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16th August 2018

Professor Alexander Steinbüchel
Editor-in-Chief
Applied Microbiology and Biotechnology

Dear Professer Steinbüchel,

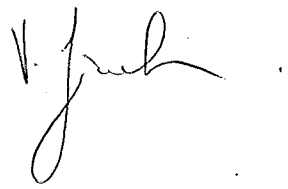
Many thanks again for the original invitation to develop this mini-review. We trust the submission entitled 'Measures to improve wine malolactic fermentation' meets your expectations and is acceptable for publication in Applied Microbiology and Biotechnology.

The review summarises the latest research on MLF including; impediments to successful MLF, microbial succession and growth of LAB in wine, un-inoculated MLF *vs* inoculated MLF, enhancement of the MLF process and sensory outcomes. We also highlight directions that future research may take.

This work has not been previously published and has not been submitted to another journal.

We thank you again for your patience in waiting for this review. It is much appreciated.

Kind regards,



Measures to improve wine malolactic fermentation

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Abstract

This review focuses on the considerable amount of work directed at improvement of malolactic fermentation (MLF) efficiency and reliability. From this work it is clear that reliable MLF is essential for process efficiency and prevention of spoilage in the final product. While most reports relate to wine made from grapes, MLF may be conducted in other beverages and foods, such as pineapple juice, cider, durian pulp fermentation, cherry wine, and many others. Nevertheless, this review focuses on what is known about the impediments to successful MLF in wine, the bacterial succession that occurs during the process and how this may affect MLF outcome. Coverage is given to how the MLF process may be enhanced including via inoculation strategies and the use of alternate species. An update of how this information may be used to enhance and improve sensory outcomes through metabolite production during MLF and suggestions for future research priorities for the field are also provided.

Keywords

Oenococcus oeni, *Lactobacillus*, malolactic fermentation, wine

Introduction

During winemaking the initial conversion of grape must to wine is an alcoholic fermentation (AF) carried out by one or more strains of yeast, typically *Saccharomyces cerevisiae*. After the alcoholic or primary fermentation, a secondary fermentation known as malolactic fermentation (MLF) is often undertaken, depending on the style of wine that the winemaker seeks to achieve. Malic acid is one of the predominant organic acid in grapes, occurring in amounts of the order of 3 $g.L^{-1}$ (Palma and Barroso 2002). When grapes are crushed and fermented by wine yeast, malic acid content is largely unaffected. This compound can therefore contribute to the acidity, pH and mouthfeel of wine and can be a nutrient for several spoilage organisms. Accordingly, a malolactic fermentation (MLF) by which L-malic acid is decarboxylated to L-lactic acid serves several purposes: to reduce the harsh acidity of malic acid, give a concomitant modest increase in pH as well as increase wine microbial stability. The former of these outcomes are of greatest importance in sparkling and some white wines, whereas most red wines undergo MLF for stability. Subsequent effects due to MLF also include impacts on both aroma and visual profile (Burns and Osborne 2013; Sumby et al. 2010).

Typically MLF is conducted by lactic acid bacteria (LAB), particularly *Oenococcus oeni*, a Gram-positive organism that is thought to have evolved to exclusively survive in the fermented beverage environment (Campbell-Sills et al. 2017a). *O. oeni* usually grows more slowly than other LAB, but ultimately triumphs in wine owing to its greater tolerance to the combination of ethanol and acid found in wine (Lonvaud-Funel, 1999). Despite this advantage, MLF can be uncertain and protracted thereby lengthening processing time and reducing winery throughput. All the while, wines are left with minimal protection from the preservative SO_2 so as to encourage MLF, increasing the risk of wine oxidation or contamination with spoilage organisms.

Greater process reliability as well as an ability to tailor wine composition are attributes keenly sought by the wine industry in any new measures to improve MLF. To this end, the selection or generation of more robust strains, identification of alternate LAB such as *Lactobacillus* sp., use of LAB-yeast co-inoculation instead of sequential inoculation strategies, characterisation of yeast-LAB interactions, alternate biomass introduction/removal methods, and a greater attention to the sensory influences of these organisms are all approaches by which researchers have sought to

meet this demand. A summary of key recent findings under several of these strategies is included here, along with a discussion of future opportunities. For details of specific strain improvement methodologies the reader is referred our earlier review (Betteridge et al. 2015).

Impediments to successful MLF

Efficient control of MLF requires an extensive knowledge of the response of LAB to the stressful conditions found in wine. The ability of a LAB to undergo MLF is influenced by many factors including pH, temperature, wine inhibitor content (e.g. ethanol, SO_2, medium chain fatty acids (MCFA's)), nutrient limitation, other potential as yet unknown factors, the yeast strains carrying out AF and interactions with the indigenous microflora of the fermentation (Cinquanta et al. 2018; Guzzon et al. 2009; Liu et al. 2017b). Survival in wine under such multi-stress conditions requires the maintenance of the functionality of the cell membrane, in order to control ion permeability and regulate solute and nutrient exchange between the cell and the external medium. Ethanol is considered to be the main stressor in wine, because it can injure cell membrane integrity and impact cell viability. Ethanol tolerance is widely reported to be strain specific and the ethanol stress response is complex and well-studied (reviewed by Bonomo et al. 2018).

The second most important stressor in wine is low pH. Most wines have a pH ranging from 3.8-3.2, with wines at the higher values being more prone to microbial spoilage as well as biogenic amine formation (Cinquanta et al. 2018). The physiological effect of pH on LAB is also well defined. RNA sequencing revealed the differential expression of several genes related to the metabolism of amino acids, carbohydrates, membrane transport and energy metabolism as part of the genetic response of *O. oeni* strain SD-2a to low pH (3.0 *vs* 4.8; Liu et al. 2017a). More recently Cinquanta et al. (2018) studied the effect of pH in two Italian wines (Falanghina and Tintilia) inoculated with *O. oeni*, *Lb. plantarum* or a 50:50 mix of both. The duration of MLF was influenced by the pH and the LAB strain used, with both *O. oeni* and *Lb. plantarum* completing at pH 3.8, neither completing MLF at pH 3.2 and *Lb. plantarum* failing to complete fermentation in Falanghia wine at pH 3.4. Interestingly an evaluation of the capacity of a new *Lb. plantarum* V22 starter culture to complete MLF at the laboratory and semi-industrial scale revealed that bacterial

survival was related more to pH evolution during MLF, than the initial pH of the must (Lerena et al. 2016). In all cases the musts showed initial pH values over 3.6 but the pH showed dynamic behaviour, changing as MLF progressed. In fermentations in which pH increased over time, *Lb. plantarum* V22 successfully metabolised most malic acid originally present in the must. By contrast, when the pH decreased over time, bacterial counts declined accordingly, as did the rate of malic acid consumption. This decrease in pH may be due to increased acetic acid production (if the *Lb. plantarum* strain was facultatively heterofermentative) or due to interactions with the yeast strain (which may produce various organic acids during fermentation (Henick-Kling 1993)). Such changes in must/wine pH and their impact on MLF kinetics should be further to allow better tailoring of strain performance and wine conditions.

Where bacterial growth or MLF are initiated despite a high ethanol content, or unfavourable pH values, the risk of stuck MLF remains. Key contributors are high total SO_2, lack of nutrients or phage infection, but there may be other as yet unknown factors such as specific inhibitory yeast metabolites. Although SO_2 produced by yeast during AF will exist in the bound form immediately after fermentation (mostly to acetaldehyde), the total SO_2 is still inhibitory to MLF because bacteria can metabolise the acetaldehyde fraction, releasing a proportion of SO_2 that is inhibitory (Osborne et al. 2000; Wang et al. 2018). A lack of nutrients can also be problematic and can be mitigated by the addition of commercially available substitutes, however, this should be used with caution as it is not advisable to leave nutrients in the wine for other spoilage microorganisms to utilise.

An additional impediment to successful MLF is the effect of phage on the LAB strain conducting MLF. There has been reinvigorated interest in this over the last couple of years and it has been reported that the low pH and high ethanol conditions found in wine can affect the lytic activity of phage (Costantini et al. 2017; Henick-Kling et al. 1986). This effect could be due to the modification of the bacterial cell surface induced by stress conditions. However, as phage have been isolated from wines having difficult MLF, other factors such as sensitivity of phage to ethanol, pH, etc. may also play a role in *O. oeni* resistance. Phage can potentially lyse bacterial cells at the start of AF and thereby interfere with MLF. Interestingly SO_2 is reported to have an antiviral activity (Henick-Kling et al. 1986; Philippe et al. 2017) and *O. oeni* strains that are more resistant to SO_2 may be further benefited by protection from phage attack with small SO_2 additions (5 $g.hL^{-1}$).

Finally it is possible that strain specific differences in *mleA* (malolactic enzyme) expression and L-malic acid consumption are due to the individual strain's ability to adapt to increased ethanol concentrations. Miller et al. (2011) reported that whilst malic acid and low pH increased *mle* expression in *Lb. plantarum*, increased ethanol concentration reduced *mle* expression. Accordingly, very low levels of malic acid could also be a reason MLF doesn't proceed to completion. Certainly ethanol has been reported on numerous occasions to be an inhibitor of MLF (Gockowiak and Henschke 2008; Vailiant et al. 2008), thus a decrease in *mleA* expression could be the basis for MLF failure. More recently Betteridge et al. (2018) reported that *O. oeni* strain A90, derived from directed evolution experiments to be better adapted to high ethanol, showed an initial drop in *mleA* expression (1 h of ethanol exposure), but ultimately had higher *mleA* expression than the parent (SB3) after 24 h in high ethanol conditions. This isolate also consumed L-malic acid faster than the parent in the presence of ethanol.

These impediments provide ready targets for strain optimisation or culture/fermentation management approaches to help improve the success of MLF. However the challenge remains significant given the diversity of MLF conditions and practices as well as the microflora involved.

Grape, must and wine ecology

A diverse community of microorganisms is present on grapes and therefore transfers into winemaking with the possibility of influencing wine processing and sensory properties. Of these, LAB not only contribute to decarboxylation of malic acid but also produce other benefits such as the liberation of aroma precursors and positive enzymatic activities (proteases, lipases, esterases, tannases, glycosidases, etc., Grimaldi et al. 2005a, 2005b; Matthews et al. 2006). In attempting to define the microbiology of grapes and wine, the use of culture-based or culture-independent methods to reveal these microorganisms determines apparent population complexity, with the latter generally uncovering more species, some in very small numbers.

In a review by Barata et al. (2012), over 50 microbial species were identified on grape berries. Of these, the number of LAB (mostly *Lactobacillus* spp. and *Pediococcus* spp.) was limited to a few species totalling only of the order of 10^2 cfu.g^{-1} on sound grapes. LAB species isolated from grapes include *Lb. plantarum*, *Lb. casei*

and *Lb. hilgardii*. Grape must contains a greater diversity of species and in addition to the former includes *Lb. brevis*, *Pediococcus damnosus*, *P. parvulus*, *P. pentosaceus*, *Leuconostoc mesenteroides* and *O. oeni* (Davis et al. 1985, 1986, 1988; Miranda-Castilleja 2016). LAB densities in crushed grapes are about 10^2 cfu.ml^{-1} to 10^4 cfu.ml^{-1}, depending on climatic conditions towards the end of grape maturation, which is inversely correlated with must acidity (Lonvaud-Funel et al. 1999). The frequency of detection on grapes specifically of *O. oeni* is much lower and requires adequate methods to promote the development and identification of minority populations (Franquès et al. 2017; Renouf et al. 2005, 2007). The general consensus is that *O. oeni* cannot be detected on grapes and they are often not detected by culture until the end of alcoholic fermentation (AF) and during MLF (Ultee et al. 2013). One exception to this (Franquès et al. 2017) involved an extended 15 days of semi-selective cultivation in MRS medium supplemented with L-malic acid, fructose, nystatin, sodium azide, L-cysteine and tomato juice, implying that the low numbers make the selection method critical when trying to isolate *O. oeni* present prior to MLF.

There is limited information regarding the succession of LAB in fermentations where the traditional approach of inoculating for MLF after the primary fermentation is used. Further in this context, the interactions of the inoculum with the indigenous microflora are poorly defined. By comparison it is known that when LAB are introduced earlier in the process and co-inoculated with yeast there is often a drop in cfu (of between 10^1-10^4 cfu.ml^{-1}) during the first 24 h followed by rapid growth toward numbers required to carry out MLF (Ong 2010; Bartle, unpublished data 2017; Figure 1). During fermentations that are not inoculated, population profiles vary depending on SO_2 additions prior to crushing but the general trend remains the same i.e. *O. oeni* and some species of *Lactobacillus* can survive during AF, whereas *Pediococcus* and other LAB gradually decline (Figure 1). Thus during the first days of AF the number of LAB usually increases to near 10^4 cfu.ml^{-1} and then decreases to around 10^2 cfu.ml^{-1} at the end of AF, due mainly to competition from yeast and sensitivity to SO_2 and ethanol. After AF, LAB (almost exclusively *O. oeni*, but some *Lactobacillus* sp. may be present) increase and MLF begins when cells reach 10^6 cfu.ml^{-1} (Figure 1). *O. oeni* is largely responsible for MLF given it is the species best adapted to wine.

Early, culture-based studies on this topic reported the succession of bacterial populations through stages of wine fermentation, thereby offering novel insights into these microbial communities. More recently-conducted surveys of this type have been completed with metagenomic approaches, the richness of data attesting to the superiority of NGS over classical methods (David et al. 2014). Such genomic methods for determining the grape and wine microbiome (reviewed by Morgan et al., 2017), have in fact led to an exponential increase in information on species abundance both before and during un-inoculated fermentation (Bokulich et al. 2016; David et al. 2014; Marzano et al. 2016; Piao et al. 2015; Portillo et al. 2016). For example, Pinto et al. (2015) used high-throughput sequencing to fully characterise both eukaryotic and prokaryotic communities in samples collected from six Portuguese wine regions and reported a clear relationship between the microbial community and fermentation stage. As expected the biodiversity decreased for both prokaryotic and eukaryotic communities as the selectivity of the environment increased with progression of fermentation. LAB were detected at low abundances and *O. oeni* was not detected. Among the LAB, high numbers of *Lactobacillus* (Lactobacillaceae), *Leuconostoc* (Leuconostocaceae), *Lactococcus* and *Streptococcus* (Streptococcaceae) were present. In a survey of over 200 commercial wine fermentations, the presence of multiple species of yeast and bacteria throughout the ferment was observed, although only reported as relative abundance (Bokulich et al. 2016). Therefore, as the researchers increase the use of genomics to study wine fermentations an opportunity arises from the deposition of raw data into publicly accessible databases to allow comparisons between studies.

Actual numbers aside, greater insights into bacteria in fermentations are being reported. Portillo et al. (2016) showed that acetic acid bacteria and LAB are more abundant than previously thought in a Grenache wine fermentation study, with similar results arising from low-sulfured or unsulfured wine fermentations (Bokulich et al. 2015). Additionally, NGS analysis has confirmed that bacteria not previously described in this context may also be present during the process (Godálová et al. 2016). Results such as the above are likely to vary depending on SO_2 additions. As expected, SO_2 additions affect microbial diversity in a dose-dependent manner, with 25 $mg.L^{-1}$ being cited by Bokulich et al. (2015) as the ideal concentration to achieve microbial stability when wine pH is sufficiently low. These other species may as yet be implicated in successful/unsuccessful fermentation outcomes and further study as

to their impact on both AF and MLF is needed. For example some *Lactobacillus* sp. have been implicated in causing stuck AF (Bokulich et al. 2016), and different strains of *O. oeni* have been shown to interact in either a negative or positive way depending on the strains tested (Brandam et al. 2016). Additionally Ramakrishnan et al. (2016) reported the ability of LAB strains to induce a metabolism-modifying prion [GAR+] in *S. cerevisiae*, hampering early yeast dominance in the fermentation and delaying the rapid depletion of amino acids by the yeast thereby enabling proliferation of bacteria present in the juice.

It is therefore possible that the inherent microbial diversity could lead to an increased risk of incomplete fermentation. Depending on the microbes present some may produce inhibitory compounds detrimental to other wine microbes (Bisson 1999; Fleet 2003; Knoll et al. 2008; Ultee et al. 2013). With the rise in popularity of indigenous (un-inoculated) fermentations the interactions of the wine microbial community as a whole is becoming more important and deserves to be studied in greater detail. More data on how microbes including *Saccharomyces* and non-*Saccharomyces* yeast and LAB interact with each other and (in the case of indigenous fermentations) other microbes will enable winemakers to develop winemaking practices (e.g. amount of SO_2 on grapes pre-ferment) that encourage selection of appropriate microbial communities. Further, there is room for improvement of current inoculation procedures to conduct successful fermentations.

Un-inoculated MLF *vs* inoculated MLF

MLF will often occur during the typical succession that transpires in most wine fermentations. However MLF by indigenous strains may be slow or incomplete and it is often more expedient to inoculate with an MLF starter culture. Multiple inoculation strategies can be used but there are two main ones, sequential and co-inoculation (reviewed in Sumby et al. 2014). In the context of MLF, sequential inoculation refers to the practice of allowing alcoholic fermentation (AF) to complete before addition of LAB to initiate MLF. Co-inoculation for MLF involves the addition of an LAB culture early in the primary fermentation, often 24-48 hours post-yeast inoculation (Figure 1). Where LAB starter cultures are used there are also a number of formulations and application methods. For example, starter cultures that have received proprietary treatment to prepare the cell membrane to cope with the

stresses found in wine can be added directly to the fermentation. Alternatively, a freeze-dried starter (e.g. *O. oeni*) is reactivated and adapted (with or without the addition of a specific activator) followed by acclimatisation to the wine. A third method involves propagation and adaptation in the winery, as is often done for low pH and acidic sparkling or white wines. In this case the bacteria are propagated under progressively increasing stress over several days. Regardless of the inoculation strategy chosen fermentations are a complex environment where yeast and LAB can interact either by cell-to-cell contact or by production of molecules that can influence the survival and activity of other cells in that environment. Study of these interactions is providing fascinating insights.

The traditional MLF management practice adopted by most wineries is sequential inoculation whereby LAB are inoculated after AF completion. However, more recent work indicates that co-inoculation is a viable option with multiple effects on wine composition (summarised by Sumby et al. 2014). Co-inoculation is also gaining in popularity because it can help secure and improve the MLF and reduce the risk of microbial spoilage by shortening the time between AF and MLF (Lasik-Kurdyś et al. 2017; Guzzon et al. 2013). For example the spoilage yeast *Brettanomyces bruxellensis* can be found at most stages of the fermentation but is particularly problematic post AF, during MLF and wine barrel aging. *Brettanomyces* produces organoleptically unpleasant volatile phenols. Periods of wine processing with low levels of SO_2, such as occur when seeking to encourage MLF, can favour the growth of *Brettanomyces*. More rapid completion of MLF so that protective levels of SO_2 can be established are therefore highly desirable. Alternatively, the isolation or development of LAB that can grow and function against a background of at least moderate amounts of SO_2 may represent a strain development opportunity worthy of pursuit.

Another opportunity exists around reducing/eliminating SO_2 additions during winemaking by identifying novel biological alternatives such as the potential of indigenous mixed cultures in the control of *B. bruxellensis* (Berbegal et al. 2017). Different strains of *S. cerevisiae*, non-*Saccharomyces* yeasts and *O. oeni* were co-inoculated under multiple strategies. With regards to the interaction between *S. cerevisiae* and *O. oeni* co-inoculated into spiked red must (*B. bruxellensis* added at 1 x 10^3 cfu.ml^{-1}) the results showed a decrease in 4-ethyl guaiacol and 4-ethyl phenol to below their sensory perception threshold at 21 days after commencement of AF

compared to fermentation with the *S. cerevisiae*-only control (Berbegal et al. 2017). Interestingly, the *B. bruxellensis* population was also reduced in cell density in the presence of *O. oeni*. What remains to be tested is whether this difference persists over time.

Optimal yeast-LAB combinations may differ for simultaneous *vs* sequential MLF (Muñoz et al. 2014; du Plessis et al. 2017). For example, although non-*Saccharomyces* yeast strains had a beneficial effect on the progress of a sequential MLF, during simultaneous inoculation some *Candida zemplinina* and *Lachancea thermotolerans* strains had a negative impact on LAB growth and MLF (du Plessis et al. 2017). There is as yet only limited information on how *O. oeni* competes in an indigenous fermentation with either non-*Saccharomyces* yeast or other LAB. Based on the ability of *O. oeni* to increase from being often undetectable levels to become the dominant species in wine, there are potentially some very interesting interactions occurring between *O. oeni* and the indigenous microflora throughout the winemaking process.

Enhancement of the MLF process

It is now well documented that there are a number of strain specific differences in response to the stressors found in wine. *O. oeni* strains have a compact genome of 1.8 Mb and several metabolic pathways related to growth in enological environments. Its genome size most likely reflects a high level of organisation and simplicity that may be the basis for its adaptation to the wine environment (Zé-Zé et al. 1998, 2000; Mills et al. 2005; Sternes and Borneman, 2016). Even so, there is still room for improvement of *O. oeni* isolates that conduct MLF.

Alternative strains

Bacterial species that can carry out MLF in wine

There are a number of LAB that have been utilised as MLF starters belonging to the species *O. oeni*, *Lactobacillus plantarum*, *Lb. hilgardii*, *Lb. brevis*, *Lb. casei* and *Pediococcus* sp (Table 1). Each have demonstrated different properties, with significant strain dependence in such characteristics. *O. oeni* has been the most utilised because of its ability to survive in the harsh wine conditions of high ethanol and low pH. But in the last decade increased research has been directed towards other

LAB species that could provide novel attributes to wine. For example, Bou and Krieger (2012) described the use of LAB strains of the genera *Lactobacillus* and *Pediococcus* that were capable of initiating and completing MLF after direct introduction, without a prior acclimatisation step.

Further analysis of indigenous strains able to complete MLF under regional- or varietal-specific conditions, has the potential to offer up new strains with increased genetic diversity and better adapted to local conditions. Indigenous LAB isolated at the end of MLF from 16 different Chilean wineries were shown to be genetically different from commercial strains and lacked genes conferring detrimental properties as well as genes encoding enzymes linked to aroma compounds (Romero et al. 2018). One strain in particular, 139, had several promising oenological properties including glucosidase activities (Romero et al. 2018).

Some new strains of *Lb. plantarum* are thought to have a greater sensory impact on wines since they can produce enzymes such as β-glucosidases, proteases, esterases and decarboxylases with potential benefits for wine composition (Matthews et al. 2004). It is anticipated that these activities will be reflected in the characteristics of the resulting wines and the fruity characters that are enhanced after MLF performed by these organisms. More recently, nine *Pediococcus* spp. isolated from commercial wines were studied for their impact on the chemistry, microbiology, and sensory quality of Pinot Noir wine. The strains studied demonstrated a range in production of the important flavour compound diacetyl, with some yielding concentrations above 12 $mg.L^{-1}$ and only one isolate producing measurable levels of the biogenic amine histamine (3.3 $mg.L^{-1}$). However, wine conditions may not have been optimal for biogenic amine production and this result will need to be tested in multiple wines to define any matrix-specific effect.

A number of isolates reduced colour in red wines (measured at 520_{nm}) by over 10% while polymeric pigment content declined by almost 30% in wines inoculated with one strain of *P. parvulus* (Strickland et al. 2016). Such impacts may be undesirable depending on the wine style sought i.e. rosé *vs* red wine. However, desirable sensory descriptors such as ‘floral’, ‘overall fruit’, and ‘red fruit’ were often higher in wines where *Pediococcus* sp. had grown compared to the control, indicating that growth of theses bacteria may not always result in spoiled wine (Strickland et al. 2016). Based on the limited information about these LAB it is clear that further

characterisation of *Pediococcus* species and strains and their enzymatic potential will help in understanding the impact that these bacteria may have on wine.

L-malic acid enzymatic pathways in LAB

It is possible that the presence of and expression of duplicate L-malic acid degradation pathways in LAB may affect their ability to efficiently conduct MLF. Most LAB can degrade L-malic acid (L-malate) to L-lactic acid (L-lactate) by a direct decarboxylation, which is catalysed by the malolactic enzyme (MLE). The MLE operon (Figure 2) consists of three genes encoding; MLE (*mleA* in *O. oeni; mleS* in *Lactobacillus* sp.), an L-malate transporter (*mleP*), and a LysR-type transcriptional regulator (*mleR*) that controls the transcription of both genes (Landete et al. 2013). A few LAB, however, can utilise the malic enzyme (ME) for L-malic acid degradation. ME converts L-malic acid into pyruvate that can be diverted to energy production via glycolysis or enter the gluconeogenic pathway (Figure 2). Therefore, ME enables growth with L-malic acid as a carbon source (Landete et al. 2010). Even though both pathways utilize the same substrate, the transcription of the corresponding genes is independently regulated (Landete et al. 2013). The genes involved in the ME pathway are arranged in two operons, *maePE* and *maeKR* (Figure 2). They encode a putative L-malic acid transporter (MaeP), an ME (MaeE), and a two-component signal transduction system (TCS, MaeK and MaeR), which has been studied in *Lb. casei* and *Lb. rhamnosus* (Landete et al. 2013; Miguel-Romero 2017; Monedero et al. 2017). In the study of Landete et al. (2013), the ME pathway in *Lb. casei* was related to higher growth rate by energy generation, while the function of the MleA pathway was reported to be solely deacidification. *O. oeni* contains putative ME (OEOE_RS02010, OEOE_RS002015) and TCS operons (OEOE_RS00545, OEOE_RS08540), but the function of these in *O. oeni* is not yet known. Sternes et al. (2017) observed that OEOE_RS02010 (*maeE*) was up-regulated in one (AWRIB419) out of three *O. oeni* strains tested (AWRB419, AWRIB551 and AWRIB552). AWRIB419 took 16 days to complete MLF whereas AWRIB551 and AWRIB552 took four and six days, respectively (Sternes et al. 2017). The efficiency of these pathways is yet to be fully compared and it may be that the MleA pathway has a higher rate of L-malic acid degradation. Whilst MleA has been studied well, ME has so far gained less attention. More information is required to delineate the different pathways of MLF, including the functions and influential factors of the ME pathway as well as a comparison

between ME and MleA. Further study of these pathways would improve both the knowledge and methods for inducing successful and controllable MLF and would provide clear targets for selection/development of new starter cultures such as those with higher MleA activity.

Non-Saccharomyces *yeasts*

Saccharomyces cerevisiae has long been recognised as a poor metaboliser of extracellular malic acid, due to a lack of a mediated transport system, low substrate affinity for L-malic acid and the mitochondrial location of the malic enzyme MAE1, which catalyses the oxidative decarboxylation of malate to pyruvate (Boles et al. 1998). However, several non-*Saccharomyces* species are capable of metabolising the L-malic acid found in wine by converting it into ethanol through malo-ethanolic deacidification. They could therefore be an alternative to traditional MLF. This approach may cause a small increase in final ethanol content, but this may be acceptable given the aroma and flavor of lactic acid produced by LAB. It has long been known that yeasts like *Schizosaccharomyces pombe* have cytosol-located malic enzyme and are especially efficient at converting L-malic acid (Volschenk et al. 2003). More recently this knowledge has been the basis for evaluating the feasibility of using other non-*Saccharomyces* yeast for 'MLF' in wine making. When *Schizosaccharomyces pombe* and *Lachancea thermotolerans* were used in combination in Tempranillo wine, *S. pombe* was able to completely metabolise L-malic acid while *L. thermotolerans* produced lactic acid to increase the acidity in otherwise low acidity musts (Benito et al. 2015). These wines were compared with control wines that had undergone classical MLF. The former were reported to be fruitier and contain less acetic acid and biogenic amines. However, most non-*Saccharomyces* yeasts have poor ethanol tolerance and often must be used in conjunction with *Saccharomyces* yeasts to complete wine fermentation, thereby casting doubt over their ability to effectively remove all malic acid. The sensory effect of using non-*Saccharomyces* yeasts instead of LAB to conduct MLF is also unclear and needs to be studied further.

Improved LAB strains

The improvement of LAB strains for more reliable fermentation was described in some detail in a previous review (Sumby et al. 2014) and as such only a short summary (Table 2) and new research will be described here.

There are many methods to improve wine bacterial strains for more reliable MLF. Amongst these is the preferred non-GMO method of directed evolution (DE). In the first study reporting the use of DE to improve *O. oeni*, Betteridge et al. (2018) conducted continuous culture of commercial strain SB3 in the presence of increasing

ethanol to yield a more ethanol tolerant isolate, A90. Although this strain had superior fermentation performance in laboratory MRSAJ (de Man, Rogosa and Sharpe medium + apple juice) medium, the ethanol resistant phenotype was not carried over when inoculated in the complex, multi-stressor environment of Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM; Jiang et al. 2018). This was true at both low inoculation rates and those analogous to recommendations to inoculate high ethanol wines, suggesting that A90 had evolved to a particular niche and may have limited abilities outside of this.

In a follow-up study (Jiang et al. 2018) continuous culture was used to evolve strain A90 in a multi-stress environment resembling wine. Accordingly the strain was grown for ~350 generations in RFCDGJM before increasing the proportion of red wine mixed with RFCDGJM thereby raising the levels of stressors (e.g. low pH, ethanol, and SO_2). Three strains were selected through the course of the DE experiment based on their ability to consume more L-malic acid than the parent strain (which became stuck) when evaluated in a RFCDGJM/wine blend with 15.1% (v/v) ethanol, 26 $mg.L^{-1}$ SO_2 at pH 3.35. Further assessment of these selected strains in four different red wines (pH values of 3.37-3.55; ethanol 13.9-16.7 % (v/v)) revealed they also fermented faster and/or achieved a greater population than the parent. In this way the effectiveness of using DE to improve *O. oeni* performance and reliability under winemaking conditions was convincingly demonstrated.

Whilst EMS or UV mutagenesis were not used in the previous study, the DE approach could possibly be enhanced further by pre-stressing strains and applying mutagens before initiation of DE. Still further opportunities to improve on DE methods arise by applying what is known about the cross-stress behaviour and DNA repair in LAB. Accordingly, Machielsen et al. (2010) demonstrated that even though mutation frequency was unaffected in *Lb. plantarum* during high temperature, low pH, osmotic, or starvation stress, it was increased by a factor of 100 after exposure to sub-lethal levels of H_2O_2. Interestingly, preadaptation at 42 °C, a non-mutagenic condition, reduced the mutagenic effect of oxidative stress (Machielsen et al. 2010). Evidence of cross-stress behaviour has been well documented throughout the microbial kingdom. Early work on glucose- and nitrogen-starved *Escherichia coli* cells showed increased survival rates after heat shock or hydrogen peroxide (H_2O_2) mediated stress compared with non-stressed cells (Jenkins et al. 1988). Other studies have used DE of LAB primarily to study the biology of these organisms. Genetic

analysis of changes arising in *Lb. rhamnosus* strain GG following 1,000 generations of growth in a rich medium under four different conditions (stress free, salt stress, bile stress, shear stress) showed that mutation rates were low under all conditions (Douillard et al. 2016). Deletion events, however, mediated by activation of IS-elements arose during bile and shear stress (Douillard et al. 2016).

It would also be of fundamental interest to investigate why *O. oeni* improves so rapidly during DE with few detrimental phenotypes developing in the DE process i.e. is it more than just the absence of the DNA repair mechanism MutS/MutL? In a study investigating *mutS* mutants Overbeck et al. (2017) improved the DE of *Lb. casei* to increase lactic acid resistance at low pH, through the deliberate use of mutator cells. This was achieved by transient inactivation of DNA mismatch repair gene *mutS* in *Lb. casei*. A two-step gene replacement method was used to delete *mutS* before strains were subjected to a 100-day DE process to increase lactic acid resistance at low pH (Overbeck et al. 2017). Genome sequencing confirmed that inactivation of *mutS* decreased DNA replication fidelity during DE and thereby allowed mutants to arise that grew better and produced more lactic acid at low pH than with wild-type cells undergoing the same DE process. However, some caution is warranted as hypermutation of the *mutS* mutants could potentially affect other genes associated with replication fidelity and prevent the restoration of a stable genotype.

Regardless of the improvement strategy used, it is also necessary to improve methods of selecting for improved MLF strains. As mentioned, it is difficult to culture environmental samples of *O. oeni* thus selection strategies tend to target wines undergoing MLF. Both Betteridge et al. (2018) and Jiang et al. (2018) used 96-well microplate (300 μL) screening methods to highlight their improved isolates in the medium used for DE. This approach is very useful when dealing with hundreds of DE isolates. However, results do not always translate to the larger scale and methods that utilise larger screening volumes, such as the automated 96x 100 ml-flask fermentation platform described by Peter et al. (2018) appear to deliver results more representative of those at the larger, pilot scale.

Enhancement of sensory outcomes through LAB metabolite production

Although acid reduction is the principle sensory effect of LAB during MLF (Volschenk et al. 2006), Davies et al. (1985, 1988) showed that LAB strains have a specific sensory effect on wine. It is now clear that during MLF, LAB can produce or release several compounds that can have either a positive or negative effect on the sensorial properties of the wine, depending on the nature of these compounds, their concentration, and the physical-chemical properties of the wine. MLF can be used to modulate sensory attributes such as buttery aroma (diacetyl), fruity aroma, mouth-feel and colour (Swiegers et al. 2005). The underlying mechanisms involve the production of enzymatic activities that can have both aroma and/or visual effects. For example, anthocyanin pigments may be impacted by glycosidases (Vivas et al 1997; Grimaldi 2005a; Burns and Osborne 2013, 2015). Aroma compounds that can increase or decrease during MLF include esters (Antalick et al. 2012; Pozo-Bayón et al. 2005; Sumby et al. 2010, 2013a), aldehydes (Osborne et al. 2000), alcohols (e.g. 2-phenylethanol), aglycones (including terpenols and C13-norisoprenoids) from their glycosylated precursor (Boido et al. 2002), and 2,3-butanedione (diacetyl) (Nielsen and Richelieu 1999; Bartowsky and Henschke 2004). There are conflicting results about the ability of LAB to synthesise higher alcohols in amounts that could be sensorially significant. Certainly Ugliano and Moio (2005) reported negligible effect of *O. oeni* on higher alcohols when four different strains were tested. Beyond strain choice, it appears the sensory impact of MLF can also be influenced by the timing of MLF inoculation (i.e. sequential *vs* co-inoculation), pre-MLF wine matrix conditions (e.g. pH) or nutrient supplementation (Abrahamse and Bartowsky 2012; Costello et al. 2012; Knoll et al. 2012; Maarman 2014). There is also some question as to the effect that inoculation rate may have on the final sensory outcome. The latter needs further investigation as to whether over-inoculation reduces the effect of strain specific differences, i.e. if strains are over-inoculated do they grow less, produce fewer metabolites and largely conduct MLF, thereby having little effect on the aroma profile? (Sumby et al. 2018). If this is the case this approach may be preferred by winemakers seek a reduction in L-malic acid with less of a sensory effect.

LAB strains therefore have enormous potential to change the volatile composition and the aroma profile of the wine, however, there is still debate over the impact of specific strains (Cappello et al. 2017; Sumby et al. 2018). For a recent review on the variability of bacterial enzymes, including glycosidases, esterases, proteases and other enzymes that can generate a wide spectrum of sensorially

significant compounds in wine refer to Cappello et al. (2017). Gámbaro et al. (2001) reported sensory differences between MLF and no-MLF in different grape clones of Tannat wine, but since only one strain was tested, strain specific differences could not be evaluated. Costello et al. (2012) found variation between strains in overall fruit flavour in Cabernet Sauvignon wines, but a large variation within the replicates requires that this effect is investigated further. As another consideration, Gammacurta et al. (2017) reported that although significant, the impact of bacteria on wine aroma was lower than the impact of yeast.

Diacetyl

A major compound produced during MLF in wine is diacetyl, which is described as having a buttery or butterscotch aroma and flavour. Diacetyl is formed through the metabolism of citric acid with the first step catalysed by citrate lyase (*citE*), which cleaves citrate into acetate and oxaloacetate. Oxaloacetate is then decarboxylated to pyruvate, leading on to the formation of diacetyl. The organoleptic impact of diacetyl in wine has been debated for many years. At low concentrations it is considered to contribute to the wine's bouquet, while higher concentrations have a negative impact. For these reasons *citE* has been investigated in various wine LAB species (Mills et al. 2005).

The role of strain is regarded as important for the initial yield of diacetyl, but once produced diacetyl can be enzymatically reduced to 2,3-butanediol. Thus co-inoculated MLF would be expected to produce wines with ultimately less diacetyl since yeast can metabolise diacetyl to acetoin and 2,3-butandiol (Mink et al. 2013). In addition, a faster MLF tends to result in lower diacetyl concentrations (Sternes et al 2017). Lactobacilli associated with winemaking have been investigated for their potential to utilise citrate, however, it has been demonstrated that not all lactobacilli have all three genes necessary for citrate metabolism and there is question about whether the citrate metabolism pathway is active in these species. (Mtshali et al. 2010, 2012).

The sensory perception of diacetyl is dependent not only on concentration (high concentrations will be overtly buttery and undesirable) but also on the presence of other wine compounds. For example butteriness can be masked by strong oak or very fruity characters (Martineau et al. 1995; Bartowsky et al. 2004). Post-MLF processing can also affect the final diacetyl content and influence the sensory profile

of the wine. Aging on lees can degrade the diacetyl, and bâtonnage (resuspending of yeast lees) can reduce or even eliminate the buttery aroma. If the wine is in contact with oxygen, acetolactate will be oxidised into diacetyl. Nielsen and Richelieu (1999) showed that the reduction of diacetyl into acetoin and 2,3-butanediol depends on the redox potential of the wine and the accumulation of diacetyl in a semi-aerobic environment could be six-times higher than in a completely anaerobic environment.

Acetaldehyde

Acetaldehyde can be consumed by LAB during MLF, thereby limiting its conjugation with anthocyanins in wine and in turn, colour stabilisation (Liu and Pilone 2000; Osborne et al. 2000). As a result, a loss of colour after MLF is one potentiality (Burns and Osborne 2015). Conversely some LAB have the ability to produce acetaldehyde (Liu and Pilone 2000), which might be expected to benefit wine colour, although this does not seem to have been specifically examined. More recently Wang and collegaues (2018) reported that *O. oeni* strains tested in model wine increased the level of acetaldehyde in at the beginning of MLF, but that a dramatic decrease of acetaldehyde was observed after 4 days of MLF. However, when the model wine medium contained *Lb. plantarum*, a continuous accumulation of acetaldehyde was observed throughout MLF with the final acetaldehyde concentration 2–3 times higher than that in the medium with *O. oeni* alone. Both tested *Lb. plantarum* strains, however, did not fully complete MLF and produced large amounts of lactic acid. This effect and possibly the effect of co-inoculation (*O. oeni* and *Lb. plantarum* together) on acetaldehyde levels could be explored further.

Esters

A comprehensive review on the reported changes in ester concentration during wine fermentation, including changes during MLF appeared in 2010 (Sumby et al. 2010). Whilst the berry fruit aroma characteristics of red wine vary according to grape variety and winemaking, the fruit aroma of red wine is a complex interaction between fruity esters, norisoprenoids, dimethyl sulfide, ethanol and other components. Strains of *O. oeni* have been shown to vary in their ability to generate or release these compounds.

Recent research has focused on characterising the enzymes that are responsible for ester synthesis and hydrolysis during MLF (Costello et al. 2013;

Esteban-Torres et al. 2014; Sumby et al. 2009, 2013a, 2013b). This group characterised intracellular esterases from *O. oeni* and *Lactobacillus hilgardii* under wine-like conditions (Sumby et al. (2009; 2013a; 2013b). Two heterologously expressed and purified esterases were stable and active under conditions that would be encountered in wine and therefore offer the potential to reduce short-chain ethyl esters such as ethyl acetate. Following on from this, *O. oeni* strain AWRI B551 was shown to produce significant levels of ethyl hexanoate and ethyl octanoate following growth in an ethanolic test medium (Costello et al. 2013). Cell free extracts of LAB strains were also successfully tested for ester synthesizing capabilities in a phosphate buffer albeit with strain-dependent variation (Costello et al. 2013).

Characterisation of esterase enzymes from *Lb. plantarum* strain Lp_1002 defined the first arylesterase from a wine LAB under wine-like conditions (Esteban-Torres et al. 2014). The enzyme was reported able to withstand ethanol, sodium metabisulfite, and tartaric, lactic and citric acids, with only malic acid slightly inhibiting activity. Whilst it is generally accepted that MLF has a significant influence on the ester composition of wines, there is as yet no consensus on the effect of individual bacterial strains on ester concentration and whether the strain specific effects that can be seen via GC/MS analysis translate to sensorially significant differences in the same wines. For example, after MLF using two different commercial *O. oeni* starters compared with un-inoculated MLF, the branched hydroxylated esters, ethyl 2-hydroxy-3-methylbutanoate and ethyl 2-hydroxy-4-methylpentanoate were the only compounds to be influenced by strain choice (Gammacurta et al. 2018) and no LAB effect was detected irrespective of the vineyard or the vintage considered.

Clearly more research is necessary to determine if sensory differences, related to ester concentrations, reported between strains are due to true strain differences or if there are other causes such as a matrix effect. It's clear that oenological parameters such as pH, ethanol and SO_2 (Knoll et al. 2011a) affect ester metabolism by LAB but further research is called for.

Phenolics

The presence of volatile phenols has been related to the action of *Brettanomyces* yeast particularly in relation to winemaking practices involving wine aging in oak barrels. Their presence is considered detrimental for wine aroma and

flavor. Volatile phenols, including 4-ethyl guaiacol and 4-ethyl phenol, may also increase after MLF because some *Lactobacillus* species are able to produce low concentrations of these compounds relative to *Brettanomyces* yeast (Brizuela et al. 2018; Couto et al. 2006). A greater understanding of this metabolic pathway is needed and a screening of strains for their production propensity should be conducted.

Volatile sulfur compounds

Volatile sulfur compound (VSC) production by wine-related LAB is still not well documented despite there being numerous reports of this from the dairy industry (e.g. Curioni et al. 2002; Al-Attabi et al. 2008). VSCs can contribute positively to wine aroma, but some compounds are considered detrimental to wine quality, depending on their concentration. As an example, the metabolism of the sulfur-containing amino acid, methionine, has an impact on wine aroma and has been studied for the production of VSCs (Pripis-Nicolau et al. 2004; Vallet et al. 2008). Both *Lactobacillus* sp. and *O. oeni* can metabolise methionine to form methanethiol and dimethyl disulfide (Pripis-Nicolau et al. 2004; Antalick et al. 2012). However, 3-(methylsulfanyl)propan-1-ol and 3-(methylsulfanyl) propionic acid were formed in more significant quantities by *O. oeni* than *Lactobacillus* (Pripis-Nicolau et al. 2004). In water, 3-(methylsulfanyl) propionic acid descriptors are chocolate and roasted, but these are not observed in wine with increasing amounts of this compound, possibly due to interactions/masking by other compounds present in the wine (Pripis-Nicolau et al. 2004). The cloning and characterisation of an *O. oeni* enzyme able to degrade sulfur-containing amino acids has been reported (Knoll et al. 2011b). Amongst other activities the ability to demethiolate methionine to methanethiol, an unpleasant VSC in terms of wine aroma, was observed. Even though the activity of the enzyme was low under wine conditions a fuller characterisation is required.

Another sulfur compound, 3-sulfanylhexan-1-ol (3SH), needs to be considered due to its important contribution to fruity notes of wine. Although 3SH has been reported not to change significantly during MLF with *O. oeni* (Antalick et al. 2012) it may be produced by other LAB species. Takase and group (2018) tested for 3SH production amongst seven LAB strains (including one *O. oeni* strain) and reported that *Lb. plantarum* strains could catalyse the transformation of 3SH-S-conjugates to produce 3SH through a single cleavage step and/or multiple cleavage steps via 3SH-S-cys. Such findings were confirmed in fermented grape juice, however, the enzyme

catalysing this transformation was not identified and further genetic and enzymatic studies are needed.

Yeast:bacteria interactions

The aroma of wine is derived from the combined influence of the microorganisms present during both inoculated and un-inoculated fermentations. Understanding these microbial interactions is essential to understanding the impact of microorganisms on wine. In the case of MLF, cross-kingdom interactions between bacteria and yeast have been reported (Jarosz et al. 2014; Ramakrishnan et al. 2016). However, the full impact of these mechanisms on wine fermentation kinetics and sensory outcomes remains ill-defined. What is known is that the timing of MLF induction can have a significant effect on the chemical and sensory properties of wine and in fact a greater effect on the sensory properties than the yeast treatment alone (du Plessis et al. 2017).

The optimal MLF strategy for each yeast strain or yeast combination to improve wine flavour and quality appears to be strain dependent, with variation in wine composition not always amounting to perceivable sensory differences. MLF co-inoculations often lead to modifications of both volatile and sensory outcomes when compared to sequential inoculations (Cañas et al. 2012; Delaquis et al. 2000; du Plessis et al. 2017; Versari et al. 2016). du Plessis et al. (2017) reported that wine flavour profiles could be modified depending on the non-*Saccharomyces* yeast strains and MLF strategies used. Wines that underwent simultaneous MLF scored higher for certain sensory descriptors than wines that underwent sequential MLF, but some yeast combinations yielded better wines with sequential MLF (du Plessis et. al. 2017).

Concentrations of many volatile compounds including esters, alcohols and fatty acids can be modified by wine LAB, therefore altering the overall sensory attributes of wines. However, the extent of these modifications depends on the combination of yeast and LAB used, the timing of LAB inoculation and the grape cultivar (Maicas et al. 1999; Delaquis et al. 2000, Ugliano and Moio 2005). Thus despite many studies revealing the importance of yeast-LAB combinations on sensory outcomes for wines, in-depth knowledge offering predictability of sensory outcomes based on yeast, LAB and grape variety selections is lacking. The interactions between *Saccharomyces*, non-*Saccharomyces* and LAB are complex and will take time to be delineated.

Future possibilities: Using MLF to influence wine style

Through ongoing research, there is now a better understanding of how MLF could be used to influence wine style. The choice of LAB strain, as well as timing of bacterial inoculation, make it possible to modulate MLF sensory influence in wine. Future work should likely focus on diversity profiling and the sensory differences between uninoculated and inoculated MLF at different inoculation rates, by way of metabolomics (Bokulich et al. 2016; Lee et al. 2009) and genomics (Bartowsky and Borneman 2011; Sternes et al 2017).

Future work may also include evaluation of the effect of strain origin. For example, Campbell-Sills et al. (2017b) sequenced 14 isolates from red and white wine and determined that that they share a common ancestor that probably colonised two different substrates. Testing the capacity of the sequenced strains to perform MLF and modify the volatile profile of a Chardonnay wine using a non-targeted metabolomics approach was undertaken. The volatile composition varied between the strains and was dependent on their group of origin. Other authors have also reported that there appears to be two groupings of *O. oeni* strains (Sternes and Borneman 2016; Zé-Zé et al. 2008). El Khoury et al. (2017) reported that strains could be grouped according to the beverage they were isolated from, and there was no correlation with geographical origin. This has interesting implications for the concept of terroir.

Conclusion

Although we have come a long way in recent years toward our understanding of MLF and how best to undertake it when desirable, much remains unknown. It is clear that the influx of ‘omics data over the next few years as this type of analysis becomes more commonplace will be massive. It is expected that the study of interactions between microbes in both inoculated and uninoculated ferments will uncover a range of new information on how best to control both AF and MLF. We now know how to conduct MLF within a range of conditions. But what can we do when we have problematic ferments, and how can we predict these? It would appear that it is first necessary to obtain a better understanding of the response of *O. oeni* cells to ethanol and to investigate the presumably synergistic effect of wine stressors and why some cells are more affected than others.

Potential future research topics may include;

- Isolation of improved strains suited to a region's unique climate. We know that there are differences in *O. oeni* isolated from white *vs* red wine. Thus are there also differences amongst the grape varieties? Is there a 'super strain' suited all conditions/varieties awaiting discovery?
- How best to encourage indigenous strains. Research on these potentially fastidious organisms is likely to benefit from a use of natural media such as grape must and wines rather than synthetic or defined laboratory media.
- Alternate strategies to improve LAB other than *Oenococcus*. Improvement of strains by using DE has been very successful in *O. oeni*. If this is due to their ineffective DNA repair systems, it is likely that other strategies e.g. mutagenesis will be needed for other LAB (e.g. UV or EMS mutagenesis followed by screening in a competitive environment).
- Investigation of methods to improve the expression of *mleA*. How important is repression by ethanol alone or in a multi-stressor environment?
- Further development of transformation systems for *O. oeni*. It is not yet possible to routinely modify gene presence and expression in *O. oeni*. The ability to do so will help to rapidly delineate metabolic pathways and stress-response systems.
- Future possibilities with the potential to eliminate additives or processing steps during winemaking;
    - Bacteriocin producing strains to increase their competitiveness over indigenous, potentially unwanted LAB.
    - Alternatives to SO_2, e.g. addition of prophage to selectively inhibit growth of unwanted microorganisms during fermentation (Bondy-Denomy et al. 2016).

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Figure 1: Inoculation strategies and an amalgamation of the growth of the two main LAB species present during wine fermentation (un-inoculated). Data taken from Bokulich et al. (2016); Davis (1986); Marzano et al. (2016); Ong et al. (2010); Wibowo et al. (1985). Other LAB species would be expected to reach a higher cell number if the pH is above 3.5 (Davis 1986). Chains of purple cocci - *Oenococcus*; Chains of pink rods – *Lactobacillus*.

Figure 2: Representation of genes and characterised pathways involved in metabolism of L-malic acid in LAB. (**A** & **B**) Schematic representation of the genetic organization of (**A**) *mleA*/*mleS* and (**B**) *mae* orthologous group gene clusters present in LAB (NCBI (https://www.ncbi.nlm.nih.gov/); Landete et al. 2013; Miguel-Romero 2017; Monedero et al. 2017). (**C**) Characterised pathways in LAB (Landete et al., 2010; Landete et al., 2013). Import of malic acid by *O. oeni* occurs through the malic acid transporter MleP, whereby L-malic acid is subsequently decarboxylated by maltate decarboxylase MleA producing L-lactic acid and CO_2. MleA also requires NAD^+ and manganese ions as co-factors. *Lactobacillus* also has malic acid transporters: MaeP & MleP. In contrast to *O. oeni*, *Lactobacillus* sp. has two pathways where malic acid may be utilised. Similar to *O. oeni*, *Lactobacillus* sp. has a malate decarboxylase MleS that is able to decarboxylate malic acid in the same way O. oeni does. The alternative pathway in Lactobacillus involves conversion of malic acid to pyruvate and CO_2 by malate dehydrogenase MaeE, however this can be repressed by glucose. When MaeE is not repressed, the pyruvate produced can be utilised for growth and other cellular processes, or as displayed here, converted to lactic acid by lactate dehydrogenase.

Table 1: LAB that have been utilised as MLF starters, both industrially and experimentally.

Species	Application	Positives	Potential negatives
O. oeni	• Co-inoculation, sequential inoculation and un-inoculated fermentation	• Co-inoculation may reduce BA* formation[1] • Generally the most reliable species over a wide range of conditions • May reduce undesirable metabolites in high acid must[2]	• Possible BA production - strain dependent[3,4] • Possible acetic acid production from glucose metabolism during co-inoculation due to heterofermentation[5] • Decrease in stable polymeric pigments[6]
Lactobacillus plantarum	• Pre-AF[7], co-inoculation[8,9,10], and un-inoculated fermentation [11]	• BA degradation ability[12] • Some strains are homofermentative (cannot synthesise acetic acid from hexose) • More diverse enzymatic profile[13] • Enhancement of floral notes in white wine at pH higher than 3.8[3]	• Increased risks of stuck or sluggish AF[14] • Possible BA production - strain dependent[3] • Possible volatile phenol production[15]
Lactobacillus sp. (other)	• Pre-AF[7], co-inoculation[7] and un-inoculated fermentation [11,16]	• Yet to be fully determined, but may add to the overall complexity of the wine.	• Possible BA production (strain dependent) • Stuck MLF[19#] • 'Bitterness' due to the production of acrolein[20,21]
Pediococcus sp.	• Un-inoculated fermentation • Only experimentally from un-inoculated ferment, pH 3.7[11,16], for co-inoculation[17] and sequential inoculation[18]	• Yet to be fully determined, but may add to the overall complexity of the wine. • Potential bacteriocin production (only useful if not directed against *O. oeni*)	• Ethyl carbamate, stuck MLF. Spoilage in low acid high pH wines. • Excessive synthesis of diacetyl[11,18] • Possible BA production (not all strains)[22] • β-glucan formation ('ropy' wine) • Loss of colour[18] • Synthesis of vinyl phenols and possible stimulation of *B. bruxellensis* growth[18]

* BA, biogenic amine; # tentatively identified as *Lactobacillus* sp. later identified as *Lb. kunkeei* (Edwards et al. 2002). [1]Smit 2012; [2]Lasik-Kurdyś et al. 2017; [3]Cinquanta et al. 2018; [4]Lucas et al. 2008; [5]Guzzon et al. 2013; [6]Burns & Osborne 2013; [7]Lucio et al. 2017; [8]du Toit et. al. 2011; [9]Iorizzo et al. 2016; [10]Lerena et al. 2016; [11]Davis et al. 1986; [12]Capozzi et al. 2012; [13]Matthews et al. 2004; [14]Liu et al. 2017b;[15]de las Rivas 2009; [16]Mesas et al. 2011; [17]Juega et al. 2014; [18]Strickland et al. 2016; [19]Huang et al. 1996; [20]Pasteris and Strasser de Saad 2009; [21]Bauer et al. 2010; [22]Landete et al. 2007.

Table 2: Methods that could be used to improve MLF strains.

Approach	Advantages	Disadvantages
Field selection	• Easy to identify and isolate microbes showing specific and desirable traits	• Cannot improve microbial characteristics beyond what is found in the originating environmental population
Random mutagenesis	• Easy to apply • Knowledge of targeted genetic pathway not required	• Relies on an efficient screening method to identify improved mutants • Multiple mutations possible (positive and negative phenotypes may be produced)
Recombinant techniques: Traditional and CRISPR/cas9	• Potential for substantial improvement for specific traits	• Substantial knowledge of targeted genetic pathways is required • Has not been implemented in all potential target organisms (e.g. *O. oeni*) • Regulatory uncertainty (CRISPR/cas9) • Potential risks and ethical debates linked with the use of GMOs
Directed evolution	• Non recombinant DNA manipulation • High potential of improvement	• Can be difficult to establish appropriate selective conditions to produce desired phenotype • Can require several months cultivation before producing desired mutants (i.e. long timeframes) • Complex phenotypes and multiple genetic changes likely

Figure 1

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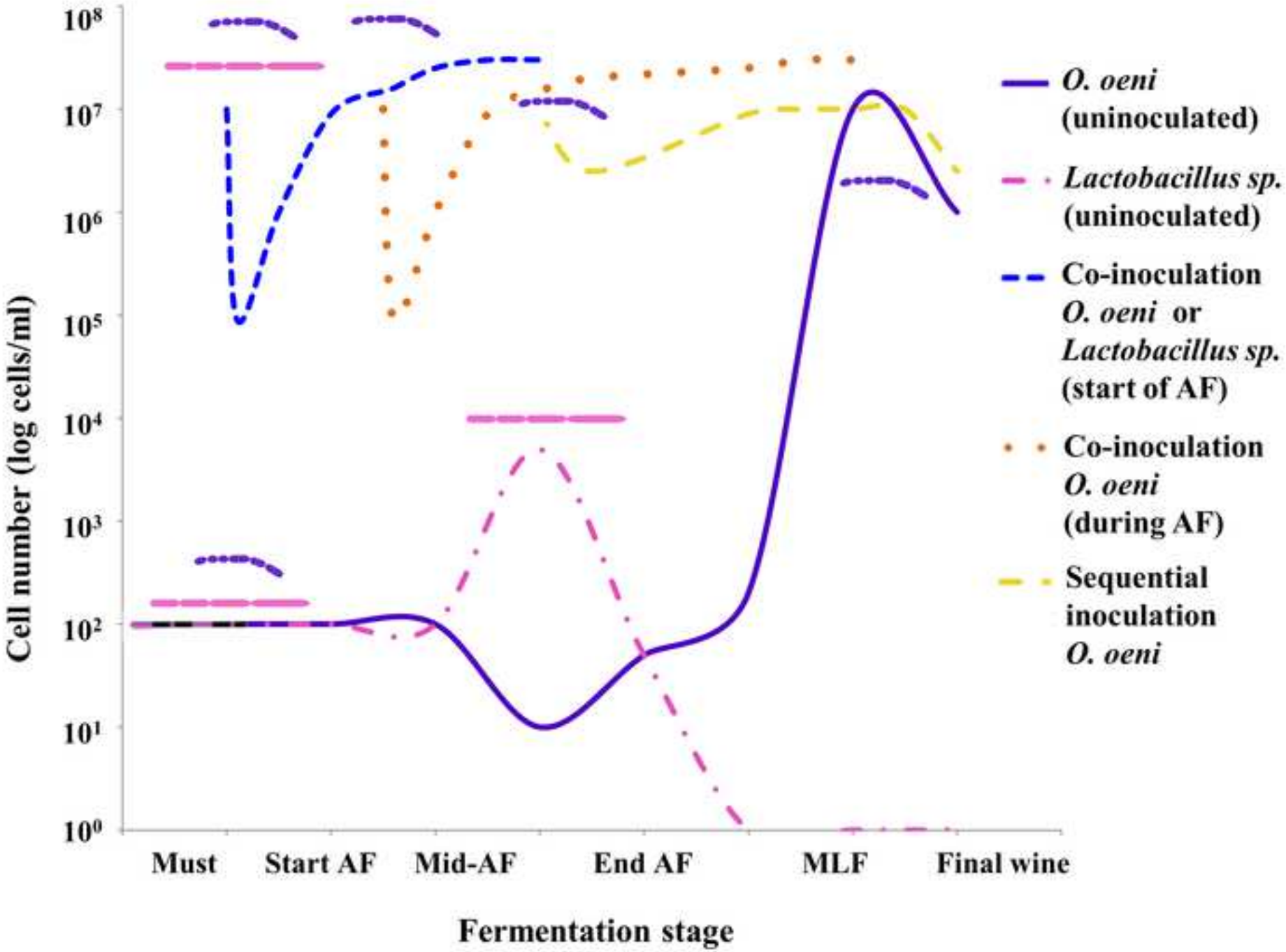

Figure 2A&B

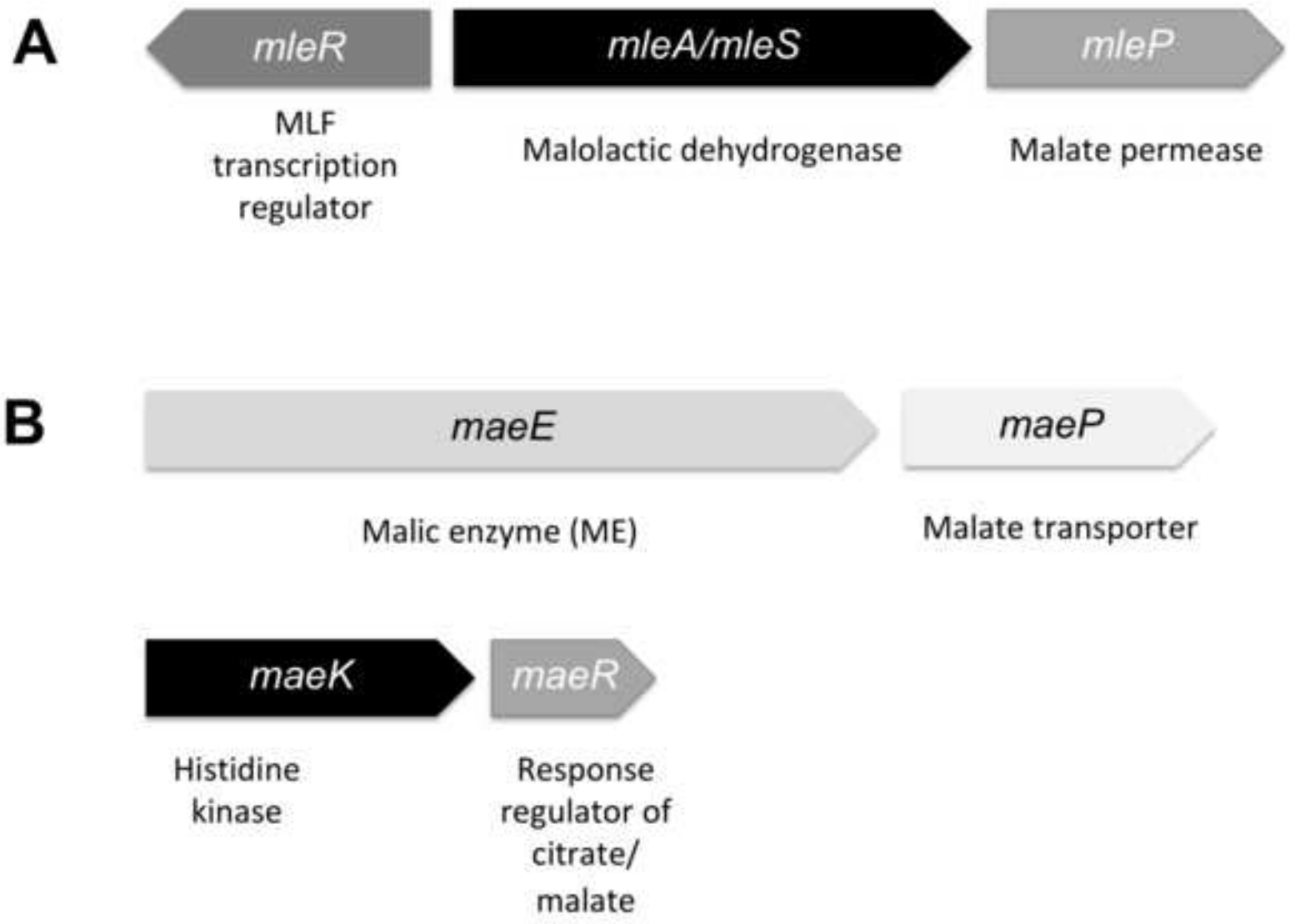

Figure 2C

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