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Dehydration stress responses of yeasts *Torulaspora delbrueckii, Metschnikowia pulcherrima* and *Lachancea thermotolerans*: Effects of glutathione and trehalose biosynthesis

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ABSTRACT

In food industry and winemaking, the use of active dehydrated yeast (ADY) *Saccharomyces cerevisiae* is a frequent practice because of the long-term stability and high efficiency of ADY. Nowadays, there is an increasing interest for new yeasts strains, such as *Torulaspora delbrueckii* (Td), *Metschnikowia pulcherrima* (Mp) and *Lachancea thermotolerans* (Lt). However, the yeasts transformation processes into the solidified form generate several stresses that reduce the cell viability. In this case, understanding the phenomena of yeast cell resistance before, during and after dehydration is of great importance. In this study we analyzed two compounds associated with resistance to stress and produced by cells, glutathione (total, oxidized and reduced) and trehalose, at different stages of the process. The impact of growing and dehydration conditions on cell viability was analyzed by flow cytometry and two-photon laser scanning microscopy. The results showed that cells naturally enriched in glutathione or trehalose acquired resistance to dehydration, preventing the oxidation of glutathione in a growth/dehydration condition dependent manner. This is the first time that simultaneous metabolic and dehydration responses were observed in three non-*Saccharomyces* strains. These findings represent an opportunity to better understand the yeast's dehydration resistance phenomena and thus to promote the efficient industrial production of new dried yeasts.

1. Introduction

The understanding of the fundamental mechanisms of microbial resistance to environment changes (temperature, oxygen level, pH, nutrients contents …) is of great importance for the food industry. This is particularly important for industries using microbial transformations, such as fermentation. The understanding of the complexity of reactions that may play roles in yeasts survival remains a big scientific challenge. From some years, significant advances in comprehension of the physical, biochemical, adaptive and genomic phenomena linked to the resistance of yeasts to stresses have already been reported using the yeast model, *Saccharomyces cerevisiae* (Sc), such as Nguyen et al. (2017) for heating stress, Da Silva Pedrini et al. (2014) and Câmara et al. (2016) for osmotic perturbations, Lemetais et al. (2012) for air drying process, Câmara et al. (2018) for nutrients availability, and Davey and Hexley (2010) for ethanol concentrations.

Non-conventional yeasts, e.g. *Torulaspora delbrueckii* (*T. delbrueckii*), *Metschnikowia pulcherrima* (*M. pulcherrima*) and *Lachancea thermotolerans* (*L. thermotolerans*), are of particular interest for the wine industry. These yeasts could improve the wine color profile, anthocyanin composition and the complexity of volatile compounds (Chen et al., 2018). If *T. delbrueckii* has already been associated with food spoilage microorganism (Kurtzman, 2011), this yeast is considered one of the best candidates for improve the aromatic wine profile and quality. For example, this yeast plays an important role in producing higher alcohols and esters (Chen and Liu, 2016). Use of *M. pulcherrima* in sequential inoculation with *S. cerevisiae* enhances the concentration of higher alcohol, esters and terpenols as well as the fruity and floral aroma (Sadoudi et al., 2017, 2012). The yeast *L. thermotolerans* is the least known among these yeast strains. However, it can improve the production of lactic acid for the correction of grape must acidity (Kapsopoulou et al., 2007). These three yeasts can also enhance the

Abbreviations: Sc, *Saccharomyces cerevisiae*; Td, *Torulaspora delbrueckii*; Mp, *Metschnikowia pulcherrima*; Lt, *Lachancea thermotolerans*; GSHt, total glutathione; GSSG, glutathione disulfide; GSH, reduced glutathione; TRE, trehalose

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wine aroma profile (Gschaedler, 2017) and reduce the use of chemicals as sulfites for must protection or wine conservation (Simonin et al., 2018). Generally, non-*Saccharomyces* yeasts are naturally present on grapes or in musts at the beginning of the fermentation process, and its viability decreases as the alcohol concentration increases (Sadoudi et al., 2012). Nowadays, in order to improve reproducibility and quality, winemakers have been substituting spontaneous process by controlled inoculation with dehydrated and active yeasts (Benito, 2018a, 2018b). Some of these yeasts have been isolated and are now currently marketed in dry form for use in the co-fermentation of grape must.

Conservation and commercialization of yeast cultures in fresh liquid or pressed forms are not economically advantageous. Besides the shorter storage time compared to the dehydrated form, the maintenance of yeasts in suspension for long periods can generate genetic derivatives (Jenkins et al., 2010). Long-term storage of yeasts in broth or on agar reduces cell viability and genetic stability of strains, often associated with loss of cell robustness in industrial fermentation processes (Boulton and Quain, 2007). In this case, dehydrated yeasts present several advantages, such as stability, ease of handling, lower cost with maintenance, transport and storage (Luna-Solano et al., 2003). Nevertheless, the dehydration or the drying of the yeasts represents highly sensitive transformation processes for microorganisms that can lead to cell death and/or a significant decrease in cell activity potential (Rapoport, 2017). Different research on the dehydration of microorganisms has shown that the kinetics of the water transfer (or the water potential variation kinetics of the medium) and the final volume of the cells induced by dehydration-rehydration cycles influence the cells survival (Gervais and Beney, 2001). The long-term preservation of dried yeasts is ensured when residual moisture reaches 5–8% (Dupont et al., 2014). An explanation of the yeast mortality is the modification of plasma membrane fluidity during the dehydration-rehydration cycles which affects the membrane structure and induce cell mortality (Dupont et al., 2010). Also, decrease in the cell volume combined with increase of contact surface of the cells with air during dehydration induces accumulation of reactive oxygen species (ROS) – superoxide anion ($O2^{\bullet -}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and hydroperoxides (R—OOH) – and contributes to suppression of several enzymatic reactions, and thus leads to cell death (Garre et al., 2010).

In the struggle for survival, yeasts are able to synthesize some substances, e.g. glutathione and trehalose, throughout the biomass formation. These molecules protect cells from subsequent stresses, such as dehydration (Câmara et al., 2018). Glutathione (L-gamma-Glutamyl-L-Cysteinylglycine) is a tripeptide antioxidant formed by three amino acids – cysteine, glutamic acid and glycine – it is present in large amounts in yeasts and it can be found in the reduced (GSH) or oxidized (GSSG) forms. Indeed, glutathione plays a key role in redox equilibrium reactions, i.e. either by allowing formation of native disulfide bonds or by scavenging free radicals present in the cytosol, protecting cell from oxidative stress (Chakravarthi et al., 2006). These reactions are mediated *via* glutathione reductase and glutathione peroxidase (Rahman et al., 2007). Trehalose is a well-known non-reducing disaccharide participating in cell desiccation tolerance (Tapia et al., 2015). Its major role is the maintenance of the cell structure by replacing water in membranes during dehydration (Golovina et al., 2010, 2009). Also, trehalose has been reported as an antioxidant component by reducing oxidation reactions rates while enhancing viscosity of cell cytoplasm (Herdeiro et al., 2006). It is interesting to note that yeasts are able to synthetize both molecules, depending on medium composition and growing conditions (Câmara al., 2018; François and Parrou, 2001; Lorenz et al., 2015). However, the majority of studies addressing these two molecules and their association with dehydration resistance refer only to the yeast *S. cerevisiae*. Precise information regarding the relationship of glutathione and trehalose accumulation and mechanisms of cell resistance to dehydration using non-*Saccharomyces* yeasts remain currently scarce.

This study aims to elucidate some of dehydration resistance phenomena associated with glutathione and trehalose production in three non-*Saccharomyces* strains. Yeasts naturally synthesized these molecules during biomass formation. Yeast cells viability before and after dehydration kinetics was evaluated by flow cytometry and by two-photon laser scanning microscopy. As well, glutathione (total, oxidized and reduced) and trehalose contents were determined in different stages of the process. The results presented in this work can be used to formulate new high performance dehydrated non-*Saccharomyces* yeasts for food and beverages applications. The cultivation and dehydration parameters presented here could serve as a basis for designing new drying technologies, more economical and respectful of the environment.

2. Materials and methods

2.1. Yeast strains and media composition

Yeasts *Saccharomyces cerevisiae* CBS8066, *Torulaspora delbrueckii* CBS4865, *Metschnikowia pulcherrima* CBS5833 and *Lachancea thermotolerans* CBS6340 were studied. They are named in this study, respectively Sc, Td, Mp and Lt. Two growing media were used to induce glutathione or trehalose production, named GSM or TSM, respectively (Câmara et al., 2018). The GSM composition is (g l^{-1}): glucose, 30; yeast extract, 30; KH_2PO_4, 0.6; cysteine, 0.6. The TSM composition is (g l^{-1}) glucose, 30; yeast extract, 3; KH_2PO_4, 0.6. YPD medium was used for standard growing condition (control).

2.2. Culture conditions

For each strain, three isolated colonies were grown in 50 ml of GSM, YPD or TSM in a 250 ml conical flask and incubated in a rotary shaker (Lab Therm, Kühner AG, Basel, Switzerland) at 30 °C for 24 h. Cells at stationary phase were then harvested by centrifugation (5810 R, Eppendorf, Hamburg, Germany) at 20 °C and 2500 × *g* for 5 min. Harvested cells were washed twice in water-NaCl solution (0.9% wt) and suspended in the same salt solution (final cell density = 2×10^{10} cells ml^{-1}) before drying kinetics.

2.3. Dehydration kinetics parameters

Dehydration kinetics has a high impact on yeast survival. Thus, in order to achieve highest cell viability after dehydration, time-temperature parameters were based on a previously study using a pre-pilot fluidized bed drying (Câmara et al., 2018). Two dehydration kinetics KA and KB presented in Table 1 were carried out inside of hermetically sealed plastic chambers (Fig. 1). The water activity (a_w) was controlled using different saturated salt solutions of potassium nitrate, sodium bromide or potassium acetate. A RH-temperature data logger (EasyLog EL-USB-2-LCD, Lascar Electronics, Pennsylvania, USA) was placed inside the chamber in order to check the stability of the desired parameters. The kinetics were carried out in three stages. Initially, 10 drops of 10 µl of each sample were placed separately in two sterile polypropylene supports which were both transferred to the first drying chamber, kept under controlled conditions and being transferred successively at end of first cycle to the second one and third chamber until the end of whole kinetics. At the end of the process, cells were rehydrated with 5 ml of 0.9% water-NaCl solution (a_w = 0.99) at 38 °C during 5 min and then vigorously stirred during 10 s (final cell density = 4×10^8 cells ml^{-1}).

2.4. Measurement of yeast cell functionality and membrane integrity using flow cytometry

Cell functionality and membrane integrity measurements were assessed using a flow cytometer after a double staining cells with

Table 1
Performed kinetics profiles. Each kinetics, KA or KB, was performed in three steps (time-temperature) using different saturated salt solutions to control the a_w (water activity).

Kinetics profile		Step	a_w	Temperature	Time
KA		1	0.95[a]	17 °C	20 min
		2	0.54[a]	31 °C	45 min
		3	0.20[b]	45 °C	25 min
KB		1	0.93[a]	27 °C	15 min
		2	0.49[a]	41 °C	25 min
		3	0.18[b]	60 °C	20 min

Saturated salt solution used to control a_w: Step 1, KNO_3; Step 2, NaBr; and, Step 3, CH_3COOK.
[a] Greenspan (1977)
[b] Labuza et al. (1985).

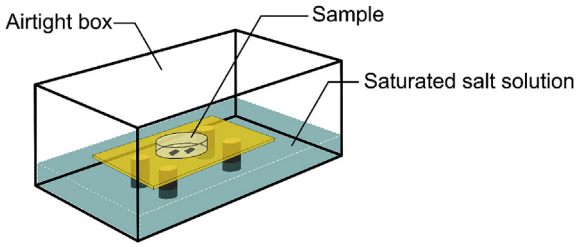

Fig. 1. Scheme of the airtight box with controlled relative humidity. The saturated salt solutions of KNO_3, NaBr or CH_3COOK cover the lower part of the chamber. The polypropylene supports containing the yeast samples were deposited on a rack. The RH-temperature data logger was placed inside the chamber during kinetics.

fluorescein diacetate (FDA) (Sigma-Aldrich, Missouri, USA) and propidium iodide solution (PI) (Sigma-Aldrich, Missouri, USA). FDA stock solution was prepared diluting 10 mg of FDA into 1 ml acetone and stored at −20 °C until analyses. For the analysis, the FDA stock solution was thawed for 15 min at 25 °C and the volume was adjusted to 5 ml with acetone and kept at 4 °C (solution stable up to 20 min protected from light). After rehydration, 800 µl of cell suspension was transferred to 5 ml polypropylene tube and 200 µl of FDA solution was added (final concentration of 0.01 mg FDA ml^{-1} cell suspension). After 10 min kept in the dark, 2 µl of PI (1 mg ml^{-1}) was added to the sample tube immediately before analysis and vigorously stirred for 5 s. A sample not submitted to the drying kinetics was used as positive control (25 °C for 90 min or 60 min) and a dead yeast cells suspension (heated at 90 °C for 20 min) as a negative control. Flow cytometry measurements were carried out using a BD FACS Aria II flow cytometer analyzer (BD Biosciences, San Jose, USA) equipped with two lasers (excitation lines at 488 nm and 633 nm). The FDA and PI fluorescence was detected with at least 10,000 events in each analysis and data was compensated based on the staining of each individual fluorochrome alone and corrected for autofluorescence with unstained cells. Results were expressed as % of viable cells (positive FDA and negative PI cells)/total cells. The percentage of events within a gate is expressed as the mean ± standard deviation of results from at least three replicate experiments.

2.5. Microscopic observations

After rehydration (if necessary), 160 µl of cell suspension was transferred to a 1.5 ml tube and 40 µl of FDA was added in the same conditions than those of flow cytometry measurements. After 10 min and immediately before microscopic analysis, 4 µl of PI was added to samples. Samples were protected from light during all procedure. A two-photon Nikon A1-MP laser-scanning microscope (Nikon, Tokyo, Japan) equipped with a × 60 (NA: 1.27) Plan Apochromat water-immersion objective (Nikon) was used to acquire images from FDA and PI signals. Excitation was performed using an IR laser (Chameleon, Coherent) at 720 nm. Emission signals were collected on two detection channels (channel 2 (green): FF03-525/50–25 filter [500–550 nm]; and, channel 4 (red) FF01-629/56–25 filter [601–657 nm]).

2.6. Glutathione content

After rehydration, if necessary, cells were harvested by centrifugation and suspended in sulfosalicylic acid solution (5% wt). Glutathione extraction was carried out after disrupting cells with FastPrep 24 (MP Biomedicals, California, USA) – 6 cycles of 45 s at 5 m s^{-1}. After disruption, samples were kept on ice for 15 min. Supernatants were collected by centrifugation during 10 min at 10,000 × *g* and 4 °C (5471 R, Eppendorf, Hamburg, Germany) and stored at −80 °C. The total glutathione (GSHt) was determined by using the glutathione assay kit CS0260 (Sigma-Aldrich, Missouri, USA), according to the manufacturer's instructions. Quantification was assessed by reading the evolution of absorbance during 5 min at 412 nm. The total glutathione content in samples was calculated based on an external calibration. Oxidized glutathione (GSSG) content was measured using the method of Griffith (1980) with modifications. Samples were mixed with 0.9 M 2-vinylpiridine to avoid oxidation from GSH to GSSG. After 1 h at room temperature, excess of 2-vinylpiridine was neutralized with 1.3 M triethanolamine. Quantification of GSSG from derivatized samples was carried out as described above. The GSSG content in samples was calculated based on an external oxidized glutathione calibration. Levels of reduced glutathione (GSH) were calculated as follows: GSHt = GSH + 2GSSG (Rahman et al., 2007). Results were expressed as µg total glutathione 10^{10} $cells^{-1}$ and GSSG:GSH ratio.

2.7. Trehalose content

Rehydrated cells were harvested by centrifugation and resuspended in distilled water. Extraction/disruption was carried out as described above. The samples were maintained at 80 °C for 15 min and supernatants were collected by centrifugation. Trehalose (TRE) contents were determined using the trehalose enzymatic kit ref. 016 (Biosentec, Toulouse, France), according to the manufacturer's instructions. Quantification was performed at 340 nm using a spectrophotometric plate reader (Paradigm, Beckman Coulter, California, USA). TRE concentration in samples was calculated based on an external standard

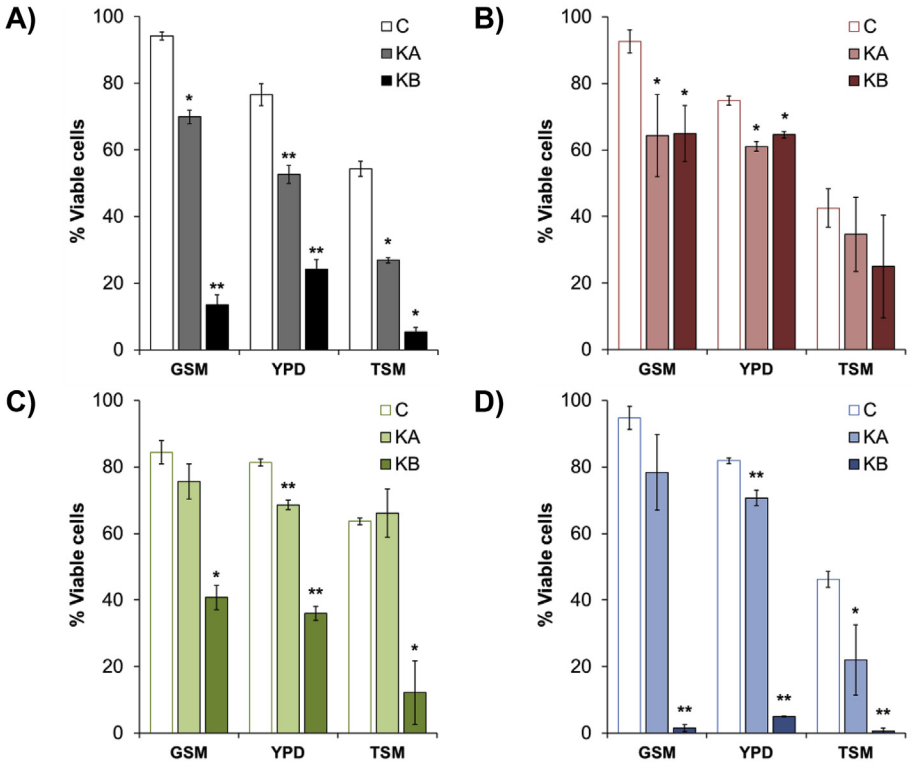

Fig. 2. Cell viability of *S. cerevisiae, T. delbrueckii, M. pulcherrima* and *L. thermotolerans* yeasts. Cell viability was assessed by flow cytometry and double staining cells with FDA and PI. Yeasts were grown in GSM, YPD or TSM. Dehydration kinetics KA and KB were carried out in airtight boxes and using saturated salt solutions. For each strain and growing condition, a fresh cell suspension not submitted to the treatment was used as control (□C). Letters correspond to: (A) *S. cerevisiae* cells, black bars; (B) *T. delbrueckii* cells, red bars; (C) *M. pulcherrima* cells, green bars; and, (D) *L. thermotolerans* cells, blue bars. Values are based on at least three independent replicates ± standard deviation. Significant differences with the corresponding control condition were tested according to Tukey's HSD post-hoc at: $^{*}p < 0.05$ or $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

calibration.

2.8. Statistical analysis

Statistically significant differences were analyzed by the Tukey HSD post-hoc test or by one-way analysis of variance (ANOVA) using the STATISTICA® software (Statsoft, Oklahoma, USA).

3. Results

3.1. Cell viability

In order to investigate the physiological behavior of the three non-*Saccharomyces* strains during drying, cell viability was evaluated before and after dehydration kinetics (KA or KB) and was compared to the yeast *S. cerevisiae* (Sc, control strain). Cells were double stained with FDA and PI to indicate with precision if cells were viable and metabolically active. For all strains, better viability was found when cells were cultivated in GSM (Fig. 2). When these cells were dehydrated under KA, Sc and Td cells exhibited viability of 70% and 64%, respectively (gray and light red bars, Fig. 2A and B). No significant differences were found for Mp and Lt cells. However, when applying KB, cell viability was 13% for Sc, 41% for Mp and 2% for Lt yeasts (black, green and blue bars, Fig. 2A, C and D). Td cells could better resist to this dehydration condition than the former (red bar, Fig. 2B). Cells cultivated in TSM showed reduction in cell viability levels for all tested strains. Compared to GSM controls, up to two-fold less viable cells were found for TSM in control conditions (white bars, Fig. 2A–D). These results suggest an adaptive nutritional behavior for all strains. Cells growing in TSM showed lower resistance to KA than GSM cells, especially for Sc and Lt that showed respectively 27% and 22% of cell viability (gray and light blue bars, Fig. 2A and D). However, Mp and Td grown in TSM showed no significant difference in this dehydration condition. On the other hand, very low viability levels were found for all strains cultivated in TSM and dehydrated with KB, with 5% for Sc, 25% for Td, 12% for Mp and < 1% for Lt. In order to compare results obtained with GSM and TSM grown cells, cell viability was also evaluated using another culture medium traditionally employed for yeast cultures, YPD medium. Cell viability of yeasts cultivated on YPD was similar to that obtained with cultured cells on GSM. Significant cell viability differences between control and dehydrated cells was observed when applying KA, which corresponded 53% for Sc, 69% for Mp and 71% for Lt (gray, light green and light blue bars, Fig. 2A, C and D). Similar behavior was found when using KB, with 24% for Sc, 36% for Mp and 5% for Lt (black, green and blue bars, Fig. 2A, C and D). Td cells grown in YPD showed 61% and 65% of cell viability in both dehydration conditions KA and KB (light and dark red bars, Fig. 2B). These results suggest that yeasts growing in rich-nutrient media (GSM or YPD) can acquire dehydration resistance, more particularly when applying the dehydration kinetics KA. Cell cultures in nutrient-poor medium (TSM) reduced cell viability even before dehydration, probably due to a limited nutritional environment.

3.2. Yeasts metabolic activity and membrane permeability analysis by two-photon microscopic observations

In order to examine most important differences between growing conditions (GSM and TSM) and yeasts strains, we choose to observe cells only after the most drastic dehydration kinetic, KB. After a dehydration-rehydration cycle, cells were stained with both FDA and PI. Representative images are presented in Fig. 3. After growing in GSM, high proportion of non-permeabilized and esterase active yeasts, i.e. viable cells, was observed for Td and Mp strains, 73% and 58% of viable cells respectively (Fig. 3C and E). Conversely, when these same cells were grown in TSM they showed an opposite behavior, with a large proportion of red cells, i.e. dead cells. Only 13% and 17% of cells were viable, respectively. Sc cells seemed to be less resistant after exposure to KB, whatever the growing medium (Fig. 3A and B). Lt cells were the most affected by the KB, after grown in both media (Fig. 3G and H) with cell viability < 4% after treatment. These findings were comparable to those obtained with flow cytometry analysis and suggested that viability of the cells during dehydration was not only affected by the

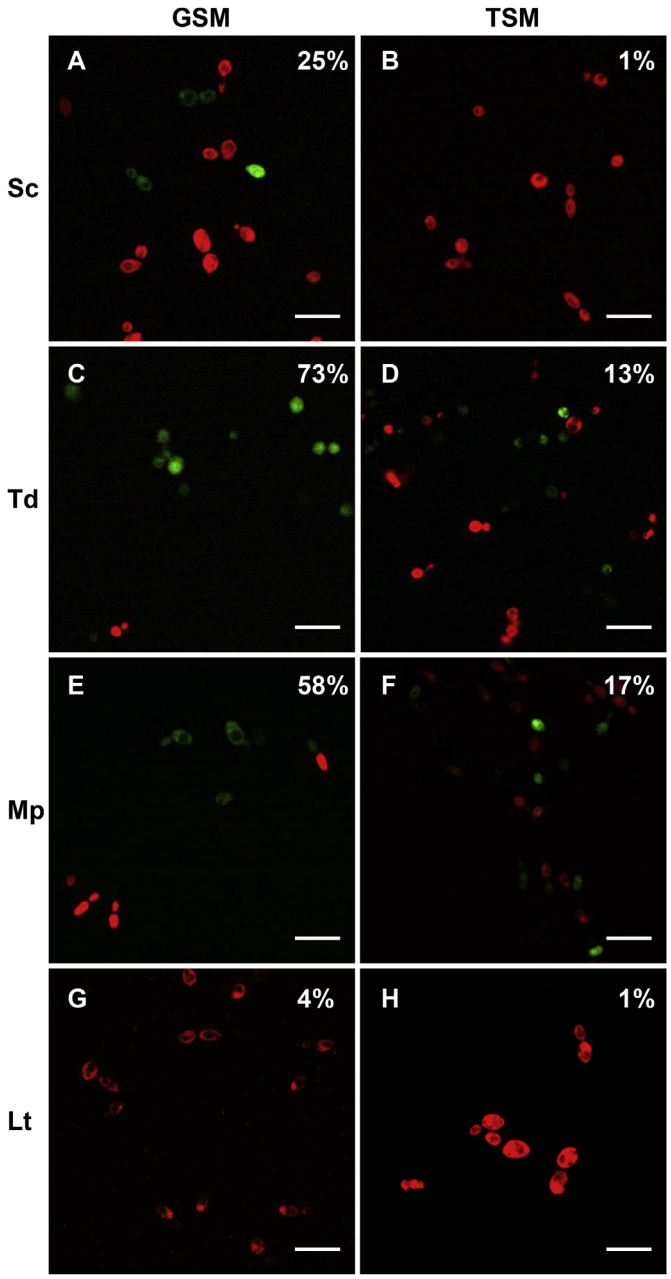

Fig. 3. Impact of dehydration with the kinetics KB and growing conditions (GSM or TSM) on yeast cell metabolic activity and plasma membrane permeability. Representative two-photon fluorescence imaging of yeasts stained with FDA and PI after dehydration-rehydration under controlled conditions. Letters correspond to: (A) and (B) *S. cerevisiae* cells; (C) and (D) *T. delbrueckii* cells; (E) and (F) *M. pulcherrima* cells; and, (G) and (H) *L. thermotolerans* cells. The percentages represent the non-permeabilized and esterase active yeasts (viable and green cells) for each growing condition and strain. Scale bar = 12 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

growing conditions, but were strongly related to the intrinsic physiological characteristics of each yeast strains.

3.3. Glutathione content

3.3.1. Total glutathione

In order to evaluate the effect of nutrient availability on glutathione production and impact of dehydration on GSHt, the four yeast strains were grown in GSM, TSM and YPD media and dehydrated according to both KA and KB. Before dehydration, all cells produced important amounts of GSHt, which were 278, 202, 213 and 242 µg for Sc, Td, Mp and Lt, respectively (white bars, Fig. 4A–D). When Sc, Td, Mp and Lt were grown in TSM they produced respectively 3.3, 1.7, 2.3 and 1.9 times lower content of GSHt (white bars, Fig. 4A–D). A similar behavior was found for cells cultivated in YPD, where Sc, Td, Mp and Lt produced 3.6, 2.8, 2.0 and 6.3 times less GSHt than in GSM. When yeasts were dehydrated with the kinetics KA, no significant differences in the amount of GSHt for all strains were observed regardless the growing condition, except to Td cells grown in TSM and Lt cells grown in YPD (light color bars, Fig. 4A–D). Conversely, all yeasts grown in GSM or TSM and dehydrated with kinetics KB showed lower GSHt content, e.g. up to four times lower for Lt cells (dark color bars, Fig. 4A–D). We did not observe this same comportment for Td and Mp cells grown in YPD which showed no significant differences between control and dehydrated cells (red and green bars, Fig. 4B and C). These findings suggest that cells are able to accumulate much more GSHt under nutrient-rich growing condition (GSM), probably due to the presence of cysteine and 10 times more nutritional sources (yeast extract). Moreover, the kinetics KB led to the reduction of the detected GSHt levels. It was possible that the higher temperature in this condition affected the intracellular glutathione content, in relation with a reduced cell viability.

3.3.2. GSSG:GSH ratio

The GSSG:GSH ratio is a good indicator of cell metabolic stress and oxidation. Results showed higher GSSG:GSH ratio for all tested conditions. Significant ratio enhancement was found for cells cultivated in TSM and dehydrated through kinetics KB (dark color bars, Fig. 5A–D). In this growing condition, cells not only produced less GSHt (Fig. 4A–D) but glutathione would be more oxidized during dehydration (e.g. ratio up to eight times higher for Lt cells, blue bar, Fig. 5D). It is interesting to note that glutathione produced by Sc (the best GSHt producer among the four strains) grown in the YPD medium was mostly in the reduced form (Fig. 5A). Conversely, for all yeasts grown in YPD lower GSSG:GSH ratio was found after dehydrating cells only with the kinetics KB (dark color bars, Fig. 5A–D). These results suggest that cell culture and dehydration conditions influenced the yeast metabolic state and the cell oxidation. Glutathione enzymatic system was probably affected in such conditions.

3.4. Trehalose content

Cell growing in TSM provided an enhancement of TRE content for all tested strains (Fig. 6A–D). These cells showed from two to three times higher TRE contents than cells grown in GSM or YPD. The highest TRE content was found for Sc and Td cells, 173 µg and 195 µg, respectively (white bars, Fig. 6A and B). Although a two-fold increase in TRE content for Mp grown in TSM (green bars, Fig. 6C) was observed, in comparison with culture on GSM or YPD media, overall amount was reduced if compared to the others yeasts strains. Dehydration kinetics KA and KB did not affect the accumulation of TRE by the cells, except for Td and Lt cells grown in TSM that showed TRE content of 1.7 and 1.3 times lower, respectively (dark red and dark blue bars, Fig. 6B and D). These results point out that cells growing in TSM have undergone an induction of the accumulation of large amounts of TRE, most probably due to a nutritional stress environment. This mechanism allows the cells to accumulate sugars as reserve in order to survive a possible period of subsequent stress, i.e. the dehydration.

4. Discussion

4.1. Yeast dehydration resistance is related to growth conditions and strains

The growing and dehydration conditions performed in this study affected yeasts cell viability. During cultivation in the nutrient-rich medium GSM, all yeasts strains showed highest viability, compared to

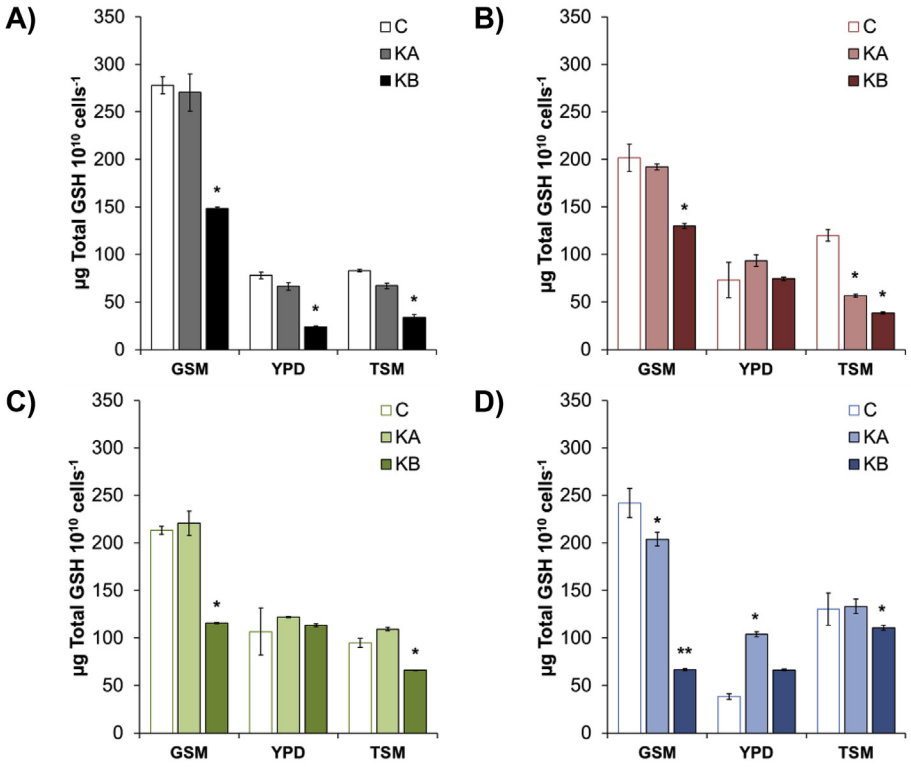

Fig. 4. Total glutathione (GSHt) produced by yeasts *S. cerevisiae, T. delbrueckii, M. pulcherrima* and *L. thermotolerans*. GSHt content was estimated before (□C) and after dehydration kinetics KA or KB. Yeasts growing were carried out in GSM, YPD or TSM. Letters correspond to: (A) *S. cerevisiae* cells, black bars; (B) *T. delbrueckii* cells, red bars; (C) *M. pulcherrima* cells, green bars; and, (D) *L. thermotolerans* cells, blue bars. Data are the mean values of three independent experiments ± standard deviation. Significant differences with the corresponding control condition were tested according to Tukey's HSD post-hoc at: $^{*}p < 0.05$ or $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the cells grown in the nutrient-poor medium, TSM (Figs. 2 and 3). A similar behavior was found for cells cultivated in YPD medium. This result suggests two cell resistance mechanisms. The first one is related to the nutrient availability effect on the maintenance and preservation of cell life (Beker and Rapoport, 1987). For example, the importance of regulating both carbon (glucose) and nitrogen content (ammonium, glutamine) in the growing media was highlighted for extend yeast *S. cerevisiae* chronological life span (Santos et al., 2016). Moreover, a previous study has already demonstrated that supplementing medium with Ca^{2+} or Mg^{2+} allows cells to increase their resistance to dehydration (Trofimova et al., 2010). In this nutritional context, high amounts of essential amino acids, peptides, vitamins and carbohydrates

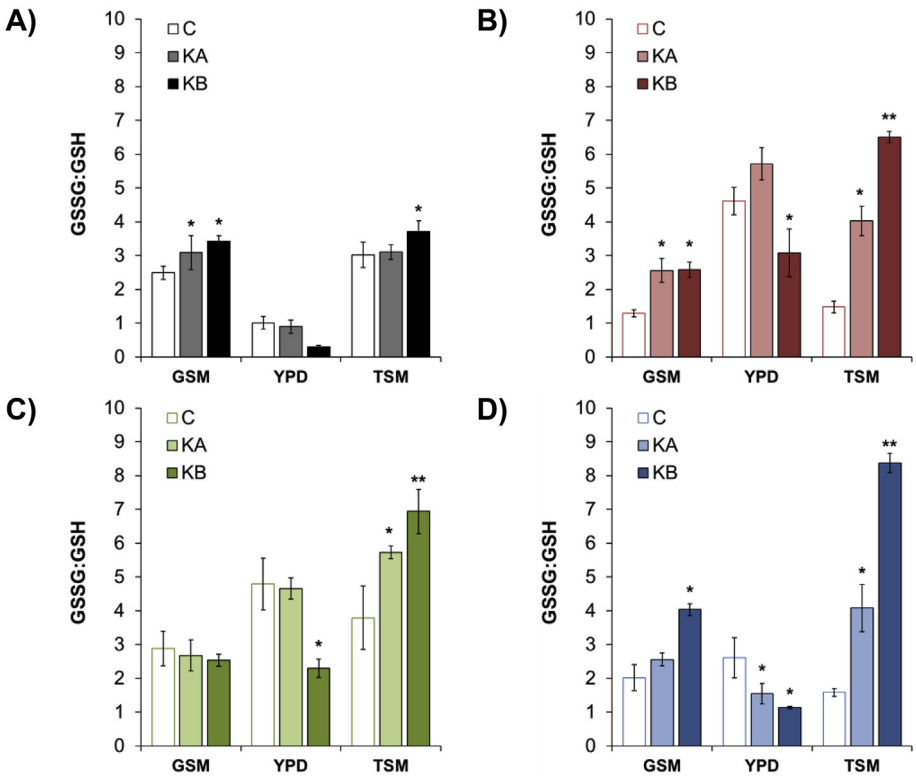

Fig. 5. GSSG:GSH ratio determinations for yeasts *S. cerevisiae, T. delbrueckii, M. pulcherrima* and *L. thermotolerans* during biomass formation and after dehydration kinetics. GSSG (oxidized glutathione) and GSH (reduced glutathione) contents were determined before (□C) and after dehydration kinetics KA or KB. Yeast cells were grown in GSM, YPD or TSM. Letters correspond to: (A) *S. cerevisiae* cells, black bars; (B) *T. delbrueckii* cells, red bars; (C) *M. pulcherrima* cells, green bars; and, (D) *L. thermotolerans* cells, blue bars. Data are the mean values of three independent experiments ± standard deviation. Significant differences were tested according to Tukey's HSD post-hoc at: $^{*}p < 0.05$ or $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

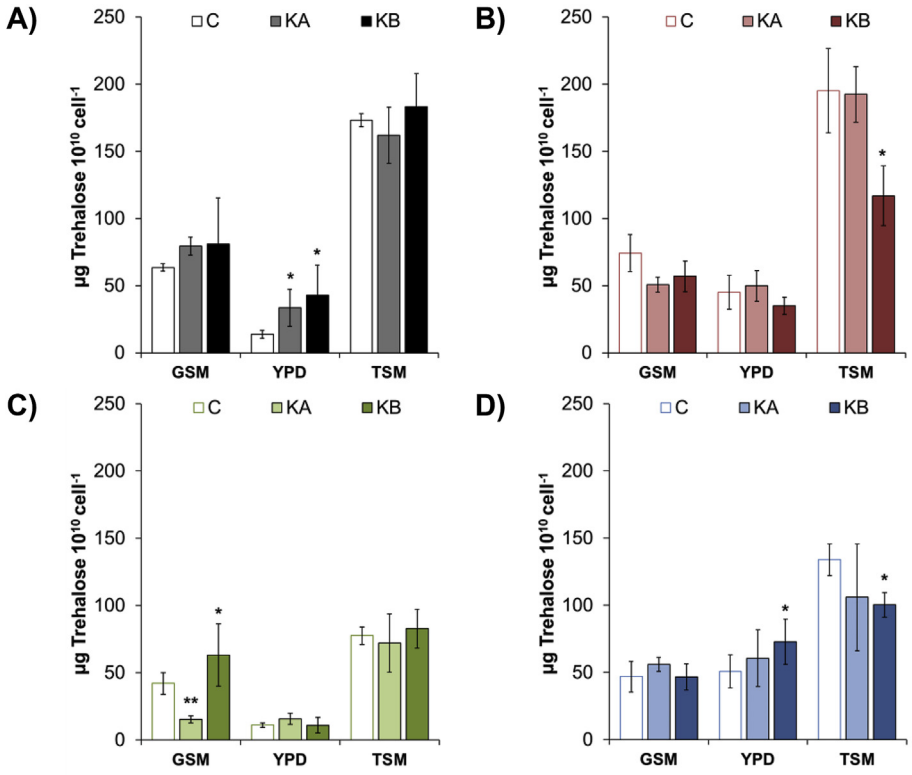

Fig. 6. Trehalose content in *S. cerevisiae, T. delbrueckii, M. pulcherrima* and *L. thermotolerans*. Cells were grown in GSM, YPD or TSM. Intracellular trehalose content was determined before (□C) and after dehydration through kinetics KA or KB. Letters correspond to: (A) *S. cerevisiae* cells, black bars; (B) *T. delbrueckii* cells, red bars; (C) *M. pulcherrima* cells, green bars; and, (D) *L. thermotolerans* cells, blue bars. Data are the mean values of three independent experiments ± standard deviation. Significant differences were tested according to Tukey's HSD post-hoc at: $^{*}p < 0.05$ or $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

are present in yeast extract (Kasprow et al., 1998). Indeed, increasing yeast extract proportion in the growing medium, from 3 to 12 g l^{-1} (final nitrogen concentration 0.141 mol l^{-1}), enhanced up to 20% the fermentative performance of the yeast *S. cerevisiae* (Bafrncová et al., 1999). Thus, in the composition of GSM, YPD and TSM media, the yeast extract represents respectively 49%, 20% and 8% of the mass of solids, so this can partially explain the better cell viability after dehydration for GSM > YPD > TSM cells (Figs. 2 and 3). Moreover, the presence of cysteine in GSM may have contributed to improve cell viability after dehydration. This assumption is supported by the fact that cysteine is one of the most important amino acids precursor of glutathione synthesis (Wen et al., 2004). About two-fold enhancement in biomass production was found when 2 mM cysteine was added to the growing media (Wen et al., 2006). These results lead us to infer that this favorable growing condition (GSM) may be directly related to cell viability before and after dehydration, especially after the dehydration kinetics KA. In a recent study, we demonstrated the relationship between the growing of *S. cerevisiae* in nutrient-rich media and the enhancement of GSHt content and biomass. We also showed that this growing condition improves cell protection to fluidized bed drying (Câmara et al., 2018).

The second mechanism refers to the specific characteristics of each yeast strains. These strains belongs to genera that are not genetically close to each other (du Plessis et al., 2017). Thus, it was expected to obtain distinct stress responses between the four tested strains. On the other hand, the complex yeast metabolic machinery is the result of millions years of evolution under stressful conditions (Marsit and Dequin, 2015). Thus, it is evident that it is pretentious, if not misleading, to generalize the yeast stress resistance mechanisms with only a few physiological characteristics of the cell. Nevertheless, some of the non-*Saccharomyces* yeasts characteristics may help to better understand the resistance phenomena observed in this study.

The yeast *T. delbrueckii* has a strong tolerance to environments with high osmotic pressure, such as products with low water activity (Deák and Deak, 2007). This osmotolerant particularity is attributed to the high-synthesized intracellular levels of trehalose (Alves-Araújo et al., 2004; D'Amore et al., 1991). Additionally, high levels of residual nitrogen were reported after fermentation with isolated *T. delbrueckii* strains (Bely et al., 2008). Authors showed that this higher contents linked to the lower nutritional demand of this yeast or because *T. delbrueckii* is a greater amino acid releaser. These characteristics suggest that the yeast ability to better withstand dehydration is connected with its intrinsic metabolic and physiological factors (Benito, 2018a). With respect to oxidation resistance, the yeast *M. pulcherrima* is able to tolerate high doses of H_2O_2. This tolerance is associated with the high catalase activity of this yeast (Barbosa et al., 2018; Mestre Furlani et al., 2017). This characteristic could explain the resistance of the tested strain to dehydration even under oxidizing conditions (high GSSG content). Concerning the stress resistance, the yeast *L. thermotolerans* is described to be highly frozen tolerant because of the important trehalose content produced by cells. During the stationary phase, cells were up to 500 times more resistant to freezing than cells in the exponential phase. This result was related to the higher trehalose content produced by cells in the growing phase (Hino et al., 1990). This high production of trehalose can partially explain the good viability of the tested strain grown even under nutritional stressful conditions (TSM). Moreover, *L. thermotolerans* showed great tolerance to Zn and Cr (Raspor and Zupan, 2006). Similarly to *T. delbrueckii*, the yeast *L. thermotolerans* showed high fermentative power compared to other non-*Saccharomyces* yeasts. It also demonstrated a glucosidase activity (Comitini et al., 2011).

4.2. Impact of dehydration kinetics on yeast viability

The magnitude of dehydration kinetics has a high impact on cell viability. In this study, it was clear that the kinetics KA has a weaker negative impact on yeasts cell viability, if compared to KB (Figs. 2 and 3). During dehydration, temperature and time amplitudes can affect cell viability (Rapoport, 2017). It was already shown that the gradual and slower rise in temperature (slope) could improve cells resistance to dehydration. In contrast, heat shock dramatically affected the yeast *S. cerevisiae* cell viability (Guyot et al., 2015). On the other hand, the rate of water efflux trough plasma membrane to outside cell followed by an

increase in osmotic pressure have been related to yeast cell dehydration resistance (Dupont et al., 2014; Gervais and Beney, 2001). Indeed, plasma membrane is one of the most affected cell structures during dehydration. Lateral reorganization of plasma membrane during osmotic fluctuations in the environment have been also associated to cell death (Dupont et al., 2010). Thus, the slowest dehydration rate (due to lower temperature) during the kinetics KA has probably led to a slower water transfer from inside of the cell to outside, causing less damages to cell membranes and to structural components that are essential for maintaining cell viability.

4.3. Glutathione and trehalose enrichment to improve cell dehydration resistance

The increase in total glutathione (GSHt) content was evident for all strains grown under better nutritional conditions (GSM medium) (Fig. 4). Nutrients availability can affect cell viability and resistance before, during and after dehydration (Câmara et al., 2018; Nguyen et al., 2017; Schmidt and Henschke, 2015). The presence of cysteine (a glutathione precursor) in GSM may have influenced both GSHt production and cell resistance to dehydration (Figs. 2 and 4). It was shown that the two-fold increase in yeast glutathione content was correlated to the addition of 2–3 mM cysteine to the culture medium (Espindola et al., 2003). One interesting aspect of the study carried out by Espindola and coworkers (2003) was that the addition of glutathione monoethyl ester (a derivate of GSH) to a glutathione deficient Sc mutant strain (Δ*gsh1*) restored cell viability to normal levels. It has been shown that the lack of the glutathione synthetase (GSH1) activity, enzyme that participates in the first stages of glutathione synthesis, and glutathione reductase (Glr1p) which participates in cellular redox balance by recycling GSSG prevent the acquisition of resistance to heat shock or oxidation (H_2O_2) (Berry et al., 2011). It seems likely that ensure cell regrowth, survival and shielding nuclear functions are the main roles of glutathione after stress (Dardalhon et al., 2012; Hatem et al., 2014). For all tested cells, our results showed that the glutathione content in its reduced form was proportionally higher in GSM grown cells, compared to TSM grown cells (Fig. 5). Similar results were found by Hatem et al. (2014). These authors monitored the glutathione depletion on protein-thiol oxidation by using the redox-sensitive yellow fluorescent protein (rxYFP) and showed that glutathione was found predominantly in the reduced form under non-nutritionally stressful conditions.

Different mechanisms have already been proposed to elucidate the role of trehalose on yeasts viability/vitality/cultivability. Nowadays it is clearly known that it is necessary for yeasts (and other cells) to synthesize considerable amounts of trehalose to ensure and/or to improve their resistance to dehydration (Eleutherio et al., 2015). Our results showed that cells cultivated under nutrient-limited condition (TSM) have produced up to eight times more trehalose than in GSM or YPD (Fig. 6). Cultivation of yeasts with low nitrogen, vitamins and minerals (yeast extract) and high carbon (glucose) availability induces yeasts cells to accumulate large amounts of trehalose in order to survive a possible subsequent period of stress (Lillie and Pringle, 1980). During growing under limited nitrogen conditions, yeasts seem to strive to compensate for slow growth by enhancing carbon stocks through trehalose (Hu et al., 2014). Over the years, scientific findings have associated trehalose with various mechanisms of cell protection, such as enhancing membranes stabilization through interaction with hydrogens bonding and phospholipids, increasing the cytosol viscosity through the formation of a vitreous state, reducing lipid peroxidation and acting as an antioxidant (Crowe et al., 1984; de Jesus Pereira et al., 2003; Dupont et al., 2014; Eleutherio et al., 2015). The persistence of high levels of viability (for the tested strains of *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima*) under conditions of possible cell starvation (TSM) may be associated with the high amount of trehalose synthesized by the cells (Figs. 2 and 6). Our data are in agreement with those of López-Martínez et al. (2014) that have supplemented cells with trehalose and improved cell viability of 20%. However, additional studies are necessary to define the minimum levels of trehalose needed to overcome the application of the kinetics KB, especially with the strain *L. thermotolerans*, as the trehalose content seems to be not sufficient to maintain cell viability at the same levels than the other strains. It is important to emphasize that all the references mentioned above used the yeast *S. cerevisiae*. For the first time, we approached the phenomenon of glutathione and trehalose synthesis for non-*Saccharomyces* strains under different culture conditions and their correlation with its resistance to dehydration.

Furthermore, during cultivation in TSM, the enzymatic system of glutathione synthesis may have been affected due to the nutritional stress and dehydration kinetics. Sebollela et al. (2004) demonstrated that glutathione reductase (GSH_R) activity could be suppressed by trehalose in an increasing-dependent manner (reaching up to 70% of inhibition). Indeed, suppression of GSH_R activity leads to an accumulation of oxidized glutathione (GSSG) in cells, affecting intracellular redox balance, causing oxidative stress and reducing cell viability (Espindola et al., 2003; Nguyen et al., 2017). These findings are in agreement with our results. We observed that *S. cerevisiae*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* cells produced much more trehalose in TSM than in GSM/YPD media (Fig. 6). Consequently, this production increased the GSSG:GSH rate (especially for *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* strains, Fig. 5) and reduced the cell viability (Figs. 2 and 3). Our results are in agreement with those of Gamero-Sandemetrio et al. (2014), where a similar behavior was found with the commercial *S. cerevisiae* T73 strain. Cells showed high trehalose and GSSG contents after dehydration, and reduced GSH_R activity.

4.4. Can glutathione or trehalose reduce oxidation of yeast cells during dehydration?

During dehydration, the oxygen in the air has a significant effect on the oxidation of important cellular components and on intracellular ROS accumulation. This effect appears mainly in the final stages of dehydration, due to the exposure of solidified cells to oxygen in the air (Dupont et al., 2014). One of the reasons for cell death during dehydration-rehydration cycles was related to the oxidative stress processes to which cells were subjected (França et al., 2005). These observations may explain the increase of oxidized glutathione (GSSG) during prolonged drying kinetics KA (Fig. 5). Moreover, glutathione reductase may have been negatively affected by the higher temperature used during the kinetics KB, leading to GSSG accumulation by failure of the glutathione enzyme recycling system. In addition, during biomass propagation the cells may be subjected to both osmotic stress resulting from high initial solids concentration, and oxidative stress caused by the growing media aeration (Gibson et al., 2007; Gómez-Pastor et al., 2010). This explains why the cells had a significant GSSG:GSH ratio even before dehydration (Fig. 5).

In contrast, it is interesting to note that *S. cerevisiae* and *T. delbrueckii* cells, which synthesized large amounts of trehalose, showed both lower GSSG:GSH rate before dehydration and after KA (Figs. 5 and 6). Probably in these cases, the trehalose content may have been sufficient to ensure the reducing environment in the cell, leading to a lower accumulation of GSSG (less impact with ROS and glutathione reductase) and maintaining a good level of cell viability. The antioxidant activity of trehalose has been reported during yeast dehydration (Eleutherio et al., 2015). Indeed, trehalose protects lipids and proteins from oxidation and is assumed to be related to the increase in the viscosity of the medium, which reduces the biochemical reactions and inhibits the release of ROS (Dupont et al., 2014).

Thus, these observations lead us to believe that the presence of glutathione and trehalose protects the cells from oxidation during dehydration. However, there are other stress responses mechanisms that are still poorly understood, especially those involved with the adaptation of cells to nutrient-rich or poor media and their association with

oxidative stress during dehydration. Also, when we enter the field of non-*Saccharomyces* yeasts these mechanisms are even more unknown. Therefore, in order to clarify these hypotheses, future studies should investigate the adaptation of non-*Saccharomyces* yeasts to nutrient availability and the correlation with both glutathione/trehalose production and the oxidative stress response (accumulation of ROS, lipid peroxidation, etc.) during dehydration.

5. Conclusion

The phenomena of yeast cell adaptation to stress are very complex. Multiple responses occur during cell growing and during cell dehydration. In this study we are focused on the glutathione and trehalose synthesis in three non-*Saccharomyces* strains. These molecules were described over the years as the main agents of cell protection to dehydration in the reference yeast *Saccharomyces cerevisiae*. Our results showed that yeasts grown in nutrient-rich medium (GSM) promoted the best glutathione accumulation and a higher resistance, when submitted to the dehydration kinetics KA. The nutrient-poor medium (TSM) induced the cells to accumulate large amounts of trehalose, which partially protected them from GSSG accumulation. However, it was not enough to ensure the viability of the cells at the same levels as in the GSM or YPD media. In addition, the negative impact of the kinetics KB on all parameters analyzed was clearly demonstrated. These results can be used to understand certain physiological responses of non-*Saccharomyces* yeasts to dehydration, in order to promote the production and the marketing of new high performance dehydrated non-*Saccharomyces* yeasts strains for food and beverages elaboration.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.008.

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