ANTIOXIDANT EFFECT OF MELATONIN ON SACCHAROMYCES AND NON-SACCHAROMYCES WINE YEASTS

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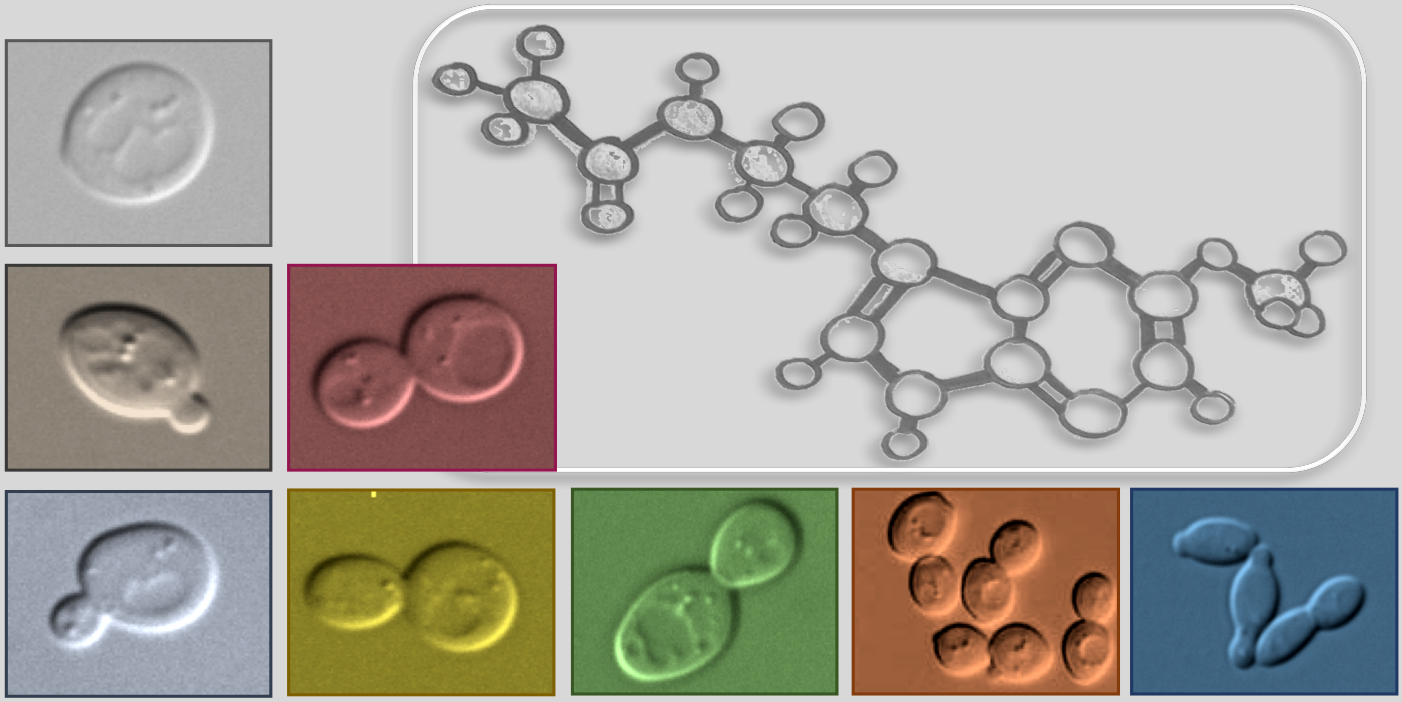

DOCTORAL THESIS
2017

Jennifer Vázquez González

“Antioxidant effect of melatonin on *Saccharomyces* and non-*Saccharomyces* wine yeasts”

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WE STATE,

That the present study, entitled "**Antioxidant effect of melatonin on *Saccharomyces* and non-*Saccharomyces* wine yeasts**", presented by **Jennifer Vázquez González** for award of the degree of Doctor with International Mention, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university.

Tarragona, 4th September 2017

Dr. Mª Jesús Torija Martínez

Dr. Gemma Beltran Casellas

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Aunque parezca un tópico, soy consciente y con la madurez cada vez más, que amigos se cuentan con los dedos de la mano. En este aspecto me siento muy afortunada pues a Tarragona me vine con un saquito lleno de las mejores. Con nuestros defectos y virtudes, parece ser que esto ya es inquebrantable. Saber que cuando es necesario estamos, me lleva a la verdadera definición del valor de la amistad, a ponerle nombre propio: Anna, Carmen, Carol, Cristina, Elena, Nerea ¡Gracias por vuestros ánimos en esta etapa!

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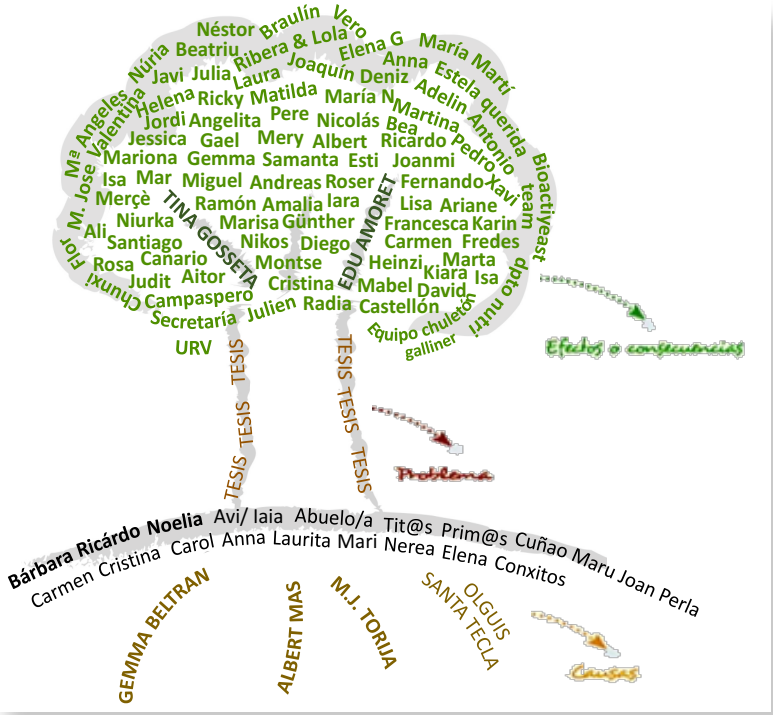

Figura 1. Árbol de causas y efectos de la tesis doctoral. A todos, ¡GRACIAS!

Dedicada a mis padres y a mi hermana,

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OBJECTIVES AND OUTLINE OF THESIS

The aim of the present work was to obtain a PhD degree, and it was performed from 2013 to 2017 in the Oenological Biotechnology research group, at the Department of Biochemistry and Biotechnology in the Faculty of Oenology at Rovira i Virgili University (URV). During this period, I held a grant 2013PMF-PIPF-09 from the Martí Franquès program (URV).

This thesis was created within the framework of the BIOACTIYEAST project entitled "Production and physiological effects of bioactive compounds derived from aromatic amino acids in yeast populations", which has given continuity to the existing SYNBIOFERM project entitled "Metabolism and protective effects of indole compounds in yeasts of food interest". Both projects have been funded by the Spanish Ministry of Economy and Competitiveness (AGL2013-47300-C3-1-R and AGL2016-77505-C3-3-R, respectively). The primary goal of these two projects was to improve our knowledge about the synthesis of these bioactive molecules by yeasts during alcoholic fermentation by studying the effects and production of these bioactive compounds, with special attention to melatonin. This molecule modulates circadian rhythms and exerts multiple pleiotropic functions in humans. One of these functions is a powerful antioxidant activity through which melatonin protects against oxidative stress by acting as a direct scavenger to detoxify free radicals or indirectly by increasing the activities of endogenous cellular antioxidant defenses. Melatonin has recently been detected in wine, and as is the case for other indolic compounds, it seems to come mostly from the tryptophan metabolism of the yeasts that participate in the alcoholic fermentation. However, neither its physiological effects nor the metabolic reason for its synthesis by yeast is completely understood.

Therefore, the working hypothesis of the present thesis was as follows: **Melatonin acts as an antioxidant compound in both *Saccharomyces* and non-*Saccharomyces* yeasts, reducing the oxidative stress damage.**

To test this hypothesis, the general objective was to study the possible role of melatonin against oxidative stress in different yeast species with food or oenological interest as well as the molecular mechanisms involved in this response. To achieve this general aim, three specific objectives were developed:

<u>Objective 1:</u> Analysis of tolerance to oxidative stress in *Saccharomyces* and non-*Saccharomyces yeasts*. (Chapters 1 and 3)

To complete this objective, the yeast response to oxidative stress (as induced with hydrogen peroxide, H_2O_2) was evaluated in *S. cerevisiae* and non-*Saccharomyces* strains. The non-*Saccharomyces* strains belonged to the following four wine yeast species: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Starmerella bacillaris* and *Hanseniaspora uvarum*. In Chapter 1, we focused primarily on changes in the lipid composition as one of the primary components of biological membranes and its relation to the tolerance of the different *Saccharomyces* and non-*Saccharomyces* strains to oxidative stress from hydrogen peroxide (H_2O_2). Fatty acids (FAs) and the composition of individual sterols were analyzed by gas liquid chromatography (GLC and GC-MS, respectively). Phospholipids (PLs) were separated by two-dimensional thin layer chromatography (TLC) and quantified by estimating the amount of phosphates in each PL. Additionally, the H_2O_2 resistance of these strains was assessed using the agar diffusion method, and the production of ROS species was tracked by flow cytometry.

In Chapter 3, the lipis damage produced by oxidative stress in *Saccharomyces* and non-*Saccharomyces* strains was evaluated by measuring lipid peroxidation using thiobarbituric acid reacting substances (TBARS). Furthermore, the catalase activity and peroxisomes proliferation were determined by measuring the decrease in H_2O_2 absorbance at 240 nm and performing a western blot analysis against Fox1p, respectively.

<u>Objective 2:</u> Evaluation of the antioxidant role of melatonin in *Saccharomyces cerevisiae*. (Chapters 2, 3 and 4)

To complete this objective, the effect of melatonin on *S. cerevisiae* was evaluated in the presence and absence of oxidative stress from H_2O_2 (2 mM).

In Chapter 2, we evaluated the effect of melatonin (5 µM) in a commercial wine strain of *S. cerevisiae* (QA23) by measuring the ROS production using flow cytometry, by measuring the intracellular levels of reduced and oxidized glutathione by fluorescence detection, and by measuring the expression of genes related to antioxidant defense

systems such as glutathione, catalase, superoxide dismutase, glutaredoxin and thioredoxin by qPCR. In addition, in Chapter 4, we performed a transcriptomic analysis in the same strain (QA23) to create a global approach to the primary genes or families affected by the presence of melatonin with and without oxidative stress. Moreover, intracellular melatonin was quantified with a liquid chromatograph coupled to a triple quadrupole mass spectrometer (GL-TQMS). Finally, in Chapter 3, we analyzed the lipid damage caused by the oxidative stress, also in the presence of melatonin, to determine the catalase and TBARS activity (as explained in objective 1) in six strains of *S. cerevisiae*.

Objective 3: Evaluation of the role of melatonin as an antioxidant in non-*Saccharomyces* yeasts. (Chapter 3)

To complete this objective, the possible antioxidant effect of melatonin was studied in four non-*Saccharomyces* species (*T. delbrueckii*, *M. pulcherrima*, *S. bacillaris* and *H. uvarum*) in the presence and absence of H_2O_2. For this purpose, we determined the catalase and TBARS activity, as described in objective 1.

Thus, these three objectives were developed in the following four chapters of this thesis (**Figure 1**):

CHAPTER 1: The role of the membrane lipid composition in the oxidative stress tolerance of different wine yeasts. Results submitted to *Food Microbiol*.

CHAPTER 2: Melatonin reduces oxidative stress damage induced by hydrogen peroxide in *Saccharomyces cerevisiae.* Results published in *Front. Microbiol*. 8:1066 (2017).

CHAPTER 3: Melatonin minimizes the impact of the oxidative stress induced by hydrogen peroxide in *Saccharomyces* and non-*Saccharomyces* yeasts. Results submitted to *Front. Microbiol*.

CHAPTER 4: Transcriptomic insights into the melatonin effect on oxidative responses in *Saccharomyces cerevisiae.* Manuscript in preparation to be submitted to *J. Pineal Res*.

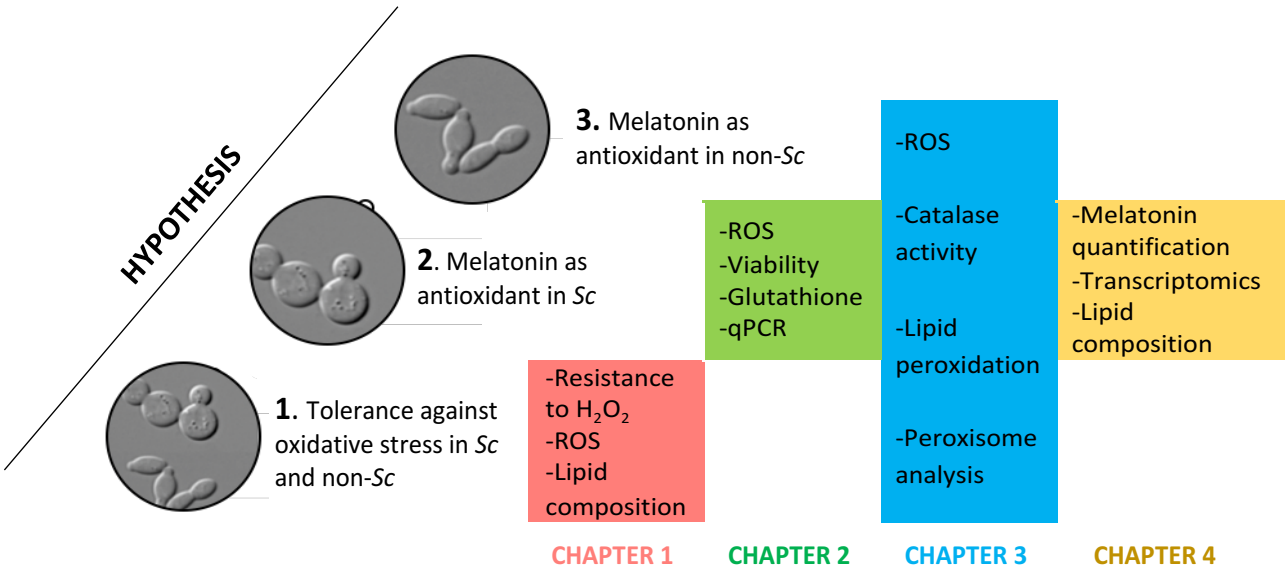

Figure 1. Schematic summary of objectives and outline of thesis.

INTRODUCTION

1. YEASTS AND WINE FERMENTATION

Yeasts have an enormous impact on food and beverages production. A scientific and technological understanding of their roles in this production began to emerge in the mid-1800s, starting with the pioneering studies of Louis Pasteur on the microbiology of wine fermentations, which demonstrated that fermentation is initiated by living organisms. Although it was a breakthrough to show that fermentation resulted from the action of microorganisms, this finding did not explain the basic nature of the fermentation process (Barnett, 2000).

Success came in 1897, when Eduard Buchner found that the fermentation of carbohydrates results from the action of different enzymes contained in yeast and not from the yeast cell itself. His discoveries, for which he received the Nobel Prize in 1907, showed that the enzyme zymase can be extracted from yeast cells and that it causes sugar to break up into carbon dioxide and alcohol (Barnett and Lichtenthaler, 2001).

These findings opened the way to elucidating the metabolic pathways of alcoholic fermentation in yeasts. During the twentieth century, this research generated major advances in biochemistry, with very important economic applications (Barnett, 2003). Thus, alcoholic fermentation was gradually revealed until reaching the knowledge of the current day.

1.1. Wine yeast ecology

Wine is a natural product that results from several biochemical reactions. Wine production starts with the ripening and harvesting of the grapes, and it continues with the alcoholic fermentation, clarification and bottling.

As early as 1876, Louis Pasteur wrote that '*the taste and properties of the wine could depend on the special nature of yeasts which develop during the fermentation of the grapes*'. Since then, countless studies have focused on understanding the ecology, biochemistry, physiology and molecular biology of the yeasts involved in the fermentation process and to select specialized yeast strains to improve the quality of wine (Fleet, 2003, 2008; Pretorius, 2000; Swiegers et al., 2005).

The current taxonomy recognizes 149 yeast genera that comprise nearly 1500 species (Kurzman et al., 2011). Of these, more than 40 species have been isolated from grape must (Ciani et al., 2010; Jolly et al., 2006). During traditional winemaking, there is a sequential succession of yeasts engaged in spontaneous fermentation (**Figure 1**). During the process, non-*Saccharomyces* yeasts play a substantial role starting from the vineyard until *S. cerevisiae* establishes its anaerobic conditions and increases the ethanol concentration in the environment, until approximately until mid-fermentation (Fleet, 2006). *S. cerevisiae* is the primary party responsible for the catalytic conversion of grape sugar into alcohol and CO_2. In addition, this species plays an important role in the formation of secondary metabolites and the release of aroma precursors (Bely et al., 1990; Jolly et al., 2014). However, the presence of non-*Saccharomyces* during early stages of fermentation also has an impact on the wine composition; and consequently, their contribution during the fermentation process cannot be ignored.

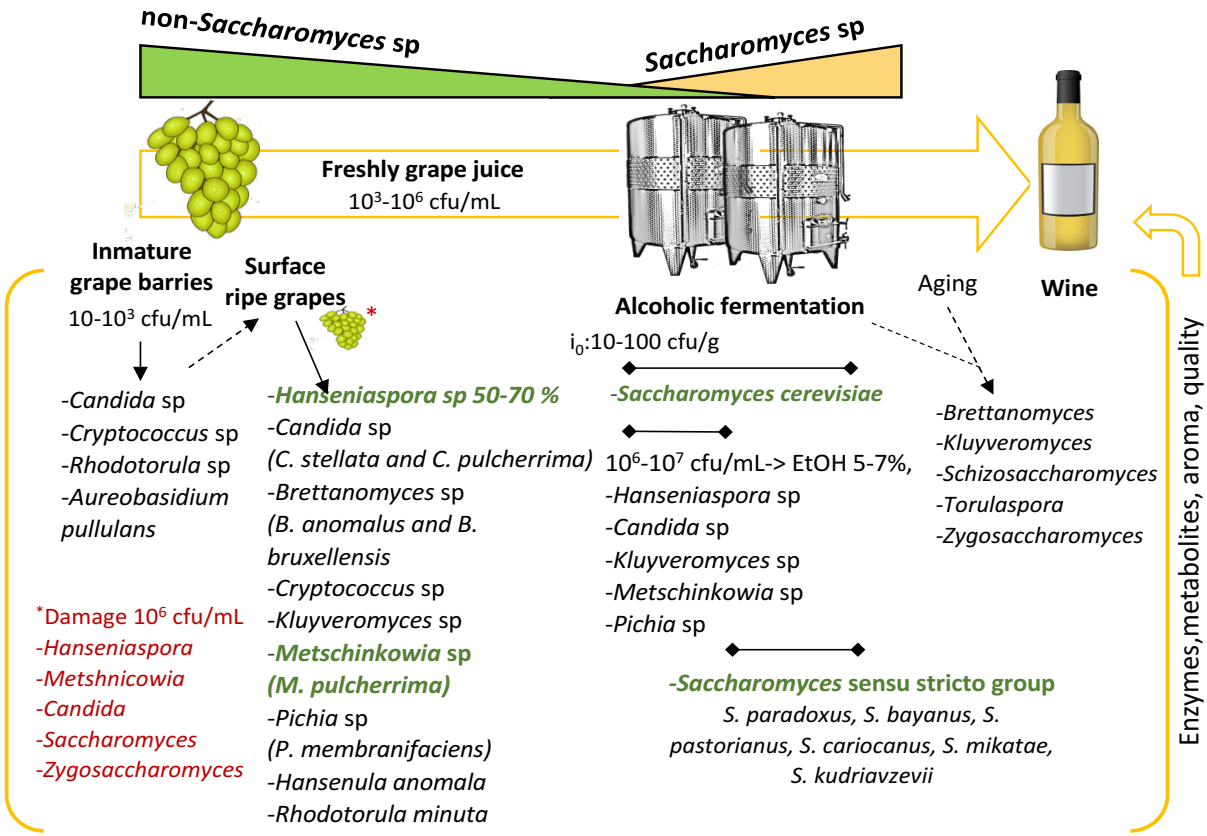

Figure 1. Sequential presence of different yeasts during the winemaking process (Adapted from Fleet, 2006).

S. cerevisiae, which is often simply known as the wine yeast par excellence, has been selected and optimized for commercial use as a starter culture (Ribereau-Gayon, 1985; Fleet and Heard, 1993). The primary rationale for selected starters is to produce wines with a uniform quality during different years, thus avoiding the variability associated with spontaneous fermentations and the risk of spoilage (Beltran et al., 2002).

As shown in **Figure 2**, despite the advantages of using pure cultures of *S. cerevisiae*, wines produced with yeast monocultures lack the complexity that the presence of various non-*Saccharomyces* species can offer. Thus, non-*Saccharomyces* yeasts are used now with the intention of producing consumer-directed wines with differentiated styles. Although these yeasts are not able to consume all of the sugar present in grape must because they are unfortunately incapable of completing alcoholic fermentation, several studies have demonstrated that some non-*Saccharomyces* yeasts used with sequential inoculation techniques can positively contribute to the aroma profile, sensory complexity and color stability of the resulting product (Fleet, 2008; González-Royo et al., 2015; Padilla et al., 2016a; Pretorius, 2000).

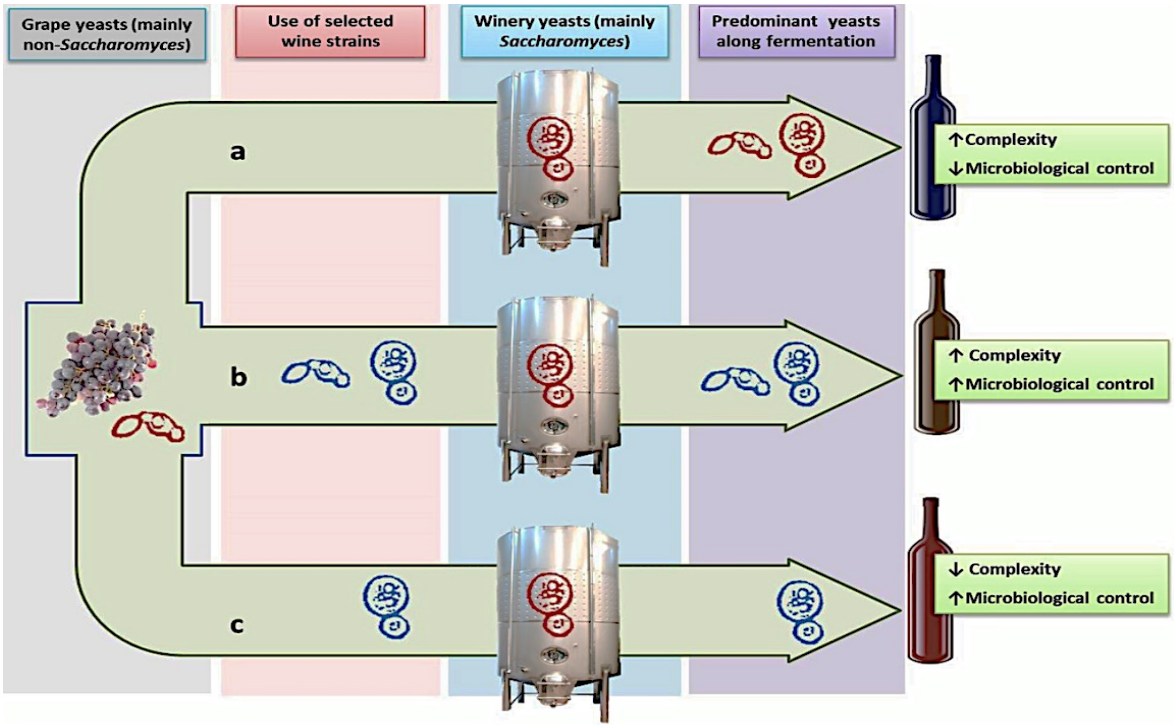

Figure 2. Use of selected *Saccharomyces* and non-*Saccharomyces* yeasts strains in winemaking (Padilla et al, 2016b). (a) Spontaneous fermentation allows for the development of indigenous yeasts from grapes (primarily non-*Saccharomyces*) and wineries (primarily *Saccharomyces*). (b) Mixed cultures of selected *Saccharomyces* and non-*Saccharomyces* strains. (c) Inoculation with a selected strain of *Saccharomyces.*

Thus, non-*Saccharomyces* species can influence the organoleptic properties of wines, increasing the volatile compounds or secondary metabolites such as gyclcerol, aromatic alcohols, esters, and acetates (Belda et al., 2017; Jolly et al., 2014; Romano et al., 2003). For exemple, *Torulaspora delbrueckii* has been proposed to reduce the volatile acidity produced by *Saccharomyces* (Bely et al., 2008), whereas *Metschnikowia pulcherrima* is recommended for the release of some volatile thiols and terpenes in white wines, which increases the aromatic intensity (Belda et al., 2017). Other species such as *Starmerella bacilaris* have been reported to contribute by producing higher amounts of glycerol (Mas et al., 2016). The *Hanseniaspora* species have been suggested to increase the wine quality (Masneuf-Pomarede et al., 2016).

1.2. Metabolic traits of wine yeasts

Saccharomyces and non-*Saccharomyces* species have similarly conserved the metabolic pathways that are involved in central carbon metabolism. However, the mechanisms of nutrient uptake and those involved in regulating respire-fermentative metabolism differ significantly (Flores et al., 2000).

Glycolysis and the TCA cycle are the central pathways of yeast metabolism, producing energy and reducing equivalents in the form of ATP, NADH or NADPH and providing building blocks to synthesize other biomolecules. As shown in **Figure 3**, both respiration and fermentation start during the glycolysis pathway to convert glucose into two molecules of pyruvate and ATP. During fermentation, pyruvate is transformed in ethanol without producing additional ATP. Conversely, the NADH released by glycolysis is recycled into NAD^+ by the action of alcohol dehydrogenase, which catalyzes the reduction of acetaldehyde into ethanol. By contrast, during respiration, pyruvate is converted into acetyl-CoA to be oxidized into CO_2 during the tricarboxylic (TCA) cycle and to undergo oxidative phosphorylation (OXPHOS), where it yields additional ATP but requires oxygen (Flores et al., 2000; García et al., 2016).

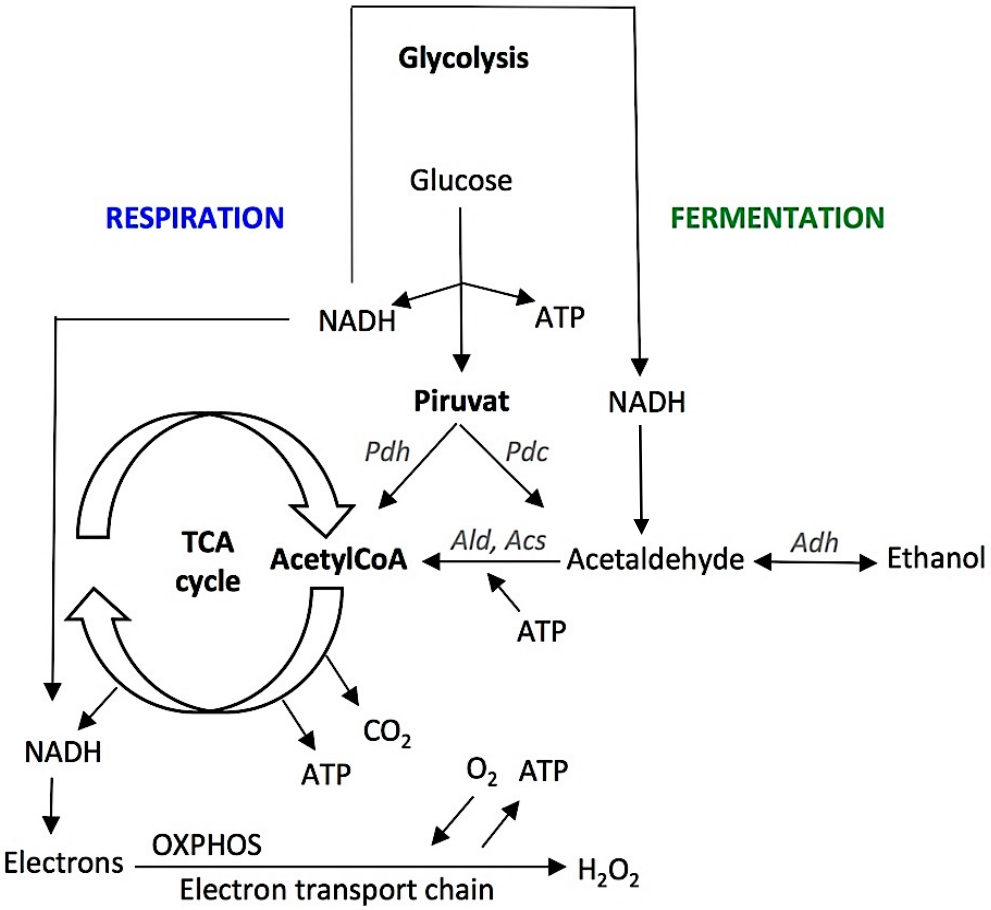

Figure 3. Yeast energy metabolism (García et al., 2016).

In terms of yeast biomass production, the Crabtree effect is a distinctive physiological phenomenon for the classification of yeasts as Crabtree-positive or negative on the basis of their cultivation conditions and pyruvate destination (**Table 1**). The yeasts classified as Crabtree-positive produce ethanol even under aerobic conditions, with high external glucose concentrations, resulting in lower biomass production because a fraction of sugars is converted into ethanol (**Figure 4**). Because only a fraction of sugar is used for the biomass and energy production, this could theoretically result in lower growth rate in Crabtree-positive yeasts, being out-competed by Crabtree-negative yeasts and other microorganisms. However, ethanol is used by the Crabtree-positive yeasts as a tool to slow down and control the proliferation of other competitive microorganisms (Dashko et al., 2014; De Deken, 1966; Pronk et al., 1996).

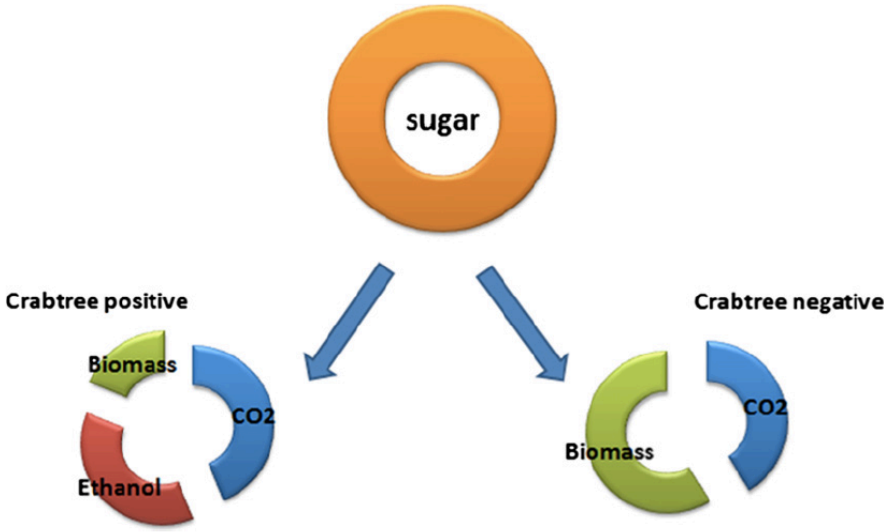

Figure 4. Crabtree effect (Dashko et al., 2014)

Thus, glycolysis is accelerated by glucose, which results in the production of ATP through the phosphorylation of the substrate. At the same time, the lower need for oxidative phosphorylation via electron transport chain decreases the oxygen consumption (**Figure 3**). This phenomenon is observed in most species of *Saccharomyces*.

Table 1. Mode of fermentation for different wine yeasts (Mas et al., 2016).

Mode of fermentation	
Crabtree-positive	Crabtree-negative
Saccharomyces cerevisiae	*Hanseniaspora uvarum*
Zygosaccharomyces balilii	*Pichia anomala*
Brettanomyces intermedius	*Candida utilis*
Torulopsis glabrata	*Hansenula neofermentans*
Schizosaccharomyces pombe	*Kluyveromyces marxianus*
Hanseniaspora guillermondi	*Debaryomyces hansenii*
Candida stellata	
Torulaspora delbrueckii*	

*Although *T. delbrueckii* is normally described as Crabtree-positive by several authors, respiration makes a higher contribution to metabolism in this species than in *S. cerevisiae* (Alves-Araújo et al., 2007; González et al., 2013; Merico et al., 2007)

One of the main problems an organism faces under anaerobic conditions is the lack of the final electron acceptor in the respiratory chain. This reduces or eliminates the activity of Krebs cycle, respiratory chain, and mitochondrial ATP generation. As a

response to hypoxic and anaerobic conditions, organisms have developed several processes to optimize the utilization of oxygen and even reduce the dependence on the presence of oxygen. According to their dependence on oxygen during the life cycle, yeasts are classified as: (1) obligate aerobes displaying exclusively respiratory metabolism, (2) facultative anaerobes, displaying both respiratory and fermentative metabolism, and (3) obligate anaerobes (Fleet, 2006; Merico et al., 2007). Most yeasts are aerobic and few can tolerate strictly anaerobic conditions. The ability of yeasts to grow under oxygen-limited conditions seems to be strictly dependent on the ability to perform alcoholic fermentation (Dashko et al., 2014).

S. cerevisiae is a facultative fermentative yeast and it requires oxygen for the synthesis of oleic acid and ergosterol (Andreasen and Stier, 1953, 1954). Although the concentration of molecular oxygen is particularly low during wine fermentation, several practices that are employed during the first stages of winemaking, such as pumping over or micro-oxygenation, can transiently but significantly increase the O_2 concentration.

2. OXIDATIVE STRESS

During the winemaking process, yeasts must respond to environmental changes primarily produced by fluctuations in the dissolved oxygen concentration, pH, osmolarity, ethanol concentration, nutrient starvation and temperature. Furthermore, practices such as using selected natural yeasts have been created and commercialized as active dry yeasts to use as an inoculum for must fermentations, increasing the magnitude of the stresses to which yeasts are subjected (Gómez-Pastor et al., 2012; Pretorius, 2000).

Oxygen is a highly reactive molecule that can be partially reduced to generate reactive species oxygen (ROS), including superoxide anions ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radicals (OH^-) or hydrogen peroxide (H_2O_2) (**Figure 5**). In a biological context, ROS are the natural byproducts of the normal metabolism of oxygen, and they have important roles in cell signaling. However, the ROS levels can increase dramatically, thereby disturbing the imbalance between the systemic manifestation of ROS and the biological systems to readily detoxify the reactive intermediates or repair the resulting damage. Cumulative ROS is known as oxidative stress.

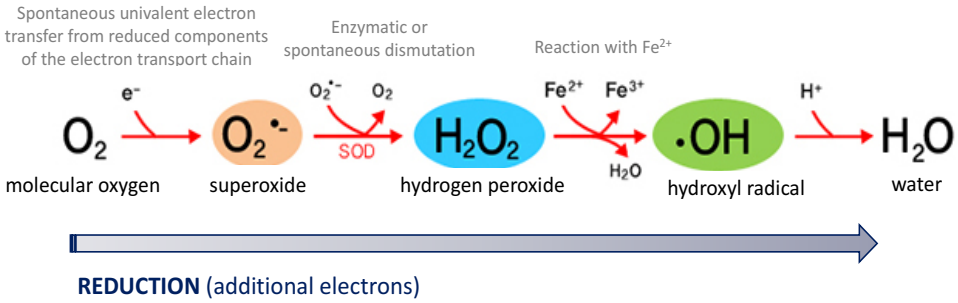

Figure 5. Formation of reactive oxygen species (Kiley and Storz, 2004).

The oxidative stress response is triggered when cells sense an increase in ROS, which may result from the following:

- The transition from anaerobic to aerobic conditions
- An increase in the mitochondrial respiratory chain activity (e.g., when yeasts change from a fermentable to a non-fermentable carbon source)
- The exposure of the cells to low concentrations of oxidants such as H_2O_2 or drugs that generate superoxide radicals
- The decrease in antioxidant defenses

Excessive ROS can overwhelm the cellular mechanism, damage cellular structures by oxidizing lipids, proteins, carbohydrates and nucleic acids and even lead cells to death (Halliwell and Gutteridge, 1990).

Although alcoholic fermentation is an anaerobic process, oxidative stress is considered one cause of the early mortality of yeasts during fermentation. However, it is important to highlight that other types of stresses can generate ROS. For exemple, the mechanism of ethanol toxicity includes a generalized increase in oxidative stress, and ethanol accumulation during batch fermentation leads to the generation of hydrogen peroxide and superoxide as the primary ROS (Pérez-Gallardo et al., 2013). Furthermore, there is a considerable overlap between the yeast oxidative stress responses and other stress responses, including starvation, heat shock, osmotic shock and resistance to heavy metals such as cadmium (Jamieson, 1998).

As such, the presence of oxygen plays an important role during the winemaking process throughout the following uses:

- Biomass propagation: Active dry yeast (ADY)

The ADY that is used as a starter in alcoholic fermentation must be functionally and metabolically active to facilitate the quick-start of the process (Poirier et al., 2002). To this end, ADYs are subjected to a multiple-stage process that involves continuous oxidative stress steps primarily due to the maintenance of production under respiratory metabolism, which could negatively affect the yeast performance further (Pérez-torrado et al., 2005, 2009).

To initiate the biomass propagation step, the cells are firstly inoculated into the batch while using molasses as a carbon source, and ammonia, urea, phosphoric acid and magnesium sulfphate meet the nitrogen, phosphorous and magnesium requirements (**Figure 6**). Then, the process initiates a sequence of consecutive batch and fed-batch fermentations in increasing volumes until being finished during the "commercial" fermentation (Gómez-Pastor et al., 2011; Degre, 1993; Chen and Chiger, 1985). This methodology is aimed at favoring respiratory metabolism to obtain higher biomass rates.

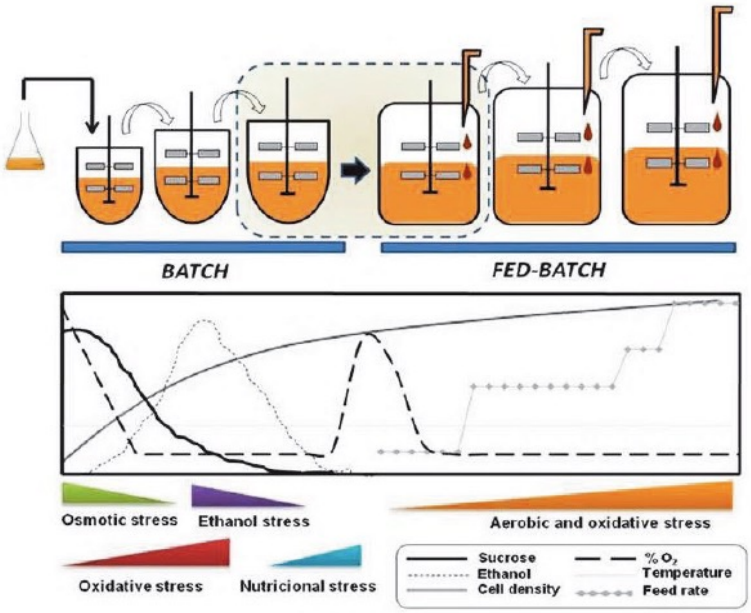

Figure 6. Diagram of the different stages in the industrial yeast biomass propagation process (Gómez-Pastor et al., 2011).

In addition, as cells are aerated to minimize the fermentative process, the oxidative stress is accentuated. Nevertheless, aeration is important for promoting the synthesis of unsaturated fatty acid and ergosterol, the absence of which might affect plasma membrane functions, increasing ethanol sensitivity (Degre, 1993). Cells within the maturation step experience 2 h of rest time, and then they reach stationary phase, during which they accumulate metabolites such as glycogen and trehalose. These metabolites will help them to better resist the rest of the drying process and maintain a suitable fermentative capacity after rehydration. At the end of the biomass propagation, the yeasts are separated from the fermented media by centrifugation. The resulting yeast paste is dehydrated by using rotatory and press vacuum filters until the product displays less than 8% of its residual humidity and is then packed under a vacuum or inert atmosphere and stored for long periods (Chen and Chiger, 1985; Gómez-Pastor et al., 2011).

- Replicative and chronological lifespan

Cellular ageing can be measured using two models as follows: replicative ageing is defined by the number of daughter cells produced by a mother cell before senescence (replicative lifespan, RLS), and chronological ageing measures the time in stationary phase during which a cell can maintain its viability in a non-dividing state (chronological lifespan, CLS) (**Figure 7**; Kaeberlein, 2010). ROS are known to have a direct role in cellular ageing (Gutteridge and Halliwell, 2000). In the replicative model, both ageing and age-related damage are inherited by the daughter cell from the mother cell, and the daughter retains its full replicative capacity until the end of the RLS of the mother cell. In the chronological model, damage is accumulated over time within a non-dividing cell until the cell is no longer able to re-enter the cell cycle (**Figure 7**; Kaeberlein, 2010). Although the CLS has a negative impact on the RLS, the CLS becomes increasingly significant during the winemaking process because fermentation is performed mostly by non-dividing cells. As explained above, modern winemaking practices use the inoculation of grape juice with ADY at high concentrations to initiate the alcoholic fermentation; under these conditions, yeast divides only 4-6 times, far from the 20 divisions as a mean of natural isolates. Therefore, the RLS is not a limiting factor for the yeast performance, unlike the CLS, which is 3-4 times longer than the growth phase

under winemaking conditions (Orozco et al., 2012). Moreover, the oxidative stress effect on the CLS has a paradoxical role because greater ROS production has a negative impact but is also essential for extending the lifespan, increasing cell tolerance to oxidative stress (Fabrizio and Longo, 2003; Orozco et al., 2012).

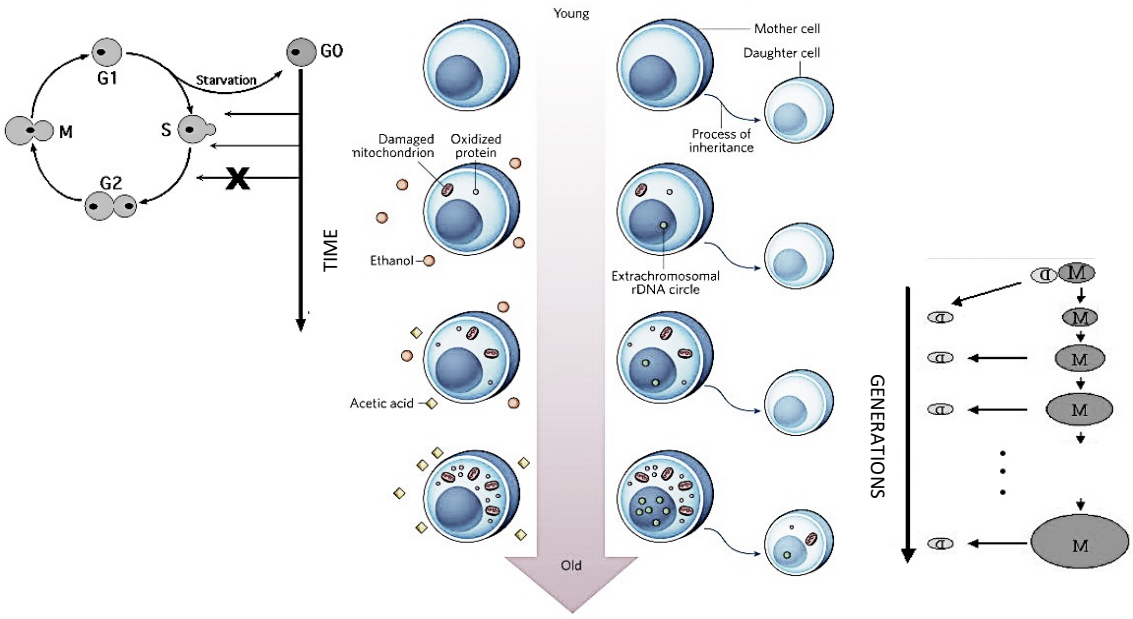

Figure 7. Damage accumulation during yeast ageing (Modified from Kaeberlein, 2010).

- Micro-oxygenation

Micro-oxygenation is a common winemaking treatment that is used to introduce oxygen into wine in a controlled manner, with the aim of improving the red wine color development and diminish the vegetal aroma, among other effects (**Figure 8**). Thus, it is not a "real" oxidative stress, but oxygen does play an important role; consequently, the micro-oxygenation impacts the yeast redox balance, releasing ROS during alcoholic fermentation (Salmon, 2006). A correct addition of oxygen can confer higher cellular tolerance to ethanol and fermentative activity. In general, yeasts need 5-10 mg/L of oxygen during the initial growing phase. At the beginning of the winemaking process, grape must is saturated with oxygen, but at the end of the growing phase, the yeast still needs oxygen; this is the key moment for oxygen addition (Han et al., 2017; Salmon, 2006). Under anaerobic conditions, yeast growth normally requires added oxygen to synthesized lipids (sterols and unsaturated fatty acids (UFA)) which are essential for

plasma membrane integrity (Andreasen and Stier, 1953, 1954). Oxygen availability during the first stages of alcoholic fermentation favored the persistence of several non-*Saccharomyces* yeasts (Hansen et al., 2001). By contrast selective oxygen addition at the end of growth phase during mixed culture fermentations of grape musts favored the *Saccharomyces* strain (Salmon, 2006).

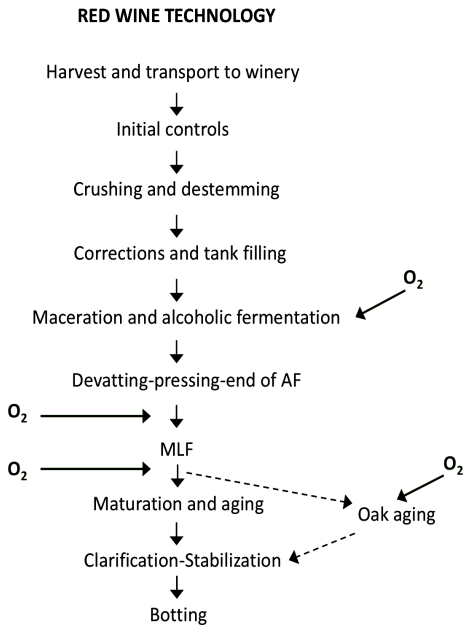

Figure 8. Diagram of red wine technology and possible oxygen additions (From Gómez-Plaza and Cano-López, 2011).

3. YEAST OXIDATIVE STRESS RESPONSE

Yeasts have developed mechanisms to respond to oxidative stress. These mechanisms are obviously aimed at protecting yeast from the detrimental effects of redox imbalance and at repairing possible damage but also at acquiring cellular oxidative stress tolerance.

The oxidative stress response involves sensor systems and the activation of signal transduction pathways that adjust the genomic expression program to activate the most efficient response. In addition to activating a specific response against oxidative stress, yeasts have developed a common molecular mechanism for cellular protection that is provoked when cells are exposed to different stressful stimuli. Thus, exposure to one type of stress leads to tolerance to other types of stress as well (Costa and Moradas-Ferreira, 2001; Gibson et al., 2007) (**Figure 9**).

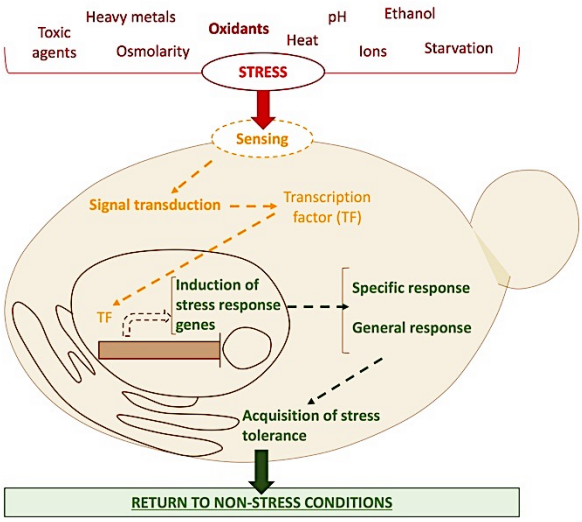

Figure 9. Schematic representation of the different molecular and physiological responses of yeast to changes in the environment (Modified from Costa and Moradas-Ferreira, 2001).

Thus, both the specific and general stress responses are initiated by oxidative challenges (Costa and Moradas-Ferreira, 2001). Adaptations to stress conditions involve early responses to provide almost immediate protection against sublethal stress conditions, and late responses provide more efficient protection against a severe stress and allow cells to return to non-stress conditions (**Figure 9**). Early responses result in the post-translational activation of pre-existing defenses, and the activation of signal transduction pathways that initiate late responses, namely, the de novo synthesis of stress proteins and antioxidant defenses.

There are many studies on the response mechanisms of *S. cerevisiae* to the different drugs that are used to generate oxidative stress. Among them, hydrogen peroxide has been commonly used as an oxidative stress promoter model. This molecule is formed during normal aerobic respiration but also after exposure to several environmental factors. It not only generates oxidative stress damage but also serves as a signaling molecule for the regulation of several biological processes (Veal et al., 2007).

3.1. Antioxidant defense systems

Antioxidant defense systems are present in cells under physiological conditions; however, they confer a limited capacity to resist sudden oxidative aggression. Thus, cells induce the antioxidant defenses and protective factors needed to survive. These

antioxidant defense systems are grouped into enzymatic (**Table 2**) and non-enzymatic (**Table 3**) systems that operate at different levels (Costa and Moradas-Ferreira, 2001; Jamieson, 1998; Moradas-Ferreira and Costa, 2000). The defense systems include several enzymes that are able to remove oxygen radicals and their products and/or to repair the oxidative damage. By contrast, non-enzymatic defense systems consist of small molecules that act as radical scavengers (Jamieson, 1998).

Table 2. The most relevant enzymatic antioxidant systems in *S. cerevisiae* (adapted from Costa and Moradas-Ferreira, 2001; Estruch, 2000; Grant, 2001; Jamieson, 1998; Moradas-Ferreira et al., 1996; Moradas-Ferreira and Costa, 2000; Walker and Van Dijck, 2006).

Enzymatic system	Protein	Localization	Function
Catalases	Cta1p	Peroxisome	Decomposition of H_2O_2 to H_2O and O_2
	Ctt1p	Cytosol	
Superoxide dismutases	Sod1p	Cytoplasm	Dismutation of superoxide anion to H_2O and O_2
	Sod2p	Mitochondria	
Enzymes of pentose phosphate pathway	Zwf1p	Cytoplasm	Reduction of $NADP^+$ to NADPH
	Tkl1p	Cytoplasm	
	Rpe1p	Cytosol	
Glutathione reductase	Glr1p	Cytosol Mitochondria	Reduction of oxidized glutathione (GSSG)
Glutathione peroxidases	Gpx1p	Mitochondria Peroxisome Cytosol	Reduction of H_2O_2 and organic peroxides using reduced glutathione (GSH)
	Gpx2p	Cytoplasm Mitochondria Nucleus	
	Gpx3p	Mitochondria Peroxisome Cytosol	
Thioredoxin peroxidases	Tsa1p	Cytosol	Reduction of H_2O_2 and alkyl hydroperoxides
	Tsa2p	Cytosol	
	Ahp1p	Cytosol	
	Prx1p	Mitochondria	
	Dot5p	Nucleus	
Thioredoxin reductase	Trr1p	Cytosol	Thioredoxin oxidases reduction
	Trr2p	Mitochondria	

Table 3. The most relevant non-enzymatic antioxidant systems in *S. cerevisiae* (adapted from Costa and Moradas-Ferreira, 2001; Estruch, 2000; Grant, 2001; Jamieson, 1998; Moradas-Ferreira et al., 1996; Moradas-Ferreira and Costa, 2000; Walker and Van Dijck, 2006).

Non-enzymatic system	Protein	Localization	Function
Glutathione	Glutamate-cysteine ligase	Cytosol Mitochondria	Synthesis of reduced glutathione (GSH). Reduction of protein disulphides, scavenging of free radicals, conjugation with electrophilic substrates, binding of Cd
	Glutathione synthase		
Glutaredoxin	Grx1p	Cytoplasm	Glutathione peroxidase activity Glutathione S-transferase activity
	Grx2p	Cytoplasm Mitochondria	
	Grx3p Grx4p	Nucleus Nucleous	
	Grx5p	Mitochondria	
	Grx6p Grx7p	Endoplasmic reticle	
Thioredoxins	Trx1p	Cytosol	Reduction of protein disulphides
	Trx2p		
	Trx3p	Mitochondria	
Poliamines			React with ROS, specially with $O_2^{\cdot-}$. Protection of lipids from oxidation
Methallothioneins	Cu-MT (1-1)	Cytosol	Avoid ROS production by Fenton reaction. Store metals in a non-toxic form
	Cu-MT (1-2)		

Among its enzymatic strategies, *S. cerevisiae* has developed a sophisticated mechanism to detoxify ROS as follows: one group of enzymes acts directly as ROS detoxifiers, and the second group consists of enzymes that act as redox regulators of protein thiols and help maintain the redox balance of the cell (Herrero et al., 2008). However, both strategies overlap (**Figure 10**).

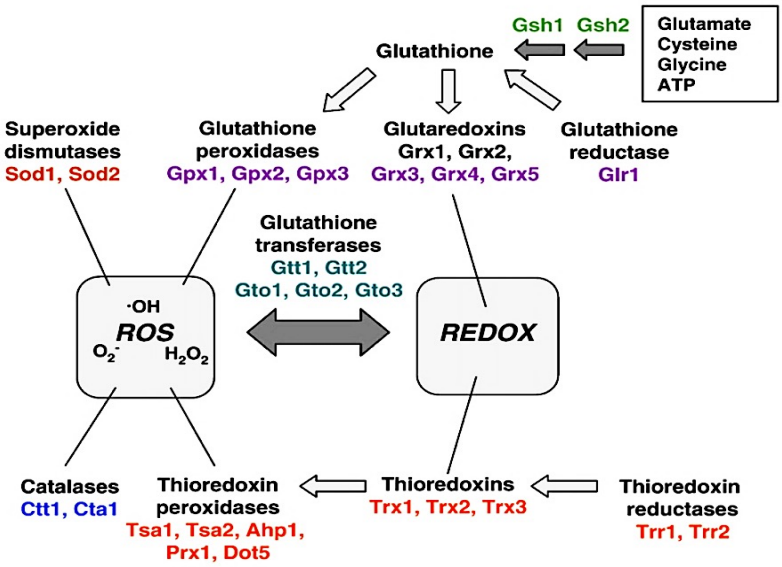

Figure 10. Primary interrelationships between different enzymatic systems involved in detoxification and the control of the redox state in *S. cerevisiae* (From Herrero et al., 2008).

- Superoxide dismutases (SODs)

Yeast cells possess two intracellular SODs, the cytoplasm-located Cu/Zn-Sod, which is encoded by the *SOD1* gene, and the mitochondria-located Mn-Sod, which is encoded by the *SOD2* gene (Jamieson, 1998). Both SODs catalyze the disproportionation of the superoxide anion into H_2O_2, and their activity requires redox-active metal ions (Herrero et al., 2008). Because of the slower growth of Sod2 null mutants under respiratory conditions, Mn-Sod seems to be essential for eliminating the superoxide anions generated by the respiratory chain (Jamieson, 1998; Moradas-Ferreira et al., 1996). By contrast, Sod1 seems to be essential for targeting externally added oxidants, and it plays a role in protecting cells against respiration-derived superoxide anions (Herrero et al., 2008). In fact, Sod1 mutants show many oxygen-related growth defects, such as cysteine, methionine and lysine auxotrophy, when they are grown in air. These Sod1 defects can be suppressed by the overexpression of metallothioneins, which directly minimizes OH radicals through the sequestration of transition metals such as copper and iron (Halliwell, 1996; Kumari et al., 1998).

- Catalases

Catalases catalyzes the breakdown of H_2O_2 into O_2 and H_2O, and as SODs, they depend on the redox properties of the metal group associated with the enzyme (Herrero et al.,

2008). *S. cerevisiae* has two catalases: catalase A (Cta1) is encoded by the *CTA1* gene, which localizes at peroxisomes, and catalase T (Ctt1) is encoded by *CTT1* gene, which is cytosolic (Cohen et al., 1988; Hartig and Ruis, 1986). Catalase A is involved in the detoxification of the H_2O_2 generated by acyl-CoA oxidase during fatty acids β-oxidation in peroxisomes. The role of catalase T at the cytosol is less clear because null Ctt1 mutants display wild type exponential growth under aerobic conditions and are not more sensitive to H_2O_2 than are wild-type cells (Izawa et al., 1995, 1996). However, their expression is regulated by oxidative and osmotic stresses and even by starvation (Ruis and Schüller, 1995). Furthermore, the double Cta1 and Ctt1 mutants are hypersensitive to H_2O_2 during the stationary phase, and both single and double catalase mutants are unable to mount an adaptive response to H_2O_2 (Izawa et al., 1996; Martinez-Pastor et al., 1996).

- Peroxidases

Unlike catalases and SODs, the role of peroxidases as ROS detoxifiers does not depend on the redox properties of the metal group associated with the enzyme. Peroxidases act by reducting of organic and inorganic peroxides into alcohol with the help of active site cysteine thiols. Thus, the electrons of thiols are essential for peroxidase activity. Depending on this property, two types of peroxidases exist: glutathione peroxidases (GPXs), which use GSH, and thioredoxin (TRX) peroxidases (also named peroxiredoxins (PRXs)), which use TRXs as reductants (Herrero et al., 2008). Three GPXs have been described in *S. cerevisiae*, and they are called Gpx1, Gpx2 and Gpx3. However, further studies have shown that GPX proteins are similar in sequence, structure, and function to phospholipid hydroperoxide glutathione peroxidases (PHGPxs), the primary enzymes for repairing membrane lipid peroxidation (Avery et al., 2004; Avery and Avery, 2001). Thus, in addition to protecting cells directly from peroxides during oxidative stress, they also regulate and act as a hydroperoxide sensor for the oxidative stress transcription factor Yap1 (Avery et al., 2004; Delaunay et al., 2002; Ma et al., 2007).

- Glutathione/glutaredoxins and thioredoxins systems

The glutathione/glutaredoxin and thioredoxin systems are two of the most important antioxidant defenses for maintaining the reduced environment of the cell, and they play an especially significant role in defending the cell against oxidative stress (**Figure 11**).

These antioxidant systems have been described as essential under both aerobic and anaerobic conditions (Auchère et al., 2008; Grant, 2001; Herrero et al., 2008).

The tripeptide glutathione (γ-glutamylcysteinylglycine, or GSH) is well known as the primary and most abundant endogenous antioxidant in the cells, consisting of a ubiquitous low-molecular-mass thiol with high reducing power due to its free sulfhydryl. GSH directly reacts with ROS, donating an electron to neutralize them and becoming reactive itself, and GSSG, its oxidized state, is formed through the combination of two reactive forms of GSH. Thus, the presence of ROS results in a decrease in GSH and an increase in GSSG, for which cells try to compensate through the action of glutathione reductase (Glr1), which reduces GSSG to GSH in an NADPH-dependent process, and through the synthesis of new GSH, which involves two ATP-dependent steps that are catalyzed by γ-glutamylcysteine synthetase (*GSH1*) and glutathione synthetase (*GSH2*) (**Figure 11**). During the first step, *GSH1* catalyzes the formation of γ-glutamylcysteine from glutamic acid and cysteine. In the second step, *GSH2* catalyzes the ligation of glycine with the dipeptide (Grant et al., 1997; Izawa et al., 1995; Jamieson, 1998; Moradas-Ferreira et al., 1996).

Glutaredoxins (GRXs) are small heat-stable proteins with an active site containing two redox-sensitive cysteines. These enzymes act as thiol oxidoreductases that are responsible for reducing protein disulfides or glutathione-protein mixed disulfides. The GRX system also includes NADPH, and because of GRX, Glr1 is reduced by GSH using electrons donated by NADPH. GRXs are divided in two families. The first one protects the cells against superoxide anions (Grx1) and H_2O_2 (Grx2). Both GRXs have been demonstrated to be active as glutathione peroxidases and glutathione S-transferases (Collinson and Grant, 2003). The second one includes six other members (Grx3-8), which are present in different subcellular compartments. Grx3/4 are involved in intracellular iron trafficking. Grx5 has a potential role in the protecting against oxidative stress during growth under normal conditions and after exposure to oxidant agents such as H_2O_2 and menadione (Estruch, 2000; Morano et al., 2012). Grx6/7 are thought to be involved in the regulation of sulfhydryl in the early secretory pathways under stress conditions. Finally, Grx8 does not seem to have a function in the oxidative stress response (Morano et al., 2012).

Thioredoxins are small oxidoreductases; as GRXs, they possess two redox-sensitive cysteines in their active sites. The primary difference between them is whether their thioredoxins are directly reduced by NADPH through the thiorredoxin reductase (Trr1, Trr2). Thus, the thioredoxin system consists of thioredoxins (Trx), thioredoxin reductases (Trr) and NADPH. Yeasts possesses one thioredoxin system in the cytoplasm (Trx1, Trx2 and Trr1) and the other system in the mitochondria (Trx3 and Trr2). The Trx3 oxidation state might depend on Trr2 in cells that are exposed to oxidative stress, whereas under normal growing conditions, they stay in a reduced form, even in the presence of the *TRR2* gene. The suppression of *TRX1* and *TRX2* has been shown to result in a decreased resistance to H_2O_2 (Estruch, 2000; Trotter and Grant, 2005).

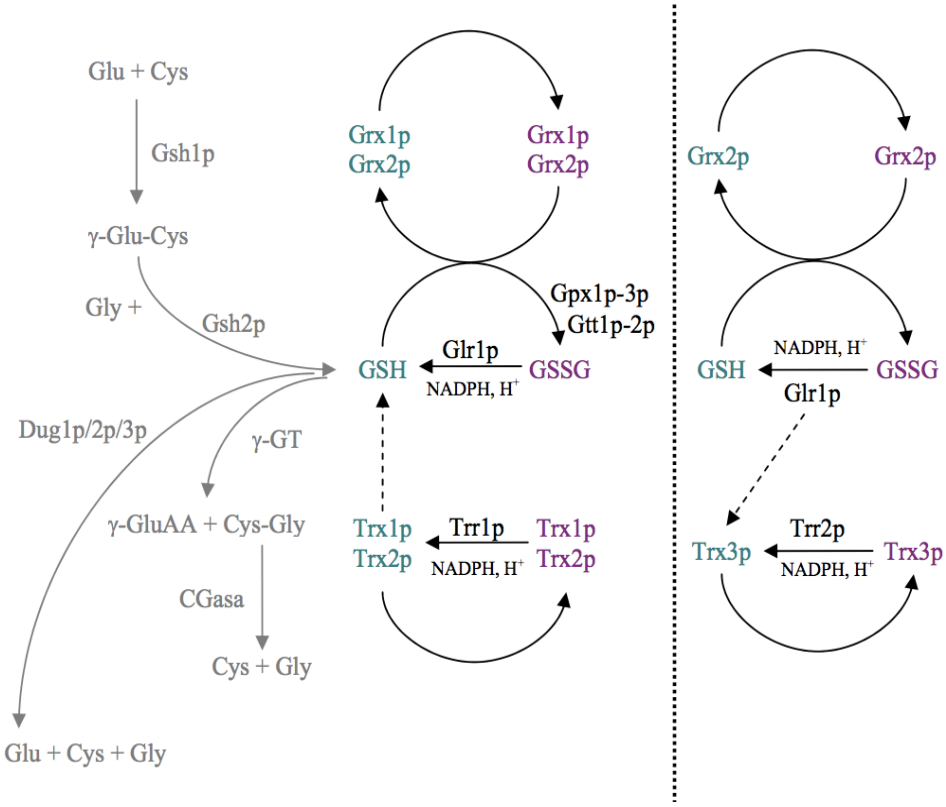

Figure 11. Scheme of the cytosolic and mitochondria glutathione/glutaredoxin and thioredoxin systems (From the doctoral thesis of García, 2008). (■) reduced form, (■) oxidized form).

As shown in **Figure 11**, glutathione, glutarredoxins and thioredoxins share common operational traits. The redox status of GSH may provide a functional link between the glutathione/glutaredoxin and thioredoxin systems because cytoplasmic thioredoxins function along with Glr1 to maintain the high intracellular GSH/GSSG ratio (Trotter and

Grant, 2002, 2005). Furthermore, thioredoxins indirectly influence glutathione/glutaredoxin through Yap1 transcription factor, which regulates several genes involved in the oxidative stress response, including genes for the glutathione synthesis (Estruch, 2000). Oxidation of Yap1 avoids its export from the nucleus activating then, the expression of numerous genes including *TRR1* and *TRX2*. Thioredoxin system is responsible for reducing and deactivating Yap1. It is such a loop of self-regulation where oxidative stress activates Yap1 which activates recovery systems which in turns inactivate Yap1 to restore the equilibrium (Delaunay et al., 2000; Izawa et al., 1999; Temple et al., 2005).

3.2. Regulation of oxidative stress response

3.2.1. Transcription factors

The specific strategies of different cellular antioxidant defense systems are related to their differential regulation, by which specific genes increase their expression under oxidative conditions as part of an oxidative stress response pathway (**Table 4**). The transcription factors Yap1, Skn7, Msn2 and Msn4 are the primary regulators of the *S. cerevisiae* oxidative stress response and of cells that grow aerobically. The Hap1 transcription factor is also included in this group, and it regulates the genes encoding components of the mitochondrial respiratory chain through the CGGnnnTAnCGG consensus core sequence (Godon et al., 1998; Costa and Moradas-Ferreira, 2001). Yap1 is a member of the Yap family that binds to YRE (consensus TTACTAA or TGACTAA), conferring the ability to tolerate oxidants such as H_2O_2 through the up-regulation of genes that encode most of the antioxidant enzymes and components of the cellular thiol-reducing pathways. Skn7 is a transcription factor that co-regulates many target proteins in response to H_2O_2 in association with Yap1. Unlike Yap1, this transcriptional regulator does not participate in the regulation of metabolic pathways that regenerate glutathione and NADPH. Msn2 and Msn4 (Msn2/4) activate genes that are involved in the general stress response, the promoters of which contain the stress response element (STRE:CCCCT). These elements respond to a wide variety of stresses, including nutritional, osmotic, acidic and oxidative stresses (Costa and Moradas-Ferreira, 2001; Delaunay et al., 2000; Godon et al., 1998; Hasan et al., 2002).

Table 4. Compilation of most of the genes induced by oxidative stress and their regulation by transcription factors (TF) (Costa and Moradas-Ferreira, 2001; Hohmann and Mager, 1997).

Gene	Function	Hydrogen peroxide		Respiratory growth	
		Induction	TF	Induction	TF
Glutathione system					
GSH1	Glutamate-cysteine ligase	+	Yap1		
GSH2	Glutathione synthetase	+	Yap1		
GLR1	Glutathione reductase	+	Yap1	+	Yap1;?
GPX1	Glutathione persoxidase	+	?	+	?
GPX2	Glutathione peroxidase	+	Yap1		
GRX1	Glutaredoxin	+	Msn2/4	+	?
GRX2	Glutaredoxin	+		+	?
Thioredoxin system					
TRX2	Thioredoxin 2	+	Yap1;Skn7		
TRR1	Thioredoxin reductase	+	Yap1;Skn7		
TSA1	Thiol peroxidase	+	Yap1;Skn7		
AHP1	Thiol peroxidase	+	Yap1;Skn7		
mTPx	Thiol peroxidase	+	?		
cTPII	Thiol peroxidase	+	Yap1;Skn7		
Other antioxidants					
CCP1	Cytochrome-c peroxidase	+	Yap1;Skn7	+	?
CTA1	Catalase A, peroxisomal	+	Yap1	+	?
CTT1	Catalase T, cytosolic	+	Yap1;Skn7,	+	Msn2/4
			Msn2/4;	+	?
SOD1	Superoxide dismutase	+	Yap1;Skn7	+	
SOD2	Superoxide dismutase	+	Yap1;Skn7		Mns2/4p; HAP2/3/4/5
CUP1	Metallothionein			+	?
Drug transporters					
FLR1	ATP transporter	+	Yap1		
ATR1	ATP transporter	+	Yap1		
Carbohydrates metabolism					
ZWF1	G-6-P dehydrogenase	+	Yap1		
NTH1	Neutral trehalase	+			
TSL1	Trehalose-6-P synthase	+	Msn2/4		
TPS1	Trehalose-6-P synthase	+	Yap1		
TPS2	Trehalose-6-P synthase	+			
UGA2	Succinate semialdehyde dehydrogenase	+	Msn2/4		
ALD3	Aldehyde dehydrogenase	+	Msn2/4		
Heat shock proteins					
SSA1	HSP70 family	+	Yap1;Skn7		
SSA3	HSP70 family	+			
SSA4	HSP70 family	+			
HSP12	HSP	+	Skn7;Msn2		
HSP26	HSP	+			
HSP42	HSP	+			
HSP82	HSP	+	Yap1;Skn7		
HSP104	HSP	+			

3.2.2. Signaling oxidative stress

Yeasts have developed a large and complex regulatory network to mediate oxidative stress. The system of regulation involves a sensing step followed by the transduction signal to different pathways, which overlap between them to activate the best oxidative stress response. The TOR, RAS, and cell wall integrity (CWI) signaling pathways have been well characterized as routes that play important role in transducing the oxidative signals in *S. cerevisiae* (Torre-Ruiz et al., 2012).

Mtl1 is a transmembrane protein and a homologue of Mid2 that is localized to the cell periphery. It has been described as being responsible for detecting environmental changes and, more specifically, acting as an oxidative stress sensor and nutritional starvation signal (Petkova et al., 2010; Vilella et al., 2005). Although Mtl1 is the best candidate to act as a cell surface sensor, others have been described, such as actin or the ROS produced in the mitochondria itself, which can act as sensors/transmitters of oxidative stress (Torre-Ruiz et al., 2012).

The CWI pathway involves a mitogen-activated protein kinases (MAPKs) cascade, which participates in sensing and transmitting several extracellular signals and stresses, including cell wall, osmotic, mating and nutritional stresses (**Figure 12**). The MAPK pathway includes three components that activate each other through the sequential phosphorylation of specific residues of conserved motifs (MAPKKK, MAPKK and MAPK). Six plasma membrane proteins (Wsc1-Wsc4, Mid2 and Mtl1) containing a single transmembrane segment have been identified as being important for activating the CWI pathway. They transmit signals to Rom2, which activates Rho1, which in turn activates the protein kinase Pkc1. Rho1, similar to Cdc24, can interact with phosphates in the head groups of membrane phospholipids. Pkc1 is then responsible for activating the sequence of MAPKs in the following sequence: Bck1 (MAPKKK) phosphorylates Mkk1 and Mkk2 (MAPKK), and both activate Slt2. Activated Slt2 is also a regulator of gene expression via the direct phosphorylation and activation of the transcription factor Rlm1 (Chen and Thorner, 2007; Torre-Ruiz et al., 2012). The upper elements of cell integrity are involved in the organization of the actin cytoskeleton under different conditions, including cell wall and nutritional stresses, oxidative stress and pH (Chen and Thorner, 2007; Helliwell et al., 1998; Motizuki et al., 2008; Torres et al., 2002; Vilella et al., 2005).

Although CWI is the best-characterized MAPK in response to oxidative stress, other paths within MAPK signaling modify the expression of genes related to mating, the cell cycle response, and osmolality as well as filamentous growth in response to oxidative stress (**Figure 12,** Zhao et al., 2015). For exemple, it has been suggested that H_2O_2-mediated signaling increases the level of *CDC28* level, favoring a G2/M delay and filamentation (Starovoytova et al., 2013). However, anoxic conditions and some preservatives have been shown to have repressive effects on invasive growth (Zupan and Raspor, 2010).

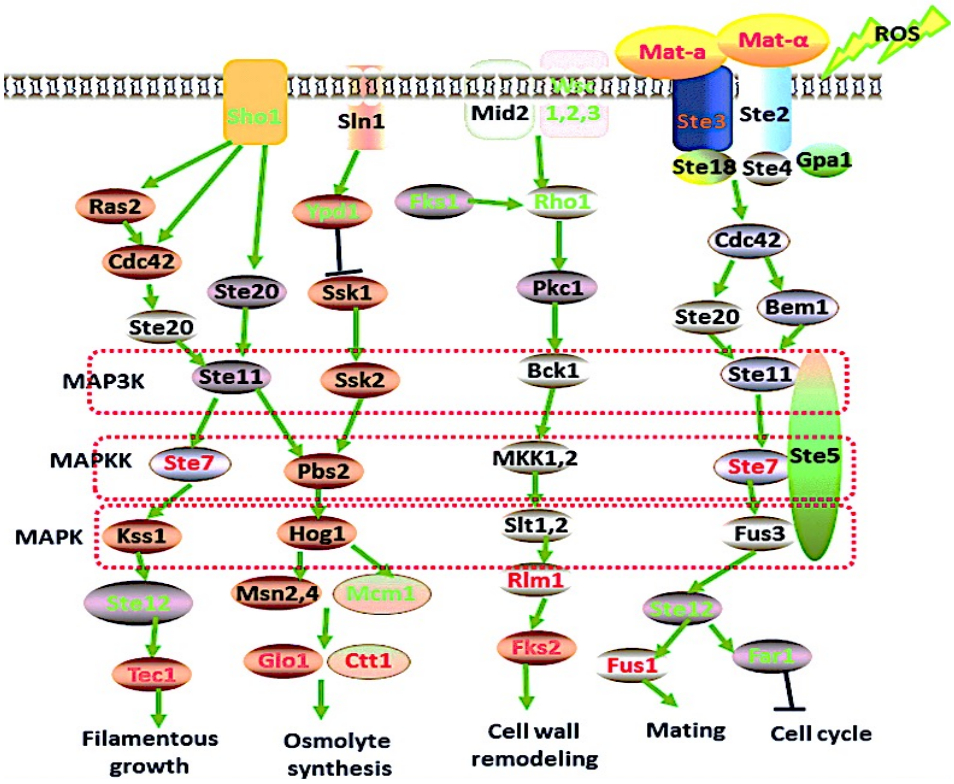

Figure 12. MAP Kinase and PKA signaling pathways. Red and green colors indicated the up- and down-regulated genes, respectively, of a mutant strain of *S. cerevisiae*, which improve stress tolerance relative to the wild-type (From Zhao et al., 2015).

Phosphatidylinositol (PI) plays an important role in signaling regulation. PI forms PI-4-kinase, which is encoded by *STT4* and localizes to the plasma membrane (**Figure 13**). This kinase functions upstream of Pkc1 in both the regulation of polarized growth and in the cell wall integrity pathway. The sequential actions of Stt4 and Mss4 at the cell surface generate PI4,5-kinase, which recruits Rom2 to the plasma membrane. The

sensors activate Rom2, which in turn activates Pkc1 (Levin, 2005; Strahl and Thorner, 2007).

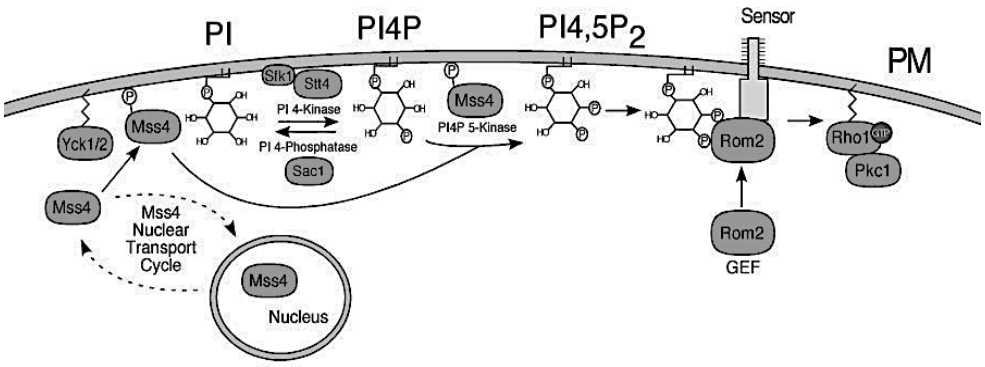

Figure 13. Phosphoinositide signaling system at the plasma membrane (From Levin, 2005).

The Pkc1 pathway is also related to the TOR pathway. Yeast possesses two different TOR protein complexes, TORC1 and TORC2, which are encoded by *TOR1* and *TOR2*, respectively. TORC1 mediates the rapamycin-sensitive signaling branch, whereas TORC2 signaling is rapamycin-insensitive and is required for the organization of the actin cytoskeleton (**Figure 14**). In a global sense, TOR inhibits the transcription of stress-responsive elements, the nitrogen pathway, starvation genes, and genes related to the retrograde response through the sequestration of transcription factors in the cytoplasm. Moreover, TOR modulates translation initiation, inhibits protein turnover and represses the transcription factors of genes related to nutrient starvation. The TOR function also regulates ribosomal protein expression in response to environmental conditions via PKA (Loewith et al., 2002; Wullschleger et al., 2006).

Mitochondrial retrograde signaling (RTG) is the pathway for communication between mitochondria and the nucleus under normal and dysfunctional mitochondrial conditions. RTG is linked to ageing, the chronological life span, mitochondrial DNA maintenance, TOR signaling and nutrient sensing pathways (Liu and Butow, 2006). RTG contains three proteins (Rtg1, Rtg2 and Rtg3). When this pathway is activated, Rtg1 and Rtg3 form a transcription factor that translocates, with the help of Rtg2, to the nucleus, where it controls the expression of a set of genes that code for mitochondrial proteins. The Lst8 component of the TOR complex (**Figure 14**) negatively regulates the RTG

pathway at the Rtg2 level. Mitochondrial dysfunction leads to a decrease in the intracellular ATP concentration, which may favor Rtg2-Mks1 interactions and allow Rtg1-Rtg3 activation. One target of the RTG pathway is the *CIT2* that encodes a peroxisomal isoform of citrate synthase, which enables the cells to utilize acetate or ethanol as the sole carbon sources (Liao et al., 1991).

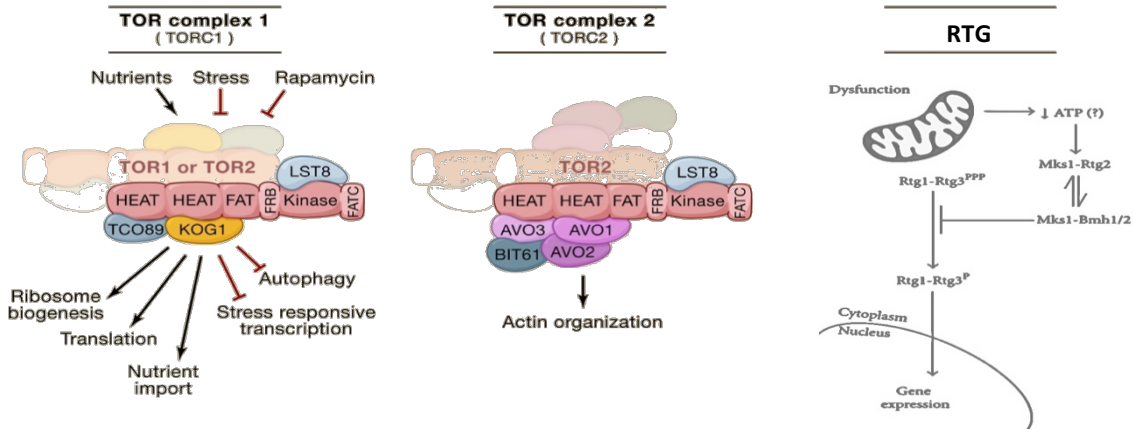

Figure 14. TOR1 and TOR2 complexes and mitochondrial retrograde signaling (RTG) (From Da Cunha et al., 2015; Wullschleger et al., 2006).

The RAS/cAMP pathway acts immediately downstream of cAMP-dependent protein kinase to control adaptations to nutrient limitations. It controls the reprogramming of the metabolism at the diauxic transition when glucose becomes limiting, some of the subsequent adaptations during both the post-diauxic phase, when cells grow respiratively on ethanol, and entry into the stationary phase, which is negatively controlled by the RAS/cAMP. Thus, under a normal carbon source, the pathway is activated by GTPases called Ras1 and Ras2, which signal the protein kinases PKA and cAMP, thereby repressing the function of the general stress transcription factor Msn2/Msn4. By contrast, nutrient starvation or/and oxidative stress negatively regulate RAS/cAMP.

The protein kinase encoded by *SCH9* increases the ROS through respiratory metabolism activation, which in turn decreases the life span and increases DNA damage (Madia et al., 2009). To extend the life span, it is necessary to down-regulate the Sch9 signal and reduce the TOR pathway function in order to decrease the mitochondrial activity (Pan and Shadel, 2009).

As noted at the beginning of the section, these signaling routes cooperate with one another to lead to the best response according to the needs of the cell. **Figure 15** schematizes and summarizes the complex interrelations between the different pathways described before in response to oxidative stress.

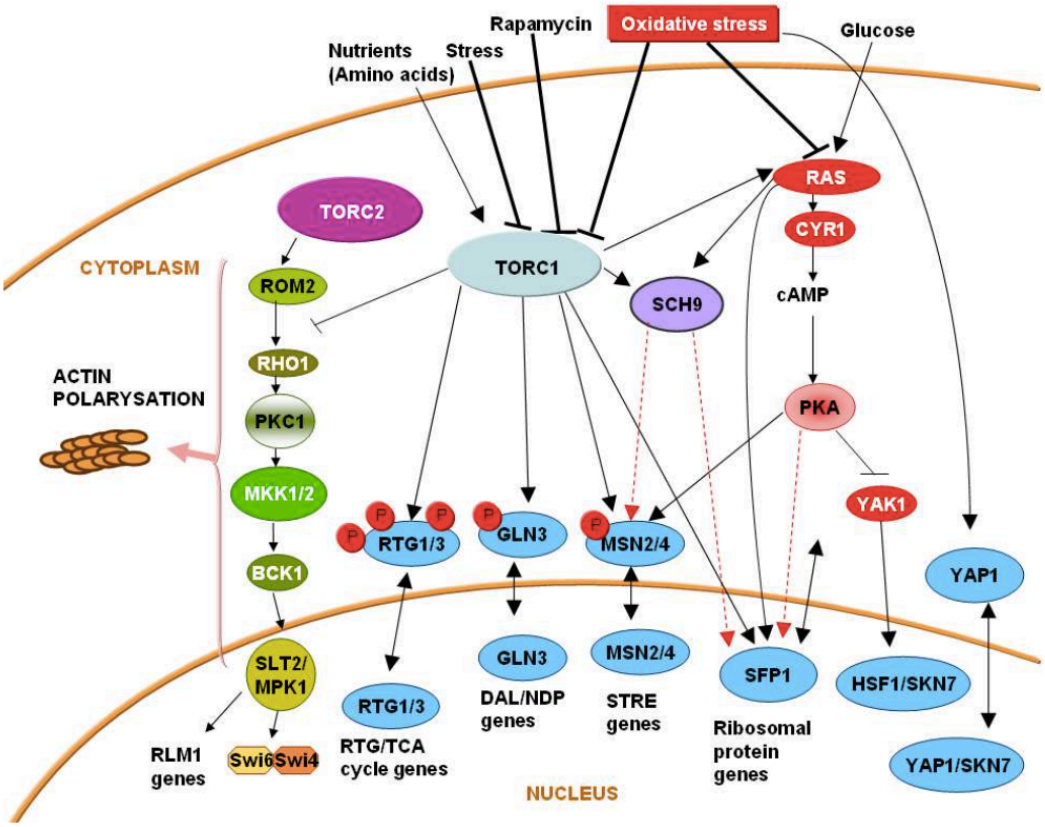

Figure 15. Oxidative stress response signaling network in *S. cerevisiae* (From Torre-Ruiz et al., 2012). After oxidative stress is sensed, complex Torc11 and Ras activities are negatively regulated. Torc1 responds to nutrient availability and is inhibited by rapamycin and stress conditions. When Torc1 is activated, it promotes the sequestration of specific transcription factors in the cytoplasm. Torc1 and Ras/cAMP pathways activate the Sfp1 transcription factor, including ribosomal gene expression. Both TORC1 and RAS converge in Sch9. RAS signals Pka kinase, which inhibits both Yak1 kinase and Msn2/Msn4. Yak1 in turn activates the Skn7/Hsf1 transcription factor that is required for an oxidative response. For this response, Torc1 signals to Ras activation. Torc inhibits the CWI activity. However, the Torc2 complex signals to cytoplasm elements of the CWI pathway to organize the actin cytoskeleton. CWI, cell wall integrity; STRE, stress-responsive element; DAL, degradation of urea and allantoin; NDP, nitrogen discrimination pathway; and RTG, retrograde pathway.

4. YEAST LIPID COMPOSITION AND OXIDATIVE STRESS

Biological membranes are selectively permeable lipid bilayers with associated and embedded proteins, and they have long been proposed as one of the prime sites of vulnerability or tolerance to stress (Ernst et al., 2016; Hunter and Rose, 1972; Steels et

al., 1994; van der Rest et al., 1995). Their lipid component has a vital role in yeast tolerance to oxidative stress and to other physiological stressors because, under unexpected changes in environmental conditions, lipids are able to change the organizational and dynamic structure of the membrane to stabilize the plasma membrane and maintain its functions (Arroyo-López et al., 2010; Beltran et al., 2008; Ding et al., 2009; Redón et al., 2009; Renaud and Lorgeril, 1992; Rodríguez-Vargas et al., 2007; Torija et al., 2003).

4.1. Lipid composition

The bulk of total cellular lipids is found in biological membranes and can be roughly divided into the following classes:

- Fatty acids (FAs)

FAs are the basic components of complex lipids. Free FAs are carboxylic acids with hydrocarbon chains that vary in chain length. Depending on their double bonds contents, they can be classified as saturated (SFAs that contain no double bonds), monounsaturated (MUFAs containing one double bond) and polyunsaturated (PUFAs containing more than one double bond) (**Figure 16**; Boyle J. Lehninger, 2005).

The major FAs in *S. cerevisiae* are palmitoleic (C16:1) and oleic (C18:1) acids, followed by palmitic (C16:0) and stearic (C18:0) acids (Klug and Daum, 2014). Unlike other non-*Saccharomyces* yeast species, such as *M. pulcherrima*, *S. cerevisiae* does not have PUFAs in its fatty acid composition (Rozès et al., 1992).

FAs influence in the function of cellular membranes. The primary function of UFA is to modulate the physical properties of membranes, especially their fluidity. Thus, the fatty acyl composition varies in response to environmental stress (Hunter and Rose, 1972; Lands and Davis, 1984). In addition, other functions are associated with FAs, including roles as signaling molecules, transcriptional regulators and posttranslational modifiers of proteins. FAs also play a role in cell secretion and provide energy through the β-oxidation pathway (Duplus et al., 2000; Trotter, 2001; van Meer et al., 2008; van Roermund et al., 1998, 2003).

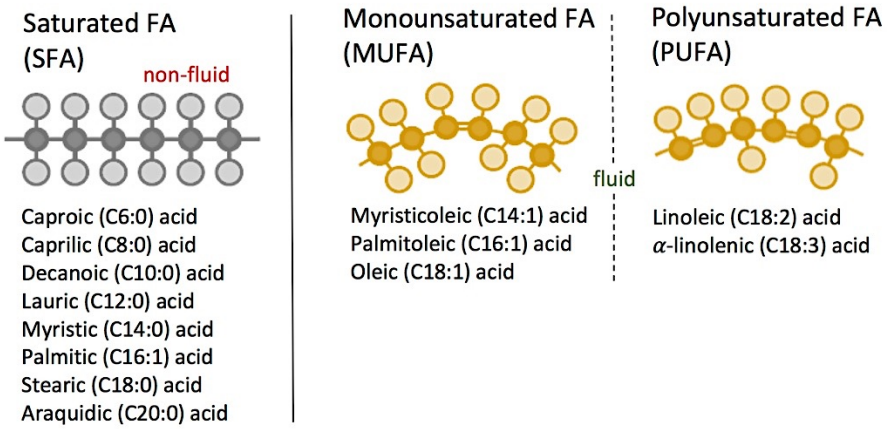

Figure 16. Representation and classification of saturated, mono- and poly-unsaturated fatty acids.

- Phospholipids (PLs)

Because of their amphiphilic characteristic, PLs serve as precursors and structural compounds of membranes, and they are the most abundant membrane lipids (Ejsing et al., 2009). The structure of PLs is based on a non-polar part (tails), consisting of two FAs attached to a polar head through an ester bond to the first and second carbons of glycerol. This glycerol links with its third carbon to the phosphate group through a phosphodiester bond. PLs are subdivided into different classes according to their polar head group (**Figure 17**). Although the PL composition can vary according to the culture conditions, the major PLs in the total yeast cell extracts are phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS). However, their distribution varies depending on the organelle, e.g., cardiolipin (CL) is an important minor PL predominantly found in the mitochondria (Flis et al., 2015; Joshi et al., 2009; Klug and Daum, 2014). Each phospholipid class comprises a multitude of molecular species that are defined by the length and degree of saturation in their acyl chains (De Kroon et al., 2013; Holthuis and Menon, 2014).

The cellular role of PLs is not only limited to a structural function. They can also provide precursors for the synthesis of other molecules, act as a reservoir of lipid messengers (Exton, 1994), be involved in the activity of membrane proteins (Fairn et al., 2011; Suetsugu et al., 2014), engage in fission and fusion events (Grillitsch et al., 2014), be involved in the transport to the plasma membrane (Opekarová et al., 2002), maintain the mitochondrial structure and function (Joshi et al., 2009), and take part in mRNA localization (Hermesh et al., 2014).

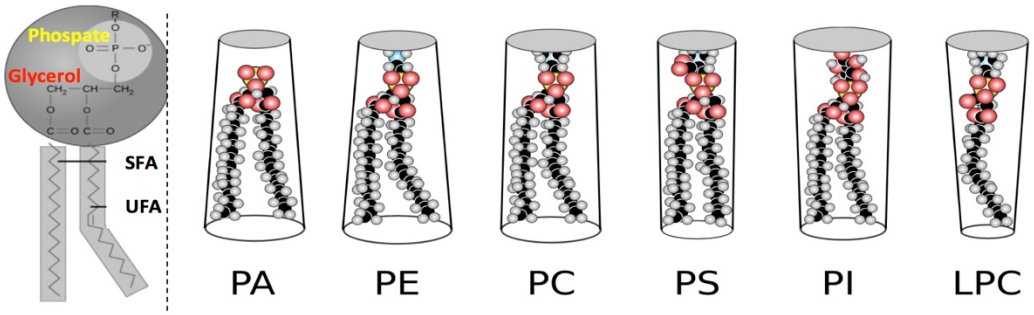

Figure 17. Representation of the phospholipid (PL) structure and the shape of each PL class (Modified from Zanghellini et al., 2010).

- Sterols

Sterols are essential structural lipid constituents of yeast membranes, and ergosterol is the major sterol present in yeast. Ergosterol is derived from its precursor squalene, and it is the final product of the yeast sterol biosynthetic pathway. Its structure consists of four fused rings, an acyl side chain and a hydrophilic hydroxyl group that facilitates insertion into membranes (**Figure 18**). Ergosterol serves many of the functions that cholesterol has in animal cells. Free sterols are predominantly present in the plasma membrane, which controls its physical state by modulating its bilayer fluidity and permeability (Nes et al., 1993; Sharma, 2006).

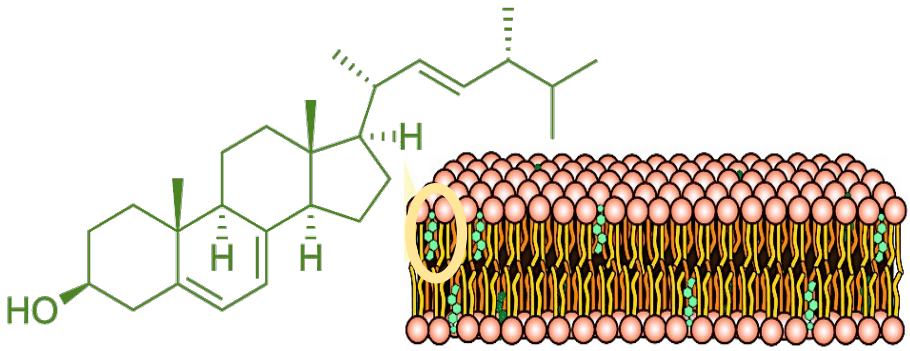

Figure 18. Representation of the ergosterol structure as the primary sterol plasma membrane.

- Sphingolipids

Sphingolipids are basically very long chain fatty acids that serve as structural components at the cell surface. They contain saturated or trans-unsaturated acyl chains linked to a serine backbone. Furthermore, sphingolipids serve as key signaling roles such

as regulating endocytosis, ubiquitin-dependent proteolysis and cell cycle control (Klug and Daum, 2014).

- Membrane properties depending on the lipid composition

Physical membrane properties are highly influenced by the lipid composition (**Figure 19**). Lipid packaging predetermines the fluidity of the membranes, meaning the viscosity of the lipid bilayer underlying the cell membrane, and it can affect the rotation and diffusion of proteins and other molecules within the membrane (Holthuis and Menon, 2014; Quinn, 1981; van Meer et al., 2008).

The head group and acyl chain composition of PLs influence the physical properties of the membrane (De Kroon et al., 2013). PC usually contains one cis-unsaturated acyl chain, such as oleic (C18:1) acid, which lowers the packing density of the acyl chains and increases the membrane fluidity (Koynova and Caffrey, 1998). With facilitation by its cylindrical shape (**Figure 17**), PC self-assembles spontaneously into closed bilayers, which adopts a liquid crystalline state. Thus, PC provides a stable and fluid matrix for cellular membranes. By contrast, PE possesses a small polar head group, and it is a conically shaped PL that imposes negative curvature stress on the membrane. The non-bilayer propensity of PE increases with acyl chain unsaturation, creating lipid packaging defects that facilitate membrane fusion and influence the binding and activity of peripheral membrane proteins (Holthuis and Menon, 2014; Marsh, 2007; Frolov et al., 2011). Although they are in lower abundance than PC and PE, PS and PI are key determinants of the membrane surface charge and mediate functional interactions with positively charged regions of peripheral and integral membrane proteins. Thus, higher membrane fluidity is promoted by lipids with short, unsaturated fatty acids (Ernst et al., 2016). The double bonds introduce kinks that lower the packing density of the acyl chains and inhibit the transition of the membrane from fluid to solid gel phases.

By contrast, sterols rigidify fluid membranes by reducing the flexibility of unsaturated acyl chains and increasing the membrane thickness and impermeability to solutes (Brown and London, 1998). High levels of packing defects are found in lipids with unsaturated acyl chains and small head groups. The surface charge is determined by the presence of anionic lipids such as PS and PI (Ernst et al., 2016; Holthuis and Menon, 2014).

The curvature is determined by the lipid shape. Lipids with a small polar head-to-acyl chain ratio (creating a cone shape) induce a negative curvature. Lipids with an equal head-to-chain ratio (creating a cylinder shape) are neutral, and those with a much larger head compared with the acyl chain area (creating an inverted cone shape) induce a positive curvature (Antonny, 2011; Bigay and Antonny, 2012) (**Figure 19**).

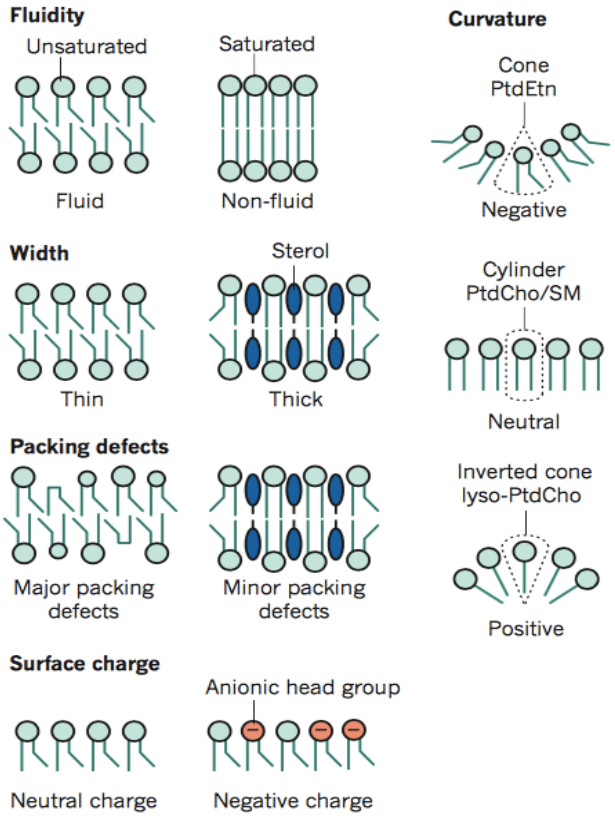

Figure 19. Membrane physical properties depend on the lipid compositions (From Holthuis and Menon, 2014).

4.2. Lipid biosynthesis

Lipid metabolism is quite complex and involves a very large number of metabolic reactions in different cellular compartments, resulting in the formation of a diverse group of chemical compounds. Despite the wide chemical variety of lipids, they all have the same key carbon precursor, acetyl-CoA, and all of the initial steps of lipid biosynthesis occur in the cytosol (**Figure 20**; Nielsen, 2009).

The lipid biosynthesis pathway involves the decarboxylation of pyruvate into acetaldehyde, which is then converted into acetate and acetyl-CoA. Although acetaldehyde is primarily converted into ethanol under fermentative conditions,

sufficient acetaldehyde is converted to acetyl-CoA to ensure an efficient lipid biosynthesis. Furthermore, this pathway is functional under fully respiratory conditions (Ernst et al., 2016; Nielsen, 2009). Lipid biosynthesis basically involves two branches from acetyl-CoA: one leading to sterols and the other leading to FAs that serve as building blocks for the biosynthesis of TAG, phospholipids, steryl esters and sphingolipids (**Figure 20**).

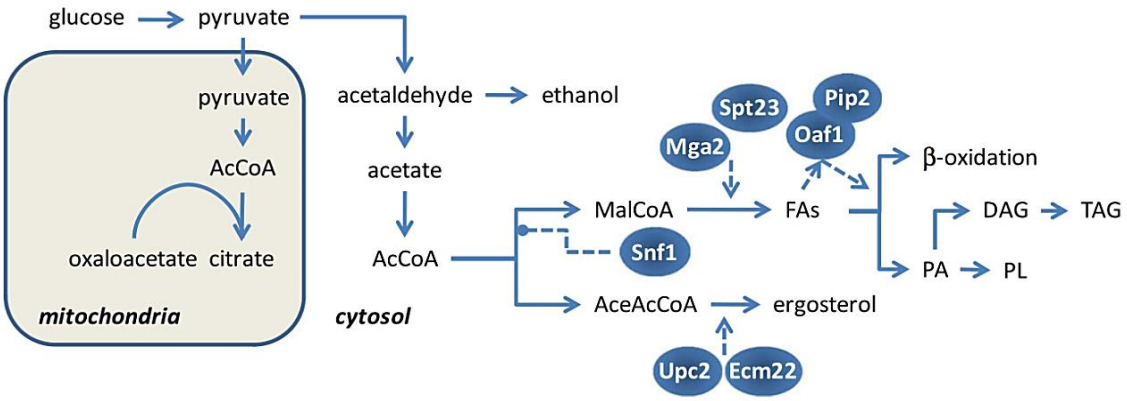

Figure 20. Representation of lipid biosynthesis (From Nielsen, 2009).

In the branch towards sterols, the first step is the condensation of two acetyl-CoA molecules that yield acetoacetyl-CoA. Later, after several reactions, squalene is formed from two molecules of farnesyl pyrophosphate (FPP), and through several oxygen-dependent reactions, squalene is transformed into lanosterol, zymosterol, fecosterol, episterol and ergosterol, which is finally synthesized in the endoplasmic reticulum (ER) and is then transported to the plasma membrane to prevent its accumulation (Baumann et al., 2005). In the other branch of lipid biosynthesis, acetyl-CoA is converted into malonyl-CoA, which serves as a precursor for further FA synthesis reactions, as catalyzed by FA synthetases (FAS). The primary product of FAS is palmitic acid (C16:0), which can be further converted into stearic acid (C18:0). They can then be converted into their corresponding MUFAs, palmitoleic (C16:1) and oleic (C18:1) acids, respectively, by Δ9-desaturase encoded by *OLE1* in yeasts. *S. cerevisiae* lacks the ability to convert oleic acid into PUFAs, such as linoleic (C18:2) and linolenic (C18:3) acids. FA synthesis occurs in the cytosol and mitochondria, and it is restricted to occasions when there is a high energy load in the cells (Tehlivets et al., 2007).

FAs can be degraded by β-oxidation inside peroxisomes, or they can enter the route towards PLs synthesis, during which two FAs are added to glycerol-3 phosphate to form phosphatidic acid (PA), which serves as a central metabolite in the synthesis of PLs (De Kroon et al., 2013). PA can either be dephosphorylated to diacylglycerol (DG), which contributes to PE and PC synthesis, or it can be converted into cytidine diphosphate-diacylglycerol (CDP-DG), which is converted into PI or PS in the ER and phosphatidylglycerol phosphate (PGP) in the mitochondria. PS can be decarboxylated into PE, which can subsequently be methylated to form PC (Klug and Daum, 2014). The PGP in the mitochondria produces phosphatidylglycerol (PG) to form CL. The insertion of additional double bonds performed by specific desaturases that are present in the ER membrane occurs when FAs are present as phospholipids. By contrast, elongation occurs only when the FAs are present as CoA-esters (Nielsen, 2009).

4.3. Oxidative stress and lipid composition

ROS production can initiate changes in the lipid layer composition, resulting in a lipid peroxidation process in which unsaturated lipids are converted into polar lipid hydroperoxides. ROS, in particular hydroxyl radical, react with lipid membranes and generate reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), in three phase reactions (**Figure 21**). PLs are particularly susceptible to the oxidative damage mediated by ROS due to their polyunsaturated FAs (PUFAs) content, which are more sensitive to peroxidation than monounsaturated FAs (MUFAs) (Ayala et al., 2014; Howlett and Avery, 1997). Extensive lipid peroxidation has been correlated with membrane disintegration and cell death.

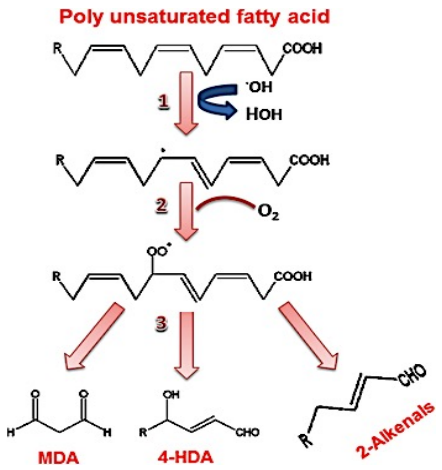

Figure 21. Major products of lipid peroxidation (From Shah et al., 2014).

Membrane dysfunction as a consequence of ROS is not systematically observed because yeasts are able to compensate for these changes by modulating the membrane fluidity and phase transitions through lipid composition modification. These changes in lipid composition, which are used by yeasts as a defense mechanisms, are fundamental for maintaining membrane integrity and functionality after stress exposure and for conferring a higher survival rate to cells when they are further exposed to the same stress (Beney and Gervais, 2001; Los and Murata, 2004). In fact, several authors have suggested a relationship between the membrane composition and tolerance to stress (Landolfo et al., 2010; Rodríguez-Vargas et al., 2007). Thus, the lipid composition is the result of a sum of complex phenomena for maintaining the optimal viability of the cell under different conditions.

In yeasts, studies of adaptive responses have primarily been focused on tolerance to ethanol and low or high temperatures (Beltran et al., 2008; Redón et al., 2009; Torija et al., 2003). Furthermore, cells with adaptive response to oxidative stress primarily face changes in the systems responsible for repairing the H_2O_2-induced damage in *S. cerevisiae*.

Alterations in yeast during the stress tolerance reactions induced by H_2O_2 have been observed to decrease the long chain fatty acid unsaturation, basically through decreased levels of oleic acid. These changes end with a decrease in the fluidity and permeability of cellular membranes (Pedroso et al., 2009). However, this adaptation mechanism seems to depend on the oxidizing agent because several studies have highlighted the ability of cells to increase the concentration of unsaturated fatty acids, raising the oleic acid in the plasma membrane as the principal mechanism for adapting to ethanol stress (Dinh et al., 2008).

An increase in the PC/PE ratio has also been reported as another yeast adaptation to oxidative stress. As described before, PC has an overall cylindrical shape and is organized as a bilayer, which makes it ideally suited for preserving the membrane integrity. Increases in the PC/PE ratio and the squalene levels of the plasma membrane in cells under oxidative stress have been suggested to decrease the membrane permeability against H_2O_2 (Pedroso et al., 2009). In fact, phospholipid model vesicles have shown that decreasing ratios of PC/PE are highly correlated with increasing permeability for glucose.

Multiple studies have shown that compositional changes in UFAs are regulated by either the transcriptional or post-transcriptional modification of desaturases (Cossins et al., 2002). An increase in the UFA percentage within yeast cells was correlated with a decrease in the responsiveness of the stress response promoter element (STRE) in *S. cerevisiae* (Chatterjee et al., 2000).

However, the yeast lipid composition is highly influenced by the aerobic or anaerobic yeast growth as well as by the nature of the yeast's own metabolism (**Table 1**). Anaerobic cellular growth has lower levels of total lipids, decreased phospholipids and sterol components, and increased hydrocarbon content. Oxygen deficiency is recognized as the reason for sterol and UFA auxotrophy in *S. cerevisiae* (Rattray et al., 1975; Rosenfeld and Beauvoit, 2003). Anaerobic growth has also been characterized by lower PE contents and higher PC and PI levels, especially at the entrance of the stationary growth phase (Rattray et al., 1975).

5. ANTIOXIDANTS IN WINE

For many years, yeasts have been recognized as a source of antioxidant compounds (Forbes et al., 1958). More recently, knowledge of natural antioxidant compounds has been directed to the screening of microbial sources to replace the synthetic ones that are currently in use as food antioxidants. For this reason, yeast constituents are considered as compounds that possess nutritional value for humans and higher animals.

The interest of antioxidant compounds of wine started in 1992 with the publication of the "French Paradox" theory in which Renaud and de Lorgeril associated the French habit to drink red wine with lower mortality from heart disease caused by high consumption of saturated fats. Since then, regular moderate wine consumption in the daily diet is considered to be protective against oxidative stress-associated diseases (German and Walzem, 2000). Its benefits have been attributed to higher well-known levels of antioxidant compounds, such as vitamins C and E and polyphenolic flavonoids such as catechins, quercetin, resveratrol and, as more recently discovered, the bioactive compound melatonin (Auger et al., 2005; Mercolini et al., 2008, 2012; Minussi et al., 2003; Olas and Wachowicz, 2002; Ross and Kasum, 2002).

5.1. Melatonin

Melatonin is an indoleamine that is produced from the amino acid tryptophan, and it is known as a neurohormone because it was originally believed that this molecule was unique to the pineal gland of vertebrates. However, over the last two decades, it has been identified in a wide range of invertebrates, plants, bacteria and fungi (Hardeland and Poeggeler, 2003). Therefore, melatonin is currently considered a ubiquitous molecule that is present in most living organisms.

Melatonin was isolated and identified in the bovine pineal gland by Lerner and colleagues in 1958, their aim was to treat skin diseases such as vitiligo. However, their finding was that melatonin had the ability to change the color of the skin in amphibians and reptiles. Hence, its name is composed of “mela”, for its bleaching effect, and “tonin” because it derives from serotonin.

In the mid-1970s, Lynch and collaborators demonstrated that the production of melatonin exhibited a circadian rhythm in the human pineal gland. Since the early 1980s, numerous physiological functions have been attributed to melatonin, such as regulating circadian rhythms and synchronizing the reproductive cycle and oncostatic, anti-aging, antioxidant and anti-inflammatory activities; and even the modulation of neural, endocrine and immune functions (Eghbal et al., 2016; Romero et al., 2014). Thus, melatonin has revealed itself to be not only as a ubiquitously distributed but also a functionally diverse molecule.

Several studies have uncovered the presence of melatonin in grapes and wine. Since Iriti and colleagues first detected melatonin in the berry skins of grapes, other studies have measured this indolamine in all berry tissues (Vitalini et al., 2011). Furthermore, the melatonin concentration in grapes has been observed to be dependent on the maturation stage (Murch et al., 2010) or to fluctuate with the harvest schedule (Boccalandro et al., 2011).

Melatonin was firstly reported in Italian wines, ranging from approximately 0.4 – 0.5 ng/ mL (Mercolini et al., 2008). In addition to showing higher melatonin concentrations in wines (74-420 ng/mL), an additional study related its presence to the activity of the yeast involved in the fermentation process, specifically with the *S. cerevisiae* species

(Rodriguez-Naranjo et al., 2011a, 2011b). However, regarding the presence or production of melatonin in yeast, Sprenger et al., (1999) were the pioneers in relating the presence of melatonin to *S. cerevisiae*. Recently, other studies have reported the production of melatonin by non-*Saccharomyces* species, which are significantly present in grapes and at the beginning of alcoholic fermentation (Fernández-Cruz et al., 2017; Vigentini et al., 2015).

5.1.1. Molecular characteristics

Melatonin is also known as N-acetyl-5-methoxytryptamine (**Figure 22**), and it belongs to the family of methoxyindoles, which are synthesized from tryptophan.

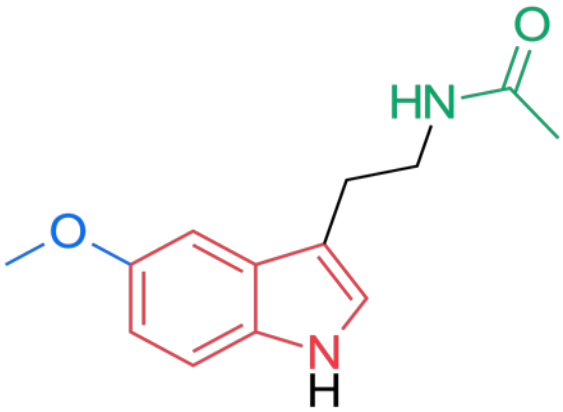

Figure 22. Chemical structure of melatonin.

Melatonin possesses an indolic-type ring with two functional groups, a methoxy group in position 5 and an N-acetyl group in position 3. These two groups not only contribute to its antioxidant properties, but they are also decisive in the specificity of receptor binding and for its amphipathic character. Its amphipathy enables melatonin to cross all biological membranes and remain at a sufficiently high proportion in the aqueous phase, giving a fundamental advantage relative to other relevant radical scavengers, which are either hydrophilic or lipophilic (Costa et al., 1995; Hardeland et al., 2006; Poeggeler et al., 2002; Shida et al., 1994).

5.1.2. Synthesis and regulation

Melatonin is primarily synthetized and secreted in the pineal gland located in the epithalamus, near the center of the brain between both hemispheres (**Figure 23**).

In the pineal gland, melatonin synthesis follows a rhythm determined by the suprachiasmatic nucleus (SCN), the biological clock. This route is dramatically repressed

by light during the day. During the night, the external signal processed by the retina is transmitted to the SCN and subsequently, neural signals are sent to the superior cervical ganglia (SGC) through a multisynaptic pathway. The noradrenaline liberated from postganglionic fibers binds to adrenoceptors in the membrane of the pinealocyte, leading to increased levels of cAMP, a second messenger that activates the arylalkylamine *N*-acetyltransferase enzyme (AA-NAT), which is the rate-limiting step in melatonin synthesis (Reiter et al., 2010, 2016).

The initial precursor of melatonin biosynthesis is the amino acid tryptophan. Tryptophan is captured from the bloodstream and hydroxylated enzymatically by tryptophan hydroxylase (TPH) in the mitochondria. The aromatic L-amino acid decarboxylase (AAAD) subsequently converts 5-hydroxytryptophan into serotonin in the cytosol, which is then acetylated by AA-NAT, forming N-acetylserotonin. Finally, N-acetylserotonin is converted into melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT). Alternatively, but at lower flux rates, melatonin can be formed via the O-methylation of serotonin and the subsequent N-acetylation of 5-methoxytryptamine or by the O-methylation of tryptophan followed by decarboxylation and N-acetylation (Hardeland et al., 1993; Sprenger et al., 1999; Tan et al., 2007). Melatonin is then released into the vascular system to access the remaining tissues and exert its pleiotropic functions (Guerrero et al., 2007).

Nevertheless, melatonin production is not limited to vertebrates; rather, it has been observed in almost all living organisms including invertebrates, plants, bacteria, etc., and none of them possess a pineal gland. Therefore, although the pineal gland is the largest producer of melatonin, its association with the pineal gland of vertebrates may be due to the need to produce and regulate melatonin in a circadian manner by neural information from light perception.

Although little information is available on melatonin biosynthesis in organisms other than vertebrates, the pathway and enzymes involved in yeast seem to be similar to the ones described in vertebrates (Mas et al., 2014; Sprenger et al., 1999). Most melatonin studies in yeasts are based on production; however, its functions and effects in yeast are completely unknown.

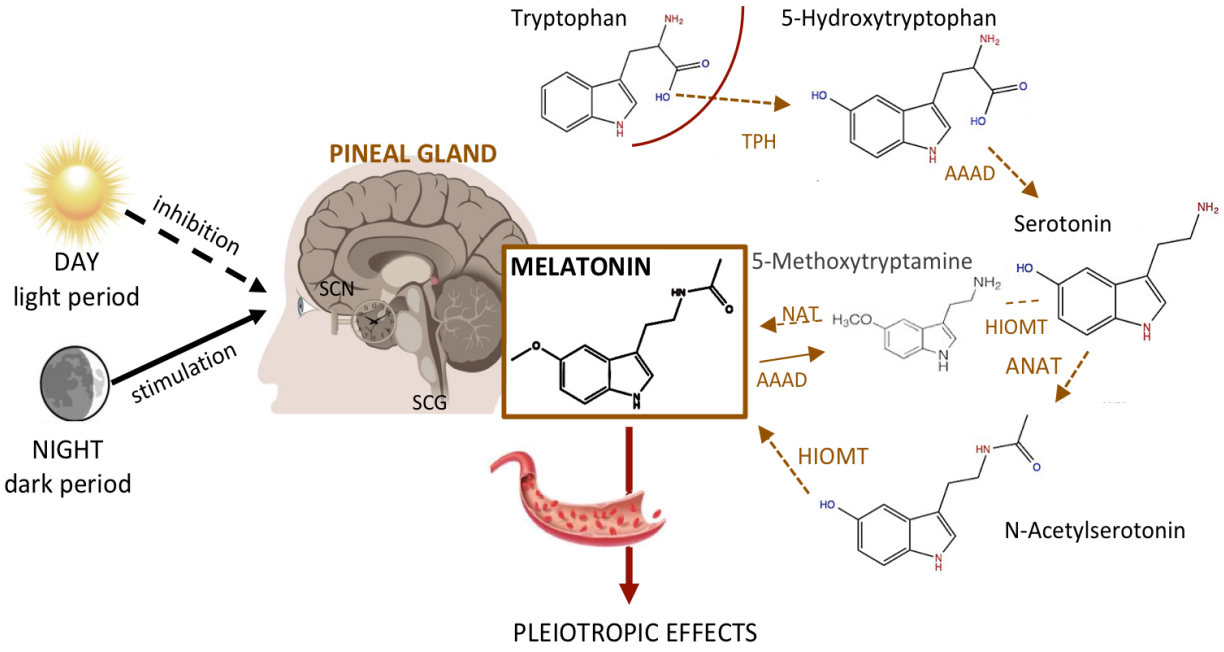

Figure 23. Physiological regulation of circadian melatonin production and the synthesis of melatonin from tryptophan (Adapted from Reiter et al., 2016). Abbreviations: SCN, suprachiasmatic nucleus; SCG, superior cervical ganglia; TPH, tryptophan hydroxylase; AAAD, aromatic amino acid decarboxylase; NAT, N-acetyltransferase; AANAT, arylalkylamine NAT; and HIOMT, hydroxyindole O-methyltransferase

5.1.3. Melatonin as an antioxidant

Since melatonin was discovered over 50 years ago, it has been linked to a wide range of functions in addition to its primary function as synchronizer of the biological clock, with its powerful antioxidant activity being amongst its best-studied attributes (Reiter et al., 2000, 2010; Štětinová et al., 2002).

In fact, melatonin has been compared with classic antioxidants such as glutathione, mannitol or vitamins C and E, with melatonin always performing in a superior manner (Martín et al., 2000; Qi et al., 2000). Hence, it is reasonable to believe that melatonin plays a vital role in the body's defenses by fighting free radical-related diseases.

- Melatonin's physiological weapons

Through a wide variety of different means, melatonin is exceptionally efficient in decreasing oxidative stress under a remarkably large number of circumstances. Furthermore, it is important to highlight that melatonin oxidation leads to the formation of other metabolites that are also biologically active, such as cyclic 3-hydroxymelatonin (3OHM), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-

methoxykynuramine (AMK) (Hardeland and Pandi-Perumal, 2005; Reiter et al., 2016). The multiple actions of melatonin and its metabolites against oxidative stress are summarized in **Figure 24**.

Due to its amphiphilic nature, melatonin easily reaches cell compartments, such as the nucleus and mitochondria, where it is able to act as a direct scavenger of oxygen-centered radicals and toxic ROS (Hardeland et al., 1993, 1995; Hardeland and Rodríguez, 1995). In addition, melatonin can indirectly reduce oxidative stress by stimulating the production and activation of endogenous antioxidant enzymes, interacting synergistically with other antioxidants to increase the antioxidant efficiency (Antolín et al., 1996; Gitto et al., 2001; López-Burillo et al., 2003; Rodriguez et al., 2004) and increasing the efficiency of the mitochondrial electron transport chain (León et al., 2005; López et al., 2009; Martín et al., 2000).

Because of these properties, melatonin is involved in different functions at the cellular and subcellular levels. Melatonin stabilized biological membranes, especially at the mitochondrial level; it protected DNA against oxidation and prevented cellular apoptosis. Furthermore, these antioxidant properties may be applicable to situations involving heightened cellular damage related to a dramatic increase in ROS, such as ageing.

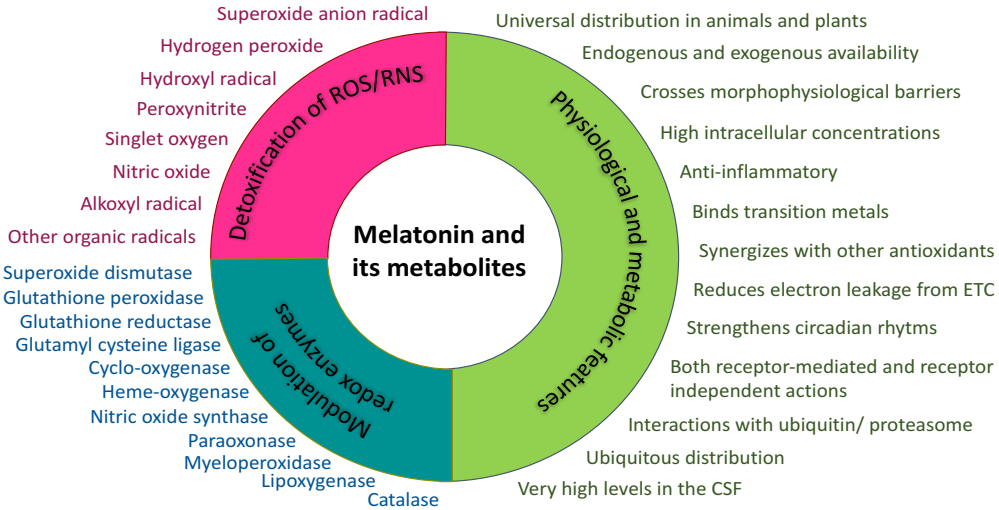

Figure 24. Multiple actions of melatonin in reducing oxidative stress (Reiter et al., 2016). The pink area indicates the reactive oxygen (ROS) and reactive nitrogen species (RNS) that are neutralized by melatonin and its metabolites. The blue area identifies enzymes that impact the redox state of the cell. The green area lists characteristics that aid melatonin in terms of its ability to quench free radicals and reduce oxidative damage.

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RESULTS

CHAPTER 1

The role of the membrane lipid composition in the oxidative stress tolerance of different wine yeasts

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Abstract

Oxidative stress is a common stress in yeasts during the stages of the winemaking process in which aerobic growth occurs, and it can modify the cellular lipid composition. The aim of this study was to evaluate the oxidative stress tolerance of two non-conventional yeasts (*Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) compared to *Saccharomyces cerevisiae*. Therefore, their resistance against H_2O_2, the ROS production and the cellular lipid composition were assessed. The results showed that the non-*Saccharomyces* yeasts used in this study exhibited higher resistance to H_2O_2 stress and lower ROS accumulation than *Saccharomyces*. Regarding the cellular lipid composition, the two non-*Saccharomyces* species studied here displayed a high percentage of polyunsaturated fatty acids, which resulted in more fluid membranes. This result could indicate that these yeasts have been evolutionarily adapted to have better resistance against the oxidative stress. Furthermore, under oxidative stress, non-*Saccharomyces* yeasts were better able to adapt their lipid composition as a defense mechanism by decreasing their percentage of polyunsaturated fatty acids and squalene and increasing their monounsaturated fatty acids.

Keywords: *Saccharomyces cerevisiae*, non-conventional yeast; ROS; oxidative stress; lipids, phospholipids, fatty acids, sterols

1. INTRODUCTION

Our understanding of the response and adaptation of yeasts to external environmental changes is very important within the biotechnological, pharmaceutical, food and beverage industries. Changes in the temperature, pH and osmotic pressure, nutrient starvation, ethanol toxicity, prolonged anaerobiosis, exposure to chemical preservatives and oxidative stress are the primary causes for the decrease in yeast viability and vitality in industrial processes (Briggs et al., 2004; Walker and Dijck, 2006; Gibson et al., 2007).

Oxidative stress is the result of an imbalance between the presence of reactive oxygen species (ROS) and the capacity of cells to detoxify these reactive intermediates of molecular oxygen, or to repair the resulting damage. Disturbances in the normal redox state of cells can damage all of their components, including lipids, carbohydrates, proteins and nucleic acids, and they may even induce programmed cell death (Moradas-Ferreira et al., 1996; Costa and Moradas-Ferreira, 2001; Gibson et al., 2008). Under normal physiological conditions, yeasts are able to effectively defend themselves against the direct consequences of stress exposure and damage by immediate cellular enzymatic and non-enzymatic responses, and finally, the adapted cells can resume proliferation (Jamieson, 1998; Moradas-Ferreira and Costa, 2000; Herrero et al., 2008).

Biological membranes are primarily made of proteins and phospholipids, and they form the first barrier that separates yeast cells and their organelle compartments from their external environment. Fatty acids (FAs), both free and as part of complex lipids, play a number of key roles in metabolism. They can be incorporated into phospholipids (PLs), which are considered as primary structural elements of biological membranes and sphingolipids, or they can serve as an energy reservoir in the form of triacylglycerols and steryl esters (Klug and Daum, 2014). Another important and essential group of lipids for maintaining the membrane integrity is the sterols, and ergosterol is the primary sterol in yeast (Daum et al., 1998; Klug and Daum, 2014). Membrane dysfunction can be associated with a loss of viability (Avery, 2011). Excessive ROS production can overwhelm the detoxifying mechanism and initiate changes in the lipid layers composition, resulting in a lipid peroxidation process, in which unsaturated lipids are converted into polar lipid hydroperoxides. PLs are particularly susceptible to oxidative damage mediated by ROS due to their content of polyunsaturated FAs (PUFAs), which

are more sensitive to peroxidation than monounsaturated FAs (MUFAs) (Howlett and Avery, 1997; Ayala et al., 2014). Extensive lipid peroxidation has been correlated with membrane disintegration and cell death. However, lethal consequences are not systematically observed because yeasts are able to compensate for these changes by modifying the membrane fluidity and phase transitions and by activating the cellular control of the chemical membrane composition. These changes in lipid composition are used by yeast as a defense mechanism, and they are important for conferring resistance to oxidative stress (Beney and Gervais, 2001; Los and Murata, 2004).

Yeast species, and even different strains of the same species, can exhibit variations in their membrane lipid composition (Hunter and Rose, 1972). In fact, yeast membranes are structurally and functionally dependent on the growth conditions, e.g., *Saccharomyces cerevisiae* is auxotrophic for oleic acid and ergosterol under strict anaerobic conditions (Walker and Dijck, 2006). Thus, the lipid composition should not be considered a fixed and static characteristic of a single yeast strain (Hunter and Rose, 1972; Torija et al., 2003; Beltran et al., 2008).

S. cerevisiae is the primary yeast species involved in wine fermentation (Ribéreau-Gayon, 1985; Fleet and Heard, 1993); however, many other yeast species can participate in different stages of the process (Beltran et al., 2002). Currently, non-*Saccharomyces* yeasts are studied to produce final products with improved organoleptic characteristics (Jolly et al., 2014, González-Royo et al., 2015). In general, these yeasts are not able to complete the alcoholic fermentation, several studies have demonstrated that some non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* used with sequential inoculation techniques, can positively contribute to the aroma profile, sensory complexity and color stability of the resulting product (Pretorius, 2000; Fleet, 2008; González-Royo et al., 2015; Mas et al., 2016). Nevertheless, despite the importance of these yeasts, there is still a lack of knowledge about the non-*Saccharomyces* species compared with *S. cerevisiae*. Therefore, studies on the effect of oxidative stress on non-*Saccharomyces* yeasts are interesting, not only for the investigating their cellular physiology but also to acquire a better understanding of the adaptations of non-conventional yeasts in response to the changes imposed by oxidative stress.

The goal of this study was to compare the effects of oxidative stress between *S. cerevisiae* and two species of non-*Saccharomyces* (*T. delbrueckii* and *M. pulcherrima*) by focussing on the effects on the lipid composition. To accomplish this goal, we evaluated the H_2O_2 resistance, intracellular ROS production and the lipid composition (FAs, PLs and sterols) in these species before and after oxidative stress exposure via H_2O_2.

2. MATERIAL AND METHODS

2.1. Yeast strains and growth conditions

The yeast strains used in this study were as follows: two strains of *S. cerevisiae* (the laboratory strain BY4742, (EUROSCARF collection, Frankfurt, Germany) and a commercial wine strain (QA23®)), two strains of *Torulaspora delbrueckii* (BIODIVA® (TdB) and Tdp) and two strains of *Metschnikowia pulcherrima* (FLAVIA® (MpF) and Mpp). Commercial *Saccharomyces* and non-*Saccharomyces* wine strains QA23, FLAVIA and BIODIVA were provided by Lallemand S.A. (Montreal, Canada), and the other two non-*Saccharomyces* strains (Tdp and Mpp) were isolated from natural musts that were taken from the Priorat Appellation of Origin (Catalonia, Spain) (Padilla et al., 2016; Padilla et al., 2017) and deposited in the Spanish Type Culture Collection (CECT) as CECT 13135 and CECT 13131, respectively.

The commercial strains were in active dry yeast form and were rehydrated according to the manufacturer's instructions. For all experiments, precultures for biomass propagation were prepared in YPD liquid medium (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain)) and incubated for 24 h at 28ºC with orbital shaking (120 rpm).

2.2. Resistance to hydrogen peroxide (H_2O_2)

Yeast resistance to H_2O_2 was assessed using the agar diffusion method (Bauer et al., 1966; Acar, 1980). Approximately $5x10^6$ cells were seeded with glass beads on YPD plates, and 6 mm blank disks were impregnated with 10 μL of 30% (v/v), 15% (v/v), 3% (v/v) or 0.3% (v/v) H_2O_2 (Perdrogen™, Sigma-Aldrich, MO, USA) and placed on the agar surface after drying. One disk impregnated with 10 μL of H_2O was used as the negative control. After 48 h of incubation at 28 ºC, the diameter of the inhibition haloes, including the disk, was measured with a ruler and photographed using a ProtoColHr automatic

colony counter (Microbiology International, Frederick, USA). The means of three biological replicates were calculated.

2.3. Determination of reactive oxygen species (ROS)

The effect of H_2O_2 on the intracellular ROS concentration was evaluated in the six yeast strains. The yeast cells were inoculated into 50 mL of YPD broth ($5x10^5$ cells/mL) and grown for 6 h (early exponential phase) at 28ºC with orbital shaking at 120 rpm. The cells were then exposed to different concentrations of H_2O_2 (from 2 mM to 8 mM) for 1 h, and the ROS were determined and compared to the control (sample without exposure to H_2O_2). Three biological replicates were set up for each condition. An ROS determination was performed according to the method described by Vázquez et al., (2017) using dihydrorhodamine 123 (DHR 123; Sigma-Aldrich) as an ROS indicator.

2.4. Experimental conditions for lipid analysis

Cells from each of the six yeast strains were inoculated into 450 mL of YPD broth to obtain an initial population of $5x10^5$ cells/mL and grown at 30ºC with orbital shaking at 130 rpm. After 6 h (early exponential phase), sublethal oxidative stress was induced in each strain by adding 2 mM H_2O_2 to the yeast culture. The optical density at 600 nm (OD_{600}) was measured at 6h (before stress) and 18 h after the stress exposure to allow the cells to respond/adapt to this stress (thus, 24 h from the beginning of the experiment). At these same time points, the total cells pellets (50 mL) were harvested for subsequent lipid analysis. Two biological replicates were set up for each strain.

2.5. Lipid analysis

2.5.1. Cell homogenates, protein quantification and lipid extraction

Homogenates of the yeast cells were obtained by extracting the pellets in the presence of glass beads using a Disruptor Genie® (Scientific Industries, Inc., NY, USA) at 4 ºC for 10 min. Proteins from the homogenates were then precipitated with 10% (v/v) trichloroacetic acid and quantified with the Folin phenol reagent (Lowry et al., 1951). The total lipids were extracted from cell fractions corresponding to 1 mg, 3 mg or 0.5 mg of total cell protein during the FA, PL or sterol assays, respectively, according to Folch et al., (1957).

2.5.2. Fatty acids

The cell FA composition was analyzed by gas liquid chromatography (GLC) according to Ruβmayer et al., (2015). In brief, the total FAs from lipid extracts (1 mg of total cell protein) were converted to methyl esters by methanolysis with sulfuric acid (2.5% in methanol (v/v)) and heating at 80 ºC for 90 min. These FA methyl esters were then extracted twice with light petroleum and water (3:1; v/v) by shaking on a Vibrax® orbital shaker (IKA, Staufen, Germany) for 30 min, and separated by GLC on a Hewlett-Packard 6890 gas-chromatograph (Agilent Technologies, CA, USA) using an HP-INNOWax capillary column (15 m x 0.25 mm x 0.50 μm film thickness) with helium as a carrier gas. Finally, the FAs were identified by comparing with a commercial FA methyl ester standard mix (NuCheck, Inc., MN, USA) and quantified using pentadecanoic acid (C15:0, Sigma-Aldrich) as an internal standard. Two analytical replicates were used for each biological replicate.

2.5.3. Phospholipids

The PLs were separated by two-dimensional thin layer chromatography (TLC) on Silica Gel 60 plates (Merck) using chloroform: methanol: ammonia solution (25%) (65:35:5; per vol.) as the first dimension solvent and chloroform: acetone: methanol: acetic acid: water (50:20:10:10:5; per vol.) as the second dimension solvent (Athenstaedt et. al., 1999). Individual PLs were visualized on TLC plates by staining with iodine vapor and then scraping the spots off the plate, which were quantified by measuring the amount of phosphate (Broekhuyse, 1968). The phosphate quantity was calculated as a relative amount of the total phosphate (%). Two analytical replicates were taken for each biological replicate.

2.5.4. Sterols

The individual sterol composition was determined by gas-liquid chromatography-mass spectrometry (GC-MS) after the alkaline hydrolysis of the lipid extracts (0.5 mg of total cell protein) (Quail and Kelly, 1996). GLC-MS was performed on a Hewlett-Packard 5690 Gas Chromatograph equipped with an HP 5972 mass selective detector using a capillary column (HP 5-MS; 30 m x 0.25 mm i.d. x 0.25 μm film thickness). The injection was set at 270 ºC using helium as the carrier gas with a constant flow rate set to 0.9 mL min^{-1}.

To identify the mass fragmentation pattern of each sterol, a cholesterol solution was used as an internal standard. The determinations were performed in duplicate.

2.6. Data analysis

The data were subjected to a one-way analysis of variance (ANOVA) and Tukey's post-hoc test to evaluate the effect of each treatment. The results were considered statistically significant at a p-values less than 0.05 (IBM SPSS Inc, XLSTAT Software). A Principal Component Analysis (PCA) was performed to visualize a 2D plot of the first two principal components (PCs) and heatmap of relative changes in lipid composition using XLSTAT Software.

3. RESULTS

3.1. Yeast resistance to hydrogen peroxide

The six yeast strains described above were plated on YPD medium, and the inhibition haloes around the disks that had been previously soaked with 3% (v/v) H_2O_2 were measured. The inhibition haloes for the *S. cerevisiae* strains (BY4742 and QA23) were 2.90 ± 0.19 cm and 1.80 ± 0.12 cm, respectively (**Figure 1A, B**). By contrast, the size of the inhibition haloes was significantly smaller for all the non-*Saccharomyces* strains. The *M. pulcherrima* strains, and especially Mpp, had the highest resistance against 3% (v/v) of H_2O_2 (**Figure 1E, F**; 1.2 ± 0.05 cm (MpF) and 0.75 ± 0.01 cm (Mpp)). Both *T. delbrueckii* strains exhibited similar inhibition haloes, with an intermediate size between the *S. cerevisiae* and *M. pulcherrima* strains (TdF, 1.40 ± 0.17; Tdp, 1.40 ± 0.02; **Figure 1C, D**).

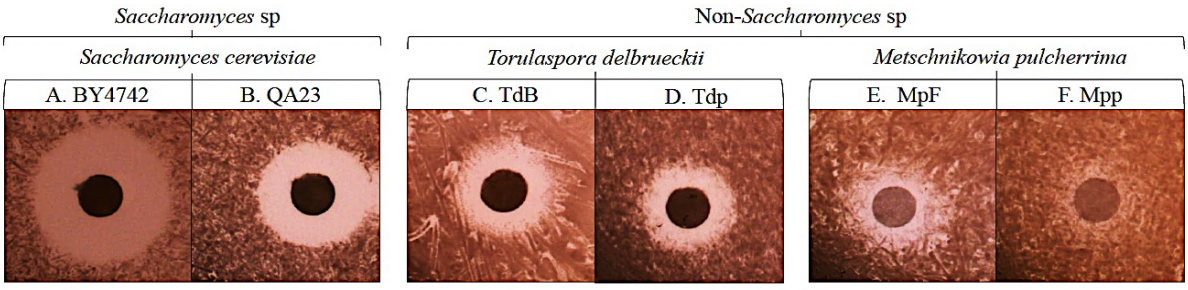

Figure 1. Resistance to H_2O_2 (10 µL from H_2O_2 3% (v/v)) by disk diffusion method for six yeast strains grown on YPD plates over 48 h. *Saccharomyces cerevisiae*: (A) BY4742 and (B) QA23 strains. *Torulaspora delbrueckii*: (C) TdB and (D) Tdp strains. *Metschnikowia pulcherrima*: (E) MpF and (F) Mpp strains.

At lower concentrations of H_2O_2 (0.3%), only BY4742 showed a small inhibition halo, while exposure to higher concentrations of H_2O_2 (15% and 30%) resulted in an increase in the sizes of inhibition haloes for all the strains (**Figure S1**). As with the 3% (v/v) H_2O_2, the *S. cerevisiae* strains were the most affected ones by high concentrations of this oxidant.

3.2. Determination of reactive oxygen species

For all the yeast species, the intracellular ROS levels were measured with and without H_2O_2 stress at the early exponential phase. Under these stress conditions, *S. cerevisiae* strains accumulated higher amounts of ROS than *non-Saccharomyces* species (**Figure 2**).

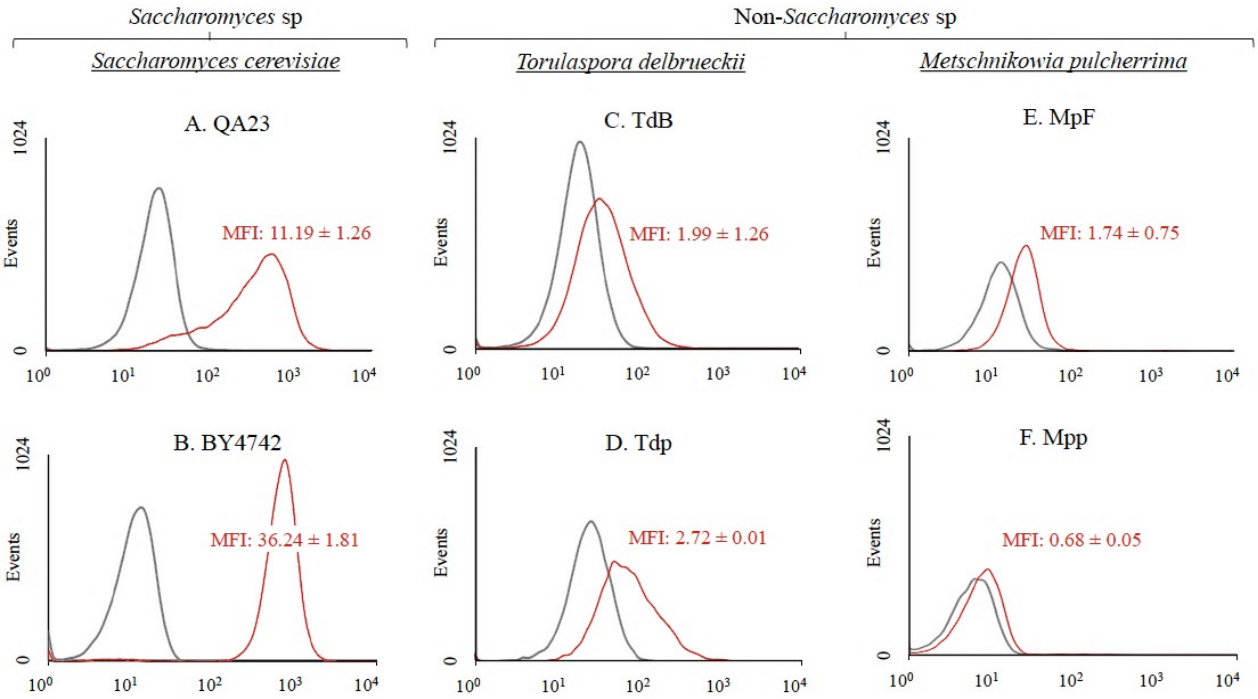

Figure 2. Effect of H_2O_2 on ROS accumulation as evaluated in six yeast strains with and without stress. The flow cytometry histogram profile expressed as the number of events with 0 mM (–) and 2 mM (–) of H_2O_2. The mean fluorescence index (MFI) was calculated according to Boettiger et al., (2001) as follows: [(geometric mean of the positive fluorescence) – (geometric mean of the control)] / (geometric mean of the control).

BY4742 was the least H_2O_2-resistant strain (**Figure 1A**), and it showed the highest levels of ROS (**Figure 2B**) followed by QA23 (**Figure 2A**) and the non-*Saccharomyces* strains (**Figure 2C-F**), with *M. pulcherrima* Mpp having the lowest levels of endogenous ROS (**Figure 2F**). Exposure to increasing concentrations of H_2O_2 from 2 mM to 8 mM resulted

in a gradual increase in ROS accumulation in *S. cerevisiae*. Instead, in non-*Saccharomyces*, practically no change in ROS accumulation was observed until the applied H_2O_2 concentrations reached 8 mM (depending on the species), showing very low MFI values at all concentrations (**Figure S2**).

3.3. Lipid composition before and after stress exposure

First, the FA, PL and sterol compositions in the six strains in this study were evaluated after 6 h of growth in a rich medium to study the differences in lipid composition between the three species used here (*S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima*). The cells were then subjected to oxidative stress (2 mM of H_2O_2), and the lipid composition of these six strains was analyzed after 18 h to determine how the different species could modify their lipid composition to better resist the oxidative stress exposure.

Fatty acid, phospholipid and sterol composition before stress exposure

FAs typically make up parts of complex lipids, and they are important structural components of biological membranes. An FA analysis of the total cell extracts showed differences between the species (**Table 1**). In the *S. cerevisiae* strains (QA23 and BY4742), MUFAs (palmitoleic (C16:1) and oleic (C18:1) acids) and palmitic acid (C16:0) represented almost 90% of the FA in the cell extracts. By contrast, the non-*Saccharomyces* strains contained a lower percentage of C16:1 (especially in the *M. pulcherrima* strains), which was compensated by the presence of linoleic acid (C18:2), a PUFA. Moreover, in the case of the *M. pulcherrima* strains, a low percentage of linolenic acid (C18:3) was also present. As a result of this fatty acid pattern, the *T. delbrueckii* strains presented higher UFA/SFA ratios than the other studied species.

PLs are major structural components of cell membranes and are essential for vital cellular processes. The PL percentages of the homogenates showed a similar composition in all the studied yeasts, with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) representing approximately 50% and 24% of the total PLs, respectively (**Table 1**). However, there were also small shifts in some PLs between different yeast species. In general, all the non-*Saccharomyces* strains showed a significantly lower percentage of dimethyl phosphatidylethanolamine (DMPE), and the *T. delbrueckii* strains had the lowest amounts of lysophospholipids (LP). Of the strains

studied here, Mpp showed the most different PL composition, resulting in the highest PC/PE and the lowest phosphatidylinositol/phosphatidylserine (PI/PS) ratios. In fact, the highest PI/PS ratio was found in the QA23 strain.

Table 1. Fatty acid (FA), phospholipid (PL) and sterol compositions of different strains after 6 h of growth in YPD medium. The QA23 and BY4742 strains are *S. cerevisiae* species, the TdB and Tdp strains belong to *T. delbrueckii,* and the MpF and Mpp strains belong to *M. pulcherrima*. Different letters in superscripts indicate significant differences between strains for each studied compound (P< 0.05).

	% Lípid composition	Yeast strain					
		QA23	BY4742	TdB	Tdp	MpF	Mpp
Fatty acids (FAs)	Myristic (C14:0) acid	2.58 ± 0.34^{a}	2.45 ± 0.59^{a}	1.30 ± 0.34^{b}	1.55 ± 0.09^{b}	0.98 ± 0.02^{c}	0.89 ± 0.30^{c}
	Palmitic (C16:0) acid	23.17 ± 1.75^{a}	23.55 ± 0.67^{a}	18.30 ± 0.30^{b}	20.03 ± 0.26^{c}	23.27 ± 1.47^{a}	21.76 ± 0.80^{a,d}
	Palmitoleic (C16:1) acid	36.29 ± 3.14^{a}	42.64 ± 0.63^{b}	21.14 ± 2.28^{c}	19.81 ± 0.21^{c}	2.95 ± 0.09^{d}	3.69 ± 0.72^{d}
	Stearic (C18:0) acid	7.64 ± 1.57^{a}	8.36 ± 1.16^{a}	8.09 ± 0.19^{a}	7.40 ± 0.56^{a}	9.37 ± 0.34^{a}	8.62 ± 0.19^{a}
	Oleic (C18:1) acid	30.32 ± 0.53^{a}	22.99 ± 1.14^{b}	24.21 ± 0.52^{b}	25.59 ± 0.86^{b,c}	17.21 ± 0.61^{d}	26.43 ± 1.10^{b,c}
	Linoleic (C18:2) acid	n.d.	n.d.	26.95 ± 2.21^{a}	25.63 ± 0.26^{a}	40.17 ± 1.29^{b}	34.67 ± 1.05^{d}
	Linolenic (C18:3) acid	n.d.	n.d.	n.d.	n.d.	6.05 ± 0.16^{a}	3.95 ± 0.15^{b}
	Total FAs [#]	86.25 ± 1.14^{a}	87.84 ± 1.48^{a}	76.53 ± 9.34^{a}	91.9 ± 2.72^{a,b}	65.75 ± 6.17^{c}	80.49 ± 0.80^{a,d}
	C16:1/ C18:1	1.20 ± 0.08^{a}	1.86 ± 0.12^{b}	0.87 ± 0.11^{c}	0.77 ± 0.03^{c}	0.17 ± 0.00^{d}	0.14 ± 0.03^{e}
	UFA/SFA	2.01 ± 0.33^{a}	1.91 ± 0.04^{a}	2.61 ± 0.06^{b}	2.45 ± 0.05^{b}	1.98 ± 0.16^{a}	2.20 ± 0.13^{a}
	UI[*]	0.67 ± 0.03^{a}	0.66 ± 0.00^{a}	0.99 ± 0.03^{b}	0.97 ± 0.00^{b}	1.19 ± 0.03^{c}	1.11 ± 0.02^{d}
Phospholipids (PLs)	PI (Phosphatidylinositol)	12.49 ± 1.31^{a}	12.19 ± 0.61^{a}	14.63 ± 2.01^{a}	12.93 ± 1.13^{a}	10.10 ± 0.46^{b}	8.94 ± 1.44^{b}
	PS (Phosphatidylserine)	4.01 ± 0.21^{a}	6.19 ± 0.76^{b}	5.82 ± 1.61^{a,b}	5.22 ± 1.29^{a,b}	6.45 ± 1.42^{b}	6.91 ± 0.63^{b,c}
	PC (Phosphatidylcoline)	43.47 ± 0.51^{a}	40.63 ± 1.07^{b}	44.23 ± 1.55^{a}	46.65 ± 2.00^{a}	44.10 ± 4.56^{a}	51.53 ± 0.93^{c}
	PE (Phosphatidylethanolamine)	24.61 ± 1.38^{a}	24.07 ± 1.66^{a}	23.34 ± 2.64^{a}	23.32 ± 4.19^{a}	21.71 ± 0.17^{a,b}	20.39 ± 1.03^{b}
	CL (Cardiolipin)	5.66 ± 0.23^{a}	2.86 ± 0.18^{b}	6.02 ± 1.33^{a}	6.87 ± 1.24^{a}	7.03 ± 0.23^{a,c}	4.71 ± 0.94^{a}
	DMPE (Dimethyl-phosphatidylethanolamine)	3.76 ± 1.12^{a}	5.39 ± 0.10^{b}	0.99 ± 0.25^{c}	1.18 ± 0.62^{c}	1.60 ± 0.37^{c}	1.02 ± 0.36^{c}
	PA (Phosphatidic acid)	2.00 ± 0.28^{a}	4.48 ± 2.57^{a}	4.18 ± 1.27^{a,b}	3.39 ± 1.37^{a}	4.67 ± 0.67^{a,b}	3.75 ± 0.11^{a,b}
	LP (Lysophospholipids)	4.01 ± 0.21^{a}	4.19 ± 2.06^{a}	0.79 ± 0.25^{b}	0.43 ± 0.54^{b}	4.35 ± 1.48^{a}	2.75 ± 0.29^{a,c}
	PI/PS	3.11 ± 0.16^{a}	2.00 ± 0.34^{b}	2.56 ± 0.36^{b}	2.52 ± 0.40^{b}	1.61 ± 0.43^{b,c}	1.31 ± 0.33^{c}
	PC/PE	1.77 ± 0.12^{a}	1.69 ± 0.16^{a}	1.90 ± 0.14^{a}	2.02 ± 0.27^{a}	2.03 ± 0.19^{a}	2.53 ± 0.17^{b}
Sterols	Squalene	18.51 ± 2.41^{a}	2.91 ± 0.52^{b}	33.15 ± 3.19^{c}	36.37 ± 3.93^{c}	6.24 ± 0.09^{d}	n.d.
	Zymosterol	16.78 ± 0.80^{a}	8.39 ± 0.29^{b}	4.16 ± 0.56^{c}	6.15 ± 1.19^{d}	0.91 ± 0.33^{e}	n.d.
	4-methylzymosterol	1.21 ± 0.08^{a}	n.d.	n.d.	1.71 ± 0.87^{a}	n.d.	n.d.
	Fecosterol	6.48 ± 0.05^{a}	14.11 ± 0.32^{b}	9.36 ± 0.37^{c}	6.02 ± 0.07^{d}	n.d.	2.24 ± 1.16^{e}
	14-methylfecosterol	n.d.	n.d.	n.d.	1.17 ± 0.10	n.d.	n.d.
	Episterol	1.38 ± 0.06^{a}	n.d.	1.08 ± 0.07^{b}	4.51 ± 0.89^{c}	n.d.	n.d.
	Lanosterol	2.75 ± 0.04^{a}	3.72 ± 0.22^{b}	11.26 ± 1.31^{c}	5.67 ± 0.89^{d}	3.91 ± 1.32^{a,b,d}	1.22 ± 1.03^{e}
	Ergosterol	52.89 ± 1.58^{a}	70.88 ± 0.77^{b}	40.99 ± 2.00^{c}	38.39 ± 1.70^{c}	88.95 ± 1.56^{d}	96.54 ± 1.89^{d}
	Total sterols [#]	26.92 ± 0.69^{a}	16.52 ± 0.40^{b}	27.94 ± 0.87^{a}	24.97 ± 4.69^{a}	3.78 ± 0.86^{c}	8.16 ± 0.50^{d}
	Ergosterol/Squalene	2.88 ± 0.46^{a}	24.78 ± 4.71^{b}	1.24 ± 0.18^{c}	1.07 ± 0.16^{c}	13.95 ± 0.05^{d}	-

[#] (µg/ mg protein)

[*]UI, unsaturation index. The unsaturation index was defined as follows: ((percentage of C16:1 + percentage of C18:1) + 2(percentage of C18:2) + 3(percentage of C18:3)) /100 (Rodríguez-Vargas, et al., 2007).

Sterols are essential lipid constituents of membranes, and they were also analyzed from yeast homogenates (**Table 1**). The total sterol content was significantly lower in both *M. pulcherrima* strains (4-8 μg sterol/mg total protein) compared to the other studied strains (approximately 15-25 μg sterol/mg total protein). The primary sterol in all the strains was ergosterol, although the percentages varied markedly between species (38-96%). In the *M. pulcherrima* strains, practically the only sterol that was quantified was ergosterol. Instead, the *T. delbrueckii* strains exhibited the lowest percentage of ergosterol (38-40%) and the highest levels of squalene (33-36%) and lanosterol (6-11%). For *S. cerevisiae*, the strains used in this study showed significant differences in their sterol compositions. Thus, without accounting for the ergosterol, QA23 had a higher percentage of squalene and zymosterol, whereas BY4742 contained a higher proportion of fecosterol than the other strains.

Fatty acid, phospholipid and sterol composition after stress exposure

Differences in the cellular lipid compositions before and after stress exposure are shown in **Figure 3**. QA23 and BY4742 showed only a few changes in the FA composition (**Figure 3**). In QA23, slightly decreased amounts of C16:0 and C18:0 and increased amounts of C16:1 were found, leading to an increase in the unsaturated FA/saturated FA (UFA/SFA) ratio and in the unsaturation index (UI) (**Figure 3**). By contrast, non-*Saccharomyces* species experienced highly modified FA compositions after stress. The percentage of PUFAs (C18:2, and for Mp also C18:3) and SFA (C16:0 and C18:0) decreased, whereas the MUFAs (C16:1 and C18:1) strongly increased. In fact, under these stress conditions, the percentages of C18:1 in non-*Saccharomyces* strains were higher than they were in *S. cerevisiae* (**Table 2**), unlike what we observed under the control conditions. These variations resulted in a higher UFA/SFA ratio and a lower UI in non-*Saccharomyces* species (**Figure 3** and **Table 2**).

The PL composition was slightly affected by stress, but the total PL profile remained similar between species (**Table 2**). The PC and PE persisted as the primary PLs in all the yeast strains, and although all the strains showed increased PC/PE ratios after stress (**Figure 3**), this increase was statistically higher in the TdB and Mpp strains (**Table 2**). Moreover, the PI/PS ratio decreased in non-*Saccharomyces* strains but increased greatly in *S. cerevisiae* (**Figure 3**) due to the increased PI and the decreased PS in QA23 and

BY4742. Notably, there was a significant decrease in the cardiolipin (CL) content under stress exposure in all the strains except for Mpp, the most H_2O_2-resistant strain, which increased the CL content under these conditions (**Figure 3**). Moreover, both *M. pulcherrima* strains showed significantly decreased amounts of lysophospholipids (LP), whereas the *S. cerevisiae* strains, especially BY4742, were the ones with higher LP contents after stress (**Table 2**).

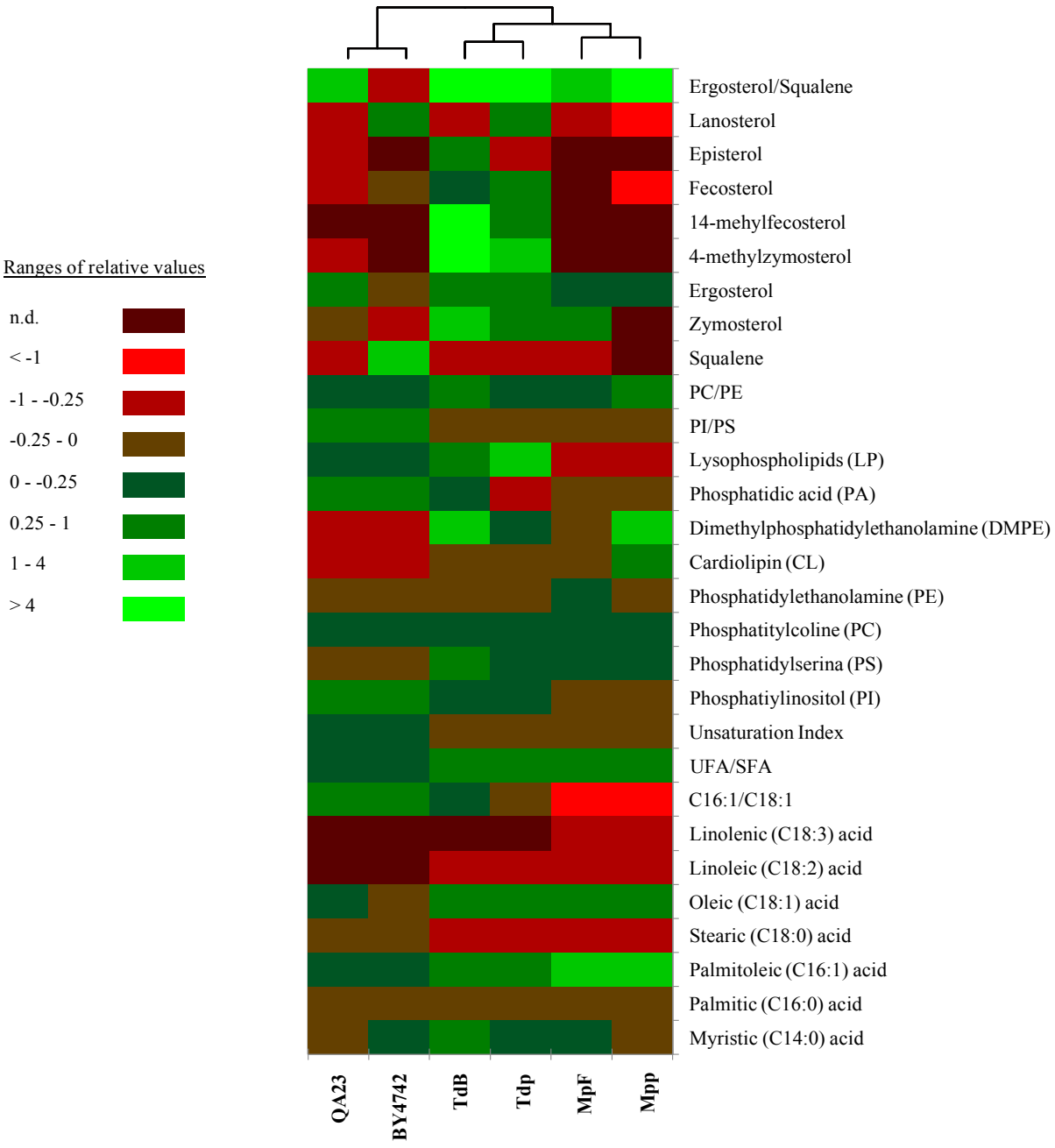

Figure 3. Heatmap representing the fold changes in the lipid composition of cells following stress exposure relative to the cells before stress exposure. *S. cerevisiae* strains: QA23 and BY4742; *T. delbrueckii* strains: TdB and Tdp; and *M. pulcherrima* strains: MpF and Mpp.

Table 2. Fatty acid (FA), phospholipid (PL) and sterol compositions of different strains after stress exposure at 24 h of growth in YPD medium. The QA23 and BY4742 strains belong to the *S. cerevisiae* species, the TdB and Tdp strains to *T. delbrueckii* and MpF and Mpp strains to *M. pulcherrima*. Different letters in superscripts indicate significant differences within a line and asterisks indicate differences between cells before (Table 1) and after stress exposure (P< 0.05).

	% Lípid composition	Yeast strain					
		QA23	BY4742	TdB	Tdp	MpF	Mpp
Fatty acids (FAs)	Myristic (C14:0) acid	2.03 ± 0.71^{a}	2.35 ± 0.35^{a}	1.83 ± 0.19^{a}	1.68 ± 0.09^{a,b}	1.12 ± 0.07^{c}	0.68 ± 0.16^{d}
	Palmitic (C16:0) acid	19.88 ± 0.20^{a,*}	23.63 ± 0.73^{b}	13.94 ± 2.11^{c,*}	15.18 ± 0.08^{c,*}	21.75 ± 0.93^{b}	17.17 ± 0.63^{c,*}
	Palmitoleic (C16:1) acid	40.44 ± 0.33^{a,*}	43.28 ± 0.89^{b}	33.32 ± 0.58^{c,*}	31.07 ± 0.02^{d,*}	10.81 ± 0.45^{e,*}	8.59 ± 0.29^{f,*}
	Stearic (C18:0) acid	6.61 ± 0.86^{a}	8.47 ± 1.05^{a}	3.91 ± 0.41^{b,*}	4.03 ± 0.02^{b,*}	4.14 ± 0.61^{b,*}	3.48 ± 0.08^{b,c*}
	Oleic (C18:1) acid	30.53 ± 0.32^{a}	22.27 ± 1.25^{b}	39.96 ± 1.24^{c,*}	41.98 ± 0.73^{c,*}	31.28 ± 1.16^{a,*}	52.23 ± 0.56^{d,*}
	Linoleic (C18:2) acid	n.d.	n.d.	7.03 ± 0.89^{a,*}	6.07 ± 0.76^{a,*}	30.00 ± 0.80^{b,*}	17.32 ± 0.39^{c,*}
	Linolenic (C18:3) acid	n.d.	n.d.	n.d.	n.d.	0.90 ± 0.16^{a,*}	0.54 ± 0.05^{b,*}
	Total FAs [#]	86.26 ± 3.70^{a}	68.12 ± 4.15^{b,*}	101.08 ± 0.43^{c,*}	93.42 ± 3.53^{a}	88.02 ± 0.34^{a,*}	74.2 ± 1.43^{d,*}
	C16:1/ C18:1	1.33 ± 0.00^{a,*}	1.95 ± 0.07^{b}	0.83 ± 0.01^{d}	0.74 ± 0.01^{e}	0.35 ± 0.03^{f,*}	0.16 ± 0.00^{g}
	UFA/SFA	2.49 ± 0.02^{a,*}	1.91 ± 0.18^{b}	4.13 ± 0.71^{c,*}	3.79 ± 0.00^{c,*}	2.70 ± 0.03^{d,*}	3.69 ± 0.16^{c,*}
	UI [*]	0.71 ± 0.01^{a,*}	0.66 ± 0.02^{a}	0.87 ± 0.04^{b,*}	0.85 ± 0.01^{b,*}	1.05 ± 0.01^{c,*}	0.97 ± 0.01^{d,*}
Phospholipids (PLs)	PI (Phosphatidylinositol)	15.86 ± 3.40^{a}	15.83 ± 2.37^{a,*}	14.52 ± 2.82^{a}	13.48 ± 0.50^{a}	7.71 ± 0.46^{b,*}	8.37 ± 0.19^{a}
	PS (Phosphatidylserine)	3.46 ± 0.21^{a,*}	5.09 ± 0.63^{b}	7.32 ± 1.69^{b}	5.76 ± 1.65^{a,b}	6.35 ± 0.54^{b}	7.37 ± 0.42^{b}
	PC (Phosphatidylcoline)	46.55 ± 2.85^{a,*}	41.38 ± 1.80^{b}	45.90 ± 2.37^{a}	51.73 ± 1.87^{b,*}	51.12 ± 1.69^{b,*}	53.90 ± 3.05^{b}
	PE (Phosphatidylethanolamine)	23.06 ± 0.54^{a}	23.82 ± 0.67^{a}	19.73 ± 2.88^{b,*}	21.36 ± 2.97^{a,b}	22.13 ± 1.11^{b}	17.01 ± 1.01^{c,*}
	CL (Cardiolipin)	3.44 ± 0.45^{a,*}	1.57 ± 0.07^{b,*}	4.67 ± 0.12^{c,*}	4.29 ± 0.22^{c,*}	5.93 ± 0.50^{c,*}	6.64 ± 1.48^{c}
	DMPE (Dimethyl-phosphatidylethanolamine)	2.34 ± 0.15^{a,*}	3.63 ± 0.82^{b,*}	1.61 ± 0.73^{c}	1.28 ± 0.28^{c}	1.10 ± 0.44^{c}	1.62 ± 0.74^{c}
	PA (Phosphatidic acid)	2.87 ± 0.60^{a}	4.54 ± 0.76^{a,b}	3.97 ± 2.08^{a,b}	1.33 ± 1.37^{a}	3.90 ± 0.70^{a,b}	3.65 ± 0.19^{a,b}
	LP (Lysophospholipids)	2.42 ± 0.27^{a,*}	4.14 ± 0.11^{b}	1.28 ± 1.07^{c}	0.75 ± 0.80^{c}	1.77 ± 0.06^{c,*}	1.44 ± 0.16^{c,*}
	PI/PS	4.64 ± 0.35^{a,*}	3.10 ± 0.26^{b,*}	1.99 ± 0.07^{c,*}	2.33 ± 0.13^{d}	1.21 ± 0.03^{e}	1.13 ± 0.09^{e}
	PC/PE	2.02 ± 0.08^{a,*}	1.74 ± 0.03^{b}	2.36 ± 0.16^{c,*}	2.40 ± 0.22^{c}	2.32 ± 0.19^{c}	3.18 ± 0.36^{d,*}
Sterols	Squalene	6.37 ± 1.75^{a,*}	11.01 ± 0.86^{b,*}	3.06 ± 0.15^{c,*}	3.34 ± 1.52^{c,*}	2.51 ± 0.49^{c,*}	n.d.
	Zymosterol	13.90 ± 0.57^{a,*}	6.10 ± 1.16^{b,*}	11.48 ± 1.56^{a,*}	9.09 ± 0.27^{c,*}	1.28 ± 0.02^{d,*}	n.d.
	4-methylzymosterol	0.90 ± 0.07^{a,*}	n.d.	2.29 ± 0.53^{b,*}	2.77 ± 0.86^{b,*}	n.d.	n.d.
	Fecosterol	4.06 ± 0.17^{a,*}	11.09 ± 1.30^{b,*}	11.78 ± 0.21^{b,*}	9.63 ± 0.77^{b,*}	n.d.	n.d.
	14-methylfecosterol	n.d.	n.d.	1.91 ± 0.34^{a,*}	1.77 ± 0.38^{a,*}	n.d.	n.d.
	Episterol	0.31 ± 0.44^{a,*}	n.d.	1.59 ± 0.08^{b,*}	1.73 ± 0.30^{c,*}	n.d.	n.d.
	Lanosterol	1.63 ± 0.17^{a,*}	6.05 ± 0.34^{b,*}	7.88 ± 0.23^{c,*}	8.97 ± 1.38^{c,*}	1.76 ± 0.59^{a}	n.d.
	Ergosterol	72.83 ± 1.48^{a,*}	65.15 ± 1.94^{b,*}	60.89 ± 3.34^{b,*}	62.69 ± 2.81^{b,*}	94.47 ± 1.69^{c,*}	100 ± 0.00^{d,*}
	Total sterols [#]	40.91 ± 2.10^{a,*}	20.00 ± 2.17^{b,*}	43.41 ± 2.48^{a,*}	27.80 ± 1.60^{c}	8.52 ± 1.00^{d,*}	9.99 ± 1.02^{d,*}
	Ergosterol/Squalene	11.90 ± 3.49^{a,*}	2.88 ± 0.46^{b,*}	19.92 ± 2.04^{c,*}	21.13 ± 2.46^{c,*}	38.26 ± 2.46^{d,*}	-

[#] (µg/ mg protein)

[*]UI, unsaturation index. The unsaturation index was defined as follows: ((percentage of C16:1 + percentage of C18:1) + 2(percentage of C18:2) + 3(percentatge of C18:3)) /100 (Rodríguez-Vargas, et al., 2007).

After the stress, ergosterol remained the primary sterol in all the studied yeasts and the only one in Mpp (**Table 2**). However, a different behavior was observed between the wine yeast strains and laboratory strain BY4742. All the wine yeasts showed increased ergosterol contents and decreased squalene contents under stress (resulting in an increase of the ergosterol/squalene ratio; **Figure 3**). However, BY4742 showed the opposite behavior, with increasing squalene and decreasing ergosterol contents, resulting in a decrease in the ergosterol/squalene ratio (**Figure 3**). In fact, BY4742 showed the highest value for this ratio before stress and the lowest after stress (**Table 2**). However, the *T. delbrueckii* strains showed the highest ergosterol/squalene ratios under stress (mostly due to the drop in squalene content), and they were the strains that had more diverse sterol compounds and the only species that exhibited methyl fecosterol. In fact, whereas both *S. cerevisiae* strains experienced decreases in their zymosterol and fecosterol percentages under stress, the *T.delbrueckii* strains increased their component (**Figure 3**).

3.4. Principal component analysis (PCA)

PCA was applied to correlate the different variables (lipid composition, inhibition haloes and ROS levels (MFI)) and highlight some grouping patterns within the different species under different conditions. Before stress (**Figure 4A**), the species were clearly separated into three groups by their lipid composition (**Table 1**), with *M. pulcherrima* being the most diverse compared to *S. cerevisiae* and *T. delbrueckii*. Both *M. pulcherrima* strains (MpF and Mpp) were different from the other strains in that they exhibited higher ergosterol and PS percentages and lower PI and PE. Furthermore, the percentage of total PUFAs (C18:2 and C18:3) was clearly higher in the *M. pulcherrima* strains (the *T. delbrueckii* only showed low levels of C18:2, and *S. cerevisiae* had no PUFAs in its lipid composition). Both *S. cerevisiae* strains were characterized by high levels of myristic acid (C14:0) and oleic acid (C16:1), DMPE, PI/PS ratios and zymosterol and low UI values. The lowest LP, C16:0 and ergosterol contents and the highest squalene content and UFA/SFA ratio were characteristics of the *T. delbrueckii* species, which showed similarities with the other non-*Saccharomyces* but also with *Saccharomyces*.

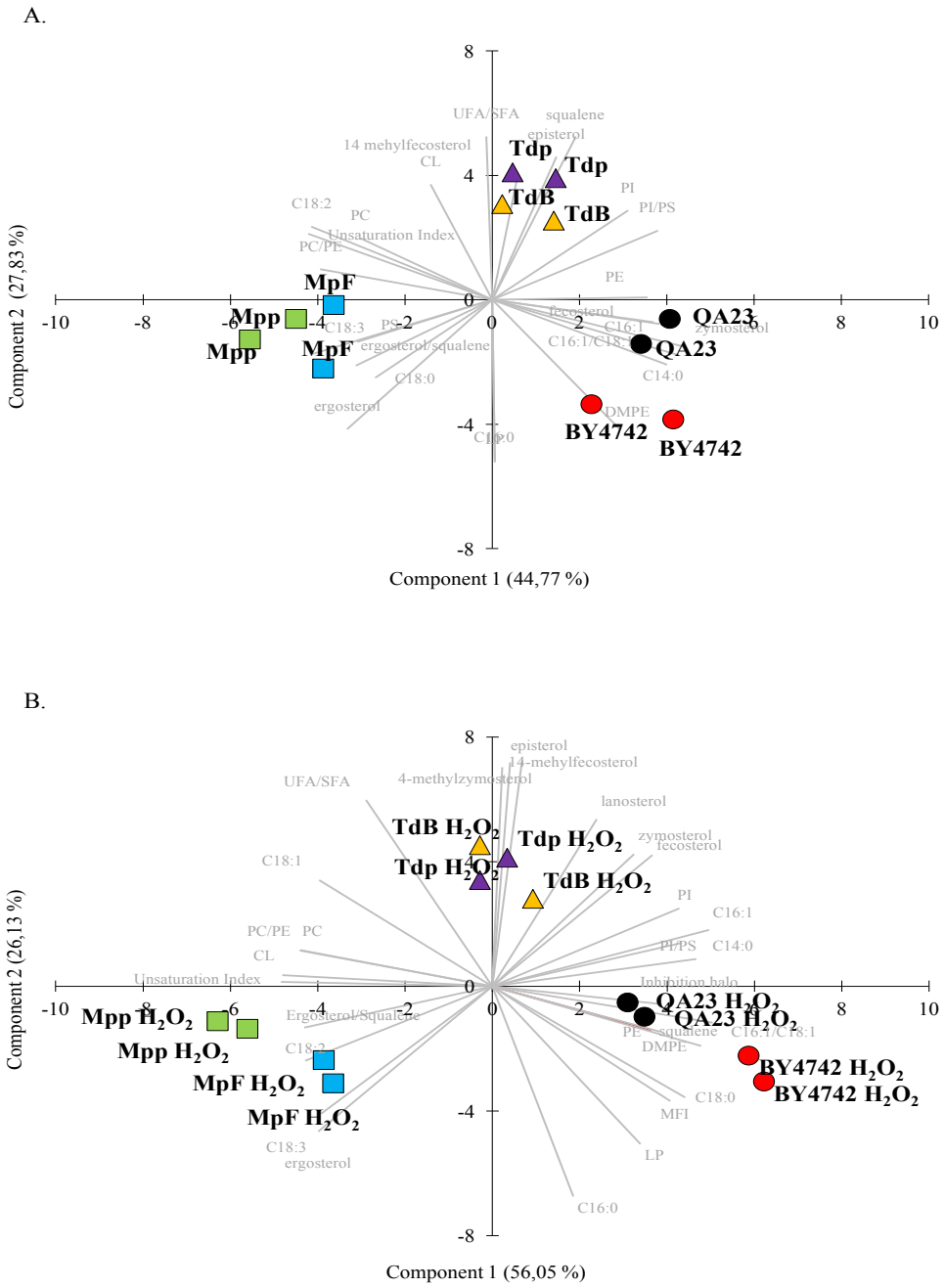

Figure 4. Biplots of principal components analysis (PCA) using fatty acids (FAs), phospholipids (PLs), sterols, inhibition halo measures and ROS accumulation markers (MFI) as variables. *S. cerevisiae* strains: QA23 (●) and BY4742 (●); *T. delbrueckii* strains: TdB (△) and Tdp (▲); *M. pulcherrima* strains: MpF (■) and Mpp (■). The explicative variables were distributed along the PCA as follows: (A) Biplot with 72.60% of the variance before the oxidative stress was applied. Component 1: (+); phosphatidylinositol (PI), phosphatidylethanolamine, (PE), PI/ phosphatidylserine (PI/PS) ratio, myristic (C14:0) and palmitoleic (C16:1) acids, C16:1/oleic (C16:1/C18:1) ratio, fecosterol and zymosterol. (-); PS, phosphatidylcholine (PC), PC/PE ratio, stearic (C18:0), linoleic (C18:2) and linolenic (C18:3) acids, ergosterol, unsaturated index and ergosterol/squalene ratio. Component 2: (+); unsaturated/saturated (UFA/SFA) ratio, cardiolipin (CL), squalene, 14-methylfecosterol and episterol. (-); dimethylphosphatidylethanolamine (DMPE), lysophospholipids (LP) and palmitic (C16:0) acid. (B) Biplot with 82.18% of the variance after oxidative stress (2 mM H_2O_2) was applied. Component 1: (+); inhibition halos, MFI, C14:0, C16:0, C16:1, C18:0, and C16:1/ C18:1 ratios, squalene, fecosterol, zymosterol, PI, PE, DMPE and PI/PS. (-); C18:1, C18:2, C18:3, ergosterol, ergosterol/squalene ratio, PC, CL, PC/PE ratio and unsaturation index. Component 2: (+); 4-methilzymosterol, 14-methylfecosterol, episterol, lanosterol and UFA/SFA ratio. (-); LP and C16:0.

The key indicative features of oxidative stress were the higher ROS accumulation (**Figure 2**) and higher inhibition haloes (**Figure 1**), which were positively correlated with the percentage of C14:0, stearic acid (C18:0), C16:1, PI, PE, DMPE, LP and squalene, and the PI/PS and C16:1/C18:1 ratios (**Figure 4B**, positive component 1 and **Table S1**). However, high ROS and inhibition haloes were negatively correlated with the UFA (C18:1, C18:2 and C18:3), UI, CL, PC and ergosterol contents, and with the ratios of PC/PE and ergosterol/squalene (**Figure 4B**, negative component 1 and **Table S1**). Thus, both *S. cerevisiae* strains were clearly different from both non-*Saccharomyces* species because they were grouped on the positive side of component 1, which is indicative of less stress tolerance (with BY4742 having higher positive values). *M. pulcherrima* strains were placed on the opposite side (negative component 1), with Mpp being the strain that exhibited more negatives values, indicating a higher resistance to stress. Thus, the component 1 places the strains according to their resistance to oxidative stress, with the less H_2O_2-tolerant strain BY4742 on one side, and Mpp on the opposite/negative side, which was the most resistant to this stress.

Although the differences between species in terms of lipid composition increased under stress, it is important to highlight that the primary differences were already observed before stress exposure (**Figure S1** and **Table S2**).

4. DISCUSSION

Although *S. cerevisiae* is the wine yeast par excellence due to its fermentative capacity, there is currently a strong interest that is being driven by consumer and industry demand for wines with improved characteristics to study the possibility of using non-conventional yeasts with peculiar features in industrial fermentations. Under these conditions, yeasts are exposed to a variety of stresses. Many studies on stress resistance have been performed in *S. cerevisiae*, but few have addressed other yeast species, which have also shown a significant impact on food and beverage production (Pretorius, 2000). In this study, we evaluated oxidative stress tolerance in selected non-*Saccharomyces* wine strains, namely, *T. delbrueckii* and *M. pulcherrima*, and we compared it to the *S. cerevisiae* response.

Our findings clearly indicated that these non-conventional yeasts are more tolerant to oxidative stress than *S. cerevisiae*. As reported elsewhere (Moradas-Ferreira et al., 1996;

Jamieson, 1998; Moradas-Ferreira and Costa, 2000), exposing yeast to H_2O_2 was associated with a rapid ROS generation and a loss of viability, at least until the yeast manages to adapt to the new environmental conditions, i.e., after the activation of defense mechanisms to maintain a proper redox state. Under our conditions, the *M. pulcherrima* species, and especially the autochthonous strain (Mpp), exhibited the greatest resistance to oxidative stress (low ROS generation and higher H_2O_2 tolerance). Both *T. delbrueckii* strains, also showed a higher oxidative resistance compared with *S. cerevisiae* as reported by Alves-Araújo et al., 2004 in a baking industry study. Furthermore, all the wine yeasts tested here were clearly more resistant to oxidative stress than the laboratory strain, probably due to their adaptive evolution to adverse stress conditions (Querol et al., 2003, Guillamón and Barrio, 2017). In fact, the BY4742 strain grew poorly after stress was applied, achieving only one more generation after stress exposure in liquid medium (data not shown). BY4742 is part of a set of deletion strains derived from S288C (Branchmann et al., 1998) with very poor fermentation capacity (Rossouw et al., 2013). Unlike wine yeast strains, BY4742 is not adapted to withstand adverse growth conditions such as those found during the fermentation process (Carrasco et al., 2001).

The cell's first barrier against stress is the cellular membrane, and lipids are one of its primary components. In this study, we evaluated the differences in lipid composition between the species before and after stress exposure. Our results showed that the cellular lipid composition differed widely between species, and thus it may be involved with their different abilities to resist and tolerate oxidative stress. Regardless of stress, the primary feature was the high fatty acid unsaturation rate observed in both non-*Saccharomyces* species, which was basically due to the presence of PUFAs, resulting in high membrane fluidity. It is well known that *S. cerevisiae* cannot synthesize PUFAs because it only contains one desaturase, Δ9 fatty acid desaturase (*OLE1*), which can only produce MUFAs of 16- and 18-carbon compounds (Stukey et al., 1990). However, *S. cerevisiae* can incorporate exogenous PUFAs into its cell membranes (Rosi and Bertuccioli, 1992; Beltran et al., 2008). Instead, in yeasts such as *Kluyveromyces lactis*, oleic acid (C18:1) is subsequently desaturated to linoleic acid (C18:2) and then to α-linolenic acid (C18:3) by Δ12 and omega (Δ15) fatty acid desaturases, respectively

(Ratledge and Evans, 1989; Kainou et al., 2006, Santomartino et al., 2017). In our case, both non-*Saccharomyces* species presented PUFAs in their lipid compositions, although linolenic acid (C18:3) was a unique feature of the *M. pulcherrima* strains. By contrast, the ratio $C_{16:1}/C_{18:1}$ ratio was higher in the *S. cerevisiae* strains, with the highest content of palmitoleic acid (C16:1), the primary UFA in aerobically grown *S. cerevisiae* strains (Steels et al., 1994), which is correlated with higher membrane rigidity (Redón et al., 2009). Many *S. cerevisiae* studies have reported a correlation between an increase in membrane fluidity (due to an increase in the degree of unsaturation) and a higher tolerance to various types of stresses, such as cold or ethanol stress (Guerzoni et al., 1997; Suutari and Laakso, 1994; Casey and Ingledew, 1986, Beltran et al., 2008). Therefore, according to the unsaturation degree, the studied non-*Saccharomyces species* were also expected to be more resistant to oxidative stress. In fact, the introduction of the gene encoding the Δ12 fatty acid desaturase gene (*FAD2*) in the *S. cerevisiae* strains reportedly resulted in a higher resistance to ethanol (Kajiwara et al., 1996), to NaCl and freezing (Rodríguez-Vargas et al., 2007). Moreover, the introduction of both desaturases (*FAD2* and *FAD3* (ω3 fatty acid desaturase) from *K. lactis*) into a strain of *S. cerevisiae* has been reported to increase the alkaline pH tolerance (Yazawa et al., 2009). Therefore, although PUFAs seem to increase yeast tolerance to stress, they can also be toxic to cells because of their susceptibility to peroxidation (Cipak et al., 2006, Johansson et al., 2016). In fact, the heterologous production of PUFAs in *S. cerevisiae* has been shown to increase oxidative stress (Ruenwai et al., 2011), and in non-*Saccharomyces* strains, a higher proportion of C18:2 acid does not assure increased tolerance to ethanol stress (Aguilera et al., 2006, Archana et al., 2015). Under our conditions, i.e., under oxidative stress, high levels of C18:2 acid were positively correlated with low ROS generation and high H_2O_2 tolerance. Nevertheless, the amounts of PUFAs decreased in all the non-*Saccharomyces* strains after stress exposure, probably indicating that the strategy of these species was a reduction of the PUFA content due to their high sensitivity to peroxidation (Ayala et al., 2014, Johansson et al., 2016). This effect could be a mechanism in non-conventional yeasts to withstanding the oxidative stress better without compromising membrane integrity. The other principal mechanism used by non-*Saccharomyces* yeasts to cope with oxidative stress was the modulation of their FA composition, by raising the proportion of MUFAs, such as

palmitoleic acid and oleic acid, and by decreasing the amounts of SFA, such as palmitic acid and stearic acid. Oleic acid has been suggested as a membrane fluidity sensor, and it seems to be the most important UFA for counteracting the toxic nature of ethanol by increasing the membrane stability and antagonizing the fluidity caused by ethanol (You et al., 2003). Furthermore, palmitoleic acid is induced by stress in high-density fermentations, and it has a protective function against damage (Ding et al., 2009). According to Redón et al. (2009), the supplementation of palmitoleic acid in wine yeast culture has a positive effect on the yeast viability and the fermentation kinetics. However, although the UFA/SFA ratio increased, the results showed a decrease in the unsaturation index in non-*Saccharomyces* species, indicating how yeasts try to maintain their membrane fluidity.

Regarding the phospholipid composition, PC and PE are the primary PLs of yeast membranes, representing up to 60-70% of total PLs (Schneiter et al., 1999). The PC/PE ratio is an important parameter for the biophysical status of the membrane, and low PC/PE ratios (in combination with low UFA/SFA and high amounts of ergosterol) cause high membrane rigidity (high anisotropy values) (Flis et al., 2015). The large quantities of PC in non-conventional yeasts could lead to an increase in membrane fluidity (Flis et al., 2015). Furthermore, an increase in the PC concentration correlates with an enhanced oxidative stress tolerance by non-conventional yeasts in a similar way to what has been observed in *S. cerevisiae* ethanol tolerance (Vendramin et al., 1995, Chi and Arneborg, 1999). The PI/PS ratio is another important parameter for cell function (Xia et al., 2011). The synthesis of these PLs is closely correlated, because both require the same precursor cytidyldiphosphate diacylglycerol (CDP-DAG) precursor. Moreover, PS can be a precursor for the synthesis of PE and PC (Voelker and Frazier, 1986). However, PI is considered essential for *S. cerevisiae* because the lack of this PL can reduce cell viability (Becker and Lester, 1977). Our results show that the *S. cerevisiae* strains exhibited a high PI/PS ratio, especially after stress exposure, whereas the *M. pulcherrima* strains, which had the highest resistance to H_2O_2 stress, exhibited the lowest values, especially after stress exposure. In fact, this ratio was negatively correlated with tolerance to oxidative stress. Our results also demonstrate that similar amounts of PI and PS seemed to confer lower sensitivity to H_2O_2.

Normal mitochondrial function is required for resistance to oxidative stress, and the maintenance of a stable respiratory chain strongly prevents the generation of mitochondrial ROS (Barros et al., 2003). CL, a mitochondrial PL found in the inner mitochondrial membrane (De Kroon et al., 2013), plays a key role in the stabilization of electron transport chain complexes and the resistance against oxidative stress during respiratory growth (Chen et al., 2008). Most of the strains in this study, and especially those of *S. cerevisiae*, decreased their CL content after stress exposure, which could indicate a destabilization of the respiratory chain. However, despite this decrease, non-*Saccharomyces* species displayed higher CL values, suggesting a better maintenance of functional mitochondria during H_2O_2 stress as previously described for ethanol stress (Chi and Arneborg, 1999).

Sterols are necessary for maintaining membrane integrity and essential for cell viability (Daum et al., 1998). Consistent with the results obtained by Murakami et al., (1996) in freezing-tolerant strains, our study demonstrated that the sterol content was low in the most H_2O_2-tolerant strains (*M. pulcherrima* species). However, a direct correlation between the sterol content and H_2O_2 tolerance was not observed because, e.g., BY4742 was the most sensitive strain, but it also contained low levels of sterols. Ergosterol is the primary yeast sterol and the end product of the yeast sterol biosynthetic pathway (Daum et al., 1998, Klug and Daum, 2014). Although *M. pulcherrima* showed the highest ergosterol percentage (but the lowest content), this parameter could not be correlated with the oxidative stress either.

By contrast, high amounts of squalene, the ergosterol precursor, led to a low ergosterol/squalene ratio and was positively correlated with less tolerance to stress. The growth of the BY4742 strain was clearly affected after stress exposure (data not shown), and it showed the highest squalene content and lowest ergosterol/squalene ratio after stress exposure. This result is consistent with the finding of Spanova et al., 2012, who hypothesized that the presence of squalene in membranes, especially in the plasma membrane, may affect yeast growth and/ or cause sensitivity to external stress.

High ratios of UFA/SFA, low amounts of ergosterol and high PC/PE ratios in membranes are known to lead to high membrane fluidity (Flis et al., 2015). In the natural strains of this study, these parameters also seem to lead to higher tolerance against to H_2O_2.

5. CONCLUSIONS

In conclusion, our results suggest that non-conventional yeasts are best at resisting induced oxidative stress. The highest stress tolerance was associated with the non-conventional yeasts' abilities to maintain a high proportion and level of unsaturated fatty acids, particularly linolenic acid and linoleic acid. Furthermore, the large variability in the fatty acid composition can result from adaptive responses to changes in external physico-chemical parameters.

Acknowledgements

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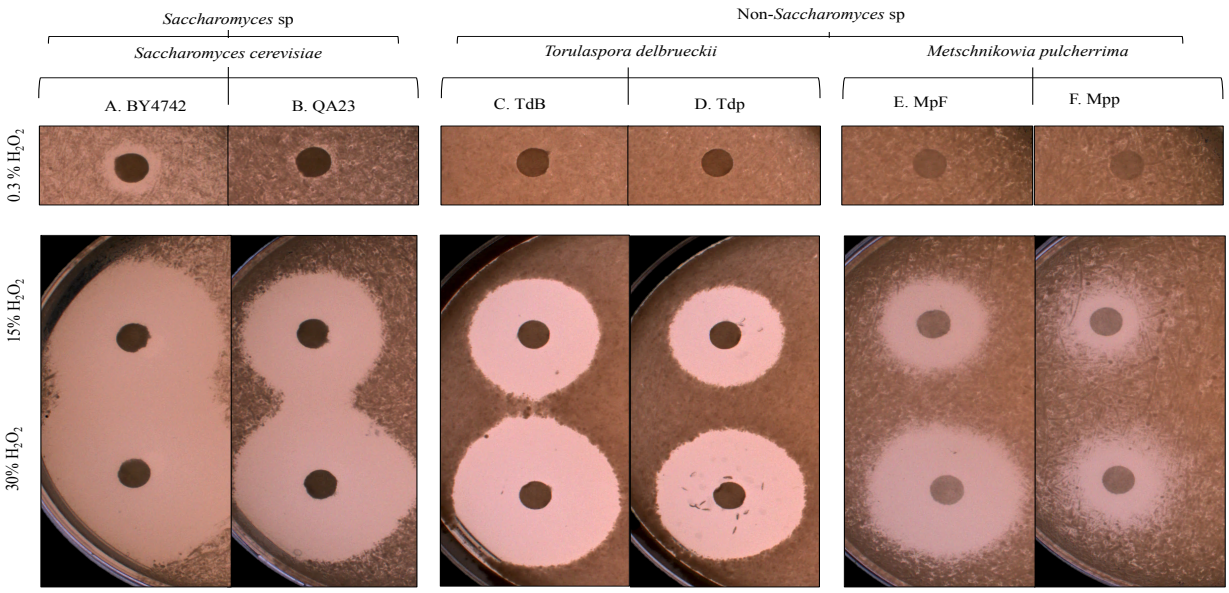
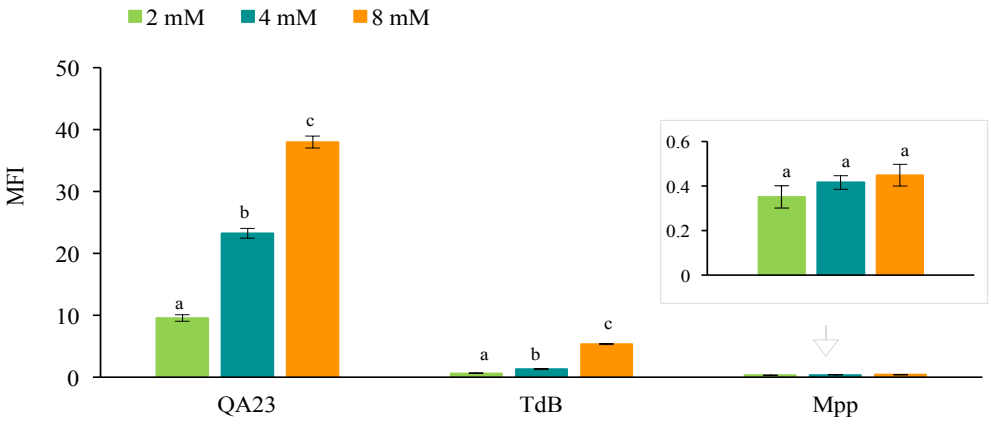
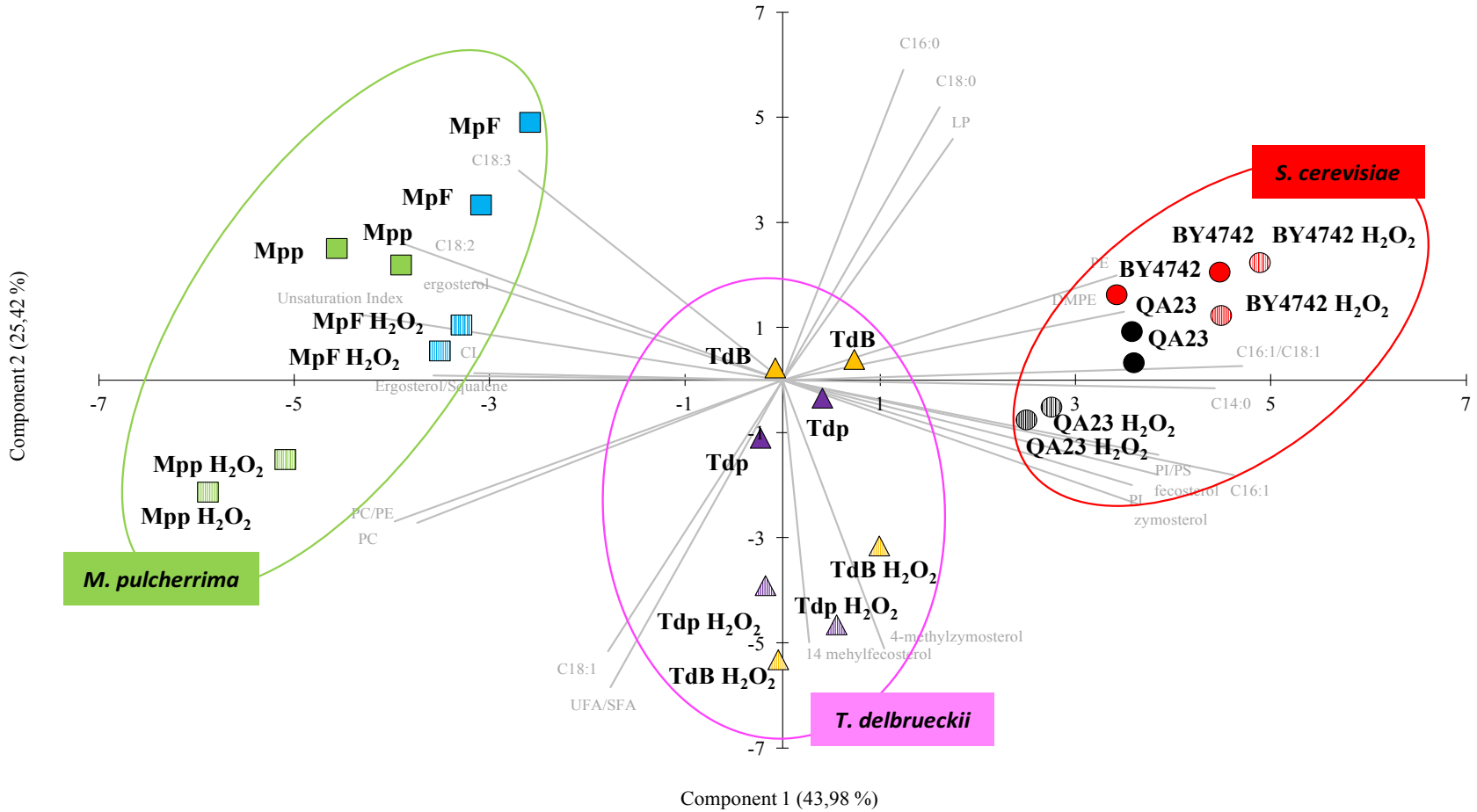
Appendix A. Supplementary data



Supplementary Figure 1. Resistance to H_2O_2 (10 µL from H_2O_2 0.3, 15 and 30% (v/v)) by disk diffusion method from six yeast strains grown on YPD plates over 48 h. *Saccharomyces cerevisiae*: BY4742 and QA23 strains. *Torulaspora delbrueckii*: TdB and Tdp strains. *Metschnikowia pulcherrima*: MpF and Mpp strains.



Supplementary Figure 2. Effect of H_2O_2 (from 2 mM to 8 mM) on the ROS accumulation as evaluated in three yeast strains with and without stress (*S. cerevisiae* QA23, *T. delbrueckii* TdB and *M. pulcherrima* Mpp). The mean fluorescence index (MFI) was calculated according to Boettiger et al., (2001) as follows: [(geometric mean of the positive fluorescence) – (geometric mean of the control)] / (geometric mean of the control).



Supplementary Figure 3. Biplot of principal components analysis (PCA; 69.4% of variance) using the fatty acid (FA), phospholipid (PL) and sterol composition in the *S. cerevisiae, T. delbrueckii* and *M. pulcherrima* strains before and after stress exposure with 2 mM of H_2O_2. Component 1: (+); phosphatidylinositol (PI), phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE), myristic (C14:0) and palmitoleic (C16:1) acids, palmitoleic/oleic (C16:1/C18:1) ratio, phosphatidylinositol/ phosphatidylserine (PI/PS) ratio, zymosterol and fecosterol. (-); Phosphatidylcholine (PC), cardiolipin (CL), linoleic (C18:2) acid, PC/PE ratio, ergosterol, ergosterol/ squalene ratio and unsaturation index. Component 2 (+): lysophospholipids (LP) and palmitic (C16:0), stearic (C18:0) and linolenic (C18:3) acids. (-); C18:1 acid, unsaturated/saturated (UFA/SFA) ratio, 4-methylzymosterol, and 14 mehylfecosterol.

Supplementary Table 1. Correlation of the ROS (MFI) and inhibition haloes variables with the FA, PL and sterol variables obtained with a Pearson correlation matrix from the PCA after oxidative stress exposure using H_2O_2 (**Figure 4B**).

	MFI	**Inhibition halo**
MFI	**1**	0.417
Inhibition halo	0.417	**1**
PI (Phosphatidylinositol)	0.488	0.673
PC (Phosphatylcoline)	-0.749	-0.532
PE (Phosphatidylethanolamine)	0.543	0.583
CL (Cardiolipin)	-0.834	-0.632
DMPE (Dimethylphosphatidylethanolamine)	0.737	0.382
LP (Lysophospholipids)	0.897	0.412
C14:0 (Mirystic acid)	0.654	0.663
C16:0 (Palmitic acid)	0.721	0.298
C16:1 (Palmitoleic acid)	0.630	0.753
C18:0 (Stearic acid)	0.924	0.646
C18:1 (Oleic acid)	-0.778	-0.612
C18:2 (Linoleic acid)	-0.525	-0.651
C18:3 (Linolenic acid)	-0.365	-0.544
UFA/SFA (unsaturated/saturated ratio)	-0.786	-0.518
PI/PS	0.508	0.810
PC/PE	-0.658	-0.645
squalene	0.915	0.597
zymosterol	0.082	0.751
ergosterol	-0.337	-0.452
4-methylzymosterol	-0.398	0.066
14-mehylfecosterol	-0.414	-0.182
fecosterol	0.409	0.214
episterol	-0.405	-0.051
lanosterol	0.131	0.001
ergosterol/squalene	-0.487	-0.667
C16:1/C18:1	0.892	0.686
Unsaturation Index (IU)	-0.769	-0.740

Supplementary Table 2. Pearson correlation matrix from the variables used in the PCA before and after oxidative stress was applied (Figure S3). PLs: phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), dimethylphosphatidylethanolamine (DMPE), and lysophospholipids (LP). FAs: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids, unstaturated/saturated (UFA/SFA) ratio, zymosterol (A), ergosterol (B), 4-methylzymosterol (C), 14-methylfecosterol (D), fecosterol (E), ergosterol/squalene ratio (F), C16:1/C18:1 (G), and unsaturation index (E).

	PI	PC	PE	CL	DMPE	LP	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	UFA/SFA	PI/PS	PC/PE	A	B	C	D	E	F	G	E
PI	1	-0.536	0.259	-0.442	0.234	-0.031	0.482	-0.204	0.720	0.158	-0.157	-0.565	-0.480	0.035	0.746	-0.443	0.639	-0.694	0.387	0.259	0.663	-0.637	0.651	-0.56
PC	-0.536	1	-0.514	0.288	-0.695	-0.588	-0.583	-0.474	-0.552	-0.606	0.656	0.340	0.100	0.567	-0.359	0.828	-0.375	0.467	0.147	0.176	-0.573	0.653	-0.702	0.48
PE	0.259	-0.514	1	-0.354	0.329	0.259	0.612	0.442	0.480	0.443	-0.574	-0.275	-0.213	-0.566	0.645	-0.892	0.353	-0.498	-0.007	-0.088	0.390	-0.656	0.578	-0.42
CL	-0.442	0.288	-0.354	1	-0.504	-0.313	-0.565	-0.144	-0.720	-0.131	0.137	0.671	0.341	0.161	-0.456	0.349	-0.319	0.052	0.024	0.032	-0.608	0.137	-0.751	0.68
DMPE	0.234	-0.695	0.329	-0.504	1	0.634	0.693	0.397	0.696	0.246	-0.197	-0.692	-0.297	-0.352	0.201	-0.518	0.445	-0.103	-0.083	-0.160	0.554	-0.246	0.761	-0.75
LP	-0.031	-0.588	0.259	-0.313	0.634	1	0.420	0.764	0.192	0.538	-0.502	-0.184	0.361	-0.678	0.011	-0.443	0.091	0.274	-0.454	-0.526	0.050	-0.069	0.368	-0.29
C14:0	0.482	-0.583	0.612	-0.565	0.693	0.420	1	0.267	0.848	0.178	-0.232	-0.769	-0.503	-0.298	0.648	-0.656	0.704	-0.476	0.234	0.075	0.645	-0.568	0.838	-0.84
C16:0	-0.204	-0.474	0.442	-0.144	0.397	0.764	0.267	1	-0.001	0.722	-0.729	0.087	0.341	-0.949	-0.010	-0.521	-0.107	0.177	-0.626	-0.711	-0.146	-0.118	0.287	-0.13
C16:1	0.720	-0.552	0.480	-0.720	0.696	0.192	0.848	-0.001	1	0.022	-0.045	-0.931	-0.706	-0.064	0.729	-0.557	0.774	-0.521	0.330	0.179	0.785	-0.565	0.927	-0.94
C18:0	0.158	-0.606	0.443	-0.131	0.246	0.538	0.178	0.722	0.022	1	-0.873	0.173	0.414	-0.863	0.167	-0.601	-0.041	-0.198	-0.449	-0.486	0.110	-0.260	0.290	-0.03
C18:1	-0.157	0.656	-0.574	0.137	-0.197	-0.502	-0.232	-0.729	-0.045	-0.873	1	-0.252	-0.390	0.845	-0.154	0.733	0.033	0.230	0.402	0.400	-0.129	0.474	-0.314	-0.03
C18:2	-0.565	0.340	-0.275	0.671	-0.692	-0.184	-0.769	0.087	-0.931	0.173	-0.252	1	0.721	-0.091	-0.627	0.302	-0.742	0.316	-0.350	-0.174	-0.643	0.311	-0.801	0.96
C18:3	-0.480	0.100	-0.213	0.341	-0.297	0.361	-0.503	0.341	-0.706	0.414	-0.390	0.721	1	-0.305	-0.485	0.142	-0.496	0.537	-0.368	-0.282	-0.574	0.319	-0.585	0.70
UFA/SFA	0.035	0.567	-0.566	0.161	-0.352	-0.678	-0.298	-0.949	-0.064	-0.863	0.845	-0.091	-0.305	1	-0.153	0.655	0.037	0.033	0.578	0.664	0.032	0.243	-0.355	0.15
PI/PS	0.746	-0.359	0.645	-0.456	0.201	0.011	0.648	-0.010	0.729	0.167	-0.154	-0.627	-0.485	-0.153	1	-0.588	0.717	-0.657	0.412	0.166	0.468	-0.671	0.645	-0.66
PC/PE	-0.443	0.828	-0.892	0.349	-0.518	-0.443	-0.656	-0.521	-0.557	-0.601	0.733	0.302	0.142	0.655	-0.588	1	-0.410	0.548	0.051	0.114	-0.520	0.766	-0.686	0.47
A	0.639	-0.375	0.353	-0.319	0.445	0.091	0.704	-0.107	0.774	-0.041	0.033	-0.742	-0.496	0.037	0.717	-0.410	1	-0.555	0.562	0.262	0.479	-0.589	0.578	-0.71
B	-0.694	0.467	-0.498	0.052	-0.103	0.274	-0.476	0.177	-0.521	-0.198	0.230	0.316	0.537	0.033	-0.657	0.548	-0.555	1	-0.475	-0.389	-0.641	0.762	-0.482	0.38
C	0.387	0.147	-0.007	0.024	-0.083	-0.454	0.234	-0.626	0.330	-0.449	0.402	-0.350	-0.368	0.578	0.412	0.051	0.562	-0.475	1	0.880	0.324	-0.323	0.032	-0.20
D	0.259	0.176	-0.088	0.032	-0.160	-0.526	0.075	-0.711	0.179	-0.486	0.400	-0.174	-0.282	0.664	0.166	0.114	0.262	-0.389	0.880	1	0.372	-0.206	-0.074	-0.01
E	0.663	-0.573	0.390	-0.608	0.554	0.050	0.645	-0.146	0.785	0.110	-0.129	-0.643	-0.574	0.032	0.468	-0.520	0.479	-0.641	0.324	0.372	1	-0.477	0.767	-0.65
F	-0.637	0.653	-0.656	0.137	-0.246	-0.069	-0.568	-0.118	-0.565	-0.260	0.474	0.311	0.319	0.243	-0.671	0.766	-0.589	0.762	-0.323	-0.206	-0.477	1	-0.561	0.39
G	0.651	-0.702	0.578	-0.751	0.761	0.368	0.838	0.287	0.927	0.290	-0.314	-0.801	-0.585	-0.355	0.645	-0.686	0.578	-0.482	0.032	-0.074	0.767	-0.561	1	-0.89
E	-0.567	0.482	-0.428	0.685	-0.757	-0.296	-0.842	-0.138	-0.946	-0.032	-0.039	0.964	0.707	0.152	-0.661	0.471	-0.716	0.380	-0.204	-0.014	-0.656	0.397	-0.899	1

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CHAPTER 2

Melatonin reduces oxidative stress damage induced by hydrogen peroxide in *Saccharomyces cerevisiae*

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Abstract

Melatonin (N-acetyl-5-methoxytryptamine), which is synthesized from tryptophan, is formed during alcoholic fermentation, though its role in yeast is unknown. This study employed Saccharomyces cerevisiae as an eukaryote model to evaluate the possible effects of melatonin supplementation on endogenous cellular defense systems by measuring its effects on various cellular targets. Cell viability, intracellular reduced and oxidized glutathione levels (GSH and GSSG, respectively), reactive oxygen species (ROS) production, and expression of genes related to antioxidant defense in yeast, such as the glutathione system, catalase, superoxide dismutase, glutaredoxin and thioredoxin, were assessed. Melatonin alone decreased GSH, increased GSSG, and activated antioxidant defense system genes, which reached maximum levels in the stationary phase. These results indicate that melatonin supplementation enables cells to resist better the stress generated in the stationary phase. However, when cells were subjected to oxidative stress induced by H_2O_2, melatonin was able to partially mitigate cell damage by decreasing ROS accumulation and GSSG and increasing GSH; this was followed by enhanced cell viability after stress exposure, mostly when occurring in the early stationary phase. Additionally, under such conditions, most genes related to endogenous antioxidant defense continued to be up-regulated with melatonin supplementation. The findings demonstrate that melatonin can act as antioxidant in *S. cerevisiae.*

Keywords: *Saccharomyces cerevisiae*; melatonin; glutathione; ROS; oxidative stress response; gene expression

1. INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) (MEL) is synthesized from tryptophan and exhibits various biological activities in humans. One such activity is its antioxidant capacity: MEL protects various biomolecules against damage caused by free radicals by acting as a direct scavenger to detoxify reactive oxygen and nitrogen species (Reiter et al., 2001; Anisimov et al., 2006; Reiter et al., 2016). In addition, MEL can indirectly reduce oxidative stress by increasing the activities of antioxidative defense systems, stimulating the synthesis of other important intracellular antioxidants such as glutathione (Antolín et al., 1996; Rodriguez et al., 2004), increasing the efficiency of the mitochondrial electron transport chain (Martín et al., 2000; León et al., 2005; López et al., 2009) and interacting synergistically with other antioxidants (Gitto et al., 2001; López-Burillo., et al 2003). Most studies to date confirm the antioxidant properties of MEL, but it might also exert a pro-oxidant effect in specific situations, e.g., cancer cell killing, even though this is not well documented *in vivo* (Zhang and Zhang, 2014).

MEL can be found in small quantities in wines (74-420 ng/mL, Rodriguez-Naranjo et al., 2011), because it is present in grapes (Iriti et al., 2006; Stege et al., 2010; Murch et al., 2010) and is also synthesized by yeast during alcoholic fermentation (Rodriguez-Naranjo et al., 2012; Mas et al., 2014; Wang et al., 2016; Fernández-Cruz et al., 2017). Despite very little information is available on melatonin biosynthesis in yeast, its pathway is supposed to be similar to the one described in vertebrates, in which four enzymes are involved in the conversion of tryptophan to melatonin, via serotonin and N-acetylserotonin intermediates (Mas et al., 2014). However, the role of MEL in yeast remains unknown. *Saccharomyces cerevisiae* is the simplest eukaryote model and is also the main yeast used in the winemaking process, where is exposed to a number of stressors, each with the potential to cause cellular damage and impair fermentation performance (Pretorius, 2000; Gibson et al., 2007). One such stressor is oxidative stress, whereby yeast cells need to manage the toxic effects of reactive oxygen species (ROS) formed from molecular oxygen, including superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radical (OH^-) or hydrogen peroxide (H_2O_2) (Gibson et al., 2008; Moradas-Ferreira et al., 1996). ROS are generated endogenously during normal cellular metabolism, and their production can also be stimulated by the presence of pro-

oxidants. Under normal physiological conditions, yeast cells are able to maintain a reduced intracellular redox environment. However, ROS become harmful when their concentration exceeds the ability of the cells to remove them, causing respiratory deficiencies that result in oxidative stress. This oxidative stress can damage lipids, carbohydrates, proteins and nucleic acids, potentially leading to cell death (Gibson et al., 2008; Moradas-Ferreira et al., 1996; Costa and Moradas-Ferreira, 2001).

Yeast cells are constantly monitoring ROS concentrations in an attempt to maintain them at a basal level by invoking antioxidant defense mechanisms, which are grouped into enzymatic and non-enzymatic systems that operate at different levels (Costa and Moradas-Ferreira, 2001; Jamieson, 1998; Moradas-Ferreira and Costa, 2000). Enzymatic systems, which include catalase, superoxide dismutase and glutathione peroxidase, are primary defenses that function to neutralize ROS. In contrast, non-enzymatic systems, such as the glutathione, glutaredoxin family or thioredoxins, are secondary defenses that repair or remove the products of oxidative damage (Jamieson, 1998; Moradas-Ferreira et al., 1996). To eliminate ROS, cells need to be equipped with regulatory molecules that rapidly sense and respond to oxidative stress. In yeast, the parallel glutathione/glutaredoxin and thioredoxins pathways (essential under aerobic and anaerobic conditions) (Herrero et al., 2008) make a large contribution to protection against oxidative damage by reacting with ROS (Auchère et al., 2008). Glutathione (GSH) is well known as the main and most abundant endogenous antioxidant in cells (Moradas-Ferreira et al., 1996; Jamieson, 1998; Izawa et al., 1995). GSH reacts with ROS, donating an electron to neutralize them and becoming reactive itself, resulting in the formation of GSSG, the oxidized state, through the combination of two reactive forms of GSH. Thus, the presence of ROS results in a decrease in GSH and an increase in GSSG (Herrero et al., 2008; Auchère et al., 2008). The enzymes directly implicated in the maintenance of the GSH/GSSG redox balance are as follows: γ-glutamylcysteine synthetase, which catalyzes the first step in the biosynthesis of glutathione; glutathione reductase, which reduces GSSG to GSH in an NADPH-dependent process; glutathione peroxidase, which reduces H_2O_2 by oxidizing GSH to GSSG; and glutaredoxins, which regulate the protein redox state using GSH and NADPH. Similar to glutaredoxins, thioredoxins are thiol oxidoreductases; however, in this case, they are not glutathione dependent but are only

reduced by NADPH and thioredoxin reductase. Although cytosolic thioredoxin, encoded by *TRX2*, is the most important enzyme required for defense against externally added hydroperoxides, cytosolic glutaredoxin, encoded by *GRX2*, also contributes to resistance to hydroperoxides (Herrrero et al., 2008; Auchère et al., 2008; Gómez-Pastor et al., 2012). Furthermore, *S. cerevisiae* possesses two catalases and two superoxide dismutases, which also act as enzymatic antioxidants. Catalase A and catalase T decompose H_2O_2 to oxygen and water in the peroxisome and the cytosol, respectively. Superoxide dismutases catalyze the conversion of superoxide anion to oxygen and H_2O_2 in the cytoplasm (Cu/ZnSOD) and in mitochondria (Mn/ZnSOD) (Costa and Morades-Ferreira, 2001; Jamieson, 1998; Auchère et al., 2008). Most of these antioxidant mechanisms are considered universal in living organisms, and regulating expression of the genes involved as well as enzyme activity is crucial for cell survival.

The goal of this study was to evaluate the effect of MEL on *S. cerevisiae* and its possible role as an antioxidant. To accomplish this, we evaluated ROS production, intracellular glutathione levels (GSH/GSSG), and expression of certain genes involved in the oxidative stress response in a commercial wine yeast strain in both the presence and absence of MEL (5 μM) and oxidative stress (addition of 2 mM H_2O_2). Furthermore, as several studies have demonstrated that yeast cells in the stationary phase exhibit a significant degree of resistance toward oxidants (Gibson et al., 2008; Jamieson, 1998), the effect of MEL was evaluated in both the exponential and stationary phases.

2. MATERIAL AND METHODS

2.1. Yeast strains and growth conditions

The wine yeast QA23, a commercial strain of *S. cerevisiae* (Lallemand, Montreal, Canada), was used in this study. For all experiments, after yeast rehydration, precultures for biomass propagation were prepared in YPD liquid medium (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract) and incubated for 24 h at 28ºC with orbital shaking (120 rpm).

2.2. Determination of reactive oxygen species (ROS)

A preliminary test to evaluate the concentration of H_2O_2 and MEL to be used in different experiments was carried out by determining their effect on the intracellular concentration of ROS.

The effect of H_2O_2 was first examined. Yeast cells were inoculated into 100 mL of YPD broth (5 x 10^5 cells/mL) and grown for 6 h (until cells reached the exponential phase) at 28ºC with orbital shaking at 120 rpm. The cells were then exposed to different concentrations of H_2O_2, (from 2 mM to 4 mM, Perdrogen™, Sigma-Aldrich, Misuri, USA) for 1 h, and intracellular ROS were determined and compared to the control (without exposure to H_2O_2). Another assessment to fix MEL concentrations was performed. In this case, the same procedure was followed with the cells grown in the presence of different concentrations of MEL (0, 5 μM, 25 μM or 50 μM) for 6 h.

Reactive oxygen species determination was carried out according to a modified version of the method described by Madeo et al. (1999) using dihydrorhodamine 123 (DHR 123; Sigma-Aldrich) as a ROS indicator. Cells were stained with 10 μg DHR 123 (stock solution of 2.5 mg/mL) per mL of cell culture for 15 min at 120 rpm in darkness. After incubation, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and the fluorescence intensity was analyzed by flow cytometry at a low flow rate with excitation and emission settings of 488 and 525-550 nm (filter FL1), respectively. FloMax software (Quantum Analysis GmbH, Münster, Germany) was used for instrument control and data acquisition, and the captured files were processed using WinMDI 2.9 software (Joseph Trotter, Salk Institute for Biological Studies, CA, USA). The mean fluorescence index (MFI) was calculated according to Boettiger et al., 2001: [(geometric mean of the positive fluorescence) – (geometric mean of the control)] / (geometric mean of the control). Moreover, cells were visualized using a Leica fluorescence microscope (DM4000B, Stuttgart, Germany) with a 40X lens.

2.3. Experimental conditions

Yeast cells were inoculated into 600 mL of YPD broth with and without supplementation of 5 μM MEL (MEL and Control, respectively) to obtain an initial population of 5x10^5 cells/mL and grown for 24 h at 28ºC with orbital shaking at 120 rpm. Both conditions were carried out in triplicate, and yeast growth was controlled by measuring the optical density at 600 nm (OD_{600}) every 2 h, at which 1 x 10^8 cells were transferred to 2-mL Eppendorf tubes and centrifuged. The pellets were washed twice with PBS (pH 7.4), frozen in liquid nitrogen and stored at -80ºC for glutathione assays.

Sublethal oxidative stress was induced under both conditions by adding 2 mM hydrogen peroxide (H_2O_2) to the yeast cultures. In different phases of the growth curve (the early exponential phase (6 h), early stationary phase (16 h) and late stationary phase (30 h)), the Control and MEL conditions were divided into two flasks of 100 mL of culture each. Stress was induced in one flask of each condition with 2 mM H_2O_2 for 120 min to generate four conditions: Control and MEL (without stress); H_2O_2 and MEL H_2O_2 (with stress). Samples were collected before and after stress exposure (0, 10, 45, 90 and 120 min for glutathione quantification; 0, 45 and 120 min for gene expression) and stored as previously described. Three biological replicates were performed for each condition.

2.4. Evaluation of yeast viability after stress exposure

Yeast viability after exposure to stress (MEL H_2O_2 and H_2O_2) in comparison with cells without stress (MEL and control) was evaluated by a microplate bioassay in which 96-well plates were prepared by dispensing 250 μL of YPD broth inoculated with cells of each condition into each well to obtain an initial OD_{600} of 0.050. The microplate was incubated at 28ºC for 24 h, and OD_{600} was measured every 30 min using a microplate reader (Omega Polarstar, BMG Labtech Gmbh, Ortenberg, Germany). OD max, growth rate and generation time were calculated from growth curves data, according Warringer et al., 2003. Moreover, the relative viable fraction was calculated using the formula described by Murakami et al., 2008:

$$Vn = \frac{1}{2^{(\frac{\Delta t_n}{\delta})}}$$

where *Vn* = viability of the cultures exposed to stress (MEL H_2O_2 and H_2O_2) relative to cultures before stress exposure (MEL and Control, respectively), Δt_n = time shift between the stressed and unstressed outgrowth curves to reach OD = 0.5, and δ = doubling time in each condition.

2.5. Determination of glutathione levels

Samples (1 x 10^8 cells) were rapidly thawed in a water bath at 37ºC. For glutathione extraction, a modified version of the method described by Borrull et al., 2016 was used. Pellets were weighed and three volumes of 5% 5-sulfosalicylic acid (SSA) were added and vortexed. The cell suspensions were then frozen in liquid nitrogen and thawed at

37ºC in a water bath three times, incubated for 5 min at 4ºC and centrifuged at 800xg for 10 min at 4ºC. For quantification, GSSG first needs to be reduced to GSH. This enzymatic reduction was performed using glutathione reductase (GR; Sigma-Aldrich) and the cofactor NADPH (Sigma-Aldrich). Briefly, 50 µL of homogenate and 3 units of GR solution were dissolved in 950 µL PBS (pH 7.8) with 16 mg/mL NADPH and incubated at 25ºC for 10 min. Total glutathione (GSHtot) and GSH were determined using the method described by White et al., 2003. Briefly, 20 µL of supernatant was transferred to a 96-well plate designed for fluorescence detection, and 180 µL of 2,3-naphthalenedicarboxyaldehyde (NDA) derivatization solution (50 mM Tris, pH 10, 0.5 N NaOH, and 10 mM NDA in Me_2SO, v/v/v; Sigma-Aldrich) was added. The microplate was shaken for 10 min at 150 rpm and at 20 ± 2ºC in darkness, as recommended by Lewicki et al., 2006, to maintain stability of the NDA-GSH adduct. After this incubation, fluorescence intensity was measured (488 ex / 530 em) using a fluorescence plate reader. For total and reduced glutathione quantification, linear regression curves were generated using GSSG and GSH standard solutions (Sigma-Aldrich). The concentration of GSSG was calculated by subtracting reduced GSH from total GSH and dividing this value by 2. Other pellet ($1x10^8$ cells) was previously dried at 28ºC for 48 h and weighed. Thus, the results are expressed as µM of glutathione per mg of dry weight.

2.6. Gene expression analysis by quantitative PCR (qPCR)

Expression levels of specific genes (**Table 1**) were determined using qPCR. Total RNA from 1 x 10^7 cells was isolated using a PureLink® RNA Mini kit from Ambion Life Technologies (Massachusetts, USA) as recommended by the manufacturer. To remove DNA, a DNAse (Qiagen, Barcelona, Spain) step was performed at 37ºC for 15 min before washing. Reverse transcription and qPCR reactions were performed as described by Beltran et al., 2004. cDNA was synthesized from 320 ng/µL RNA using SuperScript® III Reverse Transcriptase (Invitrogen) and Oligo (dT) 20 Primer (Invitrogen). qPCR was performed using the Applied Biosystems 7300 Fast Real-Time PCR system (Applied Biosystems, CA, USA). Samples were prepared as follows: 2 µL cDNA, 0.4 µL each primer, 0.4 µL ROX, 10 µL SYBR Green (Takara® SYBR Green master mix) and H_2O q.s.p 20 µL. Relative gene expression was calculated 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 in Ct of the gene of interest and the housekeeping gene (*ACT1*), and ΔΔCt is the difference between ΔCt of the condition MEL at 6 h or 16 h and the Control at 6 h or 16 h (see figure legends for relative expression details). Three biological replicates were analyzed for each time point and condition.

Table 1. Primers used in this study based on Verbelen et al., 2009, Auchère et al., 2008 and Gómez-Pastor et al., 2012 (supplied by Invitrogen)

Gene description	Primer	Nucleotide sequence (5' to 3')
Cu/Zn superoxide dismutase	*SOD1_F*	TGATCAAGCTTATCGGTCCTACCT
	SOD1_R	GCCGGCGTGGATAACG
Mn superoxide dismutase	*SOD2_F*	GCAAGCTGGACGTTGTTCAA
	SOD2_R	AGAGGAACTAGTGGGCCTGTGA
Peroxisomic catalase A	*CTA1_F*	GGACAGCAAAAGAACTTGGCATA
	CTA1_R	TGAGGACAGGCGCCTTCTA
Cytosolic catalase T	*CTT1_F*	GTCAGGCTCCCACCCTGAT
	CTT1_R	TTTTCGCCATTTTGCAATTG
Glutathione peroxidase I	*GPX1_F*	GGGAAGTCTGGAATAAAAATGATAAA
	GPX1_R	TTCTTCTGGTGGTTGATTCAGTA
γ-glutamylcysteine synthetase	*GSH1_F*	GACACCGATGTGGAAACTGA
	GSH1_R	CCCTTTTTGGCATAGGATTG
Glutathione-disulfide reductase	*GLR1_F*	AGGTTGTCGGTCTGCACATT
	GLR1_R	CCTTAGTGGCACCCATCTTT
Glucose-6-phosphate dehydrogenase	*ZWF1_F*	CCAGAGGCTTACGAGGTGTT
	ZWF1_R	GGTGAATATGCCCCAACTGA
Glutaredoxin	*GRX2_F*	GGCCAAAAGGAAGTGTTTGT
	GRX2_R	TTCAATTCTTGGAAGAGGGTAGA
Thioredoxin	*TRX2_F*	AAATCCGCTTCTGAATAC
	TRX2_R	CTATACGTTGGAAGCAATAG

2.7. Data analysis

Data were subjected to one-way analysis of variance (ANOVA) and Tukey's post hoc test to evaluate the effect of each treatment. The results were considered statistically significant at a p-value less than 0.05 (IBM SPSS Inc, XLSTAT Software). Furthermore, a Principal Component Analysis (PCA) was performed at 6 h, 16 h and 30 h (XLSTAT Software). PCs were assessed using glutathione levels (GSH and GSSG, at 10, 45, 90 and 120 min), and growth data (OD max, and maximum growth rate calculated at 5, 10, 15, 20 h).

3. RESULTS

3.1. Effect of melatonin on reactive oxygen species (ROS)

To evaluate the possible role of MEL as an antioxidant agent in *S. cerevisiae*, we determined the levels of ROS in stressed and unstressed cells (using H_2O_2 as the oxidative agent) in the presence and absence of MEL in the growth medium (**Figure 1**).

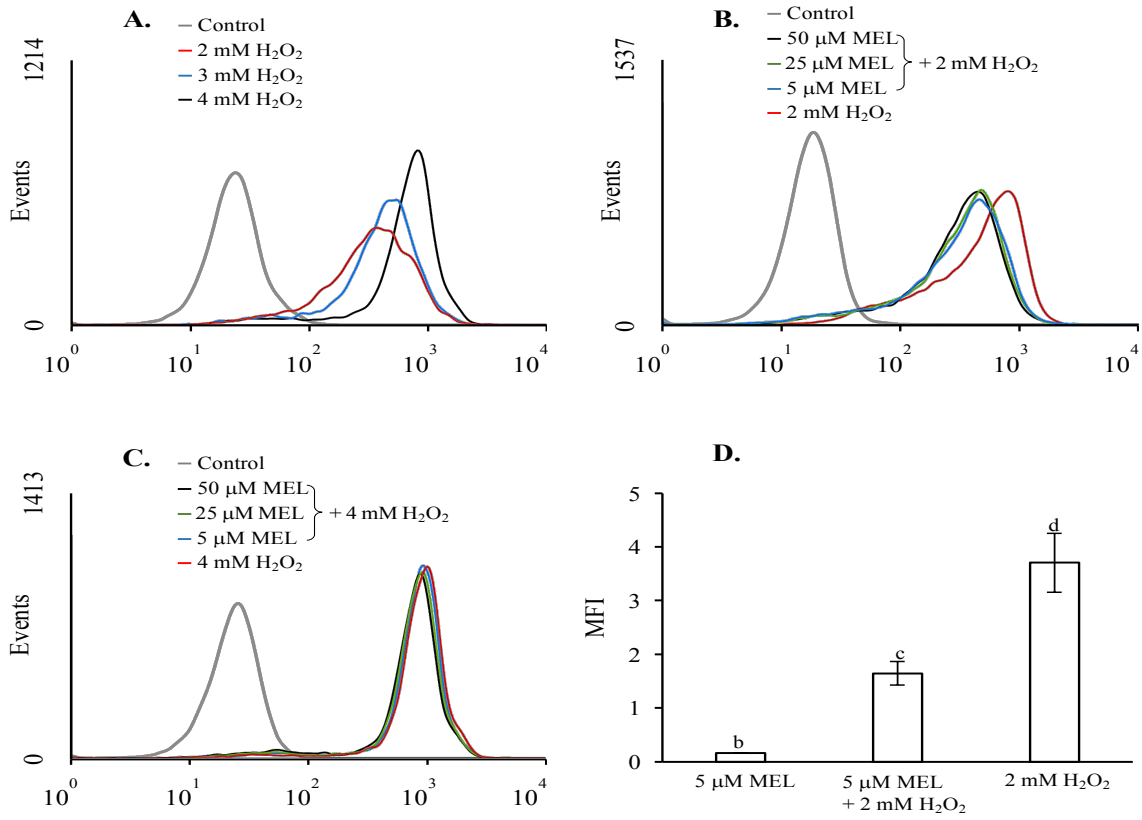

Figure 1. Effect of H_2O_2 and melatonin on ROS accumulation, as evaluated in the exponential phase. Flow cytometry histogram profile expressed in number of events with 0 mM, 2 mM, 3 mM and 4 mM H_2O_2 (A) in the absence or presence of melatonin (5 µM, 25 µM and 50 µM) in cells under oxidative stress with 2 mM H_2O_2 (B) or 4 mM H_2O_2 (C). The control condition corresponds to unstressed cells without melatonin. (D) Mean fluorescence intensity (MFI) of unstressed and stressed cells (2 mM H_2O_2) in the presence of melatonin (5 µM) compared with the control condition. Error bars represent SD of n=3 by ANOVA and Tukey's post-test $^{(b, c, d)}$, $p<0.05$.

Although *S. cerevisiae* can synthetize MEL, we have previously observed that in these conditions, the concentration of MEL in the extracellular medium is negligible (below 0.4 nM, data not shown). Preliminary experiments were conducted to select a sublethal dose of H_2O_2 and the concentration of MEL with a possible antioxidant effect. Exposure to increasing concentrations of H_2O_2 resulted in an increase in ROS (**Figure 1A**).

However, when the oxidative stress was applied to cultures growing with MEL (from 5 μM to 50 μM), a reduction in ROS was only observed at 2 mM H_2O_2 (**Figure 1B**); this reduction was dependent on MEL addition but independent of the MEL concentration in the medium. In contrast, no effect on ROS accumulation was observed when cells were exposed to higher H_2O_2 concentrations (4 mM, Figure 1C). Thus, we chose the lowest assayed dose of MEL (5 μM) and H_2O_2 (2 mM) for the ensuing experiments. As shown in Figure 1D, cells exposed to oxidative stress (2 mM H_2O_2) exhibited strong increases in total ROS, with four times higher levels than unstressed cells (**Figure 1D**). However, ROS accumulation in cells under the same oxidative stress conditions but previously grown in presence of MEL was significantly lower (only 2-times higher than unstressed cells). Conversely, a low dose (5 μM) of MEL alone, without the presence of the oxidative agent, resulted in slightly increased total ROS.

3.2. Effect of melatonin on the glutathione redox status of the *S. cerevisiae* wine strain

To further study the role of MEL in yeast cells, we evaluated the effect of its supplementation (5 μM MEL) on intracellular glutathione levels by analyzing both reduced (GSH) and oxidized (GSSG) glutathione over 24 hours of growth (**Figure 2**). Although similar growth curves were observed for both conditions (with and without MEL), with MEL, cells grew faster during the exponential phase (**Figure 2A**). Total GSH remained almost constant until the mid-exponential phase, when it began to increase, with a similar pattern observed in both conditions; however, GSHtot levels were slightly lower in the presence of MEL (**Figure 2B**). Despite the lack of significant changes in GSHtot when MEL was added, the glutathione ratio (GSH/GSSG) with and without MEL supplementation differed. In the presence of MEL, cells exhibited low levels of GSH and high levels of GSSG during the first 20-22 h (**Figure 2C, D**). GSH evolution showed similar trends with and without MEL until 22 h. As for GSHtot, the concentration of GSH was almost constant until the mid-log phase and then increased to reach its maximum value at 20-22 h. After this point (22 h), the level of GSH decreased without MEL but remained constant with MEL (**Figure 2C**). Although the GSSG content was higher in the culture supplemented with MEL, its concentration remained essentially unchanged during the 24 h of study. Without MEL, the GSSG concentration increased after the mid-log phase and was higher under this condition at 22-24 h than in the presence of MEL (**Figure 2D**).

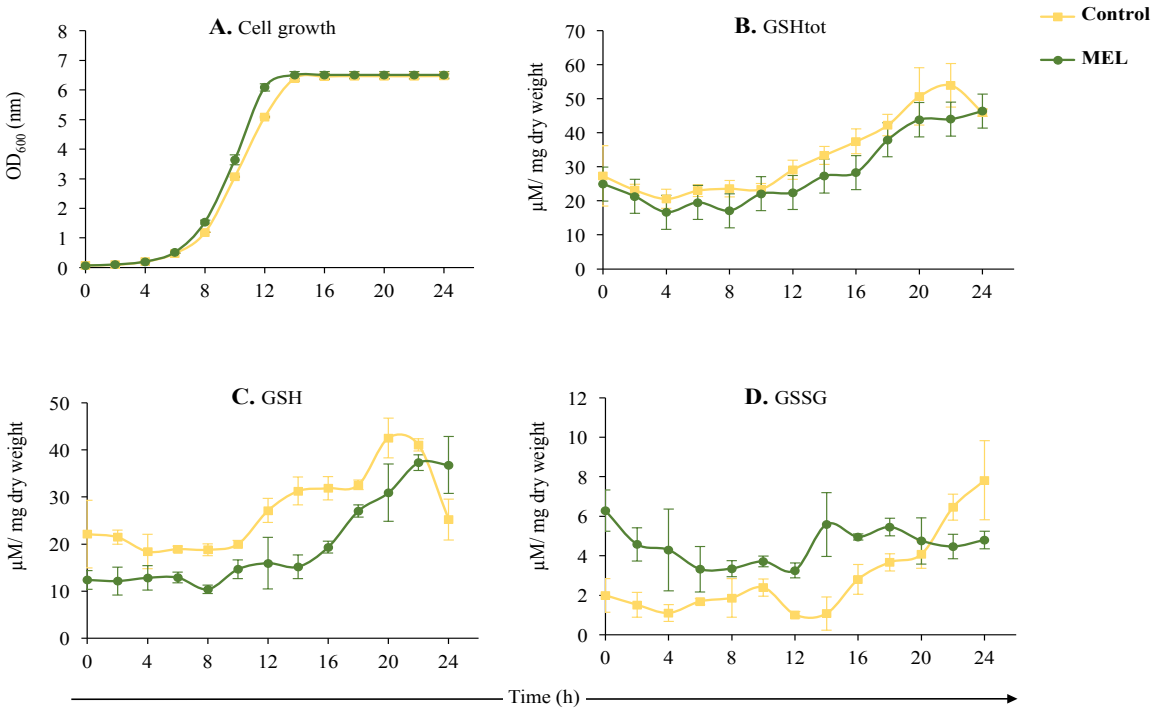

Figure 2. Effect of the presence (MEL; 5 µM) or absence (Control) of melatonin on intracellular glutathione levels in *S. cerevisiae* (QA23 strain) over 24 h of growth in YPD. Error bars represent ±SD of n=3. (A) Growth of the QA23 strain (OD, optical density). (B) Total glutathione (GSHtot) levels. (C) Reduced glutathione (GSH) levels. (D) Oxidized glutathione (GSSG) levels.

3.3. Effect of melatonin on expression levels of genes related to the antioxidant response

Quantitative PCR was used for transcriptional analysis of certain genes implicated in endogenous antioxidant defense, such as *CTA1* and *CTT1* (catalase A and T, respectively), *SOD1* and *SOD2* (cytoplasmic and mitochondrial superoxide dismutase, respectively), *GRX2* (glutaredoxin), and *TRX2* (thioredoxin) and in glutathione metabolism, such as *GSH1* (γ-glutamylcysteine synthetase), *GLR1* (glutathione reductase), *GPX1* (glutathione peroxidase) and *ZWF1* (glucose-6-phosphate dehydrogenase, which reduces $NADP^+$ to NADPH). The effect of MEL supplementation (5 µM) on the expression levels of all these genes was determined in the early exponential (6 h) and early stationary (16 h) phases (**Figure 3**). After 6 h in the presence of MEL, *CTT1*, *CTA1, SOD1* and *GRX2* expression was higher than that under the control condition, whereas expression of *GSH1*, *GPX1,* and

especially *TRX2,* was lower. Conversely, expression of *GLR1, ZWF1* and *SOD2* was not affected.

Upon entry into the stationary phase (16 h), expression of all genes increased significantly under the control condition (**Figure 3**), with the highest levels found for catalase genes *(CTT1* and *CTA1*; 1000 and 600 times, respectively) and the lowest for *GLR1* and *ZWF1* (2 and 3 times, respectively). Moreover, at this time point, the presence of MEL resulted in a greater increase in the expression level of most genes (*GSH1*, *GPX1*, *GLR1*, *CTA1*, *SOD1*, *SOD2* and *GRX2),* between 3 and 5 times higher than under the control condition (**Figure 3, inset**). Exceptions were *CTT1* and *ZWF1*, expression of which was similar to the control, and *TRX2*, expression of which remained lower than the control.

MEL activated the cytosolic catalase gene (*CTT1*) but only in the early exponential phase. Instead, at the stationary phase (16 h) expression of *CTT1* was highly up-regulated, regardless of the presence of MEL (**Figure 3**).

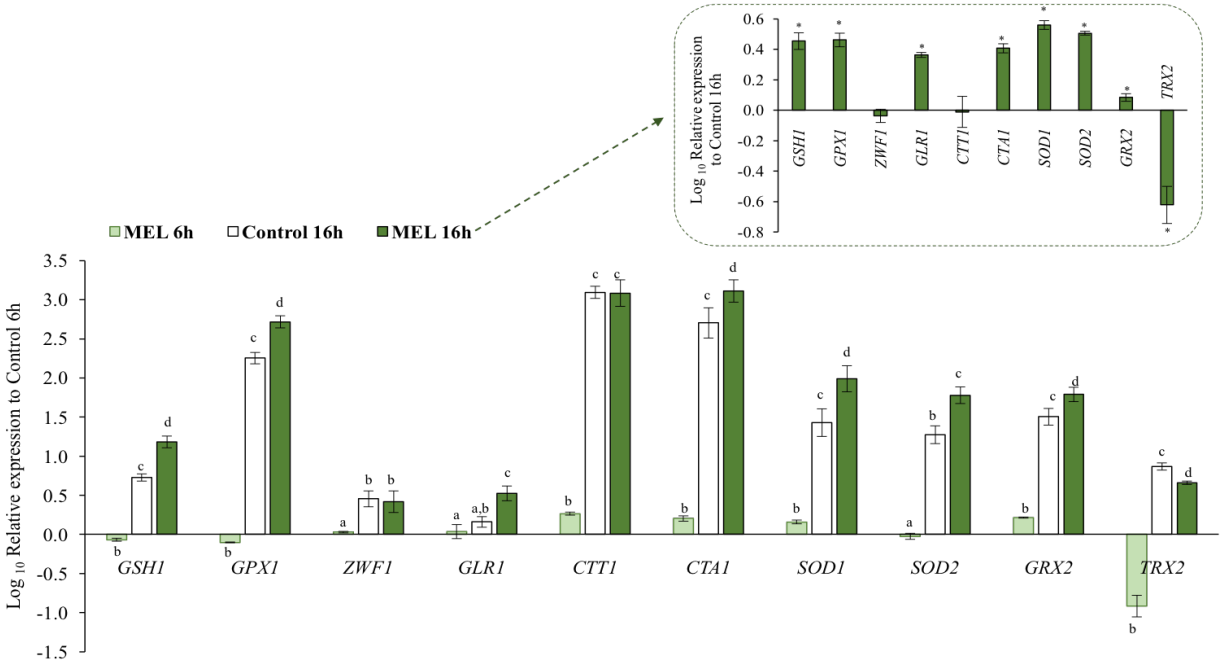

Figure 3. Effect of melatonin on expression of genes encoding enzymes involved in the response to oxidative stress: γ-glutamylcysteine synthetase (*GSH1*), glutathione peroxidase (*GPX1*), glucose 6-phosphate dehydrogenase (*ZWF1*), glutathione reductase (*GLR1*), catalase T and A (*CTT1* and *CTA1*, respectively), Cu/ZnSOD (*SOD1*), MnSOD (*SOD2*), glutaredoxin (*GRX2*) and thioredoxin (*TRX2*). Gene expression was determined at the early exponential (6 h) and early stationary (16 h) phases with and without melatonin (MEL and Control, respectively). The values are expressed relative to expression at 6 h without melatonin (Control 6 h). Relative expression of all genes in MEL 16 h relative to Control 16 h are also shown in the inset. Error bars represent ± SD of n=3 by ANOVA and Tukey's post-test [a,b,c,d or *] $p<0.05$.

3.4. Effect of melatonin on the glutathione redox status under oxidative stress

To evaluate the effect of MEL on the glutathione redox balance in yeast under oxidative stress, the response to the addition of an oxidant compound such as H_2O_2 in the presence or absence of MEL was assessed at different phases of growth. Glutathione levels (GSH and GSSG) were analyzed before and after stress induction with 2 mM H_2O_2 (at 0, 10, 45, 90 and 120 min of exposure) and at different stages of growth (early exponential (6 h), early stationary (16 h) and late stationary (30 h) phases). To determine the ability of cells to grow after stress exposure, cells were reinoculated in YPD, and growth was followed for 24 h. The levels of GSH/GSSG and cell growth recovery differed depending on the time at which the stress was applied (**Figure 4 A-I**). The presence of MEL at the early exponential phase, as shown in **Figure 2**, caused small differences in intracellular glutathione levels, slightly decreasing GSH and increasing GSSG (**Figure 4A, B**), and these changes did not alter cell growth recovery (**Figure 4C**). When oxidative stress was applied (H_2O_2), the redox balance changed, and a significant increase in GSSG/decrease in GSH was detected. Moreover, the relative viable fraction, calculated from cell growth curves, dramatically decreased until 43.0 $\pm$ 1.2 % (considering 100% the value of Control condition), indicating that the initial viability was highly affected by stress. In consequence, cell growth was highly affected, presenting a longer lag phase, higher generation time and lower final cell concentration (**Figure 4C**). This damage was slightly mitigated when stress was applied in the presence of MEL (MEL H_2O_2), with lower GSSG accumulation and slightly higher GSH than in stressed cells in the absence of MEL. Although the viable fraction was also affected, this value was higher with MEL supplementation (51.9 $\pm$ 1.8 %), resulting in a slight improvement of cell growth (**Figure 4C**).

At the early stationary phase, the total amount of glutathione (GSH and GSSG) was higher under all conditions (**Figure 4D, E**), as shown in **Figure 2B**. When stress was applied, larger differences between stressed and non-stressed cells were observed, with again lower levels of GSH and higher levels of GSSG in stressed cells. Moreover, recovery of cell growth and viability was also strongly affected by stress exposure (**Figure 4F**), presenting a relative viable fraction of 60.0 $\pm$ 1.5 %. Instead, the presence of MEL under oxidative stress significantly decreased GSSG and increased GSH, reaching similar levels

as non-stressed cells (**Figure 4D, E**). In addition, the relative viable fraction in presence of MEL was significantly higher (80.9 ± 2.9 %), greatly enhancing cell growth after stress, with a growth curve similar to non-stressed cells (**Figure 4F**).

Finally, when stress was applied at 30 h, GSH/GSSG levels were similar for all conditions, with the only significant differences found at 120 min after stress exposure, i.e., lower GSH (**Figure 4G**) and higher GSSG (**Figure 4H**) levels. In this case, the decrease of the relative viable fraction was similar within both stressed conditions (MEL H_2O_2: 94.8 ± 1.9 %; H_2O_2: 91.8 ± 1.5 %) and much higher than in early exponential and stationary phases, allowing the cells to normally recover growth after stress exposure (**Figure 4I**). The presence of MEL did not significantly modify the glutathione profile or the growth curve at this stage. In fact, under all conditions (Control, MEL, MEL H_2O_2 and H_2O_2), similar population sizes were achieved at the stationary phase, which were lower than those after oxidative stress exposure at 6 h or 16 h (**Figure 4 I**).

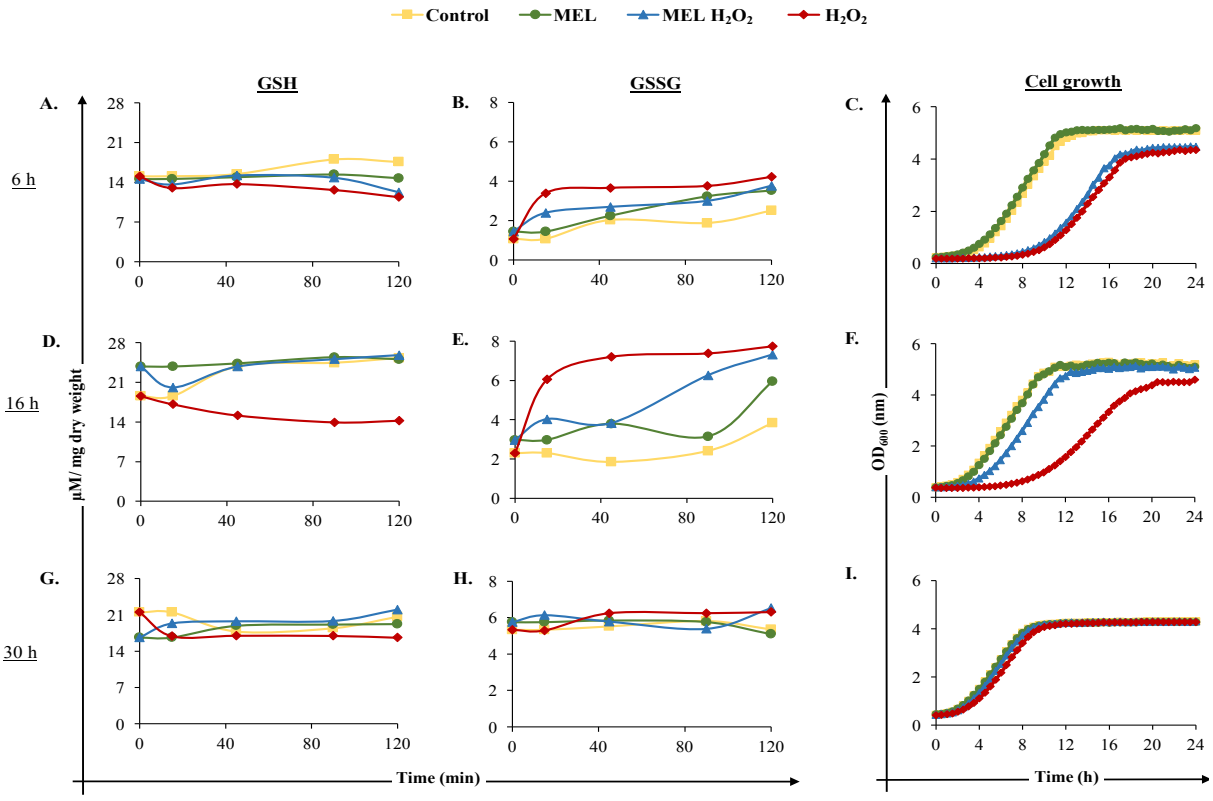

Figure 4. Effect of the presence of melatonin on reduced glutathione (GSH: A, D, G), oxidized glutathione (GSSG: B, E, H) and viability (C, F, I) of unstressed and stressed cells before and at 10, 45, 90 and 120 min after oxidative stress induction. Control: control cells; MEL: 5 µM melatonin; MEL H_2O_2: 5 µM melatonin and 2 mM H_2O_2; H_2O_2: 2 mM H_2O_2. Stress was applied in the early exponential phase (6 h, A, B, C), in the early stationary phase (16 h, D, E, F), and in the late stationary phase (30 h, G, H, I). For the viability test, cells from the four conditions were reinoculated in YPD fresh medium.

For further analysis of these data (**Figure 4**), a PCA was applied to correlate the different variables (reduced and oxidized glutathione and growth curves data) and highlight if there were grouping patterns within the different conditions (Control, MEL, MEL H_2O_2, H_2O_2) at 6 h, 16 h and 30 h (**Figure 5**).

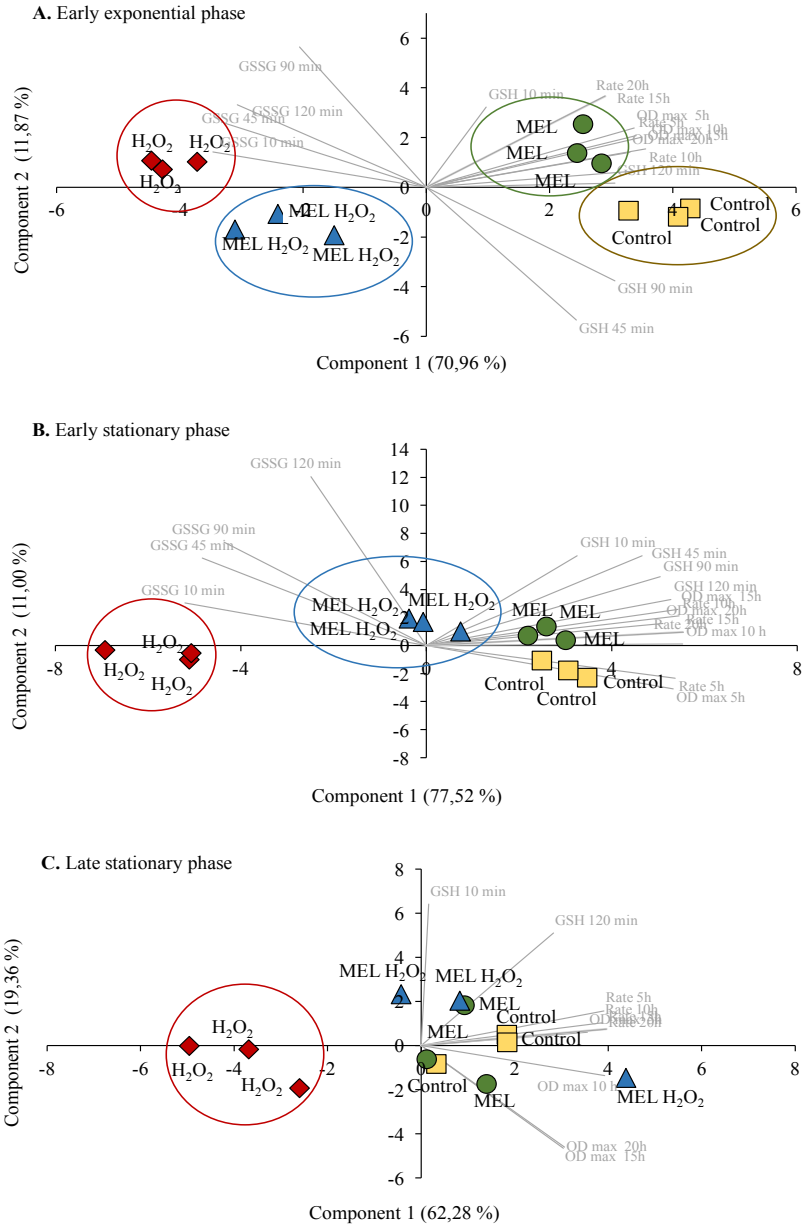

Figure 5. Bi-plots of principal components analysis (PCA) using the following variables: intracellular reduced GSH, oxidized GSH (GSSG), maximum optical density (ODmax) and maximum growth rate values calculated at 5, 10, 15 and 20 h based on cell growth obtained in the viability test. Control: control cells; MEL: 5 µM melatonin; MEL H_2O_2: 5 µM melatonin and 2 mM H_2O_2; H_2O_2: 2 mM H_2O_2. (A) Oxidative stress applied at early exponential phase (6 h). Component 1: (+); GSH (10, 45, 90 and 120 min), OD max and growth rate (5, 10, 15 and 20 h). (-): GSSG (10, 45 and 120 min). Component 2: (+); GSSG 90 min. (B) Oxidative stress applied at early stationary phase (16 h). Component 1: (+); GSH (10, 45, 90 and 120 min), OD max and rate (5, 10, 15 and 20 h). (-); GSSG (0, 45 and 90 min). Component 2: (+); GSSG 120 min. (E) Oxidative stress applied at late stationary phase (30 h). Component 1; (+); GSH 120 min, OD max (5, 10, 15 and 20 h), growth rate (5 and 10 h). Component 2: (+); GSH 10 min.

In the resulting PCA plot at 6 h (**Figure 5A**) and 16 h (**Figure 5B**), the PCs explained 82.83% and 88.52% of the variance, respectively. In both PCA, parameters indicating greater viability (higher rate and OD max) were positively correlated with higher levels of GSH (positive component 1) and negatively correlated with higher levels of GSSG (negative component 1) (**Figure 5A, B**). Thus, when stress was applied in the early exponential phase (6h, **Figure 5A**), the different conditions were clearly separated into four groups, where cells without stress presented better growth, higher GSH and lower GSSG levels than stressed cells. MEL condition was grouped apart from the control due to a higher oxidized state (higher GSSG and lower GSH). In contrast, in MEL H_2O_2, a decrease in the oxidized state in comparison to stressed cells resulted in a shift in the component 1 towards the unstressed conditions, being this shift even greater when the stress was applied at 16 h (**Figure 5B**). Finally, in the late stationary phase (30 h), the PCs explained 81.64% of the variance (**Figure 5C**), but merely by the cellular growth variables and GSH at 120 min (positively correlated within the positive component 1). Only stressed cells without MEL were grouped together and separated from the other conditions, presenting the lowest GSH levels at 120 min, rate growth and OD max, what indicated that MEL also has a slight effect when the stress was applied at 30 h.

3.5. Effect of melatonin on expression levels of genes related to the antioxidant response under oxidative stress

Expression of selected genes implicated in endogenous antioxidant defense was also determined at 45 and 120 min after oxidative stress (2 mM H_2O_2) applied in the early exponential (6 h) or early stationary (16 h) phase and in the presence or absence of MEL (**Figure 6**).

In the early exponential phase (**Figure 6A**), the expression levels of all genes increased significantly at 120 min after stress exposure, with six of increasing already at 45 min (*GSH1*, *CTT1*, *CTA1*, *SOD1*, *SOD2*, and *GRX2*). The presence of MEL in stressed cells resulted in faster up-regulation of most genes (except for *CTT1* and *GRX2*), as the levels obtained at 45 min with MEL (MEL H_2O_2) were similar to those obtained at 120 min without MEL (H_2O_2). Indeed, at 120 min, the expression levels of all genes (except *GRX2*) were significantly higher with MEL than without. Exposure to stress at 6 h caused a 30-fold increase in expression of the *CTT1* gene at 45 minutes, an activation that was much

lower than that observed upon entry into the stationary phase (Figure 3). However, at 120 min, expression remained constant in the presence of MEL but declined in its absence. In general, the levels of gene up-regulation obtained by exposure to stress during the log phase (6 h) were lower than those obtained upon entry into the stationary phase (**Figure 3**), except for *TRX2*, the levels of which were higher under stress in the presence of MEL.

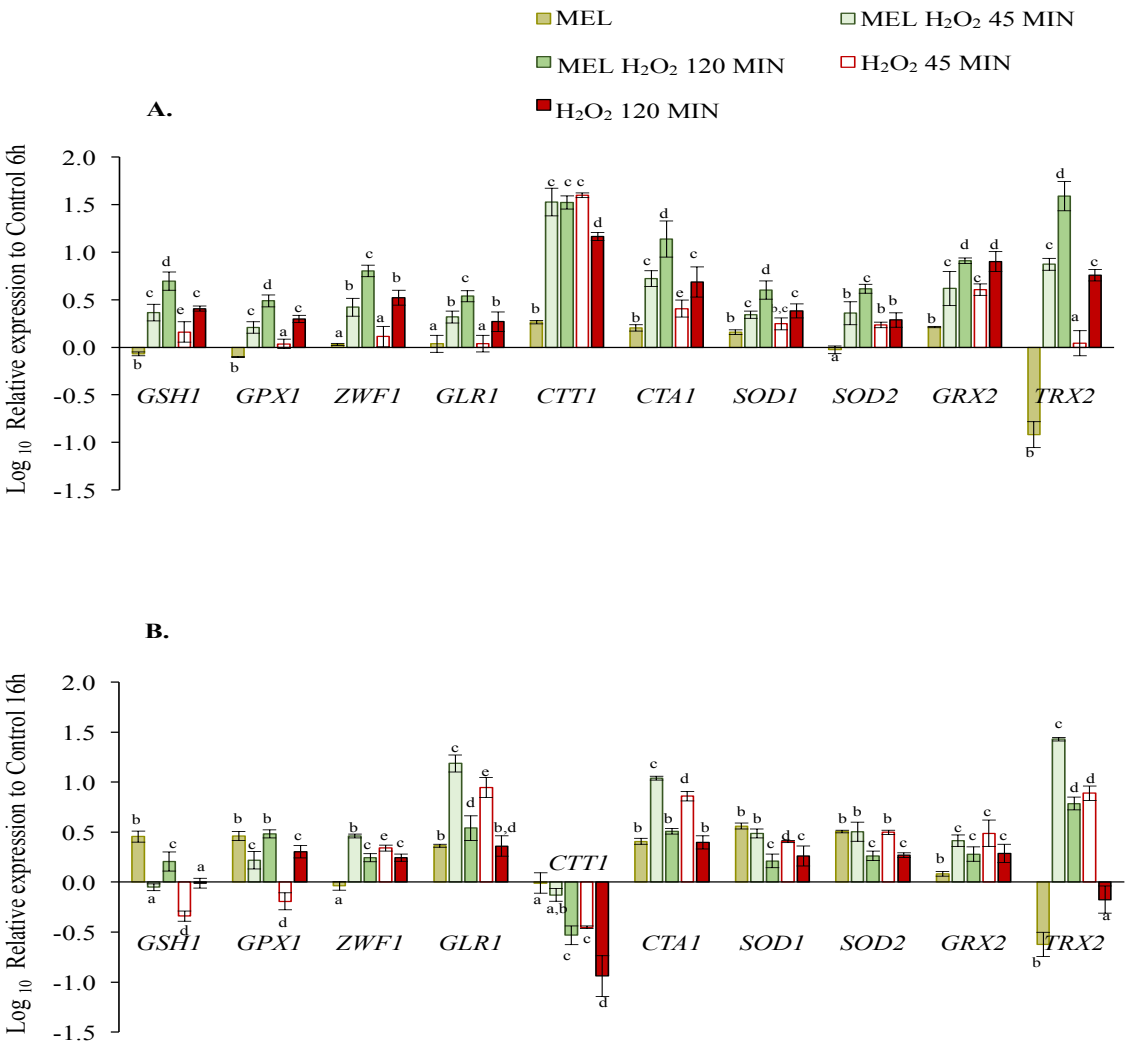

Figure 6. The effect of melatonin on expression of genes involved in the oxidative stress response, as determined before and after 45 and 120 min after stress exposure (2 mM H_2O_2), in cells previously grown with MEL (5 μM) and without MEL. (A) Gene expression in the early exponential phase, when stress was applied at 6 h. (B) Gene expression in the early stationary phase, when stress was applied at 16 h. Changes are expressed relative to gene expression of the Control condition at time 6 h or 16 h. Error bars represent ± SD of n=3 by ANOVA and Tukey's post-test $^{(a, b, c)}$, $p<0.05$.

The observed expression profile was different in cells stressed in the stationary phase (16 h) (**Figure 6B**). In this case, most of the genes (*ZWF1*, *GLR1*, *CTA1*, *SOD1*, *SOD2*, *GRX2*

and *TRX2*) were quickly activated after stress exposure, with higher values at 45 minutes, though their expression levels decreased over time. The presence of MEL increased expression of some of these genes (*ZWF1*, *GLR1, CTA1, SOD1* and *TRX2*) but only during the first 45 min. At 120 min, expression of only *GSH1*, *GPX1* and *TRX2* remained high in the presence of MEL (MEL H_2O_2). Compared to non-stressed cells, *CTT1* expression at the stationary phase was lower in stressed cells, with the lowest levels in the absence of MEL. *GSH1* and *GPX1* also exhibited lower levels of expression than the control condition at 45 min after stress exposure, though these levels were up-regulated over time, with higher levels of expression in the presence of MEL.

As mentioned above, MEL alone was able to up-regulate certain genes in cells not exposed to stress, yet this increase in expression due to the presence of MEL at 6 h was much lower than the levels observed after stress exposure. For most genes (*GSH1*, *GPX1*, *GLR1*, *CTA1*, *SOD1*, and *SOD2*), this increase at 16 h was similar or even higher than that obtained after 120 min of stress exposure.

4. DISCUSSION

Recently, it has been described that *S. cerevisiae* synthetizes bioactive compounds derived from aromatic amino acids such as MEL during alcoholic fermentation. The role of MEL in cells has been extensively studied in humans and other organisms (Hardeland and Poeggeler, 2003; Tan et al., 2015), and its antioxidant capacity is among the most important biological activities described. However, its role in yeast is unknown. Therefore, in this study, the possible effect of MEL in protecting against oxidative stress was evaluated in *S. cerevisiae,* a well-established eukaryotic model and considered the wine yeast par excellence.

Exposure to oxidative stress generates ROS, which adversely affect cells when their capacity to eliminate these reactive species is exceeded. Therefore, cells need to be equipped with regulatory molecules to rapidly sense and respond to oxidative stress. We have focused our research on GSH as the main and most abundant endogenous antioxidant in cells (Moradas-Ferreira et al., 1996; Jamieson, 1998), and accordingly, the effect of MEL on the glutathione status with and without stress was evaluated in *S. cerevisiae* in the current study. Our results show that the presence of MEL at low doses (5 μM) alters basal glutathione levels with a slight increase in ROS accumulation. ROS

accumulation with a decrease in GSH due to MEL has been exclusively reported in the *in vitro* response in human cells, mostly in cancer cells in which MEL exhibits pro-oxidant properties (Osseni et al., 2000; Wölfer et al., 2001; Albertini et al., 2006). Interaction between calmodulin and MEL might represent the mechanism involved in the stimulation of ROS by MEL (Radogna et al., 2009). We also observed that MEL repressed the *GSH1* and *GPX1* genes, which encode γ-glutamylcysteine synthetase and glutathione peroxidase, respectively, in the early exponential phase. Such repression by the presence of MEL has not previously been reported. These results confirm that the reduced ratio of GSH:GSSG corresponds to a decrease in mRNA levels of γ-glutamylcysteine synthetase. Many studies have documented the effects of MEL on gene regulation in human cells, and the mechanism by which MEL alters expression of antioxidant genes in *S. cerevisiae* may also be mediated by MEL receptor activation (Rodriguez et al., 2004; Tomás-Zapico and Coto-Montes, 2005). In this way, MEL would act indirectly on the glutathione system by decreasing GSH. Furthermore, thioredoxin, encoded by *TRX2,* which is specialized in protection against ROS, was strongly repressed in the presence of MEL, even though its mRNA levels were derepressed over time. This fact could explain the observed slight accumulation of ROS. Furthermore, very low doses of ROS (non-toxic levels) can serve as signaling molecules for cells to adapt and become more resistant to a subsequent lethal exposure.

Simultaneously, MEL activates genes involved in primary defense, which are normally repressed or present at very low levels during anaerobic growth in high-glucose culture media (Jamieson, 1998; Belazzi et al., 1991; DeRisi et al., 1997; Boy-Marcotte et al., 1998; Puig et al., 2000; Büyükavci et al., 2006), including those encoding both catalases (*CTT1* and *CTA1*) and cytosolic superoxide dismutase (*SOD1*). However, in the early stationary phase, when glucose was consumed by the cells (data not shown), all genes examined in the study were derepressed, and their expression increased. The transition of *S. cerevisiae* from fermentative to respiratory metabolism increases ROS production and involves modulation of the antioxidant system that confers cell resistance to oxidants. For this reason, as our results confirmed, cells are more susceptible to stress during the exponential phase than during the stationary phase. This also agrees with the viability results of cells after stress exposure, where the viability increased when the

stress was applied in early and late stationary phase. In addition to activation of these genes in the early stationary phase, a clear effect of MEL was observed because it potentiated expression of many genes involved in primary (*GPX1*, *CTA1*, *SOD1*, *SOD2*) and secondary (*GSH1*, *GLR1, GRX2*) defense systems. ROS accumulation and changes in the basal glutathione balance followed by activation of stress genes in the presence of MEL had no effect on *S. cerevisiae* viability, indicating a lack of correlation with cytotoxicity or apoptosis (Osseni et al., 2000; Büyükavci et al., 2006; Girish et al., 2013). Our results appear to indicate that MEL prepared the cells to better endure stress generated in the stationary phase by inducing an increase in mRNA levels of antioxidant genes.

Nonetheless, exposure to oxidative stress (2 mM of H_2O_2) caused an increase in ROS, which activated defense mechanisms to maintain a proper redox state. Upon H_2O_2 challenge, yeast cells activate various antioxidant functions, including a gene expression program mediated largely by the transcription factors Msn2p/4p in a general stress response and Yap1p and Skn7p in a specific response to oxidative stress. (Moradas-Ferreira et al., 1996; Costa and Moradas-Ferreira, 2001; Jamieson, 1998; Moradas-Ferreira and Costa, 2000). Skn7p factor controls a subset of genes involved in the thioredoxin system, whereas Yap1p is required for the induction of all the oxidative responsive genes (Gómez-Pastor, et al., 2010). The effect of stress on *S. cerevisiae* cells was higher in the early exponential (6 h) and stationary (16 h) phases than in the late exponential phase (30 h). At 30 h, the cells appeared to have already prepared their defense mechanisms and be more resistant to oxidants. Under such stress conditions, our results clearly showed, as have the vast majority of studies in humans (Reiter et al., 2016; Tan et al., 2015), that MEL has an antioxidant effect on *S. cerevisiae* in the early exponential and early stationary phases. Although MEL mitigated ROS accumulation generated by low concentrations of H_2O_2 in *S. cerevisiae*, this effect was not observed at higher concentrations of H_2O_2 (4 mM), likely because the oxidative stress exceeded the endogenous antioxidant capacity. Additionally, MEL-treated cells exhibited increased GSH and decreased GSSG concentrations. This state of lower oxidative stress resulted in a clear enhancement in cell viability in the early stationary phase. In humans, MEL displays multiple mechanisms to protect cells against oxidative stress, e.g., it possesses

direct free radical scavenging activity, whereby it is able to detoxify $^{\cdot}OH$ produced by H_2O_2 (Tan et al., 2000). In our case, analysis of expression of certain stress-related genes under oxidative stress appeared to indicate that MEL in *S. cerevisiae*, as in humans, might interact with different components of antioxidant defense systems. In fact, the amphiphilic characteristics of MEL allow it to cross all morpho-physiological barriers, reaching any subcellular structure and guaranteeing its ability to act as a free radical scavenger (Tan et al., 2000; Reiter et al., 2007). Although MEL is able to act as a direct radical scavenger in *S. cerevisiae*, our results showed that MEL might also function as an indirect antioxidant by increasing the transcription level of genes related to the antioxidant response. Furthermore, stimulation of expression of genes encoding antioxidant enzymes by MEL via receptor activation can occur at nanomolar concentrations in cultured cells (Rodriguez et al., 2004; Mayo et al., 2002; Kotler et al., 1998).

As discussed above, an intense change in gene activity occurred within minutes after exposure to H_2O_2 in both the early exponential and stationary phases, and our results evidenced how low concentrations of MEL influenced even more changes in gene expression. A number of *in vitro, in vivo* and *ex vivo* studies in humans have documented the ability of MEL to increase expression of multiple antioxidant stress genes, including copper zinc and manganese superoxide dismutases, glutathione peroxidase, catalase and γ-glutamylcysteine synthase (Kotler et al., 1998; Esparza et al., 2005; Gómez et al., 2005; Mauriz et al., 2007; Fischer et al., 2013). Moreover, this effect on antioxidant enzyme genes appears to be consistent with the ability of MEL to up-regulate the activities of these enzymes as well as glutathione reductase and glucose-6-phosphate dehydrogenase. In the current study, as in mammalian studies, the presence of MEL induced high levels of *GSH1*, *GPX1*, *CTT1*, *CTA1*, *SOD1* and *SOD2* mRNA (Tomas-Zapico and Coto-Montes, 2005; Mayo et al., 2002; Kotler et al., 1998). Furthermore, our results showed that in *S. cerevisiae*, MEL also up-regulated expression of *GLR1*, *ZWF1* and particularly *TRX2*, which encodes the most important hydrogen peroxide-eliminating enzyme. Conversely, *CTT1* expression exhibited a different profile: it was rapidly activated by H_2O_2 in the early exponential phase, but it was repressed in the early stationary phase, showing an earlier decrease in gene expression in the absence of MEL.

CTT1, which encodes cytosolic catalase T, is involved in the primary antioxidant defense induced by H_2O_2. This gene is not only induced by hyperoxidant conditions but also by starvation and heat or osmotic shock (Moradas-Ferreira et al., 1996; Costa and Moradas-Ferreira, 2001; Jamieson, 1998). Our results showed that induction of this gene occurred mainly during the exponential phase and that the effect of MEL on *CTT1* was significantly reduced after this phase. Nonetheless, some effect was still observed because lower levels were reached in the absence of MEL. *ZWF1* stimulation by MEL has been suggested in a few studies, as activation of the enzyme glucose-6-phosphate dehydrogenase was observed in the presence of the molecule (Pierrefiche and Laborit, 1995; Hardeland, 2005). The overexpression of *ZWF1* due to MEL under stress conditions observed in our study appears to highlight the importance of this gene in GSH recycling, as NADPH is a necessary cofactor for glutathione reductase and provides reducing equivalents for redoxin systems. *TRX2*-encoded thioredoxin, the mRNA levels of which were up-regulated with MEL addition under oxidative stress conditions, is specialized in protecting against ROS and is essential for YAP1-dependent resistance to hydroperoxides (Herrero et al., 2008; Gómez-Pastor et al., 2012; Kuge and Jones, 1994); indeed, this gene (*TRX2*) is one of the first targets of the major oxidative stress transcription factor Yap1p. *TRX2,* besides to be required to regulate redox state and levels of glutathione in response to oxidants, it can be also upregulated in response to changing growth conditions, providing a first line of defense against oxidative stress (Gómez-Pastor et al., 2012). Therefore, our results indicate that MEL indirectly increases the resistance of *S. cerevisiae* to oxidative stress via overexpression of *TRX2,* enhancing the fermentative capacity and viability of this wine strain under vinification conditions.

MEL has frequently been compared with vitamins C and E in terms of antioxidant properties (Reiter et al., 2007). The effect of MEL on *S. cerevisiae* may also be comparable to the effect of resveratrol on yeast (Escoté et al., 2012), which induces Yap1p activity (the major regulator of genes involved in the oxidative stress response) to reduce ROS levels under oxidative stress.

The literature contains many references regarding the biological properties of MEL as a hormone and its antioxidant properties, among other beneficial effects in vertebrates. Based on our results, MEL synthesis by *S. cerevisiae* during wine production could be

related to the ability of yeast cells to adapt to and endure the hostile environment of the wine-making process and probably counteract the prooxidant effects of ethanol. Furthermore, *S. cerevisiae* in active dry yeast form, used as starter in biotech and food industries, can suffer oxidative stress during biomass propagation and dehydration steps of their production, which could negatively affect yeast performance. Oxidative stress also influences the replicative lifespan of yeast, particularly important in re-pitching practices. Thus, protective treatments against oxidative damage with natural antioxidants, may have important biotechnological implications.

5. CONCLUSIONS

MEL presents antioxidant properties in *S. cerevisiae*. However, these antioxidant properties were dependent on the dose and the phase at which stress was induced. In the absence of stress, MEL exposure appears to prepare cells for further oxidant assaults, whereby MEL clearly reduces oxidative damage in *S. cerevisiae* by decreasing ROS and oxidized glutathione (GSSG) levels. Our analysis offers insight into the effect of MEL on antioxidant defense systems in *S. cerevisiae*. However, the findings also raise a number of intriguing questions related to the regulation of gene expression by oxidative stress as a complex process controlled by different key regulators and intercommunication with different stress response pathways. Evaluation of the impact of MEL on transcription factors (especially Yap1p and Snk7p), or their influence on gene expression related to MEL receptors in *S. cerevisiae* could offer a productive avenue for further research. Furthermore, the effect of lower concentrations of MEL, closer to the ones found in fermented beverages, should also be assessed.

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CHAPTER 3

Melatonin minimizes the impact of the oxidative stress induced by hydrogen peroxide in *Saccharomyces* and non-*Saccharomyces* yeasts

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Abstract

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized from tryptophan by *Saccharomyces cerevisiae* and non-conventional yeast species. Antioxidant properties have been suggested as a possible role of melatonin in a *Saccharomyces cerevisiae* wine strain. However, the possible antioxidant melatonin effect on non-*Saccharomyces* species and other strains of *S. cerevisiae* must be evaluated. The aim of this study was to determine the antioxidant capacity of melatonin in eight *S. cerevisiae* strains and four non-conventional yeasts (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Starmerella bacillaris* and *Klockera appiculata*). Therefore, the ROS formation, lipid peroxidation, catalase activity and peroxisome proliferation were investigated. The results showed that the presence of melatonin increases peroxisome accumulation and slightly increases the catalase activity. When cells that were grown in the presence of melatonin were exposed to oxidative stress induced by H_2O_2, lower ROS accumulation and lipid peroxidation were observed in all tested strains. As a consequence, the increased catalase activity that was a consequence of oxidative stress was lower in the presence of melatonin. These findings demonstrate that melatonin can act as an antioxidant compound in both *S. cerevisiae* and non-*Saccharomyces* yeasts.

Keywords: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Starmerella bacillaris*, *Klockera appiculata*, ROS, TBARS, catalase, peroxisomes.

1. INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) (MEL) is not only known as a neurohormone in vertebrates, but it is as well considered as a ubiquitous molecule that is present in most living organisms (Hardeland & Poeggeler, 2003). Sprenger et al., (1999) were the first authors to associate the production of MEL with *Saccharomyces cerevisiae*. Later, other reports showed high quantities of MEL being produced by *S. cerevisiae*, and by other non-conventional yeast species such as *Torulaspora delbrueckii* and *Zygosaccharomyces bailii* (Rodriguez-Naranjo et al., 2011; Vigentini et al., 2015). Although only limited information is available on MEL biosynthesis in organisms other than vertebrates, the pathway in yeasts is thought to be similar to the synthetic route described in vertebrates. Four enzymes are involved in the conversion of tryptophan into serotonin and N-acetylserotonin intermediates and finally into MEL (Mas et al., 2014).

The functions of MEL have been extensively studied in mammals and animals, and they are primarly related to the regulatory mechanisms involved in circadian rhythms. However, the role of MEL in yeasts still needs to be elucidated. Recently, we have reported that MEL is able to act as an antioxidant compound in one commercial wine strain of *S. cerevisiae* (Vázquez et al., 2017). As is the case in humans, MEL might protect various biomolecules from damages caused by free radicals by acting as a direct scavenger, detoxifying reactive oxygen and nitrogen species (Anisimov et al., 2006; Reiter et al., 2001, 2016) and indirectly increases the activities of the antioxidant defense systems. It could also act by stimulating the synthesis of other important intracellular antioxidants such as glutathione peroxidase and superoxide dismutase (Antolín et al., 1996; Rodriguez et al., 2004).

Oxidative stress is the outcome of an imbalance between the presence of reactive oxygen species (ROS) and the capacity of cells to detoxify these reactive intermediates of molecular oxygen, or to repair the resulting damage. ROS are constantly generated during normal metabolism, and they exert physiologic actions. However, when produced in excess, ROS cause detrimental effects and can damage cell macromolecules, such as DNA, lipids or proteins (Gutteridge and Halliwell, 2000; Halliwell, 2006). Among these targets of ROS, lipid peroxidation leads to one of the most

damaging consequences for cells when unsaturated lipids are converted into polar lipid hydroperoxides because it allows the propagation of free radical reactions that could affect membrane integrity and even result in cell death (Ayala et al., 2014; Howlett and Avery, 1997). However, ROS formation is accompanied by an increase in yeast antioxidant defenses, with the aim of protecting the cells against noxious ROS. One system for neutralizing the excessive ROS formation in cells is to degrade them with antioxidant enzymes such as catalase, gluthathione peroxidase and superoxide dismutase. By contrast, non-enzymatic systems such as glutathione, glutaredoxins and thioredoxins repair or remove the products of oxidative damage (Auchère et al., 2008; Costa and Moradas-Ferreira, 2001; Herrero et al., 2008; Jamieson, 1998).

Due to its high fermentation capacity, *S. cerevisiae* is the yeast that is traditionally used in the biotechnology, food and beverage industries. However, non-*Saccharomyces* yeasts are now gaining higher interests for industries; in fact, several studies have demonstrated that the presence of non-conventional yeasts during the winemaking process can contribute to the aroma profile, sensory complexity and color stability (Jolly et al., 2014). During these industrial processes, yeasts are involved in different stages that can lead to oxidative stress for the cells, which could negatively affect the yeast performance (Gómez-Pastor et al., 2012; Pérez-Gallardo et al., 2013; Pretorius, 2000). Thus, protective treatments against oxidative damage with natural antioxidants may have important biotechnological implications.

The goal of this study was to evaluate the possible antioxidant effect of MEL on different yeast species. To this end, we evaluated the ROS production, lipid peroxidation and intracellular catalase activity in sixteen yeast strains of different species. We evaluated the response to oxidative stress response induced by H_2O_2 and analyzed the possible protective activity of MEL supplementation.

2. MATERIAL AND METHODS

2.1. Yeast strains and experimental conditions

The yeast strains used in this study were eight *S. cerevisiae* and eight non-*Saccharomyces*. The *S. cerevisiae* strains included three laboratory strains (BY4741, BY4742 and Sigma 1278b from the EUROSCARF collection, Frankfurt, Germany), three commercial wine strains (QA23®, Uvaferm HPS® and the hybrid VIN7 (*S. cerevisiae* x *S.*

kudriavzevii AWRI1539®)) and two commercial strains used for animal nutrition (Levucell® SC20 and SB20). The non-*Saccharomyces* species included two wine strains of *Torulaspora delbrueckii* (BIODIVA® (TdB) and Tdp), two wine strains of *Metschnikowia pulcherrima* (FLAVIA® (MpF) and Mpp), two wine strains of *Starmerella bacillaris* (Cz4 and Cz11) and two wine strains of *Hanseniaspora uvarum* (Hu4 and Hu35). The commercial *Saccharomyces* and non-*Saccharomyces* strains QA23, Uvaferm HPS, SC20, SB20, FLAVIA and BIODIVA were provided by Lallemand S.A. (Montreal, Canada), and VIN7 was provided by AWRI (Glen Osmond, Australia). The other six non-*Saccharomyces* strains (Tdp, Mpp, Cz4, Cz11, Hu4 and Hu35) were isolated from natural musts from the Priorat Appellation of Origin (Catalonia, Spain) (Padilla et al., 2016). The Tdp, Mpp, Cz4 and Hu4 were deposited in the Spanish Type Culture Collection (CECT) as CECT 13135, CECT 13131, CECT 13129 and CECT 13130, respectively.

All commercial strains were provided as active dry yeasts and were rehydrated according to the manufacturer's instructions. For all the experiments, precultures for biomass propagation were prepared in YPD liquid medium (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain)) and incubated for 24 h at 28ºC with orbital shaking (120 rpm). Yeast cells were subsequently inoculated into 50 mL of YPD broth (initial population $5x10^3$ cells/mL) with and without supplementation of 5 μM MEL (two flasks for each condition) and grown until the cells reached the initial exponential phase at 28ºC with orbital shaking at 120 rpm. Sublethal oxidative stress was then induced in one flask for each condition with 2mM of H_2O_2 for 1 h to generate the following four conditions: Control and MEL (without stress); and H_2O_2 and MEL H_2O_2 (with stress). The MEL and H_2O_2 concentrations were chosen from our previous study in the QA23 strain (Vázquez et al., 2017). Three biological replicates were tested for each condition.

2.2. Determination of reactive oxygen species (ROS)

The effect of H_2O_2 (2 mM) with and without MEL (5 μM) on the intracellular ROS was evaluated in the sixteen yeast strains. Furthermore, ascorbic acid (25 μM), a well-known antioxidant, was used as a positive control (Saffi et al., 2006). ROS was detected using dihydrorhodamine 123 (DHR 123; Sigma-Aldrich), according to the method used by (Vázquez et al., 2017). In brief, the cells were stained with 10 μg of DHR 123 per mL of

cell culture for 15 min at 120 rpm in the dark. Then, cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4), and the fluorescence intensity was measured by flow cytometry. The captured files were processed using the WinMDI 2.9 software (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA) and the ROS were represented as the mean fluorescence index (MFI) and calculated according to Boettiger et al., (2001) as follows: [(geometric mean of the positive fluorescence) – (geometric mean of the control)] / (geometric mean of the control).

2.3. Thiobarbituric acid-reacting substances (TBARS)

The degree of lipid peroxidation was measured in unstressed and stressed cells with and without MEL supplementation in terms of TBARS content (Buege and Aust, 1978; Aust 1994). Following a treatment using 2 mM H_2O_2 for 1 h, $1x10^7$ yeast cells from each condition were mechanically homogenized over three cycles of alternating sonication and liquid nitrogen (10/10 sec). The samples were then mixed with 250 μL of trichloroacetic acid (10% v/v), incubated for 15 min on ice, and after centrifugation at 2200 g for 15 min at 4ºC, 200 μL of the supernatant was mixed with 200 μL of thiobarbituric acid (6.7 g/ L). These samples were then incubated in a boiling water bath for 10 min and cooled at room temperature. Finally, the absorbance was measured at 532 nm with a microplate reader (Omega Polarstar, BMG Labtech Gmbh, Ortenberg, Germany). The TBARS content was estimated by referring to a standard curve prepared with 1,1,3,3-tetramethoxypropane, and the results were expressed as nmol of TBARS per mg of protein.

2.4. Catalase activity

The catalase activity was evaluated in unstressed and stressed cells with and without MEL. First, $1x10^7$ yeast cells were suspended in PBS (50 mM, pH 7.0) with one tablet of protease inhibitor per 10 mL of extraction solution (cOmplete™; Roche), and they were disrupted using glass beads with six cycles alternating cycles of cooling and shaking (30/30 sec) and centrifuged at 14.000 rpm for 2 min. The assay was performed according to the method described by Aebi in 1984. In brief, cells extracts were exposed to 10 mM of H_2O_2, and the decrease in absorbance at 240 nm due to H_2O_2 decomposition was monitored for 4 min, with measurements every 30 sec a constant temperature (25 ºC) using a microplate reader (Omega Polarstar, BMG Labtech Gmbh, Ortenberg, Germany).

The catalase activity was expressed as units of catalase per mg of protein. One unit of catalase activity decomposes 1 mmol of H_2O_2 per min.

2.5. Protein estimation

The total protein levels were determined using the Bradford method (Bradford, 1976) by spectrophotometric determination at 545 nm, with bovine serum albumin (BSA, Sigma-Aldrich) as a standard. The absorbance was measured an Omega Polarstar microplate reader spectrophotometer.

2.6. Western Blot (immunoblot) analysis

The immunological characterization of of QA23 and TdB strains homogenates from the four conditions (Control, MEL, MEL H_2O_2 and H_2O_2) was performed by Western blot analysis as described by Haid and Suissa (1983). In brief, the cells were disrupted with glass beads using a Disruptor Genie® (Scientific Industries, Inc., NY, USA) at 4 ºC for 10 min and centrifuged at 4ºC at 500g for 5 min. After TCA precipitation of supernatants, protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%), and later transferred to the nitrocellulose sheets according to standard procedures (Laemmli, 1970). Finally, a western blot analysis was performed using a primary rabbit antibody against Fox1p protein (multifunctional β-oxidation protein from peroxisomal membranes), a marker of peroxisomes organelles. Immunoreactive bands were visualized using a peroxidase-conjugated secondary antibody according to the manufacturer's instructions (SuperSignal ™, Pierce Chemical Company, IL, USA). The cytosolic protein GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control, and isolated peroxisomes from *S. cerevisiae* were the positive control. The identified bands were quantified using ImageJ software (National Institutes of Health, MD, USA) and normalized to the positive control.

2.7. Data analysis

The data were subjected to a one-way analysis of variance (ANOVA) and Tukey's post-hoc test to evaluate the effect of each treatment. The results were considered statistically significant at a p-values less than 0.05 (IBM SPSS Inc, XLSTAT Software).

3. RESULTS

3.1. Reactive oxygen species (ROS)

To evaluate the possible role of MEL as an antioxidant agent in the *Saccharomyces* and non-*Saccharomyces* species, the intracellular ROS levels were measured in stressed cells with and without 5 μM of MEL (Vázquez et al., 2017). Stressed cells with 2 mM of H_2O_2 and without MEL were used as a positive control, cells without stress and without MEL were used as a negative control. Cells treated with 25 μM of ascorbic acid were the as positive antioxidant control (**Figure 1**).

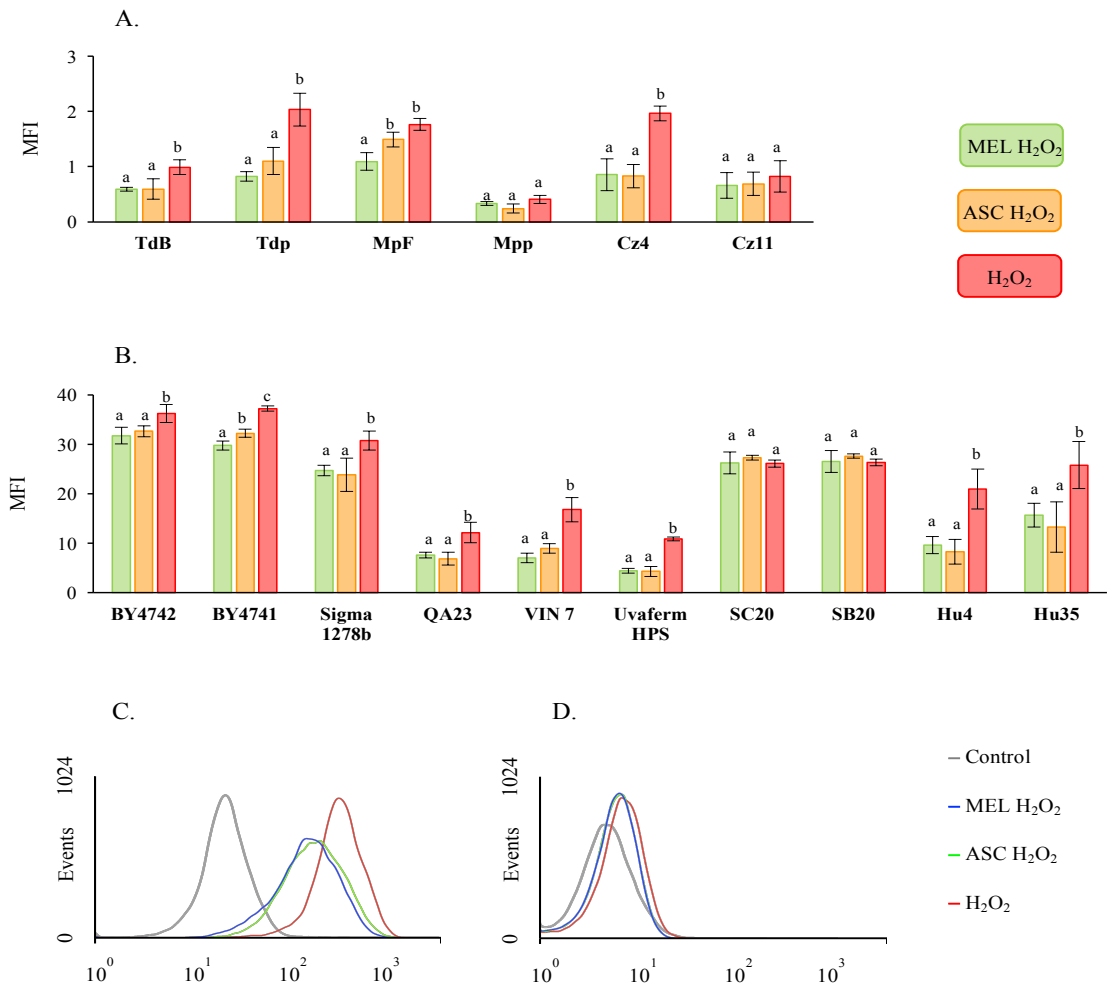

Figure 1. Melatonin (MEL, 5 μM) effect on ROS accumulation as evaluated in sixteen yeast strains under oxidative stress that was induced with 2 mM of H_2O_2. Cells without MEL and H_2O_2 were used as negative control, cells with 2 mM of H_2O_2 were used as the positive control and cells treated with ascorbic acid (25 μM) served as the positive antioxidant control. (A) The mean fluorescence index (MFI) of the *T. delbrueckii* (TdB and Tdp), *M. pulcherrima* (MpF and Mpp) and *C. zemplinina* (Cz4 and Cz11) strains. (B) The MFI of the *S. cerevisiae* (BY4742, BY4741, Sigma 1278b, QA23, VIN 7, SC20, SB20 and Uvaferm HPS) and *H. uvarum* (Hu4 and Hu35) strains. (A and B) Different letters indicate significant differences between conditions within each strain at $p<0.05$. (C and D) Flow cytometry histogram profiles as expressed as the number of events in VIN 7 (C) and Cz4 (D) with MEL (5 μM) or ascorbic acid (ASC, 25μM) and with 2mM of H_2O_2.

The results showed that cells that had been exposed to oxidative stress (2 mM H_2O_2) exhibited an increase in the total ROS. However, the ROS accumulation was species-dependent, with *M. pulcherrima*, *S. bacillaris* and *T. delbrueckii* exhibiting the lowest levels of endogenous ROS (**Figure 1A**). By contrast, the *Saccharomyces* and *H. uvarum* strains presented the highest levels of ROS (**Figure 1B**). For the *S. cerevisiae* strains, clear differences were observed between the wine strains and laboratory and animal nutrition strains, with the wine strains exhibiting the lower levels of endogenous ROS. The antioxidant effects of MEL were very similar to those of ascorbic acid for all the investigated strains. MpF was the only exception where no statistically significant protective effect was seen. However, there were few cases in which none of the antioxidants had any protective effects (*S. cerevisiae* strains SC20 and SB20 and *S. bacillaris* Cz11 (**Figure 1**)).

3.2. Lipid peroxidation

The effect of MEL on oxidative damage in the membranes was evaluated in all the yeasts by measuring the lipid peroxides in the TBA derivative form (**Figure 2A**). Most strains studied here suffered from a significant increase in lipid peroxidation after stress exposure, with the Mpp strain being the only one in which its lipid peroxidation was not triggered by H_2O_2. In fact, the lipid peroxidation results were positively correlated with ROS accumulation (**Figure 2B**, R^2=0.85863). Thus, the *M. pulcherrima*, *S. bacillaris* and *T. delbrueckii* strains, which showed lower ROS accumulation, also exhibited lower lipid peroxidation and vice versa. Strains with higher ROS accumulation showed higher lipid peroxidation (**Figure 2B**). The positive effect of MEL supplementation was clearly observed under stress conditions (**Figure 2A**, MEL H_2O_2), in which MEL seems to protect *Saccharomyces*, *T. delbrueckii* and *H. uvarum* cells against H_2O_2 damage by decreasing lipid peroxidation. In *M. pulcherrima* and *S. bacillaris*, no MEL effect was observed on lipid peroxidation. However, no significant differences were observed in the lipid peroxidation between unstressed cells with or without MEL, although in some strains, there was an increasing trend in lipid peroxidation in the presence of MEL (**Figure 2A** and **Table S1**, Control and MEL).

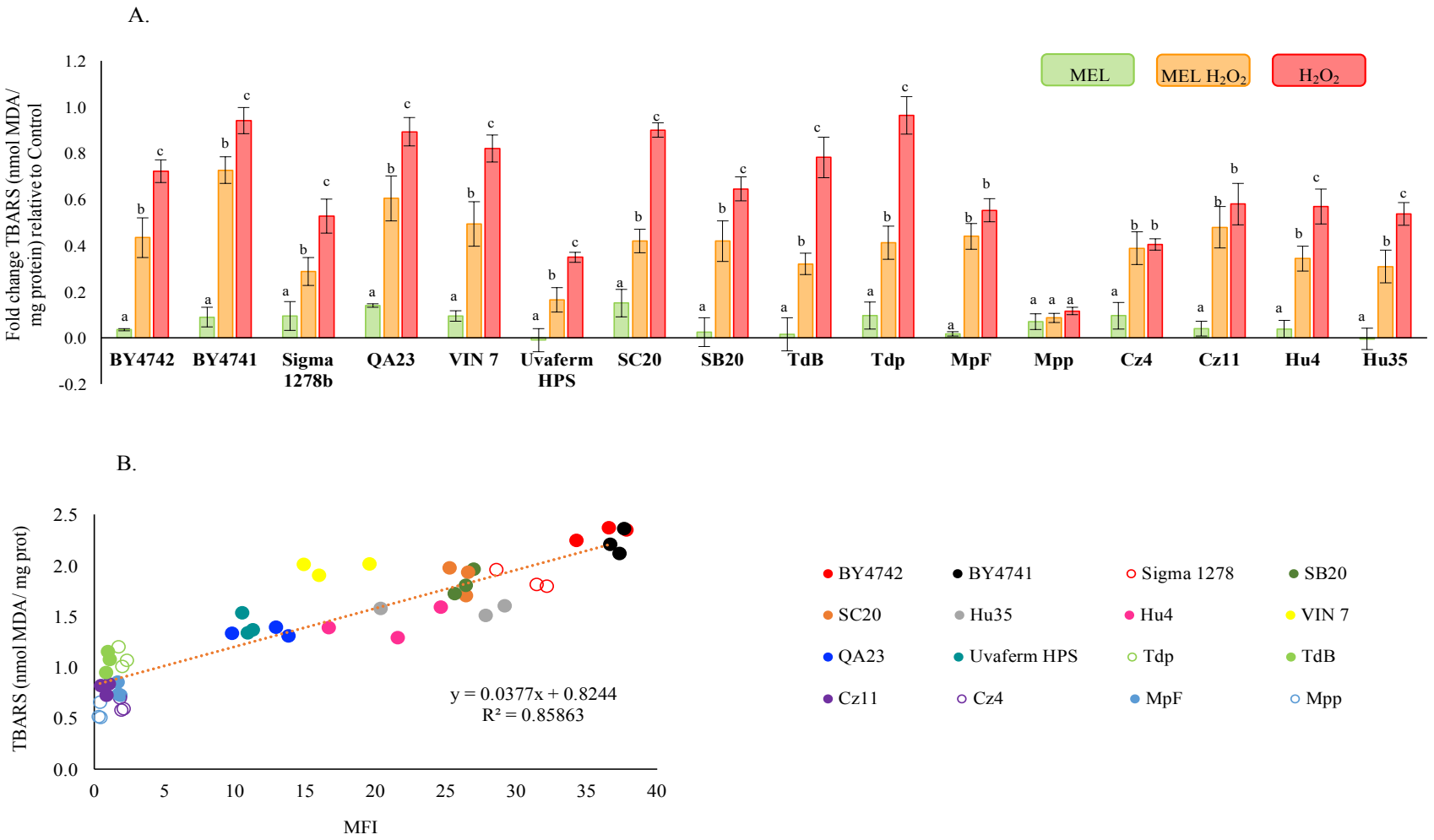

Figure 2. (A) Lipid peroxidation in unstressed and stressed yeast cells with 2 mM of H_2O_2, growing with and without 5 µM melatonin (MEL). The strains analyzed here (n=3 of each strain) were *S. cerevisiae* (BY4742, BY4741, Sigma 1278b, QA23, VIN 7, SC20, SB20 and Uvaferm HPS), *T. delbrueckii* (TdB and Tdp), *M. pulcherrima* (MpF and Mpp), *C. zemplinina* (Cz4 and Cz11) and *H. uvarum* (Hu4 and Hu35). Error bars represent ± SD of n=3 by ANOVA. Different letters indicate significant differences between conditions within each strain, p<0.05. (B) The correlation between ROS accumulation (MFI, Figure 1) and lipid peroxidation (TBARS, Table S1) in the sixteen strains (n=3 of each strain) after stress exposure with H_2O_2 (2 mM).

3.3. Catalase activity

To further study the role of MEL in yeasts, the effect of its supplementation (5 μM MEL) on catalase activity was evaluated in unstressed and stressed cells (Figure 3). The control condition (without stress and without melatonin) for non-conventional yeasts showed higher catalase activity than did the *Saccharomyces* species (**Table S1**). When MEL was added in the absence of stress, the catalase activity of *Saccharomyces*, *T. delbrueckii* and *H. uvarum* slightly increased (**Figure 3** and **table S1**). However, when cells were exposed to H_2O_2, the catalase activity clearly increased in all the strains except for Mpp and *S. bacillaris* (**Figure 3**). However, this activity was significantly reduced when the cells had been grown in the presence of MEL before the stress was applied (**Table S1**). Under these stress conditions, no numeric correlation was found between the catalase activity and ROS accumulation or TBARS assay (data not shown).

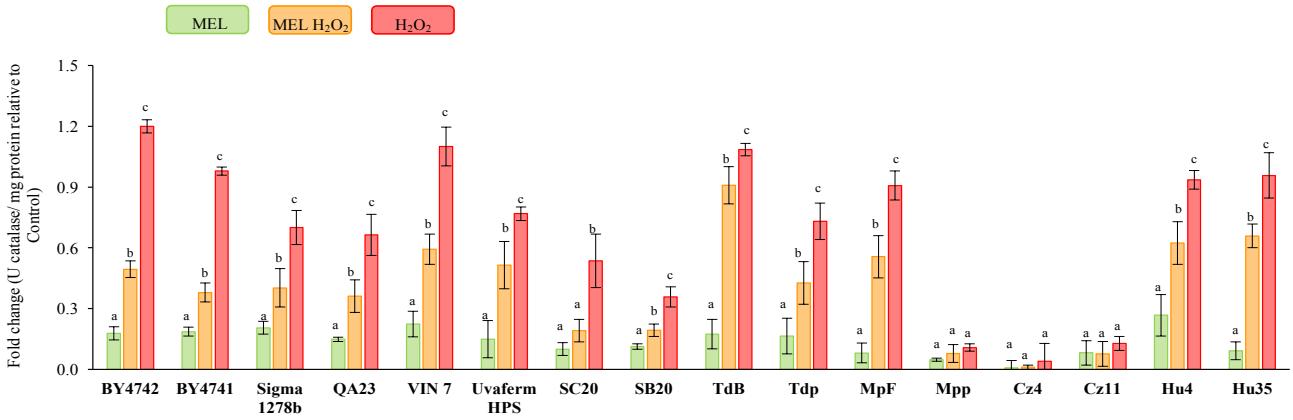

Figure 3. Catalase activity in unstressed and stressed yeast cells with 2 mM of H_2O_2, growing with and without 5 μM of melatonin (MEL). The strains used here were as follows: *S. cerevisiae* (BY4742, BY4741, Sigma 1278b, QA23, VIN 7, SC20, SB20 and Uvaferm HPS), *T. delbrueckii* (TdB and Tdp), *M. pulcherrima* (MpF and Mpp), *C. zemplinina* (Cz4 and Cz11) and H. uvarum (Hu4 and Hu35). The error bars represent ± SD of n=3 by ANOVA. Different letters indicate significant differences between conditions within each strain, $p<0.05$.

3.4. Analysis of peroxisomes proliferation

A western blot analysis using the direct antibody against the multifunctional β-oxidation protein from the peroxisomal membranes (Fox1) was performed with QA23 and TdB homogenates, with and without stress exposure, and in the presence or absence of MEL. As shown in **Figure 4A and B**, the enrichment of Fox 1p was higher in *T. delbrueckii* than in *S. cerevisiae* under both stressed and unstressed conditions. Under the control

condition, Fox1p was undetectable in *S. cerevisiae,* but its detection increased in the presence of H_2O_2. Instead, *T. delbrueckii* showed a high number of peroxisomes independent of stress exposure. MEL induced the proliferation of peroxisomes in the absence of stress, especially in *S. cerevisiae*. Under stress conditions, MEL seemed to decrease the peroxisomes accumulation slightly in both species.

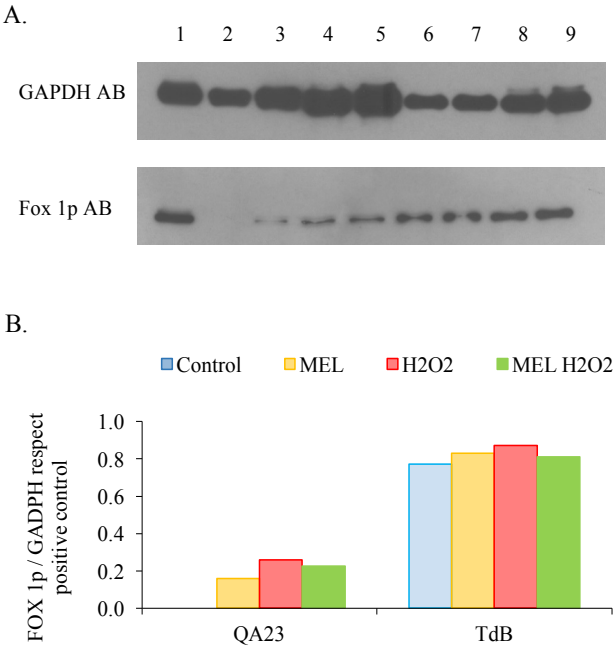

Figure 4. Western blot analysis of homogenates analyzed with an antibody against peroxisome marker Fox1p using antibody GDPDH antibody as the loaded protein control. (A) Isolated peroxisomes from *S. cerevisiae* were used as the positive control (1). Homogenates from *S. cerevisiae* QA23 (2-5) and *T. delbrueckii* BIODIVA® (6-9) in cells without treatment (Control: 2 and 6), cells without stress and 5µM of melatonin (MEL, 3 and 7), cells with 2 mM H_2O_2 (4 and 8) and cells with 2 mM H_2O_2 and 5µM of MEL (5 and 9). (B) Fox1p/GADPH quantification from the bands identified in Figure 4A were normalized to the positive control.

4. DISCUSSION

The role of MEL in cells has been extensively studied in humans and other organisms (Hardeland & Poeggeler, 2003; Tan et al., 2015), and its antioxidant capacity is one of the most important biological activities described to date. *S. cerevisiae* synthetizes MEL from tryptophan during alcoholic fermentation (Mas et al., 2014), but very little information is available on MEL biosynthesis and its bioactive functions in yeast. Recently, we reported that MEL is able to act as an antioxidant compound in a wine *S. cerevisiae* strain (Vázquez et al., 2017); however, its antioxidant role in other

Saccharomyces strains and other non-conventional yeast species is still unknown. Therefore, sixteen strains from six different yeast species were used to evaluate if the protective effect of MEL against oxidative stress is provided due to an intra or interspecific response.

As expected, ROS formation positively correlated with lipid peroxidation. Exposure to oxidative stress caused an increase in intracellular ROS that resulted in a loss of membrane integrity due to the peroxidation of unsaturated fatty acids by ROS because the polyunsaturated fatty acids are more prone to oxidation than monounsaturated fatty acids (Ayala et al., 2014; Johansson et al., 2016). However, the *M. pulcherrima*, *T. delbrueckii* and *S. bacillaris* species, which include polyunsaturated fatty acids as native constituent in their biological membranes, have exhibited higher resistance to oxidative stress (Rozès et al., 1992) together with lower ROS formation and lower lipid peroxidation. Cipak et al., (2008) reported that even if a PUFA-producing *S. cerevisiae* yeast was initially more sensitive to oxidative stress than the wild-type strain, this transgenic strain became more resistant to H_2O_2 after some time of cultivation time had passed, indicating that there was an adaptation to the endogenous oxidative stress due to the presence of PUFAs. The authors hypothesized that the presence of those PUFAs during aerobic growth generated low but significant levels of lipid peroxidation products (specifically 4-hydroxynonenal, or HNE), even in the absence of exogenous stress, which can act as a signaling molecule to activate the stress response and prepare the cells for subsequent stresses (Chen et al., 2006; Cipak et al., 2008). At sublethal concentrations, the accumulation of lipid peroxidation products stimulates the defense network, triggering the early response enzymes (antioxidative and detoxifying enzymes) and induces an adaptive response to cope with the forthcoming oxidative stress (Chen et al., 2006). A similar stress response mechanism might explain the higher resistance of these yeasts species of our study that characteristically contained membranes rich in PUFAs.

The results obtained here show that under unfavorable conditions that affect the redox balance, *Saccharomyces*, *T. delbrueckii* and *H. uvarum* clearly take advantage of MEL supplementation in the growth medium, reducing the toxic effects of H_2O_2 (decreasing the ROS levels and lipid peroxidation). These results are in accordance with several studies in humans (Taysi et al., 2003; Tesoriere et al., 1999; Ündeğer et al., 2004) and

with our previous studies with a wine *S. cerevisiae* strain (Vázquez et al., 2017) in which the protective action of MEL might be attributed to its ability to scavenge ROS particles and consequently prevent cellular damage. MEL is able to act as a direct free radical scavenger and as an indirect antioxidant, detoxifying for numerous ROS including H_2O_2, hydroxyl radicals ($^{\cdot}OH$), peroxyl radicals ($ROO^{\cdot}$), singlet oxygens ($^1O_2$) and also RNS (Romero et al., 2014).

Catalases are clearly important for proper resistance toward H_2O_2. However, the role of catalases enzymes in yeasts is not fully understood. Catalase A is located in the peroxisome and is primarily responsible for detoxifying H_2O_2 formed by acyl-CoA oxidase during β-oxidation, whereas the physiological role of the cytosolic catalase T is less clear. However, the expression of the *CTT1* gene, which encodes this enzyme, is regulated by oxidative and osmotic stress (Jamieson, 1998; Krantz et al., 2004). The process of β-oxidation is exclusively housed by peroxisomes in yeast. Here, peroxisomal oxidases such as Pox1p/Fox1p pass electrons directly to oxygen to generate H_2O_2, which is decomposed into water and oxygen by catalase A with the concomitant release of energy as heat. β-oxidation per se does not depend on a functional peroxisomal catalase (Hiltunen et al., 2003).

Non-conventional yeasts showed slightly higher catalase activity than *Saccharomyces* strains under the control condition (without stress or MEL). Cipak et al., (2008) uncovered related PUFA production with an increase in the catalase activity, pinpointing cytosolic catalase T as essential for the survival of cells against oxidative stress, and peroxisomal catalase A was important in adaptating to this stress. Therefore, higher catalase activity in non-*Saccharomyces* strains prior to stress occurs can also be induced as a response to the presence of PUFA in the membrane composition, resulting in a faster adaptation and a better tolerance to the stress. Although the catalase activity increased in the presence of oxidative stress with H_2O_2, no direct correlation between catalase activity and ROS or lipid peroxidation was observed in our results, suggesting that catalase, which is as a primary enzymatic defense, is quickly activated in the presence of H_2O_2 with the aim of avoiding cellular damage and neutralizing ROS. Furthermore this finding could indicate that other antioxidant primary defenses such as superoxide dismutase and glutathione peroxidase (not determined in this study), which

rapidly sense and respond to oxidative stress, may also be contributing to the maintenance of the ROS concentrations at a basal level (Costa and Moradas-Ferreira, 2001; Jamieson, 1998; Moradas-Ferreira and Costa, 2013).

Moreover, MEL supplementation increased their catalase activity in the *Saccharomyces*, *T. delbrueckii* and *H. uvarum* strains. Together with our previous results in the QA23 strain (Vázquez et al., 2017) in which we also observed that MEL slightly increased the ROS amount as well as the mRNA levels of *CTT1* and *CTA1* (genes enconding catalase T and catalase A, respectively) and other enzymes involved in primary defense, these current results seem to confirm the role of MEL as a prooxidant that prepares the cells to better endure subsequent stress. As expected, the catalase activity was even higher in cells exposed to H_2O_2. When cells exposed to H_2O_2 were pretreated with MEL catalase activity significantly decreased. Similar results were obtained by Saffi et al., (2006), but by using L-ascorbic acid as an antioxidant and paraquat as an oxidative agent. The authors hypothesized that the reduced catalase activity caused by the presence of L-ascorbic acid could indicate that L-ascorbic acid has sequestered part of the ROS generated by paraquat, thereby reducing the need for catalase biosynthesis. Therefore, the presence of antioxidant compounds such as MEL would reduce the amount of ROS when an oxidative stress is applied and would modulate the catalase levels in yeast cells.

Peroxisomes play important roles in yeast metabolism, mostly in the β-oxidation of fatty acids and in the degradation of toxic hydrogen peroxide via catalase and other antioxidant enzymes (Hiltunen et al., 2003; Schrader and Fahimi, 2006). The amount of peroxisomes in the cell (proliferation or degradation) is modulated in response to nutritional and environmental stimuli. Our results showed higher peroxisome proliferation in cells under stress coinciding with higher catalase activities, indicating a direct relationship between both parameters. In fact, the responses to oxidative stress in *S. cerevisiae* seem to be co-regulated, similar to the increased ROS and lipid peroxidation, which activates the proliferation of peroxisomes. The observed increase in peroxisome proliferation comes hand in hand with elevated catalase activitiy. To shed cellular organelles from harmful ROS, yeasts sequester ROS in peroxisomes, an organelle specialized and perfectly enzymatically equipped for detoxification of harmful molecules such as H_2O_2. In fact, peroxisomes are considered a source of oxidative stress

due to the generation of ROS in its respiratory pathway. However, peroxisomes can also respond to oxidative stress and ROS when they are generated at other intra- or extracellular locations, protecting the cell against oxidative damage (Schrader and Fahimi, 2006). Higher amounts of peroxisomes were observed in the TdB strain (together with higher amounts of catalase activity and lower ROS levels) in comparison to QA23. Although several authors have described *T. delbrueckii* as Crabtree-positive, its respiratory metabolism makes greater contribution to the overall metabolism than in *Saccharomyces* (Alves-Araújo et al., 2007; Merico et al., 2007). Moreover, genes encoding for peroxisomal β-oxidation in *S. cerevisiae* are repressed by glucose, even in the presence of both oleate and oxygen, which are two inducers of the peroxisomes proliferation (Hiltunen et al., 2003; Schrader and Fahimi, 2006). Therefore, this higher peroxisomal activity in the TdB strain, even before stress, together with the lower levels of ROS, indicates that *T. delbruekii* (TdB strain) could have established a sophisticated strategy to maintain an equilibrium between the production and scavenging of ROS. Peroxisomes proliferation was induced by MEL, even without stress and primarily in *S. cerevisiae* (QA23 strain). Those results suggest a possible role for MEL as a pro-oxidant because it seems capable to prepare the cells for better enduring later oxidative stress, as observed by Vázquez et al., (2017).

Our results indicate that MEL presents antioxidant properties against hydrogen peroxide stress in all the studied yeasts. To the best of our knowledge, the antioxidant effect of MEL in non-*Saccharomyces* yeasts was not previously investigated. Furthermore, in terms of antioxidant properties, MEL is comparable to vitamin C (Reiter et al., 2007), and its effect was even higher under our conditions, because similar results under ROS reduction but at a lower MEL concentration.

5. CONCLUSIONS

In conclusion, the present results provide a significant advance in our understanding of the in vivo antioxidant activity of MEL in *Saccharomyces* and non-*Saccharomyces* species. MEL can serve to mitigate oxidative stress and reduce oxidative damage by leading to a decrease in the intracellular ROS content and TBARS levels under unfavorable conditions. Furthermore, MEL previously activated the catalase activity, reducing the need for its biosynthesis against future oxidative redox changes.

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Appendix A. Supplementary data

Supplementary Table 1. Catalase activity and lipid peroxidation (TBARS) in unstressed and stressed yeast cells with 2 mM of H_2O_2, growing with and without 5 μM of melatonin (MEL). The strains used here were as follows: *S. cerevisiae* (BY4742, BY4741, Sigma 1278b, QA23, VIN 7, SC20, SB20 and Uvaferm HPS), *T. delbrueckii* (TdB and Tdp), *M. pulcherrima* (MpF and Mpp), *C. zemplinina* (Cz4 and Cz11) and H. uvarum (Hu4 and Hu35). Different letters in superscripts indicate significant differences between the conditions, $p<0.05$.

	Catalase (U catalase/ mg protein)				**TBARS** (nmol MDA/ mg protein)			
	Control	**MEL**	**MEL H_2O_2**	**H_2O_2**	**Control**	**MEL**	**MEL H_2O_2**	**H_2O_2**
BY4742	69.86 ± 4.71^{a}	81.93 ± 4.18^{b}	103.91 ± 2.18^{c}	153.02 ± 1.25^{d}	1.35 ± 0.01^{a}	1.40 ± 0.01^{b}	1.93 ± 0.12^{c}	2.32 ± 0.07^{d}
BY4741	80.67 ± 1.11^{a}	95.42 ± 1.74^{b}	110.97 ± 8.46^{c}	159.28 ± 1.68^{d}	1.15 ± 0.05^{a}	1.25 ± 0.05^{a}	1.98 ± 0.07^{b}	2.23 ± 0.12^{c}
Sigma 1278b	80.95 ± 1.79^{a}	97.40 ± 2.71^{b}	113.34 ± 8.93^{c}	137.40 ± 6.59^{d}	1.21 ± 0.05^{a}	1.33 ± 0.08^{a}	1.56 ± 0.07^{b}	1.85 ± 0.09^{c}
QA23	53.18 ± 1.01^{a}	60.83 ± 0.86^{b}	72.12 ± 5.87^{c}	88.17 ± 5.38^{d}	0.71 ± 0.07^{a}	0.87 ± 0.11^{a}	1.14 ± 0.11^{b}	1.34 ± 0.04^{c}
VIN 7	64.02 ± 5.03^{a}	78.32 ± 6.67^{b}	101.90 ± 6.37^{c}	134.38 ± 8.37^{d}	1.08 ± 0.08^{a}	1.19 ± 0.02^{b}	1.62 ± 0.10^{c}	1.97 ± 0.06^{d}
Uvaferm HPS	52.16 ± 1.43^{a}	59.74 ± 0.75^{b}	78.75 ± 8.00^{c}	91.94 ± 2.06^{d}	1.05 ± 0.08^{a}	1.01 ± 0.06^{a}	1.22 ± 0.05^{b}	1.41 ± 0.11^{c}
SC20	73.82 ± 4.93^{a}	82.32 ± 2.60^{b}	89.08 ± 6.32^{b}	114.87 ± 8.90^{c}	0.98 ± 0.07^{a}	1.12 ± 0.06^{a}	1.40 ± 0.05^{b}	1.87 ± 0.15^{c}
SB20	73.20 ± 4.22^{a}	82.42 ± 2.55^{b}	88.37 ± 1.80^{c}	100.58 ± 4.60^{d}	1.11 ± 0.04^{a}	1.14 ± 0.07^{a}	1.58 ± 0.10^{b}	1.83 ± 0.12^{c}
TdB	89.77 ± 6.54^{a}	103.69 ± 1.56^{b}	168.62 ± 4.17^{c}	184.35 ± 3.43^{d}	0.59 ± 0.05^{a}	0.60 ± 0.06^{a}	0.78 ± 0.07^{b}	1.06 ± 0.10^{c}
Tdp	96.30 ± 9.99^{a}	115.18 ± 4.79^{b}	140.94 ± 7.32^{c}	171.22 ± 17.13^{d}	0.56 ± 0.05^{a}	0.64 ± 0.05^{a}	0.78 ± 0.04^{b}	1.09 ± 0.10^{c}
MpF	92.56 ± 6.47^{a}	99.48 ± 3.73^{a}	143.26 ± 8.86^{b}	175.67 ± 7.02^{c}	0.47 ± 0.09^{a}	0.48 ± 0.06^{a}	0.67 ± 0.03^{b}	0.77 ± 0.07^{b}
Mpp	134.51 ± 10.91^{a}	142.54 ± 6.34^{a}	146.80 ± 7.13^{a}	$150.70 \pm 1.93^{a,b}$	0.46 ± 0.10^{a}	0.53 ± 0.08^{a}	0.56 ± 0.12^{a}	0.56 ± 0.08^{a}
Cz4	90.33 ± 9.93^{a}	91.78 ± 9.71^{a}	82.46 ± 1.78^{a}	94.64 ± 18.14^{a}	0.42 ± 0.03^{a}	0.47 ± 0.02^{a}	0.58 ± 0.06^{b}	0.62 ± 0.06^{b}
Cz11	88.09 ± 7.00^{a}	92.42 ± 4.99^{a}	91.97 ± 2.96^{a}	96.48 ± 1.16^{a}	0.50 ± 0.04^{a}	0.52 ± 0.08^{a}	0.74 ± 0.13^{b}	0.79 ± 0.06^{b}
Hu4	88.80 ± 5.93^{a}	112.16 ± 6.17^{b}	143.85 ± 10.98^{c}	171.55 ± 3.75^{d}	0.91 ± 0.09^{a}	0.94 ± 0.03^{a}	1.22 ± 0.05^{b}	1.42 ± 0.07^{c}
Hu35	93.71 ± 1.28^{a}	102.29 ± 2.88^{b}	155.49 ± 5.32^{c}	183.52 ± 10.27^{d}	1.02 ± 0.06^{a}	0.92 ± 0.09^{a}	1.33 ± 0.07^{b}	1.56 ± 0.05^{c}

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CHAPTER 4

Transcriptomic insights into the melatonin effect on oxidative responses in *Saccharomyces cerevisiae*

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Abstract

Melatonin (N-acetyl-5-methoxytryptamine) is a ubiquitous indolamine that plays important roles in various aspects of biological processes in mammals. Antioxidant properties have been determined to be a possible role of melatonin in *Saccharomyces cerevisiae*, and it acts by modulating some of the genes involved in endogenous defenses systems. Thus, melatonin might be involved in multiple processes in yeast that affect on genome-wide gene expression. The aim of this study was to unravel the role of melatonin at the transcriptional level in *S. cerevisiae* in the presence and absence of oxidative stress. We found that exogenous melatonin was able to cross cellular and subcellular membranes at nanomolar concentrations and modulate the expression of genes related to stress responses to cadmium and other metal ions. However, when cells were subjected to oxidative stress induced by H_2O_2, 777 genes that were differentially expressed in response to melatonin were identified by transcriptomic analysis. Melatonin conferred antioxidant and oxidoreductase activity to cells; furthermore, several pathways were markedly affected by melatonin treatment, indicating that cells experience increased stress tolerance in the presence of melatonin.

Keywords: *Saccharomyces cerevisiae*; melatonin; oxidative stress; transcriptome.

1. INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a versatile indolamine that is better known as a neurohormone in vertebrates. Since it was 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 has anti-ageing, antioxidant and anti-inflammatory activities. It can even modulate a variety of neural, endocrine and immune functions (Eghbal et al., 2016; Romero et al., 2014). The ways in which melatonin acts as an antioxidant in mammals have been extensively studied (Reiter et al., 2016; Zhang and Zhang, 2014). Thus, melatonin can act as a direct scavenger to detoxify reactive oxygen species (ROS) and other free radicals, but it can also act indirectly by activating antioxidant enzymes, increasing the efficiency of the mitochondrial electron transport chain and interacting synergistically with other antioxidants (Antolín et al., 1996; León et al., 2005; Reiter et al., 2000; Rodriguez et al., 2004).

Even since Sprenger et al., 1999 related melatonin production to *Saccharomyces cerevisiae*, several studies have reported on the ability of yeasts to synthetize melatonin (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015). Melatonin biosynthesis in yeasts is believed to have a similar route relative to that described in vertebrates, in which melatonin is synthesized using a four-steps pathway from its precursor, tryptophan (Mas et al., 2014). However, the role of melatonin in yeasts is still unknown. We recently reported that melatonin acts as an antioxidant molecule in a wine strain of *S. cerevisiae* (Vázquez et al., 2017). Exogenously applied melatonin enabled cells to resist the stress generated by hydrogen peroxide, enhancing cell viability after ROS, decreasing the oxidized glutathione (GSSG) and increasing the reduced glutathione (GSH). Furthermore, melatonin activated certain genes that were involved in the oxidative stress response such as glutathione/glutaredoxin and thioredoxin and catalase and superoxide dismutase.

S. cerevisiae has a number of inducible adaptive stress responses to oxidants such as H_2O_2, superoxide anion and lipid peroxidation products. The oxidative stress responses

are regulated at the transcriptional level, and there is a considerable overlap between them and the stress responses associated with other types of stresses (general stress response) (Costa and Moradas-Ferreira, 2001; Jamieson, 1998). The oxidative stress response is thus not mediated by an isolated linear metabolic or signaling pathway. Rather, cells are able to reprogram gene expression to optimize their signaling 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). Consequently, the physiological changes in yeast by melatonin supplementation and the ways in which yeasts respond to oxidative stress suggest that melatonin might be involved in multiple biological processes in yeasts.

To gain insight into the antioxidant role and regulatory mechanism of melatonin in yeasts, we evaluated the effect of melatonin on gene transcription after analyzing the ability of the yeast to incorporate exogenous melatonin into the cell. For this purpose, we measured the 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.

2. MATERIAL AND METHODS

2.1. Yeast strain and experimental conditions

The wine yeast QA23, which is a commercial strain of *S. cerevisiae* (Lallemand, Montreal, QA, Canada), was used in this study. For all the experiments, yeast rehydration was performed according to the manufacturer's instructions, and yeast precultures were prepared in YPD liquid medium (2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract (Panreac, Barcelona, Spain)) and incubated at 28ºC for 24 h with orbital shaking (120 rpm). The yeast cells were subsequently inoculated into 175 mL (melatonin detection assay) or 50 mL (transcriptomic assay) of YPD broth (initial population $5x10^3$ cells/mL) with (5 μM) and without melatonin supplementation (5MEL and control condition, respectively), and grown until the cells reached the initial exponential phase (OD 0.5 – 0.6), at 28ºC with orbital shaking at 120 rpm. The sublethal oxidative stress was then induced with 2 mM H_2O_2 for 1 h (5MELH and H condition, respectively). The melatonin and H_2O_2 concentrations were chosen from our previous study on the QA23

strain (Vázquez et al., 2017). Three biological replicates were employed for each condition.

2.2. Intracellular melatonin quantification

Intracellular melatonin was extracted by adapting the boiling buffered ethanol method as previously described by Gonzalez et al., 1997. In brief, a volume of culture corresponding to 1 x 10^8 cells was sampled before and after 1 h of stress exposure and centrifuged at 5000 rpm for 15 min at 4ºC. A 1 mL volume of a solution containing 75% (v/v) boiling absolute ethanol buffered with 0.5M-HEPES (pH 7.5) was added to the cell pellet (final concentration 70 mM HEPES). The mix was subsequently incubated for 3 min at 80ºC, and the extract was concentrated by evaporation at 45ºC using a SpeedBack (Concentrator *plus*, Eppendorf, Eppendorf Ibérica, Madrid, Spain). The final intracellular content was resuspended in 1 mL of milliQ water and centrifuged at 5000 rpm for 10 min to remove the insoluble particles. The melatonin in the supernatants was analyzed by liquid chromatography-mass spectrometry, as described by Rodriguez-Naranjo et al., 2011, using a liquid chromatograph coupled to a triple quadrupole mass spectrometer (Agilent G6410A; Agilent Technologies, Waldbronn, Germany). Separations were performed on a Phenomenex Luna C18 column (250 x 4.6 mm, 5 μM). Melatonin was identified by comparing the 233/174 transition MS data of the sample and the melatonin standard. In parallel, 1 x 10^8 cells were sampled and dried at 28ºC for 48 h to determine the dry weights of the samples. Thus, melatonin was expressed in nM/mg dry weight.

2.3. RNA isolation and quantification

The total RNA was extracted from the cells without (Control and 5MEL conditions) and with (5MELH and H conditions) oxidative stress (2 mM of H_2O_2). Furthermore, under these stress conditions, the culture with higher melatonin supplementation (25 μM) was added (25MELH). Then, 1 x 10^7 cells from each condition were isolated using a Trizol® Plus RNA Purification Kit from Ambion Life Technologies (Woburn, MA, United States) as recommended by the manufacturer, but by repeating the chloroform step before the upper phase containing the RNA was transferred to a fresh RNase-free tube to add the ethanol (70% v/v). Furthermore, to remove the DNA, a DNAse (Qiagen, Barcelona, Spain) step was performed at 37ºC for 15 min before washing with wash buffer II. The RNA

samples were quantified by NanoDrop 1000 ™ (Thermo Scientific, USA) and the integrity was analyzed with an RNA 2100 Bioanalyzer (Agilent Technologies Inc, CA, USA), using the RNA 6000 Nano kit and the Plant RNA Nano protocol in the Agilent 2100 Expert software.

2.4. Microarray analysis

The gene expression levels were assessed using a Yeast Gene Expression Microarray (8x15K format) containing 6256 *S. cerevisiae* probes. Fifteen samples (three biological replicates of each condition for the Control, MEL, 5MELH, 25MELH and H) were chosen to hybridize. Each sample was labeled with Cy3 and hybridized using a 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. An Agilent scan control in version A.8.5.1 software was used to scan the 3-μm resolution slides using the Agilent G2565CA microarray scanner system with sure scan high resolution technology. Feature extraction version 12.0.1.1 software (Agilent Technologies) was used for data extraction.

2.5. Analysis of the sterols, fatty acids and phospholipids

The composition of sterols, fatty acids (FAs) and phospholipids (PLs) were determined in stressed and unstressed cells, which were untreated and treated with 5 μM of melatonin (Control, MEL, 5MELH and H). First, yeast cell homogenates were obtained using glass beads and a Disruptor Genie® (Scientific Industries, Inc., NY, USA) for 10 min at 4ºC. The total lipids were extracted from cell fractions corresponding to 1 mg, 0.5 mg or 3 mg of total cell protein for FA, sterol or PL assays, respectively, according to the method by Folch et al (1957). The individual sterol composition was then determined by gas-liquid chromatography-mass spectrometry (GC-MS) after the alkaline hydrolysis of the lipid extracts (Quail and Kelly, 1996). The FA composition was determined by gas-liquid chromatography (GLC) (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 (Broehuyse, 1968). Two biological replicates were used.

2.6. Data analysis

Data from the melatonin quantification and lipid content analysis were subjected to a one-way analysis of variance (ANOVA) and *Tukey's post hoc* test. The results were considered statistically significant at a p-value < 0.05 (XLSTAT Software). The statistical transcriptomic analysis for finding the 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 a Benjamini-Hochberg multiple testing correction was used. Genes with p-values < 0.05 were designated as being differentially expressed. Venn diagrams were created with the web application Genevenn (Pirooznia et al., 2007), and the molecular functions, biological processes and cellular components were determined with Gene Ontology (CC-BY 4.0, Carbon et al. 2009). Specific pathways in the differentially expressed genes were analyzed with the KEGG pathway mapping database (Kanehisa et al., 2002).

3. RESULTS

3.1. Intracellular melatonin

To evaluate whether the QA23 strain was able to incorporate exogenous melatonin (5μM) into the cell, intracellular melatonin was quantified in cells grown with and without melatonin supplementation, and with and without oxidative stress exposure (**Figure 1**).

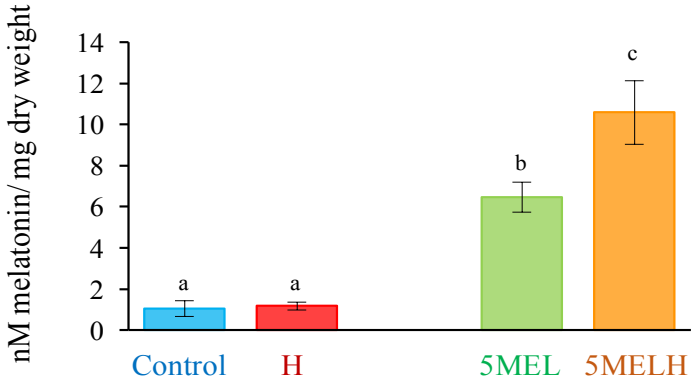

Figure 1. Intracellular melatonin quantification in cells untreated and treated with 5μM of exogenous melatonin, before (Control and 5MEL) and after (H and 5MELH) being exposed to oxidative stress with 2 mM of H_2O_2.

The intracellular melatonin amounts significantly increased when the cells had been growing with melatonin supplementation, independent of oxidative stress, indicating that *S. cerevisiae* (QA23 strain) was able to take up exogenous melatonin at nanomolar concentrations. However, the highest levels of intracellular melatonin were reached when oxidative stress was applied to cells that were previously grown in the presence of melatonin.

3.2. Differential gene expression profiling

To obtain an overview of the gene expression profile involved with melatonin supplementation in *S. cerevisiae*, a comparative transcriptomic analysis was performed between cells that were untreated and treated with melatonin (5 μM), with and without oxidative stress (2mM H_2O_2) (Control, 5MEL, H and 5MELH conditions). Furthermore, under oxidative stress, the effect of the melatonin concentration was evaluated by adding other conditions with 25 μM of melatonin (25MELH). A representation of the changes in global gene expression (FC $\geq$ 1, p-value < 0.05) is shown in **Figure 2**. A total of 21 genes were differentially expressed by exogenous melatonin treatment, including 13 up and 8 down-regulated genes (**Figure 2A**; 5MEL-Control). A total of 4224 genes were differentially expressed by the oxidative stress induced with H_2O_2, including 1687 up and 2537 down-regulated genes (**Figure 2A**; H-Control). From those results, the expression of 1466 up (**Figure 2B**) and 2185 down (**Figure 2C**)-regulated genes were common under the stress condition (H) with exogenous melatonin (5MELH). Nevertheless, melatonin clearly changed the expression profile of the cells under stress, showing 492 up and 285 down-regulated genes (**Figure 2A, 5MELH - H**). All these conditions showed four genes in common between them, and under the control conditions (**Figure 2B and C**), there were two up-regulated (*RTS3* and *DIP5,* encoding a putative component of protein phosphatase type 2A, and a dicarboxylic amino acid permease, respectively (Regenberg et al., 1999; Samanta and Liang, 2003)) and two down-regulated genes (YOR338W and *AQR1,* encoding a putative protein and a plasma membrane transporter, respectively (Slattery et al., 2006; Velasco et al., 2004;)). The effect of the higher melatonin concentration (**Figure 2A**, 25MELH-5MELH) showed 40 up- and 42 down-regulated genes.

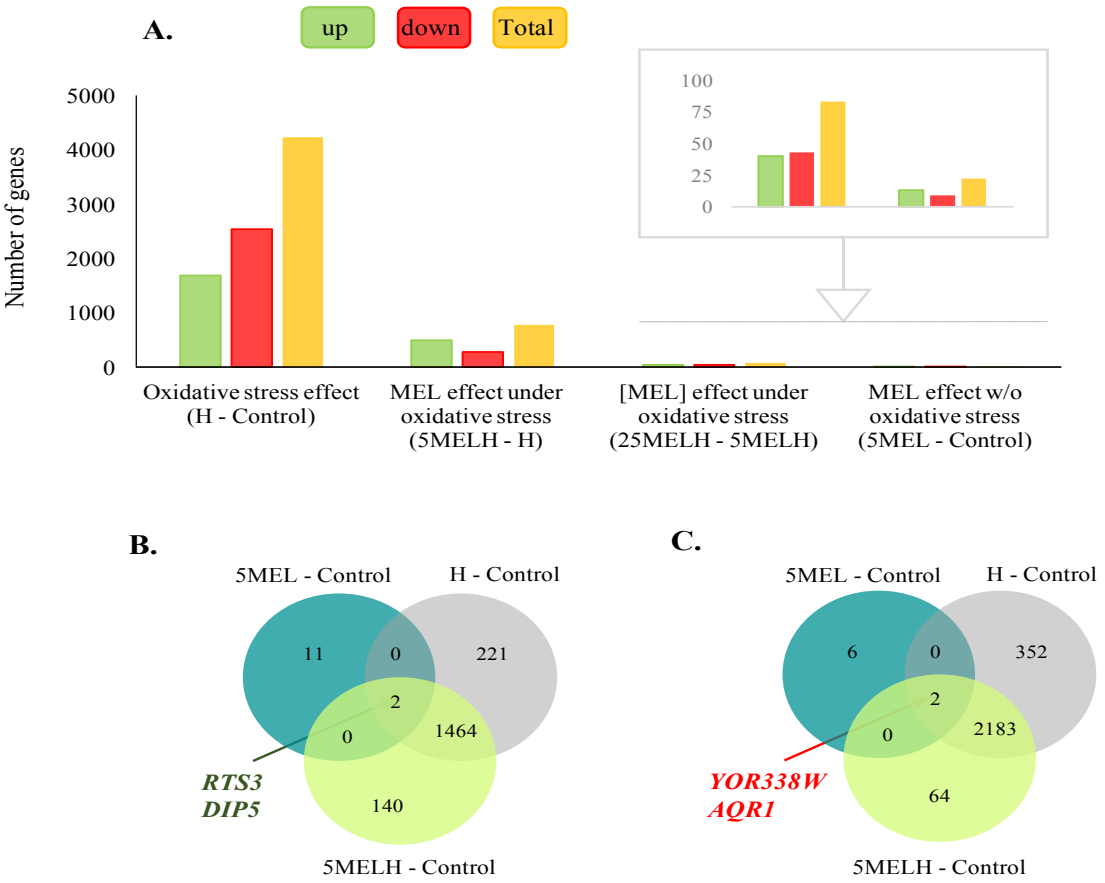

Figure 2. Distribution of differentially expressed genes in yeast cells grown with and without melatonin (5 μM or 25 μM), in stressed (2 mM of H_2O_2) and unstressed conditions (Control, 5MEL, 5MELH, 25MELH and H). (A) The number of differentially expressed genes between different conditions (fold change ≥1; p-value < 0.05). (B and C) Venn diagram showing the number of genes found in common between comparisons 5MEL-Control, 5MELH-Control and H-Control (Up- (B) and down-(C) regulated genes)

3.3. Classification of differentially expressed genes into functional categories

A Gene Ontology (GO) enrichment analysis was performed to study the transcriptional regulatory mechanism of melatonin to analyze which GO terms (molecular function, biological process, and cellular component) were over-represented among all the differentially expressed genes in the presence of melatonin or stress. Of all 21 genes that were differentially expressed in 5MEL with respect to the Control condition (**Table 1**), both metallothioneins *CUP1-1* and *CUP1-2,* which are the cytosolic copper and cadmium-binding proteins (Fogel and Welch, 1982) that are up-regulated in melatonin, were significantly representing a 100% of the total genes involved in stress response to cadmium, copper and other metal ions, and their detoxification (p-value ≤ 1.36E-04).

Both genes were also involved in superoxide dismutase activity, and its respective oxidoreductase activity acted on superoxide radicals as the acceptor and antioxidant activity (p-value ≤ 1.23E-03). Furthermore, six genes represented 7.36% of the fold enrichment for the ion transmembrane transporter activity (p-value 4.35E-02). The genes that were up-regulated by melatonin were *MPC3*, *DUR3* and *DIP5,* encoding a subunit of the mitochondrial pyruvate carrier (MPC, Zamboni et al., 2012), a plasma membrane transporter for both urea and polyamines (Uemura et al., 2007) and a dicarboxylic amino acid permease, which mediates the high-affinity and capacity transport of L-glutamate and L-aspartate, respectively (Regenberg et al., 1998). By contrast, melatonin down-regulated *AQR1*, *CTR1* and *PHO84*. *CTR1* encodes a high-affinity copper transporter in the plasma membrane (Dancis et al., 1994), and *PHO84* encodes a high-affinity inorganic phosphate transporter (Bun-Ya et al., 1991).

Table 1. Genes differentially expressed (fold change (FC) ≥1; p-value < 0.05) by the presence of 5 μM of melatonin (5MEL) respect Control condition.

Gene standard name	Name description	FC	p-value
PHO84	Phosphate transporter	-2.34	1.03E-04
CTR1	High-affinity Cu transporter	-2.11	4.51E-05
YOR338W	Hypothetical protein	-2.08	4.06E-05
GPX2	Glutathione peroxidase	-2.07	9.30E-05
YFL051C	Hypothetical protein	-1.83	4.37E-05
NDE1	NADH-ubiquinone reductase (H(+)-translocating)	-1.67	7.68E-05
GUT2	Glycerol-3-phosphate dehydrogenase	-1.64	8.27E-05
AQR1	Plasma membrane transporter	-1.56	1.26E-04
ATF1	Alcohol O-acetyltransferase	1.65	7.90E-05
MPC3	Pyruvate transporter	1.86	1.16E-04
RTS3	Component phosphatase type 2A complex	1.88	3.61E-05
DIP5	Dicarboxylic amino acid permease	1.91	3.83E-05
IZH4	Protein involved in zinc ion homeostasis	1.96	1.50E-04
ISF1	Increasing suppression factor	1.96	1.21E-04
DUR3	Plasma membrane transporter (urea and polyamines)	1.97	1.30E-04
CUP1-2	Metallothionein	2.19	4.63E-05
ANB1	Translation elongation factor eIF-5A	2.24	1.52E-05
TIR4	Cell wall mannoprotein	2.32	2.89E-05
CUP1-1	Metallothionein	2.36	2.04E-05
YHR022C	Hypothetical protein	2.51	1.52E-05
DAN1	Cell wall mannoprotein	6.14	8.22E-07

The effect of melatonin was notoriously higher under oxidative stress. Interestingly, although different gene expression profiles were observed under higher concentrations of melatonin (**Figure 2A**, 25MELH–5MELH, **Table S1**), no significant differences were observed in molecular functions or biological processes. The molecular functions that were over-represented under the 5MELH condition and all the genes involved here are listed in **Table 2**. Of all the up-regulated genes, 2.71% were involved in antioxidant activity and 11.48% were involved in oxidoreductase activity, with those two molecular functions being the only ones over-represented ones under this condition. These percentages were representative of 36.11% and 15.63%, respectively, of the total genes involved in these molecular functions in *S. cerevisiae*. The 13 genes involved in antioxidant activity that were transcriptionally induced by melatonin encoded 2 signaling molecules (*ECM4* and *GTO1*), 4 peroxidases (*DOT5, HYR1, GPX1* and *CTT1*), 4 oxidoreductases (*TRX1, TRX2, GRX1* and *GRX2*), both metallothioneins (*CUP1-1* and *CUP1-2*) and sulfiredoxin (*SRX1*). Moreover, these genes were also included within the 57 genes involved in oxidoreductase activity (**Table 2**). These genes have been described to be important against oxidative stress. For instance, *HYR1* is similar in sequence, structure and function to the phospholipid hydroperoxide glutathione peroxidases (PHGPxs) (Avery et al., 2004; Avery and Avery, 2001). This peroxidase plays an important role against oxidative stress functioning as a hydroperoxide receptor, sensing intracellular hydroperoxide levels and transducing the redox signal to the oxidative stress transcription factor Yap1p (Delaunay et al., 2002; Ma et al., 2007) Furthermore, *HYR1* protects cells directly from peroxides during oxidative stress by acting on glutathione, phospholipid hydroperoxides and thioredoxins as substrates (Delaunay et al., 2002; Avery et al., 2004). *TRX1* and *TRX2*, encode thioredoxins and they are specialized in protecting cells against ROS. They are essential for YAP1-dependent resistance to hydroperoxides (Gómez-Pastor et al., 2012; Herrero et al., 2008; Kuge and Jones, 1994), whereas glutaredoxins (*GRX1* and *GRX2*) regulate the protein redox state by using GSH and NADPH. *SRX1* is a sulfiredoxin that reduces cysteine-sulfinic acid groups, and it is formed upon exposure to oxidants (Biteau et al., 2003; Tkach et al., 2012). The methionine-S-sulfoxide reductase is encoded by *MXR1,* which protects iron-

sulfur clusters from oxidative inactivation (Sideri et al., 2009). The sphinganine C4-hydroxylase encoded by *SUR2* is involved in sensitivity to H_2O_2 (Berry et al., 2011).

Among the down-regulated genes, the over-represented molecular functions were as follows: helicase activity (with 6.07% of the genes within this molecular function being down-regulated), organic cyclic compound binding (with 41.14%), and binding (with 58.21%) (**Table 2**).

Table 2. Molecular function enrichment from Gene ontology (GO) analysis of differentially expressed genes up- and down-regulated (fold change ≥1; p-value < 0.05) among stressed cells in absence or presence of melatonin (5 μM) (5MELH – H).

	GO term molecular function (p-value <0.05)	**Gene names (|2| ≤ FC ≥ |1|)**
Up- regulated	Oxidoreductase activity (2.46E-04)	YKL071W, *QCR9, COX5A, EUG1, HFD1, GPX1, GDH3, AAD10, DOT5, TSC13, QCR7, COX7, FDH1, ECM4, THI4, HYR1, MXR1, COX2,* YCR102C*, TRX1,TRX2, DLD3, COX1, ETR1, PRM4, SER33, FRE3, GRX1, SDH4, GRX2, COX8, COQ11,* YJR096W*, CTT1, GAL80, CUP1-2, AYR1, COX3, HOM6, GLT1, TRX1,TRX2, FRE6, TPA1, GTO1, CUP1-1,* YLR456W*, ALD3,* YKL107W*, SUR2, MPD1, COX6, POX1,* YPR127W*, HBN1, SRX1, ALD5*
	Antioxidant activity (5.94E-03)	*GPX1, DOT5, HYR1, ECM4, GTO1, TRX1, TRX2, GRX1, GRX2, CTT1, CUP1-1, CUP1-2, SRX1*
Down-regulated	Helicase activity (1.60E-02)	*MSS116, SNF2, YKU80, DHH1,* YBL113C*, DBP1, ARP5,* YLL067C*, MPH1,* YHL050C*, DBP7, YRF1-8,* YEL077C*, YRF1-7, YRF1-6, YRF1-5, DBP3*
	Binding (2.48E-02)	*LAS17, ALT2, YME1, VTS1, UBX2, AIM10, SLX5, SEC1, CRN1, NFS1, TOS8,* YGR054W*, UTP15, MSS116, ERG11, MRM1, CWC2, PCL8, RTT101, RRP14, NOG2, PKH1, INN1, UFO1, YCL001W-B, INP1, EAF1, RIO1, CBK1, CCM1, SNF2, YJR084W, EPS1, CMP2, MSK1, GPB1, STE12, YKU80, POP2, VAC17, MLP1, RAD23, UPC2, CPS1, SPA2, CHS5, YSC84, MSL5, RCK1, FET5, TEL1, YCL001W-A, ILV3, PUS7, MLP1, ERF2, NRD1, SEY1, UBC11, PUF4, YLF2, ADA2, LSG1, CDD1, SRO9, NTH1, INO2, RAD27, ELP2, MRX1, AFT1, MRE11, FKH1, RRP6, SCH9, MYO1, CAB3, GEP3, TRF5, POG1, MGA1, SPS18, DHH1, XDJ1, PSP2, ECM2, MET4, MPT5, DBP1, SSC1, PEX1, SFP1, ATM1, PEX8, PKP1, HCM1, CAR2, SHS1, MPS3, VPS16, ILV1, NOP14, MRN1, YLL067C, MPH1, NPL3, NUP2, MDL1, TUP1, BDF1, HPR1, PUS1, SLT2, CBP2, EXO5, YTA12, HEM1, YOR338W, YHP1, DPS1, NTH1, CDC20, MAE1, YHL050C, DBP7, STB3, BRO1, DUS4, WTM2, WHI3, PTC5, SPS1, KAR1, YRF1-8, BI4, YEL077C, RCL1, CDC9, GZF3, CDC11, MUD2, PTC3, RRP9, BRE5, RSP5, FRA1, YBR238C, HAP4, HOS4, YHM2, CMR1, CNA1, COQ1, YRF1-7, SMI1, CTR9, YRF1-6, BIK1, YCK3, ETP1, DBP3, YRF1-5*
	Organic cyclic compound binding (2.59E-02)	*ALT2, YME1, VTS1, AIM10, NFS1, TOS8,* YGR054W*, UTP15, MSS116, ERG11, MRM1, CWC2, RRP14, NOG2, PKH1, EAF1, RIO1, CBK1, CCM1, SNF2,* YJR084W*, MSK1, STE12, YKU80, POP2, MLP1, RAD23, UPC2, MSL5, RCK1, TEL1, PUS7, MLP1, ERF2, NRD1, SEY1, UBC11, PUF4, YLF2, ADA2, LSG1, SRO9, NTH1, NO2, RAD27, MRX1, AFT1, MRE11, FKH1, RRP6, SCH9, MYO1, CAB3, GEP3, POG1, MGA1, DHH1, PSP2, ECM2, MET4, MPT5, DBP1, SSC1, PEX1, SFP1, ATM1, PKP1, HCM1, CAR2, SHS1, MPS3, ILV1, NOP14, MRN1,* YLL067C*, MPH1, NPL3, MDL1, BDF1, HPR1, PUS1, SLT2, CBP2, EXO5, YTA12, HEM1,* YOR338W*, YHP1, DPS1, MAE1, YHL050C, DBP7, STB3, DUS4, WHI3, SPS1, YRF1-8, BI4, YEL077C, RCL1, CDC9, GZF3, CDC11, MUD2, RRP9, BRE5,* YBR238C*, HAP4, YHM2, CMR1, YRF1-7, SMI1, CTR9, YRF1-6, YCK3, DBP3, YRF1-5*

The GO enrichment analysis indicated that several biological processes were significantly affected by melatonin treatment under oxidative stress (**Figure 3**, **Table S2**). It is important to highlight that the most frequently represented biological processes were classified by up-regulated genes (**Table S2**), which were involved in mitochondrial electron transport (approximately 60% of the genes involved in this biological process were up-regulated), followed by processes related to cellular detoxification (>30%), oxidation-reduction and transport by vesicles (approximately 15% for each one) (**Figure 3**). The cellular components associated with up- and down- regulated melatonin genes under stress included the cell wall and plasmatic membrane, cytoplasm and different organelles (membrane and sub compartment), with the inner mitochondrial membrane protein complex and the respiratory chain being the most representative (**Figure 4**). It is important to highlight that melatonin up-regulated essential genes for the maintenance of normal mitochondrial morphology in yeast, such as *TOM7*, which is required for the insertion of morphogenesis factors into the outer membrane (Dimmer et al., 2002), and *ETR1,* which is essential for a respiratory competence and is involved in FA biosynthesis (Torkko et al., 2001).

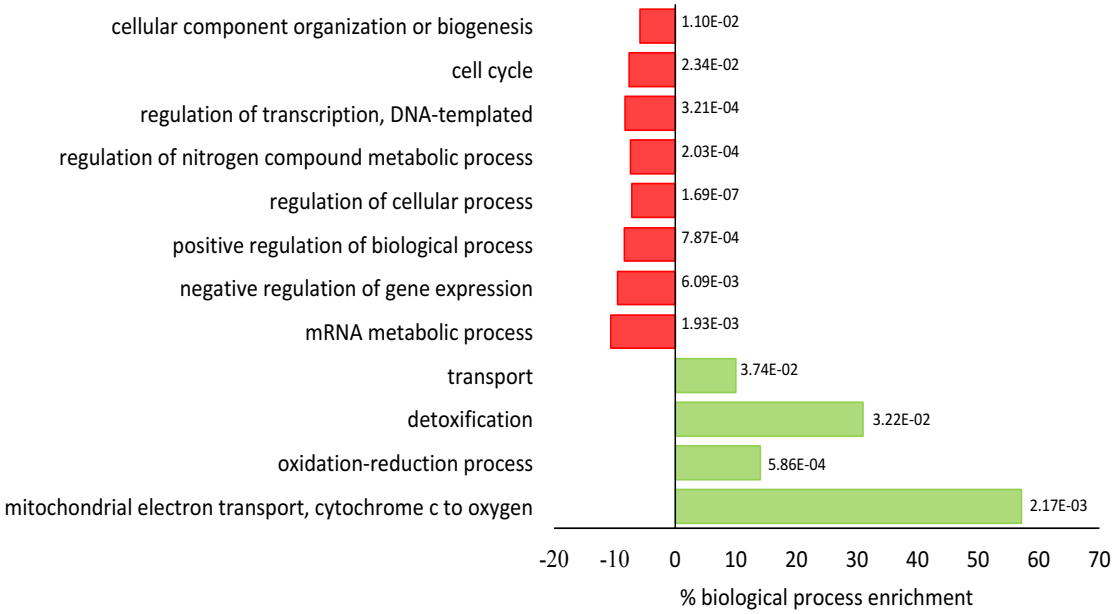

Figure 3. Biological process enrichment from Gene ontology (GO) analysis of differentially expressed genes up- (■) and down- (■) regulated (fold change ≥1; p-value < 0.05) among stressed cells in absence or presence of melatonin (5 μM). Percentages are calculated in relation to the total genes involved in each biological process in *S. cerevisiae*.

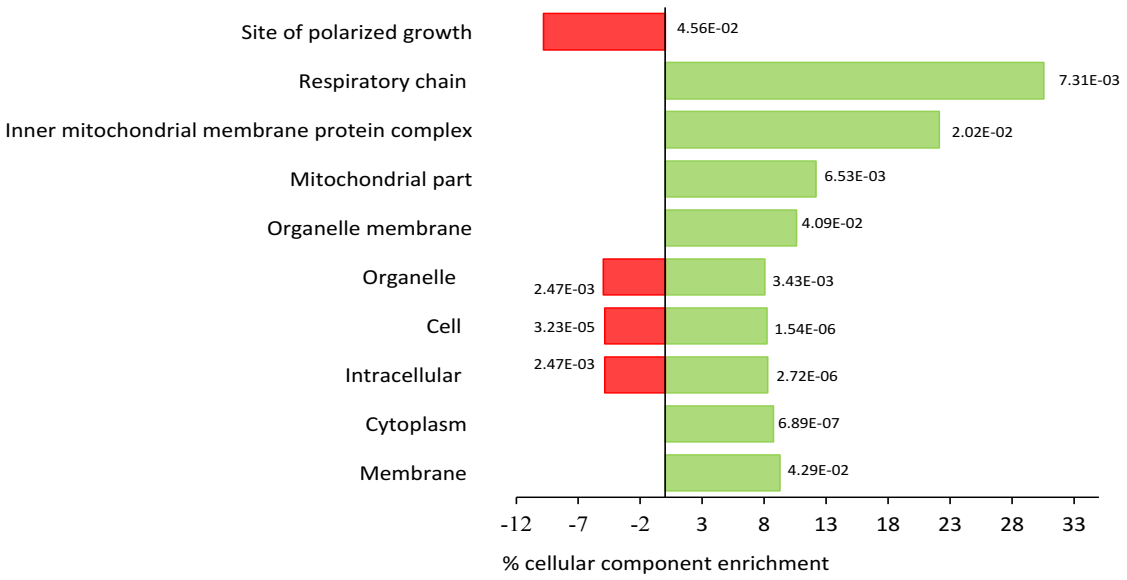

Figure 4. Cellular component enrichment from Gene ontology (GO) analysis of differentially expressed genes up- (■) and down- (■) regulated (fold change ≥1; p-value < 0.05) among stressed cells in absence or presence of melatonin (5 μM). Percentages are calculated in relation to the total genes involved in each cellular component in *S. cerevisiae*.

3.4. Metabolic pathways affected by melatonin

The KEGG database was used to understand the basic mechanism of melatonin in yeast and the primary enriched metabolic or signaling pathways. Of the 21 genes (**Table 1**), whose expression was modified when comparing 5MEL and Control conditions, only glutathione peroxidase (*GPX2*) and glycerol-3-phosphate dehydrogenase (*GUT2*), both of which were down-regulated genes, were involved in different pathways. These pathways pertained to arachidonic acid (20%) and glutathione metabolism (4.17%) in the case of *GPX2*, and glycerophospholipid metabolism (2.70%) in for *GUT2*.

As expected, many pathways were involved in the cellular response to oxidative stress (H-Control). Under these stress conditions, the transcriptional response was different depending on melatonin supplementation. In the transcriptional response of 5MELH (compared to H, 777 genes with modified expression), 92 enriched pathways involved in metabolism, genetic information processing, environmental information processing and cellular processes were identified. Most pathways were represented by more than 10% of the total genes within the pathway (**Table 3**). The primary differences were classified into the following different pathways:

Table 3. Enriched pathways from KEGG analysis database of genes differentially expressed among stressed cells in absence or presence of melatonin (5 μM) (5MELH – H). Percentages are calculated in relation to the total genes involved in each pathway in *S. cerevisiae*.

Pathway	% of total genes	Gene name (up-regulated)	Gene name (down-regulated)
METABOLISM			
Global and overview maps			
Carbon metabolism	13.04	*ACS1, CIT2, GPM2, SDH4, EMI2, NQM1, CTT1, SER33, MET17, FDH1*	*ALT2, ILV1, MAE1, MET12*
2-Oxocarboxylic acid metabolism	11.43	*CIT2, BAT1*	*ALT2, ILV3*
Fatty acid metabolism	18.18	*ETR1, TSC13, POX1, PHS1*	
Biosynthesis of amino acids	9.76	*CIT2, GPM2, GLT1, NQM1, BAT1, SER33, HOM6, MET17, ARO7*	*ALT2, ILV1, ILV3*
Carbohydrate metabolism			
Glycolysis / Gluconeogenesis	10.34	*ACS1, GPM2, EMI2, HFD1, ALD3*	*ALD5*
Citrate cycle (TCA cycle)	6.25	*CIT2, SDH4*	
Pentose phosphate	7.14	*NQM1*	
Fructose and mannose metabolism	8.70	*EMI2, PMI40*	
Galactose metabolism	8.33	*EMI2, IMA3*	
Starch and sucrose metabolism	9.75	*EMI2, IMA3, GPH1*	*NTH1*
Amino sugar and nucleotide sugar	9.38	*EMI2, PMI40, CHS1*	
Inositol phosphate metabolism	15.00	*STT4, PLC1*	*INP51*
Pyruvate metabolism	12.82	*ACS1, DLD3, HFD1*	*ALD5, MAE1*
Glyoxylate and dicarboxylate	11.54	*CIT2, CTT1, FDH1*	
Propanoate	7.69	*ACS1*	
Energy			
Oxidative phosphorylation	23.61	*COX1, ATP8, COX2, COX3, COX5A, COX6, COX7, COX8, COX17, SDH4, QCR7, QCR9, ATP14, ATP15, ATP18, ATP20*	*BI4*
Methane metabolism	14.29	*ACS1, GPM2, SER33, FDH1*	
Nitrogen metabolism	57.14	*GDH3, GLT1, NCE103*	YJR149W
Sulfur metabolism	20.00	*CYC7, MET14, MET17*	
Lipid metabolism			
Fatty acid elongation	37.5	*ETR1, TSC13, PHS1*	
Fatty acid degradation	15.79	*POX1, HFD1*	*ALD5*
Steroid biosynthesis	11.76		*ERG11, ARE2*
Glycerolipid metabolism	7.69	*HFD1*	*ALD5*
Glycerophospholipid metabolism	5.41	*AYR1, CPT1*	
Ether lipid metabolism	28.57	*AYR1, CPT1*	
Sphingolipid metabolism	7.14	*SUR2*	
Arachidonic acid metabolism	40.00	*HYR1, GPX1*	
alpha-Linolenic acid metabolism	33.33	*POX1*	
Biosynthesis of unsaturated fatty acids	27.27	*TSC13, POX1, PHS1*	
Nucleotide metabolism			
Purine metabolism	8.08	*YSA1, RPB5, RPC11, DPB4, RPA12, MET14, RPB10*	*RPA34*
Pyrimidine metabolism	11.43	*RPB5, RPB10, RPB11, RPB12, DPB4, DCD1*	*RPA34, CDD1*

Pathway	% of total genes	Gene name (up-regulated)	Gene name (down-regulated)
Amino acid metabolism			
Arginine biosynthesis	11.76	*GDH3*	*ALT2*
Alanine, aspartate and glutamate metabolism	10.00	*GDH3, GLT1, ASP3-1*	*ALT2*
Glycine, serine and threonine metabolism	15.15	*GPM2, SER33, HOM6*	*HEM1-5, ILV1*
Cysteine and methionine metabolism	12.50	*BAT1, HOM6, SPE4, MET17, SAM4, ACS1*	
Valine, leucine and isoleucine degradation	23.08	*BAT1, HFD1*	*ALD5*
Valine, leucine and isoleucine biosynthesis	25.00	*BAT1*	*ILV1, ILV3*
Lysine biosynthesis	8.33	*HOM6*	
Lysine degradation	14.29	*HFD1*	*ALD5*
Arginine and proline metabolism	19.04	*SPE4, HDF1*	*ALD5, CAR2*
Histidine metabolism	21.42	*HDF1, ALD3*	*ALD5*
Tyrosine metabolism	7.14	*ALD3*	
Phenylalanine metabolism	9.09	*ALD3*	
Tryptophan metabolism	17.65	*CTT1, HFD1*	*ALD5*
Metabolism of other amino acids			
β-Alanine metabolism	30.77	*SPE4, HFD1*	*ALD5*
Phosphonate and phosphinate	25.00	*CPT1*	
Cyanoamino acid	12.50	*ASP3-1*	
Glutathione metabolism	16.67	*HYR1, GPX1, SPE4*	
Glycan biosynthesis and metabolism			
N-Glycan biosynthesis	13.33	*ALG14, OST4, ALG6, OST2*	
Various types of N-glycan biosynthesis	13.33	*ALG14, OST4, OST2*	*VAN1*
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	12.00	*GPI18, GPI19, GPI13*	
Metabolism of cofactors and vitamins			
One carbon pool by folate	6.67		*MET12*
Thiamine metabolism	21.05	*THI4, THI80, THI6*	*NFS1*
Vitamin B6 metabolism	8.33	YPR127W	
Nicotinate and nicotinamide metabolism	5.00	*PNC1*	
Pantothenate and CoA biosynthesis	20.00	*BAT1*	*ILV3, CAB3*
Lipoic acid metabolism	33.33	*AIM22*	
Folate biosynthesis	12.50	*FOL2*	
Porphyrin and chlorophyll 1 metabolism	11.76		*HEM3, HEM1-5*
Metabolism of terpenoids and polyketides			
Terpenoid backbone biosynthesis	15.79	*STE14, IDI1*	*COQ1*
GENETIC INFORMATION PROCESSING			
Transcription			
RNA polymerase	16.67	*RPB5, RPC11, RPA12, RPB10*	*RPA34*
Basal transcription factors	6.25	*TAF2*	*NPL3*
Spliceosome	6.33	*LSM2, SSA4, BRR2, SMD1, SMD2, HSH155, LSM7, DIB1*	*ECM2, BUD31, ISY1, MUD2, PRP19*
Translation			
Ribosome biogenesis in eukaryotes	10.99	*POP4, POP6, POP7, FCF1, EMG1*	*LSG1, UTP15, NOG2, RCL1, RIO1, MRP4*

Pathway	% of total genes	Gene name (up-regulated)	Gene name (down-regulated)
Ribosome	8.91	*MRPS5, MRPS5, MRPL3, RPL27B, RSM18, RPS27B, RPS14B, MRP17, RPS27A, RPS30A, RPL18B, RPS9A, RTC6, RPL7B, MRP2*	*MRP4*
RNA transport	7.53	*POP7, POP4, POP6, GCD2, NUP133, RPM2*	*MLP1*
Aminoacyl-tRNA biosynthesis	0.89		*AIM10, DPS1, MSK1*
mRNA surveillance	8.69	*CFT1, SKI7*	*CDC55, RTS1*
Folding, sorting and degradation			
RNA degradation	14.52	*LSM2, LSM7, SKI7, SKI3*	*DHH1, SSC1, TRF5, POP2 , RRP6*
Proteasome	17.14	*RPN6, SEM1, RPN9, PRE4, RPN12, PRE9*	
Protein export	4.55	*SRP102*	
Ubiquitin mediated proteolysis	10.02	*UBC8, GRR1, NUBC12, UBC7*	*RSP5, CDC20, PRP19, UBC11*
Sulfur relay system	14.29		*NFS1*
SNARE interactions in vesicular transport	15.00	*SNC1, TLG2, SNC2*	
Protein processing in endoplasmic reticulum	16.28	*HSP26, YET3, OST4, HSP42, EUG1, SSA4, EMP47, SSM4, UBC7, OST2, MPD1*	*RAD23, EPS1, UBX2*
Replication and repair			
DNA replication	12.90	*RFC5, DPB4*	*CDC9, RAD27*
Base excision repair	16.67	*DPB4*	*CDC9, RAD27*
Nucleotide excision repair	18.92	*RFC5, DPB4, RAD26, RAD10, RAD14*	*CDC9, RAD23*
Mismatch repair	15.00	*RFC5, PMS1*	*CDC9*
Homologous recombination	15.00	*SEM1*	*TEL1, MRE11*
Non-homologous end-joining	30.00		*RAD27, YKU80, MRE11*
ENVIRONMETAL INFORMATION PROCESSING			
Membrane transport			
ABC transporters	33.33	*PXA1*	*ATM1*
Signal transduction			
MAPK signaling	10.53	*CDC28, YPD1, CTT1, STT4*	*TUP1, MKC7, PKH1, RSP5, SLT2, STE12, SPA2, SLG1*
Phosphatidylinositol signaling system	9.10	*STT4, PLC1*	*INP51*
CELLULAR PROCESSES			
Cell growth and death			
Cell cycle	7.94	*CDC28, GRR1*	*CLN3, MRC1, TUP1, YHP1, SCC4, CDC20, CDC55, SLK19*
Meiosis	7.69	*CDC28, HMRA1, HXT6, HXT5*	*CLN3, RGT2, SPS1, CDC20, RTS1, SLK19*
Transport and catabolism			
Autophagy	16.67	*SEC17, ATG8, ATG12, ATG9, ATG31, MON1, TOR1, YPT7, ATG5, ATG29*	*PRB1, SLT2, SCH9, VPS16*
Mitophagy	19.51	*ATG8, FMC1, TOR1, VPS1*	*SLT2, BRE5, SLG1, YME1*
Endocytosis	12.16	*SSA4, MVB12, PEP8, ARC18, YPT7, IST1, VPS28*	*RSP5, LAS17*
Phagosome	2.94	*YPT7*	
Peroxisome	15.79	*PEX19, YAT2, POX1, CTT1, PXA1*	*PEX1*
ORGANISMAL SYSTEMS			
Aging			
Longevity regulating pathway - multiple species	19.44	*SSA4, PNC1, SNF4, CTT1, TOR1, ATG5*	*SCH9*

Carbohydrate metabolism

The 5MELH and H conditions significantly differed in terms of the genes involved in the metabolic flux via enhanced glycolysis, pyruvate and TCA cycle metabolism. For exemple, genes such as *ACS1*, which encodes acetyl-CoA synthetase (De Jong-Gubbels et al., 1997); *GPM2,* which is involved in converting 3-phophoglycerate into 2-phosphoglycerate during glycolysis (Heinisch et al., 1998); *DLD3 ,* which is a D-lactate dehydrogenase that reduces the pyruvate to D-lactate (Chelstowska et al., 1999); *CIT2,* which encodes citrate synthase and is involved in the RTG pathway (Liao & Butow 1993); and *SDH4* which is involved in coupling the oxidation of succinate from TCA (Chapman et al., 1992), were up-regulated. In the pyruvate pathway, *MAE1,* which catalyzes the oxidative decarboxylation of malate to pyruvate, was down-regulated (Boles et al., 1998).

Energy metabolism

Melatonin primarily enriched the oxidative phosphorylation pathway, which is used to reform ATP in mitochondria, with 23% of the genes up-regulated under 5MELH compared to H. In fact, many of the genes that were up-regulated by melatonin under oxidative stress were involved in mitochondrial processes, namely electron transport and oxidative phosphorylation. These genes were taking part in the succinate dehydrogenase (complex II, *SDH4*), in cytochrome c reductase (complex III, *QCR7, QCR9,* and *CBP6*) and in cytochrome c oxidase (complex IV, *COX1, COX2, COX3, COX5A, COX6, COX7, COX8, COX17, ATP8, ATP14, ATP15, ATP18 ATP20,* and *STF2*) of the mitochondrial respiratory chain.

Lipid metabolism and peroxisome

These pathways were clearly modified by the presence of melatonin under stress conditions, especially the genes involved in arachidonic acid metabolism (with 40% of the genes in this pathway being up-regulated), the fatty acid elongation (37.5%) and the biosynthesis of unsaturated fatty acids (27%), which were up-regulated. Thus, the expression of genes such as *HYR1* and *GPX1* were involved in the conversion of arachidonic acid into 5-HETE (5S-hydroxy-6,8,11,14-eicosatetraenoic acid), *POX1,* which encodes fatty-acyl coenzyme A oxidase and is involved in β-oxidation; *PXA1,* which encodes the peroxisomal transporter; and *PEX19,* which contributes to peroxisome

partitioning, and that were up-regulated in the presence of melatonin under stress conditions (Hiltunen et al., 2003). By contrast, genes involved in steroid biosynthesis, such as *ERG11,* which encodes lanosterol 14-alpha-demethylase (Karst et al., 1977), and *ARE2,* which encodes acyl-CoA:sterol acyltransferase genes (Zweytick et al., 2000), were down-regulated. The isomerase enzyme encoded by *IDI1* that is involved in the biosynthesis of isoprenoids and the sterol precursor squalene (Chemler et al., 2006) was up-regulated by the presence of melatonin and *CPT1*, which is involved in phosphatidylcholine biosynthesis and has been related to stress tolerance (McMaster and Bell, 1994).

Metabolism of cofactors and vitamins

Genes related to the biosynthesis of thiamine (*THI4, THI6,* and *THI80*), spermidine (*SPE4*) and folate (*FOL2*) were up-regulated during their growth with melatonin. Thiamine possesses antioxidative effects, and its protective properties seem to be necessary for the effectiveness of the defense mechanisms (Wolak et al., 2014). *THI4* is reportedly required for mitochondrial genome stability in response to DNA damaging agents (Machado et al., 1997; Chatterjee et al., 2007). One of the functions of spermidine is to protect cells from damage caused by ROS produced by H_2O_2 (Rider et al., 2007; Valdés-Santiago and Ruiz-Herrera, 2013). Folic acid has been related to sulfamethoxazole resistance, regardless of whether the strains were folate utilizers or not (Bayly et al., 2001). In addition, melatonin up-regulated *AIM22* involved in lipoic acid metabolism which confers resistance to oxidative stress (Schonauer et al., 2009). By contrast, melatonin down-regulated the neutral trehalase encoded by *NTH1*, which degrades the trehalose required for thermotolerance but is also related to abnormal nuclear morphology and cell cycle progression (Niu et al., 2008; Schepers et al., 2012; Zähringer et al., 1997).

Signaling pathways

Significant transcriptional differences were observed in the MAP kinases (MAPKs) (**Table 3**). These kinases were up-regulated, and they included cyclin-dependent kinase, as encoded by *CDC28,* which is a regulator of the mitotic and meiotic cell cycle pathway (**Table 3**); cytosolic catalase T, encoded by *CTT1*, with a role in H_2O_2 damage protection; and *YPD1*, which encoded tyrosine phosphatase, which is involved in high osmolality

signaling. By contrast, the transcription factor encoded by *STE12*, which is involved in mating or pseudohyphal/invasive growth pathways, was down-regulated. Because *CDC28* was up-regulated and *SLG1*, *STL2*, *SPA*2 and *PKH1* were down-regulated by melatonin, melatonin seems to favor G2/M delay and filamentation (Starovoytova et al., 2013). In addition, melatonin down-regulated genes were involved in nutrient starvation (*MKC7* and *STE12*) and up-regulated genes were involved in MAPK signaling by high osmolality and included *YPD1* and *CTT1*.

Additionally, melatonin was involved in the phosphatidylinositol signaling pathway, which is connected to the MAPK pathway by phosphatidylinositol-4-kinase ($PI4P_2$), which is encoded by *STT4* and was up-regulated (**Table 3**). *STT4* is an essential gene for cell viability (Audhya et al., 2000; Yoshida et al., 1994) and it catalyzes the synthesis of $PI4P_2$. Its activity is required for the maintenance of vacuole morphology, cell wall integrity and actin cytoskeleton organization (Audhya et al., 2000), and it acts either upstream or in parallel to cell wall stress signaling (Gustin et al., 1998; Levin, 2005).

Pathways involved in **replication and repair** for nucleotide excision and mismatch repair were also enriched by melatonin as well as pathways involved in **yeast transport and catabolism**. The important genes involved in regulating oxidative stress were, among others, *TOR1* and *SCH9*. The protein kinase subunit of TORC1 (which is encoded by *TOR1*), was involved in regulating cell growth, autophagy, and the cellular response to DNA, and the longevity regulation pathways were down-regulated under both stressed conditions when compared to the control. However, the expression of this subunit was significantly different between them, being up-regulated in the presence of melatonin compared to the H condition. However, the protein kinase encoded by *SCH9*, which was involved in the TORC1 and RAS-cAMP pathway, was clearly down-regulated with melatonin. This enzyme seems to regulate the protein kinase A (PKA) directly by phosphorylating Bcy1, and therefore, it decreases the activity of PKA (Zhang et al., 2011). Moreover, Sch9 is proposed to be the downstream effector of TORC1, but this kinase seems to act antagonistically against TORC1 and to induce several stress defense genes that are normally repressed by TORC1 (Smets et al., 2008).

Under stress conditions, the increase in the melatonin concentration (25 µM MEL) did not modify any additional pathways relative to the ones that were already affected by 5

µM MEL; thus, no significantly differences were observed between them (25MELH-5MELH).

3.5. Physiological changes in the lipid composition

Because melatonin modulated the expression of several genes involved in lipid metabolism, we tested if these transcriptomic modifications resulted in changes in the cell lipid composition, measuring the sterol, FA and PL contents of the yeast cells under four conditions (Control, 5MEL, 5MELH and H, **Figure 5**).

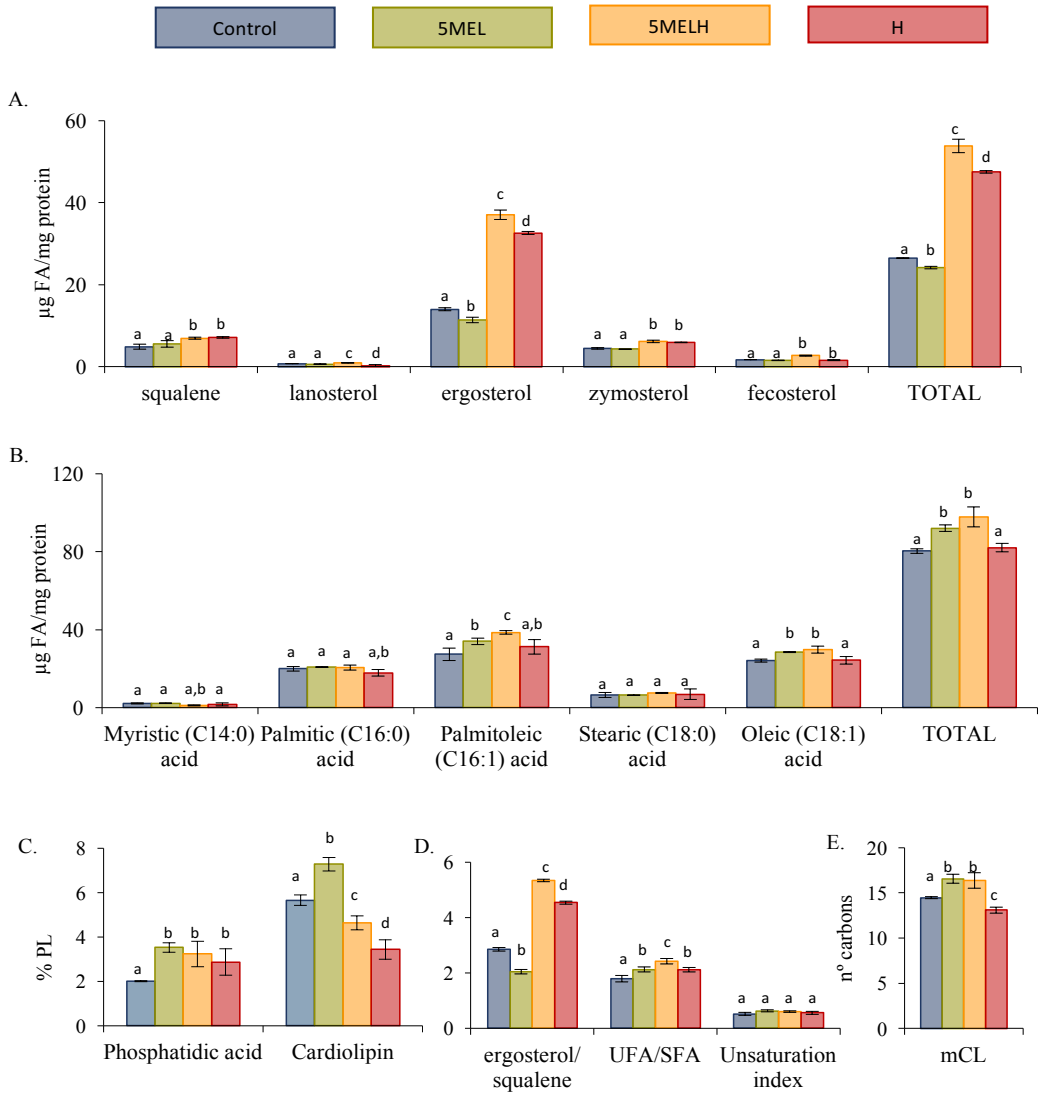

Figure 5. Effect of melatonin supplementation (5 µM) on lipid composition in cells exposed and unexposed to oxidative stress with 2 mM of H_2O_2. (A) Sterols. (B) Fatty acids (FA) (C) Phospholipids. (D) ergosterol/squalene ratio, unsaturated FA / saturated FA (UFA/SFA) ratio and unsaturation index defined as follows: ((% C16:1 + % C18:1) + 2 (% C18:2) + 3 (% C18:3)) /100. (E) Medium length chain of FA calculated as follows: mCL= Σ (% FA x Nº FA carbons)/100.

In the absence of oxidative stress, the melatonin treatment showed lower total sterol levels compared to the control condition primarily in a lower ergosterol content (**Figure 5A**), leading to a lower ergosterol/squalene ratio (**Figure 5D**). By contrast, the opposite was found under oxidative stress, with increases in the total sterols from the increased ergosterol, leading to a higher ergosterol/squalene ratio (**Figure 5A, D**). Changes in the FA produced by melatonin were practically equal independent of the stress, with higher total FA contents from the higher oleic and palmitoleic acids contents, both leading to higher ratios of UFA/SFA and a higher percentage of medium chain lengths (mCL) (**Figure 5B, D, E**). The only changes observed in the PL contents were in phosphatidic acid (PA) and cardiolipin (CL). The PA was only higher in the absence of stress, and although the cardiolipin decreased in the presence of oxidative stress, it was higher under both conditions supplemented with melatonin (**Figure 5C**).

4. DISCUSSION

In previous studies, we analyzed the antioxidant effects of exogenous melatonin on an *S. cerevisiae* strain at the physiological level. Our data showed a slight increase in ROS and GSSG with melatonin supplementation when no stress was induced. In contrast, when the cells were under oxidative stress, the melatonin activated some of the genes in the yeast antioxidant defense systems, and the cells reduced the ROS accumulation, thus enhancing the cellular viability (Vazquez et al., 2017). In this study, we wanted to investigate the effect of melatonin on the global transcriptomic response of cells to gain insight into the antioxidant role and the regulatory mechanism of melatonin in yeast.

In vertebrates, several melatonin functions 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 the intracellular compartments (Galano et al., 2011). As reported in mammals (Reiter et al., 2007; Rodriguez et al., 2004), *S. cerevisiae* was able to incorporate exogenous melatonin at nanomolar concentrations, and, among other possible roles, it could modulate gene expression independently of oxidative stress. Although the levels of incorporated melatonin were also at nanomolar concentration, these melatonin levels were higher under oxidative stress, probably because H_2O_2 induces rapid changes in both plasma

membrane permeability and its gradient, which might promote changes in cellular transport (Folmer et al., 2008) that could favor the entry of melatonin into the cell.

Due to its amphipathic nature, melatonin is speculated to pass the cell membrane with ease; however, the exact mechanisms by which melatonin enters into cells remains unknown. Recently, it was reported that a facilitated diffusion was involved in melatonin's transmembrane transportation in human cancer cells, either through glucose transporters (Hevia et al., 2015), or oligopeptide transporters (Huo et al., 2017). In *S. cerevisiae*, melatonin overexpressed the membrane transporter for both urea and polyamines which possess a similar structure to that of melatonin. This permease could be a candidate to mediate melatonin transportation, and thus further studies could be performed to relate the role of these permeases to melatonin transportation.

As described in lines of human tumor cells lines (Alonso-Gonzalez et al., 2008), melatonin activated both metallothioneins in *S. cerevisiae,* with antioxidant and superoxide dismutase activities being involved in the detoxification of metal ions and the removal of superoxide radicals. Melatonin has been shown to have a protective role in counteracting the toxic effects of metal exposure in human cells (Romero et al., 2014). In our previous results (Vázquez et al., 2017), in which we analyzed different genes related to yeast antioxidant defenses using qPCR, the glutathione peroxidase encoded by *GPX1* was slightly down-regulated by melatonin in cells that were at the early exponential phase. Furthermore, the cytosolic dismutase (Cu/ZnSOD) encoded by *SOD1* was slightly up-regulated, which could be in accordance with metallothioneins overexpression. Our results seem to indicate that the protective role of melatonin against metals also works in *S. cerevisiae* because melatonin is able to activate the different cellular defense mechanisms, even without the presence of metals to better endure further stresses. However, physiological changes have been observed with melatonin supplementation in the absence of stress, which could not be uncovered by a transcriptomics assay that indicated factors such as a higher FA content and lower sterol content. The higher FA content could be consisitent with the higher peroxisome proliferation observed with melatonin. In absence of stress, these examples could be melatonin mechanisms for preparing cells for additional possible stresses (Vázquez et al., 2017).

Cells that were pretreated with melatonin under oxidative stress showed greater changes at the transcriptional level. The genes associated with antioxidant defenses systems such as glutathione/glutaredoxins and thioredoxins systems, cytosolic catalase and glutathione peroxidase were transcriptionally induced by melatonin. In fact, these results are consistent with our previous results where genes such as *GPX1*, *GRX2*, *TRX2* and *CTT1* as idintified by qPCR were also up-regulated by melatonin (Vázquez et al., 2017). Furthermore, other genes that were evaluated and up-regulated by melatonin, such as *GSH1*, *ZWF1*, *GLR1*, *SOD1*, *SOD2* and *CTA1*, were not significantly different in the transcriptomic assays, but their expression tended to be higher in the presence of melatonin. *GPX1* is a paralogue of *HYR1* that functions as a hydroperoxide receptor to transduce the redox signal to Yap1p and it seem to be important to activate arachidonic pathway at transcriptional level. Higher amounts of arachidonic acid (AA) have been correlated with more viability at higher temperature in yeast (Mejía-Barajas et al. 2017). AA can be produced from linoleic and linolenic acid through the coexpression of Δ5- and Δ6-desaturase with Δ6-elongase (Chemler et al., 2006) however, although *S. cerevisiae* can incorporate exogenous PUFAs, as it only possesses Δ9 desaturase AA was not present in its FA composition. As described in human cells, arachidonate potentiation by melatonin could be a parallel immediate and transient intracellular free radical stimulation pathway (Godson and Reppert, 1997, Radogna et al., 2009, 2010).

Mitochondria are believed to be the biological targets of melatonin in human cells (Reiter et al., 2016), and they are the primary ATP-generating organelles in eukaryotic cells. Furthermore, mitochondria are both the source and the site for the detoxification of reactive oxygen species in yeast (Chevtzoff et al., 2010; Rhoads et al., 2006). As reported in humans (León et al., 2005; Martín et al., 2000), our results showed that melatonin modulated mitochondria at the transcriptional level in *S. cerevisiae*, increasing the expression of genes related to the respiratory chain (complex II, III and IV) and oxidative phosphorylation, which are required genes for ATP synthesis. Furthermore, melatonin up-regulated essential genes for the maintenance of normal mitochondrial morphology in yeast, such as *TOM7* and *ETR1* (Torkko et al. 2001; Dimmer et al. 2002). Mitochondrial function is required for yeast resistance to oxidative stress (Grant et al., 1997), and the electron transport chain has been identified as being vital

for H_2O_2 tolerance by *S. cerevisiae* (Thorpe et al., 2004). In addition, cardiolipin (CL), the signature lipid of inner mitochondrial membranes, was also higher, with melatonin supplementation indicating the stabilization of transport chain complexes and resistance against oxidative stress (De Kroon et al., 2013; Joshi et al., 2009). Our results indicate that, as in vertebrates (Reiter et al., 2016), melatonin acts as a mitochondria-targeted antioxidant in *S. cerevisiae*, both at the physiological level, by reducing ROS accumulation (Vázquez et al., 2017), and at the transcriptional level, by activating genes related to mitochondrial function and maintenance.

Energy is necessary for cell growth and viability, and for processes such as the repair of damaged proteins, detoxification of lipoperoxidation products and transport of oxidized molecules, especially under environmental stress conditions (Gasch et al., 2000; Grant et al., 1997; Zhao et al., 2015). In fact, melatonin also activated genes involved in glycolysis and the TCA cycle, indicating the importance of choosing the most efficient route to generate and maintain energy reserves in cells. The presence of melatonin increased the total FA levels, specifically, the monounsaturated FA such as palmitoleic and oleic acids, leading to higher UFA/SFA ratios, which have been related to a higher tolerance to H_2O_2 (Serrazanetti et al., 2015). Furthermore, genes such as *ETR1* are related to FA biosynthesis. *TSC13* and *PHS1* are both involved in long chain FA elongation and were up-regulated with melatonin. Those results are consistent with the activation of genes related to FA synthesis and elongation. However, melatonin also increased the total sterols when cells were exposed to H_2O_2, showing higher levels of ergosterol and its precursor lanosterol as well as a higher ergosterol/squalene ratio, which is related to higher tolerance to H_2O_2 and other stress conditions (Henderson and Block, 2014). However, the higher ergosterol levels cannot be explained by changes in the expression of biosynthetic genes. In fact, some genes involved in its synthesis, such as *ERG11,* were down-regulated in the presence of melatonin, probably due to the tight regulation at the transcriptional level through a feedback mechanism that responds to high amounts of the end product ergosterol, as previously described by Yuan and Ching 2015 and Servouse and Karst 1986. Thus, this increase in the ergosterol content might be due to either posttranscriptional enzyme activation or to higher oxygen availability in the presence of melatonin, as required for ergosterol biosynthesis (Zavrel et al., 2013).

Another possibility would be a higher ergosterol uptake under melatonin, but sterol import in *S. cerevisiae* is specific to anaerobic growth, a phenomenon known as aerobic sterol exclusion (Lorenz and Parks, 1987; Zavrel et al., 2013). Moreover, melatonin up-regulated some of the genes involved in the biosynthesis of phosphatidylcholine and sphingolipids, which are related to stress tolerance. Berry et al. (2011) demonstrated that a sur2Δ mutant showed high sensitivity to an oxidizing agent as H_2O_2, indicating a role for this gene in the acquisition of tolerance to that stress. In parallel, the Acyl-CoA oxidase encoded by the *POX1* gene was up-regulated, indicating that melatonin could increase the β-oxidation of FAs inside peroxisomes. This gene confers the ability of yeast to grow on oleic acid as a sole carbon source (Hiltunen et al., 2003). These results are consistent with our previous studies in peroxisomes, which also showed that melatonin increases their proliferation.

MAPK pathways can be activated by a number of extracellular and intracellular stimuli. In humans, the ROS and cellular stimuli that are able to induce ROS production have been postulated to be one of the primary activators of this pathway. Moreover, it has been reported that the direct exposure of cells to exogenous H_2O_2 leads to the activation of MAPK pathways, whereas the presence of antioxidants and inhibitors of ROS-producing enzymatic systems block the activation of the MAPK pathway (Son et al., 2011). Under the stress conditions in this study, *STE11* was up-regulated. *STE11* is one of the core genes in the MAPK kinase cascade, being involved in the yeast pheromone response, pseudohyphal/invasive growth pathways and high-osmolarity response pathway, therefore activating the MAPK pathway. Instead, under stress conditions in the presence of melatonin, the down-regulation of *STE12* occurs. *STE12* is a transcription factor that regulates the mating genes and the filamentous growth, although after it forms a hetero-multimer with TEC1, it seems to confirm that the presence of melatonin, which implies a reduction of ROS content, can reduce the activation of this pathway.

In our study, Tor1 was down-regulated by stress, supporting the notion that TORC1 activity is regulated by nutrient abundance and inhibited by noxious stress (Urban et al., 2007). Instead, under the 5MELH condition (when compared to H), this gene was up-regulated. However, *SCH9* was down-regulated; it is a key downstream effector of ROS and the Chronological life span (CLS) and TOR-mitochondria pathways. The kinase Sch9

is known to activate respiratory metabolism during the quiescent phase, thus increasing the ROS concentration and DNA damage (Wei et al., 2009). Therefore, the sch9 down-regulation by melatonin resulted in an extension of the CLS (Pan and Shadel, 2009).

This study shows how melatonin apparently appears to indirectly provide yeast with highly efficient machinery to maintain a reduced environment. As Zhao et al. (2015) noted in a mutant strain with higher tolerance to H_2O_2, some of the genes involved in carbohydrate metabolism, fatty acid degradation, glycolysis/gluconeogenesis, the peroxisomal matrix, pyruvate metabolism, amino acid metabolism and nucleotide repair pathways were crucial to oxidative stress tolerance. Furthermore, as described in humans, melatonin may stimulate other antioxidant molecules such as thiamines, spermidines, folic acid or lipoic acid.

5. 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, once inside, it acted on a genome-wide gene expression level. In the absence of stress, melatonin exposure appears to prepare cells for further oxidant assaults, and melatonin clearly modulated genes related to antioxidant defenses systems, conferring a higher power of detoxification against oxidative stress to the cell. Under environmental stress conditions, yeast reprograms its cellular machinery for better adaptation to stress. In this sense, melatonin might enhance the energetic efficiency and signal transduction, conferring higher H_2O_2 tolerance to *S. cerevisiae*.

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Appendix A. Supplementary data

Supplementary Table 1. Genes differentially expressed (fold change (FC) ≥1; p-value < 0.05) by the increase of melatonin concentration (25 μM and 5 μM) in cells under oxidative stress with 2 mM of H_2O_2 (25MELH – 5MELH)

Gene standard name	Name description	FC	p-value
YDL114W	Putative short-chain dehydrogenase/reductase	2.36	3.35E-02
YKL070W	Putative protein of unknown function	2.00	1.44E-02
ERR1	Enolase-related repeat	1.92	1.78E-02
YOR387C	Putative protein of unknown function	1.88	1.50E-02
YGL118C	Putative protein of unknown function	1.83	1.37E-03
YPL257W	Putative protein of unknown function	1.75	1.67E-02
YJL027C	Putative protein of unknown function	1.68	2.74E-02
IRC4	Increased recombination centers	1.65	3.80E-02
FIG1	Factor induced gene	1.56	4.43E-02
YDL151C	BUD site selection	1.55	4.72E-02
YNL194C	Integral membrane protein	1.52	4.01E-02
YCL001W-A	Putative protein of unknown function	1.40	2.10E-02
FRM2	Fatty acid repression mutant	1.39	3.49E-02
YPR038W	Increased recombination centers	1.39	3.59E-02
AGA2	a-Aglutinin	1.36	2.71E-02
PIR3	Protein containing internal repeats	1.33	2.02E-04
DAK2	Dihydroxyacetone kinase	1.32	1.30E-02
YGR115C	Dubious open reading frame	1.32	7.46E-03
MSH4	Protein involved in meiotic recombination	1.30	3.56E-02
YLL017W	Non-essential Ras guanine nucleotide exchange factor	1.29	3.71E-02
YOR338W	Putative protein of unknown function	1.25	3.84E-03
PDC5	Pyruvate decarboxylase	1.25	1.44E-02
SRX1	Sulfiredoxin	1.24	4.51E-02
CRF1	Co-repressor with *FHL1*	1.24	2.84E-02
YML057C-A	Dubious open reading frame	1.22	4.85E-02
YPR077C	Dubious open reading frame	1.22	4.43E-02
SEG2	Stability of eisosomes guaranteed	1.21	4.50E-02
NPL3	Nuclear protein localization	1.20	1.02E-02
TSL1	Trehalose synthase long chain	1.18	2.97E-03
SFK1	Supressor of four kinase	1.18	4.53E-02
YEL077C	Helicase-like protein	1.17	3.42E-02
EAF1	Esa1p-associated factor	1.16	1.37E-02
DYS1	Deoxyhypusine synthase	1.15	1.91E-02
GAD1	Glutamate decarboxylase	1.14	1.99E-02
CWC23	Complexed with Cef1p	1.13	1.42E-02
YCR061W	Protein of unknown function	1.11	3.71E-02
GLO4	Glyoxalase	1.10	4.88E-02
RSC8	Remodel the structure of chromatin	1.10	4.19E-02
CIS3	Clk1 suppressing	1.10	3.24E-02
YNL046W	Putative protein of unknown function	1.09	4.36E-02
MEF2	Mitochondrial elongation factor	-1.09	4.00E-02
ESBP6	Protein with similarity to monocarboxylate permeases	-1.09	3.36E-02
VMA22	Vacuolar membrane Atpase	-1.09	3.47E-02

Gene standard name	Name description	FC	p-value
LOS1	Loss of suppression	-1.09	4.90E-02
YKR070W	Putative protein of unknown function	-1.10	4.23E-02
PLC1	Phospholipase C	-1.10	3.46E-02
EMC5	ER membrane protein complex	-1.10	4.97E-02
AVT7	Amino acid vacuolar transport	-1.11	4.35E-02
AIM20	Putative protein of unknown function	-1.11	3.52E-02
YOR093C	Putative protein of unknown function	-1.12	2.51E-02
AXL2	Integral plasma membrane protein	-1.12	3.59E-02
MET18	Methionine requiring	-1.12	2.24E-02
CTO1	Protein required for cold tolerance	-1.12	2.47E-02
ERG8	Ergosterol biosynthesis	-1.12	3.43E-02
YGR016W	Putative protein of unknown function	-1.12	2.45E-02
YCR064C	Dubious open reading frame	-1.12	2.62E-02
YNL092W	S-adenosylmethionine-dependent protein methyltransferase	-1.12	3.83E-02
REF2	RNA-binding protein	-1.13	3.41E-02
RTP1	Required for the nuclear transport of RNA Pol II	-1.13	2.55E-02
SEC59	Dolichol kinase	-1.13	2.63E-02
DAD4	Duo1 and Dam1 interacting	-1.13	4.70E-02
MIF2	Mitotic fidelity of chromosome transmission	-1.13	4.80E-02
TEL2	Telomere meintenance	-1.13	3.27E-02
TDA11	Topoisomerase I damage affected	-1.13	2.12E-02
YKR051W	Putative protein of unknown function	-1.13	4.39E-02
NGL1	Putative endonuclease	-1.14	3.86E-02
MNE1	Protein involved in splicing	-1.14	3.12E-02
OST4	Oligosaccharyltransferase	-1.14	2.22E-02
PCI8	Proteasome-COP9 signalosome (CSN)-eIF3	-1.14	2.32E-02
YBL096C	Non-essential protein of unknown function	-1.14	4.13E-02
AEP2	ATPase expression	-1.16	4.16E-02
APL2	Clathrin adaptor protein complex large chain	-1.16	2.71E-02
SPT20	Supressor of Ty	-1.18	3.00E-02
YBL070C	Dubious open reading frame	-1.19	2.40E-02
YDR467C	Dubious open reading frame	-1.20	2.39E-02
STE11	Signal transducing MEK kinase	-1.21	4.64E-02
CRC1	Carnitine carrier	-1.26	4.48E-02
SPL2	Suppressor of PLc1 deletion	-1.27	3.82E-02
YBL094C	Dubious open reading frame	-1.41	3.16E-03
YLR217W	Dubious open reading frame	-1.50	2.85E-02
DAL1	Degradation of allantoin	-1.61	4.58E-02
YLL030C	Putative protein of unknown function	-2.55	8.28E-03

Supplementary Table 2. Biological process enrichment from Gene ontology (GO) analysis of differentially expressed genes up- and down-regulated (fold change ≥1; p-value < 0.05) among stressed cells in absence or presence of melatonin (5 μM) (5MELH – H).

	GO term biological process (p-value)	Gene names (|2| ≤ FC ≥ |1|)
Up- regulated	Mitochondrial electron transport, cytochrome c to oxygen (2.17E-02)	*COX5A, COX7, COX2, COX1, CYC7, COX8, COX3, COX6*
	Oxidation-reduction process (5.86E-04)	YKL071W, *QCR9, RGI1, COX5A, EUG1, HFD1, GPX1, GDH3, AAD10, DOT5, TSC13, QCR7, COX7, FDH1, ECM4, THI4, HYR1, GPH1, MXR1, COX2, MIX14,* YCR102C, *TRX1,TRX2, DLD3, COX1, ETR1, PRM4, CYC7, SER33, FRE3, GRX1, SDH4, GRX2, COX8, COQ11,* YJR096W, *CTT1, GAL80, AYR1, COX3, HOM6, GLT1, FRE6,, TPA1, GTO1, YLR456W, ALD3,* YKL107W, *MET14, SUR2, COQ10, ALG6, ACS1, MPD1, COX6, POX1, YPR127W, CIT2, HBN1, SRX1, ALD3, CIR1*
	Detoxification (3.22E-02)	*GPX1, DOT5, ECM4, HYR1, TRX1, TRX2, GRX1, GRX2, CTT1, CUP1-2, GTO1, CUP1-1, SRX1*
	Transport (3.74E-02)	*HUT1, YPT7, NTF2, JEN1, CHS7, BAT1,YBT1, ATP18, HOT13, GET1, ATP14, VPS68, CDC31, ATP8, QCR9, TVP18, ROY1, ECM10, COX5A, SEC3, GFD1, YGL140C, MAL31, ATG8, GYP8, MFM1, PDR16, OPT1, SRP102, VPS1, COG1, SIS1, RER1, ATG31, PBI2, SNC1, CRC1, VPS28, RAV2, YET2, YIP3, GET4, NUP133, QCR7, COX7, AQY2, SEM1, EGD1, CDC28, IST1, TIM8, DSS4, HSP30, SIW14, ATP15, RTA1, TLG2, COX2, TRX1,TRX2, HXT6, VHC1, COX1, TRS33, NDL1, GMH1, CNL1, COS7, ACB1, FRE3, ATG9, EMP47, CCC2, SNC2, ATG5, HSP10, YPT6, BLS1, COX8, CUR1, PEX19, TOM7, IST3, ERV14, PHS1, SGE1, YSP2, ATP20,* YLL053C, *ATG12, PEX25, MRS4, COX3, SEC17, TPM2, TVP15, FRE6, ADY2, COX17, YET3, PFY1, YIF1, SPL2, ROD1, SEC66, YAT2, ERP5, COG3, BLI1, MST28, PTH1, HXT5, YOP1, BTN2, PAM17, SNX41, IRC6, PEP8, ATG29, COX6, COG7, PEP12, OM14, PMP2, COF1, MON1, SSA4, MVB12, ARV1, YRO2, YIP1, DNF3, PXA1*
Down-regulated	mRNA metabolic process (1.93E-03)	*LAS17, SLK19,* YOL019W, *YME1, VTS1, VAN1, UBX2, AIM10, MRP4, SLX5, SEC1, CRN1, NFS1,* YGR054W, *IFH1, UTP15, MSS116, ERG11, CAF4, CWC2, MRM1, MMT1, RTT101, PCL8, RRP14, INP51, INN1, PKH1, UFO1,* YCL001W-B, *INP1, EAF1, RIO1, PET9, CBK1, EXO84, PIR3, CCM1, SNF2,* YJR084W, *EPS1, STD1, CMP2, MSK1, GPB1, STE12, YKU80, VAC17, POP2, HOS3, EAR1, MLP1, RAD23, UPC2, BCK2, MED1, CPS1, UBP13, IPT1, SPA2, UBP10, CHS5, YSC84, ERC1, MSL5, RCK1, MKC7, TMN2, FET5,* YCR061W, *TEL1,* YCL001W-A, *USO1, ILV3, PUS7, MLP1, ERF2, NRD1, SEY1, DSN1, UBC11, KTR7, MSO1, PUF4, FAR10, ADA2, LSG1, CDD1, RPA34, SRO9, NTH1, BUD8, SCC4, INO2, CDC55, ELP2, RAD27, MRX1, GID8, AFT1, MRE11, FKH1, FYV8, RRP6, EPL1, HMT1, SCH9, MYO1, CAB3, GEP3, TRF5, POG1, IRC4, DHH1, SPS18, XDJ1, AIM14, ERF2, ECM2, MET4, RGT2, DBP1, SSC1, UTR2, PEX1, BUD31, MRC1, SFP1, ATM1, ARP5, PEX8, PKP1, BUD27, HCM1, HLR1, SDS23, BMT6, BUD23, CAR2, SHS1, MPS3, MSS51, VPS16, SPO23, ILV1, CLN3, NOP14, MRN1, LDS2, MPH1, PSR2, NPL3, NUP2, TUP1, BDF1, RTS1, GAS1, HPR1, PUS1, SLT2, DSE1, CBP2, EXO5, YTA12, HEM1,* YOR338W, *SPO21, YHP1, SDA1, DPS1, NTH1, ALD5, CDC20, MAE1, DBP7, STB3, BRO1, LGE1,* YNL190W, *DUS4, MMS1, WTM2, SUL2, WHI3, PRP19, PSR1, MNN4, ARE2, PTC5, MET12, SPS1, SLX4, ISY1, IZH3, KAR1, FRD1, BI4, RCL1, VPS53, CDC9, GZF3, CDC11, MUD2, PTC3, IRS4, RRP9, BRE5, HEM3, RSP5,* YBR238C, *FRA1, UTH1, HAP4, YHM2, SLG1, HOS4, CMR1, IMH1, ALD5, CNA1, YRF1-7, COQ1, PRB1, SMI1, NHA1, CTR9, YRF1-6, BIK1, YCK3, ETP1, YRF1-5, DBP3*
	Negative regulation of gene expression (6.09E-03)	*LAS17, SLK19, VTS1, VAN1, SLX5, SEC1, CRN1, RFU1, NFS1, TOS8,* YGR054W, *IFH1, UTP15, MSS116, CAF4, MMT1, PCL8, RTT101, PKH1, INN1,* YCL001W-B, *EAF1, RIO1, CBK1, SNF2,* YJR084W, *EPS1, STD1, CMP2, GPB1, STE12, YKU80, POP2, HOS3, MLP1, RAD23, UPC2, BCK2, MED1, UBP13, SPA2, UBP10, CHS5, YSC84, RCK1, MKC7, TMN2, FET5,* YCR061W, *TEL1,* YCL001W-A, *MLP1, ERF2, NRD1, UBC11, PUF4, FAR10, ADA2, NTH1, INO2, CDC55, ELP2, GID8, AFT1, MRE11, FKH1, RRP6, EPL1, SCH9, HMT1, TRF5, POG1, MGA1, SPS18, DHH1, XDJ1, AIM14, MET4, MPT5, DBP1, RGT2, SSC1, PEX1, MRC1, SFP1, ATM1, ARP5, PET111, BUD27, HCM1, SDS23, MPS3, MSS51, VPS16, SPP41, CLN3, MRN1, MPH1, NPL3, NUP2, TUP1, BDF1, RTS1, GAS1, HPR1, SLT2, DSE1,* YOR338W, *SDA1, YHP1, CDC20, STB3, BRO1, LGE1, MMS1, WTM2, SUL2, WHI3, MNN4, PTC5, SPS1, SLX4, IZH3, RCL1, VPS53, GZF3, CDC11, PTC3, IRS4, BRE5, RSP5, FRA1, UTH1, HAP4, HOS4, SLG1, CMR1, CNA1, YRF1-7, SMI1, NHA1, CTR9, YRF1-6, BIK1, YCK3, YRF1-5*
	Positive regulation of biological process (7.87E-04)	*LAS17, SLK19, VTS1, SEC1, CRN1, IFH1, UTP15, RIO1, SNF2, STD1, GPB1, STE12, POP2, HOS3, MLP1, UPC2, BCK2, MED1, SPA2, PUF4, ADA2, NTH1, INO2, CDC55, GID8, AFT1, FKH1, EPL1, SCH9, HMT1, MET4, MPT5, SSC1, SFP1, PET111, HCM1, MSS51, NPL3, BDF1, HPR1, SLT2,* YOR338W, *SDA1, CDC20, STB3, BRO1, WTM2, WHI3, GZF3, CDC11, RSP5, UTH1, HAP4, SLG1, CTR9*
	Regulation of cellular process (1.69E-07)	*LAS17, SLK19, VTS1, SLX5, SEC1, CRN1, RFU1, TOS8,* YGR054W, *IFH1, UTP15, MSS116, CAF4, MMT1, PCL8, RTT101, PKH1, EAF1, RIO1, CBK1, SNF2,* YJR084W, *EPS1, STD1, CMP2, GPB1, STE12, YKU80, POP2, HOS3, MLP1, RAD23, UPC2, BCK2, MED1, SPA2, UBP10, CHS5, YSC84, RCK1, MKC7, MLP1, UBC11, PUF4, FAR10, ADA2, NTH1, INO2, CDC55, ELP2, GID8, AFT1, MRE11, FKH1, EPL1, SCH9, HMT1, POG1, MGA1, DHH1, AIM14, MET4, MPT5, RGT2, SSC1, MRC1, SFP1, ARP5, PET111, BUD27, HCM1, SDS23, MPS3, MSS51, VPS16, SPP41, CLN3, MRN1, MPH1, NPL3, NUP2, TUP1, BDF1, RTS1, GAS1, HPR1, SLT2, DSE1,* YOR338W, *SDA1, YHP1, CDC20, STB3, BRO1, LGE1, MMS1, WTM2, WHI3, SPS1, SLX4, GZF3, CDC11, PTC3, IRS4, BRE5, RSP5, FRA1, UTH1, HAP4, HOS4, SLG1, CMR1, CNA1, SMI1, CTR9, BIK1, YCK3*

	GO term biological process (p-value)	Gene names (|2| ≤ FC ≥ |1|)
Down-regulated	Regulation of nitrogen compound metabolic process (2.03E-04)	*VTS1, SLX5, RFU1,* YGR054W, *TOS8, IFH1, UTP15, MSS116, CAF4, PCL8, PKH1, EAF1, RIO1, SNF2, STD1, GPB1, STE12, YKU80, POP2, HOS3, MLP1, UPC2, RAD23, MED1, UBP10, CHS5, PUF4, ADA2, NTH1, INO2, CDC55, ELP2, AFT1, MRE11, FKH1, EPL1, SCH9, HMT1, POG1, MGA1, DHH1, MET4, MPT5, SSC1, MRC1, SFP1, ARP5, PET111, BUD27, HCM1, MPS3, MSS51, SPP41, CLN3, MRN1, MPH1, NPL3, NUP2, TUP1, BDF1, HPR1, GAS1,* YOR338W, *YHP1, CDC20, STB3, BRO1, LGE1, WTM2, WHI3, SLX4, GZF3, CDC11, PTC3, IRS4, RSP5, FRA1, UTH1, HAP4, CTR9, SMI1*
	Regulation of transcription, DNA-templated (3.21E-04)	*VTS1, SLX5,* YGR054W, *TOS8, IFH1, UTP15, MSS116, CAF4, PCL8, EAF1, RIO1, SNF2, STD1, STE12, YKU80, POP2, HOS3, MLP1, UPC2, RAD23, MED1, UBP10, CHS5, PUF4, ADA2, NTH1, INO2, CDC55, ELP2, GID8, AFT1, MRE11, FKH1, EPL1, SCH9, HMT1, POG1, MGA1, DHH1, MET4, MPT5, MRC1, SFP1, ARP5, PET111, BUD27, HCM1, MPS3, MSS51, SPP41, CLN3, MRN1, NPL3, NUP2, TUP1, BDF1, HPR1, GAS1,* YOR338W, *YHP1, STB3, LGE1, WTM2, WHI3, GZF3, IRS4, RSP5, FRA1, UTH1, HAP4, CTR9, SMI1*
	Cell cycle (2.34E-02)	*SLK19,* YOL019W, *CWC2, RTT101, INN1, RIO1, HOS3, MLP1, BCK2, SPA2, CHS5, DSN1, MSO1, FAR10, LSG1, BUD8, SCC4, CDC55, GID8, AFT1, MRE11, FKH1, EPL1, MYO1, TRF5, POG1, BUD31, MRC1, HCM1, BUD23, SHS1, MPS3, SPO23, CLN3, LDS2, RTS1, DSE1,* YOR338W, *SPO21, SDA1, YHP1, CDC20, LGE1, MMS1, WTM2, WHI3, SPS1, KAR1, CDC9, CDC11, UTH1, SLG1, CTR9, BIK1*
	Cellular component organization or biogenesis (1.10E-02)	*LAS17, SLK19,* YOL019W, *YME1, VAN1, UBX2, SLX5, CRN1, NFS1, IFH1, UTP15, CAF4, MRM1, CWC2, RRP14, NOG2, PKH1, INN1,* YCL001W-B, *INP1, EAF1, RIO1, CBK1, EXO84, PIR3, CCM1, SNF2,* YJR084W, *YKU80, VAC17, HOS3, MLP1, UBP13, SPA2, UBP10, CHS5, YSC84, MKC7,* YCR061W, *TEL1, USO1, YCL001W-A, PUS7, ERF2, SEY1, DSN1, KTR7, MSO1, PUF4, ADA2, LSG1, RPA34, SCC4, CDC55, RAD27, AFT1, MRE11, FKH1, RRP6, EPL1, SCH9, HMT1, MYO1, GEP3, DHH1, XDJ1, ECM2, SSC1, UTR2, PEX1, MRC1, SFP1, ARP5, PEX8, PET111, BUD27, HCM1, HLR1, BMT6, BUD23, SHS1, MPS3, MSS51, VPS16, CLN3, NOP14, MRN1, LDS2, MPH1, NPL3, NUP2, TUP1, BDF1, RTS1, GAS1, SLT2, DSE1, EXO5, YTA12,* YOR338W, *SPO21, SDA1, DBP7, BRO1, LGE1, WHI3, PRP19, SPS1, ISY1, KAR1, RCL1, ALB1, CDC9, MUD2, IRS4, RRP9, RSP5, UTH1, HOS4, YHM2, SLG1, CNA1, YRF1-7, SMI1, YRF1-6, BIK1, DBP3, YRF1-5*

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GENERAL DISCUSSION

In recent years, melatonin has attracted a great deal of attention due to its potentially beneficial effects on human health. These benefits have been attributed to melatonin's multiple functions, including its powerful antioxidant effect, which is one of its best-studied attributes in mammals (Reiter et al., 2016). The Wine Biotechnology group (yeast subgroup) at Rovira i Virgili University, where the present thesis was performed, is one of the first groups to relate the production of melatonin by *Saccharomyces cerevisiae* to wine alcoholic fermentation (Rodriguez-Naranjo et al., 2011, 2012). Since then, most studies on melatonin in yeasts have focused their attention on melatonin production and detection (Fernández-Cruz et al., 2017; Muñiz-Calvo et al., 2017; Vigentini et al., 2015), but the role of melatonin in yeast is completely unknown.

In addition to being the simplest eukaryote model, *S. cerevisiae* has been considered the primary yeast for use in the winemaking process. Even though it remains the best candidate for performing alcoholic fermentation, there is an increasing interest in the utilization of non-*Saccharomyes* species, with a view towards developing consumer-directed wines with differentiated styles (González-Royo et al., 2015; Padilla et al., 2016). The winemaking process is a hostile environment for yeasts, especially for non-*Saccharomyces* that are considered less resistant species than *S. cerevisiae* for enduring this process, which is traditionally associated with their lower ethanol tolerance. Nevertheless, the stress induced by ethanol is not the only one that yeasts have to resist during alcoholic fermentation. Other stresses, such as oxidative stress, are also involved in this process (Gómez-Pastor et al., 2012; Pretorius, 2000).

Knowledge of melatonin's effects on yeast will help to control its synthesis for uses as natural antioxidants, which could have important biotechnological implications such as diminishing cellular oxidative damage during the biotechnological production of dry starters (Gamero-Sandemetrio et al., 2015) or even for use as potential therapeutic targets for several oxidative stress-related diseases (Escoté et al., 2012; Gutteridge and Halliwell, 2010; Halliwell, 2006). We focused our studies on the possible antioxidant effect of melatonin on both *Saccharomyces* and non-*Saccharomyces* yeasts. However, given the small amount of information that is available about how non-*Saccharomyces* yeasts respond to oxidative stress, we were compelled to initiate a study in which we

compared the oxidative stress responses between *Saccharomyces* and non-*Saccharomyces* wine yeast species.

- Effect of oxidative stress on *Saccharomyces* and non-*Saccharomyces* yeasts

Different approaches have been commonly employed to determine the effect of induced oxidative stress on yeasts. In the present work, we aimed to compare the response of different yeast species (*S. cerevisiae*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Starmerella bacillaris* and *Hanseniaspora uvarum*) to oxidative stress (2 mM H_2O_2) by evaluating their resistance to the oxidizing agent, their levels of reactive oxygen species (ROS), catalase activity, lipid peroxidation and changes in their lipid composition. Thus, the results give us a general view of the consequences of oxidative stress in yeast, including free radical production by oxidative stress, endogenous antioxidant defenses to scavenger ROS, damage produced by ROS and adaptive responses by yeast.

Our findings indicated that polyunsaturated fatty acids (PUFAs) play an important role in the resistance and tolerance of yeast to oxidative stress. *M. pulcherrima*, *T. delbrueckii* and *S. bacillaris* species, which contain PUFAs in their lipid composition (Rozès et al. 1992), were able to better resist an induced oxidative stress compared with *S. cerevisiae* and *H. uvarum*, which do not contain PUFAs in their membranes (Rozès et al. 1992). The PUFA synthesis strategy used by these species to acquire oxidative tolerance could be related to hydroperoxide signaling. In the absence of stress, these cells accumulate higher PUFA contents, which in turn generate low but significant amounts of lipid peroxidation products. Those products are able to act as signaling molecules to activate the enzymes involved in yeast antioxidant defense systems (Chen et al., 2006; Cipak et al., 2008), such as cytosolic and peroxisomal catalases (Cipak et al., 2008). As a consequence, these yeasts would be better prepared for subsequent stresses, including oxidative stress. In fact, the catalase activity was higher in non-conventional yeasts in the absence of stress. However, no clear differences were observed in this enzyme under oxidative stress, which could be an indication that other primary antioxidant defenses systems in *M. pulcherrima*, *T. delbrueckii* and *S. bacillaris* are cooperating with catalase to respond quickly to this stress, scavenging ROS molecules and subsequently resulting in a lower lipid peroxidation. Thus, although PUFAs are the lipids that are more

susceptible to peroxidation (Cipak et al., 2006; Johansson et al., 2016), PUFA-containing cells are better equipped to face oxidative stress.

Moreover, under oxidative stress conditions non-*Saccharomyces* yeasts exhibited the greatest increase in monounsaturated fatty acids (MUFAs) with a concomitant decrease in saturated fatty acids (SFA) to cope with oxidative stress. These changes led to a higher UFA/SFA ratio, an indicator of more fluid membranes (You et al., 2003). As described for ethanol tolerance, oleic acid also seemed to be the most important UFA at counteracting the toxic nature of oxidative stress in non-conventional yeasts. Furthermore, palmitoleic acid has been reported to possess a positive effect on yeast viability (Ding et al., 2009; Redón et al., 2009).

Additionally, the most resistant strains, namely, *M. pulcherrima* and *T. delbrueckii,* showed higher PC/PE ratios, which have been related to an enhanced ethanol tolerance in *S. cerevisiae* (Chi and Arneborg, 1999; Vendramin-Pintar et al., 1995). By contrast, a higher PI/PS ratio was observed in the *S. cerevisiae* species. The PI has been considered essential for maintaining cellular viability in *S. cerevisiae* (Becker and Lester, 1977; De Kroon et al., 2013). However, our results negatively correlated tolerance to stress with a high PI/PS ratio as well as with the squalene content, which was higher in the most ROS-producing strains and the most affected strains by oxidative stress. Thus, squalene accumulation may compromise yeast growth and increase its sensitivity to external oxidizing agents (Spanova et al., 2012).

Thus, considering that the primary differences correlated with higher stress tolerance were already observed before stress exposure, the adaptive evolution of each yeast may influence the way it copes with stress. For example, the preference for respiratory or fermentative metabolism as well as the oxygen requirements of each yeast could explain why peroxisomes proliferated markedly in *T. delbrueckii* compared to *S. cerevisiae*. It is well known that anaerobic growth of *S. cerevisiae* requires oxygen to synthesize UFAs such as oleic acid, which is needed to induce yeast peroxisomes (Grillitsch et al., 2011; Kohlwein et al., 2013). Moreover, although both species were classified as Crabtree-positive, *T. delbrueckii* has been reported to exhibit a higher respiratory contribution to its metabolism than *S. cerevisiae* (Alves-Araújo et al., 2007; González et al., 2013; Merico et al., 2007;). Thus, in the absence of stress, *S. cerevisiae* did not exhibit peroxisome

proliferation, which was surely due to the glucose repression of genes encoding peroxisome oxidation (Hiltunen et al., 2003). Instead, *T. delbrueckii* needs functional peroxisomes as a major site of oxygen utilization. After stress exposure, the peroxisomes of the TdB strain were more frequently induced, probably by the capacity to synthesize oleate, which is able to induce the enzymes in the peroxisomal oxidation cycle. Even before stress, this higher peroxisomal activity indicates that *T. delbrueckii* has a greater ability to maintain the equilibrium between the production and scavenging of ROS. This respiratory-deficient condition of *S. cerevisiae* may also influence the oxygen-sensitive process in the conversion of squalene to ergosterol as noted before.

- Effect of melatonin on *Saccharomyces* and non-*Saccharomyces* yeasts

S. cerevisiae has been the most studied yeast in this work. Because it is considered a model for eukaryotic cell biology, there is much more information available to drive studies of transcriptomics, functional genomics and systems biology. Thus, in our study, the melatonin effect was first evaluated in a *S. cerevisiae* wine strain (QA23) and after, compared with other *Saccharomyces* strains and non-*Saccharomyces* species.

As reported in mammals (Rodriguez et al., 2004), our results showed that melatonin easily enters the *S. cerevisiae* cell and is detected intracellularly at nanomolar concentrations. This property has been attributed to its amphiphilic nature, which allows it to cross all membranes and arrive at the intracellular compartments, where it may directly exert its antioxidant actions (Galano et al., 2011; Rodriguez et al., 2004). Nevertheless, melatonin has also been reported to be transported by glucose or oligopeptide transporters (Hevia et al., 2015; Huo et al., 2017). In this study, we observed that melatonin activated the genes of some permeases in *S. cerevisiae,* which could be candidate melatonin transporters in yeast, opening the doors to further research.

The cells that were previously grown with exogenous melatonin (5 μM), and in the absence of oxidative stress, showed few changes with respect to their cellular redox state. During the exponential phase, *S. cerevisiae* slightly increased both the ROS production and lipid peroxidation, which may in turn promote a decrease in the reduced to oxidized glutathione ratio (GSH/GSSG). Very low doses of ROS and/or hydroperoxides

(non-toxic levels) could serve as signaling molecules to prepare antioxidant defense systems, such as catalase activity, amongst others (Chen et al., 2006; Cipak et al., 2008). The catalase activity slightly increased in the presence of melatonin. Furthermore, the phosphatidic acid (PA), cardiolipin and peroxisomes proliferation were significantly higher in the presence of melatonin; indicating that melatonin may act by increasing the electron transport chain activity in mitochondria, and consequently increasing β-oxidation in peroxisomes. In addition, significant changes were observed in other lipids: melatonin decreased the total sterol levels, basically by lowering the amounts of ergosterol, and increased the total fatty acid composition, by increasing oleic and palmitoleic acids, leading to higher ratios of UFA/SFA and higher amounts of medium chain-length FAs. In parallel, as in human cells (Romero et al., 2014), our results seems to indicate that melatonin may also have a protective role against metals in *S. cerevisiae,* since different cellular defense mechanisms were activated, even without the presence of metals. Regardless of melatonin, when cells entered in the stationary phase they derepressed all the tested genes encoding antioxidant enzymes when the glucose was exhausted. Nevertheless, the presence of melatonin maintained the genes encoding glutathione production (*GSH*), glutathione peroxidase (*GPX1*), glutathione reductase (*GLR1*), peroxisomal catalase (*CTA1*), both Sod dismutases (*SOD1* and *SOD2*), glutaredoxin (*GRX2*) and thioredoxin (*TRX2*), highly expressed. This up-regulation seems to indicate that cells with melatonin were more efficient detoxifying ROS and subproducts of lipid peroxidation. In fact, shortly after 16 h had passed, the GSH:GSSG ratio increased in the presence of melatonin.

Nonetheless, exposure to oxidative stress with H_2O_2 (2 mM) caused an increase in ROS, as well as in lipid peroxidation, which severely affected the cellular viability in further inoculations, especially when stress was applied during the early exponential and stationary phases. Under these redox imbalance conditions, the cells initiated a remodeling process of their cellular machinery, which involved changes at the transcriptional level to fully activate all the genes involved in the oxidative stress response as well as the general stress response to maintain a proper redox state (Costa and Moradas-Ferreira, 2001; Jamieson, 1998; Moradas-Ferreira and Costa, 2013). In addition, as discussed in the previous objective, an adaptive response to oxidative stress

was observed in the different yeast species, which involved changes in their lipid composition.

Under these adverse conditions, the presence of melatonin was clearly able to mitigate ROS accumulation and lipid peroxidation. In fact, stressed cells that incorporated exogenous melatonin enhanced their viability when reinoculated into fresh medium. This protective effect of MEL was especially significant when stress was applied during the early stationary phase. These results confirm that the cells are more susceptible to oxidative stress during the early exponential phase (DeRisi, 1997; Jamieson, 1998; Puig and Pérez-Ortín, 2000) and in the role in which melatonin prepares cells to better resist oxidative stress. Moreover, the antioxidant function was one of the most important molecular functions attributed to melatonin in our transcriptomic assay.

It is important to highlight that cells previously treated with melatonin which are exposed to oxidative stress have up-regulated, among others, genes associated with the gluthathione/ glutaredoxin and thioredoxin systems, which have been correlated with higher levels of reduced glutathione and lower levels of its oxidized form. Therefore, melatonin seems to activate these glutathione/glutaredoxin and thioredoxin systems, which are considered essential under aerobic and anaerobic conditions (Herrero et al., 2008), and which provide the most important protection against oxidative stress generated by ROS and hydroperoxides in *S. cerevisiae* (Auchère et al., 2008; Gómez-Pastor et al., 2012). These defense mechanisms for detoxifying ROS can act in both cytosolic and mitochondrial compartments. Thus, melatonin could also cross mitochondrial membranes acting as a mitochondria-targeted antioxidant in *S. cerevisiae* both at the physiological level, by reducing ROS accumulation, and at the transcriptional level, by activating genes related to mitochondrial function and maintenance. Furthermore, the content of cardiolipin, a phospholipid that is primarily localized in the inner mitochondria membrane, was higher in cells treated with melatonin, suggesting a more functional mitochondrion during H_2O_2 stress, as has been previously described for ethanol stress (Chi and Arneborg, 1999).

In response to oxidative stress, melatonin may not only contribute by generating energy in the mitochondria (ATP), but it also produces the acetyl-CoA required for the biosynthesis of FAs. Cells grown in presence of melatonin maintained higher MUFAs,

such as palmitoleic and oleic acids, leading to higher UFA/SFA and higher medium chain-length fatty acids after oxidative stress exposure. Regarding sterols, melatonin also increased the total levels of sterols, leading to a higher ergosterol/squalene ratio. As discussed before, both the UFA/SFA and ergosterol/squalene ratios have been correlated with higher tolerance to oxidative stress. As a consequence, melatonin could increase the β-oxidation within peroxisomes. Moreover, melatonin produced cellular changes at the transcriptomic level, which would imply changes in fatty acid degradation, the glycolysis/gluconeogenesis pathway, peroxisomal matrix, pyruvate metabolism, amino acid metabolism and nucleotide repair. According to Zhao et al. (2015), these transcriptomic changes could be crucial, among others functions, for detoxifying lipoperoxidation products, thus reaching higher oxidative stress tolerance.

The mechanisms of action exerted by melatonin as described until the present could be extrapolated to other strains of *S. cerevisiae* and to non-*Saccharomyces* species because our results seem to indicate a similar protection effect by melatonin against oxidative stress in all the tested strains. However, further research is needed to evaluate its effect on the enzymatic and non-enzymatic defense systems of those species, especially in the more resistant ones. In any case, the melatonin in unstressed cells also seemed to activate catalase activity indirectly in *S. cerevisiae*, *T. delbrueckii* and *H. uvarum*. In stressed cells, melatonin partially mitigated oxidative damage by decreasing ROS production and/or lipid peroxidation without increasing the catalase activity. This finding could indicate that melatonin has a direct effect on ROS scavengers or the simultaneous actions of other defense systems such as glutathione/glutaredoxin and thioredoxins systems, which could decrease the need for catalase activity.

In summary, regarding the antioxidant activity of melatonin, we were able to show its powerful antioxidant properties in yeasts. When exogenous melatonin was added (5 μM) in the absence of stress, our results indicated that melatonin supplementation enables cells to better resist further stresses. However, when cells were subjected to oxidative stress, melatonin exerted a prominent antioxidant activity. Our results showed that melatonin can act at different levels in yeast to reduce oxidative stress (**Figure 1**), (1) as an antioxidant that directly scavenges ROS (2) by modulating the gene expression to indirectly stimulate the antioxidant enzyme production and (3) by increasing the

effectiveness of mitochondrial functions, refining the oxidative phosphorylation in the mitochondrial respiratory chain, which would further decrease the peroxidation of membrane lipids.

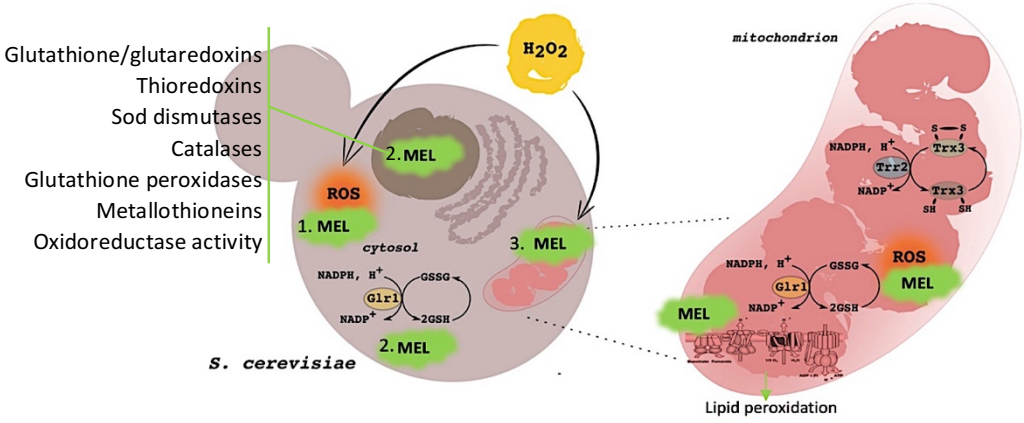

Figure 1. Primary melatonin effect as an antioxidant in *S. cerevisiae* (figure modified from Gostimskaya and Grant 2016 and adapted for melatonin effects).

In many respects, the mechanisms used by melatonin to exert its antioxidant capacity in yeasts are similar to the ones described in several studies in mammals, which show the strong direct and indirect radical-scavenging potential of melatonin (León et al., 2005; Reiter et al., 2000, 2016; Rodriguez et al., 2004; Zhang and Zhang, 2014). Its antioxidant effect seems to be even greater than the one observed with ascorbic acid, well known antioxidant compound (Montilla-López et al., 2002).

Therefore, the results presented in this thesis indicate that our initial hypothesis was correct because melatonin is able to act as an antioxidant compound in both *Saccharomyces* and non-*Saccharomyces* yeasts by indirectly interacting with yeast endogenous defense systems.

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GENERAL CONCLUSIONS

- ✓ *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* and *Starmerellera bacillaris* wine yeasts are best at resisting the oxidative stress induced by hydrogen peroxide.

- ✓ A high proportion of unsaturated fatty acids, particularly linolenic and linoleic acids, are associated with higher stress tolerance in non-conventional yeast.

- ✓ Oleic acid and the PC/PE and ergosterol/squalene ratios are important indicators of oxidative stress tolerance in yeast strains.

- ✓ *S. cerevisiae* can incorporate exogenous melatonin at nanomolar concentrations, which act on genome-wide gene expression and interfere with the regulation of specific and general stress responses.

- ✓ In the absence of oxidative stress, melatonin prepares cells to better endure further stresses by activating cellular antioxidant defense systems in both *S. cerevisiae* and non-*Saccharomyces* species.

- ✓ Under oxidative stress, melatonin exerts antioxidant properties in yeast, partially mitigating the damage produced by oxidative stress by decreasing intracellular ROS and lipid peroxidation, which enhances yeast viability.

- ✓ Melatonin modulates the gene expression in *S. cerevisiae*, stimulating the genes encoding antioxidant enzymes, such as glutathione/glutaredoxin and thioredoxin systems, catalases, superoxide dismutases, methallothioneins and oxidoreductases, which play an important role in detoxifying free radicals and mitigating the damage they produce.

- ✓ Melatonin indirectly stimulates the glutathione system to enhanced the redox cellular state, by increasing reduced glutathione under oxidative stress.

- ✓ Melatonin enhances the mitochondrial functions by activating genes related to its metabolism and maintenance, which results in higher cardiolipin contents.

- ✓ Melatonin improves stress tolerance to stress in *S. cerevisiae* by increasing the total fatty acid content, primarily unsaturated fatty acids, and the ergosterol/squalene ratio. Furthermore, melatonin increases peroxisome proliferation independently of oxidative stress.

No reniego de mi naturaleza, no reniego de mis elecciones, de todos modos he sido afortunada.

Muchas veces en el dolor se encuentran los placeres más profundos, las verdades más complejas, la felicidad más certera.

Tan absurdo y fugaz es nuestro paso por el mundo, que sólo me deja tranquila el saber que he sido auténtica, que he logrado ser lo más parecida a mi que he podido.

(Frida Kahlo)

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