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MINI-REVIEW

Implications of new research and technologies for malolactic fermentation in wine

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Abstract The initial conversion of grape must to wine is an alcoholic fermentation (AF) largely carried out by one or more strains of yeast, typically *Saccharomyces cerevisiae*. After the AF, a secondary or malolactic fermentation (MLF) which is carried out by lactic acid bacteria (LAB) is often undertaken. The MLF involves the bioconversion of malic acid to lactic acid and carbon dioxide. The ability to metabolise L-malic acid is strain specific, and both individual *Oenococcus oeni* strains and other LAB strains vary in their ability to efficiently carry out MLF. Aside from impacts on acidity, LAB can also metabolise other precursors present in wine during fermentation and, therefore, alter the chemical composition of the wine resulting in an increased complexity of wine aroma and flavour. Recent research has focused on three main areas: enzymatic changes during MLF, safety of the final product and mechanisms of stress resistance. This review summarises the latest research and technological advances in the rapidly evolving study of MLF and investigates the directions that future research may take.

Keywords *Oenococcus oeni* · *Lactobacillus* · Malolactic fermentation · Wine

Introduction

Wine is a complex mixture of hundreds of compounds, many of which contribute substantially to the colour, mouthfeel or aromatic properties of this beverage. Many variables affect the characteristic aroma of wine including the grape variety, viticultural and winemaking practices along with wine maturation and storage conditions. The initial conversion of grape must to wine is an alcoholic fermentation (AF) largely carried out by one or more strains of yeast, typically *Saccharomyces cerevisiae*. After the AF, a secondary or malolactic fermentation (MLF) is often undertaken, depending on the style of wine that is being produced. MLF is carried out by lactic acid bacteria (LAB), most commonly *Oenococcus oeni* (Carr et al. 2002), which is acidophilic and indigenous to wine and similar environments and is generally thought to be best suited to the harsh environment of wine. *Lactobacillus* spp. and *Pediococcus* spp. can also carry out MLF, but not always to completion (Renouf et al. 2008). However, recent red wine trials have shown that strains of *Lactobacillus plantarum* have the potential to conduct an efficient MLF and also produce desirable sensory attributes in red wines (Lerm et al. 2011; Bravo-Ferrada et al. 2013).

The MLF involves the bioconversion of malic acid to lactic acid and carbon dioxide and improves the biological stability of wine by preventing the utilisation of malic acid by other microorganisms in bottled wine (Davis et al. 1985). LAB do not use the Krebs cycle and a terminal electron transport system for metabolism and energy generation but instead obtain their energy by carbohydrate fermentation coupled to substrate level phosphorylation. *O. oeni* is an obligate heterofermentative bacterium (Kandler 1983; Papagianni 2012). The ability to metabolise L-malic acid is strain specific, and both individual *O. oeni* strains and other LAB strains vary in their ability to efficiently carry out MLF (Sieiro et al. 1990; Vailiant et al. 1995; Zapparoli et al. 2004). Aside from impacts on acidity, LAB can also metabolise other precursors present in wine during fermentation and, therefore, alter the chemical composition of the wine resulting in an increased complexity of wine aroma and flavour. The best described example is the compound diacetyl; however, the production of esters, alcohols and other carbonyl compounds results in buttery, fruity, spicy, vanilla and smoky notes, as well as a softer mouthfeel

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(Swiegers et al. 2005; Malherbe et al. 2012). As a consequence of this, strains selected for commercial use are usually chosen for their ability to both efficiently metabolise malic acid and to confer desirable sensory properties on the wine. Different strains, both in nature and available commercially, show various properties in terms of sensory compounds formed (Malherbe et al. 2012). This has led to a great number of studies in recent years attempting to unravel the complex interaction between LAB and the wine environment with a view to developing superior MLF starter cultures and obtaining more tailored and consistent outcomes.

MLF can be one of the most problematic processes that a winemaker has to manage, but is nonetheless considered a vital process in the production of many wines, impacting on safety, quality, microbial stability and operational efficiency. Being conducted in almost all red wines, some white wines and in sparkling wine bases, it is a process of great significance. For example, red wine varieties made up 52 % of the 2013 production in Australia with a recorded crush size of 945,586 tonnes (http://www.wfa.org.au/resources/1/Reports/WFA_Vintage_Report_2013.pdf) and 57 % of the 4 million tonnes crushed in California in 2012 (CDFA and California Agricultural Statistics 2013). An understanding of MLF and how to improve it are therefore of great importance to the wine industry. The increase in studies in this area over the past few years indicates that the changes occurring in wine during the MLF are more complex than first thought. Our recent unpublished survey of Australian winemakers revealed that most were concerned with the ability of a LAB strain to rapidly conduct MLF (24 %) and its ability to ferment efficiently in the presence of high ethanol concentrations (20 %). Tolerance to the normal stresses found in wine, such as low pH and high sulfur dioxide (SO_2) concentrations, was the next key concern (16 %). The specific attributes of selected LAB strains deemed to be the most important were low acetic acid production (27 %), enhancement of wine mouthfeel (26 %) and not contributing to the aromatic profile (i.e. neutrality) (22 %). Only 10 % of winemakers were interested in enhancement of wine colour (5 %) or contribution to the aromatic profile (5 %).

The research community has focused on three main areas: enzymatic changes during MLF, safety of the final product and mechanisms of stress resistance. It is now widely accepted that LAB strains carrying out MLF can affect the wine aromatic profile and final wine colour in a strain-specific manner. Indeed, some companies that supply starter cultures for MLF also include general predictions of each strain's ability to alter certain compounds in the wine that are regarded as being linked to quality. For example, low diacetyl production is often considered a desirable trait. There has also been an increasing number of publications on the ability of LAB to withstand wine-like stresses and the mechanisms for such resistance, with the final aim being to aid selection of superior LAB strains to conduct a more reliable MLF. Finally, the safety of the final product is always of importance when intended for human consumption. Accordingly, there is much interest in this topic, primarily concerned with biogenic amine and ethyl carbamate formation during or after MLF. This review summarises the latest research in the rapidly evolving study of MLF and investigates the directions that future research may take.

Enzyme activity and sensory changes during MLF

LAB have a considerable array of metabolic pathways and enzymes that are capable of altering wine aroma during MLF by hydrolysing and synthesising volatile secondary compounds at concentrations above their odour detection threshold. Examples of secondary compounds that have been shown to change during MLF include ethyl and acetate esters, higher alcohols, carbonyls, volatile fatty acids and sulfur compounds (Bartowsky 2005; Siebert et al. 2005; Sumby et al. 2013). As previously described, strain-specific variation in metabolic capabilities can impact on both the types and concentration of the compounds produced (Matthews et al. 2004; Bartowsky 2005; Lee et al. 2009; Sumby et al. 2013). The following section summarises the current knowledge of enzymatic activities of LAB in wine.

Malolactic enzyme

The first report of cloning of the malolactic (*mleA*) gene of *O. oeni* was in 1996 and expression was performed in both *Escherichia coli* and *S. cerevisiae* (Labarre et al. 1996). However, the MleA protein expressed in *E. coli* was only active against L-malic acid after protein fractionation and concentration. Labarre et al. (1996) also reported the presence of a malate carrier protein (*mleP*) downstream of *mleA* suggesting that in *O. oeni* the genes that encode for MLF are clustered and that malic acid is actively transported into the cell. As evidence, the *mleP* gene encoding a 34-kDa protein with characteristics of a carrier protein was cloned into dicarboxylic acid transport-deficient *E. coli* mutants revealing energy-dependent L-malate transport (Labarre et al. 1996). Schümann et al. (2013) also characterised the *mleA* gene from *O. oeni* via expression into *E. coli* with the crude extract having a specific activity of 14.9 U/mg of protein and the purified recombinant protein having a specific activity of 280 U/mg of protein, much higher than that previously reported (Labarre et al. 1996). But the exact reaction mechanism of MleA remains in question and needs to be studied in further detail (Schümann et al. 2013).

Recognising the often poor growth of *O. oeni*, further work with the *O. oeni* malolactic enzyme involved expression in *L. plantarum* to determine if MLF could be improved above

the level of the wild-type strain (Schümann et al. 2013). However, induction of the expression vector at low pH and even in the presence of diluted wine decreased the expression level. At pH 4.0, activity was undetectable and conversion of the added L-malic acid (5 g/l) was the same as the wild-type strain (Schumann et al. 2012). This is probably at least partly due to expression vector loss at low pH, as the selective maker used had minimal activity at low pH, and therefore, the bacteria likely dispose of the plasmid. To overcome this, *L. plantarum* cells were cultured and induced in a medium at pH 6.0, where cells showed highest malolactic activity and then were harvested and directly inoculated to L-malic acid solutions. The authors used pre-induced cells as starter cultures and reported that the recombinant strain converted L-malic acid more efficiently than the wild-type strain (Schumann et al. 2012). Nevertheless, the malolactic activity of the recombinant *L. plantarum* was only slightly above that of adapted wild-type cells and the modified wine solution used was at a pH of 5.0, which is well above typical wine pH. The authors suggested using a food-grade selection marker, such as the pSIP vectors with the alanine racemase (*alr*) gene as the plasmid selection marker instead of the erythromycin gene (Schumann et al. 2012). This system has successfully been applied for overexpression of a β-galactosidase in a modified *L. plantarum* strain (Nguyen et al. 2011). This expression vector is however unstable when induced in the presence of D-alanine. As wine contains this amino acid and other inhibitory factors such as low pH, further study will be needed to determine if *alr* can be utilised as a selection marker in *Lactobacillus* spp. for the purpose of overexpression of *O. oeni* genes in wine. Gene expression studies of *mle* in LAB have also added to the knowledge of how *mle* functions when LAB are in the wine environment. For example, Augagneur et al. (2007) reported that the presence of L-malate in low pH media resulted in an increase in *mleP* expression leading to the suggestion that *mleP* encodes a carrier protein responsible for L-malate uptake. Following on from this, Miller et al. (2011) investigated the effect of pH, ethanol and malic acid concentration on expression of the structural *mle* gene of *L. plantarum* to find induction at low pH values and when malic acid was present in the medium. Increasing ethanol concentrations and/or pH decreased *mle* expression with increased pH appearing to have a greater effect than high ethanol concentrations. These results suggest that under certain conditions such as high pH (wine pH can exceed 4.0), the added effect of high ethanol concentrations will have a negative impact on the completion of MLF. However, the normalisation of messenger RNA (mRNA) quantity in this experiment was conducted with ribosomal RNA (rRNA) and could contain a bias, as 16S rRNA transcripts do not reflect the overall mRNA within *O. oeni* and should therefore not be used as an internal control to study gene expression (Desroche et al. 2005). Beltramo et al. (2006) have also previously reported that low pH resulted in increased *mle* expression. MLF plays a role in the regulation of the intracellular pH; thus, LAB could achieve a biological advantage by increasing *mle* expression in acidic conditions (Miller et al. 2011). It will be of interest to investigate if low pH causes increase in *mle* expression due to an increase in the undissociated form of malic acid, which would enable it to pass through the cellular membrane, or whether the previously reported increase in mleP activity (Augagneur et al. 2007) and therefore increased active transport of malic acid across the membrane are more important. At pH 3.2, 64 % of L-malic acid is undissociated and the initial rate of passive diffusion represents 50 % of the total uptake rate (Tourdot-Maréchal et al. 1993). However, *O. oeni* cells respond to ethanol by increasing membrane rigidity (Tourdot-Marechal et al. 2000; Chu-Ky et al. 2005), and this could potentially affect passive diffusion of L-malic acid into the cell.

Glycosidases

Glycosylated aroma and flavour compounds can be liberated enzymatically by microbial glycosidases. Wine-related LAB have been shown to possess the ability to hydrolyse various synthetic glycosides (Grimaldi et al. 2000, 2005a, b), and high variations in glycosidase activities have been reported amongst isolates of *O. oeni* (Ugliano and Moio 2006; Gagne et al. 2011). The activity of these enzymes can aid release of numerous aroma compounds, including monoterpenes, norisoprenoids and aliphatic compounds, all of which contribute to the fruity and floral attributes of wine. Capaldo et al. (2011a, b) reported the cloning and characterisation of two phospho-β-glucosidases from *O. oeni*. The first gene (*bglD*) is found in a putative β-glucosidase operon encoding four genes designated *bglA* to *bglD*. The *bglA*, *B* and *C* genes are thought to be phosphoenolpyruvate-dependent phosphotransferase system components IIC, IIA and IIB, which regulate the uptake, phosphorylation and translocation of β-glucosides across the cytoplasmic membrane (Capaldo et al. 2011a). High activity towards the phosphorylated β-glucoside *para*-nitrophenol-β-D-glucopyranoside-6-phosphate substrate was observed with purified BglD. The enzyme was not active against non-phosphorylated β-glucosides. The second gene characterised was phospho-β-glucosidase *celD*, which is in a putative PEP-PTS operon in *O. oeni* (Capaldo et al. 2011b). CelD is proposed to be an intracellular enzyme that also acts on phosphorylated β-glucosides. More recently, a purified recombinant glucosidase and an arabinosidase from *O. oeni* were reported to release monoterpenes from natural substrates but under optimal conditions. This indicates that these intracellular enzymes might hydrolyse aroma precursors during MLF (Michlmayr et al. 2012), but information about strain variation in these genes will likely prove more useful than

direct application of these enzymes, as their activity was very low in wine.

Wine LAB, in particular *O. oeni*, are capable of releasing attractive aroma compounds during MLF, and LAB might be a promising source of novel glycosidases with oenological potential. Whilst numerous fungi and bacteria produce glycosidases, there is variation in the abilities of these microbes to function efficiently under the high alcohol and low pH encountered in winemaking. Given the potential impact of glycosidases on the sensory profile of wine, it is envisaged that further characterisation of glycosidase systems from LAB will provide information to aid winemakers in tailoring wine aroma, colour and overall complexity.

Esterases

Sumby et al. (2009, 2012a, 2013) reported the characterisation of esterase enzymes from wine LAB. These esterases were demonstrated to retain activity under conditions relevant to winemaking (Sumby et al. 2009, 2012a). The *O. oeni* Ooeni28 esterases EstB28 and EstA2 were also shown to have both hydrolysis and synthesis activity with natural substrates (Sumby et al. 2013). Further investigation of these enzymes would provide a better understanding of the role they play in ester profile modification during MLF. The reason for strain-specific changes in esters during MLF was also investigated, and strains could be characterised in a manner that concurred with their previously observed activity against artificial ester substrates (Sumby et al. 2013). The strength of this correlation, however, will need to be investigated further. Additional experiments on these esterase genes from a strain designated as having high esterase hydrolysis activity (Sumby et al. 2012b) revealed that they are induced in the presence of substrates. More detailed investigation of these activities in wine will benefit winemaking by allowing a more informed choice of MLF culture to be made based on activities other than just the decarboxylation of malic acid.

The study of these enzymes under 'wine-like' conditions is currently only of academic value as direct application of bacterial enzymes in winemaking is not presently possible. The major obstacle to this is the necessity for recombinant enzyme production. Consumer preferences preclude the use of recombinant techniques in the food industry. However, an interesting alternative could be the use of LAB as GRAS/food-grade expressions systems, which are also recombinant but generally more acceptable. There has been much interest in designing LAB 'food-grade' gene expression systems over the past decade (Gosalbes et al. 2000; Sørvig et al. 2003; Maischberger et al. 2010; Nguyen et al. 2011). In order for these systems to be accepted for use in food products, they are designed with specific attention to self-cloning strategies, food-grade selection markers, plasmid replication and chromosomal gene replacements (Peterbauer et al. 2011). Even though *O. oeni* is not easily genetically manipulated due to poor transformability, it could be possible to express the genes of interest in other more easily manipulated LAB such as *Lactobacillus* spp. This would enable winemakers to utilise the benefits of these characterised *O. oeni* malolactic, esterase and glycosidase genes.

Potential negative impacts of MLF

Volatile phenols

Volatile phenols are aromatic compounds that are reported to be mainly produced by the yeast genus *Brettanomyces* (Curtin et al. 2013). However, there is some evidence that LAB can also produce these compounds (Chatonnet et al. 1995, 1997; Silva et al. 2011; Fras et al. 2014). The formation of volatile phenols in wine is the result of the sequential activity of two enzymes. The first enzyme, hydroxycinnamate decarboxylase, catalyses the decarboxylation of non-volatile, odourless hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acids) forming hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol). The second enzyme a vinylphenol reductase can then sometimes reduce the hydroxystyrenes to their corresponding ethyl derivative forms (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol) (Heresztyn 1986; Chatonnet et al. 1992; Harris et al. 2009; Carrillo and Tena 2007; Godoy et al. 2008; Tchobanov et al. 2008; Buron et al. 2012). Both the ethyl and vinyl derivatives of hydroxycinnamic acids are of concern in wine and are considered to be the most significant molecules linked to olfactory defects in wine. If the concentration of volatile phenols is greater than 425 μg/l, they can potentially mask any desirable aromas and the corresponding wine can often be described as smelling of 'animal' or 'horse sweat' (Heresztyn 1986; Chatonnet et al. 1995).

LAB produce volatile phenols via the activity of decarboxylase enzymes, which can be enhanced with increased phenolic composition of the medium (Silva et al. 2011). Couto et al. (2006) investigated the ability of 35 strains of LAB (20 species including two *O. oeni* strains) to produce volatile phenols in a culture medium. It was shown that 13 strains produced volatile phenols from *p*-coumaric acid and only three (9 %; *Lactobacillus brevis*, *Lactobacillus collinoides* and *L. plantarum*) of these could produce 4-ethylphenol (Cuto et al. 2006). There is so far only limited evidence that LAB can produce 4-ethylphenol from 4-vinylphenol and this evidence is also strain dependent (Silva et al. 2011). Buron et al. (2012) reported that four different *L. collinoides* strains were able to produce 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol in synthetic media and in cider with amounts increasing when caffeic acid and *p*-coumaric acid were added

to the cider. More recently, Fras et al. (2014) investigated the ability of *L. plantarum* (strain NCFB 1752) to produce volatile phenols in the presence of *p*-coumaric and ferulic acids in de Man–Rogosa–Sharpe media (MRS) and in MRS supplemented with wine (red and white). There was no evidence of ferulic acid metabolism by *L. plantarum*, but there was production of 4-vinylphenol and 4-ethylphenol in varying amounts depending on the ratio of wine in the medium, suggesting that the presence of wine leads to the accumulation of 4-vinylphenol and potentially inhibits the production of 4-ethylphenol (Fras et al. 2014). The conversion yields of hydroxycinnamic acids were also still lower than those seen with *Brettanomyces*. The ability of LAB to produce phenolic off-flavours in real wine is therefore still to be investigated.

Indole

High levels of indole are mainly only reported in wines under sluggish fermentation conditions and are linked to 'plastic' off-aromas (Capone et al. 2010). Indole formation in wine still is not fully understood, but is thought to be linked to tryptophan metabolism. Four strains of LAB (*O. oeni*, *Lactobacillus lindneri*, *Pediococcus cerevisiae* and *Pediococcus parvulus*) have been reported to generate indole during MLF in defined media supplemented with 100 mg/l tryptophan and only minimal amounts without tryptophan supplementation (Arevalo-Villena et al. 2010). Indole has an aroma detection threshold of 23 mg/l in white wine (Capone et al. 2010), and all four tested LAB strains had accumulated indole at the end of MLF at concentrations ranging from 70 to 370 mg/l. It has previously been reported that yeast can release tryptophan back into wine in the later stages of AF (Henschke and Jiranek 1993) at which point it might act as a substrate for bacteria to produce indole at concentrations detrimental to wine quality. A fuller investigation of indole formation during MLF in wine by LAB is required.

Biogenic amine formation

Biogenic amines are low molecular weight organic nitrogenous bases that can be formed in fermented foods and beverages by decarboxylation of free amino acids. In wine, this can be facilitated by the action of microbial decarboxylase enzymes, from the corresponding amino acid precursors. This reaction is usually strain dependent rather than species specific, and it has been suggested that horizontal gene transfer is responsible for the variation in decarboxylation abilities amongst LAB (Marcobal et al. 2006; Coton and Coton 2009). High concentrations (i.e. 1–100 mg/l) of biogenic amines can cause undesirable physiological effects in sensitive humans, especially when alcohol and acetaldehyde are present (Maintz and Novak 2007; Spano et al. 2010). Putrescine, histamine, tyramine and cadaverine have been identified as the most abundant biogenic amines found in wine (Beneduce et al. 2010; Bartowsky and Stockley 2011; Patrignani et al. 2012; Costantini et al. 2013; Martuscelli et al. 2013). It is now widely believed that the main source of biogenic amines in wine is from LAB metabolism either during or after MLF (Lonvaud-Funel 2001; Polo et al. 2011; Smit et al. 2012). For a review of the source of amines in wine, including during MLF, refer to Ancín-Azpilicueta et al. (2008).

Choosing MLF starter culture strains that do not produce biogenic amines and/or can degrade biogenic amines formed in wine is therefore desirable. Whilst *O. oeni* has been reported to rarely degrade wine biogenic amines (García-Ruiz et al. 2011), recent studies have pointed to the potential of *Lactobacillus* strains in particular *L. plantarum* (Capozzi et al. 2012) and *L. casei* (García-Ruiz et al. 2011) to do so through the production of amine oxidase enzymes. García-Ruiz et al. (2011) reported that in a model system, the greatest biogenic amine-degrading ability was exhibited by nine strains belonging to the lactobacilli and pediococci groups, with most able to simultaneously degrade to some extent at least two of the three studied biogenic amines. The ability of LAB to reduce biogenic amines was negatively affected by the wine matrix (Capozzi et al. 2012; García-Ruiz et al. 2011). Only one LAB strain (*L. casei*) was able to significantly degrade histamine, tyramine and putrescine in a contaminated wine but at lower percentages than in culture media. Additionally, the ability of these strains to complete MLF is yet to be tested, and it is possible that they may need to be inoculated alongside *O. oeni* to ensure MLF completion. Only one of the 42 *O. oeni* strains tested showed 10 % or more degradation of biogenic amines in control media, but this strain showed no significant degradation during wine MLF.

It has also been reported that co-inoculation of *O. oeni* starter cultures at the beginning of AF simultaneously with yeast has been more effective in avoiding the production of biogenic amines than either spontaneous or conventional inoculation (Cañas et al. 2012; Smit and du Toit 2013). This is most likely due to the inoculated starter culture dominating the fermentation and reducing the impact that indigenous lactobacilli have on biogenic amine formation (Smit et al. 2012; Smit and du Toit 2013). This highlights the need to screen any potential starter culture for decarboxylase activity to minimise the production of biogenic amines. Physiochemical factors such as pH, temperature, SO_2 and alcohol concentration, along with the nutrients present in wine, influence bacterial development and, as a consequence, amine production. Complete MLF is necessary to avoid the risk of increased biogenic amines during aging (Polo et al. 2011). Smit et al. (2013) reported that cold maceration (when compared to conventional maceration) during red wine

production inhibited the formation of high levels of biogenic amines under the conditions tested. However, further investigations are necessary to determine the reasons for this.

Ethyl carbamate

Wine, like most fermented foods and beverages, can contain trace amounts of ethyl carbamate (EC), a known animal carcinogen (Canas et al. 1994). EC is formed by a reaction between ethanol and N-carbamyl compounds at acidic pH and can be due to the production of certain precursors during AF (such as urea) and MLF (citrulline and carbamyl phosphate). Its formation is dependent on reactant concentration and is favoured by high temperature and low pH (Ough et al. 1988). The EC content is therefore higher in wines that have been stored for a long time without temperature control. Some LAB can degrade the arginine present in must and wine via the arginine deiminase pathway (Liu et al. 1995). When arginine is not completely catabolised, intermediate products of the pathway, citrulline and carbamyl phosphate, can accumulate in the medium. Both compounds can react with ethanol and produce EC. EC-producing LAB strains include all heterofermentative lactobacilli, *O. oeni*, *Pediococcus pentosaceus* and some strains of *Leuconostoc mesenteroides* and *L. plantarum* (Araque et al. 2009).

In most LAB, the three genes involved in arginine catabolism are clustered in an operon-like structure (Fig. 1). The catabolic enzyme arginine deiminase, encoded by *ArcA*, converts arginine into citrulline and ammonia. Citrulline can then be further metabolised by ornithine transcarbamylase (*ArcB*) into carbamoyl-P, which can then be utilised by carbamate kinase (*ArcC*) to produce CO_2, ammonia and ATP (Tonon et al. 2001; Spano et al. 2004; Araque et al. 2009). The timing of MLF induction on EC accumulation in wine has no influence (Masqué et al. 2011). EC levels at the end of MLF were quite low (<3 μg/l) and increased after storage. It is therefore difficult to determine if the increase in EC is from remnants of either yeast or bacterial metabolism and, hence, making it important to quantify citrulline and carbamyl phosphate levels in studies investigating EC production during MLF (Masqué et al. 2011). Significant differences between two LAB species (*L. brevis* and *P. pentosaceus*) in citrulline production in the presence of ethanol have been reported (Araque et al. 2013). *ArcC* expression (carbamate kinase) was repressed in *L. brevis* making this the basis for the suggestion that this species is more likely to produce EC precursors.

Patrignani et al. (2012) reported that there was no significant difference observed in EC concentration after MLF between samples of wine produced by uninoculated fermentation and wine that was inoculated. However, citrulline and carbamyl phosphate levels were not quantified in the tested wines and the wines were not accessed for EC accumulation after storage. Arena et al. (2013) analysed citrulline production in Malbec wine and reported that formation was correlated with its low glucose, fructose, citric and phenolic acid concentrations. Further analysis of other wines with lower concentrations of these sugars and acids in relation to the formation of ethyl carbamate precursors could provide more information on wines at risk of increased EC formation.

Chemical impediments to successful MLF

One of the most important requirements of MLF is that the process is reliably completed in a timely manner therefore reducing the risk of the proliferation of spoilage microorganisms. A potential cause of stuck or sluggish MLF is the fastidious nutritional requirements of malolactic bacteria whose growth typically depends on the availability of nutrients left after AF. This is an area that has however not received much attention in the literature, and a study of the minimum nutritional needs of LAB species in wine would help eliminate the need for costly nutrient supplementation. When *O. oeni* is added to wine, it encounters multiple stressors, amongst which are low pH and temperature and high ethanol concentrations. The tolerance to ethanol varies from strain to strain, and it is generally accepted that all *O. oeni* strains grow in a medium containing 10 % ethanol and that small quantities of ethanol [3–5 (Britz and Tracey 1990) or 7 % (Alegría et al. 2004)] can stimulate their growth. Sulfur dioxide is an additional stressor that is often added to wine as an antioxidant and

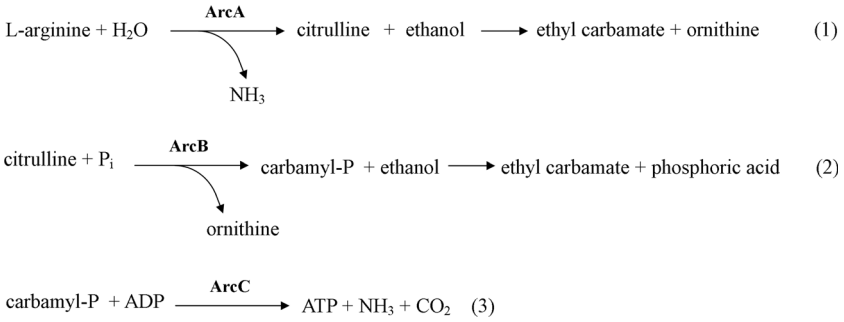

Fig. 1 Pathways for ethyl carbamate (EC) formation in wine during MLF and via acid catalysed alcoholysis. ArcA (arginine deiminase), ArcB (ornithine transcarbamylase) and ArcC (carbamate kinase) adapted from Tonon et al. (2001), Spano et al. (2004) and Araque et al. (2009)

to reduce the risk of microbial spoilage during primary fermentation. The yeast carrying out the primary fermentation can also produce it. The presence of any one of these stressors in the wine may inhibit the LAB and cause a sluggish or stuck MLF.

Other factors in the wine that might cause a slow or stuck MLF include medium chain fatty acids, phenolic compounds (e.g. phenolic acids, tannins), pesticides, metal ions (such as copper), bacteriocins produced by other LAB species (e.g. nisin, pediocin and plantaricin), inhibitors produced by yeasts during fermentation, bacteriophage infections and lysis of the malolactic bacteria. Recent research has indicated that certain grape phenolic compounds including phenolic acids that can increase membrane permeability in LAB (Campos et al. 2003, 2009) and tannins can have a negative influence on *O. oeni* (Figueiredo et al. 2008). Tannins are naturally occurring high molecular weight (M_r>500) polymers of phenolic compounds flavonoids and non-flavonoids, which form stable complexes with proteins and other plant polymers (Ribereau-Gayon et al. 2000). Tannins derived from flavonoids are naturally present in the grape skins, seeds and stems. Non-flavonoid tannins are derived from the pulp of the grape and can also be extracted from oak sources (barrels, staves, chips) during fermentation and aging. Certain red cultivars can have more difficulty undergoing MLF due to tannins (Vivas et al. 2000). Research is ongoing into this observation, as the exact nature of these tannins and their concentrations are still not well defined.

In years when the incidence of *Botrytis* infection of grapes is high, residues of systemic fungicides/pesticides have the potential to be problematic for LAB thereby influencing MLF success. Cabras et al. (1994) reported that the presence of dichlofluanid reduced the ability of *O. oeni* to conduct MLF in proportion to its concentration, but the other pesticides tested (benalaxyl, carbendazim, triadimefon and vinclozolin) did not influence MLF. The insecticide dicofol has also been reported to reduce the activity of *O. oeni*, whilst other fungicides and insecticides had only a minor effect (Vidal et al. 2001; Ruediger et al. 2005). Whilst the addition of yeast hulls or ghosts may help relieve this toxicity, inhibition of MLF by pesticides is increased in the presence of ethanol (Vidal et al. 2001). The commonly used fungicide, copper, apparently does not affect malolactic activity directly (Vidal et al. 2001), but rather through the decrease in cellular viability that accompanies increasing copper. There is also a synergistic effect when LAB are grown in media containing copper and other stressors of wine such as ethanol, SO_2, tartaric acid or low pH (Vidal et al. 2001). These findings aside, the interactions between LAB and metal ions in wine are not fully investigated.

There have been a number of reports that compounds of peptidic or proteic nature produced by yeasts can inhibit LAB growth (Comitini et al. 2005; Osborne and Edwards 2007; Mendoza et al. 2010; Nehme et al. 2010; Branco et al. 2014). For example, Mendoza et al. (2010) found that the *S. cerevisiae* strain tested inhibited wine LAB growth by a synergistic effect of a peptidic compound of low molecular size (3–10 kDa) and fermentation metabolites. Although this yeast inhibited *O. oeni* growth, it did not affect the malolactic activity. More extensive fractionation studies are called for to determine the full range of inhibitory compounds and mechanisms affecting LAB grown in newly fermented wines.

Sulfur dioxide

Sulfur dioxide is used as both an antimicrobial and antioxidant agent. Winemakers can add it at different stages during wine production. When it is added in the form of either sodium or potassium metabisulfite, these compounds release SO_2, which reacts with water to form sulfites. Yeast will also commonly produce some bisulfite during fermentation through the reduction of sulfate as part of the production of essential sulfur-containing compounds such as cysteine and methionine (Dott et al. 1976).

In solution, the different forms of sulfites are at equilibrium; however, the lower the pH, the more heavily the reaction shifts towards molecular SO_2 and bisulfite (together termed free SO_2), which make up the vast majority (99.99 % at pH 3.4) of the sulfite compounds present in wine. Sulfites will also react with other wine components such as sugars, carbonyl compounds (such as acetaldehyde) and phenolic compounds. Such ‘bound’ sulfites can no longer take part in the equilibrium reaction. Only the molecular form of SO_2 can enter through the cell membrane causing disruption to enzyme activity. It is therefore the concentration of molecular SO_2 that is considered to control microbial growth in wine.

Bacterial inhibition by SO_2 bound to other wine components is often reported as being as a consequence of the release of SO_2 following degradation of the binding compound by the bacteria (Osborne et al. 2000, 2006; Jackowetz and Mira de Orduña 2012, 2013). Larsen et al. (2003) reported that *O. oeni* was inhibited in wines with high bound SO_2 concentrations and suggested that inhibition of MLF by bound SO_2 is more significant than previously thought. The authors also reported that inhibition of *O. oeni* was stronger when SO_2 was bound to compounds other than acetaldehyde (Larsen et al. 2003). More recently, Wells and Osborne (2011) investigated the impact of the production of SO_2 and SO_2-binding compounds by wine yeast on MLF. Samples were taken from the fermentations at different time points, filter sterilised and inoculated with *O. oeni* to induce MLF. Significant differences between the yeast strains in the amount of SO_2, acetaldehyde and pyruvic acid produced were reported and high total SO_2 concentration inhibited MLF. However, for all yeast strains tested, insignificant free SO_2 was measured, indicating that bound SO_2 rather than free SO_2 was responsible for MLF

inhibition (Wells and Osborne 2011). At almost all time points of the AF, acetaldehyde-bound SO_2 was determined to be the dominant species of bound SO_2 present, suggesting that MLF inhibition by bound SO_2 was due to this species. In a subsequent investigation of the degradation of SO_2 bound to acetaldehyde and pyruvic acid, by several LAB species including *O. oeni*, no correlation with growth of the bacteria was observed (Wells and Osborne 2012). The bacteria were still metabolically active in media containing bound SO_2 even when no growth was observed, indicating that bound SO_2 is bacteriostatic rather than bacteriocidal (Wells and Osborne 2012). In accordance with the results reported by Larsen et al. (2003), the authors also reported that *O. oeni* was the most sensitive of the LAB tested against pyruvic acid-bound SO_2. Therefore, is it now evident that SO_2 bound to acetaldehyde or pyruvic acid is inhibitory to the growth of wine LAB and must be considered both when choosing a yeast strain for conducting AF and, subsequently, when choosing a LAB strain to conduct MLF.

Response of *O. oeni* to stress

There are now a number of tools available to study the effect of either individual or multiple stresses on *O. oeni* and other LAB cells, including metabolomic analysis, proteomics, genomics and transcriptomics. These techniques generate large datasets, making it possible to perform comprehensive analyses of biological systems to generate new knowledge and help to address research questions when applied to any organism. For example, there is a large amount of omics data available for wine yeast, and a detailed metabolic map of wine yeast in a model wine fermentation is currently being developed (Chambers 2011). This approach is yet to be applied to wine LAB and could provide valuable insight into how they function in wine during MLF and could also help develop informed models for the in silico design of new and/or improved industrial strains. As an example, Bon et al. (2009) used comparative genome subtractive hybridization to propose that the presence of eight stress-responsive genes was associated with high MLF performance thereby suggesting a relationship between genome variation and malic acid metabolism. The eight genes were annotated and encode for the Dps gene homolog, *fnr* (DNA binding protein), copper chaperone, maltose phosphorylase, oxo-acyl carrier protein reductase, P-type ATPase, YP_809753 (hypothetical protein) and NP_786185 (hypothetical protein). They also identified six different regions of plasticity, resulting from recombination or InDel events and suggested that IS*30*-related elements play a role in *O. oeni* genome plasticity.

In order to overcome the multiple stressors that are encountered during MLF in wine, LAB display numerous adaptive responses. In recent years, analysis of gene expression under different stress conditions have led to reports of many potential survival strategies and metabolic properties that may enable LAB to effectively compete in the wine environment (Table 1). For example, Maitre et al. (2014) proposed a model whereby *O. oeni* can adapt to ethanol stress through the synthesis of the small heat shock protein Lo18 and the subsequent modification of phospholipid content of the cell membrane. Other potentially interesting stress responses include carotenoid production, which has been shown to reduce membrane fluidity in *Staphylococcus aureus* (Clauditz et al. 2006) and increase multistress tolerance when carotenoid biosynthesis genes are overexpressed in *Lactococcus lactis* (Hagi et al. 2013). This is worthy of further investigation in *O. oeni* as geranylgeranyl pyrophosphate synthase (Table 1), an enzyme involved in carotenoid biosynthesis, is overexpressed in *O. oeni* in response to ethanol (Cafaro et al. 2014). The existence of such unique features can be viewed as evolutionary adaptation to the wine environment (Beltramo et al. 2006). The response of LAB to stress relies on the coordinated expression of genes that alter specific cellular processes (e.g. membrane biogenesis, transport, DNA metabolism, etc.). A network of regulators allows the cell to react to complex environmental changes and achieve a coordinated response. Although several studies have analysed mechanisms that enable LAB to withstand stress conditions (Table 1), more information about the mechanisms of LAB adaptation to stress conditions found in wine is required. A better understanding of the regulatory networks involved will help explain how particular LAB strains adapt to wine and help predict a strain's ability to complete MLF in a given wine. Methods allowing for identification of gene expression changes associated with differential microbial behaviour under different stress conditions include fluorescent differential display (FDD) (Sico et al. 2009), microarray analysis and RNA sequencing. For a detailed review of stress response in *O. oeni*, see Wen-ying and Zhen-kui (2013).

Genetic diversity and molecular approaches to identify LAB

Within, LAB there is a large degree of phenotypic heterogeneity and different strains vary in their capacity to adapt to wine and degrade malic acid. An increasing interest in the use of malolactic starter cultures in winemaking has produced numerous publications on this subject. LAB species may be differentiated by several DNA fingerprint profiling methods, such as restriction endonuclease analysis pulsed-field gel electrophoresis (REA-PFGE), patterns of low-frequency restricted genomes (Viti et al. 1996; Zapparoli et al. 2000; Lopez et al. 2008b; González-Arenzana et al. 2013b), random amplification of polymorphic DNA (RAPD) (Reguant and

Table 1 Studies reporting genes involved in the adaptation and stress response in wine

Gene	Function	Stress					
		Ethanol	Heat shock	Oxidative (H_2O_2)	Acid	Osmotic shock	Synthetic wine
arcR	Regulatory protein, arginine deiminase pathway		Bourdineaud (2006)			Bourdineaud (2006)	
atp—β and α subunits	H^+-ATPase				Fortier et al. (2003), Beltramo et al. (2006)		
cfa	Cyclopropane fatty acid synthase	Beltramo et al. (2006), Grandvalet et al. (2008)			Grandvalet et al. (2008)		Beltramo et al. (2006)
citE	Citrate lyase β subunit	Olguín et al. (2009)					
clpL	ATP-dependent protease	Beltramo et al. (2004a), Grandvalet et al. (2005)	Beltramo et al. (2004a), Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005), Beltramo et al. (2006)		
clpP	ClpP protease	Beltramo et al. (2004a)	Beltramo et al. (2004a), Desroche et al. (2005)				Beltramo et al. (2006)
clpX	ATPase regulation component of ClpP		Guzzo et al. (2000), Desroche et al. (2005)				Beltramo et al. (2006)
ctsR	CtsR, heat shock transcriptional regulator	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005)		Beltramo et al. (2006)
ftsH	Membrane protease		Bourdineaud et al. (2003)			Bourdineaud et al. (2003)	
ggpps	Geranylgeranyl pyrophosphate synthase	Cafaro et al. (2014)					
groES	GroES, heat shock chaperone class I	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)				Beltramo et al. (2006)
grpE	GrpE, heat shock chaperone class II	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005)		Beltramo et al. (2006)
hsp18	Small heat shock protein (Lo18)	Fiocco et al. (2007), Maitre et al. (2014)	Jobin et al. (1997), Guzzo et al. (2000), Desroche et al. (2005), Fiocco et al. (2007)		Beltramo et al. (2006)		
maeP	Putative citrate transporter	Olguín et al. (2009)					
mleA	Malolactic enzyme				Beltramo et al. (2006)		Beltramo et al. (2006)
omrA	ABC-type transporter		Bourdineaud et al. (2004)			Bourdineaud et al. (2004)	
rmlB	DtdP-glucose-4,6-dehydratase	Da Silveira et al. (2004)					
trxA	Thioredoxin		Jobin et al. (1999), Guzzo et al. (2000)	Jobin et al. (1999)	Beltramo et al. (2006)		Beltramo et al. (2006)

Bordons 2003; Solieri and Giudici 2010; Sánchez et al. 2012), amplified fragment length polymorphism (AFLP) (Cappello et al. 2008) or ribotyping analyses (Zavaleta et al. 1997; de las Rivas et al. 2004; Rodas et al. 2005). More recent methods include a combination of PFGE and multilocus sequence typing (MLST) (González-Arenzana et al. 2014), PCR-RFLP (Ilabaca et al. 2014) and variable number of tandem repeat analysis (VNTR) (Claisse and Lonvaud-Funel 2012, 2014).

Advances in DNA sequencing technology have led to rapid increases in sequencing throughput and a decrease in sequencing cost leading to more comparative studies of the whole genomes of many related species. This is also driving an interest in utilising this knowledge to design metabolic pathways in many LAB used in the production of fermented foods. This generally involves targeting the synthesis of specific compounds to produce the desired sensorial, textural and nutritional attributes. Genome analyses can provide valuable insights into key trait variations including evolutionary mechanisms, diversity and adaptability of life to environmental changes. Intra-species genetic diversity and the genomic basis of strain-specific differences amongst industrially relevant LAB have received much attention. Direct comparisons of complete genome data, subtractive hybridizations and array-based comparative hybridization have detected numerous insertion and deletion events (InDels) (Boekhorst et al. 2004; Bon et al. 2009; Borneman et al. 2010; Bartowsky and Borneman 2011; Borneman et al. 2012a, b). Genomic diversity can be increased by the presence of prophages, which generate large-size structural polymorphisms among *O. oeni* genomes (Ze-Ze et al. 2008; Jaomanjaka et al. 2013) and insertion sequences (IS) (Ze-Ze et al. 2008; El Gharniti et al. 2012). IS are autonomous transposable elements which encode a transposase gene mediating their transposition to other loci in the genome (Mahillon and Chandler 1998). IS are widely distributed in both eukaryotic and bacterial genomes and may disrupt genes, but may also activate downstream genes and fine tune gene expression through transposition-mediated genome inversions.

Inoculation, strain selection and optimisation

In order to achieve better control over MLF, many wineries now inoculate with a commercial MLF starter culture. They do this with the hope that the commercial strains will be more reliable than the indigenous microbiota, and indeed, many strains are now promoted as performing well under different stress conditions. However, there is still some contention over this as even with the use of commercial starter cultures, MLF is not always successful. Allowing the indigenous microbiota to conduct MLF is considered to have the advantage that these organisms have previous adaptation to wine. Much of the work on wine LAB in recent years has focused on *O. oeni* as it is historically considered to be the best adapted to the wine environment, but this is not always the case. Of 40 indigenous strain isolates evaluated, two of the three strains possessing desirable traits were *L. plantarum* and only one was *O. oeni* (Izquierdo et al. 2004). Further screening will undoubtedly reveal additional strains with desirable properties. In addition to strain selection, strain improvement and optimisation strategies use two broad approaches, recombinant and non-recombinant methods. Recombinant methods have the advantage of being highly specialised and controlled but are often focused on the addition or deletion of specific genes. This requires an intricate knowledge of gene identity, function and interactions prior to manipulation. Non-recombinant approaches require little prior knowledge of the genetic basis of a trait, but can be time consuming, often random and with pleotropic effects.

Co-inoculation of *O. oeni* and yeast

Prolonged or delayed MLF can increase the risk of spoilage by other microorganisms and the production of undesirable compounds such as biogenic amines (discussed above). In recent years, co-inoculation of bacterial cultures with yeast into the must at the beginning of fermentation has been proposed as a solution for obtaining fast and reliable MLF, particularly for grape juices or musts with high acidity or sugar concentration with a concomitant high level of ethanol on the completion of AF (Jussier et al. 2006; Zapparoli et al. 2009). The justification for this approach is that bacteria better adapt to the physiochemical conditions of the must rather than the wine (Abrahamse and Bartowsky 2012), and the limiting factors increase gradually according to the evolution of AF. There is, however, still some reluctance amongst winemakers to use co-inoculation based on the belief that there is a risk of producing acetic acid in high sugar juices through the heterofermentative metabolism of *O. oeni*. Also, it is though undesirable metabolites are produced because of interactions between the yeast and inoculated malolactic bacteria. Indeed, many studies report negative interactions between yeast and inoculated malolactic bacteria (Bisson 1999; Comitini et al. 2005; Comitini and Ciani 2007; Rodriguez and Thornton 2008).

More recent studies indicate, however, that co-inoculation is a viable option with multiple effects on wine composition. As summarised in Table 2, acetate and ethyl esters, acids and alcohols can show increase, decreases or no change. Abrahamse and Bartowsky (2012) investigated the influence of co-inoculation on Shiraz composition and reported that the presence of bacteria during AF did not affect the efficiency of primary fermentation and produced a distinct volatile profile along with differences in anthocyanin and pigmented polymer composition when compared with wines produced with bacteria inoculated late or post-AF. The authors also reported that

Table 2 Volatile compounds reported to change when yeast and malolactic bacteria are co-inoculated, compared to inoculation post-AF

Volatile compound measured	Shiraz[a]	Tempranillo[b]	Merlot[b]	Riesling[c]	Cabernet Franc[d]
Acetate esters					
Ethyl acetate	⇔	⇑	⇑/⇔	⇓	⇑
2-Methylpropyl acetate	⇔	⇓	⇑	NR	NR
2-Methylbutyl acetate	⇑	NR	NR	⇔	NR
3-Methylbutyl acetate	⇑	⇔	⇓/⇔	⇑	⇔
Hexyl acetate	⇑	⇓	⇔	⇑/⇔	⇑
Phenylmethyl acetate	NR	⇓	⇓	NR	NR
2-Phenylethyl acetate	⇔	⇓/⇑	⇑/⇓	⇓/⇔	⇓
Ethyl esters					
Ethyl propanoate	⇓	NR	NR	⇓	NR
Ethyl 2-methylpropanoate	⇓	NR	NR	NR	NR
Ethyl butanoate	⇔	⇑	⇑/⇔	⇑	⇑
Ethyl hexanoate	⇔	⇔	⇑/⇔	⇔/⇑	⇑
Ethyl lactate	⇑	⇑	⇑	⇑	⇑
Ethyl ocatnoate	⇑	⇔/⇑	⇑/⇔	⇔	⇑
Ethyl decanoate	⇔	⇔	⇔	⇔	⇔
Diethyl succinate	NR	⇑	⇑	⇔/⇑	⇑
Acids					
Hexanoic Acid	NR	⇑	⇑/⇔	⇔/⇑	⇔
Octanoic Acid	NR	⇔/⇑	⇑/⇔	⇔	⇔
Decanoic Acid	NR	⇔	⇔	⇔/⇑	⇔
Alcohols					
2-Methylpropanol	⇔	NR	NR	⇑	⇔
Butanol	⇔	NR	NR	⇔	NR
2-Methylbutanol	⇔	NR	NR	⇑/⇔	NR
3-Methylbutanol	⇔	NR	NR	⇔	⇔
Hexanol	⇔	⇑	⇑/⇔	⇔	⇔
2-Phenylethanol	⇔	⇑	⇑/⇓	⇔	⇓

⇑/⇔ = increased in one strain, but no difference with the other yeast strain

NR not reported

[a] Abrahamse and Bartowsky (2012)

[b] Cañas et al. (2012)

[c] Knoll et al. (2012)

[d] Cañas et al. (2014)

the time point at which bacteria were inoculated into the must or wine did not affect the final concentration of acetic acid. Differences in yeast and bacterial metabolism at various stages in fermentation were purported to be the drivers for changes in volatile chemical composition (Abrahamse and Bartowsky 2012).

In a large study, Guzzon et al. (2012) compared co-inoculation with post-AF inoculation using five pairs of commercial oenological starters, in four red musts with low nitrogen content. Whilst co-inoculation caused a slowdown in the activity of yeasts, the MLF was completed, whereas some failures in the degradation of malic acid were observed in the sequentially inoculated fermentations. The authors did note an increase in acetic acid production with some of the pairs tested, but in most cases, this was minor (only two of the 20 combinations were above the sensory threshold of acetic acid in wine of 700 mg/l) and not in all four of the tested wines.

By contrast, Cañas et al. (2012, 2014) noted that co-inoculation of yeast and *O. oeni* in Tempranillo, Merlot and Cabernet Franc resulted in reduced fermentation time with no or minimal increase in volatile acidity. A lower concentration of the volatile phenol 4-vinylguaiacol was also observed in the Cabernet Franc (Cañas et al. 2014), whilst volatile phenols were lower in Tempranillo wines but higher in some Merlot wines produced by co-inoculation (Cañas et al. 2012). The

pairing of yeast strain VRB and *O. oeni* C22L9 in Merlot wine was consistently linked with an increase in volatile phenols (Cañas et al. 2012) highlighting the strain and juice dependence of this outcome. Wines produced by co-inoculation were also lower in biogenic amines, particularly cadaverine and tyramine, whereas significant increases in hexanoic and octanoic acid production occurred in co-inoculated Tempranillo fermentations whilst only some of the Merlot fermentations had increased octanoic acid (Cañas et al. 2012). These latter increases may be biologically significant as medium chain fatty acids are known inhibitors of LAB growth and malolactic activity and fatty acids can have a synergistic inhibitory effect when present together (Lonvaud-Funel et al. 1988; Capucho and San Romão 1994).

Delving further into the interactions between yeast and bacteria, Rossouw et al. (2012) studied co-inoculations of *S. cerevisiae* and *O. oeni* in synthetic must. Many genes were differentially expressed when the yeast transcriptome was compared between co-inoculated and yeast-only fermentations. Some of these genes appeared to be responding to chemical changes in the fermenting must that were linked to bacterial metabolic activities. In general, there was an up-regulation of genes involved in nutrient uptake such as transporters for amino acids (*DIP5*), sulfate (*SUL1*), hexoses (*HXT13*, *HXT17*) and ammonium (*MEP1*, *MEP2*) (Rossouw et al. 2012). Up-regulation of several stress response genes might indicate a direct response of the yeast to the presence of a competing organism (Rossouw et al. 2012). *O. oeni* may also release antagonistic chemicals since the yeast gene *FYV12*, required for survival upon exposure to K1 killer toxin, was also up-regulated in the yeast during co-inoculation (Rossouw et al. 2012). It is possible that the bacteria inhibit yeast metabolism and/or physiology or that there is a direct competition for nutrients.

Further research is required to determine how best to minimise acetic acid production whilst utilising the benefits of co-inoculation of LAB with yeast during AF. An examination of a large matrix of yeast and bacterial combinations and juices will help delineate the propensity of given combinations to yield undesirable and desirable processing, sensory and compositional outcomes and thereby provide some guidance for winemakers in strain selection.

LAB other than *O. oeni*

In recent years, there has been an increased interest in other LAB species and their ability to carry out MLF either alone or alongside *O. oeni*. Although *O. oeni* is by far the most studied LAB species of oenological origin and the most used initiator of MLF, several studies have stated the potential of the facultatively homofermentative *L. plantarum* (du Toit et al. 2011; Lerm et al. 2011; Bravo-Ferrada et al. 2013) and more recently *Pediococcus damnosus* (Juega et al. 2014) as starter cultures. Although LAB other than *O. oeni* are generally still considered to be spoilage organisms, they have been isolated from wines that were not considered spoiled (Lerm et al. 2011; Bravo-Ferrada et al. 2013; Juega et al. 2014). *L. plantarum* strains can grow in wines (du Plessis et al. 2004; Lopez et al. 2008a); however, in a mixed culture fermentation, *O. oeni* often takes over as ethanol content increases (Lopez et al. 2008a; González-Arenzana et al. 2013a, b). There are some *L. plantarum* isolates that also display the ability to survive harsh wine conditions and commercial cultures of this LAB species are available. More recently, a mixed bacterial starter culture (*O. oeni* and *L. plantarum*) has also been released (Lerm et al. 2011). The use of *Lactobacillus* sp. increases the pool of potential desirable traits that can be selected to tailor the end result, such as an increase or decrease in individual esters (Sumby et al. 2010). More in-depth studies of viability during MLF as well as the oenological properties of *L. plantarum* and alternative LAB isolates at industrial scale vinifications, therefore, have the potential to improve MLF outcomes.

Strain improvement

Recombinant methods

This technology involves the expression of foreign genes or overexpression or deletion of native genes in the organism of interest. Such expression is often achieved via introduction of a plasmid; however, unlike other LAB, transformation is problematic in *O. oeni* and researchers have had only limited success. The first report of successful electroporation of the plasmid pGK13 into *O. oeni* strains PSU-1, ML-34 and 19CI (Dicks 1994) is yet to be confirmed in other laboratories. Another method which used ethanol as a membrane-fluidising agent prior to transformation led to the successful introduction of a foreign vector (pGID052) encoding a truncated form of the ClpL2 protein into *O. oeni* ATCC BAA-1163 (Assad-García et al. 2008). However, pGID052 has a low copy number and this work has also not led to increased publication of molecular transformations in *O. oeni*. A further plasmid, pCB42, was isolated and found to be capable of replicating successfully within *O. oeni*; however, the transformation frequency was low (Eom et al. 2010). It is clear that novel strategies are needed for successful transformation in *O. oeni*. For example, strains of *O. oeni* have been shown to contain several native plasmids (Shareck et al. 2004), some of which may have higher copy numbers and are able to replicate themselves within *O. oeni*. Generation of a custom expression vector using the origin of replication from these native plasmids, genetic markers and the genes of interest may generate a plasmid more effective for overexpression.

Another method of expression of foreign genes is transduction, which is the process by which bacteriophages carry bacterial genes from one cell to another. As bacteriophages

can be one of the causes of a failed MLF (Davis et al. 1985), this is theoretically possible in *O. oeni*; however, the mechanisms of infection have not yet been fully elucidated and this method needs further research. The final method of genetic manipulation is the use of conjugative transposons. Problematically, the current methods of conjugation for *O. oeni* do not allow for gene replacement, as the transfer frequency is lower than the recombination frequency (Zúñiga et al. 2003; Beltramo et al. 2004b).

It is difficult to apply any of these techniques to *O. oeni*, as they are most beneficial when allowing the removal or addition of genes, and therefore, the individual genes associated with optimization of stress resistance would first need to be identified. For example, stress resistance during MLF involves multiple genes at multiple loci (Table 1), which are broadly distributed throughout the genome making targeted genetic manipulation highly complex. The main stresses affecting MLF interact at a physical level and potentially also at a genetic level. If strains are improved for only a single stress, it is possible that their ability to survive other stresses will be adversely affected.

Directed evolution

Non-recombinant methods of improving bacterial strains for industrial processes are gaining increased attention. One method, directed evolution (DE) also known as adaptive evolution, has been applied successfully in the species of *Lactobacillus* (Teusink et al. 2009; Bachmann et al. 2012; Zhang et al. 2012; Wu et al. 2014). DE is a genome-wide method that manipulates and diversifies a population without the necessity for detailed knowledge of gene networks. The premise of DE is that an organism will adapt to its environment when placed under continuing stress conditions. Mutations that allow the organism to thrive and propagate under the specific stress are selected for by a gradual increase in the stress condition (Dragosits and Mattanovich 2013). The process by which this occurs is not yet fully understood with three possible models presented in the literature: firstly, the directed mutation model, in which mutations might target specific genes to relieve the stress factors; secondly the hypermutation model, in which mutation rates increase genome-wide so that both adaptive and non-adaptive mutations are stimulated; and finally, the cryptic growth model suggests that mutation rates do not increase at all, but that extra DNA replications simply let the normal rate of mutation acting on multiple DNA copies to give the appearance of an enhanced mutation rate (Foster 1999; Rosenberg 2001). An advantage of DE is that when an organism is adapted within its primary niche, it lacks the deleterious side effects of modern recombinant techniques (Sauer 2001). Genetic changes or mutations within a given gene will potentially affect the viability and productivity of that organism in unforeseen ways. Therefore, the major benefit of DE is that the organism must stay viable and functional throughout the process or it will simply vanish from the population.

O. oeni has been shown to be a rapidly evolving organism (Yang and Woese 1989), and this, combined with the inhibitory properties of wine, may in fact make it a perfect candidate for this method of optimization. Genome data from *O. oeni* PSU-1 (Mills et al. 2005) has revealed that *mutS* and *mutL*, which encode two key enzymes in the mismatch repair (MMR) pathway (Makarova et al. 2006; Makarova and Koonin 2007), are absent in *O. oeni*. The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species; therefore, a defect in the MMR system leads to an increase in the mutation frequency. One possible reason for the loss of MMR is that a high mutation rate generated beneficial mutations during adaptation to a restrictive environment such as wine (Marcobal et al. 2008). An increased mutation rate is initially beneficial; however, once an organism has adapted to a particular environment, an increased mutation rate can lead to a loss of fitness due to an increased load of deleterious mutations within the population (Taddei et al. 1997). In DE, mutations that cause deleterious effects on fitness arise due to genome erosion more often than advantageous mutants (Perfeito et al. 2007). When this occurs, a population remains viable within its specific environment, but its fitness is reduced for growth in any other environment and is therefore highly specialised. The application of this approach to improve *O. oeni* growth and MLF ability has been recently investigated by growing a continuous culture of *O. oeni* with increasing ethanol concentration over many generations (Betteridge et al. 2013). The evolved population not only survived under higher ethanol conditions but completed MLF in MRS with 15 % ethanol in almost half the time of the parent strain. This study confirmed that DE can be successfully used as a technique for developing new strains and is a promising method to improve other LAB strains for use in wine.

Technological advances

Immobilised cells

The use of high densities of immobilised cells to carry out MLF has been suggested as a method to combat the effect of high ethanol or low pH (mainly in cool climate wines) on the bacterial cells. Another advantage of using immobilised cells is that they can be reused for several cycles. The study of immobilisation methods for conducting MLF has increased over the years as an awareness of the impact that climate change is having on grape and wine quality grows. Increased heat during grape maturation has being linked to an increase in sugar content and decomposition of acid (Jones

et al. 2005; Mira de Orduña 2010; Bock et al. 2011). This leads to higher pH and also higher ethanol levels in the final wine. High temperatures may also inhibit malolactic bacterial growth particularly in high alcohol wines. Many matrices for bacterial immobilisation have been proposed, and the advantages and disadvantages of each are still under debate.

Two main immobilisation methods have been utilised: encapsulation of the bacterial cells (Spettoli et al. 1982; Crapisi et al. 1987; Guzzon et al. 2012; Rodríguez-Nogales et al. 2013) and attachment/adsorption onto a support (Maicas et al. 2001; Agouridis et al. 2008; Genisheva et al. 2013). Two recent encapsulation studies have used Ca-alginate microbeads coated with an organo-silica membrane (Guzzon et al. 2012) and polyvinyl alcohol-based hydrogel 'Lentikats®' (Rodríguez-Nogales et al. 2013). Evaluation of the MLF capabilities of entrapped *O. oeni* cells in Lentikats® matrix showed that the relative percentage of malic acid degradation at 14 % ethanol was increased by using immobilised cells. Relative degradation at 14 % ethanol was also increased using immobilised cells with the added challenge of either low pH or increased temperature. Furthermore, the cells could be used five times without loss of activity. Further studies will determine the full scope of the impact on wine composition and sensory properties when using this method to immobilise cells and to conduct MLF.

Immobilisation does not always prove advantageous when applied to wine. Guzzon et al. (2012) performed 50 l MLF trials using cells encapsulated in Ca-alginate microbeads. Both the effect of yeast and bacterial co-fermentation and sequential fermentation were tested, and in each case, the free cells degraded malic acid faster than the immobilised ones. However, one advantage when lysozyme was added to control the growth of unwanted LAB was that the immobilised cells could still degrade the malic acid, whereas the free cell fermentations were inhibited (Guzzon et al. 2012). More recently, Servetas et al. (2013) have reported the use of a two-layer composite biocatalyst containing both yeast and LAB for simultaneous AF and MLF in wine. The yeast and LAB species were physically separated by the use of delignified cellulosic material and a starch gel as a method to reduce species competition. Whilst improved performance was reported, further investigation comparing wines fermented with free cells vs. immobilised cells was not reported (Servetas et al. 2013) and is necessary before this technique can be adopted.

Immobilised MleA enzyme

Although numerous studies have reported the use of free or immobilised LAB to conduct MLF, direct bioconversion with free or immobilised MleA has scarcely been studied. This is most likely because the process requires the availability of both manganese (Mn^{2+}) and NAD^+, which act as cofactors (Schümann et al. 2013). Immobilised enzymes allow for continuous processing, which in turn can lead to lower production costs and energy consumption. A further benefit when compared with free enzymes in solution is that immobilised enzymes are more robust and are often more stable and resistant to environmental changes (Krajewska 2004). Wine naturally contains Mn^{2+} at a concentration that is considered sufficient for enzyme activity (Formisyn et al. 1997). However, the optimal pH for MleA activity is 6.0 (Schümann et al. 2013), and the enzyme still requires the addition of NAD^+ to the reaction system.

A membrane reactor using free *O. oeni* enzyme has also been reported (Formisyn et al. 1997). The design involved the use of two compartments separated by a polysulfone membrane, one for MleA and NAD^+ cofactor and one for the wine. This arrangement generated a pH gradient to ensure activity of the enzyme; however, this also resulted in a strong dilution of wine in the system. One potential solution to this problem is the use of synthetic enzyme cascades. For example, Köhler et al. (2013) reported a method for efficient concurrent tandem catalysis that could potentially be used to enable utilisation of Mle catalysis with regeneration of NAD^+. Given the significant degree to which the physical and chemical properties of the support influence catalytic performance, an appreciation of materials science, beyond the scope of this review, is required for the successful development of an immobilised enzyme catalyst. For more information on microcapsules, refer to Dähne and Peyratout (2004), and for the one-step multicomponent encapsulation method, see Chen et al. (2008).

Monitoring MLF

Many winemakers regard the timely determination of the completion of MLF to be of primary importance so that the wine can be promptly stabilised. There is concern that any LAB remaining in wine after the end of MLF could promote the production of undesirable volatile compounds and the formation of biogenic amines. Therefore, following on from MLF, the winemaker will often remove or inhibit the bacterial biomass, typically through the addition of sulfites. Several methods are available to monitor and identify the completion of MLF, such as enzymatic analysis kits, paper chromatography, thin layer chromatography and high-performance liquid chromatography. As all of these methods require time and resources in the winery for periodic sampling of tanks or barrels and for analysis, there is increased interest in monitoring MLF in real time. Various methods have been proposed including the use of electrochemical biosensors (Gamella et al. 2010), a wireless sensor bung (Di Gennaro et al. 2013) and ultrasonic velocity measurements (Novoa-Díaz et al. 2014).

Electrochemical biosensors can provide up to 500 measurements using the same biosensor; however, the need for manual sampling and dilution of the wine sample to ensure a liner response make this system more time consuming (Gamella

et al. 2010). A real-time solution is proposed through the use of a wireless sensor bung (WSB) system based on the detection of an increase in pH during MLF (Di Gennaro et al. 2013). Whilst the results of this preliminary study are very promising by showing good agreement between malic acid degradation and pH increase, the means by which the endpoint of MLF is defined, other than a plateau in the rise in pH, needs to be determined. This is particularly important in order to rule out the possibility that pH has stopped rising merely because the MLF has stuck. The same can be said of the use of ultrasonic velocity measurements to monitor changes in malic acid and lactic acid concentrations during MLF (Novoa-Díaz et al. 2014). Whilst possibly more sensitive, the ultrasonic approach also relies on a cease in the change of the measurement to indicate an end of MLF or a stuck MLF. In each case, these methods do not obviate the need for the winemaker to check the MLF and perhaps conduct traditional malic/lactic acid determinations off-line, but they do at least reduce the number of barrel samplings to potentially a single one. Both systems show potential for use as decisional support systems (DSS), indicating to the winemaker the correct time to conduct the final laboratory analysis. Further investigation of both of these methods will also ensure that they are flexible and accurate enough to be applied to a range of wine styles, fermentation and cellar conditions.

Expression of malolactic enzyme in yeast

In yeast, the malic enzyme predominately converts malic acid into pyruvic acid, which is further metabolised to ethanol and carbon dioxide under fermentative conditions via the malo-ethanolic (ME) pathway (Volschenk et al. 2003). Due to the lack of a specific malate transporter, uptake by *S. cerevisiae* is considered to occur by simple diffusion. In addition, the *S. cerevisiae* malic enzyme has low substrate affinity (Husnik et al. 2007) and is subject to catabolite repression (Redzepovic et al. 2003), resulting in this yeast having a limited ability to metabolise extracellular malate (Volschenk et al. 1997a, b). Two tactics have been utilised to express malolactic enzyme in *S. cerevisiae*: co-expression of the malate permease gene and surface display of malolactic enzyme. The malate permease gene (mae1) of the fission yeast *Schizosaccharomyces pombe* has been co-expressed with either the *L. lactis* malolactic gene (*mleS*) or the *O. oeni* malolactic gene (*mleA*) in *S. cerevisiae* (Volschenk et al. 1997a; Husnik et al. 2006, 2007). The industrial *S. cerevisiae* wine strain ML01 was so constructed by insertion of a malolactic cassette containing the malate transport (*MAE1*) gene from the yeast *S. pombe* (Grobler et al. 1995) and the malolactic enzyme (*mleA*) from *O. oeni* (Husnik et al. 2006). Both genes were constitutively expressed under control of the *S. cerevisiae* PGK1 promoter and terminator sequences. ML01 can complete MLF within the first 9 days of AF.

Main et al. (2007) investigated malic acid reduction using ML01 and two naturally selected yeast (ICV-GRE and 71B). Malolactic bacteria were also added at the end of an additional fermentation with ICV-GRE and general wine parameters were measured. Although ML01 successfully converted all the malic acid to lactic acid, there was also an increase in SO_2 production when compared to the other fermentations (Main et al. 2007). The other yeast investigated reduced the malic acid content in wine by 18 % (ICV-GRE) and 33 % (71B). The wine fermented with ICV-GRE+*O. oeni* had lower lactic acid and titratable acidity than ML01 because of malic consumption by the yeast before MLF, presumably via the ME pathway (Volschenk et al. 2003) as no L-lactic acid was produced at the end of AF and the final ethanol concentration was increased when compared to ML01. Expression of *MleA* in a low SO_2-producing strain has not been reported. Additionally, expression of malolactic enzyme in yeast affected the flavour profile of the wine resulting, for example, in decreased mouthfeel due to decreased ethyl lactate metabolism (Husnik et al. 2007).

In an alternative approach, the α-factor secretion signal encoding sequence has been used to display the mleA enzyme from *O. oeni* on the surface of *S. cerevisiae* cells (Zhang et al. 2013). The resulting yeast strain could degrade 21 % of L-malate after only 12 h; however, no details were provided on the capabilities of this strain over a longer period of time. The authors also used the laboratory yeast strain AH109 whose oenological properties have not been specified. Thus, although this is a promising solution, it requires further validation in the genetic background of a wine yeast.

Each of these methods offer promise but need to have their full impact on wine quality assessed. Of course, a further feature of these systems, which may be unattractive to some producers, is the fact that they rely on recombinant organisms.

Conclusion

Through ongoing research, a better understanding of the important changes occurring during MLF and how to use MLF to influence wine style will be achieved. Both the choice of LAB strain and timing of bacterial inoculation can be used to modulate modifications in wines from MLF aside from deacidification such as buttery or fruity-berry aromas and improved mouthfeel attributes. The ability of LAB to conduct MLF and produce desirable aroma compounds has been shown to differ between both LAB genera and across strains of the same species. An increased understanding of the enzymes and how they are involved in changes in wine composition during MLF along with increased understanding of the genes and metabolic pathways involved in adaptation to the stressful wine environment will lead to novel approaches for the characterisation and development of starter cultures.

Additional new strategies such as co-inoculation, using mixed LAB cultures, directed evolution of LAB strains, immobilised cells or immobilised enzymes have the potential to reduce the duration of MLF and risks associated with sequential MLF. It is becoming clear that these techniques can contribute positively to the aroma of wine without the excessive production of acetic acid. Increased knowledge of safety considerations will also impact strain selection and how we view MLF, and careful selection and use of selected strains will enable more reliable and safe MLF. Comparative genomics and transcriptomics will increase our understanding of regulatory mechanisms and, in turn, help improve MLF performance and success. Increased analysis of the coordinated expression of genes that alter specific cellular processes will help identify molecular markers for the selection of improved strains. Additionally, an increased understanding of the genes involved in production of undesirable aromas or compounds that are of safety concern to consumers, such as biogenic amines and ethyl carbamate, will enable improved screening of new strains prior to industrial use.

Future studies may involve the use of enzyme cascades to enable direct application of malolactic enzyme to wine. As is evident by recent studies, the use of different genera of LAB has the potential for future use in starter culture preparations. This will increase the genetic diversity of inoculated LAB and, if properly tested, could provide novel and desirable outcomes. Alternatively, the use of DE to evolve LAB strains shows much promise to develop new strains better suited to growth in wine and to provide an increased understanding of the metabolic processes that are important during MLF, thereby informing new strategies for generating and screening evolved strains. Additionally, new technologies for monitoring MLF in real time will enable winemakers to monitor the process more efficiently and reduce the incidence of wine production delays. This will have a knock-on effect in allowing subsequent fermentations to proceed as scheduled without the need to delay fruit harvest or wine processing. Overall, cost reductions associated with reliable MLF and more efficient wine production will ensure a better quality product.

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