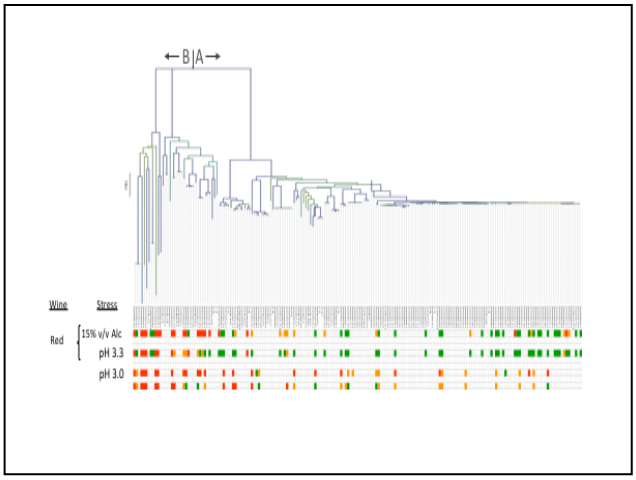
Australian Government

Australian Grape and Wine Authority

The Australian Wine Research Institute

Efficient and reliable malolactic fermentation to achieve specification wine style



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **AWR1303**

Principal Investigators: **Eveline Bartowsky until July 2016; Simon Schmidt from August 2016**

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Author: Dr Simon Schmidt

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1. Abstract:

Malolactic fermentation (MLF) is a fundamental aspect of winemaking that can be difficult and lengthy. This project characterised the diversity of bacterial fitness that remained undiscovered in the rich resource of the AWRI wine microorganism culture collection (AWMCC). Strains were identified that lend themselves well to challenging environmental conditions. Nutrient additions and other treatment regimens were explored that might facilitate robust and reliable malolactic fermentations. Finally, the characterisation of genetic factors responsible for bacterial fitness and wine quality attributes through gene expression analysis was attempted. Candidate genes that contribute to diacetyl production were identified.

2. Executive summary:

The conversion of malic acid to lactic acid by lactic acid bacteria (known as malolactic fermentation or MLF) is a fundamental aspect of winemaking. MLF is important because it helps provide microbial stability to the finished product through the removal of a potential carbon source for spoilage organisms (malic acid), it reduces acidity and can impart various sensory characteristics, some subtle, others less so.

This project's primary aim was to improve the reliability of MLF. This goal was approached from multiple angles. The first was to better understand and characterise the bacterial resources available to winemakers. By defining the limits of what was possible using available resources, areas for development were identified. Specifically, the conduct of MLF in low pH or higher ethanol wines was identified as a high priority for achieving tangible improvements in winemaking, given the limited fundamental knowledge and practical resources available to draw upon.

A program of genetic and physiological screening was undertaken to identify strains of *O. oeni* that could perform better in such conditions, particularly low pH. Pilot and industry trials with a range of bacterial isolates led to information about winemakers' MLF requirements and enabled the identification of specific bacterial isolates that can conduct efficient MLF at industrial scale.

The second approach to improvement of MLF reliability was through an investigation of process options that had the potential to substantially reduce the overall time taken to get a wine to a point where is can be stabilised and protected through SO_2 addition and barrel topping. While MLF is traditionally conducted following completion of alcoholic fermentation, it is also possible for MLF to be conducted concurrently. This is an option increasingly being used by winemakers, but its successful deployment requires a complete understanding of when and where it is most effective such that risks can be managed. Work conducted in this project identified substantial time savings that can be achieved in the production of red wine. Co-inoculation was a reliable and efficient strategy regardless of bacterial strain used. However, the same cannot be said for white and sparkling wine production and some work is still required to better understand how to get co-inoculation to work effectively in these environments.

Other process-driven interventions were also explored, specifically, the use of nutrient additives to support MLF and the impact of oxygen. Nutrient addition has the potential to be stimulatory in nutrient-limiting conditions. Several industry trials over two vintages failed to identify any improvement resulting from nutrient addition. Oxygen or air addition to ferments can be used to stimulate alcoholic fermentation but has the potential to have a negative impact on MLF. Experiments conducted to determine if aeration of alcoholic fermentation could affect subsequent MLF found no inhibitory or stimulatory effects. Work on the potential impact of oxygen use on co-inoculated ferments is ongoing. This area is especially important to winemakers wanting to use oxygen additions in their ferments without adversely affecting MLF, especially when MLF is performed after completion of primary fermentation.

The third approach was to identify genetic markers that might help in more efficiently identifying robust bacterial strains from microbial surveys carried out by others. Both gene expression studies and genome wide association approaches were used. Candidate genes that could be used as markers for diacetyl production and others that indicated potential variation in strain-specific sugar utilisation in genomically highly related strains were identified. Furthermore, variations in metabolic pathways were found that indicated varied capacity of bacterial strains to utilise citric acid.

The key benefit to industry of this work is the provision of evidence-based advice on how to conduct and manage malolactic fermentation under challenging conditions. In this project, different approaches to the conduct of MLF have been investigated, some substantially outside of standard industry practice, with the aim of pushing the boundaries of what is achievable in MLF. Such risks can be undertaken in a research environment so that commercial risks taken by Australian winemakers can be objectively minimised. This approach contributes to the maintenance of a sustainable and profitable Australian wine industry.

The project benefitted substantially from the involvement of several industry partners including Yalumba, Treasury Wine Estates through their Wolf Blass winery, Hardy's Wines through their Tintara winery and Pernod Ricard through their Orlando winery at Rowland Flat.

3. Background:

Malolactic fermentation (MLF) is conducted in all red wines and in many white and sparkling base wines. Even though pH, alcohol concentration, SO_2 and temperature of wine are useful guides to predicting the success of an MLF, this winemaking step remains unreliable, and can add extra costs to the winery through suboptimal performance of MLF bacteria, microbial spoilage and quality downgrades.

The AWRI has helped the Australian industry improve MLF efficiency in red wines by developing co-inoculation strategies (yeast and bacteria inoculated together) that are more efficient than standard sequential inoculations. However, difficulties remain for MLFs in both red and white winemaking. Currently there are no MLF starter strains available that are specifically suited to Australian winemaking conditions; commercially available strains are largely targeted to overseas markets.

Improving MLF efficacy in winemaking is a significant opportunity for the Australian wine industry as highlighted through numerous articles in industry journals and reviews. AWRI studies on co-inoculation in red wine have assisted in addressing this problem (Abrahamse and Bartowsky 2012), but there are limited studies in white wine. Several studies detailing the impact of *O. oeni* and MLF on wine aroma and wine style have been conducted (Swiegers et al. 2005), yet there is limited research undertaken on specific flavour compounds, the pathways involved and their modulation through winemaking. Recent studies by the AWRI have identified the role of MLF in modulating fermentation-derived volatile compounds that impact on 'fruity' and 'berry' aromas in red wine (Costello et al. 2012, 2013). Research by the AWRI and others on glycosidases have demonstrated that MLF bacteria possess these enzymes which are typically absent in yeast (D'Incecco et al. 2004, Grimaldi et al. 2005). However, the knowledge of winemaking conditions required for the expression of such enzymes involved in liberation of the latent aroma compounds is missing. Genome sequencing of 12 *O. oeni* strains by AWRI has revealed the large genetic diversity amongst strains (Borneman et al. 2012) and the link between genome and phenotype is only beginning to be unravelled (Bartowsky 2017).

MLF is used in production of all red and some white wines. A large amount of time, energy and profit is lost through stuck or sluggish MLF. Slow MLF increases wine spoilage risks (such as *Brettanomyces* spoilage) and leads to increased costs (e.g. due to extended periods of heating tanks/barrel halls). Ensuring timely MLF completion is therefore paramount to reliable, cost-effective, quality wine

production. In addition, MLF has been shown to affect wine style but research is required to determine how this can be reliably harnessed by winemakers to tailor their wines.

4. Project aims and performance targets:

This project was designed to take promising candidate O. oeni strains (identified in Project AWR 1305), including Australian isolates, and evaluate their performance under difficult winemaking conditions. Evaluations were to be performed in red, white and sparkling base wines. Additionally, genomic data generated in AWR 1305, was to be used to identify enzymatic pathways involved in the formation of desirable aroma compounds, leading to the development of genetic markers for the isolation of individual strains with flavour-enhancing properties that provide winemakers with the opportunity to shape wine style. Together, the findings were to be captured in a comprehensive knowledge base of the impact of MLF strain, inoculation regime and compatibility with a broad range of yeast strains on MLF efficiency and flavour development.

The specific project aims were to:

a. Identify *O. oeni* strains that tolerate MLF-limiting wine parameters, grow rapidly and efficiently metabolise malic acid

b. Validate performance of target strains in a wide range of conditions typically found in red, white and sparkling winemaking

c. Identify additional factors that ensure an efficient MLF and augment aroma and flavour development, such as MLF inoculation regime (in white wines), impact of winemaking additives (e.g. nutrients), and compatibility with current and 'in development' yeast strains

d. Identify genes for metabolic processes that impact on wine aroma and flavour

e. Determine winemaking practices and/or conditions required to modulate aroma and flavour compounds and their impact on wine style

f. Develop a comprehensive knowledge base of potential metabolic pathways in *O. oeni* that can be used to shape wine style

g. Provide the wine industry with knowledge generated from the above, particularly with regard to strategies for improving MLF outcomes

h. Develop commercial MLF bacterial strains that are suited to Australian winemaking conditions.

5. Method:

5.1. *O. oeni* strain selection

Using data from Project FPA 390, *O. oeni* strains from the AWRI germplasm collection recognised as having potential for use in MLFs were assessed for tolerance to extreme MLF-limiting wine parameters found in red, white and sparkling wine production. Candidate strains were trialled at lab-, pilot- and winery-scale for MLF efficiency in red and white wines under Australian winemaking conditions. Pilot- and winery-scale wines underwent sensory analyses to ensure sound wines were produced with the candidate strains.

5.2. Chemical analysis of wine

Wine ethanol, pH, TA and glucose plus fructose were determined by Fourier-transform spectroscopy (mid-IR) using a Winescan FT2 (Foss) apparatus calibrated for table wine. Free and total sulfur dioxide concentration were determined by flow injection analysis (FIA, Lachat). In some cases, ethanol was determined by near infrared (NIR) spectroscopy

(Alcolyser, MEP). The initial L-malic acid concentration of test wines was determined enzymatically (Randox) using a Daytona automated spectrophotometric analyser (Randox).

5.3. RNA extraction and transcriptomic analysis

Transcriptome analysis was conducted as reported in Sternes et al. (2017).

5.4. Phenotypic screening

Prior to inoculation into test wine, bacterial strains were adapted to wine conditions by pre-culture through two successive stages. In the first, strains were cultured in a modified MRS (mod.MRS) medium prepared by supplementing MRS with D-fructose (10 g/L), DL-malic acid (6 g/L), ethanol (5 % v/v), and cysteine (1.6 g/L or 0.5 g/L for experiments conducted in either Cabernet Sauvignon or Shiraz wines, respectively), with final pH 4.5. Strains were cultured aerobically at 27˚C for 7-10 days, after which time they were subcultured (1-2 % v/v) into a second pre-culture stage (mod.MRS/wine) comprising mod.MRS medium (50% v/v) and wine used for screening (Cabernet Sauvignon or Shiraz) (50% v/v). Strains in this second stage were cultured anaerobically (anaerobic chamber; Coy, USA) at 22˚C for 10-12 days, and cell biomass determined by absorbance (600 nm, 1 cm path length). Using a pre-determined standard curve correlating absorbance (600 nm, 1 cm) values and cell counts (determined microscopically using a bacterial counting chamber, 1000x magnification) of representative strains (stationary phase cultures in mod.MRS/wine), the biomass of each culture was standardised to achieve $1x10^8$ cells/mL by appropriate dilution with mod.MRS/wine medium.

5.5. Tolerance screening of malolactic bacteria

Screening for tolerance to ethanol or low pH stress was conducted in two red wines:

(i) Cabernet Sauvignon: ethanol 13.9 % v/v; pH 3.49; total acidity (TA), 6.9 g/L; total SO2 <4 mg/L, glucose plus fructose, 1.1 g/L; L-malic acid, 1.2 g/L

(ii) Shiraz: ethanol, 13.2 % v/v; pH 3.41; TA, 9.2 g/L; total SO2, <4 mg/L; glucose plus fructose, 0.4 g/L; L-malic acid, 3.4 g/L).

Cabernet Sauvignon was obtained as commercially vinified wine (Barossa Valley, South Australia) from the 2013 vintage; Shiraz wine was prepared from grapes (Robe, South Australia) from the 2015 vintage and vinified at the Hickinbotham Roseworthy Wine Science Laboratory. Wines were obtained after completion of alcoholic fermentation and prior to MLF, and stored at 0-2˚C prior to use. Prior to screening, the L-malic acid content of the Cabernet Sauvignon wine was adjusted to 2.0 g/L. Three sub-lots of respective test wines were prepared from each wine by adjustment to the following parameters: (i) 12 % v/v ethanol, pH 3.5 (reference wine); (ii) 15 % v/v ethanol, pH 3.5 (ethanol stress wine), and (iii) pH 3.3, 12 % v/v ethanol (low pH stress wine). Media and test wines were sterilised by filtration using a sterile membrane filter (0.22 µm pore size).

5.6. Evaluating the effect of nutrient additives

The influence of winemaking additives, such as nutrients, and compatibility with yeast strains was investigated to improve MLF performance.

6. Results and discussion:

6.1. Understanding the genetic basis of malolactic bacterial contribution of wine flavour

Comparative genomic studies of hundreds of *O. oeni* isolates have demonstrated substantial genomic variation between strains of *O. oeni*, with up to 10% variation in protein coding genes observed between any two strains, including those predicted to be involved in sugar utilisation and transport, exopolysaccharide biosynthesis, amino-acid biosynthesis and natural

competence (Bartowsky and Borneman 2011, Borneman et al. 2012). However, little is understood regarding the *O. oeni* transcriptome. To characterise how the *O. oeni* genome interacts with its environment, RNAseq analysis of this important industrial microbe, including the global mapping of operon architecture, was undertaken.

MLFs were initiated separately for three *O. oeni* strains, AWRIB551, AWRIB552 and AWRIB419. Samples were taken at three stages of MLF for RNAseq and metabolite analyses, MLF-start (~ 2.1 g/L L-malic acid), mid-MLF (~ 1.6 g/L L-malic acid) and late-MLF (~0.5 g/L L-malic acid). The three *O. oeni* strains completed MLF at different rates: finishing after 5, 8 and 20 days respectively.

This study has progressed the understanding of genetic variability for *O. oeni* by conducting the first whole transcriptome analysis of this bacterium. There were 47 such novel RNAs identified, of which two were highly abundant. These two most abundant novel RNAs were also the longest in length, comprising 880 nt and 1086 nt (average novel RNA length of 118 nt). In general, these 47 novel RNAs did not appear to encode functional proteins; for example, the 880 nt novel RNA contained an open reading frame (ORF) that encoded part of galactose mutarotase and the 1083 nt novel RNA contained no substantive ORFs.

Given the potential variation that is present between strains of *O. oeni*, *de novo* transcript assembly was employed to investigate genes that may be lacking from the reference strain PSU-1, but present in the three strains used in this study. Analysis of the *de novo* transcripts revealed the presence of a single operon in AWRIB419 that was absent from the PSU-1 genome and which consisted of ORFs encoding a putative NADH-flavin reductase, a transposase and a threonine dehydrogenase followed by a downstream inverted repeat of the first two genes.

In AWRIB551 and AWRIB552 this transposon appears to have inserted into an alternate genomic locus, residing within a large, multi-genic operon, disrupting an ORF that is predicted to encode an arginine deiminase. Insertion of the fGI173 transposon into this alternate genomic locus in AWRIB551 and AWRI552 has resulted in the gene expression of threonine dehydrogenase involved in vitamin B6 metabolism, reducing its abundance between 1.7- to 3.2-fold relative to AWRIB419 across all sampling points.

Pair-wise comparisons between sampling points within individual strains failed to reveal any significant difference in gene expression when comparing T1 and T2, however the comparison of T1 and T3 yielded 10, 24 and 7 differentially expressed genes in AWRIB551, AWRIB552 and AWRIB419, respectively. This shows that individual *O. oeni* strains do not respond dramatically during progression of MLF.

Despite the relative temporal stability of the transcriptional profile for a strain over time, substantial differences between strains were observed. Most notably, the IIA subunit of a non-specific phosphotransferase system was highly upregulated in AWRI419. The bacterial phosphotransferase system is a major mechanism for the uptake of various sugars including extracellular D-glucose, arbutin and salicin. In the same strain fifteen amino-acyl transfer RNAs (tRNAs) were observed to be significantly downregulated. tRNAs play a variety of roles in cell biology. Whether the substantially reduced expression of them in AWRIB419 is a cause or a consequence of the slow progression of malolactic fermentation in this strain is worthy of further exploration.

Dramatic differences were observed between AWRIB419 and the other two strains in the expression of genes related to diacetyl metabolism. This result is somewhat expected since the strains were chosen, at least in part, because of their different profiles of diacetyl production, with AWRIB419 being the higher producer. The most significant difference was in an open reading frame encoding L-lactate dehydrogenase, a gene that is lacking in the other two strains. This gene allows the production of pyruvate from L-lactate which is a potential

substrate in the production of diacetyl. This would explain the different in diacetyl production between AWRIB419 and the other two strains.

Identification of differentially expressed genes in *O. oeni* lays the foundation for further transcriptome profiling of a larger cohort of strains. In conjunction with publicly available comparative genomic analysis, further transcriptomic profiling of *O. oeni* will aid the identification of fit-for-purpose strains which exhibit desirable combinations of genetic characteristics. The final results of this work were published in Sternes et al (2017).

6.2. Enhancing malolactic bacterial performance through process management

Evaluating inoculation regimes as a tool in the management of malolactic fermentation duration

Different winemakers and wineries have different needs from malolactic fermentation. Depending on the situation, faster is not always better. A slow yet steady malolactic fermentation provides the time and space to take care of other matters during the hectic vintage months, giving some assurance that wines have a level of protection resulting from the slow release of CO_2 while they are in barrel. Yet for others rapid completion of malolactic fermentation is a primary scheduling issue that enables efficient use of limited winery resources. Different inoculation strategies can result in substantially different completion times for wines, as demonstrated by Abrahamse and Bartowsky (2012). With co-inoculation, completion of malolactic fermentation can occur concurrently with alcoholic fermentation. At the other end of the spectrum sequential inoculation can result in malolactic fermentation occurring over a period of months. However, these two extremes are not always predictable and can depend on several factors including yeast strain used for alcoholic fermentation and matrix conditions such as pH, SO_2 and temperature of fermentation.

The effect of inoculation regime has been progressively explored across the duration of this project. This has occurred primarily through the vehicle of pilot- and winery-scale trials with winery-scale trials being conducted in collaboration with industry partners.

Trials over three vintages in 2015, 2016 and 2017 assessed the impact of co-inoculation and sequential inoculation at pilot- and industry-scale. The aim was to extend the observations that were made at laboratory scale by Abrahamse and Bartowsky (2012). These trials looked at three different systems: Shiraz, Chardonnay and sparkling base. Over the three vintages different *O. oeni* strains were trialled to take account of varying conditions.

Evaluation of inoculation regimes - 2015 vintage trials

In 2015 three strains – one commercially available and two non-commercially available – were trialled in in Shiraz and Chardonnay (AWRIB551, AWRIB710 and AWRIB714). The duration of malolactic fermentation in Shiraz ferments (12 days) were similar regardless of whether they were initiated as part of a co-inoculation or sequential inoculation regimes (Figure 1A). The overall process time was therefore reduced in the co-inoculated ferments by 20 days from 42 to 22 days. All three strains performed similarly, with regional isolates demonstrating a performance profile equivalent to that of an industry 'workhorse' strain.

In contrast, bacterial strain differences were evident in malolactic fermentation of Chardonnay (Figure 1B). While all strains performed similarly in sequentially inoculated ferments, with a total process time of 36-40 days from the beginning of alcoholic fermentation, only one strain efficiently completed co-inoculated fermentation with a total process time of 18 days (B710). The other two strains (B706 and B551), one of which is the commercial benchmark in this work, took longer to complete MLF in the co-inoculation regime than in the sequential inoculation regime (74 days). The cause of MLF delay in the co-inoculation regime remains undetermined.

Concentrations of diacetyl, a key compound contributing to the perception of butteriness in wines, did not appear to be related to either co-inoculation or sequential inoculation regimes, but rather appeared to increase in concentration when the duration of MLF became extended, especially in Chardonnay. However, even at their highest concentrations the Chardonnay concentration of diacetyl was 10-fold lower than that which was observed in Shiraz (Figure 2A). Despite this overall lower concentration of diacetyl, the relative perception of butteriness was higher in the Chardonnay for those Chardonnay wines that had the highest concentration of diacetyl (Figure 2B).

In summary, chemical and sensory analysis of the 2015 pilot and industry trials showed that, except in one case, novel *O. oeni* strains produced sound wines with no obvious faults. One strain produced wine with a slight reduced character (data not shown). Red wines exhibited higher diacetyl concentrations than white wines. Strains apparently differed in their suitability for co-inoculation work.

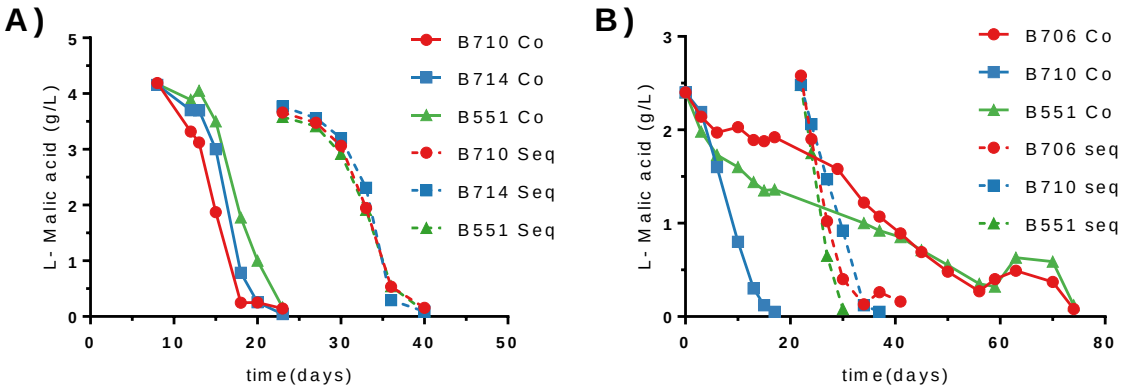


Figure 1. Progress of MLF in Shiraz (A) and Chardonnay (B) using co-inoculation and sequencing inoculation regimes with three different bacterial strains**.**

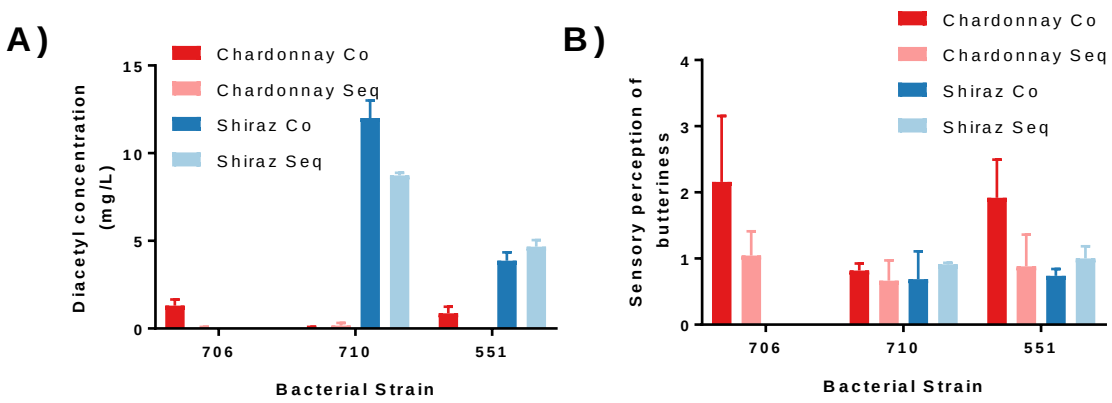


Figure 2. Effect of inoculation regime and wine type on diacetyl concentration and perception of butteriness.

Evaluation of inoculation regimes - 2016 vintage trials

Three trials were undertaken in the vintage of 2016. These trials again examined MLF inoculation regimes at pilot- and winery-scale, focusing on two novel *O. oeni* strains that were chosen based on data from AWR 1305 and trials undertaken in the previous year (data not shown). Strain AWRIB447 was identified in laboratory-scale trials as a high ethanol tolerant strain and was therefore chosen as a candidate for red wine MLF trials. AWRIB706 showed tolerance to low pH in the same laboratory trials and good performance in sequential inoculation pilot-scale vintage trials in 2015 and was therefore selected as a promising candidate for industry trials in Chardonnay table wine and sparkling base wine production.

Shiraz fermentation was undertaken in open tank fermenters inoculated with yeast AWRI796. Co-inoculation MLF treatments were inoculated with AWRIB447 48 hours post-initiation of alcoholic fermentation. At 0° baumé wines were pressed to barrel and left to complete MLF. Sequential treatments were inoculated post-pressing in barrel at 0° baumé at standard bacterial addition rates. Co-inoculation of AWRIB447 was extremely beneficial (Figure 3A). MLFs were complete almost concurrently with alcoholic fermentation. This contrasts with sequential inoculation which, for this strain, required 80 days before any indication of malic acid utilisation was apparent. The co-inoculated fermentation eventually finished after 120 days.

MLF of Chardonnay was carried out in triplicate in 20 L stainless steel kegs. Co-inoculation with AWRIB706 was initiated 48 hours post-initiation of alcoholic fermentation by addition of Qa23. Fermentations were carried out at 17°C. Alcoholic fermentation was completed, at which point sequential inoculation was initiated in a separate replicate set of 20 L wines. The pH values of these wines were between 3.3 and 3.4. In a pattern that reflected the 2015 trial with this bacterial strain (Figure 1B), both sequentially inoculated MLFs finished 6 days prior to co-inoculated wines (24 and 31 days respectively). This data indicates that in relatively soft conditions AWRIB706 is not well suited to co-inoculation.

Finally, trials with AWRIB706 in the production of sparkling base wine showed the inverse of previous observations of this strain. Trials were performed in duplicate, 300 L stainless steel barrels. As with previous trials co-inoculated MLFs were initiated 48 hours post-yeast inoculation whereas sequential MLFs were initiated after the ferments had become ‘sugar dry’. Although co-inoculated sparkling base MLFs showed no sign of malic acid utilisation for almost 60 days (compared to 25 days for the sequential ferments) these MLFs ultimately finished substantially faster (82 days) than sequentially inoculated MLFs, which failed to complete malic acid utilisation (0.8 g/L after 125 days).

In summary, it is difficult to discern a consistent pattern of performance of individual bacterial strains. Co-inoculation of red fermentation was generally reliable with a variety of bacterial strains and substantial reductions in the overall duration of the combined alcoholic and malic acid fermentations were observed. This was not always the case for white wine MLFs where the duration of co-inoculated ferments often exceeded that of sequentially inoculated MLFs. These observations were consistent over several bacterial strain/juice matrix/vintage combinations. The one bacterial strain that demonstrated a consistent pattern of performance in co-inoculated and sequentially inoculated red and white fermentations was strain AWRIB710. However, this strain suffered from sensory defects that precluded it from further study but may warrant closer attention in future work.

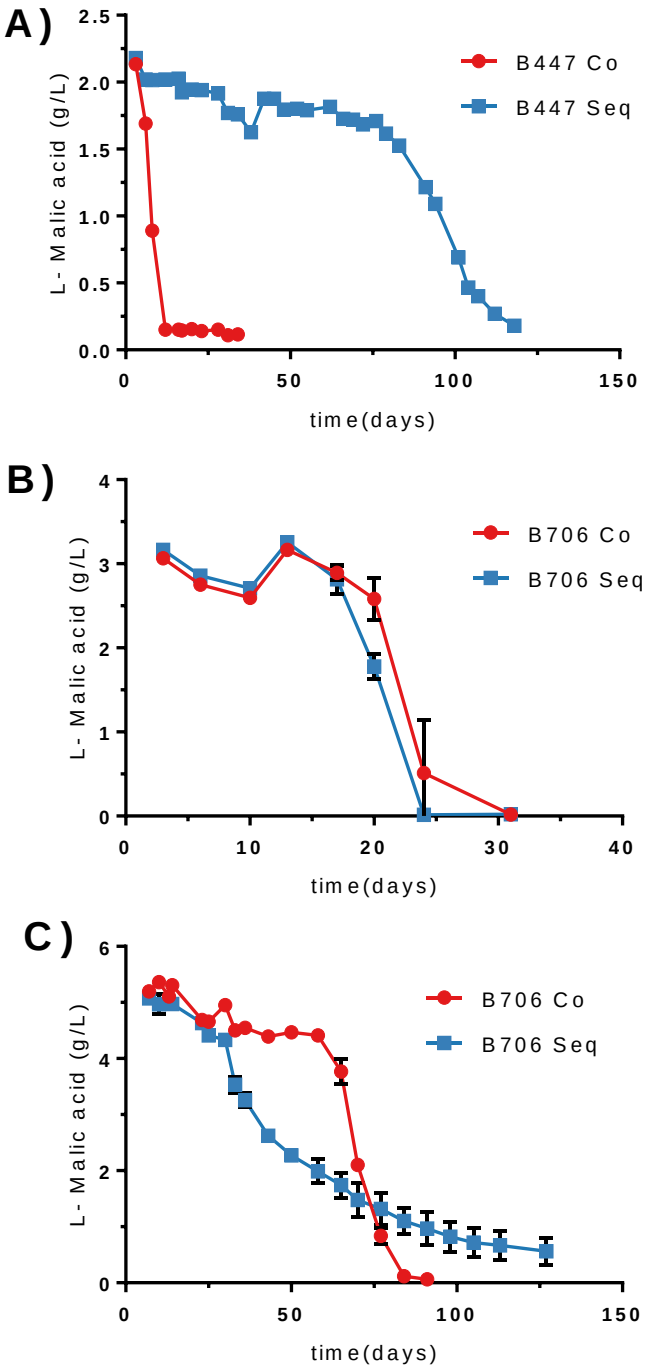


Figure 3. 2016 vintage co-inoculation and sequential inoculation MLF trials in Shiraz (A), Chardonnay (B) and sparkling base (C). These trials were conducted in 10 tonne, 20 L and 300 L volumes respectively.

Nutrient addition as an aid to malolactic fermentation

Just as with yeast nutrient additives, additives claiming to support reliable malolactic fermentation are increasingly becoming available via a number of commercial nutrient suppliers. Technical information provided by inactive dry yeast suppliers generally indicates that these products provide a source of bacterial nutrients such as amino acids and parietal polysaccharides. Some brands may also include other specific components such as yeast ghosts which, evidence suggests, facilitate absorption of toxic medium chain fatty acids produced by yeast. Proprietary recommendations for use of IDY bacterial nutrients include cases where, for example, nutrient status of the wine may be limiting, conditions are difficult for MLF induction, and where MLF is slow or stuck.

At the initiation of this work it was unclear under what circumstances nutrient addition would be helpful in malolactic fermentations and whether individual strains might respond differently to nutrient supplementation.

In the development of a small volume bacterial screening method used for comparison of bacterial strains in section 6.3, several different MLF nutrient additives were evaluated against eight different bacterial strains. In this work, which evaluated strain performance in a range of different matrices and with nutrients from a number of different suppliers, almost all strains benefitted from the addition of nutrients. Without nutrient addition, most bacterial strains were only capable of partial MLF in most of the conditions tested. An example of this is given in Figure 4A. Nutrient addition to small-scale MLFs facilitated completion of MLF by all but two strains under these conditions (Figure 4B). While the magnitude of the effect of some additives was greater than others, the general trend was consistent, that nutrient addition helped. As a result, MLF nutrients have become a standard additive in the AWRI's small-scale bacterial assessment protocol.

Based on the results observed in small-scale trials, industry trials were undertaken over two successive vintages. Two nutrient additives with two *O. oeni* strains (one that responded well to nutrient addition in previous trials and an industry reference) were trialled in a Shiraz wine with low free alpha-amino nitrogen (considered as stressful for *O. oeni*). Neither nutrient addition increased the rate of MLF for either of the two strains. This suggests that the stress of low alpha-amino nitrogen in this wine was insufficient to compromise MLF. In the second vintage, a commercial nutrient was used to treat Shiraz wine and sparkling wine that were undergoing MLF in two independent trials. Both of these wines were challenging and MLF was slow in both cases. Shiraz took 55-65 days to complete and sparkling base between 37 and 63 days. In neither case did nutrient addition reduce the duration of MLF.

In summary, nutrient additions were observed to be beneficial in small-scale MLF fermentations where wines were highly clarified and the dissolved oxygen concentration of the wines can be presumed to be high. However, in barrel or tank MLF of red or white wine no evidence for enhancement of MLF was observed over two vintages.

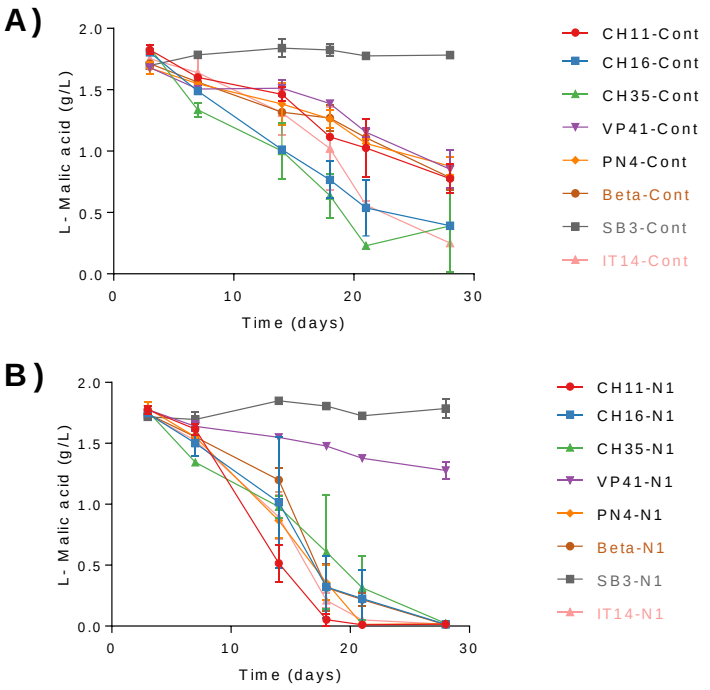


Figure 4. Effect of nutrient addition on progress of malolactic fermentation in small scale fermentations of Chardonnay wine by eight different strains of *O. oeni*. Control (A) and nutrient treatment (B).

Oxygen and malolactic fermentation

Data derived from trials in the application of oxygen during fermentation of red wines in rotary fermenters suggested that the oxygen exposure history of a ferment could influence the performance of subsequent malolactic fermentations (AWRI Project 3.3.2). While malolactic fermentation was a feature of that trial, the trial was not designed to detect changes to malolactic fermentation as a consequence of fermentation treatments. To explore the impact of fermentation oxygen treatments on malolactic fermentation more rigorously, a set of experiments was conducted in which potential confounding influences could be better controlled.

Four Chardonnay wines were made with two different oxygen addition regimes during fermentation with two different exposure levels with each of the two regimes. Ferments experienced a high level of exposure using a regime of either 2 hours or 72 hours, or a low level of exposure using a regime of 2 hours or 24 hours. Alcoholic fermentation completed after nine days for the high-level exposure regime and three days later for the low-level exposure regime. In each case, wines were inoculated with *O. oeni* strain VP41 immediately upon completion of alcoholic fermentation. The completed alcoholic ferments were transferred to a separate vessel under anaerobic conditions. Malolactic fermentation was carried out on yeast lees. Progress of malolactic fermentation was monitored through enzymatic assay. The results of this work are presented in Figure 5. There was no consistent relationship between the type of oxygen treatment applied and the progress of malolactic fermentation. In all cases, complete consumption of malic acid occurred extremely quickly, less than 0.1 g/L L-malic acid was reached in 8 – 11 days. Similar results were obtained in replications and variations of this experiment.

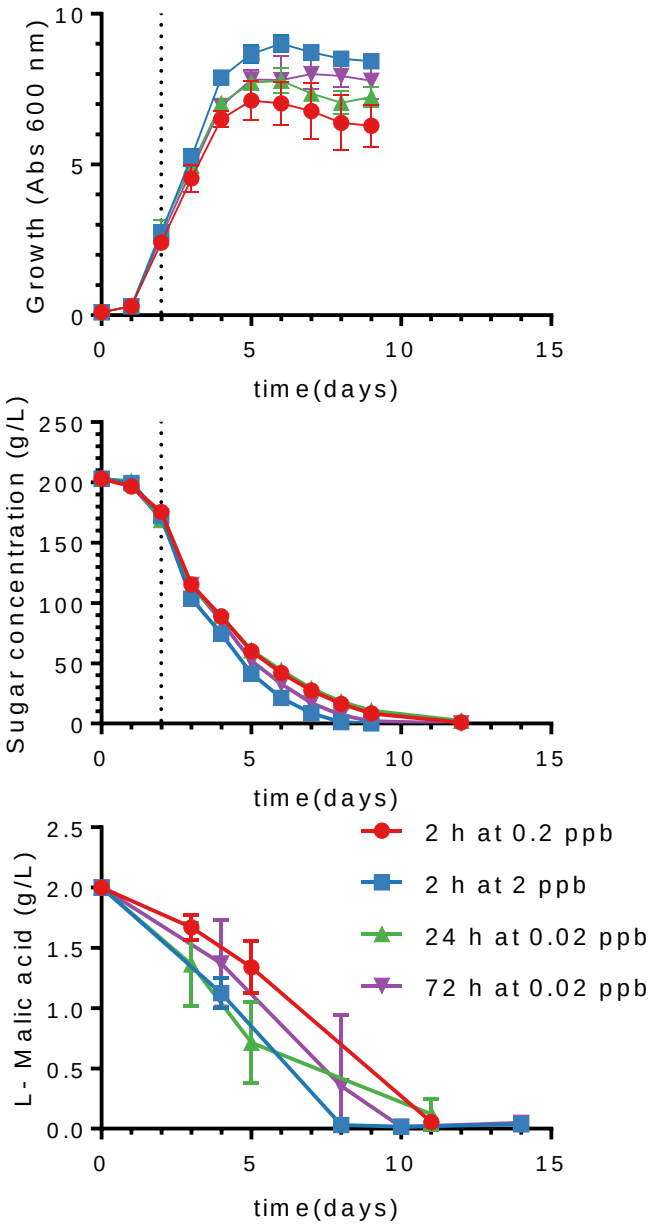


Figure 5. Effects of oxygen addition on alcoholic and malolactic fermentation in Chardonnay. Two regimes were used to deliver high level exposure: 2 hours at 2.0 mg/L (red) and 72 hours at 0.02 mg/L (purple), and another two regimes were used to deliver a low level exposure: 2 hours at 200 mg/L (blue) and 24 hours at 0.02 mg/L (green).

6.3. Isolation and characterisation of robust malolactic bacterial strains from Australian wine regions

Characterisation of *O. oeni* genetic diversity through whole genome sequencing and comparative genomics in project AWR1305 revealed substantial inter-strain genetic variation with large clusters of highly related strains (Borneman et al. 2012, Sternes and Borneman 2016). In general *O. oeni* can be split into two groups (shown as A and B in Figure 6) with group A being the least diverse and comprised largely of strains isolated in Australia. The knowledge gained through these comparative genomics efforts was employed to evaluate the breadth of oenologically useful traits. To this end, an *O. oeni* subset was created to reflect the broader genetic diversity of this species. The source of many of these strains was the AWRI wine microorganism culture collection (AWMCC), which is a repository for strains isolated over many decades. The representative subset was characterised using a high throughput phenotyping approach (Costello et al. 2017) and their ability to complete malolactic fermentation under a range of challenging, wine relevant conditions recorded. An overview of this work is presented in Figure 6.

The most alcohol-tolerant strains were found amongst group A and this physiological trait appeared to be the most common among all the characterised strains. Tolerance to low pH was neither as common or as well clustered, with a broader cross section demonstrating tolerance to low pH. Nevertheless, group B strains also tended to be almost uniformly sensitive to low pH.

Individual strains that demonstrated robustness to a range of conditions were selected for more detailed analysis through small-scale laboratory work and pilot-scale trials. The small-scale trials approximated moderately tough conditions of pH and SO_2. Three strains outperformed all others including a commercially available strain used for comparison (Figure 7).

One of these strains (AWRIB1062) was evaluated in industry trials in the 2017 vintage. This strain was compared with a commercially available reference strain and two previously trialled regional isolates in MLF of sparkling base in barrel. The strain was evaluated in co-inoculation and sequential inoculation regimes (Figure 8A and B). This strain performed competitively with the commercial reference strain when used in a co-inoculation and outperformed all other strains in sequential inoculation. This indicates a robust tolerance of AWRIB1062 toward low pH and cooler temperatures, which is consistent with earlier small-scale laboratory trials.

In summary, large-scale small volume screening followed by larger-scale laboratory trials have identified several candidate bacterial strains that demonstrated robust performance characteristics in industry trials. Strains varied in their capacity to perform in both co-inoculation and sequential inoculation regimes and it may be that different strains will need to be used depending on the inoculation regime employed. At least one strain was identified that outperformed an industry benchmark strain in low pH/low temperature MLF of sparkling base wine, a style of winemaking that for many requires short process times.

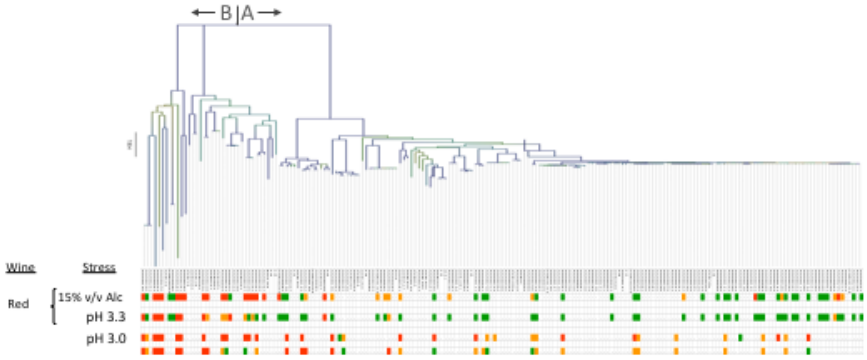


Figure 6. Bacterial strain MLF performance in wine relative to a baseline condition. Green - top 25% of performers; orange - intermediate performers, red - did not complete MLF. Performance profiles in high alcohol (15%), pH 3.3 and pH 3.0 is aligned against a phylogenetic tree that illustrates the relationship between *O. oeni* strains.

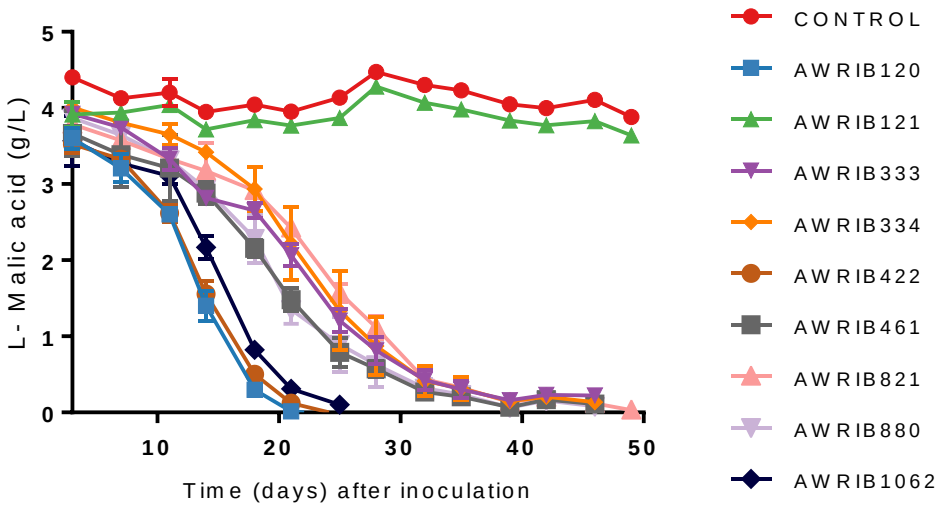


Figure 7. Comparison of *O. oeni* performance in 2 L Chardonnay wine. Sterile filtered Chardonnay wine (12.5% ethanol, 28 mg/L total SO_2, pH 3.18) was inoculated with acclimatised starter cultures at ~1 x 10^7 cells/mL and ferments were carried out at 22°C. Progress of MLF was monitored enzymatically. A commercially available strain was included for comparison (control).

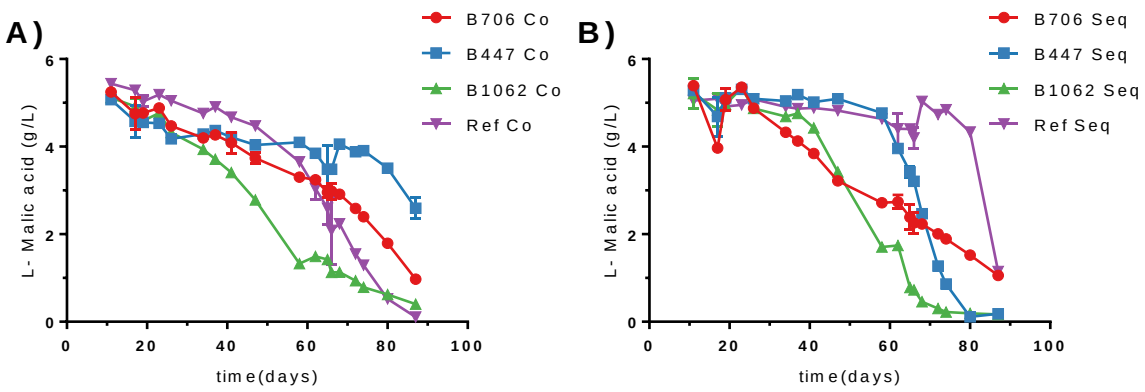


Figure 8. Comparison of novel isolate performance with a commercially available reference strain in MLF of sparkling base wine in barrel. MLFs were performed in duplicate.

7. Outcome/conclusion:

7.1. Performance against planned outputs

Identify O. oeni *strains that tolerate MLF-limiting wine parameters, grow rapidly and efficiently metabolise malic acid; Validate performance of target strains in a wide range of conditions typically found in red, white and sparkling winemaking*

Through an extensive evaluation of bacterial traits using novel screening methodologies developed during this project, numerous strains were identified that are tolerant of specific conditions generally considered to be inhibitory to MLF. Some of these strains are available through commercial suppliers, others were isolated from industrial fermentations over several decades. Target conditions in which strains needed to perform well were primarily high ethanol concentration (>15%), low pH (<3.1) and high SO_2. While many strains capable of sustaining effective MLF in higher ethanol wines were identified, far fewer were able to tolerate low pH. Few wines with a low pH are required to undergo MLF (primarily sparkling wines) and because this represents a niche application it is likely that selection for strains that can perform well in such conditions has not been a priority for commercial suppliers.

Performance of strains identified as robust in various conditions was validated through larger-scale laboratory fermentations (2 – 20 L). At this scale characterisation also involved sensory assessment using the AWRI's quality panel. A prerequisite for the progress of strains to industry trials is that they should not only efficiently consume L-malate but that the resultant wines should be free of faults or taints. A small number of strains were noted as imparting sulfidic aroma profiles to wines. The basis and degree of volatile sulfur compound production in wine by *O. oeni* and the strain-specific nature of that contribution is poorly understood. As such, this is an area worthy of further research. Strains that consistently performed well in these conditions and were fault-free were subsequently assessed in industry trials varying in scale from replicated barrel fermentations to singular 10-tonne fermentations. Making the transition from 20 or 50 L fermentations to >5000 L remains a challenge in evaluation work. Preparation of sufficient quantities of acclimatised starter culture places a burden on project resources and this naturally limits the number of such trials that can be undertaken in any given vintage. Future work will look to minimise the number of such trials through increased focus on specific experimental questions and optimise production of bacteria at scales that facilitate interaction with industry partners.

Determine winemaking practices and/or conditions required to modulate aroma and flavour compounds and their impact on wine style; Identify additional factors that ensure an efficient MLF and augment aroma and flavour development, such as MLF inoculation regime (in white wines), impact of winemaking additives (e.g. nutrients), and compatibility with current and 'in development' yeast strains

Several winemaking practices were evaluated from both performance and wine style perspectives. The MLF regime employed can have a substantial impact on both MLF efficiency and sensory qualities. The specifics are highly wine matrix dependent.

In red wines, co-inoculation regimes generally proceeded reliably with a substantial reduction in total process time. Many of the bacterial strains evaluated in red wine performed equivalently and while the production of a key metabolite, diacetyl, did vary substantially in a strain-dependent manner, this variation did not have large impacts on the sensory quality of the finished wine.

This was not the case in white wines. In white wines, there could be large differences in total process time between co-inoculated and sequentially inoculated ferments but not in a way that was predictable. While some co-inoculated strains were able to complete MLF in a time comparable with sequentially inoculated strains, therefore reducing the overall process time, other strains took substantially longer, thereby extending total process time beyond that which was required for sequentially inoculated strains. MLFs that became sluggish in this way also tended to have higher concentrations of diacetyl. While the concentrations of this metabolite were much lower than what was observed in red wines, the sensory impact appeared to be much higher, with perception of buttery characters doubling over that of wines that proceeded efficiently. These results indicate that co-inoculation of white and sparkling base wines is still a risker operation than it is for red wines.

Other treatments investigated included nutrient addition and oxygen treatment. Neither treatment had a measurable effect on MLF performance, either positive or negative. Nutrient addition carries the risk that if the nutrients are not consumed by *O. oeni*, residual nutrient in the finished wine could act as a source of sustenance for spoilage organisms. Oxygen use is more commonly associated with stimulation of alcoholic fermentation and that was at least in part the inspiration for this series of experiments. This work demonstrated that oxygen could be used during alcoholic fermentation without negative consequences for subsequent MLF. This work is being continued as part of ongoing projects investigating the use of oxygen during winemaking.

Identify genes for metabolic processes that impact on wine aroma and flavour; Develop a comprehensive knowledge base of potential metabolic pathways in O. oeni that can be used to shape wine style

The contribution to wine aroma and flavour by *O. oeni* and the genetic factors that underpin that contribution have been approached through the extensive sequencing of more than 200 *O. oeni* strains and the characterisation of physiological traits in a subset of those strains. Gene expression profiling of three strains throughout malolactic fermentation identified likely candidate genes that could be used as markers for diacetyl production and others that indicated potential variation in strain-specific sugar utilisation in genomically highly related strains. Furthermore, variations in metabolic pathways indicating varied capacity to utilise citric acid were identified. Extending these findings to a broader range of bacterial strains requires further work as does the testing of predictions made on the basis of a single gene expression study. However, the experiments undertaken in the course of this project have provided a framework for a deeper understanding of MLF and have laid the foundation of future work in this area.

Provide the wine industry with knowledge generated from the above, particularly with regard to strategies for improving MLF outcomes

The primary vehicle for industry engagement and extension activities has been the AWRI roadshow program. Several talks are routinely given that aim to inform industry stakeholders about specific project activities, progress of industry trials and also more broadly about how learnings can be put into practice to minimise risk of problem MLFs. These roadshow activities are supported through the provision of fact sheets that were either created or updated upon completion of the project and are available through a number of channels including the AWRI helpdesk, the AWRI library and AWRI website.

Develop commercial MLF bacterial strains that are suited to Australian winemaking conditions

Through an extensive evaluation of bacterial traits, several promising bacterial strains were identified that exhibited strong performance in either higher ethanol concentrations or low pH conditions. Some of these strains have been trialled extensively at pilot and industry scale and represent promising candidates for commercialisation.

7.2. Could changing the methodology/technology have improved the outcome?

The approaches to specific aspects of the work undertaken in this project have, naturally, evolved over the duration of the projects as different elements were found to work or not. For example, preparation of starter cultures for industry trials began by simply growing bacterial strains in larger and larger volumes of commercially sourced juice until sufficient biomass was generated to inoculate a large volume of wine. However, wine conditions are substantially different to those of the growth media used to generate biomass and experiments initiated in this way generated unreliable outcomes. Starter culture preparation then evolved to include increasingly extensive acclimatisation procedures to get the strains ready for the wine they were to encounter in the trial. Acclimatisation of starters came at the cost of staff time and limited the capacity to be responsive to rapidly changing vintage realities. Long lead times were required as well as samples of equivalent wine prior to the wine being ready. The development of methods to prepare cultures in the inter-vintage period and store them as frozen stocks dramatically shortens the lead time involved in starter culture preparation during vintage periods, improves the ability to respond to evolving vintage needs and reduces commitment of staff time during an already busy period. While these changes are now in place at the end of the project, it is likely that some of the vintage trials that were undertaken during the project would have benefitted substantially had they been in place earlier.

Much of the screening work was performed in wine. When work such as this takes place over many vintages the wine matrix can change, or if a particular source is exhausted and a new one sought, it will be completely different. This occurred during this project making a continuous data set difficult to compile. This would occur in almost any context if, after completion of a project, additional work was performed to extend the findings. It would have been better had the initial phenotyping work been undertaken in a defined wine matrix rather than an actual wine made from grape juice. The results can always be validated in real wine matrices after initial findings have been made but it is difficult or impossible to extend a set of data from one real wine to another to obtain a consistent foundational data set that spans multiple matrices.

7.3. Practical implications

The molecular markers identified in this project will facilitate more efficient screening of novel bacterial isolates. Potential sources of novel isolates include ongoing Wine Australia projects such as AWR1501. Given the difficulty and time involved in performance screening of *O. oeni* isolates, methods that help to streamline bacterial strain characterisation will be particularly beneficial.

All of the work on co-inoculation, nutrient addition and the effects of oxygen has generated a dataset that will form the basis for advice on how best to apply different winemaking practices and the risks and rewards associated with them. This information is being disseminated through multiple channels and will become a resource for researchers and industry practitioners alike.

7.4. Benefits to the industry

The key benefit to industry is the provision of evidence-based advice on how to conduct and manage malolactic fermentation under challenging conditions. In this project, different approaches to the conduct of MLF have been investigated, some substantially outside of standard industry practice, with the aim of pushing the boundaries of what is achievable in MLF. Such risks can be undertaken in a research environment so that commercial risks taken by Australian winemakers can be objectively minimised. This approach contributes to the maintenance of a sustainable and profitable Australian wine industry.

7.5. Recommendations

One area of malolactic performance that was only partially addressed in this project was the constraining effect of SO_2 concentration in juice and wine on MLF. This will become a more important consideration in light of increased adoption of co-inoculated MLFs. If it is considered at all, analysis of juice will focus on free SO_2 because of the potentially inhibitory effect on primary fermentation. However, *O. oeni* and other lactic acid bacteria can strip components from bound SO_2, thus liberating free SO_2 from the total. The degree to which this is a fundamental consideration for co-inoculated fermentation regimes and the mechanisms by which *O. oeni* resists SO_2 are natural progressions of the current work.

8. Appendix 1: Communication:

Outcomes and knowledge generated during the progress of this project have been communicated to peers through peer-reviewed publications, conference presentations, and annual meetings with researchers at the University of Adelaide. These are itemised in sections 9.1, 9.2 and 9.3. Representatives of Wine Australia attended these meetings and therefore have had the opportunity to hear updates on project progress outside of the six- monthly reports that were provided for the duration of the project.

Information has been extended to industry stakeholders through articles in trade journals, presentations in the AWRI roadshow program, webinars and workshops at both the 2013 and 2016 Australian Wine Industry Technical Conferences. Updated fact sheets have been prepared that provide the latest information to winemakers and are available upon request from the AWRI helpdesk or as a download from the AWRI website.

8.1. Journal publications

Bartowsky, E.J. 2017. *Oenococcus oeni* and the genomic era. *FEMS Microbiol. Rev.* 41: S84–S94.

Bartowsky, E.J., Costello, P.J., Chambers, P.J. 2015. Emerging trends in the application of malolactic fermentation. *Aust. J. Grape Wine Res.* 21: 663–669.

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Costello, P.J., Siebert, T.E., Solomon, M.R., Bartowsky, E.J. 2013. Synthesis of fruity ethyl esters by acyl coenzyme A: alcohol acyltransferase and reverse esterase activities in *Oenococcus oeni* and *Lactobacillus plantarum*. *J. Appl. Microbiol.* 114: 797–806.

Schmidt, S.A., Costello, P.J. 2014. Nutrients to support primary and secondary fermentations: what are they and do they work? *AWRI Tech. Rev.* 211: 6-11.

Sternes, P.R., Borneman, A.R. 2016. Consensus pan-genome assembly of the specialised wine bacterium *Oenococcus oeni*. *BMC Genom*. 17(308): 1-15.

Sternes, P.R., Costello, P.J., Chambers, P.J., Bartowsky, E.J., Borneman, A.R. 2017. Whole transcriptome RNAseq analysis of *Oenococcus oeni* reveals distinct intra-specific expression patterns during malolactic fermentation, including genes involved in diacetyl metabolism. *Int. J. Food Microbiol.* 257: 216–224.

8.2. Conference Presentations

Genomic analysis of 80 *Oenococcus oeni* strains and connecting the genome with winemaking properties. Presenter: E. Bartowsky; 11th Lactic Acid Bacteria Symposium, Egmond aan Zee, The Netherlands, 31 Aug-3 September 2014.

Genomic analysis of 80 *Oenococcus oeni* strains and connecting the genome with winemaking properties. Presenter: E. Bartowsky; Crush 2014 - Grape and Wine Science Symposium, Adelaide, 25-26 Sepembert 2014.

High-throughput phenotyping of malolactic bacteria. Presenter: P. Costello; 16th Australian Wine Industry Technical Conference, Adelaide, 24-28 July 2016.

High-throughput phenotyping of malolactic bacteria. Presenter: P. Costello; 16th Australian Wine

Industry Technical Conference, Adelaide, 24-28 July 2016 (MLF Workshop).

Wine Fermentation – Research and innovation with an age-old process. Presenter: P. Costello; AIFST Seminar – Sydney, October 2015.

8.3. Extension presentations

Titles of AWRI roadshow presentations

- Can I improve MLF performance and reliability?
- Avoiding spoilage issues caused by wine bacteria: prevention is better than cure
- Novel *O. oeni* strains with appropriate winemaking properties
- Can you influence wine styles through MLF?

Fact sheets – available from the AWRI website

Updates for the AWRI fact sheet 'Achieving successful MLF' have been prepared for upload to the AWRI website.

A new protocol 'Preparation of a liquid malolactic fermentation (MLF) starter from an agar slant culture of *Oenococcus oeni*', will also be available for wineries wishing to culture 'in-house' or other malolactic strains from the AWRI wine microorganism culture collection.

A new AWRI fact sheet 'MLF in white and sparkling base wines' has been prepared for upload to the AWRI website.

9. Appendix 2: Intellectual Property

This project has generated knowledge, know-how and data on the subject of malolactic fermentation and wine quality which will be published and communicated as part of the project outcomes.

10. Appendix 3: References

Abrahamse, C.E., Bartowsky, E.J. 2012. Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. *World J. Microbiol. Biotechnol.* 28: 255–265.

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Costello, P.J., Siebert, T.E., Solomon, M.R., Bartowsky, E.J. 2013. Synthesis of fruity ethyl esters by acyl coenzyme A: alcohol acyltransferase and reverse esterase activities in *Oenococcus oeni* and *Lactobacillus plantarum*. *J. Appl. Microbiol*. 114: 797–806.

D'Incecco, N., Bartowsky, E., Kassara, S., Lante, A., Spettoli, P., Henschke, P. 2004. Release of glycosidically bound flavour compounds of Chardonnay by *Oenococcus oeni* during malolactic fermentation. *Food Microbiol.* 21: 257–265.

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Sternes, P.R., Costello, P.J., Chambers, P.J., Bartowsky, E.J. Borneman, A.R. 2017. Whole transcriptome RNAseq analysis of *Oenococcus oeni* reveals distinct intra-specific expression patterns during malolactic fermentation, including genes involved in diacetyl metabolism. *Int. J. Food Microbiol.* 257: 216–224.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S. 2005. Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11: 139–173.

11. Appendix 4: Staff

Peter Costello, Eveline Bartowsky, Anthony Borneman, Paul Chambers

12. Appendix 5:

Nothing additional to report

13. Appendix 6: Budget reconciliation.

The project's budget reconciliation statement will be submitted separately.