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Glutathione production by non-*Saccharomyces* yeasts and its impact on winemaking: A review

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ABSTRACT

Glutathione (GSH) is a non-protein thiol naturally present in grape berries and produced by yeasts during fermentation. It has a strong antioxidant activity; thus, the addition of pure GSH during winemaking is recommended to limit the oxidative phenomena of wine, preserving sensory characteristics and stability, ultimately promoting a healthier product by reducing the need for SO_2 addition. A promising alternative approach considers the use of yeast starter cultures high producers of this compound *in situ*, during the fermentation process, in substitution of external GSH addition. Recent research showed that multistarter fermentations with non-*Saccharomyces* yeasts produce even higher concentrations of GSH compared to single *Saccharomyces cerevisiae*. Accumulation of GSH in yeast cells is also considered valuable during the growth and dehydration of biomass for starter production, aiding strains to overcome the stressful conditions of industrial process. Moreover, a current trend in oenology is the use during fermentation of inactivated dry yeasts preparations as a source of nutrients, and many of them contain GSH-enriched cells. The aim of this review was to assess the significance of GSH production for the exploitation of wine-related non-*Saccharomyces* yeasts, both in starter biomass production and during fermentations, which were until now studied in detail for *S. cerevisiae*. This compendium highlights an interesting new feature of non-conventional yeasts and upgrade the strategy of multistarter fermentation as a valuable tool to positively modulate wine composition.

1. Introduction

Winemaking is an ancient fermentation process that has been part of human traditions for at least a few millennia (Pretorius, 2020). The transformation of grape must to wine through alcoholic fermentation is naturally carried out by a wealthy yeast population, either native to vineyard and winery or inoculated by winemakers. Spontaneous fermentations are usually characterized by a huge diversity of yeast species and strains, which ideally bring more complexity and a certain "sense of place" to wine, but equally vast nowadays is the range of selected starter cultures available to winegrowers worldwide (Gonzalez & Morales, 2021). These carefully selected cultures can be tailored to the needs and expectations associated with the many different regions and styles of wine. The choice of the fermentation strategy has a remarkable effect on wine quality, and often it is dictated by consumer preferences (Querol et al., 2018). As the market trends continue to evolve, also the criteria for selecting and developing new yeast starters are at constant improvement.

Saccharomyces cerevisiae, long regarded as 'the wine yeast', has unparalleled abilities to completely convert grape sugars into ethanol and carbon dioxide, with the concomitant depletion of nutrients, increase of temperature, and production of potentially inhibitory compounds, which favor the dominance over the native must microbiota (Carrau & Henschke, 2021). Important intra-species diversity is on the core of that multitude of commercial strains available. Nevertheless, to face the current challenges of wine industry, such as climate change, environmental sustainability, increased competition, and health concerns, among others, *S. cerevisiae* can be helped by the biodiversity of other yeast species (Benito et al., 2019).

Non-conventional yeasts, often referred to as 'non-*Saccharomyces*' yeasts (NSY), attract an ever-growing interest from researchers, starter producers and end-users, thanks to valuable features, such as a broader utilization of substrates, to produce interesting compounds via uncommon metabolic pathways (Binati et al., 2021b; Navarrete & Martínez, 2020). For winegrowers, many NSY became available as starter cultures in the last years, mostly represented by, but not limited to, the species *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia* sp. They are generally not able alone to complete the process of alcoholic

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fermentation, but are rather valued in multistarter fermentations alongside *S. cerevisiae* (Vejarano & Gil-Calderón, 2021). The spreading of multistarter fermentations is mainly aimed to achieve improved winemaking process and wine sensory characteristics, diversifying the pool of secondary metabolites usually associated with *S. cerevisiae* (Jolly, Varela, & Pretorius, 2014; Mateo & Maicas, 2016; Roudil et al., 2020).

Among the many compounds produced by yeasts during alcoholic fermentation, the natural antioxidant glutathione is receiving growing attention. This molecule is the most abundant low molecular weight thiol in biological systems, and it is present in the cells of almost all living organisms, including grapevines, bacteria, yeasts, and wine drinkers (De Vero, Bonciani, Verspohl, Mezzetti, & Giudici, 2017; Lavigne, Pons, & Dubourdieu, 2007). As far as we are concerned, most of the existing research focuses on *S. cerevisiae* and there is still scarce information about the biosynthesis, accumulation, and release of glutathione by wine NSY, and the implications of improving its production in wine-related strains (Bonciani, De Vero, Mezzetti, Fay, & Giudici, 2018; Câmara, Maréchal, Tourdot-Maréchal, & Husson, 2019a; Gamero-Sandemetrio, Payá-Tormo, Gómez-Pastor, Aranda, & Matallana, 2018; Penninckx, 2002).

Thus, this review starts dealing with the natural presence of glutathione in grapes and wine, then evaluates the effects of its addition during winemaking based on the most recent reports. A broad vision is given on the GSH metabolism in yeasts, making comparisons between *S. cerevisiae* and NSY regarding the genes and pathways involved, the functions of GSH for the cell homeostasis, and the production of pure GSH using cell factories. Finally, the importance of selecting NSY starters that are high producers of this compound is discussed in-depth with the division in three parts, covering all the literature citations which studied GSH in wine-related NSY: inactivated dry yeasts with guaranteed glutathione levels to be added as fermentation nutrients; active dry yeast starters containing more robust GSH-rich cells; and multistarter fermentations inoculated with GSH-producing yeasts. As the application of glutathione and mixed-culture fermentations gains increased attention in wine industry, we aim to provide new insights and possibly expand the toolkit available to winemakers to innovate their traditions.

2. Glutathione in grapes and wine

Glutathione is a biologically active sulphur tripeptide composed of L-γ-glutamyl-L-cysteinyl-glycine, which inside the cells is prevalent in its reduced form (GSH), but it could also be oxidized (GSSG) or bound to other molecules (GS-S-CoA and GS-S-Cys). The reaction of reduced GSH and peroxide to form oxidized GSSG is catalyzed in procaryotic and eucaryotic cells by glutathione peroxidase, while the reduction of GSSG to GSH involves glutathione reductase, using NADPH as a cofactor (Fig. 1; Penninckx, 2002; Margalef-Català et al., 2016).

Starting from grapes, GSH participates within the plant cells in the essential roles of antioxidation, sulphur metabolism and detoxification of xenobiotic compounds. It is also associated with the biosynthesis of some non-volatile aroma precursors, e.g., in Sauvignon Blanc where precursors of varietal thiol compounds are essential to the grape's aromatic profile. Some GSH-conjugates identified in the pulp and skin of grape berries, such as S-3-(hexan-1-ol)-glutathione and S-4-(4-methylpentan-2-one)-glutathione, are potential precursors for the volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercaptohexyl acetate (3MHA) which are released during fermentation by the action of yeasts (Clark & Deed, 2018; Gabrielli, Aleixandre-Tudo, Kilmartin, Sieczkowski, & du Toit, 2017; Pons, Lavigne, Darriet, & Dubourdieu, 2015).

Glutathione level varies according to many factors, as grape variety, vintage, climate, geographical location, ripeness, and viticultural practices. Such fluctuation is not well understood, although it is thought to be correlated with the vine nitrogenous nutrition and sugar accumulation during grape maturation. Afterwards, the natural glutathione content of grapes will remain in must, albeit at low concentrations of around a few milligrams per liter (Dubourdieu & Lavigne-Cruège, 2004; Kritzinger, Bauer, & du Toit, 2013a).

However, GSH amount can be greatly modified during the various steps of winemaking. At harvesting and pre-fermentation phases, as pressing and maceration, exposure of split grape berries to oxygen will greatly determine GSH content, depending on the oxidative/reductive treatments taking place. As berry skin is the major glutathione *reservoir* in grapes, skin contact during maceration has a prominent impact on GSH level in fermenting must. Finally, further changes on GSH concentration come from chemical and biological reactions during alcoholic fermentation, comprising yeast metabolic pathways of GSH assimilation and secretion. Concentration in wine can range from non-detectable to over 100 mg/L (Christofi, Katsaros, Mallouchos, Cotea, & Kallithraka, 2021; De Vero et al., 2017; Kritzinger, Bauer, & du Toit, 2013b; Pons et al., 2015; Schmidt, Bekker, Sanders, Cuijvers, Kulcsar, Capone, Puglisi, & Jeffery, 2020).

The main effects of GSH in must and wine are shown in Fig. 2. Through the sulfhydryl group (-SH) of its cysteine residue, GSH can react with hydrogen peroxide and carbonyl compounds and scavenge *o*-quinones, molecules related to oxidative reactions, regenerating phenolic compounds (Comuzzo et al., 2015; Díaz, Castro, Ubeda, Loyola, & Laurie, 2021; Marchante et al., 2020). Hence, the main role played by GSH during winemaking is as a strong antioxidant, inhibiting polymerization of phenolic compounds and limiting the development of sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) and 2-aminoacetophenone, which are related to browning reactions during aging and off-flavors perception, respectively (Kritzinger et al., 2013a; Mezzetti, Fay, Giudici, & De Vero, 2017).

Moreover, glutathione can protect some interesting aroma compounds produced by yeasts, such as esters, terpenes, and volatile thiols (Bonciani et al., 2018; Rodríguez-Bencomo et al., 2014). An additional

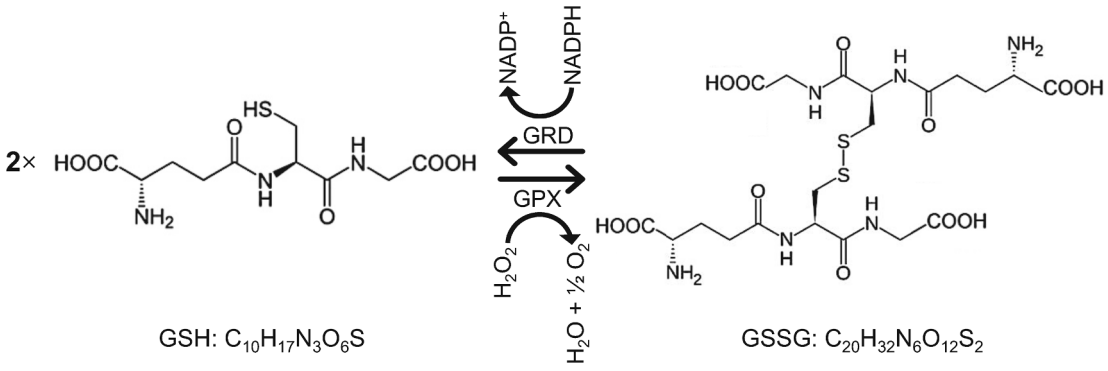

Fig. 1. Chemical structures and redox cycle of reduced glutathione (GSH; MW: 307.33 g/mol) and oxidized glutathione (GSSG; MW: 612.66 g/mol); GRD: glutathione reductase; GPX: glutathione peroxidase.

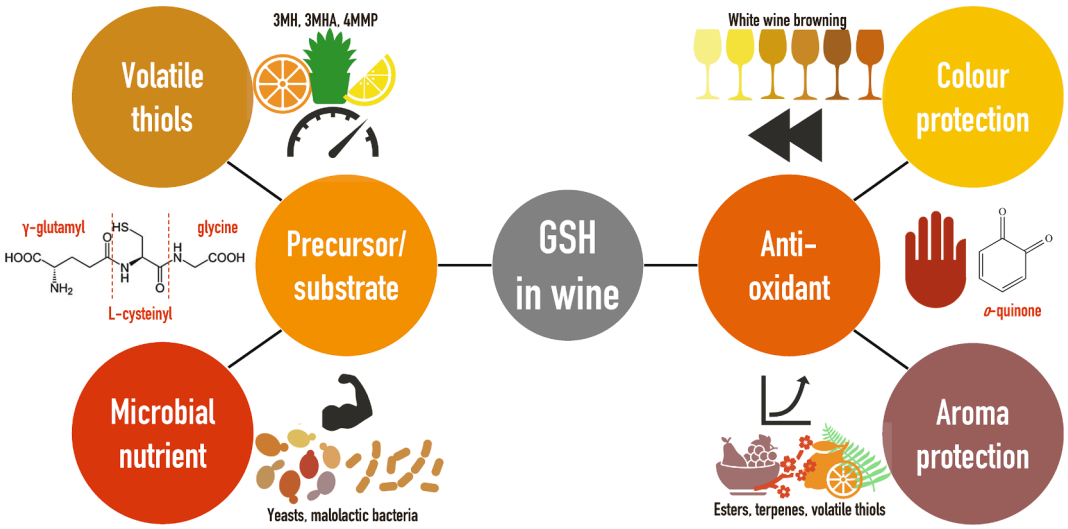

Fig. 2. Main effects of glutathione addition and/or natural accumulation in winemaking. 3MH: 3-Mercaptohexan-1-ol; 3MHA: 3-Mercaptohexylacetate; 4MMP: 4-Mercapto-4-methylpentan-2-one.

effect that could interest some styles of wine is related to the uptake of GSH by *Oenococcus oeni*, the main bacteria responsible of malolactic fermentation in wine, which can use GSH but does not synthetize it. In this bacterium, GSH helps to improve the resistance to stress factors, such as high ethanol and low pH, promoting its growth (Margalef-Català et al., 2016).

Thus, glutathione addition is a valued strategy, especially in white wine production technology, to control detrimental color changes and impaired aromatic profile associated with uncontrolled oxidation phenomena (Kritzinger et al., 2013a). Traditionally, sulphur dioxide (SO_2) is the antioxidant of choice in winemaking, thanks also to its efficient antimicrobial effect. Nevertheless, some health issues associated with SO_2, especially for sensitive subjects, have been gaining *momentum* in recent years, therefore wine industry is actively searching alternative strategies to replace SO_2 addition (Badea & Antoce, 2015; Capece, Pietrafesa, Siesto, & Romano, 2020; Checchia et al., 2021; Lisanti, Blaiotta, Nioi, & Moio, 2019; Organization Internationale de la Vigne et du Vin, 2020). In this context, increase of glutathione content in wine is seen as a promising approach to partially reduce the need for SO_2 input, thus helping to meet consumer demand for healthier and more sustainable products and processes (De Vero et al., 2017; Gabrielli et al., 2017). Although a more robust body of research is being accumulated in recent years to prove the usefulness of GSH in wine, there is still a strong debate among scientists and winemakers about the practical suitability of glutathione as a substitute for SO_2, especially in view of the concentration needed and associated risks of off-flavor formation (Schmidt et al., 2020).

2.1. Pure glutathione addition

Direct addition of pure glutathione is a fast way to increase GSH concentration in wine, although this strategy might encompass relatively high costs for wineries (De Vero et al., 2017; Liu et al., 2020). Recognizing the positive effects of GSH to restrain oxidation, the International Organisation of Vine and Wine (OIV) has recently approved the addition of reduced glutathione in must and wine in its International Oenological Codex, but to a maximum of 20 mg/L, and classified it as an additive rather than a processing aid (Organization Internationale de la Vigne et du Vin, 2015, 2018a). Nonetheless, the formal authorization for use of any food product is subject to national laws of individual wine-producing countries, which did not happen yet for pure glutathione (Bahut et al., 2020; Schmidt et al., 2020).

The pure compound can be produced chemically or enzymatically, but at industrial scale the principal pathway is *via* microbial fermentation, frequently using mutants of *S. cerevisiae* or NSY, such as *Cyberlindnera jadinii* (synonym *Candida utilis*). Those yeasts have the advantage to rapidly grow at high cell densities using affordable carbon sources. Pure glutathione is commercialized as a white crystalline powder soluble in water, with more than 98% GSH content (Organization Internationale de la Vigne et du Vin, 2017b; Schmacht, Lorenz, & Senz, 2017).

Several authors have tested GSH addition in must and wine, mainly focusing on the antioxidant effects, but with some contrasting findings. Considering trials in Sauvignon Blanc grape juice, Lyu, Del Prado, Araujo, Quek, and Kilmartin (2021) reported significant protective effect against oxidation and increased concentrations of volatile thiols in wine, while opposite effects were described by Patel et al. (2010). Regarding wine after the end of alcoholic fermentation, Díaz et al. (2021) did not find protective effects of glutathione against oxidation, but, on the contrary, Tomašević, Gracin, Ćurko, and Ganić (2017), Cojocaru and Antoce (2019), and Christofi et al. (2021) observed significant antioxidative effects of GSH during ageing, quantified as higher concentrations of esters, terpenes, and/or varietal thiols, compared to the control. Furthermore, these protective effects were stronger in the first months and diminished afterwards, which might be caused by a progressive decline of glutathione concentrations during ageing, as also reported by other authors (Andújar-Ortiz, Pozo-Bayón, Moreno-Arribas, Martín-Álvarez, & Rodríguez-Bencomo, 2012; Ferreira-Lima, Burin, Caliari, & Bordignon-Luiz, 2016).

2.1.1. Bioprocess engineering

Thanks to the multiple biotechnological applications of glutathione, as in medicine, cosmetics, pharmaceutical, food and beverage industries, the global annual production of this molecule is increasing in recent years: it was estimated at 200 tons at the beginning of last decade (Orumets, Kevvai, Nisamedtinov, Tamm, & Paalme, 2012). As stated previously, yeast cell factories are currently the main source of glutathione; therefore, it can be expected that bioprocess optimization to enhance accumulation, usually introducing amino acid precursors during fermentation, and engineering strategies for obtaining high-producing strains are important topics in this field (Kresnowati, Ikhsan, Nursa'adah, Santoso, & Susanto, 2019; Kurylenko et al., 2019; Schmacht et al., 2017; Suzuki et al., 2011).

Most research was dedicated to *S. cerevisiae* (De Vero et al., 2017; Orumets et al., 2012; Patzschke et al., 2015), but also other species were subject of investigation, including *Komagataella pastoris* (synonym *Pichia*

pastoris; Fei, Wang, & Chen, 2009), *Ogataea polymorpha* (synonym *Hansenula polymorpha*; Ubiyvovk, Ananin, Malyshev, Kang, & Sibirny, 2011), *Rhodotorula diobovata* (synonym *Rhodosporidium diobovatum*; Kong, Wang, Tian, Tang, & Zhang, 2017). To give a more recent example, a genetically engineered strain of *O. polymorpha* was developed by overexpression of key genes involved in glutathione biosynthesis and successfully produced five times more glutathione than its parental strain. Additionally, cells of the engineered strain showed a higher viability during storage of dried cells, possibly correlated with the protective effects of glutathione overproduction against oxidative stress during dehydration (Kulikova-Borovikova et al., 2018; Kurylenko et al., 2019). This characteristic is one of the main reasons that support targeting glutathione production as a marker in the screening of novel yeast starters, as it will be detailed in the following Section 4.2.

Taking sustainability into account, the use of substrates from renewable sources and recovered wastes for the bioproduction of value-added compounds became a priority in many industrial sectors. Tallian et al. (2019) described a successful production of glutathione by an engineered strain of *S. cerevisiae* using glucose enzymatically recovered from cellulose fiber manufacture. In developing circular economy solutions, NSY are certainly in a privileged position thanks to their many unusual metabolic features (Binati et al., 2021b).

3. Glutathione in yeasts

3.1. Metabolism and functions

In yeast cells, glutathione content is typically in the range of 0.1–1% cell dry weight and the most important form is the reduced one, with a cytosolic ratio of GSH to GSSG of 30-100:1 (Ask, Mapelli, Höck, Olsson, & Bettiga, 2013). GSSG is formed by two molecules of GSH interlinked with a disulfide bond (Fig. 1). The life cycle of GSH within the cell includes the reactions of cytoplasmic biosynthesis, transport, compartmentalization, degradation, regeneration, and consumption (De Vero et al., 2017). Kinetics of glutathione transport across most intracellular membranes, regulation and signaling network are rather complex and not yet fully understood (Oestreicher & Morgan, 2019; Torrellas, Rozès, Aranda, & Matallana, 2020). Thanks to the relevance of glutathione for cell homeostasis, it can be expected a tight control of many genes throughout its biosynthesis, conjugation, and degradation pathways, involving some transcription factors as Aft2p, Met4p, and Yap1p (Dhaoui et al., 2011; Wheeler, Trotter, Dawes, & Grant, 2003).

GSH is synthesized in the cytosol via two consecutive ATP-dependent reactions: firstly, γ-glutamyl-L-cysteine synthetase (GSH1, encoded by the gene *GSH1*) links the amino group of L-cysteine to the side-chain γ-carboxylate group of L-glutamate forming the dipeptide γ-glutamyl-L-cysteine; secondly, L-γ-glutamylcysteine-glycine-γ-ligase, also known as glutathione synthetase (GSH2, encoded by *GSH2*), bonds the amino group of a L-glycine to the cysteine carboxylate group of that intermediary, completing the formation of GSH (Mezzetti et al., 2017; Oestreicher & Morgan, 2019; Patzschke et al., 2015). The first reaction in the synthesis pathway is generally considered the rate-limiting step due to the feedback inhibition by GSH, controlled at transcriptional and post-translational level (Orumets et al., 2012; Schmacht et al., 2017).

GSH can also be assimilated by cells from the external environment by specific oligopeptides, such as the high-affinity *S. cerevisiae* glutathione transporter Opt1p/Hgt1p (*OPT1/HGT1*) (Bourbouloux, Shahi, Chakladar, Delrot, & Bachhawat, 2000; De Vero et al., 2017; Penninckx, 2002). Orthologs were identified in other yeast species, such as *Kluyveromyces lactis*, *Pichia guilliermondii*, *Schizosaccharomyces pombe*, *Sc. japonicus* (Thakur & Bachhawat, 2010; Zimdars, Schrage, Sommer, Schieber, & Weber, 2019). Secretion of GSH in *S. cerevisiae* was shown to be assisted by the GSH/proton antiporters (GEX, encoded by *GEX1-2*), members of the major facilitator superfamily (MFS) of transporters and which were detected in both the plasma and vacuolar membranes (Dhaoui et al., 2011).

GSH degradation is catalyzed by γ-glutamyl-transpeptidase (γ-GT, encoded by *CIS2*), which detaches L-glutamate from GSH, releasing L-cysteinyl-glycine (Mehdi, Thierie, & Penninckx, 2001). Finally, the last step of GSH hydrolysis reported in *S. cerevisiae* is catalyzed by a L-cysteinyl glycine dipeptidase (CGase), part of the proteins Dug1p/Dug2p/Dug3p degradosomal complex (*DUG1-3*), generating L-cysteine and glycine (Kaur, Kumar, Junot, Toledano, & Bachhawat, 2009; Penninckx, 2002).

Among the many important roles played by GSH within yeast cells, the main functions are as an antioxidant and a detoxifier of xenobiotic compounds; it is involved in redox buffer, preservation of membrane integrity, gene expression, iron homeostasis, protein folding, and as a source of nutrients like nitrogen and sulphur depending on cell needs (De Vero et al., 2017; Kurylenko et al., 2019; Lavigne et al., 2007; Patzschke et al., 2015; Penninckx, 2002).

The protection against oxidative stress, which mechanism is similar in *Saccharomyces* and in NSY, is based on directly preventing the formation of reactive oxygen species (ROS) via GSH thiol moiety, and indirectly through antioxidant enzymes, as glutaredoxin (GRX, encoded by *GRX1-8*) and glutathione peroxidase (GPX, encoded by *GPX1-3*). The reduction of peroxides by glutathione peroxidase generates oxidized GSSG, therefore levels of GSSG tend to increase following oxidative stress. Regeneration of GSH from GSSG, at the expense of NADPH, occurs through glutathione reductase (GRD, encoded by *GLR1*). Hence, to maintain an optimal redox balance, cells may induce glutathione synthetase and glutathione reductase activity, aiming to raise GSH and the GSH/GSSG ratio (Fig. 1Espindola, Gomes, Panek, & Eleutherio, 2003; Morano, Grant, & Moye-Rowley, 2012; Penninckx, 2002; Torrellas et al., 2020; Wheeler et al., 2003).

The detoxification of xenobiotics is targeted, for example, on heavy metals, such as cadmium, copper, zinc, silver, and lead, lipophilic compounds, and aldehydes (De Vero et al., 2017). Formation of chelation complexes of glutathione with heavy metals is catalyzed by a group of glutathione S-transferases (GST, encoded by *GTT1-2*, *GTO1-3*) (Penninckx, 2002; Lemos Junior et al., 2021b). Transportation of GSH conjugates to the vacuole is performed by a family of low-affinity glutathione S-conjugate export pumps (GS-X pumps), such as the yeast cadmium factor 1 (YCF1, encoded by *YCF1*) (Li et al., 1997). This activity could be useful to prevent negative effects of excessive copper concentrations in must, which impair yeast activity. It was shown that GSH addition mitigated the acetaldehyde accumulation and increased yeast vitality in a copper-rich grape must (Zimdars et al., 2019). In particular, detoxification mechanisms involving GSH were described in NSY, such as formaldehyde detoxification in some methylotrophic species of the genera *Pichia*, *Candida*, and *Ogataea*, and cadmium response in *Sc. pombe* (Penninckx, 2002).

3.2. Genetic aspects

Genes involved in GSH metabolism are well-known in *S. cerevisiae* (Lemos Junior et al., 2021b). Nonetheless, identification and comparison of homologues found in NSY could give important insights about the significance of this compound in wine-associated strains. Penninckx (2002) reported similarities around 40% between many GSH-related genes described in *S. cerevisiae* and *Sc. pombe*. Some important differences were also acknowledged, such as the presence of only one gene encoding for GPX (*GPX1*) in *Sc. pombe* genome, while *S. cerevisiae* has three genes (Penninckx, 2002).

In the study of Lemos Junior et al. (2021b), the genomes of two *Starmerella bacillaris* strains were compared with a model strain of *S. cerevisiae* (EC1118^{TM}/S288c). Among 24 orthologous GSH-related genes found in *St. bacillaris*, the authors reported a high number of SNPs (single nucleotide polymorphisms) in 20 of them, potentially affecting their function. None of the genes related to glutathione plasma membrane or vacuolar transport could be identified in *St. bacillaris* genomes. When comparing the amino acidic sequences, substitutions were

observed in four proteins. Comparison between the two *St. bacillaris* strains highlighted some differences in genes encoding for glutathione peroxidase, reductase, and S-transferase, which could be related with diverse glutathione content in synthetic must fermented by these strains (Lemos Junior et al., 2021b).

A similar approach was used to investigate another two wine relevant NSY, namely *L. thermotolerans* and *Metschnikowia* spp. (our unpublished data). In a previous research, genome sequences of the *L. thermotolerans* strains COLC27 and SOL13 were analyzed to shed light on the important metabolism of L-lactic acid, which is remarkably variable in this species and has interesting implications on wine quality (Gatto et al., 2020). The *Metschnikowia* sp. strain DBT012 (FIANO12) genome was sequenced with the goal to clarify its taxonomic position within the *M. pulcherrima* clade, as traditional phylogenetic markers did not give an unambiguous classification to members of this group (unpublished data).

Table 1 shows the main GSH-related genes analyzed and their similarities among these NSY. The genomes of the three strains harbor all genes involved in GSH metabolism. SFA1, which is annotated as S-(hydroxymethyl)glutathione dehydrogenase, is the protein with the highest similarity with *S. cerevisiae* S288c in each of the three strains. In *L. thermotolerans* strains COLC27 and SOL13, the enzymes annotated as thiosulfate glutathione S-transferase display the lowest similarity compared to *S. cerevisiae*, while in *Metschnikowia* sp. strain DBT012 the glutathione S-transferase 1 showed low similarity both with *S. cerevisiae* as well as with *L. thermotolerans* strains.

Interestingly, each of the strain under investigation includes the genes *OPT1* and *YCF1*, involved in GSH transport, which were not found in the *St. bacillaris* genomes investigated by Lemos Junior et al. (2021b). This genetic background could help to explain the different behavior of *L. thermotolerans* and *Metschnikowia* spp. compared to *St. bacillaris* strains in wine fermentation trials, as described in Section 4.3.

4. Oenological significance of glutathione in non-*Saccharomyces* yeasts

The selection of NSY with superior GSH production could have multiple oenological and biotechnological implications of remarkable significance (Fig. 3). First, NSY could be exploited to produce GSH-enriched inactivated dry yeasts, additives gaining popularity in oenology to be supplemented in fermenting must and wine (Bonciani et al., 2018; De Vero et al., 2017; Gabrielli et al., 2017). Secondly, wine yeasts with enhanced glutathione formation and metabolism might be better adapted to the stresses associated with industrial production of dry biomass for starter cultures, a limiting step for NSY commercialization, and later could show better survival and efficiency during wine fermentation (Gamero-Sandemetrio et al., 2018; Mezzetti et al., 2017; Penninckx, 2002). Finally, starter cultures capable of producing high levels of glutathione during winemaking process could be valuable to naturally increase GSH content during the first steps of multistarter fermentations (Binati, Lemos Junior, & Torriani, 2021a) or throughout storage thanks to cell autolysis, improving the wine protection against oxidation phenomena.

Genetic engineering strategies were successfully applied to increase the yield of pure GSH produced in yeast cell factories. Nevertheless, the exploitation in wine industry of genetically modified organisms (GMO) as active yeast starters or inactivated yeast nutrients is still a topic generally avoided, mainly due to regulatory issues as well as to consumer behavior often reluctant towards food products obtained with GMO technology (Çakar, Turanlı-Yıldız, Alkım, & Yılmaz, 2012; De Vero et al., 2017; Schmacht et al., 2017). Thus, bioprospecting of food-grade NSY to exploit this potential of higher GSH accumulation is one of the most sustainable strategies to be chased in the next years.

4.1. GSH-enriched inactivated dry yeasts

Besides pure glutathione, another possibility to increment GSH content in wine consists of using inactivated dry yeasts (IDYs)

Table 1
Annotation of proteins involved in GSH metabolism in *S. cerevisiae* S288c and analysis of their presence in *L. thermotolerans* strains COLC27 and SOL13 and in *Metschnikowia* sp. strain DBT012 (FIANO12).

Gene ID	Gene *S. cerevisiae* S288c	Protein	*L. thermotolerans* COLC27	*L. thermotolerans* SOL13	*Metschnikowia* sp. DBT012
			Similarity (%)		
850605	*DUG1*	Cys-Gly metallodipeptidase DUG1	72.92	73.18	67.57
851386	*SFA1*	S-(hydroxymethyl)glutathione dehydrogenase	80.31	80.05	75.13
851713	*YCF1*	ATP-binding cassette glutathione S-conjugate transporter YCF1	67.45	67.51	57.63
852124	*GRX2*	Glutaredoxin-2	52.21	52.94	52.94
852215	*PRX1*	Peroxiredoxin PRX1, mitochondrial	66.67	66.67	57.73
852546	*GPX2*	Glutathione peroxidase-like peroxiredoxin 2	68.94	68.32	67.92
852584	*DUG2*	Probable di- and tripeptidase DUG2	51.70	51.70	38.04
853218	*OPT1*	Oligopeptide transporter OPT1	72.40	72.40	39.65
853316	*NIT2*	Deaminated glutathione amidase	59.80	59.47	41.58
853344	*GSH1*	Glutamate-cysteine ligase	65.12	65.43	49.77
853377	*YJL068C*	S-formylglutathione hydrolase	63.18	62.50	49.65
853781	*TEF4*	Elongation factor 1-gamma 2 (GST-like domain)	73.30	73.30	53.48
853951	*ECM4*	Putative glutathione S-transferase	66.02	66.57	57.14
854108	*GSH2*	Glutathione synthetase	51.47	51.68	50.10
854205	*GLO4*	Hydroxyacylglutathione hydrolase, mitochondrial	63.03	63.03	47.04
854459	*RDL1*	Thiosulfate:glutathione S-transferase	43.07	43.07	34.78
854855	*HYR1*	Glutathione peroxidase-like peroxiredoxin HYR1	73.29	72.67	70.44
854856	*GTT1*	Glutathione S-transferase 1	70.94	71.79	33.48
855009	*GLO1*	Lactoylglutathione lyase	66.36	66.36	51.10
855492	*URE2*	Transcriptional regulator URE2 (GST-like domain)	72.08	72.08	70.39
855530	*DUG3*	Probable glutamine amidotransferase DUG3	77.59	77.59	60.48
855669	*POR1*	Mitochondrial outer membrane protein porin	66.55	66.55	52.14
856014	*GLR1*	Glutathione reductase	73.03	73.03	63.67
856048	*GRX5*	Monothiol glutaredoxin-5, mitochondrial	70.15	70.15	64.54
856910	*GCG1*	Glutathione-specific gamma-glutamylcyclotransferase	53.48	53.04	43.30

Gene IDs derive from the annotation of *Saccharomyces cerevisiae* S288c genome sequence (Accession Number: NC001133-NC001148).

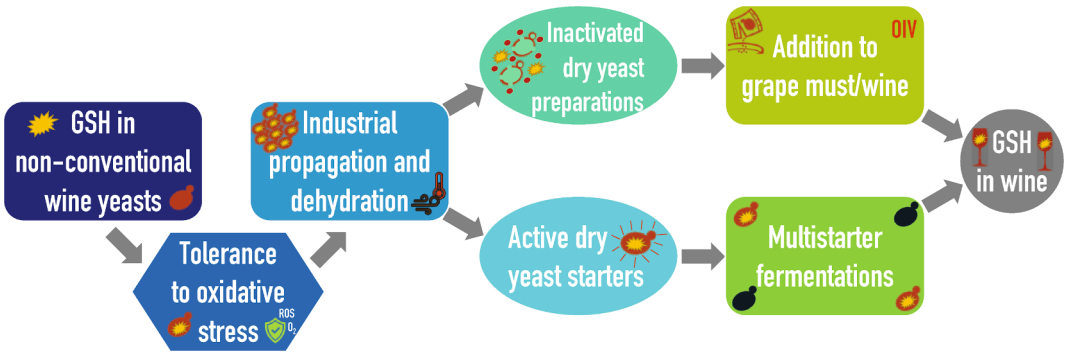

Fig. 3. Framework to evaluate the implications of optimized GSH production in wine-related non-conventional yeasts.

preparations (Bonciani et al., 2018; Gabrielli et al., 2017). Among the many different classes of yeast derivative products, widely and increasingly used in winemaking to improve fermentation management, sensory perception, and wine stabilization, the OIV and national food standard codes approve the use of inactivated yeasts with guaranteed glutathione levels (G-IDYs). They can provide nutrients for the active yeasts conducting fermentation and reduce ochratoxin-A formation during wine maturation and clarification. Additionally, G-IDYs have the antioxidant effects related to GSH, such as color and aroma protection (Alfonzo et al., 2021; López-Solís et al., 2017; Organization Internationale de la Vigne et du Vin, 2018b; Rigou, Mekoue, Sieczkowski, Doco, & Vernhet, 2021; Rodríguez-Bencomo et al., 2014).

G-IDYs were first proposed in a patent in 2005 (PCT/FR2005/000115) and are characterized by naturally having a greater GSH content than other standard IDYs, achieved by proper selection of high-producing strains and an optimized bioprocess aimed to maximize accumulation of intracellular GSH before inactivation (Bahut et al., 2019). Importantly, no antibiotics or additives other than the essential nutrients for yeast growth can be added to the culture medium, thus, G-IDYs have only the glutathione and its precursors naturally produced by yeasts. Besides a minimum level of GSH (1% w/w), there might be a certain amount of cysteine (maximum 0.3% w/w) and γ-glutamyl-L-cysteine (maximum 1% w/w) (Organization Internationale de la Vigne et du Vin, 2017a, 2018b).

When it comes to the yeast species, IDYs could technically be prepared from *S. cerevisiae* and/or NSY (Organization Internationale de la Vigne et du Vin, 2018b). Although yeast-based products have been in the market for more than two decades, in the catalogue of the most important suppliers of oenological products worldwide, to the best of our knowledge, all IDYs are declared to be obtained from *S. cerevisiae* cells. The more recent G-IDYs are increasingly becoming available in those portfolios, but, also in this case, all products whose origin is specified are manufactured with *S. cerevisiae*. Anyhow, a recent study reported the use of an experimental IDY prepared from a *T. delbrueckii* strain. It was added to a Macabeo base wine during the elaboration of traditional method Cava, which had better foaming properties and a higher appreciation in sensory analysis than the control wine without IDY addition (Medina-Trujillo et al., 2017).

An advantage of G-IDYs compared to pure glutathione is the avoidance of cost- and time-consuming purification steps; besides, GSH-enriched yeasts can be priced around double of the normal yeast price (Kresnowati et al., 2019). Nonetheless, a combined addition of pure GSH and G-IDYs can be performed at the start or during alcoholic fermentation, at the beginning of wine maturation or during storage, provided that the dosage used does not exceed 20 mg/L. This maximum concentration of GSH and a sufficient level of assimilable nitrogen should be ensured to avoid risks of glutathione consumption, reduction, and strong yeasty aromas (Organization Internationale de la Vigne et du Vin, 2017a, 2018b).

Glutathione and its building blocks could be subject to yeast β-lyase activity, generating thiol compounds (Gabrielli et al., 2017; Rigou et al., 2021); or be metabolized by nitrogen-starved yeasts, resulting in the formation of hydrogen sulphide (H_2S) and riskily increasing the sensory perception of sulphur-based off-flavors (Schmidt et al., 2020; Ugliano et al., 2011; Wegmann-Herr, Ullrich, Schmarr, & Durner, 2016). Further compounds with antioxidant activity and aromatic repercussions could be released from G-IDYs, produced by the yeast strains and/or generated by thermal and oxidative degradation during the industrial biomass propagation and inactivation (Bahut et al., 2020; Pons-Mercadé et al., 2021; Rodríguez-Bencomo, Andújar-Ortiz, Sánchez-Patán, Moreno-Arribas, & Pozo-Bayón, 2016). Hence, addition of G-IDYs in winemaking may have an organoleptic impact beyond protection from oxidation, even if not fully understood yet as there is a great diversity of oenological conditions and IDYs composition (Andújar-Ortiz, Chaya, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2014; Bahut et al., 2019; López-Solís et al., 2017; Šuklje et al., 2016).

4.2. *Active dry yeast starters*

The development and large-scale production of active dry yeasts (ADYs), beginning after the Second World War, are among the most important keystones of wine industry, thanks to the ease of supplying, long-term stability, and reliability of these dehydrated pure cultures of yeast cells. They enter a state of temporary suspension of the metabolism without water, called anhydrobiosis, in which cells can survive long periods, but, as soon as normal hydric conditions are restored, those yeasts reactivate their regular metabolism (Rapoport, Turchetti, & Buzzini, 2016; Câmara, Maréchal, Tourdot-Maréchal, & Husson, 2019b; Kurylenko et al., 2019; Lemos Junior et al., 2021a).

Differently from beer brewing and bread baking, winemaking is a seasonal activity, therefore most commercial wine producers need to rely on ADYs with proven good oenological attributes. However, industrial production of ADYs was mainly optimized for *S. cerevisiae*, hence the conditions might not be ideal for NSY, which are more difficult and stress sensitive, leading to low biomass yields, cell death or unbearable loss of activity (Câmara et al., 2019a; Gamero-Sandemetrio et al., 2018; Matallana & Aranda, 2017; Pérez-Torrado et al., 2015; Torrellas et al., 2020).

Among the many pressures associated with ADYs production, oxidative stress is the main challenge to be overcome in yeast cells to have a high biomass yield and subsequent satisfactory fermentation performance. In the biomass propagation step, yeasts are proliferated in batch or fed-batch large-scale fermenters using sugar-rich substrates. Respiratory metabolism is stimulated to generate more biomass, but it also causes internal oxidative stress. Biomass is then concentrated and dehydrated with one of various possible technologies, as hot air bed, spray drying, freeze-drying, air-blast drying, which trigger again some ROS accumulation. Furthermore, during long-term storage, oxidation of membrane lipids affects viability of dried cultures (Kim, Lee, Jeon, & Park, 2019; Matallana & Aranda, 2017; Pérez-Torrado et al., 2015).

Cellular oxidative stress is caused by the endogenous production of ROS, mainly in the mitochondria, following metabolic unbalances. It could cause multiple damages to cellular components and metabolites, such as proteins, nucleic acids, and lipids. The mechanisms involved in ROS scavenging and redox balance are mainly based on gene expression regulation, enzymatic activities, and protective molecules, including glutathione (Gamero-Sandemetrio, Gómez-Pastor, Aranda, & Matallana, 2019; Herrero, Ros, Bellí, & Cabiscol, 2008; Matallana & Aranda, 2017).

In both *S. cerevisiae* and NSY, the production of GSH was correlated with better response to ADY production conditions (Câmara et al., 2019a; Gamero-Sandemetrio et al., 2018; Torrellas et al., 2020). It is well-established that, by protecting cells from oxidative damage through formation of disulfide bonds in GSSG or scavenging of free radicals, glutathione is pivotal in the tolerance to dehydration stress and survival during storage (Espindola et al., 2003; Kim et al., 2019; Kulikova-Borovikova et al., 2018).

As novel species and strains of NSY are being highlighted for their interesting oenological properties, the great inter- and intra-species variability also become evident; thus, further studies are necessary to better comprehend the response of different NSY to the stressful conditions of ADY production, helping the development of optimized conditions to increase the availability of this new generation of yeast starters (Binati et al., 2020; Torrellas et al., 2020).

Table 2 summarizes the main results obtained with studies published up to date on the glutathione production by NSY correlated with their performance after the oxidative stress of biomass propagation/dehydration.

In the first investigation on the performance of NSY under ADY production, Gamero-Sandemetrio et al. (2018) obtained a general low biomass yield after growing the strains in a molasses-based medium, except for *Starmerella stellata* (synonym *Candida stellata*). As for the fermentative capacity assayed with the rehydrated cells, the best results were shown by *T. delbrueckii*. GSH production was the highest in *St. stellata* and lowest in *T. delbrueckii*, suggesting that deficient GSH synthesis causing low oxidative defense might be associated with low biomass yield, while poorer fermentative capacity is intrinsically displayed in some species, independently of oxidative stress. However, the authors could not find a clear correlation between antioxidant responses, biomass yield and fermentation performance, proposing additional physiological determinants for NSY (Gamero-Sandemetrio et al., 2018).

Câmara et al. (2019a) showed a better tolerance to dehydration stress in the NSY with higher production of glutathione during the growing phase. Moreover, GSH accumulation was enhanced in a nutrient rich medium, containing cysteine, respect to a conventional one. Some degree of variability was found among the species, with a higher glutathione production, and correlated higher viability after dehydration, for *L. thermotolerans*, followed by *M. pulcherrima* and finally *T. delbrueckii* (Câmara et al., 2019a).

In the study by Torrellas et al. (2020), the highest biomass yield was obtained with *Zygosaccharomyces bailii*, while *M. fructicola*, *M. pulcherrima*, and *St. bacillaris* had similar values. As for the survival after dehydration, NSY showed a significant variability, with viability as low as around 2% for *Hanseniaspora vineae*, while *Metschnikowia* sp. and *St. bacillaris* maintained more than 80% of viable cells. A great variability was present in both reduced (GSH) and oxidized (GSSG) glutathione contents, and the latter species presented the highest GSH accumulation after dehydration, confirming a more effective protection against the dehydration stress. Interestingly, *St. bacillaris* displayed the lowest GSH content in fresh cells, thus suggesting that this species could have used different strategies to respond the oxidative stress during aerobic propagation. All strains evaluated had a loss of fermentative capacity in rehydrated cells compared to the fresh ones, and generally the most affected were those with lower viability (Torrellas et al., 2020).

Lemos Junior et al. (2021a) investigated the growth of three NSY in a culture medium that maximize biomass and GSH production, but which was optimized for *S. cerevisiae*. Biomass measured as dry cell weight was similar, with a higher value for *L. thermotolerans*, while GSH production was much lower in *St. bacillaris*, in agreement with Torrellas et al. (2020). Authors concluded that diverse mechanisms of response to oxidative stress during aerobic growth and regulation of GSH metabolism might be present in each species.

Notwithstanding, it is well-known that NSY generally present low fermentative capacity and ethanol tolerance, but they are not expected to complete the alcoholic fermentation in wine. They are rather part of multistarter fermentations with *S. cerevisiae*, who is co-inoculated at the fermentation start or sequentially added a few days later, giving the NSY enough time to trigger significant changes in wine profile (Binati et al., 2020). Thus, it is not crucial that ADYs of NSY have a high fermentative capacity as it is for *S. cerevisiae*. The former should instead be selected, and the industrial production optimized, with a focus on achieving high cell numbers and viability after dehydration, which was proved to be strongly linked with an outstanding GSH metabolism.

4.3. Multistarter fermentations

Besides external addition of the pure compound and G-IDYs, which add costs to winemaking and might impact other aspects of wine quality (Rodríguez-Bencomo et al., 2014), the GSH production *in situ* from high-producer starter cultures is a valuable alternative for glutathione supplementation in wine fermentation. These are inoculated to perform the alcoholic fermentation and have the advantage of naturally secreting a high concentration of glutathione (De Vero et al., 2017). To the best of our knowledge, only a few laboratory studies were conducted to test this strategy, focusing on *S. cerevisiae*. There is one commercially available strain of *S. cerevisiae* which is claimed to generate increased glutathione, thanks to an adaptive evolution strategy focused on specific selection pressures linked to its release (AEB, 2021). This strain accumulates glutathione during alcoholic fermentation, and the wine content will increase afterwards with cell lysis. Moreover, NSY have a huge untapped potential and could be part of the research efforts, as high GSH producing strains in multistarter fermentations with *S. cerevisiae* could result in wine with a higher GSH concentration than monocultures of *S. cerevisiae*.

In a recent research effort by Binati et al. (2021a), nine NSY belonging to three different species, *L. thermotolerans*, *Metschnikowia* spp., and *St. bacillaris*, were tested in multistarter fermentations of Pinot Grigio, sequentially inoculated with *S. cerevisiae*. Glutathione content was measured in both the wine supernatants and yeast lees at the end of fermentation. Interestingly, some intra-species variability besides inter-species was associated with this trait. As regards the GSH dissolved in wine, two out of three strains of both *L. thermotolerans* and *Metschnikowia* spp. resulted in higher GSH content in multistarter fermentations compared to singly inoculated *S. cerevisiae*, while all the three strains of *St. bacillaris* achieved lower values. The highest GSH concentration was measured in the fermentation with *Metschnikowia* sp. COLR7, leading to an increment of around 10 mg/L of GSH. This is remarkable, considering that the addition of pure GSH is limited at 20 mg/L according to the OIV (Organization Internationale de la Vigne et du Vin, 2017a), and that a relatively low concentration (1–2 mg/L) of GSH is released from the application of GSH-IDYs at the recommended dosage of 0.3 g/L (Rodríguez-Bencomo et al., 2016).

Furthermore, GSH produced by yeasts during fermentation could remain trapped inside cells and not be immediately released to the surrounding liquid. Binati et al. (2021a) found the highest intracellular GSH accumulated in the yeast lees of the multistarter fermentations with *St. bacillaris*, which could be related with the lowest secreted GSH measured in those ferments. Corroborating this hypothesis, the lowest intracellular GSH was detected in the fermentations that achieved the highest GSH in the supernatant. Interestingly, these results correlate with the genomic analysis (Section 3.2), which showed presence of genes involved in GSH transport in *L. thermotolerans* and *Metschnikowia*

Table 2
Glutathione production by wine-related non-*Saccharomyces* yeasts in growth conditions associated with ADY production (data from Gamero-Sandemetrio et al., 2018; Câmara et al., 2019a; Torrellas et al., 2020; Lemos Junior et al., 2021a).

Species	Strain	Growth conditions	Biomass yield	Glutathione production	Cell viability after dehydration	Fermentative capacity
Hanseniaspora guilliermondii	CECT 11027	[1]	OD_{600} 9.20	~1.0 nmol/mg dry cells	nd	2.81 mL $CO_2/10^7$ rehydrated cells
Hanseniaspora osmophila	CECT 1474	[1]	OD_{600} 10.17	~0.7 nmol/mg dry cells	nd	3.81 mL $CO_2/10^7$ rehydrated cells
Hanseniaspora vineae	from Lallemand Inc. collection	[3]	OD_{600} 7.72 0.06 g/g sucrose	~0.5 nmol/mg fresh cells ~0.5 nmol/mg dry cells	1.83%	15.45 mL $CO_2/10^7$ fresh cells 8.03 mL $CO_2/10^7$ rehydrated cells
Kluyveromyces wickerhamii	from Lallemand Inc. collection	[3]	OD_{600} 10.12 0.06 g/g sucrose	~3.5 nmol/mg fresh cells ~1.5 nmol/mg dry cells	23.18%	4.40 mL $CO_2/10^7$ fresh cells 1.85 mL $CO_2/10^7$ rehydrated cells
Lachancea thermotolerans	CBS6340	[2]	nd	242 μg/10^{10} fresh cells ~200 μg/10^{10} dry cells	~78%	nd
	SOL13	[4]	3.82 g/L (24 h) 4.81 g/L (96 h)	0.43 nmol/mg fresh cells (24 h) 2.57 nmol/mg fresh cells (96 h)	nd	4.87 g CO_2/100 g synthetic must
Metschnikowia sp.	*M. pulcherrima* from Lallemand Inc. collection	[3]	OD_{600} 7.35 0.05 g/g sucrose	~2.5 nmol/mg fresh cells ~5.5 nmol/mg dry cells	80.29%	4.11 mL $CO_2/10^7$ fresh cells 1.83 mL $CO_2/10^7$ rehydrated cells
	M. fructicola from Lallemand Inc. collection	[3]	OD_{600} 8.30 0.07 g/g sucrose	~4.0 nmol/mg fresh cells ~6.0 nmol/mg dry cells	88.69%	3.54 mL $CO_2/10^7$ fresh cells 1.96 mL $CO_2/10^7$ rehydrated cells
	M. pulcherrima CBS5833	[2]	nd	213 μg/10^{10} fresh cells ~220 μg/10^{10} dry cells	~75%	nd
	Metschnikowia sp. FIANO12	[4]	3.66 g/L (24 h) 3.74 g/L (96 h)	0.93 nmol/mg fresh cells (24 h) 2.83 nmol/mg fresh cells (96 h)	nd	0.70 g CO_2/100 g synthetic must
Pichia fermentans	CBS7435	[1]	OD_{600} 8.73	~1.7 nmol/mg dry cells	nd	1.23 mL $CO_2/10^7$ rehydrated cells
Starmerella bacillaris	from Lallemand Inc. collection	[3]	OD_{600} 18.41 0.07 g/g sucrose	~0.5 nmol/mg fresh cells ~3.5 nmol/mg dry cells	80.46%	1.85 mL $CO_2/10^7$ fresh cells 1.55 mL $CO_2/10^7$ rehydrated cells
	MALV45	[4]	3.53 g/L (24 h) 3.51 g/L (96 h)	0.08 nmol/mg fresh cells (24 h) 0.08 nmol/mg fresh cells (96 h)	nd	3.31 g CO_2/100 g synthetic must
Starmerella stellata	CECT 11108	[1]	OD_{600} 25.84	~2.7 nmol/mg dry cells	nd	1.77 mL $CO_2/10^7$ rehydrated cells
Torulaspora delbrueckii	from Lallemand Inc. collection	[3]	OD_{600} 13.52 0.09 g/g sucrose	~1.0 nmol/mg fresh cells ~1.0 nmol/mg dry cells	56.24%	8.95 mL $CO_2/10^7$ fresh cells 8.09 mL $CO_2/10^7$ rehydrated cells
	CBS4865	[2]	nd	202 μg/10^{10} fresh cells ~190 μg/10^{10} dry cells	~64%	nd
	D91 from Lallemand Inc. collection	[1]	OD_{600} 10.03	~0.6 nmol/mg dry cells	nd	11.33 mL $CO_2/10^7$ rehydrated cells
Wickerhamomyces anomalus	from Lallemand Inc. collection	[3]	OD_{600} 14.91 0.09 g/g sucrose	~1.5 nmol/mg fresh cells ~1.0 nmol/mg dry cells	33.76%	3.49 mL $CO_2/10^7$ fresh cells 0.98 mL $CO_2/10^7$ rehydrated cells
Zygosaccharomyces bailii	from Lallemand Inc. collection	[3]	OD_{600} 20.60 0.15 g/g sucrose	~0.5 nmol/mg fresh cells ~0.5 nmol/mg dry cells	37.93%	16.28 mL $CO_2/10^7$ fresh cells 7.46 mL $CO_2/10^7$ rehydrated cells

CBS: Centraal Bureau voor Schimmelcultures (Westerdijk Fungal Biodiversity Institute).
CECT: Spanish Type Culture Collection.
nd: non determined.
[1] Gamero-Sandemetrio et al., 2018: 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 (0.5 mg/L D-biotin, 1 mg/L calcium pantothenate and 1 mg/L thiamine hydrochloride). Incubation at 30 °C with shaking (180 rpm), for 24 h.
[2] Câmara et al., 2019a: GSM medium with 30 g/L glucose, 30 g/L yeast extract, 0.6 g/L KH_2PO_4, 0.6 g/L cysteine. Incubation at 30 °C in a rotary shaker for 24 h.
[3] Torrellas et al., 2020: 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 (0.5 mg/L D-biotin, 1 mg/L calcium pantothenate and 1 mg/L thiamine hydrochloride). Incubation at 30 °C with shaking (180 rpm), for 24 h.
[4] Lemos Junior et al., 2021a: Medium optimized for glutathione production (MGSH) composed of 54 g/L glucose, 50 g/L yeast extract, 12 g/L MgSO4, pH 5.0, supplemented 7 h after inoculation with 18 mM glycine, 10 mM glutamate, and 3.35 mM cysteine. Incubation at 27 °C with shaking (200 rpm), for 96 h.

sp., while absent in *St. bacillaris*. In all cases, GSH was higher in the yeast lees of multistarter fermentations then in the control (Binati et al., 2021a).

Similarly, Lemos Junior et al. (2021a) investigated the GSH accumulation in the yeast lees at the end of multistarter fermentations using the same NSY, but in synthetic and pasteurized grape juice. GSH content was much higher in the pasteurized juice compared to the synthetic one, even if biomass produced was similar, highlighting that the matrix composition could affect the GSH metabolism in yeast cells. The highest GSH accumulation was found in the yeast lees of the mixed-culture fermentation with *St. bacillaris*, in accordance with Binati et al. (2021a). A further study with only single-culture fermentations of *St. bacillaris* strains and *S. cerevisiae* showed a higher intracellular glutathione content in the former, in synthetic grape must (Lemos Junior et al., 2021b).

Further studies are necessary to evaluate the long-term effect of this GSH increment associated with multistarter fermentations, following the evolution of aromatic compounds and oxidation impact during ageing and storage. It was reported that *S. cerevisiae* achieve the maximum level of intracellular GSH at the end of exponential phase of growth, being subsequently secreted when cells approach stationary phase (Perrone, Grant, & Dawes, 2005). The release of glutathione and amino acids continues when dead cells start the autolytic process (Dubourdieu & Lavigne-Cruège, 2004). Thus, it would be interesting to investigate if a higher GSH content accumulated in yeast lees of multistarter fermentations compared to single *S. cerevisiae* could lead to a higher GSH release in wine during lees ageing.

5. Conclusions

The diversity of wine styles, protocols for GSH additions, composition of the tested products and methods of analysis make it difficult to generalize any conclusions from the scientific literature about the impact of glutathione in winemaking. Nevertheless, new formulations are increasingly coming to the market, and it seems that more winemakers are willing to give it a try, not least because they acknowledge the consumer pressure to reduce SO_2 inputs. Hence, it is very likely that more data will help to expand this oenological strategy in future.

There is more agreement regarding the critical role of glutathione in yeast metabolism during the industrial production of biomass for starter cultures. Focusing on new species and strains with increased GSH natural production, the optimization of conditions for biomass cultivation and GSH accumulation in NSY are imperative to obtain superior yeast products with guaranteed glutathione levels.

Even if genetic engineering can be successfully exploited for obtaining wine yeasts with enhanced GSH production, its acceptance in wine industry is still an issue to be dealt with in future. Therefore, high GSH producing yeasts must be mined from the natural biodiversity, confirming the urge to expand and improve selection protocols aimed to take advantage of this feature and unlock the potential of wine relevant NSY.

CRediT authorship contribution statement

Renato L. Binati: Writing – original draft, Visualization, Writing – review & editing. **Ilaria Larini:** Investigation, Visualization. **Elisa Salvetti:** Investigation, Writing – original draft. **Sandra Torriani:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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