITOCHONDRIA

The term *mitochondrion* is derived from the Greek words *mitos* (thread) and *chondros* (grain), as they appear as threads of granules (Westermann, 2012). The most favourable model to explain the origin of mitochondrion is through the endosymbiosis theory. This theory explains that mitochondria are the remnants of an ancient α-proteobacteria that became established in a nucleus-containing cell (Figure 15). Therefore, its genome has been partly lost or transferred to the nucleus throughout evolution (Gray et al., 1999).

Mitochondria are the respiratory organelles. For some time, mitochondria were thought to degenerate and lose their function in anaerobiosis, but it is currently known that although there is markedly less mitochondrial mass and mitochondria are less active in anaerobic than aerobic conditions (glucose transcriptionally represses respiratory enzymes and mitochondrial development), they are still present in anaerobiosis, as mitochondria have functions other than respiration (reviewed in Trumbly, 1992; O'Connor-Cox et al., 1996; Ribéreau-Gayon et al., 2006; Westermann, 2012).
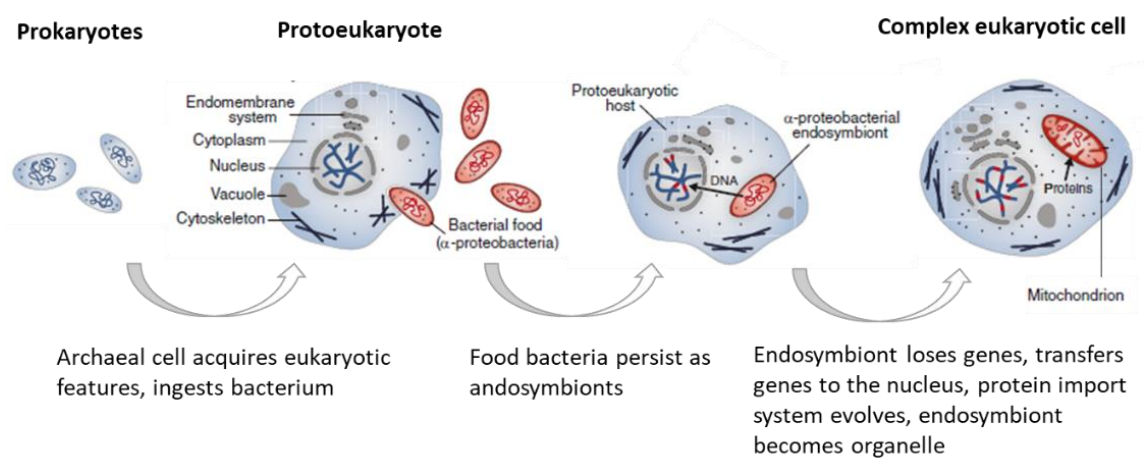
Figure 15. Schema of the traditional hypothesis of the origin of eukaryotic cells and their mitochondria (endosymbiosis) (adapted from Archibald, 2015).

Importance of mitochondria

Mitochondria are considered the cell's energy source because they are where most of the cellular ATP is produced through respiratory metabolism (they host the TCA cycle and oxidative phosphorylation). However, only 15% of the mitochondrial proteins are involved in energy metabolism (Malina et al., 2018), so mitochondria are involved in other cellular processes, such as fatty acid oxidation, amino acid degradation, metabolism of intermediate metabolites, synthesis of iron-sulfur clusters and heme, which provide metabolic precursors for the generation of macromolecules, such as lipids, proteins, DNA and RNA (Baccolo et al., 2018; Malina et al., 2018). Moreover, mitochondria have a key role in the regulation of Ca^{2+} homeostasis, apoptosis, mitophagy, ageing and maintaining the cellular redox state (Figure 16) (Westermann, 2010; Mishra and Chan, 2016; Baccolo

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et al., 2018; Malina et al., 2018; Liu et al., 2021; Loncke et al., 2021; Wanderoy et al., 2021).
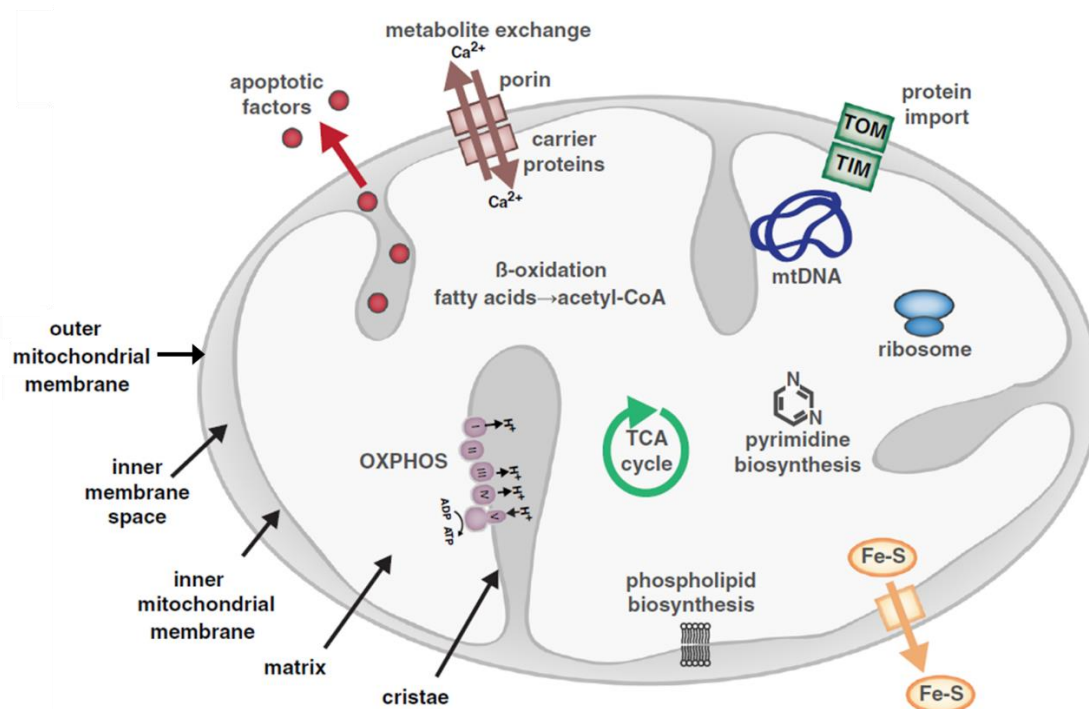
Figure 16. The form and functions of yeast mitochondria. In addition to ATP production via oxidative phosphorylation (OXPHOS), mitochondria play critical roles in phospholipid biosynthesis, metabolite exchange/buffering, β-oxidation of fatty acids, iron-sulfur cluster biogenesis, pyrimidine biosynthesis and the storage and release of apoptotic factors. TCA, tricarboxylic acid (Lackner, 2014).

Mitochondria are the centre of a "vicious circle", since they are the main site of ROS production but also the main target of ROS. ROS produced by the ETC damage mitochondria, leading to increased ROS production and mitochondrial dysfunction (Venditti and Di Meo, 2020). During respiration, ROS are formed in the ETC (Figure 17) due to the leakage of electrons from their carrier systems, which leads to an incomplete reduction of oxygen and the generation of superoxide ($O_2^{\cdot-}$), which is converted to hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), as explained previously (Beckman and Ames, 1998; Baccolo et al., 2018; Malina et al., 2018). These ROS species are released into the intermembrane space and the mitochondrial matrix (Malina, 2018). However, as $O_2^{\cdot-}$ cannot cross membranes at substantial rates, its products ($\cdot OH$ and H_2O_2) are those

that attack cellular components (such as lipids, proteins or DNA, explained above) and damage mitochondria (reviewed in Hardeland, 2017; Malina et al., 2018).

Functional mitochondria are essential for basal resistance to both endogenous and exogenous oxidative stress not only during aerobic growth but also during fermentation (Grant et al., 1997; Elliott and Volkert, 2004). This is because mitochondria host many antioxidant enzymes (Jamieson, 1998; Ukai et al., 2011; Volkov et al., 2011; Larosa and Remacle, 2018) and are the main source of ATP needed to combat ROS (Grant et al., 1997). Moreover, mitochondria activate signalling mechanisms in response to low and high levels of ROS that protect cells and mitochondria against dysfunction by triggering the antioxidant system to prevent the inactivation of ETC components, the TCA cycle and other proteins or even mitochondrial dysfunction, as explained below (Westermann, 2010; de la Torre-Ruiz et al., 2015; Venditti and Di Meo, 2020).

As mitochondria play a key role in energy metabolism and the maintenance of cellular homeostasis, it is very important that they remain in communication with the nucleus and cytoplasm to coordinate the response to extra and intracellular changes (Guaragnella et al., 2018). Therefore, there are very complex signalling pathways that control mitochondrial gene expression from the nucleus (anterograde regulation) and nuclear gene expression from the mitochondria (retrograde regulation). The anterograde pathway mainly involves signalling pathways in response to nutritional changes (such as carbon catabolite repression), such as the cAMP/PKA, TOR-Sch9 and Snf1-Mig1, which regulate mitochondrial biogenesis and activity and consequently cell growth and proliferation. The retrograde pathway (RTG) mainly involves the response to stress, generally activated by mitochondrial dysfunction caused by environmental changes that trigger alterations in the mitochondrial membrane potential, mutants in TCA or OXPHOS genes, deletions of mtDNA, ATP depletion or ROS production. Dysfunctional mitochondria can trigger organelle degradation (mitophagy) or even cell death (apoptosis), regulating different cellular processes and cellular stress response pathways, such as ageing or cell quality control, to restore metabolic fitness (de la Torre-Ruiz et al., 2015; Guaragnella et al., 2018).

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Therefore, mitochondria have a role in protecting the cell against fermentation-associated stresses, mainly those that produce ROS, such as oxidative and ethanol stresses, as well as dehydration stress (Picazo et al., 2015; Chen et al., 2021).

3.1. Mitochondria structure

Mitochondria are organelles surrounded by two membranes, the inner membrane (IMM) and the outer membrane (OMM), with an intermembrane space between them. The membranes are physically connected by various protein complexes. The IMM, which delimits the mitochondrial matrix, has *cristae*, which are large membrane invaginations where respiratory chain complexes are accommodated and significantly enlarge the surface of the IMM (Figure 16) (Ribéreau-Gayon et al., 2006; Unger et al., 2017).

3.1.1. Mitochondrial membranes

The mitochondrial membranes are mainly composed of phospholipids. The major classes of phospholipids in these membranes are similar to those of other membranes, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), but some are exclusive to the mitochondrial membrane, such as cardiolipin (CL) (Zinser and Daum, 1995; Schenkel and Bakovic, 2014). Cardiolipin is necessary for the activity and organization of the ETC, since it binds complexes III and IV and helps to form respiratory supercomplexes (Joshi et al., 2009; Luévano-Martínez et al., 2013).

Most mitochondrial proteins are synthesized in the cytoplasm. In fact, only eight major proteins are synthesized within the mitochondria in *S. cerevisiae*, including some subunits of respiratory complexes III, IV and V (Foury et al., 1998). Therefore, the remaining proteins (approximately 1000), encoded in the nucleus, must be imported into their respective mitochondrial compartment (Malina et al., 2018). For this process, the translocase of the outer (TOM) and inner (TIM) membrane complexes (Figure 16) and the presequence-associated motor (PAM) are needed (Muro et al., 2003; Malina et al., 2018). On the other hand, mitochondria are involved in a number of different pathways that require the exchange of metabolites between the cytosol and mitochondrial matrix; therefore, many metabolites are imported (FAD, NAD^+, adenosine diphosphate (ADP), Pi,

pyruvate, iron) and exported (intermediates of the TCA cycle, ATP and ornithine) through mitochondrial membranes. Metabolites cross OMM using the porins contained in this membrane, whereas they use the mitochondrial carrier family (MCF) to cross IMM, since it is highly impermeable to most molecules and only allows the free diffusion of small uncharged molecules, such as oxygen and carbon dioxide (Palmieri et al., 2006; Rutter and Hughes, 2015; Malina et al., 2018; Pfanner et al., 2019).

Oxidative phosphorylation

Oxidative phosphorylation (OXPHOS) results from the coupling between the ETC and F_1F_0-ATP synthase (complex V). The OXPHOS system converts, through redox reactions, the high-energy electrons of NADH and FAD into ATP (2 molecules per pair of electrons), which can be used in the mitochondrial matrix or transported to different mitochondrial or cellular compartments. The ETC is located in the IMM *cristae* and consists of the following enzymatic systems: NADH dehydrogenases, succinate dehydrogenase (complex II), cytochrome reductase (complex III) and cytochrome oxidase (complex IV), linked by two electron transport systems (ubiquinone/coenzyme Q and cytochrome *c*) (Figure 17) (Ribéreau-Gayon et al., 2006; Baccolo et al., 2018).

Electrons can enter the ETC using two pathways. The first is by oxidation of NADH via NADH dehydrogenases (also called type II NADH:ubiquinone oxidoreductases). This system replaces complex I of mammalian cells in yeast. The other pathway is through succinate dehydrogenase, which links the ETC and the TCA. In this pathway, succinate is oxidized to fumarate in the TCA cycle and generates $FADH_2$ as a part of the ETC. The electrons from both oxidations are transferred to ubiquinone, which is reduced to ubiquinol. The electrons are then passed to cytochrome *c* reductase, which reduces cytochrome *c*. Finnally, cytochrome *c* oxidase, the last step in the flux of electrons, oxidizes the reduced cytochrome *c* and performs a four-electron reduction of the molecular oxygen, producing two molecules of water (Figure 17). The transfer of electrons by complexes III and IV is accompanied by the translocation of protons from the matrix into the intermembrane space, named proton pumping. This generates a proton motive force across the membrane, generating an electrochemical gradient that is exploited by

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ATP synthase (Complex V; F_1F_0 ATPase) to drive the phosphorylation of ADP to ATP (Figure 17) (de Vries and Marres, 1987; Sakamuru et al., 2016; Baccolo et al., 2018).
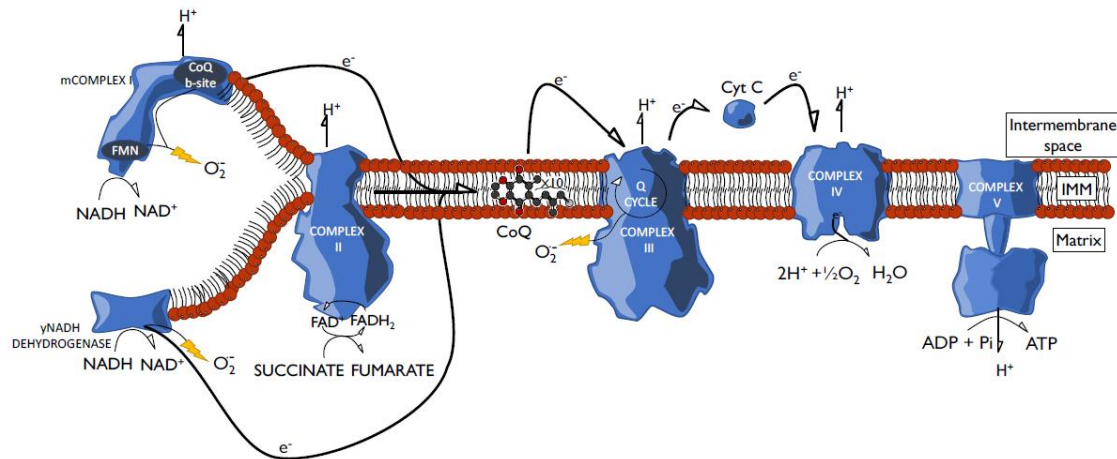
Figure 17. Schematic representation of the oxidative phosphorylation (OXPHOS), including the electron transfer along the electron transport chain, ATP production, and the main sites involved in ROS generation. CoQ B-site, CoQ biding-site; CoQ, coenzyme Q; Cyt C, cytochrome *c*, FMN, flavin mononucleotide; IMM, inner mitochondrial membrane, mCOMPLEX I, mammal complex I, yNADH DEHYDROGENASE, yeast NADH dehydrogenase (Baccolo et al., 2018).

Complexes III and IV can form supercomplexes that assemble or dissociate depending on physiological conditions, conforming to different associations: a dimer of III (III_2) with one (III_2IV) or two (III_2IV_2) copies of complex IV (Figure 18) (Schägger and Pfeiffer, 2000; Böttinger et al., 2012; Acin-Perez and Enriquez, 2014). Their assembly, which depends on the supercomplex assembly factors Rcf1 and Rcf2 and their association with cardiolipin, affects complex IV activity, optimizing the respiratory rate (Zhang et al., 2005; Acin-Perez and Enriquez, 2014; Strogolova et al., 2019). Complex IV (cytochrome *c* oxidase) plays an important role in ETC regulation. On the one hand, it is the rate-limiting step of the ETC (Piccoli et al., 2006), and on the other hand, its activity seems to be an indicator of the oxidative capacity of cells since its defect causes an ROS increase, promoting a signalling pathway that blocks cell growth (Piccoli et al., 2006; Srinivasan and Avadhani, 2012; Bode et al., 2013).
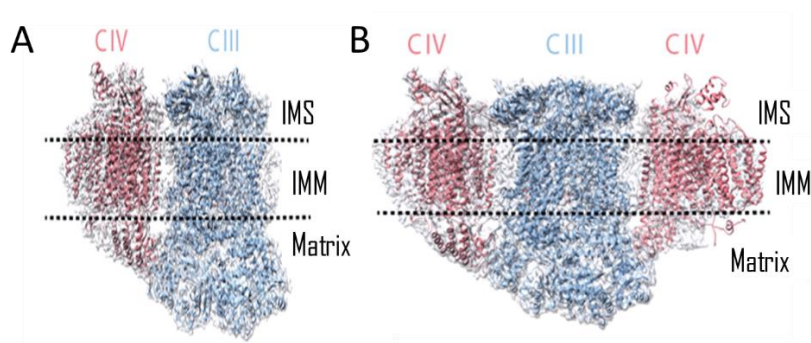
Figure 18. Cyro-EM structures of different classes of the *S. cerevisiae* supercomplex. (**A**) Supercomplex class 1 (III$_2$IV), (**B**) supercomplex class 2 (III$_2$IV$_2$). The structural models fitted into the density of complex III (CIII) or complex IV (CIV) are colored in blue and red, respectively. IMM, inner membrane; IMS, intermembrane space, The transmembrane region is indicated by two black dashed lines (adapted from Rathore et al., 2019).

Mitochondrial membrane potential

The electrochemical gradient across the inner membrane produced by ETC proton pumping generates the mitochondrial membrane potential ($\Delta\Psi_m$), a key parameter to evaluate mitochondrial function (Sakamuru, 2016). A decrease in $\Delta\Psi_m$ is a common and dangerous situation associated with OXPHOS impairment for several reasons including the following: (i) $\Delta\Psi_m$ powers ATP synthesis by Complex V; (ii) it drives the insertion of some proteins into the IMM; and (iii) it drives the import of ions and proteins into the mitochondria, such as Ca^{2+}, regulator of mitochondrial respiration or Fe^{2+}, required for the biogenesis of Fe-S clusters (Fe-S clusters are required for OXPHOS function) (Chacinska et al., 2009; Malina et al., 2018; Zorova et al., 2018). Several oxidants (such as H_2O_2 or menadione) decrease $\Delta\Psi_m$ (Trendeleva et al., 2011). In fact, $\Delta\Psi_m$ appears to be tightly regulated, since dysfunction in OXPHOS activates responses to maintain $\Delta\Psi_m$, and a decrease in $\Delta\Psi_m$ could be the signal to activate some signalling pathways, such as the RTG pathway (Suhm and Ott, 2017; Liu et al., 2021).

3.1.2. Mitochondrial matrix

The mitochondrial matrix is the hub for metabolic processes, such as the TCA cycle; the synthesis of some amino acids, mitochondrial lipids or hemes; iron-sulfur cluster

biogenesis; and the oxidation of fatty acids (Ribéreau-Gayon et al., 2006; Malina et al., 2018). Moreover, it also encloses mitochondrial DNA (mtDNA), which represents on average 15% of the total cellular DNA and contains 35 genes encoding eight mitochondrial proteins. Seven of those are subunits of respiratory complexes (cytochrome *b* of cytochrome *c* reductase, central subunits 1, 2 and 3 of cytochrome *c* oxidase and subunits 6, 8 and 9 of ATP synthase), and one is a ribosomal protein of the small subunit (Ribéreau-Gayon et al., 2006; Malina et al., 2018). Mitochondria also have the machinery for the synthesis of proteins encoded in the mitochondria, such as mitoribosomes (which are anchored in the membrane) (Malina et al., 2018; Carlström et al., 2021).

3.2. Mitochondria morphologies and dynamics

The morphology of mitochondria consists of tubular networks that dynamically fuse and divide, causing changes from an elongated filamentous network to fragmented mitochondrial structures (Figures 19, 20) (Scott and Youle, 2010; Westermann, 2012).
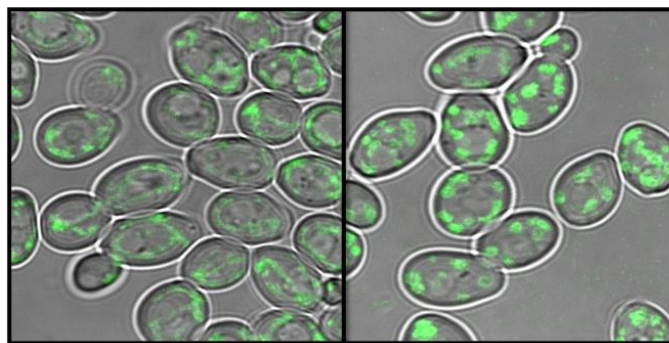
Figure 19. Microscopy images of the mitochondrial structure. Filamentous network (left) and fragmented network (right).

The cycles of fusion and fission favour the maintenance of mitochondrial integrity and functionality, preventing cell damage, and allowing mitochondria to modify their morphology to adapt to physiological changes (Venditti and Di Meo, 2020). Therefore, these dynamic processes are essential to have a healthy mitochondrial population, which affects cell health (Westermann, 2010; Mishra and Chan, 2016; Venditti and Di Meo, 2020).

An increase in fusion builds extended and interconnected mitochondrial networks (Figure 20), enabling the mitochondrial compartment to mix and unify for a more functional and structurally homogenous network. This more structured network is beneficial in active respiratory cells for several reasons including the following: (i) it allows efficient mixing of metabolites and enzymes throughout the entire mitochondrial network, enabling constituents of the respiratory machinery to cooperate more efficiently, as well as providing a better antioxidant defence organization; (ii) it allows the complementation of gene products in cells that carry different mtDNA mutations, decreasing the manifestation of respiratory deficiencies (Westermann, 2010, 2012; Malina et al., 2018); and (iii) it generates extended electrically unified networks that facilitate the dissipation of energy in the form of membrane potential, enabling the production of ATP, even in remote parts of the cell, where oxygen supply is low (Figure 20) (Skulachev, 2001). Moreover, the maintenance of mtDNA depends on fusion, since when mitochondria are fragmented, there is a loss of mtDNA (Wang et al., 2014b; Venditti and Di Meo, 2020; Viana et al., 2020). Therefore, fusion contributes to the maintenance of respiratory capacity.
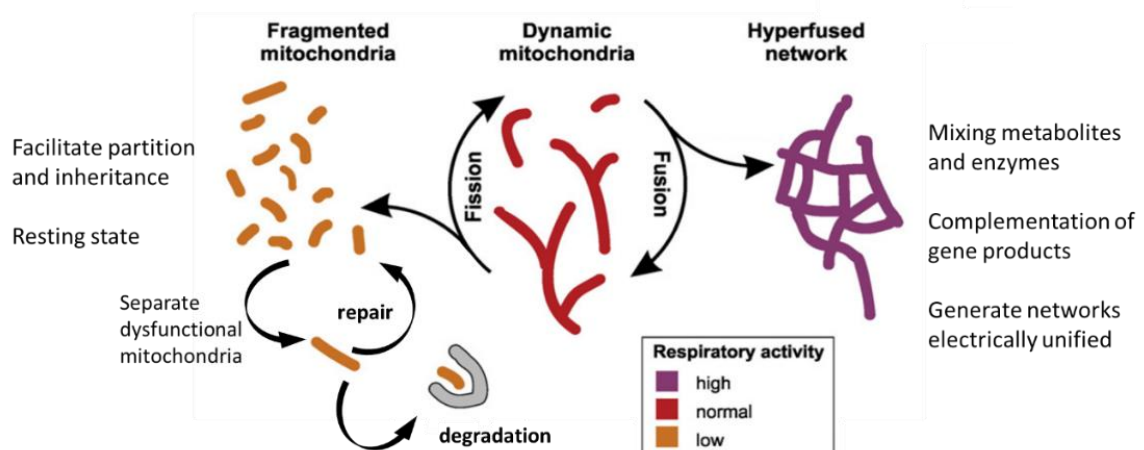
Figure 20. Schema showing dynamic fusion and fission of mitochondria, with the benefits of fragmented (left) or hyperfused (right) network. When a mitochondria is dysfunctional, it can get repaired and mix with the rest of the network of be cleared, normally by mitophagy (adapted from Westermann, 2012; Lackner, 2014).

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An increase in fission generates numerous morphologically and functionally distinct small spherical organelles (Figure 20) (Westermann, 2010). Fission has several functions, such as facilitating the partition and inheritance of organelles during cell division, generating transportable mitochondrial units to move along the cytoskeleton, and maintaining bioenergetic capacity in cells when high respiratory activity is not required (such as in resting cells) (Figure 20) (Skulachev, 2001; Klecker et al., 2013). Additionally, fission serves to separate dysfunctional mitochondria (for example, those that accumulate ROS) from the network, allowing them to be repaired or eliminated by mitophagy (if they are irreversibly damaged) (reviewed in Westermann, 2010, 2012; de la Torre-Ruiz et al., 2015). Therefore, fission seems to regulate apoptosis and ageing and appears to be a feature of programmed cell death. This last function seems to constitute a mechanism to control mitochondrial quality that contributes to the bioenergetic capacity maintenance (Westermann, 2010; Venditti and Di Meo, 2020).

Therefore, mitochondrial morphology will change depending on the state of the cell, such as carbon metabolism or the cell growth phase (Westermann, 2010). During fermentation, the mitochondrial network consists of relatively few tubules and branches, while during respiration, the structure becomes much larger and more ramified (Kitagaki and Takagi, 2014). During the exponential phase, mitochondria are highly dynamic (up to 2.5 fusion and fission events per minute) and are elongated and located throughout the periphery near the point of oxygen entry. In the stationary phase, the network is fragmented, forming many small, round mitochondria (Westermann, 2012). Moreover, several stresses induce mitochondrial fragmentation (acetic acid, ethanol, H_2O_2) since fission seems to provide stress resistance, although it is not clear whether it is through the regulation of $\Delta\Psi_m$ or directly through mitochondrial dynamics (Fannjiang et al., 2004; Pozniakovsky et al., 2005; Kitagaki et al., 2007; Knorre et al., 2008; Westermann, 2010; Galkina et al., 2020; Chen et al., 2021).

As fission and fusion machineries (scheme in Figure 21) are always active, even when the other is absent (Westermann, 2010), the regulation of mitochondrial dynamics is very complex, since a right balance must be maintained between both machineries. Therefore, the activity of the key proteins of mitochondrial dynamics is regulated at

multiple levels, such as transcription, posttranslational modification, and possibly the direct response to the bioenergetic state of mitochondria (decrease in membrane potential causes fission), ER/mitochondria contacts (dynamics are spatially coordinated at ER membrane contact sites) or perhaps the availability of nucleotide triphosphate (Chang and Blacksone, 2010; Abrisch et al., 2020; Galkina et al., 2020; Chen et al., 2021).
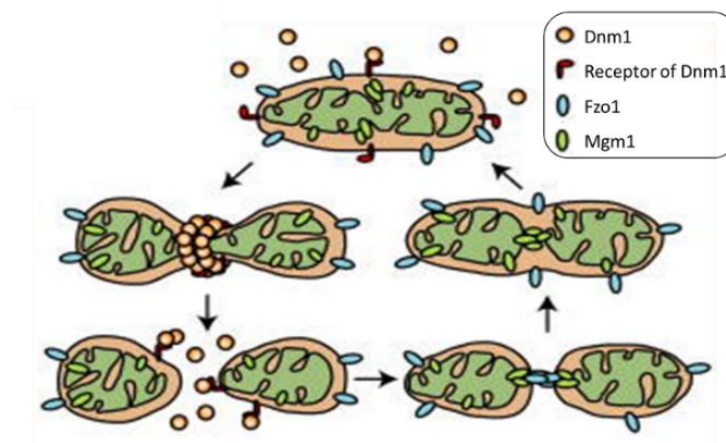
Figure 21. Scheme of the machineries of fusion-division cycle in yeast. The proteins Fzo1 and Mgm1 are the main proteins responsible for fission of the outer and inner membrane, respectively. The GTPase Dnm1 is the main protein responsible for fission of both mitochondrial membranes (adapted from Tamura, 2011).

4. BIOACTIVE COMPOUNDS IN WINE

Wine has traditionally been produced and consumed in the Mediterranean area, and some studies suggest that it contributes to the beneficial health properties of the Mediterranean diet (Fernández-Mar et al., 2012). Since the study of the "French Paradox", in which it was confirmed that a dietary intake of wine decreased the risk of death by cardiovascular disease (Renaud and Lorgeril, 1992), intense research has been carried out to establish the relationship between wine and health. To date, studies suggest that a regular diet with a daily supplementation of 1–2 glasses of red wine reduces cardiovascular risk and improves the antioxidant capacity in plasma (Fernández-Mar et al., 2012; Vilela, 2019). Research has revealed that the main compounds responsible for these effects are polyphenols, such as resveratrol, gallic acid, caffeic acid and catechin (Minussi et al., 2003; Fernández-Mar et al., 2012; Vilela, 2019). In fact, as polyphenols are

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highly related to both wine quality (colour, flavour, taste) and health-promoting properties (antioxidant, cardioprotective), the wine industry is highly interested in studying how the phenolic composition can be modulated (Gutiérrez-Escobar et al., 2021).

Grape must contain a variety of nitrogen compounds, among which the most important are amino acids, ammonium ions and small peptides (Pretorius, 2000). Among them, aromatic amino acids contribute to the aroma of wine, especially with the synthesis of aromatic higher alcohols by the Ehrlich pathway, which can also modulate physiological processes or signalling pathways in yeast. Moreover, there are other metabolites derived from aromatic amino acids, such as melatonin, serotonin or hydroxytyrosol, that are putative bioactive molecules with interesting properties (Mas et al., 2014; Hornedo-Ortega et al., 2016). In this introduction, we will focus on melatonin and hydroxytyrosol.

4.1. Melatonin

Melatonin, or N-acetyl-5-methoxytryptamine, is an indoleamine, also known as a neurohormone because it was first discovered in the bovine pineal gland (Lerner et al., 1958, 1959) and initially considered unique in vertebrates. However, melatonin is a ubiquitous molecule that is present in most living organisms, including vertebrates, invertebrates, vesicular plants, protozoa, bacteria, algae, or fungi (Hardeland and Poeggeler, 2003; Que et al., 2020).
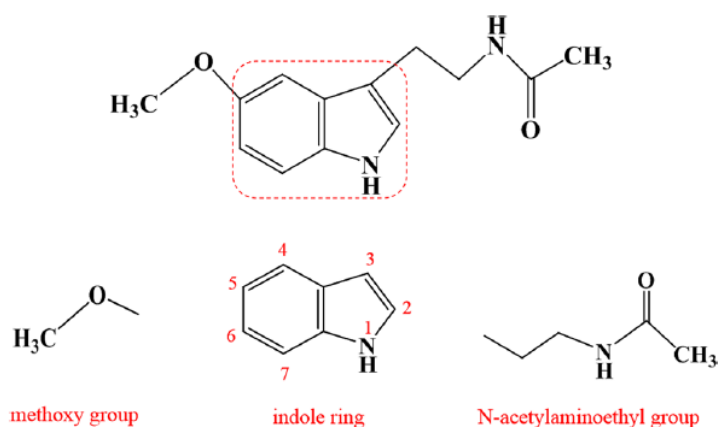
Figure 22. Structure of melatonin (Que et al. 2020).

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In mammals, melatonin production is regulated according to light and dark conditions by the suprachiasmatic nucleus (SCN) of the hypothalamus, which maintains the physiological circadian rhythm (reviewed in Bhattacharya et al., 2019; Kennaway, 2019; Chitimus et al., 2020; Ferlazzo et al., 2020). In the absence of light, the SCN stimulates the production of melatonin (Claustrat et al., 2005), which is why its best-known role is as a regulator of sleep and circadian rhythm.

Melatonin can be found in several body parts because it is distributed by the blood and cerebrospinal fluid and because it is synthesized in extrapineal tissues (gastrointestinal tract, ovaries, lymphocytes, macrophages, bone marrow, retina, heart, liver, placenta, skin). Moreover, due to its amphipathic structure and low molecular weight (Figure 22), melatonin can easily diffuse through cell membranes, so it spreads both in intracellular and extracellular spaces, and is able to perform its effect locally (Tan et al., 2015; Bhattacharya et al., 2019; Chitimus et al., 2020; Ferlazzo et al., 2020). Melatonin can also cross membranes through the transporters of oligopeptides (such as PEPT1/2) or glucose (Hevia et al., 2015; Huo et al., 2017; Mayo et al., 2018).

In addition to regulating circadian rhythms, melatonin exhibits numerous physiological functions in several parts of the body. Among them, its powerful antioxidant activity is the most relevant, although it can also modulate the anti-inflammatory, anti-ageing and autophagy inhibition responses (Acuña-Castroviejo et al., 2003; Reiter et al., 2016, 2017). Melatonin is normally considered an immune stimulatory agent; however, there is growing evidence of its dual role as both a pro- and anti-inflammatory regulator, although what determines which immune response model is promoted is not fully understood (Hardeland, 2017; Chitimus et al., 2020; Ferlazzo et al., 2020). Moreover, the potential role of melatonin in the treatment of various pathologies, which have in common alterations in the redox state and inflammation, has recently been evidenced due to its antioxidant and anti-inflammatory properties. Thus, melatonin can modulate a variety of neuronal, endocrine, immune, cardiovascular and reproductive functions (Figure 23) (Romero et al., 2014; Eghbal et al., 2016; Chitimus et al., 2020; Ferlazzo et al., 2020), playing a role in the prevention and treatment of diseases such as osteoporosis, obesity, cardiovascular diseases (atherosclerosis), neurodegenerative diseases

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(Alzheimer's and Parkinson's disease), infertility or immune disorders (Hornedo-Ortega et al., 2016; Cerezo et al., 2017; Ferlazzo et al., 2020). For the latter, due to the current pandemic of COVID-19, a beneficial effect of melatonin has recently been postulated against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Ferlazzo et al., 2020; Zhang et al., 2020). Additionally, melatonin also exhibits specific actions that do not completely rely on its antioxidant and anti-inflammatory properties (Ferlazzo et al., 2020; Reiter et al., 2020).
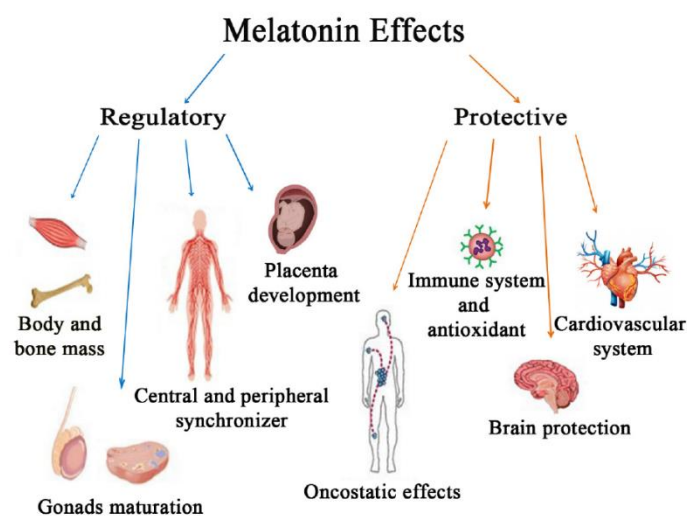
Figure 23. Pleiotropic actions of melatonin in human body (Ferlazzo et al., 2020).

4.1.1. Melatonin as an antioxidant

Melatonin is a powerful antioxidant that is more effective than classical antioxidants, such as vitamins E and C (Martín et al., 2000b; Qi et al., 2000), and acts both intracellularly and extracellularly and protects against highly toxic oxygen and nitrogen free radicals (ROS and RNS).

The antioxidant function of melatonin is believed to be exerted in all the organisms in which it is present (Tan et al., 2015; Que et al., 2020). Indeed, to date, it has been found to exert antioxidant action on several species, such as plants, the macroalga *Ulva* sp. (Tan et al., 2015) or yeasts (Vázquez et al., 2017, 2018). Melatonin was speculated to neutralize the toxic molecular oxygen derivatives produced during photosynthesis in early

cyanobacteria (more than 3.5-3.2 billion years ago), suggesting that being an antioxidant was its first role. Thus, melatonin synthetic machinery in eukaryotes would have been inherited from bacteria as a result of endosymbiosis (Manchester et al., 2015; Tan et al., 2015; Reiter et al., 2017).
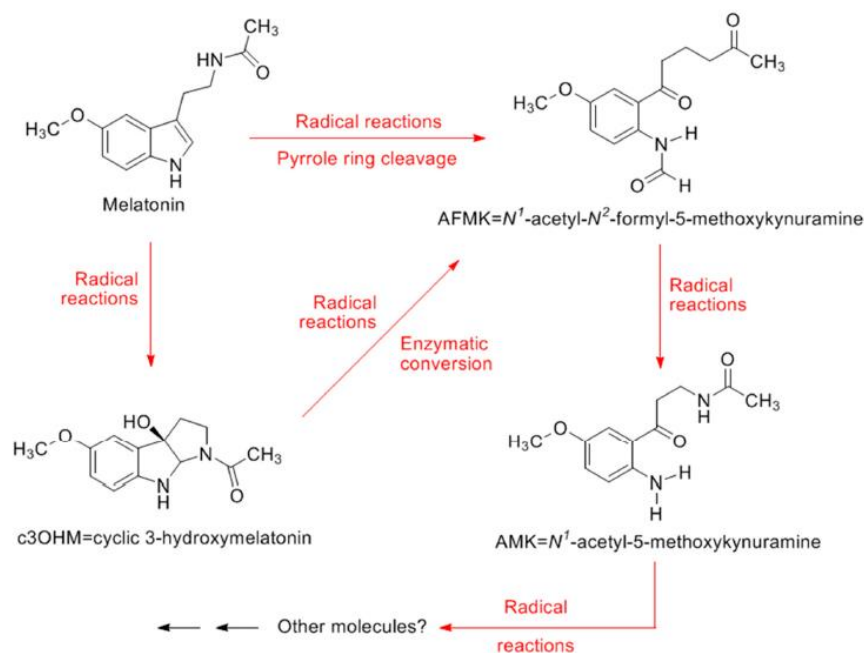
Figure 24. Structure of melatonin and some of its metabolites that detoxify reactive oxygen and nitrogen species (melatonin's antioxidant cascade), shown in the sequence by which the molecules are formed (Reiter et al., 2018).

Melatonin has antioxidant effects that are exerted through direct and indirect mechanisms (Chitimus et al., 2020; Ferlazzo et al., 2020). The direct mechanisms consist mainly of free radical scavenging, for which melatonin penetrates into the cell and intracellular compartments (Galano et al., 2011). Indeed, melatonin reactions with ROS and RNS lead to a cascade reaction mechanism with amplified antioxidant effects, since the generated molecules (such as AFMK (N1-acetyl-N2-formyl-S-methzykynuramine) or AMK (N1-acetyl-5-methozykynuramine)) (Figure 24) also have antioxidant functions that limit oxidative damage, prevent DNA and protein damage and lipid peroxidation, or modulate mitochondrial metabolism (reviewed in Reiter et al., 2007; Tan et al., 2015; Chitimus et al., 2020; Ferlazzo et al., 2020). This cascade is a feature that is not reported in classical antioxidants and increases the antioxidant capacity of melatonin, since one

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molecule can scavenge up to ten ROS. Moreover, melatonin has other direct effects, such as interactions with nonradical oxidants (such as H_2O_2, $^1O_2$ (singlet oxygen), HNOO (peroxynitrite)), inhibiting metal-induced oxidation or chelating metals (iron, lead, zing, aluminium, copper), leading to a chelating cascade that contributes to the reduction of oxidative stress (Manchester et al., 2015; Chitimus et al., 2020).

The indirect mechanisms consist of the activation of signalling pathways, either mediated by its membrane receptors (MT1 and MT2) (Slominski et al., 2012) or by direct interaction with other molecules (Amaral and Cipolla-Neto, 2018). Through these signalling pathways, melatonin stimulates the expression and activity of endogenous antioxidant enzymes and molecules related to antioxidant activity (such as GSH). Moreover, melatonin can also cooperate with other antioxidants, which increases its efficiency (Tan et al., 2016; Chitimus et al., 2020; Ferlazzo et al., 2020).

Melatonin in the mitochondria

Mitochondria are the main target of melatonin in mammals and several other species (Venegas et al., 2012; Reiter et al., 2016) because they are the site of the highest production of free radicals (Cardinali and Vigo, 2017). In humans, melatonin is produced in the mitochondria or imported through transporters, such as the oligopeptide transporter PEPT1/2 (Huo et al., 2017; Suofu et al., 2017). This confers an advantage to melatonin in comparison to other antioxidants, since it can react faster to mitochondrial oxidative stress, as it is already in the mitochondria (Reiter et al., 2017, 2020).

Therefore, the antioxidant activity of melatonin has a pivotal role in preventing mitochondrial dysfunction (Tan et al., 2016), since it also influences specific processes of the mitochondria (Figure 25). As the main target and producer of ROS is the ETC, melatonin modulates the activity of its complexes (mainly complex IV) by increasing or restoring them from the effects of stress. The preservation of ETC activity is associated with the improvement of respiratory efficiency and the reduction of superoxide formation, resulting in ATP production. Moreover, melatonin contributes to the maintenance of the optimal mitochondrial membrane potential, limits mitochondrial-related apoptosis, and regulates mitochondrial biogenesis and dynamics to preserve

mitochondrial functions (reviewed in Acuña-Castroviejo et al., 2003; Tan et al., 2016; Hardeland, 2017; Reiter et al., 2018, 2020; Chitimus et al., 2020).

The preservation of mitochondrial morphology and function is important for healthy cells, as several studies have reported that melatonin production is important for regulating normal mitochondrial functionality, but it is also beneficial for reducing disease progression from disorders associated with mitochondrial dysfunction, such as Parkinsonism, Huntington's disease, multiple sclerosis, cancer or Alzheimer's disease (reviewed in Reiter et al., 2016, 2017; Tan et al., 2016; Hardeland, 2017). Moreover, the blockade of melatonin production by several disorders, such as inflammation, can have repercussions on mitochondrial metabolism (Ferlazzo et al., 2020).
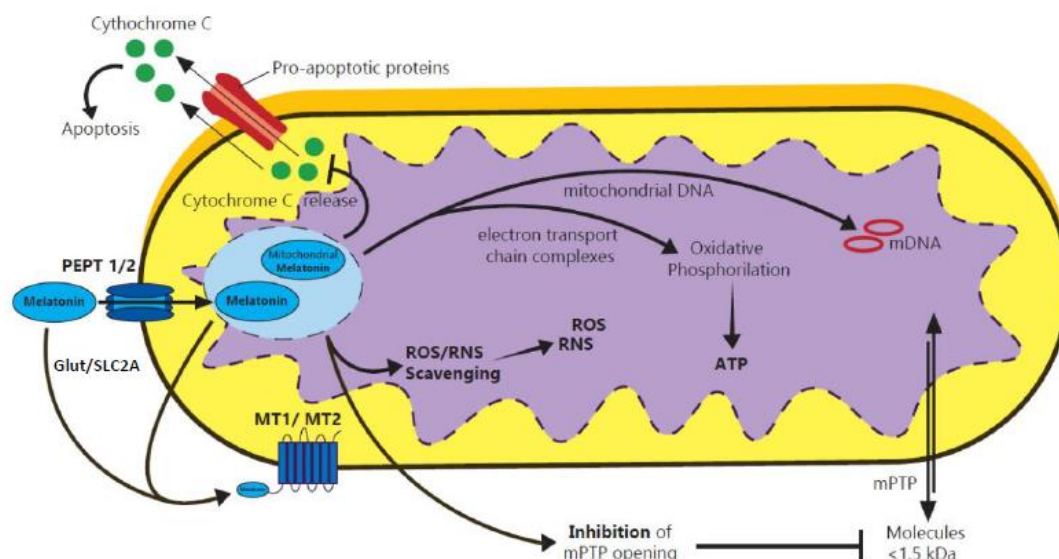
Figure 25. Effects of melatonin in the mitochondria. ROS, reactive oxygen species; RNS, reactive nitrogen species; mPTP, mitochondrial permeability transition pore (Chitimus et al., 2020).

4.1.2. Melatonin in wine

Melatonin has been found in several food products derived from fermentation (wine, beer, bread), and to date, red wine is the fermented product with the highest concentration of melatonin reported (Muñiz-Calvo et al., 2020a; Que et al., 2020). In fact, red wine supplemented with melatonin has been demonstrated to represent a dietary

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source, since the intake of a glass of melatonin-enriched red wine increased the serum levels of this indolamine (Varoni et al., 2018). Indeed, synthetic melatonin has been approved as a dietary supplement for the treatment of insomnia by the American Academy of Family Physicians (Savage et al., 2021), whereas the European Food Safety Authority (EFSA) gave a scientific opinion evidencing a relationship between melatonin consumption (a dose between 0.5 and 5 mg) and the reduction of jet lag and the improvement of sleep quality (EFSA, 2010). Therefore, interest in the study of melatonin production during winemaking has increased.

In 1999, Sprenger et al. (1999) showed that yeast could synthesize melatonin under laboratory conditions, and since then, many studies have reported the presence of this molecule in fermented beverages, such as wine or beer (Table 2) (Rodriguez-Naranjo et al., 2011b; Garcia-Moreno et al., 2013; Kocadağli et al., 2014; Vigentini et al., 2015; Fernández-Cruz et al., 2020). However, it was not until 2011 that Rodríguez-Naranjo et al. (Rodriguez-Naranjo et al., 2011a) demonstrated that *S. cerevisiae* plays a role in melatonin synthesis during alcoholic fermentation. Since then, several studies have evidenced the importance of *Saccharomyces* and non-*Saccharomyces* yeasts in melatonin production during fermentation (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018, 2019; Valera et al., 2019; Vilela, 2019; Morcillo-Parra et al., 2020a), detecting melatonin both intracellularly and extracellularly (Fernández-Cruz et al., 2019; Morcillo-Parra et al., 2019b, 2020b; Muñiz-Calvo et al., 2019). Moreover, during malolactic fermentation, the lactic acid bacteriun *Oenococcus oeni* can also produce melatonin (Fracassetti et al., 2020).

Increasing research on this topic have suggested that melatonin synthesis depends upon several variables, such as the yeast strain or species, its metabolic state or growth phase, the fermentation time, or the cultivar (Rodriguez-Naranjo et al., 2012; Fernández-Cruz et al., 2017, 2018, 2019; Valera et al., 2019). Furthermore, melatonin production depends on the availability of some of its precursors, mainly tryptophan but also N-acetyl serotonin or 5-methoxytryptamine (Sprenger et al., 1999; Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2019; Muñiz-Calvo et al., 2019), and on the composition of the growth medium, such as reducing sugar or nitrogen contents

(Rodriguez-Naranjo et al., 2012; Valera et al., 2019; Morcillo-Parra et al., 2020a). However, to date, a clear condition to increase melatonin synthesis is still unknown (Muñiz-Calvo et al., 2020a). The presence of melatonin during fermentation follows a zigzag pattern, appearing and disappearing throughout yeast growth, but in most yeast species, melatonin has been detected during the lag phase in the intracellular medium and during the exponential and late stationary phases in the extracellular medium (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018; Morcillo-Parra et al., 2019b, 2020b).

Table 2. Concentration of melatonin in various fermented food and beverages (Muñiz-Calvo et al., 2020a).

Fermented product	Melatonin concentration	References
Sangiovese and Trebbiano wine	0.4-0.5 ng/mL	(Mercolini et al., 2008)
Chardonnay, Malbec and Cabernet Sauvignon wine	0.16-0.32 ng/mL	(Stege et al., 2010)
Beer	51.8-169.7 pg/mL	(Maldonado et al., 2009)
Petit Verdot, Shyrah, Cabernet Sauvignon, Prieto Picudo and Tempranillo wine	140.5-277.5 pg/mL; 5.1-129.5 ng/mL	(Rodriguez-Naranjo et al., 2011b)
Cabernet Sauvignon, Merlot, Palomino Fino, Syrah, Tempranillo, Tintilla de Rota wine	74.13-423.01 ng/mL	(Rodriguez-Naranjo et al., 2011a)
Gropello, Merlot wine	5.8-8.1 ng/mL	(Vitalini et al., 2011)
Alban, Sangiovese, Trebbiano wine	0.3-1.5 ng/mL	(Mercolini et al., 2012)
Pomegranate wine	0.54-5-50 ng/mL	(Mena et al., 2012)
Red, white and dessert wines	0.05-0.62 pg/mL	(Vitalini et al., 2013)
Beer	58-169 pg/mL	(Garcia-Moreno et al., 2013)
Fermented orange juice	3.15-21.80 ng/mL	(Fernández-Pachón et al., 2014)
Beer	94.5 pg/mL	(Kocadağli et al., 2014)
Bread	138.1-341 pg/g	
Yogurt	126 pg/g	
Mulberry wine	3.41-14.2 ng/mL	(Wang et al., 2016)
Tempranillo, Garnacha wine	0.03-161.83 ng/mL	(Marhuenda et al., 2016)
Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Vijiriega and Tempranillo wine	0.07-322.70 ng/mL	(Fernández-Cruz et al., 2018)
Nebbiolo wine	0.038-0.063	(Fracassetti et al., 2019)

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Melatonin biosynthesis and detection

Melatonin is synthesized from the aromatic amino acid tryptophan. The synthesis route in mammals and plants is well-described and is shown in Figure 26. In mammals, tryptophan is converted to serotonin through hydroxylation and decarboxylation reactions, and then, serotonin is converted to N-acetyl-serotonin by the enzyme AANAT (arylalkylamine *N*-acetyltransferase), which in turn is converted to melatonin by HIOMT (hydroxyl-indole-*O*-methyltransferase) (red arrows in Figure 26) (Ferlazzo, 2020). However, despite efforts to unravel the route in yeast, to date, only one gene has been described and characterized as being involved in melatonin production (*PAA1*, homologue to the mammalian AANAT) (Ganguly et al., 2001; Liu et al., 2005), and a putative biosynthetic pathway has been proposed, which includes some steps described in plants, such as the synthesis of serotonin from tryptophan through tryptamine instead of 5-hydroxytryptophan (Figure 26) (Muñiz-Calvo et al., 2019).

Melatonin is found in very low concentrations in natural samples (femtomole range in mammal samples and nanomole range in wine (Table 2)), in which compounds with a similar structure can also be present, so the detection methods must be highly sensitive and specific (Kennaway, 2019). Since melatonin was first isolated in 1960 (Lerner et al., 1960), several methods for melatonin detection and quantification have been developed based on bioassays, chromatography separation (such as liquid chromatography (LC), gas-chromatography-mass spectrometry (GC–MS) and LC–MS) or the use of specific antibodies against melatonin as antigen (such as radioimmunoassay (RAI) and enzyme-linked immunosorbent assay (ELISA)). Indeed, commercial assay kits, mainly based on immunological techniques, have recently been developed (reviewed in Kennaway, 2019).

The most common techniques used to detect and quantify melatonin in wine samples are based on GC-MS and high-performance liquid chromatography (HPLC), but the most powerful technique currently used is based on ultrahigh performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS) (Muñiz-Calvo et al., 2020a) since this technique has the lowest detection and

quantification limits, which allows the detection and quantification of tryptophan-derived compounds present in very low concentrations (Fernández-Cruz et al., 2016).
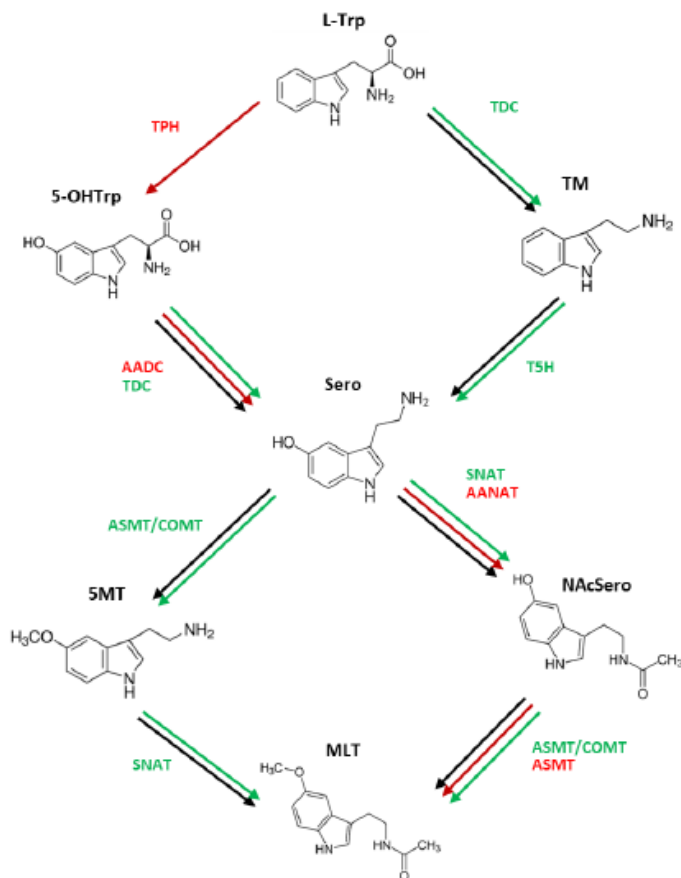
 Figure 26. Biosynthetic pathway of melatonin from tryptophan for mammals (red arrows), plants (green arrows) and yeast (black arrows). MLT, melatonin; L-Trp, tryptophan; 5-OHTrp, 5-hydroxytryptophan; Sero, serotonin; NAcSero, N-acetylserotonin; TM, tryptamine; 5MT, 5-methoxytryptamine; TPH, tryptophan hydroxylase; TDC, tryptophan decarboxylase; AADC, aromatic amino acid decarboxylase; T5H, tryptamine 5-hydroxylase; SNAT, serotonin N-acetyltransferase; AANAT, aromatic amino acid N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; COMT, caffeic acid O-methyltransferase (Muñiz-Calvo et al., 2020a).

Additionally, rapid detection methods for melatonin and other indolic compounds have recently been developed to meet the demand for faster and simpler techniques for routine analysis (Muñiz-Calvo et al., 2020a). Of these methods, it is worth mentioning a method based on voltammetry of immobilized particles (VIMP) (Muñiz-Calvo et al., 2017), and two bioassays that use yeast cells (Shaw et al., 2019) or cell lines (Morcillo-Parra et al., 2019a) with mammalian melatonin receptors as sensors.

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Physiological role of melatonin in yeast cells

According to the abovementioned pattern of melatonin accumulation in the intra- and extracellular medium, the production of melatonin in the lag phase has been proposed to have a role in signalling yeast adaptation to new conditions (Rodriguez-Naranjo et al., 2012; Morcillo-Parra et al., 2019b, 2020b). Moreover, it has recently been deciphered that melatonin binds to glycolytic proteins within the cell (Morcillo-Parra et al., 2019b, 2020b) but only in Crabtree-positive yeasts. On the other hand, these glycolytic proteins were previously reported to form a macromolecular complex that binds the mitochondrial surface, with a possible role in regulating the channelling of different substrates into the mitochondria (such as pyruvate) (Brandina et al., 2006). As melatonin has been related to the mitochondria of mammalian cells, where its synthesis and antioxidant activity occur (Acuña-Castroviejo et al., 2003; Suofu et al., 2017; Reiter et al., 2018; Chitimus et al., 2020), it could act as a signalling molecule of this glycolytic complex in a fermentative capacity-dependent manner (Morcillo-Parra et al., 2020b).

However, the main known role of melatonin in yeast cells is its antioxidant activity (Vázquez et al., 2017, 2018; Bisquert et al., 2018). Great efforts are being made to decipher its mechanisms, and to date, the antioxidant response on *S. cerevisiae* cells appears to be similar to that of mammalian cells as follows: melatonin acts as a direct antioxidant by scavenging ROS and indirectly modulating antioxidant enzymes and molecules that confer protection against oxidative stress, leading to better growth performance (Figure 27) (Bisquert et al., 2018; Vázquez et al., 2018; Zampol and Barros, 2018). Indeed, in nonstressed cells, melatonin is suggested to have a prooxidant role by inducing a higher oxidized state in cells as follows: yeast cells treated with melatonin have a slight increase in oxidative stress indicators, such as ROS accumulation or lipid peroxidation, and several antioxidant genes and enzymes are also activated (Vázquez et al., 2017, 2018). Thus, melatonin seems to prepare cells to endure subsequent stress situations. Several mechanisms induced by melatonin have been reported in stressed cells. First, melatonin neutralizes the increase in ROS caused by oxidative stress. Second, it activates genes involved in the response to oxidative stress, such as SOD, GSH/GRX, TRX and catalase, and modulates nonenzymatic systems, such as GSH, leading to a reduction

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in lipid peroxidation (Figure 27). The presence of melatonin influences other cell parameters related to antioxidant effects, such as membrane fatty acid composition or the effectiveness of peroxisome functions (Vázquez et al., 2017, 2018; Bisquert et al., 2018). Melatonin has also been reported to play a role in mitochondria by enhancing mitophagy and positively regulating ETC function at the complex IV level (Zampol and Barros, 2018). Indeed, melatonin has been shown to have antioxidant power similar to or better than vitamin C in yeasts (Vázquez et al., 2018).

The antioxidant effect of melatonin has also been observed in some non-*Saccharomyces* species, such as *Torulaspora delbrueckii* and *Hanseniaspora uvarum* (Vázquez et al., 2018). In addition, the presence of melatonin in a fermentative medium has been reported to enhance the survival of some non-*Saccharomyces* (such as *T. delbrueckii*) and their fermentative capacity (Valera et al., 2019). Melatonin also seems to protect yeast cells against UVC light (ultraviolet light in a wavelength range of 200 to 280 nm) damage, as it modulates the expression of genes encoding DNA repair, which improves viability and growth performance after UVC radiation stress (Bisquert et al., 2018). Therefore, these studies suggest that melatonin may have a signalling role in yeast cells, as it does in mammalian cells.
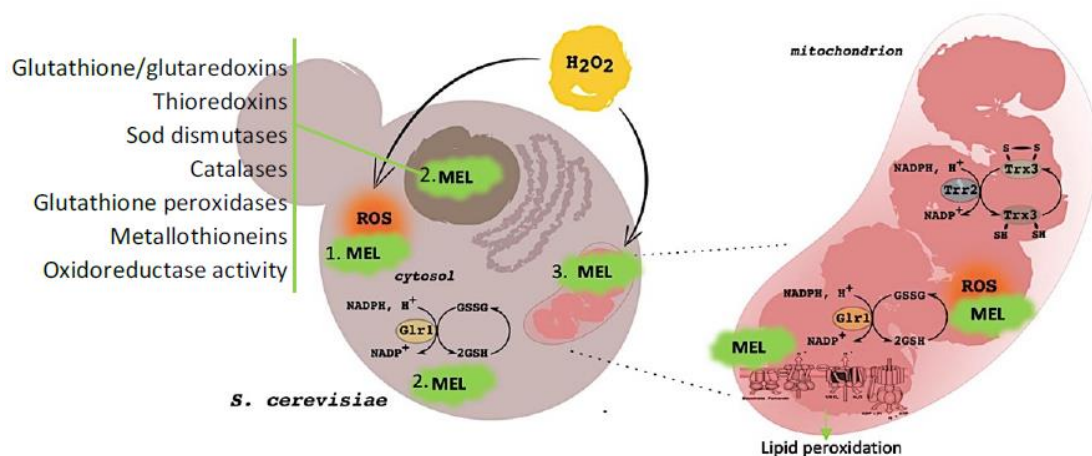
Figure 27. Primary melatonin effect as an antioxidant in *S. cerevisiae* (Vázquez, 2017).

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4.2. Hydroxytyrosol

Hydroxytyrosol (or 4-(2-hydroxyethyl)1,2-benzenediol, HT) is a phenolic alcohol, that consists of a benzene ring bearing two hydroxyl groups. HT is synthesized from oleuropein during the ripening of olives and during the storage and elaboration of table olives (Charoenprasert and Mitchell, 2012) (Figure 28), components of the Mediterranean diet. The consumption of virgin olive oil is highly recommended due to its beneficial health effects that protect against cardiovascular diseases, but also prevents the development of metabolic syndrome, obesity, diabetes, and several types of cancers, and even it exerts beneficial effects in healthy individuals (Karković Marković et al., 2019). Indeed, when looking for the compounds responsible for these effects in virgin olive oil, the compounds that stand out are polyphenols, mainly sercoirioid derivatives, such as oleuropein, oleocanthal and oleacein, and the phenols tyrosol and HT (Karković Marković et al., 2019). Among them, HT is an excellent food supplement because it has good absorption in the human body, and it is one of the most powerful dietary antioxidants, making it the most researched natural phenol for nutritional and food applications (Vissers et al., 2002; Hu et al., 2014; Bertelli et al., 2020).
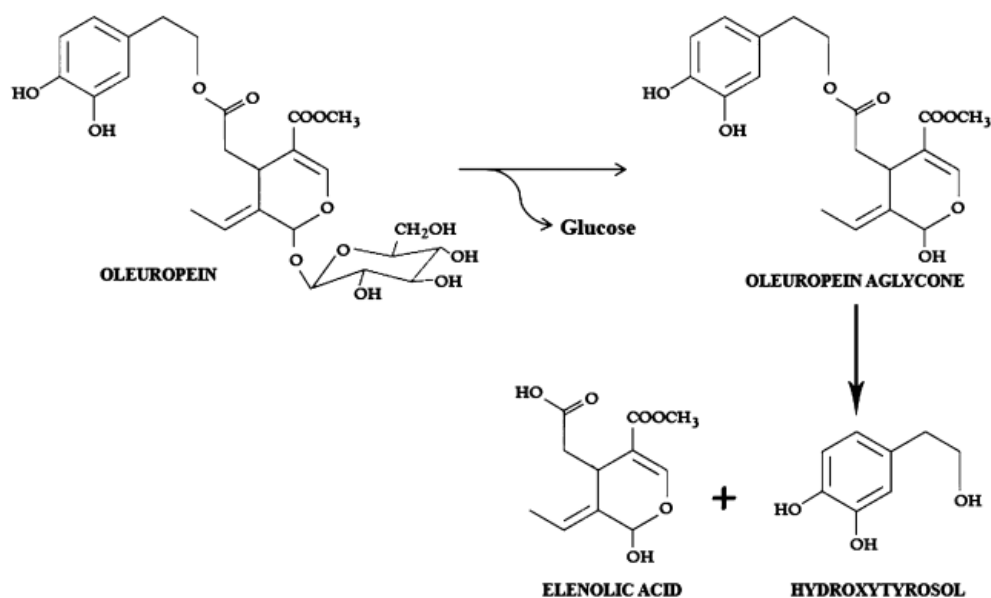
Figure 28. Origin of hydroxytyrosol from oleuropein (Granados-Principal et al., 2010).

In fact, EFSA approved in 2011 a health claim recommending the daily consumption of 20 g of extra virgin olive oil containing 5 mg of HT and its derivatives provided by moderate amounts of extra virgin olive oil in the context of a balanced diet (EFSA 2011; European Commission, 2012). Later, EFSA also considered the addition of HT to some fish and vegetable oils (up to 215 mg/kg) and margarine (up to 175 mg/kg) safe (EFSA, 2017). Moreover, its derivatives and precursors, such as tyrosol, oleuropein, oleocanthal and oleacein, also exert beneficial health properties, exhibiting antioxidant or anti-inflammatory activity, which has been deeply reviewed in Karković Marković et al. (2019).

4.2.1. Importance of hydroxytyrosol in mammal health

There has been great interest in the properties of HT in the context of olive oil consumption, so the studies performed normally used pure HT extracts but also the extracts of olive oil or food products enriched with this compound, and the effects were mainly attributed to HT. The wide variety of HT biological activities are associated with its strong and widely reported antioxidant activity (Karković Marković et al., 2019).

The most relevant antioxidant property of HT is its high free radical scavenging activity, both in extracellular and intracellular media, which mainly neutralizes superoxide, H_2O_2 and hypochlorous acid (Robles-Almazan et al., 2018). The hydroxyl radicals in the ortho position donate a hydrogen atom to the peroxyl radicals, so the peroxyl radicals are replaced with hydroxytyrosol radical, which is unreactive due to the presence of an intramolecular hydrogen bond in the phenoxy radical. Therefore, they break the chain of reactions triggered by free radicals (Figure 29) (Visioli and Galli, 1998; Tripoli et al., 2005).
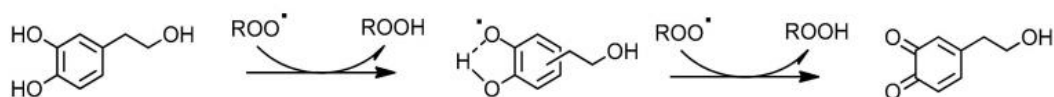
Figure 29. Mechanism of free radical scavenging by hydroxytyrosol (Karković Marković et al., 2019).

HT has also been proposed to confer additional antioxidant protection through other mechanisms, such as acting as a metal chelator mainly for iron (Visioli and Galli,

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1998; Manna et al., 2000; Paiva-Martins and Gordon, 2005; Granados-Principal et al., 2010) or increasing endogenous defence systems against oxidative stress (reviewed in Karković Marković et al., 2019; Bertelli et al., 2020). In addition, HT activates several signalling pathways to decrease the accumulation of free radicals from the environment. Some of the actions of HT are to activate enzymes to detoxify ROS (such as SOD or catalase), repair structures damaged by free radicals (such as DNA or peroxidated lipids), modulate endogenous antioxidant molecules (such as GSH), or stimulate mitochondria function (Robles-Almazan et al., 2018).

Surprisingly, in several cancer cell types, HT increases oxidative stress, by inducing the generation of ROS. This effect damages the cancer cell, increasing the widely reported anti-tumour effect of HT, which consists of mechanisms such as blocking the cyclin-dependent kinases and messengers involved in cell proliferation, (Granados-Principal et al., 2010; Robles-Almazan et al., 2018; Karković Marković et al., 2019; Rosignoli et al., 2016). The dichotomy of the pro-oxidant and antioxidant effects of HT on normal or cancer cells suggests that it has selective antitumour capacity.

Inside the body, HT and its precursors are enzymatically converted into oxidized and/or methylated derivatives, which together with HT exert antioxidant properties in several body parts (reviewed in Vissers et al., 2002; de Bock et al., 2013; Karković Marković et al., 2019; Bertelli et al., 2020). Therefore, as in the case of melatonin, the antioxidant effect of HT seems to confer protection against several disorders, such as respiratory, cardiovascular, metabolic and neurodegenerative disorders (Robles-Almazan et al., 2018; Bertelli et al., 2020). Indeed, one of the studies considered in the EFSA claim concluded that HT protected against oxidation of low-density lipoprotein *in vitro* (EFSA, 2011), which contributed to the cardioprotective effect. Other activities related to cardiovascular dysregulation have been reported by HT (Cerezo et al., 2019; Karković Marković et al., 2019).

In addition to its antioxidant role, HT has other effects, both pleiotropic (such as important anti-inflammatory effects (Robles-Almazan et al., 2018; Bertelli et al., 2020)) and specific to counteract the abovementioned disorders. For example, HT and olive tree

leaf extracts exhibit *in vitro* anti-HIV activity (Bedoya et al., 2016) and antimicrobial activity against several fungi, bacteria and protozoa and specifically against some foodborne pathogens, such as *Escherichia coli, Clostridium perfringens, Salmonella enterica* or *Staphylococcus aureus* (Medina et al., 2006; Robles-Almazan et al., 2018; Karković Marković et al., 2019; Bertelli et al., 2020). Moreover, a recent study demonstrated that HT has a relevant role in the prevention and treatment of biofilm-based infections (Crisante et al., 2015). However, there is ongoing controversy about the antimicrobial role of HT, which could depend on the concentration, strain and culture media (Medina-Martínez et al., 2016). On the other hand, HT also exerts neuroprotective effects by protecting against processes associated with brain degeneration, making it a promising compound for the treatment of neurodegenerative diseases, such as Parkinson's or Alzheimer's disease (Gallardo-Fernández et al., 2019, 2020; Hornedo-Ortega et al., 2018a, 2018b; Robles-Almazan et al., 2018; Karković Marković et al., 2019).

In summary, HT has several beneficial effects in humans, such as anticarcinogenic, cardioprotective, antidiabetic, and neuroprotective effects and immune system modulation, the improvement of endothelial dysfunction, or skin protection (Robles-Almazan et al., 2018; Bertelli et al., 2020). Moreover, HT has an excellent safety profile, as it is nonmutagenic, nongenotoxic and suitable for long-term consumption (Bertelli et al., 2020). Therefore, due to all these biological properties, HT has great potential for pharmacological, therapeutic and clinical applications in several diseases, such as cardiovascular or neurodegenerative diseases and cancer prevention (Karković Marković et al., 2019).

4.2.2. Hydroxytyrosol in wine

Although the highest concentrations of HT are found in extra virgin olive oil (Tripoli et al., 2005) (and fruit and leaves of the olive tree), it is also present at lower concentrations in other fermented products, such as red and white wines and beer (Table 3) (Di Tommaso et al., 1998; Minussi et al., 2003; Proestos et al., 2005; Boselli et al., 2006; de La Torre et al., 2006; Minuti et al., 2006; Piñeiro et al., 2011; Boronat et al., 2020).

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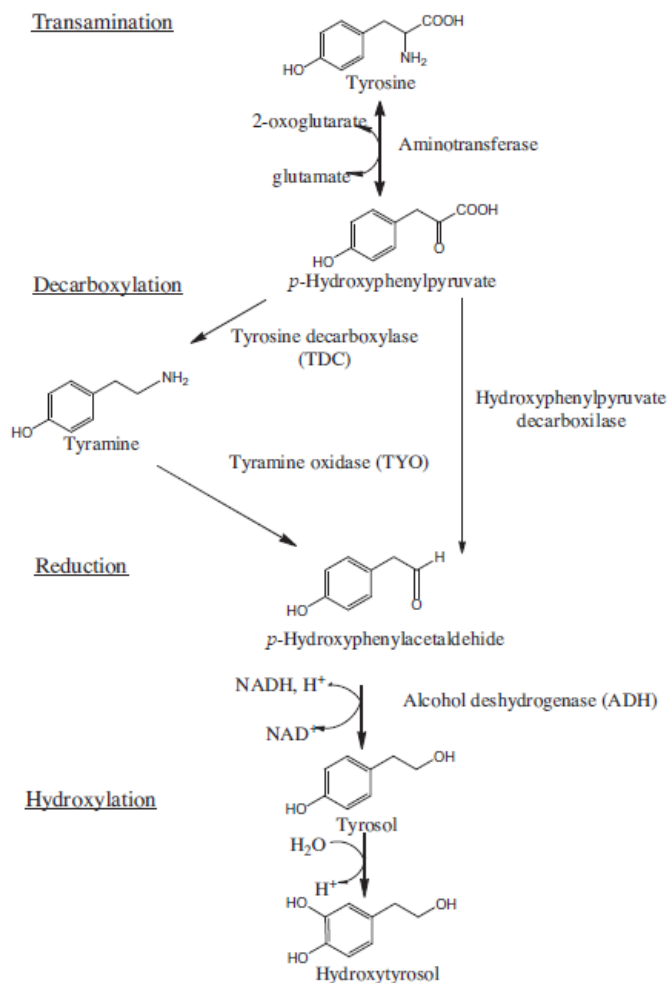
Figure 30. Ehrlich pathway for the production of hydroxytyrosol (Álvarez-Fernández et al., 2018).

In wine, HT can be synthesized from tyrosol by the polyphenol oxidase present in grapes (Garcia-Moreno et al., 2013) but also by yeast metabolism during alcoholic fermentation (Álvarez-Fernández et al., 2018; Bordiga et al., 2016, Rebollo-Romero et al., 2020) from tyrosine through the well-established Ehrlich pathway (Figure 30). In this pathway, amino acids are first transaminated into α-keto acids, followed by decarboxylation to aldehydes, which in turn are reduced to higher alcohols. In this case, the obtained higher alcohol, tyrosol, can then be hydroxylated to HT (Dickinson et al., 2003; Hazelwood et al., 2008; Muñiz-Calvo et al., 2020b). Therefore, HT is a secondary metabolite produced from tyrosine during alcoholic fermentation, and its production depends on the yeast strain, must composition, or fermentation characteristics, such as

temperature or alcoholic degree (Álvarez-Fernández et al., 2018; Bordiga et al., 2016, Rebollo-Romero et al., 2020).

The synthesis of this molecule during winemaking is important, mainly because of its beneficial properties for human cells. Moreover, the matrix of its administration is important for its bioavailability, and absorption with ethanol seems to promote endogenous HT generation, since ethanol interacts with dopamine, which induces the formation of HT (de La Torre et al., 2006; Karković Marković et al., 2019). There is a growing interest in testing different HT-related products to look up synthetic derivatives of HT to improve absorption, distribution, metabolism, excretion, stability and biological health features (reviewed in Bernini et al., 2015). Indeed, the great importance of this molecule is highlighted by all the research related to the improvement of its production, by purification from olive or plant products or by synthesis using chemical and biotechnological methods (Britton et al., 2019). On the latter, there are several studies to overproduce this compound using bacteria as a cell factory, and recently Muñiz-Calvo et al. constructed a *S. cerevisiae* strain with increased production of this compound in a medium supplemented with tyrosine or tyrosol (Muñiz-Calvo et al., 2020b)

Table 3. Concentration of hydroxytyrosol in various fermented beverages and yeast (adapted from Muñiz-Calvo, 2021).

Sample	Hydroxytyrosol concentration (mg/L)	Analytical method	Reference
Red and white wines	1.72-4.2	GC-MS	(Di Tommaso et al., 1998)
Red wine	0.28-5.02	HPLC-F and HPLC-PDA	(Piñeiro et al., 2011)
Wine	0.53-35.11	UHPLC-MS/MS	(Marhuenda et al., 2017)
Wine	0.2-5.2	HPLC-MS/MS	(Bordiga et al., 2016)
Wine and intracellular yeast	0.235-0.4 (wine) 0.086-1.062 (yeast)	UHPLC-HRMS	(Álvarez-Fernández et al., 2018)
White wine	0.007-0.288	UHPLC-HRMS	(Rebollo-Romero et al., 2020)

CHAPTER I

Transcriptomic insights into the effect of melatonin in *Saccharomyces cerevisiae* in the presence and absence of oxidative stress

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Mercè Sunyer Figueres

Transcriptomic study

Abstract: Melatonin is a ubiquitous indolamine that plays important roles in various aspects of biological processes in mammals. In *Saccharomyces cerevisiae*, melatonin has been reported to exhibit antioxidant properties and to modulate the expression of some genes involved in endogenous defense systems. The aim of this study was to elucidate the role of supplemented melatonin at the transcriptional level in *S. cerevisiae* in the presence and absence of oxidative stress. This was achieved by exposing yeast cells pretreated with different melatonin concentrations to hydrogen peroxide and assessing the entry of melatonin into the cell and the yeast response at the transcriptional level (by microarray and qPCR analyses) and the physiological level (by analyzing changes in the lipid composition and mitochondrial activity). We found that exogenous melatonin crossed cellular membranes at nanomolar concentrations and modulated the expression of many genes, mainly downregulating the expression of mitochondrial genes in the absence of oxidative stress, triggering a hypoxia-like response, and upregulating them under stress, mainly the cytochrome complex and electron transport chain. Other categories that were enriched by the effect of melatonin were related to transport, antioxidant activity, signaling, and carbohydrate and lipid metabolism. The overall results suggest that melatonin is able to reprogram the cellular machinery to achieve tolerance to oxidative stress.

Keywords: yeast; melatonin; oxidative stress; antioxidant; hydrogen peroxide; bioactive compound; hypoxia; mitochondria.

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1. INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine; Mel) is a versatile indolamine that is best known as a neurohormone in vertebrates. Since it was first discovered in the bovine pineal gland (Lerner et al., 1958), it has been found in most living organisms (Hardeland and Poeggeler, 2003). In humans, melatonin has numerous physiological functions, such as regulating circadian rhythms and synchronizing the reproductive cycle, and it exhibits anti-aging, antioxidant and anti-inflammatory activities. It can even modulate a variety of neural, endocrine and immune functions (Romero et al., 2014; Eghbal et al., 2016).

Since Sprenger et al. (1999) initially related melatonin production to *Saccharomyces cerevisiae*, several studies have reported the ability of yeasts to synthetize melatonin during alcoholic fermentation, with levels in wine ranging bewteen 0.3–1800 nM (Rodriguez-Naranjo et al., 2011a, 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018, 2019; Valera et al., 2019). Little information on melatonin biosynthesis in yeast is available. Although a synthetic route similar to that described for vertebrates was initially reported (Sprenger et al., 1999), Muñiz-Calvo et al. (2019) recently proposed a putative biosynthetic pathway including some steps described in plants, such as the synthesis of serotonin from tryptophan through tryptamine instead of 5-hydroxytryptophan. Another issue that remains to be deciphered is the physiological role of melatonin in yeasts. Recent studies have demonstrated a protective function of melatonin against oxidative stress and UV radiation (Vázquez et al., 2017, 2018; Bisquert et al., 2018) and indicated a hypothetical role as a signaling molecule (Rodriguez-Naranjo et al., 2012). The recent finding that melatonin interacts with glycolytic proteins during alcoholic fermentation reinforces the latter role, which seems to be linked to fermentative metabolism (Morcillo-Parra et al., 2019b, 2020b).

Oxidative stress is provoked by an imbalance in reactive oxygen species (ROS) resulting in ROS-mediated molecular and functional impairment and ultimately leading to cell death. To prevent these effects, cells employ antioxidant defense mechanisms in which enzymatic and nonenzymatic systems take part, neutralizing free radicals before they affect essential elements of the cell (Jamieson, 1998; Moradas-Ferreira and Costa, 2000; Cardinali and Vigo, 2017). In yeast, exogenously applied melatonin has been reported to protect various biomolecules from damage caused by free radicals both directly, by scavenging ROS, and indirectly, by decreasing oxidized glutathione and

activating genes that are involved in the oxidative stress response, such as glutathione/glutaredoxin, thioredoxin, catalase and superoxide dismutase genes (Vázquez et al., 2017, 2018). ROS produce changes in the lipid bilayer composition resulting in lipid peroxidation, which is correlated with membrane disintegration and cell death. Recently, it has been shown that under oxidative stress, *Saccharomyces* takes advantage of melatonin supplementation by reducing the lipid peroxidation provoked by ROS, leading to an increase in total fatty acids and a higher proportion of unsaturated fatty acids, resulting in a higher tolerance to hydrogen peroxide (H_2O_2) (Vázquez et al., 2018).

In humans, the action of melatonin is mitochondrion targeted, as the electron transport chain (ETC), where higher ROS production occurs, is located in the mitochondria (Cardinali and Vigo, 2017). Therefore, melatonin not only reduces ROS damage by scavenging ROS and increasing antioxidant enzyme activities but also improves the efficiency of the ETC and ATP production (reviewed in Reiter et al., 2020). Zampol and Barros (2018) revealed that the addition of melatonin to yeast cells improved respiration when the cells were challenged with a compound that induces oxidative stress (menadione), mainly by affecting complex IV activity, suggesting that melatonin may also exhibit mitochondrion-specific activity in yeast.

S. cerevisiae exhibits a number of inducible adaptive stress responses to oxidants such as H_2O_2, superoxide anions and lipid peroxidation products. The oxidative stress responses are regulated at the transcriptional level, and there is considerable overlap between them and the stress responses associated with other types of stresses (general stress response) (Jamieson, 1998; Costa and Moradas-Ferreira, 2001). Thus, the oxidative stress response is not mediated by an isolated linear metabolic or signaling pathway. Instead, cells are able to reprogram gene expression to optimize signal transduction for more efficient and effective adaptation, setting up a general stress response that encompasses a much larger stress signaling network and integrating information from many pathways (Causton et al., 2001; Thorpe et al., 2004; Zhao et al., 2015). The physiological changes induced in yeast by melatonin supplementation and the ways in which yeast cells respond to oxidative stress suggest that melatonin might be involved in multiple biological processes in yeast, and it is interesting to investigate whether melatonin acts as a signaling molecule that triggers a molecular and physiological response to cope with stress situations. Therefore, obtaining an accurate

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idea of how melatonin supplementation modulates gene expression is critical for understanding the signaling events that it triggers.

To gain insight into the antioxidant role and regulatory mechanism of melatonin in yeast, we evaluated the effect of melatonin on gene transcription after analyzing the ability of yeast to incorporate exogenous melatonin into the cell. For this purpose, we measured intracellular melatonin and performed a transcriptomic study in a commercial wine yeast strain of *S. cerevisiae*, in the presence and absence of both melatonin and oxidative stress. After analyzing the results, we validated the effect of melatonin on the lipid composition and mitochondria via physiological studies.

2. MATERIALS AND METHODS

2.1. Yeast strain and experimental conditions

The wine yeast QA23, a commercial strain of *S. cerevisiae* (Lallemand, Montreal, QC, Canada), was used in this study. Yeast precultures were prepared in YPD broth [2% (*w/v*) glucose, 2% (*w/v*) peptone and 1% (*w/v*) yeast extract (Panreac, Barcelona, Spain)], incubated at 28 °C with orbital shaking (120 rpm) for 24 h, and monitored by measuring the OD at 600 nm. Then, an initial population of 5×10^5 cells/mL (OD_{600nm} 0.05) was inoculated into YPD broth (175 mL for melatonin quantification, 50 mL for transcriptomic assays, 300 mL for time course assays, 450 mL for lipid analysis and 60 mL for the quantification of mitochondria) with or without melatonin [TLC grade, purity ≥98%, Sigma-Aldrich (St. Louis, MO, USA)] supplementation (5 μM; Mel and Control conditions, respectively) and grown until the cells reached the initial exponential phase (OD_{600nm} 0.5–0.6) (designated time 0). Then, sublethal oxidative stress was induced with 2 mM H_2O_2 (MelH and H conditions, respectively), and samples were taken after 1 h by harvesting the cells via centrifugation at $28,672 \times g$ for 5 min at 4 °C. Then, the pellet was washed with Milli-Q water (Q-PODTM Advantage A10, Millipore, Burlington, MA, USA) and stored at −80 °C until use. The variations in this general procedure are specified below. The melatonin and H_2O_2 concentrations were chosen on the basis of our previous study in the QA23 strain (Vazquez et al., 2017). A culture with 25 μM melatonin supplementation under stress conditions was added (25MelH) in the microarray analysis. Three biological replicates were employed in all assays.

2.2. Intracellular melatonin quantification

For intracellular melatonin quantification, 10^8 cells were harvested from the samples at 0 h (just before stress induction) and 1 h after the stress induction by centrifugation at 4700 × g for 15 min. Intracellular melatonin quantification was performed as previously described by Morcillo-Parra et al. (2019b). In brief, melatonin was extracted by adapting the boiling buffered ethanol method described by Gonzalez et al. (1997) and quantified by liquid chromatography mass spectrometry using a liquid chromatograph coupled to a triple quadrupole mass spectrometer (LC-MS/MS Agilent G6410A; Agilent Technologies Inc, Santa Clara, CA, USA). In parallel, 10^8 cells were dried at 28 °C for 48 h to determine the dry weight of the samples, and the melatonin concentration was expressed as nM/mg dry weight.

2.3. Assessment of global gene expression by microarray analysis

RNA was isolated from 10^7 cells from each condition (Control, Mel, H, MelH, 25MelH) using a TRIzol® Plus RNA Purification Kit from Ambion Life Technologies (Woburn, MA, USA), following the instructions of the manufacturer, but the chloroform step was repeated twice before transferring the upper phase containing the RNA to a fresh RNase-free tube. Furthermore, a DNAse (Qiagen, Barcelona, Spain) incubation step at 37 °C for 15 min was included to remove the remaining DNA. The RNA samples were quantified with a NanoDrop 1000 TM spectrophotometer (Thermo Scientific, Waltham, MA, USA), and their integrity was analyzed with an RNA 2100 Bioanalyzer (Agilent Technologies Inc.) using the RNA 6000 Nano kit and the Plant RNA Nano protocol in Agilent 2100 Expert software. Gene expression levels were assessed using a Yeast Gene Expression Microarray (8 × 15 K format) containing 6256 *S. cerevisiae* probes. Fifteen samples (three biological replicates of each condition) were analyzed. Each sample was labeled with Cy3 and hybridized through one-color microarray-based exon analysis (Low Input Quick Amp WT Labeling kit protocol version 2.0, Agilent Technologies) according to the manufacturer's instructions.

2.3.1. Microarray data analysis

Agilent Scan Control version A.8.5.1 software was used to scan 3-μm-resolution slides using the Agilent G2565CA Microarray Scanner System with SureScan High-Resolution Technology. Feature extraction version 12.0.1.1 software (Agilent Technologies) was used for data extraction. Statistical transcriptomic analysis for

identifying significant changes between the conditions was performed using Gene Spring GX Software v.13.1.1 from Agilent Technologies. The signal for each spot was normalized at the 75% percentile, and the moderated t-test with Benjamini-Hochberg multiple testing correction was used to designate the differentially expressed genes. The genes that were differentially expressed between two conditions (p-value < 0.05) were selected for further analysis: Venn diagrams were generated at the website http://bioinformatics.psb.ugent.be/webtools/Venn/, regulatory networks were generated with the PheNetic web tool (De Maeyer et al., 2015) and the molecular functions, biological processes and cellular components were determined with Gene Ontology (GO) (CC-BY 4.0, (Carbon et al., 2009)). The specific pathways involved in the differentially expressed genes were analyzed with the KEGG pathway mapping database (Kanehisa, 2002), and significantly enriched pathways were determined with the DAVID tool (Huang et al., 2009a, 2009b). The results of transcriptomic analysis were deposited in the Gene Expression Omnibus (GEO) repository, with GEO accession number: GSE154702 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154702).

2.4. Gene expression by qPCR analysis

The expression of genes of interest was evaluated via qPCR before stress (0 h) and after exposure to stress (1, 4, 12 and 24 h for Control and Mel; 0.5, 1, 4, 12, 24 and 36 h for H and MelH). RNA was isolated from 10^7 cells using the Universal RNA Purification Kit from EURx (Gdańsk, Poland) with some modifications: to improve cellular lysis, cells were resuspended with a mixture of 1% β-mercaptoethanol in lysis buffer [PureLink RNA mini kit (Invitrogen, Carlsbad, CA, USA)], added to a 2 mL screw-cap tube with 1 g of 0.5 mm-diameter glass beads, and lysed using a MBB-16 Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) (5 cycles of 30 s in the Mini-Beadbeater + 30 s on ice). The suspension was centrifuged for 5 min at 21,952 × g at 4 °C and transferred to a homogenization spin column. Thereafter, the protocol was followed as recommended by the manufacturer, with optional on-column DNase digestion with DNase I from EURx. The RNA samples were quantified with a NanoDrop 1000 TM spectrophotometer (Thermo Scientific) and stored at −80 °C. The obtained RNA, together with that obtained as described in section in 2.3, was converted to cDNA to evaluate the expression of genes of interest by qPCR (Table 1.1). The samples were prepared as follows: 12 µL of 320 ng/µL RNA, 1 µL of an Oligo (dT)20 primer (Invitrogen), 1 µL of dNTPs (10 mM), 4 µL of buffer, 1 µL of DTT and 1 µL of SuperScript IV Reverse Transcriptase (Invitrogen), and

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amplification was performed according to the instructions of the manufacturer using a 2700 Thermal Cycler (Applied Biosystems, Waltham, MA, USA) and stored at −20 °C.

Table 1.1. Primers used in this study for the analysis of gene expression by qPCR.

Gene name	Nucleotide sequence of forward primer (5' to 3')	Nucleotide sequence of reverse primer (5' to 3')
ACT1	TGGATTCCGGTGATGGTGTT	CGGCCAAATCGATTCTCAA
ADY2	TTTCAGCCTTCGCGTTGAC	CTTGCGCTCTCGCATTGA
ATP20	GGGTCTTCAACCACCAACTGTT	GGCTCTGCTTATAAAGGTTCGAAT
CIS1	CCCATCGGGTTAGTTTCAAAAA	GACATGCTACCCACTCTGCAATAG
COX2	TCGTTGTAACAGCTGCTGATGTT	CCAGGAGTAGCATCAACTTTAATACCT
GDH3	CACCGGGTTCGGCTTAGTT	GCCGTTTGTTGCATAATCGA
HEM2	TTCCGCTATTCATCTCCGATAATCCAG	ACAGACATCGCAAATAATATACAGTTCAGG
MGA1	ATGGGCAGTCCCGTCCATTACT	TCGCATCATGTTCACCGTGGGT
MMT1	GCGTTGTTTGCGGATGCTA	GCAAAGTCAACAAGTCAGAAACCA
SDH6	ACTTCACCACCATTGAACACTTGT	GGGTGTGAAAAGGTGGCAATT
SRX1	CCTGTGTTGGATCCTCAA	GGCATAATATAGCGTCTGTC
TAF10	ATATTCCAGGATCAGGTCTTCCGTAGC	GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG

Primer design was performed using Primer Express software (Primer Express 3.0 Applied Biosystems). The primer ACT1 was previously reported by Beltran et al. (2004), and those for HEM2 and TAF10 were previously reported by Teste et al. (2009).

The primers for each evaluated gene (Table 1.1) were designed using Primer Express software (Primer Express 3.0 Applied Biosystems), and we employed the genes *ACT1, HEM2* and *TAF10* as endogenic controls. All primers were supplied by Invitrogen, and a standard curve was performed for each pair of primers. qPCR was performed using 2 µL of cDNA diluted 10-fold, 0.4 µL of each primer, 0.08 µL of ROX [SYBR Premix Ex Taq II (TaKaRa Bio Inc, Shiga, Japan)], 10 µL of SYBR Green (SYBR Premix Ex Taq II) and 7.12 µL of sterile Milli-Q water. Amplification was conducted using a QuantStudio5 Real Time PCR system (Thermo Fisher Scientific) as follows: one cycle of 95 °C for 1 min and 40 cycles of 95 °C for 5 s and 60 °C for 35 s, followed by a dissociation step. Relative gene expression was calculated with Thermo Fisher Cloud software (Thermo Fisher Scientific) using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is

determined to be statically significantly above background; ΔCt is the difference between the Ct of the gene of interest and the mean value for the endogenous controls, and ΔΔCt is the difference between the ΔCt values under the different conditions (see figure legends for relative expression details). Two biological replicates were analyzed under each condition.

2.5. Analysis of sterols, fatty acids and phospholipids

Yeast cell homogenates were obtained, and the protein content was quantified as described by Vázquez et al. (2019). Total lipids were extracted from cell fractions corresponding to 0.5 mg, 1 mg or 3 mg of total cell protein for sterols, fatty acid (FA) or phospholipid (PL) assays, respectively, according to the method described by Folch et al. (1957). The composition of each lipid was determined as described by Vázquez et al. (2019). In brief, the composition of individual sterols was determined via gas-liquid chromatography-mass spectrometry (GC-MS) after the alkaline hydrolysis of the lipid extracts as described by Quail and Kelly (1996); to this aim a Hewlett-Packard 5690 Gas Chromatograph (Agilent Technologies) equipped with an HP 5972 mass selective detector using a capillary column (HP 5-MS; 30 m × 0.25 mm i.d. × 0.25 μm film thickness) was employed. The FA composition was determined by gas-liquid chromatography (GLC) using a Hewlett-Packard 6890 gas chromatograph (Rußmayer et al., 2015), and the PLs were first separated by two-dimensional thin layer chromatography (TLC) (Athenstaedt et al., 1999). Later, individual PLs were scraped off the plate and quantified by estimating the amount of phosphates (Broekhuyse, 1968). Two biological replicates were performed.

2.6. Quantification of mitochondria

Mitochondria were stained with the fluorescent dye MitoTracker Green (Thermo Fisher Scientific). Cells were grown in YPD with or without melatonin (5 and 50 μM) until the cells reached the initial exponential phase, and oxidative stress was then induced (with 0, 2 or 5 mM H_2O_2) for 1 h. A total of 10^7 cells were harvested and directly (without a freezing step) resuspended in 1 mL of PBS (phosphate-buffered saline) with a final concentration of 100 nM MitoTracker Green and incubated for 10 min at room temperature protected from light. Fluorescence was measured via flow cytometry, and data acquisition was performed with FloMax software (Quantum Analysis GmbH, Münster, Germany) and processed with WinMDI 2.9 software (Joseph Trotter, Salk

Institute for Biological Studies, La Jolla, CA, USA). The mean fluorescence index (MFI) was calculated according to Boettiger et al. (2001): [(geometric mean (Gmean) of the positive fluorescence)-(Gmean of the control)]/(Gmean of the control).

2.7. Data analysis

Data obtained from the intracellular melatonin quantification, lipid content, mitochondrial quantification and qPCR analyses were subjected to analysis of variance (ANOVA) and Tukey's post hoc test using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and XLSTAT 2019 software (NY, USA). All the results were considered statistically significant at a p-value < 0.05. To merge standard deviations, the program "combine means and SDs into one group program" of the StatsToDo website (https://www.statstodo.com/index.php) was used.

3. RESULTS AND DISCUSSION

In previous studies, we analyzed the antioxidant effects of exogenous melatonin on *S. cerevisiae* at the physiological level (Vázquez et al., 2017, 2018). Our data showed slight increases in ROS and oxidized glutathione under melatonin supplementation when no stress was induced. In contrast, when cells were under oxidative stress, melatonin activated some genes involved in the yeast antioxidant defense systems, thus reducing ROS accumulation and increasing cellular viability (Vázquez et al., 2017). In this study, we wanted to investigate the effect of melatonin on the yeast global transcriptomic response to gain insight into its antioxidant role and regulatory mechanisms.

3.1. Differential gene expression profiling

To obtain an overview of the gene expression profile associated with melatonin supplementation in *S. cerevisiae*, a comparative transcriptomic analysis was performed between cells that were grown with and without melatonin supplementation (5 µM) and with and without oxidative stress exposure (2 mM H_2O_2) (Control, Mel, H and MelH conditions). The overall results obtained from the transcriptomic analysis can be found in Datasets S1–S10: differentially expressed genes (p-value < 0.05) are listed in Datasets S1–S5, and the results for all genes are provided in Datasets S6–S10 and at the GEO repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1547 02). The numbers of genes showing significant changes in global gene expression (with a p-value

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< 0.05) under each condition are represented in Figure 1.1. A total of 649 genes were differentially expressed under exogenous melatonin treatment (Mel *vs* Control), mainly downregulated (422 genes), while oxidative stress resulted in the altered expression of 4427 genes (Figure 1.1A; H *vs* Control). Among these genes, 3658 were affected by stress independent of the presence of melatonin (common to H *vs* Control and MelH *vs* Control but not Mel *vs* Control) (Figures 1.1B, C). However, melatonin clearly altered the gene expression profile of stressed cells, with 775 genes being differentially expressed (MelH *vs* H), most of which were upregulated (498 genes), in contrast with the downregulation effect of melatonin on expression in nonstressed cells (Figure 1.1A). Furthermore, the effect of the melatonin concentration under oxidative stress was evaluated by testing a higher concentration (25 µM melatonin, 25MelH). Its effect was weak, with only 85 genes exhibiting altered expression due to the higher concentration of melatonin (Figure 1.1A, 25MelH *vs* MelH, Datasets S5 and S10). Thus, 5 µM melatonin was sufficient to modulate gene regulation in yeast cells.

Our results showed 189 genes were differentially expressed under all three conditions (Mel, H, MelH) in comparison with the control (Figure 1.1B(i), Figure 1.1C(j)), most of which were downregulated and were involved in mitochondrial function (Table S1). Additionally, 24 genes were regulated by melatonin regardless of the stress treatment (common in Mel *vs* Control and MelH *vs* Control, Figure 1.1B(a), Figure 1.1C(c)). Most of them were also downregulated and were mainly involved in nutrient regulation (*NRD1*, *SNZ2* and *RGM1*) or gene transcription (*GCD14*, *NOG1* and *NRD1*) (Figure 1.1E(c)). On the other hand, when we focused on the effect of melatonin in the cell, under stressed and nonstressed conditions, we found 69 genes that were commonly up- or downregulated by melatonin in both conditions (Figure 1.1D, groups e and f) and 22 genes that were regulated in opposite direction in stressed or nonstressed cells (Figure 1.1D, groups g and h). The upregulated genes were involved in the response to ROS (including both metallothioneins, *CUP1-1* and *CUP1-2*) and water deprivation, protein folding, oxidation-reduction process (*COX1*), copper binding (metallothioneins and *CCC2*), zinc homeostasis (*IZH4*) and the transport of maltose (*MAL31*), oligopeptides (*OPT1*) and ions (*HSP30*) (Figures 1.1D, E, group e, Table S1). The genes downregulated by melatonin were mainly related to transcription and the regulation of gene expression (Figures 1.1D, E, group f, Table S1). On the other hand, several mitochondrial genes presented opposite behavior in relation to melatonin treatment; i.e., they were downregulated in nonstressed cells and upregulated in

stressed cells, mainly being involved in the ETC and ATP synthesis, including genes related to cytochrome *c* oxidase (*COX5A, COX8*) and reductase activity (*QCR9*), ATP synthase (*ATP14*), and mitochondrial organization and stability. In this group, there were also genes related to thiamine metabolic processes and RNA processing (Figures 1.1D, E, group g, Table S1).
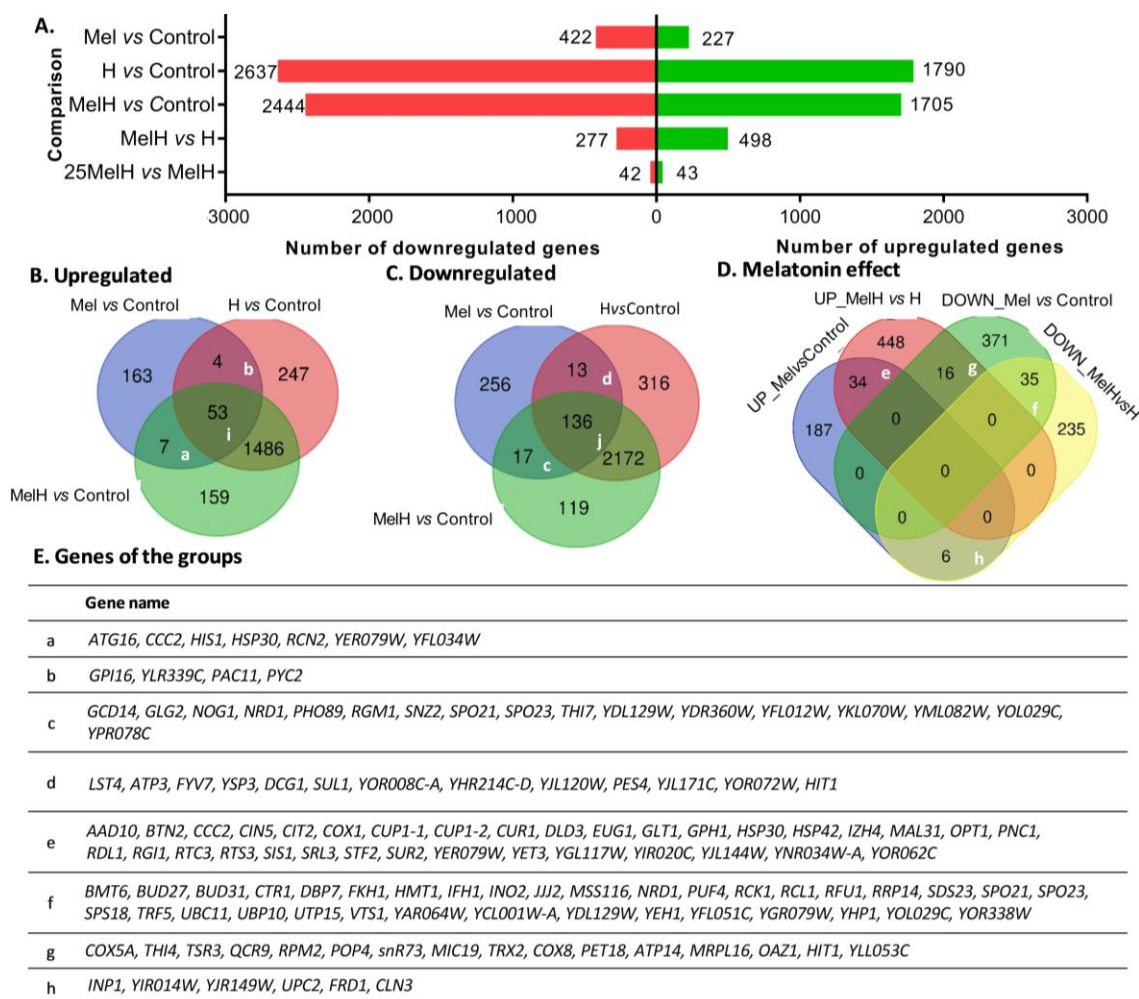
Figure 1.1. Distribution of differentially expressed genes in yeast cells grown with and without melatonin (5 µM or 25 µM), in stressed (2 mM of H_2O_2) and in unstressed conditions (Control, Mel, MelH, 25MelH and H). (**A**) The number of differentially expressed genes between different conditions (fold change $-1 \leq FC \geq 1$; *p*-value < 0.05). (**B**) and (**C**) Venn diagram showing the number of common genes found among the Mel *vs* Control, MelH *vs* Control and H *vs* Control comparisons (up- (**B**) and down (**C**) regulated genes). (**D**) Venn diagram showing the number of common genes found among the up- and downregulated genes identified in the Mel *vs* Control and MelH *vs* H comparisons. (**E**) List of genes in some groups indicated in (**B**), (**C**) and (**D**). The list of genes and the GO enrichment analysis of all the groups is shown in Table S1.

3.2. Classification of differentially expressed genes into functional categories

To understand the role of melatonin supplementation in yeast, GO enrichment analysis was performed to determine which molecular functions, biological processes, cellular components and pathways were overrepresented among the differentially expressed genes under the different conditions. In this work, we mainly focus on the results obtained under conditions with the addition of melatonin with or without stress. The overall results obtained from the GO enrichment analysis with the genes included in each category can be found in the supplementary material (Datasets S11–S19). Figure 1.2 and Table 1.2 show the main biological annotations significantly associated with genes that were upregulated or downregulated by melatonin.

In the presence of melatonin (Mel *vs* Control), the oxidation-reduction process and the responses to abiotic stimuli and chemicals were the biological processes that were most significantly represented among the upregulated genes, followed by the response to decreased oxygen levels, which showed the highest enrichment (50%), including 4 upregulated genes (*YLR256W, FRD1, MGA2, UPC2*) (Figure 1.2A and Dataset S13). Additionally, molecular functions related to transport activity and transmembrane transport were significantly overexpressed in the presence of melatonin, with 29 genes upregulated being found in this category (Table 1.2). Several pathways related to metabolism, especially that of carbohydrates (sucrose and citrate cycle (TCA)) and sphingolipids, were also enriched (Figure 1.2C). In the case of downregulated genes, the most affected categories were related to mitochondrial electron transport and oxidative phosphorylation, with 50 % of the cytochrome complex being downregulated, mostly represented by genes associated with complex III (80% enriched) and cytochrome *c* reductase activity therein (Figure 1.2, Table 1.2). Moreover, categories related to ribosome biogenesis and the structures in which it takes place (preribosomes and nucleolus) were also highly enriched among the downregulated genes, together with categories related to RNA processing (Table 1.2, Figure 1.2).

Table 1.2. (next page) Molecular function enrichment according to the Gene Ontology (GO) analysis of differentially expressed genes that were up- or downregulated (fold change ≥ 1; *p*-value < 0.05) among nonstressed and stressed (2 mM H_2O_2) cells in the absence or presence of melatonin (5 µM) (Mel *vs* Control, MelH *vs* H). Percentages of enrichment are calculated as the ratio of the number of up- or downregulated genes in relation to the total genes involved in each molecular function in *S. cerevisiae*.

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GO term molecular function (*p*-value)	% enrichment	Gene names
Mel *vs* Control		
Upregulated (227 genes)		
Transporter activity (0.00174)	7.21	*OPT2, MEP1, SWH1, VMA11, YHK8, AGP1, COX5B, FET4, AZR1, LAM5, FET3, PTR2, VMA3, HES1, GAP1, OPT1, FCY21, MPC3, VPS73, STE6, CCC2, YMR279C, AAC3, MAL31, DIP5, COX1, MUP3, HSP30, DUR3*
Trasmembrane transporter activity (0.01011)		
Oxireductase activity (0.01995)	6.96	*YGL039W, HEM14, CUP1-2, COX1, SUR2, MDH2, GPD2, RNR3, HEM13, TSA2, CUP1-1, FRD1, AAD10, EUG1, MPO1, DLD3, HMG2, FET3, YJR149W, YGL185C, GLT1, TDH3, COX5B, SHH4*
L-serine ammonia-lyase activity (0.02)	75.00	*YIL168W, CHA1, YIL167W*
Structural constituent of cell wall (0.053)	16.28	*TIR4, TIR1, SED1, PIR5, DAN1, YBR067C, DAN4*
Downregulated (422 genes)		
Ubiquinol-cytochrome-*c*-reductase activity (2.11 × 10⁻⁵)	77.78	*COR1, QCR10, QCR8, QCR2, QCR9, QCR6, YEL024W*
Electron transfer activity (2.57 × 10⁻⁵)	28.07	*COX8, COX12, CYT1, QCR9, QCR6, COX4, CYB5, YEL024W, COX5A, QCR2, COR1, CIR2, DRE2, QCR8, CYC1, QCR10,*
Inorganic molecular entity transmembrane transporter (0.09017)	11.72	*MPH3, MMP1, AQR1, ATP3, ATP14, YBR294W, VBA3, ATP7, OAC1, YLL053C, MIR1, PHO89, COX12, COX8, HNM1, TAT2, YPR124W, PHO84, FEX2, ATP17, CTR3, CTR1, COX4, CTP1, KCH1, PRM6, ATP5, YBR219C, COX5A*
MelH *vs* H		
Upregulated (498 genes)		
Oxireductase activity (2.24 × 10⁻⁶)	15.74	*GDH3, COX8, QCR9, AYR1, ALD3, SDH4, COX5A, GLT1, POX1, COX1, CUP1-2, HYR1, YKL071W, CUP1-1, MPD1, TRX2, CYC7, GPX1, HOM6, FRE6, COX6, DLD3, TRX1, COX3, MXR1, YKL107W, FRE3, COX7, COQ11, GTO1, CTT1, TSC13, GIS1, CIR1, YPR127W, YCR102C, DOT5, TPA1, GAL80, SER33, SUR2, SRX1, PRM4, HBN1, COX2, QCR7, AAD10, FDH1, ETR1, GRX2, GRX1, EUG1, HFD1, YJR096W*
Electron transfer activity (0.00877)	24.56	*CIR1, GRX2, GRX1, COX7, COX8, QCR9, CYC7, PRM4, COX6, COX5A, QCR7, COX2, COX3, COX1*
Antioxidant activity (0.01019)	32.26	*CUP1-2, DOT5, HYR1, GRX2, CUP1-1, GRX1, GPX1, SRX1, GTO1, CTT1*
Cytochrome-*c* oxidase activity (0.02878)	41.18	*COX1, COX3, COX7, COX2, COX5A, COX6, COX8*
Oxidoreductase activity acting on a sulfur group of donors (0.08819)	25.64	*TRX1, GTO1, SRX1, PRM4, EUG1, GRX2, MPD1, TRX2, GRX1, MXR1*
Downregulated (277 genes)		
Helicase activity (0.0165)	14.16	*YRF1-6, YRF1-7, DBP1, ARP5, YRF1-5, YHL050C, MSS116, MPH1, DBP7, YLL067C, YEL077C, YRF1-8, DHH1, SNF2, DBP3, YKU80*

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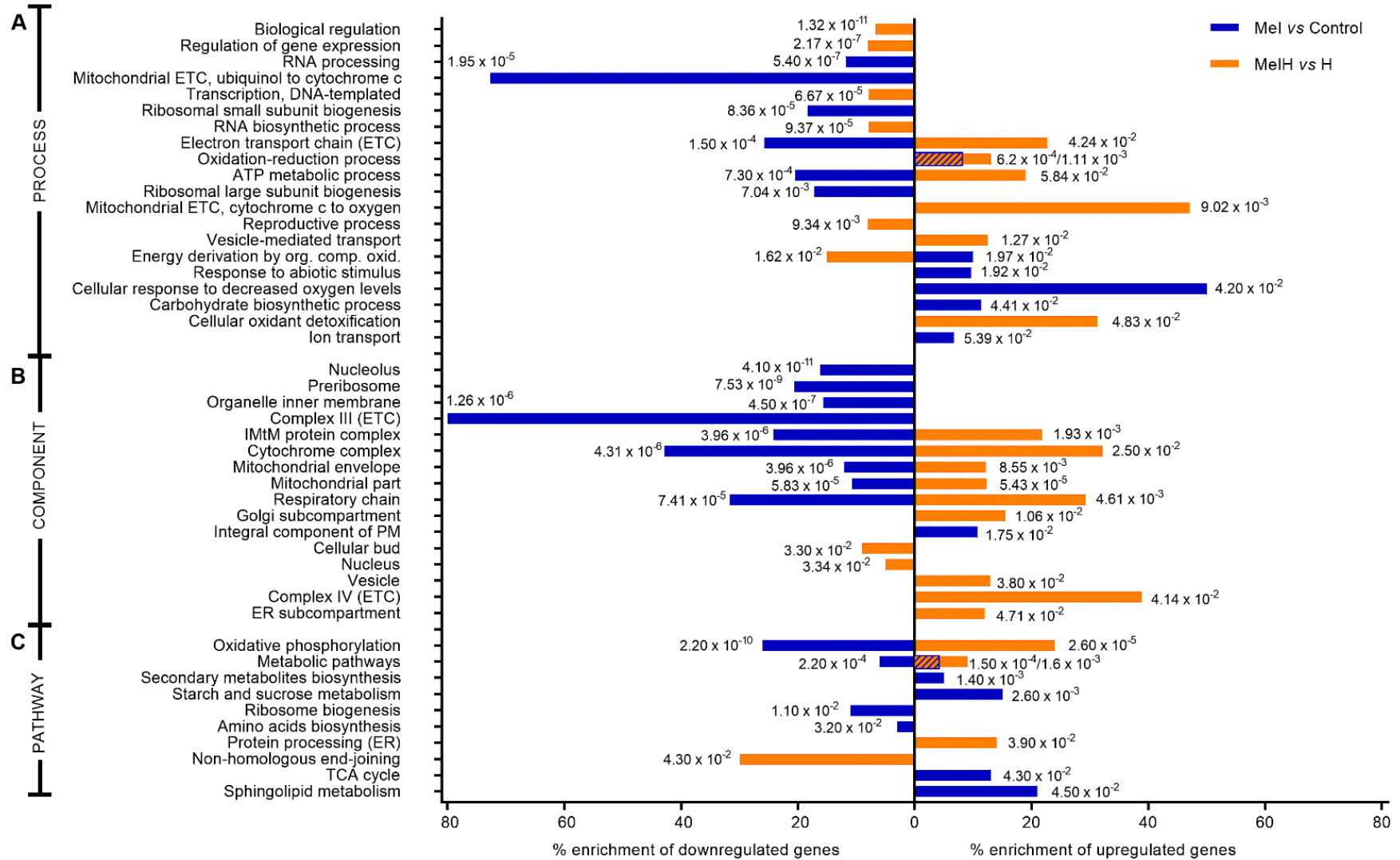
Figure 1.2. (previous page) Categories enriched by differentially expressed genes that were up- (values to the left) and down- (values to the right) regulated (1 ≤ fold change ≥1; *p*-value < 0.05) among nonstressed (blue) and stressed (orange) (2 mM H_2O_2) cells in the absence or presence of melatonin (5 µM). Percentages of enrichment are calculated as the ratio of the number of up- or downregulated genes in relation to the total number of genes involved in each molecular function or pathway in *S. cerevisiae*. The numbers correspond to the *p*-values. When two *p*-values are presented, the left one corresponds to Mel *vs* Control and the right to MelH *vs* H. (**A**) Biological process function enrichment from Gene Ontology (GO) analysis, (**B**) cellular components enriched according to GO analysis, (**C**) pathway enrichment analyzed with the DAVID tool. Abbreviations: ETC, electron transport chain; ER, endoplasmic reticulum; TCA, citrate cycle, IMtM, inner mitochondrial membrane.

Categories related to mitochondria were also highly enriched in the MelH *vs* H comparison, as observed in the Mel *vs* Control comparison; however, in this case, these categories were enriched in upregulated genes, such as complex IV and associated cytochrome *c* oxidase activity, which was the most enriched component (40%), resulting in 32% enrichment of the cytochrome complex (Figure 1.2, Table 1.2). Categories related to antioxidant function and detoxification were also significantly overrepresented (Figure 1.2A, Table 1.2). The downregulated genes were enriched mainly in categories related to gene transcription, reproduction, and the regulation of these processes (Table 1.2, Figure 1.2).

Some of the GO categories and genes regulated by melatonin with or without stress will be discussed in more detail in the following sections. In the case of higher concentrations of melatonin (25MelH *vs* MelH), although different gene expression profiles were observed, no significant differences were detected in the GO enrichment analysis.

3.3. Effect of melatonin on transport and membrane composition

Our transcriptomic study showed that the melatonin-upregulated genes were involved in transmembrane transporter activity in nonstressed cells, including transporters of ions [*HSP30* (also upregulated by melatonin in stressed cells)*, CCC2, VMA3, YHK8, FET3, FET4, VMA11*], amino acids (*DIP5, GAP1, AGP1, MUP3*), oligopeptides (*OPT1, OPT2, PTR2*) and urea (*DUR3*), among others (Table 1.2). Moreover, pathways related to lipid metabolism and peroxisomes were clearly altered in the presence of melatonin in both nonstressed and stressed cells. The melatonin-upregulated genes were involved in fatty acid (FA) elongation (13% and 38% enrichment in nonstressed and stressed cells,

respectively) and the biosynthesis of unsaturated fatty acids (UFAs) (10% and 30%) and sphingolipids (21% and 7%, Figure 1.2) (*SUR2,* independent of the presence of oxidative stress, and *LAC1* and ISC1 in nonstressed cells) (Table S2). Genes related to peroxisome and β-oxidation were also affected (13%, 15%) by melatonin and were both up- and downregulated.

In vertebrates, several of the functions of melatonin are mediated by its membrane receptors (Slominski et al., 2012), but others are receptor-independent, such as antioxidant activity, for which melatonin is required to penetrate the cell and enter intracellular compartments (Galano et al., 2011). In humans, melatonin is suggested to cross membranes by passive diffusion and through transporters of glucose (Hevia et al., 2015) or oligopeptides (Huo et al., 2017; Mayo et al., 2018). Moreover, as the membrane is the first barrier that separates the cell from the environment, it is one of the main targets of oxidative stress, which alters its lipidic composition. Yeast cells can sense oxidative stress and change their membrane composition to achieve tolerance against stress, and this response varies among yeast strains and species (Vázquez et al., 2019). Indeed, the diversity in the membrane composition in different yeast species and strains seems to lead to different levels of tolerance against oxidative stress (Vázquez et al., 2019). Moreover, melatonin modulates the FA composition and peroxisome proliferation in stressed and nonstressed cells, suggesting that it could influence the lipidic composition of cell membranes to achieve tolerance to oxidative stress (Vázquez et al., 2018).

Therefore, because melatonin modulated the expression of several genes involved in membrane transport and lipid metabolism, it was of interest to determine whether yeast cells are able to incorporate exogenous melatonin, whether this incorporation is altered by oxidative stress and whether the presence of melatonin in the medium affects the yeast membrane composition, either to prepare the cell to tolerate oxidative stress or to deploy a response that could lead to better tolerance against this stress.

3.3.1. Intracellular melatonin

To evaluate whether the QA23 strain was able to incorporate exogenous melatonin into the cell, intracellular melatonin was quantified under our four different conditions (Control, Mel, H and MelH) (Figure 1.3). Intracellular melatonin levels significantly increased when the cells were grown with melatonin supplementation independent of oxidative stress, indicating that *S. cerevisiae* (QA23 strain) was able to take up exogenous

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melatonin at nanomolar concentrations. Similar results have been previously reported in yeast (Bisquert et al., 2018) and mammals (Rodriguez et al., 2004; Reiter et al., 2007). Our results also showed that the highest levels were reached in stressed cells that were previously grown in the presence of melatonin (Figure 1.3). The higher melatonin levels in stressed cells may be due to the changes induced by H_2O_2 in both plasma membrane permeability and the gradient thereof, which might promote changes in cellular transport (Folmer et al., 2008) favoring the entry of melatonin into the cell. Another possible route through which melatonin may enter the cell is potentially through some membrane transporters, such as those for urea or polyamines (*DUR3*) (as they present a similar structure to melatonin), the oligopeptide transporter *OPT1*, or the maltose permease *MAL31*, the last two of which were upregulated by melatonin in presence and absence of oxidative stress (Figure 1.1E).
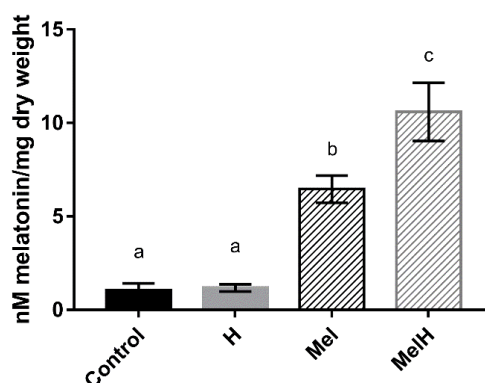
Figure 1.3. Intracellular melatonin quantification in cells untreated and treated with 5 µM of exogenous melatonin, before (Control and Mel) and after (H and MelH) being exposed to oxidative stress with 2 mM of H_2O_2. Error bars represent standard deviation, letters show statistical differences with *p*-value < 0.05.

Huo et al. (2017) recently described the possible involvement of *PEPT1/2* oligopeptide transporters in the uptake of melatonin in mammalian cells, and a new line of evidence has shown that glucose transporters are linked to melatonin uptake in human cells (Hevia et al., 2015; Mayo et al., 2018). Indeed, our results showed that some genes encoding carbohydrate and carbon source transporters were upregulated by the presence of melatonin in stressed cells (*HXT5, HXT6, HUT1, JEN1*, Dataset S4), which could also be related to melatonin internalization. Nevertheless, further studies should be performed to elucidate the possible role of these transporters in melatonin uptake by yeast cells.

3.3.2. Physiological changes in the lipid composition

To test whether the abovementioned transcriptomic modifications resulted in changes in the cell lipid composition, we measured the sterol and PL contents of the yeast cells under the four different conditions (Control, Mel, MelH and H, Figure 1.4). In a previous study, we measured the changes in the fatty acid composition under these four conditions (Vázquez et al., 2018).

In nonstressed cells, melatonin supplementation resulted in lower total sterol levels than in the control condition, primarily because of a lower ergosterol content (Figure 1.4A), leading to a lower ergosterol/squalene ratio (Figure 1.4C). The opposite situation was found under oxidative stress, with higher levels of total sterols, mainly due to increased ergosterol, leading to a higher ergosterol/squalene ratio (Figures 1.4A, C). A study that compared the membrane composition of different yeast strains showed that the most H_2O_2-tolerant strains exhibited a low sterol content before stress and an increased ergosterol/squalene ratio after oxidative stress (Vázquez et al., 2019), behavior that we observe with melatonin supplementation in the present study. Moreover, an increase in total sterols has been related to higher tolerance to H_2O_2 and other stress conditions (Henderson and Block, 2014). This suggests that one of the mechanisms by which melatonin confers resistance against H_2O_2 could be alteration of the ergosterol composition. The observed physiological behavior seems opposite to the pattern of the regulation of genes involved in sterol biosynthesis and transport, as these genes were upregulated in nonstressed cells (*ARE1, DAN1, IZH4, FHN1, HES1, UPC2*) and downregulated in stressed cells (*ARE2, ERG11, SWHI1, UPC2*). This could be explained by the regulatory role of Upc2p, a transcription factor that activates genes involved in sterol biosynthesis and transport and anaerobic genes and is activated under sterol depletion and anaerobic conditions (reviewed by Joshua and Höfken (2017)). Therefore, its targets would be downregulated in the presence of high sterol concentrations (in stressed cells, melatonin downregulated *ERG11*) and upregulated in the presence of low sterol concentrations (in non-stressed cells, melatonin upregulated *FHN1*, *ARE1*, *HES1* and particularly *DAN1*, a cell wall mannoprotein and sterol transporter). Thus, it seems that the increase in the ergosterol content in the presence of melatonin could be produced during the first hour of oxidative stress, and genetic regulation could be a response to the sterol concentration. This increase in the ergosterol content could be driven by either

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posttranscriptional enzyme activation or higher oxygen availability, which is required for ergosterol biosynthesis (Zavrel et al., 2013).
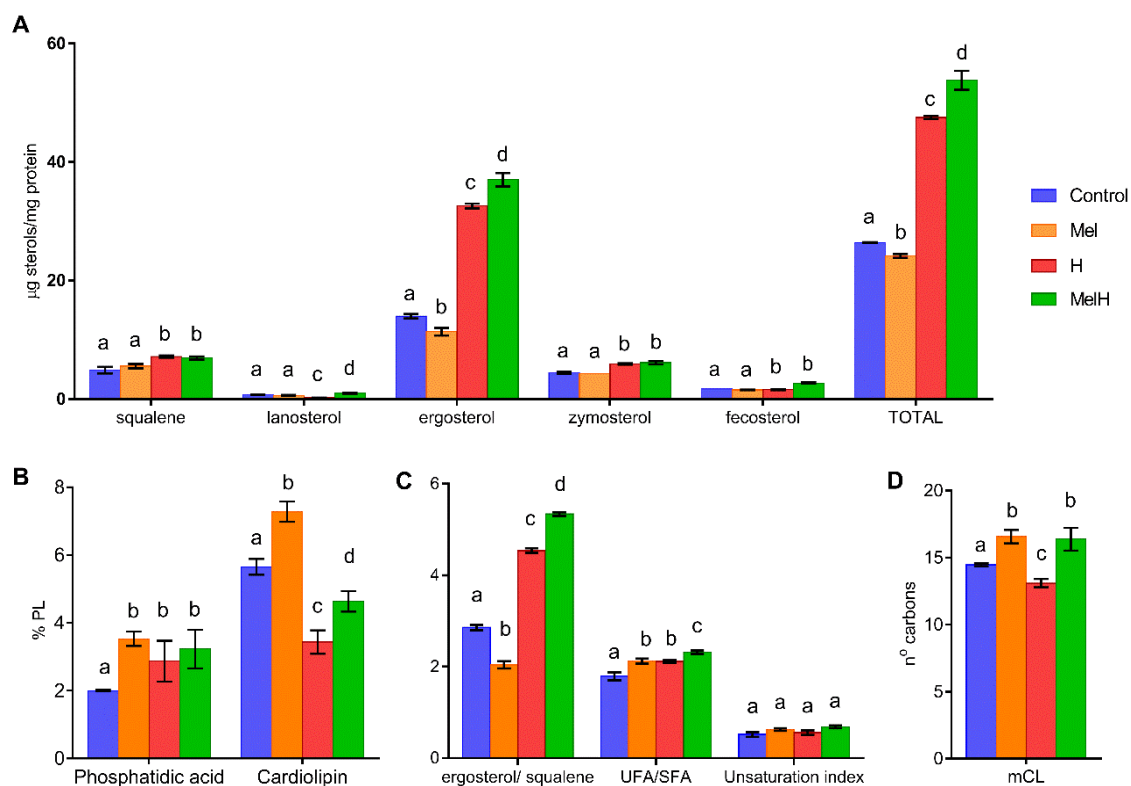
Figure 1.4. Lipid composition of nonstressed and stressed yeast cells with 2 mM of H_2O_2, grown with and without 5 µM of melatonin (Control, Mel, H, MelH). (**A**) Sterols. (**B**) Phospholipids. (**C**) UFA/SFA ratio (from Vázquez et al., 2018) and unsaturation index defined as follows: [(% C16:1 + % C18:1) + 2 (% C18:2) + 3 (% C18:3)]/100. (**D**) Medium-chain-length of FA (mCL, from Vázquez et al., 2018).

On the other hand, the changes in FAs produced by melatonin were independent of stress, with higher oleic and palmitoleic acid contents (Vázquez et al., 2018) leading to a higher total FA level, a higher UFA/SFA ratio and an increased percentage of medium chain length (mCL) FAs in Mel and MelH conditions (Figures 1.4C, D). This increase was correlated with the induction of the genes involved in the FA synthesis and elongation pathways (*TES1* in the absence of oxidative stress and *TSC13*, *PHS1*, *ETR1* in the presence of oxidative stress), among which the thioesterase gene *TES1*, which is involved in the last step of the synthesis of several UFAs (such as oleic acid), and the *TSC13* and *PHS1* genes, which are involved in long-chain FA elongation, were the most affected by melatonin treatment. Moreover, high UFA/SFA ratios have been related to higher tolerance to H_2O_2 (Serrazanetti et al., 2015). In fact, yeast strains with higher H_2O_2 tolerance showed higher

UFA/SFA ratios before and after stress (Vázquez et al., 2019), with increases in palmitoleic and oleic acid levels being observed after stress, as observed under melatonin treatment. These results suggest that melatonin changes in the membrane FA composition improve the tolerance to oxidative stress. Genes related to β-oxidation and peroxisomes were mostly upregulated by melatonin under oxidative stress in this study (*POX1*, *PXA1*, *PEX19*), which suggests that melatonin could increase the β-oxidation of FAs inside peroxisomes. These results are consistent with the higher FA content observed under melatonin treatment, but they are not consistent with the increased peroxisome proliferation observed in nonstressed cells and the decreased peroxisome proliferation observed in stressed cells (Vázquez et al., 2018).

Finally, the only observed changes in the PL content were related to phosphatidic acid (PA) and cardiolipin (CL). Melatonin only increased PA levels in the absence of stress, and although the CL level decreased in the presence of oxidative stress, it was higher under both conditions involving melatonin supplementation (Figure 1.4B). Vázquez et al. (2019) observed that all investigated strains showed a decrease in CL after stress exposure, but the strains that were most tolerant to H_2O_2 displayed higher CL values, as observed under melatonin treatment. In humans, melatonin prevents the peroxidation of cardiolipin to protect mitochondria from damage provoked by aging (Cardinali and Vigo, 2017). However, these results were not correlated with gene expression data, which revealed that in stressed cells, melatonin upregulated some of the genes involved in the biosynthesis of phosphatidylcholine (*CPT1*) and sphingolipids (*SUR2*), which are related to stress tolerance.

3.4. Response to oxidative stress

Our results showed that 32% of the genes involved in antioxidant activity were upregulated by melatonin in stressed cells (Table 1.2), including glutaredoxins (*GRX1*, *GRX2*), sulfiredoxin (*SRX1*) and four peroxidases (*DOT5, HYR1, GPX1* and *CTT1*), together with other genes with oxidoreductase activity, such as thioredoxins (*TRX1*, *TRX2*) and methionine-S-sulfoxide reductase (*MXR1*) (Table 1.2). These results were consistent with our previous studies (Vázquez et al., 2017), in which we observed that genes such as *GPX1*, *GRX2*, *TRX2* and *CTT1* were also induced by melatonin in *S. cerevisiae* stressed cells. Instead, in non-stressed cells, melatonin upregulated the cytoplasmic thioredoxin peroxidase *TSA2* but downregulated its paralog *TSA1* and the thioredoxin *TRX2*, mitochondrial superoxide dismutase *SOD2* and glutathione peroxidase *GPX2* genes

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(Dataset S1). Therefore, melatonin seems to downregulate the antioxidant response in the absence of oxidative stress. Other studies point in the same direction, as they have also shown that the expression of *GPX1*, *SOD2*, *GPX3* and *CTA1* was downregulated in the presence of melatonin (Vázquez et al., 2017; Bisquert et al., 2018).

To confirm our transcriptomic data, some of the genes related to stress tolerance showing higher expression ratios in MelH *vs* H condition were verified by qPCR analysis using the same conditions as in the transcriptomic assay: *SRX1*, which encodes a sulfiredoxin that contributes to protection against oxidative stress (Biteau et al., 2003; Allan et al., 2016); *ADY2*, which encodes a carboxylic acid transporter of the plasma membrane implicated in acetic acid tolerance (Zhang et al., 2017b); and *MGA1*, a stress-responsive gene that regulates Cis1p (explained in Section 3.6). In spite of the results of the Mel *vs* Control comparison, which were nonsignificant, the MelH *vs* H results showed tendencies that supported the results of the microarray analysis (Table S4).

Moreover, independent of the application of stress, melatonin upregulated the copper transporter *CCC2* and both metallothioneins (*CUP1-1* and *CUP1-2*), which have antioxidant and superoxide dismutase activity, and are involved in the detoxification of metal ions and removal of superoxide radicals, and downregulated the copper transporters *CTR1* and *CTR3* (the latter only in nonstressed cells) (Figure 1.1, Table 1.2). *FET3*, which contributes to resistance to copper toxicity, was upregulated by melatonin in the absence of oxidative stress (Table 1.2, Dataset S1). In a previous study, we observed that melatonin upregulated other metal-related antioxidants, such as *SOD1* (Vázquez et al., 2017). Therefore, our results seem to indicate that melatonin activates a response against the toxic effects of metal exposure in *S. cerevisiae*, as reported in human cells (Alonso-Gonzalez et al., 2008; Romero et al., 2014), even without the presence of metals, as a mechanism for better enduring further stresses.

3.5. Effect of melatonin on the mitochondria

Many of the genes regulated by melatonin were located in mitochondria (89 genes in Mel *vs* Control and 107 in MelH *vs* H), mainly in the respiratory chain complexes and mitochondrial envelope (Figure 1.2, Table 1.3); these results suggested that the mitochondria could be the biological target of melatonin, as observed in humans (Reiter et al., 2016).

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Table 1.3. List of mitochondrial genes regulated by melatonin in nonstressed cells (Mel *vs* Control) and stressed cells (MelH *vs* H). The fold change and *p*-values are provided in Datasets S1.1 and S1.4. They are classified into different categories, and the genes that were upregulated and downregulated in each condition are listed.

Category/Element	Mel *vs* Control		MelH *vs* H	
	Upregulated	**Downregulated**	**Upregulated**	**Downregulated**
Complex II of ETC	SHH4		SDH4, SDH8, SDH6	
Complex III of ETC		RIP1, CYT1, COR1, QCR2, QCR6, QCR9, QCR10, QCR8	QCR7, QCR9	
Complex IV of ETC	COX1, COX5B	COX5A, COX10, COX15, COX4, COX8, COX12	COX1, COX2, COX3, COX5A, COX6, COX7, COX8, COX17	
Complex V of ETC		ATP3, ATP5, ATP7, ATP17, ATP14	ATP8, ATP14, ATP15, ATP18, ATP19, ATP20	
Other genes related to ETC	RCF2	CYC1, CYC3, CYT2, COX10, COX15, NDE1	RCF1, CYC7, COQ10, COA1, CMC2	ALD5, COQ1
Mitochondrial protection	HSP78	SOD2, HSP60, GPX2	CIS1/YLR246C, GDH3, GPX1, TRX1, HYR1	
Mitochondrial import		PAM16, PAM18	TOM7, TIM8, HOT13, PAM17	XDJ1, MSK1, YJR045C
Mitochondria morphology		MIC19, MIC12, FMP30, MDV1	MIC19, MIC26, GET1	
Mitoribosome	PP1	MRPL7, MRPL16, MRPL51, MEPL15, MRPL19, MRPL6, MRPS28, MRP21	MRPL16, MRPL44, MRPL32, MRPL27, YNL122C, MRP2, MRP17, MRPS5, RSM18, MRPS8	GEP3, MRP4
Mitochondrial translation		RPO41, RMD9, RPM2, MSS116, MTG2	CBP6, RPM2, RTC6, PET54	MSS116, MSS51, PET111, PET9
Intergenomic signaling		COR1, QCR2, QCR6, COX5A, COX8, ATP3, ATP5, ATP7, ATP14, ATP17, BNA4, MIR1, SOD2	COX6, COX5A, COX7, COX8, ATP14, ATP20, QCR7, SDH4	
Mitochondrial transporters	MPC3, AAC3	MIR1, CTP1, RIM2, YAT1, OAC1	HSP10, HXT6, OM14, MRS4, CRC1	MMT1, MDL1, GNP1, ATM1, YHM2
TCA cycle	MDH2, CIT2, SHH4	LSC2	CIT2, SDH4, SDH8, SDH6	
Metabolism (other)	HEM13, HEM14, CHA1, ICT1, SYM1	GUT2, ARG8, EHT1, CLD1, FOL1, UPS2, DLD1	YHR208W, AYR1, NCE103, CPT1, HFD1, ETR1	MAE1, HEM1
Other genes ubicated in the mitochondria membrane	RDL1, ISC1	COQ8, YGR266W, OMS1	ZEO1, OM45, RDL1	YBR238C, UTH1
Protein modification		MAS2	PTH2, ISA2	YTA12, UBX2, PTC5, YME1
Other	ISU1, MMF1, VPS73	CIR2, UBP16, PUF3, AIF1, AIM36, MIX17, POS5, DRE25	YBL059W, YPT7, FYV4, ISU2, YSC83, CIR1, AIM19, VPS1, YCR028C-A, ATG9, FMC1, YSP2, MIX14, FMP33, HMF1, ECM10	PKP1, CAF4

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However, the effects of melatonin on mitochondrial genes were opposite in stressed and nonstressed conditions, since melatonin mainly downregulated mitochondrial genes in nonstressed cells but upregulated these genes in stressed cells (Table 1.3).

In non-stressed cells, most of the repressed genes were related to the ETC, particularly to cytochrome *c* reductase [complex III, including two catalytic (*RIP1, CYT1*) and six additional subunits], as well as complexes IV, V and other ETC-related elements, such as NADH dehydrogenase, a cytochrome *c* isoform and enzymes involved in the process of obtaining the heme groups of ETC complexes. Melatonin also downregulated most of the genes related to other mitochondrial functions, such as protection, morphology, transport, mitoribosome and intergenomic signaling between the nucleus and mitochondria. However, some genes were also upregulated, such as *SHH4* of complex II of the ETC, a respiratory supercomplex factor, and genes involved in the TCA cycle and heme synthesis (Table 1.3). On the other hand, in stressed cells, the induced mitochondrial genes were mainly related to cytochrome *c* oxidase (complex IV), including not only genes that encode the central units (*COX1-3*) but also genes related to assembly (such as *ATP20*), stability, regulation or enzyme activity. Melatonin also upregulated genes associated with other ETC complexes as well as a respiratory supercomplex factor or a cytochrome *c* isoform and genes related to mitochondrial functions such as protection, morphology, mitoribosomes, intergenomic signaling or the TCA cycle. However, in the transport, translation and metabolism categories, there were both up- and downregulated genes.

3.5.1. Gene validation

As mitochondria were the most enriched component, to confirm our transcriptomic data, the expression of some mitochondrion-related genes showing higher expression ratios in the MelH *vs* H comparison were verified through qPCR analysis. We chose genes related to different mitochondrial functions, such as genes involved in the respiratory chain (*COX2, SDH6, ATP20*), structure (*ATP20*), mitochondria protection (*GDH3, CIS1*) and transport (*MMT1*) (Figure 1.5).

Gene expression was analyzed at the same sampling points used in the microarray assays and at different growth times under the four conditions (Control, Mel, H, MelH), and the results are shown in Figure 1.5.
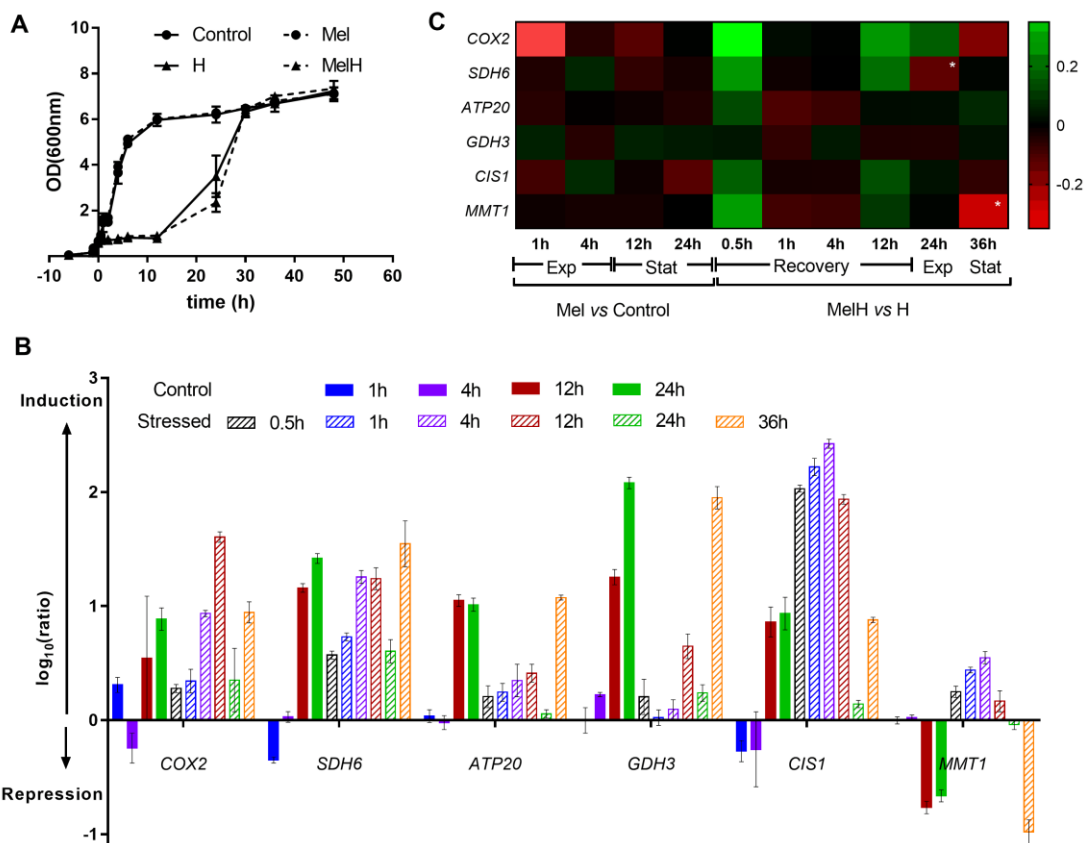
Figure 1.5. Expression of six selected genes over a time course. (**A**) Kinetics of yeast growth according to OD_{600nm} values. The cells were incubated with or without melatonin (5 µM) for 6 h, and two conditions (Mel, MelH) were subjected to 2 mM H_2O_2 treatment (0 h). (**B**) Expression of each gene of interest at different time points in the nonstressed and stressed conditions (Control, H), expressed as $\log_{10}$(ratio). The ratio represents the $2^{-\Delta\Delta Ct}$ value corrected to the $2^{-\Delta\Delta Ct}$ value of the Control at 0 h. (**C**) Heat map of the effect of melatonin on the expression of the six genes during the time course analysis in nonstressed and stressed cells. The color corresponds to the $\log_{10}$ of the ratio. The ratio represents the $2^{-\Delta\Delta Ct}$ value for the condition with melatonin corrected to the $2^{-\Delta\Delta Ct}$ value for the condition without melatonin. Genes are presented in rows, and the time points under both conditions are presented in columns. Exp, exponential phase; stat, stationary phase. High-intensity colors refer to values >0.35 (green) or < −0.35 (red) * significance level of < 0.05. Error bars represent the standard deviation, and the statistical analysis of B and C is presented in Table S3.

Most of the selected genes were upregulated either by entry into the stationary phase or by the response to oxidative stress exposure (which inhibited cell growth for 12 h, Figure 1.5A), following a general environmental stress response profile (Gasch et al., 2000). In nonstressed cells (Control condition), almost no changes in the expression of these genes were observed during the exponential phase (1 and 4 h) (Figures 1.5A, B), and only some of the genes were slightly downregulated (*SDH6, CIS1*) or upregulated (*GDH3*).

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However, the expression of all the genes (except *MMT1*) increased significantly in the stationary phase (12 and 24 h) (Figures 1.5A, B). Exposure to oxidative stress upregulated the expression of all genes, and these changes were significant for some of them at 1 h and for all the rest, except for *GDH3*, at 4 h (Table S3); their expression then decreased during the exponential phase (24 h), reaching the same levels observed before the stress in some cases, and significantly increased at the stationary phase (36 h) (Figure 1.5B). An exception was observed for *MMT1*, whose expression decreased in the stationary phase, as observed in the control condition.

The effect of melatonin on the expression of these genes in stressed and nonstressed cells is presented in Figure 1.5C. In nonstressed cells (Mel *vs* Control), melatonin slightly decreased the expression of most of the genes in the early exponential phase (1 h), but practically no changes were observed in the exponential and stationary phases (Figure 1.5C). In stressed cells (MelH *vs* H), melatonin induced most of the genes immediately after stress was applied (0.5 h), indicating an additive effect to the stress, after which the expression of most genes was slightly lower in MelH than in H conditions (1–4 h), followed by an increase just before entering the exponential phase (12 h). Thereafter, no important changes were observed in the exponential and stationary phases, except in *SDH6* and *MMT1*, respectively, whose expression was lower under MelH conditions (Figure 1.5C). Overall, the qPCR results supported those of the microarray analysis (Figure 1.5C, Table S4).

3.5.2. Physiological effect of melatonin in mitochondria

To determine whether the high impact of melatonin on mitochondrial gene expression revealed physiological effects such as an increase in the concentration or activity of mitochondria, we analyzed the number of mitochondria per cell using MitoTracker Green, a dye that accumulates in the active mitochondria of living cells, regardless of the mitochondrial membrane potential. When the cells were subjected to oxidative stress (2 mM H_2O_2) for 1 h, fluorescence in the cells increased, indicating a greater number of mitochondria per cell. This increase was greatest, and the difference was statistically significant in the presence of a high concentration of H_2O_2 (5 mM) (Figure 1.6). Moreover, in nonstressed cells or stressed cells in the presence of a low concentration of the oxidative compound (2 mM), the presence of melatonin increased the number of mitochondria, which became even higher when the melatonin concentration was increased (50 µM). However, when the stress was increased (5 mM),

the presence of melatonin did not affect the number of mitochondria per cell, which was already quite high due to the intensification of oxidative stress (Figure 1.6). These tendencies were also observed in the presence of 10 mM H_2O_2 (data not shown).
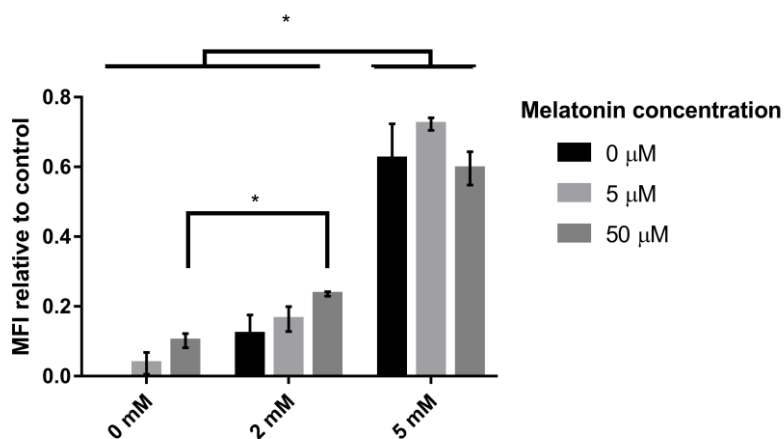
Figure 1.6. Effect of H_2O_2 and melatonin on the number of mitochondria per cell. The values are expressed as the mean fluorescence intensity (MFI) of the different conditions compared to the control. The cells were incubated with different melatonin concentrations (0 μM, 5 μM and 50 μM) and then subjected to treatment with different H_2O_2 concentrations (0 mM, 2 mM and 5 mM). Error bars represent the standard deviation, and * indicates statistically significant differences between media ($p < 0.05$).

The observation that melatonin affects complexes in different ways depending on the presence or absence of oxidative stress is supported by the validation analysis of two genes necessary for the growth during respiratory metabolism, *COX2* and *ATP20*: melatonin upregulated these genes after oxidative stress and downregulated them in non-stressed cells. *COX2* encodes a central subunit of complex IV necessary to assemble a functional complex, and *ATP20* encodes a subunit of Complex V that produces a functional association with complex IV, which is essential to achieve maximum levels of complex IV activity (Boyle et al., 1999).

This, together with the increase in complex IV activity observed by Zampol and Barros (2018), shows similar behavior to that of melatonin in human cells, in which melatonin increases the activity and gene expression of complex IV (Acuña-Castroviejo et al., 2003; Petrosillo et al., 2013). In mammals, one of the main roles of melatonin is the maintenance of the respiratory chain flux, mainly by increasing the activity of complexes I and IV (Martín et al., 2000a; Xia et al., 2019). Since *S. cerevisiae* lacks Complex I, it seems that the action of melatonin could be focused on complex IV, possibly due to the

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important role of complex IV in ETC regulation. Defects in complex IV increase ROS production, so this complex seems to be an indicator of the cell oxidative capacity and thus to be pivotal in ETC regulation (Piccoli et al., 2006; Srinivasan and Avadhani, 2012; Bode et al., 2013). On the other hand, complex III was the most affected by melatonin in non-stressed cells, in which most of the genes related to this complex were repressed. In mammalian cells, despite the clear antioxidant function of melatonin, several works have reported that melatonin *per se* can act as a prooxidant to induce ROS generation in several cell lines (Osseni et al., 2000; Büyükavci et al., 2006; Bejarano et al., 2010), an effect that has also been suggested in yeast cells (Vázquez et al., 2017). Recently, it was revealed that the ROS generation induced in mammalian cells by melatonin at micromolar concentrations occurs via complex III (Zhang et al., 2011). Moreover, Hardeland (2017) noted in a review that complex III activity has been found to be unchanged or upregulated by melatonin in different studies in different cell types. However, in yeast, melatonin seems to repress the expression of this complex; therefore, the effect of melatonin in this complex must be further studied.

Melatonin differentially regulates the expression of genes involved in mitochondrial protection. *GDH3*, which encodes a protein involved in the glutathione system and ROS reduction in the stationary phase, is upregulated in non-stressed cells and downregulated in stressed cells (1 and 12 h) by melatonin. In contrast, melatonin downregulated the expression of *CIS1* in nonstressed cells but upregulated its expression after stress exposure (0.5 and 12 h) (Figure 1.5). Cis1p, a mitochondrial protein whose biological role is unknown in yeast, has been recently reported to participate in the mitochondrial import surveillance mechanism, alleviating protein import stress (Weidberg and Amon, 2018). Mitochondrial import stress has been associated with an increase in the mitochondrial mass and with mitochondrial stress conditions, such as high levels of ROS (Weidberg and Amon, 2018). Therefore, the observed induction of *CIS1* in conditions that resulted in an increase in the mitochondrial content per cell (H and MelH, Figures 1.5 and 1.6) supported the hypothesis that this induction could be a response to maintain active mitochondrial import and, thus, mitochondrial function. Moreover, *CIS1* is induced by the transcription factor *PDR3*, which also mediates a multidrug resistance (MDR) response, and in our work, the conditions of stress and melatonin treatment (in stressed cells) also upregulated *PDR1,* another transcription factor related to MDR (reviewed in Kolaczkowska and Goffeau, 1999). Therefore, one of the actions of melatonin could be the triggering of the MDR response.

Our results indicated that melatonin plays a very important role in mitochondria, suggesting that it could be the biological target of melatonin, as observed in humans (Reiter et al., 2016), because in both organisms, mitochondria are the site of higher production of ROS and reactive nitrogen species (RNS) (Cardinali and Vigo, 2017) and therefore the site where the reported antioxidant effect of melatonin is most needed. Nevertheless, melatonin seems to play roles other than counteracting the effects of oxidative stress (as seen in mammalian cells (Tan et al., 2016)) because while the effect of melatonin is focused on the ETC, this stress mainly affects the mitoribosomes and the inner membrane (Datasets S11–S19), suggesting that the targets of melatonin in mitochondria could be different from those observed in association with oxidative stress. This is supported by the observation that melatonin affects different complexes and genes in different ways depending on the presence or absence of oxidative stress.

3.6. Effect of melatonin on cell signaling

The transcription factors involved in the different conditions were determined using PheNetic, a web tool that uses publicly available interactomics data to create networks and reveal possible relevant regulators. An interaction network was created using all the genes that were found to be differentially expressed ($-1 < FC > 1$, p-value < 0.05) in the Mel *vs* Control and MelH *vs* H comparisons to identify the relevant regulators of the effects of melatonin (Figures S1A, B). As melatonin seemed to have a great effect on mitochondria, another network was created using only the mitochondrial genes that were differentially expressed in these conditions (Figures 1.7A, B).

The analysis highlighted Cin5p and Ste12p as the central transcription factors among the genes regulated by melatonin in stressed and non-stressed cells (Figure S1). *CIN5*, which was upregulated by melatonin in all conditions, is a member of the Yap family and is upregulated after the application of an external stimulus such as oxidative or osmotic stress (reviewed in Rodrigues-Pousada et al. (2010)). Cin5p induces genes upregulated by melatonin in stressed cells (such as *SRX1*, *SET6* or *HSP30*) and in non-stressed cells (such as *HSP42*, *ISF1*, *GSY1*, *PIG2*, *HSP30* or *GAC1*) (MacIsaac et al., 2006; Hu et al., 2007; Rodrigues-Pousada et al., 2019). Both *CIN5* and *ADY2,* which were upregulated by melatonin in stressed cells in our study (Figure S1B, Table S4, Dataset S4), are known to be activated by Msn2/4p stress-responsive transcription factors (Alonso-Gonzalez et al., 2008; Batova et al., 2010). This suggests that melatonin could increase the response to

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oxidative stress in stressed cells, indicating an additive effect to the stress, which supports the results of Section 3.4 regarding the response to oxidative stress.

Ste12p, Mga1p (a node in MelH *vs* H, Figure S1B) and Gat3p (a node in Mel *vs* Control, Figure S1A) regulate filamentation and activate genes related to pseudohyphal/invasive growth (Liu et al., 1993; Borneman et al., 2006). These transcription factors seemed to be downregulated by melatonin, which suggests that melatonin could modulate filamentous growth in *S. cerevisiae*, as reported for some aromatic alcohols (such as phenylethanol and tryptophol) (Chen and Fink, 2006; González et al., 2018) produced (similarly to melatonin) through aromatic amino acid metabolism.

When we focused only on mitochondrial genes, the same transcription factors were identified together with some additional transcription factors, such as Hap4p and Yrm1p in both stressed and nonstressed cells and Hap1p and Rox1p only in the Mel *vs* Control comparison (Figure 1.7). Hap1p, Hap4p and Rox1p are heme-dependent transcriptional regulators. In the presence of heme, the Hap2/3/4/5 complex (in which Hap4p provides the activation domain) and Hap1p activate and upregulate genes required for aerobic growth, and Hap1p is also responsible for activating Rox1p, a repressor of hypoxic-related genes.

Therefore, in low-oxygen conditions (hypoxic growth), Rox1p is not expressed, and hypoxia-related genes are upregulated (reviewed by Siso et al., 2012). In our study, melatonin downregulated genes related to aerobic conditions in non-stressed cells (mitochondrial or nonmitochondrial) that are targets of Hap1p (*NDE1*, *CYC1* but also *CIR2* and *CLD1*), Hap2/3/4/5 complex (*COX5A*, *COX4* but also *INH1*) or both factors (*CYT1*, *RIP1*) (Figure 1.7, Dataset S1) (MacIsaac et al., 2006; Lee et al., 2013).

Under the same conditions, melatonin also upregulated several hypoxia-related genes that are targets of Rox1p, including three of the most upregulated genes (*DAN1*, *HEM13* and *ANB1*), several mitochondrial genes (*COX5B*, *AAC3* or *RCF2*) and other nonmitochondrial genes with high fold-changes (FCs) (*FET4*, *ARE1*, *GAC1*, *SUR2*, *FET3* and *LAC1*) (Figures 1.2, 1.7A, Dataset S1) (MacIsaac et al., 2006; Hu et al., 2007). All of these results suggest that melatonin could activate a hypoxic response in yeast cells, despite the slight upregulation of the transcription factors *HAP1*, *HAP4* and *ROX1* (which was only significant for *HAP1*) (Dataset S1). Indeed, Hap1p is known to be regulated by its interaction with heme at the protein level but not at the transcriptional level (reviewed at Siso et al. (2012)).

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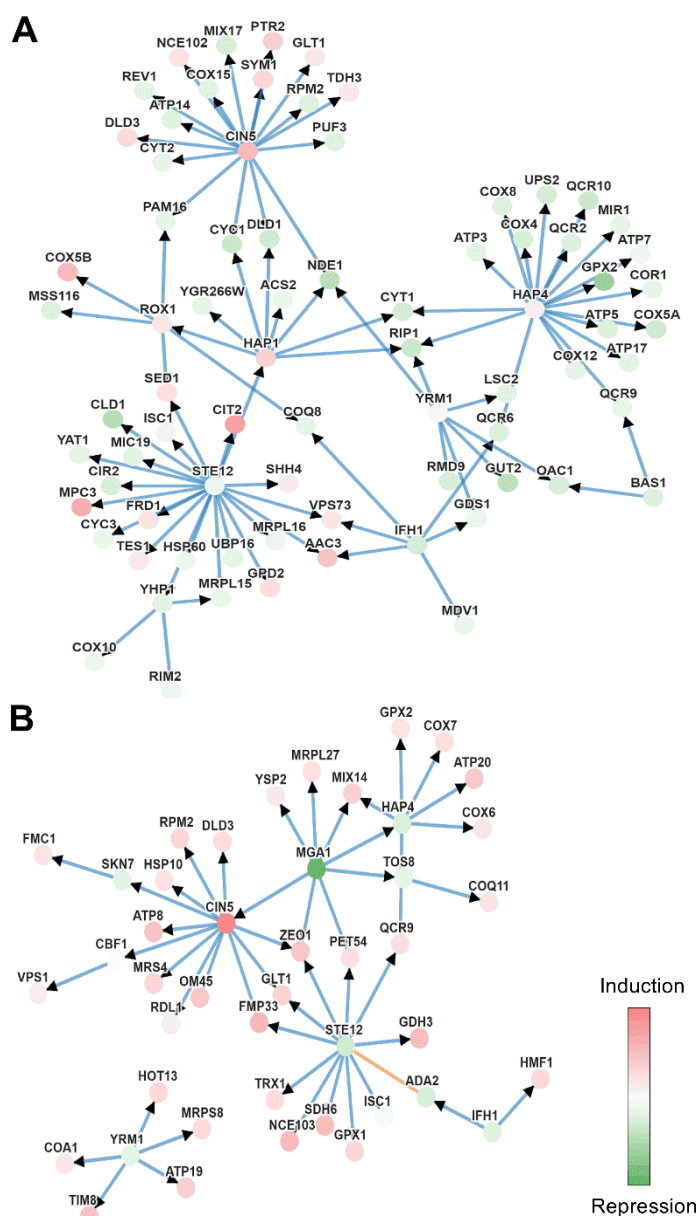
Figure 1.7. Genetic interactions given by Phenetics using mitochondrial genes with $-1 <$ FC < 1, *p*-value < 0.05. Set of genes used in each comparation: (**A**) Mel *vs* Control. (**B**) MelH *vs* H. Nodes represent molecular entities, and the intensity of its color represents the level of differential expression, green being under-expression and red over-expression. The edges indicate the interaction between the genes (blue indicates protein-DNA and orange protein-protein), and the arrows the direction of the interaction.

Other results obtained also supported the notion that melatonin could trigger a hypoxic response in nonstressed cells, such as the enrichment of the "response to decreased oxygen levels" among the upregulated genes in the Mel *vs* Control comparison (Figure 1.2, Dataset S13). Among these genes, we found *UPC2*, a sterol regulatory element that controls the expression of an anaerobic sterol transport system. The regulation of

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sterol import is tightly connected to anaerobic conditions, as it is specific to anaerobic growth (Lorenz and Parks, 1987; Zavrel et al., 2013). Under anaerobic conditions, Upc2p is upregulated and induces the expression of the *DAN/TIR* genes, a group of eight cell wall mannoproteins that are expressed under anaerobic conditions (Abramova et al., 2001). Indeed, our results showed the upregulation of four genes of this complex, *DAN1, DAN4, TIR1* and *TIR4*, which are associated with the enriched "structural constituent of the cell wall" molecular function category (Table 1.2). *DAN1*, a well-known hypoxic-related gene, was the gene showing the highest FC in the Mel *vs* Control comparison (Dataset S1). Some of our results agreed with those of Bendjiali et al. (2017), which describes a response specific to hypoxia.

Indeed, it has been reported that hypoxia induces the formation of foci containing glycolytic enzymes, which increases the incorporation of carbon into pyruvate and oxaloacetate (Miura et al., 2013). Recently, melatonin has been shown to bind a complex of glycolytic enzymes (Morcillo-Parra et al., 2019b), and this binding would be related to yeast fermentative capacity (Morcillo-Parra et al., 2020b). *S*everal glycolytic genes found in this complex, such as *PYC2* and *TDH3*, were upregulated by melatonin in non-stressed cells (Figure 1.1B, E, Dataset S1). Therefore, it seems that melatonin could trigger a hypoxia-like response in non-stressed *S. cerevisiae* cells regulated by the Hap complex, which could increase their fermentation performance. Nevertheless, more studies are needed to examine this hypothesis.

4. CONCLUSIONS

This study is the first to reveal the yeast transcriptional response in the presence of exogenous melatonin. *S. cerevisiae* was able to incorporate exogenous melatonin, which affected genome-wide gene expression levels upon entering the cell. In the absence of stress, melatonin exposure appears to activate genes related to transport, oxidoreductase activity and hypoxia and to downregulate genes related to mitochondria and the ETC (mainly complex III), triggering a hypoxia-like response, which could enhance fermentation performance. Indeed, some heme-dependent transcriptional regulators (Hap1p, Hap4p, Rox1p), together with the stress response factor Cin5p, seem to play a crucial role in the effect of melatonin at the transcriptional level. These changes in gene expression could also prepare yeast cells for coping with additional possible stresses such as oxidative stress. It is well established that under environmental stress conditions, the cellular machinery of yeast is reprogrammed to achieve better adaptation to stress,

affecting not only genes involved in antioxidant defenses but also genes involved in lipid metabolism and reproduction. In this context, melatonin might enhance energetic efficiency and signal transduction, conferring higher H_2O_2 tolerance to *S. cerevisiae*. Under oxidative stress, melatonin upregulates genes related to antioxidant activity, cellular detoxification, oxidoreductase activity and the respiratory chain (mainly complex IV), inducing transcriptional and physiological changes in yeast mitochondria. Thus, in stressed cells, melatonin supplementation seems to contribute to the stabilization of the mitochondrial electron chain, as observed in humans. However, as also shown in humans (reviewed in Cardinali and Vigo (2017)) melatonin can play other roles in the yeast mitochondria: it increases the cardiolipin concentration and acts as a mitochondrion-targeted antioxidant at both physiological and transcriptional levels, activating genes related to mitochondrial function and maintenance.

SUPPLEMENTARY MATERIAL

All the supplementary materials except for Figure S1 and Table S4 are not included in the present thesis due to format incompatibility, and are available online at https://www.mdpi.com/2076-3921/9/10/947/s1: **Table S1**: Categories enriched according to GO analysis of the common genes identified in different comparisons, as shown in a Venn diagram (Figure 1); **Table S2**: Enriched pathways according to a KEGG database analysis of genes that were differentially expressed among cells in Mel vs. Control and in MelH vs. H; **Table S3**: Statistical results of the ANOVA test for the data in Figure 5. **Datasets S1–S10**: Microarray analysis data of the different comparisons, statistics (moderated t-test) and annotations for each gene. Genes filtered with p-value < 0.05 (S1–S5) or no filtered by p-value (S6-S10): S1,S6: Mel vs Control; S2,S7: H vs. Control, S3,S8: MelH vs. Control; S4,S9: MelH vs. Control; S5,S10: 25MelH vs. MelH; **Datasets S11–S19**: Results of the GO enrichments [molecular function (11, 14, 17), cellular component (12, 15, 18) and biological process (13, 16, 19)] of the genes differentially expressed in Mel vs. Control (11–13), MelH vs. H (14–16), H vs. Control (17–19).

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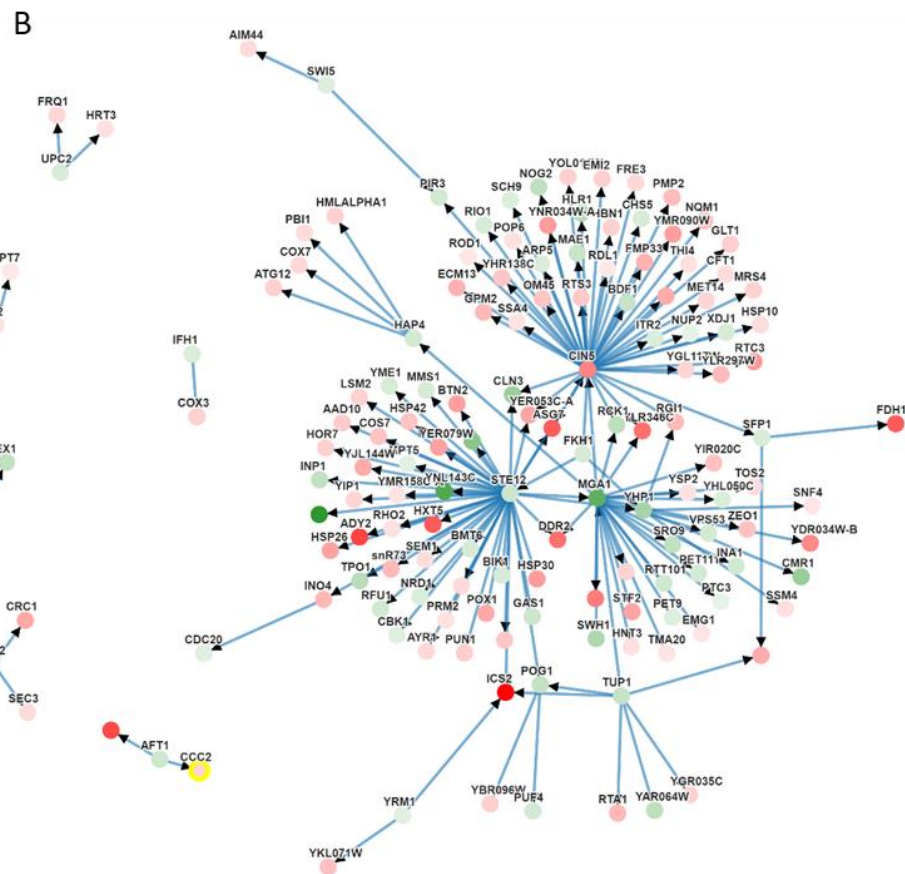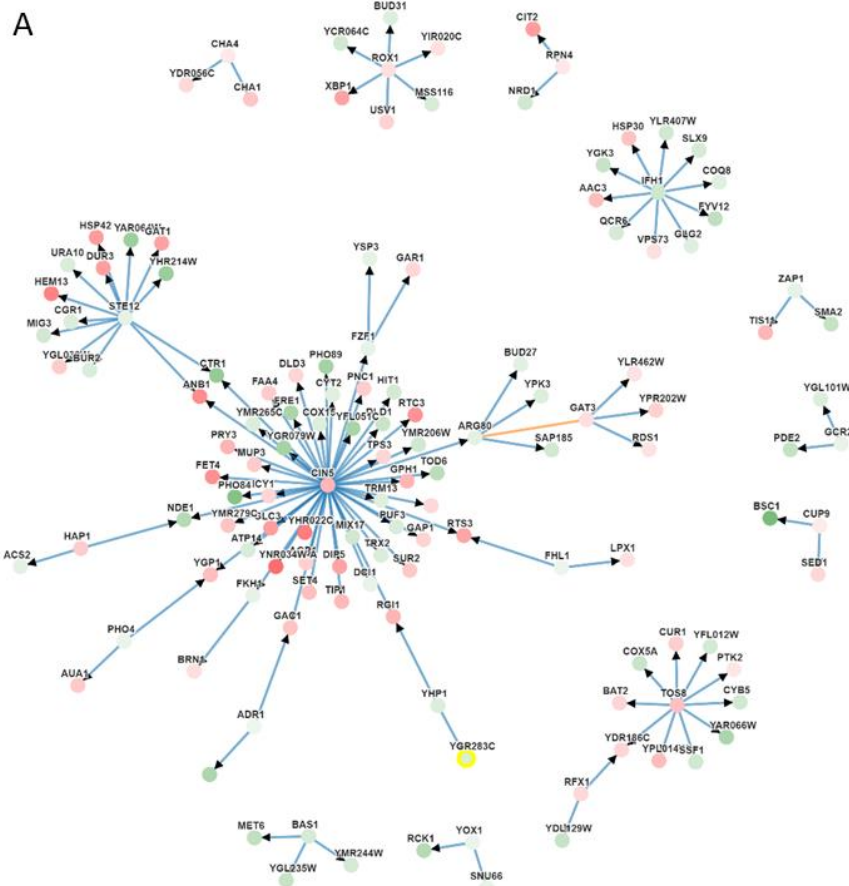
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Figure S1. (previous page) Genetic interactions given by Phenetics using genes with -1 < FC < 1, *p*-value < 0.05. Set of genes used in each comparation: (**A**) Mel *vs* Control, (**B**) MelH *vs* H. Nodes represent molecular entities, and the intensity of its color represents the level of differential expression, gree being under-expression and red over-expression. The edges indicate the interaction between ghe genes (blue indicates protein-DNA and orange protein-protein), and the arrows the direction of the interaction.

Table S4: Gene expression values representing the effect of melatonin on the nine selected genes in nonstressed and stressed cells. The transcriptomic (array) and validation (qPCR) analyses were performed using the same samples, and the results are represented as the $\log_{10}$ of the ratio of the $2^{-\Delta\Delta Ct}$ values for the condition with melatonin corrected to the $2^{-\Delta\Delta Ct}$ values for the condition without melatonin. * and + indicate significance levels of < 0.05 and < 0.1, respectively.

Gene name	Mel array	Mel qPCR	MelH array	MelH qPCR
COX2	0.014	-0.475*	0.088*	0.095
SDH6	-0.032	0.154	0.108*	0.206
ATP20	-0.048	-0.069	0.091*	0.149[+]
GDH3	-0.049	-0.326*	0.109*	0.056
CIS1	0.080[+]	0.078	0.226*	0.161
MTT1	-0.019	-0.570	-0.049*	0.024
MRPL44	-0.080[+]	-0.026	0.052*	-0.034
SRX1	-0.086	0.033	0.189*	0.212
ADY2	0.055	-0.012	0.296*	0.137[+]
MGA1	-0.035	0.029	-0.247*	-0.060
LDS2	-0.066	0.112	-0.189*	-0.152

CHAPTER II

Effects of oxidative stress and melatonin on mitochondria of *Saccharomyces cerevisiae* under fermentative conditions

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Abstract: Mitochondria are the principal targets of reactive oxygen species and their principal production sites but are also the main source of mechanisms to protect against oxidative stress caused by these compounds. Melatonin has antioxidant roles in several organisms, including yeasts, and in humans, its antioxidant action is mainly targeted in the mitochondria. The aim of this study was to examine the effects of an exogenous source of oxidative stress (H_2O_2) on the mitochondria of yeast grown under fermentative conditions. Additionally, the role of melatonin supplementation on the mitochondria of yeast cells in the presence and absence of stress was also studied. To achieve that, the effect of melatonin supplementation and H_2O_2 exposure on mitochondria of *S. cerevisiae* was evaluated by monitoring oxygen consumption, membrane potential, mitochondrial morphology and the electron transport chain function by further determining the abundance and activity of complexes III and IV and its assembly as a supercomplex. In general, oxidative stress exposure reduced oxygen consumption, membrane potential, and complex III and IV activities and promoted mitochondrial fission. Melatonin supplementation did not affect oxygen consumption, the membrane potential, or the activities of complexes III and IV in stressed or in nonstressed cells. However, in nonstressed cells, melatonin triggered mitochondrial fission. The results showed that exposure to hydrogen peroxide induced mitochondrial dysfunction in yeast grown under fermentative conditions, but the antioxidant role of melatonin previously observed under these conditions did not have a protective effect on mitochondrial functions.

Keywords: yeast, H_2O_2, oxidative stress, ROS, mitochondria dynamics, melatonin

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1. INTRODUCTION

All organisms are exposed to reactive oxygen species (ROS) during normal aerobic metabolism or following exposure to radical-generating compounds, such as hydroperoxides or thiol-reactive compounds (Halliwell, 2006). ROS are toxic agents that can damage a wide variety of cellular components, resulting in lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA (Morano et al., 2012). Yeast cells utilize a variety of antioxidant systems to protect against ROS, including detoxification systems, adaptation responses and regulation of protein synthesis at different levels (Morano et al., 2012; Picazo and Molin, 2021).

Mitochondria, mainly the electron transport chain (ETC), are the principal site of ROS production in the cell (Beckman and Ames, 1998). During aerobic respiration, ETC complexes generate superoxide ($O_2^{\bullet-}$), which cannot cross membranes at substantial rates but can produce hydroxyl radicals ($\cdot HO$) and hydrogen peroxide (H_2O_2), which cross membranes and attack cellular components, such as lipids, proteins or DNA (Hardeland, 2017). Paradoxically, mitochondria are also the main target of ROS, and therefore, damaged mitochondria produce higher levels of ROS, triggering cellular dysfunctions and resulting in degenerative processes underlying diseases and ageing (Venditti and Di Meo, 2020). Preventing mitochondrial damage is important to maintain cellular homeostasis. Therefore, mitochondria have mechanisms to protect themselves and the cell against this dysfunction, such as an antioxidant system to eliminate ROS, preventing the inactivation of mitochondrial components, or mechanisms to induce mitochondrial clearance (Venditti and Di Meo, 2020).

Mitochondria are reticula that dynamically fuse and divide, causing changes in morphology between an elongated filamentous network and fragmented individual mitochondrial structures (Scott and Youle, 2010). The cycles of fusion and fission favour the maintenance of mitochondrial integrity and functionality, preventing cell damage, and allowing mitochondria to modify their morphology to adapt to physiological changes (Venditti and Di Meo, 2020). For example, during fermentation, mitochondria have a tubular shape, whereas during respiration, their structure is branched (Kitagaki and Takagi, 2014). Additionally, mitochondria can be fragmented, separating by fission the parts where ROS accumulate, to prevent damaging the rest of the network. These fragmented mitochondria can be repaired and fused with the rest of the network or cleared from the cell by mitophagy, which is the specific pathway of autophagy for

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damaged or dysfunctional mitochondria (Venditti and Di Meo, 2020). As mitochondrial dysfunction is a characteristic of several human diseases, there is an increasing interest in treatments that lower ROS production and increase energy production. Therefore, the role of different antioxidants in mitochondrial functionality is being investigated, and recent studies have unveiled that several polyphenols, such as resveratrol or hydroxytyrosol, induce mitochondrial function or biogenesis (Pérez-Torres et al., 2021; Venditti and Di Meo, 2020).

Melatonin (N-acetyl-5-methoxytriptamine) is a bioactive molecule present in most living organisms (Hardeland and Poeggeler, 2003) with widely studied beneficial properties in humans through numerous physiological functions (Acuña-Castroviejo et al., 2003; Reiter et al., 2016, 2017). Mitochondria are the main target of melatonin in mammalian cells (Venegas et al., 2012), where it can be produced or imported through transporters, such as the oligopeptide transporter PEPT1/2 (Huo et al., 2017; Suofu et al., 2017). The fact that melatonin is targeted in the mitochondria confers an advantage in removing ROS faster than other antioxidants (Reiter et al., 2017, 2020). Therefore, it is beneficial for the prevention of some disorders related to mitochondrial dysfunction, such as Parkinsonism, Huntington's disease, multiple sclerosis, diabetes mellitus, cancer, Alzheimer's disease or septic shock (Reiter et al., 2017; Tan et al., 2016).

In fact, several cell protective actions of melatonin, which are well known in humans (reviewed in Acuña-Castroviejo et al., 2003; Cardinali and Vigo, 2017; Hardeland, 2017; Reiter et al., 2018; Tan et al., 2016), have been recently described in yeast (Vázquez et al., 2017, 2018, Bisquert et al., 2018). In both organisms, its main protective effect acts as an antioxidant, and is mitochondria-targeted in humans. In yeast, several studies have pointed in the same direction, since under H_2O_2 exposure, melatonin reduces ROS accumulation (Vázquez et al., 2017) and activates genes related to mitochondrial defence, function and maintenance (Chapter I of the present thesis). Moreover, melatonin also regulates cardiolipin concentrations in yeast membranes (Chapter I of the present thesis) and enhances mitophagy in yeast (Zampol and Barros, 2018). Recently, Zampol and Barros (2018) demonstrated that melatonin is beneficial for maintaining the efficiency of respiratory chain function in yeast, evidencing the existence of a conserved mechanism for melatonin in mitochondrial quality control and biogenesis. Therefore, these data suggest that melatonin also acts at the mitochondrial level in yeast cells, as this organelle

is also the site of higher production of ROS/reactive nitrogen species (RNS); therefore, it is the site where the antioxidant protections of melatonin are most needed.

Melatonin has been widely reported to be synthesized by yeast during alcoholic fermentation (Fernández-Cruz et al., 2017, 2018, 2019; Morcillo-Parra et al., 2020b; Rodriguez-Naranjo et al., 2012; Valera et al., 2019; Vigentini et al., 2015). Although little information is available on the synthetic route in yeasts, a recent study proposed a putative biosynthetic pathway including some steps described in plants (Muñiz-Calvo et al., 2019). Moreover, recent studies have deciphered that melatonin is involved in multiple biological processes in yeasts (Chapter I of the present thesis), gives protection to stresses other than oxidative stress (UV, ethanol) (Bisquert et al., 2018; Chapter IV of the present thesis) and could have a signalling role in fermentative metabolism (Morcillo-Parra et al., 2019b, 2020a, 2020b; Rodriguez-Naranjo et al., 2011a), which suggests that melatonin could give some advantage for fermentation performance. Melatonin has been proposed to be involved in the formation of a glycolytic complex in fermentative yeasts (Morcillo-Parra et al., 2019b, 2020b), and a glycolytic complex has been related to the channelling of different substrates, such as pyruvate, into the mitochondria (Brandina et al., 2006; Graham et al., 2007); therefore, taken this together with the well-known role of melatonin in the mitochondria of mammalian cells, the interest in the possible role of this molecule on yeast mitochondria during fermentation heightens. The presence of mitochondria is critical during fermentation, since its defect may lead to multiple cellular dysfunctions that increase cell sensitivity against fermentation-associated stresses, such as osmotic, ethanol or oxidative stress (Burphan et al., 2018; Grant et al., 1997; Hutter and Oliver, 1998). This suggests that mitochondria may have roles other than energy production during fermentation (Ernandes et al., 1993; Kitagaki and Takagi, 2014; Lodolo et al., 1999; O'Connor-Cox et al., 1996; O'Connor-Cox et al., 1993).

Therefore, the aim of this study was to evaluate, first, the effect of oxidative stress induced by H_2O_2 on the mitochondrial performance of *Saccharomyces cerevisiae* grown in fermentative conditions and then, the effect of melatonin on the functionality of mitochondria in the presence and absence of oxidative stress. For that, we monitored oxygen consumption, ETC complexes activities, mitochondrial protein abundance, membrane potential and mitochondrial morphology in cells under fermentative conditions in the absence or presence of oxidative stress and melatonin.

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2. MATERIALS AND METHODS

2.1. Yeast strains and experimental conditions

In this study, two strains of *S. cerevisiae* were used: the commercial wine strain QA23 (Lallemand Inc., Montreal, QA, Canada) and the lab strain BY4743 (EUROSCARF collection, Frankfurt, Germany). Additionally, for the mitochondrial morphology visualization, in the lab BY4743 strain, the Om45 protein was tagged with the green fluorescent protein (GFP) (BY4743 Om45-GFP, see section 2.2).

Yeasts were grown in complete medium (YP; 1% (w/v) yeast extract, 2% (w/v) peptone (Bacto, BD Biosciences, Franklin Lakes, NJ, USA)) supplemented with different carbon sources: 2% (w/v) glucose (YPD) (Sigma-Aldrich, St Louis, MO, USA) or 2% (v/v) glycerol (VWR, Radnor, PA, USA) (YPG). Yeast precultures were prepared in YPD broth for 24 h at 28 °C and 170 rpm, with a successive preculture in YPG for experiments using this medium. Then, yeasts were inoculated at OD $_{600nm}$ 0.05 in fresh YPD or YPG broth, with or without melatonin supplementation (5, 50, 100 µM). When the cells reached the initial exponential phase (OD$_{600nm}$ 0.5 – 0.6, considered throughout all the manuscript as time 0 h), they were exposed to oxidative stress, 2 mM of H_2O_2 (Sigma-Aldrich) for 48 h, monitoring the growth by measuring OD$_{600nm}$. A nonstressed control was performed for each condition, so, if not specified otherwise, four conditions were performed for each assay: nonstressed cells (Control, C), stressed cells (H), nonstressed cells supplemented with 5 µM melatonin (M) and stressed cells supplemented with 5 µM melatonin (MH).

For the assays of membrane potential, mitochondrial morphology, oxygen consumption rate and mitochondrial protein levels, experiments were performed in a volume of 60 mL of the medium, and cells were sampled after different times of exposure to stress (explained in the following sections). For the assays in which a mitochondria-rich fraction was needed, such as in the determination of complex III and IV activities and supercomplex assembly, 2 L of the yeast cultures were used. Three biological replicates were employed in all assays.

2.2. Construction of BY4743 Om45-GFP strain

The BY4743 Om45-GFP strain was constructed by inserting GFP in the C-terminal region of the OM45 gene. Briefly, a DNA cassette encoding GFP with HIS3 from *Schizosaccharomyces pombe* as a selective marker was amplified using pYM28 (Janke et

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al., 2004) as a template plasmid, and the primers hybridized with the C-terminal domain of the OM45 gene of *S. cerevisiae* (Table S2.1). After checking and purifying the PCR product, it was used to transform BY4743 yeast cells following the protocol of Schiestl and Gietz (1989). Positive transformants were selected on SD agar plates without histidine. Correct integration was determined by PCR analysis of genomic DNA, with the primers specified in Table S2.1.

2.3. Measurement of the yeast oxygen consumption rate

The oxygen consumption rate was measured in QA23 cells grown in YPD and YPG media at different time points. In YPD, samples were taken before (C and M) and after (H and MH) exposure to oxidative stress at different phases of the growth curve, just 1 h after the stress and in the early exponential and early stationary phases. In YPG, samples were taken at time 0 h with and without melatonin (C and M). Oxygen consumption by yeast was quantified with a Clark-type oxygen electrode, as described in Toth et al. (2020). The electrodes were calibrated using $NaSO_3$ (considered 0% oxygen) and YPD (considered 100% oxygen). In brief, 2 mL of QA23 culture was transferred into the measurement chamber, and the oxygen concentration was measured for at least 1 min. Oxygen consumption was normalized by cellular mass (measured by OD_{600nm}) and was subsequently normalized to the mean oxygen consumption of nonstressed cells grown in YPD medium without melatonin to present fold change values. Cultures were diluted in YPD prior to measurements if required.

2.4. Analysis of mitochondrial proteins by immunoblotting in whole cells

The mitochondrial protein levels were determined in whole cell extracts of samples taken before (C, M) and after (H, MH) exposure to oxidative stress for 1 h and at different phases of the growth curve as follows: at early exponential, early stationary and late stationary phase for QA23 cells grown on YPD; at early exponential phase for QA23 cells grown on YPG; and at early stationary phase for BY4743 cells grown on YPD. Cells (OD_{600} 2.5) were harvested by centrifugation at 2,300 g for 1 min, followed by a lysis step with 0.1 M NaOH for 5 min. After lysis, the samples were centrifuged at 16,000 g for 2 min, and the pellet was resuspended in protein extraction buffer (50 mM Tris-HCl pH 6.8, 2% SDS (sodium dodecyl sulfate), 10% glycerol, 100 mM DTT (dithiothreitol)). Then, 25 μL of the samples was boiled for 3 min at 95 °C, separated by standard SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and transferred to a nitrocellulose

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membrane (Roth, Karlsruhe, Germany). Finally, blots were probed using rabbit antibodies against Cox2 and Qcr7 (Gruschke et al., 2012) diluted 1:10,000. The antibody against Tom70 (Hildenbeutel et al., 2014) was used as loading control. Primary antibody detection was performed using goat anti-rabbit (BioRad, Hercules, CA, USA) (1:10,000) and WesternBright® Quantum® Chemiluminescent HRP substrate (Advansta, San Jose, CA, USA), following the manufacturer's instructions.

2.5. Measurement of the mitochondrial transmembrane potential in whole cells

To monitor changes in mitochondrial transmembrane potential and cell dead (analyzed as the loss of plasma membrane integrity), the protocol of Aufschnaiter et al. (2018) was followed. In brief, 2×10^6 cells of a culture of QA23 grown on YPD medium before (0 h, C, M) and 1 h (H, MH) after stress were collected by centrifugation in 96-well plates. The pellet was resuspended in 250 µL of phosphate buffered saline (PBS, 25 mM potassium phosphate, 0.9% NaCl, pH 7.2) containing 200 nM MitoTracker Red CMXRos (ThermoFisher Scientific, Whaltham, MA, USA) for the mitochondrial transmembrane potential assay and 100 µg/L propidium iodide (PI, ThermoFisher Scientific, Whaltham, MA, USA) for cell death. After incubation for 10 min in darkness at room temperature, cells were washed once in YPD and analyzed with a Guava easyCyte flow cytometer (Merck Millipore, Burlington, MA, USA) or visualized via confocal laser scanning microscopy (protocol explained in Section 2.6 of the present chapter). Flow cytometric data was analyzed with Guava InCyte software (Merck Millipore). Mitochondrial transmembrane potential was quantified as the mean fluorescence intensity and cell death was evaluated as the percentage of PI positive cells.

2.6. Determination of the mitochondrial morphology by microscopy analysis

Cultures of BY4743 cells expressing GFP-tagged Om45 were incubated on YPD medium supplemented with 5, 50 or 100 µM melatonin, using the condition without melatonin addition as a control. Samples were taken before (0 h, nonstressed) and after (1 h, stressed) stress exposure. The samples were analysed using confocal microscopy as described in Toth et al. (2020). In brief, cells were immobilized on 3% agar slides and images were obtained with a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany), equipped with a Zeiss Plan-Apochromat 63x NA 1.4 DIC M27 oil immersion objective. Samples were excited at 555 nm and emission between 580 and 610 nm was detected. Images were recorded as Z stacks using 56x56x330 (x/y/z) nm sampling, and

subsequently processed using the open-source software Fiji (Schindelin et al., 2012). The treatment of the images consisted of the application of three-dimensional Gaussian filtering (xσ = yσ = zσ = 1), followed by background subtraction (rolling ball radius = 50) and the adjustment of brightness and contrast. Z-projections were created using the maximum-intensity projection method. All pictures within an experiment were captured and processed with the same settings. The classification of the mitochondrial network morphology followed the criteria reported in previous studies (Okamoto and Shaw, 2005; Toth et al., 2020; Westermann, 2010).

2.7. Mitochondria isolation

Mitochondrial isolation from yeast cells was performed as described in Meisinger et al. (2006) with some modifications as follows: cells were harvested by centrifugation for 10 min at 6,000 g, washed with distilled water and incubated with 2 mL MP1 buffer/g cell weight (0.1 M Tris, 10 mM DTT) at 30 °C for 10 min. Afterwards, the cells were washed with 1.2 M sorbitol and spheroplasts were obtained by incubation with Zymoliase 20T (3 mg/g cell weight; Amsbio, Cambridge, MA, USA, prepared in buffer MP2 (1.2 sorbitol, 20 mM potassium phosphate, pH 7.4)) for 1 h at 30 °C. From this point, all the procedures were performed in the cold. After centrifugation, spheroplasts were washed with lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.6 M sorbitol and 0.2% BSA) and then lysed in the same buffer by resuspending forty times with a cut 5-mL pipette tip. Then, three successive centrifugations at 2,000 g for 5 min were performed to eliminate the pellet, and the resulting supernatant was centrifuged at 17,000 g for 12 min to precipitate mitochondria. The pellet was resuspended in SH buffer (0.6 M sorbitol, 20 mM HEPES, 1 mM EDTA), measured the protein concentration by the Bradford method (Bradford, 1976) (Sigma-Aldrich) and stored at -80 °C until use. The mitochondrial concentration was approximated by dividing the amount of protein by the initial cell weight.

2.8. Measurement of complexes III and IV activities in mitochondrial extracts

The activities of complexes III and IV were determined in the mitochondria-rich extracts of QA23 and BY4743 cells grown in YPD medium before (0 h, conditions C and M) and 1 h (conditions H and MH) after oxidative stress. Additionally, the activity of complex IV in mitochondrial extracts of QA23 cells grown on YPG medium before stress (0 h, conditions C and M) was also measured. For these analyses, isolated mitochondria were

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lysed using the protocol of cytochrome *c* oxidase assay kit (Sigma-Aldrich): 2 mg/mL of mitochondria protein content (measured by Bradford method (Bradford, 1976) (Sigma-Aldrich)) were resuspended in enzyme dilution buffer containing 1 mM n-dodecyl-β-maltoside (DMM) and incubated on ice for 10 min, slightly mixing every 3 min. Lysed mitochondria were used directly or diluted in enzyme dilution buffer.

For complex III activity, the protocol of Spinazzi et al. (2012) was used. Briefly, 15 µL of lysed mitochondria was added to a cuvette together with 980 µL of buffer containing 25 mM Tris (pH 7.5), 0.4 mg/mL oxidized cytochrome *c* (cytochrome *c* from equine heart, Sigma–Aldrich), 0.5 mM KCN, 0.1 mM EDTA and 0.02% Tween 20. Immediately, the reaction was initiated by the addition of 5 µL of 20 mM decyl-ubiquinol (coenzyme Q (Decylubiquinone, Sigma-Aldrich) reduced with sodium borohydride), and the absorbance at 550 nm was monitored every 2 s for 8 min in a SpectroStar NANO spectrophotometer (Bmb Labtech, Germany). This measure reported an increase in reduced cytochrome *c* concentration.

For complex IV activity, cytochrome *c* oxidase assay kit (Sigma-Aldrich) was used. Briefly, 940 µL of assay buffer was mixed with 10 µL of lysed mitochondria, and just after adding 50 µL of reduced cytochrome *c* (2.7 mg/mL), the absorbance at 550 nm was measured every 2 s for 8 min in a SpectroStar NANO spectrophotometer (Bmb Labtech, Germany). This measure reported a decrease in reduced cytochrome *c* concentration.

For both assays, we performed a blank with water instead of mitochondria for each sample. To ensure the specificity of the measured activity, for each sample, we performed a negative control by adding to the reaction a compound that blocked complex III (antimycin A at 20 mg/mL) or complex IV (KCN at 20 µg/mL) and measuring the activities III and IV, respectively. In all cases the final reaction volume was 1 mL. For each assay, the slope (ΔAbsorbance/min) was calculated and used to obtain the enzymatic activity of each complex according to the following equation (Spinazzi et al., 2012):

Enzyme activity (nmol min^{-1} mg^{-1}) = (ΔAbs$_{550nm}$/min × reaction volume (mL))/[(Ɛ× sample volume (mL)) × (sample protein concentration (mg ml^{-1})) × path length (cm)], where Ɛ was the extinction coefficient for reduced cytochrome *c* at 550 nm and 18.5 mM^{-1} cm^{-1}.

2.9. Analysis of mitochondrial supercomplex assembly by blue-native PAGE and immunoblotting

Mitochondrial supercomplex assembly was analysed in mitochondria-rich extracts of QA23 cells grown on YPD medium before (0 h, C, M) and 1 h after (H, MH) the application of stress. All mitochondrial lysis protocols were performed under cold conditions. Briefly, 100 µg of isolated mitochondria was centrifuged at 10,000 g for 10 min, and the pellet was resuspended in lysis buffer (50 mM Bis-Tris, 25 mM KCl, 2 mM aminohexanoic acid, 12% glycerol, 1 mM PMSF, 2% digitonin and protease inhibitor cocktail (cOmplet^{TM}, Roche, Penzberg, Germany)) and incubated for 10 min. After centrifugation at 16,000 g for 10 min, the supernatant was mixed with 0.5% sample additive (5% G-250 Sample Additive; Thermo Fished Scientific) and loaded on a 3–12% precast native gel (Invitrogen, Waltham, MA, USA), which was subsequently stained with Coomassie (0.125 % (w/v) Commassie Brilliant Blue R250, 45 % (v/v) ethanol, 10 % (v/v) acetic acid). Finally, for the immunoblot analysis, the gel was transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA), which was probed with an antibody against Cox1 (Abcam, Cambridge, UK) (1:10,000). Primary antibody detection was performed using goat anti-rabbit and WesternBright® Quantum® Chemiluminescent HRP substrates as indicated in section 2.4 of the present chapter.

2.10. Data analysis

Data obtained from all the assays were subjected to analysis of variance (ANOVA) and *Tukey's post hoc* test using GraphPad Prism 7 (GraphPad Software, CA, USA). The results were considered statistically significant at a p-value < 0.05. To calculate combined standard deviations, the StatsToDo website (China) was used.

3. RESULTS

3.1. Effect of melatonin and oxidative stress on the yeast oxygen consumption rate

The rate of oxygen consumption of QA23 cells during growth in YPD medium was measured in the presence or absence of melatonin and oxidative stress (2 mM H_2O_2) (Figure 2.1). In nonstressed cells, the oxygen consumption rate was maintained during the exponential phase, regardless of the presence or absence of melatonin. However, this value was halved when cells reached the stationary phase (Figures 2.1A, S2.1A). On the other hand, oxidative stress provoked a total inhibition of oxygen consumption, which

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was gradually restored when cells recovered from stress and started growing. Even the oxygen consumption levels at the stationary phase were similar to those before stress (Figures 2.1A, S2.1A). Melatonin presence did not modify oxygen consumption rate in the absence or presence of stress (Figure 2.1A). As expected, the oxygen consumption rate was higher in a respiratory medium, such as YPG, being 1.5-fold higher than that in fermentative conditions (YPD). Melatonin did not affect this indicator in respiratory metabolism (Figure 2.1B).

Our results showed that the application of oxidative stress completely inhibited oxygen utilization by the cells just after stress. Therefore, as oxidative stress may affect mitochondria, even under fermentative conditions, and probably the ETC, as the main site of oxygen consumption in the cell, we wanted to deepen the understanding of the effect of oxidative stress and melatonin on different mitochondrial functions.
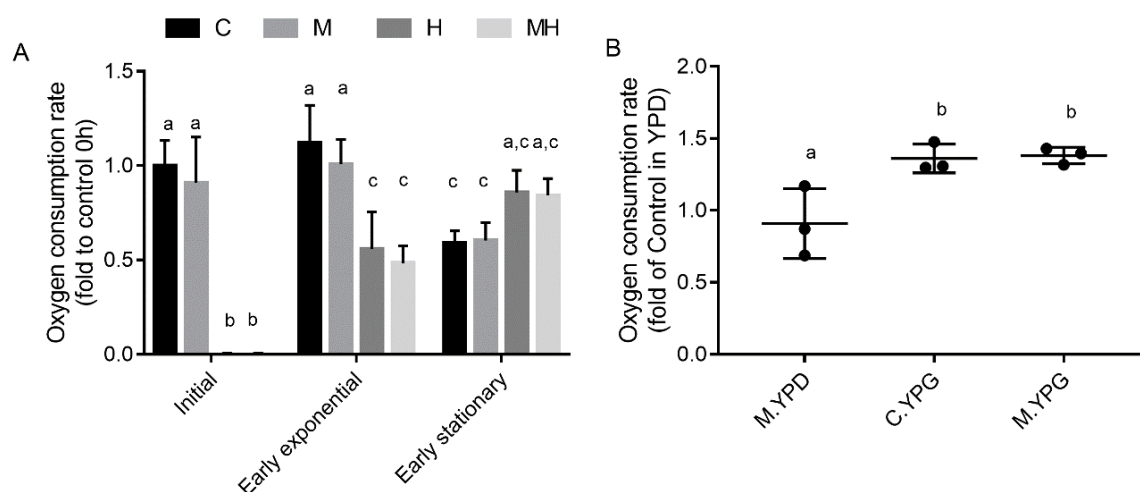
Figure 2.1. Cellular oxygen consumption rate of QA23 cells. Oxygen consumption was calculated as the difference on oxygen saturation of the culture (in %) for 1 minute, normalized to cellular growth (OD_{600nm}); values are normalized to the oxygen consumption rate of cells grown in YPD medium without melatonin and before being exposed to oxidative stress (C.YPD). (**A**) Oxygen consumption measured in QA23 cells in presence or absence of 5 µM of exogenous melatonin, before (C and M) and after (H and MH) being exposed to oxidative stress (2 mM H_2O_2) in different phases of the growth curve: just 1 h after stress (initial), at early exponential and early stationary phases. (**B**) Comparison of oxygen consumption rate between nonstressed QA23 cells grown in fermentative (YPD) and non-fermentative (YPG) medium in presence or absence of melatonin (5 µM). Bars indicate deviations, and letters indicate significative differences (p-value<0.05).

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3.2. Effect of oxidative stress and melatonin on mitochondrial protein levels

The levels of some mitochondrial proteins during the growth of QA23 cells in fermentative medium (YPD) in the presence or absence of melatonin and oxidative stress (2 mM H_2O_2) were evaluated. For that reason, the subunits Qcr7 of complex III and Cox2 of complex IV were monitored by immunoblotting in a time-course experiment. Although oxidative stress negatively affected the cell growth on YPD (Figure S2.1A), it did not change the abundance of the mitochondrial proteins analysed in comparison to the control condition, regardless of the presence or absence of melatonin (Figure 2.2). However, the abundance of these proteins was modified due to yeast growth. In fact, Cox2p levels gradually increased during the exponential and stationary phases, showing the highest level at the late stationary phase in all conditions. Instead, Qcr7p abundance initially decreased in the exponential phase to slightly increase in the stationary phase, and the late stationary phase was the point of maximal accumulation (Figure 2.2). Similar results were obtained for lab strain BY4743, with no effect on mitochondrial protein content due to the presence of melatonin or to the application of oxidative stress (Figure S2.1B). Nevertheless, in this strain, Cox2p abundance decreased in the stationary phase, opposite to the behaviour observed in QA23 (Figure S2.1B).
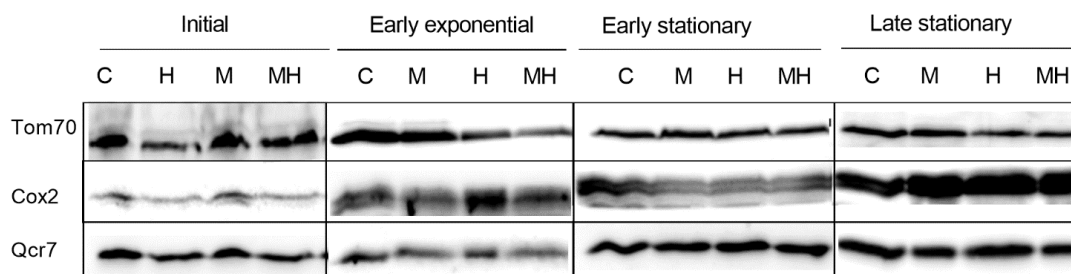
Figure 2.2. Monitoring of mitochondrial protein levels by immunoblotting using antibodies against Cox2 and Qcr7 in QA23 cell extracts grown in YPD medium in presence or absence of 5 μM of exogenous melatonin, before (C and M) and after (H and MH) being exposed to oxidative stress (2 mM H_2O_2) at different phases of the growth curve: just 1 h after stress (initial), at early exponential, early stationary and late stationary phases. Antibody against Tom70 was used as control.

In the case of respiratory medium (YPG), no differences were detected in these proteins between conditions, neither by oxidative stress or melatonin nor by yeast growth phase (Figure S2.1C). Indeed, under this condition, less effect was also observed on cell growth due to oxidative stress compared to YPD medium (Figure S2.1A).

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3.3. Effects of oxidative stress and melatonin on mitochondrial membrane related parameters

We evaluated the effects of oxidative stress (exposure for 1 h) and melatonin on the mitochondrial membrane potential of *S. cerevisiae* cells using a membrane potential-sensitive fluorescent dye, CMXRox. PI analysis showed a very low number of dead cells; therefore, Mitotracker Red CMXRos dye accumulation due to loss of membrane permeability was considered negligible. The exposure of cells to 2 mM H_2O_2 for 1 h clearly lowered the membrane potential, but no differences were reported by melatonin incubation in the cytometry analysis (Figures 2.3; S2.2).
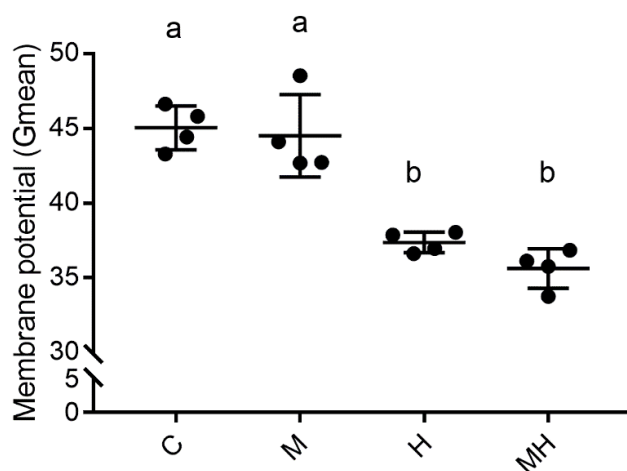
Figure 2.3. Quantification of mitochondrial transmembrane potential of QA23 cells grown on YPD in presence or absence of 5 µM of exogenous melatonin, before (C and M) and after (H and MH) being exposed for 1h to oxidative stress (2 mM H_2O_2). Mean fluorescence intensities of mitotracker CMXRos-stained cells quantified by flow cytometry are presented. Error bars represent standard deviation, letters show significative differences (p-value<0.05).

Additionally, we wanted to monitor the effect of H_2O_2 and melatonin on mitochondrial morphology by visualizing the mitochondrial network in the BY4743 Om45-GFP strain. This strain was constructed by inserting green fluorescent protein (GFP) in the C-term domain of *OM45*, encoding the major constituent of the outer mitochondrial membrane, to observe whether there were changes in the shape and conformation of the mitochondrial network due to the presence or absence of melatonin in stressed and nonstressed cells. Microscopic analysis revealed that the cells subjected to oxidative stress presented an unequivocal fragmented mitochondrial network, while in the nonstressed cells, the network morphology was tubular, confirming that oxidative stress promoted mitochondrial fission (Figure 2.4). In nonstressed cells, treatment with 5 and 50

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μM melatonin also promoted mitochondrial fission. Interestingly, in stressed cells, the preincubation with melatonin had no significant effect on mitochondrial morphology (Figure 2.4).
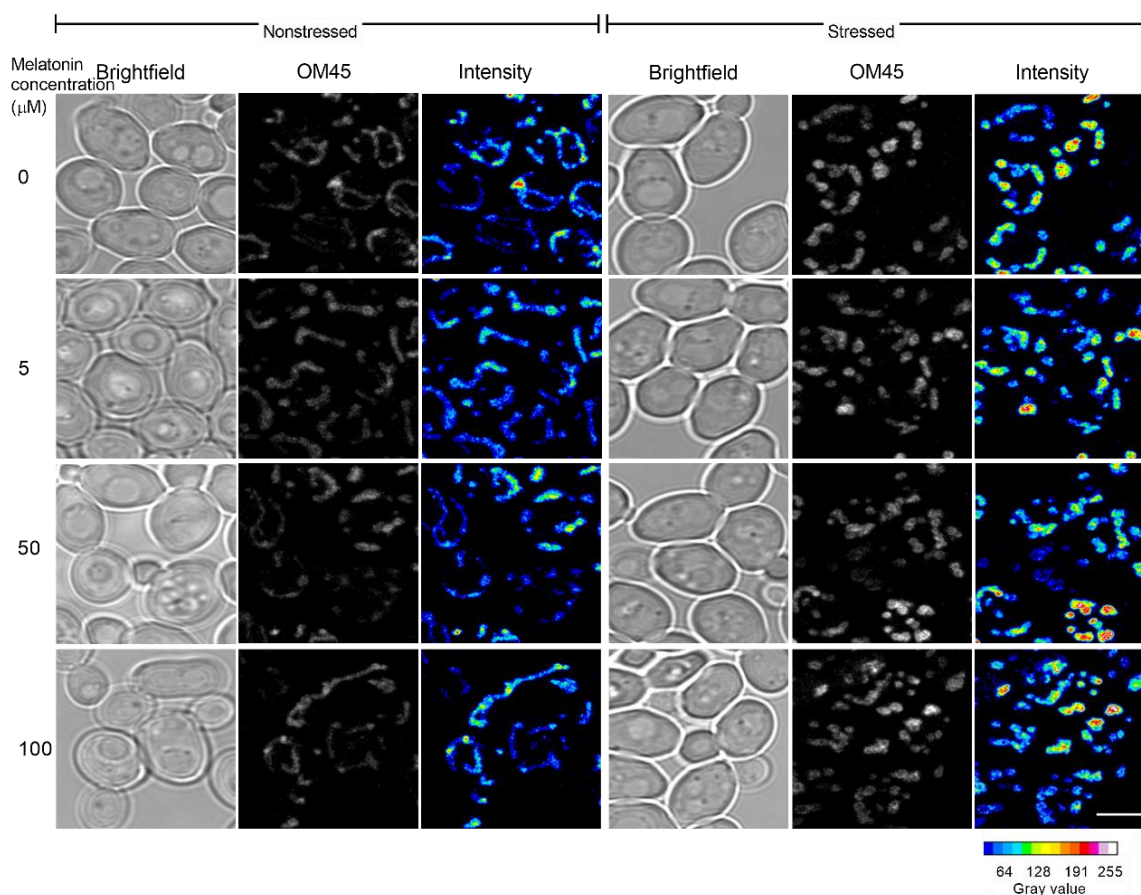
Figure 2.4. Visualization of mitochondrial network morphology of cells of BY4743 Om45-GFP strain grown on YPD in presence (5, 50 and 100 μM) or absence of exogenous melatonin, before (nonstressed) and after (stressed) being exposed to oxidative stress (2 mM H_2O_2) for 1 h. Scale bar is 5 μm.

3.4. Effect of oxidative stress and melatonin on ETC complexes

Next, we evaluated the effect of oxidative stress and melatonin on the enzymatic activity of mitochondrial respiratory complexes III and IV in mitochondrial-enriched extracts QA23 and BY4743 cells grown in fermentative medium.

In nonstressed cells, the activities of both complexes were higher in the wine strain than in the lab strain by 4-fold and 8-fold for complexes III and IV, respectively (Figure 2.5). Incubation for 1 h in the presence of 2 mM H_2O_2 did not affect the activity of complex

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III. However, this oxidative stress produced a large decrease in complex IV activity in both strains, which was higher in the lab strain (23-fold *vs.* 3-fold), and, there was hardly any activity of this complex after stress (Figure 2.5B). However, the activity of both complexes was unaltered by melatonin supplementation. Complex IV activity was also measured in nonstressed QA23 cells grown in respiratory medium (YPG), and it was slightly lower than that in fermentative QA23 cells (Figure S2.3A). However, under this condition, the mitochondrial abundance per cell obtained from mitochondrial isolation was 4 times larger than that in cells grown in fermentative medium (in both QA23 and BY4743 cells) (Figure S2.3B). No effect of melatonin supplementation was reported on cells grown on nonfermentative metabolism.
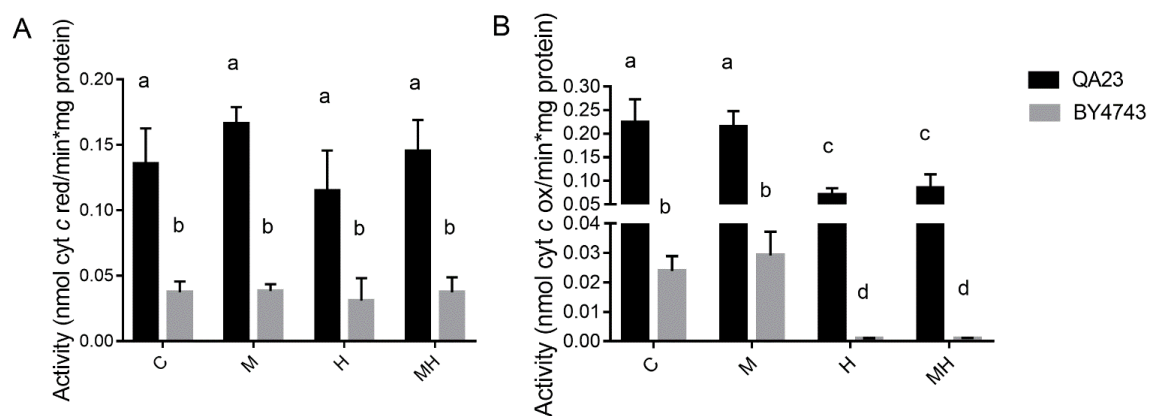
Figure 2.5. Activities of complex III (**A**) and complex IV (**B**) in mitochondrial-enriched extracts from cells of strain QA23 (black) and BY4743 (grey) grown on YPD. Four conditions were tested, in presence or absence of 5 µM of exogenous melatonin and before (C and M) and after (H and MH) of being exposed to oxidative stress (2 mM H_2O_2) for 1 h. The activities are expressed as nmol (cyt *c* ox-red)/min/mg protein. Bars represent standard deviation, and significative differences are indicated with different letters (p-value<0.05). Cyt *c* stands for cytochrome *c*, ox for oxidized and red for reduced.

Then, we determined the formation of respiratory chain supercomplexes by lysing mitochondria with digitonin and analysing the protein complexes by blue native electrophoresis and immunodecoration. Neither of the complexes altered their blue native mobility upon stress or melatonin treatment, indicating that H_2O_2 and melatonin did not affect the large oligomeric assembly of the ETC (Figure S2.4). Surprisingly, immunodecoration with Cox1, subunit 1 of complex IV, revealed a lower presence of complex IV in respiratory supercomplexes under oxidative stress.

4. DISCUSSION

4.1. Effect of oxidative stress on yeast mitochondria under fermentative conditions

Fermentative conditions are common for yeast cells, particularly in biotechnological processes, and often involve exposure to oxidative stress (Matallana and Aranda, 2017; Moradas-Ferreira and Costa, 2000; Vamvakas and Kapolos, 2020). The effects of exogenous oxidative stress on the mitochondria of yeast cells under respiratory conditions have been previously reported and showed that oxidative stress induces mitochondrial fragmentation and decreases the transmembrane potential and oxygen consumption, mainly caused by dysfunction of complexes II and III of the ETC (Cortés-Rojo et al., 2007; Zampol and Barros, 2018). It has also been reported that the lethality induced by H_2O_2 in yeast is mediated by a mitochondrial process (Elliott and Volkert, 2004) and that functional mitochondria are essential for a basal level of resistance to oxidative stress, both in fermentative and nonfermentative conditions (Grant et al., 1997). Our previous studies showed that under fermentative conditions, H_2O_2 induces ROS production and the expression of genes involved in mitochondrial antioxidant systems, as well as other mitochondrial functions, and modulates the composition of mitochondrial membranes (Vázquez et al., 2017, 2019; Chapter I of the present thesis). However, the specific effects of this stress on yeast mitochondria under fermentative conditions have not been extensively explored. Therefore, we wanted to evaluate the deleterious effect of oxidative stress induced by H_2O_2 on yeast mitochondria under fermentative conditions. Even though no single oxidant is truly representative of the totality of oxidative stress, H_2O_2 has been widely used in studies of oxidative stress responses (Cortés-Rojo et al., 2007; Morano et al., 2012; Picazo and Molin, 2021; Vázquez et al., 2019).

Our results showed that H_2O_2 immediately decreased the oxygen consumption of yeast cells, an effect also reported in isolated yeast mitochondria exposed to increasing concentrations of H_2O_2, even at the lowest concentration tested (0.125 µM) (Cortés-Rojo et al., 2007). These results suggested that oxidative stress affected the capacity to consume oxygen, mostly driven by ETC. This inhibition of oxygen consumption is also related to growth arrest. Interestingly, stressed cells recovered their oxygen consumption after growth restoration, achieving, in stationary phase, higher rates of oxygen consumption than nonstressed cells. This difference was not due to sugar consumption,

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since in both conditions, glucose was depleted (data not shown) but could be explained by the delay of stressed cells entering the stationary phase, which caused a decrease in oxygen consumption.

When comparing fermentable (glucose, YPD) and nonfermentable (glycerol, YPG) carbon sources, we observed that in glucose, yeast cells grew faster and had a lower oxygen consumption capacity, but they also had a higher growth inhibition under H_2O_2 exposure. This could be explained because some of the genes related to the antioxidant defence system (mitochondrial superoxide dismutase, cytochrome-c-peroxidase and glutathione peroxidase) are already activated under respiratory metabolism but repressed in fermentative carbon sources, with the cells better prepared to face H_2O_2 and superoxide anions under respiration (Grant et al., 1997; Liu and Barrientos, 2012).

As it is well known that oxidative stress affects mitochondrial membranes (Kamei et al., 2018; Lefevre et al., 2012; Trendeleva et al., 2011; Wang et al., 2014c), in this study, we evaluated the effect of H_2O_2 exposure on mitochondrial morphology and transmembrane potential under fermentative conditions. On the one hand, regarding mitochondrial morphology, nonstressed cells presented tubular nonbranched mitochondria, similar to what was reported previously for the first stages of fermentative growth (Kitagaki and Takagi, 2014). Instead, oxidative stress clearly induced mitochondrial fission, leading to a fragmented mitochondrial network. Damaged or dysfunctional mitochondria can be removed by fission and mitophagy to maintain a functional mitochondrial network and avoid cell damage (reviewed by Venditti and Di Meo, 2020). Indeed, several stresses (acetic acid, ethanol or exposure to pheromones) have been described to induce mitochondrial fragmentation in yeast grown on fermentative media (Fannjiang et al., 2004; Kitagaki et al., 2007; Pozniakovsky et al., 2005; Westermann, 2010). On the other hand, mitochondrial membrane potential is a key parameter to evaluate mitochondrial function (Sakamuru et al., 2016). The proton motive force produced by ETC proton pumping generates an electrochemical gradient through the inner membrane, which results in the mitochondrial membrane potential. This parameter was reduced in the oxidative stress-treated cells compared to control cells, in accordance with the effect of H_2O_2 and other prooxidants on isolated mitochondria reported in Trendeleva et al. (2011). This decrease in membrane potential may be due to a decrease in proton pumping performed by the ETC (Böttinger et al., 2012; Strogolova et al., 2019)

or due to the removal of damaged mitochondria by mitophagy (Venditti and Di Meo, 2020).

Therefore, we evaluated the effect of oxidative stress on ETC performance by determining the mitochondrial protein abundance and the activity of complexes III and IV. In the ETC, complexes III and IV transfer electrons from ubiquinol to cytochrome c (complex III, bc_1 complex) and from reduced cytochrome c to O_2 (complex IV, cytochrome c oxidase). Both processes are accompanied by the uptake of electrons and protons from opposite sides of the inner mitochondrial membrane (proton pumping), which results in the proton translocation used for ATP synthase to synthetize ATP (de Vries and Marres, 1987; Sakamuru et al., 2016). Oxidative stress did not affect the abundance of these proteins in the total cell extracts, regardless of whether they were nuclear (such as Cbp6 (data not shown), Qcr7) or mt-DNA (Cox2) encoded subunits. These results did not agree with those obtained in the transcriptomic study (Chapter I of the present thesis), where a downregulation of complex IV genes, such as *COX2*, and an upregulation of complex III genes, such as *QCR7*, were evidenced; therefore, these differences in gene expression did not translate into changes at the protein level. Indeed, Fam et al. (2018) also did not detect variations in the protein abundance of complex IV by H_2O_2 exposure. However, when protein abundance was evaluated in mitochondrial extracts, an effect of oxidative stress was noticed on Cox1, subunit 1 of complex IV, which resulting in lower protein levels, which was probably due to an increase in mitochondrial mass under oxidative stress (Chapter I of the present thesis) and caused lower levels of complex IV proteins per mitochondria but the same levels per cell.

Then, we analysed the activity of complexes III and IV in the presence or absence of stress. Although Dagsgaard et al. (2001) reported that complex IV was not active in yeast grown in anaerobic conditions, in our study, both complexes (III and IV) were active during fermentation. However, oxidative stress clearly decreased the activity of complex IV but did not change the activity of complex III, so the decrease in oxygen consumption detected after oxidative stress may be related to selective damage to complex IV. Surprisingly, these results were opposite to those obtained by Cortés-Rojo et al. (2007), in which complex III appeared to be the target of H_2O_2 while complex IV was unaffected. However, Cortes-Rojo et al. (2007) applied stress directly to isolated mitochondria, whereas in our study, H_2O_2 treatment was applied in cell culture. Therefore, these results suggest that H_2O_2 affects different complexes when applied to cells or isolated mitochondria,

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evidencing that experiments in isolated mitochondria may not reflect physiological conditions, since these organelles may be differentially energized *in vitro* and *in vivo*. The dysfunction of complex IV has been reported to produce an increase in ROS, which induces a signalling pathway that blocks cell growth (Bode et al., 2013; Srinivasan and Avadhani, 2012), and is an indicator of the oxidative capacity of the cell. Our results agree with these studies, since exposure to H_2O_2 for 1 h resulted in an arrest of cell growth together with the abovementioned inhibition of oxygen consumption and the increase in ROS accumulation reported in previous studies (Vázquez et al., 2017, 2019). Additionally, a decrease in complex IV activity has also been associated with a decrease in membrane potential in fermentable and nonfermentable growth, probably due to a decrease in proton pumping (Böttinger et al., 2012; Strogolova et al., 2019). Moreover, in Dubinski et al. (2018), yeast with compromised complex IV presented, in addition to the decrease in membrane potential, fragmented mitochondria, suggesting that a damaged ETC can also modify the mitochondrial network. This relationship between decreased membrane potential and fragmentation has been outlined in more studies in which H_2O_2 has been proposed to promote fragmentation by signalling or by membrane depolarization rather than at the transcriptional level (Venditti and Di Meo, 2020; Wang et al., 2014c). All these previous results agree with ours, since, in our study, stressed cells exhibited a decrease in complex IV activity and membrane potential together with mitochondrial fragmentation.

Complexes III and IV form supercomplexes that can assemble and dissociate depending on physiological conditions (Acin-Perez and Enriquez, 2014; Schägger and Pfeiffer, 2000). The supercomplex assembly factors Rcf1 and Rcf2 have been described to maintain the integrity of complex IV by association with lipids (Acin-Perez and Enriquez, 2014; Strogolova et al., 2019) and contribute to H_2O_2 resistance (Chen et al., 2012). Moreover, the presence of cardiolipin has also been reported to stabilize supercomplexes (Acin-Perez and Enriquez, 2014). In a previous study (Chapter I of the present thesis), we observed that oxidative stress caused a decrease in the cardiolipin content and in the expression of the *RCF1* and *RCF2* genes, suggesting that this stress could affect the assembly of the supercomplexes. However, in this study, no differences were observed in the assembly of these supercomplexes due to oxidative stress.

Therefore, oxidative stress mainly affects complex IV, its abundance per mitochondria and its activity, but more research is needed to decipher how this stress exactly acts on this complex, if at the transcriptomic or proteomic level, or if it only affects

the functionality of Cox1p but not of Cox2p. Cortés-Rojo et al. (2007) observed that the loss of complex III activity was related to complex modification by oxidation of -SH groups, so the effect on complex IV observed in this case could be similar.

In previous assays, different tolerances against oxidative stress were reported between the two strains used in this study as follows: wine strain QA23, which produced low levels of ROS species when exposed to oxidative stress, and lab strain BY4743, which was highly affected by oxidative stress (Vázquez et al., 2018). Similar behaviour was observed under ethanol stress (Chapter IV of the present thesis). Here, we observed that the lab strain BY4743 exhibited lower levels of complex III and IV activities, the latter being almost totally inhibited just after oxidative stress was applied. Therefore, a higher effect on the mitochondria due to oxidative stress was also observed in the lab strain in comparison to the wine strain, as previously reported by Vázquez et al. (2018, 2019) for other stress parameters, such as ROS accumulation, catalase activity or the fatty acid composition of cell membranes.

4.2. Effect of melatonin on yeast mitochondria under oxidative stress

Recent studies have described the protective effects of melatonin against oxidative stress induced by H_2O_2 exposure in yeast (Vázquez et al., 2017, 2018), which were in line with the antioxidant properties described in humans. In human cells, melatonin has a mitochondria-targeted role (Reiter et al., 2017, 2018; Tan et al., 2016), and recent studies have suggested a possible role of melatonin related to mitochondria in yeasts under respiratory (Zampol and Barros, 2018) or fermentative conditions (Morcillo-Parra et al., 2019b, 2020b; Vázquez et al., 2017; Chapter I of the present thesis). In this study, we wanted to evaluate the possible effect of melatonin on yeast mitochondria under fermentative conditions and oxidative stress and whether this effect was related to its antioxidant properties, reversing the damage caused by H_2O_2 in the mitochondria.

During fermentation, the presence of melatonin did not modify oxygen consumption, activities of ETC complexes, mitochondrial membrane potential or abundance of mitochondrial proteins in stressed or nonstressed cells. Therefore, the transcriptional changes triggered by melatonin under these conditions, mainly by downregulating complex III genes in nonstressed cells and upregulating complex IV genes in stressed cells (Chapter I of the present thesis), were not observed at the protein level, as already observed in the results of oxidative stress. Moreover, these results did not

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agree with those reported in humans and in yeasts under respiratory conditions, in which melatonin had a great impact on ETC by increasing enzymatic activities or restoring those damaged by oxidative stress, contributing to the improvement of respiratory efficiency, ATP formation and reduction of superoxide formation (Hardeland, 2017; Zampol and Barros, 2018). In particular, in yeast grown on nonfermentable carbon sources, melatonin enhanced oxygen consumption and complex IV activity and reversed the damage caused by α-synuclein on those indicators and in complex III activity (Zampol and Barros, 2018). The differences in the results between both studies could be due to the different experimental designs, since Zampol and Barros (2018) used a strain with elevated efficiency of mitochondrion-related metabolic functions (W303-1B) (Montanari et al., 2014) under respiratory conditions, and we used a wine strain (QA23) under fermentative conditions.

In mammalian cells, the effect of melatonin on the maintenance of the optimal mitochondrial membrane potential occurs mainly through the activation of uncoupling proteins (UCPs, which balance the potential and are the first line of mitochondrial antioxidant defence) or by reducing the opening of the mitochondrial permeability transition pore (MPTP) (oxidative stress causes its opening, which dissipates the membrane potential) (Reiter et al., 2018; Tan et al., 2016). However, *S. cerevisiae*, to the best of our knowledge, does not have UCPs (Jarmuszkiewicz et al., 2010), and H_2O_2 does not seem to form an MPTP (Trendeleva et al., 2011), which could explain why melatonin did not modify the mitochondrial membrane potential in yeast cells.

Although some factors contributing to supercomplex organization (Acin-Perez and Enriquez, 2014; Schägger and Pfeiffer, 2000; Zhang et al., 2002) were increased by melatonin in nonstressed and stressed cells, such as cardiolipin accumulation and mRNA levels of *RCF1* (in stressed) and *RCF2* (in nonstressed) (Chapter I of the present thesis), our results showed no modifications on the formation of those supercomplexes due to the presence of melatonin in the medium.

In several organisms, melatonin helps counteract the loss of control of electrons on the ETC, which are the main cause of oxidative stress in the cell (Acuña-Castroviejo et al., 2003; Hardeland, 2017; Reiter et al., 2018). This effect was also reported in yeast grown on nonfermentable carbon sources (Zampol and Barros, 2018) but not on fermentable carbon sources. This suggested that melatonin has a specific benefit over the respiratory

chain when it is the principal source of ATP, but its action changes in fermentable metabolism.

Melatonin modified the morphology of the mitochondrial network in the lab strain by triggering mitochondrial fission in nonstressed cells. Therefore, melatonin in yeast under fermentative conditions seemed to regulate mitochondrial dynamics by promoting fission, which is the opposite effect of that reported in mammals, in which melatonin has been described to reduce fission and increase fusion (Reiter et al., 2018; Tan et al., 2016). Moreover, the mechanisms by which melatonin regulates mitochondrial dynamics in mammals are highly complex and imply regulation at the transcriptional and especially proteomic levels (Tan et al., 2016). However, in our transcriptomic study, melatonin did not modify the transcription of the genes involved in mitochondrial dynamics in yeast (Chapter I of the present thesis).

Recently, it has been demonstrated that melatonin induces mitophagy in mammalian cells and in yeast respiring cells, although the exact mechanism of action is not known. In mammalian cells, it seems to occur through the regulation of signal response pathways, such as AMPK or mTOR pathways (Tan et al., 2016; Zampol and Barros, 2018). We have reported that melatonin triggers mitochondrial fission, and mitochondrial dynamics have been previously suggested to be involved in mitophagy signalling (Liu and Okamoto, 2021). Therefore, this could suggest that during fermentation, melatonin could activate mitophagy due to its probable prooxidant effect, as previously reported (Bisquert et al., 2018; Vázquez et al., 2018). Nevertheless, no effect on mitochondrial membrane potential was observed by melatonin. This was also observed with resveratrol, an antioxidant with similar properties to melatonin in yeast (Dani et al., 2008; Escoté et al., 2012; Gharwalova et al., 2017), which reduces lipid peroxidation, activates antioxidant enzymes and modulates mitochondrial dynamics without modifying the mitochondrial membrane potential (Wang et al., 2014c). Moreover, Galkina et al. (2020) suggested that membrane potential was not a decisive factor in the regulation of mitochondrial dynamics, and other mechanisms, such as nucleotide triphosphate availability could determine the equilibrium between fusion and fission.

In conclusion, under fermentative conditions, H_2O_2 stress caused an increase in ROS (Vázquez et al., 2017, 2019), which resulted in a decrease in complex IV activity, oxygen consumption rate and membrane potential. Additionally, mitochondrial fragmentation

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was also observed after oxidative stress exposure. Thus, in this case, fragmented mitochondria could be the result of fission and mitophagy pathway activation for dysfunctional mitochondrial clearance, to prevent ROS accumulation and cell damage and maintain the quality of cell mitochondria. Melatonin has an antioxidant role in many organisms, including yeast, and its action seems to be mainly targeted in the mitochondria by scavenging ROS and activating the antioxidant machinery. However, melatonin supplementation only affected mitochondrial fragmentation but not the other indicators, suggesting that the wide protective effects of melatonin against oxidative stress were not due to effects on the respiration chain. Therefore, under fermentative metabolism, melatonin appears to confer protection to yeast through different pathways and mechanisms than that of human cells and yeast cells in respiratory metabolism. Thus, more studies are needed to unravel the mechanisms triggered by melatonin to protect yeast from oxidative stress under fermentative conditions.

SUPPLEMENTARY MATERIAL

Table S2.1. Oligonucletodies used in this study (5'-3')

Function	Sequence (5'-3')
C- terminal tagging of OM45 with GFP (forward)	5'-ATTCAAAGAATGGAATGATAAGGGTGATGGTAAATTCTGGAGTCGAAAAAGGACCGTACGCTGCAGGTCGAC-3'
C- terminal tagging of OM45 with GFP (reverse)	5'-GAATATGTATATATGTTATGCGGGAACCAACCCTTTACAATTGCTATCTAACTAATCGATGAATTCGAGCTCG-3'
Control PCR OM45 (forward)	5'-GCCAGAGGTTTAGAAGGATGGGG-3'
Control PCR OM45 (reverse)	5'-GTCGACCTGCAGCGTACG-3'

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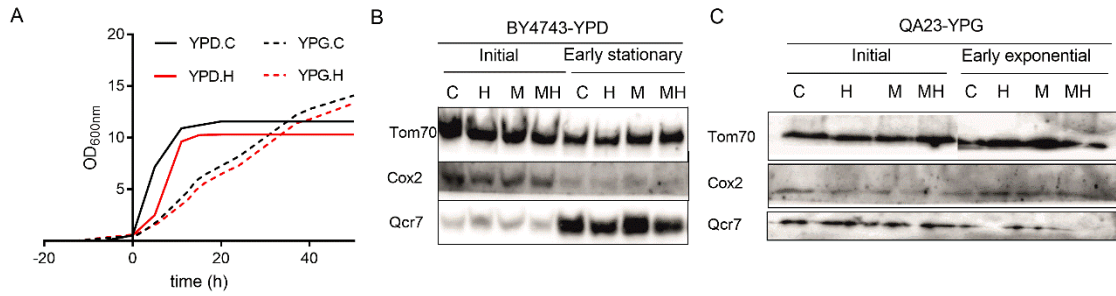
Figure S2.1. (**A**) Growth curve of QA23 cells grown on YPD (continuous line) or YPG (discontinuous line) submitted to 2 mM of H_2O_2 (red). The nonstressed controls are indicated in black. Time 0 h indicates the time when stress was submitted. (**B**) and (**C**). Monitoring of mitochondrial protein levels by immunoblotting using antibodies against Cox2 and Qcr7. Cell extracts grown in different medium (BY4743 in YPD in B, QA23 in YPG in C) in presence or absence of 5 μM of exogenous melatonin, before (C and M) and after (H and MH) being exposed to oxidative stress (2 mM H_2O_2) in different phases of the growth curve, just 1 h after stress (initial; B and C) and in early exponential (C) and early stationary (B) phase.
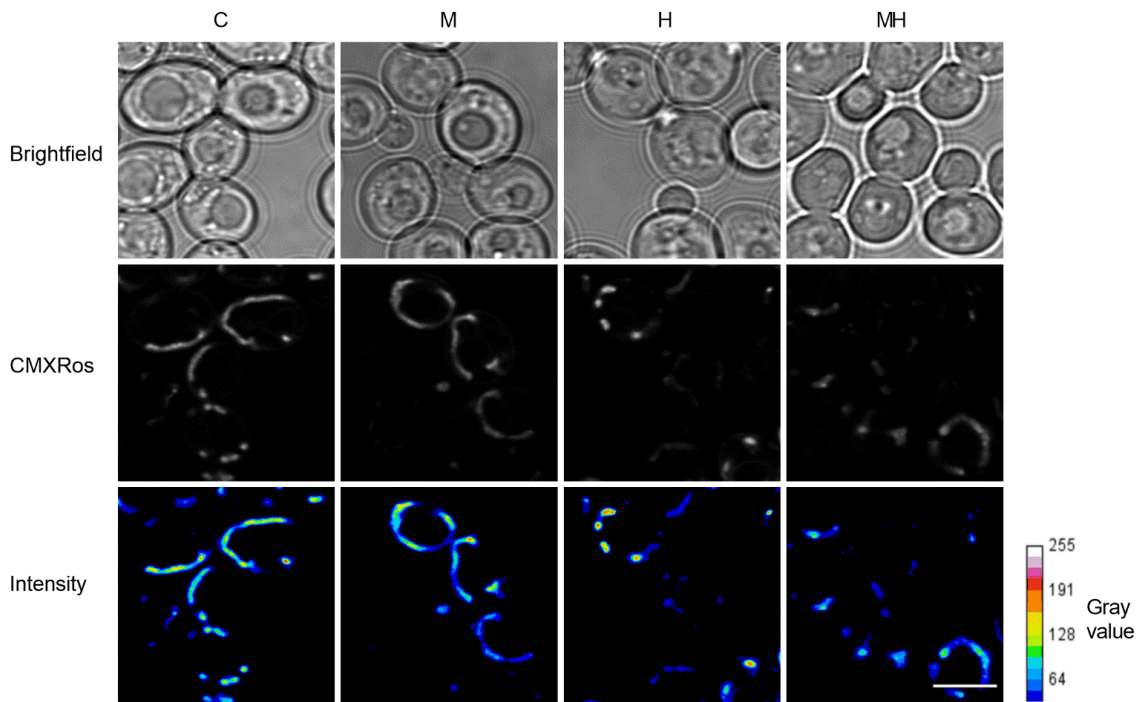
Figure S2.2. Visualization of mitochondrial transmembrane potential of QA23 cells untreated and treated with 5 μM of exogenous melatonin, before (C and M) and after (H and MH) being exposed to oxidative stress for 1h with 2 mM H_2O_2. Z-projections of representative confocal micrographs of Mitotracker CMXros-stained cells. Scale bar is 5 μm.

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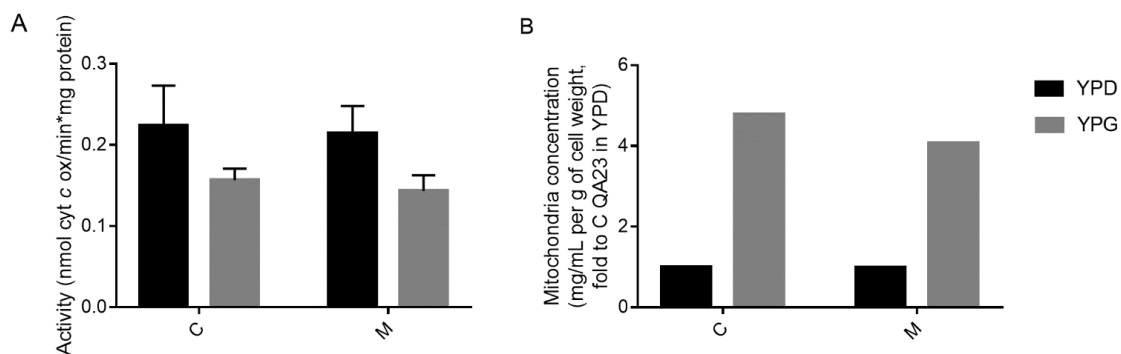
Figure S2.3. Activity of complex IV and concentration of mitochondria of QA23 cells grown on YPD (black) and YPG (grey) in presence (M) or absence (C) of 5 µM of exogenous melatonin, at early exponential phase (OD 0.5, time 0 h). (**A**) Activity of complex IV in mitochondrial-enriched extracts, expressed as nmol (cyt *c* ox)/min/mg protein. Scale bars represent standard deviation, and cyt *c* ox stands for cytochrome *c* oxidized. (**B**) Mitochondria concentration obtained in the mitochondria isolation, approximated by dividing the final protein concentration by the initial cell weight and expressed as fold of cells grown on YPD and untreated with melatonin (YPD C).
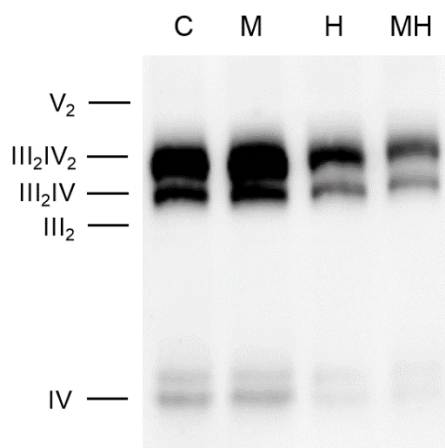
Figure S2.4. Determination of electron transport chain supercomplexes by blue native electrophoresis of isolated mitochondria of QA23 cells grown on YPD untreated and treated with 5 µM of exogenous melatonin, before (C and M) and after (H and MH) being exposed to oxidative stress for 1 h with 2 mM H_2O_2. Blot was probed with antibody against Cox1 to monitor complex IV.

CHAPTER III

Effects of melatonin on the growth of *Saccharomyces cerevisiae* under fermentation-associated stresses

Manuscript in preparation

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Abstract: During alcoholic fermentation, yeast cells are exposed to several stresses, such as osmotic stress, low pH, or the accumulation of ethanol, which affects yeast growth and disturbs the fermentation performance. Yeast cells respond by activating mechanisms to tolerate the stress, which can be general or specific to a certain stress. Melatonin is a bioactive molecule with widely reported beneficial effects in human cells, and it has recently been reported to have antioxidant role in yeast cells. This molecule is synthesized by yeast cells during alcoholic fermentation. The aim of this study was to carry out a screening to determine whether melatonin confers protection to *Saccharomyces cerevisiae* yeast cells against fermentation-associated stresses. To achieve that, yeast was pretreated with melatonin (5 or 50 μM) and exposed to osmotic (which was induced by glucose, sorbitol or salt (NaCl)), acid and ethanol stresses to determine the role of melatonin in cell growth and recovery. Generally, the concentrations of stressors used delayed or inhibited yeast growth, but only the higher concentrations disrupted cell recovery after exposure to the stressors for 2 h on fresh media. Melatonin supplementation enhanced the growth and recovery of cells exposed to ethanol stress, but no effect was reported for the cells subjected to osmotic or acid stress. The protection against ethanol accumulation could rely on the antioxidant role of melatonin, as ethanol exposure produces oxidative stress in yeast cells.

Keywords: yeast, ethanol, melatonin, fermentation, stress, osmotic

1. INTRODUCTION

Melatonin is a bioactive molecule with many beneficial properties in mammals. Among the properties, melatonin has a well-documented role in the regulation of the circadian rhythm but also in numerous physiological functions in many tissues and organs and in modulating neural, endocrine, immune, cardiovascular and reproductive functions. Therefore, melatonin has been proposed to confer protection against several human disorders (Romero et al., 2014; Eghbal et al., 2016; Chitimus et al., 2020; Ferlazzo et al., 2020). These protection mechanisms rely mainly on the antioxidant capacity of melatonin, which has been reported in most melatonin-producing organisms, including animals, plants, protozoa, bacteria and fungi (Hardeland and Poeggeler, 2003; Tan et al., 2015; Reiter et al., 2017; Que et al., 2020). Moreover, in some organisms, melatonin synthesis appears to be triggered by conditions of stress, such as in plants subjected to desiccation stress or in the dinoflagellate *Gonylaux polyedra* exposed to low temperature, which suggests that melatonin presence could be a mechanism of defence against specific stresses encountered by organisms (Tan et al., 2015).

Saccharomyces cerevisiae and other wine yeasts can produce melatonin during alcoholic fermentation, but the synthesis pathway and the mechanisms that trigger its production, although intensively studied, are far from being fully understood (Rodriguez-Naranjo et al., 2011a, 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018, 2019; Valera et al., 2019; Vilela, 2019; Muñiz-Calvo et al., 2020a). Melatonin production during fermentation depends on several factors related to the yeast (yeast strain or species, its metabolic state or the initial cell population) or to physical factors (such as temperature), but the factor that seems to affect the most is the must composition (Rodriguez-Naranjo et al., 2012; Fernández-Cruz et al., 2017, 2018, 2020; Valera et al., 2019; Morcillo-Parra et al., 2020a). The availability of melatonin precursors (tryptophan, N-acetyl serotonin and 5-methoxytryptamine) and the sugar content have a great impact on melatonin synthesis (Rodriguez-Naranjo et al., 2012; Fernández-Cruz et al., 2017, 2018; Valera et al., 2019; Fernández-Cruz et al., 2020; Morcillo-Parra et al., 2020a). Morcillo-Parra et al. suggested that the changes in melatonin synthesis by different glucose concentrations could be due to the osmotic stress produced by high glucose concentrations (Morcillo-Parra et al., 2020a). Moreover, the same authors also proposed a role of melatonin in the regulation of carbon metabolism, since melatonin only binds to glycolytic proteins under fermentative conditions (Morcillo-Parra et al., 2019b, 2020b).

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During alcoholic fermentation, the immediate environment occupied by yeasts is constantly changing (Walker and Basso, 2020) and becomes exposed to several stresses. At the beginning of fermentation, yeasts must cope with the high sugar concentration in the must, which creates osmotic stress for the cells along with the low pH of the media; thus, yeasts also have to deal with acid stress (Brandt et al., 2021). Later, during the process, cells encounter more stressful situations, such as nutrient starvation, the presence of ethanol and other toxic compounds, such as sulfites, and possible physical disturbances in temperature and aeration (Auesukaree, 2017; Matallana and Aranda, 2017; García-Ríos and Guillamón, 2019). Oxidative stress also affects yeast during alcoholic fermentation, as it is a consequence of other present stresses, such as osmotic and ethanol stress (Auesukaree, 2017; Matallana and Aranda, 2017). Each of these stresses is regulated by general and specific stress responses, but when they act simultaneously (as in the case of fermentation), the resistance of yeast to individual stresses is reduced (Brandt et al., 2021). Thus, yeasts have a network of coordination between stress-specific responses and a common response to several stresses (such as osmotic, extreme pH, ethanol, oxidative or heat shock), which is called the environmental stress response (Gasch et al., 2000; Gasch, 2003; Teixeira et al., 2011; Auesukaree, 2017; Matallana and Aranda, 2017).

Hyperosmolarity, pH and ethanol, the parameters strictly linked to winemaking, have a detrimental impact on yeast viability, growth and physiological processes, which negatively affects the fermentation performance (Auesukaree, 2017; Guan et al., 2017; Matallana and Aranda, 2017; Betlej et al., 2020). Therefore, during winemaking conditions, there is a correlation between high stress tolerance and good fermentative capacity, which is why there is increasing research on ways to relieve yeast stress (Matallana and Aranda, 2017). Melatonin is a well-reported antioxidant in yeast, that also protects it against UV radiation and has a possible prooxidant role in nonstressed yeast (Vázquez et al., 2017, 2018; Bisquert et al., 2018). This suggests that melatonin could activate a stress response to prepare yeast against several stresses, which, together with the fact that melatonin production during alcoholic fermentation may be modulated by sugar levels, opens the door to study whether yeast synthesizes melatonin to withstand the osmotic stress or other stresses present during alcoholic fermentation (Vázquez et al., 2018).

Thus, the aim of this study was to carry out a first screening to determine whether melatonin confers protection to yeast against fermentation-associated stresses (osmotic, low pH and ethanol). For that, yeasts were exposed to each stress in the presence and absence of melatonin, and its effect on yeast growth and recovery was studied.

2. MATERIALS AND METHODS

2.1. Yeast strains and experimental conditions

The wine yeast QA23, a commercial wine strain of *S. cerevisiae* (Lallemand, Montreal, QA, Canada), was used in this study. Yeast was precultured in YPD broth (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain); pH 6.8) at 28 °C with orbital shaking (120 rpm) for 24 h. Then, an initial OD_{600nm} of 0.05 was inoculated in 60 mL of YPD broth with or without melatonin supplementation (5 and 50 μM). Once the cultures with or without melatonin supplementation reached the initial exponential phase (OD_{600nm} 0.5 – 0.6), they were centrifuged at 10,000 rpm for 3 min, and the pellets were immediately reinoculated in YPD in the presence of the different stressors, adjusting the OD_{600nm} at 0.2. The stressors used were glucose (250, 300, 350, 400, 450 and 500 g/L) (Panreac, Barcelona, Spain), sorbitol (150, 200 and 300 g/L) (Sigma-Aldrich, St Louis, MO, USA) and NaCl (20, 30 and 50 g/L) (PanReac) for osmotic stress; pH 3.5, 2.5 and 2 for acid stress, adjusted with HCl (Panreac); and 6%, 8%, 10%, 12% and 14% (v/v) of absolute ethanol (AnalaR NORMAPUR, France) for ethanol stress. Three biological and five technical replicates were done in all assays, and nonstressed controls using YPD with and without melatonin were performed for each assay.

2.2. Determination of yeast growth

The control and stressed cultures with and without melatonin were incubated in a microplate (250 μL) at 28 °C and 120 rpm, and the growth was monitored by measuring OD_{600nm} every 30 min in a SpectroStar NANO microplate reader (Bmb Labtech, Germany).

Additionally, the growth recovery of the cells after being stressed with the different stressors for 2 h was determined by inoculating the stressed cells in fresh YPD medium at OD_{600nm} 0.05. The different stress concentrations used for checking the cell recovery were the following: 350 g/L, 450 g/L and 500 g/L of glucose, 150 g/L and 200 g/L and 300 g/L of sorbitol, and 30 g/L and 50 g/L of NaCl for osmotic stress, pH 3.5, 2.5 and 2 for acid stress

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and 8% and 10% (v/v) of absolute ethanol for ethanol stress. Yeast growth was monitored every 30 min using a SpectroStar NANO microplate reader (Bmb Labtech).

2.3. Data analysis

From the growth curves, different parameters were evaluated: OD max, growth rate and the area under the OD-time curve (AUC) or growth potential (Todor et al., 2014; Lairón-Peris et al., 2021). The growth rate was calculated with the following formula: rate $= (\log(OD_t)-\log(OD_0))/t-t_0$, and the estimate of AUC was calculated as a metric of the OD distribution as a function of time t.

Data obtained from all the assays were subjected to analysis of variance (ANOVA) and *Tukey's post hoc* test using GraphPad Prism 7 (GraphPad Software, CA, USA). The results were considered statistically significant at a p-value < 0.05. To calculate combined standard deviations, the StatsToDo website (China) was used.

3. RESULTS

3.1. Effect of different stresses on cell growth

The effect of the main fermentation-associated stresses (high osmotic pressure, high ethanol concentration and low pH) on yeast growth was determined by cultivating the QA23 strain in YPD medium with different concentrations of glucose (250, 300, 350, 400, 450, 500 g/L), sorbitol (150, 200, 300 g/L), NaCl (20, 30, 50 g/L), ethanol (6, 8, 10, 12, 14%) or at different pHs (3.5, 2.5 and 2).

In general, the presence of stressors in the medium caused a growth delay, which was longer at higher stressor concentrations, although the effect was different between the stresses (Figures 3.1, 3.2; Table S3.1). In terms of osmotic stress, glucose exerted a different behaviour on yeast growth than that of the other osmotic stressors (high salt and sorbitol concentrations). The glucose-stressed cells exhibited a prolonged lag phase compared to that of the control, mostly for cells grown at 400 – 450 g/L, but the total cell growth was significantly higher (except for 500 g/L, which totally inhibited the growth). Moreover, the growth rate of cells exposed to 250 – 350 g/L glucose was higher than that of the control condition, although this was only significant for the lowest glucose concentration (250 g/L), and for the range 400 – 450 g/L the rate was lower than the control condition. The values of glucose concentration and the growth parameters

presented a linear inverse correlation rate (r^2=0.906 for rate and r^2=0.852 for OD max) (Table S3.1).
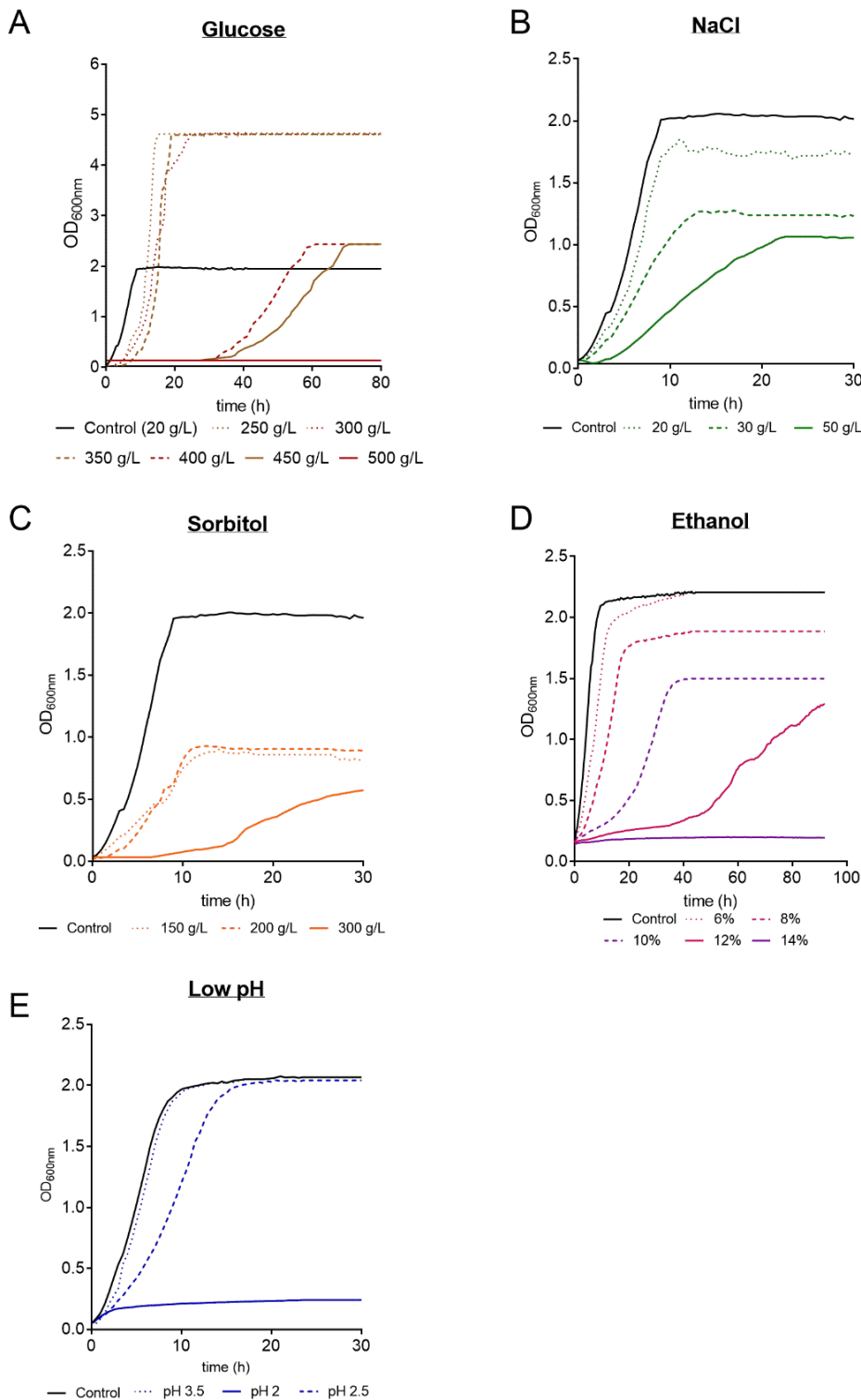
Figure 3.1. Effect of different stresses on the growth of *S. cerevisiae* QA23: glucose (**A**, brown, 250 g/L, 300 g/L, 350 g/L, 400 g/L, 450 g/L, 500 g/L), NaCl (**B**, green, 20 g/L, 30 g/L, 50 g/L), sorbitol (**C**, orange, 150 g/L, 200 g/L, 300 g/L), ethanol (**D**, purple, 6%, 8%, 10%, 12%, 14%) and low pH (**E**, blue, 3.5, 2.5, 2). The control conditions (YPD 20 g/L glucose, pH 6.8) are indicated in black.

The presence of NaCl and sorbitol in the medium caused a decrease in the growth rate and the maximum OD. For NaCl, a linear inverse correlation was observed between the increase in NaCl concentration and both the rate (r^2=0.994) and the OD max (r^2=0.824) (Figure 3.2, Table S3.1). In the case of sorbitol, the growth inhibition was similar at 150–200 g/L, but much higher at 300 g/L (Figures 3.1, 3.2). Interestingly, cells subjected to 150–200 g/L of sorbitol presented a similar OD max to cells exposed to the highest NaCl concentration (50 g/L), but a clearly higher growth rate, which evidenced that the effects of osmotic stress on cell growth inhibition depended on the compound used to trigger this stress (Figure 3.2, Table S3.1).
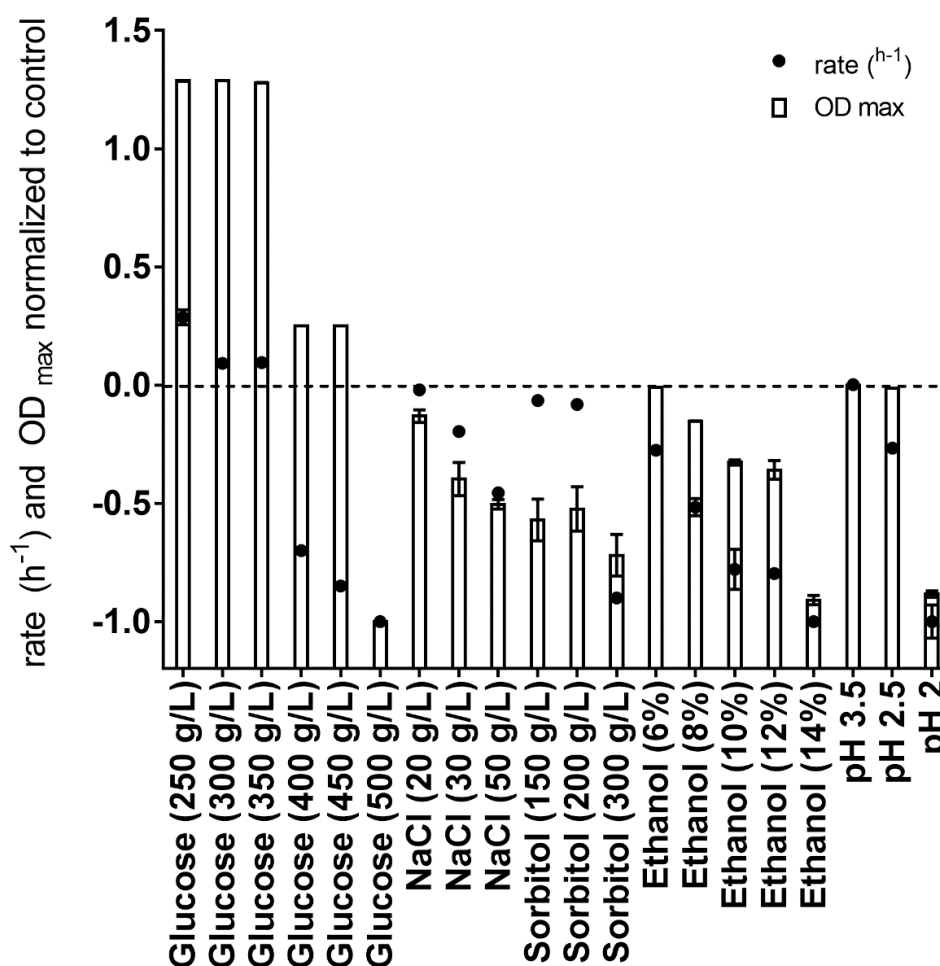
Figure 3.2. Effect of different stresses on the growth of *S. cerevisiae* QA23: glucose (250 g/L, 300 g/L, 350 g/L, 400 g/L, 450 g/L, 500 g/L), NaCl (20 g/L, 30 g/L, 50 g/L), sorbitol (150 g/L, 200 g/L, 300 g/L), ethanol (6%, 8%, 10%, 12%, 14%) and low pH (3.5, 2.5, 2). The parameters analyzed were the growth rate (h^{-1}, circles) and the maximum OD (OD_{max}, bars). Values were normalized to the control condition (YPD 20 g/L glucose, pH 6.8).

Cells subjected to ethanol stress showed a decrease in the growth rate and OD max by increasing the ethanol concentration (Figure 3.2), and this inverse correlation was linear between 6% and 10% ethanol (r^2 of 0.999 for rate and 0.996 for OD max) (Table S3.1). On the other hand, cell exposure to a medium with low pH (2.5) resulted in a lower growth rate than the control (pH 6.8) and the grape must pH (3.5, which presented the same growth than the control), but the OD max was not affected. Moreover, both 14% ethanol and pH 2 conditions completely inhibited cell growth (Figure 3.1).

3.2. Effect of different stresses on growth recovery

Growth recovery was tested in *S. cerevisiae* cells after exposure to different stressors for 2 h. For this assay, yeast cultures grown until the early exponential phase were exposed to the same stressors as in the previous experiment, although in some cases, only the highest concentrations were tested as follows: glucose (350, 450 and 500 g/L), NaCl (30 and 50 g/L), sorbitol (150, 200, 300 g/L), low pH (3.5, 2.5, 2) or ethanol (8, 10%). After 2 h of stress exposure, the cells were recovered and reinoculated into fresh YPD medium, and cell growth was monitored.

In general, no significant effect of the different stressors was observed on growth recovery after stress exposure, except for the highest concentration of ethanol, glucose, sorbitol and the lowest pH (Figures 3.3 and 3.4). In fact, the condition that most impacted cell growth recovery was 10% ethanol, in which the growth rate and AUC were significantly decreased. In contrast, in cells exposed to high glucose concentrations for 2 h, a significant increase in OD max was observed, indicating that exposure to high glucose allowed some intracellular energy storage. Nevertheless, a delay in growth was observed in cells exposed to high glucose (longer lag phase). A slight but significant decrease was observed in AUC at 300 g/L sorbitol (due to the lower OD max) and at pH 2 (due to the longer lag phase). Interestingly, although not significant, the cells submitted to sorbitol (150–300 g/L) and to the lowest concentration of glucose (350 g/l) exhibited an increased growth rate compared to that of the control (Figure 3.4).
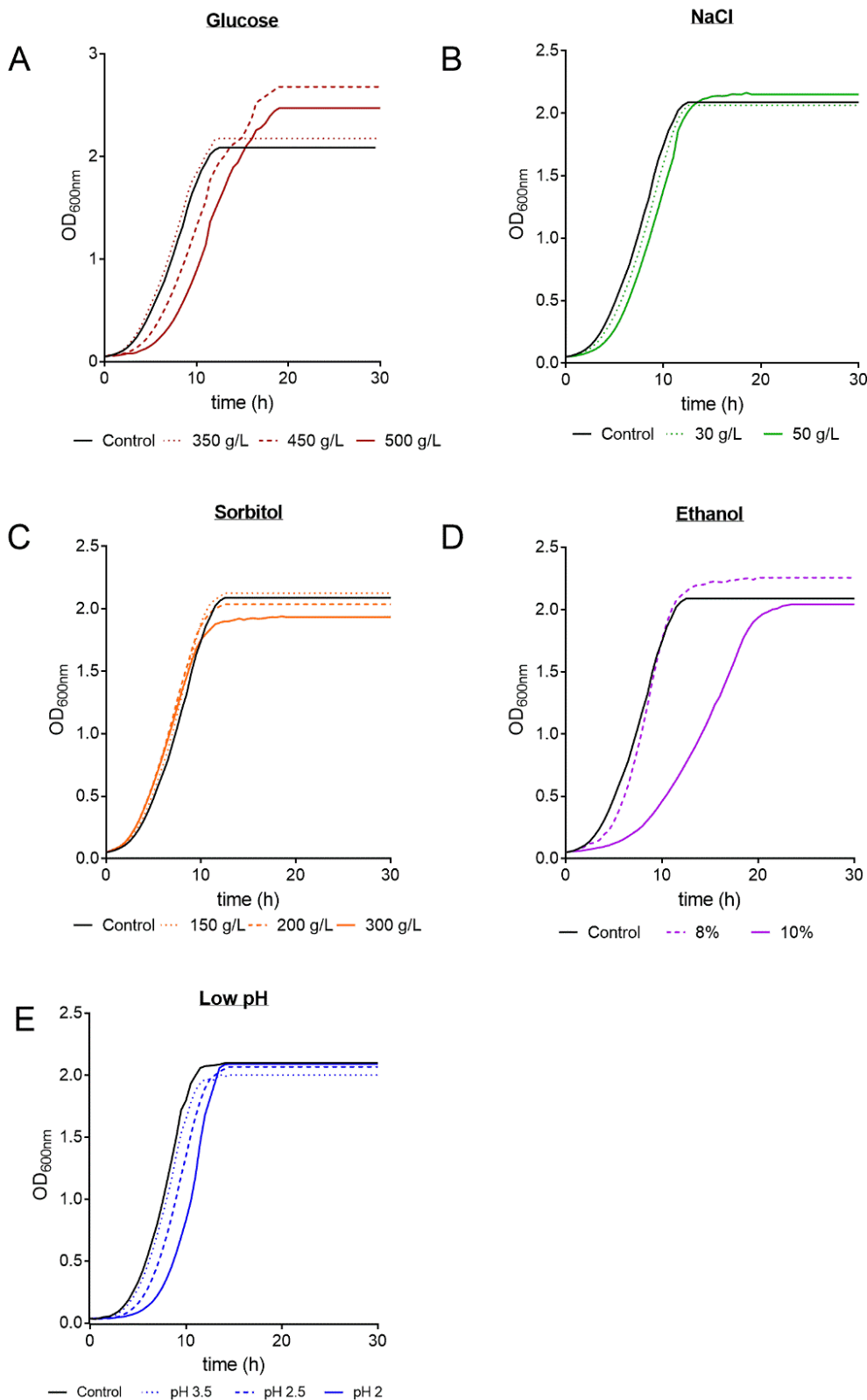
Figure 3.3. Growth of QA23 cells previously exposed to different stresses and recovered after 2 h. The stressors were glucose (**A**, brown, 350 g/L, 450 g/L, 500 g/L), NaCl (**B**, green, 30 g/L, 50 g/L), sorbitol (**C**, orange, 150 g/L, 200 g/L, 300 g/L), ethanol (**D**, purple, 8%, 10%) and low pH (**E**, blue, 3.5, 2.5, 2). The control conditions (YPD 20 g/L glucose, pH 6.8) are indicated in black.

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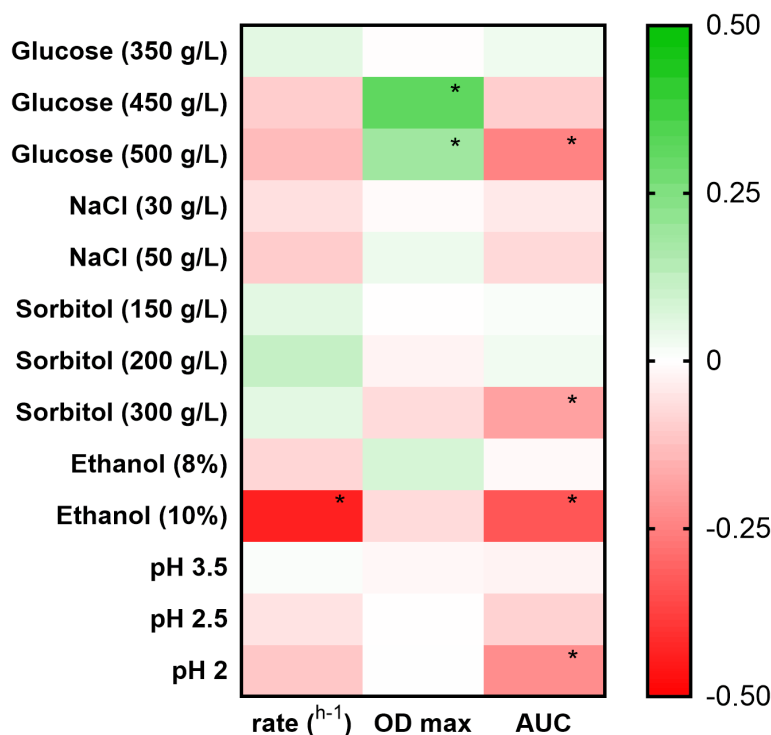
Figure 3.4. Effect of different stresses on the growth of *S. cerevisiae* QA23 cells previously exposed to the stresses and recovered after 2 h. The stressors were glucose (350 g/L, 450 g/L, 500 g/L), NaCl (30 g/L, 50 g/L), sorbitol (150 g/L, 200 g/L, 300 g/L), ethanol (8%, 10%) and low pH (3.5, 2.5 and 2). The parameters analyzed were growth rate (h^{-1}), maximum OD (OD$_{max}$) and area under the curve (AUC) calculated until 20h of growth. Values were normalized to the control condition (YPD 20 g/L glucose, pH 6.8). * indicates significative differences between the condition stressed and the nonstressed (p-value <0.05).

3.3. Effect of melatonin on the cell growth and recovery of cells exposed to stresses

Next, the effect of melatonin supplementation on the growth curve of cells exposed to different stressors was evaluated by incubating the *S. cerevisiae* cultures in the presence of 50 µM melatonin and by applying these stressors when the cells reached the early exponential phase (Figure 3.5). A intermediate concentration of each stressor that did not inhibit growth was selected from the previous growth experiment. The chosen concentrations were 350 g/L for glucose, 30 g/L for NaCl, 200 g/L for sorbitol, 8% for ethanol and pH 2.5 for low pH. No significant effect of melatonin pretreatment was observed on the growth of cells subjected to the different stressors except for 8% ethanol. Under this condition, supplementation with 50 µM melatonin significantly increased the total cell growth, which was even higher than that on nonstressed cells and also increased the AUC (Table S3.2).
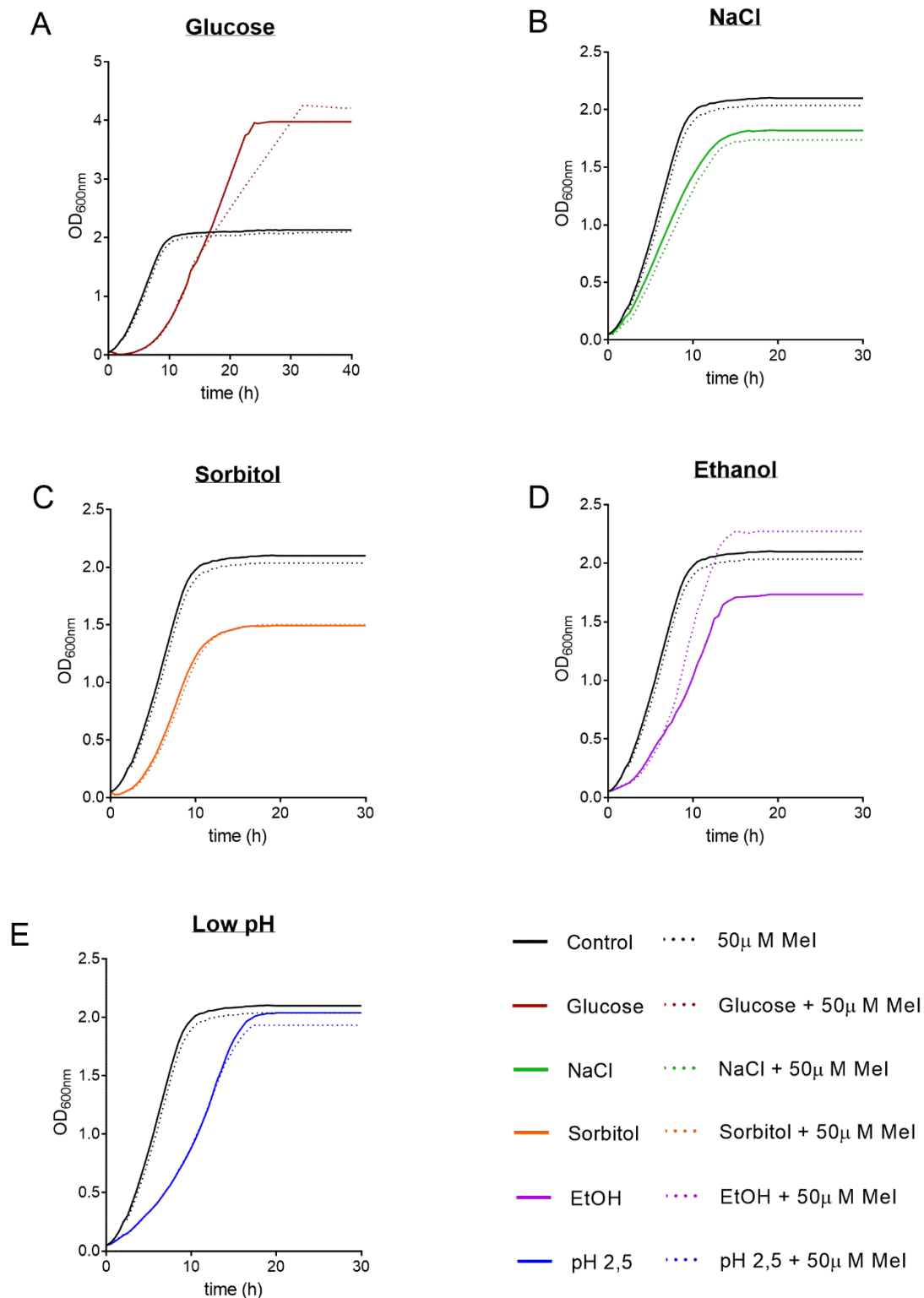
Figure 3.5. Effect of melatonin (Mel) supplementation (50 μM, in dotted lines) on the cell growth of QA23 cultures in absence of stress (YPD 20 g/L glucose, pH 6.8, black lines) or exposed to 350 g/L glucose (**A**, brown), 30 g/L NaCl (**B**, green), 200 g/L sorbitol (**C**, orange), 8% ethanol (**D**, purple) and pH 2.5 (**E**, blue).

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In general, the presence of 50 µM melatonin resulted in a slight delay in yeast growth, indicated by a decrease in both the growth rate and OD max, although those differences were not significant (Figure 3.5, Table S3.2). In the case of glucose, although there was also a slight increase in the OD max in the presence of melatonin, a clear delay in growth was observed from the mid-exponential phase, which resulted in a lower AUC (Figure 3.5 and Table S3.2). The absence of an effect was observed with a higher melatonin concentration (100 µM) respect to the control condition (data not shown).

Finally, the effect of melatonin supplementation (5 and 50 µM) on the growth recovery of cells exposed to ethanol (8, 10%) stress for 2 h was evaluated. When cells were exposed to 10% ethanol, the presence of melatonin improved the growth, increasing the growth rate, the OD max and the AUC, although not significantly (Figure 3.6, Table S3.3). However, melatonin supplementation had less effect on cells stressed with 8% ethanol, and only the low concentration (5 µM) produced a slight enhancement of growth.
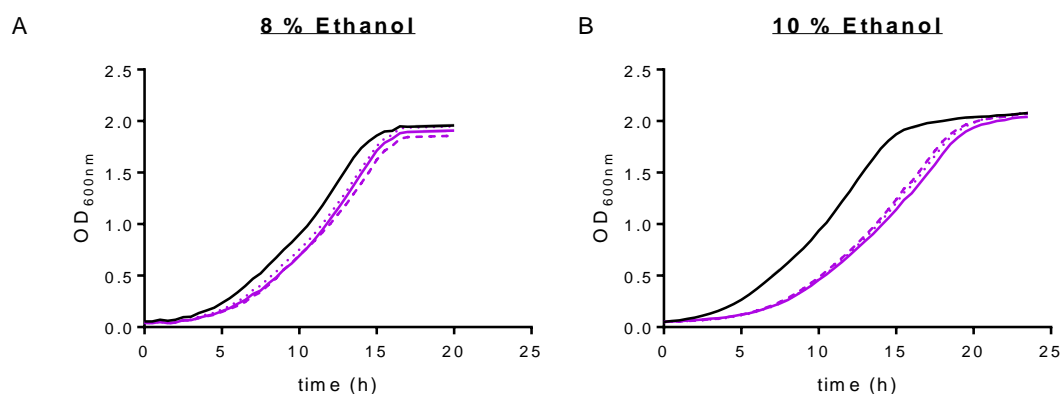
Figure 3.6. Effect of melatonin supplementation (0 µM in continuous lines, 5 µM in dotted lines, 50 µM in dashed lines) on the recovery of *S. cerevisiae* QA23 strain after exposure to 8% (**A**) or 10% (**B**) ethanol (purple) for 2h. The nonstressed conditions are indicated in black.

4. DISCUSSION

Melatonin is a pleiotropic molecule that exerts a wide range of functions in several organisms, but its most relevant role is to confer resistance to cells against different stresses, mainly oxidative stress (Romero et al., 2014; Tan et al., 2015; Eghbal et al., 2016). In yeast, this indole compound is synthesized during alcoholic fermentation and has recently also been shown to provide protection against oxidative and UV stress (Rodriguez-Naranjo et al., 2012; Fernández-Cruz et al., 2017, 2018, 2019; Vázquez et al., 2017, 2018; Bisquert et al., 2018). Furthermore, ongoing research is trying to unravel the

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mechanisms and pathways of melatonin synthesis in yeast pointing out that its synthesis depends, among other factors, on must composition, being especially important for its content of sugars and nitrogen compounds (Rodriguez-Naranjo et al., 2012; Muñiz-Calvo et al., 2019; Valera et al., 2019; Fernández-Cruz et al., 2020; Morcillo-Parra et al., 2020a). Therefore, as previous research has suggested that melatonin could be produced in response to stresses associated with fermentation, a first screening was performed in this study to evaluate the possible role of melatonin in improving the resistance of the wine *S. cerevisiae* strain QA23 against different stresses.

In general, the presence of compounds that induce osmotic, acid or ethanol stresses in the medium delayed yeast growth, or even, in extreme situations, completely inhibited cell growth, as widely reviewed elsewhere (Auesukaree, 2017; Matallana and Aranda, 2017; García-Ríos and Guillamón, 2019). In contrast, cultures exposed for 2 h to the same stress concentrations observed restored normal growth in most cases when inoculated in fresh medium, highlighting that cells can easily recover from growth impairment if the stress disappears.

The osmotic stress produced by high concentration of sugars is one of the most important stresses encountered by yeasts during fermentation due to the high sugar content of grape musts (normally between 180 and 240 g/L) (Aranda et al., 2011; Auesukaree, 2017). In this study, QA23 was able to grow in high-glucose concentrations, up to 450 g/L, which is in agreement with Watanabe et al. (2010), who reported that in media containing up to 400 g/L glucose, yeast can still grow and ferment. Despite the growth observed, cells exposed to high concentrations of glucose exhibited a prolonged lag phase due to the time period required for cells to adapt to this osmotic shock, in which viability was reduced (Sommer, 2020). However, once adapted, stressed cells are able to grow normally, reaching higher populations than that in the control condition. This higher OD is probably related to the higher availability of carbon sources, which resulted in higher biomass production. The same behaviour was reported when cells were exposed to high glucose concentrations for 2 h and then grown on fresh media without stress, suggesting that cells can accumulate carbon sources during this short period, which enables them to grow and achieve higher populations.

When osmotic stress was induced by compounds that are not carbon sources for yeast, such as sorbitol and salts, cell growth was more affected, decreasing the growth rate, OD max and AUC. This deleterious effect of NaCl and sorbitol on cell growth has been

previously reported (Watson, 1970; Wei et al., 1982; Kaino and Takagi, 2008). The stress caused by exposure to high concentrations of substances (sugars or salts) is mainly because it reduces the cellular available water and concentrates biomolecules and ions, resulting in a decrease in water activity (a_w). Water activity is defined as the chemical potential of free water in solution and must be lower than that of the surrounding medium to maintain a proper cellular volume and biochemical reactions. However, low a_w limits cellular growth (Tamás and Hohmann, 2003; Auesukaree, 2017; Guan et al., 2017; Matallana and Aranda, 2017). Therefore, the a_w induced by the different osmolytes must be considered when these stressors are compared. In our case, osmolytes were compared at concentrations (250 g/L glucose, 50 g/L NaCl and 300 g/L sorbitol) with similar water activity (a_w =0.97), (Chen, 1989; Comesaña et al., 2001) which resulted in the same degree of osmotic stress, but nevertheless, they exerted different effects on *S. cerevisiae* cell growth and recovery. This was in agreement with Gomar-Alba et al. (2015), who reported that the yeast response to osmotic stress differed depending on the stress agent responsible for this stress, with differences in all the stages involved in these molecular responses, from the activation of the high-osmolarity glycerol (HOG) pathway to the synthesis of functional proteins required for metabolic or cell wall reorganization.

Cells exposed to an acidic environment exhibited delayed (pH 2.5) or totally inhibited (pH 2) growth compared to those grown in neutral pH (6.8, Control) or in the pH of the grape must (3.5). This effect has been previously reported and reviewed in several studies (Orij et al., 2011; Rogers et al., 2016; Matallana and Aranda, 2017; Lucena et al., 2020). Moreover, Lucena et al. (2020) reported that a low pH (range of pH 1.5-2.5) did not affect cell mortality, and cells exposed to acidic conditions for some time were capable of restoring growth when inoculated in a medium with pH 5, coinciding with the results of the present study. As in our study, Lucena et al. (2020) adjusted the pH of the medium with a strong inorganic acid. The addition of strong acids has a limited effect on intracellular pH since fully ionized compounds cannot diffuse over biological membranes (Orij et al., 2011), but Lucena et al. (2020) reported that extremely low extracellular pH values (1.2 to 2) can cause a decrease in intracellular pH, leading to complete growth arrest, as observed in our study at pH 2.

The increase in ethanol concentrations produced the strongest effects on yeast growth and recovery until the inhibitory concentration of 14% ethanol was reached. Previous studies have already reported this effect (Lourenço et al., 2013; Pérez-Gallardo

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et al., 2013; Cheng et al., 2016; Navarro-Tapia et al., 2017). Indeed, ethanol is one of the major inhibitors of yeast growth during fermentation, as it can cause sluggish and even stuck fermentations. Moreover, ethanol is also described as one of the main causes of death in non-*Saccharomyces* species during wine production (Jolly et al., 2014; Albergaria and Arneborg, 2016). The main mechanisms of ethanol toxicity are membrane permeabilization, protein and enzyme denaturation, and the production of reactive oxygen species (ROS), which trigger oxidative stress (Ristow et al., 1995; Ma and Liu, 2010a; Stanley et al., 2010; Auesukaree, 2017; García-Ríos and Guillamón, 2019; Vamvakas and Kapolos, 2020).

In general, melatonin pretreatment in cells exposed to different stressors modulated their growth slightly but not significantly. Melatonin (50 µM) exhibited no effect on cells exposed to acid or osmotic stresses. However, it produced a significant increase in the growth rate and the OD max in ethanol-stressed cells. Moreover, a slight improvement in growth recovery was also observed in cells exposed to 10% of ethanol in the presence of melatonin.

Therefore, melatonin did not exhibit a clear protective effect against the osmotic and acid stresses tested in this study. However, melatonin appeared to enhance the growth of cells exposed to ethanol stress. Protection against ethanol stress could be related to the antioxidant properties of melatonin previously reported in yeast (Vázquez et al., 2017, 2018; Bisquert et al., 2018) because, as explained above, ethanol stress induces the production of ROS, triggering oxidative stress (Stanley et al., 2010; Auesukaree, 2017). Therefore, the cellular response to ethanol stress seems to rely partly on the activation of response pathways against oxidative stress to alleviate the cell from the consequences of ROS accumulation (Teixeira et al., 2011). Thus, the mechanisms that melatonin triggers in response to oxidative stress could help cells cope with ethanol stress. However, this is a preliminary study, and more research is needed to unravel whether the antioxidant properties of melatonin can confer protection against ethanol stress and to study whether melatonin modulates other mechanisms that confer protection against ethanol exposure.

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SUPPLEMENTARY MATERIAL

Table S3.1. Effect of different stresses on the growth of *S. cerevisiae* QA23: glucose (250 g/L, 300 g/L, 350 g/L), NaCl (20 g/L, 30 g/L, 50 g/L), sorbitol (150 g/L, 200 g/L), low pH (2.5, 2) and ethanol (6%, 8%, 10%, 12%, 14%). The parameters analyzed were the growth rate (h^{-1}), the OD max, and the area under the curve (AUC), calculated until 30h of growth. The linear regression value (r^2) was calculated for all the range of concentrations in glucose and NaCl, and for the range of 6-10% for ethanol. * indicates significative differences between the stressed and the nonstressed YPD 20 g/L glucose, pH 6.8) conditions (p-value <0.05).

		PARAMETERS			r^2 OF LINEAR REGRESSION	
Stressor	Concentration	Rate (h^{-1})	OD max	AUC	Rate	OD max
Control		0.174	2.073	319.826		
Glucose	250 g/L	0.224*	4.742*	566.882*	0.906	0.852
	300 g/L	0.190	4.744*	468.364*		
	350 g/L	0.191	4.725*	457.069*		
	400 g/L	0.052*	2.346*	0*		
	450 g/L	0.026*	2.456*	0*		
	500 g/L	0*	0*	0*		
NaCl	20 g/L	0.171	1.800*	256.017*	0.994	0.824
	30 g/L	0.140*	1.249*	182.941*		
	50 g/L	0.095*	1.029*	115.990*		
Sorbitol	150 g/L	0.163	0.892*	124.684*	0.903	0.793
	200 g/L	0.160	0.987*	136.664*		
	300 g/L	0.017*	0.569*	85.642*		
Low pH	pH 3.5	0.171	2.072	315.954	0.891	0.585
	pH 2.5	0.128*	2.070	271.446		
	pH 2	0.000*	0.240*	39.531*		
Ethanol	6%	0.126*	2.053	267.345	0.999	0.996
	8%	0.084*	1.758*	199.287*		
	10%	0.038*	1.395*	77.725*		
	12%	0.035*	1.330*	38.524*		
	14%	0.000	0.189*	29.387*		

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Table S3.2. Effect of melatonin (Mel) supplementation (0, 50 and 100 µM) on the growth of QA23 cultures exposed to 350 g/L glucose, 30 g/L NaCl, 200 g/L sorbitol, pH 2.5 and 8% ethanol. A nonstressed condition (YPD 20 g/L glucose, pH 6.8) was performed as control. The parameters analyzed were the growth rate (h^{-1}), the maximum OD (OD_{max}) and the area under the curve (AUC) calculated until 30h of growth. * indicates significant differences between the condition in presence and absence of melatonin (p-value <0.05).

	Rate (h^{-1})		OD max		AUC	
Mel Conc	0 µM	50 µM	0 µM	50 µM	0 µM	50 µM
Control	0.257	0.222	2.136	2.082	207.157	197.918
Glucose	0.279	0.249	3.816	4.192	191.004	179.594
NaCl	0.212	0.204	1.834	1.759	166.700	158.608
Sorbitol	0.240	0.226	1.523	1.540	135.970	134.950
pH	0.167	0.153	2.088	2.016	164.264	159.064
Ethanol	0.189	0.173	1.893	2.523*	150.474	199.842*

Table S3.3. Effect of melatonin (Mel) supplementation (5, 25 µM) on the recovery of *S. cerevisiae* QA23 strain after exposure to ethanol (8% or 10 %, EtOH) for 2h. The parameters analyzed were growth rate (h^{-1}), maximum OD (OD_{max}) and area under the curve (AUC) until 12 h of growth. * indicates significative differences between the condition treated and not treated with melatonin (p-value <0.05).

	Mel conc	0 µM	5 µM	50 µM
Control	Rate (h^{-1})	0.157	0.158	0.159
	OD_{max}	1.793	1.953	2.076
	AUC	28.115	39.470*	47.011*
10% Ethanol	Rate (h^{-1})	0.117	0.122	0.122
	OD_{max}	2.036	2.077	2.048
	AUC	11.226	11.657	12.317
8% Ethanol	Rate (h^{-1})	0.19	0.20	0.20
	OD_{max}	2.25	2.30	2.24
	AUC	37.19	39.83	34.41

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CHAPTER IV

Protective effects of melatonin on *Saccharomyces cerevisiae* under ethanol stress

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PROTECTIVE EFFECTS OF BIOACTIVE COMPOUNDS AGAINST FERMENTATION-ASSOCIATED STRESSES IN SACCHAROMYCES CEREVISIAE
Mercè Sunyer Figueres

Melatonin ethanol stress

Abstract: During alcoholic fermentation, *Saccharomyces cerevisiae* is subjected to several stresses, among which ethanol is of capital importance. Melatonin, a bioactive molecule synthesized by yeast during alcoholic fermentation, is reported to have an antioxidant role and is proposed to contribute to counteracting fermentation-associated stresses. The aim of this study was to unravel the effect of melatonin on protecting yeast cells subjected to ethanol stress. For that, the effect of ethanol concentrations (6 to 12%) on two strains of *S. cerevisiae*, a wine and a lab strain, was evaluated, monitoring the viability, growth capacity, mortality, and several indicators of oxidative stress over time, such as reactive oxygen species (ROS) accumulation, lipid peroxidation, and the activity of catalase and superoxide dismutase enzymes. In general, ethanol exposure reduced the cell growth of *S. cerevisiae*, and increased mortality, ROS accumulation, lipid peroxidation and antioxidant enzyme activity. Melatonin supplementation smoothened the effect of ethanol, enhancing cell growth and decreasing the oxidative damage by lowering ROS accumulation, lipid peroxidation and antioxidant enzyme activities. However, the effects of melatonin were dependent on strain, melatonin concentration, and growth phase. The results of this study propose that melatonin has a protective role against mild ethanol stress, mainly by reducing the oxidative stress triggered by this alcohol.

Keywords: ethanol stress, yeast antioxidant response, melatonin supplementation, ROS accumulation, catalase activity, superoxide dismutase

1. INTRODUCTION

Saccharomyces cerevisiae is the main yeast involved in alcoholic fermentation and is widely used not only in industrial fermentation of products such as wine, beer, and bread, but also in the production of bioethanol, a sustainable and clean transportation fuel (Ma and Liu, 2010a). During fermentation, yeasts face several stresses, such as osmotic, oxidative and acidic stresses, nutrient starvation and the presence of ethanol and other toxic molecules. As these stresses can compromise fermentation performance, an increase in yeast tolerance is a way to enhance the process (reviewed in Auesukaree, 2017; Matallana and Aranda, 2017). Therefore, to cope with these fermentation-associated stresses, yeasts have developed specific responses to each stress as well as a general response, the environmental stress response (ESR). These responses are coordinated, and thus the mechanism triggered by one stress can induce a protective response against others, causing a phenomenon called cross-protection (reviewed in Matallana and Aranda, 2017).

Among all fermentation-associated stresses, ethanol is of capital importance, as its presence is unavoidable in the medium and can lead to a reduction in cell viability, resulting in sluggish or even stuck fermentations (Malherbe et al., 2007; Auesukaree, 2017). Therefore, the effects of this stress and the mechanisms to overcome it have been widely studied (reviewed in Ma and Liu, 2010a; Snoek et al., 2016; Vamvakas and Kapolos, 2020). Due to its structure, ethanol is soluble in both aqueous and lipid phases. For this reason, it can penetrate inside cells but can also be incorporated into cell membranes, increasing membrane fluidity and permeability (Lloyd et al., 1993; Marza et al., 2002). The accumulation of ethanol in the cell inhibits its growth due to the inhibition of cell division and the intracellular acidification and denaturation of proteins and enzymes, thereby resulting in altered metabolism (Ristow et al., 1995; reviewed in García-Ríos and Guillamón, 2019; Ma and Liu, 2010a; Stanley et al., 2010). Moreover, ethanol causes the generation of reactive oxygen species (ROS) and acetaldehyde, which can induce lipid peroxidation, DNA damage and oxidative stress (Alexandre et al., 2001; Yang et al., 2012; Pérez-Gallardo et al., 2013).

The oxidative stress response is based on different defense mechanisms that try to maintain cellular ROS concentrations at a basal level. These mechanisms are grouped into enzymatic and nonenzymatic systems (Jamieson, 1998). The glutathione system

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encompasses both enzymatic and nonenzymatic mechanisms, playing a pivotal role in the yeast antioxidant response, although Costa et al. (1993) reported that this system is not needed to acquire tolerance to ethanol stress. The main enzymatic defenses against oxidative stress include catalase and superoxide dismutase (SOD). Yeast cells have two catalases (catalase A and catalase T) that decompose H_2O_2 to water in the peroxisome and in the cytosol, respectively, and two SODs (Cu/ZnSOD and Mn/ZnSOD) that catalyse the conversion of superoxide anion to oxygen and H_2O_2 in the cytoplasm and in the mitochondria, respectively (Jamieson, 1998). Generally, the importance of cytoplasmic SOD in the antioxidant response is much higher than the one of the mitochondrial isoforms; however, one exception is ethanol-induced stress (Zyrina et al., 2017). In this condition, although both SODs are activated, the mitochondrial isoform seems to be more necessary to face ethanol stress, probably related to the fact that during ethanol stress, mitochondrial ROS are the primary source of damage (Pérez-Gallardo et al., 2013; Zyrina et al., 2017; Jing et al., 2020). In fact, in the case of ethanol-mediated stress, the deletion of *SOD2*, the gene encoding the mitochondrial isoform, has been described to strongly reduce yeast resistance to high ethanol concentrations, while the deletion of *SOD1*, the gene encoding the cytoplasmic isoform, has practically no effect (Costa et al., 1997). Moreover, mitochondrial SOD is also essential for Yap1p (the main transcription factor for yeast response to oxidative stress) activation in ethanol stress (Zyrina et al., 2017). However, other studies pointed towards a less essential role of this transcription factor in response to ethanol stress, indicating that other mechanisms, such as glycogen and trehalose protection, also contributed (Bleoanca et al., 2013). Trehalose and glycogen are upregulated by ethanol and are induced by the ESR (Gasch et al., 2000; Alexandre et al., 2001; Ding et al., 2009). Therefore, the cellular response to ethanol stress is a complex mechanism mediated by gene expression reprogramming due to a coordinated action of the ESR, the specific responses to oxidative stress and heat shock (Teixeira et al., 2011) and some specific responses to ethanol stress (Stanley et al., 2010), mostly activated by mitochondrial dysfunction (Zyrina et al., 2017; Jing et al., 2020).

Melatonin (N-acetyl-5-methoxytriptamine) is a bioactive molecule present in most living organisms (Hardeland and Poeggeler, 2003) with widely studied beneficial properties in humans (Reiter et al., 2016, 2017). Among the numerous physiological functions of melatonin in humans, several are related to the prevention of some of the disorders related to high ethanol consumption, such as the regulation of circadian rhythms; analgesic, anti-inflammatory or antistress properties; and modulation of

immune functions (Eghbal et al., 2016; reviewed in Kurhaluk and Tkachenko, 2020; Romero et al., 2014). Melatonin also protects human cells, tissues, and organs from ethanol stress mainly by its antioxidant properties (ROS scavenger action and the activation of the endogen defense system) (Kurhaluk and Tkachenko, 2020). Melatonin synthesis by yeast during alcohol fermentation has been reported in several studies (Fernández-Cruz et al., 2017, 2018, 2019; Morcillo-Parra et al., 2020a; Rodriguez-Naranjo et al., 2012; Valera et al., 2019; Vigentini et al., 2015). However, little information is available on the synthetic route in yeasts. The first studies reported a route similar to that described for vertebrates (Sprenger et al., 1999), but a recent study proposed a putative biosynthetic pathway including some steps described in plants, such as the synthesis of serotonin from tryptophan through tryptamine instead of 5-hydroxytryptophan (Muñiz-Calvo et al., 2019).

A topic under research is whether melatonin confers some advantage to yeast cells during fermentation. Melatonin is reported to act as an antioxidant in *Saccharomyces cerevisiae* (Vázquez et al., 2017; Chapter I of the present thesis) and non-*Saccharomyces* (Vázquez et al., 2018) yeasts and to protect yeast cells against UV radiation (Bisquert et al., 2018). Moreover, recent studies have deciphered that melatonin is involved in multiple biological processes in yeasts (Chapter I of the present thesis) and could have a signaling role in fermentative metabolism (Morcillo-Parra et al., 2019b, 2020a, 2020b; Rodriguez-Naranjo et al., 2011a). In the response against oxidative stress, melatonin acts as a direct antioxidant by scavenging ROS and as indirect antioxidant by decreasing oxidized glutathione and activating genes involved in the oxidative stress response, such as catalase, SOD, glutathione/glutaredoxin, and thioredoxin, leading to reduced lipid peroxidation and higher tolerance to H_2O_2 (Vázquez et al., 2017, 2018).

The production of melatonin by yeast during alcoholic fermentation, together with its protective effect against oxidative stress, suggests the possibility that yeast produces this molecule to protect against fermentation-associated stresses. In fact, the protection against ethanol stress relies partly on antioxidant mechanisms (Bleoanca et al., 2013), and melatonin confers protection against oxidative stress in human and yeast cells and against ethanol stress in human cells (Kurhaluk and Tkachenko, 2020). Therefore, it seems interesting to evaluate whether melatonin has a protective effect against ethanol stress in yeast cells. Therefore, the aim of this study was to assess first the effect of different ethanol concentrations on yeast cells and second the effect of melatonin in protecting *S.*

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cerevisiae cells subjected to ethanol stress. For that, we evaluated mortality, cell recovery, ROS accumulation, lipid peroxidation and catalase and SOD activities in cells exposed to ethanol stress for different times in the presence and absence of melatonin.

2. MATERIALS AND METHODS

2.1. Yeast strains and experimental conditions

In this study, two strains of *S. cerevisiae* were used: the wine commercial strain QA23 (Lallemand, Montreal, QA, Canada) and the lab strain BY4743 (EUROSCARF collection, Frankfurt, Germany). Yeasts were precultured in YPD broth (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain)) by incubation at 28 °C with orbital shaking (120 rpm) for 24 h. Then, yeasts were inoculated at an initial OD_{600nm} of 0.05 in fresh YPD broth (60 mL for growth curve, recovery, mortality and ROS accumulation and 180 mL for catalase, SOD and lipid peroxidation assays) with or without melatonin supplementation (5, 25, 50 μM) and grown until the cells reached the initial exponential phase (OD_{600nm} 0.5 − 0.6). At this point, the cultures were submitted to different ethanol concentrations using absolute ethanol (AnalaR NORMAPUR, France) and incubated in the presence of stress. The effect of ethanol was determined by comparing stressed and unstressed cells in the same growth phase (lag, early exponential, mid-exponential, early stationary or stationary). Therefore, samples were taken at different growth phases depending on the experiment (specified in the results section). For the assays of lipid peroxidation and catalase and SOD activities, 10^8 cells were harvested by centrifugation at 4,700 rpm for 5 min at 4 °C, and the pellets were washed with Milli-Q water (Millipore Q-PODTM Advantage A10), centrifuged at 16,000 rpm for 5 min at 4 °C, fast-frozen with liquid nitrogen and stored at -80 °C until use. Three biological replicates were employed in all assays, and a nonstressed control without melatonin was performed for each assay.

2.2. Determination of yeast growth

For both strains, once the cultures with or without melatonin supplementation reached the initial exponential phase (OD_{600nm} 0.5 − 0.6), cells were immediately reinoculated in fresh YPD in the presence of different ethanol concentrations (6, 8, 10, 12, 14% (v/v)) at OD_{600nm} 0.2. Yeast growth was monitored for 96 h by measuring OD_{600nm} every 30 min in a SpectroStar NANO microplate reader (Bmb Labtech, Germany). For each

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biological triplicate, five technical replicates were analyzed. From the obtained growth curves, different parameters were evaluated: OD max, growth rate and the area under the OD-time curve (AUC) or growth potential (Todor et al., 2014; Lairón-Peris et al., 2021). The growth rate was calculated with the following formula: rate = $(\log(OD_t)-\log(OD_0))/t-t_0$, and the estimate of AUC was calculated as a metric of the OD distribution as a function of time t.

Additionally, the growth recovery of the cells after being stressed with different ethanol concentrations (8, 10% (v/v)) and exposure times was evaluated by inoculating those cells in fresh YPD medium at OD_{600nm} 0.05. Yeast growth was monitored every 30 min using a SpectroStar NANO microplate reader. For each triplicate, five technical replicates were analyzed. From the obtained growth curves, the different parameters explained above were calculated.

2.3. Determination of mortality rate

Cell mortality was monitored using the propidium iodide (PI) fluorescent staining dye (Invitrogen, Waltham, MA, USA), as specified in the manufacturer's instructions, with some modifications. Briefly, aliquots of 1 mL of culture were mixed with 1 µg of PI and incubated in darkness at room temperature for 10 min. Then, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and fluorescence intensity was measured with the flow cytometer CyFlowspace (Partec, Norderstedt, Germany). Data were acquired with FloMax software (Quantum Analysis GmbH, Münster, Germany) and processed to calculate the percentage of dead cells with WinMDI 2.9 software (Joseph Trotter, Salk Institute for Biological Studies, CA, USA).

2.4. Quantification of ROS

Reactive oxygen species (ROS) were determined using the fluorescent probe dihydrorhodamine 123 (DHR 123), as described in Vázquez et al. (2017). Briefly, samples (0.5 mL) were stained with DHR 123 (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 10 µg/mL in darkness for 20 min at 120 rpm and 28 °C. Then, the cells were harvested and washed twice with PBS (pH 7.4); ROS were immediately quantified by measuring the fluorescence intensity (geometric mean, Gmean) with a CyFlowspace flow cytometer. Data were acquired with FloMax software, and the median fluorescence intensity was quantified with WinMDI 2.9 software. The mean fluorescence index (MFI)

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was calculated according to (Boettiger, 2001): [(Gmean condition)-(Gmean control)]/(Gmean control).

2.5. Lipid peroxidation

The degree of lipid peroxidation was evaluated by the colorimetric determination of thiobarbituric acid reacting substances (TBARS) described in Buege and Aust (1978), with some modifications. Briefly, cell pellets were resuspended in 450 µL of TCA (trichloroacetic acid 10% (v/v)) in PBS and broken using glass beads with five cycles alternating shaking and cooling (30/30 s) using an MBB-16 Mini-Beadbeater (BioSpec Products, Inc., OK, USA). Then, the cells were incubated for 15 min on ice and centrifuged at 2,200 g for 15 min at 4 °C. After this, the protocol of Vázquez et al. (2018) was followed: 200 µL of the supernatant was mixed with 200 µL of 2-thiobarbituric acid (TBA, 6.7 g/L) (Sigma-Aldrich) and incubated at 95 °C for 10 min. After cooling at room temperature, the absorbance was measured at 532 nm using the microplate reader SpectroStar NANO. The concentration of TBARS was estimated by referring to a standard curve of 1,1,3,3-tetramethoxylpropane (Sigma-Aldrich), and the results were expressed as nmol of TBARS per mg of protein.

2.6. Antioxidant enzyme activities

Protein extracts were obtained following the cell disruption protocol described in Vázquez et al. (2018), with minor alterations. Cell pellets were resuspended in 0.5 mL of precooled PBS 50 mM (pH 7) containing one tablet of protease inhibitor cocktail per 10 mL of extraction solution (cOmplet^{TM}; Roche, Penzberg, Germany) and disrupted by alternating five cycles of shaking and cooling (30/30 s) using an MBB-16 Mini-Beadbeater in presence of glass beads. Then, homogenates were centrifuged at 14,000 rpm for 5 min at 4 °C, and the supernatant was used to immediately perform the assays in triplicate.

Total protein content was estimated according to the Bradford method (Bradford 1976). Briefly, 10 µL of the protein extract was incubated for 15 min with 240 µL of Bradford reagent (Sigma-Aldrich), and absorbance at 595 nm was determined using a SpectroStar NANO microplate reader. Protein content was calculated using a standard curve constructed with bovine serum albumin (BSA; Sigma-Aldrich).

Catalase activity was determined by measuring the decomposition of H_2O_2 after 10 min in the presence of the protein extract, according to Góth (1991), modified by Hadwan

and Abed (2016) and with some further modifications. Briefly, 15 µL of protein extract was exposed to 40 µL of H_2O_2 (16 mM; Sigma-Aldrich); after incubation for 10 min at 37 °C, 200 µL of ammonium heptamolybdate (32,4 mM; Millipore, Burlington, MA, USA) was added, and the absorbance at 374 nm was measured using a SpectroStar NANO microplate reader. A standard curve was generated with different H_2O_2 concentrations (range of 0.5-16 mM) in PBS, and a negative control was assayed for each cell extract.

Superoxide dismutase (SOD) activity was measured by inhibiting the tetrazolium salt WST-1 (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) reduction by O_2^- generated by the xanthine/xanthine oxidase system using a commercial assay kit (SOD assay kit, Sigma-Aldrich), as specified by the provider. Briefly, cell extracts diluted 20-fold were mixed with a solution containing WST-1. Then, the xanthine oxidase enzyme solution was added, and the mix was immediately incubated at 37 °C for 20 min, monitoring the increase in absorbance at 450 nm every 1.5 s, using a SpectroStar NANO microplate reader. SOD activity of protein extracts was estimated using a standard curve prepared with known amounts of bovine SOD (range 0-5 U/mL) (Sigma-Aldrich), and the results were expressed as units of SOD per mg protein.

2.7. Data analysis

Data obtained from all the assays were subjected to analysis of variance (ANOVA) and *Tukey's post hoc* test using GraphPad Prism 7 (GraphPad Software, CA, USA). The results were considered statistically significant at a p-value < 0.05. To calculate combined standard deviations, the StatsToDo website (China) was used.

3. RESULTS

3.1. Effect of different ethanol concentrations on *S. cerevisiae* growth

The effect of ethanol concentration on yeast growth was determined in two *S. cerevisiae* strains, a wine commercial (QA23) and a lab (BY4743) strain. For that, both strains were cultivated in YPD medium in the presence of different ethanol concentrations (from 6% to 14%), using a medium without ethanol as a control. The presence of ethanol resulted in a prolonged lag phase and a decrease in the growth rate in both strains (Figures 4.1, S4.1, Table S4.1). As expected, the higher the ethanol concentration was, the longer the yeast growth delay. Indeed, there was a direct correlation between ethanol concentration (from 6% to 10%) and growth rate for both strains (Table S4.1, Figure S4.1).

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The ethanol concentration that totally suppressed the growth was 12% for the lab strain and 14% for the wine strain. Moreover, the ethanol concentration also affected the OD max obtained. In the case of QA23, the maximum yeast growth decreased almost linearly with the increase in ethanol content, from 6% to 10% ethanol (Figure S4.1). Surprisingly, in BY4743, low ethanol concentrations (6% and 8%) resulted in higher values of OD max (Figure 4.1, S4.1), and the decrease was observed only at 10%, as growth was suppressed at 12% and 14%.
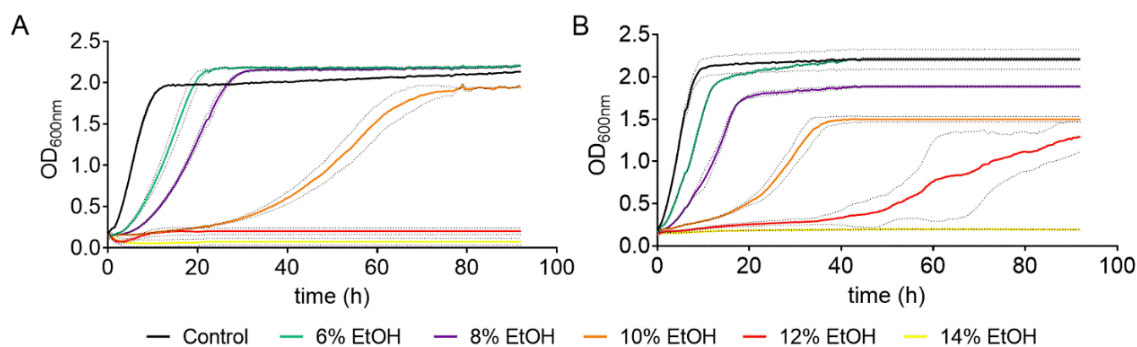

Figure 4.1. Effect of different ethanol concentrations on the cell growth of *S. cerevisiae* strains BY4743 (**A**) and QA23 (**B**). Ethanol concentrations: 0% (black), 6% (green), 8% (purple), 10% (orange), 12% (red), and 14% (yellow).

After this first assay, three ethanol concentrations (8%, 10% and 12%) were chosen to evaluate the effect of ethanol stress in *S. cerevisiae* strains, monitoring different parameters such as cell mortality, cell recovery after stress and ROS accumulation.

3.2. Effect of ethanol concentration on cell mortality and growth recovery

Cell mortality of the cultures was evaluated on cells exposed to ethanol stress at different concentrations and times. As expected, a higher ethanol content in the medium resulted in an increase in dead cells for both strains, being higher in BY4743 (Figure 4.2A). As an example, an ethanol concentration of 10% for 20 h resulted in 11.7% of dead cells in QA23 and 38.9% in BY4743. Interestingly, this last percentage of dead cells was similar to that obtained for QA23 with 12% ethanol, indicating a higher tolerance to ethanol stress for the wine strain.

After that, the growth recovery of the cells after being stressed with different ethanol concentrations and exposure times was evaluated by inoculating those cells in fresh YPD. As the growth curves in the presence of ethanol were delayed in relation to the

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control (Figures 4.1, S4.3), cells were recovered and reinoculated into fresh media according to their growth phase (lag, early exponential and early stationary phase).

The recovery of the growth of the stressed cells was affected by the intensity of the stress (ethanol concentration) and by the exposure time to this stress (Figure 4.2B-G, Figure S4.2, Table S4.2).
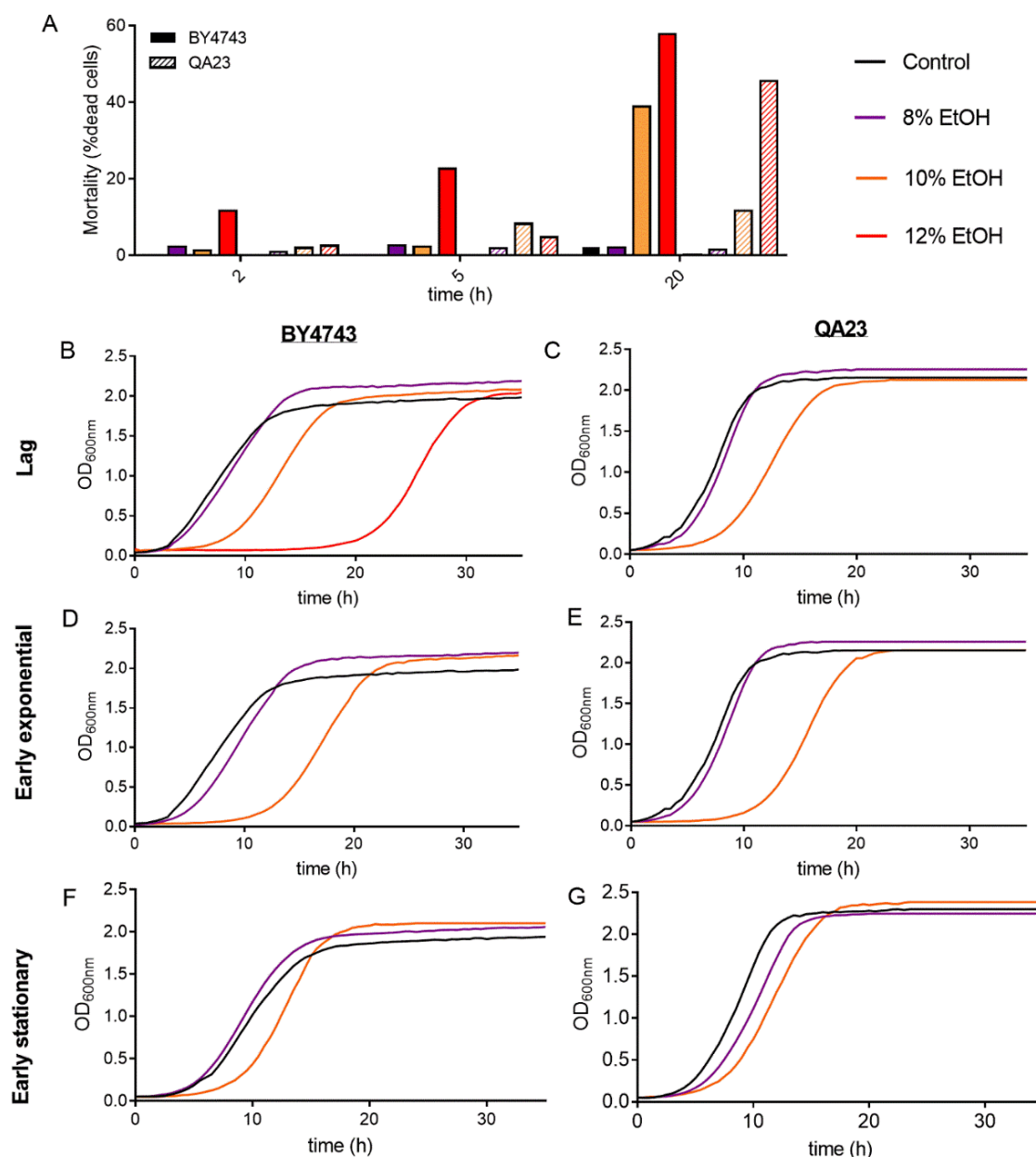
Figure 4.2. Effect of different ethanol concentrations (0% (black), 8% (purple), 10% (orange)) on the *S. cerevisiae* strains BY4743 (A, B, D, F) and QA23 (A, C, E, G). (**A**) Mortality rate expressed as the percentage of dead cells (solid columns, BY4743; stripped columns, QA23); (**B-G**) growth of cells previously exposed to ethanol and recovered at different growth phases: lag phase (**B-C**); early exponential phase (**D-E**); early stationary phase (**F-G**).

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The higher the ethanol concentration was, the more time yeast cells needed to recover normal growth, resulting in a longer lag phase, mainly after exposure to 10% and 12% ethanol (Figure 4.2, Table S4.2). Indeed, after 8% ethanol exposure, yeast cells grew similarly to nonstressed cells, showing only a slight growth delay under some conditions (in cells recovered at early exponential phase for BY4743 or at early stationary phase for QA23). Our results also showed that the growth phase reached by those stressed cells clearly affected growth recovery (Figure S4.2). After being exposed to 10% ethanol, cell growth recovery was more delayed when cells came from the lag phase and early exponential phase, and less affected when cells came from the stationary phase (Figure 4.2, Table S4.2).

3.3. Effect of ethanol on oxidative stress response

ROS production in the presence of different ethanol concentrations was evaluated by flow cytometry in both *S. cerevisi*ae strains using nonstressed cells as controls. The fluorescence values (Gmean) in nonstressed cultures increased in the mid-exponential phase and reached the maximum fluorescence in the late stationary phase (30-40 h) (Figure 4.3A, C).
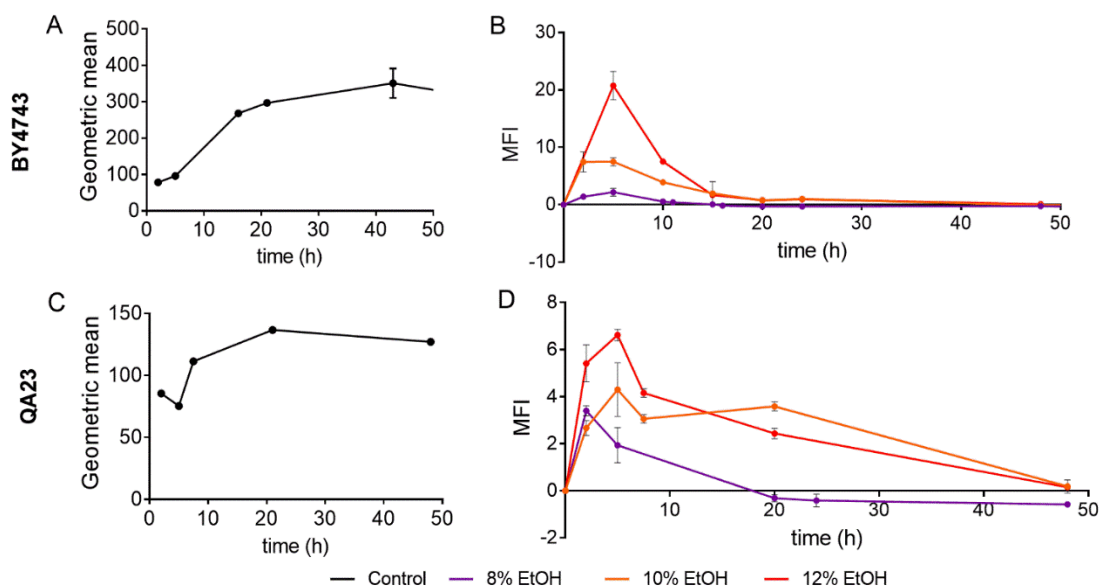
Figure 4.3. Effect of different ethanol concentrations (0% (black), 8% (purple), 10% (orange), 12% (red)) on ROS (reactive oxygen species) accumulation over time in the BY4743 (**A, B**) and QA23 (**C, D**) strains. (**A, C**) ROS accumulation in the nonstressed cells, expressed as the geometric mean (Gmean). (**B, D**) ROS accumulation in stressed cells normalized to nonstressed cells and expressed as Mean Fluorescence Intensity (MFI), [(Gmean stressed cells)-(Gmean control)]/(Gmean control). Error bars represent standard deviation.

Both strains followed a similar ROS accumulation pattern, although ROS production was clearly higher in the lab strain (Figure 4.3A). Ethanol treatments exacerbated ROS generation, presenting the highest fluorescence signal after 5 h of stress exposure (Figure 4.3B, D). The higher the ethanol concentration was, the higher the ROS production, and this production was especially remarkable in the lab strain treated with 12% of ethanol. In fact, under this condition, BY4743 presented high mortality and practically no growth (Figures 4.1A, 4.2A). In both strains, similar ROS amounts were accumulated in stressed and nonstressed cells in the stationary phase, which was more evident in strain BY4743 (Figure 4.3).

As we observed that 10% and 12% ethanol seriously compromised the growth and functionality of the lab strain, we analyzed the following oxidative stress indicators (lipid peroxidation, catalase and SOD activities) with a concentration of 8% ethanol in both strains.

Both strains presented a similar profile of lipid peroxidation, with similar levels of TBA reactive substances, in nonstressed cells (Figure 4.4A). Lipid peroxidation increased with the entry of yeast cells into the stationary phase due to the increase in oxidative stress in this phase. The only difference between strains was that these levels decreased when the stationary phase progressed in the wine strain but were maintained in the lab strain (Figure 4.4A). Cells exposed to ethanol stress presented a lipid peroxidation profile similar to that of control cells until the early exponential phase and slightly higher levels in the mid-exponential phase. However, in contrast to control cells, practically no changes were observed in TBARS levels by entrance in stationary phase in any of the strains in the presence of ethanol. Moreover, surprisingly, the QA23 strain sharply increased these levels in the stationary phase, while no changes were detected in BY4743 (Figure 4.4A).

As ethanol stress induces ROS accumulation (Figure 4.3), catalase and SOD activities were measured in the presence and absence of 8% ethanol (Figure 4.4B, C) to evaluate the effect of ethanol on these key enzymes for cell antioxidant defense. Our results showed that the catalase activity of nonstressed cells increased when cells entered the stationary phase, in concordance with the other studied parameters (ROS concentration, lipid peroxidation), and with the increase in oxidative stress due to alcoholic fermentation. Although a similar pattern was observed in both strains, the catalase activity in the wine strain was approximately twenty times higher than that in the lab strain. When cells were exposed to ethanol, the general profile of catalase activation was not modified in relation

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to nonstressed cells, and was highly activated during the stationary phase, although some considerations could be made.
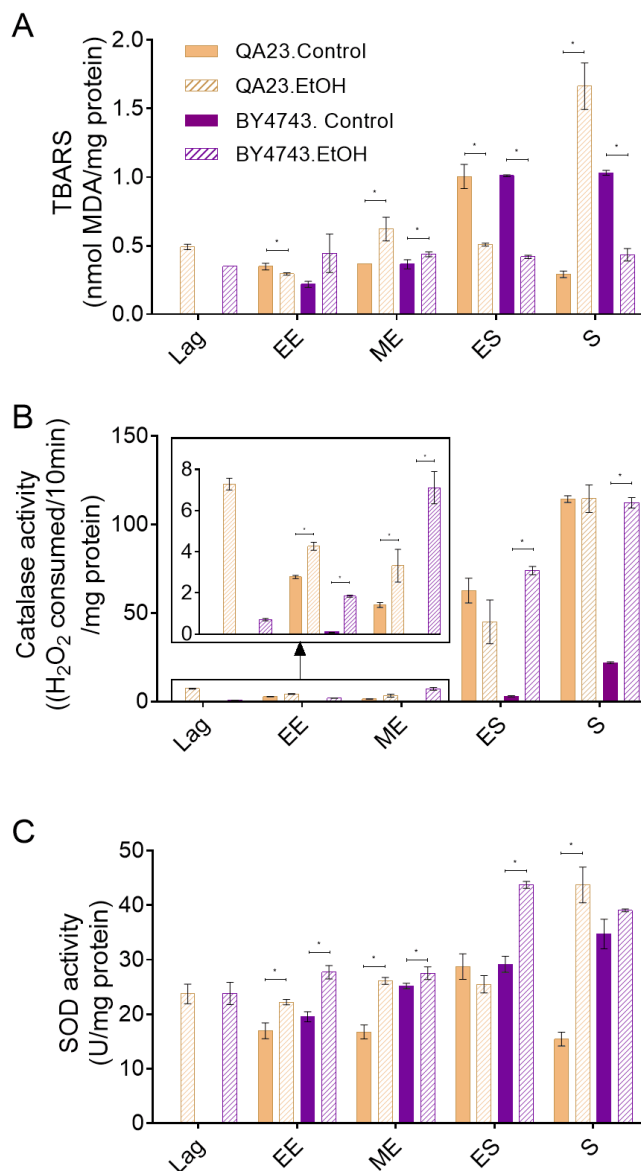
Figure 4.4. Effect of 8% ethanol on the *S. cerevisiae* strains BY4743 (purple) and QA23 (orange) on (A) lipid peroxidation (nmol TBARS/mg protein), (B) catalase activity ((H_2O_2 consumed/10 min)/mg protein) and (C) superoxide dismutase (SOD) activity (U/mg protein). The parameters were calculated at different growth phases after the stress exposure: lag, early exponential (EE), mid-exponential (MD), early stationary (ES) and stationary(S). Solid columns (nonstressed cells), stripped columns (stressed cells). Lag phase was not observed for nonstressed cells. Error bars represent standard deviation, and * indicates significant differences between stressed and nonstressed conditions (p-value < 0.05). TBARS stands for thiobarbituric acid-reacting substances.

In the case of QA23, exposure to ethanol induced catalase activity during the lag phase, remaining higher than in nonstressed cells during the exponential phase but achieving a similar final activity at the stationary phase (Figure 4.4B). On the other hand, in BY4743, the catalase activity in cells exposed to ethanol was clearly higher than that in nonstressed cells throughout the entire process, finishing with similar activity levels to those detected for QA23, both in stressed and nonstressed cells, but five times higher than the activity of nonstressed BY4743 cells (Figure 4.4B).

In nonstressed cultures of strain BY4743, SOD activity increased over time, following a similar pattern to catalase activity, although the initial levels were clearly higher; therefore, the increase due to entrance into the stationary phase was less important, just 1.2-fold (Figure 4.4B, C). In QA23, SOD activity remained mainly unchanged during the exponential phase, increasing only in the early stationary phase. As in BY4743, QA23 presented high levels of SOD activity from the beginning of the growth; thus, the entrance to stationary phase provoked only a 1.7-fold activity induction. In both strains, ethanol exposure did not much change the activity profile of SOD, being maximal in the stationary phase, although with higher values than in the control condition. Moreover, a displacement of this maximum activity was observed, which was in the early stationary phase for BY4743 and in the stationary phase for QA23 (Figure 4.4C).

Although exposure to 8% ethanol was not lethal for the studied strains, it clearly affected their cell growth and mortality, their cell oxidative state and the activity of some enzymes associated with the antioxidant response. The presence of melatonin has previously been described to have an antioxidant role in yeast cells (Vázquez et al., 2017, 2018). For these reasons, we evaluated the effect of melatonin in yeast cells exposed to ethanol stress at the lag phase, early exponential phase and early stationary phase.

3.4. Effect of melatonin on cell viability under ethanol stress

To evaluate the effect of melatonin on the viability of yeasts exposed to ethanol stress, *S. cerevisiae* cultures were grown in the presence of different melatonin concentrations (5, 25, 50 µM). When the cultures reached the early exponential phase, cells were stressed with 8% of ethanol to analyze cell recovery and mortality after different incubation times in the presence of ethanol. Moreover, cultures grown in the presence of melatonin were transferred to fresh media containing 8% ethanol, and cell growth was monitored.

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Melatonin had a slight effect on the growth of cells exposed to ethanol by increasing (50, 25 µM) or decreasing (5 µM) the area under the curve (AUC), although these changes were not significant (Figures 4.5A, S4.4A, Table S4.3). Melatonin pretreatment also modified the OD max obtained in the QA23 strain with a slight increase when cells were grown with 25 and 50 µM melatonin and a decrease with 5 µM melatonin (Figure S4.4A, Table S4.3).

We also tested the effect of melatonin on the growth recovery of stressed cells at different exposure times (Figure 4.5B, Figure S4.4B, Table S4.3). In BY4743 cells, all melatonin concentrations significantly shortened the lag phase at the early exponential phase (Figure 4.5B). For strain QA23, similar growth curves were obtained for stressed cells treated with or without melatonin, although the values of AUC and OD max were slightly increased by melatonin (Figure S4.4B, Table S4.3). These results suggest that melatonin could modulate the growth recovery of cells exposed to ethanol stress.

Low concentrations of melatonin (5 µM) significantly decreased the mortality triggered by 8% ethanol in lag and early exponential phases in BY4743, while high concentrations (50 µM) also decreased it in the early stationary phase (Figure 4.5C). A similar effect was observed in QA23 (Figure S4.4C). Therefore, in both strains, the presence of melatonin decreased the cell mortality of cells exposed to ethanol stress, mainly during the initial growth phases.
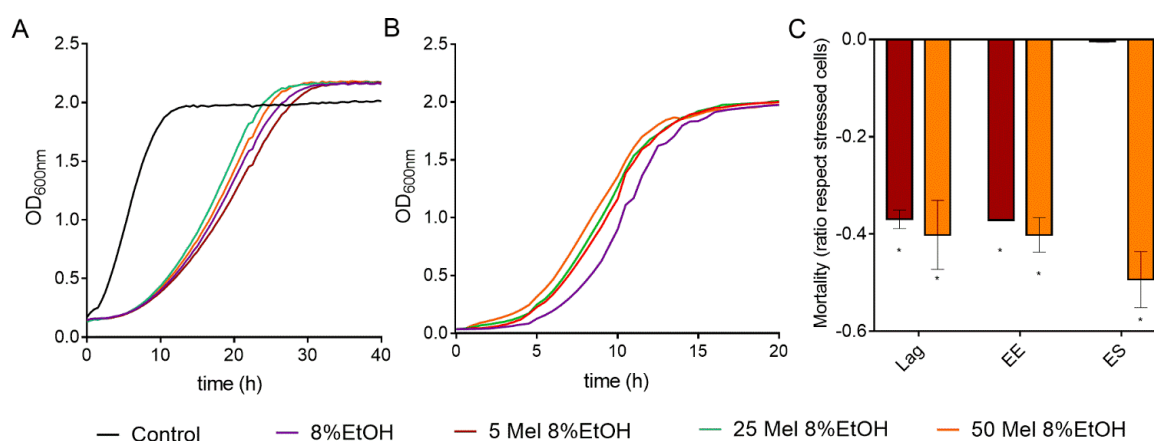
Figure 4.5. Effect of melatonin (Mel) supplementation on BY4743 cells exposed to 8% ethanol on (A) cell growth, (B) growth of cells previously exposed to ethanol and recovered at exponential phase and (C) mortality of cells exposed to ethanol until lag, early exponential (EE) and early stationary (ES) phase, (ratio of mortality in stressed cells with Mel vs stressed cells without Mel). Nonstressed cells (black) and stressed cells with Mel supplementation: 0 (purple), 5 (maroon), 25 (green) or 50 (orange) µM. Error bars represent standard deviation, and * significant differences between stressed cells with and without melatonin.

3.5. Effect of melatonin as an antioxidant shield under ethanol stress

Once evidenced that melatonin improved the viability of ethanol-treated cells and ethanol induced oxidative stress, we wanted to determine whether melatonin could protect cells from this oxidative stress caused by the presence of ethanol (8%). Therefore, we studied the effect of melatonin on different parameters related to oxidative stress.

Figure 4.6. Effect of melatonin (Mel) supplementation (5 (red) or 50 (orange) µM) on BY4743 (A, C) and QA23 (B, D) cells exposed to 8% ethanol until lag, early exponential (EE), mid exponential (ME), early stationary (ES) and stationary (S) phases, on (A, B) ROS accumulation (ratio of MFI of stressed cells with Mel vs stressed cells without Mel), and (C, D) lipid peroxidation (ratio of TBARS of stressed cells with Mel vs stressed cells without Mel). Error bars represent the standard deviation, * indicates significant differences with respect to stressed cells without melatonin (p-value < 0.05). ROS stands for reactive oxygen species, and TBARS stands for thiobarbituric acid-reacting substances.

Both melatonin concentrations tested (5 and 50 µM) decreased ROS accumulation during the initial growth of BY4743 in the presence of ethanol, which was significant at entry to the stationary phase (Figure 4.6A). At this point, stressed cells treated with melatonin presented even lower ROS accumulation than nonstressed cells (Figure 4.3B). However, as the stationary phase progressed, melatonin-treated cells increased ROS

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levels until reaching levels similar to those of stressed cells without melatonin (Figure 4.6A). The profile of ROS generation in QA23 was similar to that in BY4743, with a significant reduction in ROS accumulation during the mid-exponential and early stationary phases at both concentrations of melatonin (Figure 4.6B). However, these same melatonin concentrations had a lower effect in cells submitted to higher ethanol concentrations (10%), as although a significant ROS reduction was observed in the early exponential phase, the ROS levels increased during the stationary phase (data not shown).

Melatonin, regardless of the concentration, reduced lipid peroxidation provoked by ethanol stress in both strains (Figure 4.6C, D). This effect was significant in the lag phase and decreased over time, except for BY4743 in the early stationary phase in the presence of 5 µM of melatonin.

Once showed that melatonin has some protective effect on cells stressed with ethanol, by a decrease in oxidative damage, we wanted to assess whether melatonin also affected the activity of enzymes related to antioxidant capacity, such as catalase and SOD.

In BY4743 cells, the presence of low melatonin concentrations (5 µM) provoked a progressive decrease in catalase activity over time compared to the stressed cells without melatonin, which was significant in the early stationary phase. In contrast, the presence of high melatonin concentrations (50 µM) rapidly decreased catalase activity in the lag phase, and increased it during the early exponential phase, while no difference was observed at the early stationary phase (Figure 4.7A). On the other hand, in QA23, a low melatonin concentration initially decreased catalase activity at the lag phase but increased catalase activity at the early exponential and early stationary phases, where its activity was higher than that in stressed cells without melatonin (Figure 4.7B). Similar results were obtained with a lower melatonin concentration in the medium (1 µM), while higher concentrations practically did not modify the catalase activity in this strain (data not shown).

For SOD activity, both melatonin concentrations triggered the same behavior in BY4743 cells; melatonin slightly increased SOD activity at lag phase and decreased it at early exponential and stationary phases (Figure 4.7C). Interestingly, in the wine strain, melatonin treatment of stressed cells caused an inverse profile: SOD activity decreased at the lag phase (especially significant for low melatonin concentrations), and afterwards, this activity increased until the early stationary phase (Figure 4.7D).
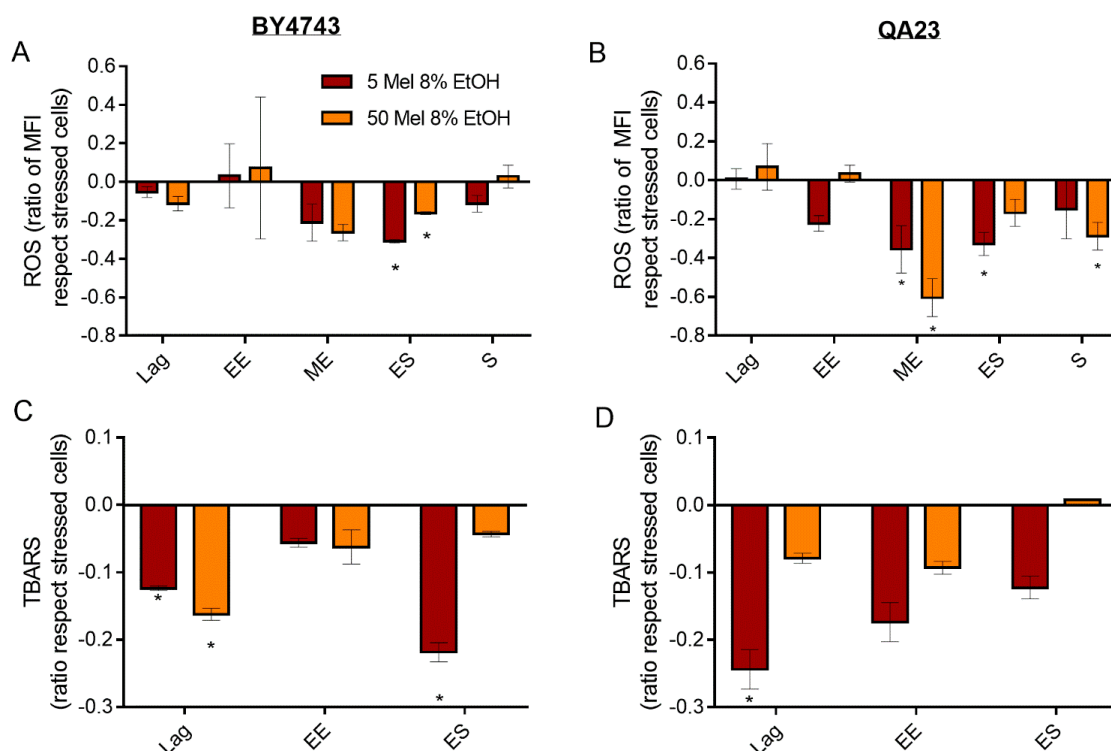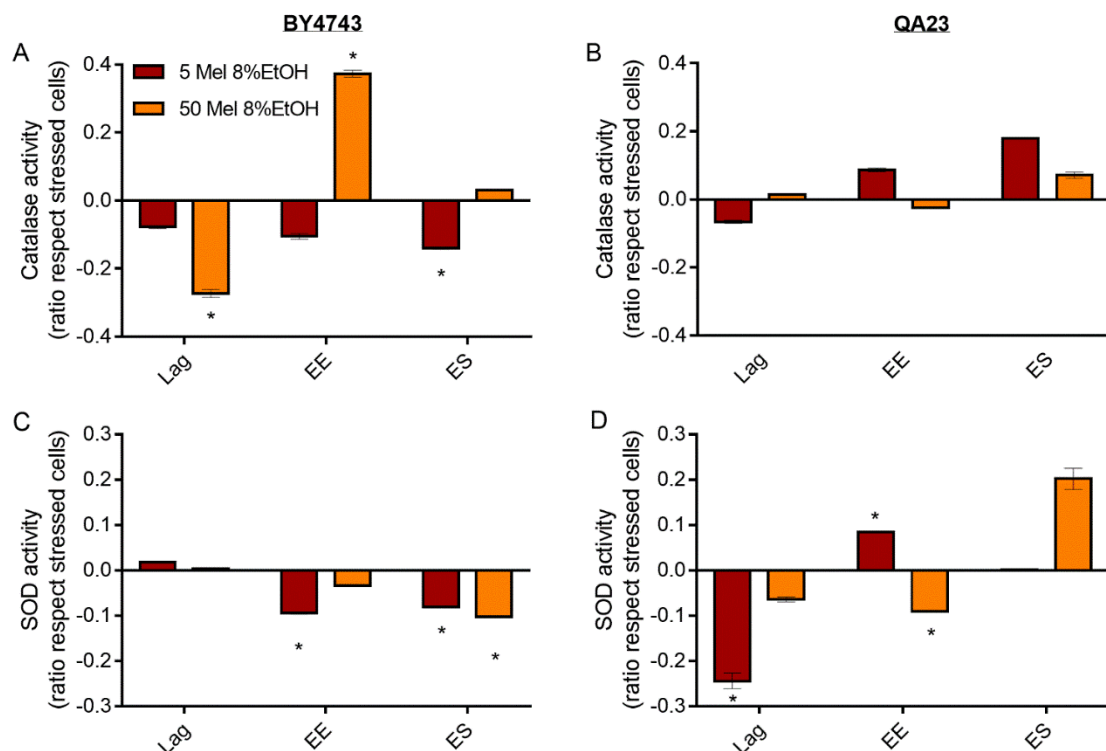
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Figure 4.7. Effect of melatonin (Mel) supplementation (5 (red) or 50 (orange) µM) on BY4743 (**A, C**) and QA23 (**B, D**) cells exposed to 8% ethanol until lag, early exponential (EE) and early stationary (ES) phase, on (**A, B**) catalase activity and (**C, D**) superoxide dismutase (SOD) activity (ratio of the enzymatic activity of stressed cells with Mel vs stressed cells without Mel). Error bars represent the standard deviation, * indicates significant differences with respect to stressed cells without melatonin (p-value < 0.05).

DISCUSSION

Melatonin exhibits antioxidant properties in different organisms, including humans and yeast (Romero et al., 2014; Eghbal et al., 2016; Vázquez et al., 2017, 2018). In yeasts, this molecule has been reported to be produced during fermentation to face the associated stresses (Fernández-Cruz et al., 2017, 2018, 2019; Vázquez et al., 2017, 2018; Morcillo-Parra et al., 2020a). One of the main stress factors that yeast cells encounter during fermentation is the production and accumulation of ethanol in the medium, which could be toxic to yeast cells. Although *S. cerevisiae* possesses inherent tolerance to ethanol, some toxic effects have been associated with ethanol accumulation, such as an increase in oxidative stress, inactivation of related enzymes, dysfunctional mitochondrial metabolism and interference on the cellular membrane and wall (Costa et al., 1997; Alexandre et al., 2001; Kitagaki et al., 2007; Teixeira et al., 2009; Ma and Liu, 2010a;

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Stanley et al., 2010; Lairón-Peris et al., 2021). Moreover, ethanol accumulation is one the main causes described to justify the death of non-*Saccharomyces* species during wine production (Jolly et al., 2014; Albergaria and Arneborg, 2016). The accumulation of some compounds in the media, such as polyphenols or amino acids, has been related to increased yeast tolerance to ethanol (Cheng et al., 2019; Cheng et al., 2016; Gharwalova et al., 2017; Hirasawa et al., 2007; Ohta et al., 2016; Takagi et al., 2016). Resveratrol has been the most studied polyphenol, showing that this compound is able to increase yeast tolerance to ethanol by decreasing lipid peroxidation and SOD activity and by regulating the membrane composition (Gharwalova et al., 2017). Several amino acids, such as arginine or proline, have also been reported to confer tolerance to ethanol by decreasing ROS accumulation (Cheng et al., 2016; Takagi et al., 2016). Thus, as high ethanol concentrations induce oxidative stress, it seems plausible that melatonin, due to its role as an antioxidant, could protect against this stress. Moreover, this molecule is reported to protect humans from alcoholism effects by regulating the immune system, optimizing homeostasis, and providing protection against the oxidative stress provoked by ethanol on tissues and organs (reviewed in Kurhaluk and Tkachenko, 2020). Therefore, we evaluated whether melatonin also had a protective effect against the oxidative stress provoked by ethanol in yeast cells. For this reason, we first studied the effect of different ethanol concentrations in two *S. cerevisiae* strains (a wine and lab strain, QA23 and BY4743, respectively) on different parameters related to cell viability and oxidative stress and then studied the possible protective effect of two melatonin concentrations (5 and 50 µM) on cells exposed to ethanol.

As commented previously, ethanol is a metabolite produced by yeast that can become toxic when a threshold is surpassed, and different yeast strains can show very different abilities to grow and survive in the presence of ethanol (Snoek et al., 2016; Morard et al., 2019; Lairón-Peris et al., 2021). Therefore, the two *S. cerevisiae* strains were first confronted with a range of ethanol concentrations to determine their cellular response and tolerance to this stress. As expected, increasing ethanol concentrations produced stronger effects on yeast viability, cell recovery and ROS accumulation (also reported in several studies, such as Cheng et al., 2016; Martínez-Alcántar et al., 2019; Navarro-Tapia et al., 2017) until reaching the inhibitory concentration, at which point cell growth was totally inhibited. Similar behavior in response to ethanol stress was observed in the two strains, although the wine strain exhibited higher tolerance to high alcohol concentrations than the lab strain. In the case of the lab strain, 12% ethanol totally

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suppressed its growth, whereas in the wine strain, 14% of ethanol was necessary to totally inhibit it. Additionally, the wine strain presented a lower percentage of dead cells; more QA23 cells endured and thrived in ethanol stress, requiring a higher percentage of ethanol to achieve a similar percentage of dead cells than in BY4743. These results agree with those of Lairón-Peris et al. (2021), who reported that wine yeast strains were among the most ethanol-tolerant *S. cerevisiae* strains. Additionally, the wine strain also generated a lower quantity of ROS, both at entry into the stationary phase and in response to ethanol treatment. These results confirmed that the wine strain was better adapted to withstand ethanol stress and other stresses originating during wine fermentation, as also reported in Pais et al. (2013) with other wine and lab strains.

In general, ethanol-stressed cells exhibited higher mortality and oxidative stress (shown by ROS accumulation, lipid peroxidation, and the activation of some antioxidant enzymes) and lower cell growth and recovery capacity, than nonstressed cells. There was only one exception: entry in the early stationary phase, in which nonstressed cells increased the markers of oxidative damage even more than stressed cells. Cells in the stationary phase are stressed by the lack of nutrients and by the accumulation of toxic metabolites (Galdieri et al., 2010; Nussbaum et al., 2014), leading to an increase in oxidative stress markers (Chen et al., 2005; Owsiak et al., 2010) and the activation of the ESR (Gasch et al., 2000; Teixeira et al., 2011; Matallana and Aranda, 2017), which includes mechanisms for stress resistance, such as modulation of *SOD2* (Galdieri et al., 2010). Therefore, this response is triggered in the stationary phase in nonstressed cells but earlier in stressed cells. During alcoholic fermentation, ethanol accumulates over time, and a clear correlation has been established between ethanol and oxidative stress in yeast (Costa et al., 1997; Alexandre et al., 2001; Du and Takagi, 2007). In fact, the principal mechanism of ethanol toxicity on yeast cells has been reported to be an oxidative burst, mainly produced by ROS generation in the mitochondria (H_2O_2 and $O_2^{\cdot-}$) (Du and Takagi, 2007; Kitagaki et al., 2007; Pérez-Gallardo et al., 2013; Jing et al., 2020): therefore, it was not surprising that ethanol stress induced a quick formation of ROS and lipid peroxidation in both strains. This ROS generation started just after the application of the stress, reaching its maximum after 5 h of the treatment with a subsequent decline. ROS are toxic to yeast, as they inhibit metabolic processes and prevent cellular growth, so the fact that ROS levels were higher in cells under early exponential growth could be the reason why these cells need more time to recover normal growth. Other studies also reported an accumulation of H_2O_2 and $O_2^{\cdot-}$ in mitochondria after short ethanol exposure times (Pérez-

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Gallardo et al., 2013; Charoenbhakdi et al., 2016; Martínez-Alcántar et al., 2019), and in poorer media, this ROS increase can last longer (Du and Takagi, 2007; Yang et al., 2012). The induction of membrane lipid peroxidation by ethanol stress was also reported in Gharwalova et al. (2017), Fierro-Risco et al. (2013) and Gupta et al. (1994). Thus, ethanol seemed to accelerate ROS formation, mainly in the mitochondria, causing lipid peroxidation in the membranes that was maintained over time. Instead, the rapid decrease in ROS levels after reaching the maximum in the early exponential phase could be explained by the fact that ROS can stimulate mitophagy, leading to the elimination of damaged and dysfunctional mitochondria and thereby contributing to a decrease in H_2O_2 and $O_2^{\cdot-}$ (Jing et al., 2020). Thus, this could result in the protection of the cell from ROS damage, a decrease in cell mortality and an increase in tolerance to ethanol.

Additionally, the response to oxidative damage triggers the synthesis of enzymes that are able to detoxify ROS, such as catalases (encoded by *CTT1* and *CTA1*) or SOD (encoded by *SOD1* and *SOD2*), among others (Herrero et al., 2008; Ayer et al., 2013). Furthermore, antioxidant defenses have been described to be mainly repressed by glucose during exponential growth and derepressed when cells approach the diauxic shift to prepare for the increase in oxidative stress (Costa et al., 1997; Moradas-Ferreira and Costa, 2000). All these data are concordant with our results, as in nonstressed cells, antioxidant enzymes, especially catalase, are mostly activated in the entry to stationary phase, when the sugar present in the medium is low and the oxidative stress is higher. On the other hand, the cells treated with ethanol activated the antioxidant machinery earlier, to counteract the effects of oxidative stress at the beginning of growth, thereby exhibiting higher oxidative stress tolerance (Moradas-Ferreira and Costa, 2000). The induction of these enzymes by ethanol has been widely reported (Costa et al., 1997; Fierro-Risco et al., 2013; Gharwalova et al., 2017; Pérez-Gallardo et al., 2013; Wang et al., 2014a), highlighting the importance of both SODs and the cytosolic catalase for ethanol tolerance (Costa et al., 1993; Du and Takagi, 2007; Kitagaki et al., 2007; Bleoanca et al., 2013; Wang et al., 2014a; Cheng et al., 2016). These enzymes are pivotal for ethanol tolerance because they are responsible for eliminating the ROS generated by ethanol (Costa et al., 1993; Kitagaki et al., 2007). In this study, catalase was clearly activated during the stationary phase, but SOD also presented high activity during the exponential phase, with a profile similar to that of lipid peroxidation, suggesting that SOD is activated before catalase. This is not surprising because SOD is in charge of the detoxification of the O_2^{-} anion by

transforming it in H_2O_2, which is subsequently detoxified by catalases (Pérez-Gallardo et al., 2013; Jing et al., 2020).

In general, the presence of melatonin lightened the effect of 8% ethanol on cell growth. Cells treated with a range of 5 to 50 μM melatonin exhibited improved cell viability when submitted to 8% ethanol. In cells pretreated with melatonin and exposed to ethanol in early growth stages, cell mortality was decreased, and better cell recovery was obtained. When the cells were exposed to ethanol until the stationary phase, those effects of melatonin were lessened, probably due to an adaptation of the cells and an activation of the ESR, which was reflected in a lower mortality due to ethanol presence. These results seem to point towards an early protective response of melatonin against ethanol stress, similar to the one observed against oxidative stress, which was activated after 45 min (Vázquez et al., 2017).

Our results also showed that cells grown with melatonin and exposed to ethanol stress had less oxidative damage than stressed cells without melatonin, as ROS accumulation and lipid peroxidation were lower. Similar results were previously reported for cells subjected to oxidative stress in the presence and absence of melatonin (Vázquez et al., 2017, 2018) or resveratrol (Gharwalova et al. 2017). A higher concentration of melatonin did not always confer higher protection, as was observed for oxidative stress in Vázquez et al. (2017).

Melatonin has been described to interfere with cellular antioxidant activities and the transcriptional machinery, more specifically, modulating the genes of the antioxidant response (Vázquez et al., 2017; Bisquert et al., 2018; Chapter I of the present thesis). These antioxidant activities generally decreased by melatonin in stressed cells, although in the wine strain, after an initial decrease, the activities started to increase over time. In a previous study, an increase in catalase activity was observed in nonstressed cells treated with melatonin, suggesting a pro-oxidant role of this compound (Vázquez et al., 2018). In this study, similar results were obtained, as melatonin in nonstressed QA23 cells induced a similar pattern than that of ethanol-stressed cells, by increasing catalase activity in the exponential phase and decreasing catalase activity in the stationary phase. These results again suggest a possible pro-oxidant effect of melatonin, which could confer resistance to further oxidative stress exposure (Bisquert et al., 2018; Vázquez et al., 2018). Therefore, as those cells were previously grown in melatonin before the stress was applied, the presence of melatonin could have activated the antioxidant response, making them more

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prepared to endure ethanol stress and reducing the need to activate those enzymes against future stresses. Gharwalova et al. (2017) found a similar decrease in SOD activity in ethanol-stressed cells treated with resveratrol, and Estruch et al. (2011) found a similar decrease in healthy men after red wine intake, suggesting a reduction in enzyme activity when not necessary to save energy. The induction of SOD in the wine strain by melatonin in the early stationary phase seems to validate this idea. In this strain, the presence of melatonin caused a fast activation of antioxidant machinery after ethanol stress, neutralizing the stress and quickly relaxing the defense system. Therefore, when cells entered stationary phase, they needed to again activate the antioxidant machinery. Instead, in the lab strain, the response activated by melatonin was less efficient, and antioxidant defense were still activated when cells entered stationary phase.

The results of this study suggest that melatonin has a protective role against mild ethanol stress, mainly by reducing the oxidative stress triggered by this alcohol. However, other important targets of ethanol stress should be analyzed to decipher whether melatonin can also enhance ethanol tolerance by affecting other response mechanisms. One of these targets of ethanol stress is lipid membranes. Indeed, ethanol stress induces changes in yeast lipid composition as an adaptation mechanism to face this stress (Ishmayana et al., 2017; Lairón-Peris et al., 2021). Vázquez et al. (2018) reported that the presence of melatonin also modulated the membrane composition, leading to a higher unsaturated fatty acids/saturated fatty acids (UFA/SFA) ratio, mainly increasing the content of oleic and palmitoleic acids. In fact, oleic acid is considered a membrane fluidity sensor, as it appears to be the most important UFA for counteracting the toxicity of ethanol by increasing the membrane stability to compensate for the fluidity caused by ethanol (You et al., 2003). Resveratrol has also been described to act on the lipid composition of the membrane as a mechanism to enhance ethanol tolerance, although its mechanism is based on increasing the proportion of SFAs to stabilize the membrane (Gharwalova et al., 2017). Another important cell target of ethanol toxicity is mitochondria, which have a dual key role in tolerance to ethanol. On the one hand, they are the site of ROS production but on the other hand, they are required for the activation of the oxidative stress response mediated by Yap1p, since mitochondrial SOD and the retrograde signaling pathway (activated by mitochondrial dysfunction) are involved in Yap1p activation (Zyrina et al., 2017). Moreover, Jing et al. (2020) reported that clearance of damaged mitochondria by mitophagy contributes to improving ethanol tolerance. Mitochondria have been widely described as targets of melatonin in human cells

(Cardinali and Vigo, 2017; Reiter et al., 2020), and recently, it was elucidated that melatonin modulates the transcription of several mitochondrial genes in yeast (Chapter I of the present thesis); therefore, it would be interesting to study the effect of melatonin on mitochondria in cells exposed to ethanol stress. Moreover, the accumulation of reserve carbohydrates, such as glycogen and trehalose, has been related to ethanol stress tolerance, as they stabilize or repair proteins denatured by ethanol (reviewed in Ding et al., 2009; Ma and Liu, 2010a). In a recent study (Chapter I of the present thesis), we found that some carbohydrates biosynthesis (such as glycogen and trehalose) pathways were regulated by the addition of melatonin, suggesting that this could be another topic to focus on further research.

In conclusion, ethanol increased the production of ROS and lipid peroxidation and triggered the activation of antioxidant defense, which was consistent with previous studies. However, in cells treated with melatonin, such damage was attenuated by the antioxidant capacity of this molecule, which was able to scavenge ROS, reducing their noxious effects, such as lipid peroxidation, and increasing ethanol tolerance. The mitigation of oxidative stress by the presence of melatonin reduced the activity of some antioxidant enzymes, thereby resulting in a lower oxidative stress response. Therefore, these results suggest that cells grown in the presence of melatonin are better prepared to endure ethanol stress. However, to fully understand the mechanism by which melatonin confers protection against ethanol stress, the effects of this molecule on the lipid membrane and mitochondria need to be evaluated.

SUPPLEMENTARY MATERIAL
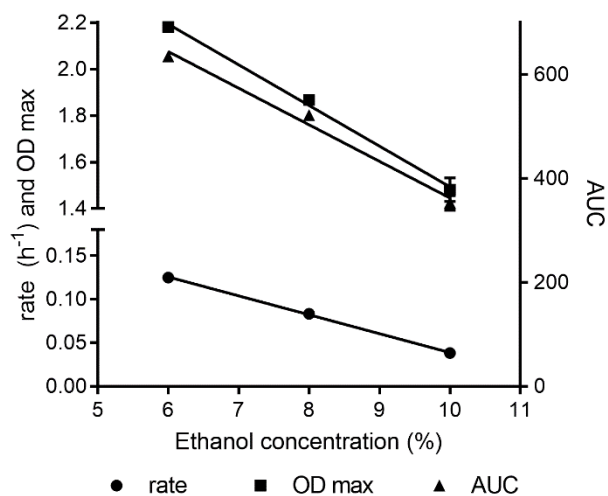
Supplementary Figure S4.1. Linear regressions correlating ethanol concentration (6-10%) with growth rate (circles, h^{-1}), maximum OD (squares, OD max) and area under the curve (triangles, AUC) obtained from the growth curves of the QA23 strain.
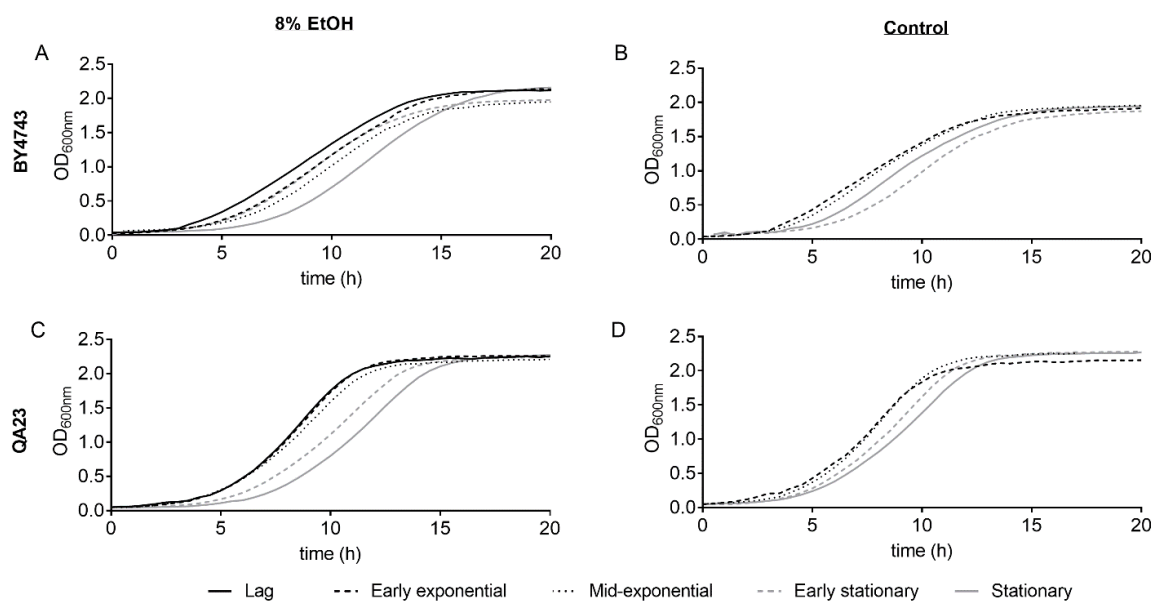
Supplementary Figure S4.2. Growth of BY4743 (**A, B**) and QA23 (**C, D**) cells previously exposed to ethanol (0%, **B, D**; 8%, **A, C**) and recovered at different growth phases: lag phase, early exponential phase, mid-exponential phase, early stationary phase and stationary phase. No lag phase was observed for nonstressed cells.

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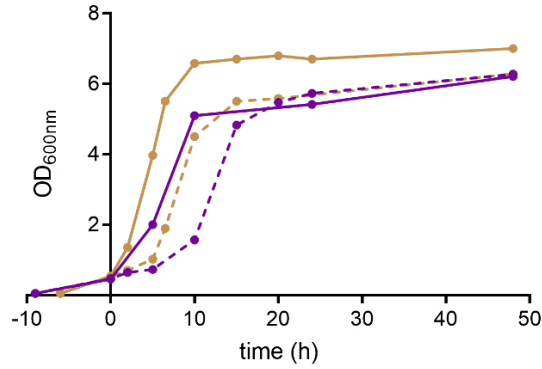
Supplementary Figure S4.3. Growth curve of BY4743 (purple) and QA23 (orange) strains with 0% (continuous line) and 8% (discontinuous line) ethanol. Time 0 h represents the moment in which ethanol stress was applied.

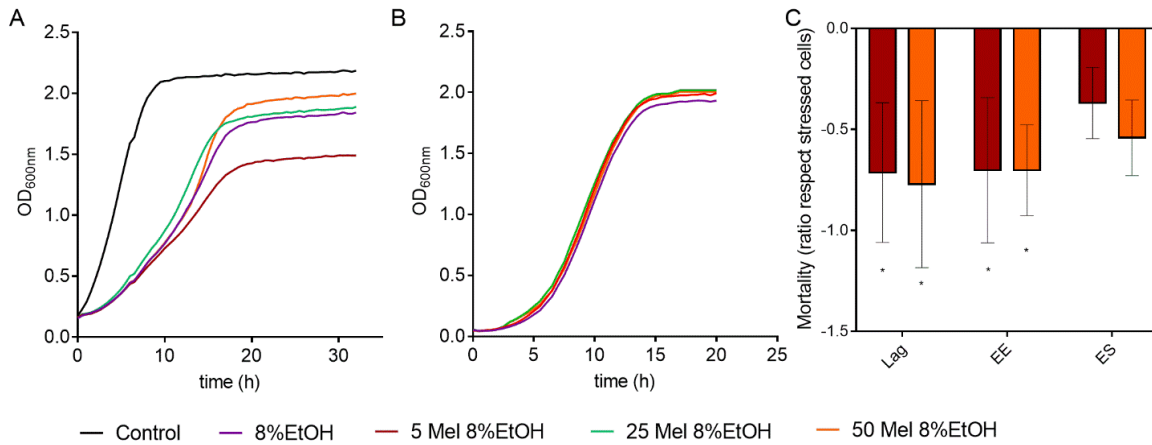**Supplementary Figure S4.4.** Effect of melatonin (Mel) supplementation on QA23 cells exposed to 8% ethanol on (**A**) cell growth, (**B**) growth of cells previously exposed to ethanol and recovered at exponential phase and (**C**) mortality of cells exposed to ethanol until lag, early exponential and early stationary phase (ratio of mortality in stressed cells with Mel vs stressed cells without Mel). Nonstressed cells (black) and stressed cells with Mel supplementation: 0 (purple), 5 (maroon), 25 (green) or 50 (orange) μM. Error bars represent standard deviation, and * significant differences between stressed cells with and without melatonin.

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Supplementary Table S4.1. Effect of different ethanol concentrations on the growth of *S. cerevisiae* QA23 and BY4743. The parameters analysed were growth rate (h^{-1}), maximum OD (OD_{max}) and area under the curve (AUC) calculated until 80 h of growth. The linear regression values (slope and r^2) were calculated for concentrations in the range of 6-10% ethanol.

ETHANOL CONCENTRATIONS (%)	RATE (h^{-1}) BY4743	QA23	OD_{MAX} BY4743	QA23	AUC BY4743	QA23
0	0.148	0.173	2.132	2.202	592.626	689.847
6	0.090	0.125	2.204	2.181	579.542	634.705
8	0.065	0.083	2.200	1.868	532.495	521.683
10	0.025	0.038	1.955	1.481	261.923	353.108
12	0.005	0.035	0.464	1.413	141.599	155.192
14	0.004	0.015	0.423	0.201	104.676	60.694
LINEAR REGRESSION VALUES						
SLOPE	-0.016	-0.022	-0.062	-0.175	-79.405	-70.399
r^2	0.983	0.999	0.760	0.996	0.858	0.987

Supplementary Table S4.2. Effect of different ethanol concentrations (0%, 8%, 10%) on the growth of BY4743 and QA23 cells previously exposed to ethanol and recovered at different growth phases: lag phase; early and mid-exponential phases; early stationary and stationary phases. The parameters analysed were growth rate (h^{-1}), maximum OD (OD_{max}) and area under the curve (AUC) calculated until 12 h of growth. Lag phase was not observed for nonstressed cells (indicated with a slash "-"). ND stands for not determined.

time (h)	Control Rate	OD_{max}	AUC	8% EtOH Rate	OD_{max}	AUC	10% EtOH Rate	OD_{max}	AUC
BY4743									
Lag	-	-	-	0.20	2.22	34.53	0.15	2.38	12.34
Early exponential	0.21	2.00	37.80	0.21	2.32	28.92	0.15	2.35	4.90
Mid-exponential	0.21	2.04	35.79	0.16	2.05	25.31	ND	ND	ND
Early stationary	0.17	1.96	24.35	0.18	2.07	26.53	ND	ND	ND
Stationary	0.19	2.03	30.19	0.18	2.26	17.22	ND	ND	ND
QA23									
Lag	-	-	-	0.19	2.25	37.19	0.16	2.21	10.96
Early exponential	0.20	2.15	41.54	0.20	2.26	36.56	0.18	2.06	20.36
Mid-exponential	0.20	2.25	40.38	0.20	2.27	33.90	0.16	2.26	4.78
Early stationary	0.21	2.33	34.76	0.19	2.29	24.02	0.16	2.39	16.69
Stationary	0.19	2.30	30.26	0.18	2.27	16.99	ND	ND	ND

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Supplementary Table S4.3. Effect of melatonin supplementation (0, 5, 25, 50 µM) on the growth curve and recovery of *S. cerevisiae* strains BY4743 and QA23 after exposure to 8 or 10% ethanol. The parameters analysed were growth rate (h^{-1}), maximum OD (OD_{max}) and area under the curve (AUC) until 20 h (growth curve) and 12 h (recovery) of growth. These parameters were calculated at different growth phases after the stress exposure: lag, early exponential (EE), and early stationary (ES). ND: Not determined.

Parameter		Rate (h^{-1})				OD_{max}				AUC			
Melatonin concentration (µM)		0	5	25	50	0	5	25	50	0	5	25	50
Growth curve													
BY4743		0.07	0.06	0.07	0.07	2.16	2.17	2.17	2.18	44.2	41.6	49.6	46.3
QA23		0.09	0.08	0.09	0.08	1.88	1.52	1.94	2.25	73.6	63.6	80.8	80.7
Recovery													
BY4743	Lag	0.20	0.21	0.20	0.19	2.22	2.33	2.28	2.27	34.5	34.6	31.8	29.4
	EE	0.21	0.22	0.21	0.22	2.32	2.34	2.33	2.29	29.0	38.3	40.7	45.8
	ES	0.18	0.19	0.18	0.17	2.07	2.12	2.10	1.99	26.6	31.5	28.0	27.7
QA23	Lag	0.19	0.20	ND	0.20	2.25	2.30	ND	2.24	37.2	39.8	ND	34.4
	EE	0.20	0.20	0.20	0.19	2.26	2.29	2.43	2.33	36.6	39.6	40.2	38.6
	ES	0.19	0.19	0.19	0.19	2.29	2.31	2.42	2.30	24.0	22.7	27.8	23.7

CHAPTER V

Protective role of hydroxytyrosol as an antioxidant in *Saccharomyces cerevisiae*

Manuscript in preparation

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Mercè Sunyer Figueres

Hydroxytyrosol oxidative stress

Abstract: Hydroxytyrosol (HT) and melatonin are bioactive molecules that have widely reported beneficial effects on human health, mainly due to their strong antioxidant capacity. They are synthesized by yeast during alcoholic fermentation, and although the antioxidant role of melatonin in yeast is well reported, nothing is known about the role of HT in those organisms. The aim of this study was to evaluate the possible antioxidant effect of HT on *Saccharomyces cerevisiae* cells using a commercial wine strain. First, the impact of HT presence (ranging from 0.5 to 100 µM) on yeast growth was monitored in different media, and no effect was found. Then, yeast cells were supplemented with a low concentration of this compound (5 µM) and exposed to mild oxidative stress (induced by H_2O_2) to decipher the effects on cell viability (reporting cell growth and recovery) and on indicators of oxidative stress, such as reactive oxygen species (ROS) accumulation, lipid peroxidation and the activities of catalase and superoxide dismutase (SOD) enzymes. For the indicators of oxidative stress, the effects were compared to those provoked by 5 µM melatonin supplementation. Although HT did not affect the growth of cells exposed to H_2O_2, it enhanced the recovery when the stressed cells were in the lag phase. Moreover, HT smoothed the increase in ROS and lipid peroxidation provoked by oxidative stress, mimicking the effects of melatonin. However, supplementation with HT and melatonin affected catalase activity in the opposite way as follows: H_2O_2 exposure increased catalase activity, which was further increased by HT supplementation but was decreased by melatonin. No effect was reported on SOD after H_2O_2 exposure. This study indicated that HT has a protective role against oxidative stress in *S. cerevisiae* cells, by reducing oxidative damage and modulating the endogenous antioxidant machinery.

Keywords: hydroxytyrosol, yeast, oxidative stress, antioxidant, melatonin

CHAPTER V

1. INTRODUCTION

Hydroxytyrosol (or 4-(2-hydroxyethyl1,2-benzenediol), HT) is a phenolic alcohol with many beneficial effects on human health by mainly protecting against cardiovascular diseases and acting as an anticarcinogenic, antidiabetic, neuroprotective or modulator of the immune system (Cerezo et al., 2019; Karković Marković et al., 2019; Bertelli et al., 2020). In fact, HT is one of the most powerful dietary antioxidants (Vissers et al., 2002; Hu et al., 2014; Bertelli et al., 2020), even though the European Food Safety Authority (EFSA) recommends its daily consumption in the context of a balanced diet, allowing its addition in some food products (EFSA, 2011; 2017).

Many of the beneficial properties of HT rely on its strong antioxidant capacity (Karković Marković et al., 2019). The most relevant antioxidant property is its high free-radical scavenging activity, which mainly neutralizes superoxide, hydrogen peroxide and hypochlorous acid and breaks the chain of reactions triggered by free radicals (Visioli and Galli, 1998; Tripoli et al., 2005; Robles-Almazan et al., 2018). Moreover, HT administration in mammals is also suggested to confer antioxidant protection by other mechanisms. For example, HT can act as a metal chelator, repair structures damaged by free radicals, such as DNA or peroxidized lipids, activate signalling pathways to decrease the accumulation of free radicals or increase endogenous defence systems against oxidative stress (antioxidant enzymes such as catalase or superoxide dismutase (SOD) or endogenous antioxidant molecules such as glutathione) (reviewed in Visioli and Galli, 1998; Paiva-Martins and Gordon, 2005; Robles-Almazan et al., 2018; Karković Marković et al., 2019; Bertelli et al., 2020).

HT is one of the major polyphenolic compounds in olives and olive oil (around the range of 550–1000 and 50–445 mg/kg, respectively) (Tripoli et al., 2005; Marsilio et al., 2006; EFSA, 2011), but it has also been found in wine (ranging from 0.28 to 9.6 mg/L), both as a component of grape must and as a product of yeast metabolism during alcoholic fermentation, synthesized from tyrosine through the Ehrlich pathway (Di Tommaso et al., 1998; Proestos et al., 2005; Boselli et al., 2006; Piñeiro et al., 2011; Garcia-Moreno et al., 2013; Bordiga et al., 2016; Álvarez-Fernández et al., 2018; Rebollo-Romero et al., 2020). A moderate intake of red wine has been reported to reduce cardiovascular risk and improve the antioxidant capacity of plasma, properties that have been attributed to the presence of polyphenols, such as resveratrol or gallic and caffeic acid, in wine (Renaud

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and Lorgeril, 1992; Minussi et al., 2003; Fernández-Mar et al., 2012; Vilela, 2019). These compounds have well-reported antioxidant effects but very low bioavailability. Recently, several studies have shown that some bioactive molecules, such as melatonin, HT or serotonin, produced by yeast as secondary metabolites of alcoholic fermentation also exert beneficial effects in humans. Moreover, melatonin and HT have a higher absorption than the abovementioned wine polyphenols, and recent studies suggest that the synergic action of these molecules could intensify their beneficial effects, pointing towards the idea that they could be partially responsible for the benefits of red wine in human health (Mas et al., 2014; Hornedo-Ortega et al., 2016; Marhuenda et al., 2017).

Oxidative stress occurs when the concentration of reactive oxygen species (ROS) in a cell increases beyond its antioxidant buffering capacity. ROS can be produced endogenously (mainly during oxidative phosphorylation in the mitochondria) or by exposure to exogenous agents, such as ionizing radiation, H_2O_2, or high concentrations of ethanol. Reactive species damage cellular components by oxidizing DNA, proteins and lipids, which increases the oxidized state of the cell and causes impairment of several cellular processes, which in turn leads to cellular dysfunction and finally to cell death (Jamieson, 1998; Moradas-Ferreira and Costa, 2000; Halliwell, 2006; de la Torre-Ruiz et al., 2015; Picazo and Molin, 2021; Toledano et al., 2003). Therefore, cells have enzymatic (such as catalases, SOD and glutathione peroxidases) and nonenzymatic (such as the glutathione and thioredoxin systems) defence mechanisms to protect the cellular components against oxidative stress (Jamieson, 1998; Costa and Moradas-Ferreira, 2001). *S. cerevisiae* has two SOD enzymes, ubicated in the cytoplasm (Cu/Zn-SOD) and in the mitochondria (Mn-SOD), which catalyse the conversion of superoxide anion into H_2O_2 and O_2. It also has two catalases, which scavenge H_2O_2 by its reduction to H_2O and O_2 and are in the peroxisome (catalase A) and cytoplasm (catalase T) (Jamieson, 1998; Herrero et al., 2008; Auesukaree, 2017; Toledano, 2003).

Recent studies have reported that melatonin also has antioxidant properties in yeast cells, acting directly, by reducing the ROS accumulation produced by H_2O_2 exposure, and indirectly, by modulating gene expression related to the cell response to oxidative stress, among other pathways. Thus, melatonin could contribute to conferring protection to yeast cells against fermentation-associated stresses, such as ethanol stress, and enhance their fermentation performance (Vázquez et al., 2017, 2018; Bisquert et al., 2018; Chapters I and IV of the present thesis). The goal of this study was to evaluate the possible

antioxidant effect of HT on yeast. To this end, we first determined the effect of HT on yeast growth and then its effect on cells exposed to oxidative stress by monitoring different oxidative stress indicators, such as ROS accumulation, degree of lipid peroxidation, and some antioxidant enzymatic activities (catalase and SOD). Additionally, the effect of HT on oxidative stress indicators was compared with that of melatonin.

2. MATERIALS AND METHODS

2.1. Yeast strain and culture media

In this study, the commercial wine strain *S. cerevisiae* QA23 (Lallemand, Montreal, QA, Canada) was used. Four different media were used in this experiment: YPD (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain)), YNB (Difco^{TM} Yeast Nitrogen Base, BD, Sparks, MD, USA) with 2% (w/v) (YNB-2) or 5% (w/v) (YNB-5) of glucose, and synthetic must (Beltran et al., 2004).

Cultures were supplemented with HT (0.5 - 100 µM, Sigma-Aldrich, St Louis, MO, USA) or melatonin (5 µM, Sigma-Aldrich), and submitted to oxidative stress, using 2 mM H_2O_2 (Sigma-Aldrich). The specific concentrations used for each assay and the experimental conditions are detailed in the following sections.

2.2 Determination of yeast growth

Yeast growth in the presence and absence of HT was evaluated through different experiments. Yeast was precultured in YPD or YNB-2 (only when specified) broth by incubation at 28 °C with orbital shaking (120 rpm) for 24 h. Then, an initial OD_{600nm} of 0.05 was used to inoculate the media and growth was monitored by measuring OD_{600nm} every 30 min in a SpectroStar NANO microplate reader (Bmb Labtech, Germany). A culture without HT nor melatonin was used as a control, and three biological and five technical replicates were employed in all the assays.

Firstly, the growth of *S. cerevisiae* was evaluated in different media (YPD, YNB-2, YNB-5, synthetic must) supplemented with several HT concentrations (0.5, 5, 100 µM), by inoculating yeast from YPD preculture (for growth in YPD) or from YNB-2 preculture (for growth in YNB-2, YNB-5 and synthetic must). Cell growth was monitored for 90 h.

Secondly, yeast growth under oxidative stress was determined. Once the cultures in YPD with or without HT supplementation (0 and 5 µM HT) reached the initial exponential

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phase (OD$_{600nm}$ 0.5 − 0.6), they were immediately inoculated into fresh YPD in the presence of 2 mM H$_2$O$_2$ and growth was monitored for 40 h.

Thirdly, the growth recovery of the cultures with or without HT supplementation (0 and 5 µM HT) after being stressed with 2 mM H$_2$O$_2$ for different exposure times (specified in the results section) was determined by inoculating the cultures at OD$_{600nm}$ 0.05 in fresh YPD medium and monitoring growth for 40 h. In this experiment, the stress was subjected to cultures in the early exponential phase (OD$_{600nm}$ 0.5 − 0.6) but also in the stationary phase (OD$_{600nm}$ 6).

From the growth curves, different parameters were extracted: OD max, growth rate and the area under the OD-time curve (AUC) or growth potential (Todor et al., 2014; Lairón-Peris et al., 2021). The growth rate was calculated with the following formula: rate = (log(OD$_t$)-log(OD$_0$))/t-t$_0$, and the estimate of AUC was calculated as a metric of the OD distribution as a function of time t.

2.3. Determination of oxidative stress indicators

Yeasts were precultured in YPD broth by incubation at 28 °C with orbital shaking (120 rpm) for 24h. Then, an initial OD$_{600nm}$ of 0.05 was inoculated in 60 mL of YPD broth supplemented with or without 5 µM of HT or melatonin and incubated until the cells reached the early exponential phase (OD$_{600nm}$ 0.5-0.6). Then, cultures were subjected to oxidative stress, using 2 mM H$_2$O$_2$, and incubated in the presence of stress for 1 h. For lipid peroxidation, catalase and SOD activities assays, 10^8 cells were harvested by centrifugation at 4,700 rpm for 5 min at 4 °C. Then, cells were washed with Milli-Q water (Millipore Q-POD^{TM} Advantage A10), centrifuged at 16,000 rpm for 5 min at 4 °C, fast-frozen with liquid nitrogen and stored at -80 °C until use. Three biological replicates were carried out in all assays, and a nonstressed control without HT or melatonin was performed for each assay.

Reactive oxygen species (ROS) were quantified using the fluorescent probe dihydrorhodamine 123 (DHR123; Sigma-Aldrich), as described in Vázquez et al. (2017) and in Chapter IV of the present thesis. The fluorescence intensity was measured with a CyFlowspace flow cytometer (Partec, Norderstedt, Germany), and the data were acquired with FloMax software (Quantum Analysis GmbH, Münster, Germany) and processed with WinMDI 2.9 software to quantify mean fluorescence intensity (Joseph, Trotter, Salk

Institute for Biological Studies, CA, USA). The results were expressed as fold of fluorescence intensity normalized to nonstressed cells without HT.

The degree of lipid peroxidation was measured based on Buege and Aust (1978), which uses the colorimetric determination of thiobarbituric acid reacting substances (TBARS), but including some modifications as described in Chapter IV of this thesis based on the protocol of Vázquez et al., (2018). TBARS concentration was estimated by referring to a standard curve of 1,1,3,3-tetramethoxylpropane (Sigma-Aldrich), and the results were expressed as fold of concentration normalized to nonstressed cells without HT.

The enzymatic activities of catalase and SOD in presence or absence of oxidative stress and HT or melatonin were determined following the protocol described in Chapter IV of this thesis, which is based on Vazquez et al (2018) for catalase activity and uses the SOD assay kit (Sigma-Aldrich) for SOD activity. The catalase and SOD activities of the protein extracts were estimated using a standard curve prepared with known amounts of catalase (range 0-6 µg/mL) and bovine SOD (range 0-5 U/mL) (Sigma-Aldrich), and the results were expressed as fold of activity normalized to nonstressed cells without HT.

2.4. Data analysis

Data obtained from all the assays were subjected to analysis of variance (ANOVA) and Tukey's post hoc test using GraphPad Prism 7 (GraphPad Software, CA, USA). The results were considered statistically significant at a p-value < 0.05.

3. RESULTS

3.1. Effect of hydroxytyrosol on *S. cerevisiae* growth

The effect of HT concentration on *S. cerevisiae* growth was determined in different media. For accomplishing that, yeast cells were cultivated in YPD, YNB-2, YNB-5 and synthetic must. The composition of the medium clearly affected the growth of yeast cells (Figure 5.1A). Surprisingly, cells grown in minimal medium (YNB-2) reached a higher OD max than those grown in rich medium (YPD), despite having the same glucose concentration (2%) (Figure 5.1A). The presence of a higher initial glucose concentration (YNB-5 and synthetic must) resulted in a prolonged lag phase, which was longer when more sugar was in the medium. Additionally, this high concentration of sugar present in synthetic must (20%) decreased the growth rate and OD max, an effect not observed in YNB-5 (5%).

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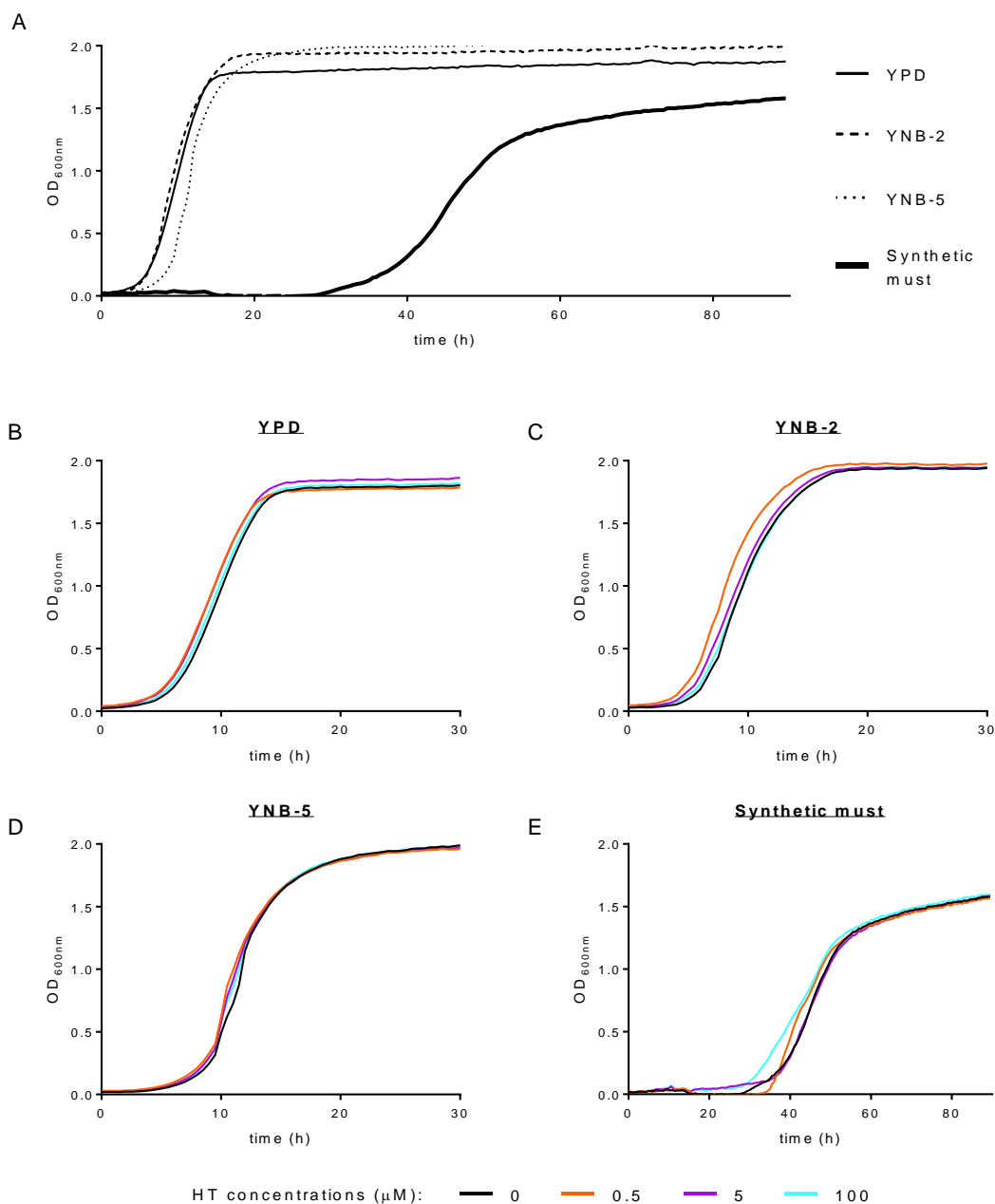
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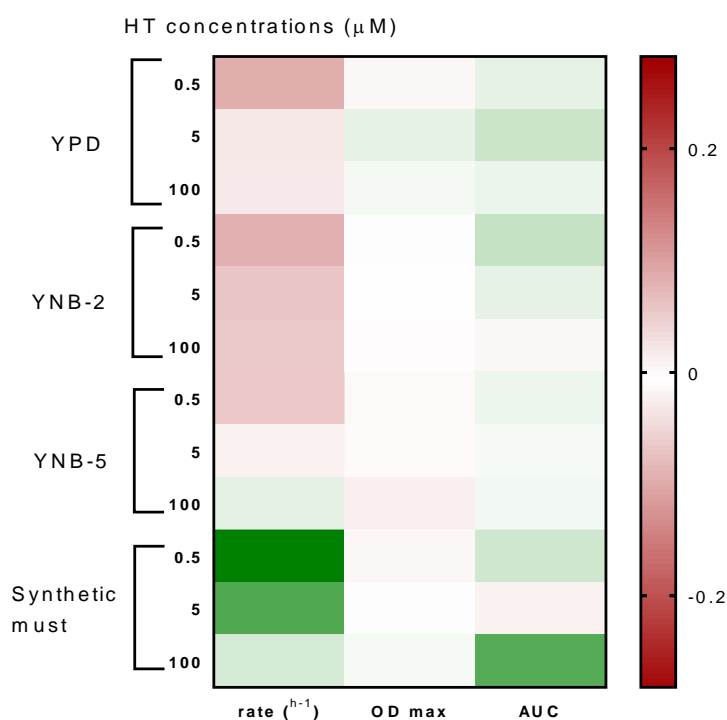**Figure 5.1.** Effect of different hydroxytyrosol (HT) concentrations on the cell growth of *S. cerevisiae* in different media: YPD (**B**), YNB with 2% glucose (YNB-2, **C**), YNB with 5% glucose (YNB-5, **D**) and synthetic must (**E**). In **A**, growth curve of yeast cells without HT supplementation in the different media. HT concentrations: 0 (black) 0.5 (orange), 5 (purple), 100 (blue).

Supplementation with HT (0.5–100 µM) did not show a significant effect on the growth rate or OD max when compared to that of the control condition in the respective medium (Figures 5.1, 5.2). However, a slight decrease in the lag phase was observed in some conditions, which explains the increase in the AUC. Interestingly, the cultures grown with the lowest HT concentration tested (0.5 µM) showed the greatest drop in the growth

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rate in all the media, except in synthetic medium, which exhibited the opposite behaviour (Figures 5.1, 5.2). In this medium, all HT concentrations increased the growth rate and, in some cases, such as 100 µM, the AUC increased due to a shorter lag phase (Figures 5.1, 5.2).

These results showed that HT did not have a toxic effect on yeast growth and that the effect of this compound did not depend on the medium composition. Therefore, to decipher whether HT has a protective role against oxidative stress in yeast, we analysed several indicators of oxidative stress in yeast cells grown in rich medium (YPD). Moreover, to compare the results of this study with those previously reported for melatonin (Vazquez, 2017; 2018), we chose the same HT concentrations as those used in the melatonin studies, 5 µM.

Figure 5.2. Heat map of the effect of different hydroxytyrosol (HT) concentrations on the cell growth of *S. cerevisiae* in different media (YPD, YNB with 2% glucose (YNB-2), YNB with 5% glucose (YNB-5) and synthetic must). The parameters analyzed were growth rate (h^{-1}), maximum OD (OD$_{max}$) and area under the curve (AUC) calculated until 30 h of growth except for synthetic must, for which it was calculated from 30 to 60 h of growth. The color corresponds to the values normalized to the control condition without HT supplementation. Conditions are expressed in rows, and the parameters are presented in columns.

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3.2. Effect of hydroxytyrosol on cell viability under oxidative stress

To evaluate the effect of HT on the viability of *S. cerevisiae* after exposure to oxidative stress, yeast cultures were grown in YPD medium supplemented with 5 µM HT to the early exponential phase and then transferred to fresh media containing 2 mM H_2O_2, and their cell growth was monitored. As in nonstressed cells, the presence of HT in stressed cells had a minimal effect, causing only a slight increase in the OD max (Figure 5.3).
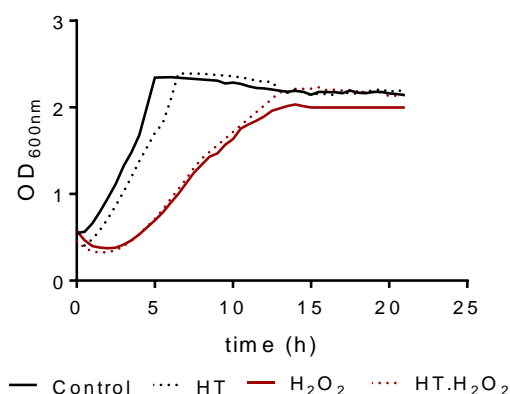
Figure 5.3. Effect of 5 µM hydroxytyrosol (HT) supplementation on the growth of yeast cells exposed to 2 mM H_2O_2. Nonstressed cells (black, Control) and stressed cells (red, H_2O_2) without HT supplementation (continuous line) or with HT supplementation (discontinuous line).

Then, the effect of 5 µM HT on growth recovery was evaluated in *S. cerevisiae* cells exposed to oxidative stress in early exponential and early stationary phases for 1 h and reinoculated in fresh media. The growth recovery in stressed cells was delayed in relation to the nonstressed cells, but this difference diminished when cells were stressed in the stationary phase compared to the exponential phase, mainly due to a delay in the growth recovery in nonstressed cells in stationary phase (Figure 5.4). In nonstressed cells, HT supplementation did not affect cell recovery under any of the conditions. However, supplementation with 5 µM HT significantly affected the recovery of cells exposed to stress during the exponential phase, increasing the OD max and the AUC in cells recovered during the lag phase after H_2O_2 exposure (1 h, Figures 5.4A, S5.1). Additionally, when stress was applied for 1 h to cells in the early stationary phase, supplementation with HT had no significant effect on cell recovery (Figure 5.4B).
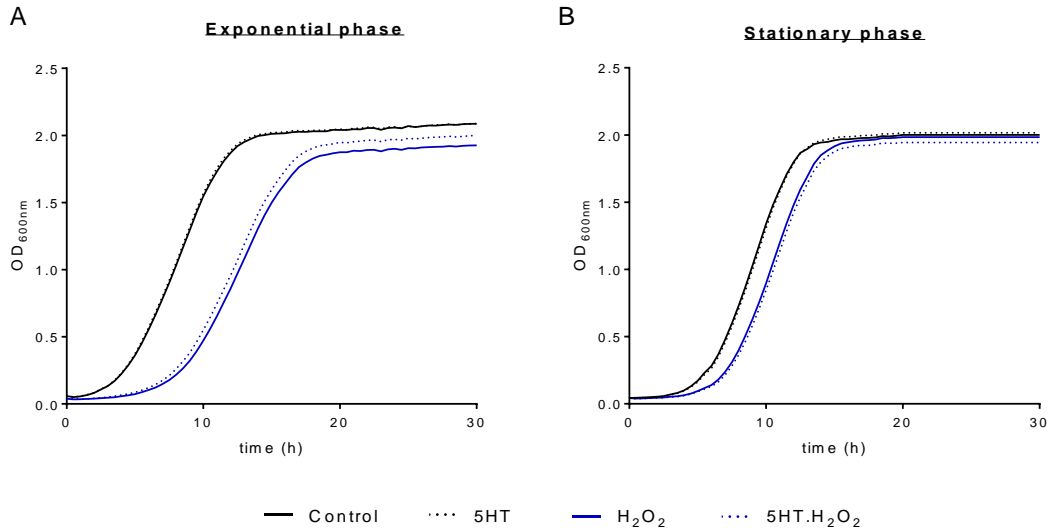
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Figure 5.4. Effect of hydroxytyrosol (HT) supplementation on growth recovery of yeast cells previously exposed to 2 mM H_2O_2 at early exponential (**A**) and early stationary (**B**) phases for 1 h. Nonstressed cells (black) and stressed cells (blue) with HT supplementation: 0 (continuous lines) or 5 (dotted lines) μM.
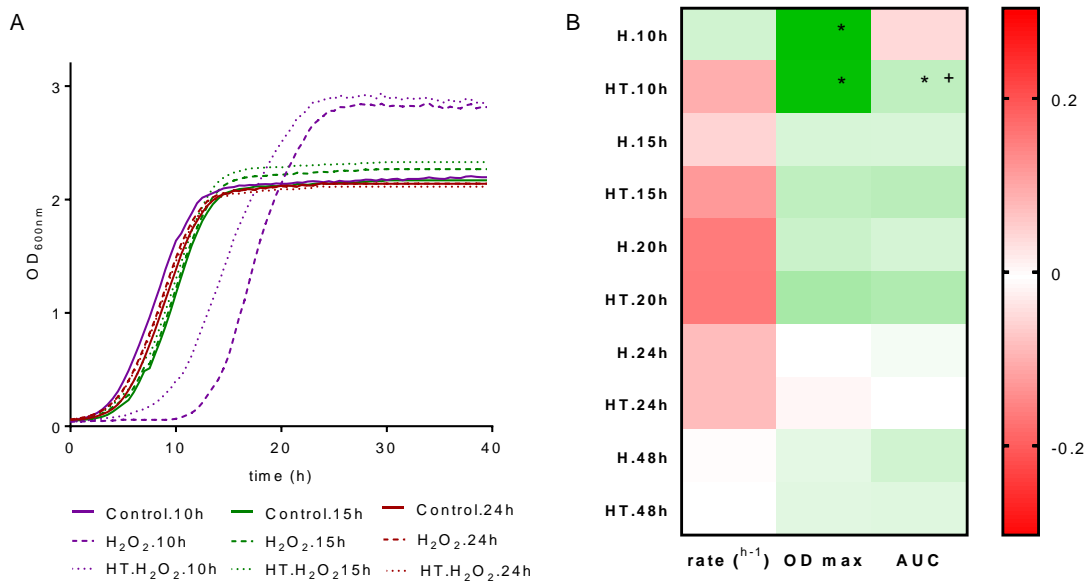

Figure 5.5. Effect of hydroxytyrosol (5 μM, HT) supplementation on the recovery of yeast cells exposed to 2 mM H_2O_2 for different time periods. In (**A**), curves of growth recovery after different periods: 10 h (purple), 15 h (green) and 24 h (red). Nonstressed (continuous line), stressed without HT (discontinuous line) and stressed with HT supplementation (dotted line). In (**B**), heat map representing the parameters of growth rate (h^{-1}), maximum OD (OD_{max}) and area under the curve (AUC) calculated until 40 h of growth. The color corresponds to the values normalized to the control condition without HT supplementation. Conditions are expressed in rows (H means stressed cells and HT, stressed cells with HT supplementation), and the parameters are presented in columns. * indicates significant differences between stressed and nonstressed conditions, and + indicates

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significant differences between stressed conditions without or with HT supplementation (p-value < 0.05).

Subsequently, we monitored the effect of oxidative stress and 5 μM HT supplementation on the growth recovery of cells exposed to H_2O_2 at the early exponential phase and reinoculated in YPD after different times of exposure (at the end of lag phase (10 h) and at the start and end of exponential (15 and 20 h) and stationary (24 and 48 h) phases). Regarding the effect of oxidative stress, a growth delay was observed in cells exposed to H_2O_2 during the lag phase (10 h, Figure S5.1), in which growth was still inhibited due to a significant increase in the lag phase, although, surprisingly, they reached a higher ODmax (Figure 5.5). On the other hand, when cells were exposed for a longer time to H_2O_2 and recovered in exponential (15 h, 20 h of H_2O_2 exposure) or stationary (24 h, 48 h of H_2O_2 exposure) phases, the growth in the new medium was almost identical between stressed and nonstressed cells (Figure 5.5).

Therefore, HT supplementation had a significant impact on the recovery of cells at late lag phase (10 h), improving the growth, with a shorter lag phase and a higher OD max (Figure 5.5), which was similar to the effect reported at early la phase (1 h) after of H_2O_2 exposure (Figures 5.4, 5.5). After longer exposure times to H_2O_2 (15 and 20 h, exponential phase), HT supplementation slightly increased the OD max and the AUC, but not significantly, and no differences were observed when cells were recovered after 24–48 h of exposure (stationary phase) (Figure 5.5).

3.3. Effect of hydroxytyrosol on oxidative stress indicators

Once evidence showed that HT improved cell viability after mild oxidative stress was applied in the exponential phase, we wanted to determine whether HT affected parameters related to this stress, such as ROS accumulation and the degree of lipid peroxidation, as well as the cellular antioxidant response, such as catalase and SOD activities. For that, cultures were grown on YPD in the presence and absence of 5 μM HT, and when cells arrived at early exponential phase, oxidative stress was induced by exposing the cells to H_2O_2 for 1 h, and the abovementioned parameters were analysed. Stressed cells treated with 5 μM melatonin were used as a control.

The results showed that cells exposed to oxidative stress exhibited an increase in total ROS and lipid peroxides (Figure 5.6). A positive effect of HT supplementation was clearly observed since it decreased ROS accumulation and lipid peroxidation in stressed cells, having antioxidant effects similar to those of melatonin.

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Regarding the activities of catalase and SOD, both enzymatic activities increased upon exposure to H_2O_2, but only significantly in the case of catalase. Surprisingly, those activities were further increased when cells were previously incubated with HT, showing an opposite behaviour to that observed for melatonin, which caused a decrease in both activities. However, the changes in SOD activity caused by supplementation with melatonin and HT were nonsignificant (Figure 5.6).
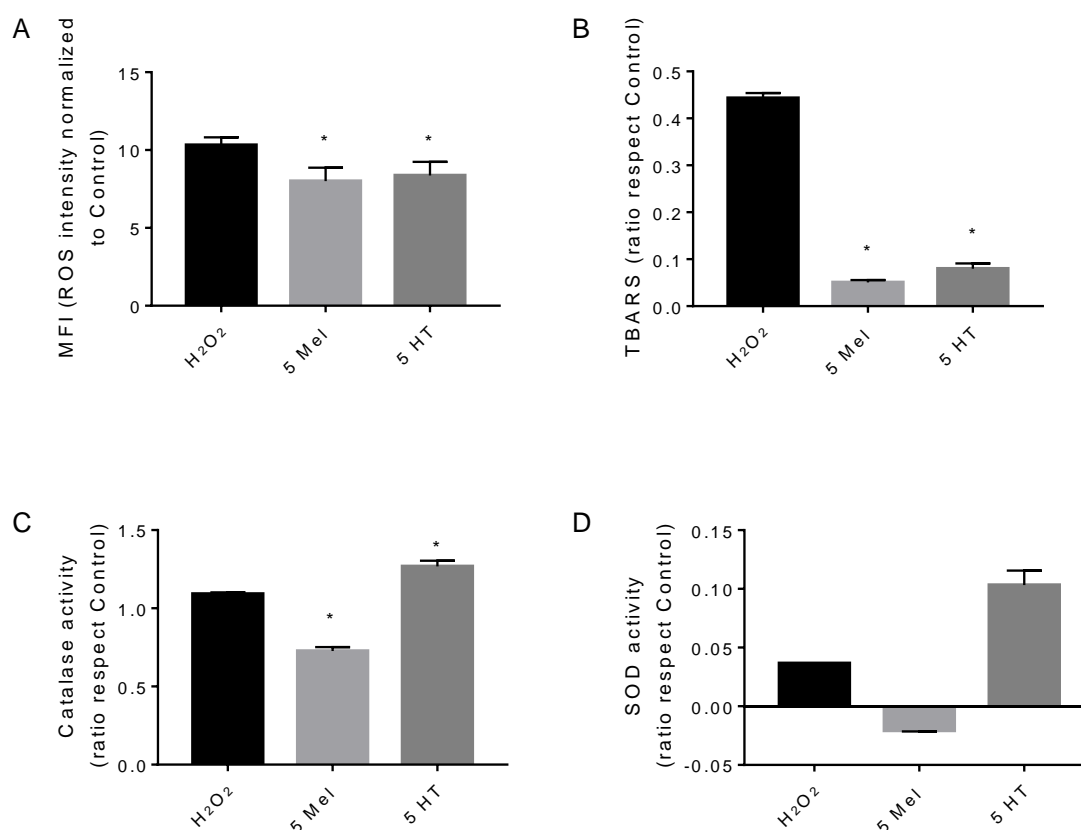

Figure 5.6. Effect of hydroxytyrosol (5 µM, HT) and melatonin (5 µM, Mel) on oxidative stress parameters of *S. cerevisiae* cells exposed to 2 mM H_2O_2 during 1 h: ROS accumulation (**A**), lipid peroxidation (**B**), catalase activity (**C**) and superoxide dismutase (SOD) activity (**D**). Data are expressed as the ratio compared with nonstressed cells in absence of HT or Mel, which is represented by value 0. Error bars represent standard deviation, * indicates significative difference respect to stressed cells without HT nor Mel (p-value < 0.05). ROS stands for reactive oxygen species, and TBARS, for thiobarbituric acid reacting substances.

4. DISCUSSION

HT is synthesized by yeasts during alcoholic fermentation in the winemaking process (Bordiga et al., 2016; Álvarez-Fernández et al., 2018; Rebollo-Romero et al., 2020). This bioactive compound has numerous beneficial properties in mammals, which is why EFSA

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recommends the consumption of food products supplemented with HT (EFSA, 2017). Indeed, the consumption of this compound in a matrix with ethanol (such as wine) seems to promote the endogenous generation of HT, since ethanol interacts with dopamine, which induces the formation of the phenolic alcohol (de la Torre-Ruiz et al., 2015; Karković Marković et al., 2019). Therefore, there is great interest in improving the production of this compound during alcoholic fermentation and unravelling its role and interaction with yeast cells. In fact, although the antioxidant role of HT is well reported in mammals, its role in yeast is unknown. Therefore, this study evaluated the possible effect of HT on the protection of *S. cerevisiae* cells against oxidative stress, considered the wine yeast par excellence. For this, we used the commercial wine strain QA23, which was reported to be more resistant to oxidative stress than laboratory strains of *S. cerevisiae* (Vázquez et al., 2018, 2019).

Exposure of yeast cells at the early exponential phase to H_2O_2 was associated with a loss of viability, as reported elsewhere (Vázquez et al., 2017, 2018; reviewed in Jamieson, 1998; Moradas-Ferreira and Costa, 2000). Therefore, peroxide-stressed cells exhibited lower recovery capacity, mainly during the lag phase. Exogenous H_2O_2 is rapidly consumed inside the cell due to the presence of H_2O_2-removing enzymes, mainly cytosolic catalase and mitochondrial cytochrome *c* peroxidase (Minard and McAlister-Henn, 2001; Morano et al., 2012; Marinho et al., 2013). Therefore, when cells are exposed to external H_2O_2 they activate those defence mechanisms to maintain a proper redox state to adapt to new environmental conditions. Once the cells managed to grow in the presence of H_2O_2 and were in the exponential or stationary phases, the peroxide-stressed cells had a similar recovery to the nonstressed cells, showing that oxidative stress had little effect on cell recovery, probably due to the previous decomposition of external H_2O_2 by antioxidant enzymes (Minard and McAlister-Henn, 2001) adaptation of the cells to oxidative conditions and activation of the environmental stress response. The effect on the recovery was even milder when the cells were exposed to stress during the stationary phase, as reported previously (Vázquez et al., 2017).

Exposure to oxidative stress is well known to generate ROS, which are responsible for a loss of membrane integrity due to the peroxidation of fatty acids (Ayala et al., 2014; Johansson et al., 2016; Vázquez et al., 2017). In the present study, H_2O_2 exposure triggered the accumulation of ROS and lipid peroxidation, so the results are in accordance with previous studies (Vázquez et al., 2017, 2018, 2019). Yeast cells have regulatory molecules

that sense and respond to damage caused by oxidative stress by modulating, among others, the synthesis of enzymes that can detoxify ROS, such as catalase and SOD (Herrero et al., 2008; Ayer et al., 2013). Our results reported an increase in catalase activity after H_2O_2 exposure (also reported in Vázquez et al., 2018), but SOD activity remained unchanged. Catalases are ubiquitous heme-containing enzymes that catalyse the dismutation of H_2O_2 into H_2O and O_2 and have been shown to play an important role in the acquisition of peroxide resistance after being pretreated with low doses of H_2O_2 (Izawa et al., 1996; Morano et al., 2012). On the other hand, SODs convert the superoxide anion to H_2O_2 and are particularly necessary during the stationary phase, which may be linked to superoxide generation from mitochondrial respiration but are less involved in the detoxification of H_2O_2 (Morano et al., 2012). In previous studies, the presence of H_2O_2 triggered the transcription of genes encoding catalases and SODs, but the overexpression was higher for catalases than for SODs (Vázquez et al., 2017), suggesting a different modulation of both enzymes. Moreover, although exposure to H_2O_2 has been reported to increase *de novo* synthesis of these enzymes, the increase or decrease in their activities appears to depend on the yeast strain and oxidant dose, with SOD being more affected and even inactivated at high H_2O_2 concentrations (Bayliak et al., 2006; Semchyshyn and Lozinska, 2012). These authors also reported a positive correlation between the activation of catalase and SOD triggered by H_2O_2 exposure, pointing out that these enzymes are somehow involved in regulating and protecting each other against oxidative stress (Bayliak et al., 2006; Semchyshyn and Lozinska, 2012). Therefore, the absence of an effect of H_2O_2 on SOD activity could be due to the dose of peroxide used or to the specificity of the strain.

The presence of HT in the medium did not affect the growth of cells under fermentative conditions, regardless of the composition of the medium and the glucose concentration (2, 5 or 20%), highlighting that HT, in a concentration range of 0.5–100 µM or even higher (data not shown), was not toxic to *S. cerevisiae* cells under fermentative metabolism. The pretreatment of stressed cells with 5 µM HT did not affect cell growth but improved the cell recovery of cultures exposed to H_2O_2 for a short period of time. In contrast, HT supplementation had no effect on growth recovery after longer exposure times to H_2O_2.

Therefore, we monitored the effect of HT on oxidative stress markers after 1 h of H_2O_2 exposure, mimicking our previous studies on the antioxidant role of melatonin

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(Vázquez et al., 2017, 2018) to compare the effects of both compounds. HT has been described to have ROS scavenging activity (reviewed in Robles-Almazan et al., 2018). This was also observed in our study, since cells treated with HT exhibited lower ROS accumulation and lipid peroxidation. Similar results were previously reported for cells treated with melatonin (Vázquez et al., 2017, 2018), which also improved cell recovery and reduced ROS accumulation and lipid peroxidation in cells exposed to oxidative stress for short periods. Thus, these results seem to suggest an early protective response of HT against oxidative stress activated after 1 h, similar to that observed for melatonin by Vázquez et al. (2017, 2018) and Bisquert et al. (2018). Indeed, the present study shows that both compounds reduce ROS accumulation and lipid peroxidation to the same extent. Moreover, higher HT concentrations (25 µM) conferred the same radical scavenging activity (data not shown), as also reported with higher doses of melatonin (Vázquez et al., 2017), but they did not affect the growth recovery of stressed cells.

Once showed that HT can reduce the oxidative state of the cell through direct antioxidant activities on yeast cells, as previously reported by melatonin in yeast and by both compounds in mammals (Fernández-Mar et al., 2012; Vázquez et al., 2017, 2018), we wanted to decipher whether HT also modulates the endogenous defence systems in yeast, as observed in human cells (Fernández-Mar et al., 2012). HT supplementation increased the catalase activity of stressed yeast cells, the opposite behaviour to that observed after melatonin supplementation, in which when yeast cells exposed to H_2O_2 were pretreated with melatonin, catalase activity decreased (Vázquez et al., 2018). This suggests that the effects of HT and melatonin on the endogenous defence system are different. The increase in catalase activity triggered by HT could contribute to the detoxification of the cells from the presence of H_2O_2. Indeed, previous reports suggest that the antioxidant capacity of melatonin relies on part of its possible pro-oxidant effect, as melatonin induces the accumulation of ROS and the activation of catalase activity in nonstressed cells (Bisquert et al., 2018; Vázquez et al., 2018). In that case, treatment with melatonin seems to activate the antioxidant response prior to stress exposure, conferring resistance to further oxidative stress and reducing the need to activate this response when oxidative stress occurs, particularly catalase and SOD enzymes (Vázquez et al., 2018; Chapter IV of the present thesis). Thus, the results of the present study suggest that HT does not act as a pro-oxidant, but modulates the endogenous response of the cell when oxidative stress occurs. However, more research is needed to decipher which mechanisms are regulated by HT in stressed *S. cerevisiae* cells.

In summary, this study shows that *S. cerevisiae* seems to take advantage of HT supplementation under unfavourable conditions that affect the redox balance, mitigating the toxic effects of H_2O_2 and reducing oxidative damage, which results in a decrease in intracellular ROS content and lipid peroxidation. These results are in accordance with studies in humans, in which HT acts as a direct antioxidant due to its ability to scavenge ROS molecules and, consequently, prevent damage. The effect of HT is also similar to that reported for melatonin in yeast, although the regulation of some indirect antioxidant responses, such as catalase activity, appears to be different between the two compounds. Thus, to the best of our knowledge, this is the first study on the impact of HT supplementation on yeast cells, indicating a beneficial role, which opens the door to deepen its study. Moreover, this study supports the argument of Vázquez et al. (2017) that natural antioxidants could have an importance on biotechnological implications in conferring protective treatments against oxidative damage.

SUPPLEMENTARY MATERIAL
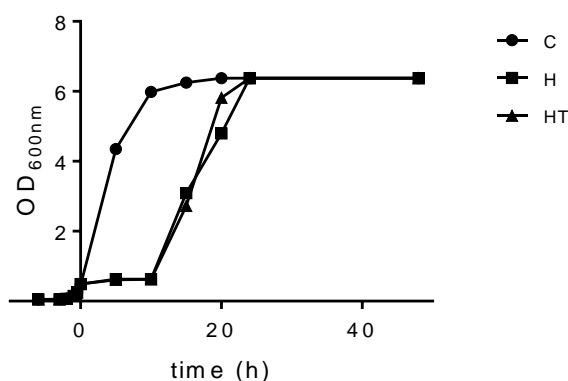
Figure S5.1. Effect of 2 mM H_2O_2 (H) and 5 µM hydroxytyrosol (HT) on the growth curve of *S. cerevisiae* over time. 0 h represents the time in which cells were subjected to hydrogen peroxide stress. Nonstressed cells (C) are represented in circles, and stressed cells are represented in squares (without HT supplementation) and in triangles (with HT supplementation).

GENERAL DISCUSSION

Organisms must cope with constantly changing environmental conditions, which affect the optimal performance of cells and even threaten their survival, so they respond to these stresses by adapting their metabolism and physiology (Hohmann and Mager, 2003; Sommer, 2020). The disruption of cell functions can affect the system to which it belongs, and in the case of human cells, this can cause diseases. In the case of wine yeast, this affecs fermentation performance, causing sluggish, or even stuck fermentations. Therefore, to achieve homeostasis, all organisms govern their activities and modulate their interactions with the environment, which is a process known as the stress response (Hohmann and Mager, 2003). Indeed, there are some molecules that enhance tolerance against these stresses either by neutralizing the agent that produces the stress, reverting the damage caused by this stress or by activating the cellular machinery to confront it. Molecules that protect against oxidative stress, are considered antioxidants. The *Biotecnologia Enològica* group of the *Universitat Rovira i Virgili* was one of the first groups to show that melatonin, a molecule with well-reported protective effects in human cells, basically as an antioxidant, is synthesized during alcoholic fermentation by wine yeasts (Rodriguez-Naranjo et al., 2011a, 2012). This group was also the first to demonstrate that melatonin can act as an antioxidant compound in cells of *Saccharomyces* and non-*Saccharomyces* wine yeasts (Vázquez et al., 2017, 2018).

After those first steps, the relationship of melatonin with alcoholic fermentation has been widely investigated, as reviewed by Muñiz-Calvo et al. (2020a). In brief, melatonin is synthesized by *Saccharomyces* and non-*Saccharomyces* yeasts during alcoholic fermentation and by the lactic acid bacteria *Oenococcus oeni* during malolactic fermentation (Vigentini et al., 2015; Fernández-Cruz et al., 2017, 2018, 2019; Valera et al., 2019; Vilela, 2019; Fracassetti et al., 2020). Although the synthesis pathway in yeast is not yet known (Muñiz-Calvo et al., 2019), there is intense research about the conditions that trigger the production of melatonin. Moreover, apart from its antioxidant role, Bisquert et al. (2018) reported that melatonin also protects *Saccharomyces cerevisiae* against other stresses, such as UV radiation. Another bioactive compound also synthesized by yeast during alcoholic fermentation is hydroxytyrosol (HT). This compound is accepted to be produced as a secondary metabolite from tyrosine during alcoholic fermentation (Dickinson et al., 2003; Álvarez-Fernández et al., 2018) and to have broad beneficial effects on human health (Fernández-Mar et al., 2012; Bordiga et al., 2016; Álvarez-

GENERAL DISCUSSION

Fernández et al., 2018; Gallardo-Fernández et al., 2019; Karković Marković et al., 2019; Rebollo-Romero et al., 2020), but its role in yeast cells is still unknown.

To better understand the conditions that favour the production of these compounds, it is important to decipher why yeasts produce them, since the reason for their production could be related to the advantage that their presence provides to yeast performance. Therefore, in this thesis, we wanted to validate the hypothesis that melatonin and HT have protective functions in *S. cerevisiae* against stresses associated with alcoholic fermentation. To interpret the effects of these molecules on yeast metabolism, we studied the effects of the stresses on *S. cerevisiae* yeast cells prior to determining the effect of melatonin or HT.

MECHANISMS OF MELATONIN PROTECTION AGAINST OXIDATIVE STRESS IN *S. cerevisiae*

Melatonin supplementation protects *S. cerevisiae* yeast cells against oxidative stress (Vázquez et al., 2017, 2018; Bisquert et al., 2018). The physiological changes that melatonin induces, together with the way yeast cells respond to oxidative stress, suggest that melatonin might be involved in multiple biological processes, acting as a signalling molecule that triggers molecular or physiological responses to cope with stress situations. Therefore, with the aim of deciphering the molecular mechanisms by which melatonin interferes with oxidative stress, in this thesis, we described for the first time the yeast transcriptional response in the presence and absence of exogenous melatonin and oxidative stress.

The intracellular melatonin levels increased significantly when *S. cerevisiae* QA23 cells were grown in the presence of melatonin, and the concentration inside the cell were proportional to the concentration added to the medium (in the range of 0.1 − 50 µM, results not shown), indicating that yeast cells were able to internalize exogenous melatonin at nanomolar concentrations. Moreover, the internalized concentration increased when cells were exposed to oxidative stress, which could be due to changes induced by H_2O_2 in plasma membrane permeability, as reported previously (Vázquez et al., 2018, 2019), suggesting that melatonin could cross membranes by passive diffusion. In mammalian cells, melatonin is believed to cross membranes by passive diffusion and also through transporters of glucose or oligopeptides (Hevia et al., 2015; Huo et al., 2017; Mayo et al., 2018). The present thesis reports that melatonin supplementation (5 µM) in

S. cerevisiae induces the expression of genes encoding some membrane transporters, such as those for urea or polyamines (which have a similar structure to that of melatonin) (*DUR3*), oligopeptides (*OPT1*, *OPT2*, *PTR2*) or carbohydrate and carbon sources (*HXT5*, *HXT6*, *HUT1*, *JEN1*), which suggests that in yeast cells, these membrane transporters could be related to melatonin internalization. However, further studies should be performed to elucidate the possible role of these transporters in melatonin uptake by yeast cells. Unexpectedly, supplementation with 5, 25 or 50 µM melatonin showed little difference in the response of cells exposed to H_2O_2, which also occurred when cells were exposed to ethanol stress.

The internalization of melatonin by yeast cells suggests that it could modulate cellular transcriptional responses, as reported in Vázquez et al. (2017) and in Bisquert et al. (2018). Moreover, the transcriptional study performed showed that, as expected, in stressed cells, melatonin upregulated a great number of genes involved in antioxidant function and detoxification, which was likely, in part, caused by modulating the activity of the transcription factor Cin5p, a member of the Yap family. In contrast, in nonstressed cells, melatonin downregulated several genes of the antioxidant response, supporting the hypothesis that in the absence of stress, melatonin could have a prooxidant role to prepare cells to cope with further stresses, and in the presence of oxidative stress, melatonin induces antioxidant defence mechanisms (Vázquez et al., 2017, 2018; Bisquert et al., 2018). Furthermore, the present thesis suggests that melatonin also activates a response against the toxic effects of metal exposure, to better endure future stresses, as reported in human cells (Alonso-Gonzalez et al., 2008; Romero et al., 2014).

Lipid metabolism genes were also highly modulated by melatonin supplementation, which was in accordance with the changes in yeast lipid composition observed in previous results in the presence of melatonin (Vázquez et al., 2018). The present study suggests that melatonin could confer resistance against H_2O_2 to yeast cells by altering their ergosterol and fatty acid composition, which was observed both at the transcriptional level (by changes in the expression of the genes involved in those pathways) and at the physiological level, by increasing the proportion of unsaturated fatty acids (UFAs) to saturated fatty acids (SFAs). These changes in lipid composition may improve tolerance to oxidative stress (Vázquez et al., 2019), as observed in tolerance to low temperatures or ethanol stress, in which more fluid membranes (higher UFA/SFA ratio) confer higher stress tolerance to yeast cells (Torija et al., 2003; Beltran et al., 2008; Lairón-Peris et al., 2021).

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GENERAL DISCUSSION

The families of genes that were more enriched by melatonin supplementation were those related to the mitochondria, especially the respiratory chain complexes, mitochondrial envelope and mitochondria protection. These data were similar to those observed in respiring yeast cells (Zampol and Barros, 2018) and in human cells (Venegas et al., 2012; Tan et al., 2016; Hardeland, 2017; Reiter et al., 2017, 2020), in which melatonin plays a very important role in mitochondria. In fact, the principal target of melatonin in human cells is suggested to be the mitochondrial electron transport chain (ETC), since it is the main site of reactive oxygen species (ROS) production, so where the antioxidant effect of melatonin is more needed (Hardeland, 2017). Therefore, we first studied the effect of exogenous H_2O_2 exposure on the mitochondria of *S. cerevisiae* cells grown under fermentative conditions and then the effect of melatonin on this organelle by analysing both transcriptomic and physiological responses. Our results showed that exposure to H_2O_2 affects mitochondrial functionality, since this stress increased mitochondrial mass, decreased oxygen consumption, and modulated the expression of several mitochondrial genes, which were mainly involved in mitochondrial antioxidant systems, ETC complexes or mitochondrial envelopes. Therefore, we studied in depth the physiological effects of this oxidative stress on these mitochondrial components.

Oxidative stress had an impact on the composition of mitochondrial membranes by decreasing the levels of cardiolipin, which is considered the signature lipid of mitochondrial membranes, and on mitochondrial physiology by inducing mitochondrial fission, which leads to a fragmented mitochondrial network, as previously reported for yeast cells exposed to different stresses (Fannjiang et al., 2004; Kitagaki et al., 2007; Westermann, 2010). In general, oxidative stress upregulated the expression of genes involved in complex III (cytochrome *c* reductase) of the ETC and downregulated those involved in complex IV (cytochrome *c* oxidase). Nevertheless, although these transcriptomic results were not correlated with changes in protein levels, an important decrease in complex IV activity was observed in the mitochondrial extracts, accompanied by a lower accumulation of a central protein of this complex, Cox1p. This suggests that the decrease in oxygen consumption induced by oxidative stress may be related to selective damage to complex IV of the ETC, which could disturb proton pumping through the inner mitochondrial membrane, also supported by the decrease in membrane potential triggered by oxidative stress. Therefore, these results seem to point towards a relationship between the decrease in complex IV activity, the decrease in membrane potential and mitochondrial fragmentation, as already reported in other studies

(Böttinger et al., 2012; Strogolova et al., 2019; Venditti and Di Meo, 2020). However, this connection is not fully understood, and fragmented mitochondria could also result from fission and mitophagy pathways activated for dysfunctional mitochondrial clearance, to prevent ROS accumulation and cell damage, thus maintaining the quality of cellular mitochondria.

On the other hand, melatonin supplementation (5–50 μM) caused a slight increase in mitochondrial mass, and this increase was higher when oxidative stress was stronger (from 0 to 5 mM H_2O_2), but did not modify oxygen consumption. Moreover, melatonin supplementation affected the expression of a great number of ETC-related genes. In nonstressed cells, the presence of melatonin mainly repressed complex III genes, a behaviour that supported the proposed prooxidant effect, since the function of this complex is pivotal to maintain a proper redox state. In human cells, malfunction of this complex can cause electron leakage, which is related to ROS production, and in yeast cells, complex III plays an important role in ROS detoxification (Wang et al., 2014b; Hardeland, 2017). However, Hardeland (2017) pointed out that in mammals, the activity of complex III could be either upregulated or unchanged by melatonin depending on the cell type, and the latter case is the one observed in this study for yeast cells under fermentative conditions. Complex IV activity was mainly upregulated by melatonin in humans and yeasts under respiratory conditions (both in the presence and absence of stress), so melatonin was proposed to contribute to the maintenance of respiratory chain flux, counteracting the loss of electrons, which is the main cause of oxidative stress in the cell (Martín et al., 2000a; Acuña-Castroviejo et al., 2003; Petrosillo et al., 2013; Xia et al., 2019). In yeast that was grown on fermentable carbon sources and exposed to oxidative stress, melatonin mainly upregulated complex IV genes but did not alter this complex activity. The results suggest that under these conditions, melatonin did not help counteract the loss of electrons in the ETC provoked by H_2O_2 exposure. These data were supported by the lack of alterations in membrane potential. Therefore, melatonin in yeast cells appears to have a specific benefit on the ETC in respiratory conditions (Zampol and Barros, 2018) but not in fermentative metabolism, since this effect is only observed when the respiratory chain is the principal source of energy in the form of ATP. Thus, this study demonstrated that the broad protective effects of melatonin against oxidative stress reported in yeast under fermentative conditions did not seem to be due to its effect on the respiratory chain.

Melatonin modulated the mitochondrial membrane composition by increasing the cardiolipin content, as previously described in humans (Cardinali and Vigo, 2017). Moreover, in nonstressed cells, melatonin triggered the fission of mitochondria, inversely to the behaviour reported in human cells (Tan et al., 2016; Reiter et al., 2018). Therefore, these data supported its possible prooxidant role in yeast because mitochondrial fission is normally triggered by stressful conditions, as reported above for oxidative stress. Thus, our results suggest that the protective mechanisms that trigger melatonin in yeast under respiratory or fermentative metabolism are different, and the latter does not seem to be closely related to the effects of oxidative stress on mitochondrial performance.

Several studies have suggested that melatonin possibly has a signalling role in yeast cells (Rodriguez-Naranjo et al., 2012; Morcillo-Parra et al., 2019b, 2020b). In fact, it has been proposed that the intracellular production of melatonin during the lag phase of yeast growth is a signal for yeast cells to adapt to new conditions (Rodriguez-Naranjo et al., 2012; Morcillo-Parra et al., 2020b). Moreover, melatonin has recently been reported to bind glycolytic proteins but only in Crabtree-positive yeasts, suggesting a role as a signal molecule for this glycolytic complex, which seems to be dependent on the yeast fermentative capacity (Morcillo-Parra et al., 2019b, 2020b). On the other hand, our transcriptomic results pointed to the activation by melatonin, in nonstressed cells, of a hypoxia-like response regulated by the Hap complex through the transcription factors Hap1p and Rox1p and the complex Hap2/3/4/5. In fact, several genes related to anaerobic or aerobic conditions were up- or downregulated after melatonin supplementation, respectively. Moreover, our transcriptomic results also suggest that melatonin could modify the activity of the transcription factor Cin5p, which in turn is regulated by oxidative and osmotic stress and is also under the control of Msn2/4p, the transcription factor of the environmental stress response. Therefore, all these results seem to point towards a role of melatonin related to fermentation performance, perhaps through the abovementioned binding to glycolytic proteins or by conferring resistance against some stresses associated with fermentation, such as osmotic or ethanol stress. As we discussed in the Introduction, there is considerable overlap between yeast responses to oxidative stress and other fermentation-related stresses.

ROLE OF MELATONIN AGAINST FERMENTATION-ASSOCIATED STRESSES

Melatonin has previously been reported to affect the growth of *S. cerevisiae* cells, but the effect was higher on cells exposed to stress, such as oxidative stress, induced by H_2O_2 and UV radiation (Vázquez et al., 2017, 2018; Bisquert et al., 2018). Moreover, the production of melatonin depended on exogenous conditions, such as sugar concentration and temperature (Morcillo-Parra et al., 2020a). Therefore, we assessed the effects of different stresses that yeast could encounter during alcoholic fermentation on the growth of *S. cerevisiae* in the presence or absence of melatonin. We observed that exposure of *S. cerevisiae* yeast cells to osmotic, acid, ethanol or oxidative stress provoked growth impairment, which mainly delayed cell growth. Indeed, this delay was higher with the increase of the stressor concentration until concentrations that totally inhibited the growth (pH 2, 14% ethanol, 500 g/L glucose). The different osmolytes used for osmotic stress, such as sugar (glucose), salt (NaCl) and sorbitol, had different effects on *S. cerevisiae* growth, as reported by Gomar-Alba et al. (2015). This was also observed when different oxidants were used (such as menadione, H_2O_2, paraquat or plumbagin) (data not shown) (Osorio et al., 2003; Todorova et al., 2009) or when acid stress was triggered by strong inorganic or weak organic acids (Takagi and Kitagaki, 2015).

In fact, the present thesis focused on the effect of H_2O_2 (2 mM) and ethanol (8%) on several oxidative stress indicators. The effect of these stresses on yeast cells is reported to be similar, as one of the principal damaging mechanisms of ethanol is the accumulation of ROS, causing oxidative stress (Ma and Liu, 2010a; Bleoanca et al., 2013). Indeed, we observed that both stresses generated an increase in ROS levels, which caused lipid peroxidation in the membranes, supporting the idea that ethanol triggers oxidative stress, as previously reported (Du and Takagi, 2007; Pérez-Gallardo et al., 2013; Vázquez et al., 2018, 2019). However, the increase in ROS and lipid peroxidation levels was higher when the stressor was H_2O_2 but more gradual when the stressor was ethanol. This difference could be because H_2O_2 is a reactive species itself, but during ethanol exposure, ROS are endogenously produced, so H_2O_2 could produce oxidative damage faster than ethanol stress. After ethanol exposure, ROS accumulation reached a peak in the early exponential phase, followed by a decrease in its levels, while lipid peroxidation reached the maximum levels in the mid-exponential phase and was maintained overtime. This decrease in ROS concentration after the peak in the early exponential phase could be explained by two mechanisms. On the one hand, high levels of ROS can stimulate mitophagy, leading to the

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clearance of damaged mitochondria; since ROS are mainly produced in mitochondria after ethanol exposure, their elimination contributes to a decrease in ROS (Jing et al., 2020). On the other hand, exposure to ethanol could trigger the activity of antioxidant defence systems, which contribute to ROS clearance.

Indeed, in this study, exposure to ethanol activated the activity of catalase and superoxide dismutase (SOD) enzymes. In the case of catalase, which detoxifies H_2O_2 decomposing it to H_2O (Jamieson, 1998; Herrero et al., 2008), its activity profile was similar in response to ethanol and oxidative stress (reported in Vázquez et al. (2018) for oxidative stress); however, for SOD activity, which catalyses the dismutation of the superoxide anion in O_2 and H_2O_2, the increase was significant in response to ethanol stress, but not to oxidative stress, although the genes encoding both enzymes were upregulated by oxidative stress in Vázquez et al. (2017). Therefore, these results indicate that cell responses to oxidative and ethanol stress have some different traits, as reported previously (Bleoanca, 2013; Stanley, 2010).

In addition, this study on the effect of oxidative and ethanol stress was carried out with two strains, the commercial wine strain QA23 and the lab strain BY4743. The wine strain presented a better tolerance to high concentrations of ethanol and oxidative stress than the lab strain, confirmed by its greater ability to grow and survive in the presence of stress. In nonstressed cells, the lab strain exhibited lower catalase activity, higher ROS accumulation and lower levels of mitochondrial ETC complexes III and IV activities, suggesting that it could be less prepared to face exogenous stresses. The presence of ethanol induced higher ROS accumulation and increased more the catalase activity in this strain compared to QA23 strain, but lipid peroxidation was the same in both strains, which was a behaviour also reported for oxidative stress in Vázquez et al. (2018). Moreover, oxidative stress also affected complex IV of the mitochondrial ETC to a greater extent in the lab strain: it was almost completely inhibited. Therefore, all these data suggest that the commercial wine strain was better adapted to withstand these stresses, as also reported in Pais et al. (2013), Vázquez et al. (2018) and Lairón-Peris et al. (2021).

Once the effects of fermentation-associated stresses on the growth of *S. cerevisiae* cells were determined, we studied the potential beneficial role of melatonin supplementation against them. Thus, we showed that melatonin pretreatment had no effect on the growth of cells exposed to osmotic and acid stress and clearly increased the growth, recovery and viability of cells exposed to ethanol stress. Therefore, we studied

whether melatonin attenuates the oxidative damage induced by ethanol exposure and evaluated which mechanisms are most affected by this molecule. Cells grown with melatonin reduced the oxidative damage caused by ethanol by decreasing ROS accumulation and lipid peroxidation, similar to the effect reported in yeast cells exposed to oxidative stress (Vázquez et al., 2017, 2018; Bisquert et al., 2018). Furthermore, melatonin generally modulated cellular antioxidant activities, although the effects were different depending on the strain and the growth phase. After 2 h of ethanol exposure, the presence of melatonin levelled the increase in catalase activity provoked by ethanol, which was an effect that was significant only in the lab strain, mimicking the effect of melatonin in both strains after H_2O_2 stress. In the wine strain, the presence of melatonin levelled the increase in SOD activity triggered by ethanol but had no impact when subjected to oxidative stress, which was in accordance with the absence of change in *SOD1* and *SOD2* gene expression reported after 1 h of H_2O_2 exposure (Vázquez et al., 2017; Bisquert et al., 2018). Therefore, the presence of melatonin during alcoholic fermentation can enhance the alcohol tolerance of yeasts, thereby improving their performance.

EFFECT OF HYDROXYTYROSOL ON *S. CEREVISIAE*

Although the antioxidant role of HT in mammals is well reported, the physiological role of HT in yeast cells is still unknown (Robles-Almazan et al., 2018; Karković Marković et al., 2019; Bertelli et al., 2020). In this study, we reported for the first time that the presence of HT in the range of 0.5–100 µM was not toxic to *S. cerevisiae* cells. On the other hand, although HT supplementation (5 µM) did not affect the growth of cells exposed to mild oxidative stress, it improved their recovery after being exposed to H_2O_2 (2 mM) for short periods of time, mimicking the effect reported by melatonin in Vázquez et al. (2018). HT also had the same effect as melatonin in lowering ROS and lipid peroxidation levels induced by exposure to H_2O_2, suggesting a radical scavenging role, as previously reported for HT in mammals and for melatonin in both organisms (Tripoli et al., 2005; Galano et al., 2011; Fernández-Mar et al., 2012; Manchester et al., 2015; Vázquez et al., 2017; Robles-Almazan et al., 2018). Furthermore, HT also showed an effect on catalase activity, but opposite to that observed in melatonin, since HT increased its activity and melatonin decreased it, indicating that both molecules seem to involve different molecular mechanisms for the antioxidant response. Therefore, *S. cerevisiae* seems to take advantage of HT supplementation under unfavourable conditions that affect the redox balance, suggesting that this compound, together with melatonin, has antioxidant

mechanisms in both human and yeast cells. Moreover, this compound levelled the decrease in cell growth provoked by ethanol stress (10%, results not shown), which opens a promising field of study in analysing the role of this molecule as a protection against the most relevant stresses encountered during alcoholic fermentation, as proposed in the case of melatonin.

In summary, regarding the protective effects of melatonin in yeast, we were able to confirm that the previously reported antioxidant effects had a molecular basis, but this protective effect did not seem to be related to modifications in mitochondrial functionality. Moreover, the addition of exogenous melatonin enables cells to better resist ethanol stress, which plays a pivotal role in alcoholic fermentation. On the other hand, HT, another compound derived from an aromatic amino acid and produced by yeast during alcoholic fermentation, also exhibited antioxidant properties in yeast. Nevertheless, more research is needed to explore the beneficial effect of these bioactive compounds on yeast performance during alcoholic fermentation.

Therefore, the results shown in this thesis support our initial hypothesis, mainly in the case of melatonin, since its antioxidant properties help *S. cerevisiae* cells cope with oxidative and ethanol stresses, which is crucial for ensuring a good performance during alcoholic fermentation.

GENERAL CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
PROTECTIVE EFFECTS OF BIOACTIVE COMPOUNDS AGAINST FERMENTATION-ASSOCIATED STRESSES IN SACCHAROMYCES CEREVISIAE
Mercè Sunyer Figueres

GENERAL CONCLUSIONS

1. *S. cerevisiae* cells can incorporate exogenous melatonin, which affects gene expression levels and modulates the regulation of specific and general stress responses.

   • In the absence of oxidative stress, melatonin triggers a hypoxia-like response and prepares yeast cells to cope with stress, which could enhance the fermentation performance. Melatonin also downregulates genes related to the mitochondrial ETC.

   • Under oxidative stress, melatonin upregulates genes related to antioxidant activity and mitochondria, especially the cytochrome complex and electron transport chain.

2. Melatonin regulates the expression of genes related to lipid metabolism, as well as membrane lipid composition, which could confer *S. cerevisiae* cells tolerance against H_2O_2 by mainly increasing the unsaturated fatty acids/saturated fatty acids ratio and altering the ergosterol composition.

3. Oxidative stress exerts damaging effects on the mitochondria of *S. cerevisiae* under fermentative conditions by reducing oxygen consumption, disrupting the electron transport chain and mitochondrial membrane potential, and causing mitochondrial fragmentation.

4. Melatonin modifies the expression of genes related to the functioning and maintenance of mitochondria but does not affect the functioning of the respiratory chain. Therefore, mitochondria do not appear to play a central role in the pathways regulated by melatonin to confer protection to *S. cerevisiae* cells against oxidative stress under fermentative conditions.

5. Ethanol and H_2O_2 exposure cause similar effects in yeast cells, increasing ROS production and lipid peroxidation and triggering the activation of catalase enzyme. Furthermore, ethanol also modifies superoxide dismutase activity.

6. Yeast cells grown with melatonin are better prepared to withstand ethanol stress but not osmotic or acidic stresses.

7. The antioxidant capacity of melatonin attenuates the damage caused by ethanol stress in *S. cerevisiae* cells by reducing ROS and lipid peroxidation and by modulating the activity of antioxidant enzymes, such as catalase or superoxide dismutase.

8. Under oxidative stress, hydroxytyrosol exerts antioxidant properties in yeast and reduces the damage produced by oxidative stress by decreasing intracellular ROS and lipid peroxidation, which enhances yeast viability and increases catalase activity to a greater extent than oxidative stress.

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ANNEX I: OTHER ARTICLES

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Short communication

Analysis of ribosomal RNA stability in dead cells of wine yeast by quantitative PCR

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ARTICLE INFO

ABSTRACT

During wine production, some yeasts enter a Viable But Not Culturable (VBNC) state, which may influence the quality and stability of the final wine through remnant metabolic activity or by resuscitation. Culture-independent techniques are used for obtaining an accurate estimation of the number of live cells, and quantitative PCR could be the most accurate technique. As a marker of cell viability, rRNA was evaluated by analyzing its stability in dead cells. The species-specific stability of rRNA was tested in Saccharomyces cerevisiae, as well as in three species of non-Saccharomyces yeast (Hanseniaspora uvarum, Torulaspora delbrueckii and Starmerella bacillaris). High temperature and antimicrobial dimethyl dicarbonate (DMDC) treatments were efficient in lysing the yeast cells. rRNA gene and rRNA (as cDNA) were analyzed over 48 h after cell lysis by quantitative PCR. The results confirmed the stability of rRNA for 48 h after the cell lysis treatments. To sum up, rRNA may not be a good marker of cell viability in the wine yeasts that were tested.

1. Introduction

Microorganisms are the main actors in wine industry. Consequently, microbial analyses are critical for understanding fermentation process, for detecting spoilage microorganisms, and for further controlling or improving wine quality (Mills et al., 2002). In the last century, culture-independent methods have been developed to detect and quantify the main microorganisms (Cocolin et al., 2013; Hierro et al., 2006). However, it is an issue to consider whether these methods could accurately detect viable microorganisms without including dead cells.

During wine fermentation, microorganisms undergo a series of stresses (decreases in nutrients, the appearance of toxic new compounds such as ethanol, and interactions with other microorganisms) that challenge the viability of yeast cells (Wang et al., 2016). Due to these challenges, live cells can exist in different states; it is important to detect and quantify all live cells, because they can affect the progress of alcoholic fermentation and the final wine quality (Fleet, 2008). Specifically, the Viable But Not Culturable (VBNC) state has been defined as metabolically active cells that cannot undergo cellular division in growth medium (Oliver, 1993). But VBNC cells are included in viable cells because they have the potential of resuming growth and achieving full metabolic activity, which can affect the wine-making process. In wine, the presence of cells in the VBNC state has been attributed to the use of fungistatic and bacteriostatic compounds, such as SO$_2$ (Divol and

Lonvaud-Funel, 2005), as well as to the interactions between different wine yeasts (Wang et al., 2016). After removing the two factors by resuscitation assays, VBNC yeast cells can recover to the normal state (Salma et al., 2013; Wang et al., 2016). Therefore, VBNC state can be considered as a transition state of yeast from culturable cells to dead cells (Branco et al., 2015; Wang et al., 2016). During this transition process, yeast cells lose the ability to form colonies, and the permeability of cell membrane change as well as the intracellular pH (Andorrà et al., 2010; Branco et al., 2015; Wang et al., 2015). Therefore, due to the lack of growth of VBNC cells and dead cells, how to distinguish them without underestimating or overestimating dead cells is the question. It cannot be realized by traditional culture-dependent methods without resuscitation; the only alternative is to find culture-independent methods that include VBNC cells and exclude dead cells when quantifying the total viable population. These types of techniques can detect cells despite their growth abilities, and they are faster, more sensitive, and more accurate (Cocolin et al., 2013). Therefore, these culture-independent methods can be used as a tool to better understand the true microbial diversity and allowing the accurate study of microbial populations (Cocolin et al., 2013). Since 2000, several culture-independent techniques have been used to monitor wine fermentation; all of these techniques target genetic material. It was found that some wine yeasts not recovered on culture plates were detected when using culture-independent techniques (Cocolin and Mills, 2000). This opened a

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P. Varona Rubio

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