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Basal catalase activity and high glutathione levels influence the performance of non-Saccharomyces active dry wine yeasts

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Abstract:	Non- Saccharomyces wine yeasts are useful tools for producing wines with complex aromas or low ethanol content. Their use in wine would benefit from their production as active dry yeast (ADY) starters to be used as co-inocula alongside S. cerevisiae . Oxidative stress during biomass propagation and dehydration is a key factor in determining ADY performance, as it affects yeast vitality and viability. Several studies have analysed the response of S. cerevisiae to oxidative stress under dehydration conditions, but not so many deal with non-conventional yeasts. In this work, we analysed eight non- Saccharomyces wine yeasts under biomass production conditions, and studied oxidative stress parameters and lipid composition. The results revealed wide variability among species in their technological performance during ADY production. Also, for Metschnikowia pulcherrima and Starmerella bacillaris , better performance correlates with high catalase activity and glutathione levels. Our data suggest that non- Saccharomyces wine yeasts with an enhanced oxidative stress response are better suited to grow under ADY production conditions.

On behalf of the four authors I am submitting the manuscript entitled “Basal catalase activity and high glutathione levels influence the performance of non-Saccharomyces active dry wine yeasts” for publication in Food Microbiology.

Highlights

Production of active dry wine yeast is not optimized for non-*Saccharomyces* yeasts

High lipid peroxidation in dry respiratory yeast cells does not cause low viability

Total fatty acids composition can be correlated to active dry yeast performance

Antioxidant protection is relevant for non-*Saccharomyces* active dry yeast performance

Title:

Basal catalase activity and high glutathione levels influence the performance of non-*Saccharomyces* active dry wine yeasts

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Abstract

Non-*Saccharomyces* wine yeasts are useful tools for producing wines with complex aromas or low ethanol content. Their use in wine would benefit from their production as active dry yeast (ADY) starters to be used as co-inocula alongside *S. cerevisiae*. Oxidative stress during biomass propagation and dehydration is a key factor in determining ADY performance, as it affects yeast vitality and viability. Several studies have analysed the response of *S. cerevisiae* to oxidative stress under dehydration conditions, but not so many deal with non-conventional yeasts. In this work, we analysed eight non-*Saccharomyces* wine yeasts under biomass production conditions, and studied oxidative stress parameters and lipid composition. The results revealed wide variability among species in their technological performance during ADY production. Also, for *Metschnikowia pulcherrima* and *Starmerella bacillaris*, better performance correlates with high catalase activity and glutathione levels. Our data suggest that non-*Saccharomyces* wine yeasts with an enhanced oxidative stress response are better suited to grow under ADY production conditions.

Keywords: Non-*Saccharomyces,* Active dry yeasts, Antioxidant defences, Fatty acids, Glutathione metabolism

1. Introduction

The use of commercial starter cultures for grape must fermentations is a well-established practice in modern wineries as they provide a dominant yeast from the beginning of fermentation, produce wines with a fixed and reproducible quality, and avoid sluggish or stuck fermentations. In recent years, interest in producing wines with enhanced and complex aromas and flavour profiles, or with reduced ethanol content, has grown. This can be achieved by using non-*Saccharomyces* wine yeasts, which

have positive impact on wine character (reviewed by Jolly et al., 2014), when present in early fermentation stages.

There are examples of using non-*Saccharomyces* wine yeasts, belonging to different genera, in mixed and sequential fermentations to improve organoleptic properties (Belda et al., 2015; Benito et al., 2015; Englezos et al., 2016; Garavaglia et al., 2015; Lleixà et al., 2016; Renault et al., 2015), to diminish ethanol content (Ciani et al., 2016; Contreras et al., 2014a, 2014b; Tronchoni et al., 2018), and also as biocontrol agents (Comitini et al., 2004; Freimoser et al., 2019; Oro et al., 2014). Several yeasts are commercially available, such as *Torulaspora delbrueckii, Lachancea thermotolerans* or *Metschnikowia pulcherrima*, from Lallemand Inc and Laffort, and *Pichia kluyveri*, from Hansen. However, the ADY production process has been optimised for the baker's yeast *Saccharomyces cerevisiae* (Reed and Nagodawithana, 1991) and non-*Saccharomyces* yeasts are not always well adapted (Jolly et al., 2014; Varela and Borneman, 2017).

Oxidative stress plays a key role during the industrial biomass propagation and dehydration processes as it affects biomass yield and technological ADY performance (reviewed by Matallana and Aranda, 2017). In the fed-batch phase of industrial growth (Gómez-Pastor et al., 2010a; Pérez-Torrado et al., 2005) and during dehydration (Garre et al., 2010), many oxidative stress-related genes are induced. Main defence mechanisms include enzymatic activities (catalase, superoxide dismutase, glutathione reductase) and protective molecules (trehalose, glutathione), that help to maintain redox balance or to scavenge reactive oxygen species (ROS) (Dupont et al., 2014; França et al., 2007). Recently we described a set of biochemical markers for prediction of better *S. cerevisiae* ADY performance, which include catalase and glutathione reductase activities, trehalose and glutathione (Gamero-Sandemetrio et al., 2014). The still scarce literature on non-*Saccharomyces* wine yeasts under biomass production conditions shows a correlation between a better oxidative stress response and

improved toleration to dehydration, and focus on the importance of trehalose and glutathione (Câmara et al., 2019a; Gamero-Sandemetrio et al., 2018; Kim et al., 2019). However, there is still much to uncover, especially in the characterisation of different wine species and strains under ADY production conditions.

Oxidative stress causes direct damage to membrane lipids and severe membrane damage is associated with loss of viability (Avery, 2011). The main fatty acids (FA) in yeasts are oleic, palmitoleic, stearic and palmitic acid (Daum et al., 1998). The degree of FA unsaturation influences membrane fluidity insofar as the more unsaturated the membrane is, the more fluid it will become, and it will be able to adapt to unfavourable changes in the environment, such as the conditions that yeasts face during dehydration. Studies in *S. cerevisiae* have revealed a correlation between greater membrane fluidity and better tolerance to different stresses (Beltran et al., 2008; Casey and Ingledew, 1986; Sakamoto and Murata, 2002). Recently, the importance of membrane unsaturation when facing oxidative stress has been underlined in two non-*Saccharomyces* yeasts (Vázquez et al., 2019).

Understanding how non-*Saccharomyces* yeasts behave under ADY production conditions will be useful to develop suitable inocula. The aim of this work was to characterise a set of non-*Saccharomyces* wine yeasts under ADY production conditions to understand the role of the oxidative stress response in the process. We simulated industrial growth and desiccation conditions on the laboratory scale and we analysed the technological performance of the produced biomass. Then, we tested the aforementioned set of oxidative stress biomarkers in fresh and dry biomasses, and we extended the analysis to the total lipid composition of two selected strains.

2. Materials and Methods

2.1 Strains and cultivation conditions

Eight non-*Saccharomyces* species provided by Lallemand Inc. (Montreal, Canada) were tested: *Zygosaccharomyces bailii, Torulaspora delbrueckii, Kluyveromyces wickerhamii, Wickerhamomyces anomalus, Hanseniaspora vineae, Metschnikowia pulcherrima, Metschnikowia fructicola* and *Starmerella bacillaris*. The commercial *Saccharomyces cerevisiae* strain Lalvin T73 (Querol et al., 1992) was used as reference strain.

Precultures for biomass propagation were prepared in liquid YPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose) and incubated at 30ºC with shaking (180 rpm). The biomass propagation experiments were performed in molasses medium diluted to 60 g/L sucrose supplemented with 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4$ and 10 mL/L vitamin solution. The vitamin solution contained 0.5 mg/L D-biotin, 1 mg/L calcium pantothenate and 1 mg/L thiamine hydrochloride. Cells were cultivated at 30ºC with shaking (180 rpm) for 24 h. Synthetic grape must (MS300) (Riou et al., 1997; Viana et al., 2014) was used for the fermentative capacity measurements.

2.2 Biomass dehydration and rehydration conditions.

Yeast biomass was separated from the molasses medium by centrifugation at 4,000 rpm and several washes with sterile distilled water were performed. The biomass paste was recovered and placed as thin noodles inside a tabletop fluid bed dryer (Sherwood Scientific, Cambridge, UK). Biomass was dehydrated at 37ºC until it reached approximately 8% relative humidity, as determined by weight loss. It was then stored at 4ºC. For rehydration, the dry biomass was placed in sterile distilled water (1 part biomass : 9 parts water) at 37ºC for 10 min, followed by 10 min with shaking at 140 rpm.

The biomass from the molasses culture (fresh cells) and the rehydrated biomass (dry cells) were diluted, plated and then grown on YPD plates for 24 h at 30ºC, and colony-

forming units (CFU) were counted. The survival percentage was calculated by taking the CFU of the fresh cells as 100% survival.

2.3 Fermentative capacity measurement.

Fresh and dry cells were rehydrated (when necessary) and inoculated in MS300 medium as previously described (Gamero-Sandemetrio et al., 2013). CO_2 production was measured in 10-minute intervals for 6 h in a Gas Production System from Ankom Technology (Macedon, USA). Fermentative capacity was expressed as mL of CO_2 produced by 10^7 cells. Experiments were carried out in triplicate.

2.4 Resistance to stress in plates

Resistance to heat and oxidative stress was tested in YPD agar (2 % (w/v)) plates. Cells were grown in YPD medium at 30ºC for 24 h. Then cultures were serially diluted (10^{-1} to 10^{-4}) and 5 µL drops were plated. Cells were grown for 24 h at 30ºC and 37ºC for thermal stress, and at 30ºC in plates containing 3.5 mM H_2O_2 for oxidative stress.

2.5 Thiobarbituric Acid-Reacting Substances (TBARS) assay.

Quantification of lipid peroxidation in cells was measured using the reaction of thiobarbituric acid with malondialdehyde (MDA) (Buege and Aust, 1978), a product of fatty acid breakage, as previously described (Gómez-Pastor et al., 2010b). MDA content was estimated by using a standard curve with known commercial MDA concentrations. Lipid peroxidation was expressed as pmoles of MDA mg of cells^{-1}.

2.6 Catalase and Glutathione Reductase Activity.

Extracts were obtained from fresh and dry cells, and were assayed spectrophotometrically as previously described by Jakubowski et al., (2000). Catalase activity was expressed as µmol of H_2O_2 min^{-1} mg of protein $^{-1}$ (U / mg prot). Glutathione reductase (GR) was assayed as previously described by Murshed et al., (2008), and activity was expressed as µmol of GSSG min^{-1} mg of protein^{-1} (U/mg protein).

2.7 Trehalose determination

Cell extracts were obtained form 100 mg of cells and were used for trehalose and glutathione determinations. Trehalose levels were determined as previously described (Gamero-Sandemetrio et al., 2014; Parrou and François, 1997); in short, cells were resuspended in 250 mM of Na_2CO_3 and incubated at 95ºC for 4 h. Afterwards they were centrifuged at 12,000 rpm for 30 s. The supernatant was recovered and incubated overnight with 8.4 mU of commercial trehalase (Sigma) at 37ºC. The released glucose was measured by the glucose-oxidase/peroxidase method. Trehalose content was estimated using a standard curve of trehalose with known concentrations. Trehalose levels were expressed as µg of trehalose mg cells^{-1}.

2.8 Glutathione determination.

Glutathione determination was carried out as previously described (Gómez-Pastor et al., 2010b; Griffith, 1980). Cells were collected and resuspended in HCl 8 mM, 1.3% 5-sulphosalicylic acid. Cells were broken by vortexing at 4ºC with glass beads. Cell debris was pelleted and supernatants were used for the glutathione determinations. Total glutathione was determined using 200 µL of the supernatant, while 40 µL of 1 M 2-vinylpiridine were added to determine GSSG content. The reduced glutathione content was calculated as the difference between the total and oxidised glutathione. Glutathione levels were expressed as nmol mg of cells $^{-1}$.

2.9 Total fatty acids, sterol and squalene determinations.

The fatty acid methyl ester analysis was performed as reported by Romero et al., (2018). First 1 mL of HCl 1.25 N in methanol, 10 µL heptanoic acid (C7, 1 g/L) and 10 µL heptadecanoic acid (C17, 4 g/L) were added to glass tubes that contained a yeast pellet of around $2{\cdot}10^8$ cells. Samples were heated to 90 °C for 60 min and were then cooled to room temperature. After cooling, 1 mL of NaCl 0.9% (w/v) in water and 300 µL hexane were added. The extraction was repeated twice. Between each extraction

phase, tubes were centrifuged at 3,000 × g for 5 min to allow the best phase separation. Analytical GC was carried out in an Agilent 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies). The extract (2 µL) was injected (splitless, 0.75 min) into an FFAP-HP column of 30 m × 250 µm × 0.25 µm phase thickness (Agilent Technologies) with an automatic injector (Agilent). Relative amounts of the given fatty acids were calculated from their respective chromatographic peak areas after normalization with internal standards (C7 or C17).

Sterols and squalene were determined using the method reported by Quail and Kelly (1996). Briefly around 2 10^8 cells were re-suspended with 1.5 mL methanol (MeOH), 1 mL of pyrogallol (0.5% (w/v) in MeOH), 1 mL KOH solution (60% (w/v) KOH in distilled water) and 10 µL of α-cholestane (internal standard (IS), 1 mg /mL in hexane) into a glass tube and saponified at 90 ºC during 2 hours. Finally, sterols and squalene were extracted two times with 500 µL hexane and the extract was dried in a SC110 speed vacuum system SC110 (Savant Instruments, USA). The dried residue was dissolved in 100 µL of hexane. From the collected organic phase, 2 mL were injected in pulsed splitless mode (70 psi, 0.10 min) into a DB-5HT column (30 m x 0.25 mm x 0.1 µm, Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). The identification of each compound was carried out by comparing the mass fragmentation pattern of peak with those of the injection of available standard or described by Quail and Kelly (1996). The relative abundance of each identified compound was calculated according to the respective chromatographic peak areas corrected with respect to the IS peak area. The results are expressed as individual percentage of total sum of identified sterols and squalene.

2.10 Statistical analysis.

Sample averages were compared using a Student's *t*-test. The samples denoted (a) significantly differed from those labelled (b) with a $p < 0.05$, and also differed from those denoted (c) with a $p < 0.05$. The samples labelled (ab) did not significantly differ

from (a) and (b), but did significantly differ from (c). The samples denoted (*) were significantly different from one another.

Heatmaps were generated using the *heatmap.2* function of the *R* software.

3. Results

3.1 Different technological performance is observed in ADY production for the analysed wine yeast species

Eight non-*Saccharomyces* wine yeast species from the Lallemand Inc. collection were selected by their desirable traits as co-starters in wine fermentations and analysed in laboratory-scale simulations of ADY production (see Table 1). Previous studies in our lab used simulations of the industrial yeast growth, but the desiccation method (described in Gamero-Sandemetrio et al., 2014) differed from the industrial conditions. In this work, we adapted the dehydration conditions using a bench-top fluid bed dryer that better simulates the industrial process. When grown in molasses for 24 h, only *Z. bailii and S. bacillaris* reached similar OD levels to the *S. cerevisiae* commercial strain. The rest of the non-*Saccharomyces* species presented less growth than *S. cerevisiae*. *H. vineae*, *M. pulcherrima and M. fructicola* reached the lowest OD. Apart from their growth, biomass yield (g biomass/g sucrose consumed) was calculated. Only *Z. bailii* showed a higher biomass yield than the control, while *K. wickerhamii, H. vineae, M. pulcherrima* and *S. bacillaris* presented lower biomass yields than the control, and the rest showed no differences.

We also studied cell viability after dehydration (Table 1). *M. pulcherrima*, *M. fructicola* and *S. bacillaris* displayed viability values upwards of 80%, which was higher than the reference strain, which showed around 50% of viable cells after dehydration. *T. delbrueckii*, *W. anomalus* and *Z. bailii* had similar viability values to *S. cerevisiae*, while *K. wickerhamii* and *H. vineae* had less viability. This was especially significant for *H.*

vineae, which was most affected by this process as we were only able to recover about 2% of viable cells.

Fermentative capacity in synthetic grape must was also assayed as indicative of the technological performance of the ADY (Table 1). All the non-*Saccharomyces* species showed less fermentative capacity than *S. cerevisiae* in both fresh and dry cells, and underwent fermentative capacity loss after dehydration, which, in most cases, was related with their viability after the process. In the dry cells, *T. delbrueckii* showed the highest fermentative capacity, followed by *Z. bailii*. The other species presented very poor fermentative capacities.

3.2 Tolerance to oxidative and heat stresses is generally low in non-conventional wine yeasts

Thermotolerance and oxidative stress experiments in YPD agar plates were also performed (Figure 1). Under the control conditions, *Z. bailii* showed less growth than the other species. The thermotolerance assays at 37 ºC revealed that only *W. anomalus* was capable of growing, albeit considerably less than for the control condition. When grown in the presence of 3.5 mM H_2O_2, all the non-*Saccharomyces* strains showed slightly reduced growth compared to their controls, except for *Z. bailii* and *K. wickerhamii*, which were most affected and showed almost no growth after 24 h. *S. bacillaris* displayed intermediate behaviour.

3.3 Dehydration causes high oxidative damage to lipids in respiratory wine yeasts

We measured lipid peroxidation in the fresh and dry cells as an indicator of oxidative damage. The MDA levels were similar in the fresh cells and lipid peroxidation increased after dehydration for all the species (fig. 2A). *M. pulcherrima* and *M. fructicola* reached the highest MDA levels in the dry cells, probably due to their respiratory nature (Quirós et al., 2014).

3.4 Protective antioxidant enzymatic activities display high variability between wine yeast species

The enzymatic activities of catalase and glutathione reductase were assayed to gain further insight into the capacity of yeast cells to deal with oxidative stress. As seen in figure 2B, *M. pulcherrima, M. fructicola* and *S. bacillaris* showed catalase activities that were more than 10-fold higher than the other species in both the fresh and dry cells, consistently with their high viability. Catalase activity in *Z. bailli, K. wickerhamii, H. vineae* and *M. pulcherrima* was induced after desiccation, as in the control strain. The increase in catalase activity in the dry cells was similar in all the species (between 1.4- and 2.4-fold), except for *Z. bailii* which was the highest (3.74-fold).

Basal glutathione reductase activity in the fresh cells of non-*Saccharomyces* species was higher than *S. cerevisiae* (fig. 2C). After dehydration, GR activity displayed the most activation in *W. anomalus, M, pulcherrima, M. fructicola* and *S. bacillaris*. No variation in GR activity was observed for the other species.

3.5 Non-*Saccharomyces* yeasts show deficient trehalose accumulation.

Trehalose intracellular levels were measured in both the fresh and dry cells (fig. 2D). In the fresh cells, only *T. delbrueckii* and *S. bacillaris* showed similar levels to the control *S. cerevisiae* strain. We were able to detect low trehalose levels in the dry cells of *W. anomalus*, *M. pulcherrima* and *M. fructicola,* but levels were still significantly low (5–10 µg/mg cell). Trehalose content rose in *T. delbrueckii* and *S. bacillaris* dry cells, as well as in *S. cerevisiae*. *K. wickerhamii* and *H. vineae* did not show trehalose accumulation in neither the fresh nor the dry cells.

3.6 Both GSH/GSSG ratio and GR activity are relevant for oxidative stress tolerance.

We analysed the total glutathione (GSH_{TOT}), reduced glutathione (GSH) and oxidised glutathione (GSSG) levels in the fresh and dry cells (Figure 3). The GSH_{TOT} levels

vastly varied among species for both conditions. We observed an increase in GSH_{TOT} after dehydration in *M. pulcherrima, M. fructicola* and *S. bacillaris,* which also showed the highest overall glutathione levels in the dry cells, correlating with their high viability after the process. These three species also had increased GSSG and GSH contents in the dry cells (Figs. 3B and C). The GSH/GSSG ratio (Fig 3D) rose after drying in *M. pulcherrima* and *S. bacillaris*, suggesting stronger protection against oxidative stress, and correlating with their high GR activity. We observed a slight drop in the GSH/GSSG ratio in *M. fructicola*.

The GSH_{TOT} levels in *Z. bailli*, *T. delbrueckii* and *H. vineae* did not vary after dehydration. Notwithstanding, the GSH/GSSG ratio was lower in the dry cells for *Z. bailli* and *T. delbrueckii*, which indicates greater oxidative damage. Coincidentally, these strains had the lowest total glutathione levels of all the studied non-*Saccharomyces* yeasts and also displayed poor GR activities. A decrease in GSH_{TOT} was observed in *S. cerevisiae, K. wickerhamii* and *W. anomalus*. Despite this decrease, the GSSG level rose in the dry cells, but the GSH levels lowered, except in *W. anomalus*, which had lower GSSG levels in the dry than in the fresh cells.

In *Z. bailii, T. delbrueckii* and *K. wickerhamii*, the GSH/GSSG ratio lowered after dehydration as happened with the *S. cerevisiae* strain. The other non-*Saccharomyces* species showed higher GSH/GSSG ratios in the dry cells, which suggests a better response to the oxidative stress. Coincidentally, the species with increased GSH/GSSG ratios were those with greater GR activity.

3.7 Total levels of fatty acids and ergosterol vastly vary among species

Based on the technological efficiency results obtained for the dry biomass, we selected the two species with the most dissimilar viability after desiccation, namely *H. vineae* and *M. pulcherrima.* As their differences in oxidative defence parameters did not fully explain their differences in technological performance, total fatty acid composition was

investigated due to its important role in the structural integrity of biological membranes. The study of total fatty acids in *S. cerevisiae*, *H. vineae* and *M. pulcherrima* revealed large differences in the FA content among species. As seen in Fig. 4, the unsaturated fatty acids palmitoleic (C16:1) and oleic (C18:1) acid made up most of the total FA content of *S. cerevisiae* (around 35 - 40 % each). In contrast, *H vineae* showed larger amounts of palmitoleic (C16:1) (50 %) and lower levels of oleic (C18:1) (25 %) acid. *M. pulcherrima* had the smallest amount of linoleic acid (around 8%), which was compensated for by a higher percentage of oleic acid (near 50%) and the presence of the polyunsaturated fatty acids linoleic acid (C18:2) (25%) and small amounts of gamma linoleic acid (C18:3). No polyunsaturated fatty acids were detected in either *S. cerevisiae* or *H. vineae*. Only minor differences were observed in the FA composition between the fresh and dry cells (Fig. 4 B and D).

Ergosterol is the main sterol found in yeast membranes and it plays a critical role in membrane fluidity. *M. pulcherrima* showed the highest percentage of ergosterol (85%,) with lower percentages in *S. cerevisiae* and *H. vineae* (70–75%) (Fig. 4, C and D). In *S. cerevisiae,* zymosterol was the secondary sterol detected, while squalene was the second most abundant sterol in *H. vineae.* The clear differences observed in lipid compositions among species help to explain the observed technological performance differences. Dehydration did not change the pattern.

3.8 Hierarchical clustering reveals similarities among species

In order to obtain further insight into the differences and similarities among species, we performed hierarchical clustering of all the species using the values of the different parameters we had studied compared to those of the control strain in both the fresh and dry cells. The heatmaps generated are shown in Figure 5. We observed that *T. delbrueckii* was the most similar species to *S. cerevisiae* for both conditions. The almost identical behaviour of the closely related *Metschnikowia* species was significant under the fresh and dry conditions. In the fresh cells, *S. bacillaris* showed similarities to

S. cerevisiae and *T. delbrueckii*, which would explain their close behaviour when grown in molasses. They were also the only species in which high trehalose accumulation was detected. For the fresh and dry conditions, *Z. bailii* shared a resemblance to *H. vineae* and *K. wickerhamii*, despite displaying better technological performance, which indicates there may be other factors that come into play.

The heatmap obtained with the data from the dry cells shows that the species with similar ADY performance share like traits in the studied oxidative stress-related parameters. We observed that the species with wide viability (*M. pulcherrima, M. fructicola* and *S. bacillaris*) clustered together. It is worth noting that while *S. bacillaris* showed more similarity to the control strain under the fresh conditions, the changes observed in the different parameters due to dehydration made it closer to the response of the *Metschnikowia* species. These three species all shared marked catalase and glutathione reductase activities, high GSH_{TOT} levels and a high GSH/GSSG ratio compared to the control strain. *W. anomalus* was the species that clustered the closest to *S. cerevisiae* and *T. delbrueckii* in the dry cells. On the opposite end of the spectrum, we found the species with less viability, namely *Z. bailii, K. wickerhamii* and *H. vineae*. In these species, we observed lower trehalose levels (a trait shared with most non-*Saccharomyces* species), and this lack of trehalose accumulation was generally accompanied by lower GSH_{TOT} levels and less catalase activity.

4. Discussion

Interest in wines with complex aroma and flavour profiles has exponentially grown in recent years. In order to meet the demand for these wines, it is necessary to produce non-*Saccharomyces* as dry yeast inocula. However, the industrial biomass propagation process is optimised to produce *S. cerevisiae*, so non-*Saccharomyces* yeasts can be faced with suboptimal growth conditions. Recent works describe the response of different non-*Saccharomyces* wine yeasts to dehydration-related stress (Câmara et al.,

2019a, 2019b; Kim et al., 2019). Notwithstanding, only previous works by our group (Gamero-Sandemetrio et al., 2019, 2018) have studied non-*Saccharomyces* yeasts in simulations under ADY production conditions similar to those used herein, although culture type strains were then used and the drying technology has now been improved.

We analysed eight non-*Saccharomyces* wine species belonging to the Lallemand Inc. non-commercial collection. All these species have a positive effect on wine in mixed fermentations. Our data show that most of these non-*Saccharomyces* species present diminished growth in molasses compared to commercial strain Lalvin T73, in accordance with previously published results (Gamero-Sandemetrio et al., 2018). Only *Z. bailli* and *S. bacillaris* reached similar OD_{600} levels to those of *Saccharomyces*. It is also worth noting that *Z. bailli* was the only strain to exhibit a higher biomass yield than the commercial strain. It has already been established that *Z. bailii* is capable of growing in high sugar environments and is are able to vigorously ferment hexose sugars (Martorell et al., 2007). So it would seem logical that it would be able to grow in molasses.

Yeast viability is the main technological parameter of ADY, especially in non-*Saccharomyces* species, in which high fermentative capacity may not necessarily be a desirable trait. Under our conditions, *M. pulcherrima, M. fructicola* and *S. bacillaris* displayed extremely wide viabilities, which went higher than the 50% of the viable cells observed in the control strain. Although their dehydration kinetics did not equal our conditions, these findings agree with those of (Câmara et al., 2019b; Kim et al., 2019), who reported wide viability in the dry cells of *M. pulcherrima* and *S. bacillaris*. ADY from *T. delbrueckii, Z. bailii* and *W. anomalus* displayed similar viability to the control. The only two strains with a significantly lower viability than the control strain were *K. wickerhamii,* and most notably *H. vineae,* with viability as low as 2%. The dehydration and rehydration conditions strongly impact yeast viability (Dupont et al., 2014; Rodríguez-Porrata et al., 2008), and optimising dehydration kinetics to each species

could be useful for optimising ADY production. Furthermore, it could be helpful to incorporate treatments that have proven useful in improving the survivability of yeasts to different dehydration conditions, such as adding antioxidant agents during growth (Gamero-Sandemetrio et al., 2015), adding protectants like skimmed milk (Lee et al., 2016; Romano et al., 2015) or immobilising cells during dehydration (Borovikova et al., 2014).

The ability of different species to respond to oxidative stress influences how well they can adapt to the dehydration process. We studied a set of biochemical parameters that served as indicators of redox state, which have been previously used in our lab for different *S. cerevisiae* strains (Gamero-Sandemetrio et al., 2014). Greater oxidative damage to lipids is related to worse technological performance. Notwithstanding, we detected no major differences in MDA accumulation among species. As previously mentioned, *M. pulcherrima, M. fructicola* and *S. bacillaris* had the widest viability of the studied yeasts, but curiously enough they had the highest MDA levels. It is likely that membrane composition played a role in the amount of detected MDA. As described in Section 3.7, *M. pulcherrima* is able to produce PUFA, which makes it more sensitive to lipid peroxidation than a MUFA-rich membrane (Ayala et al., 2014; Howlett and Avery, 1997). We expect the same to be true for the closely related *M. fructicola*. We were unable to trace a direct correlation between MDA levels and ADY performance for the studied species.

We have previously described how marked basal catalase activity and its induction after desiccation correlate with better technological performance (Gamero-Sandemetrio et al., 2014). We found exceptionally marked catalase activity in *M. pulcherrima, M. fructicola* and *S. bacillaris,* which correlated with their wide viability after drying, and underlines the importance of this enzymatic activity during dehydration (França et al., 2005). Other species showed catalase induction in the dry cells, but no direct correlation between catalase induction and ADY viability was observed. Oxidative

stress response is a complex interplay of multiple factors (Herrero et al., 2008), and despite species like *K. wickerhamii* or *H. vineae* having similar catalase activities to *S. cerevisiae,* they lack other important defence mechanisms, such as trehalose, which may explain their worse performance.

Glutathione regulation is a complicated process involving a complex signalling network that is not yet fully understood. However, its important role in dehydration tolerance has long since been known (Espindola et al., 2003). After dehydration, GSSG levels are expected to raise due to the oxidative stress that cells face and, as a result, the GSH/GSSG ratio lowers. Notwithstanding, cells may be able to maintain an optimal redox balance by keeping a high GSH/GSSG ratio. This can be achieved by recycling GSSG through great glutathione reductase activity or by inducing glutathione synthesis. We observed how GSSG content increased in almost all the species. However, the GSH/GSSG ratios increased in *W. anomalus, H. vineae, M. pulcherrima* and *S. bacillaris,* which may be due to a combination of the GR activity induction detected in these species and induced GSH_{TOT} accumulation. Once again, the species with wider ADY viability had high glutathione levels and GSH/GSSG ratios. In contrast, the other species displayed lower GSH/GSSG ratios in the dry cells, which is an indicator of an imbalanced intracellular redox state. Accordingly, our results are similar to the findings of (Câmara et al., 2019a) for *S. cerevisiae* and *T. delbrueckii*. In these species, other factors, such as high trehalose accumulation, that we detected could be sufficient for protecting cellular components of oxidative damage.

It has been established that trehalose plays a critical role in desiccation tolerance (reviewed in Eleutherio et al., 2015). In this work, we observed considerable differences in trehalose levels among species. Only *T. delbrueckii* and *S. bacillaris* accumulated trehalose to levels that came close to *S. cerevisiae*. The other species showed almost no accumulation in the fresh cells, and only after desiccating did we observe an increase in trehalose levels, but total levels were very low. Recent works

have reported a correlation between higher levels of trehalose and better desiccation tolerance in different non-*Saccharomyces* wine species (Câmara et al., 2019a; Gamero-Sandemetrio et al., 2018), and that trehalose can be used as a protectant during dehydration to improve the viability of non-*Saccharomyces* yeasts (Kim et al., 2019). With *K. wickerhamii* and *H. vineae,* a direct correlation appears between lack of trehalose accumulation and their poor ADY viability. It is worth noting that we were unable to find trehalose metabolism genes when checking the available genomic data for *K. wichermanii* and *H. vineae*, which explains the lack of trehalose accumulation. Nonetheless, it is meaningful that we were unable to detect high trehalose levels in both *Metschnikowia* species, despite them showing the widest ADY viability along with *S. bacillaris*. These results prove the complexity of oxidative stress response and desiccation tolerance. In these species, deficiency in trehalose accumulation may be balanced out by other factors, such as marked catalase activity and high glutathione levels. The results we obtained in the analysed oxidative stress response parameters allowed us to speculate that oxidative stress response in non-*Saccharomyces* species may not be parallel to that of *S. cerevisiae* because other factors may play an important role in complementing the clear differences that these species have compared to *S. cerevisiae*.

One of these factors may be membrane lipid composition. Works done in *S. cerevisiae* have described the importance of membrane fluidity in stress and dehydration tolerance (Beltran et al., 2008; Redón et al., 2008). For the total FA composition analysis, we selected *H. vineae* and *M. pulcherrima* due to the major differences we observed in their ADY performance, and we maintained *S.* cerevisiae as a control strain. The study of total FA composition revealed large differences among species, which may help to explain the big differences we observed in ADY technological performance. FA composition agreed with the data published for *M. pulcherrima* (Vázquez et al., 2019) and *S. cerevisiae* (Daum et al., 1998). Our data represent the

first lipid composition analysis in *H. vineae* but according to Rozès et al. (1992), it seems very close to the genus *Hanseniaspora*. Indeed these authors found equivalent percentages in C16:1 and C18:1 for *H. uvarum* (CBS 5914). *M. pulcherrima* was the only species to produce PUFAs, which confer the membrane better fluidity and help to adapt to stressful conditions. *S. cerevisiae* cannot produce PUFAs (Stukey et al., 1990), but its oleic acid (C18:1) content was higher than in *H. vineae*, whose more prevalent FA was palmitoleic acid (C16:1). Oleic acid supplementation has been used to mitigate oxidative stress during wine fermentation (Landolfo et al., 2010), while palmitoleic acid has been related to greater membrane rigidity (Redón et al., 2009). Sterols are also important in maintaining membrane integrity and fluidity (Daum et al., 1998), and ergosterol, the main sterol found in yeast, is necessary against oxidative stress as mutants lacking it are hypersensitive to oxidative stress (Higgins et al., 2003). Once again, *M. pulcherrima* presented the highest percentage of ergosterol which, in combination with its FA content, makes it better suited to face the harsh changes and stressful conditions linked to the dehydration process. Finally, the lipid composition of *H. vineae*, along with a deficient oxidative stress response, can help to explain its poor performance under ADY propagation conditions. It is not an adaptative mechanism because lipid composition did not change with dehydration.

5. Conclusions

In conclusion, our results suggest that the wide variability observed in the technological performance of non-*Saccharomyces* wine yeasts could be due to major differences in oxidative stress response parameters. We found that three species (*M. pulcherrima, M. fructicola* and *S. bacillaris*) had wider ADY viability than a commercial *S. cerevisiae* strain. Compared to the control strain, all these species showed exceptionally good catalase activity, high glutathione levels and a higher GSH/GSSG ratio. We also found that *M. pulcherrima* was rich in PUFAs and ergosterol, which may also play a role in adaptation to dehydration conditions. Conversely, the species with poor technological

performance, such as *H. vineae*, displayed similar redox parameters to the control strain, except for lack of trehalose accumulation and a different lipid composition. The response against oxidative stress in the non-*Saccharomyces* species is not parallel to what has been previously described for *S. cerevisiae* strains. The apparent differences that we observed in oxidative stress defence mechanisms between non-*Saccharomyces* and *S. cerevisiae* seemed to be balanced by other factors, and the interplay among all them appeared to be determinant for ADY viability.

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Tables

Table 1. Physiological parameters after 24 h of growth in molasses. Fermentative capacity was measured in the fresh and dry cells. All the measurements were taken in triplicate. Different letters denote a significant difference among species with a $p < 0.05$.

Species	OD_{600}	Biomass yield (g biomass / g sucrose)	% CFU after dehydration	Fermentative capacity, fresh cells (mL CO_2 / 10^7 cells)	Fermentative capacity, dry cells (mL CO_2 / 10^7 cells)	Fermentative capacity loss (%)
S. cerevisiae	20.23 (± 1.68) [ab]	0.09 (± 0.01) [b]	48.55 (± 5.11) [bc]	16.07 (± 1.97) [a]	7.56 (± 0.70) [a]	52.96
Z. bailii	20.60 (± 0.78) [a]	0.15 (± 0.01) [a]	37.93 (± 7.09) [c]	12.23 (± 2.28) [b]	2.83 (± 0.18) [c]	76.86
T. delbrueckii	13.52 (± 0.94) [d]	0.09 (± 0.01) [b]	56.24 (± 7.49) [b]	8.11 (± 1.64) [c]	5.02 (± 0.47) [b]	38.10
K. wickerhamii	10.12 (± 1.09) [de]	0.06 (± 0.02) [c]	23.18 (± 5.16) [d]	2.51 (± 0.52) [e]	1.02 (± 0.07) [f]	59.36
W. anomalus	14.91 (± 1.55) [cd]	0.09 (± 0.02) [ab]	33.76 (± 6.55) [c]	1.95 (± 0.25) [f]	1.18 (± 0.11) [d]	39.49
H. vineae	7.72 (± 0.54) [e]	0.06 (± 0.01) [c]	1.83 (± 0.51) [e]	5.27 (± 0.20) [d]	0.33 (± 0.03) [g]	93.74
M. pulcherrima	7.35 (± 1.06) [e]	0.05 (± 0.01) [c]	80.29 (± 5.07) [a]	3.19 (± 0.57) [e]	1.47 (± 0.08) [e]	53.92
M. fructicola	8.30 (± 0.92) [e]	0.07 (± 0.01) [bc]	88.69 (± 5.13) [a]	2.65 (± 0.09) [e]	1.74 (± 0.02) [d]	34.33
S. bacillaris	18.41 (± 0.56) [bc]	0.07 (± 0.01) [c]	80.46 (± 9.70) [a]	2.77 (± 0.32) [e]	1.25 (0.04) [ef]	54.87

Figure legends

Figure 1. Cell growth in YPD plates for 24 h at 30 ºC, 37 ºC and 30 ºC in the presence of 3.5 mM H_2O_2.

Figure 2. Determination of oxidative damage and defence parameters in the fresh (blue bars) and dry (yellow bars) cells. A) Lipid peroxidation measured as pmol of malondialdehyde (MDA) per mg of cells. B) Catalase activity, expressed as specific activity per mg of protein. C) Glutathione reductase activity, expressed as specific activity per mg of protein. D) Trehalose accumulation, expressed as µg of trehalose per mg of cells. Error bars correspond to the SD of three independent experiments. (*) Significantly differ from their respective fresh cell samples with a $p < 0.05$.

Figure 3. Determination of glutathione levels in the fresh (blue bars) and dry (yellow bars) cells. A) Total glutathione measured as nmol of malondialdehyde (MDA) per mg of cells. B) Oxidised glutathione (GSSG). C) Reduced glutathione (GSH). D) GSH/GSSG ratio. Error bars correspond to the SD of three independent experiments. (*) Significantly differ from their respective fresh cell samples with a $p <0.05$.

Figure 4. Determination of the total lipid content in *S. cerevisiae*, *H. vineae* and *M. pulcherrima*. The total FA percentage in A) the fresh cells and in B) the dry cells. Determination of the total sterol content in C) the fresh cells and in D) the dry cells. Error bars correspond to the SD of three independent experiments. Different letters denote a significant difference among species for the studied compound at $p < 0.05$.

Figure 5. Hierarchical clustering of yeast species based on the relative value of the different parameters (columns) compared to *S. cerevisiae* in A) the fresh cells and B) the dry cells. Total values for each parameter were made relative to the *S. cerevisiae* values and Log2 was calculated. The colour palette indicates the value of variation from the lowest (green) to the highest (red), as indicated by the colour key**.**

Figure 1

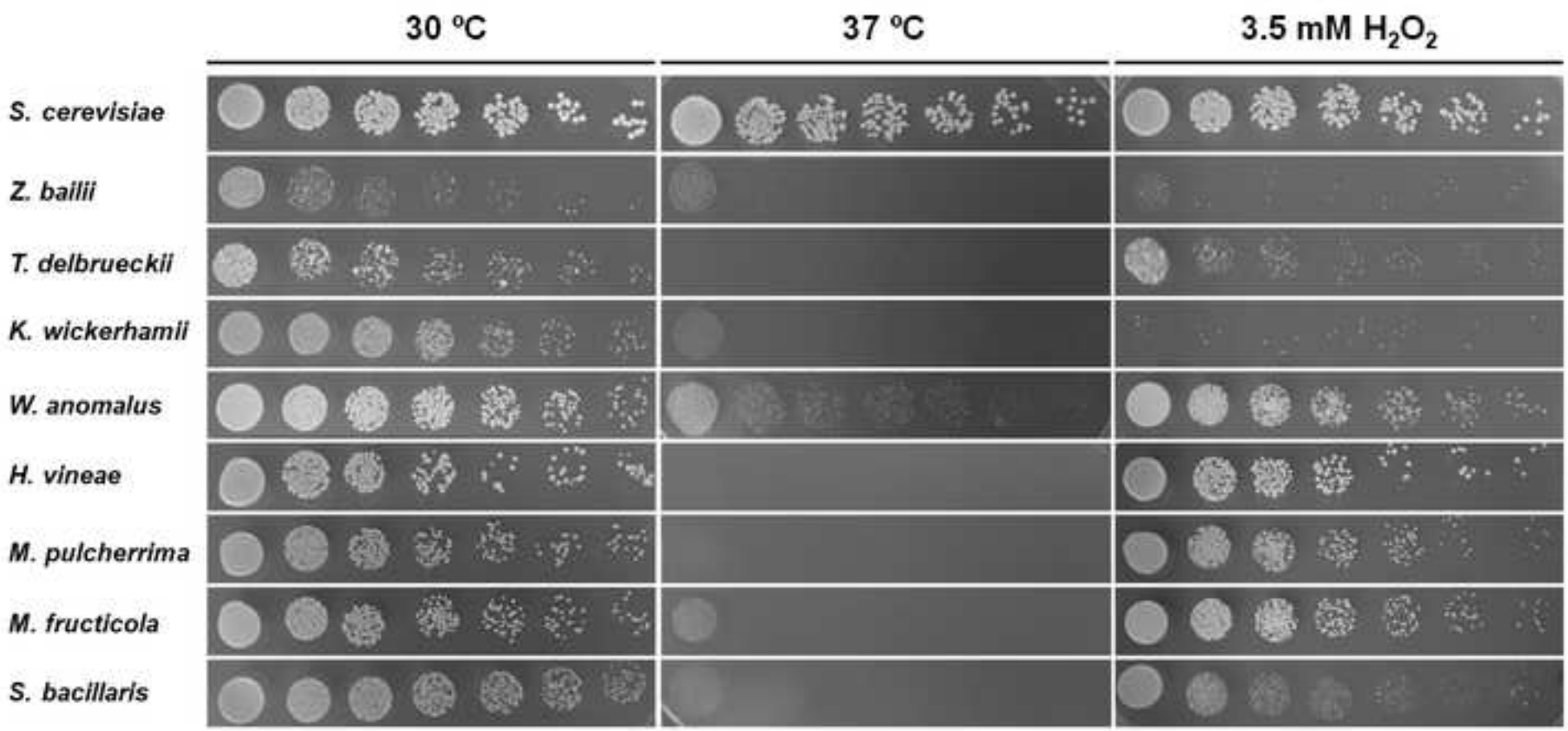


Figure 2

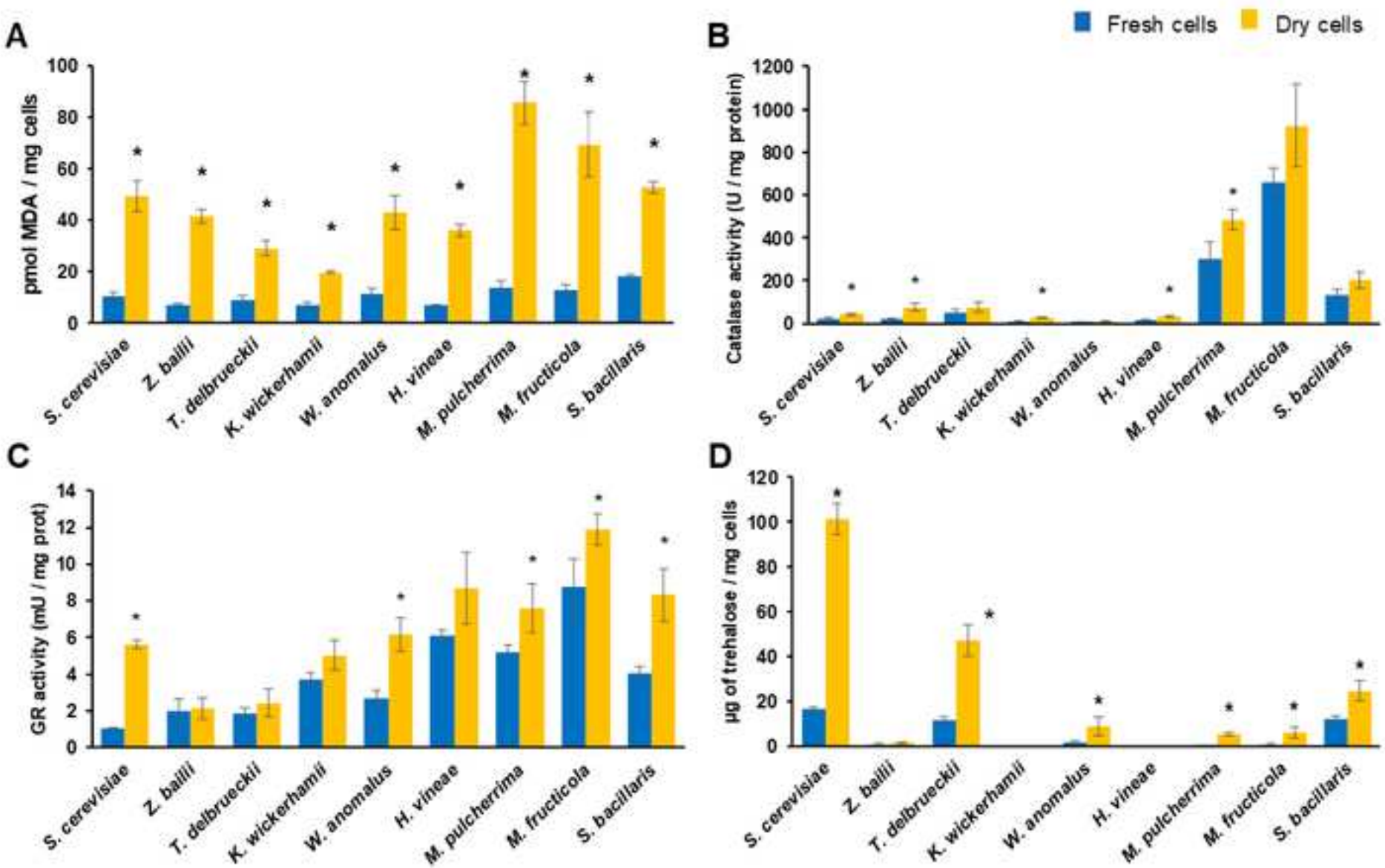


Figure 3

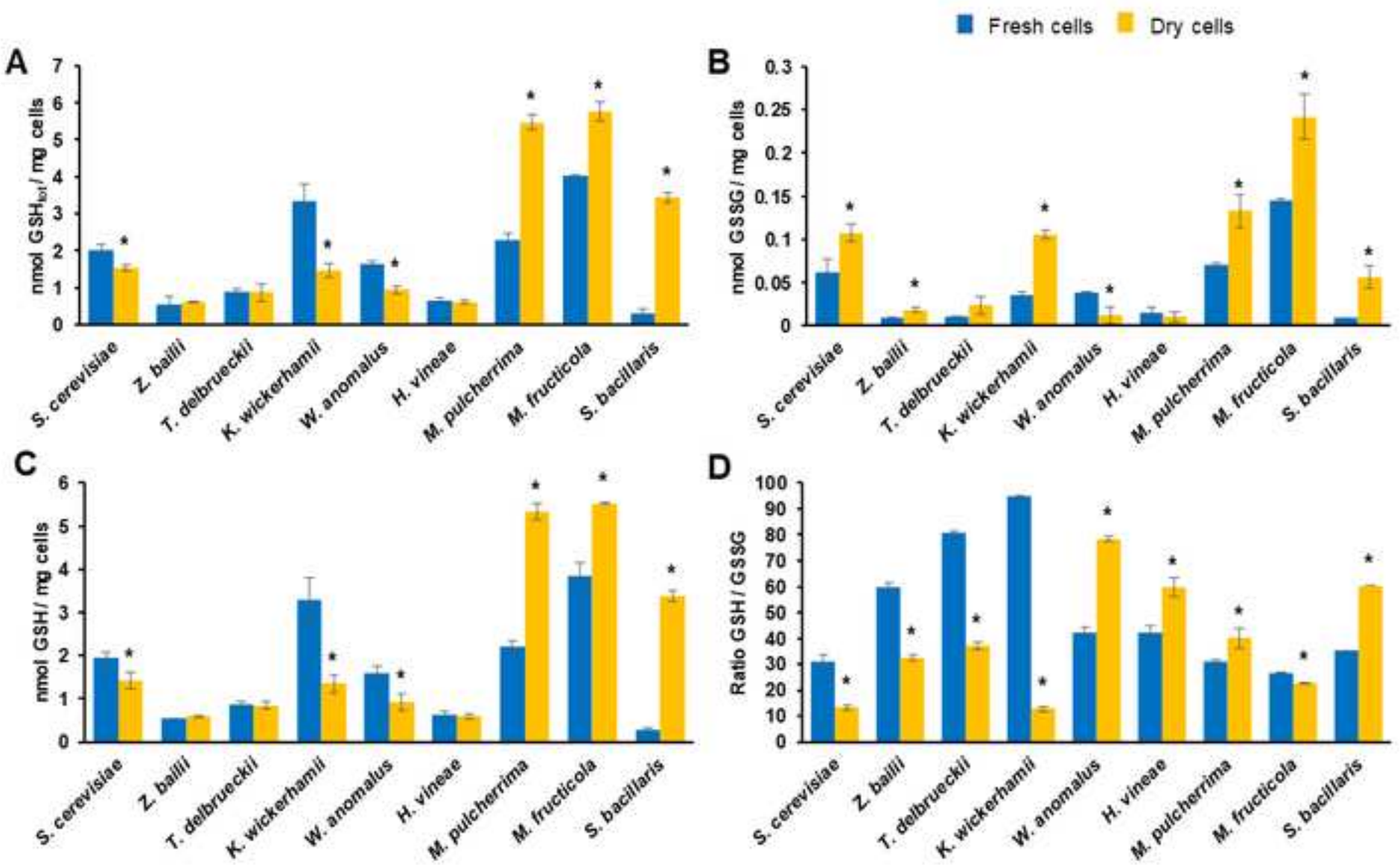


Figure 4

Click here to access/download;Figure;fig4.tif

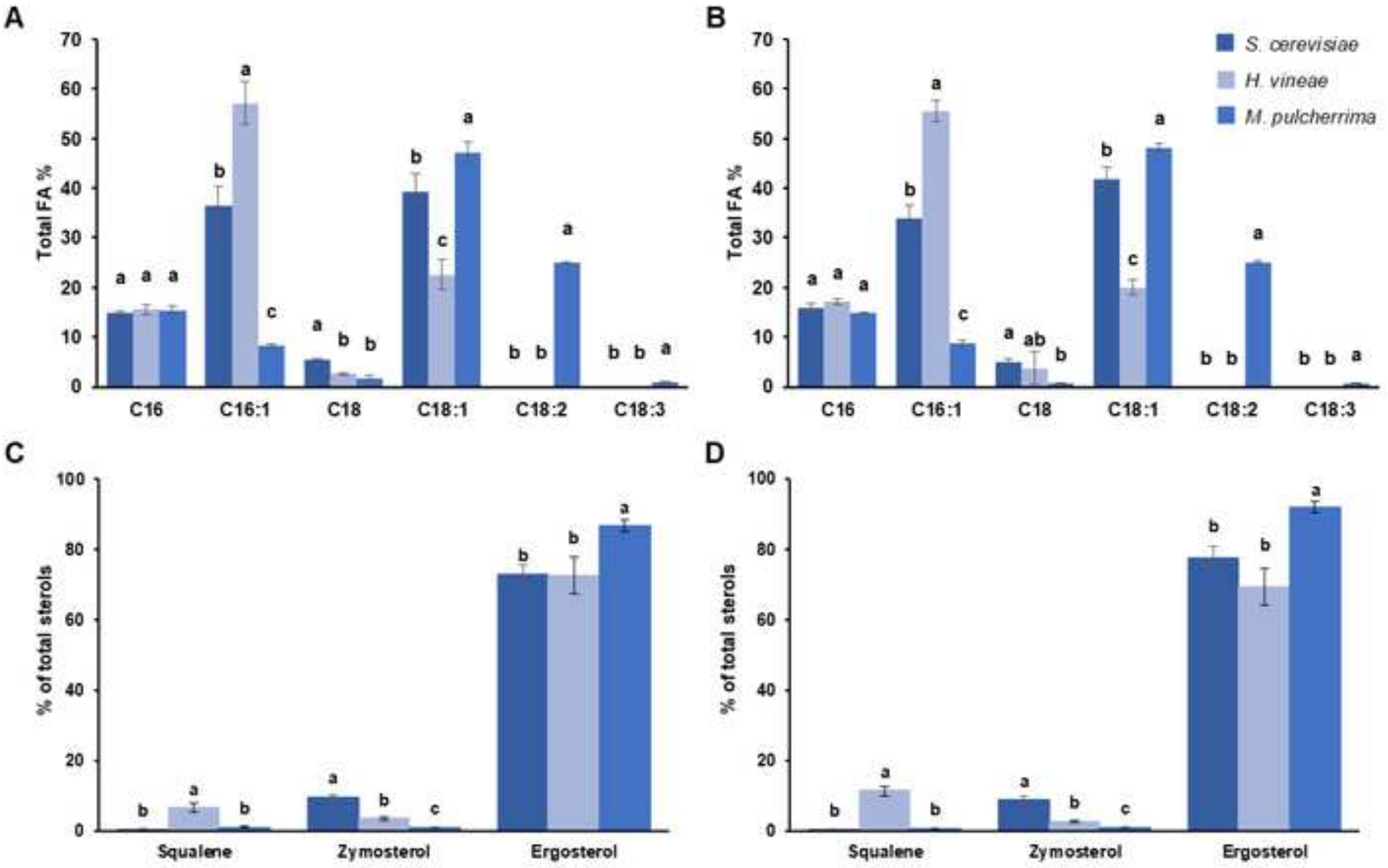
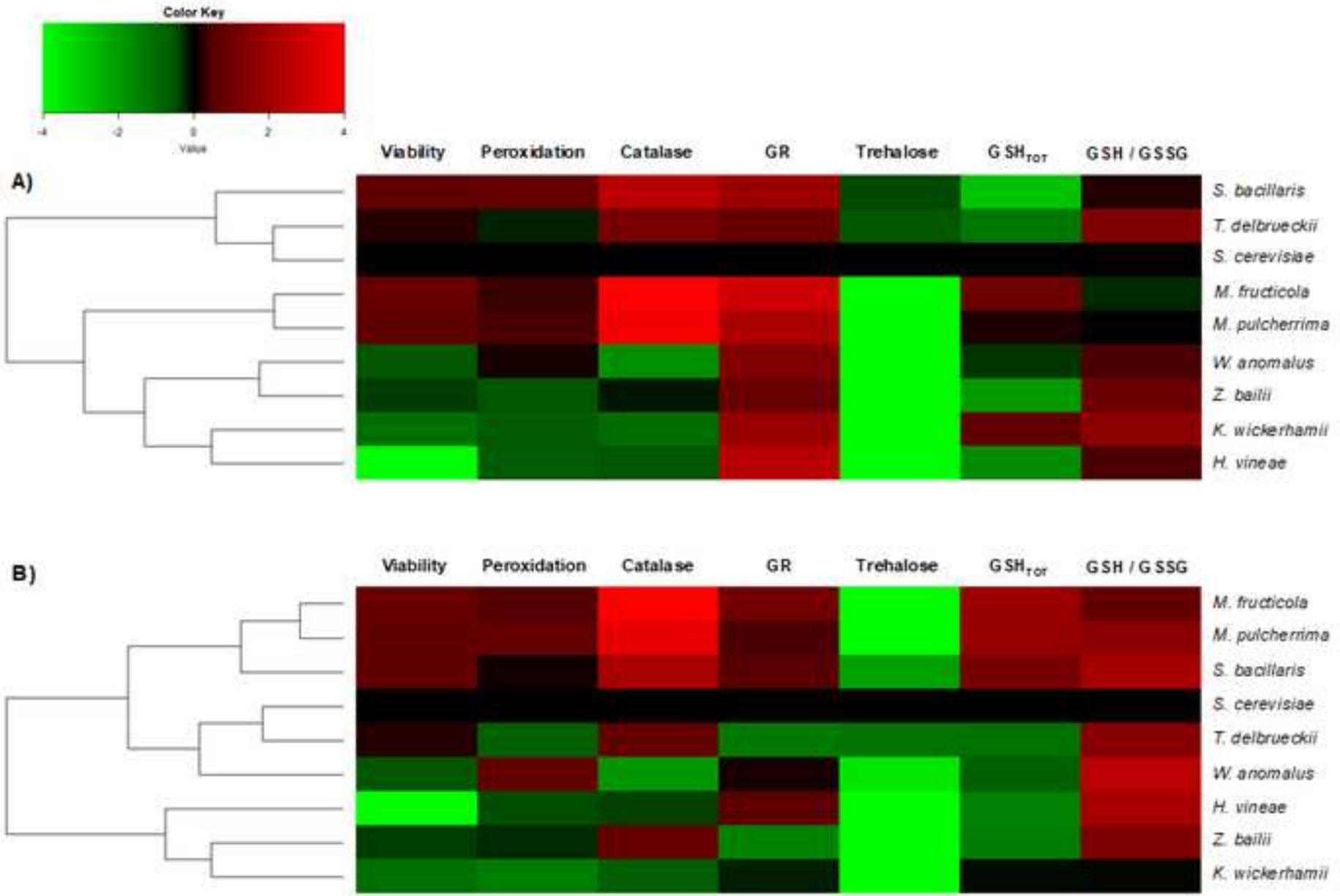


Figure 5



The authors declare that they have no conflict of interests