Contribution of non-*Saccharomyces* yeasts to increase glutathione concentration in wine

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Abstract

Background and Aims: Glutathione (GSH), a strong antioxidant naturally present in grape berries and produced by yeasts during fermentation, can be added by winemakers to control oxidative deterioration of wine. A promising approach is the inoculation of starter or co-starter yeasts that are strong producers of GSH in situ. Since little is known about this activity in non-*Saccharomyces* yeasts, the aim of this research was to evaluate the contribution of several strains of *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris* to increase the GSH concentration in wine.
Methods and Results: Pinot Grigio grape must was sequentially inoculated with the non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*. Some strains significantly increased GSH in wines, notably *Metschnikowia* spp., up to a surplus of 10 mg/L compared to the Control singly inoculated with *S. cerevisiae*.
Conclusions: These results suggest the feasibility of the proposed strategy to increase GSH concentration in wines as an alternative to addition of pure GSH or inactivated dry yeasts.
Significance of the Study: As the maximum allowed addition of GSH in wine is 20 mg/L, the strategy of mixed-culture fermentation can be a valuable tool to lower inputs, and ultimately obtain a healthier product by reducing the need for sulfur dioxide.

Keywords: *glutathione production, multi-starter fermentation, non-conventional yeasts,* Saccharomyces cerevisiae, *wine quality*

Introduction

The tripeptide L-γ-glutamyl-L-cystinyl-glycine (glutathione) is present in almost all living organisms, from prokaryotes to humans, including yeasts and grapes. In cells, glutathione is mainly present in the reduced form (GSH) and serves many important functions, such as playing roles as a redox buffer, as an antioxidant and as a detoxifier of xenobiotics, metals and acetaldehydes (Patzschke et al. 2015). In grape berries, GSH is also reported to participate in the biosynthesis of many non-volatile aroma precursors, as for example some precursors of varietal thiol compounds in Sauvignon Blanc (Kritzinger et al. 2013a, Gabrielli et al. 2017).

More recently, the exploitation of GSH in winemaking has gained increased attention. It could be useful for controlling the oxidation, especially of white wines, which leads to impaired aromatic profile, detrimental change of colour and development of off-flavours (Kritzinger et al. 2013a). Glutathione acts by inhibiting the polymerisation of phenolic substances related to browning reactions and by limiting the formation of volatile compounds that give unwanted aromas during aging, such as sotolon and 2-aminoacetophenone. It also avoids the loss of important aromatic molecules (esters, terpenes, volatile thiols) (Rodríguez-Bencomo et al. 2014, Bonciani et al. 2018). Particularly, the use of GSH could represent a healthier and safe strategy for reducing the addition of SO_2 as an antioxidant in wine, with beneficial effects for consumers (De Vero et al. 2017, Gabrielli et al. 2017).

The concentration of natural GSH is variable in must and wine, and several factors are probably responsible, including yeasts, which are able to assimilate and secrete GSH during fermentation (Kritzinger et al. 2013a). A method to increase GSH concentration is the direct addition of the pure compound in wine, although this approach tends to be costly (De Vero et al. 2017). Alternatively, it is becoming more popular recently to use preparations containing inactivated dry yeasts (IDYs) enriched with GSH, or the inoculation of *Saccharomyces cerevisiae* starter cultures which naturally produce a high concentration of GSH in situ (De Vero et al. 2017, Gabrielli et al. 2017, Bonciani et al. 2018).

The yeast *S. cerevisiae* has been the benchmark starter culture for many decades, inoculated into grape musts to overpower the native microbiota and provide a more controlled and predictable fermentation. A growing trend in oenology is, however, the use of multi-starters containing representatives of non-conventional yeasts (Gamero-Sandemetrio et al. 2018). The so-called group of non-*Saccharomyces* yeasts is becoming popular for a myriad of reasons, but the ultimate goal is to improve fermentation and obtain wines with greater complexity, distinctiveness and quality (Roudil et al. 2020). These yeasts can produce oenologically important secondary metabolites, improving and diversifying beneficial traits usually associated with *S. cerevisiae* (Mateo and Maicas 2016).

Hence, we have hypothesised that the inoculation of non-*Saccharomyces* yeast strains capable of producing GSH in multi-starter fermentations with *S. cerevisiae* will result in wines with a higher GSH concentration than single fermentations with *S. cerevisiae* alone. Some recent studies measured the production of GSH during stress-response of non-*Saccharomyces* yeast strains to the dehydration process (Gamero-Sandemetrio et al. 2018, Câmara et al. 2019), but, to the best of our knowledge, this is the first time that non-conventional yeasts were evaluated for the in situ

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production of GSH during wine fermentation. This would increase the interest in exploitation of non-conventional yeasts and represent a new aspect to be considered in their selection.

The aim of the present research was to evaluate the ability of different strains of *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris* to produce GSH in multi-starter fermentations with *S. cerevisiae*. These non-*Saccharomyces* yeasts were selected among the most studied in wine fermentations (Englezos et al. 2017, Morata et al. 2018, 2019).

Materials and methods

Wine samples

The samples analysed in this study were obtained in a previous investigation (Binati et al. 2020), in which microvinification trials were carried out by inoculating natural grape must with multi-starters composed of native non-*Saccharomyces* yeast strains and *S. cerevisiae* EC 1118 (Lallemand, Montréal, QC, Canada), following a sequential approach. Must was obtained by pressing of Pinot Grigio grapes [236 g/L reducing sugar, 235.5 mg/L yeast assimilable nitrogen (YAN)] and it received no thermal treatment or addition of SO_2. The non-*Saccharomyces* strains used were: *L. thermotolerans* COLC27, DESP53 and SOL13; *S. bacillaris* CHIAR4, MALV45 and PECO10; and *Metschnikowia* spp. COLR7, FIANO12 and SOUV1. They were previously selected based on relevant oenological parameters (Binati et al. 2019). Control fermentations were conducted with the sole inoculation of *S. cerevisiae* EC 1118, at time 0 and after 48 h. The 11 different experiments were conducted in triplicate, in sterile 200 mL glass bottles completely filled, and equipped with perforated silicon stoppers combined with 0.45 mm filters (Merck Millipore, Milan, Italy). Bottles were kept under static conditions at 22°C. Once the fermentations were finished, 50 mL aliquots of wine were collected in conical centrifuge tubes for quantification of GSH and cell dry mass (CDM). Samples were maintained at −20°C until the analysis. Viable yeast cell counts, fermentation kinetics and quality of the final wines were described in Binati et al. (2020).

Glutathione assay and biomass determination

Supernatant and pellet for each sample were separated by centrifugation at 5000*g* for 10 min, then processed separately to analyse the extracellular and intracellular GSH. Extracellular GSH concentration of grape must and wines was measured directly in the supernatants. Intracellular GSH was extracted from cells, according to Xiong et al. (2009). Briefly, after centrifugation of the wine samples, wet cell pellets were resuspended in 1 mL of physiological solution (0.9% NaCl) and washed twice with the same solution. Ethanol (500 µL 25% v/v) was 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. Glutathione was determined with the enzymatic GSH assay kit (Sigma-Aldrich, Milan, Italy), following the manufacturer's instructions. A standard curve with GSH of known concentration was prepared simultaneously with every batch of analysis.

Extracellular GSH concentration was converted to mg/L by using the molecular mass. The values of intracellular GSH obtained in nmol/mL were normalised through the CDM calculated for every sample. The CDM was determined after drying cell pellets at 60°C to a constant mass.

Statistical analysis

Data were compared by one-way ANOVA, followed by the post-hoc Tukey's honestly significant difference test, through the software PAST (Hammer et al. 2001). The threshold for statistical significance was set at $P < 0.05$.

Results

Biomass production

The CDM was measured at the end of fermentations to compare biomass production among multi-starter fermentations and to express GSH concentration as nmol/mg cells (Table 1). Sequential fermentations with *Metschnikowia* spp. strains and *S. cerevisiae* showed the highest production of CDM, although the difference was not significant with *S. bacillaris*/*S. cerevisiae* and with the Control. Fermentations with *L. thermotolerans* strains produced the lowest level of biomass.

Table 1. Total yeast population, cell dry mass and intracellular glutathione extracted from cells at the end of sequential fermentations with non-*Saccharomyces* strains and *S. cerevisiae*.

Yeast	Maximum population (Log_{10} CFU/mL)†	End population (Log_{10} CFU/mL)‡	CDM (g cell/L)	GSH (nmol/mg cell)
Lachancea thermotolerans* + *Saccharomyces cerevisiae				
COLC27	7.89 ± 0.11	6.98 ± 0.03	0.498 ± 0.011c	0.537 ± 0.011ab
DESP53	7.72 ± 0.20	7.12 ± 0.05	1.480 ± 0.113abc	0.269 ± 0.045bcd
SOL13	7.78 ± 0.18	6.88 ± 0.24	1.013 ± 0.385bc	0.205 ± 0.064bcd
Starmerella bacillaris* + *Saccharomyces cerevisiae				
CHIAR4	7.87 ± 0.15	7.29 ± 0.01	1.795 ± 0.184ab	0.718 ± 0.049a
MALV45	7.68 ± 0.17	7.37 ± 0.05	1.693 ± 0.619ab	0.716 ± 0.262a
PECO10	7.90 ± 0.00	7.17 ± 0.06	2.055 ± 0.191a	0.499 ± 0.082abc
Metschnikowia* spp. + *Saccharomyces cerevisiae				
COLR7	8.04 ± 0.08	7.13 ± 0.08	2.223 ± 0.081a	0.134 ± 0.011d
FIANO12	7.88 ± 0.09	7.18 ± 0.12	2.020 ± 0.057ab	0.193 ± 0.009bcd
SOUV1	7.92 ± 0.06	7.24 ± 0.01	1.900 ± 0.170ab	0.167 ± 0.008 cd
Saccharomyces cerevisiae				
EC 1118	8.19 ± 0.02	6.36 ± 0.10	1.858 ± 0.124ab	0.095 ± 0.014d

†Maximum population, maximum concentration of yeast cells reached during the fermentation (from Binati et al. 2020); ‡end population, concentration of yeast cells at the end of fermentations (from Binati et al. 2020), when CDM was measured. CDM, cell dry mass; CFU, colony forming unit; GSH, glutathione.

In addition, Table 1 reports the maximal and final numbers of yeast cells for each trial calculated from total viable cell counts (sum of colonies of non-*Saccharomyces* yeasts and *S. cerevisiae*) obtained previously by Binati et al. (2020). All fermentations had similar maximum cell numbers. Nevertheless, the highest cell number was observed in the single inoculation of *S. cerevisiae*, underlining a competition between yeasts, that determined a growth restraint. Interestingly, the viable population of single *S. cerevisiae* fermentation had the greatest decrease towards the end of fermentations, while fermentations in combination with *S. bacillaris* and *Metschnikowia* spp. strains maintained the highest population.

Glutathione accumulation by yeast cells

The GSH that accumulated in yeast cells was expressed as nmol GSH/mg of cells (Table 1). Cells in fermentations with the *S. bacillaris* strains achieved the highest GSH accumulation, especially CHIAR4 and MALV45 (0.72 nmol GSH/mg cells), significantly higher than all others, except *L. thermotolerans* COLC27. Fermentations with the *Metschnikowia* spp. strains had the lowest GSH accumulation among the sequential inoculations. Nevertheless, all mixed-culture fermentations produced more GSH than single *S. cerevisiae* fermentations, although not statistically significant in some comparisons. Moreover, some strain variability was observed within the species *L. thermotolerans* and *S. bacillaris*. The strain COLC27 produced more GSH than the other two, while for *S. bacillaris*, PECO10 accumulated the lowest concentration of GSH.

Glutathione in wine

Quantification of GSH in wine supernatants showed a completely different picture (Figure 1). Fermentations with the *Metschnikowia* spp. strains resulted in the highest values, most notably for strains COLR7 and SOUV1 (25.2 and 24.4 mg/L, respectively), followed by the *L. thermotolerans* strains. Wines fermented with *S. bacillaris* had similar GSH concentration, around 6.3 mg/L, all remarkably lower than all other wines.

Single inoculation with *S. cerevisiae* had an intermediate value (15.7 mg/L) between the lowest *S. bacillaris* and the highest *L. thermotolerans* and *Metschnikowia* spp. strains. Glutathione in the grape must, prior to inoculation, was 2.87 mg/L, significantly lower than all values obtained at the end of fermentation, confirming the trend of increase in GSH concentration because of yeast activity during alcoholic fermentation.

Intra-species variability was clearly observed among the *L. thermotolerans* and *Metschnikowia* spp. strains. Fermentations with COLC27 had a significantly lower GSH concentration than DESP53 and SOL13, while FIANO12 was lower than COLR7 and SOUV1.

Discussion

Because of its positive effects in restraining the oxidative phenomena in wine, addition of reduced GSH, up to 20 mg/L, was approved in must and wine by the Organisation Internationale de la Vigne et du Vin (Organisation Internationale de la Vigne et du Vin 2015) (Resolutions OIV-OENO 445-2015 and OIV-OENO 446-2015). Notwithstanding, use of IDYs or pure GSH represents additional costs to winemaking, and their efficacy and effect on other aspects of wine quality are not well understood (Rodríguez-Bencomo et al. 2014, De Vero et al. 2017).

This survey explored the capability of non-*Saccharomyces* yeasts to increase GSH concentration in wines obtained by sequential fermentations, complementing a previous study (Binati et al. 2020) that acknowledged significant differences in chemical and sensory analysis. The biomass production, of *L. thermotolerans*/*S. cerevisiae* fermentations was the lowest, while that of *S. bacillaris*/*S. cerevisiae* and *Metschnikowia* spp./*S. cerevisiae* was not significantly different to single *S. cerevisiae* fermentations. Nevertheless, Binati et al. (2020) have reported that at the end of all fermentations, only viable cells of *S. cerevisiae* were present, and at a level higher in wine sequentially inoculated than that in the single fermentation. Possibly, death and autolysis of non-*Saccharomyces* yeasts provided an extra nutrient source for *S. cerevisiae*, as suggested by Lleixà et al. (2016), and thus it was able to survive longer. Intriguingly, the lowest GSH/mg cells was in the single *S. cerevisiae* fermentations. *Saccharomyces cerevisiae* alone could have metabolised GSH as a nutritional source, in the absence of nutrients from autolysis of dead non-*Saccharomyces* cells. But it might not have been sufficient, leading to early decline of *S. cerevisiae*. Nevertheless, a lower capacity of *S. cerevisiae* EC 1118 to produce GSH compared to non-*Saccharomyces* yeasts could also be taken into account.

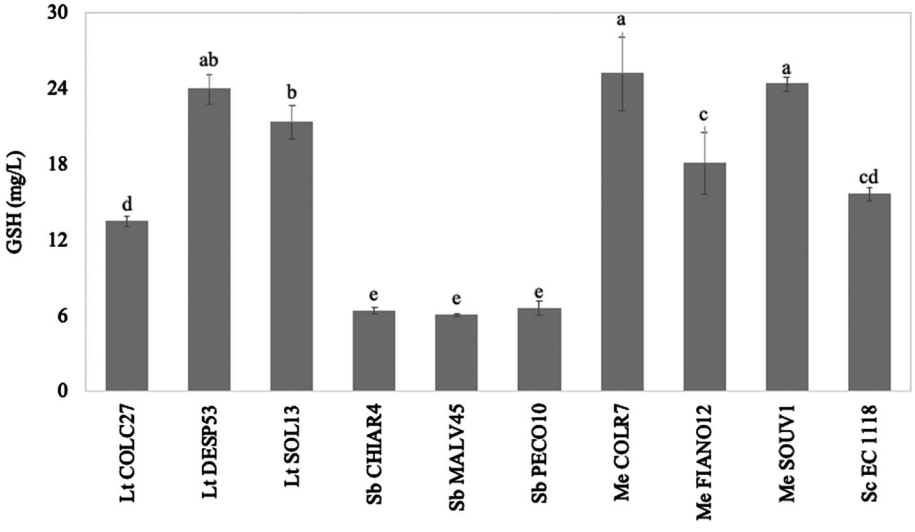

Figure 1. Glutathione concentration in wines at the end of sequential fermentations in natural grape must. Lt, *Lachancea thermotolerans*; Sb, *Starmerella bacillaris*; Me, *Metschnikowia* spp.; Sc, *Saccharomyces cerevisiae*. Mean values of three independent replicates, with error bars representing SD. Different letters indicate statistically significant difference ($P < 0.05$).

Fermentations with the *S. bacillaris* strains had the highest concentration of intracellular GSH and the lowest GSH dissolved in wine. Interestingly, Binati et al. (2020) found a significantly lower concentration of YAN at the end of fermentations with *S. bacillaris*, indicating a higher consumption of nutrients, as previously reported (Medina et al. 2012). Thus, GSH could have been accumulated in the cells as storage of nitrogen and sulfur. Considering also that cell number was highest in the *S. bacillaris*/*S. cerevisiae* fermentations, the longer survival of the cells could have contributed to the GSH results observed here.

In this survey, the range of GSH in wine was 6.04–25.20 mg/L, all showing significant increase from the initial value (2.87 mg/L). Several authors reported the impact of yeast metabolism on GSH concentration after alcoholic fermentations, in several synthetic and natural grape must matrices, but having in common the single *S. cerevisiae* inoculation. Lavigne et al. (2007) found a maximal GSH increase of 6 mg/L in white wine. Kritzinger et al. (2013b) screened 20 commercial *S. cerevisiae* starters, and found that the GSH concentration decreased from 40 mg/L in synthetic grape juice and from 15 mg/L in Sauvignon Blanc wine to less than 3.5 mg/L. In contrast, Gabrielli et al. (2017) reported that GSH increased from around 2.5 to 10 mg/L in Sauvignon Blanc wine. Further, recent investigations with synthetic grape must indicated that the GSH production by *S. cerevisiae* strains ranged between 2.11 and 4.71 mg/L (Bonciani et al. 2018) and between 5.39 and 16.28 mg/L (Guerrini et al. 2018). These results highlight that yeasts modulate GSH concentration during alcoholic fermentation in a strain-dependent manner, but also illustrate a lack of consensus on their capability to increase or decrease GSH.

Nevertheless, the effect of multi-starter fermentations on GSH concentration has not been previously reported. The interactions between strains of *Saccharomyces* and non-*Saccharomyces* yeasts can be through direct contact or changes in the medium composition. The effect of these interactions can be positive, negative or neutral to the production of certain metabolites, modulating the final wine composition (Roullier-Gall et al. 2020).

In this survey, wines fermented with the contribution of *L. thermotolerans* DESP53, *Metschnikowia* spp. COLR7 and FIANO12 had an increase of about 10 mg/L in GSH, in comparison with the Control fermentation. Considering that maximal GSH addition is limited to 20 mg/L, those results showed a great potential for the use of mixed-culture fermentations to reduce the risk and expense of external GSH addition. As observed by Gabrielli et al. (2017), addition of IDYs changed the aroma profile and sensory characteristics of wine, although most likely caused by different components released from the yeast preparations other than GSH. Furthermore, external GSH supplementation of musts could lead to the development of off-flavours, such as volatile sulfur compounds, under certain conditions (Granchi et al. 2019).

Conclusions

Multi-starter fermentations with selected native non-*Saccharomyces* strains and *S. cerevisiae* demonstrated the feasibility of this biotechnological approach to increase GSH concentration in wines by up to 10 mg/L, confirming the proposed hypothesis. It could prevent the addition of either costly pure GSH or IDYs with unknown composition, and possible negative collateral effects on wine aroma profile. Differences in metabolic activities largely reported among yeast species and strains highlight the prominence of efficient strain selection programs, where the ability to produce GSH could complement other relevant oenological parameters. Nonetheless, further studies are needed to assess the long-term effect of GSH increase in the prevention of oxidative phenomena and protection of key aroma compounds, during barrel maturation and/or bottle aging, after repetition of the fermentation trials in large scale industrial conditions.

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