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Natural glycolipids for the control of spoilage organisms in red wine

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Abstract. A natural glycolipid mixture derived from the edible mushroom *Dacryopinax spathularia* has proven effective as an antimicrobial agent in non-alcoholic beverages, offering an alternative to SO_2 in winemaking. This study investigated its efficacy in Shiraz wine, focusing on its ability to combat spoilage microorganisms such as *Brettanomyces bruxellensis*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans*. In laboratory trials, Shiraz wine free of SO_2 was inoculated with 1000 CFU/mL of these microbes and treated with 10-50 mg/L glycolipids. Results showed a reduction in viable cell counts and prevention of spoilage over six months. Glycolipids also inhibited the production of odour compounds associated with *B. bruxellensis* and acetic acid from *A. pasteurianus* and *G. oxydans*. A commercial-scale trial involved treating Shiraz wine in oak barrels with 40 and 80 mg/L glycolipids after inoculation with *B. bruxellensis*. Over 28 weeks, only untreated barrels showed significant spoilage. These findings suggest that glycolipids are a viable alternative to SO_2 for controlling microbial spoilage in wine and can be used as a processing aid during maturation or storage.

1. Introduction

The edible mushroom *Dacryopinax spathularia*, also known as "sweet osmanthus ear", is widely distributed in tropical and subtropical areas [1] and is known to be used in culinary applications. The fungus produces antimicrobial glycolipids, possibly as a strategy to fight against competition in its environment. These long-chain glycolipids can also be obtained via fermentation, using the fungus as producer strain. The primary extract is a pure mixture of very similar molecules, with three main components varying in molecular weight from 970 to 1013 Da [2].

Studies have shown these glycolipids to be poorly absorbed by the oral route, with the small primary metabolites glucose, xylose, acetate, and isovalerate expected to have a fast and high bioavailability but rapid clearance [2,3]. The beneficial safety profile was verified in a series of further *in vitro* and *in vivo* studies, based on which the European Food Safety Agency confirmed the safe use of glycolipids in food [4].

Accordingly, glycolipids have recently been approved by food standard authorities in Europe, North America, and Australia for use in non-alcoholic beverages. They are Generally Recognized as Safe (GRAS) by the FDA in the USA. Unlike SO_2, the most used antimicrobial in wine production, the glycolipid antimicrobial activity shows low only variation in relation to the pH of the beverage. This property, together with its activity against a broad range of yeast and bacteria, make them a promising candidate for applications in winemaking. For example, glycolipids could be used to achieve the objectives outlined in OIV International Code of Oenological Practices 3.5.5 and 3.4.16, and prevent the need for remedial practices such as those outlined in 3.5.18.

Brettanomyces bruxellensis is a yeast species that can produce several compounds that negatively affect wine quality, often resulting in unpleasant medicinal and phenolic characteristics. The most significant impact of this yeast species on wine sensory properties is through the production of 4-ethylphenol and 4-ethylguaiacol 5,6]. These compounds are primarily responsible for the 'Brett' off flavour found in wines affected by *B. bruxellensis*.

Brettanomyces issues are managed using a multi-faceted sanitation and inhibitory compound application strategy. However, this strategy typically does not eliminate *Brettanomyces* yeasts from wineries and relies heavily on sulfur dioxide (SO_2) to stabilise wine against this yeast [7].

Most 'Brett' strains isolated in Australia belong to one of three major clades [8], shown in Figure 1. The most

prominent of these clades contain the most sulfite-tolerant *Brettanomyces* isolates [9]. It has been proposed that the overall proportion of sulfite-tolerant strains may be increasing together with the evolution of greater SO_2 resistance [10]. In addition, a trend in recent years toward producing red wines with higher pH values means that standard SO_2 additions are less effective at managing microbial spoilage.

In this work, we explored the application of glycolipids in wine production. Specifically, we assessed whether glycolipids could kill and prevent the re-emergence of the yeast *Brettanomyces bruxellensis* and the bacteria *Acetobacter pasteurianus and Gluconobacter oxydans* in Shiraz wine. These microorganisms are responsible for most microbial spoilage issues in red wines [11,12]. The evaluations were undertaken at a laboratory scale and, in the case of *B. bruxellensis,* at large-scale winery trials in barrels.

2. Methods and Materials

2.1. Effect of glycolipids on *B. bruxellensis* growth during storage of red wine in laboratory trials

The trial was conducted in commercially produced, SO_2-free red wine (2020 Puritan Shiraz, Battle of Bosworth, McLaren Vale). The wine was filtered (0.2 µM) and mixed with a sterile glucose solution (20 g/L) to give a final glucose concentration of 5 g/L.

Glycolipids (Nagardo®, Lanxess, Germany) were added to 10 and 50 mg/L final concentrations from a 10 g/L water solution. Half of the glycolipid treatments were combined with 0.5 mg/L of molecular SO_2 to evaluate interactions between glycolipids and SO_2. SO_2 was added as a solution of potassium metabisulfite (17.4 g/L) in water. Free and total SO_2 concentrations as measured by enzymatic method on a Gallery Discrete Analyser (ThermoFischer) are provided in Table 1. Molecular SO_2 concentrations were calculated according to the following formula:

$$mSO_2 = fSO_2 / (1 + 10^{(pH - 1.8)}) \quad (1)$$

Table 1. Free and total SO_2 concentrations in wines following addition of potassium metabisulfite. All concentrations are given as mg/L. Strain numbers are AWRI culture collection numbers.

Strain	*Glycolipids*	*Total SO_2*	*Free SO_2*	*mSO_2*
1499	0	50	35	0.45
	10	53	39	0.49
	50	52	36	0.46
1608	0	51	36	0.46
	10	49	34	0.43
	50	46	32	0.41
1613	0	35	22	0.27
	10	30	19	0.24
	50	52	37	0.47

Three isolates of *B. bruxellensis* from Australian wine were chosen. AWRI 1499 and AWRI 1608 are triploid strains representing different major clades of *B. bruxellensis* [13]. AWRI 1613 is a diploid Australian isolate with reduced SO_2 tolerance relative to AWRI 1499 [10], representing the 3rd major clade. Their relationship to known *Brettanomyces* isolates and group-wise sulfite tolerance is shown in **Figure 1A** and **B**, respectively.

2.2. Effect of glycolipids on *B. bruxellensis* growth during storage of red wine in barrels

The barrel trial was undertaken using 2660 L of Shiraz wine, produced in 2020 by WIC Winemaking. The composition of the wine is provided in Table 3. Three treatments were applied as follows:

1) No treatment
2) 40 mg/L glycolipids
3) 80 mg/L glycolipids

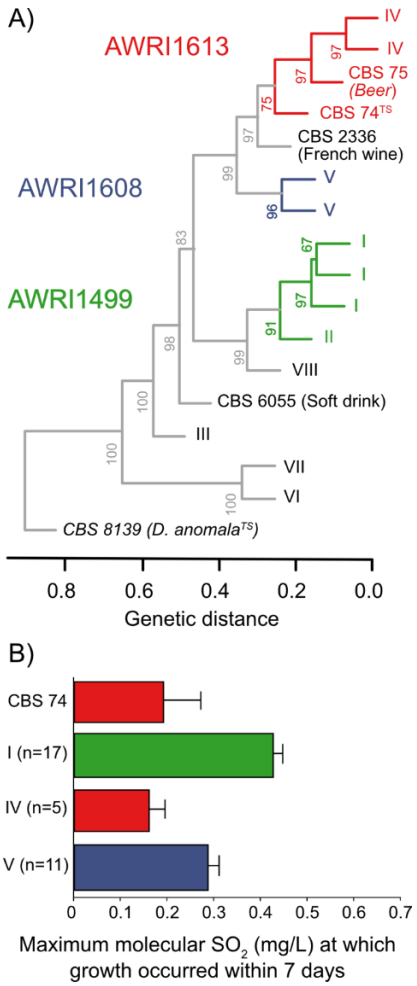

Figure 1. AFLP-based cluster analysis (A) of *B. bruxellensis* isolates [adapted from 8]. (B) The maximum molecular SO_2 concentration at which isolates from the specified genotypic group grow [adapted from 9].

Nine oak barrels were filled with Shiraz wine. The barrels used for the 'No treatment' set were of mixed capacity (2 × 225 L and 1 × 300 L), whereas all barrels used for the glycolipid treatments were of the same capacity (300 L). *B. bruxellensis* strain 1499 was

inoculated into the treatment ii) and iii) barrels at a density of 1000 CFU/mL immediately after filling (25th May 2022). This yeast strain had been conditioned to the wine before inoculation by growing it in an increasing ratio of wine : YPD media over one month. The May barrel filling is consistent with industry practice in Australia and ensured that the barrel maturation trial would experience temperature and humidity fluctuations as expected following vintage winemaking.

Glycolipids were added two days after inoculation to simulate an environment in which *Brettanomyces* is already established. The later addition of glycolipids also facilitated the establishment of a viable *Brettanomyces* population in wine, which is typically difficult to achieve. Stock solutions of glycolipid were prepared by adding 12 g/L and 24 g/L to warm water and stirring for 60 min. Glycolipids were added to treatment ii) and treatment iii) barrels by adding 1 L of the glycolipid stock solution to each barrel. 1 L of water without glycolipid was added to each of the 'No treatment' barrels. The barrels were mixed using a propeller mixer with folding blades for 5 min immediately following glycolipid addition and again 5 days after the addition. It is noted that despite the rigorous mixing protocol employed, there is still a risk of heterogeneity within the barrel due to its size (300 L). The barrels were then matured statically undercover outdoors.

Samples were taken 2, 4, 8, 16 and 28 weeks after inoculation. Cell viability and wine temperature were monitored at each sample time. Poor homogeneity within the barrels was anticipated to result in unreliable viable cell count data during the trial. Before the week 28 samples were taken, the barrels were thoroughly mixed using a mechanical stirrer. Therefore, the week 28 data most likely accurately represent the viable cell population within the barrels. Viable cell concentrations were measured by plating 50 µL of sample onto YPD with 100 mg/L cycloheximide. The LOD of the method is 20 CFU/mL. When no colonies were observed, the LOD is given in graphs and tables.

The concentration of the volatile phenols 4EP and 4EG were also measured in the week 28 samples as an alternative measure of glycolipid efficacy. Quantitation was undertaken by Affinity Labs using the method described by [14]. A portion of uninoculated wine was stored in a stainless-steel keg for the length of the trial as reference.

2.3. Effect of glycolipids on wine spoilage bacteria

The effect of three concentrations of glycolipids (0, 10 and 50 mg/L) in combination with two concentrations of SO_2 (0 and 0.6 mg/L molecular) on the growth and associated acetate production of two bacterial strains listed in Table 4 was assessed. The experiment was performed in a temperature-controlled room (22 °C), and cultures were sampled destructively at 2, 4, 8, 16 and 32 weeks.

Viable bacterial cells were enumerated by dispensing 50 µL aliquots of serially diluted (0.1 % w/v bacteriological peptone (Amyl Media, Australia)) fermentation samples onto a modified de Man, Rogosa and Sharpe (MRS, Amyl or Oxoid) agar using an automated spiral plater (WASP 2, Don Whitley Scientific, Australia). The latter medium was prepared by supplementing MRS medium with preservative-free clarified apple juice (20% v/v) and agar (1.5 - 2.4 % w/v, Amyl or Oxoid). Natamycin (50 mg/L, NataP UltraPure, Handary, Alchemy Agencies, Australia) was added to suppress yeast growth. The agar plates were incubated at 27 °C for 7-10 days, and resultant bacterial colonies were counted using a Protocol 3 colony counter (Synopsis, Don Whitley Scientific, Australia).

Acetic acid was quantified enzymatically using a Discrete Analyser (ThermoFischer). Dissolved oxygen concentration was measured using a Robust Dipping Probe fitted with a Pst3 fluorescent oxygen sensor (PreSens).

2.4. Statistical analyses

Data related to analyte concentration or ferment duration were subjected to one-way ANOVA using the aov function in R (version 4.2.1) to determine whether means differed with respect to treatment (n = 3 for all treatments). If ANOVA P values were less than 0.05 a multiple comparison with respect to treatment was undertaken using the function tukey_HSD (rstatix) to determine the mean difference, upper and lower confidence intervals for the contrasts at alpha = 0.05.

3. Results

3.1. Evaluation of glycolipid activity against *B. bruxellensis* in laboratory-scale trials

The initial composition of the wine used for the trial is provided in Table 2.

Table 2. Basic analytical parameters of Shiraz wine used in 50 mL laboratory experiments inoculated with *B. bruxellensis.*

Analyte	*Value*
Alcohol	14.3%
Residual sugar	5.6 g/L
pH	3.7
Total SO_2	3 mg/L

Results provided strong evidence that glycolipids at a concentration of 50 mg/L in red wine with residual sugar suppressed the growth of all three strains of *B. bruxellensis*. At this glycolipid concentration, the viable population of *B. bruxellensis* decreased to below 20 CFU/mL within two weeks from an inoculation density of 500 – 600 CFU/mL (**Figure 2**). The effect of 50 mg/L glycolipids was equivalent to 0.5 mg/L of molecular SO_2. Control cultures without treatment supported the growth of *B. bruxellensis* to 1×10^7 CFU/mL on average for all three strains over the same period.

Although cell growth and metabolite production are related, the production of the vinyl phenol compounds 4-ethylphenol (4EP) and 4-ethylguiacol (4EG) are the key

factors determining whether a wine is spoiled. For this reason, the concentrations of 4EP and 4EG were measured in wine containing 0 and 50 mg/L glycolipids inoculated with the *B. bruxellensis* strain 1613 (**Figure 3**). More than 2000 µg/L of 4EP and 275 µg/L of 4EG were found in wine without glycolipids. The odour thresholds for 4EP and 4EG are 440 µg/L and 33 µg/L, respectively [15].

This laboratory study shows that 50 mg/L of glycolipids can suppress the growth of a key spoilage organism in red wine and prevent its re-emergence over oenologically relevant periods. The growth suppression effect of glycolipids prevented the formation of the two major odourant compounds associated with *B. bruxellensis* spoilage.

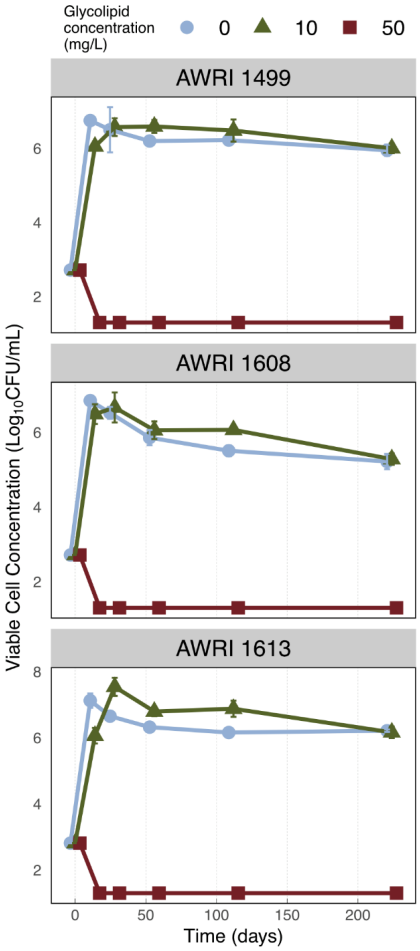

Figure 2. Effect of glycolipids on the viability of three strains of *B. bruxellensis* (AWRI 1499, AWRI 1608 and AWRI 1613) over 6 months (n=3). Error bars show standard deviation.

There was no evidence for differences in growth between strains (P > 0.1). There was also no evidence for a strain-dependent effect of glycolipids on *B. bruxellensis* growth (P> 0.05). There was weak evidence that 10 mg/L glycolipids reduced the growth of *B. bruxellensis* over the first two weeks of the experiment (P[1499] = 0.01, P[1613] = 0.0003). However, by 4 weeks, there was no evidence that 10 mg/L of glycolipids reduced the viable cell number of *B. bruxellensis*.

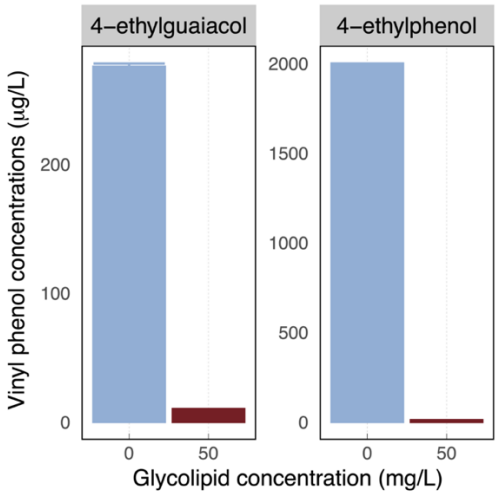

Figure 3. Effect of 50 mg/L glycolipids on the production of 4-ethylphenol and 4-ethylguaicol production in Shiraz wine after inoculation with *B. bruxellensis* strain 1613. Measurements were made 32 weeks after inoculation.

3.2. Evaluation of glycolipid activity against *B. bruxellensis* in pilot-scale trials

Following the promising laboratory trial demonstrating the effective control of *B. bruxellensis* growth and volatile phenol production in red wine, a large-scale industry trial was proposed to evaluate glycolipids in a real-world scenario. In this trial, glycolipids were used to prevent the growth of *B. bruxellensis* in barrels during wine maturation. Oak barrels are the most common environment in which *B. bruxellensis* can flourish during the wine production process. The composition of the wine is provided in Table 3. The market segment most likely to use barrels is producers of premium wine. *B. bruxellensis* can become a problem during wine maturation in barrel either because SO_2 is insufficiently applied before barrel filling or barrel topping does not sufficiently replace the SO_2 lost due to oxidation during maturation. Two properties of glycolipids make it ideally suited to this specific application: it is non-volatile, and, unlike SO_2, its efficacy is not highly dependent on wine pH.

Table 3. Basic analytical parameters of Shiraz wine in 300 L barrels inoculated with *B. bruxellensis*.

Analyte	*value*
Alcohol	13.5%
Residual Sugar	0.6 g/L
pH	3.6
Molecular SO2	0.3 mg/L

Within the first week of the experiment, the temperature inside the barrels decreased by 5.7 °C. The decrease in temperature within the barrels is consistent with the decrease in ambient temperatures at the end of May, with mean daily temperatures of 13 °C during the first 4 weeks of the experiment. Measurable viable cell concentrations dropped below 20 CFU/mL during the period (**Figure 4A**). The cold temperatures and static incubation conditions likely resulted in substantial settling of inoculated cells.

By week 16, wine temperatures in the barrels were increasing, reaching an average of 14.1 °C. At this time, viable *B. bruxellensis* could be detected in samples taken from the top of the barrels for the untreated and 80 mg/L glycolipid barrels. In both cases, viable cell counts were highly variable between treatments, ranging from 150 to 7650 CFU/mL in the untreated barrels and 150 to 1050 CFU/mL in the barrels with 80 mg/L glycolipids.

At the final sample (28 weeks), no viable cells were detected in the 40 mg/L glycolipid treated barrels, and a maximum of 300 CFU/mL was detected in one of the 80 mg/L glycolipid barrels. In contrast, viable cell concentrations increased in the untreated barrels ranging from 6×10^3 CFU/mL to 1.4×10^5 CFU/mL.

The observed variability in the viable cell data was anticipated due to the tendency for cell populations to settle and adhere to surfaces within the barrel. Therefore, vinyl phenols associated with *B. bruxellensis* spoilage were measured in all barrels after the experiment (**Figure 4B**) to provide an alternative measure of glycolipid efficacy for which heterogeneous concentrations within the barrel were not anticipated.

In all cases, 4EG concentrations were low, with the highest observed in the non-treated barrels with a mean concentration of 18.3 (SD 8) µg/L. More variation was seen in the concentrations of 4EP. The highest absolute concentration and the greatest variance in 4EP concentrations were found in the untreated barrels with a mean of 119 (SD 108) µg/L. In one of the non-treated barrels, a 4EP concentration of 237 µg/L was recorded, the highest in this data set. Concentrations of 4EP were lowest in the 40 mg/L glycolipid-treated barrels with a mean of 43 µg/L. High variability was observed across the replicates of the 40 mg/L treatment with a maximum observed 4EP concentration of 111 µg/L in one of the replicates and both others below the level of quantification (LOQ = 10 µg/L).

This trial provided evidence that glycolipids suppressed the growth of *B. bruxellensis* in oak barrels, with only the non-treated barrel set exhibiting substantial populations of *B. bruxellensis* after 28 weeks of maturation. 4EP concentrations were also beginning to accumulate in the non-treated barrels, with one of the replicates having a concentration that would be perceptible by sensitive individuals. Stochastic effects appeared to predominate in the 4EP and 4EG data sets, with no correlation between volatile phenol concentrations and viable cell population sizes.

One possibility is that the precursor concentrations in this wine were low, or that increasing the duration of the experiment may have provided increased resolution and a more definitive result. Nonetheless, this trial indicates that glycolipids have potential as an antimicrobial agent for the protection of red wine in barrels.

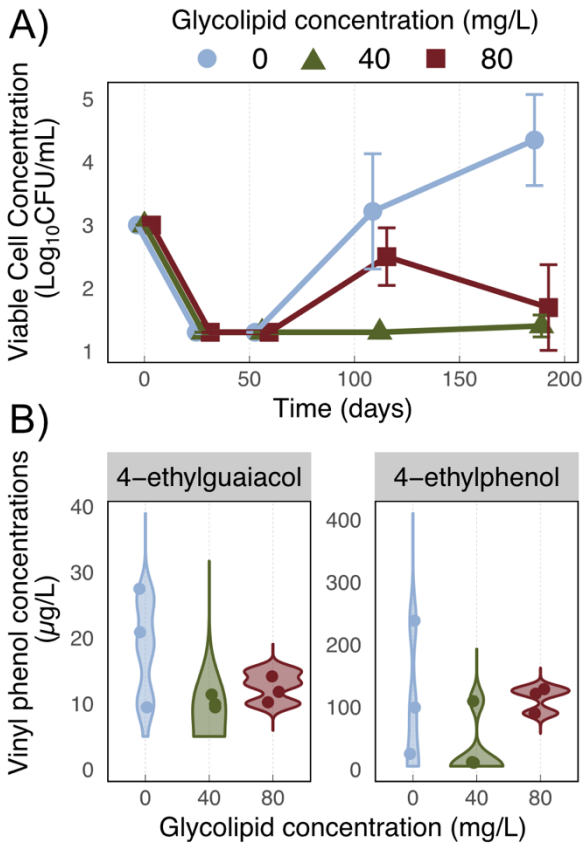

Figure 4. (A) *B. bruxellensis* viable cell concentration in glycolipid-treated wine stored in barrels. (B) Volatile phenol concentration (4EP and 4EG) in samples taken at 28 weeks from barrels inoculated with *B. bruxellensis* and subsequently treated with 0, 40 or 80 mg/L of glycolipid.

3.3. Inhibition of bacterial growth during storage of red wine on ullage by glycolipids

Wine is susceptible to microbial spoilage during storage, either in barrels or tanks. All acetic acid bacteria are considered spoilage bacteria in wine. The two species of acetic acid bacteria most commonly associated with wine spoilage are *Acetobacter pasteurianus* and *Gluconobacter oxydans*. These bacteria are aerobic and, therefore, require oxygen for growth. In the presence of oxygen, ethanol can be used as a carbon source to produce acetic acid. *Acetobacter pasteurianus* can further oxidise acetic acid to carbon dioxide and water [11].

In dry conditions, barrels can lose ~ 1 L per month from evaporation [16]. This loss leaves a headspace of air (ullage) that bacteria can colonise between barrel toppings. Tank storage can be more problematic because filling a tank may not always be possible. Large-volume tanks have a considerable area at the wine/air interface for gas exchange. While inert gas blanketing can be used to help manage oxygen contact, protection is often short-lived, with commonly used lighter gases such nitrogen providing only limited protection [17].

Maintaining inert conditions in tanks of fixed size but holding variable volumes of wine is difficult due to the ineffectiveness of gas blanketing operations. Inevitably, dissolved oxygen is introduced, providing a fertile substrate for spoilage microbes at the air/wine interface.

This trial aimed to determine whether glycolipids could inhibit bacterial spoilage during the storage of wine. We attempted to simulate red wine stored with ullage by undertaking the experiment in half-filled falcon tubes. The duration of the experiment was 6 months.

Wines were inoculated with the two most common spoilage organisms, *A. pasteurianus* and *G. oxydans* (Table 4). Inoculated wines were treated with glycolipids (10 and 50 mg/L) and/or SO_2 (0.6 mg/L mSO_2). At the pH of the wine used in this study, 0.6 mg/L molecular SO_2 equates to 45 mg/L free SO_2. The mean molecular SO_2 concentration was 0.41 mg/L and 0.4 mg/L for the bacterial strains *A. pasteurianus* and *G. oxydans*, respectively.

Table 4. Bacterial strains used in this work.

AWRI number	*Bacterial species*	*Source*
AWRI B546	Acetobacter pasteurianus	Cabernet Sauvignon
AWRI B1905	Gluconobacter oxydans	Grenache

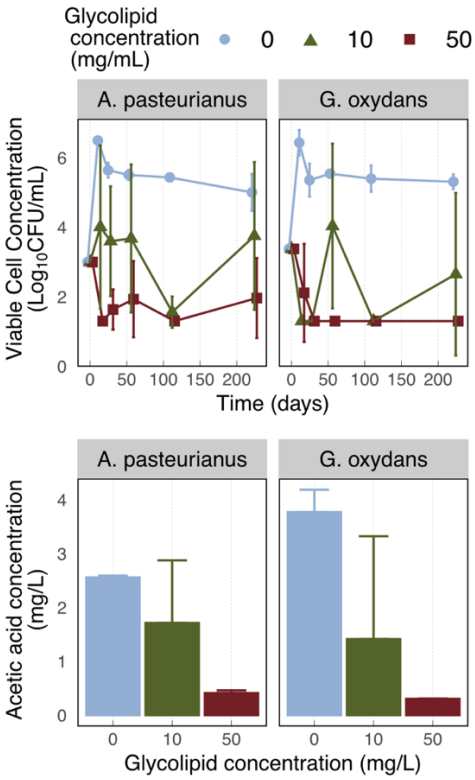

Figure 5. Effect of glycolipids on survival of, and acetic acid production by, *A. pasteurianus* and *G. oxydans*. Glycolipids were supplied at 0, 10 and 50 mg/L. The acetic acid concentration in the experimental wine was 0.37 g/L.

A. pasteurianus and *G. oxydans* reached maximum mean cell concentrations of 3.2×10^6 and 3.4×10^6 CFU/mL within two weeks in the absence of either glycolipids or SO_2 (**Figure 5**). All treatments with SO_2, with addition to between 0.4 and 0.54 mg/L in the molecular form, resulted in the death of bacterial strains and prevented their re-emergence over the entire experimental period of 32 weeks (data not shown).

Glycolipid addition at 10 mg/L was moderately inhibitory to bacterial growth. However, growth was not sufficiently inhibited to prevent acetic acid accumulation in these wines. Conversely, adding 50 mg/L glycolipids resulted in the loss of bacterial viability with similar kinetics to that observed in the SO_2 treatments. With the higher glycolipid treatment, acetic acid accumulation was also prevented for the entire experimental period (**Figure 5**).

Glycolipids were thus effective in suppressing the growth of, and acetate formation by, *A. pasteurianus* and *G. oxydans* in red wine. These two bacteria are the most common microorganisms associated with the spoilage of wine during storage. The effective glycolipid concentration was 50 mg/L.

4. Conclusion

Natural glycolipids from the edible mushroom *Dacryopinax spathularia* have proven effective as an antimicrobial agent in non-alcoholic beverages. The results of the present study show that glycolipids are also an effective antimicrobial agent in wine and may thus be used for production of wines with lower total SO_2.

Laboratory experiments showed that 50 mg/L glycolipids in Shiraz red wine protected against growth of different *Brettanomyces bruxellensis* strains over oenologically relevant periods. Importantly, the formation of the volatile vinyl phenol components 4-ethylphenol (4EP) and 4-ethylguiacol (4EG) was also prevented, which are responsible for the well-known “Brett” off-flavour in spoiled wines.

Laboratory results were confirmed in a winery trial with red wine in 300 L oak barrels, which were inoculated with 1000 CFU/mL *B. bruxellensis* before a 6-month maturation period. Here, 40 mg/L glycolipids were effective at suppressing yeast growth.

Bacterial spoilage of wine caused by the acetic acid bacteria *A. pasteurianus* and *G. oxydans* and associated formation of acetic acid was avoided by 50 mg/L glycolipids in a long-term laboratory experiment.

These results suggest that glycolipids are a viable option and alternative for SO_2 for controlling microbial spoilage in wine and can be used as a processing aid during maturation or storage (if removed by filtration before bottling) or as an additive if remaining functionally present in the wine.

5. Acknowledgements

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6. Conflicts of Interest Declaration

The AWRI undertook this research as part of a research program funded by Lanxess. The AWRI is a not-for-profit research organisation and provides research, analysis, and contract winemaking as a commercial service through Affinity Laboratories. It does not receive any royalties from the sale of the glycolipids. Andrea Bosse and Jens Bitzer are employees of Lanxess.

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