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### **► To cite this version:**

Antonio de Anchieta Câmara, Pierre-André Marechal, Raphaëlle Tourdot-Maréchal, Florence Husson. Oxidative stress resistance during dehydration of three non-Saccharomyces wine yeast strains. Food Research International, 2019, 123, pp.364-372. 10.1016/j.foodres.2019.04.059 . hal-02173093

**HAL Id: hal-02173093**

**<https://u-bourgogne.hal.science/hal-02173093>**

Submitted on 22 Oct 2021

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**Oxidative stress resistance during dehydration of three non-Saccharomyces  
wine yeast strains**

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**Keywords:** dehydration; ROS accumulation; lipid oxidation; *Torulaspora delbrueckii*;  
*Metschnikowia pulcherrima*; *Lachancea thermotolerans*

## Abstract

The development of new fermented foods and beverages requires more and more the use of new dehydrated yeasts species. In this context, the non-*Saccharomyces* (NS) yeasts *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans* are developed especially in winemaking as co-culture in the fermentation of the must or for the must bioprotection. However, during formulation-dehydration the yeast cells are exposed to several stresses that reduce cellular activity. Among these, the oxidative stress induced by the stabilization processes has been described as one of the main causes of cell death. In this study, we analyzed the effects of growth medium associated with two dehydration kinetics on the accumulation of reactive oxygen species (ROS) and lipid peroxidation levels. The cultivability of tested yeast strains was dependent on growth and dehydration conditions. The *L. thermotolerans* strain was the most sensible to dehydration when growing in nutrient-poor media, which was characterized by high levels of ROS, lipid peroxidation and reduced cultivability. In contrast, this yeast was able to restore its cultivability when growing in nutrient-rich medium before dehydration. The other NS yeast strains acquired resistance to oxidative stress similarly but in a growth-dehydration condition less dependent manner. These results showed that modulation of growing medium composition is a simple way to improve resistance to oxidative attack imposed by dehydration in NS yeasts. This was the first time that multiple quantitative and qualitative indicators of oxidative stress response in these three NS yeast strains were explored.

## **1. Introduction**

Yeasts are microorganisms capable of surviving large periods with limited water content, i.e. in the dehydrated state; once in normal hydric conditions they reactivate their metabolism and restart their normal activity. This phenomenon is known as anhydrobiosis (Rapoport, Turchetti, & Buzzini, 2016). Taking advantage of these characteristics, beverage industries have been interested in new species of yeasts stabilized in dry form (active dry yeasts: ADY), especially for winemaking. In general, for wine production the fermentation occurs spontaneously due to the indigenous flora present on grapes or in musts, which consists in different yeasts and bacteria species (Liu, Lu, Duan, & Yan, 2016). Although this practice favors the development of the wine sensorial profiles, it remains difficult to control the microbial population development during winemaking, which represents a loss of quality and productivity. Thus, the controlled application of ADY provides high quality products with guaranteed reproducibility (Rodríguez-Porrata et al., 2011).

During ADY formulation, yeast cells undergo multiple stresses which reduce their performance, e.g. cell starvation in the course of biomass propagation then heat and oxidation induced by the drying stages (Beker & Rapoport, 1987; Rapoport, 2017; Schmidt & Henschke, 2015). In fact, cells subjected to hydric variations accumulate intracellular reactive oxygen species (ROS) as result of failure of their detoxification systems and the functionality of the proteins and enzymes responsible for the ROS elimination (Dupont, Rapoport, Gervais, & Beney, 2014). In addition, the modulation of energy metabolism, from fermentation to respiration, has a great influence on lipid stability to oxidation, especially during the biomass propagation (Gómez-Pastor, Pérez-Torrado, & Matallana, 2010).

In this context, depending on the composition of growing medium, yeasts activate biosynthesis of different compounds, like glutathione which plays part in their resistance to dehydration-oxidation (Câmara et al., 2018; François & Parrou, 2001; Lorenz, Schmach, Stahl, & Senz, 2015). Glutathione (L-gamma-Glutamyl-L-Cysteinylglycine) is a tripeptide thiol antioxidant formed by the amino acids cysteine, glutamic acid and glycine. Its major role is to maintain the intracellular redox homeostasis by scavenging free radicals (ROS) in the cytosol and reducing the disulfide bonds produced during oxidative stress (Grant, 2001).

The stabilization processes of yeasts in dried form are mainly applied to the yeast *Saccharomyces cerevisiae*, which is undoubtedly the most studied eukaryotic microorganism. Numerous studies have elucidated the mechanisms of passive, genetic or adaptive response of *S. cerevisiae* during nutritional, osmotic, dehydration and oxidation stresses (Câmara et al., 2016, 2018; Da Silva Pedrini, Dupont, Câmara, Beney, & Gervais, 2014; Eleutherio et al., 2018; Garre, Raginel, Palacios, Julien, & Matallana, 2010; Nguyen et al., 2017). Nevertheless, precise reports about these mechanisms associated to other yeast species are currently scarce. The non-*Saccharomyces* (NS) yeasts *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans* aroused great interest in recent years, especially for winemaking applications or bioprotection (Benito, 2018b, 2018a; Oro, Ciani, & Comitini, 2014; Simonin, Alexandre, Nikolantonaki, Coelho, & Tournet-Maréchal, 2018). The NS yeasts are generally inoculated before the fermentation of must by *S. cerevisiae* yeast, or in a co-inoculation with *S. cerevisiae* (Sadoudi et al., 2012). These new practices in enology aim to improve the wines flavor complexity during winemaking (Sadoudi et al., 2012; Simonin et al., 2018; Suzzi et al., 2012). Moreover, NS yeasts are able to inhibit growing of undesirable yeast species, reduce

levels of ochratoxin-A – toxin possible carcinogenic to humans – and act as a biocontrol agent against spoilage fungi (García, Esteve-Zarzoso, & Arroyo, 2016).

Despite NS yeasts importance in enology, their responses to oxidation during dehydration remain currently poorly characterized. Previous results demonstrated that NS yeasts strains *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* produces large amounts of glutathione (mostly in the reduced form, GSH) when grown in nutrient-rich medium (GSM), which resulted in better resistance of the cells to dehydration (Câmara, Maréchal, Tourdot-Maréchal, & Husson, 2019).

In this study we focused on the dehydration-oxidation resistance of three wine yeast species *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* cultivated and dehydrated in distinct conditions, and compared with *S. cerevisiae*, as reference. We implemented the oxidant probe dihydroethidium and the quantification of total lipid peroxidation in order to evaluate the impact of growing conditions and dehydration kinetics in cell oxidative response. Moreover, the accumulation of reactive oxygen species (ROS) in cells was analyzed by flow cytometry and by two-photon laser scanning microscopy. The findings presented in this work enhance the comprehension of the growing, dehydration and oxidative stress resistance of these three NS wine yeast strains. They may be useful to design a more efficient process for ADY production.

## **2. Materials and Methods**

### **2.1. Yeast species and culture conditions**

The strains used were *S. cerevisiae* CBS8066, *T. delbrueckii* CBS4865, *M. pulcherrima* CBS5833 and *L. thermotolerans* CBS6340. Two growing medium were used: GSM (nutrient-rich medium) and TSM (nutrient-poor medium) (Câmara et

al., 2018). The determination of the population (count of colony forming units, CFU) was realized on YPDA medium (Nguyen et al., 2017). Three colonies previously isolated on YPDA were transferred to a 250 mL conical flask containing 50 mL of GSM (For 1 L: 30 g glucose, 30 g yeast extract, 0.6 g  $\text{KH}_2\text{PO}_4$  and 0.6 g cysteine) or TSM (30 g glucose, 3 g yeast extract, 0.6 g  $\text{KH}_2\text{PO}_4$ ). Cells were grown in a rotary shaker (Lab Therm, Kühner AG, Basel, Switzerland) at 140 rpm, 30°C for 24 h. Cells were harvested at stationary phase by centrifugation (5810 R, Eppendorf, Hamburg, Germany) at 20°C, 2,500×g for 5 min and washed twice in water-NaCl solution (0.9% w/v) and resuspended in the same physiological solution. The final cell density was  $2 \times 10^{10}$  cells  $\text{mL}^{-1}$ . This procedure was repeated for each independent experiment (n = 3).

## **2.2. Dehydration-rehydration conditions**

After cell growing, yeasts were dried with two kinetics of dehydration (named KA and KB) composed of three steps that were characterized by their water activity, temperature and time (Fig. 1). The steps were carried out in different hermetically sealed boxes in which the activity of the water and temperature were in equilibrium. The kinetics allowed to reproduce at laboratorial scale the dehydration processes applied in a previous work (Câmara et al., 2018). Procedure was carried out by disposing separately ten drops of 10  $\mu\text{L}$  on two sterile polypropylene supports, resulting in 100  $\mu\text{L}$  of cell suspension for each experiment. Cells were transferred to the first drying box until the end of the first cycle, then transferred to the second box, and to the third box until the end of the whole kinetics. After dehydration, cells were rehydrated with 5 mL of 0.9% water-NaCl solution at 38°C during 5 min. The final cell density was  $4 \times 10^8$  cells  $\text{mL}^{-1}$ .

137

### 138 **2.3. Determination of cell cultivability**

139 After dehydration-rehydration cycle, cell suspension was serially diluted with the  
140 physiological solution (0.9% water-NaCl). Aliquots (10  $\mu$ L) from  $10^{-3}$  to  $10^{-5}$  dilutions  
141 were spotted in triplicate separately onto YPDA plates. The plates were incubated at  
142 30°C for 36 h and the number of colony forming unit (CFU) was counted. The control  
143 sample consisted of a fresh cells suspension of the same yeast strain kept at 25°C  
144 for 60 min or 90 min and not submitted to dehydration. Results were expressed as  
145 log of number of CFU per L.

146

### 147 **2.4. Accumulation of ROS inside yeast cells**

148 The oxidant probe dihydroethidium (DHE) was used to observe the ROS  
149 accumulation inside yeast cells. For the DHE stock solution, 10 mL ethanol was  
150 added to 25 mg DHE (Invitrogen Molecular Probes, Eugene, USA). The solution was  
151 stored at -20°C until analysis. After dehydration-rehydration, cell suspension was  
152 mixed with 4  $\mu$ L of DHE stock solution. Flow cytometry analysis was carried out using  
153 a BD FACS Aria II flow cytometer analyzer (BD Biosciences, San Jose, USA) using  
154 excitation laser line at 488 nm and emission filter 585/42 nm. The DHE fluorescence  
155 signals were detected with at least 10,000 events in each analysis. Cells treated with  
156 H<sub>2</sub>O<sub>2</sub> (30% w/v) were used as positive control. Results were expressed as  
157 percentage of DHE positive stained cells.

158

### 159 **2.5. Two-photon microscopic observations**

160 Observations of ROS accumulation were carried out with a two-photon Nikon A1-MP  
161 laser-scanning microscope (Nikon, Tokyo, Japan). After rehydration, cell suspension



was mixed with DHE, under the same conditions as for the flow cytometry analysis. After 10 min in the dark, images were acquired with a  $\times 60$  (NA: 1.27) Plan Apochromat water-immersion objective (Nikon, Japan). Excitation was performed using an IR laser (Chameleon, Coherent) at 800 nm. Emission signals were collected on three detection channels: 2 (green): FF03-525/50-25 [500-550 nm]; 3 (orange) FF01-575/25-25 [563-588 nm] and, 4 (red) FF01-629/56-25 [601-657 nm]. The final cell density was  $10^8$  cell mL<sup>-1</sup>.

## **2.6. Quantification of total lipid peroxidation**

Total lipid peroxidation was measured with the malondialdehyde (MDA) colorimetric method (Steels, Learmonth, & Watson, 1994). The MDA (Alfa-Aesar, Karlsruhe, Germany) is one of the products of the oxidative attack (ROS) to polyunsaturated lipids present in cells. Its reaction with the thiobarbituric acid (TBA, Alfa-Aesar, Karlsruhe, Germany) forms a colorimetric compound proportional to the MDA concentration. Rehydrated cells were resuspended in distilled water and cell disruption was carried out using same protocol described above. The supernatant was collected and mixed with 0.1 mL 0.1M EDTA (Sigma-Aldrich, Missouri, USA) and 0.6 mL 1% (w/v) TBA in 0.05 M NaOH. After 15 min in water bath at 90 °C followed by 5 min on ice, the absorbance at 532 nm was measured.

## **2.7. Statistical analysis**

The data mean values of three independent replicates ( $n = 3$ ) and significant differences were analyzed by the Tukey HSD post-hoc test or by one-way analysis of variance (ANOVA) using the STATISTICA<sup>®</sup> software (Statsoft, Tulsa, USA).

### 3. Results

#### 3.1. Impact of growth and dehydration conditions on yeast cell cultivability is strain-dependent

Cell cultivability assays permitted to analyze the impact of growing conditions (GSM or TSM) and dehydration kinetics (KA or KB). No significant differences (between fresh and dried cells) were observed when yeasts were dehydrated with kinetics KA (Figs. 2A-D). Moreover, the values of cultivability obtained with fresh and dried yeasts cells *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* grown in nutrient-rich medium (GSM) were comparable with kinetics KB (Figs. 2B-D). *S. cerevisiae* cells grown in GSM showed a lower resistance when submitted to the same kinetics, with one log reduction (Fig. 2A) compared to fresh cells. *T. delbrueckii* and *M. pulcherrima* growth in nutrient-poor medium (TSM) had little effect on their cultivability, whatever the dehydration kinetics employed. *S. cerevisiae* and *L. thermotolerans* cells grown in nutrient-poor medium (TSM) showed two and three log reduction respectively after dehydration with kinetics KB, (Figs. 2A and D), compared to fresh cells. These results led us to infer that different growing conditions have a singular effect on yeast replication ability, especially for yeasts *S. cerevisiae* and *L. thermotolerans*. Moreover, the kinetics KA minimized the negative impact of dehydration on yeast cultivability, compared to kinetics KB, in the tested conditions.

#### 3.2. Better nutritional environment correlates with reduction of ROS

The intracellular accumulation of ROS was evaluated with DHE as fluorescent indicator via two different techniques: two-photon laser scanning microscopy (qualitative) and flow cytometry (quantitative). DHE is a well-known ROS probe used

211 to examine oxidative stress response in cells. The effects of growing medium and  
212 dehydration kinetics on the ROS accumulation were analyzed for each yeast strain.

213 *S. cerevisiae* yeast cultivated in nutrient-rich medium (GSM) showed slight ROS  
214 accumulation for fresh cells (Figs. 3A and Aa). We observed cells with bigger vacuole  
215 (Fig. 3A) and a significant ROS accumulation when cells were dehydrated with  
216 kinetics KA (Fig. 3Aa). In addition, almost 30% of cells were ROS positive when  
217 submitted to the dehydration kinetics KB ( $p < 0.01$ ). Cells grown in nutrient-poor  
218 medium (TSM) showed the highest ROS accumulation with approximately 80% of  
219 oxidized cells, even without dehydration stress (Figs. 3A and Aa). These data  
220 suggest that this *S. cerevisiae* strain was particularly sensible to growing and  
221 dehydration conditions applied in this study. It was also interesting to note some  
222 'green spots' in the cells grown in the nutrient-poor medium (Fig. 3A, white arrow).

223 The effect of growing and dehydration conditions to *T. delbrueckii* strain oxidative  
224 response was less evident in comparison with *S. cerevisiae* yeast. *T. delbrueckii* cells  
225 cultivated in nutrient-rich medium (GSM) showed almost no fluorescent signals in  
226 both microscopy and flow cytometry analyzes (Figs. 3B and Bb). In contrast,  
227 *T. delbrueckii* cells cultivated in nutrient-poor medium (TSM) showed a gradual  
228 accumulation of ROS (Figs. 3B and Bb). In this growing condition, the percentage of  
229 oxidized cells was significant different for both kinetics KA (46%) and kinetics KB  
230 (58%). These results correlated to microscopy analysis. Like previously observed,  
231 cells showed an increase of the 'green droplets' intensity when cultivated in nutrient-  
232 poor medium (TSM) and dehydrated with kinetics KB (Fig. 3B, white arrows),  
233 suggesting a stress response to nutrients availability and to the applied dehydration  
234 kinetics.

In the case of *M. pulcherrima* strain, when cells were cultivated in nutrient-rich medium (GSM) they exhibited insignificant accumulation of ROS before and after dehydration with kinetics KA, which represented 0.2% and 3% of oxidized cells, respectively (Figs. 3C and Cc). However, a significant percentage of oxidized cells after kinetics KB (61%) was observed. When *M. pulcherrima* cells were cultivated in nutrient-poor medium (TSM), they showed an increase in ROS accumulation for both kinetics KA (25% of oxidized cells) and KB (65% of oxidized cells). We observed separated low intensity red spots in the *M. pulcherrima* cell membrane (Fig. 3C, white arrows). In addition, the yeast *M. pulcherrima* was the one that exhibited the 'green droplets' in greater quantity and intensity than the other yeasts even before dehydration and especially when cells were cultivated in nutrient-poor medium (TSM) (Fig. 3C). These results suggest a correlation between these 'green droplets' and nutritional stress.

Accumulation of ROS in the strain *L. thermotolerans* was most important and significant for cells dehydrated with kinetics KB for both grown cells in nutrient-rich or poor medium (GSM and TSM), with 48% and 78% of oxidized cells, respectively (Figs. 3D and Dd). Moreover, *L. thermotolerans* yeast cells grown in nutrient-poor medium showed an important ROS accumulation before dehydration (29% of oxidized cells) and after kinetics KA (45% of oxidized cells). The presence of the same 'green spots' was observed for *L. thermotolerans* cells grown in nutrient-poor condition (Figs. 3D, white arrows), suggesting a stress response linked to the medium composition. The percentage of oxidized cells remained low (6% of oxidized cells) with *L. thermotolerans* cells grown in GSM and dehydrated with kinetics KA (Fig. 3Dd).

### 3.3. Can the regulation of medium composition protect lipids from oxidation?

Under specific growing-dehydration conditions, yeast cells accumulate ROS as result of failure of the enzymatic system or by contact of dehydrated cells with air (Jamieson, 1998). In this case, lipids are particular sensible to ROS accumulation in cells, because of the double bonds in the unsaturated fatty acid chains of phospholipids (Catalá, 2009). No significant difference for *S. cerevisiae* yeast grown in nutrient-rich medium (GSM) and dehydrated with kinetics KA was observed, but a strong increase in MDA content, approximately four times, was observed when applying kinetics KB (Fig. 4A). Despite the same behavior in nutrient-poor medium (TSM) and after dehydration with kinetics KA, MDA levels were three times higher than in nutrient-rich medium (GSM), as well as after kinetics KB. ROS attack to lipids of *T. delbrueckii* strain grown in nutrient-rich medium (GSM) seemed less strong. Indeed, we observed no significant differences between fresh cells and cells dehydrated with kinetics KA or KB (Fig. 4B). Even with higher MDA accumulation for fresh *T. delbrueckii* cells in nutrient-poor medium (TSM) and after kinetics KA, levels were reduced if compared with cells dehydrated with kinetics KB, which were almost eight times higher than fresh cells levels (Fig. 4B). *M. pulcherrima* and *L. thermotolerans* cells showed similar behavior after growth in nutrient-rich medium (GSM), with small amounts of MDA for fresh cells and after kinetics KA, and a strong increase (45 and 39 nmol respectively) after kinetics KB. For *M. pulcherrima* and *L. thermotolerans*, cells grown in nutrient-poor medium (TSM) showed a significant increase in MDA content for both kinetics KA or KB, from three to seven times higher (Fig. 4C) and from three to four times higher (Fig. 4D), respectively. These results led us to infer the existence of a strong correlation between cell oxidative stress (MDA and ROS) and growing-dehydration conditions performed in this study. All yeasts

were less resistant to dehydration with kinetics KB, correlated with the stronger MDA accumulation.

## 4. Discussion

### 4.1. Dehydration-oxidation affects non-*Saccharomyces* yeast cultivability

Oxidation is one of the most important stresses to which yeast cells undergo during the production and dehydration processes. This stress plays an important part on reduction of cell performance after dehydration. Indeed, yeast cultivability can be directly affected by intracellular ROS accumulation. This comportment has been described as a major inductor or starting factor of numerous cell dysfunctions in a ROS concentration-dependent manner (Ayer, Gourlay, & Dawes, 2013; Farrugia & Balzan, 2012). We observed that this behavior is not only dependent on the growing or dehydration conditions, but it was also correlated to intrinsic metabolic and physiological characteristics of each strain belonging to different genus and species. One of the findings in common with all strains showed that cells growing in a nutrient-rich medium (GSM) had the ability to replicate actively (Fig. 2), even after dehydration (kinetics KA). In fact, yeasts can adapt their energy metabolism to the nutrients available in the environment and growing conditions (Gómez-Pastor, Pérez-Torrado, Garre, & Matallana, 2011; Wu, Liu, & Huang, 2013). The presence of essential amino acids, carbon and nitrogen in sufficient amounts is primordial to extend yeast life span (Santos, Leitão-Correia, Sousa, & Leão, 2016). Another factor is the presence of O<sub>2</sub> during the biomass formation that triggers the fermentative metabolism to change to respiration and enhances biomass propagation (Gómez-Pastor et al., 2011). This could partially explain the ROS accumulation (Fig. 3) observed for *T. delbrueckii* and *M. pulcherrima* cells grown in nutrient-poor medium

(TSM). *M. pulcherrima* yeast have a Crabtree-negative behavior (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Schnierda, Bauer, Divol, Rensburg, & Görgens, 2014), unlike *S. cerevisiae*, *T. delbrueckii*, and *L. thermotolerans* yeasts. This particularity is an evolutionary benefit for Crabtree-positive yeast strains due to the higher rate of ATP production during aerobic fermentation (Pfeiffer & Morley, 2014). Furthermore, the ethanol produced by these strains can be latter used as an additional carbon source (diauxic shift), producing ATP via the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondria (Brauer, Saldanha, Dolinski, & Botstein, 2005), thus giving a fitness advantage to *S. cerevisiae*, *T. delbrueckii*, and *L. thermotolerans* yeasts (Piškur, Rozpędowska, Polakova, Merico, & Compagno, 2006). These findings suggest that a growth in nutrient-rich medium (GSM) allows an improvement of yeast robustness during and post-dehydration, especially after kinetics KA. The dehydration kinetics KA and KB, with different temperature and water activity gradients (Fig. 1), were chosen based on conditions usually employed in the ADY industry. Indeed, an increase of the dehydration gradient (kinetics KB) had a higher impact on yeasts, reducing cell cultivability (Figs. 2 and 3), probably as a consequence of oxidation (Espindola, Gomes, Panek, & Eleutherio, 2003; França, Panek, & Eleutherio, 2005). In addition, the yeasts *T. delbrueckii* and *M. pulcherrima* did not show the same behavior, as we did not observe significant differences in CFU analysis on all tested conditions. These results imply that these two yeasts strains were more resistant to dehydration than *S. cerevisiae* and *L. thermotolerans*.

#### **4.2. GSSG:GSH ratio as oxidative homeostasis indicator for wine yeasts**

Glutathione is one of the most important redox-scavenging molecules in cells. Glutathione exists in two different forms, reduced (GSH) and oxidized (GSSG,

glutathione disulfide) and the GSSG:GSH ratio indicates the physiological and oxidative state of the cell, where healthy cells have lower GSSG:GSH ratio. It was already demonstrated that GSSG:GSH ratio is an indicator of yeast *S. cerevisiae* oxidative and healthy state (Espindola et al., 2003; França et al., 2005; Rahman, Kode, & Biswas, 2007). The synthesis of large amounts of glutathione correlates with the yeast oxidative stress resistance (Câmara et al., 2019, 2018; Espindola et al., 2003; Grant, 2001; Hatem et al., 2014). In fact, glutathione participates in several functions in cells, specially scavenging free radicals (ROS) present in the cytosol. In addition, better performance and viability are frequently associated to high glutathione content in cells (Câmara et al., 2018; Espindola et al., 2003; Hatem et al., 2014).

Previous data (Câmara et al., 2019), performed with the same NS strains under cultivation and dehydration conditions identically to the present study, showed a strong GSSG:GSH ratio (up to five times higher) for yeast *L. thermotolerans* when cells were grown in nutrient-poor medium (TSM) and dehydrated with kinetics KB, compared to fresh cells. Similarly for yeast *T. delbrueckii* (five times higher) and *M. pulcherrima* (two times higher). This behavior was also found when the *T. delbrueckii* and *L. thermotolerans* cells, grown in nutrient-poor medium (TSM), were dehydrated with kinetics KA, with GSSH:GSH ratio three times higher (Câmara et al., 2019). For all cells grown in nutrient-rich medium (GSM), no remarkable differences in the increase of GSSG:GSH ratio was observed. These findings suggest an adaptive nutritional response in function of the growing medium composition and strains.

The presence of cysteine in the nutrient-rich medium (GSM) allows cells to accumulate large amounts of intracellular glutathione (Câmara et al., 2019). In fact,



cysteine is the most important amino acid for the synthesis of glutathione (Alfafara et al., 1992). Nevertheless, under physiological conditions, the free cysteine is practically inert and has low redox activity due to its relatively high  $pK_a \sim 8.5$  (Netto et al., 2007). Cells cultivated in nutrient-poor conditions (TSM), with a high GSSG:GSH ratio (Câmara et al., 2019), were much more sensible to oxidation (Fig. 3), principally when submitted to a stronger dehydration step (kinetics KB). These findings suggest that growing in nutrient-poor conditions (TSM) may have affected yeast detoxification mechanisms. Moreover, the enzymatic glutathione recycle mechanism, one of the most important ROS elimination pathways in cells, mediated by both glutathione reductase and glutathione oxidase, may have been affected during cultivation in limited nutritional conditions (TSM) (Gamero-Sandemetrio, Paya-Tormo, Gómez-Pastor, Aranda, & Matallana, 2018; Schmidt & Henschke, 2015). Indeed, glutathione can be mobilized as a source of nitrogen and sulfur under limited nutritional conditions, thus satisfying yeast growth requirements (Mehdi & Penninckx, 1997). This can partially explain why cells cultivated in nutrient-poor medium were more cultivable after dehydration with kinetics KA (Fig. 2). We observed that non-*Saccharomyces* yeasts (*T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans*) accumulated much more GSSG after kinetics KB especially after growth in nutrient-poor medium (Câmara et al., 2019), showing the impact of this drying condition on cell oxidative stress response. Indeed, temperature-time dehydration conditions have a crucial role during ADY production, as it can affect cell survival and stabilization post-dehydration (Gamero-Sandemetrio, Gómez-Pastor, & Matallana, 2014). Yeast life occurs only in the presence of water and its removal involves severe consequences for the microbial survival. In particular, the level of water activity in the intracellular medium plays an important role in metabolic reactions and in the

maintenance of cell structure (Ball, 2008). Thus, during dehydration processes, the maintenance of cell cultivability is directly correlated with the level of activity of the water reached after dehydration and the intracellular redox environment (Picazo et al., 2014). The drying kinetics and dehydration levels determine the water transfer kinetics and the final water activity level of the cells. Thus, when the dehydration gradient is high, the transfer of water from the cell to the medium is greater and faster. Thus, drying amplitude and kinetics have an impact on cell stability (Gervais & Beney, 2001; Rodríguez-Porrata et al., 2008), i.e. on the functionality of proteins, enzymes and cell structures.

#### **4.3. Yeast oxidative stress response is affected by ROS levels during dehydration**

It is well known that one of the reasons for cell death during dehydration processes is in part due to oxidative stress and accumulation of ROS inside cells (Espindola et al., 2003; França et al., 2005). During the biomass propagation, the oxidative stress resulting from aeration also leads to the accumulation of ROS (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007; Gómez-Pastor et al., 2010). In order to evaluate levels of intracellular ROS in yeasts, we used the oxidant indicator DHE (dihydroethidium). The DHE enters all cells and reacts with ion  $O_2^-$  to form a red fluorescent compound, 2-hydroxyethidium (Zielonka, Vasquez-Vivar, & Kalyanaraman, 2007). We analyzed the resulting metabolite 2-hydroxyethidium fluorescent signals using two methods: (i) qualitative with two-photon laser microscopy and (ii) quantitative by flow cytometry. A reduced number of oxidized cells when yeasts were cultivated in nutrient-rich medium (GSM) was observed (Fig. 3). These results correlated with lower GSSG:GSH ratio previously obtained

(Câmara et al., 2019), while cells growing in nutrient-poor medium (TSM) were more sensible to oxidation. In addition, dehydration via kinetics KB greatly affected the yeasts oxidation levels. In this dehydration condition, the intensity of the fluorescent signals detected by microscopy and the percentage of oxidized cells detected via flow cytometry were clearly higher, in comparison with results obtained using the kinetics KA (Fig. 3). Interestingly, cells growing in nutrient-poor medium showed irregular distribution of 'green droplets' during microscopic analysis. Probably, cells cultivated in this growing condition have produced more unsaturated lipids as consequence of nutritional limited environment (Rodríguez-Porrata et al., 2011). Oleaginous yeasts, such as yeast *M. pulcherrima*, grown under limited nitrogen conditions and in the presence of large amounts of carbon accumulate lipids in response to nutritional stress (Beopoulos et al., 2009). Another hypothesis supports that these cells were metabolically weaker, with lipids more susceptible to ROS attack leading to an enhancement of oxidation levels and intensity of detected fluorescent signals. It was previously shown that lipid droplets may arise during stressful cycles of dehydration-rehydration (Wang, 2016). The formation of these structures seems to be related to the aggregation and/or inactivation of proteins (Klug & Daum, 2014). These data support the hypothesis that the formation of these lipid droplets are also related to the restructuration of the metabolic activity and to the reparation of the cell membrane structures (Rapoport, 2017). This could explain the better resistance to dehydration-rehydration for yeasts with higher amount of these droplets, as observed for *M. pulcherrima* cells.

#### **4.4. Impact of dehydration on lipid peroxidation levels**

The lipids are one of the most important targets of oxidation damages induced by dehydration processes (Wiseman, 1993). In order to evaluate the impact of dehydration kinetics, levels of lipid peroxidation were measured. We observed a correlation between a better nutritional growing environment (GSM) and the reduction of lipid peroxidation levels, mainly after kinetics KA (Fig. 4). These results also correlate with reduced ROS levels (Fig. 3) and a reduction of the GSSG:GSH ratio previously observed (Câmara et al., 2019). However, an enhancement of lipid peroxidation for cells dehydrated with kinetics KB was obtained. Indeed, lipids can be directly affected with dehydration time-temperature parameters. Modifications in the lipid composition of the plasma membrane, as quantity of sterols or changes in fatty acid unsaturation levels, has already been correlated with yeast sensitivity to environmental modifications (Dupont, Beney, Ferreira, & Gervais, 2011). This could partially explain the observed improvement of oxidative attack to lipids. We also observed a nutritional effect to reduction of lipid peroxidation, especially for cells cultivated in nutrient-rich medium (GSM). Higher levels of reduced glutathione in the cytosol protect sulfhydryl proteins against oxidation and reduce the lipid peroxidation levels in yeast cells (Fernandes et al., 2007). This observation is also supported by the fact that yeasts growing in nutrient-rich medium (GSM) exhibit lower ROS levels (Fig. 3) and a better replication activity (Fig. 2) after dehydration.

## 5. Conclusions

To the best of our knowledge, this is the first time that multiple quantitative and qualitative oxidative stress indicators have been analyzed simultaneously for the non-*Saccharomyces* yeasts *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* during dehydration. We observed that cultivating yeasts in better nutritional environment

(GSM) led to a better cell cultivability after rehydration with lower ROS and lipid peroxidation levels. We also observed that dehydration kinetics KA led to a better post-dehydration cell survival. This fact is attributed mainly to the reduction of cell oxidative stress in this dehydration condition. These findings have proved that it is primordial to reduce the oxidative stress imposed to the cells during the formulation of the biomass and throughout the dehydration process in order to obtain new high-quality dehydrated non-*Saccharomyces* yeasts. This can be achieved by increasing the intracellular glutathione levels or reducing GSSG:GSH ratio in yeasts. Future works should focus on the modulation of the composition of the growing medium in order to promote the production of new additive-free ADY, such as sorbitan monostearate and ascorbic acid.

## **Acknowledgment**

This work was supported by the Regional Council of Bourgogne – Franche-Comté, the “Fonds Européen de Développement Régional (FEDER)” and “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”– Brazil [grant number 200101/2014-5 to A. A. C. Jr.]. The authors are deeply grateful to the Plateau Technique DlmaCell (Université Bourgogne Franche-Comté, Dijon, France) and to Camille Malnoury for technical assistance with the two-photon fluorescence imaging and to Raphaël Caire for the support with the enzymatic analysis.

## **Conflict of interest**

The authors declare no financial or commercial conflict of interest.

## **Ethical approval**

484 This article does not contain any studies with human participants or animals  
485 performed by any of the authors.

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## Figure Legends

**Fig. 1. Dehydration kinetics profiles applied to yeasts.** Each kinetics, KA or KB, was carried out in three steps (time-temperature) using KNO<sub>3</sub> (step 1), NaBr (step 2) and CH<sub>3</sub>COOK (step 3) saturated solutions to control the water activity ( $a_w$ ).

**Fig. 2 Evolution of yeasts *S. cerevisiae*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* cell cultivability during dehydration.** Yeasts were grown in GSM or TSM and cell cultivability (CFU) was estimated before dehydration (fresh) and after kinetics KA or KB. (A) CFU analysis of *S. cerevisiae* yeast. (B) CFU analysis of *T. delbrueckii* yeast. (C) CFU analysis of *M. pulcherrima* yeast. (D) CFU analysis of *L. thermotolerans* yeast. Error bars correspond to the standard deviation of three independent experiments. \* Represents significant difference from the control (fresh) with a  $p < 0.05$ .

**Fig. 3 Analysis of the oxidative stress response of yeasts *S. cerevisiae*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* cultivated in GSM or TSM and dehydrated with kinetics KA or KB.** Cells were growth in GSM or TSM and intracellular ROS were detected with dihydroethidium (DHE) using two-photon fluorescence microscopy and flow cytometry. Flow cytometry analysis and microscopic representative images of cells were acquired before dehydration in fresh cells (white bars) and after kinetics KA (gray bars) or KB (black bars). Final cell density was  $10^8$  cells mL<sup>-1</sup>. Letters correspond to: (A-Aa) *S. cerevisiae* cells; (B-Bb) *T. delbrueckii* cells; (C-Cc) *M. pulcherrima* cells; and, (D-Dd) *L. thermotolerans* cells. Error bars correspond to the standard deviation of three independent experiments.

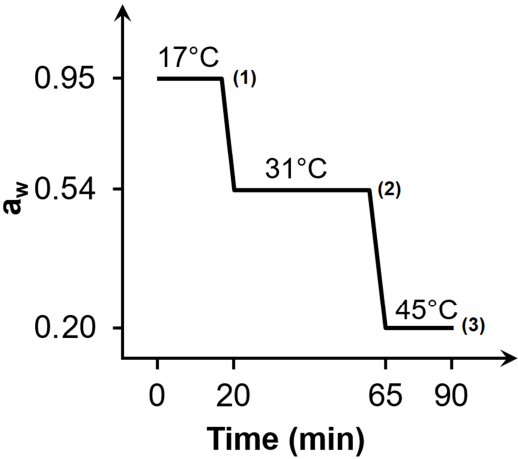
728 Tukey's HSD was used to test significant difference with the corresponding control  
729 (Fresh) at  $*p < 0.05$  or  $**p < 0.01$ . Scale bar = 10  $\mu\text{m}$ .

730

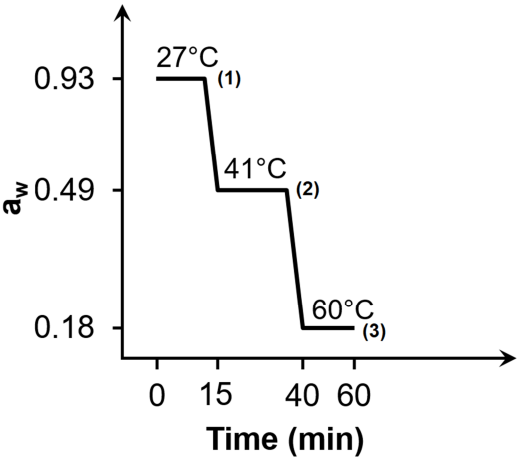
731 **Fig. 4 Lipid peroxidation measurements of yeasts *S. cerevisiae*, *T. delbrueckii*,**  
732 ***M. pulcherrima* and *L. thermotolerans*.** Cells were growth in GSM or TSM and lipid  
733 peroxidation was determined in different stages: before (fresh cells) and after  
734 dehydration with kinetics KA or KB. (A) MDA content in *S. cerevisiae* yeast. (B) MDA  
735 content in *T. delbrueckii* yeast. (C) MDA content in *M. pulcherrima* yeast. (D) MDA  
736 content in *L. thermotolerans* yeast. Error bars correspond to the standard deviation of  
737 three independent experiments. \* Represents significant difference from the control  
738 (fresh) with a  $p < 0.05$ .

Fig. 1

KA



KB



**Fig. 2**

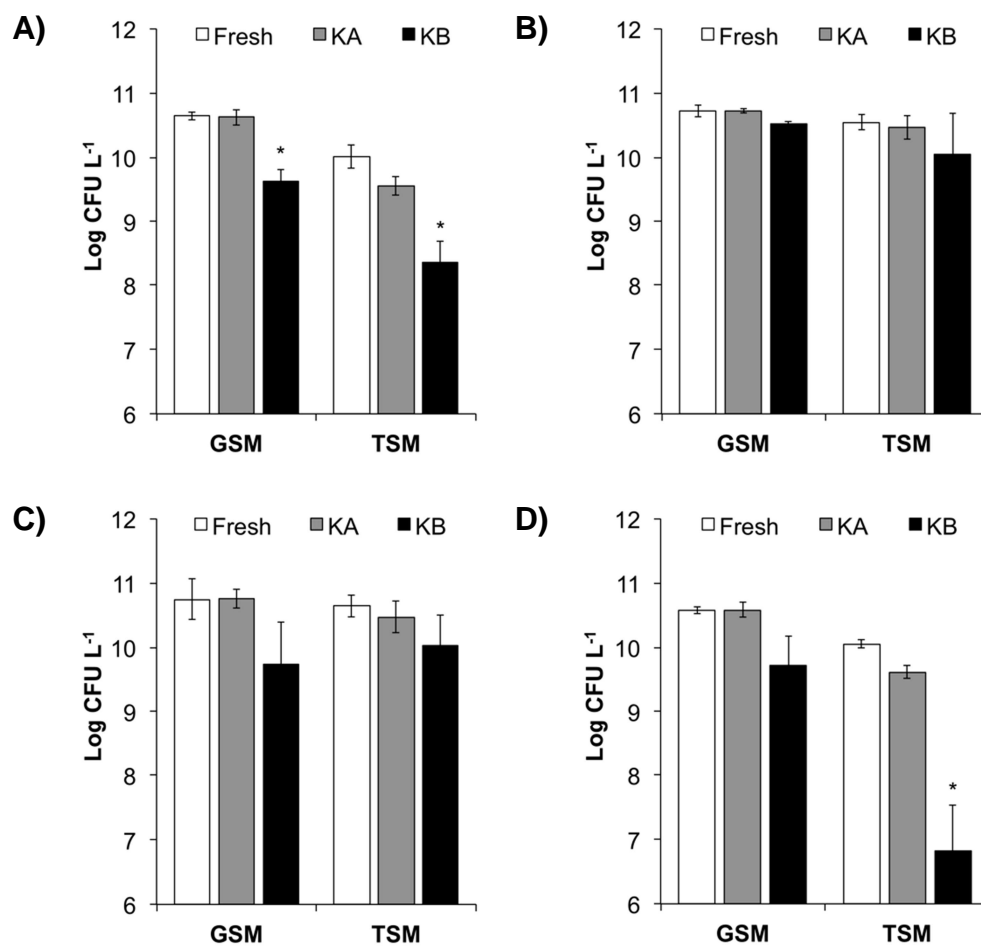


Fig. 3

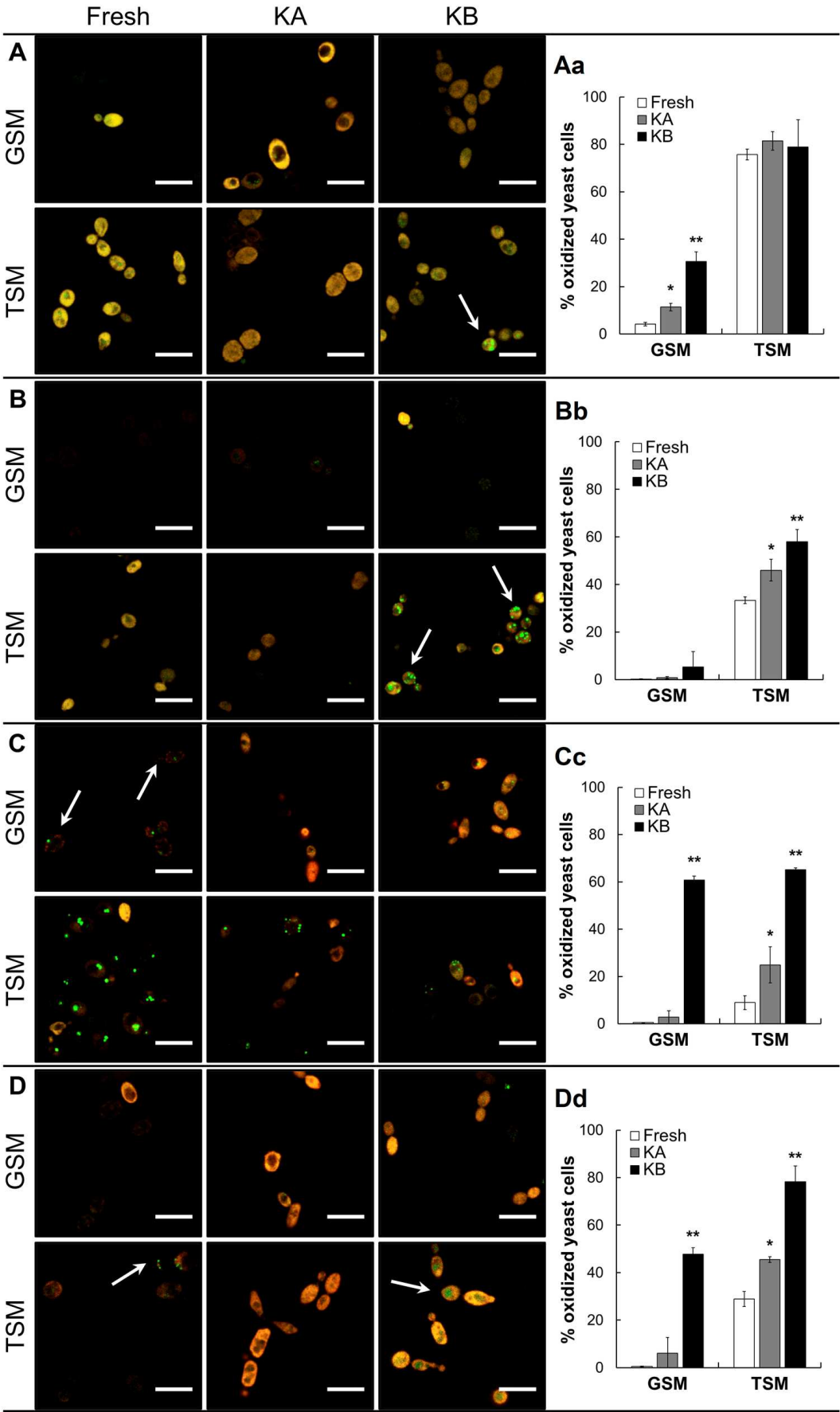




Fig. 4

