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Investigation on the Oenological Applicability of Bioprotection in Tokaj Wine Region

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Abstract

Viticulture and oenology are increasingly focused on environmental sustainability, food safety and health awareness. Reduced or zero sulphur in wine preservation is one of the key aspects of organic production, where bioprotection cultures are used to prevent oxidation, inhibit the growth of harmful microorganisms. Particular attention should be paid to the microbiological protection of noble rot raw materials, because the presence of Botyrtis cinerea fundamentally alters the composition of the associated microflora and allows bacterial growth resulting changes in chemical composition associated with increased amounts of sulphuric acid-binding ketoacids, so that there are certainly a number of advantages to be gained from reducing or replacing sulphur-dioxide by bioprotection. In the present study, the experience with the application of Erbslöh Oenoferm® MProtect Metschnikowia pulcherrima is shown in the treatment of raw materials with different degrees of noble rot in the Tokaj wine region.

Keywords: bioprotection, reduced sulphur-dioxide use, Metschnikowia pulcherrima, noble rot

1. Introduction

1.1 Bioproduction in the winery

Bioprotection (or bioconversion or biocontrol) involves the use of microorganisms or their antimicrobial products as bioprotective agents (Gianvito et al., 2022). This practice is widely used, mainly in agriculture and food industry, to protect fruits against microorganisms that cause postharvest spoilage and to extend the shelf life of food (Ferraz et al., 2019). The bioprotection strategy consists of the inoculation of living microorganisms (bioprotection cultures, BPCs) or the addition of their metabolites (bioprotection metabolites, BPMs), in purified form, during or after food production. These microorganisms prevent microbial spoilage of food through different bioprotective mechanisms, which can be divided into passive (competition for space, nutrients and oxygen) and active antagonistic strategies (production of antimicrobial molecules). The addition of BPCs early in the production process can favourably improve the organoleptic properties of fermented food, such as taste, texture and nutritional value (Gaggia et al., 2011). In the wine industry, sulphur dioxide is one of the most important and widely used excipients due to its antioxidant and antimicrobial properties. Among its many benefits, it is a labelled allergen, and more and more wine consumers are sensitive to it. The World Health Organization (WHO) is advocating alternative methods to reduce or eliminate its use in wine production (Lisanti et al., 2019). In addition to the use of lysozyme, DMDC, chitosan, nisin, allyl isocyanate-infused paraffin waxes, bioprotection can play an important role in the feasability of this effort (Giacosa et al.). Most commonly *Torulaspora delbrueckii, Lachancea thermotolerans* and *Metschnikowia pulcherrima* or a combination of these are used as bioprotective agents as shown in Figure 1. (Lebleux et al., 2023; Vejarano&Gil-Calderón, 2021). The bioprotective wild yeast strains can be added to the berries immediately or during cold maceration (Agarbati et al., 2023), followed by inoculation with Saccharomyces yeast after 24-72 hours. The predominant presence of non-Saccharomyces strains in the preferential phase results in a microbiological control, prevents the proliferation of acetic acid bacteria, attenuates mould diversity and participates in preventing undesirable oxidation processes by rapid oxygen consumption, binding metals (Cu^{2+}, Fe^{2+}) that catalyse oxidation. Acetoin formation is reduced by the reduction of *Kloeckera apiculata* and *Candida stellata* strains, which are capable of producing large amounts of acetoin from pyruvic acid through acetolactate synthesis (Figure 2.). Less nitrogen nutrient consumption occurs,

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more higher alcohols, terpenes, esters and thiols are formed, glycerol content increases, acid composition is enriched (galacturonic acid, lactic acid, succinic acid) (Vejarano&Gil-Calderón, 2021).

1.2 Metschnikowia pulcherrima strain

The wild yeast *Metschnikowia pulcherrima* is a common strain of yeast for grapes and musts. It is characterised by the production of pulcherrimin, an insoluble, non-carotenoid, red, antifungal pigment, as an antimicrobial compound. The precursor compound of pulcherrimin is pulcherriminic acid, which reacts with and binds Fe ions in the medium, so that microorganisms that require Fe for their growth are repelled (Figure 3.) (Oro et al.).

It can effectively inhibit the growth of several yeasts (*Candida tropicalis, Candida albicans, Brettanomyces/Dekkera, Hanseniaspora* and *Pichia genera*) and moulds (*Botrytis cinerea, Penicillium, Alternaria* and *Monilia spp.*) (Morata et al., 2019; Sipiczki, 2006).

MP has several enzyme activities: pectinase, protease, glucanase, β-glucosidase, cellulase, xylanase, amylase, sulfite reductase, lipase and β-lyase, making it one of the non-Saccharomyces yeast species that can produce several extracellular hydrolytic enzymes. It is also capable of rapid flavour release due to its α-arabinofuranosidase and β-D-glucosidase enzymes, helping to form volatile terpene compounds and thiols to develop fruity flavours. In the early fermentation phase, *M. pulcherrima* is able to highly affect wine quality and aroma complexity, for example by releasing hydrolytic enzymes as mentioned above, increasing production of glycerol and glutathione, and reducing yields of ethanol, acetaldehyde and acetic acid (Binati et al., 2022; Morata et al., 2019).

Oxygen plays a key role in the metabolism of *M. pulcherrima* as it does in other non-*Saccharomyces* species. It is a high oxygen user, which means that at least 17% of the sugar content is used for respiration at the start of fermentation. Thus, other non-*Saccharomyces* competitors are deprived of oxygen because M. pulcherrima consumes it from them (Morales et al., 2015).

A number of biopreparations are commercially available from food additive manufacturers that seek to exploit the benefits of non-*Saccharomyces* yeast strains in wine production. *Metschnikowia pulcherrima* is one of the most commonly used strains, and is also recommended for grapes and musts (Table 1.).

It has many beneficial properties for its oenological applications (Figure 4.):

- low alcohol tolerance, so less alcohol yield can be achieved in the early stages of fermentative process;
- there is a good ester production capability;
- promote the glycerol production together with *Saccharomyces* strains;
- low or moderate volatile acidity content;
- stop or eliminate the presence of Brettanomyces, apiculatus yeast strains and lactic acid bacteria (Puyo et al., 2023).

An important reference for my own research is the work of Binati et al., 2023, in which they investigated the application of bioprotection to overripened, withered grapes of the Italian autochthonous grape variety Garganega, where *Botrytis cinerea* activity also plays an important role, as in the Tokaj wine region. In this experimental work, *M. pulcherrima* has been shown to be effective in protecting grapes against fungal and bacterial infections during wilting and enrich the wine character with special aromas.

1.3 The specific microbiota and chemical composition of noble rotted process

The microflora of grapes affected by *Botrytis cinerea* differs greatly from the microbial composition found on the surface of healthy grapes. The composition of an intact, healthy grape is practically unaffected by the saprophytic yeasts, moulds and bacteria on its surface, since they cannot access the must localised in the flesh through the skin. However, *Botrytis cinerea* is able to enzymatically loosen and destroy the epidermis tissue, making the interior of the berry permeable to surface saprobiont microflora, various yeast strains (*Candida, Rhodotorula, Pichia, Kluyveromyces species*) (Magyar&Bene, 2006) and moulds (*Aspergillus, Penicillium sp.*) and increased growth of acetic acid bacteria (*Gluconobacter, Acetobacter*) (Figure 5.). The noble rot causes characteristic chemical changes in the composition of must: an increase in sugar and glycerol content, a change in the acid composition, an increase in polyphenol oxidases in musts, which increases the tendency of wines to browning and browning, an accumulation of phenolic acids. The formation of a characteristic botrytis odour and aromatic substances (fungal alcohol (1-octen-3-ol), a lactone called sotolone (3-hydroxy-4,5-dimethyl,2(5H)-furanone), an increase in the amount of keto acids, an increase in the concentration of biogenic amines (tyramine, agmatine, phenylethylamine) and other amines (primary aliphatic amines: i-butylamine, 2-methylbutylamine) (Bene, 2023).

Enzymatic browning is one of the most important reactions that takes place in most fruits and vegetables. These processes affect not only the colour of the food, but also its taste. It is a series of chemical reactions involving polyphenol oxidase (PPO), catechol oxidase and other enzymes that produce melanin and benzoquinone from natural phenols. Enzymatic browning (also called oxidation of food) requires exposure to oxygen. It starts with the oxidation of phenols by polyphenol oxidase to quinones, whose strong electrophilic state makes them highly sensitive to nucleophilic attack by other proteins. These quinones are then polymerised in a series of reactions, which eventually result in the formation of brown pigments (melanosis). The rate of enzymatic browning is reflected by the amount of active polyphenol oxidases present in the food. Therefore, most research on methods to inhibit enzymatic browning has focused on the inhibition of polyphenol oxidase activity.

The laccase enzyme produced by Botrytis cinerea is an oxidation enzyme consisting of a protein part and a copper atom, which is essential for its function. Laccase is a non-specific polyphenol oxidase that oxidises the polyphenols in wine, resulting in acetaldehyde production, reduction in free sulphur, depletes the richness of flavour, loss of primary aromas, darkening of white wines and increase in brownish tone in red wines. Lactase is a very stable enzyme that is not rendered inactive by alcohol, so its adverse effects, if not stopped, persist in the finished wine. In order to reduce the effects of the laccase enzyme released by Botrytis cinerea, rapid intervention is essential, as far as possible by blocking the action of oxygen in the air, reducing as much as possible the contact of must or wine with the infected skins, speeding up the maceration and clarification time (Li et al., 2021). Sulphur dioxide, sulphites are the most important and widely used chemicals to prevent wine from browning. In addition to its antioxidant properties, SO_2 also has antimicrobial properties and other important functions that have been used successfully for many years.

The presence of a laccase produced by Botrytis cinerea in the must of rotten grapes leads to the uncontrolled oxidation of phenolic compounds, which continues even in wine. The enzymes producted by M. pulcherrima might be able to block this enzyme activity by its pulcherrimin-content and in this way to propose to winemakers as an alternative to using sulphites.

2. Materials and methods

Commercially available preparations are designed for healthy grapes or must and are associated with the extraction of beneficial aromas to keep dissolved oxygen levels low. Since botrytised grapes are not healthy in terms of microbiological status and the specific aroma composition is due to the biochemical activity of *Botrytis cinerea*, I used the product *M. pulcherrima* Oenoferm® MProtect from Erbslöh GmbH for the tests being known its properties according to product sheet and suggested temperature is 5-15°C, what is important as can be found in references (Simonin et al., 2022).

The basic material for the research was the Kövérszőlő grape variety, grown in the Lapis vineyard in Bodrogkeresztúr, Tokaj wine region. For each pair of samples, 10-10 kg of grapes were harvested, treated with 2 parallel treatments (Oenoferm®MProtect and 5% sulphuric acid stock solution) at 3 different time points (14.09.2023, 27.09.2023, 14.10.2023) and in 3 different health conditions (Figure 6.):

A: completely healthy, sugar content 173 g/l (A0);

B: bunches with 40% noble rotted berries, 260 g/l sugar content (B0);

C: bunches with 80% noble rotted berries, 360 g/l sugar content (C0).

One member of the sample pairs was treated with **10 g/hl** O. MProtect (A1(MP), B1(MP), C1(MP)) and the other with 40 mg/l sulphur dioxide (A2, B2, C2). At each stage, half of the O. MProtect dose was applied to the grapes already, and the other half was applied after stemming before pressing **without rehydration**.

All three series were inoculated with 30 g/hl of Oenoferm®Wild&Pure (*Torulaspora delbrueckii*&*Saccharomyces* spp.). **Samples A were inoculated 36 h after pressing and samples B and C after 48 h.** Samples were taken on day 10 (A), day 12 (B) and day 11 (C) of the fermentation stage.

The fermentation temperature was controlled with room cooling at **12°C**.

The analysis of the samples was carried out in the laboratory of Diagnosticum Zrt. in Szerencs, using NMR (Nucleic Magnetic Resonance) method called H NMR technique (Godelmann et al., 2013). Sample preparation and assay parameters for the targeted assay were as follows: pH adjustment to pH 3.1 with an automatic BTPH system, addition of deuterium and tetramethyl silane, relaxation delay 4 s, sampling time 3.98 s, spectral width 8223.68 Hz.

For each series, microbiological inoculation was also performed on four different media before the inoculation with Oenoferm®Wild&Pure.

1. YGC (Yeast, Glucose Chloramphenicol): glucose 20g, yeast extract 5g, agar 20g, chloramphenicol 100 mg/l, distilled water up to 1000 ml

2. MRS (Man, Rogosa & Sharpe): peptone 10g, yeast extract 4g, meat extract 8g, glucose 20g, sodium acetate 2g, triammonium citrate 5g, magnesium sulphate 0,05g, manganese sulphate 0,05g, actidione 0,05g, tomato juice 250 ml, Tween-80 1ml, distilled water up to 1000 ml

3. MYP (Mannitol-Yeast Extract-Peptone): mannitol 25g, yeast extract 5g, peptone 3g, agar 12g, distilled water up to 1000 ml

4. GYP (Glucose-Yeast Extract-Peptone): glucose 30g, yeast extract 5g, peptone 2g, agar 15g, distilled water up to 1000 ml.

The YGC culture agar is for yeast and moulds, the MRS culture agar is for lactic acid bacteria, while MYP and GYP are for acetic acid bacteria, Acetobacter strains do not grow in the presence of glucose and are therefore most likely to belong to the Gluconobacter strain.

Samples were filtered through a 0.45 μm membrane and incubated at 26 ^{o}C for 3 days.

3. Results and discussion

The research work carried out is in many respects a missing activity in the Tokaj wine region and aims to help prepare for the negative changes that climate change will bring to the wine industry. The objectives are:

1. Based on the literature reference, the bioavailability of the wild yeast strain M. pulcherrima in bioprotection has been investigated so far in 8 grape varieties (4 white and 4 blue), the present research will extend the scope by investigating the autochthonous Tokaj grape variety called Kövérszőlő.

2. Healthy grapes and its must have been studied so far, but now the M. pulcherrima strain has to prove itself in a completely new medium, because the yeast, mould and bacterial composition of the botrytised raw material is completely different from the microbiota of normal grapes.

3. A key element of sustainable winemaking is the practice of reduced sulphur use in viticulture and bioprotection is intended to help this, as there is very little experience of its application, we do not know whether it can be used to replace or reduce sulphur use.

4. In the case of base materials with noble rot, it is a major struggle to limit the activity of botrytis laccase enzymes, the deep coffee brown colour of the wine cannot be removed and can only be corrected by the use of derivates at the expense of aromas. I wanted to investigate whether the bioprotection already applied in the grape state is effective in stopping enzymatic browning.

5. From the point of view of fermentation, the microbiota of wine yeasts is hampered by the components of the microbiota, in particular the number of yeasts, moulds, lactic acid bacteria and acetic acid bacteria. It was expected that bioprotection with M. pulcherrima strain could reduce the numbers of these microorganisms to a large extent, even by several orders of magnitude, even in cases where botrytis activity results in higher numbers of these microorganisms.

To achieve the above objectives, I used Oenoferm®MProtect from Erbslöh GmbH, because on the one hand the application range, temperature and dosage volume are well adaptable for Tokaj wines, and on the other hand I wanted to carry out the inoculation with the combined *Torulaspora delbrueckii+Saccharomyces cerevisiae* preparation (Oenoferm® Wild&Pure), which I had already studied (Bene&Kiss, 2023). Results are shown according to wine chemistry, enzymatic browning and microbial ecology.

3.1 Wine chemistry

The biochemical composition of each experimental setup is shown in Table 2., Figures 7., 8. and 9.
At all three health stages, the analytical composition of bioprotect-treated batches differs from that of sulphur dioxide-treated batches.
Healthy grapes (batch A) fermented to dryness in the same time, but bioprotection resulted in less acetic acid, ethyl acetate production. An increase in higher alcohols (2,3-butanediol, 2-phenyl ethanol) was observed and higher amounts of galacturonic acid, succinic acid, glycerol. Sulphuric acid-binding ketoacid, the pyruvic acid is higher than the initial value, but almost the same for the two treated batches.

For the botrytized samples (B and C), the degree of variation is greater. After the same amount of time, the bioprotected batches showed a higher sugar loss and a shift in pH towards the more acidic range. All 4 treated samples showed significantly increased values of acetic acid, acetoin, ethyl acetate, acetaldehyde, pyruvic acid compared to the initial values. The treatment with sulphur dioxide shows a greater increase, but in the range close

to bioprotection. Succinic acid and galacturonic acid, which have a favourable effect on organoleptic properties, are also high for B1(MP), B2 and C1(MP), C2 samples, showing values measurable for botrytised feedstocks.In the studies reported in the literature, using Zymaflore®EgideTDM 5 g/hl in a must inoculation, 61 mg/l ethyl acetate was measured at 12°C after 48 h pre-fermentation in a must inoculation, which was 86 mg/l in a healthy sample without sulphite (Agarbati et al., 2023). This level of variation was also observed for the Kövérszőlő grape variety test under all three botrytis infection conditions. The C sample (80% infected by Botrytis) was expected to have the highest initial ethyl acetate levels, although it had the lowest levels and the rate of increase was found to be less in both the bioprotected and sulphur dioxide treated samples. This may be explained by the fact that the size of acetic acid bacteria does not increase linearly with the degree of botrytis infection and is not associated with the formation of unfavourable volatile esters.

An increase in acetic acid content is always to be expected in botrytised raw materials, due to the specific microbiological composition of the surface of the noble rot berries (increased presence of acetic acid bacteria) and also produced by yeasts under osmotic stress. These values are also high in batches treated with bioprotection, but not as high as in those treated with sulphur dioxide. *Metschnikowia pulcherrima* is much more tolerant of sugar stress, so this is likely to account for the lower values measured for both acetoin and ethyl acetate. The concern remains, however, that if we completely abandon the use of sulphur dioxide and only use bioproduction to carry out the fermentation process, we will still face elevated levels of acetic acid, ethyl acetate, acetoine at the end of the process and bioproduction will no longer have any effect, its technological role will not have any effect on the time of vinification and ageing.

Further investigation is needed to see how much we can influence the formation of these compounds and how long shelf-life can be affected by supplementing bioprotection with reduced sulphur use.

It is important to point out that the formation of higher alcohols is also positively affected by bioprotection, with lower values being measured for batches treated with sulphur dioxide (Figure 8.).

In the study of sweet Garganega wines (Binati et al., 2023) with LEVEL²INITIATDM these ratios are observed, higher levels of acetic acid, acetoin, ethyl acetates, which is also contributed by the production of acetic acid by *Saccharomyces* yeast due to osmotic stress caused by higher sugar content.

For glycerol content, all bioprotection treated batches (A1(MP), B1(MP), C1(MP)) showed higher levels compared to sulphur dioxide treated batches (A2, B2, C2) (Figure 9).To compare with literature datas, in the case of *M.pulcherrima* AWRI Obsession in Merlot wines, the alcohol content was reduced up to 1.0 v/v% and the increase of glycerol content was observed as *M.pulcherrima* Flavia MP346 in Syrah wines (Windholtz et al., 2021).

Phenolic acids are predominantly composed of two subgroups: hydroxycinnamic acids (HCA) of the C6-C3 and C6-C1 types and hydroxybenzoic acids (HBA). The cinnamic acid derivatives without condensed skeleton (p-coumaric acid, ferulic acid, caffeic acid) are mostly present in the form of esters with tartaric acid (cutaric acid, ferulic acid, caffeic acid), of which caffeic acid (caffeyl tartrate) in particular is a preferred substrate for polyphenol oxidase enzymes in grapes. HCAs and their derivatives have antimicrobial properties, in particular against lactic acid bacteria and yeasts (e.g. *Brettanomyces bruxellensis, Dekkera spp.)* responsible for wine spoilage, increasing cell membrane permeability in wine. HBA compounds are found in lower amounts, with the most significant properties being gallic acid and chicamic acid, important antioxidants and precursors of all hydrolysable tannins.

The positive effect of bioprotection on the amounts of phenolic acids is shown on Figure 10.

The caftaric acid (kaffeoyl tartrate) is a preferred substrate for polyphenol oxidase enzymes in grapes and with its formation there is a prevented mechanism from undesirable oxidization. The beginning value of trigonelline content depends on the healthy state of grapes and both treatments could increase. The changes in shikimic acid content depends on more factors and there is a need for more examinations being able to draw the right conclusion.

Simonin et al. (2022) in their bioprotection study with Primaflora VB® *M.pulchherima* on Chardonnay grapes also found that bioprotection treatments increased the amount of phenolic acids, with the temperature range applied having the greatest influence on the rate of increase.

3.2 The appearance of enzimatic browning

The appearance of enzymatic processes associated with browning is shown in Figure 11. In all three settings, the colour of the musts treated with bioprotection is lighter and the browning processes are less pronounced.

The laccase enzyme of Botrytis cinerea is able to oxidize phenolic compounds to quinones, which results in more browning, but the enriched samples treated with Oenoferm®MProtect (B1, C1) show a lighter shade.

Based on the literature (Puyo et al., 2023a), bioprotection alone cannot prevent browning, but when supplemented with other antioxidants, such as wine tannins, it can already provide significant stabilisation. In the present samples, especially in the B setting (40% infected by Botrytis), the limitation of the enzymatic process is significant, and the effect is also visible in the C setting, but compared to the A setting, it supports the literature data that the extent of the limitation is not sufficient and that it would be necessary to add other treatment substances to the bioprotection.

Puyo et al., 2023b extended their series of experiments with tannin use in rosé winemaking, inoculating *M. pulcherrima* Primaflora VB® in the pre-fermentative stage with quebracho tannin and *M. pulcherrima* gall nut wine tannin, so they also investigated the extent to which SO_2 use can be counteracted by tannin use to promote bioprotection efficiency. The use of quebracho tannins was found to be preferable to gallotannins, but was only effective on oxygen sensitive phenolic acids e.g. coumaric acid and SO_2 could show a broader spectrum of polyphenol reduction.

3.3 Changes in microbiological composition

The intact, healthy grapes have a mixed yeast population of 10^3-10^5 cells/g, dominated by non-Saccharomyces species, for example wild yeasts. These are dominated by *Kloeckera* species (mainly *K. apiculata*) and their teleomorphic variants, the *Hansenulaspora* species. *Candida* species (*C. pulcherrima, C. stellata, C.zemplinina*) and *Pichia, Kluyveromyces, Rhodotorula* and *Cryptococcus* species are found in smaller quantities. The size of the moulds is of the order of less than 10^2 cells/g. On healthy grapes, both lactic and acetic acid bacteria are estimated to be 10^2-10^3 cells/g (Magyar, 2010).

The noble rot activity changes this composition. The yeasts do not change in size, but *Kloeckera* species are reduced and *Candida* and other sugar-tolerant species (*Zygosaccharomyces rouxii, Torulaspora delbrueckii*) become dominant. Acetic acid bacteria and lactic acid bacteria may increase by several orders of magnitude, reaching 10^5-10^6 cells/g (Bene, 2004).

In the case of three batches (A, B, C), the results of the microbiological inoculations before putting the specified yeast are shown in Table 3.

Smaller cell counts are observed in bioprotected batches, basically characterized by purification, but none of the microbial groups changes drastically in composition. No reduction in magnitude is observed for healthy grapes (A), but the initial cell counts prior to treatment are not high either. In the 40% botrytized rate samples (B), a considerable decrease in the abundance of yeast, mould, acetic acid and lactic acid bacteria was observed compared to the sulphur dioxide treated batches.

This size reduction effect cannot be demonstrated in samples of berries (C) with 80% infected by Botrytis which may be due to the fact that the efficiency of bioprotection is highly dependent on the size of the initial cell counts. The efficiency of its use highly depends on the health state of the basic material. If the botrytis infection opens up a pathway for the bacteria to the extent that their number reaches 10^5-10^6 cells/g and the combined presence of acetic acid bacteria and lactic acid bacteria is of such magnitude, then bioprotection with *M. pulcherrima* cannot produce a reduction of sufficient magnitude.

In many cases, research has shown the same results, the biocontrol can reduce the development of spoilage flora with the same effectiveness as the addition of sulphites. However, it is necessary both to maintain low temperatures and to determine the size of the original population in the grape must. High concentrations (above 10^5 CFU/mL) are a source of risk for the application of the bioprotective strain (Simonin et al., 2022; Windholtz et al., 2021).

4. Conclusions

On the one hand, the present research has contributed to the practical experience of using *M.pulcherrima* strain in bioprotection, and on the other hand, we have practical data on Oenoferm®MProtect (Erbslöh), further contributing to the knowledge of the autochthonous grape variety Kövérszőlő in the Tokaj wine region.

It was found that bioprotection with *M. pulcherrima* strain is effective in reducing the number of microorganisms in healthy grapes, for example when the microbiota size is less than 10^5 cells/g berry. In this case, it is also able to displace undesirable bacteria and other yeast strains, which are thus unable to gain ground at the expense of the *Saccharomyces* species yeasts in the competition for nutrients. In the case where this condition

changes, for example due to *Botrytis cinerea* activity, and both acetic and lactic acid bacteria exceed 10^5 cells/g berry, it can no longer function with sufficient safety and efficiency.

In the case of enzymatic oxidation, the literature suggests that bioprotection helps to prevent browning, but its use is limited, especially for rosé musts, where it is not only important to preserve the freshness of the aromatic grape varieties but also their colour after pressing, and for raw materials that have undergone noble rot, other treatments are needed in addition to bioprotection. Bioprotection with the addition of tannin can give more favourable results, but the effect of SO_2 addition is more certain. As a continuation of the present research, it is essential to extend the studies to include bioprotection with reduced sulphur use.

The treatment with sulphur dioxide can improve the situation in high doses, but its negative effect on quality is visible and its use is dangerous due to allergenic properties. It is therefore essential to change the current practice and reduce the quantities of sulphur dioxide. Bioprotection is a potential alternative, but as the study shows, it is not a complete substitute for sulphur dioxide treatment, it cannot achieve sulphur-free production, but it is a specific tool.

The temperature range at which bioprotection is applied is very important, and both the literature and the present research confirm that efficacy is enhanced by application at 10-12°C. The study is part of a series of research, a pilot work, which will be continued by extending it to more grape varieties, varying the treatment volumes, taking into account vintage particularities and applying bioprotection with reduced sulphur use.

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Appendix:

Figure 1. Numbers of commercial used non-Saccharomyces yeast strains (Source: Vejarano & Gil-Calderón, 2021)

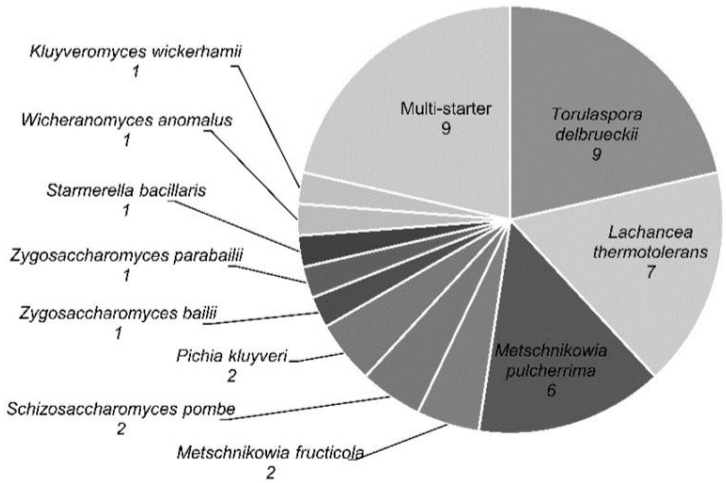

Figure 2. Pathway of yeast acetoin biosynthesis (Source: Romano & Suzzi, 1996)

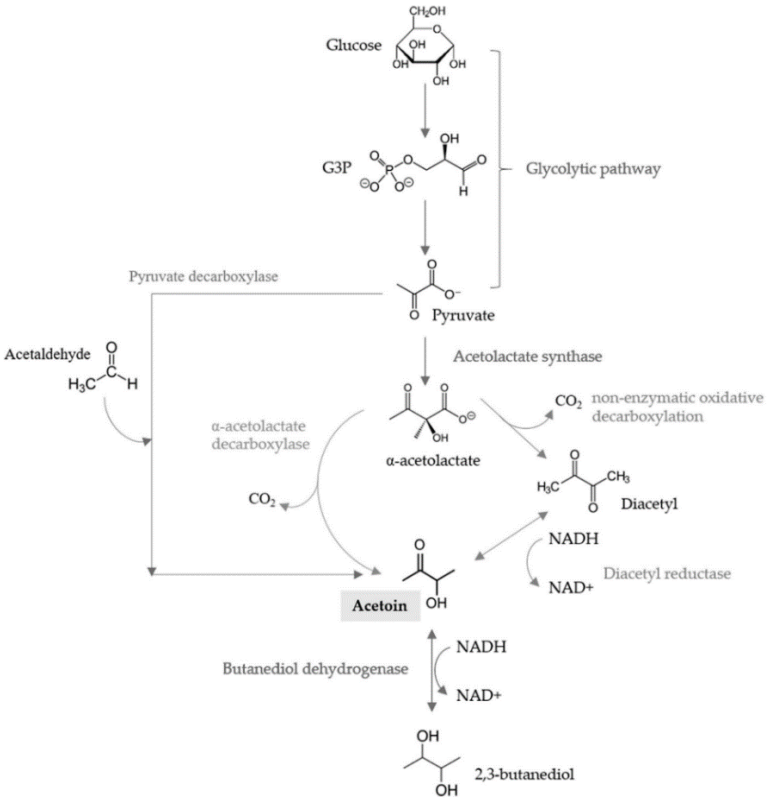

Figure 3. The metabolic pathway of pulcherriminic acid (Source: Puyo et al., 2023)

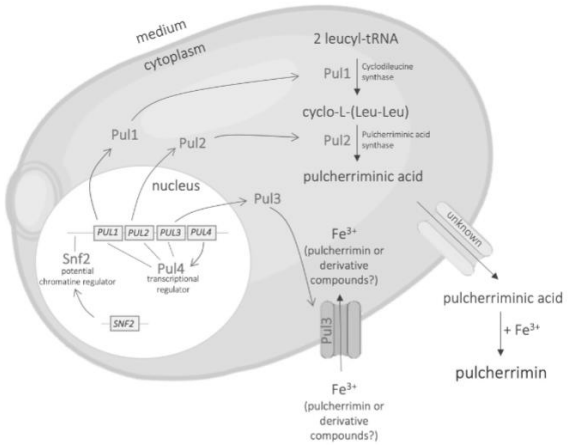

Figure 4. The applied field of use of M. pulcherrima (Source: Puyo et al., 2023)

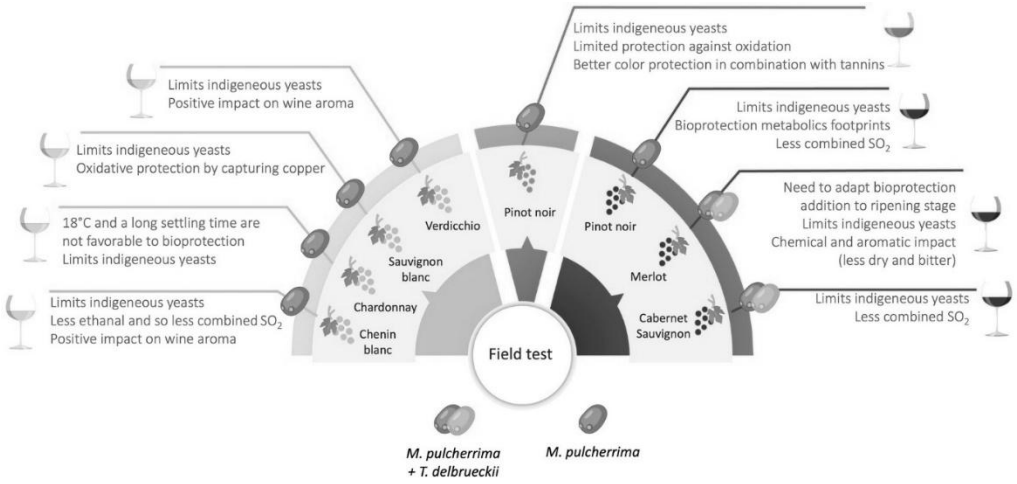

Figure 5. Scanning electron microscope view of yeast and mould strains in a 15µm band on a noble rotted berry (Source: Bene, 2004)

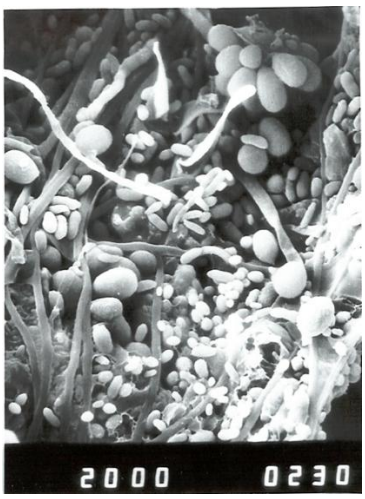

Figure 6. Several bunches harvested in different states of health (A: fully healthy, B: 40% infected by Botrytis, C: 80% infected by Botrytis)

Figure 7. Degree of formation of compounds with undesirable effects on sensory parameters

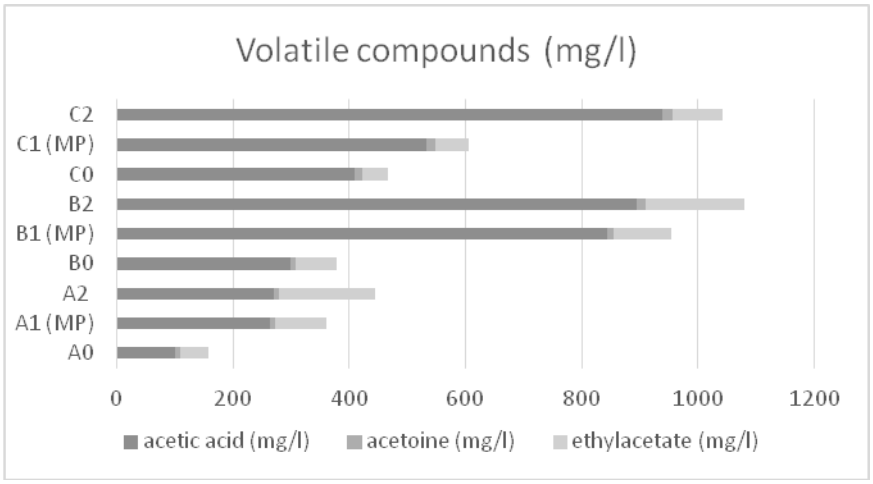

Figure 8. Values measured for higher alcohol contents

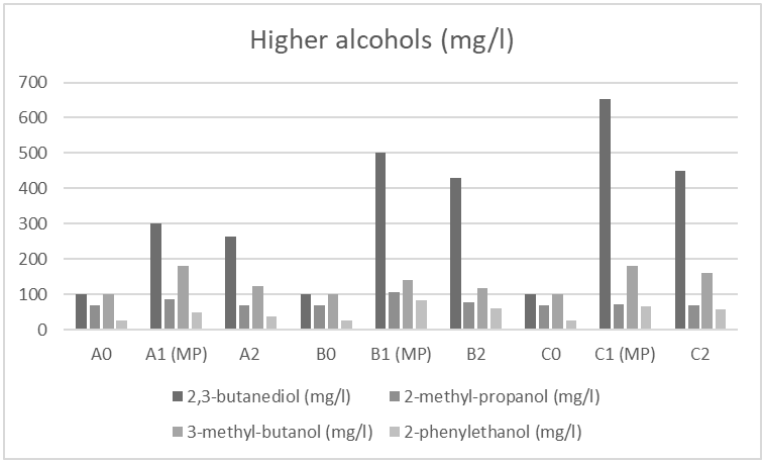

Figure 9. Variation of glycerol content depending on botrytized state

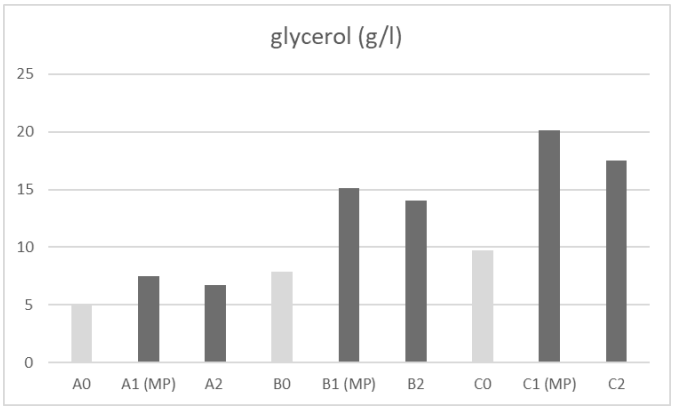

Figure 10. Differences in the phenolic acid composition

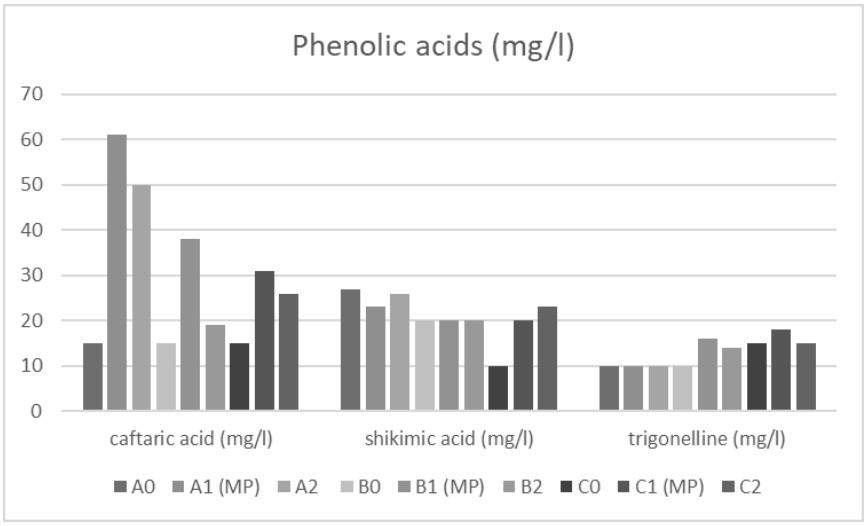

Figure 11. Enzymatic browning rate in the examined sample-pairs

Table 1. Commercial Metschnikowia pulcherrima products available on the market. Information obtained from the website of the companies that commercialize them

Product name	Producer	Applied strains	Field of use	Use of temperature	Aim of use	Dosage	Use of direction
ZYMAFLOR E® KHIOMP	Lafort (France)	Metschnikowia pulcherrima	White and rosé wines	5-15°C	protecting musts against oxidation; keeping dissolved oxygen levels low; limiting the habitat and activity of other undesirable micro-organisms	5 g/hl	use directly or after rehydration
ZYMAFLOR E® ÉGIDETDMP	Lafort (France)	Torulaspora delbrueckii és Metschnikowia pulcherrima	White, rosé and red wines	12°C	grape-prevention, use of SO2 consumption	5 g/hl	use directly or after rehydration
Flavia MP346	Lallemand (Canada)	Metschnikowia pulcherrima	White and rosé wines	18-20°C	increases the amount of varietal flavouring substances, terpenes and thiols in fruit fruits (floral, fresh fruit aromas)	5 g/hl	rehydration is required
Oenoferm®MProtect	Erbslöh (Germany)	Metschnikowia pulcherrima	White, rosé and red wines	5-15°C	microbiological control, less formation of defective by-products (volatile acid)	15 g/hl	use directly or after rehydration
Awri Obsession	AB Biotek (United Kingdom)	Metschnikowia pulcherrima	ed wines	20°C	This yeast produces more dark fruit flavour and has the capability to mask green characters.	0 g/hl	rehydration is required
LEVULIA Pulcherrima	AEB Group (Italy)	Metschnikowia pulcherrima	White and rosé wines	15-20°C	Lower alcohol yield, less volatile acid production, increased aroma production	0-30 g/hl	rehydration is required
Primaflora VB®	AEB Group (Italy)	Metschnikowia pulcherrima	White and rosé wines	15-20°C	limits the presence of undesirable Brettanyomyces, apiculatus yeasts and lactic acid bacteria; helps bind green flavours	7g/hl	rehydration is required
Excellence B-Nature	Lamothe-Abiet (France)	Metschnikowia pulcherrima	White, rosé and red wines	15-20°C	Control to natural flora when harvesting Reduce the amount of compounds that combine SO_2 Increase the wine's aromatic complexity Decrease the dosage of SO_2 on the grapes	5 g/hl	use directly or after rehydration
LEVEL²INITIA™	Lallemand (Italy)	Metschnikowia pulcherrima	White and rosé wines	4-18°C	Limits browning, Preserves aroma including those sensitive to oxidation, avoids organoleptic deviations from microbiological origins	25 g/hl	rehydration is required

Table 2. Analytical parameters of each sample based on NMR analysis

parameter/sample	A0	A1 (MP)	A2	B0	B1 (MP)	B2	C0	C1 (MP)	C2
sugar (g/l)	173	<1.0	<1.0	260	24	98	360	115	200
pH	3.00	2.90	3.00	3.22	3.09	3.22	3.33	3.21	3.33
lactic acid (mg/l)	<200	<200	<200	210	210	230	240	240	283
malic acid (g/l)	2.6	2.4	2.0	2.3	2.2	2.2	2.2	2.2	2.3
tartaric acid (g/l)	5.5	5.3	5.0	2.9	3.1	3.1	2.7	2.7	2.7
citric acid (mg/l)	240	240	244	270	399	318	280	507	307
acetic acid (mg/l)	<100	263	270	300	844	894	410	533	938
acetoine (mg/l)	<10	<10	<10	<10	11	16	12	16	19
ethylacetate(mg/l)	<50	89	166	70	100	169	45	57	85
ethyllactate (mg/l)	<150	<150	<150	<150	<150	<150	<150	<150	<150
2,3-butanediol (mg/l)	<100	300	264	<100	500	431	<100	653	449
2-methyl-propanol (mg/l)	<70	85	<70	<70	107	77	<70	73	<70
3-methyl-butanol (mg/l)	<100	181	123	<100	140	119	<100	181	160
2-phenylethanol (mg/l)	<25	48	39	<25	84	62	<25	65	57
acetaldehyde (mg/l)	<10	12	12	<10	25	33	<10	20	21
pyruvic acid (mg/l)	<20	30	32	<20	37	47	<20	29	54
galacturonic acid (mg/l)	<160	182	<160	<160	693	652	<160	750	645
succinic acid (mg/l)	<50	898	848	<50	1100	916	<50	988	865
glycerol (g/l)	5	7.5	6.7	7.9	15.1	14.0	9.7	20.1	17.5
caftaric acid (mg/l)	<15	61	50	<15	38	19	<15	31	26
gallic acid (mg/l)	<25	<25	<25	<25	28	<25	<25	<25	<25
shikimic acid (mg/l)	27	23	26	<20	<20	<20	<20	<20	23
trigonelline (mg/l)	<10	10	<10	<10	16	14	<10	18	15

Table 3. Microbiological composition of each setting

Sample	Microbiological composition	Treated by bioprotection	Treated by sulphur-dioxide
A	Yeast and mould count (CFU/10 ml)	$1.9x10^2$	$3.2x10^2$
	Acetic acid bacteria count (CFU/10 ml)	$2.2x10^3$	$4.0x10^3$
	Lactic acid bacteria count (CFU/10 ml)	$1.1x10^1$	$2.0x10^1$
B	Yeast and mould count (CFU/10 ml)	$5.7x10^3$	$4.2x10^4$
	Acetic acid bacteria count (CFU/10 ml)	$3.5x10^5$	$4.0x10^6$
	Lactic acid bacteria count (CFU/10 ml)	$1.1x10^1$	$2.0x10^3$
C	Yeast and mould count (CFU/10 ml)	$2.4x10^5$	$2.0x10^5$
	Acetic acid bacteria count (CFU/10 ml)	$9.8x10^5$	$7.0x10^6$
	Lactic acid bacteria count (CFU/10 ml)	$1.2x10^5$	$5.4x10^5$