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Investigating the glutathione accumulation by non-conventional wine yeasts in optimized growth conditions and multi-starter fermentations

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ABSTRACT

Glutathione (GSH) is the most abundant non-protein thiol in yeasts and plays an important role as antioxidant in wine. Application of selected GSH-accumulating strains can improve wine sensorial quality and stability, but most selection programs have been conducted on *Saccharomyces cerevisiae*. Therefore, the aim of the study was to evaluate the ability of different non-*Saccharomyces* yeasts, i.e. *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris*, to produce glutathione. This feature was evaluated in an optimized medium under aerobiosis (MGSH) and in microfermentations using synthetic grape juice (SGJ) and pasteurized grape juice (PGJ), in single or sequential inoculations with *S. cerevisiae*. The non-*Saccharomyces* yeasts showed a good capacity to produce GSH, particularly *Metschnikowia* spp., which achieved significantly greater concentrations. As expected, the strains presented higher growth and glutathione accumulation in MGSH than in SGJ and PGJ. In the microvinification trials, GSH production was higher in PGJ than SGJ, while cell growth was similar, indicating the influence of grape juice composition on GSH metabolism. Taken together, our results demonstrated that selected non-conventional yeast strains, with proven interesting oenological traits, have a good potential to produce GSH, and thus are more suitable and worthwhile for application as active or inactivated dry yeasts preparations.

1. Introduction

Wine is obtained from a very straightforward alcoholic fermentation of grape musts, spontaneously carried out by multifaceted indigenous microbial populations colonizing grape berries and winery equipment. Although spontaneous fermentation presumably leads to more complex and distinctive products, the unpredictability and elevated risk of spoilage have commanded the development of yeast starter cultures. Stability and reliability of pure cultures in the form of active dry yeasts (ADY) is a cornerstone of wine industry. *Saccharomyces cerevisiae* has been comfortably reigning alone for most of the time, but this picture started to change since a few decades ago, when species of non-conventional yeasts began to claim their potential to participate, as well (Bonciani et al., 2018; Câmara et al., 2019b; Gamero-Sandemetrio et al., 2018).

Three relevant examples of non-*Saccharomyces* yeast species widely studied in oenology are *Starmerella bacillaris*, *Lachancea thermotolerans* and *Metschnikowia pulcherrima* (Englezos et al., 2017; Morata et al., 2018, 2019). They are being more and more proposed as co-starter cultures with *S. cerevisiae* due to their desirable contribution to the fermentation process and sensory characteristics of wine. Indeed, important flavor and aroma compounds produced by them as secondary metabolites do not belong to *S. cerevisiae* repertoire or are produced in small amount. Nevertheless, still little is known about the formation, release and metabolism of many of these molecules by non-conventional yeasts, such as the natural antioxidant glutathione and other non-volatile thiols (Bonciani et al., 2018; Câmara et al., 2019a; Gamero-Sandemetrio et al. 2018; Jolly et al. 2014; Penninckx, 2002).

Glutathione is the most abundant low molecular thiol in biological systems (De Vero et al., 2017; Lavigne et al., 2007). This sulfur tripeptide, composed of L-γ–glutamyl-L-cystinyl-glycine, is prevalent inside the eukaryotic cells in the reduced form (GSH), but it could also be oxidized (GS-SG) or bound to other molecules (GS-S-CoA and GS-S-Cys). In yeast cells, some roles played by GSH include: protection against oxidative stress by preventing the formation of reactive oxygen species (ROS), detoxification of xenobiotic compounds, preservation of membrane integrity, protein folding, and as a source of nitrogen and sulfur during nutritional limitations (De Vero et al., 2017; Lavigne et al., 2007;

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Penninckx, 2002).

GSH is synthesized intracellularly in two ATP-dependent steps: firstly, γ-glutamyl- L-cysteine synthetase links L-cysteine to L-glutamate forming γ-glutamyl- L-cysteine; then glutathione synthetase adds L-glycine to this intermediary, completing the final product (Mezzetti et al., 2017). GSH homeostasis within the cell is controlled by the cytoplasmic biosynthesis, compartmentalization, degradation and consumption. It is strictly correlated with nitrogen and sulfur metabolism (De Vero et al., 2017).

Although being naturally present in must and wines at low concentrations (around a few milligrams per liter), GSH amount can vary greatly depending on the reactions during the fermentation process, including yeast metabolism (De Vero et al., 2017; Kritzinger et al., 2013). GSH acts as a strong antioxidant and helps to preserve wine quality by inhibiting polymerization of phenolic compounds related to detrimental change of color and by limiting development of off-flavors during aging, such as sotolon and 2-aminoacetophenone (Kritzinger et al., 2013; Mezzetti et al., 2017). It also protects important aromatic molecules (esters, terpenes, volatile thiols) produced by yeasts during fermentation (Bonciani et al., 2018; Rodríguez-Bencomo et al., 2014). Moreover, presence of glutathione in wine could partially replace sulfur dioxide addition, which is a traditional antioxidant used in winemaking, but recently associated with negative impacts on consumer preference, due to its possible adverse health effects (De Vero et al., 2017; Gabrielli et al., 2017).

There is hence a growing oenological and biotechnological interest in yeast strains with enhanced GSH production, which might be profitable in two fronts at the same time. First, differences on glutathione formation and metabolism among wine yeast strains could help to guide the selection of strains better adapted to environmental stresses occurring during industrial production of ADY and later on alcoholic fermentation (Gamero-Sandemetrio et al., 2018; Granchi et al., 2019; Mezzetti et al., 2017; Penninckx, 2002). Secondly, GSH-producing strains would also be advantageous during winemaking process and wine storage, aiding to protect wine against oxidation phenomena, either as GSH-enriched inactivated dry yeasts (IDY) or as starter cultures naturally producing high levels of glutathione *in situ* (Bonciani et al., 2018; De Vero et al., 2017; Gabrielli et al., 2017). Nevertheless, as the usage of genetically modified organisms, a commonly investigated tool for bulk GSH production, is generally not accepted by consumers in food products (De Vero et al., 2017; Schmacht et al., 2017), the GSH-enriched yeasts have to be mined from the natural biodiversity.

In this context, the present research aimed to characterize the GSH production in selected vineyard-associated native strains of *L. thermotolerans*, *Metschnikowia* spp. and *S. bacillaris*, previously characterized *in vitro* for a number of relevant oenological parameters (Binati et al., 2019), and evaluated in microvinification trials (Binati et al., 2020). The selected non-*Saccharomyces* yeasts were tested in diverse media, in single and sequential inoculation with *S. cerevisiae* EC 1118, in agitated or static conditions, mimicking both biomass-production and fermentation processes. To complement the quantification of GSH content, fermentation kinetics and population dynamics of the yeast strains were evaluated, as well.

2. Material and methods

2.1. Yeast strains and inoculum preparation

Saccharomyces cerevisiae EC 1118 and the non-*Saccharomyces* yeasts *L. thermotolerans* SOL13, *S. bacillaris* MALV45 and *Metschnikowia* sp. FIANO12 were used in this study. The main oenological features used for the selection of the native non-*Saccharomyces* yeast strains, isolated from grape musts in Italy, are indicated in Table 1. All strains are deposited in the local culture collection of the Department of Biotechnology of the University of Verona (Italy). They were routinely cultivated in YPD broth (10 g/L Yeast extract, 20 g/L bacteriological Peptone, 20 g/L Dextrose) at 27 °C and maintained at −80 °C in glycerol (25 %v/v).

Table 1
Source of isolation and main distinctive oenological traits of the vineyard-associated yeast strains used in the present study (from Binati et al., 2019, 2020).

Strain	Grape variety	Location	Distinctive oenological features
Lachancea thermotolerans SOL13	Solaris	Grumes (TN), Trentino-Alto Adige	High lactic acid and low ethanol production
Starmerella bacillaris MALV45	Malvasia Candia	Vigolzone (PC), Emilia-Romagna	Fructophilic character, high glycerol and low acetaldehyde production
Metschnikowia spp. FIANO12	Fiano Minutolo	Gravina in Puglia (BA), Puglia	Presence of enzymatic activities and high production of aromatic higher alcohols and esters

The inoculum was prepared as previously described (Binati et al., 2020). Yeasts were grown overnight in YPD broth at 27 °C with shaking to reach the early stationary phase. Then, cells were harvested by centrifugation (3000×*g*, 5 min), washed twice with physiological solution (0.9% NaCl), re-suspended in the media described below, and microscopically counted with a Burker counting chamber to adjust the concentration of the inoculum.

All reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Medium optimized for glutathione production (MGSH)

Growth in conditions to boost GSH production was carried out in the optimized medium of Santos et al. (2007) containing: 54 g/L glucose, 50 g/L yeast extract, and 12 g/L $MgSO_4$, pH 5.0, hereafter referred as MGSH. Following the indications of Wen et al. (2005), the medium was supplemented 7 h after inoculation with 18 mM glycine, 10 mM glutamate, and 3.35 mM cysteine.

The standardized inoculum of each non-*Saccharomyces* yeast and EC 1118, prepared as described above, was transferred to sterile 250-mL Erlenmeyer flasks containing 150 mL of MGSH medium, at a 5% inoculum rate. The test was performed in triplicate, and flasks were incubated at 27 °C in an orbital shaker KS 260 basic (IKA, Staufen, Germany) at 200 rpm for 96 h.

2.3. Synthetic grape juice (SGJ) and pasteurized grape juice (PGJ)

A synthetic medium to mimic the composition of grape juice (SGJ) was prepared following the indications of Bely et al. (1990) and Rossignol et al. (2003). The medium was then modified by splitting the sugar source in 100 g/L glucose and 100 g/L fructose, and by adding 2 g/L L-malic acid.

A commercial biological red grape juice (Folicello Società Agricola, Modena, Italy) was used in a second set of fermentation trials. This pasteurized grape juice (PGJ) was made with Lambrusco Grasparossa and Sangiovese grapes [225 g/L of sugar, 46.5 mg/L of YAN (Yeast Available Nitrogen) and 18 mg/L of SO_2].

Each non-*Saccharomyces* yeast strain was inoculated at a concentration of approximately 1×10^6 cells/mL in sterile 200-mL glass bottles filled to the top with SGJ or PGJ, equipped with perforated silicon stoppers combined with 0.45-mm filters (Merck Millipore, Milan, Italy), to let the carbon dioxide release and prevent contamination. The bottles were kept under static conditions at 22 °C. For the trials in SGJ, single and mixed culture fermentations with the strain EC 1118, sequentially inoculated 48 h later at the same concentration, were carried out. *S. cerevisiae* EC 1118 was also singly inoculated as a control, at time 0 and after 48 h. For the trials in PGJ, only sequential fermentations were carried out, besides the control with EC 1118. All trials were performed in triplicate.

2.4. Fermentation kinetics and yeast enumeration

Fermentation kinetics during the growth in SGJ and PGJ were monitored by daily weighing the bottles, reflecting the loss due to CO_2 release. Fermentations were stopped when the daily weight loss was below 0.05 g, and nonetheless no longer than 40 days in SGJ and 45 days in PGJ.

For yeast enumeration, samples were taken after 24, 72 and 96 h in MGSH and throughout the fermentation in SGJ and PGJ. Appropriate serial dilutions were spread plated on Wallerstein Laboratory (WL) nutrient agar (Sigma-Aldrich), and colonies were counted after incubation at 27 °C for 48 h. In mixed culture fermentations, non-*Saccharomyces* yeasts could be differentiated in the countable plates (from 30 to 300 colonies) based on the morphological particularities that distinguish them from *S. cerevisiae* (Binati et al., 2019).

2.5. Glutathione assay and biomass determination

After 24, 72 and 96 h in MGSH, and at the end of fermentations in SGJ and PGJ, samples for GSH quantification were collected and kept in 50-mL conical flasks. Intracellular glutathione was extracted from the cells, obtained after centrifugation of the samples (5000×*g*, 10 min), following the protocol described by Xiong et al. (2009): the pellet was washed twice with 1 mL of physiological solution; 500 μL of ethanol (25% v/v) were added to the tubes and they were incubated for 2 h at 30 °C; the tubes were centrifuged again (5000×*g*, 10 min), and the supernatant was used for the GSH assay. Quantification of the reduced form (GSH) was carried out with the enzymatic Glutathione Assay Kit (Sigma-Aldrich), according to the manufacturer's instructions, using a standard curve generated with known concentrations of GSH, built simultaneously to every batch of analysis.

Cell biomass concentration was determined by cell dry-weight; wet cell pellets were dried at 60 °C from 2 to 3 days to constant weight. GSH levels in nmol/mL were then normalized to nmol/mg of cells for every sample.

2.6. Statistical analysis

Data were compared by one-way ANOVA (Analysis of Variance), followed by the post-hoc Tukey's HSD (Honestly Significant Difference) test, using the software PAST 4.01 (Hammer et al. 2001). The threshold for statistical significance was set at 95% (*p-value* < 0.05).

3. Results

3.1. Growth in MGSH medium

Slight differences in population dynamics of strains were observed in MGSH medium, even though *S. cerevisiae* EC 1118 presented viable cell concentrations lower than the three non-*Saccharomyces* yeasts (Fig. 1). SOL13, MALV45 and FIANO12 showed the same counts at 72 h, but then cell viability of FIANO12 decreased around the same level as EC 1118 (8.3 $\log_{10}$ CFU/mL), while SOL13 and MALV45 reached 8.6 $\log_{10}$ CFU/mL at 96 h of incubation.

Cell dry-weight values were not significantly different ($p > 0.05$) among the strains at 24 h, but biomass of SOL13 (4.81 g/L) was significantly higher ($p < 0,05$) than the other strains at the end of incubation (Fig. 1).

The time-course of GSH accumulation is shown in Fig. 2. Different behaviors were observed among the strains. Increasing levels of GSH were produced during growth of *S. cerevisiae* and *L. thermotolerans*, reaching concentrations of 3.13 and 2.57 nmol/mg cell, respectively, at the end of incubation. Maximal GSH content (4.59 nmol/mg cell), significantly higher ($p < 0,05$), was presented by *Metschnikowia* sp. at 72 h, but, differently from the other strains, a decrease of the GSH level (2.83 nmol/mg cell) was detected after 96 h. Conversely, *S. bacillaris* produced very low amounts of GSH (maximum of 0.08 nmol/mg cell), although growth dynamic and biomass (Fig. 1) were comparable to the other yeasts.

3.2. Microvinification in synthetic grape juice

A chemically defined medium (SGJ) was prepared in laboratory to mimic the composition of grape must. The time-course of fermentation with single and sequential cultures in SGJ is shown in Fig. 3A. Any of the single cultures of the non-*Saccharomyces* yeasts was able to finish the fermentation, in accordance with their limited fermentative capacity and alcohol tolerance (Barbosa et al., 2018; Lemos Junior et al., 2019; Porter et al., 2019). In addition, fermentation of *L. thermotolerans* got stuck after 20 days.

Fermentation kinetics of the mixed cultures in SGJ revealed deleterious interactions between the non-*Saccharomyces* yeasts and EC 1118, since the fermentation length increased and the CO_2 production was lower respect to the control. However, the sequential inoculation with *Metschnikowia* sp. (which had the lowest fermentative capacity when

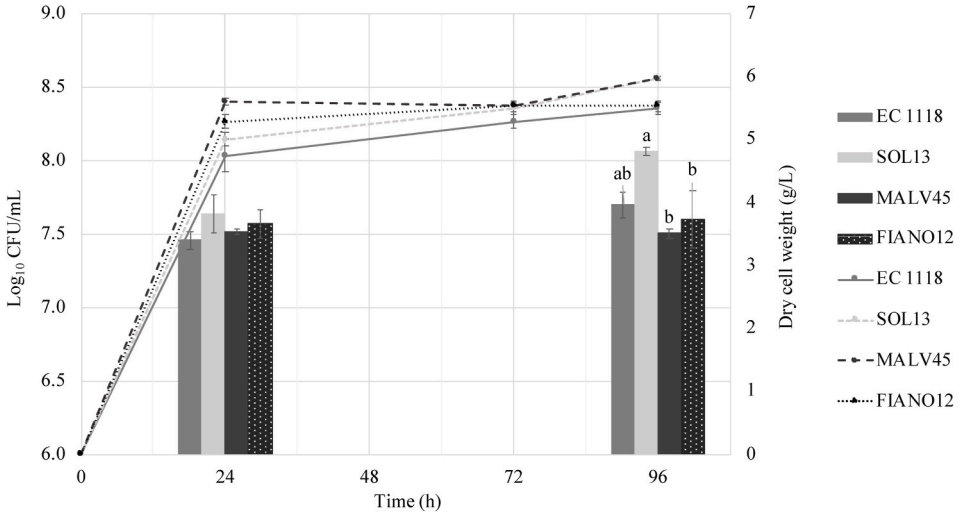

Fig. 1. Yeast population dynamics (lines) and cell dry-weight (bars) during growth of *L. thermotolerans* SOL13, *S. bacillaris* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in MGSH medium. Error bars are representative of standard deviations for three independent replicates. Different letters above the bars indicate a significant difference in Tukey's HSD test ($p < 0.05$).

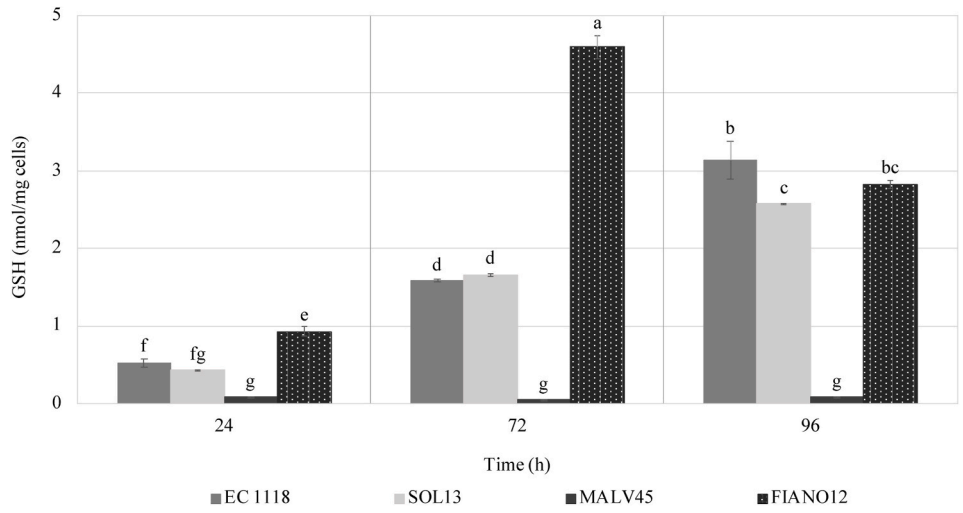

Fig. 2. GSH concentration during growth of *L. thermotolerans* SOL13, *S. bacillaris* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in MGSH medium. Error bars are representative of standard deviations for three independent replicates. Different letters above the bars indicate a significant difference in Tukey's HSD test ($p < 0.05$).

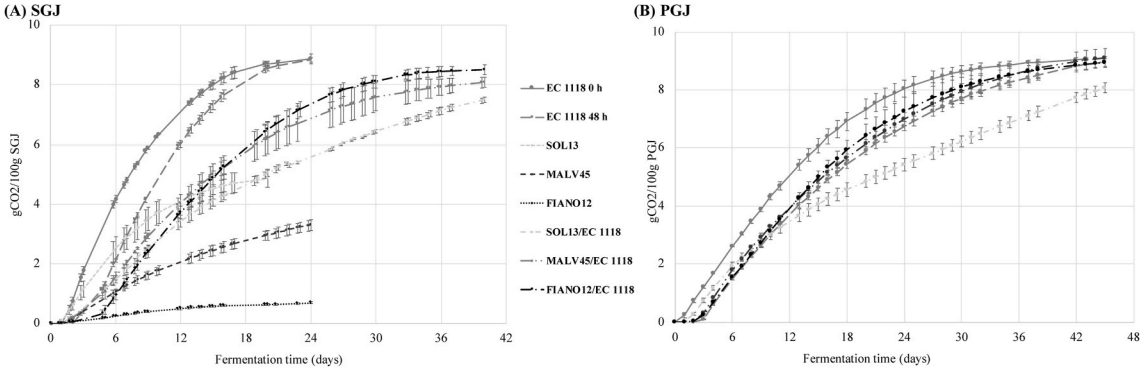

Fig. 3. Fermentation kinetics of single and sequential cultures of *L. thermotolerans* SOL13, *S. bacillaris* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in synthetic grape juice (A) and pasteurized grape juice (B). Pure culture of *S. cerevisiae* EC 1118, inoculated at time 0 or after 48 h, was used as a control. Error bars are representative of standard deviations for three independent replicates.

singly inoculated) was the closest to the behavior of single *S. cerevisiae*. On the other hand, *L. thermotolerans*, that displayed the fastest fermentation kinetics among the single non-*Saccharomyces* yeasts, in combination with *S. cerevisiae* showed the most impaired kinetics curve. Such negative interactions could have been potentialized in the synthetic fermentation medium, possibly due to competition for essential nutrients present in limiting concentrations.

The viable counts of yeast populations are shown in Fig. 4A. In single inoculations, all yeasts reached 10^7 CFU/mL in 48 h, then started to decrease until the end of fermentation, confirming that SGJ could not sustain optimal growth. FIANO12 had the most accentuated decrease in cell viability, agreeing with the slower fermentation rate. Regarding mixed culture fermentations, all strains showed lower cell concentrations when compared with their single inoculation and *S. cerevisiae* was able to overcome all the three non-*Saccharomyces* yeast strains. The most affected strain was FIANO12, which was undetectable (under 10^4 CFU/mL) after the 5th day of fermentation with EC 1118. The behavior of SOL13 and MALV45 was similar, even though the loss of viability of *L. thermotolerans* was more pronounced soon after the inoculation of EC 1118. The competitiveness between *L. thermotolerans* and *S. cerevisiae* in mixed fermentations was reported by several authors (Petitgonnet et al., 2019; Shekhawat et al., 2019).

Table 2 shows the values of biomass and GSH production measured at the end of fermentations in SGJ. Cell biomass concentration was significantly higher ($p < 0,05$) for the trial SOL13/EC 1118 (1.136 g/L) and significantly lower for FIANO12 (0.320 g/L). On the other hand, even with a lower growth and CO_2 production, FIANO12 produced significantly higher ($p < 0,05$) amount of GSH (0.191 nmol/mg cell) compared to all other trials. The sequential inoculation FIANO12/EC 1118 had the second highest value, similar to single EC 1118. The lowest GSH production (0.036 nmol/mg cell) by SOL13/EC 1118 showed that this interaction was deleterious for GSH accumulation, whereas at the same time beneficial for the biomass production.

3.3. Microvinification in pasteurized grape juice

A second microvinification trial was carried out setting up sequential fermentation in pasteurized grape juice (PGJ).

Mixed-culture fermentations of MALV45 and FIANO12 showed similar progressions and finished in 45 days, producing almost the same level of CO_2 as the control (Fig. 3B). Instead, the sequential fermentation of SOL13 was faster during the first 10 days, then slowed down highlighting again the deleterious association with EC 1118.

It can be noted that EC 1118 faced more difficulties to carry out alcoholic fermentation in PGJ respect to SGJ (Fig. 3), taking around double the time to finish the process. Although initial sugar concentration and temperature were similar in both fermentations, PGJ contained only 46.5 mg/L YAN, around six times less than SGJ. It is usually agreed

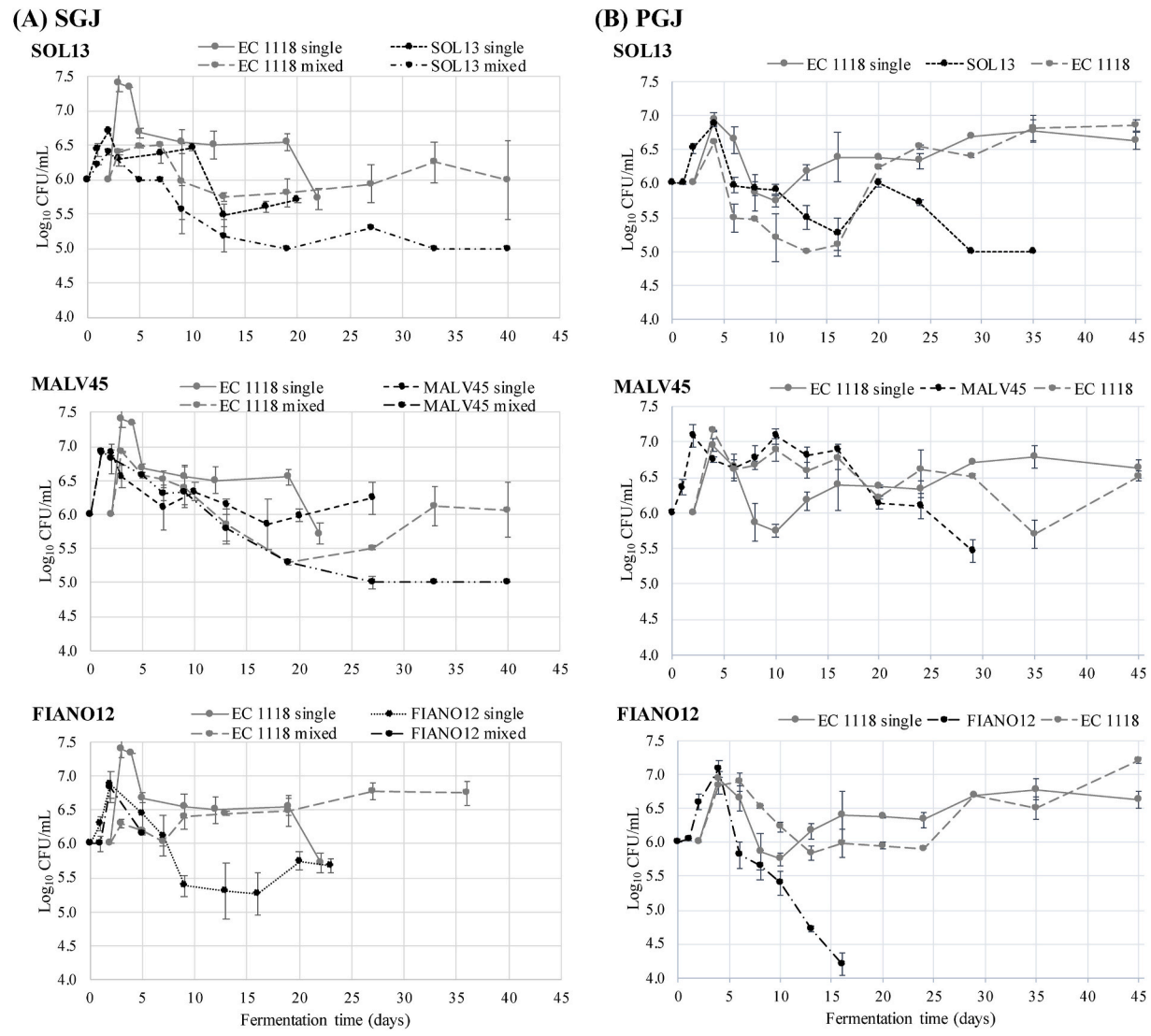

Fig. 4. Yeasts population dynamics during single and sequential fermentations of *L. thermotolerans* SOL13, *S. bacillaris* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in synthetic grape juice (A) and pasteurized grape juice (B). The curve of *S. cerevisiae* EC 1118 inoculated singly after 48 h is reported in each graph for a better comparison. Error bars are representative of standard deviations for three independent replicates.

that a minimal of 140 mg/L is necessary for an optimal fermentation performance, while lower values can lead to stuck or sluggish process (Gobert et al., 2019).

The population dynamics (Fig. 4B) highlighted clear differences among the non-*Saccharomyces* yeasts and the response of EC 1118. The three non-*Saccharomyces* yeasts showed a similar trend of exponential growth in the first days, but, following the inoculation of EC 1118 after 48 h, the populations started to drop and decreased below 10^4 CFU/mL before the end of fermentations. FIANO12 was the first to decrease under that level (after 16 days), while MALV45 reached the closest concentrations to EC 1118, as observed in SGJ. SOL13 grew less than MALV45, but caused a stronger decrease of EC 1118.

Cell biomass concentration was similar between SGJ and PGJ, although slightly higher for the single EC 1118 and lower for the mixed cultures in PGJ (Table 2). The sequential inoculation of SOL13/EC 1118 reached again the significantly highest ($p < 0,05$) cell dry-weight at the end of fermentation (0.965 g/L). GSH production was markedly higher in PGJ than SGJ for all fermentations, most notably MALV45/EC 1118 (0.627 nmol/mg cell), which had an increase of 10 times. MALV45/EC 1118 and FIANO12/EC 1118 (0.531 nmol/mg cell) were significantly higher ($p < 0,05$) than the control, while SOL13/EC 1118 showed the lowest GSH level (0.270 nmol/mg cell).

4. Discussion

Accumulation of glutathione in yeast cells is an important feature that is attracting increasing interest, especially in the oenological context. Most investigations have focused on *S. cerevisiae* (De Vero et al., 2017), and the body of knowledge about non-conventional yeasts is still scarce. Thus, in the present study, the GSH accumulation capability of selected non-conventional yeast strains was evaluated in different growth conditions, with a view not only to increase the yeast resistance to industrial production, but also to potentially increase the glutathione content directly in wine. Particularly, for the first time a medium that was optimized to promote GSH production in *S. cerevisiae* was tested with non-*Saccharomyces* yeast strains. Furthermore, the GSH production was measured in microvinification trials using mixed cultures of *S. cerevisiae* and selected native non-*Saccharomyces* yeast strains.

The first test was conducted growing the yeasts in a medium that enhances the GSH level, following the general approaches of favoring high cell densities, through respiratory metabolism, as well as increasing intracellular GSH accumulation by largely providing the essential

Table 2
Biomass and GSH accumulation at the end of single and sequential fermentations of *L. thermotolerans* SOL13, *S. bacillaris* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in synthetic grape juice and in pasteurized grape juice, represented as the mean ± standard deviation of three independent replicates.

Yeast	Synthetic grape juice		Pasteurized grape juice	
	Cell dry-weight (g/L)	GSH (nmol/mg cell)	Cell dry-weight (g/L)	GSH (nmol/mg cell)
EC 1118	0.618 ± 0.014^{bc}	0.090 ± 0.011^{bc}	0.687 ± 0.039^{b}	0.379 ± 0.022^{b}
SOL13	0.526 ± 0.065^{bc}	0.073 ± 0.001^{c}	–	–
MALV45	0.784 ± 0.102^{b}	0.041 ± 0.005^{d}	–	–
FIANO12	0.320 ± 0.057^{c}	0.191 ± 0.010^{a}	–	–
SOL13/EC 1118	1.136 ± 0.175^{a}	0.036 ± 0.002^{d}	0.965 ± 0.078^{a}	0.270 ± 0.021^{c}
MALV45/EC 1118	0.700 ± 0.074^{b}	0.064 ± 0.001^{cd}	0.620 ± 0.057^{b}	0.627 ± 0.042^{a}
FIANO12/EC 1118	0.640 ± 0.006^{bc}	0.103 ± 0.011^{b}	0.545 ± 0.018^{b}	0.531 ± 0.008^{a}

Different letters in the same column indicate a significant difference in Tukey's HSD test ($p < 0.05$).

construction blocks, especially cysteine (Câmara et al., 2019a; Li et al., 2004). Aerobic condition was achieved by flasks agitation, and the MGSH medium optimized for GSH production was singly inoculated with *L. thermotolerans* SOL13, *S. bacillaris* MALV45 and *Metschnikowia* sp. FIANO12, and *S. cerevisiae* EC 1118, as a control. Their growth and GSH accumulation were measured during 96 h incubation. All strains presented viable cell counts higher than the maximum values observed in the subsequent SGJ and PGJ trials, of at least one order of magnitude for the non-*Saccharomyces* yeasts, confirming that MGSH medium is suitable to improve growth and biomass production. The highest GSH production was obtained with *Metschnikowia* sp. after 72 h. *S. cerevisiae* and *L. thermotolerans* presented their peak of GSH at the end of cultivation (96 h), while *S. bacillaris* produced very low amounts of GSH.

Perrone et al. (2005) reported that intracellular GSH level in *S. cerevisiae* reaches the maximum at the end of exponential phase of growth, and GSH excretion starts as cells exit exponential growth and approach stationary phase. In this study, the population dynamic of FIANO12 showed a small decrease between 72 and 96 h (Fig. 1), opposite from the other strains, suggesting that cells were in the stationary phase and thus accumulated GSH could have been released to the surrounding medium. In addition, dead cells could release glutathione following the autolysis, together with amino acids (Dubourdieu and Lavigne-Cruè ge, 2004). Another possible fate for intracellular GSH is degradation to be used as endogenous sulfur (Granchi et al., 2019) or nitrogen source (Mehdi & Penninckx, 1997) in starvation conditions.

In an attempt to increase *S. cerevisiae* cell growth and GSH accumulation, Rollini and Manzoni (2006) and Santos et al. (2007) carried out full factorial experimental design with relevant fermentation parameters (medium composition and environmental factors). However, in both studies, the maximal biomass and GSH values were not found at the same conditions, rather contrasting, showing that further research is needed to better understand the mechanisms involved and find an equilibrium. The present results showed that also different yeasts can respond differently to the same growth conditions, where biomass was favored in some cases and GSH accumulation in others.

The GSH-enriched IDY preparations, increasingly used in winemaking, are usually grown under aerobic conditions in a non-limiting medium stimulating the pathways for GSH production, then cells are inactivated and dried (Bahut et al., 2019). A study about glutathione content in different IDY products showed an expected higher GSH extraction in model wine from the GSH-enriched IDY prepared in optimized nutritional features that maximize intracellular GSH concentration (Bahut et al., 2019). With the exception of MALV45, all strains tested here showed markedly higher GSH concentrations in the optimized medium (MGSH), when compared with the wine fermentation conditions (SGJ and PGJ).

Focusing on ADY production, well-known in *S. cerevisiae*, multiple stresses challenge the performance of cells during their formulation, possibly leading to ROS formation. Growth medium composition has an influence on antioxidant protection systems and on biosynthesis of protective molecules, such as GSH. This compound is important for the resistance to dehydration-oxidation stress and thus crucial for the success of industrial production of ADY (Gamero-Sandemetrio et al. 2018; Câmara et al., 2019a, 2019b). Accordingly, recent studies with non-*Saccharomyces* strains have suggested that high glutathione biosynthesis might be a limiting factor for adaptation during biomass propagation, possibly associated with a better performance and viability (Gamero-Sandemetrio et al. 2018; Câmara et al., 2019a, 2019b). Particularly, Gamero-Sandemetrio et al. (2018) tested biomass production and glutathione accumulation, among other parameters, after growth in molasses of five non-*Saccharomyces* strains, belonging to species different from this study, and a reference *S. cerevisiae*. Only *Candida stellata* showed higher biomass and GSH than *S. cerevisiae*. The total GSH level ranged from around 0.7 to 2.7 nmol/mg cell for the non-*Saccharomyces* and was 1.7 nmol/mg for *S. cerevisiae*. Low biomass yields were correlated with low oxidative response and deficient GSH synthesis. In this study, the GSH production ranged between 0.08 and 4.59 nmol/mg cell, and another non-*Saccharomyces* species, *Metschnikowia* sp., was the top producer. *S. cerevisiae* came in second place (3.13 nmol/mg cell), followed by *L. thermotolerans* and *S. bacillaris*.

Câmara et al. (2019a) analyzed the GSH content of *Torulaspora delbrueckii*, *M. pulcherrima* and *L. thermotolerans* compared to a control *S. cerevisiae*, after growth in different culture media. The nutrient-rich medium promoted the best GSH accumulation and higher resistance to dehydration for all four yeasts, and GSH level reached 278, 202, 213 and 242 μg/10^{10} cells for *S. cerevisiae*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans*, respectively. As in this study, different species showed distinct behaviors, although should be highlighted that non-*Saccharomyces* yeasts have the capacity to accumulate GSH comparable to *S. cerevisiae*.

Tirelli et al. (2010) examined the reduced cysteine content, including glutathione, in 16 *S. cerevisiae* ADY and 12 IDY preparations. GSH values ranged between 3.9 and 9.2 nmol/mg for the ADY, and 3.3–46.0 nmol/mg for the IDY. The differences could be due to the manufacturing process, with variations in culture media and strains responsible for the increased GSH found in some samples, as well as the enrichment with exogenous GSH in the IDY preparations.

Subsequently, the potential to produce GSH by non-*Saccharomyces* yeasts was assessed in microvinification trials, using two fermentation media. In mixed cultures with *S. cerevisiae*, *S. bacillaris* and *Metschnikowia* sp. were possibly higher GSH producers than *L. thermotolerans*, or their presence had a positive effect on GSH production by *S. cerevisiae*. Many grape and yeast metabolites change during alcoholic fermentation in complex microbial interactions, influenced by must initial composition, yeast genetic background, and environmental parameters (Kritzinger et al., 2013). The presence of specific compounds in PGJ not present in SGJ could be responsible for the higher GSH production in the first, or the response to some kind of stress triggered GSH accumulation. It is still not clear which are the reasons behind GSH level variations in different fermentation media, although other authors also pointed out the grape juice complexity (Kritzinger et al., 2013). It has been suggested that different *S. cerevisiae* strains can have variable ability to produce or assimilate GSH, and consequently alter GSH content in wine (Kritzinger et al., 2013; Lavigne et al., 2007; Mezzetti et al., 2017).

The present findings reinforce those complex interactions between must composition and yeast strain, but also include the non-*Saccharomyces* yeast strains as possible modulators of GSH levels during fermentation. As these are non-GMO strains selected from nature, they

could be widely accepted by consumer and thus arise great interest for wine industry.

5. Conclusions

Taken together, findings obtained here demonstrated that an appropriate selection of non-conventional yeast strains with high GSH production, coupled with positive oenological traits, could be a feasible strategy to obtain both active and inactivated dry yeasts more suitable and worthwhile for application in the wine production chain. Among the non-*Saccharomyces* yeasts tested, *Metschnikowia* sp. FIANO12 showed the highest potential for GSH accumulation. Nonetheless, further research with a maximized number of yeast species and strains could be advisable to strengthen these results and give new insights about the metabolism of glutathione. The investigation of GSH metabolism at a molecular level in non-conventional yeasts could also contribute valuable information.

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CRediT authorship contribution statement

Wilson J.F. Lemos Junior: Conceptualization, Investigation. **Renato L. Binati:** Formal analysis, Writing - original draft. **Nicolò Bersani:** Investigation. **Sandra Torriani:** Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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