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1 Oxidative stress resistance during dehydration of three non-Saccharomyces

- 2 wine yeast strains
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- 13
- 14 Keywords: dehydration; ROS accumulation; lipid oxidation; Torulaspora delbrueckii;
- 15 Metschnikowia pulcherrima; Lachancea thermotolerans

16 Abstract

The development of new fermented foods and beverages requires more and more 17 the use of new dehydrated yeasts species. In this context, the non-Saccharomyces 18 (NS) yeasts Torulaspora delbrueckii, Metschnikowia pulcherrima and Lachancea 19 thermotolerans are developed especially in winemaking as co-culture in the 20 fermentation of the must or for the must bioprotection. However, during formulation-21 dehydration the yeast cells are exposed to several stresses that reduce cellular 22 activity. Among these, the oxidative stress induced by the stabilization processes has 23 been described as one of the main causes of cell death. In this study, we analyzed 24 25 the effects of growth medium associated with two dehydration kinetics on the accumulation of reactive oxygen species (ROS) and lipid peroxidation levels. The 26 cultivability of tested yeast strains was dependent on growth and dehydration 27 conditions. The *L. thermotolerans* strain was the most sensible to dehydration when 28 growing in nutrient-poor media, which was characterized by high levels of ROS, lipid 29 peroxidation and reduced cultivability. In contrast, this yeast was able to restore its 30 cultivability when growing in nutrient-rich medium before dehydration. The other NS 31 yeast strains acquired resistance to oxidative stress similarly but in a growth-32 dehydration condition less dependent manner. These results showed that modulation 33 of growing medium composition is a simple way to improve resistance to oxidative 34 attack imposed by dehydration in NS yeasts. This was the first time that multiple 35 quantitative and qualitative indicators of oxidative stress response in these three NS 36 yeast strains were explored. 37

38 **1. Introduction**

Yeasts are microorganisms capable of surviving large periods with limited water 39 content, i.e. in the dehydrated state; once in normal hydric conditions they reactivate 40 their metabolism and restart their normal activity. This phenomenon is known as 41 anhydrobiosis (Rapoport, Turchetti, & Buzzini, 2016). Taking advantage of these 42 characteristics, beverage industries have been interested in new species of yeasts 43 stabilized in dry form (active dry yeasts: ADY), especially for winemaking. In general, 44 for wine production the fermentation occurs spontaneously due to the indigenous 45 flora present on grapes or in musts, which consists in different yeasts and bacteria 46 species (Liu, Lu, Duan, & Yan, 2016). Although this practice favors the development 47 of the wine sensorial profiles, it remains difficult to control the microbial population 48 development during winemaking, which represents a loss of quality and productivity. 49 Thus, the controlled application of ADY provides high quality products with 50 guaranteed reproducibility (Rodríguez-Porrata et al., 2011). 51

During ADY formulation, yeast cells undergo multiple stresses which reduce their 52 performance, e.g. cell starvation in the course of biomass propagation then heat and 53 oxidation induced by the drying stages (Beker & Rapoport, 1987; Rapoport, 2017; 54 Schmidt & Henschke, 2015). In fact, cells subjected to hydric variations accumulate 55 intracellular reactive oxygen species (ROS) as result of failure of their detoxification 56 systems and the functionality of the proteins and enzymes responsible for the ROS 57 elimination (Dupont, Rapoport, Gervais, & Beney, 2014). In addition, the modulation 58 of energy metabolism, from fermentation to respiration, has a great influence on lipid 59 stability to oxidation, especially during the biomass propagation (Gómez-Pastor, 60 Pérez-Torrado, & Matallana, 2010). 61

In this context, depending on the composition of growing medium, yeasts activate 62 biosynthesis of different compounds, like glutathione which plays part in their 63 resistance to dehydration-oxidation (Câmara et al., 2018; François & Parrou, 2001; 64 Lorenz, Schmacht, Stahl, & Senz, 2015). Glutathione (L-gamma-Glutamyl-L-65 Cysteinylglycine) is a tripeptide thiol antioxidant formed by the amino acids cysteine, 66 glutamic acid and glycine. Its major role is to maintain the intracellular redox 67 homeostasis by scavenging free radicals (ROS) in the cytosol and reducing the 68 disulfide bonds produced during oxidative stress (Grant, 2001). 69

The stabilization processes of yeasts in dried form are mainly applied to the yeast 70 Saccharomyces cerevisiae, which is undoubtedly the most studied eukaryotic 71 microorganism. Numerous studies have elucidated the mechanisms of passive, 72 genetic or adaptive response of *S. cerevisiae* during nutritional, osmotic, dehydration 73 74 and oxidation stresses (Câmara et al., 2016, 2018; Da Silva Pedrini, Dupont, Câmara, Beney, & Gervais, 2014; Eleutherio et al., 2018; Garre, Raginel, Palacios, 75 Julien, & Matallana, 2010; Nguyen et al., 2017). Nevertheless, precise reports about 76 these mechanisms associated to other yeast species are currently scarce. The non-77 Saccharomyces (NS) yeasts Torulaspora delbrueckii, Metschnikowia pulcherrima 78 and Lachancea thermotolerans aroused great interest in recent years, especially for 79 winemaking applications or bioprotection (Benito, 2018b, 2018a; Oro, Ciani, & 80 Comitini, 2014; Simonin, Alexandre, Nikolantonaki, Coelho, & Tourdot-Maréchal, 81 2018). The NS yeasts are generally inoculated before the fermentation of must by 82 S. cerevisiae yeast, or in a co-inoculation with S. cerevisiae (Sadoudi et al., 2012). 83 These new practices in enology aim to improve the wines flavor complexity during 84 winemaking (Sadoudi et al., 2012; Simonin et al., 2018; Suzzi et al., 2012). 85 Moreover, NS yeasts are able to inhibit growing of undesirable yeast species, reduce 86

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 95 96 species T. delbrueckii, *M. pulcherrima* and *L. thermotolerans* cultivated and dehydrated in distinct conditions, and compared with S. cerevisiae, as reference. We 97 implemented the oxidant probe dihydroethidium and the quantification of total lipid 98 peroxidation in order to evaluate the impact of growing conditions and dehydration 99 kinetics in cell oxidative response. Moreover, the accumulation of reactive oxygen 100 101 species (ROS) in cells was analyzed by flow cytometry and by two-photon laser scanning microscopy. The findings presented in this work enhance the 102 comprehension of the growing, dehydration and oxidative stress resistance of these 103 104 three NS wine yeast strains. They may be useful to design a more efficient process for ADY production. 105

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107 **2. Materials and Methods**

108 **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) 112 was realized on YPDA medium (Nguyen et al., 2017). Three colonies previously 113 isolated on YPDA were transferred to a 250 mL conical flask containing 50 mL of 114 GSM (For 1 L: 30 g glucose, 30 g yeast extract, 0.6 g KH₂PO₄ and 0.6 g cysteine) or 115 TSM (30 g glucose, 3 g yeast extract, 0.6 g KH₂PO₄). Cells were grown in a rotary 116 shaker (Lab Therm, Kühner AG, Basel, Switzerland) at 140 rpm, 30°C for 24 h. Cells 117 were harvested at stationary phase by centrifugation (5810 R, Eppendorf, Hamburg, 118 Germany) at 20°C, 2,500×g for 5 min and washed twice in water-NaCl solution (0.9% 119 w/v) and resuspended in the same physiological solution. The final cell density was 120 2×10¹⁰ cells mL⁻¹. This procedure was repeated for each independent experiment 121 (n = 3).122

123

124 **2.2. Dehydration-rehydration conditions**

After cell growing, yeasts were dried with two kinetics of dehydration (named KA and 125 KB) composed of three steps that were characterized by their water activity, 126 temperature and time (Fig. 1). The steps were carried out in different hermetically 127 sealed boxes in which the activity of the water and temperature were in equilibrium. 128 The kinetics allowed to reproduce at laboratorial scale the dehydration processes 129 applied in a previous work (Câmara et al., 2018). Procedure was carried out by 130 disposing separately ten drops of 10 µL on two sterile polypropylene supports, 131 resulting in 100 µL of cell suspension for each experiment. Cells were transferred to 132 the first drying box until the end of the first cycle, then transferred to the second box, 133 and to the third box until the end of the whole kinetics. After dehydration, cells were 134 rehydrated with 5 mL of 0.9% water-NaCl solution at 38°C during 5 min. The final cell 135 density was 4×10^8 cells mL⁻¹. 136

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138 **2.3. Determination of cell cultivability**

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

146

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

The oxidant probe dihydroethidium (DHE) was used to observe the ROS 148 149 accumulation inside yeast cells. For the DHE stock solution, 10 mL ethanol was added to 25 mg DHE (Invitrogen Molecular Probes, Eugene, USA). The solution was 150 stored at -20°C until analysis. After dehydration-rehydration, cell suspension was 151 mixed with 4 µL of DHE stock solution. Flow cytometry analysis was carried out using 152 a BD FACS Aria II flow cytometer analyzer (BD Biosciences, San Jose, USA) using 153 excitation laser line at 488 nm and emission filter 585/42 nm. The DHE fluorescence 154 signals were detected with at least 10,000 events in each analysis. Cells treated with 155 H₂O₂ (30% w/v) were used as positive control. Results were expressed as 156 percentage of DHE positive stained cells. 157

158

159 2.5. Two-photon microscopic observations

Observations of ROS accumulation were carried out with a two-photon Nikon A1-MP
 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 x60 (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 10⁸ cell mL⁻¹.

169

170 **2.6. Quantification of total lipid peroxidation**

Total lipid peroxidation was measured with the malondialdehyde (MDA) colorimetric 171 method (Steels, Learmonth, & Watson, 1994). The MDA (Alfa-Aesar, Karlsruhe, 172 Germany) is one of the products of the oxidative attack (ROS) to polyunsaturated 173 174 lipids present in cells. Its reaction with the thiobarbituric acid (TBA, Alfa-Aesar, Karlsruhe, Germany) forms a colorimetric compound proportional to the MDA 175 concentration. Rehydrated cells were resuspended in distilled water and cell 176 disruption was carried out using same protocol described above. The supernatant 177 was collected and mixed with 0.1 mL 0.1M EDTA (Sigma-Aldrich, Missouri, USA) and 178 0.6 mL 1% (w/v) TBA in 0.05 M NaOH. After 15 min in water bath at 90 °C followed 179 by 5 min on ice, the absorbance at 532 nm was measured. 180

181

182 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[®] software (Statsoft, Tulsa, USA).

186

187 **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 190 or TSM) and dehydration kinetics (KA or KB). No significant differences (between 191 fresh and dried cells) were observed when yeasts were dehydrated with kinetics KA 192 (Figs. 2A-D). Moreover, the values of cultivability obtained with fresh and dried 193 yeasts cells T. delbrueckii, M. pulcherrima and L. thermotolerans grown in nutrient-194 rich medium (GSM) were comparable with kinetics KB (Figs. 2B-D). S. cerevisiae 195 cells grown in GSM showed a lower resistance when submitted to the same kinetics, 196 with one log reduction (Fig. 2A) compared to fresh cells. T. delbrueckii and 197 M. pulcherrima growth in nutrient-poor medium (TSM) had little effect on their 198 199 cultivability, whatever the dehydration kinetics employed. S. cerevisiae and L. thermotolerans cells grown in nutrient-poor medium (TSM) showed two and three 200 log reduction respectively after dehydration with kinetics KB, (Figs. 2A and D), 201 compared to fresh cells. These results led us to infer that different growing conditions 202 have a singular effect on yeast replication ability, especially for yeasts S. cerevisiae 203 and L. thermotolerans. Moreover, the kinetics KA minimized the negative impact of 204 dehydration on yeast cultivability, compared to kinetics KB, in the tested conditions. 205

206

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

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

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

In the case of *M. pulcherrima* strain, when cells were cultivated in nutrient-rich 235 medium (GSM) they exhibited insignificant accumulation of ROS before and after 236 dehydration with kinetics KA, which represented 0.2% and 3% of oxidized cells, 237 respectively (Figs. 3C and Cc). However, a significant percentage of oxidized cells 238 after kinetics KB (61%) was observed. When *M. pulcherrima* cells were cultivated in 239 nutrient-poor medium (TSM), they showed an increase in ROS accumulation for both 240 kinetics KA (25% of oxidized cells) and KB (65% of oxidized cells). We observed 241 separated low intensity red spots in the *M. pulcherrima* cell membrane (Fig. 3C, white 242 arrows). In addition, the yeast *M. pulcherrima* was the one that exhibited the 'green 243 droplets' in greater quantity and intensity than the other yeasts even before 244 dehydration and especially when cells were cultivated in nutrient-poor medium (TSM) 245 (Fig. 3C). These results suggest a correlation between these 'green droplets' and 246 247 nutritional stress.

Accumulation of ROS in the strain L. thermotolerans was most important and 248 significant for cells dehydrated with kinetics KB for both grown cells in nutrient-rich or 249 poor medium (GSM and TSM), with 48% and 78% of oxidized cells, respectively 250 (Figs. 3D and Dd). Moreover, L. thermotolerans yeast cells grown in nutrient-poor 251 medium showed an important ROS accumulation before dehydration (29% of 252 oxidized cells) and after kinetics KA (45% of oxidized cells). The presence of the 253 same 'green spots' was observed for *L. thermotolerans* cells grown in nutrient-poor 254 condition (Figs. 3D, white arrows), suggesting a stress response linked to the 255 medium composition. The percentage of oxidized cells remained low (6% of oxidized 256 cells) with L. thermotolerans cells grown in GSM and dehydrated with kinetics KA 257 (Fig. 3Dd). 258

259

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

Under specific growing-dehydration conditions, yeast cells accumulate ROS as result 261 of failure of the enzymatic system or by contact of dehydrated cells with air 262 (Jamieson, 1998). In this case, lipids are particular sensible to ROS accumulation in 263 cells, because of the double bonds in the unsaturated fatty acid chains of 264 phospholipids (Catalá, 2009). No significant difference for S. cerevisiae yeast grown 265 in nutrient-rich medium (GSM) and dehydrated with kinetics KA was observed, but a 266 strong increase in MDA content, approximately four times, was observed when 267 applying kinetics KB (Fig. 4A). Despite the same behavior in nutrient-poor medium 268 (TSM) and after dehydration with kinetics KA, MDA levels were three times higher 269 than in nutrient-rich medium (GSM), as well as after kinetics KB. ROS attack to lipids 270 of T. delbrueckii strain grown in nutrient-rich medium (GSM) seemed less strong. 271 272 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 273 274 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 275 eight times higher than fresh cells levels (Fig. 4B). *M. pulcherrima* and 276 L. thermotolerans cells showed similar behavior after growth in nutrient-rich medium 277 (GSM), with small amounts of MDA for fresh cells and after kinetics KA, and a strong 278 increase (45 and 39 nmol respectively) after kinetics KB. For M. pulcherrima and 279 L. thermotolerans, cells grown in nutrient-poor medium (TSM) showed a significant 280 increase in MDA content for both kinetics KA or KB, from three to seven times higher 281 (Fig. 4C) and from three to four times higher (Fig. 4D), respectively. These results led 282 us to infer the existence of a strong correlation between cell oxidative stress (MDA 283 and ROS) and growing-dehydration conditions performed in this study. All yeasts 284

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

287

288 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 290 the production and dehydration processes. This stress plays an important part on 291 reduction of cell performance after dehydration. Indeed, yeast cultivability can be 292 directly affected by intracellular ROS accumulation. This comportment has been 293 294 described as a major inductor or starting factor of numerous cell dysfunctions in a ROS concentration-dependent manner (Ayer, Gourlay, & Dawes, 2013; Farrugia & 295 Balzan, 2012). We observed that this behavior is not only dependent on the growing 296 297 or dehydration conditions, but it was also correlated to intrinsic metabolic and physiological characteristics of each strain belonging to different genus and species. 298 One of the findings in common with all strains showed that cells growing in a nutrient-299 rich medium (GSM) had the ability to replicate actively (Fig. 2), even after 300 dehydration (kinetics KA). In fact, yeasts can adapt their energy metabolism to the 301 nutrients available in the environment and growing conditions (Gómez-Pastor, Pérez-302 Torrado, Garre, & Matallana, 2011; Wu, Liu, & Huang, 2013). The presence of 303 essential amino acids, carbon and nitrogen in sufficient amounts is primordial to 304 extend yeast life span (Santos, Leitão-Correia, Sousa, & Leão, 2016). Another factor 305 is the presence of O₂ during the biomass formation that triggers the fermentative 306 metabolism to change to respiration and enhances biomass propagation (Gómez-307 Pastor et al., 2011). This could partially explain the ROS accumulation (Fig. 3) 308 observed for T. delbrueckii and M. pulcherrima cells grown in nutrient-poor medium 309

(TSM). M. pulcherrima yeast have a Crabtree-negative behavior (Bely, Stoeckle, 310 Masneuf-Pomarède, & Dubourdieu, 2008; Schnierda, Bauer, Divol, Rensburg, & 311 Görgens, 2014), unlike S. cerevisiae, T. delbrueckii, and L. thermotolerans yeasts. 312 This particularity is an evolutionary benefit for Crabtree-positive yeast strains due to 313 the higher rate of ATP production during aerobic fermentation (Pfeiffer & Morley, 314 2014). Furthermore, the ethanol produced by these strains can be latter used as an 315 additional carbon source (diauxic shift), producing ATP via the tricarboxylic acid cycle 316 and oxidative phosphorylation in the mitochondria (Brauer, Saldanha, Dolinski, & 317 Botstein, 2005), thus giving a fitness advantage to S. cerevisiae, T. delbrueckii, and 318 L. thermotolerans yeasts (Piškur, Rozpędowska, Polakova, Merico, & Compagno, 319 2006). These findings suggest that a growth in nutrient-rich medium (GSM) allows an 320 improvement of yeast robustness during and post-dehydration, especially after 321 322 kinetics KA. The dehydration kinetics KA and KB, with different temperature and water activity gradients (Fig. 1), were chosen based on conditions usually employed 323 324 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 325 a consequence of oxidation (Espindola, Gomes, Panek, & Eleutherio, 2003; França, 326 Panek, & Eleutherio, 2005). In addition, the yeasts T. delbrueckii and M. pulcherrima 327 did not show the same behavior, as we did not observe significant differences in CFU 328 analysis on all tested conditions. These results imply that these two yeasts strains 329 were more resistant to dehydration than S. cerevisiae and L. thermotolerans. 330

331

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

333 Glutathione is one of the most important redox-scavenging molecules in cells. 334 Glutathione exists in two different forms, reduced (GSH) and oxidized (GSSG,

glutathione disulfide) and the GSSG:GSH ratio indicates the physiological and 335 oxidative state of the cell, where healthy cells have lower GSSG:GSH ratio. It was 336 already demonstrated that GSSG:GSH ratio is an indicator of yeast S. cerevisiae 337 oxidative and healthy state (Espindola et al., 2003; França et al., 2005; Rahman, 338 Kode, & Biswas, 2007). The synthesis of large amounts of glutathione correlates with 339 the yeast oxidative stress resistance (Câmara et al., 2019, 2018; Espindola et al., 340 2003; Grant, 2001; Hatem et al., 2014). In fact, glutathione participates in several 341 functions in cells, specially scavenging free radicals (ROS) present in the cytosol. In 342 addition, better performance and viability are frequently associated to high 343 344 glutathione content in cells (Câmara et al., 2018; Espindola et al., 2003; Hatem et al., 2014). 345

Previous data (Câmara et al., 2019), performed with the same NS strains under 346 347 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 348 cells were grown in nutrient-poor medium (TSM) and dehydrated with kinetics KB, 349 compared to fresh cells. Similarly for yeast T. delbrueckii (five times higher) and 350 M. pulcherrima (two times higher). This behavior was also found when the 351 T. delbrueckii and L. thermotolerans cells, grown in nutrient-poor medium (TSM), 352 were dehydrated with kinetics KA, with GSSH:GSH ratio three times higher (Câmara 353 et al., 2019). For all cells grown in nutrient-rich medium (GSM), no remarkable 354 differences in the increase of GSSG:GSH ratio was observed. These findings 355 suggest an adaptive nutritional response in function of the growing medium 356 composition and strains. 357

358 The presence of cysteine in the nutrient-rich medium (GSM) allows cells to 359 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 360 al., 1992). Nevertheless, under physiological conditions, the free cysteine is 361 practically inert and has low redox activity due to its relatively high pKa ~8.5 (Netto et 362 al., 2007). Cells cultivated in nutrient-poor conditions (TSM), with a high GSSG:GSH 363 ratio (Câmara et al., 2019), were much more sensible to oxidation (Fig. 3), principally 364 when submitted to a stronger dehydration step (kinetics KB). These findings suggest 365 that growing in nutrient-poor conditions (TSM) may have affected yeast detoxification 366 mechanisms. Moreover, the enzymatic glutathione recycle mechanism, one of the 367 most important ROS elimination pathways in cells, mediated by both glutathione 368 reductase and glutathione oxidase, may have been affected during cultivation in 369 limited nutritional conditions (TSM) (Gamero-Sandemetrio, Paya-Tormo, Gómez-370 Pastor, Aranda, & Matallana, 2018; Schmidt & Henschke, 2015). Indeed, glutathione 371 372 can be mobilized as a source of nitrogen and sulfur under limited nutritional conditions, thus satisfying yeast growth requirements (Mehdi & Penninckx, 1997). 373 374 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-375 Saccharomyces yeasts (T. delbrueckii, M. pulcherrima and L. thermotolerans) 376 accumulated much more GSSG after kinetics KB especially after growth in nutrient-377 poor medium (Câmara et al., 2019), showing the impact of this drying condition on 378 cell oxidative stress response. Indeed, temperature-time dehydration conditions have 379 a crucial role during ADY production, as it can affect cell survival and stabilization 380 post-dehydration (Gamero-Sandemetrio, Gómez-Pastor, & Matallana, 2014). Yeast 381 life occurs only in the presence of water and its removal involves severe 382 consequences for the microbial survival. In particular, the level of water activity in the 383 intracellular medium plays an important role in metabolic reactions and in the 384

maintenance of cell structure (Ball, 2008). Thus, during dehydration processes, the 385 maintenance of cell cultivability is directly correlated with the level of activity of the 386 water reached after dehydration and the intracellular redox environment (Picazo et 387 al., 2014). The drying kinetics and dehydration levels determine the water transfer 388 kinetics and the final water activity level of the cells. Thus, when the dehydration 389 gradient is high, the transfer of water from the cell to the medium is greater and 390 faster. Thus, drying amplitude and kinetics have an impact on cell stability (Gervais & 391 Beney, 2001; Rodríguez-Porrata et al., 2008), i.e. on the functionality of proteins, 392 enzymes and cell structures. 393

394

395 4.3. Yeast oxidative stress response is affected by ROS levels during 396 dehydration

It is well known that one of the reasons for cell death during dehydration processes is 397 in part due to oxidative stress and accumulation of ROS inside cells (Espindola et al., 398 2003; França et al., 2005). During the biomass propagation, the oxidative stress 399 resulting from aeration also leads to the accumulation of ROS (Gibson, Lawrence, 400 Leclaire, Powell, & Smart, 2007; Gómez-Pastor et al., 2010). In order to evaluate 401 levels of intracellular ROS in yeasts, we used the oxidant indicator DHE 402 (dihydroethidium). The DHE enters all cells and reacts with ion O_2^- to form a red 403 2-hydroxyethidium fluorescent compound, (Zielonka, Vasquez-Vivar, & 404 Kalyanaraman, 2007). We analyzed the resulting metabolite 2-hydroxyethidium 405 fluorescent signals using two methods: (i) qualitative with two-photon laser 406 microscopy and (ii) quantitative by flow cytometry. A reduced number of oxidized 407 cells when yeasts were cultivated in nutrient-rich medium (GSM) was observed 408 (Fig. 3). These results correlated with lower GSSG:GSH ratio previously obtained 409

(Câmara et al., 2019), while cells growing in nutrient-poor medium (TSM) were more 410 411 sensible to oxidation. In addition, dehydration via kinetics KB greatly affected the yeasts oxidation levels. In this dehydration condition, the intensity of the fluorescent 412 signals detected by microscopy and the percentage of oxidized cells detected via 413 flow cytometry were clearly higher, in comparison with results obtained using the 414 kinetics KA (Fig. 3). Interestingly, cells growing in nutrient-poor medium showed 415 irregular distribution of 'green droplets' during microscopic analysis. Probably, cells 416 cultivated in this growing condition have produced more unsaturated lipids as 417 consequence of nutritional limited environment (Rodríguez-Porrata et al., 2011). 418 419 Oleaginous yeasts, such as yeast *M. pulcherrima*, grown under limited nitrogen conditions and in the presence of large amounts of carbon accumulate lipids in 420 response to nutritional stress (Beopoulos et al., 2009). Another hypothesis supports 421 422 that these cells were metabolically weaker, with lipids more susceptible to ROS attack leading to an enhancement of oxidation levels and intensity of detected 423 424 fluorescent signals. It was previously shown that lipid droplets may arise during stressful cycles of dehydration-rehydration (Wang, 2016). The formation of these 425 structures seems to be related to the aggregation and/or inactivation of proteins (Klug 426 & Daum, 2014). These data support the hypothesis that the formation of these lipid 427 droplets are also related to the restructuration of the metabolic activity and to the 428 reparation of the cell membrane structures (Rapoport, 2017). This could explain the 429 better resistance to dehydration-rehydration for yeasts with higher amount of these 430 droplets, as observed for *M. pulcherrima* cells. 431

432

433 **4.4. Impact of dehydration on lipid peroxidation levels**

The lipids are one of the most important targets of oxidation damages induced by 434 435 dehydration processes (Wiseman, 1993). In order to evaluate the impact of dehydration kinetics, levels of lipid peroxidation were measured. We observed a 436 correlation between a better nutritional growing environment (GSM) and the 437 reduction of lipid peroxidation levels, mainly after kinetics KA (Fig. 4). These results 438 also correlate with reduced ROS levels (Fig. 3) and a reduction of the GSSG:GSH 439 ratio previously observed (Câmara et al., 2019). However, an enhancement of lipid 440 peroxidation for cells dehydrated with kinetics KB was obtained. Indeed, lipids can be 441 directly affected with dehydration time-temperature parameters. Modifications in the 442 443 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 444 environmental modifications (Dupont, Beney, Ferreira, & Gervais, 2011). This could 445 446 partially explain the observed improvement of oxidative attack to lipids. We also observed a nutritional effect to reduction of lipid peroxidation, especially for cells 447 cultivated in nutrient-rich medium (GSM). Higher levels of reduced glutathione in the 448 cytosol protect sulfhydryl proteins against oxidation and reduce the lipid peroxidation 449 levels in yeast cells (Fernandes et al., 2007). This observation is also supported by 450 the fact that yeasts growing in nutrient-rich medium (GSM) exhibit lower ROS levels 451 (Fig. 3) and a better replication activity (Fig. 2) after dehydration. 452

453

454 **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 459 peroxidation levels. We also observed that dehydration kinetics KA led to a better 460 post-dehydration cell survival. This fact is attributed mainly to the reduction of cell 461 oxidative stress in this dehydration condition. These findings have proved that it is 462 primordial to reduce the oxidative stress imposed to the cells during the formulation 463 of the biomass and throughout the dehydration process in order to obtain new high-464 quality dehydrated non-Saccharomyces yeasts. This can be achieved by increasing 465 the intracellular glutathione levels or reducing GSSG:GSH ratio in yeasts. Future 466 works should focus on the modulation of the composition of the growing medium in 467 order to promote the production of new additive-free ADY, such as sorbitan 468 monostearate and ascorbic acid. 469

470

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479

480 **Conflict of interest**

481 The authors declare no financial or commercial conflict of interest.

482

483 Ethical approval

This article does not contain any studies with human participants or animalsperformed by any of the authors.

486 6. References

Alfafara, C., Kanda, A., Shioi, T., Shimizu, H., Shioya, S., & Suga, K. (1992). Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *36*(4), 538–540. https://doi.org/10.1007/BF00170199
Ayer, A., Gourlay, C. W., & Dawes, I. W. (2013). Cellular redox homeostasis, reactive

- 492 oxygen species and replicative ageing in Saccharomyces cerevisiae. FEMS
 493 Yeast Research, 14(1), 60–72. https://doi.org/10.1111/1567-1364.12114
- Ball, P. (2008). Water as an active constituent in cell biology. *Chemical Reviews*,
 108(1), 74–108. https://doi.org/10.1021/cr068037a
- Beker, M. J., & Rapoport, A. I. (1987). Conservation of yeasts by dehydration. *Biotechnology Methods*, 35, 127–171. https://doi.org/10.1007/BFb0004428
- Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of
 mixed Torulaspora delbrueckii–*Saccharomyces cerevisiae* culture on high-sugar
 fermentation. *International Journal of Food Microbiology*, *122*(3), 312–320.
 https://doi.org/10.1016/j.ijfoodmicro.2007.12.023
- Benito, S. (2018a). The impact of *Torulaspora delbrueckii* yeast in winemaking.
 Applied Microbiology and Biotechnology, *102*(7), 3081–3094.
- 504 https://doi.org/10.1007/s00253-018-8849-0
- Benito, S. (2018b). The impacts of *Lachancea thermotolerans* yeast strains on
 winemaking. *Applied Microbiology and Biotechnology*, *102*(16), 6775–6790.
 https://doi.org/10.1007/s00253-018-9117-z
- Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.-L., Molina-Jouve, C., &
 Nicaud, J.-M. (2009). Yarrowia lipolytica as a model for bio-oil production. *Progress in Lipid Research*, 48(6), 375–387.
- 511 https://doi.org/10.1016/j.plipres.2009.08.005
- 512 Brauer, M. J., Saldanha, A. J., Dolinski, K., & Botstein, D. (2005). Homeostatic 513 adjustment and metabolic remodeling in glucose-limited yeast cultures.
- 514 *Molecular Biology of the Cell*, *16*(5), 2503–2517.
- 515 https://doi.org/10.1091/mbc.e04-11-0968
- Câmara, A. A., Dupont, S., Beney, L., Gervais, P., Rosenthal, A., Correia, R. T. P., &
 Pedrini, M. R. S. (2016). Fisetin yeast-based bio-capsules via osmoporation:
 effects of process variables on the encapsulation efficiency and internalized
 fisetin content. *Applied Microbiology and Biotechnology*, *100*(12), 5547–5558.
 https://doi.org/10.1007/s00253-016-7425-8
- 521 Câmara, A. A., Maréchal, P.-A., Tourdot-Maréchal, R., & Husson, F. (2019).
- 522 Dehydration stress responses of yeasts *Torulaspora delbrueckii*, *Metschnikowia* 523 *pulcherrima* and *Lachancea thermotolerans*: Effects of glutathione and trehalose 524 biosynthesis. *Food Microbiology*, *79*, 137–146.
- 525 https://doi.org/10.1016/j.fm.2018.12.008
- Câmara, A. A., Nguyen, T. D., Jossier, A., Endrizzi, A., Saurel, R., Simonin, H., &
 Husson, F. (2018). Improving total glutathione and trehalose contents in
 Saccharomyces cerevisiae cells to enhance their resistance to fluidized bed
 drying. *Process Biochemistry*, 69, 45–51.
- 530 https://doi.org/10.1016/j.procbio.2018.03.013
- 531 Catalá, A. (2009). Lipid peroxidation of membrane phospholipids generates hydroxy-532 alkenals and oxidized phospholipids active in physiological and/or pathological
- conditions. *Chemistry and Physics of Lipids*, 157(1), 1–11.
- 534 https://doi.org/10.1016/J.CHEMPHYSLIP.2008.09.004

- Da Silva Pedrini, M. R., Dupont, S., Câmara, A. A., Beney, L., & Gervais, P. (2014).
 Osmoporation: A simple way to internalize hydrophilic molecules into yeast.
 Applied Microbiology and Biotechnology, *98*(3). https://doi.org/10.1007/s00253 013-5386-8
- Dupont, S., Beney, L., Ferreira, T., & Gervais, P. (2011). Nature of sterols affects
 plasma membrane behavior and yeast survival during dehydration. *Biochimica et Biophysica Acta (BBA) Biomembranes*, *1808*(6), 1520–1528.
- 542 https://doi.org/10.1016/J.BBAMEM.2010.11.012
- 543 Dupont, S., Rapoport, A., Gervais, P., & Beney, L. (2014). Survival kit of
 544 Saccharomyces cerevisiae for anhydrobiosis. Applied Microbiology and
 545 Biotechnology, 98(21), 8821–8834. https://doi.org/10.1007/s00253-014-6028-5
- Eleutherio, E., Brasil, A. de A., França, M. B., de Almeida, D. S. G., Rona, G. B., &
 Magalhães, R. S. S. (2018). Oxidative stress and aging: Learning from yeast
 lessons. *Fungal Biology*, *122*(6), 514–525.
 https://doi.org/10.1016/j.funbio.2017.12.003
- 550 Espindola, A. D. S., Gomes, D. S., Panek, A. D., & Eleutherio, E. C. A. (2003). The
- role of glutathione in yeast dehydration tolerance. *Cryobiology*, *47*(3), 236–241. https://doi.org/10.1016/j.cryobiol.2003.10.003
- 553 Farrugia, G., & Balzan, R. (2012). Oxidative stress and programmed cell death in 554 yeast. *Frontiers in Oncology*, 2, 62–82. https://doi.org/10.3389/fonc.2012.00064
- Fernandes, P. N., Mannarino, S. C., Silva, C. G., Pereira, M. D., Panek, A. D., &
 Eleutherio, E. C. A. (2007). Oxidative stress response in eukaryotes: effect of
 glutathione, superoxide dismutase and catalase on adaptation to peroxide and
 menadione stresses in *Saccharomyces cerevisiae*. *Redox Report*, *12*(5), 236–
 244. https://doi.org/10.1179/135100007X200344
- França, M. B., Panek, A. D., & Eleutherio, E. C. A. (2005). The role of cytoplasmic
 catalase in dehydration tolerance of *Saccharomyces cerevisiae*. *Cell Stress* &
 Chaperones, *10*(3), 167–170. https://doi.org/10.1379/CSC-103R.1
- François, J., & Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast
 Saccharomyces cerevisiae. FEMS Microbiology Reviews, 25(1), 125–145.
 https://doi.org/10.1016/S0168-6445(00)00059-0
- Gamero-Sandemetrio, E., Gómez-Pastor, R., & Matallana, E. (2014). Antioxidant
 defense parameters as predictive biomarkers for fermentative capacity of active
 dried wine yeast. *Biotechnology Journal*, 9(8), 1055–1064.
 https://doi.org/10.1002/biot.201300448
- Gamero-Sandemetrio, E., Paya-Tormo, L., Gomez-Pastor, R., Aranda, A., &
 Matallana, E. (2018). Non-canonical regulation of glutathione and trehalose
 biosynthesis characterizes non-*Saccharomyces* wine yeasts with poor
 performance in active dry yeast production. *Microbial Cell (Graz, Austria)*, *5*(4),
 184–197. https://doi.org/10.15698/mic2018.04.624
- 574 184–197. https://doi.org/10.15698/mic2018.04.624
 575 García, M., Esteve-Zarzoso, B., & Arroyo, T. (2016). Non-Saccharomyces yeasts:
 576 biotechnological role for wine production. In Grape and Wine Biotechnology (pp. 249–271). https://doi.org/10.5772/64957
- Garre, E., Raginel, F., Palacios, A., Julien, A., & Matallana, E. (2010). Oxidative
 stress responses and lipid peroxidation damage are induced during dehydration
 in the production of dry active wine yeasts. *International Journal of Food Microbiology*, *136*(3), 295–303. https://doi.org/10.1016/j.ijfoodmicro.2009.10.018
- 582 Gervais, P., & Beney, L. (2001). Osmotic mass transfer in the yeast *Saccharomyces*
- 583 cerevisiae. CELLULAR AND MOLECULAR BIOLOGY-PARIS-WEGMANN-,
- 584 47(5), 831–840. Retrieved from

- 585 https://www.researchgate.net/publication/11627994
- Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R. R., Powell, C. D., & Smart, K. A.
 (2007). Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, *31*(5), 535–569. https://doi.org/10.1111/j.1574-6976.2007.00076.x
- Gómez-Pastor, R., Pérez-Torrado, R., Garre, E., & Matallana, E. (2011). Recent
 advances in yeast biomass production. *Biomass Detection, Production and Usage*. https://doi.org/10.5772/19458
- 593 Gómez-Pastor, R., Pérez-Torrado, R., & Matallana, E. (2010). Improving yield of 594 industrial biomass propagation by increasing the Trx2p dosage. *Bioengineered* 595 *Bugs*, *1*(5), 352–353. https://doi.org/10.4161/bbug.1.5.12384
- Grant, C. M. (2001). Role of the glutathione/glutaredoxin and thioredoxin systems in
 yeast growth and response to stress conditions. *Molecular Microbiology*, *39*(3),
 533–541. https://doi.org/10.1046/j.1365-2958.2001.02283.x
- Hatem, E., Berthonaud, V., Dardalhon, M., Lagniel, G., Baudouin-Cornu, P., Huang,
 M.-E., ... Chédin, S. (2014). Glutathione is essential to preserve nuclear function
 and cell survival under oxidative stress. *Free Radical Biology and Medicine*, 67,
 103–114. https://doi.org/10.1016/j.freeradbiomed.2013.10.807
- 603
 Jamieson, D. J. (1998). Oxidative stress responses of the yeast Saccharomyces

 604
 cerevisiae. Yeast, 14(16), 1511–1527. https://doi.org/10.1002/(SICI)1097

 605
 0061(199812)14:16<1511::AID-YEA356>3.0.CO;2-S
- Klug, L., & Daum, G. (2014). Yeast lipid metabolism at a glance. *FEMS Yeast Research*, *14*(3), 369–388. https://doi.org/10.1111/1567-1364.12141
- Liu, P.-T., Lu, L., Duan, C.-Q., & Yan, G.-L. (2016). The contribution of indigenous
 non-Saccharomyces wine yeast to improved aromatic quality of Cabernet
 Sauvignon wines by spontaneous fermentation. LWT Food Science and
 Technology, 71, 356–363. https://doi.org/10.1016/J.LWT.2016.04.031
- Lorenz, E., Schmacht, M., Stahl, U., & Senz, M. (2015). Enhanced incorporation yield
 of cysteine for glutathione overproduction by fed-batch fermentation of
 Saccharomyces cerevisiae. Journal of Biotechnology, *216*, 131–139.
 https://doi.org/10.1016/j.jbiotec.2015.10.016
- Mehdi, K., & Penninckx, M. J. (1997). An important role for glutathione and γglutamyltranspeptidase in the supply of growth requirements during nitrogen
 starvation of the yeast Saccharomyces cerevisiae. Microbiology, 143(6), 1885–
 1889. https://doi.org/10.1099/00221287-143-6-1885
- Netto, L. E. S., de Oliveira, M. A., Monteiro, G., Demasi, A. P. D., Cussiol, J. R. R.,
 Discola, K. F., ... Horta, B. B. (2007). Reactive cysteine in proteins: Protein
 folding, antioxidant defense, redox signaling and more. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146(1), 180–
- 624 193. https://doi.org/10.1016/j.cbpc.2006.07.014
- Nguyen, T. D., Guyot, S., Pénicaud, C., Passot, S., Sandt, C., Fonseca, F., ...
 Husson, F. (2017). Understanding the responses of *Saccharomyces cerevisiae* yeast strain during dehydration processes using synchrotron infrared
 anactroscopy Arghvet 142(10) 2620 2628 https://doi.org/10.1020/c7cp00257h
- 628 spectroscopy. *Analyst*, *142*(19), 3620–3628. https://doi.org/10.1039/c7an00257b 629 Oro, L., Ciani, M., & Comitini, F. (2014). Antimicrobial activity of *Metschnikowia*
- *pulcherrima* on wine yeasts. *Journal of Applied Microbiology*, *116*(5), 1209–
 1217. https://doi.org/10.1111/jam.12446
- Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect .
 Frontiers in Molecular Biosciences . Retrieved from
- https://www.frontiersin.org/article/10.3389/fmolb.2014.00017

Picazo, C., Gamero-Sandemetrio, E., Orozco, H., Albertin, W., Marullo, P., 635 Matallana, E., & Aranda, A. (2014). Mitochondria inheritance is a key factor for 636 637 tolerance to dehydration in wine yeast production. Letters in Applied Microbiology, 60(3), 217-222. https://doi.org/10.1111/lam.12369 638 Piškur, J., Rozpędowska, E., Polakova, S., Merico, A., & Compagno, C. (2006). How 639 640 did Saccharomyces evolve to become a good brewer? Trends in Genetics, 641 22(4), 183–186. https://doi.org/10.1016/j.tig.2006.02.002 Rahman, I., Kode, A., & Biswas, S. K. (2007). Assay for guantitative determination of 642 glutathione and glutathione disulfide levels using enzymatic recycling method. 643 Nature Protocols, 1, 3159. https://doi.org/10.1038/nprot.2006.378 644 Rapoport, A. (2017). Anhydrobiosis and dehydration of yeasts. In A. A. Sibirny (Ed.), 645 Biotechnology of Yeasts and Filamentous Fungi (pp. 87–116). Cham: Springer 646 International Publishing. https://doi.org/10.1007/978-3-319-58829-2 4 647 Rapoport, A., Turchetti, B., & Buzzini, P. (2016). Application of anhydrobiosis and 648 dehydration of yeasts for non-conventional biotechnological goals. World Journal 649 of Microbiology and Biotechnology, 32(6). https://doi.org/10.1007/s11274-016-650 2058-8 651 Rodríguez-Porrata, B., Lopez-Martinez, G., Redón, M., Sancho, M., Mas, A., Rozès, 652 N., & Cordero-Otero, R. (2011). Enhancing yeast cell viability after dehydration 653 by modification of the lipid profile. World Journal of Microbiology and 654 Biotechnology, 27(1), 75-83. https://doi.org/10.1007/s11274-010-0428-1 655 656 Rodríguez-Porrata, B., Novo, M., Guillamón, J., Rozès, N., Mas, A., & Otero, R. C. (2008). Vitality enhancement of the rehydrated active dry wine yeast. 657 International Journal of Food Microbiology, 126(1–2), 116–122. 658 659 https://doi.org/10.1016/j.ijfoodmicro.2008.05.016 Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Stever, D., Gallardo-Chacón, J.-660 J., Ballester, J., ... Alexandre, H. (2012). Yeast-yeast interactions revealed by 661 aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-662 663 culture of non-Saccharomyces and Saccharomyces yeasts. Food Microbiology, 32(2), 243-253. https://doi.org/10.1016/J.FM.2012.06.006 664 Santos, J., Leitão-Correia, F., Sousa, M. J., & Leão, C. (2016). Nitrogen and carbon 665 source balance determines longevity, independently of fermentative or 666 respiratory metabolism in the yeast Saccharomyces cerevisiae. Oncotarget, 667 7(17), 23033. https://doi.org/10.18632/oncotarget.8656 668 Schmidt, S. A., & Henschke, P. A. (2015). Production, reactivation and nutrient 669 requirements of active dried yeast in winemaking: theory and practice. Australian 670 671 Journal of Grape and Wine Research, 21(S1), 651–662. https://doi.org/10.1111/ajgw.12189 672 Schnierda, T., Bauer, F. F., Divol, B., Rensburg, E., & Görgens, J. F. (2014). 673 Optimization of carbon and nitrogen medium components for biomass 674 production using non-Saccharomyces wine yeasts. Letters in Applied 675 Microbiology, 58(5), 478–485. https://doi.org/10.1111/lam.12217 676 Simonin, S., Alexandre, H., Nikolantonaki, M., Coelho, C., & Tourdot-Maréchal, R. 677 678 (2018). Inoculation of *Torulaspora delbrueckii* as a bio-protection agent in winemaking. Food Research International, 107, 451-461. 679 https://doi.org/10.1016/j.foodres.2018.02.034 680 Steels, E. L., Learmonth, R. P., & Watson, K. (1994). Stress tolerance and 681 membrane lipid unsaturation in Saccharomyces cerevisiae grown aerobically or 682 anaerobically. Microbiology, 140(3), 569-576. https://doi.org/10.1099/00221287-683

- Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., & Tofalo,
 R. (2012). Multistarter from organic viticulture for red wine Montepulciano
 d'Abruzzo production. *Frontiers in Microbiology*. Retrieved from
 https://www.frontiersin.org/article/10.3389/fmicb.2012.00135
- Wang, C. W. (2016). Lipid droplets, lipophagy, and beyond. *Biochimica et Biophysica Acta Molecular and Cell Biology of Lipids*, *1861*(8), 793–805.
- 691 https://doi.org/10.1016/j.bbalip.2015.12.010
- Wiseman, H. (1993). Vitamin D is a membrane antioxidant ability to inhibit irondependent lipid peroxidation in liposomes compared to cholesterol, ergosterol
 and tamoxifen and relevance to anticancer action. *FEBS Letters*, *326*(1–3), 285–
 288. https://doi.org/10.1016/0014-5793(93)81809-E
- Wu, Z., Liu, S. Q., & Huang, D. (2013). Dietary restriction depends on nutrient
 composition to extend chronological lifespan in budding yeast Saccharomyces
 cerevisiae. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0064448
- Zielonka, J., Vasquez-Vivar, J., & Kalyanaraman, B. (2007). Detection of 2 hydroxyethidium in cellular systems: a unique marker product of superoxide and
 bydroxthiding. Nature Protocola, 2, 8, https://doi.org/10.0.4.14/paret 2007.472
- hydroethidine. *Nature Protocols*, *3*, 8. https://doi.org/10.0.4.14/nprot.2007.473

703 Figure Legends

704

Fig. 1. Dehydration kinetics profiles applied to yeasts. Each kinetics, KA or KB,
was carried out in three steps (time-temperature) using KNO₃ (step 1), NaBr (step 2)
and CH₃COOK (step 3) saturated solutions to control the water activity (a_w).

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Fig. 2 Evolution of yeasts S. cerevisiae, T. delbrueckii, M. pulcherrima and 709 L. thermotolerans cell cultivability during dehydration. Yeasts were grown in 710 GSM or TSM and cell cultivability (CFU) was estimated before dehydration (fresh) 711 and after kinetics KA or KB. (A) CFU analysis of S. cerevisiae yeast. (B) CFU 712 analysis of T. delbrueckii yeast. (C) CFU analysis of M. pulcherrima yeast. (D) CFU 713 analysis of *L. thermotolerans* yeast. Error bars correspond to the standard deviation 714 of three independent experiments. * Represents significant difference from the 715 716 control (fresh) with a p < 0.05.

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Fig. 3 Analysis of the oxidative stress response of yeasts S. cerevisiae, 718 T. delbrueckii, M. pulcherrima and L. thermotolerans cultivated in GSM or TSM 719 and dehydrated with kinetics KA or KB. Cells were growth in GSM or TSM and 720 intracellular ROS were detected with dihydroethidium (DHE) using two-photon 721 fluorescence microscopy and flow cytometry. Flow cytometry analysis and 722 microscopic representative images of cells were acquired before dehydration in fresh 723 cells (white bars) and after kinetics KA (gray bars) or KB (black bars). Final cell 724 density was 10⁸ cells mL⁻¹. Letters correspond to: (A-Aa) *S. cerevisiae* cells; (B-Bb) 725 T. delbrueckii cells; (C-Cc) M. pulcherrima cells; and, (D-Dd) L. thermotolerans cells. 726 Error bars correspond to the standard deviation of three independent experiments. 727

Tukey's HSD was used to test significant difference with the corresponding control (Fresh) at *p < 0.05 or **p < 0.01. Scale bar = 10 µm.

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













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Fig. 4
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